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THE PHYSIOLOGICAL CONSEQUENCES OF BREATH-HOLD DIVING IN MARINE MAMMALS; THE SCHOLANDER LEGACY

Topic Editor
Andreas Fahlman

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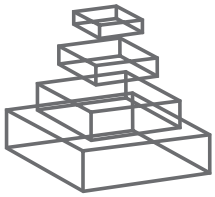

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THE PHYSIOLOGICAL CONSEQUENCES OF BREATH-HOLD DIVING IN MARINE MAMMALS; THE SCHOLANDER LEGACY

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All fish and humans: Erika Schagatay; row 2, column 1: Photo credit 3S (AnnemiekePodt); row 3, column 1: Photo Credit 3S (SannaKuningas); row 4, column 1: Photo Credit 3S (SannaKuningas); row 4, column 2: Photo Credit 3S (Paul Ensor); row 4, column 3: Photo Credit 3S (SannaKuningas); Divingrat: W. Michael Panneton, Saint Louis University; bottom weddel seal: Stephen J. Trumble.

Breath-hold diving marine mammals are able to remain submerged for prolonged periods of time and dive to phenomenal depths while foraging. A number of physiological, biochemical and behavioral traits have been suggested that enable this life style, including the diving response, lung collapse, increased O_2 stores, diving induced hypometabolism, and stroke-and-glide behavior to reduce dive metabolic cost.

Since the initial studies by Scholander in the 1940's, when most of the physiological and biochemical traits were suggested, few have received as much study as the diving response and O_2 management. The calculated aerobic dive limit (cADL) was an important concept which allowed calculation of the aerobic dive duration, and was defined as the total O_2 stores divided by the rate of O_2 consumption (metabolic rate). The total O_2 stores have been defined for several species, and studies in both forced and freely diving animals have refined the metabolic cost of diving.

Currently there appears to be little consensus about whether marine mammals perform a significant proportion of dives exceeding the cADL or not and there may be large

differences between species. The diving response is a conserved physiological trait believed to arise from natural selection. The response includes diving-induced bradycardia, peripheral vasoconstriction, and altered blood flow distribution. While the response results in reduced cardiac work, it is not clear whether this is required to reduce the overall metabolic rate.

An alternate hypothesis is that the primary role of the diving bradycardia is to regulate the degree of hypoxia in skeletal muscle so that blood and muscle O_2 stores can be used more efficiently. Scholander suggested that the respiratory anatomy of marine mammals resulted in alveolar collapse at shallow depths (lung collapse), thereby limiting gas exchange. This trait would limit uptake of N_2 and thereby reduce the risk of inert gas bubble formation and decompression sickness.

In his initial treatise, Scholander suggested that alveolar collapse probably made inert gas bubble formation unlikely during a single dive, but that repeated dives could result in significant accumulation that could be risky. Despite this, lung collapse has been quoted as the main adaptation by which marine mammals reduce N_2 levels and inert gas bubble formation. It was surprising, therefore, when recent necropsy reports from mass stranded whales indicated DCS like symptoms. More recent studies have shown that live marine mammals appear to experience bubbles under certain circumstances.

These results raise some interesting questions. For example, are marine mammals ever at risk of DCS, and if so could N_2 accumulation limit dive performance? While an impressive number of studies have provided a theoretical framework that explains the mechanistic basis of the diving response, and O_2 management, many questions remain, some widely-accepted ideas actually lack sufficient experimental confirmation, and a variety of marine mammal species, potentially novel models for elucidating new diving adaptations, are understudied.

The aim of this Frontiers Topic is to provide a synthesis of the current knowledge about the physiological responses of marine mammals that underlie their varied dive behavior. We also include novel contributions that challenge current ideas and that probe new hypotheses, utilize new experimental approaches, and explore new model species. We show that the field has recently entered a phase of renewed discovery that is not only unraveling more secrets of the natural diving response but will drive new applications to aid human exploration of the ocean depths. We also welcome comparative analyses, especially contributions that compare marine mammals with human divers.

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EDITORIAL
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The physiological consequences of breath-hold diving in marine mammals: the Scholander legacy

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Most of the physiological traits used by marine mammals to perform long and deep breath-hold dives were described in Scholander's seminal paper in 1940. Since then, several studies have provided an improved understanding of the mechanistic basis of the mammalian diving response (Scholander, 1940, 1963; Mottishaw et al., 1999; Fahlman et al., 2011), the aerobic dive limit (ADL) (Kooyman et al., 1980; Butler and Jones, 1997; Davis and Kanatous, 1999; Horning, 2012), and management of respiratory gases (Boutilier et al., 2001; Fahlman et al., 2008a; Hooker et al., 2009; Kvadsheim et al., 2012), but many questions remain. Some widely-accepted ideas actually lack experimental confirmation, and a variety of marine mammal species, potentially novel models for elucidating new diving adaptations, have not been adequately studied. The aim of this Frontiers Special Topic is to provide a synthesis of the current knowledge of the physiological responses that may explain the varied diving behavior of marine mammals. We strove to include contributions that challenge current ideas, and which propose new hypotheses, utilize new experimental approaches, and explore new model species.

Much work has been dedicated to understanding the ADL and how a species can manage its foraging within its ADL. The ADL was originally defined as the length of time an animal could remain submerged before the post-dive blood lactate levels began to increase (Kooyman et al., 1980). The calculated aerobic dive limit (cADL) was later conceived to estimate the maximum duration of aerobic metabolism by dividing the total usable O_2 stores by the rate of O_2 consumption (metabolic rate, Butler and Jones, 1997). While most species appear to dive well within their cADL, others appear to exceed the cADL on a regular basis (Costa et al., 2001). Horning proposes an interesting method to investigate the plasticity of the functional ADL using constraint lines, which may help improve our understanding of the link between behavior and physiology (Horning, 2012). On a physiological level, it is possible that dives that appear to be beyond the cADL are actually attributable to underestimating the usable O_2 stores, or overestimating the metabolic costs of diving and foraging (Hurley and Costa, 2001; Fahlman et al., 2008b; Ponganis et al., 2011). A study suggests that elephant seals possess extreme hypoxia tolerance and make use of their entire blood O_2 store during diving (Meir et al., 2009; Ponganis et al., 2011). The use of the spleen to increase hematocrit during diving has been shown to enhance breath-hold capacity in humans (Schagatay et al., 2012) and in marine mammals (Cabanac, 2000; Thornton et al., 2001). It may be that previous analyses of cADL have missed these sources of usable O_2 (Meir and Ponganis, 2009; Ponganis et al., 2011).

Logistical constraints have made it difficult to estimate metabolic rate in foraging animals (Ponganis et al., 2011). Variation in prey density or other environmental factors may alter metabolic costs of foraging. It has been hypothesized that alteration in prey species may affect the nutritional status of the predator (Rosen, 2009). Trumble and Kanatous (2012) argue that the metabolic stoichiometry between O_2 and ATP is affected by the lipid composition of the diet. As the lipid composition varies between prey species and seasons, the ingested food may alter the foraging efficiency through changes in the metabolic burden while underwater.

Weingartner et al. (2012) have shown that increased thyroid hormone levels elevate the metabolic rate during diving in harbor seals and result in higher post-dive lactate levels. This suggests that thyroid hormone could be important in modulating metabolic rate to fit the dive conditions. The higher metabolic rate resulted in a more pronounced reduction in heart rate during the dive. This provides an interesting link between endocrine and neural control of the physiological responses during diving. The hyperthyroid animals, with a more extreme diving bradycardia, may be indirect evidence of the O_2 conserving effect of the diving response (Weingartner et al., 2012). The diving response is believed to be a conserved physiological trait, which includes diving-induced bradycardia, peripheral vasoconstriction, and altered blood flow distribution (Mottishaw et al., 1999; Fahlman et al., 2011). While our understanding of the central control of the diving response is limited (McCulloch, 2012; Panneton et al., 2012), the bradycardia results in reduced cardiac work. It is not clear whether the reduced work is sufficient to significantly lower the overall metabolic burden, or whether the response serves other purposes. An alternate hypothesis is that the primary role of the diving bradycardia is to regulate the degree of hypoxia in skeletal muscle so that blood and muscle O_2 stores can be used more efficiently (Davis and Kanatous, 1999).

If marine mammals generally dive within their cADL, what other physiological constraints may limit diving? Scholander suggested that alveolar collapse (commonly called lung collapse) would limit uptake of N_2 and reduce the likelihood of decompression sickness (DCS, Scholander, 1940). However, necropsy reports from mass stranded whales indicated DCS-like symptoms

(Jepson et al., 2003; Fernández et al., 2005). A more recent study has shown that the gas bubble composition in stranded whales is similar to that from land mammals suffering DCS in experimental dive models (Bernaldo De Quirós et al., 2012). Imaging work in both live and stranded marine mammals indicates that they live with elevated inert gas tensions that cause bubbles to form under certain circumstances (Dennison et al., 2012). This raises some interesting questions: are marine mammals ever at risk of DCS, and if so, could N_2 accumulation limit dive performance (Hooker et al., 2009; Kvadsheim et al., 2012; Sivle et al., 2012)? The estimated end-dive N_2 levels suggest that a significant proportion of marine mammals should experience DCS symptoms if their responses to elevated N_2 are physiologically similar to those of humans and various species of land mammals used in diving simulations (Hooker et al., 2009). Our understanding of the anatomy and physiology of marine mammals is not well-defined in this regard. The DCS model assumptions are based on data from widely different species, which may explain the elevated predictions for marine mammals. A recent study by Costidis and Rommel (2012) provides data on the vascular anatomy in bottlenose dolphins, suggesting that certain adipose tissue compartments may be highly vascularized. The ability to exchange gases in these compartments would vastly alter our understanding of how these species manage gases underwater, and provide interesting research challenges for the future.

Since the initial studies by Scholander in the 1940's, physiologists have been fascinated by the diving traits of marine mammals, and there is a large heritage not only from Scholander, but also from other classical work following this pioneer. While most of the physiological and biochemical traits were suggested Scholander and Irving, few have received as much study as the diving response and O_2 management. The contributions to this special topic have shown that the field of diving physiology has recently entered a phase of renewed discovery that is revealing more secrets of the natural responses observed in marine mammals. While there is still a lot more to learn this special topic has focused on work progressing from this heritage, instead of re-inventing knowledge. What is becoming clear is that marine mammals may be a useful model system to understand physiological challenges in extreme environments.

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The genetic component of the forced diving bradycardia response in mammals

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We contrasted the forced diving bradycardia between two genetically similar (inbred) rat strains (Fischer and Buffalo), compared to that of outbred rats (Wistar). The animals were habituated to forced diving for 4 weeks. Each animal was then tested during one 40 s dive on each of 3 days. The heart rate (f_H) was measured before, during, and after each dive. Fischer and Buffalo exhibited marked difference in dive bradycardia (Fischer: 120.9 ± 14.0 beats min^{-1} vs. Buffalo: 92.8 ± 12.8 beats min^{-1}, $P < 0.05$). Outbred rats showed an intermediate response (103.0 ± 30.9 beats min^{-1}) but their between-animal variability in mean dive f_H and pre-diving resting f_H were higher than the inbred strains ($P < 0.05$), which showed no difference ($P > 0.05$). The decreased variability in f_H in inbred rats as compared with the outbred group indicates that reduced genetic variability minimizes variability of the diving bradycardia between individuals. Heritability within strains was assessed by the repeatability (R) index and was 0.93 ± 0.05 for the outbred, 0.84 ± 0.16 for Buffalo, and 0.80 ± 0.12 for Fischer rats for f_H during diving. Our results suggest that a portion of the mammalian diving bradycardia may be a heritable trait.

Keywords: forced diving, heart rate, rat, repeatability, quantitative genetics

INTRODUCTION

The heart and brain are organs that are vitally dependent upon a continuous supply of oxygen. Interruption of oxygen supply for only a short time, as occurs during sleep apnea, stroke, or heart disease, can cause permanent damage or death. Scholander (1963) coined the phrase "master switch of life" to describe the cardiovascular role of the diving response (bradycardia and reduced peripheral blood flow) in reducing the demand for oxygen in both forced and free-diving vertebrates. It has been suggested that the mammalian dive response is a conserved physiological trait that arises from natural selection, reflecting genotypic adaptations to prolonged apnea or asphyxia. The response is observed in both aquatic (Butler and Jones, 1997), and semi-aquatic (rat and musk rat; Signore and Jones, 1995, 1996; Mcculloch et al., 2010a; Panneton et al., 2010a,b) mammals, preventing permanent damage of heart and brain during prolonged apnea. Even fish show bradycardia when removed from water, raising the question whether bradycardia is a pre-adaptation to hypoxia or asphyxia (Davis et al., 2004) or an evolved adaptation driven by selection across evolutionary time (Scholander, 1963).

A similar, although less prominent, type of response also occurs in man (Lindholm et al., 1999), making studies on appropriate animal models particularly relevant to humans. The fact that the diving response in individual humans is repeatable over many years suggests heritability (Terblanche et al., 2004), and such heritability is widely assumed, but has never been directly demonstrated. Previous studies have shown great variability in the human diving bradycardia, and individual factors such as age and diving experience have been suggested to modify the response (Lin and Baker, 1975; Manley, 1990). It is known that several factors induce or modify these cardiovascular responses, such as arterial hypoxia and hypercapnia, cessation of respiratory movements, and face immersion (Butler and Jones, 1997; Lindholm et al., 1999). Even though the physiological mechanism of this response has been thoroughly investigated (Butler and Jones, 1997; Lundgren and Miller, 1999), we are not aware of any study that has investigated the genetic basis of this physiological trait.

Simple physiological traits that are regulated by a single gene represent only a small portion of the total genetic makeup of any organism. Most phenotypes are complex and involve interactions between many genes and environmental factors. This complicates the use of physiological traits as indicators of heritability as it is difficult to tease apart environmental from genetic variation. Reducing the genetic variation between animals is one potential strategy for dealing with complex traits. Inbred rat strains are the product of 20 or more generations of brother–sister matings, making individuals within a strain genetically identical and homozygous at most loci. This assures reduced genetic variability within strains. Therefore, any difference in a physiological variable between strains assures that a portion of the trait is genetically encoded and facilitates isolation of the genetic basis of the phenotype (Tankersley et al., 1994, 1997). Rats are an excellent animal model to study the genetic component of the heart rate responses during diving as inbred strains are readily available. In addition, several studies have concluded that they are a good research model for the dive response as they are semi-aquatic animals that are easily trained, they possess a strong diving bradycardia response during forced as well as voluntary dives (Ollenberger et al., 1998; Mcculloch et al., 2010a; Panneton et al., 2010a), and are able to

dive for as long as 60–80 s in 30°C water (Lin and Baker, 1975; Panneton et al., 2010a).

In this study, we isolated two different inbred strains with varying heart rate responses to forced diving and compared these against an outbred strain (wild type). The data from the various strains allowed us to determine (1) what proportion of the overall variability is genetic and if (2) the mammalian diving bradycardia is heritable.

MATERIALS AND METHODS

All animal and experimental protocols were reviewed and approved by the Animal Care Committee of University of British Columbia.

ASSUMPTIONS

Inbred rat strains are the product of 20 or more generations of brother–sister matings, making individuals within a strain genetically identical and homozygous at most loci (Haldane and Waddington, 1931). Consequently, inbred strains as a model to study heritable effects of physiological traits offer unique advantages because (1) the magnitude of genetic variability can be controlled, (2) environmental factors can be strictly controlled in a laboratory setting, and (3) variation between strains in the trait considered can be partitioned into its genetic and environmental components.

ANIMALS

Male inbred rats from the following strains were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA): Fischer 344 (*F*), and Buffalo (*B*). The choice of strains was made by the limited knowledge of their physiological phenotypes. Fischer F344 is a very common all-purpose strain and a large literature base exists for this strain, our "control" strain. The Buffalo strain has compromised thyroid function affecting metabolic rate. One strain of outbred Wistar rats (W), bred at the Animal care facility at UBC (UBC Animal Care Centre, Rodent breeding unit, original stock from Charles River), was also examined. The rats of the outbred strain were not crossed with their siblings and variation in the heart rate response during diving from this strain includes both genetic and environmental variation.

All animals were recently weaned pups (3–5 weeks old, ∼30–35 g), examined on receipt by the veterinary staff and housed in the animal care facility. After arrival, the rats were stabilized for 4 days, and held at 22.0 ± 0.5°C at 60% relative humidity under a 12-h light/dark cycle with *ad libitum* access to standard rat chow (Lab diet 5001, Rodent diet PMI nutrition Int., Brentwood, MO, USA) and water. The ambient room temperature during manipulations, i.e., training, surgery, dive experiments, was controlled around 20°C, but the humidity was not altered. While some studies have indicated that this air temperature is below that corresponding to minimum metabolic effort (Gordon et al., 1991), our housing complied with the National Institutes of Health guidelines for the care and use of laboratory animals. In addition, previous studies in rats have used a similar housing temperature and report normal blood pressure and heart rate values (Mcculloch et al., 2010b; Panneton et al., 2010a).

TRAINING

To reduce stress from forced submersions, the rats were progressively habituated to water immersion. Five days a week (Mon–Fri) each animal performed a training session in the morning and another in the afternoon. During the first week, the rats were placed in a bucket filled with water. Initially, the water level allowed the animals to stand on the bottom and the water level was raised during repeated sessions. When the water level was deep enough so that the animals could not stand on the bottom, they swam around searching for an exit point. Most rats began to dive voluntarily after about the third training session.

After the first week of water habituation, the rats began forced dive training. The animal was placed in a restraining device. The restrainer was varied in size as the rats grew to minimize movement. Each training session involved three repeated submersions separated by at least 5 min. Initial submersions were <5 s in duration. The duration of submergence was increased on a daily basis based on the comfort level of the rat to a maximum dive duration of 40 s. Once rats dived for 40 s, they were prepared for experimental trials by implanting subcutaneous ECG electrodes. The dive duration was limited at 40 s for all strains to reduce variation between individuals and strains. This duration appeared to be tolerated by the inbred strains during initial trials and is well within the maximum reported dive duration for rats of similar size (Panneton et al., 2010a,b).

SURGICAL PROCEDURE AND HEART RATE MEASUREMENTS

Heart rate was measured by surgically implanting two ECG electrode wires. Rats were prepared for surgery by placing the animal in a pre-anesthetization box flushed with isoflurane. Once unconscious, the animal was removed from the box and the head placed in a modified head cone to which anesthetic gas was delivered in O_2. The amount of isoflurane delivered was initially ∼3–4% isoflurane. When the rat reached a surgical plane of anesthesia (i.e., no pinch reflex or response to pain,) the isoflurane concentration was adjusted as necessary (1–2.5% isoflurane). Once properly anesthetized, analgesia (Ketoprofen, 2 mg kg^{-1}) was administered intramuscularly. Betadine was used to cold sterilize all instruments and the ECG electrodes. The incision sites were shaved and disinfected using betadine.

Using aseptic techniques, dorsal incisions were made on either side of the vertebral column. The electrodes were implanted subcutaneously on the ventral side of the animal, one on each side of the sternum. The wires were made of stainless steel with a silicone sleeve, leaving only the wire tips bare. The opposite ends of the ECG electrode wires were inserted into a 21 G needle which in turn was tunnelized subcutaneously to a third incision about 2 cm from the base of the head. The needle was removed leaving the ends in place. The electrodes were immobilized by fixing the ends subcutaneously using an absorbable suture. The external end of each wire was exposed and attached to the heart rate recorder. The incisions were closed using common suturing techniques. Before removal of the anesthetic gas, the rat received an intramuscular injection of Baytril (2.5 mg kg^{-1}). Following surgery, the animal was observed until it awoke and returned to a solitary enclosure until complete recovery (∼1–2 h) after which the rat was returned to the holding pen. Experiments were

conducted approximately 5 days after surgery when the rat had finally recovered.

The ECG was measured by connecting the external ends of the implanted electrodes to an amplifier (Gould universal amplifier model 13/4615/58). The amplified ECG signal was passed via a purpose built analog-to-digital converter to a data acquisition program (Labtech Notebook, Version 9.0, Laboratory Technologies Corp., FL, USA) where it was sampled at 800 Hz. Heart rate was determined from the ECG trace by counting the time between *R–R* peaks (AcqKnowledge V. 3.7, Biopac Systems Inc., CA, USA).

DIVE TRIALS

The rat was placed in the restraining cage and the subcutaneous electrodes attached to the pre-amplifier. The animal was allowed to settle down from the handling for 5 min followed by a forced submersion using the same approach as during the training sessions. The water temperature during all forced submersions was maintained around 30°C (±1.0°C) to reduce thermal stress due to cold water immersion (Mcculloch and Panneton, 2003). The dive duration was standardized to 40 s unless the animal appeared distressed (struggle) or if the diving bradycardia was not maintained. In those instances the rat was immediately removed from the water. At completion of the dive, the animal was first dried with a towel and then placed in a cage with a heat lamp at one end, allowing the animal access to additional heat while air drying.

Each animal performed only one dive experiment per day, but repeated the experiment on three occasions separated by at least 24 h and at most 7 days. Out of six animals used for each strain, three repeated dives with successful heart rate recordings were obtained for five Buffalo, five Fischer, and six outbred Wistar rats. One Buffalo and one Fischer rat performed one successful experiment after which one ECG electrode broke off. The data from these rats are included in **Table 1** but the number of successful trials was only 16 for the inbred as compared with 18 for the outbred strains.

DATA ASSESSMENT AND STATISTICAL ANALYSIS

Heart rate data were split up and averaged into dive time bins of 5 s. The average heart rate 30 s before the start of the dive was considered the pre-dive period. The average heart rate immediately following a dive and for the next 30 s was considered the post-dive period.

Mixed models regression using a compound symmetry covariance structure to deal with the correlation within rats (Littell et al., 1998) was used to determine the best predictive model for the relationship between heart rate (dependent variable) and four experimental variables[dive period (pre-dive, dive, post-dive), rat strain, body mass, dive time bin] as independent fixed covariates. Statistical analyses were performed using the nlme package in R (A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, v.2.5.1, 2007). Animal ID was included as a random effect, which accounts for the correlation between repeated measurements on the same rat.

Forward and backward stepwise techniques were used to search for the best model. The likelihood ratio test was used to determine significance of parameters between nested models. In this test, significance is defined by increases in the log-likelihood (LL) values of the models (i.e., significantly smaller negative LL values). Acceptance of significance was set at $P < 0.05$, and $0.05 < P < 0.1$ was considered to be important enough to warrant investigation or consideration. All values are reported as means ± SD unless otherwise stated.

HERITABILITY

Measuring the variability of a trait within and between individuals is useful to assess the genetic contribution, or heritability, of the trait of interest (Spicer and Gaston, 1999). The total variation of a physiological trait (V_t), or phenotype, is the sum of the genetic variation and environmental factors. Genetic variation is caused by differences among genes (V_a, additive variation) and interaction between genes (non-additive variation). Narrow-sense heritability (h^2) is a useful measure to predict how animals will respond to natural selection and is the ratio between the additive genetic variation and the total variation ($h^2 = V_a/V_t$). If a trait has a high narrow-sense heritability, selection can alter that trait. Repeatability (R), or the intra-class coefficient (Sokal, 1981) is a simple and useful measure to assess the h^2 of the trait of interest (Dohm, 2002). R is a measure of the proportion of variance between individuals to the summed variance both between and within individuals (Falconer and Mackay, 1996) and is usually expressed as a percentage. Consequently, a high R represents a trait where most of the variation is partitioned between individuals and is likely to respond to natural selection (Dohm, 2002). One strategy to study complex physiological traits is to reduce the genetic variation between animals by studying inbred strains (Tankersley et al., 1994, 1997). Thus, we aimed to screen different strains of inbred rats and measure the heart rate responses during forced diving. We compared the responses against an outbred strain, which enabled us to estimate the contribution of genetic and environmental variability.

RESULTS

BODY MASS

There were significant differences in the mean body mass (±SD) between strains ($P < 0.05$, mixed effects model, **Table 1**), and both Buffalo and outbred rats weighed more than the Fischer strain during the dive trials (**Table 1**).

DIVE TRAINING

Initially, most rats struggled during the forced diving but at the end of the training period most appeared calm during the submergence. In addition, most rats entered the restraining device with little difficulty, and they appeared calmer in a cage with minimal room for movement.

DIVE DURATION

The forced submersion ended before the 40 s pre-determined dive duration in 8 out of 18 dives for the outbred rats as the heart rate increased (**Figure 1**). The increase in heart rate coincided with increasing struggle. For this reason, the dive duration in the outbred strain was significantly shorter from either of the inbred strains, where a dive ended prematurely only once for a Fischer rat ($P < 0.05$, mixed effects model).

Table 1 | Total number of animals (*N*) performing three dives separated by at least 1 day, mean (±SD) body mass (M_b), and mean resting heart rate (f_H) immediately before the dive for a total of three repeated forced dives performed on different days. One Fischer and one Buffalo rat completed only one dive experiment and their data are included.

Strain	*N*	Mean M_b (g)	Resting f_H (beats min^{-1})	Mean dive f_H (beats min^{-1})	Post-dive f_H (beats min^{-1})	Mean dive duration (s)
Fischer	5 (15)	$194.5 \pm 9.5^{a,b}$	$400.0 \pm 13.1^{\dagger}$	$120.9 \pm 14.0^{\dagger a}$	410.0 ± 30.8	39.8 ± 0.8^{a}
Buffalo	5 (15)	205.3 ± 14.2^{a}	$395.0 \pm 15.6^{\dagger}$	$92.8 \pm 12.8^{\dagger b}$	400.9 ± 31.5	38.2 ± 3.1^{b}
Outbred	6 (18)	206.9 ± 16.7^{b}	416.0 ± 33.1	$103.0 \pm 30.9^{a,b}$	406.6 ± 38.9	$36.8 \pm 3.1^{a,b}$

Superscripted letters indicate significant differences between groups, i.e., groups which are different (one way ANOVA followed by Bonferroni multiple comparison).
†Variance significantly different from the outbred strain ($P < 0.05$, F-test, $F\text{-crit}_{(0.05,5,5)} = 5.05$).

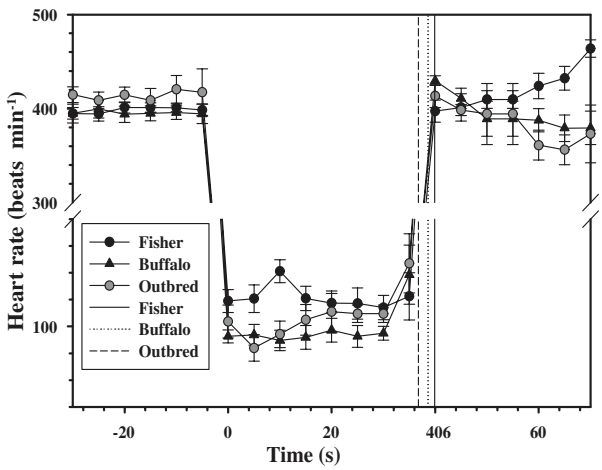

FIGURE 1 | Mean (±1 SD) heart rate every 5 s for two inbred (Fischer and Buffalo) and one outbred strain of rats ($n = 6$ rats in each strain), 30 s before, during, and 30 s after forced submergence. The mean is for a total of three repeated forced dives performed on different days. One Fischer and one Buffalo rat completed only one dive experiment which they lost their ECG electrodes, and their data are included. Solid and dotted vertical lines are the mean dive duration for each of the strains.

HEART RATES

Pre-dive, dive, or post-dive heart rates were not affected by variation in body mass either within ($P > 0.05$ for all) or between strains ($P > 0.1$, mixed effects model).

There were no temporal changes in pre-dive resting heart rates during the 30 s before the dive ($P > 0.1$, mixed effects model). In addition, the pre-dive heart rates did not differ between inbred or outbred strains ($P > 0.1$, mixed effects model, **Table 1**). However, the variance in heart rate during the resting period before the dive was higher in the outbred strain as compared with the inbred strains ($P < 0.05$, Fischer–Outbred: $F_{(0.05,\,5,\,5)} = 5.79$, Buffalo–Outbred: $F_{(0.05,\,5,\,5)} = 6.49$), but not different between inbred strains ($P > 0.1$, **Table 1**, $F_{(0.05,\,5,\,5)} = 1.14$).

For all rats for which the data were retained in the analysis, there was an immediate diving bradycardia (within 1–2 s) as the animal was submerged that persisted until the rat surfaced (**Figure 1**). The bradycardia was maintained throughout submergence, and the mean heart rate during each 5 s interval was constant for all strains ($P > 0.4$, mixed effects model). There were significant differences in the diving heart rate between the Fischer and Buffalo strains ($P < 0.05$, mixed effects model followed by Bonferroni multiple comparison; **Figure 1**), but not between inbred and outbred strains (**Table 1**).

The heart rate upon re-surfacing returned to pre-dive values within 5 s for all strains and remained constant for the next 30 s. However, there was a trend toward a post-dive tachycardia in the Fischer strain starting 25 s after surfacing (**Figure 1**; $P < 0.1$, mixed effects model). There were no differences in mean post-dive heart rate between strains over the 30 s following the dive ($P > 0.1$; **Table 1**).

Between the inbred strains only R, and its associated standard error of the mean (Sokal, 1981; Becker, 1984), was 0.99 ± 0.005 for diving f_H. Within strains, R for f_H during diving was of 0.93 ± 0.05 for the outbred, 0.84 ± 0.16 for Buffalo, and 0.80 ± 0.12 for Fischer rats.

DISCUSSION

We measured the heart rate response during forced diving. Our data are the first to suggest a genetic component of the universal diving bradycardia and we have shown that genetically distinct populations of rats demonstrate divergent heart rate responses during diving. The results also suggest that the trait is heritable and could respond to natural selection. While future analysis of the heart rate variability between strains may indicate a mechanism behind these differences, the current analysis cannot assess whether the divergent responses between strains are caused by

differences in neural control (sympathetic vs. parasympathetic control), or variation in endocrine response. In addition, the data presented here cannot determine the genetic components that control these differences.

HEART RATE CHANGES DURING FORCED VS. VOLUNTARY DIVES

The data presented in this study are limited to the heart rate response during forced submergence. As such, this represents a maximum response to an acute situation, such as asphyxiation or stroke in a human, or predator avoidance in a freely diving animal (Fedak et al., 1988). In unrestrained, trained, or free-diving marine mammals, on the other hand, the response is more variable and generally not as severe (Kooyman and Campbell, 1972; Hill et al., 1987; Thompson and Fedak, 1993; Ponganis et al., 1997; Hindell and Lea, 1998). It has been suggested that the primary role of diving bradycardia is to regulate the degree of hypoxia in skeletal muscle so that blood and muscle oxygen stores can be used more efficiently (Davis and Kanatous, 1999; Davis et al., 2004; Fahlman et al., 2009). In this scenario, the heart rate changes during voluntary diving would serve as a mechanism to manage the available O_2 within the planned dive and to maximize the aerobic dive limit (Kooyman, 1985; Davis and Kanatous, 1999; Fahlman et al., 2009). Still, the extremely low heart rates seen during forced dives are rarely seen in freely diving animals at sea. Heart rates during voluntary dives include confounding variables, such as variation in exercise level (Williams et al., 1991, 2004; Signore and Jones, 1996) or dive duration (Ponganis et al., 1997), and we therefore opted to use forced diving experiments in this study. The effect of habituation of forced diving has been variable in different species and studies (Gabbott and Jones, 1987; Jobsis et al., 2001; Mcculloch et al., 2010b; Panneton et al., 2010a,b). For example, repeated forced head submersions abolished the diving bradycardia in the duck (Gabbott and Jones, 1987) and reduced the magnitude of the response in three out of four harbor seals (Jobsis et al., 2001). In the rat, on the other hand, habituation does not appear to affect the magnitude of the diving bradycardia (Mcculloch et al., 2010b; Panneton et al., 2010a), but the mean arterial blood pressure decreases in habituated rats that dive >10 s (Mcculloch et al., 2010b; Panneton et al., 2010a). In the current study, the heart rate in the outbred strain decreased within 1–2 s after submersion by 75%, which is similar to that observed in outbred Sprague-Dawley rats of similar size (Mcculloch et al., 2010b; Panneton et al., 2010a). However, in the current study the dive ended before the pre-determined dive duration in eight out of the 18 dives, and this was associated with increasing heart rates as the rats began to struggle (**Figure 1**). In the inbred strains, the mean diving bradycardia was greater in the Buffalo strain as compared with the Fischer strain, with the outbred group being somewhere in between (**Figure 1**). In addition, the variation in the heart rate between individuals was much less varied both before and during the dive in the inbred strains as compared with the outbred rats (**Table 1**).

Unlike other dive studies in rats (Mcculloch et al., 2010b; Panneton et al., 2010a), we focused on the heart rate responses during forced diving. The dive response consists of numerous reflexes that result in a reduction in the heart rate and peripheral vasoconstriction (Kooyman, 1989; Butler and Jones, 1997). Studies in forced and voluntary diving in muskrats have shown that the diving bradycardia occurs independently of the peripheral vasoconstriction (Signore and Jones, 1995), and that the parasympathetic activity predominates the heart rate response while sympathetic influence has minimal effect (Signore and Jones, 1995, 1996). This "accentuated antagonism" therefore results in parasympathetic dominance of the heart rate response, despite considerable vagal stimulation. Whether the parasympathetic tone dominates the heart rate response in diving rats is debatable, but accentuated antagonism has been reported in diving muskrats (Signore and Jones, 1995, 1996), harbor seals (Elliott et al., 2002), and the lesser scaup (Mcphail and Jones, 1999). It was also considered a plausible mechanism to explain the heart rate responses in a previous study in forced diving rats (Mcculloch et al., 2010b). While forced diving appears to be more stressful and significantly varies the sympathetically mediated vasoconstriction as compared with voluntary dives, the diving bradycardia does not vary between modes of diving (Mcculloch et al., 2010b; Panneton et al., 2010a). Because the heart rate response, unlike the peripheral vasoconstriction, appears to be conserved and vary the least in both voluntary and forced diving rats, it was the target trait in the current study.

VARIABILITY OF THE HEART RATE RESPONSE AND HERITABILITY

The heart rate variation highlights something fundamental in physiological research. The within- and between-animal variability of the cardiac responses to diving is poorly understood. Both theoretical (Davis and Kanatous, 1999; Davis et al., 2004; Fahlman et al., 2009) and experimental (Ponganis et al., 1997) studies have suggested that the heart rate response is crucial to extend the aerobic dive limit, and is most likely influenced by genetic as well as multiple physiological factors. Therefore, it is not surprising that most studies have reported considerable variability within and between individual animals. To our knowledge, no other study has attempted to partition the within and between subject variability in the heart rate response to diving and most other studies report mean values (Panneton et al., 2010a). The average heart rate may not be representative of an individual's or species sensitivity or physiological plasticity to submergence, and may be particularly misleading, especially when the variable in question exhibits high between-individual variation (Dohm, 2002; Terblanche et al., 2004). Therefore, we suggest that studies should perform rigorous partitioning of sources of variation of the reflexes that make up the dive response. We suggest that R is a useful measure as it is an estimate of the variation between in relation to that within individuals (Sokal, 1981; Falconer and Mackay, 1996). In addition, the R of a trait is an estimate of the narrow-sense heritability and may represent the upper limit for its evolutionary significance (Dohm, 2002), and identification of variables with a high R are those most likely to respond to natural selection. While the mean and its associated error may give some important information, it cannot be used to answer questions about evolutionary importance (Terblanche et al., 2004, 2005).

While our data are limited, it provides a basic understanding about the raw material allowing this physiological trait to change by natural selection. Differential reproduction and survival are prerequisites for natural selection. For a physiological trait to be

responsive to natural selection it must show (1) a relationship between the trait and varying fitness, (2) heritability, and (3) consistent variation between individuals (Endler, 1986). In humans, a more marked diving bradycardia conserves O_2 and allows for longer duration breath-holds (Lindholm et al., 1999). In animals, a decrease in average heart rate allows for increasing time submerged (Kooyman, 1985; Fedak et al., 1988). This extends the time available for prey acquisition, which in turn increase survival. If the diving bradycardia responds to natural selection, we would expect R to be higher in the outbred strain and between inbred strains, but close to 0 within inbred strains if the genetic variation had considerable influence on the trait as $V_g \sim 0$. R was indeed high in the outbred strain but surprisingly high also within both inbred strains. This suggests an arrangement of genes that interact to alter the trait within a narrow margin, or a trait where the variation due to the environment is more important than the genetic effects. Despite this, R was high both within and between strains suggesting that the trait is able to respond to natural selection.

CONCLUSION

Our data are the first to suggest a genetic component of the heart rate response during diving, and we have shown that genetically distinct populations of rats demonstrate divergent responses. Heart rate variability in inbred rats was significantly less than in the outbred strain both before and while diving. The differences in heart rate variability between inbred and outbred rat strains show that this trait is genetically controlled and a portion of the variability in diving bradycardia is genetic. However, our data do not tell us anything about which or how many genes are involved. It has been proposed that the control system regulating and modulating heart rate changes is complex (Mottishaw et al., 1999). Thus, understanding the genetic mechanism behind these differences will be complicated. Linkage analysis has shown great success in detailing the ventilatory response to hypoxia in mice and a similar approach could be used here (Tankersley et al., 1994, 1997).

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Activation of brainstem neurons by underwater diving in the rat

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The mammalian diving response is a powerful autonomic adjustment to underwater submersion greatly affecting heart rate, arterial blood pressure, and ventilation. The bradycardia is mediated by the parasympathetic nervous system, arterial blood pressure is mediated via the sympathetic system and still other circuits mediate the respiratory changes. In the present study we investigate the cardiorespiratory responses and the brainstem neurons activated by voluntary diving of trained rats, and, compare them to control and swimming animals which did not dive. We show that the bradycardia and increase in arterial blood pressure induced by diving were significantly different than that induced by swimming. Neuronal activation was calculated after immunohistochemical processing of brainstem sections for Fos protein. Labeled neurons were counted in the caudal pressor area, the medullary dorsal horn, subnuclei of the nucleus tractus solitarii (NTS), the nucleus raphe pallidus (RPa), the rostroventrolateral medulla, the A5 area, the nucleus locus coeruleus, the Kölliker–Fuse area, and the external lateral and superior lateral subnuclei of the parabrachial nucleus. All these areas showed significant increases in Fos labeling when data from voluntary diving rats were compared to control rats and all but the commissural subnucleus of the NTS, A5 area, and RPa were significantly different from swimming rats. These data provide a substrate for more precise experiments to determine the role of these nuclei in the reflex circuits driving the diving response.

Keywords: diving response, c-Fos, medullary dorsal horn, parabrachial nucleus, nucleus tractus solitarii, rostroventrolateral medulla, neural circuits, swimming behavior

INTRODUCTION

It is an honor to pay tribute to Per Scholander, a man of immeasurable enthusiasm for science who with Laurence Irving made the original observations of the physiological changes in blood pressure to underwater submersion (Irving et al., 1942) in numerous species (Scholander and Elsner, 1968). He is considered by most the pioneer cardiovascular physiologist who initiated decades of studies on the remarkable phenomenon we call today the diving response. The work of our laboratory for the past 20 years extends his legacy by offering our observations on the neural control of the diving response.

The mammalian diving response is a powerful autonomic response comprising at least three simpler reflexes that activate the parasympathetic, sympathetic, and respiratory systems (Kooyman et al., 1981; Butler and Jones, 1982, 1997; Blix and Folkow, 1983; Elsner and Gooden, 1983; de Burgh Daly, 1984; Kooyman and Ponganis, 1998; Ferretti, 2001; Davis et al., 2004; Foster and Sheel, 2005). These simple reflexes independently can be dissociated peripherally to block the different systems associated with the response. For example, the profound bradycardia of diving induced via activation of a vagally mediated parasympathetic circuit can be eliminated with systemic administration of atropine while the sympathetic and respiratory responses persist (Lin, 1974; Nakamura and Hayashida, 1992; Yavari et al., 1996; McCulloch et al., 1997). Also, the selective peripheral vasoconstriction seen in diving can be blocked with sympatholytics while the bradycardia and apnea is maintained (Lin, 1974; Yavari et al., 1996).

These simple reflexes are not dependent on suprabulbar neurons since the diving response is still elicited in mammals after transecting the brainstem through the thalamus (Drummond and Jones, 1979), colliculi (Huxley, 1913; Martner et al., 1977; Panneton et al., 2010b), or pons (Panneton et al., 2012). Thus we speculate that the circuits driving these behaviors are intrinsic to the medulla and spinal cord, but may be modulated by higher order neurons. Indeed, it has been suggested that these reflexes can be modulated by suprabulbar neurons (Blix, 1988). Seals often show either little bradycardia when diving voluntarily (Kooyman and Campbell, 1972) or may reduce heart rate (HR) in anticipation of underwater submersion (Casson and Ronald, 1975), suggesting that suprabulbar influences may indeed impinge on these medullary reflex circuits. Similarly, the hemodynamic responses to "forced" submersions when mammals are involuntarily "dunked" underwater are subtly dissimilar to the hemodynamics of voluntary diving (Drummond and Jones, 1979; Kooyman, 1989; McCulloch and Jones, 1990; Jobsis et al., 2001; Panneton et al., 2010b).

Eliciting these reflexes of the diving response apparently is dependent on innervation of the nares, since either submersion or wetting the snout induces the diving response in muskrats (Koppányi and Dooley, 1929; Drummond and Jones, 1979), rats (Lin,

1974; Panneton et al., 2010b), cats (Martner et al., 1977), pigs (Schagatay and Van Kampen, 1995), and rabbits, sheep, and lambs (Tchobroutsky et al., 1969), while numbing either the nares or the nasal mucosa inhibits these responses (Dykes, 1974; Drummond and Jones, 1979; McCulloch et al., 1995; Yavari et al., 1996; Kratschmer, 2001). In this regard we investigated the anterior ethmoidal nerve (AEN), since its receptive fields surround the nares and innervate anterior parts of the nasal mucosa (see Panneton et al., 2006, for review). This nerve projects densely into areas of the medullary dorsal horn (MDH; Panneton, 1991a; Panneton et al., 2006) where both neurons are activated (McCulloch and Panneton, 1997; McCulloch, 2005) and the bradycardia and apneic responses due to nasal stimulation are inhibited (Panneton, 1991b; Panneton and Yavari, 1995). Neuroanatomical tracing techniques show that neurons in similar parts of the MDH project to numerous nuclei in the brainstem (Panneton et al., 2000, 2006), but these tracing techniques provide no information whether these nuclei are activated by underwater diving.

The present investigation expands those of others studying the distribution of neurons labeled with the immediate early gene Fos activated either by nasal stimulation (Anton et al., 1991; Gieroba et al., 1994; Dutschmann and Herbert, 1997; McCulloch and Panneton, 1997; Dutschmann et al., 1998; Rybka and McCulloch, 2006) or by underwater diving of awake rats (McCulloch and Panneton, 2003; McCulloch, 2005). However, the nasal mucosa either was stimulated with irritating vapors or underwater submergence between 12 and 120 times over periods up to 2 h in all of these studies; excessive stimulation is known to induce spurious labeling in the Fos technique (Bullitt et al., 1992) yet a single stimulus trial can still activate neurons (Panneton et al., 2010a). In addition, the experimental animals were anesthetized in most of these reports using nasal stimulation, and anesthesia itself induces much confounding activation of neurons (Takayama et al., 1994).

Hemodynamic data in the present study are obtained from a single voluntary trial of either swimming or submersion in awake, previously trained rats instrumented with telemetric transmitters. The cardiorespiratory responses either to diving or swimming are reported herein, as well as the quantity of neurons c-Fos-labeled in various brainstem nuclei. These experiments make progress toward our long-term goal to establish the neural circuits in the brainstem driving the reflexes comprising the diving response. Much of this data has been presented previously in abstract from Panneton et al. (2009).

MATERIALS AND METHODS

CARDIOVASCULAR DATA

Six initially immature (70–90 g) and 10 adult (∼275–325 g) Sprague-Dawley male rats were obtained commercially (Harlan, Indianapolis, IN, USA) and used in this study. All protocols were approved by the Animal Care Committee of Saint Louis University and followed the guidelines of the National Institutes of Health Guide for Care and Handling of Laboratory Animals.

Six immature rats were trained 5 days/week for 5–6 weeks and learned to swim or dive underwater through a maze (McCulloch and Panneton, 2003; Panneton et al., 2010b). Six mature rats were trained for about 2 weeks only to swim through the maze. Once these rats reached 270–290 g they were anesthetized with ketamine/xylazine (60/40 mg/kg; IP) and the catheter of a biotelemetric transmitter (Model PA-C40; Data Sciences International, DSI; St. Paul, MN, USA) inserted into their femoral arteries while the transmitter itself was implanted in their abdominal cavities. The rats healed for 5–7 days without training, but they did not forget their willingness either to swim or submerge underwater. Cardiovascular data were obtained from these rats prior to their water tasks; this served as control data. The trained rats voluntarily either swam or dove underwater and hemodynamic data were recorded for a single trial.

The transmitter's broadcast was received with a radio receiver (Model RLA3000; DSI), relayed to a Calibrated Pressure Analog Adaptor (Model R11CPA; DSI), and transferred through an A–D interface (1401 plus; Cambridge Electronic Design, CED; Cambridge, UK), stored in the computer, and analyzed using Spike 2 software (CED). Systolic, diastolic, and mean arterial blood pressure (MABP) were calculated from traces and HR was determined by counting peaks of systolic pressure. We assumed the rats made no attempt to breathe while underwater since they showed no difficulty in breathing after their experience and none drowned during submergence.

NEUROANATOMICAL DATA

Four untrained adult rats remained isolated in their home cage prior to perfusion and served as controls for the Fos counts. These rats, and all of the experimental rats 2 h after diving or swimming, were deeply anesthetized (Sleepaway, 0.1 ml/100 g; IP) and perfused through the heart with a peristaltic pump first with a saline–procaine solution, followed immediately by a fixative of 4% paraformaldehyde and 3% sucrose in 0.1 M sodium phosphate buffer (PB; pH 7.3). Brains and spinal cords were removed and refrigerated in the fixative with 20% sucrose at 4°C. The brains were blocked in the transverse plane using a precision brain slicer prior to cutting frozen transverse sections (40 μm) with a microtome.

Every third section was processed immunohistochemically overnight with antibodies against Fos (rabbit polyclonal IgG for *c-fos* p62; 1:20,000; Santa Cruz Biotechnology, Inc.) mixed in 0.1 M PB with 0.3% Triton. On the following day, the sections were washed, incubated for 1 h in goat anti-rabbit biotinylated secondary IgG (1:500; Vector Labs), washed again, and then incubated in an ABC complex (Vectastain Elite; Vector Labs) for another hour. The Fos antigen was visualized in the brainstem with the chromogen diaminobenzidine (DAB) enhanced with nickel ammonium sulfate. Sections were mounted serially on gelatin-coated slides, counter-stained with Neutral Red, dehydrated in alcohol, defatted in xylene, and coverslipped with Permount. Fos-positive neurons appeared as cells with black-labeled nuclei and were visualized with bright field optics (Nikon E800).

Fos-positive neurons in 12 nuclei/subnuclei of the brainstem were photographed digitally (MicroImager II) with Northern Eclipse Software (Empix, Inc.). Six to eight samples from sections on either side were chosen through the nucleus in all cases except for the midline commissural subnucleus of the nucleus tractus solitarii (NTS) and raphe pallidus (RPa), where three sections were chosen/case, respectively. Sections photographed bilaterally through the caudal pressor area (CPA; six photomicrographs) were at levels of, and immediately adjacent to, the caudal pole

of the lateral reticular nucleus as defined previously (Sun and Panneton, 2002, 2005). Those through the commissural subnucleus of the NTS (comNTS; three photomicrographs) were taken of both sides from three sections immediately caudal to the calamus scriptorius while those for the dorsal lateral (dlNTS; eight photomicrographs) and medial (medNTS; eight photomicrographs) subnuclei of the NTS were photographed immediately caudal to the obex, overlapping the area postrema. The MDH was photographed bilaterally in the four sections (eight photomicrographs) rostral to the calamus scriptorius, with rostral sections overlapping the caudal pole of the subnucleus interpolaris. The nucleus RPa (three photomicrographs) was analyzed just rostral to the rostral pole of the inferior olivary complex, and coincided with the caudal pole of the facial motor nucleus. The rostroventrolateral medulla (RVLM; eight photomicrographs) was photographed bilaterally in four sections immediately caudal to the facial motor nucleus, the A5 area bilaterally (six photomicrographs) when juxtaposed medially to the emerging roots of the facial nerve, and the locus coeruleus (LC; six photomicrographs) in its entirety bilaterally. The Kölliker–Fuse area (KF; six photomicrographs), and external lateral (PBel; six photomicrographs) and superior lateral (PBsl; six photomicrographs) subnuclei of the peribrachial complex were photographed bilaterally in their entirety.

Nuclear areas were outlined in Northern Eclipse software; representative outlines are shown in **Figure 7**. Neurons were considered Fos-positive if visualized microscopically with nuclei containing a black immunoprecipitate, but the nuclei were stained to various degrees making consistent counts problematic. Thus, labeled profiles were discriminated and quantified with a threshold function of the Northern Eclipse software (**Figure 1**). Similar parameters (threshold color: red range 0, 0; green range 8, 49; blue range 14, 67) were maintained for analyses to prevent biased cell counts. This procedure negated counts of lighter, presumably less optimally labeled neurons (Bullitt et al., 1992) and presumably eliminated investigator bias. Sections were drawn with a Nikon E600 microscope and Neurolucida software (MicroBrightField, Inc.). The photomicrographs were standardized using levels, brightness and contrast in Adobe Photoshop software (v.7) and aligned in Adobe Illustrator software (v.11) for figures. All nomenclature and abbreviations are from a stereotaxic rat atlas (Paxinos and Watson, 1998) except for designations of some subnuclei.

DATA ANALYSIS

Means and SEs ($M \pm \text{SE}$) were determined for experimental and control groups. HR and MABP during swimming and diving were compared to data taken just prior to submersion in all experimental rats and compared for significance (SPSS software; v. 13) using the Independent Samples T-test. Counts of discriminated data points of swimming and diving groups were compared also using the Independent Samples T-test. Data are presented as $M \pm \text{SE}$ and significance was calculated as $p < 0.05$.

RESULTS

CARDIOVASCULAR DATA

The diving rats voluntarily dove underwater and traversed the maze in an average of 16 s while swimming rats averaged 20 s. All voluntary diving rats ($n = 6$) showed a marked drop in HR and an increase in MABP to diving (**Figure 2**), but no changes were seen during swimming ($n = 6$). HR dropped significantly ($p < 0.001$) from 434 ± 11 to 101 ± 7 bpm, a bradycardia of 77%, during underwater submersion (**Figure 3**) while MABP rose significantly ($p < 0.05$), rising from 112 ± 3 to 130 ± 2 mmHg, a 14% rise (**Figure 3**).

NEUROANATOMICAL DATA

Fos labeling

Neurons labeled with Fos were visualized throughout the brainstem after swimming and underwater diving (**Figure 4**). Some areas were labeled sparsely in all rats, even control animals which just sat in their cages, and these areas were not analyzed. These nuclei included sporadic labeling in the parvocellular lateral reticular nucleus, the basal pontine nuclei, dorsal parts of the ventral cochlear nucleus, the dorsal tegmental nucleus, the central gray (pars alpha), neurons surrounding the trigeminal motor root, and a few close to the paracochlear glial substance. It appeared qualitatively that Fos-labeled neurons in some of these areas were increased subtly in diving rats over control and swimming rats in the parvocellular lateral reticular nucleus, near the trigeminal motor root, and the paracochlear glial substance, but these areas were not quantified. In addition, diving rats showed inconsistent, sporadic labeling in laminae III–IV of the MDH, along the ventral surface of the medulla, the lateral medulla, and the superior salivatory nucleus.

Neurons in 12 brainstem nuclei/subnuclei in control ($n = 4$), swimming ($n = 5$), and diving ($n = 6$) groups were then counted

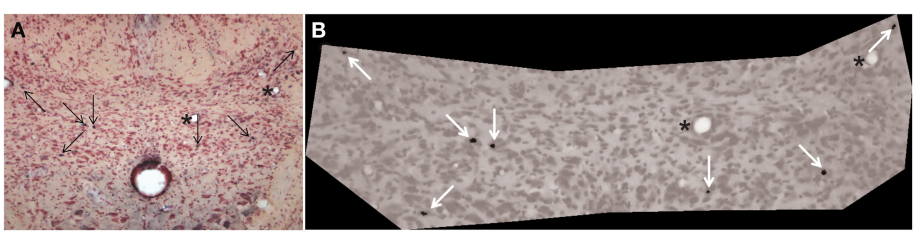

FIGURE 1 | Photomicrographs illustrating threshold determination for quantization of neurons counted as Fos-positive. The comNTS in **(A)** showed seven neurons with black precipitate in their nuclei (arrows). All seven of these neurons seen visually were counted after software discrimination [**(B)** white arrows] in this case, but this did not always happen. Neurons sometimes were excluded if they were too lightly stained or too small (<11 pixels). Asterisks show similar blood vessels in either photomicrograph.

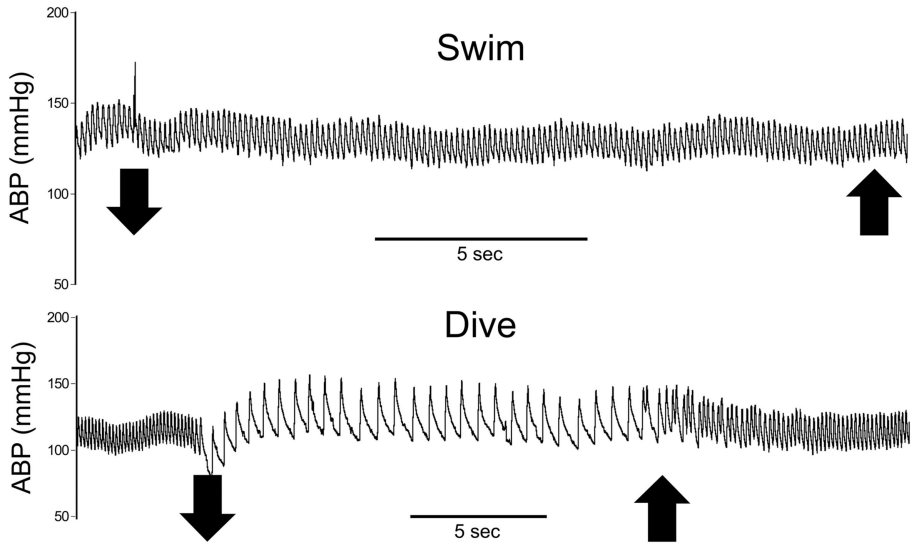

FIGURE 2 | Traces of representative cardiovascular responses of rats to either swimming on the surface or underwater diving. Note the marked bradycardia and slight increase in arterial blood pressure while rats were underwater but no change while rats swam on the surface. Large down arrows indicate when the rat entered or submerged underwater while large up arrows indicate emersion from the water. ABP, arterial blood pressure; HR, heart rate.

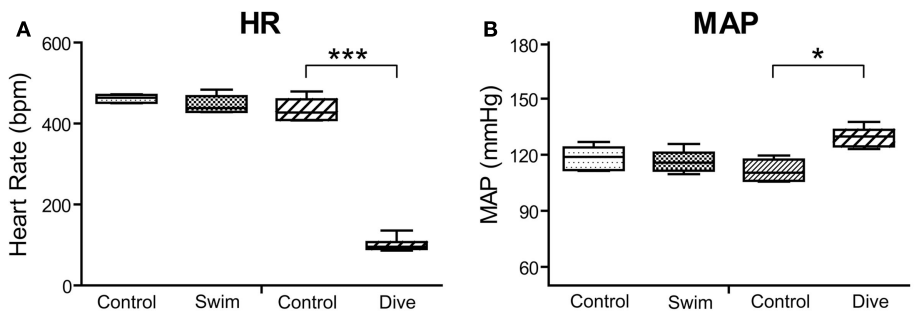

FIGURE 3 | Box plots illustrating changes in heart rate (A) and MAP (B) in rats to either swimming or underwater diving. Note that only diving behavior induced significant bradycardia and increases in mean arterial blood pressure. $***p < 0.001$; $*p < 0.05$.

after they met threshold functions of the computer (**Table 1**). These nuclei were selected since they were considered important either in the neural circuitry of the diving response or have been implicated in cardiorespiratory control. These nuclei included the comNTS, dlNTS (note: the dorsomedial subnucleus of Paxinos and Watson, 1998), and medNTS (note: the central subnucleus of Paxinos and Watson, 1998) subnuclei of the NTS, the CPA, the MDH, the RVLM, the RPa, the A5 area, the LC, the KF, and the PBel and PBsl subnuclei of the parabrachial nucleus.

Data points discriminated from all these nuclei of control rats were significantly less than those discriminated for diving rats. However, when comparing discriminated data points between swimming and diving rats (**Table 1**), those in the commNTS, the RPa, and the A5 group were not significantly different (**Figure 5A**). Discriminated data points in the dlNTS, MDH, CPA, and RVLM however were significantly different to $p < 0.05$ between swimmers and divers (**Table 1**; **Figures 5B–F** and **6**), to $p < 0.01$ in the medNTS, LC, PBel, and PBsl (**Figures 5G–L** and **6I,K,L**), and to $p < 0.001$ in the KF (**Figure 6J**).

DISCUSSION

Activation of brainstem neurons of rats were compared after three qualitatively different behaviors, i.e., resting in a cage, surface swimming through a maze, and navigating the same maze while underwater. We show that the cardiovascular parameters of HR and MABP were significantly different after exercise in diving rats versus swimming rats. This study also shows for the first time activation of brainstem neurons of rats after either swimming or diving behaviors. Moreover, significantly more profiles immunoreactive to Fos antigen were found after diving in several brainstem nuclei known to be important in cardiorespiratory behavior.

Since the independent parasympathetic, sympathetic, and respiratory components of the diving response apparently are all

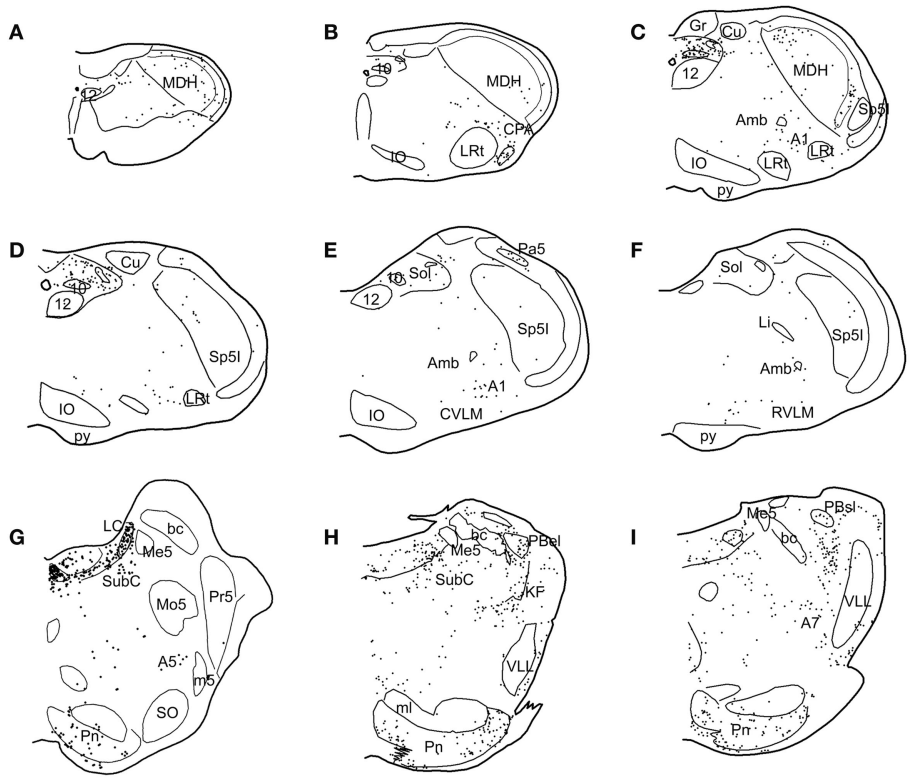

FIGURE 4 | Line drawings illustrating the distribution of neurons immunolabeled with Fos in the brainstem after underwater diving. Each dot represents a single, non-thresholded neuron observable through the microscope. Immunolabeling for Fos was discriminated by computer in twelve nuclei/subnuclei through the medulla **(A–F)** and pons **(G–I)** from caudal **(A)** to rostral **(I)** and quantified; the results are seen in **Table 1**. Abbreviations: A1, A5, A7, catecholamine groups; Amb, nucleus ambiguus; Cu, cuneate nucleus; CVLM, caudal ventrolateral medulla; DT, dorsal tegmental nucleus; Gr, gracile nucleus; IO, inferior olivary nucleus; KF, Kölliker–Fuse nucleus; LC, nucleus locus coeruleus; LI, nucleus linearis; LRt, lateral reticular nucleus; MDH, medullary dorsal horn; Me5, mesencephalic trigeminal nucleus; Mo5, motor trigeminal nucleus; Pa5, paratrigeminal nucleus; PBel, external lateral subnucleus of the peribrachial complex; PBsl, superior lateral subnucleus of the peribrachial complex; Pn, pontine nucleus; Pr5, principal sensory trigeminal nucleus; SO, superior olivary nucleus; Sol, nucleus tractus solitarii; Sp5I, Spinal 5 nucleus–subnucleus interpolaris; SubC, nucleus subcoeruleus; VLL, ventral nucleus of lateral lemniscus; 10, dorsal nucleus of the vagus nerve; 12, hypoglossal nucleus; bc, brachium conjunctivum; m5, motor root of the trigeminal nerve; py, pyramidal tract; 7n, facial nerve.

Table 1 | Counts of discriminated data points after three behaviors.

	CPA	Raphe	RVLM	comNTS	dlNTS	medNTS	MDH
Control	0.67 (0.30)	0.25 (0.16)	0.53 (0.18)	1.44 (0.92)	2.50 (0.74)	2.31 (1.15)	0.72 (0.28)
Swim	1.63 (0.40)	2.80 (0.67)	1.63 (0.37)	1.40 (0.34)	1.39 (0.38)	0.46 (0.11)	0.45 (0.33)
Diver	5.44 (5.44)	4.11 (0.79)	5.03 (1.02)	13.04 (4.56)	10.04 (2.42)	20.08 (3.40)	1.98 (0.49)

	A5	KF	Pbexl	LC	PBsl
Control	1.37 (0.50)	5.54 (1.86)	1.33 (0.90)	19.83 (10.05)	3.33 (1.99)
Swim	2.77 (0.55)	0.97 (0.97)	0.49 (0.27)	1.57 (0.38)	2.23 (0.56)
Diver	3.67 (0.67)	17.14 (1.71)	35.75 (7.91)	39.14 (8.07)	9.39 (1.84)

Counts of data points after thresholding of neurons immunolabeled with c-Fos in various brainstem nuclei in resting rats (control), swimming rats, and diving rats. Means (SE).

activated by the same peripheral stimulation, e.g., underwater submersion, the term diving response has replaced the former term the diving reflex. Nevertheless, these independent autonomic reflexes of diving still must have a peripheral sensory neuron, usually one or several central interneurons, and selective motoneurons to drive the bradycardia, peripheral vasoconstriction, and expiratory apnea. The neural circuitry driving these responses is intrinsic to the brainstem since the responses persist in neurally

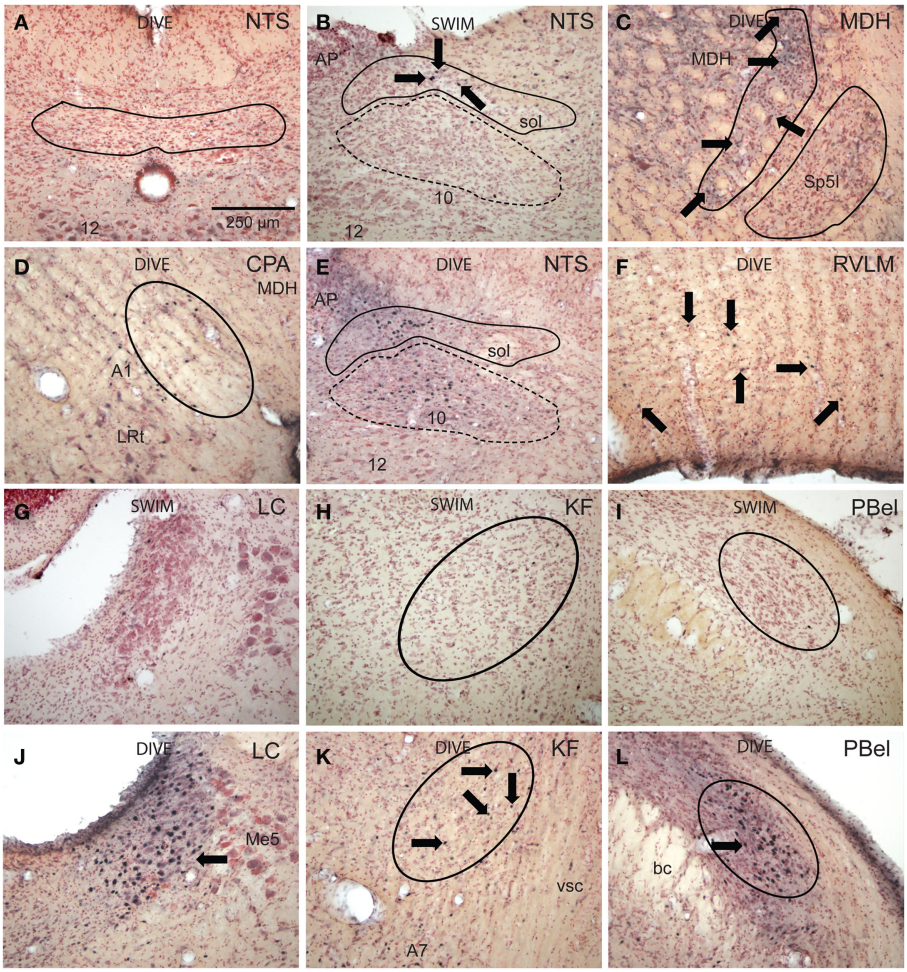

FIGURE 5 | Bright field photomicrographs of sections showing immunolabeling of Fos in selected brainstem nuclei. Diving behavior activated few neurons in subnucleus commissuralis of the nucleus tractus solitarii [**(A)** NTS, outline], but numerous neurons near the levels of the obex in its dorsolateral [**(B,E)** solid outline] and medial subnuclei [**(B,E)** dashed outline] compared to swimming rats. Voluntary diving behavior in rats induced relatively few, but significant numbers of Fos-labeled neurons in the medullary dorsal horn [**(C)** MDH, projection field of the anterior ethmoidal nerve is outlined] over swimming rats. Note these small labeled neurons (arrows) are in the substantia gelatinosa, presumably lamina II, displaced dorsally and medially by the caudal pole of the subnucleus interpolaris (Sp5I, outlined). Neurons labeled with Fos were noted in the caudal pressure area [**(D)** CPA, circled] as well as presumptive noradrenergic neurons in the juxtaposed A1 area after diving. There was a significant increase of Fos labeling (arrows) in the RVLM after diving **(F)** compared to swimming behavior. Little label was seen in the locus coeruleus **(G)**, Kölliker–Fuse area **(H)**, or external lateral subnuclei **(I)** of the parabrachial complex after swimming, but these areas were significantly labeled after diving behavior [**(J,K,L)** respectively]. Arrows point to Fos-labeled neurons; all figures at the same magnification.

truncated mammals (Martner et al., 1977; Panneton et al., 2010b, 2012). Thus the pathways comprising the diving response are like many other reflexes with complete circuits within the spinal cord or brainstem. It has been a goal of our laboratory to establish the brainstem circuitry utilized by the three reflexes comprising the diving response. The present report adds to the considerable progress toward this goal.

TECHNICAL CONSIDERATIONS

The Fos technique potentially can identify the sensory, intermediate, and motor limbs of a reflex pathway, but this technique has limitations. The synthesis of Fos protein generally follows the excitation of neurons. However, Fos protein generation may be masked in continuously firing neurons (Dragunow and Faull, 1989). Also, neurons which are inhibited in a reflex circuit will not generate Fos protein, while other neurons may not express Fos regardless of the stimulus used (Dragunow and Faull, 1989). In a previous study utilizing the Fos technique in the muskrat (McCulloch and Panneton, 1997), we could not determine significance in most nuclei from control animals, perhaps since these animals were anesthetized and had experienced prior surgery, both of which greatly cloud interpretation of data (Takayama et al., 1994; Hoskin and Goadsby, 1999). These animals also were subjected to excessive stimulation, which can activate confounding neurons (Bullitt et al., 1992). These caveats were not problematic in the present study, however, since all nuclei quantified in our awake behaving

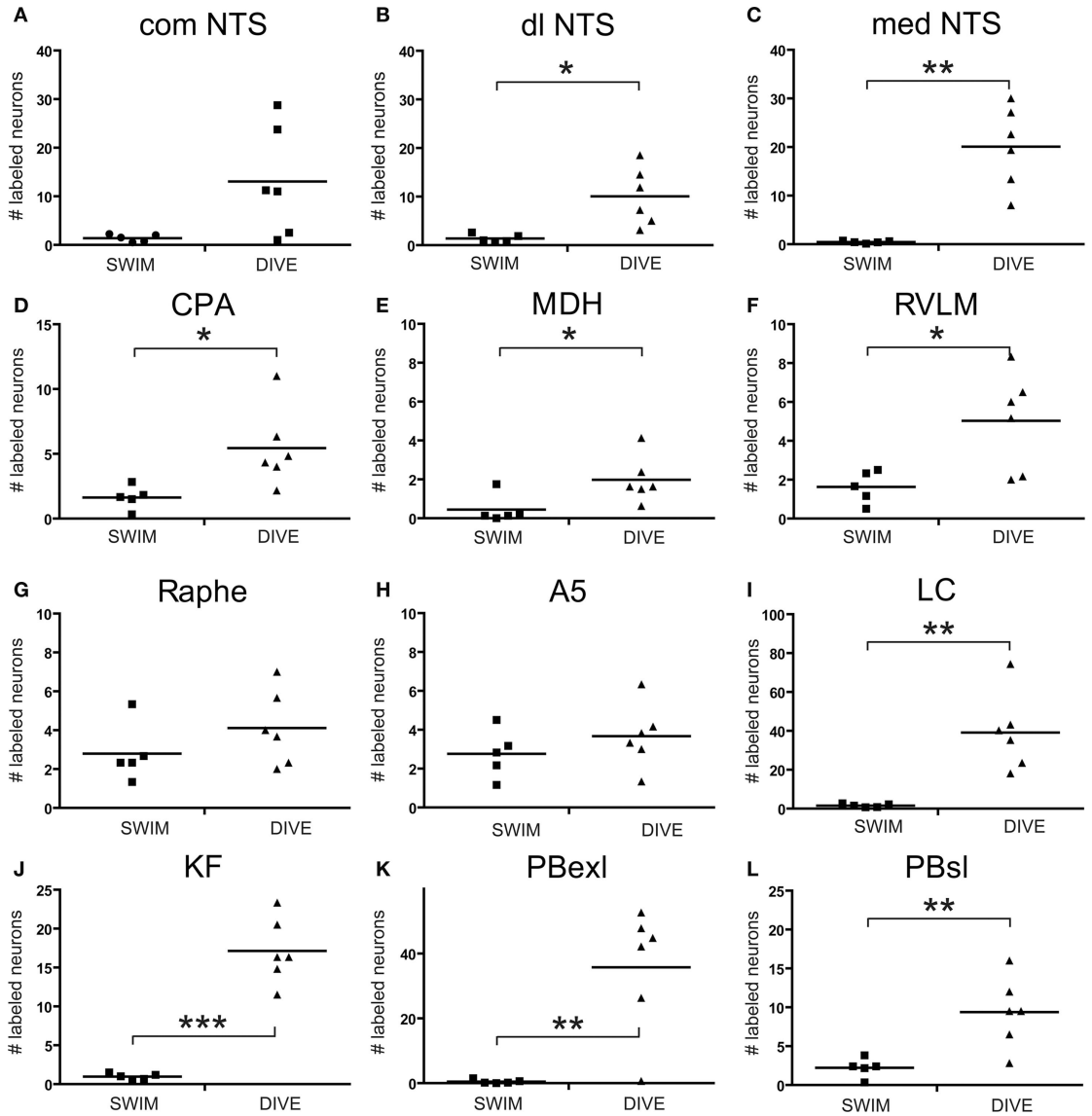

FIGURE 6 | Scatter plots comparing the number of discriminated data points determined with thresholding in rats after swimming behavior versus those that voluntarily dove underwater. Data points were discriminated from photomicrographs with software and represent neurons labeled with Fos which met color, intensity and size determinants. Note that there was a significant increase after underwater submersion of discriminated data points in all nuclei but the comNTS, raphe, and A5 area. Square symbols mark data from individual swimming rats while triangles mark that from individual diving rats. Horizontal bars represent the mean. Independent Samples *T*-test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

animals showed significant increases in labeling when voluntary diving rats were compared to control values. Moreover, significant differences were determined in several brainstem areas when data points from nuclei immunolabeled after swimming behavior were compared to diving behavior.

Quantifying neurons labeled with Fos is difficult since not all activated nuclei are stained equally in intensity, making counting lightly stained nuclei subjective. It is unknown if faint staining is due either to their relative activation or to differences in immunohistochemical processing. Thus Fos-labeled neurons in the present study were discriminated by computer similar to Graham et al. (1995) in an effort to reduce this bias. An example of such discrimination is provided (**Figure 1**) of a section through the commissural subnucleus of the NTS of a diving rat showing a typical analysis. Note that all labeled nuclei in this section (**Figure 1A**; arrows) were discriminated and counted by the computer (**Figure 1B**; arrows).

CARDIOVASCULAR DATA

There was no change in hemodynamics during swimming behavior in the present study, confirming data of others (Baker and Horvath, 1964; Whishaw and Schallert, 1977; McCulloch et al., 2010). The hemodynamic adjustments seen in the present study confirm our previous report in voluntary diving rats (Panneton et al., 2010b), but the increase in MABP was considerably less than that seen in another study (McCulloch et al., 2010). Numerous

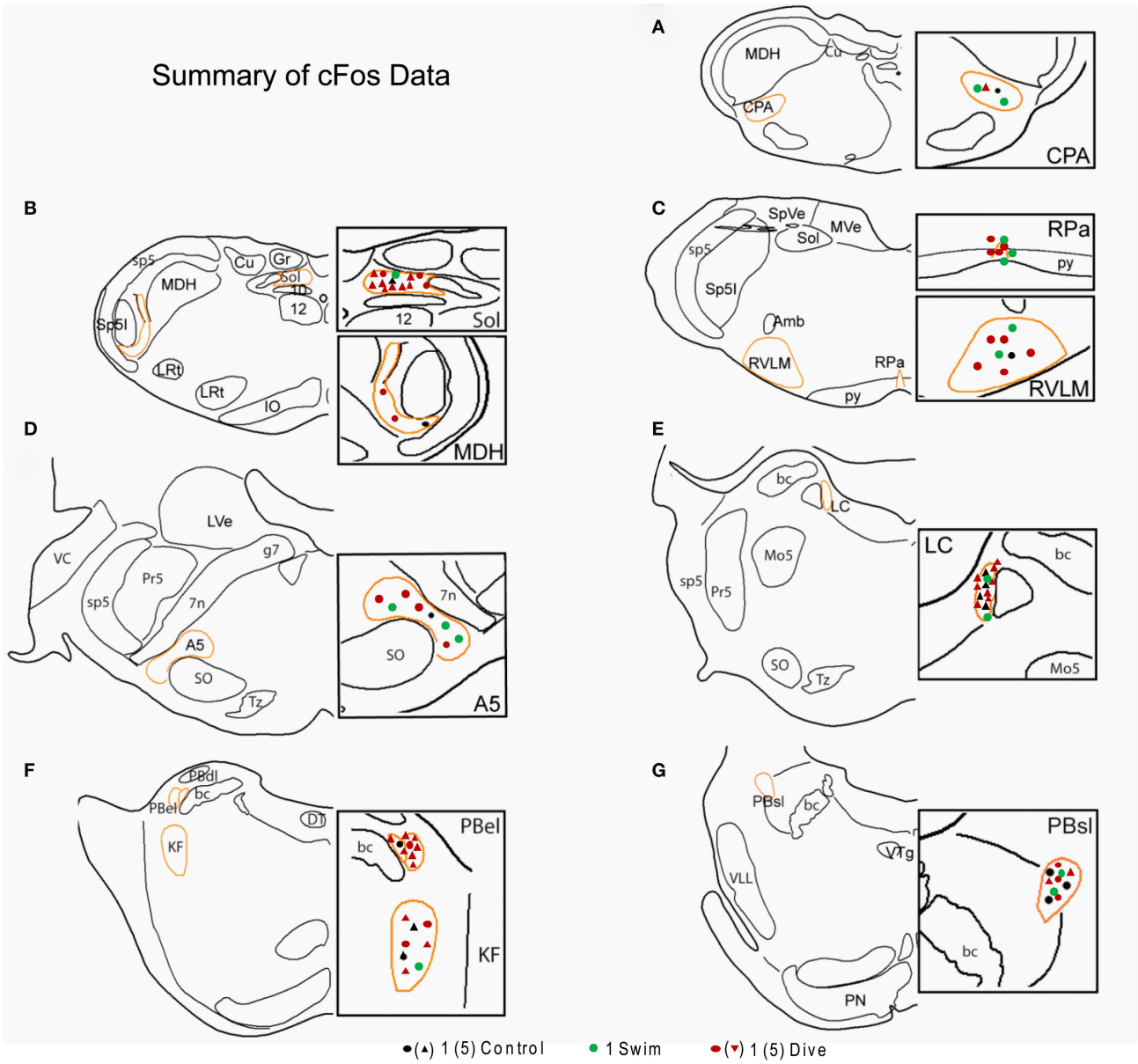

FIGURE 7 | Line drawings comparing the mean number of data points in brainstem nuclei in control, swimming, and diving rats. Sections are drawn from the caudal medulla **(A)** thru the rostral pons **(G)**. Note that underwater diving increased the number of data points in all nuclei compared to control or swimming animals. Areas circumscribed in orange outlines the area demarcated for threshold determination; these areas are magnified in the boxes on the right of the drawing. Each dot represents a single neuron while triangles represent five neurons. See **Figure 4** for abbreviations.

reports on diving mammals have shown similar discrepancies (Elsner and Gooden, 1983; de Burgh Daly, 1984; Butler and Jones, 1997) and may be related to the balance between the initiation of the parasympathetically mediated bradycardia and sympathetically mediated vasoconstriction. It also should be noted five trials/animal were used in our previous study (Panneton et al., 2010b) while only a single trial/animal was used in the present study. While there is no standard for activating Fos in neurons, we feel fewer stimulations induce less confounding data points as well as minimize stress in awake behaving animals.

THE NUCLEUS TRACTUS SOLITARII

The NTS, the primary relay for visceral afferent fibers, may contain interneurons of the reflex circuits driving the diving response. While the three subnuclei of the NTS quantified herein were labeled significantly with Fos in the diving versus control rats, only the dlNTS and medNTS were significantly different when compared to swimming rats. The comNTS receives numerous primary afferent fibers from neurons monitoring blood gases and is especially sensitive to hypoxia in peripheral blood (see Panneton et al., 2010b, for review). Apparently the short swimming and

diving behaviors utilized in the present study were not sufficient to change partial gas pressures enough to activate many comNTS neurons. However 4/6 diving rats showed much higher activation in these neurons (**Figure 6A**), perhaps an introduction to the massive immunolabeling of comNTS seen when rats are submerged beyond their aerobic dive limit (Panneton et al., 2010a).

Most labeled neurons were found in the dlNTS and medNTS and both are implicated in cardiovascular control. The dlNTS receives numerous primary afferent fibers from the carotid sinus and aortic depressor nerves (Panneton and Loewy, 1980; Housley et al., 1987; Chan and Sawchenko, 1998; Blessing et al., 1999) and contains neurons responsive to hypertensive stimuli detected neurophysiologically (Donoghue et al., 1978, 1984) as well as by activation of Fos (Chan and Sawchenko, 1994, 1998; Graham et al., 1995). Although there are but few catecholamine neurons in the dlNTS, those present are rarely double-labeled after increases of arterial blood pressure (Chan and Sawchenko, 1994, 1998). Thus these catecholamine neurons are probably not influenced by hypertensive stimuli. However, it is well known that injections of kynurenate into the NTS, which pharmacologically antagonizes excitatory amino acid receptors non-selectively, blocks the bradycardia and decrease in arterial blood pressure induced by activation of the baroreceptor reflex (Guyenet et al., 1987; Leone and Gordon, 1989); the bradycardia is mediated by both NMDA and non-NMDA receptors (Zhang and Mifflin, 1993, 1995; Frigero et al., 2000; Machado et al., 2000). Nevertheless, injections of kynurenate into dlNTS failed to block the increase in arterial blood pressure or sympathetic nerve activity induced by nasal stimulation (McCulloch et al., 1999b). Indeed, studies have shown that the baroreceptor reflex is inhibited by nasal stimulation (McCulloch et al., 1999b) and the pressor responses to nasal stimulation persist despite cutting baroreceptor nerves (Nakamura and Hayashida, 1992), suggesting the pressor response to diving may not utilize this reflex pathway during diving. Moreover, it has been shown that the cardiorespiratory components of other trigeminoautonomic reflexes also are relayed through areas other than the NTS (Kumada et al., 1977; Allen and Pronych, 1997). These data collectively argue that NTS neurons are not part of the circuit of neurons integral to the diving reflex. However, the projections originating in the ventral MDH to the dlNTS (Panneton et al., 2000, 2006) potentially could modulate these barosensitive neurons. Thus, we speculate that the significant Fos labeling in the dlNTS seen in the diving rats versus either control or swimmers possibly was due to activation of non-aminergic barosensitive neurons responding to the increase in arterial blood pressure seen with underwater submergence, but these neurons are not part of the circuit of neurons driving the diving response.

Neurons in the medNTS were significantly labeled with Fos in the diving rats and may be involved in both baro- and chemoreceptor reflexes. These neurons receive primary afferent fibers from the carotid body, carotid sinus, and aortic depressor nerves (Panneton and Loewy, 1980; Finley and Katz, 1992; Chan and Sawchenko, 1998; Blessing et al., 1999), respond electrophysiologically to chemoreceptor stimuli (Donoghue et al., 1978), and are robustly activated by hypotensive stimuli (Chan and Sawchenko, 1994, 1998; Graham et al., 1995; Dampney and Horiuchi, 2003). Moreover, blocking studies using microinjections of cobalt chloride suggest the increase in arterial blood pressure induced by electrical stimulation of the AEN is mediated by neurons in the medNTS (Dutschmann and Herbert, 1998). The medNTS contains the A2 catecholamine group and ∼70% of the A2 neurons are double-labeled with Fos in rats after underwater submersion (McCulloch and Panneton, 2003). However, few A2 neurons respond to hypertensive stimuli (Moore and Guyenet, 1985; Chan and Sawchenko, 1994; Mayne et al., 1998) and chemical depletion of amines in the NTS with 6-OHDA effects neither basal HR nor arterial pressure, nor does such depletion affect the baroreceptor reflex (Itoh et al., 1992). Nevertheless, since our diving rats experienced no hypotension, the collective data suggests medNTS is not directly part of the diving circuit *per se*, but agree with others (Rinaman, 2011) that A2 neurons modulate diverse roles, and speculate that many of them activated by diving reflects the organism's response to the acute physiological stress to underwater submersion.

THE MDH AND CPA

The MDH serves as a primary relay for sensory fibers innervating the head and may be where the first central interneuron of a medullary reflex circuit is located. Stimulation of the skin and mucosa near the nares has been shown to initiate the diving response (Koppányi and Dooley, 1929; Tchobroutsky et al., 1969; Lin, 1974; Martner et al., 1977; Drummond and Jones, 1979; Schagatay and Van Kampen, 1995), an area innervated in part by the AEN (Williams and Warwick, 1980). Moreover, stimulating the AEN electrically induces a bradycardia, an increase in MABP and apnea, responses similar to those of diving (Dutschmann and Herbert, 1997; McCulloch et al., 1999a), and is important for the bradycardia and apnea during stimulation of the nasal mucosa (Nakamura and Hayashida, 1992; Rybka and McCulloch, 2006). Primary afferent fibers carried in the AEN of the rat project to areas of the MDH (Panneton et al., 2006) where discriminated data points representing Fos-labeled neurons were found in the present study, confirming data by others (Rybka and McCulloch, 2006; Panneton et al., 2010a). When similar areas in the MDH are blocked by injections of lidocaine or kynurenate (Panneton, 1991b; Panneton and Yavari, 1995), or after the AEN is transected (Rybka and McCulloch, 2006), the bradycardic and apneic responses to either nasal or diving stimulation are inhibited. Although the data points in the MDH of diving rats were significantly higher than those of swimmers, there were relatively few neurons immunolabeled after our single trial. The reason for this is unknown, but suggests that these neurons invoke a powerful influence over cardiorespiratory behavior with little modulation.

A significant number of CPA neurons were activated and immunolabeled with Fos after underwater submersion compared to control and swimming rats in the present study. Neurons in and around the CPA have been implicated in cardiovascular regulation, in the processing of noxious information, as well as important for the exercise pressure response (Sun and Panneton, 2002, 2005; Panneton et al., 2008, 2011; Marques-Lopes et al., 2009; Takakura et al., 2011a,b). Although we have shown previously that the area of the MDH activated by voluntary diving projects to the CPA (Panneton et al., 2006), it plays no role in the cardiorespiratory adjustments seen with nasal stimulation (Panneton et al., 2008). Thus the role played by neurons in the CPA, whether integration of sensory pathways or in autonomic behavior, must still be determined.

THE RPa AND RVLM

The experimental animals in this study voluntarily dove underwater for approximately 15–20 s, and were apneic during this time. This may have induced activation of central chemoreceptors, which adjust ventilation to meet metabolic needs. Neurons in the nucleus RPa abut branches of the basilar artery and are speculated by some to be chemoreceptors monitoring ventilation (Richerson, 2004). However, injections of kynurenate into the RPa had no effect on resting arterial blood pressure, sympathetic outflow, or the responses to activation of a nasotrigeminal reflex (McCulloch et al., 1999b).

It is well known that the RVLM is an important brainstem locus for controlling arterial blood pressure. Neurons in the RVLM are *activated* when cardiorespiratory responses similar to diving are induced by nasal stimulation, despite the increases in arterial pressure (McCulloch et al., 1999b); this contrasts their silence after similar increases in arterial blood pressure when the baroreceptor reflex is activated. Indeed, present data shows a significant increase in data points in the RVLM of diving rats when compared to both control and swimming animals. We suspect this activation induces peripheral vasoconstriction via the sympathetic system in non-essential vascular beds (see Nakamura and Hayashida, 1992), similar to that seen in aquatic species. Many RVLM neurons contain catecholamines of the C1 group, specifically adrenaline, but only 29% of C1 neurons were double-labeled with Fos after diving underwater (McCulloch and Panneton, 2003). This percent is comparable to that seen in animals with induced hypertension (Chan and Sawchenko, 1994, 1998; Erickson and Millhorn, 1994; Dampney et al., 2003) but differs dramatically from that seen in hypotensive states, where more aminergic neurons are labeled. Indeed, recent reports document separate outflows from both C1 and non-aminergic RVLM neurons to the spinal cord and activation of sympathetic outflow (Burke et al., 2011). Thus we suggest that most of the Fos-labeled RVLM neurons of diving rats are non-aminergic, and may be the faster conducting barosensitive bulbospinal RVLM neurons activated by nasal stimulation (see McCulloch et al., 1999b, for discussion).

PONTINE NUCLEI

The A5 area contains sympathoexcitatory neurons implicated in cardiovascular function (Loewy et al., 1979; Neil and Loewy, 1982; Byrum et al., 1984; Guyenet, 1984; Hara et al., 1997; Maiorov et al., 1999, 2000) as well as those modulating respiration (Hilaire et al., 2004; Viemari et al., 2004). The data presented herein showed the number of A5 neurons labeled with Fos after diving underwater was insignificantly different when compared to swimmers but was different than control rats. The absolute number of labeled neurons in the A5 in the present study was small, however, and reduced greatly from that seen after repeated voluntary diving stimulations reported previously (McCulloch and Panneton, 2003). However, immunolabeling of neurons after underwater diving in the LC was significantly elevated compared to control and swimming. Since neither the A5 area nor LC are activated by hypertension (Graham et al., 1995), nor is the LC a necessary component of the diving circuit (Panneton et al., 2012), their activation may be in response to the physical stress of underwater submergence.

The peribrachial complex in the dorsolateral pons, especially the PBel and PBsl subnuclei of the parabrachial nucleus and the Kölliker–Fuse nucleus, are considered important in modulation of visceral activity. The PBel, PBsl, and KF all showed significant increased labeling with Fos after underwater submersion. Lateral parabrachial neurons are involved in the baroreceptor reflex (Hayward and Felder, 1998; Saleh and Connell, 1998; Len and Chan, 2001) as well as the chemoreceptor reflex (Koshiya and Guyenet, 1994; Haibara et al., 2002). The PBel neurons induce cardiovascular changes when stimulated (Miura and Takayama, 1991; Chamberlin and Saper, 1992) and induce Fos production (Chan and Sawchenko, 1994; Potts et al., 1997; Mayne et al., 1998) when activated by changes in arterial pressure. Neurons in the lateral peribrachial complex, including the PBsl, PBel, and KF, also are important in respiratory control (Jodkowski et al., 1994; Mizusawa et al., 1995) and respiratory rates are modulated with stimulation here (Takayama and Miura, 1993; Chamberlin and Saper, 1994). Several studies have shown increased Fos labeling in the lateral parabrachial nucleus, especially the presumptive outer portion of the PBel, after changing arterial blood pressure or activating the chemoreceptor reflex (Erickson and Millhorn, 1994; Graham et al., 1995; Potts et al., 1997), but also after nasal stimulation in anesthetized animals (Dutschmann and Herbert, 1997; McCulloch and Panneton, 1997). Moreover, it has been reported that the bradycardia and apnea induced by electrical stimulation of the AEN is mediated by neurons of the Kölliker–Fuse area (Dutschmann and Herbert, 1996) and that the KF is important in trigeminoautonomic reflexes (Dutschmann and Herbert, 1998). We have shown previously that the presumptive outer portion of the PBel as well as parts of the KF area receives primary afferent fibers from the AEN (Panneton, 1991a; Panneton et al., 2006). These derelict extratrigeminal projections recently have been confirmed with staining of the PBel nucleus in genetically manipulated mice; this study (Cavanaugh et al., 2011) shows a strong TRPV1 receptor presence in the PBel, a receptor only found on primary afferent neurons. However, these pontine nuclei must not be part of the basic reflex circuit driving the diving response, since the cardiorespiratory changes induced by nasal stimulation persist after pontine transection (Panneton et al., 2012).

PERSPECTIVES

Most studies on the diving response have been done on aquatic mammals which submerge frequently. However, we (McCulloch and Panneton, 2003; Panneton et al., 2010a,b) and others (McCulloch, 2005; McCulloch et al., 2010; Fahlman et al., 2011) have shown that the diving response is brisk even in the common laboratory rat. Can the responses seen in aquatic mammals be compared legitimately to those of non-aquatic, land-bound rats? Behaviors which serve basic vegetative functions are usually less complex and more uniform across species; this offers support for studies of autonomic functions in different species. If the diving response can be considered such a basic vegetative function, it is more easily studied in a laboratory animal rather than a large aquatic mammal. The substrate for "simple" reflex behaviors are thought to be circuits located within the brainstem and the spinal cord, and we have shown the nasotrigeminal reflex IS contained in the caudal neuraxis (Panneton et al., 2012).

We have documented neurons in several brainstem nuclei which may act as interneurons in the reflex pathways integral for the parasympathetically mediated bradycardia, the sympathetically mediated vasoconstriction, and the apnea induced in the diving response. We feel our considerable effort toward studying those circuits which are the simplest, the most organized, and the most automatic is both logical and certainly worthwhile. Moreover, the cardiorespiratory responses of voluntarily diving rats are invariable at least in our hands. We already have documented the diving response inhibits basic homeostatic reflexes such as the baroreceptor and chemoreceptor reflexes; we consider the cardiorespiratory reflexes comprising the diving response collectively make it the most powerful autonomic response known. Moreover, the complexity of an animal's behavior increases according to its place in phylogeny and is paralleled by the complexity of the neural systems driving behavior. Since neurons in the brain both coordinate and control peripheral function, including those activated during diving behavior, our laboratory has taken considerable effort toward deciphering the circuits of neurons driving the changes in respiration, HR, and peripheral vasoconstriction seen with underwater submersion as first described by Scholander and Irving. Perhaps the larger diving aquatic mammals, especially those noted for their intelligence, can use their forebrains to control this simple, highly organized autonomic reflex via suprabulbar circuits. Future studies will include investigations as to how suprabulbar neurons modulate these invariable brainstem reflexes.

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Estimated tissue and blood N_2 levels and risk of decompression sickness in deep-, intermediate-, and shallow-diving toothed whales during exposure to naval sonar

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Naval sonar has been accused of causing whale stranding by a mechanism which increases formation of tissue N_2 gas bubbles. Increased tissue and blood N_2 levels, and thereby increased risk of decompression sickness (DCS), is thought to result from changes in behavior or physiological responses during diving. Previous theoretical studies have used hypothetical sonar-induced changes in both behavior and physiology to model blood and tissue N_2 tension (P_{N_2}), but this is the first attempt to estimate the changes during actual behavioral responses to sonar. We used an existing mathematical model to estimate blood and tissue N_2 tension (P_{N_2}) from dive data recorded from sperm, killer, long-finned pilot, Blainville's beaked, and Cuvier's beaked whales before and during exposure to Low- (1–2 kHz) and Mid- (2–7 kHz) frequency active sonar. Our objectives were: (1) to determine if differences in dive behavior affects risk of bubble formation, and if (2) behavioral- or (3) physiological responses to sonar are plausible risk factors. Our results suggest that all species have natural high N_2 levels, with deep diving generally resulting in higher end-dive P_{N_2} as compared with shallow diving. Sonar exposure caused some changes in dive behavior in both killer whales, pilot whales and beaked whales, but this did not lead to any increased risk of DCS. However, in three of eight exposure session with sperm whales, the animal changed to shallower diving, and in all these cases this seem to result in an increased risk of DCS, although risk was still within the normal risk range of this species. When a hypothetical removal of the normal dive response (bradycardia and peripheral vasoconstriction), was added to the behavioral response during model simulations, this led to an increased variance in the estimated end-dive N_2 levels, but no consistent change of risk. In conclusion, we cannot rule out the possibility that a combination of behavioral and physiological responses to sonar have the potential to alter the blood and tissue end-dive N_2 tension to levels which could cause DCS and formation of *in vivo* bubbles, but the actually observed behavioral responses of cetaceans to sonar in our study, do not imply any significantly increased risk of DCS.

Keywords: decompression sickness, diving physiology, marine mammals, gas exchange, modeling

INTRODUCTION

It has been suggested that anthropogenic sound, such as naval sonar, might lead to development of tissue N_2 gas bubbles and decompression sickness (DCS; Jepson et al., 2003), and that relationships between sound and DCS could explain some unusual whale strandings (Jepson et al., 2003). Increased blood or tissue N_2 tensions (P_{N_2}) could either be caused by a change in dive behavior in response to sonar (Jepson et al., 2003), by changes in physiological responses to diving (Hooker et al., 2012) or directly by an acoustically enhanced bubble growth (Crum and Mao, 1996).

While logistical and ethical constraints have prevented physiological studies on large whales, gas exchange models have indicated that the cardiac output, blood flow distribution, and pulmonary shunt are important variables that determine the level of blood and tissue P_{N_2} (Fahlman et al., 2006, 2009). Theoretical studies have also indicated certain behavioral changes that may affect risk (Houser et al., 2001; Zimmer and Tyack, 2007; Hooker et al., 2009). It has been suggested that N_2 loading is managed by the animals through different physiological trade offs, and if a behavioral response to an unanticipated acute threat (such as man-made

noise) over-rides behaviors adapted to manage N_2, the result may be decompression injury (Hooker et al., 2012). Until recently, no data existed on behavioral changes associated with sonar exposure. Previous theoretical studies attempting to estimate the effect of physiology and behavior on tissue and blood N_2 levels in marine mammals tested a range of plausible behavioral responses, such as changes in the ascent and descent rates (Houser et al., 2001; Zimmer and Tyack, 2007; Hooker et al., 2009), the ratio between surface interval and dive duration (Fahlman et al., 2006), deep diving (Houser et al., 2001; Zimmer and Tyack, 2007; Hooker et al., 2009), and repetitive shallow diving (Houser et al., 2001; Zimmer and Tyack, 2007; Hooker et al., 2009).

Recent behavioral response studies have investigated how exposure to naval sonar signals affects the natural dive behavior in a range of species: Blainville's beaked whales (*Mesoplodon densirostris*; Tyack et al., 2011), Cuvier's beaked whales (*Ziphius cavirostris*; Southall et al., 2011), sperm whales (*Physeter macrocephalus)*, long-finned pilot whales (*Globicephala melas*), and killer whales (*Orcinus orca*; (Miller et al., 2011; Sivle et al., submitted). Beaked whales and sperm whales are expert deep divers which regularly descend to depths of >1000 m for more than 60 min (Tyack et al., 2006, 2011; Watwood et al., 2006; Sivle et al., submitted), pilot whales are intermediate divers, typically performing dives to 300–600 m but of relatively short durations (<15 min; Baird et al., 2002; Aguilar Soto et al., 2008; Sivle et al., submitted), while killer whales are shallow divers that hardly ever exceed 100 m depth and dive durations of 10 min (Baird et al., 2005; Miller et al., 2010; Sivle et al., submitted). During these behavioral response studies, the whale was equipped with a suction cup attached digital tag (Johnson and Tyack, 2003). Following tag attachment, the whale was allowed to continue diving without sound exposure for between 1 and 7 h, followed by pre-determined periods of sonar exposures. The collected data allow comparison of the natural dive behavior during the pre-exposure as compared with that during sonar exposure. These data, therefore, provide species-specific cases which can be used to estimate how changes in dive behavior may affect blood and tissue P_{N_2} levels.

We have used a previously published mathematical model (e.g., Fahlman et al., 2009) to estimate blood and tissue N_2 tension $\left(P_{N_2}\right)$ from dive data recorded from sperm-, killer-, long-finned pilot-, Blainville's beaked-, and Cuvier's beaked whales before, during and after exposure to sonar signals. Our objectives were: (1) to determine if differences in natural behavior make some species more prone to DCS (i.e., higher end-dive P_{N_2} levels), (2) to investigate if the measured sonar-induced changes in dive behavior make odontocetes vulnerable to anthropogenic disturbance, and finally (3) to investigate how a hypothetical sonar-induced physiological flight response, involving changes in cardiac output on top of the behavioral response, would affect the risk of DCS.

MATERIALS AND METHODS

PERMITS

Animal experiments on sperm whales (*Physeter macrocephalus, sw*), long-finned pilot whales (*Globicephala melas, Gm*), and killer whales (*Orcinus orca, Oo*) were conducted in Norwegian waters under permits issued by the Norwegian Animal Research Authority to Dr. Petter Kvadsheim (permits no 2004/20607 and S-2007/61201), and in compliance with ethical use of animals in experimentation. The research on Blainville's beaked whales (*Mesoplodon densirostris, Md*) was conducted under permits for marine mammal research issued by the U.S. National Marine Fisheries Service (NMFS) to Dr. Peter Tyack (Permit #981-1578), and issued by the Government of the Bahamas to the Bahamas Marine Mammal Research Organisation (Bahamas permit #01/09) and Dr. Ian Boyd (Bahamas permit #02/07 and #02/08). The research on Cuvier's beaked whales (*Ziphius cavirostris, Cv)* were conducted in U.S. waters under U.S. NMFS research permit (#14534), as well as Channel Islands National Marine Sanctuary (CINMS) permit (#2010/004) for operations within the boundaries of the CINMS. All research protocols were also approved by the University of St. Andrews Animal Welfare and Ethics Committee as well as the Woods Hole Oceanographic Institution Animal Care and Use Committee.

DIVE DATA

Dive data for this research were collected in conjunction with several different research projects studying behavioral responses of cetaceans to naval sonar signals using very similar methodology. The "*3S-project*" collected data on sperm whales, pilot whale and killer whales in the Norwegian Sea, off the coast of Northern Norway, in 2006–2009 (Miller et al., 2011). The "*AUTEC BRS-project*" collected data on Blainville's beaked whales off Andro's Island, Bahamas, in 2007–2008 (Tyack et al., 2011). The "*SOCAL BRS-project*" collected data on Cuvier's beaked whales off the coast of California, USA, in 2010 (Southall et al., 2011). In all these projects, time versus depth records were collected at 50 Hz sampling rate using a digital tag (Johnson and Tyack, 2003) attached to the whale by suction cups. In addition to the depth sensor the tag also contains acoustic sensors that can be used to measure the level of sound exposures. Following tag attachment, the whale was allowed to continue diving without sound exposure during a pre-exposure period of 1–7 h duration. This was followed by pre-determined periods of sonar exposures. During exposure the ship carrying the sonar source gradually approached the position of the whale and/or gradually increased the transmitted source level to achieve an escalation of the received sound pressure levels from initial values of 60–120 dB to maximum levels of 147–180 dB re 1 μPa (RMS values). This procedure was used to simulate an approaching naval vessel. Complete dive profiles and details of experimental procedures and calculations of received sonar levels are given in Miller et al. (2011) for sperm whales, pilot whales, and killer whales, in Tyack et al. (2011) for Blainville's beaked whales and in Southall et al. (2011) for Cuviers's beaked whales. A total of 21 dive records of >8 h were gathered (**Table 1**). Thirteen whales in the data set were exposed to LFAS (1–2 kHz) and/or MFAS (3–4 or 6–7 kHz) sonar signals and eight records contain undisturbed baseline behavior only (**Table 1**).

GAS EXCHANGE MODEL

The dive records were entered into a gas exchange model in order to estimate blood and tissue N_2 tension throughout the dives. The model was adapted from a previous breath-hold model which included exchange of N_2, O_2, and CO_2 and also the effect of pressure on pulmonary gas exchange as previously detailed in

Table 1 | Animal ID, species, assumed body size (M_b), total dive record duration, sonar exposure duration (LFAS and/or MFAS) and description of behavioral responses to sonar as reported by [1]Sivle et al. (submitted), [2]Southall et al. (2011), or [3]Tyack et al. (2011). In addition some baseline data records without sonar exposures are also included as reported by [3]Tyack et al. (2011) and [4]Miller et al. (2011).

Animal ID	Species	M_b (kg)	Tag duration (h:m)	Sonar duration (h:m)		Behavioral response to sonar
				LFAS	MFAS	
Oo08_149a	Killer whale	3500	15:43	0:50	1:22	[1]No change in dive behavior
Oo09_143a	Killer whale	3500	12:54	–	–	[4]Baseline record without exposure
Oo09_144a	Killer whale	3500	11:52	0:34	0:59	[1]Switched from deep to shallow diving during LFAS, and shallow dives became deeper
Oo09_144b	Killer whale	3500	12:43	0:34	0:59	[1]Switched from deep to shallow diving during LFAS, and shallow dives became deeper
Sw08_152a	Sperm whale	43000	9:22	1:00	1:35	[1]No change in dive behavior
Sw09_141a	Sperm whale	30000	15:23	0:40	0:52	[1]Shallower deep dives during LFAS
Sw09_142a	Sperm whale	43000	15:08	0:44	0:33	[1]Deep dives with several disrupted ascents during LFAS
Sw09_153a	Sperm whale	43000	8:36	–	–	[4]Baseline record without exposure
Sw09_160a	Sperm whale	43000	14:45	0:43	0:42	[1]Shallower deep dives during LFAS and MFAS
Gm08_154d	Pilot whale	1500	8:16	1:20	0:25	[1]Switched from deep to shallow diving during MFAS
Gm09_137a	Pilot whale	1500	8:35	–	–	[4]Baseline record without exposure
Gm09_137b	Pilot whale	1500	8:25	–	–	[4]Baseline record without exposure
Gm09_137c	Pilot whale	1500	8:23	–	–	[4]Baseline record without exposure
Gm09_138a	Pilot whale	1500	11:02	0:32	0:35	[1]No change in dive behavior
Gm09_138b	Pilot whale	1500	17:26	0:32	0:35	[1]No change in dive behavior
Gm09_156b	Pilot whale	1500	17:51	0:32	0:26	[1]Switched from deep to shallow diving during LFAS, and shallow dives became deeper
Zc10_272a	Cuvier's beaked whale	2050	18:20	–	0:30	[2]Unusual slow ascent (MFAS)
Md06_296a	Blainville's beaked whale	1150	19:23	–	–	[3]Baseline record without exposure
Md07_227a	Blainville's beaked whale	1150	17:26	–	–	[3]Baseline record without exposure
Md07_245a	Blainville's beaked whale	1150	17:31	–	0:15	[3]Unusual slow ascent (MFAS)
Md07_248a	Blainville's beaked whale	1150	17:22	–	–	[3]Baseline record without exposure

Bostrom et al. (2008), Fahlman et al. (2009), and Hooker et al. (2009) with the revisions for the current analysis summarized below. The body was partitioned into four different tissue compartments (brain, fat, muscle, and central circulation) and one blood compartment (arterial and mixed venous). The parameters used for this model is the best available information from literature survey for each species, when available information was insufficient we applied information for other relevant species. In the current study, bone was included in the fat compartment as the bone of deep diving whales appears to be high in fat content (Higgs et al., 2010). The central circulatory compartment included heart, kidney, liver, and alimentary tract while the muscle compartment included muscle, skin, connective tissue, and all other tissues (Fahlman et al., 2009). The size of each compartment was taken from Hooker et al. (2009) for beaked whales and for the sperm whale, killer whale and pilot whale was based on available data for the sperm whale (Omura, 1950; McAlpine, 1985; Rice, 1989). Body mass for each species was estimated based on data recorded from stranded animals or from length-weight equations and length estimates (beaked whale; Hooker et al. (2009), sperm whale; Lockyer, 1991); killer whale; Clark et al. (2000)).

Gas exchange was assumed to occur between lung and blood and between blood and each compartment. The same assumptions were used for the blood N_2 stores as those detailed in Fahlman et al. (2009). The total – (Q_{tot}) and fractional blood flow to each tissue were not fixed, and could be varied to mimic diving bradycardia and changes in regional blood flow due to peripheral vasoconstriction (Butler and Jones, 1997). Hence, cardiovascular changes seen in freely diving animals could be simulated.

As in previous studies (Fahlman et al., 2006, 2009; Hooker et al., 2009), in the instances in which we had no direct anatomical or physiological data for the species in this study, we used data reported for the Weddell seal (Davis and Kanatous, 1999). The model included pulmonary shunting which varied with depth and diving lung volume (Bostrom et al., 2008; Fahlman et al., 2009; Hooker et al., 2009; see section below for details). For the sperm, killer, and pilot whale, the relative size of each compartment, expressed as a per cent weight of the body mass, was 3.3% for central circulation, 0.18% for brain, 50.02% for blubber, 26.5% for muscle, and 20% for blood. For the beaked whale the muscle was 57%, central circulation 3%, brain 0.2%, blubber 19.8%, and blood 20% of the total body mass. When calculating the O_2 stores, it was assumed that the lean muscle mass was 23.9% of the body

mass for the killer, sperm, and pilot whale and 49% for the beaked whales. The mass specific cardiac output was calculated according to Fahlman et al. (2009) and was 80 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ for a 43 ton sperm whale, 151 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ for a 3500 kg killer whale, and 186 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ for a 1500 kg pilot whale. While at the surface 31% of the cardiac output was directed to the central circulation, 67% to the muscle, 1.3% to the brain, and 0.7% to the blubber. During diving, the cardiac output was decreased to half and the blood distribution was changed such that 80% was directed to the central circulation, 1% to the muscle, 12% to the brain, and 7% to the blubber (Fahlman et al., 2006, 2009; Hooker et al., 2009).

TISSUE METABOLIC RATE AND GAS STORES

While we do not report the blood and tissue O_2 and CO_2 levels in the current study, estimates of these parameters are included in the model as they affect the uptake and removal of N_2 from the lungs and thereby the overall blood and tissue P_{N_2} (Fahlman et al., 2009). The initial lung, blood, and tissue gas stores were assumed to be similar to those used in Fahlman et al. (2009). The metabolic rates for each tissue compartment were estimated from the data presented in Davis and Kanatous (1999). The O_2 available during a dive came from lung, blood and tissue stores (mainly muscle, see below). The Ostwald solubility coefficient was used to calculate the dissolved O_2 content in blood and we used a value of 0.0261 l $O_2 \cdot l^{-1}$ blood (Weathersby and Homer, 1980). The same solubility coefficient was used to estimate O_2 content of muscle and central circulation. For the fat and brain compartment we used a value of 0.133 l $O_2 \cdot l^{-1}$ tissue.

In addition to dissolved O_2, the muscle compartment was assumed to contain a significant amount of endogenous O_2 bound to myoglobin and available for muscle metabolism. When calculating the total O_2 stored in the muscle compartment, we assumed that for the pilot whale, sperm whale, and killer whale, 23.9% of the total M_b was skeletal muscle, an estimate based on data for the sperm whale (Omura, 1950; McAlpine, 1985; Rice, 1989). The same parameter was estimated at 49% for the beaked whales, i.e., the muscle compartments for the different species were composed of a variety of tissues. For beaked whales, we used the reported myoglobin concentration for *Hyperoodon* (63 g·kg^{-1} muscle; Butler and Jones, 1997), and for pilot, sperm, and killer whales we used the value reported for *P. macrocephalus* (57 g·kg^{-1} muscle, Dolar et al., 1999). For all species, an O_2-binding capacity of 1.34 ml O_2 (STPD)·g^{-1} muscle tissue (Stephenson, 2005) was assumed. The muscle was assumed to be completely saturated at the beginning of a trial run, i.e., initial conditions. The blood was assumed to have a hemoglobin (Hb) concentration of 0.26 kg·l^{-1} of blood and the same O_2-binding capacity as myoglobin (Stephenson, 2005). Initially, it was assumed that arterial blood was 97% saturated and venous blood 87% saturated.

LUNG COMPRESSION AND PULMONARY SHUNT

The lung collapse model presented by Bostrom et al. (2008) was used to estimate alveolar volume at depth (DV_A). Initial parameters used to estimate DV_A were: total lung capacity (TLC, total respiratory volume), the volume of the upper respiratory system including trachea and bronchi (V_T), and maximal alveolar volume (V_A), i.e., $TLC = V_T + V_A$. TLC was estimated as $TLC = 0.135 \cdot M_b^{0.92}$ (Kooyman, 1973; Fahlman et al., 2011). It was assumed that gas exchange occurred only in the alveoli and when $DV_A = 0$, no gas exchange occurred. Dead space volume was assumed to be 1/15 (6.7%) of TLC, the value reported for the bottlenose whale (Kooyman, 1973). It was assumed that all species dived with a lung volume (DV_L) lower than TLC and the reduction in gas volume was taken from the alveolar gas space. That is, $DV_A = DV_L - V_T$. For sperm, killer, and pilot whales, we used a $DV_L = 26.4$ ml·kg^{-1} estimated for the sperm whale (Miller et al., 2004). Thus, for a 43000 kg sperm whale diving on a $DV_L = 26.4$ ml·kg^{-1}: TLC = 2472 l, V_T = 165 l, DV_L = 1135 l, DV_A = 970 l. For Blainville's beaked whale, we assumed a DV_L estimated for this species of 13.1 ml·kg^{-1} (Zimmer and Tyack, 2007).

ESTIMATED P_{N_2} LEVELS DURING DIVING

A dive was defined as a submergence for >10 s to a depth >1 m. Dives were categorized as shallow (depth >1 m and ≤30), intermediate (depth >30 m and ≤200 m) or deep (depth >200 m) based on the maximum depth of the dive. These categories were based on the assumption that shallow dives <30 m may serve to reduce PN_2 and be potentially helpful as decompression dives (Fahlman et al., 2007). Intermediate dives are dives where there is still significant gas exchange and thus N_2 is being absorbed by the body (Kooyman and Sinnett, 1982; Fahlman et al., 2008, 2009; Hooker et al., 2009) because of the hydrostatic pressure, whereas during deep dives the alveoli will most likely be collapsed and gas exchange will have ceased (Kooyman and Sinnett, 1982; Fahlman et al., 2008, 2009; Hooker et al., 2009). Within these categories we present average maximum dive depth (the maximum depth reached during the dive), average dive depth (the average depth of the dive), and average dive duration (the time spent submerged; **Table 2**).

Tissue and blood partial pressure of N_2 were estimated throughout the entire duration of each dive series. As the N_2 equilibrium state of a diving whale is not known at the start of a dive trace, the starting tissue and blood N_2 must therefore be assumed (Zimmer and Tyack, 2007). Alternatively, the dive trace needs to be long enough such that a "quasi-equilibrium" is reached, which depends on the size of the animal and the specific dive behavior (Hooker et al., 2009). The time to equilibrium was shown to be approximately 4 h for a 1000 kg whale and 13 h for a 5000 kg whale. Consequently, none of the dive series used in this study were long enough for the sperm whales to reach equilibrium. For that reason, we initialized all tissues to two times the surface P_{N_2}, which provided us reasonable equilibrium values for all tissues. This was based on testing a range of starting P_{N_2}-values, were we determined that initializing the blood and tissue P_{N_2} to two times ambient minimized variability of the model output. Dive records with less than 2 h of pre-exposure data still had to be removed from the analysis as the pre-exposure P_{N_2} estimates became too uncertain.

RISK OF DECOMPRESSION SICKNESS (R)

The end-dive P_{N_2} values were extracted for each dive category (shallow–intermediate–deep) as the average value of the first

Table 2 | Summary of animal dive series used in the current study. Shallow dives are to depths of 1–30 m, intermediate dives to depths of 30–200 m, and deep dives are deeper than 200 m. Values are mean ± SD.

Animal ID	No. dive	Shallow				Intermediate				Deep			
		No. dives	Av DD (s)	Average depth (m)	Average max depth (m)	No. dives	Av DD (s)	Ave rage depth (m)	Average max depth (m)	No. dives	Av DD (s)	Average depth (m)	Average max depth (m)
oo08-149a	577	577	92 ± 77	3.6 ± 2.7	4.9 ± 3.9	–	–	–	–	–	–	–	–
oo09-143a	1041	1012	35 ± 32	2.7 ± 1.8	4.4 ± 3.5	29	209 ± 113	37 ± 16	64 ± 24	–	–	–	–
oo09-144a	948	900	28 ± 20	2.6 ± 1.4	3.9 ± 2.3	48	205 ± 61	48 ± 22	77 ± 30	–	–	–	–
oo09-144b	1007	955	32 ± 23	3.2 ± 1.4	4.8 ± 2.4	52	196 ± 71	54 ± 24	84 ± 32	–	–	–	–
gm08-154d	186	175	32 ± 31	3.3 ± 2.0	4.9 ± 3.2	6	186 ± 38	41 ± 11	71 ± 18	5	643 ± 341	240 ± 70	402 ± 120
gm09-137a	567	545	38 ± 47	4.3 ± 4.0	6.2 ± 5.4	21	228 ± 58	31 ± 14	52 ± 28	1	524	200	291
gm09-137b	542	520	38 ± 46	4.2 ± 3.8	6.0 ± 5.2	21	232 ± 58	31 ± 14	52 ± 29	1	524	197	287
gm09-137c	647	636	32 ± 36	3.3 ± 3.0	4.9 ± 4.6	10	190 ± 48	33 ± 24	55 ± 40	1	446	297	203
gm09-138a	870	856	29 ± 22	2.9 ± 1.5	4.1 ± 2.3	1	91	18	34	14	589 ± 156	277 ± 31	417 ± 22
gm09-138b	1250	1218	26 ± 19	3.0 ± 1.7	4.3 ± 2.6	6	126 ± 40	34 ± 19	66 ± 42	27	496 ± 68	353 ± 58	236 ± 35
gm09-156b	1222	1191	29 ± 31	3.4 ± 2.7	5.0 ± 3.9	8	253 ± 84	40 ± 31	62 ± 50	23	506 ± 76	271 ± 33	473 ± 87
sw09-141a	45	19	201 ± 318	7.9 ± 5.3	12.4 ± 8.7	15	1217 ± 570	62 ± 43	103 ± 66	11	1896 ± 266	217 ± 64	352 ± 117
sw09-142a	29	5	347 ± 360	9.6 ± 4.6	15.3 ± 8.2	12	1498 ± 501	62 ± 34	104 ± 45	12	1924 ± 523	261 ± 118	416 ± 156
sw09-153a	16	1	31	2.9	5.1	9	1564 ± 320	107 ± 20	153 ± 20	6	1890 ± 312	148 ± 10	264 ± 53
sw09-160a	23	6	108 ± 84	4.9 ± 2.0	7.5 ± 3.7	–	–	–	–	17	2642 ± 661	537 ± 258	859 ± 438
sw08-152a	21	3	20 ± 15	3.7 ± 1.5	5.6 ± 2.3	8	1239 ± 671	92 ± 21	130 ± 32	10	1792 ± 651	242 ± 66	422 ± 167
Md06-296a	69	36	466 ± 344	12.2 ± 7.9	15.7 ± 9.8	25	785 ± 212	40 ± 18	64 ± 32	8	3126 ± 985	607 ± 192	854 ± 277
Md07-227a	56	11	143 ± 169	8.4 ± 6.9	11.9 ± 9.2	35	770 ± 153	60 ± 34	86 ± 51	10	2637 ± 898	606 ± 258	1044 ± 482
Md07-245a	64	11	96 ± 156	4.8 ± 6.0	6.9 ± 8.3	38	744 ± 201	50 ± 29	74 ± 48	15	1897 ± 1178	374 ± 280	579 ± 439
Md07-248a	81	57	316 ± 346	7.8 ± 6.7	10.5 ± 9.1	17	694 ± 171	56 ± 36	91 ± 54	7	2992 ± 177	606 ± 258	877 ± 103
Zc10-272a	41	6	43 ± 24	8.0 ± 2.6	12.3 ± 3.7	4	884 ± 426	89 ± 49	126 ± 73	31	1899 ± 1075	454 ± 350	290 ± 207

10 s after the animal reached the surface. Risk of DCS following each dive was estimated as the instantaneous mixed venous supersaturation level (R):

$$R = \left(P_{N_2}\ (\text{mixed venous}) - P_{N_2}\ (\text{ambient})\right) \quad (1)$$

where P_{N_2} is given in Atmospheres Absolute (ATA) corresponding to the pressure at the sea surface (1 ATA = 101.3 kPa). The mixed venous P_{N_2} levels were chosen because they represent the overall saturation level of the animals and have previously been used as a measure of risk of DCS in other species (Berghage et al., 1979) including humans (Weathersby et al., 1984). R was extracted for each dive during the pre-exposure period and compared to the estimated R-levels during LFAS and MFAS sonar exposure. This tested the effect of potential changes in behavior on the overall risk. To test the effect of a hypothetical physiological response to sonar, we removed the dive response during sonar exposure and re-ran the model. This implied that the model was run assuming that total cardiac output and blood distribution between tissue compartments were the same during diving as before diving. The change in R was again estimated and the pre-exposure compared with the exposure period. This tested the combined effect of changes in both behavioral and physiological responses.

BEHAVIORAL "RESPONDERS"

Analyses of changes in dive behavior in response to sonar exposure have been conducted on the same dataset used here to study potential changes in risk of DCS in beaked whales, sperm whales, pilot whales, and killer whales (**Table 1**). The Blainville's beaked whale (md07_296a) and the Cuvier's beaked whale (zc10_272a) were both exposed at depth and responded in much the same manner. Echolocation based foraging ceased and the animals broke off the deep dive prematurely before performing an unusually slow ascent to the surface (Southall et al., 2011; Tyack et al., 2011; **Figure 1**). In sperm whales responses were less clear, but there was an overall trend that deep dives were shorter and shallower during LFAS exposure (Sivle et al., submitted; e.g., sw09_160a in **Figure 1**), and this was often associated with reduced echolocation rates (Miller et al., 2011). Sperm whales generally performed normal deep dives with echolocations sounds during MFAS exposure (Sivle et al., submitted). When killer whales and pilot whales were engaged in deep diving foraging behavior at the time of exposure onset, they typically ended foraging and switched to shallow diving traveling mode. Interestingly, the shallow dives also became deeper during exposure than the shallow resting dives performed between deep dives prior to exposure (Sivle et al., submitted; e.g., gm09_156b and oo09_144a in **Figure 1**). Animals that were already in shallow diving traveling mode at exposure onset, just continued without changes in the dive pattern (Sivle et al., submitted). This response was consistent during LFAS exposure but less consistent during MFAS exposure (Sivle et al., submitted).

In addition to the comparison of risk of DCS (R, Eq. 1) between the exposure- and pre-exposure periods within each dive category (shallow–intermediate–deep; **Figure 3**), we have also used the gas exchange model to look at sequential changes from pre-exposure to exposure in behavioral "responders" without considering dive categories (**Figure 4**). This analysis will capture effects of subtle behavioral change within a dive category as well as effect of behavioral changes were the animal changes dive category in response to sonar (e.g., going from deep to shallow diving). In animals which are supersaturated even a single event of having a high R, even for a short period might be enough to trigger a cascade of bubble formation. Therefore we have calculated both average and maximum R-values for dives during exposure and compared those values to maximum and average values for dives during the pre-exposure period in the behavioral "responders" (**Figure 4**).

RESULTS

Summary statistics for each species and dive series are presented in **Table 2**. Each dive trace is indicated by the species abbreviation (oo: killer whale, sw: sperm whale, gm: pilot whale, zc: cuvier's beaked whale, md: Blainville's beaked whale) and an animal ID.

ESTIMATED BLOOD AND TISSUE P_{N_2} DURING NORMAL DIVING

The blood and tissue end-dive P_{N_2} as well as the variation between tissues increased as the dive depth increased (**Figure 2**). This increase in end-dive P_{N_2} levels and tissue variance is caused by the increase in the "fast" tissues, which has low tissue time constants (brain and central circulation) with depth, while fat and muscle end-dive P_{N_2} levels were less variable with dive depth. The correlation between dive depth and end-dive P_{N_2} levels implied a higher risk to the deep divers (sperm whales and beaked whales) than the shallower divers (killer whales; **Figure 2**). Except for sperm whales, the end-dive P_{N_2} during shallow dives was highest for the fat compartment (**Figure 2**). For deep and intermediate depth dives, end-dive P_{N_2} was highest for the fast tissues (central circulation and brain) and lowest for the muscle compartment for all animals (**Figure 2**).

CHANGES IN RISK OF DCS DURING LFAS SONAR EXPOSURE

The maximum change in risk of DCS (R, Eq.1) during exposure as compared with the pre-exposure period is shown in **Figure 3** on the left hand panels, for shallow, intermediate, and deep dives. For shallow dives the changes in R were not consistent and very minor for the killer whales, pilot whales and for all but one sperm whale. R decreased significantly for sperm whale sw08_152a during the sonar exposure, but there is very few shallow dives in this record and this might therefore be a coincidence. When the dive response was removed during sonar exposure, R increased somewhat for three of the four sperm whales, but decreased for the fourth one. For dives to intermediate depth, R decreased for the killer whales oo09_144a and oo09_144b, and removal of the dive response further decreased R for oo09_144a. For the pilot whale gm08_154d and the sperm whales sw08_152a and sw09_141a, removal of the dive response during sonar increased R. During deep dives, the behavior caused varying changes in R for the sperm whales and removal of the dive response increased R.

CHANGES IN RISK OF DCS DURING MFAS SONAR EXPOSURE

For shallow dives, there was large variation in risk of DCS (R, Eq.1), and overall R decreased during MFAS exposure (**Figure 3**). However, removal of the dive response increased R for oo09_144a, gm09_138b, gm09_156b, and sw09_141a. For intermediate dives, the change in behavior reduced R and only a slight effect was

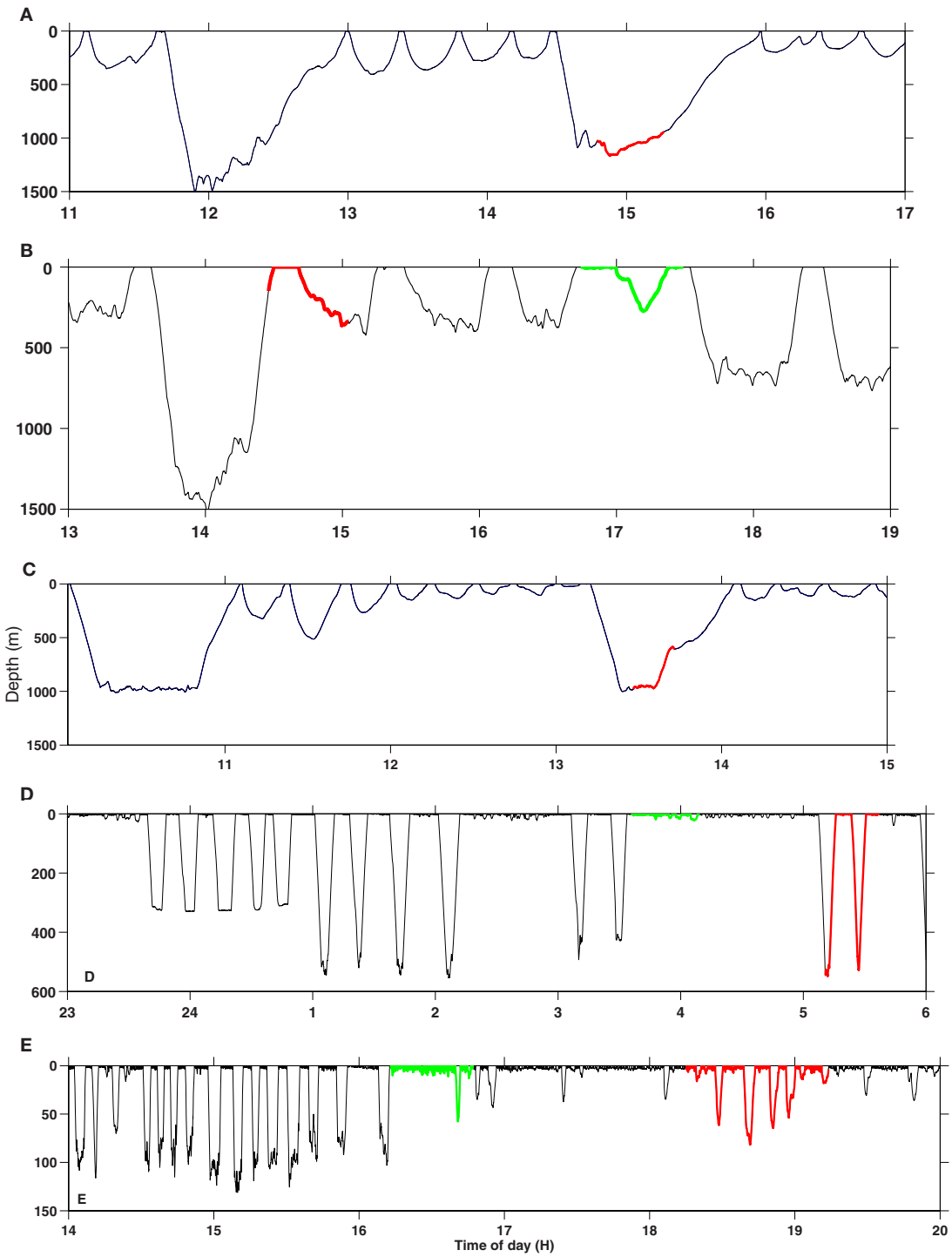

FIGURE 1 | Typical examples of changes in dive behavior in response to sonar. (A) Cuvier's beaked whale (zc10_272a), **(B)** sperm whale (sw09_160a), **(C)** Blainville's beaked whale (md07_245a), **(D)** pilot whale (gm09_156b), **(E)** killer whale (oo09_144a). The red part of the dive profile is exposure to MFAS sonar and the green to LFAS sonar. Time is in hours GMT and depth is in meters. Note the differences in depth scale between the different panels.

noticed in oo09_144a when the dive response was removed during sonar exposure. For the deep dives, MFAS exposure mostly caused a slight decrease in *R* in all species, but removal of the dive response increased *R*, especially for sw09_160a.

CHANGES IN RISK OF DCS IN "BEHAVIORAL RESPONDERS"

Typical examples of dive records of behavioral "responders" performing typical change in dive behavior in response to sonar are presented in **Figure 1** for each of the studied species. Of 13 whales

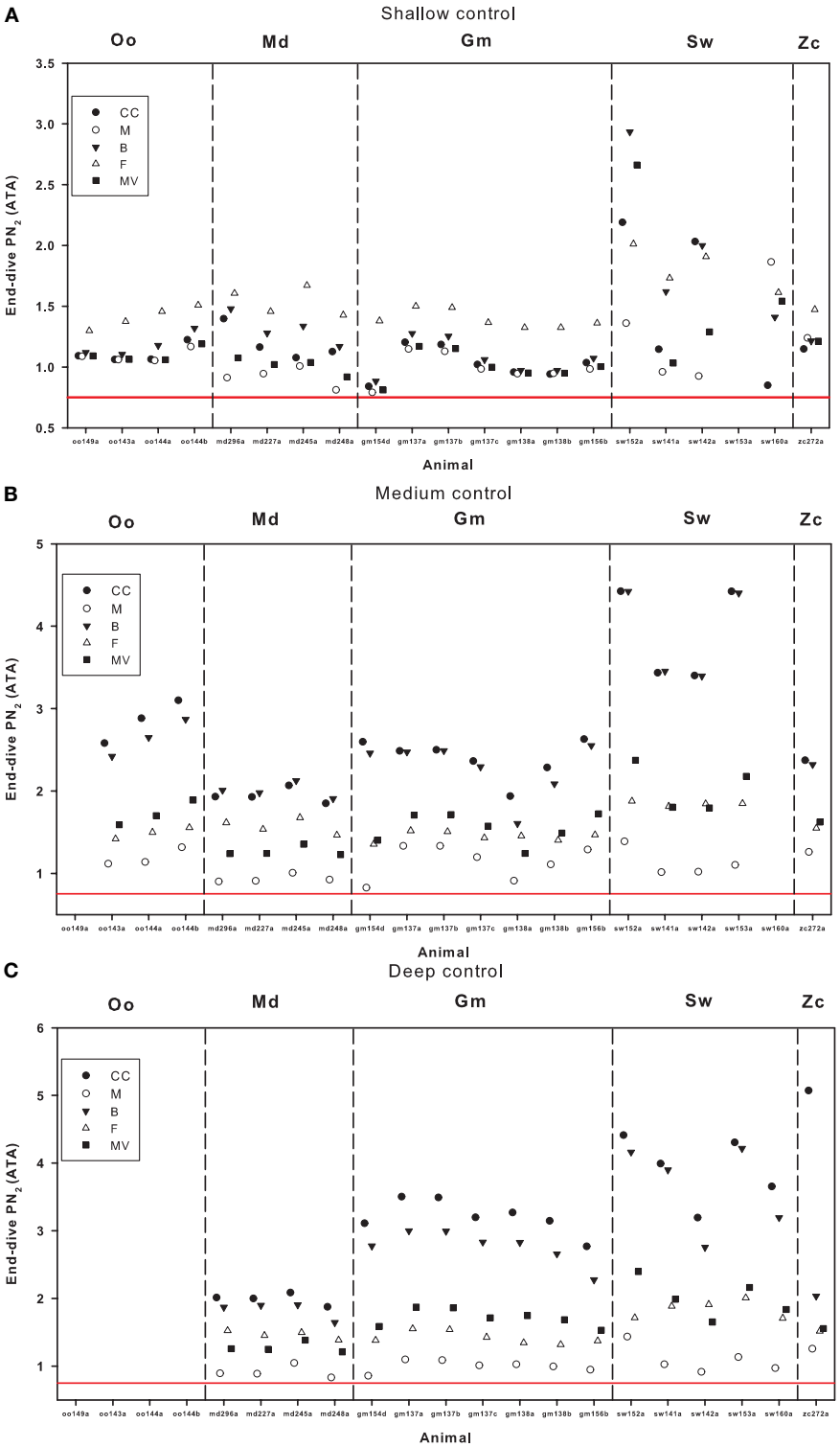

FIGURE 2 | End-dive tissue and blood N_2 tension following (A) shallow- (<30 m), (B) intermediate- (>30 m but <200 m), or (C) deep (>200 m) dives in the pre-exposure control period for killer whales (Oo), Blainville's beaked whales (Md), pilot whales (Gm), sperm whales (Sw), and Cuvier's beaked whale (Zc). Values are given for different tissue compartments; central circulation (CC), muscle (M), brain (B), fat (F), and mixed venous (MV). The red line at 0.75 ATA indicate 100% saturation at the surface (i.e., no risk of DCS).

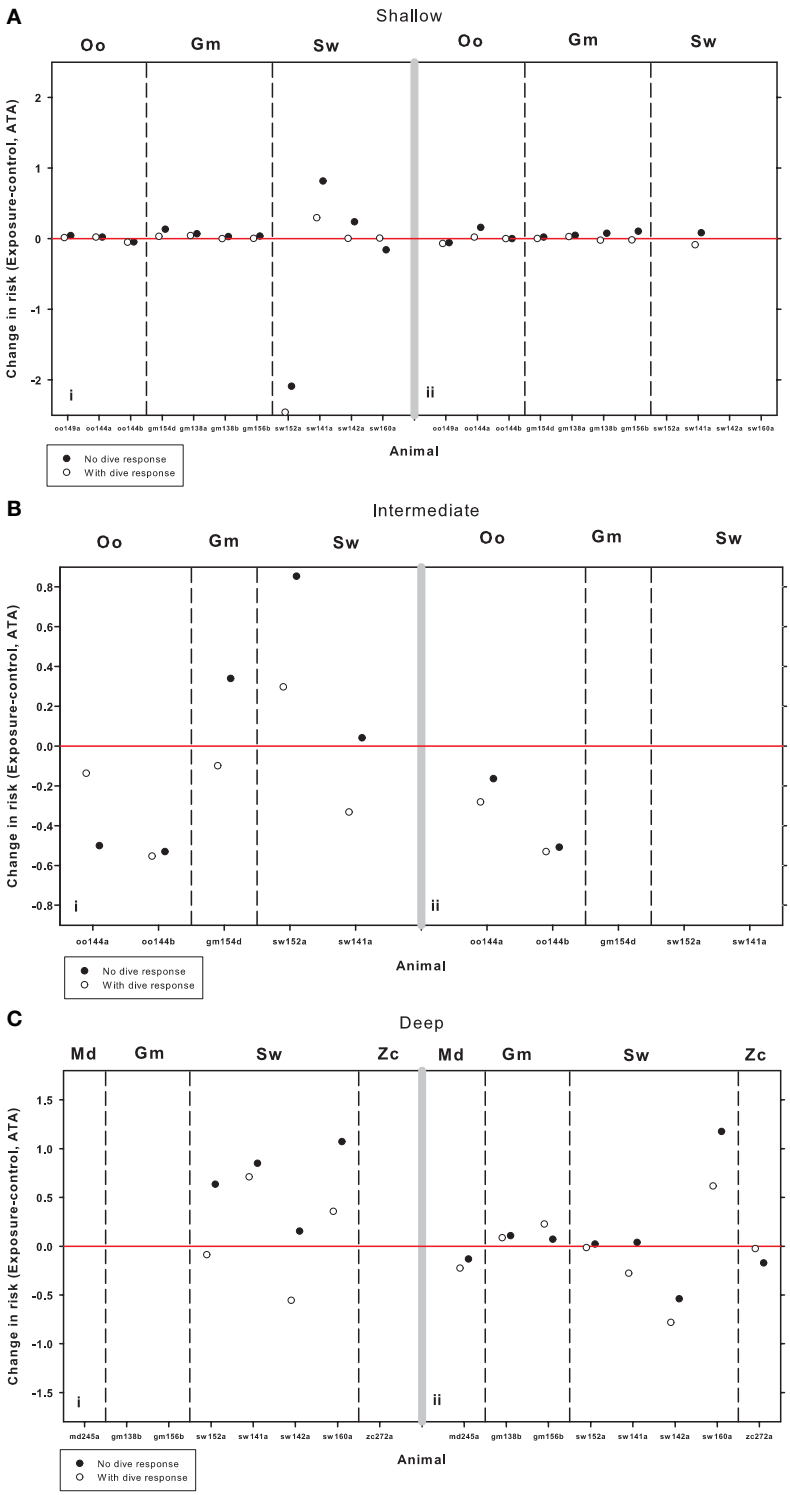

FIGURE 3 | Change in risk of DCS (*R*, Eq. 1) during sonar exposure as compared with pre-sonar control period during (A) shallow-, (B) intermediate-, and (C) deep-dives for killer whales (Oo), pilot whales (Gm), and sperm whales (Sw). The left panels are LFAS exposures **(i)** and right panel MFAS exposures **(ii)**. Open symbols indicate model output assuming normal physiological dive response, and solid symbols indicate model output when assuming a hypothetical removal of the dive response (no reduction in cardiac output and no redistribution of blood flow) in addition to the behavioral response during sonar exposure. Risk is defined as the end-dive mixed venous N_2 tension minus the ambient N_2 tension (Eq. 1). The red line indicates zero change in risk.

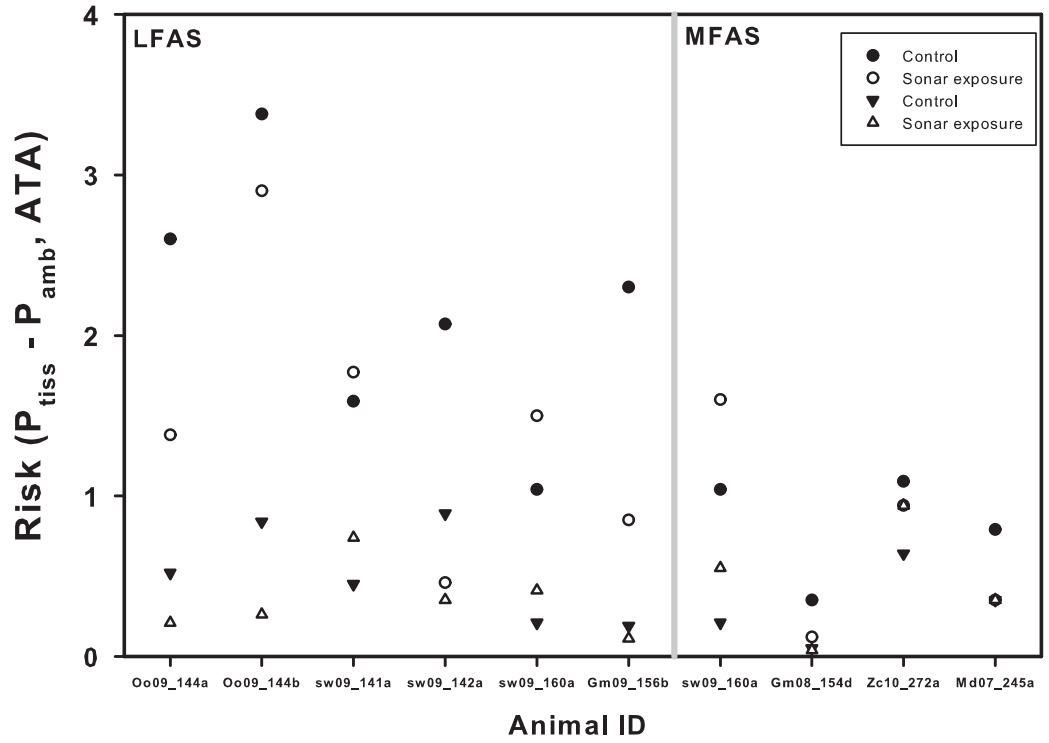

FIGURE 4 | Average (▽) and maximum (◯) risk of DCS (*R*, Eq. 1) during pre-sonar control (solid symbols) and sonar exposure periods (open symbols) in behavioral "responders". LFAS (left) and MFAS (right). Killer whales (Oo), pilot whales (Gm), sperm whales (Sw), Blainville's beaked whales (Md), and Cuvier's beaked whales (Zc). Risk is defined as the end-dive mixed venous N_2 tension minus the ambient N_2 tension (Eq. 1).

exposed to LFAS and/or MFAS, 10 showed a change in dive behavior apparently in response to the sonar (**Table 1**). This response varied from unusual slow or disrupted ascents of the deep divers to complete shifts from deep dive to shallow dive mode seen in pilot whales and killer whales (**Figure 1**). Except for the sperm whales reported to respond to sonar by shallower deep diving, *R* decreased during sonar exposure in all behavioral "responders" (**Figure 4**). In sw09_141a during LFAS exposure and for sw09_160a during both LFAS and MFAS exposure both maximum and average *R* increased (**Figure 4**).

DISCUSSION

Our model estimates suggest that shallow (killer whales), intermediate (pilot whales) and deep diving whales (sperm whales, Cuvier's beaked whale, and Blainville's beaked whale) all live with high blood and tissue P_{N_2} levels, but the deep divers seem to experience the most extreme values (**Figure 2**). The deep diving sperm whales which respond to sonar exposure by shallower but still deep diving, were found to increase risk of DCS (*R*, Eq. 1), but not beyond the normal risk range of sperm whales. We found no systematic changes in *R* during sonar exposure in the other species, thus for some animals *R* appeared to increase slightly, while for others it decreased. However, the variation in *R* increased with dive depth. Also, removal of the dive response during sonar exposure increased *R* for most whales except in a few instances, e.g., oo09_144a during LFAS exposure, but also increased the variation of *R*.

EFFECT OF DIVE DEPTH ON END-DIVE P_{N_2} LEVELS

We have shown that the estimated end-dive P_{N_2} values increased with maximum dive depth (**Figure 2**). The largest increase in P_{N_2} levels between dive categories happens between the shallow and intermediate dives, with only a moderate further increase in some tissues between intermediate and deep dives. Shallow dives (1–30 m) includes the decompression depth zone where tissue and blood P_{N_2} exceed the ambient partial pressure of N_2 and the direction of N_2 flux is therefore from the blood into the lung (N_2 removal; Fahlman et al., 2007). Intermediate dives (30–200 m) extend into the compression depth zone where pulmonary exchange still occurs (Hooker et al., 2009), but ambient pressure now exceeds tissue and blood P_{N_2} and therefore the direction of N_2 flux changes and N_2 is now being absorbed. However, in this region depth related pulmonary shunting begins to impede gas exchange (Kooyman and Sinnett, 1982; Bostrom et al., 2008). Thus, variation in dive behavior and physiological responses may cause large variation in end-dive tissue and blood P_{N_2} in this zone. The deep dives (>200 m) extend into the no-compression depth zone where lungs are completely collapsed and gas exchange ceased (Bostrom et al., 2008; Fahlman et al., 2009). Consequently, the total body N_2 load will be determined by the ratio of time spent within the compression zone and the decompression zone, whereas time spent

into the no-compression zone will not add to the total body N_2 load, but may allow time for redistribution of N_2 between different tissues.

For shallow dives, end-dive mixed venous P_{N_2} ranged between 0.8 ATA for pilot whales to values >1.5 ATA for sperm whales. For dives to medium and deep depths, mixed venous P_{N_2} were >1.0 ATA for all whales (**Figure 2**) and were close to or exceeding 2 ATA for the sperm whale and pilot whales. Although difficult to compare directly because of differences in how shallow dives where defined, these results appear to disagree with the suggestion made by Zimmer and Tyack (2007) that shallow dives increase the risk of inert gas bubbles and DCS. One possible reason for these divergent results could be related to the different assumptions on how pulmonary gas exchange is altered during diving. Empirical data in both the California sea lion and harbor seal have indicated that a pulmonary shunt develops that is related to the dive depth and diving lung volume (Kooyman and Sinnett, 1982). Despite this, previous studies made the simplistic assumption that gas exchange was perfusion limited until the alveoli collapsed, and the collapse depth was assumed to be at a pre-determined depth, e.g., 70 m (Fahlman et al., 2006; Zimmer and Tyack, 2007). It was suggested that this was a conservative approach and considered a worst-case scenario. More recent work has developed a model that predicts air volumes in the upper and lower airways, based on the structural properties of the respiratory system (Bostrom et al., 2008). The lung compression model was later coupled with the empirically derived pulmonary shunt data for pinnipeds (Kooyman and Sinnett, 1982). This made it possible to include the effect of pressure and diving lung volume on gas exchange (Fahlman et al., 2009; Hooker et al., 2009). When the lung compression/pulmonary shunt model was included in gas exchange models, the models output agreed well with measured blood and tissue N_2, CO_2 and O_2 levels (Fahlman et al., 2009). The differences in model estimates vary substantially with these varying assumptions in gas exchange models used (Fahlman et al., 2009) and may be one reason for the divergent results.

EFFECT OF BODY MASS ON END-DIVE P_{N_2} LEVELS

A previous study showed a positive correlation between predicted end-dive P_{N_2} and body mass, when the body mass was varied for each species (Hooker et al., 2009). However, when the tissue and blood P_{N_2} levels were estimated with the species-specific body mass, there were little differences in predicted N_2 levels between species. It was suggested that these results may indicate behavioral adjustments within each species that limits the end-dive P_{N_2} (Hooker et al., 2009). In the current study, there were no clear differences in end-dive blood or tissue P_{N_2} with animal size (body mass), not even between the expert deep divers (sperm whale and beaked whales). However, the variation in estimated values was much greater in sperm whales at all depths (**Figure 2**).

CHANGES IN RISK OF DCS DUE TO BEHAVIORAL RESPONSES TO SONAR

The behavioral responses to sonar differed both within species and between the species in this study. The beaked whales (*Md* and *Zc*) displayed an unusually slow ascent from the deep dive (Southall et al., 2011; Tyack et al., 2011), while sperm whales tended to continue deep diving during exposure, but shallower than before (Sivle et al., submitted). Pilot whales are intermediate divers and killer whales shallow divers as compared with the expert deep diving sperm- and beaked whales. Pilot whales typically perform bouts of relatively deep dives in between periods of very shallow diving (Sivle et al., submitted). Sonar responses in killer whales and pilot whales that were in deep diving mode prior to exposure typically involved a shift to shallow diving mode, but the shallow dives also became deeper than during normal undisturbed shallow diving (Sivle et al., submitted). These differences in response is probably largely explained by differences between species, but could also partly be explained by differences in the experimental procedures. Sperm whales, pilot whales and killer whales were all exposed using the same protocol (Miller et al., 2011), involving multiple exposures in a random behavioral context (feeding, resting, traveling) using a moving source. The beaked whales were exposed using a different and stationary source, and exposures were always conducted in a fixed behavioral context during deep feeding dives (Southall et al., 2011; Tyack et al., 2011).

BEAKED WHALES

Zimmer and Tyack (2007) reported that increased ascent rates from deep dives would decrease end-dive P_{N_2}. The actual observed response of Zc and Md to sonar was an unusually slow ascent (Southall et al., 2011; Tyack et al., 2011; **Figure 1**), and this could increase *R* because of the additional time spent in the compression zone. However, theoretical studies have suggested that a reduced ascent rate in the decompression zone coupled with a pre-surface tachycardia may reduce end-dive P_{N_2} by as much as 45% (Fahlman et al., 2006). Our results indicate that even without this physiological adjustment the actual observed decrease in ascent rate resulted in a slightly decreased *R* (**Figure 4**).

SPERM WHALES

Sperm whales sw09_141a and sw09_160a were both reported to respond to the LFAS by continuing to perform deep dives, but the deep dives became shallower (Sivle et al., submitted; **Figure 1**). In the two animals which responded this way the shallower deep dives implied switching from dives at maximum depth of 1200–1500 m pre-exposure to about 300–400 m during exposure in sw09_160a (**Figure 1**), and from 250–400 m pre-exposure to only 50 m, during exposure in sw09_141a (Miller et al., 2011). Both these animals showed an increased *R* during LFAS exposure (**Figures 3** and **4**), while for the other two sperm whales, which did not respond by shallower deep dives, *R* did not increase (**Figure 3**). During the MFAS exposure, again the sw09_160a responded by shallower deep diving (Sivle et al., submitted) and again *R* increased (**Figure 4**), while for the other three, who did not display shallower deep diving, *R* did not increase. For sw09_160a the shallower deep dives during sonar exposure were still deep enough to extend well into the no-compression zone (300–400 m), but the descent phases of these dives were much slower than for the deep dives during pre-exposure (**Figure 1**). The increase in *R* is thereby explained by the increased time spent in the compression zone during the descent phase of these dives. The shallower deep diving response of sw09_141a to the LFAS exposure is similar to the hypothetical response described to result in higher *R* also in beaked whales by Zimmer and Tyack (2007). This animal switched from dives

to depth well within the no-compression zone (250–400 m) to shallower dives during sonar exposure where most of the time was spent in the compression zone and never extended into the no-compression zone, and therefore resulted in increased *R*.

It has been proposed that the deep diving species are more at risk of suffering from decompression injury than shallower diving species (Hooker et al., 2009). Our results support this hypothesis. Even though the increase in *R* during sonar exposure was within the normal risk range of sperm whales, it is still a conspicuous observation that this increase happened in all three cases where the whales also changed dive behavior. Deep divers such as beaked whales and sperm whales probably push the physiological limits of diving in mammals and this might make them more vulnerable to human disturbance such as naval sonar (Hooker et al., 2012). Dysbaric osteonecrosis progressing with age has been reported in sperm whales (Moore and Early, 2004), and a recent study by Bernaldo de Quirós et al. (in press) showed that at necropsy of stranded animals there was a higher prevalence of gas bubbles in deep divers compared to non-deep divers.

KILLER WHALES AND PILOT WHALES

The change from deep dive mode to shallow diving mode in response to sonar seen in killer whales and pilot whales (Sivle et al., submitted; **Figure 1**), did not seem to increase the *R* (**Figures 3** and **4**). Indeed, an increase in *R* is not expected from such behavioral change since it implies that animals spend more time in the decompression zone, where N_2 may be removed, instead of in the compression zone, where N_2 is taken up. However, the deeper shallow dives which were also associated with this response could potentially increase *R* if they extended into the compression zone. Even if the dives were deeper (Sivle et al., submitted), they were still quite shallow ($<$10 m) and therefore probably still within the decompression zone. Thus, our results showed no consistent change in *R* in killer and pilot whales. The responses seen in the behavioral "responders" indicate that *R* was actually reduced.

CHANGES IN RISK OF DCS DUE TO HYPOTHETICAL PHYSIOLOGICAL RESPONSE TO SONAR

It was previously suggested that the dive response may be useful to reduce N_2 uptake during diving and thereby minimize *R* (Fahlman et al., 2006). The results in this study concur, as *R* increased for most whales when the dive response was hypothetically removed during sonar exposure, thereby increasing N_2 uptake during the dive. Still, in a few occasions the elevated cardiac output reduced *R*. This agrees with more recent work that indicate that the diving bradycardia does not always reduce N_2 levels during repeated diving, but that there are certain tissue time constants (τ) that should be avoided to reduce N_2 levels (Fahlman et al., 2007; Hooker et al., 2009). Inert gas loading is probably managed through complex trade offs between physiological and behavioral responses (Hooker et al., 2012). If a behavioral response to an unanticipated acute threat (such as man-made noise) is perceived as more immediately critical than management of N_2, it might result in decompression injury (Hooker et al., 2012). For example, metabolic demand limits the ability to adjust blood flow, and there is therefore a trade-off between the need to supply sufficient O_2 and reducing CO_2 and N_2 accumulation. As the cardiac output and blood flow distribution alter the tissue time constant (see Eq. 3 in Fahlman et al., 2006), studies are required to determine the physiological responses in deep diving whales both during undisturbed condition and during sonar exposure.

METHODOLOGICAL CONSIDERATIONS

Using mathematical models to investigate complex problems offers important insight but is also limited in scope as models are only an abstraction of the real world. For example, the model used in this study uses a pre-determined blood flow at the surface and while diving, but it is known that the heart rate, and therefore most likely the cardiac output, changes throughout a dive (Thompson and Fedak, 1993; Ponganis et al., 1997). The estimates for some of the physiological variables in the model are also taken from studies on pinnipeds, and may differ for cetaceans. In addition, understanding the effect of pressure on gas exchange is rudimentary and recent studies have suggested that there may be species variation in the depth-dependent pulmonary shunt (Bostrom et al., 2008; Fahlman et al., 2011; Moore et al., 2011). While the parameter estimates and compartment sizes for this model were not always species specific, the model has been calibrated against known blood and tissue P_{N_2}, PO_2 and PCO_2 values and resulted in good agreement between observed and predicted values (Fahlman et al., 2009). Furthermore, we have published several studies using this model where sensitivity analyses were conducted (Fahlman et al., 2006, 2007, 2009; Hooker et al., 2009). These sensitivity analysis consistently show that the variables that had the greatest impact on the model outcome were changes in rate of pulmonary gas exchange, cardiac output, and blood flow distribution with depth, all variables where data only exist in pinnipeds and shallow diving odontocetes. The results from these previous sensitivity analyses contributed to the hypotheses that sonar could cause a startle response which could affect blood flow and thereby risk of DCS. We therefore tested the effect of this potential response in the current study.

Previous theoretical studies have used hypothetical sonar-induced changes in both behavior and physiology to model blood and tissue P_{N_2} (Hooker and Baird, 1999; Houser et al., 2001; Fahlman et al., 2006, 2009; Zimmer and Tyack, 2007), but this is the first attempt to estimate the changes during actual behavioral responses to sonar. The behavioral response data were collected to determine how different species respond to anthropogenic sound. Of special interest was to determine the behavioral responses to LFAS and MFAS sonar signals, as studies have suggested that their use is related to mass-strandings (Cox et al., 2006; D'Amico et al., 2009). Jepson et al. (2003) and Fernández et al. (2005) expanded on this correlation and suggested that sonar related strandings may be associated with *in vivo* bubble formation. The large variation in decompression risk in response to sonar exposure may partly be due to the experimental design, with relative short pre-exposure periods followed by short exposures. This design is chosen to generate dose response functions, where the key is to determine acoustic dose at the threshold of response (Miller et al., 2011; Tyack et al., 2011). In addition, for some animals the sonar exposures to MFAS and/or LFAS were repeated during a single tag deployment to investigate frequency specificity of responses and habituation or sensitization during repeated exposures (Miller et al., 2011).

The uptake and removal of inert gas is commonly modeled using single exponential models where the kinetics is determined by a time constant (τ) that determines the time to equilibrium. The time constant is physiologically relevant and related to the solubility of the gas and the blood flow rate (see Eq. 3 in Fahlman et al., 2006). For tissues with a high perfusion rate, e.g., heart and brain, τ is short and time to equilibrium faster than for other tissues. For a diving animal, this means that these tissues may experience extreme P_{N_2} during the dive, but removal is also so fast that the supersaturation seldom reaches dangerous levels, and the tissue soon equilibrate when the animal has reached the surface (Kooyman et al., 1972; Fahlman et al., 2006; Houser et al., 2010). Slow tissues such as blubber, are those where the surface interval duration between repeated dives in a bout are too short for the tissues to return to equilibrium with the surface atmosphere. For these tissues, N_2 slowly accumulates to reach considerable levels. It has also been suggested that these tissues may limit the length of a dive bout, and it may be those slow tissues that put animals at risk of developing DCS (Fahlman et al., 2007). Eventually, the animal will reach a state of quasi-equilibrium where the saturation state is more or less constant between dives, but the time to this equilibrium depends on the size, physiology, and dive behavior of the animal (Hooker et al., 2009). Thus, large animals and tissues with a long τ will take longer time to respond but they will also show less variation between dives. Therefore, to accurately estimate tissue and blood N_2 levels, it is important to have data sets that contain a representative sample of the natural dive behavior and exposures that are long enough to clearly indicate the behavioral responses. The data used in the current study are therefore not ideal for modeling gas management. For the type of analysis conducted here a more optimal design would be to increase the duration of the pre-exposure and sonar exposure periods. In particular, larger animals and slow responding tissues (e.g., fat) have very long response times (Fahlman et al., 2006), and therefore short exposure durations may not allow for such tissues to reach maximum values.

CONCLUSION

We conclude that there is great variation in the behavioral responses to sonar exposure and in most cases the response does not increase decompression risk, but there may be certain situations where the risk is increased, such as the shallower deep dives seen in sperm whales. The hypothetical removal of dive response during sonar exposure increased the variation in risk of DCS (R, Eq. 1), suggesting that physiological responses to anthropogenic sound may lead to altered tissue and blood N_2 levels. Cetaceans seem to live with natural high N_2 levels, and since both behavioral and physiological responses have the potential to alter R, we have to assume that N_2 levels are managed through complex interactions between behavioral and physiological responses. We therefore can not rule out the possibility that a combination of behavioral and physiological responses to sonar have the potential to alter the blood and tissue end-dive N_2 tension to levels which could cause DCS. Our results support previous suggestions that deep divers might be more at risk of suffering from decompression injury than shallower diving species. As little is known concerning the physiological adjustments associated with diving in large whales, future work should improve our knowledge in these areas.

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Animal models for investigating the central control of the mammalian diving response

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Pioneering studies by Per Scholander indicated that the diving response consists of reflexly induced apnea, bradycardia and an alteration of blood flow that maintains perfusion of the heart and brain. More recently field physiological studies have shown that many marine animals can adjust cardiorespiratory aspects of their diving response depending upon the behavioral situation. This could suggest that the very labile heart rate during diving is under direct cortical control. However, the final control of autonomic nervous system functioning resides within the brainstem and not the cortex. Many physiologists regard the brain as a "black box" where important neuronal functioning occurs, but the complexity of such functioning leaves systematic investigation a daunting task. As a consequence the central control of the diving response has been under-investigated. Thus, to further advance the field of diving physiology by understanding its central neuronal control, it would be first necessary to understand the reflex circuitry that exists within the brainstem of diving animals. To do this will require an appropriate animal model. In this review, two animals, the muskrat and rat, will be offered as animal models to investigate the central aspects of the diving response. Firstly, although these rodents are not marine animals, natural histories indicate that both animals can and do exploit aquatic environments. Secondly, physiological recordings during natural and simulated diving indicate that both animals possess the same basic physiological responses to underwater submersion that occur in marine animals. Thirdly, the size and ease of housing of both animals makes them attractive laboratory research animals. Finally, the enormous amount of scientific literature regarding rodent brainstem autonomic control mechanisms, and the availability of brain atlases, makes these animals ideal choices to study the central control of the mammalian diving response.

Keywords: muskrat, *Ondatra zibethicus* rat, *Rattus norvegicus*, diving response, autonomic control

INTRODUCTION

The physiological and behavioral adaptations of marine animals are amazing and allow these animals to survive and thrive in their aquatic environment. The pioneering work by Per Scholander and colleagues such as Laurence Irving, revolutionized the field of diving physiology in the 1930s and 1940s (Irving, 1934, 1939; Scholander, 1940). In these studies the basic physiological responses to underwater submersion were investigated primarily by studying diving animals in a laboratory setting. Scholander and his colleagues described how the diving response consists of reflexly induced apnea, bradycardia and an alteration in blood flow that limits flow to non-exercising muscles while maintaining flow to the heart and brain.

The field of diving physiology was revolutionized again in the 1970s and 1980s, this time by Gerald Kooyman and his colleagues, who took physiology to the field by using time-depth recorders (TDRs), data loggers, and, more recently, critter-cams. What has became exceedingly apparent from these studies is that many marine animals, especially when they are in their natural environment, can exert a cortical control over their autonomic nervous system (ANS) and can adjust the cardiorespiratory aspects of their diving response, depending upon the situation and their behavioral response (Kooyman, 1989; Butler and Jones, 1997). Diving heart rate can include both anticipatory submersion bradycardia and anticipatory re-emersion tachycardia (Jones et al., 1973; Thompson and Fedak, 1993). This could suggest that marine mammals have direct suprabulbar control over their cardiovascular system (Ramirez et al., 2007). However, while it is true that cortical and sub-cortical regions of the brain can provide a modulatory afferent input, the final control of ANS functioning resides within the brainstem and not the cortex.

Many physiologists regard the brain as a "black box," where important neuronal functioning occurs but the complexity of such functioning leaves systematic investigation a daunting task. As a consequence the brainstem control of the diving response has been under-investigated. Thus, to further advance the field of diving physiology, and to really understand how diving animals have such a labile diving response, it would be first necessary to understand the neuronal circuitry that exists within the brainstem of diving

Abbreviations: ADL, aerobic dive limit; AEN, anterior ethmoidal nerve; ANS, autonomic nervous system; BAT, brown adipose tissue; BDA, biotinylated dextran amine; BMR, basal metabolic rate; BPM, beats per minute; DMR, diving metabolic rate; HIF, heat increment of feeding; HRP-WGA, horseradish peroxidase conjugated to wheat-germ agglutinin; M, mass; MDH, medullary dorsal horn; NTS, nucleus tractus solitarius; TDR, time-depth recorders.

animals. Once the brainstem circuitry has been described, then it will be possible to determine how cortical afferent signals in marine animals can modify the basic autonomic reflex. However, to do this will require an appropriate animal model.

A difficulty in studying the brainstem control of the diving response has a lot to do with the animals being studied. It is obvious that conducting neurophysiological studies in and under the open ocean has numerous logistical problems. Thus it makes sense to utilize an appropriate animal model for the problem being investigated, as some animals could facilitate the study of central control of cardiorespiratory functioning while others would make it more difficult. Indeed, Krogh's (1929) principle has been a recurrent theme in comparative physiology: for every research question there will be some animal of choice on which the problem can be most conveniently studied. Scholander himself used a variety of animals in his investigations that best suited his research question.

Semi-aquatic species that spend part of their lives in and around water and part of their lives on land are amazing animals as they exploit two separate environments: the underwater world and terrestrial world. Just because these animals spend only part of their lives in an aquatic environment should not exclude them from being considered "diving animals." Thus, to engage in land, and laboratory, based investigations of the central control of the diving response, semi-aquatic animals could be ideal animal models. In this review, two animals, the muskrat and rat, will be offered as animal models to investigate the central aspects of the diving response. Although these rodents certainly cannot be considered marine animals, natural histories indicate that both the muskrat and rat can and do exploit aquatic environments. Additionally, both the muskrat and rat possess the same basic physiological responses to underwater submersion that occur in marine animals. Indeed, in his classic 1940 monograph, Scholander used rats to show that during diving blood flow to non-exercising muscles is decreased (Scholander, 1940). To illustrate that the muskrat and rat are ideal models for investigating the central control of the diving response, the physiological responses to natural and simulated diving, as well as responses from anesthetized animals used in physiological investigations, will be reviewed.

MUSKRATS

NATURAL DIVING HISTORY

Muskrats (*Ondatra zibethicus*), the only species in the genus *Ondatra*, are small (approximately 1 kg) semi-aquatic rodents that are common in marshes and other wet-land areas from the tropics to the Arctic. They are native to North America, but have been introduced to parts of Europe, Asia, and South America. They spend much of their time in the water, are good swimmers (Dagg and Windsor, 1972; Fish, 1983, 1984), and can remain submerged for 12 min (Irving, 1939; MacArthur, 1984b; Signore and Jones, 1995). Thus on a per weight basis, muskrats can be included among the best of the breath-hold divers. Since muskrats do not store food for the winter, their foraging activity continues year-round, often under frozen ponds and lakes (MacArthur, 1978, 1979b, 1980; MacArthur and Aleksiuk, 1979). The muskrat once was an important fur-bearing animal, but is considered by many to be a pest because its burrowing causes damages to dikes and levees. Because they are non-hibernating, muskrats are readily available in their natural environment throughout the entire year, which can be an advantage for scientific research studies (Aleksiuk and Frolinger, 1971).

Field studies in Manitoba Canada using radiotracking devices indicate that muskrats have seasonal activity patterns (MacArthur, 1979b, 1980). In the summer there is a bimodal activity pattern, with major activity peaks occurring between sunset and sunrise. This nocturnal activity may correlate with cooler nighttime temperatures that could prevent potential heat stress during very warm daytime temperatures (MacArthur, 1979b, 1980). Additionally, in summer months muskrats tend to live in burrows or open nests that provide a cool microclimate (MacArthur and Aleksiuk, 1979). In the winter activity is more diurnal, with activity occurring in the late afternoon and early evening when daily temperatures reach their peak (MacArthur, 1979b, 1980). In winter months muskrats tend to live in lodges that provide a microclimate that may be 20°C warmer than external air temperature (MacArthur and Aleksiuk, 1979). Nest chambers in these frozen winter lodges may house up to five adult muskrats (MacArthur and Aleksiuk, 1979), and considering that muskrats may spend 13–14 h/day resting in a winter lodge (MacArthur, 1980), accumulation of CO_2 and depletion of O_2 could pose potential respiratory problems. Indeed, microenvironment gas measurements indicate that winter lodge CO_2 levels can reach as high as 10% and O_2 levels can reach as low as 18% (MacArthur, 1984b). Laboratory studies have shown that muskrats are generally quite tolerant of increased CO_2 levels (Irving, 1938; MacArthur, 1984b, 1986b), although breathing 10% CO_2 can significantly depress oxygen consumption (MacArthur, 1986b,c). However, whether muskrats have decreased CO_2 chemoreceptor sensitivity as an adaptation to diving or to communal living in burrows and lodges is still unknown (MacArthur, 1984b).

Field studies indicate there is a 41.7% increase in oxygen storage capacity in winter-acclimatized muskrats compared with summer-acclimatized muskrats (MacArthur, 1990). The increased oxygen storage capacity of 35.7 ml O_2/kg is accompanied by a 17 s increase in aerobic dive limit (ADL), to 57.9 s, which allows muskrats to dive up to 13 m further in their wintertime ice-covered environment (MacArthur, 1990). Winter adaptations also include significant increases in hematocrit, hemoglobin concentration, blood volume, blood oxygen capacity, and skeletal muscle myoglobin content (Aleksiuk and Frolinger, 1971; MacArthur, 1984c, 1990). In muskrats, blood comprises the major oxygen storage compartment, accounting for 57 and 65% of total oxygen stores in summer and winter, respectively (MacArthur, 1990). The average under-ice swimming speed of muskrats is 0.76 ± 0.04 m/s, although they can reach peak-burst speeds of 1.27 m/s during escape dives in response to human disturbance (MacArthur, 1992). In comparison, muskrats diving through a maze in the laboratory have an underwater swimming speed of 0.45 m/s (MacArthur, 1992). Field observations indicate that 86.5% of dives are within the ADL of muskrats (MacArthur, 1992), but under-ice transit dives lasting up to 96 s and escape dives lasting 91 ± 8 s (range 64–184 s) have been recorded (MacArthur and Karpan, 1989).

The diving patterns of muskrats in their natural environment have not yet been recorded. However TDRs were recently used with another small semi-aquatic mammal, the American mink, diving in shallow rivers in England (Hays et al., 2007). This study

shows that the diving behavior of small free-living animals that dive only a few centimeters below the surface can be monitored, and provides the feasibility of using a TDR with species such as the muskrat. Muskrats spend the majority of their time in or near lodges and feeding shelters, usually forage within 5–10 m of a lodge or push-up, and rarely move further than 150 m from their primary dwelling lodge (MacArthur, 1978, 1980; MacArthur and Aleksiuk, 1979). This restricted geographical range could facilitate the use of biologging to gather information regarding the diving behavior and physiology of free-ranging undisturbed muskrats in their natural environment (Rutz and Hays, 2009).

SUITABILITY AS LABORATORY RESEARCH ANIMALS

Within the controlled environment of the lab, detailed physiological investigations can more easily be accomplished than during field studies. Since muskrats are expert divers, their physiological adaptations to underwater submergence are of inherent interest. It is therefore fortuitous that muskrats can be easily maintained in conventional laboratory animal facilities (Nagel and Kemble, 1974; Doyle et al., 1988), and that they adjust easily to captivity (MacArthur, 1979a). Additionally, muskrats can be easily live-trapped and transported to the lab. It is advisable, however, to obtain prior authorization from conservations agencies before trapping operations begin (Nagel and Kemble, 1974). Although muskrats can be housed in standard stainless steel cages (Doyle et al., 1988), they can also be housed in simulated pond microhabitats complete with natural foliage similar to that of a marsh microhabitat (Fish, 1983; MacArthur, 1986c; MacArthur and Karpan, 1989). From a practical standpoint, the small size of muskrats eliminates the need for extensive and/or expensive housing facilities, like those required for marine mammals, and diving tanks for muskrats can be constructed using fiberglass-lined plywood. Muskrats housed in this controlled laboratory environment have been used extensively in experiments investigating the cardiovascular, respiratory, metabolic, and behavioral responses during simulated diving. Additionally, the brains of muskrats are of a relatively uniform size and possess cytoarchitectural features comparable to other mammals (Doyle et al., 1988). Thus the preparation of a brainstem atlas allows accurate stereotaxic targeting of brainstem structures in the muskrat (Panneton and Watson, 1991).

PHYSIOLOGICAL RESPONSES DURING SIMULATED DIVING IN THE LABORATORY

Diving response

Several researchers have recorded the cardiac response of muskrats during simulated voluntary diving in laboratory facilities. In studies using ECG electrodes with trailing wire connections, the heart rate of unrestrained voluntarily diving muskrats decreases from approximately 315 to 50 BPM within 1–2 s of submergence, and then falls to approximately 30 BPM after 20–40 s (Drummond and Jones, 1979; Jones et al., 1982). The first cardiac interval after submersion is usually the longest, and upon resurfacing heart rate increases to the pre-dive level within 5 s (Drummond and Jones, 1979). Qualitatively similar results have also been found using implantable heart rate transmitters (Gilbert and Gofton, 1982; MacArthur and Karpan, 1989; McCulloch and Jones, 1990; Signore and Jones, 1995, 1996; Hindle et al., 2006; Shereshkov et al., 2006; **Figure 1**). However, although all data indicate that an immediate and substantial bradycardia accompanies every voluntary dive in muskrats, the extent of the bradycardia can vary with the nature of the dive.

Under a range of simulated field conditions in the laboratory, there is a progressively greater decrease in heart rate with foraging, exploratory, escape, and forced dives (MacArthur and Karpan, 1989; McCulloch and Jones, 1990; **Figure 2**). Presumably this progression is associated with increasing degrees of stress experienced by the muskrats. Dive heart rate is highest during voluntary dives (130 ± 9 BPM; 44% of pre-dive heart rate), is lower during escape dives precipitated by human investigators (95 ± 18 BPM; 35% of pre-dive heart rate), and is lowest during forced dives (74 ± 7 BPM; 27% of pre-dive heart rate; McCulloch and Jones, 1990). In studies using implantable heart rate transmitters (MacArthur and Karpan, 1989; McCulloch and Jones, 1990), and in contrast to studies using trailing ECG wires (Drummond and Jones, 1979; Jones et al., 1982), the bradycardia for any given dive is relatively stable and does not intensify toward the end of long duration dives. It is possible that in the latter studies the presence of the researchers, who need to be present to prevent entanglement of the trailing ECG wires, affect both the muskrats' behavior and cardiac responses to diving. In animals trained to swim an underwater maze of varying length (1–16 m), telemetered dive heart rate decreases with increasing underwater swimming distances (MacArthur and Karpan, 1989). Additionally, escape dive heart rate decreases as submersion duration increases (MacArthur and Karpan, 1989). These data suggest that not only do the cardiac responses to submersion vary with the nature of the dive, but that additional stresses imposed upon muskrats can cause a cortical potentiation of the diving response. Indeed, in decorticate muskrats heart rate in escape and forced dives are similar to those seen in voluntary dives (McCulloch and

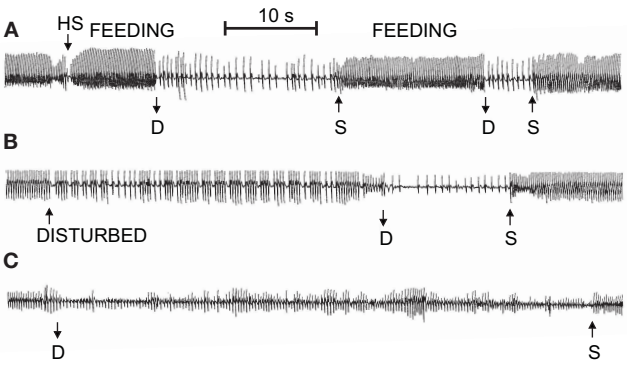

FIGURE 1 | Sample ECG recordings for free diving muskrats in the microhabitat simulation during (A) foraging activity, (B) following disturbance by the investigator, and (C) 30 min following intramuscular injection of atropine sulfate. In **(B)**, animal was disturbed by tapping on the side of the lodge. D, dive; S, surface; HS, head submersion while floating on the surface; feeding, period when animal was consuming aquatic vegetation while floating in push-up chamber (From MacArthur and Karpan, 1989).

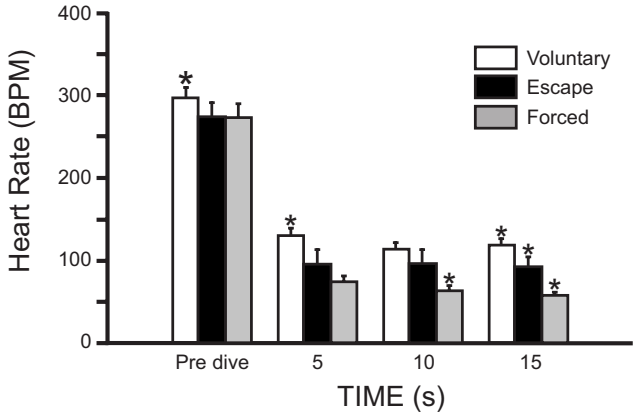

FIGURE 2 | Mean heart rate (±SEM) of muskrats during voluntary, escape, and forced dives. In all three dives there was a substantial bradycardia on submersion. Heart rates from the three dives showed separation from each other throughout their duration, but only after 15 s into the dive were all three significantly different from each other. *Indicates that heart rate is significantly different from other two heart rates at that time (Modified from McCulloch and Jones, 1990).

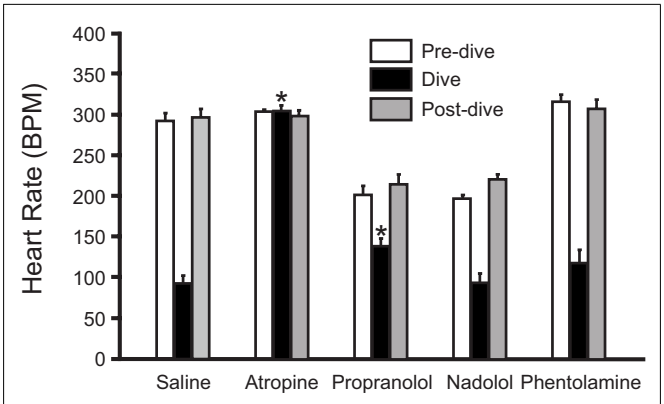

FIGURE 3 | Effect of injected drugs and submergence on mean heart rate of muskrats (±SEM; *N* = 5) in the free diving tank. Pre-dive heart rate, diving heart rate, and post-dive heart rate are shown for saline-, atropine-, propranolol-, nadolol-, and phentolamine-treated muskrats. *Indicates that diving heart rate differs significantly from diving heart rate in saline-treated animals (Adapted with permission from Signore and Jones, 1995).

Jones, 1990). However, muskrats typically do not show post-dive tachycardia or significant anticipatory changes in heart rate prior to onset or termination of spontaneous dives (Drummond and Jones, 1979; Jones et al., 1982; MacArthur and Karpan, 1989). So, although nasal receptor stimulation may be the most important factor involved in initiating reflex submersion bradycardia in muskrats (Drummond and Jones, 1979), there is a certain lability associated with the cardiac response to diving that depends, presumably, upon cortical perception of the type or condition of the dive.

Pharmacological studies indicate that the rapidly developing bradycardia that occurs during voluntary diving in muskrats is due to activation of the parasympathetic nervous system rather than sympathetic withdrawal. Injection of the muscarinic antagonist atropine eliminates diving bradycardia (MacArthur and Karpan, 1989; Signore and Jones, 1995, 1996; Shereshkov et al., 2006), while injection of β-adrenergic antagonists (nadolol or propranolol) do not (Signore and Jones, 1995, 1996; Shereshkov et al., 2006; **Figure 3**). However, β-adrenergic activation causes an increase in heart rate prior to voluntary dives, and when recovering from voluntary dives, the sympathetic nervous system helps return heart rate to pre-dive values within the first 5 s after resurfacing (Signore and Jones, 1995; Shereshkov et al., 2006). With regards to the control of heart rate during diving in muskrats, there is an accentuated antagonism between the two limbs of the ANS, such that the effects of the parasympathetic nervous system predominate over the effects of the sympathetic nervous system (Signore and Jones, 1995, 1996). Even injection of the β-adrenergic agonist isoproterenol does not cause an increase in heart rate during voluntary dives (Signore and Jones, 1996). Additionally, an increase in heart rate during underwater exercise in muskrats is due to a reduction in parasympathetic tone, whereas the increase in heart rate during exercise in air is due mainly to an increase in sympathetic tone (Signore and Jones, 1996).

During diving, bradycardia occurs in association with a peripheral vasoconstriction that is effected by sympathetic α-adrenergic control. However, diving bradycardia is unaffected by injection of the α-adrenergic antagonist phentolamine (Signore and Jones, 1995; Shereshkov et al., 2006), and thus development of diving bradycardia is independent from peripheral vasoconstriction (**Figure 3**). Both bradycardia and peripheral vasoconstriction are necessary for maximum underwater endurance (Signore and Jones, 1995). In control muskrats during forcible submergence, maximum underwater duration is 12.0 ± 1.1 min, which is reduced to 7.7 ± 0.1, 5.2 ± 0.4, and 5.2 ± 0.5 min after atropine, phentolamine, and a mixture of atropine and phentolamine, respectively (Signore and Jones, 1995). This suggests that both peripheral vasoconstriction and bradycardia greatly improve underwater duration, and that peripheral vasoconstriction is the more important of the two mechanisms. Curiously, muskrats lacking a diving response (the atropine and phentolamine group) are able to remain submerged for periods approaching their estimated ADL during voluntary dives (approximately 50 s; MacArthur, 1990), and are able to survive forcible submergence for more than 5 min (Signore and Jones, 1995). This suggests that anaerobic metabolism can play an important role during diving in muskrats (Signore and Jones, 1995).

Underwater endurance

The underwater endurance of freely diving air-breathing animals, including muskrats, depends upon the rate of oxygen usage, and the capacity to utilize oxygen stored in respiratory organs, blood, and muscles (Butler and Jones, 1997; Butler, 2004). The resting oxygen consumption of muskrats in air ranges between 0.78 and 0.85 ml O_2/g/h (MacArthur and Krause, 1989; MacArthur and Campbell, 1994; MacArthur et al., 2003), while resting oxygen consumption in thermoneutral water (29–30°C; MacArthur, 1984a) is 0.77 ± 0.04 ml O_2/g/h (Fish, 1983). In a laboratory study of recently captured animals, the allometric relationship between

mass (M) and basal metabolic rate (BMR) of field acclimatized adult muskrats is $BMR = 700M^{0.68}$ (Campbell and MacArthur, 1998). In muskrats underwater exercise is accompanied by an increase in energy expenditure which approaches that of surface swimming (Fish, 1983; MacArthur and Krause, 1989). In thermoneutral water estimated oxygen consumption of diving muskrats ranges between 2.05 and 2.49 ml O_2/g/h (MacArthur and Krause, 1989; MacArthur et al., 2003; Hindle et al., 2006), and the proportionality coefficient for diving metabolic rate (DMR) is 2.73 times that of BMR ($DMR = 1908.8M^{0.74}$; MacArthur et al., 2001). The relationship between mass and total body oxygen stores (which combines lung, blood, and muscles oxygen stores) is $33.7M^{1.09}$, giving a calculated ADL of $61.4M^{0.37}$ (MacArthur et al., 2001). The total body oxygen stores of muskrats are estimated to be 29.7 ml O_2/kg (Snyder and Binkley, 1985), and using an oxygen consumption during diving of 2.22 ml O_2/g/h (MacArthur and Krause, 1989), this gives an ADL of 48.2 s. In simulated diving environments in the lab, most dives are of relatively short duration and well within the aerobic limit (MacArthur and Krause, 1989; MacArthur et al., 2001), and only 4–6% of all dives by adult muskrats exceed their calculated ADLs (MacArthur et al., 2001).

A number of other factors can affect underwater endurance in muskrats, such as seasonal variations, ontological development and dive training, and temperature (both body and water). Winter-acclimatized muskrats have a 31% increase in BMR compared with summer-acclimatized muskrats (Campbell and MacArthur, 1998). However, compared with summer-acclimatized muskrats, winter-acclimatized adult muskrats have a 29–42% increase in total body oxygen stores, with a gain in blood oxygen accounting for most of this seasonal increase (MacArthur, 1984c, 1990; MacArthur et al., 2001). Winter-acclimatized muskrats appear to be superior divers, exhibiting greater cumulative and average dive durations and longer dive:pause ratios (MacArthur et al., 2001). Additionally, winter-acclimatized adult muskrats have an 8.6% increase in diving oxygen consumption and a 12.1% increase in calculated ADL, although these parameters are not significantly different from summer-acclimatized muskrats (MacArthur et al., 2001).

Contrary to allometric predictions, the diving abilities of muskrats do not increase with age or body size, and 1- to 2-month-old juvenile muskrats exhibit similar dive durations compared with adults (MacArthur et al., 2001). Younger muskrats likely are more dependent than adults on anaerobic metabolism during diving, as there is a greater tendency for smaller and younger muskrats to exceed their ADLs (MacArthur et al., 2001). During ontological development in muskrats there is a concurrent increase in diving experience, and so the effect of dive training was specifically investigated in a laboratory study that used a 9- to 11-week dive training protocol (MacArthur et al., 2003). MacArthur et al. (2003) found that dive training produces a 26% increase in blood oxygen stores, due mainly to increases in hematocrit and hemoglobin concentration, and a 13.5% increase in mean total body oxygen stores compared with muskrats restricted to surface swimming. However, the diving oxygen consumption of dive-trained muskrats (2.22 ml O_2/g/h) is 14.4% greater than for swim-trained muskrats (1.94 ml O_2/g/h), and consequently the calculated ADL is indistinguishable between the two groups (61.3 s for divers and 61.8 s for swimmers; MacArthur et al., 2003). Thus the relative importance of diving experience in predicting diving proficiency in muskrats is debatable.

Although muskrats actively dive beneath ice throughout winter (MacArthur, 1978, 1979b, 1980), a limitation to their wintertime diving activity may be related to thermoregulatory costs, rather than to apneic tolerance (MacArthur, 1984a). Muskrats exhibit a decrease in core body temperature when either swimming or diving in water less than 30°C (MacArthur, 1979a,b, 1984a). The extent of the decrease in body temperature and increase in post-dive oxygen consumption and recovery time is dependent upon both decreasing water temperature and increasing underwater duration (MacArthur, 1984a). Additionally, with each additional minute that a muskrat remains submerged in 3°C water, the cost of diving increases by 99 ml O_2/kg (MacArthur, 1984a). In free-ranging muskrats during winter, body temperature increases prior to foraging excursions (MacArthur, 1979b), and the metabolic heat generated during feeding (HIF – the heat increment of feeding) prior to immersion could provide a thermoregulatory benefit to muskrats by retarding the development of excessive hypothermia while diving in cold water (MacArthur, 1979b; MacArthur and Campbell, 1994). Indeed, active non-shivering thermogenesis does not occur during cold water dives in muskrats, and the primary role of interscapular brown adipose tissue (BAT) in muskrats may be to mediate rapid rewarming following repetitive foraging dives (MacArthur, 1986a). Surprisingly, muskrats do not actively exploit the body heat of nest mates by huddling to attenuate decreases in body temperature or to extend foraging time in cold water (MacArthur et al., 1997). Decreasing water temperature also produces an intensification of bradycardia during exploratory and foraging dives (MacArthur and Karpan, 1989) as well as during forced submergence (Thornton et al., 1978). Diving heart rate also decreases as core body temperature decreases (MacArthur and Karpan, 1989; Hindle et al., 2006), which may be related to a more intense abdominal vasoconstriction (MacArthur and Krause, 1989). Adult muskrats are rather tolerant of hypothermia, but do not use hypothermia to maximize underwater submergence during routine diving through a depression of metabolic rate (Hindle et al., 2006). Indeed, muskrats may defer thermoregulatory costs associated with cold water diving until the post-dive recovery period (MacArthur, 1986a; Hindle et al., 2006). However, the ability of muskrats to restore deep body temperature and acid-base balance was reduced when breathing 5–10% CO_2 during recovery from a cold water dive (MacArthur, 1986b,c).

The respiratory properties of muskrat blood generally do not differ from similarly sized mammals in terms of hematocrit, hemoglobin concentration, red blood cell count, and buffering capacity (MacArthur, 1984c; Rothstein et al., 1984; Snyder and Binkley, 1985). However, muskrat blood does have an increase in both oxygen affinity and Bohr effect, which may provide better oxygen uptake from the lung and oxygen delivery at the tissue, respectively (MacArthur, 1984c; Rothstein et al., 1984; Snyder and Binkley, 1985). Many respiratory properties of muskrat blood are enhanced during winter, which may be adaptive to the hypoxia encountered during diving and burrowing (MacArthur, 1984c). The increases in hematocrit and hemoglobin concentration in dive-conditioned muskrats could be due to the intermittent hypoxia experienced by

these muskrats during their underwater swimming (MacArthur et al., 2003).

Muskrats have significant increases in myoglobin concentration in heart, gastrocnemius, and diaphragm muscle compared with the rat (Snyder and Binkley, 1985). Skeletal muscle myoglobin concentration varies with age and is mass dependent from 250 to 600 g ($Mb = 27.7M^{1.63}$), while over 600 g muscle myoglobin concentration is independent of body size (MacArthur et al., 2001). Skeletal muscle myoglobin levels are not significantly different between muskrats trained to dive through a 16 m underwater channel and muskrats restricted to surface swimming (MacArthur et al., 2003).

Glycogen concentrations and pyruvate kinase activities in heart, brain, and gastrocnemius muscle are similar to those obtained from terrestrial animals, suggesting that muskrats tolerate submersion by adaptations primarily associated with aerobic, rather than anaerobic, metabolism (Snyder and Binkley, 1985). However enhanced buffering capacity of the hind limb swimming muscles of winter-caught muskrats implies a greater tolerance to lactic acidemia and perhaps an increased dependence on anaerobic pathways in this tissue (MacArthur et al., 2001). Isolated perfused muskrat hearts are adapted to hypoxic conditions (McKean and Landon, 1982; McKean, 1984), and have a high potential for anaerobic glycolysis (McKean et al., 1986). Also, during reoxygenation after an hypoxic insult, isolated muskrat hearts experience reduced myocardial damage compared with guinea pig hearts (McKean and Landon, 1982). Additionally, mitochondria from the left ventricle of muskrats have an increased ability to sequester calcium and maintain calcium homeostasis, which may help recovery from hypoxia, ischemia, or acidosis (McKean, 1991).

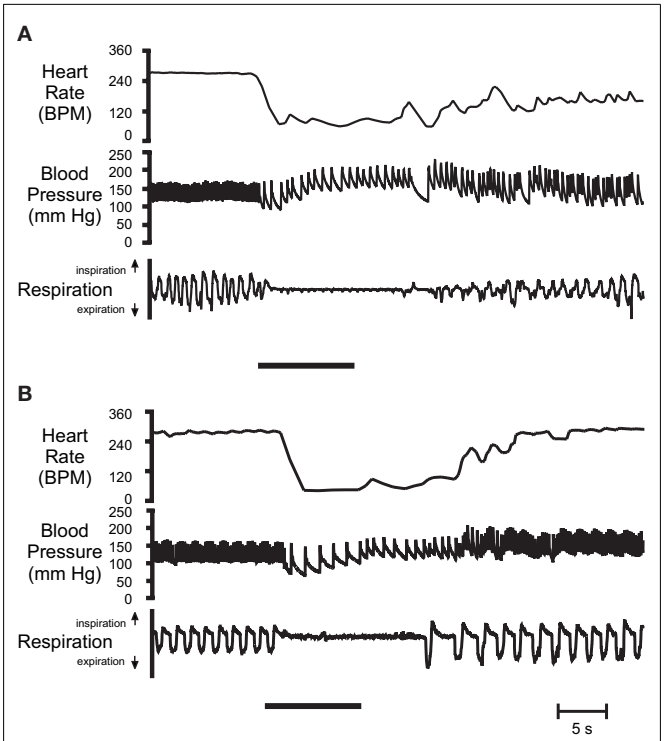

FIGURE 4 | Original cardiovascular responses of α-chloralose-urethane anesthetized muskrats after stimulation of their nasal passages with (A) nasal water flow, and (B) 50% ammonia vapors. In both cases nasal stimulation (indicated by solid bar) produced a substantial and sustained bradycardia, an increase in arterial pressure, and an apnea that lasted longer than the stimulus duration. From top: Heart rate, arterial blood pressure, and respiration (up, inspiration; down, expiration; From McCulloch and Panneton, 1997).

NEUROPHYSIOLOGICAL CONTROL OF DIVING RESPONSE

Submerging muskrats surgically anesthetized with urethane causes heart rate to immediately decrease from approximately 290 to about 80 BPM, similar to that seen in unanesthetized restrained muskrats (Drummond and Jones, 1979; Jones et al., 1982). Additionally, diving bradycardia is relatively unaffected by decerebration (Drummond and Jones, 1979; Jones et al., 1982). These experiments indicate that the cardiac responses to submersion in the muskrat are mediated within the brainstem and not at cortical levels.

The full cardiac response to submersion in anesthetized muskrats involves expression of afferent input from three groups of receptors: nasal, lung, and carotid chemoreceptors (Drummond and Jones, 1979). Pouring water on the external nares during maintained artificial ventilation causes a substantial bradycardia within 1 s, with heart rate decreasing from 292 ± 6 to 76 ± 12 BPM (Drummond and Jones, 1979). The bradycardia is eliminated if the nasal region is coated in petroleum jelly (Drummond and Jones, 1979). Bradycardia and expiratory apnea can also be caused by flowing water (Drummond and Jones, 1979; Douse and Jones, 1988; Doyle et al., 1988; Panneton, 1990; McCulloch and Panneton, 1997; **Figure 4A**) or drawing ammonia vapors (Doyle et al., 1988; Panneton, 1990, 1991b; Panneton and Yavari, 1995; McCulloch and Panneton, 1997; **Figure 4B**) through the internal nasal passages. Using saline, rather than water, attenuates the depth of the bradycardia, and anesthetizing the nasal passages with local anesthetic completely eliminates the cardiac and respiratory responses to nasal water flow (Drummond and Jones, 1979). The nasal passages are innervated by the anterior ethmoidal nerve (AEN), a branch of the ophthalmic division of the trigeminal nerve, and electrical stimulation of this nerve produces immediate and sustained bradycardia (McCulloch et al., 1999a). However, Drummond and Jones (1979) found that bilateral section of the maxillary division of the trigeminal nerve abolishes the cardiac and respiratory response to nasal water flow, whereas bilateral section of the ophthalmic division has little effect. The AEN of the muskrat contains a high concentration of small diameter fibers, 62% being unmyelinated C fibers and 27% being slightly myelinated Aδ fibers (McCulloch et al., 1999a). These small diameter fibers likely carry, from the nasal passages to the brainstem, the afferent information necessary for initiating the immediate cardiorespiratory responses seen after nasal stimulation (McCulloch et al., 1999a). In spontaneously breathing anesthetized muskrats, water stimulation of the nasal region causes apnea, with the lungs collapsing to the expiratory position (Koppanyi and Dooley, 1929; Drummond and Jones, 1979). Withdrawal of lung stretch receptor input through lung deflation enhances the cardiac response to nasal stimulation, although the response to nasal water flow is intact even when the nasal stimulation is applied during a period of maintained lung inflation (Drummond and Jones, 1979). Maintaining

artificial ventilation during the period of nasal water flow reduces the magnitude of the bradycardia, whereas lung deafferentation eliminates any direct effect of artificial ventilation on heart rate during nasal stimulation (Drummond and Jones, 1979). Stimulation of peripheral chemoreceptors by decreasing oxygen tensions could contribute to and intensify the bradycardia toward the end of a long duration dive, but chemoreceptor afferents in the muskrat are likely not involved in producing the initial bradycardia seen upon submersion (Drummond and Jones, 1979; Douse and Jones, 1988). Additionally, pre-dive exposure to 5–10% CO_2 has no effect on the establishment of the bradycardia in conscious muskrats during forced diving (MacArthur, 1986b).

Nasal water flow causes an initial decrease in mean arterial blood pressure that accompanies the onset of the bradycardia (Drummond and Jones, 1979; Douse and Jones, 1988; McCulloch and Panneton, 1997), but blood pressure usually increases to pre-stimulation values or above within 10–20 s (Drummond and Jones, 1979; Douse and Jones, 1988). Similar blood pressure responses are found in unanesthetized, anesthetized, and decerebrated muskrats during restrained dives (Jones et al., 1982). However, any afferent input by the arterial baroreceptors toward intensifying the bradycardia that develops due to nasal stimulation appears to be minimal (Drummond and Jones, 1979; Douse and Jones, 1988). Since arterial blood pressure is maintained or elevated during nasal stimulation in the muskrat, this suggests an increase in total peripheral resistance greater than the decrease in cardiac output caused by the bradycardia. This also suggests a peripheral vasoconstriction that redistributes blood flow to oxygen-dependent tissue (Scholander, 1940). Indeed, during submergence in muskrats anesthetized with pentobarbital, the proportion of cardiac output going to the brain and heart increases by factors of 15 and 5, respectively, and the proportion of cardiac output decreases to all other tissue, especially the stomach and intestines (Jones et al., 1982).

A detailed review of the central pathway of the diving response is beyond the scope of this review, and readers are instead referred to Panneton's contribution in this issue. However, a brief synopsis of studies in which muskrats have been used for this purpose will be presented. The primary afferent projections of the nerves that innervate the upper respiratory tract of the muskrat were determined by transganglionic transport of horseradish peroxidase conjugated to wheat-germ agglutinin (HRP-WGA; Panneton, 1991a). The central projections of the AEN, the nerve that innervates the nasal passages, are found within discrete areas of the ipsilateral trigeminal sensory complex, especially the ventrolateral part of superficial laminae of the spinal trigeminal nucleus caudalis (also known as the medullary dorsal horn, MDH; Panneton, 1991a). Injections of anterograde tracers [either biotinylated dextran amine (BDA), or HRP-WGA] into this specific MDH location identified brainstem projections of the secondary afferent neurons located within the MDH (Panneton et al., 1994, 2000). Additionally, transport of a virus that crosses synapses and transports in the anterograde direction [herpes simplex virus (HSV-1), strain 129] was used to follow the primary, secondary, and tertiary brainstem relays of the AEN (Panneton et al., 2000). These studies provide an anatomical base for the potential brainstem circuit of the diving response in the muskrat, especially the afferent portion of this circuit. Functionally, the MDH is an important part of the brainstem circuitry, as reversible blockade of this location with either the local anesthetic lidocaine or the glutamate receptor antagonist kynurenate abolishes the cardiorespiratory responses to nasal stimulation (Panneton, 1991b; Panneton and Yavari, 1995). Additionally, Fos, a marker of activated neurons, is immunohistologically detected within MDH secondary neurons after repetitive nasal stimulation with either nasal water flow or ammonia vapors (McCulloch and Panneton, 1997). Significant increases in Fos are also found in the dorsal reticular formation and in the area of A5 catecholaminergic group (McCulloch and Panneton, 1997). Concerning the efferent limb of the central circuitry of the diving response that would be involved in the production of the parasympathetically mediated bradycardia, cell bodies of cardiac preganglionic motoneurons of muskrats are primarily located in the external formation of the nucleus ambiguus after WGA-HRP is injected into the cardiac plexi located near the fat pads at the base of the heart (Panneton et al., 1996). However, there is sparse anatomical information that currently links the afferent limb of this circuit in the MDH to the efferent parasympathetic limb of the circuit within the external formation of the nucleus ambiguus.

RATS

NATURAL DIVING HISTORY

The common albino rat is a strain of *Rattus norvegicus*, or Norway rat, which is indicated by the fact that the two interbreed freely (Donaldson, 1912; Richter, 1954). The Norway rat, also known as the brown or sewer rat, was the first animal to have become domesticated strictly for scientific purposes (Richter, 1954). *R. norvegicus* originated in eastern Asia, and slowly migrated westward, reaching major European cities by the 1720s, and North America by 1775 (Donaldson, 1912; Robinson, 1965; Hanney, 1975; Walker, 1975; Lindsey et al., 2006). *R. norvegicus* live in nearly all parts of the world and in practically all land habitats, especially in close association with man (Jackson, 1982; Lindsey et al., 2006). Natural history of *R. norvegicus* indicates that they live in watery areas, such as sewers, ditches, and marshes (Walker, 1975; Jackson, 1982). While *R. rattus*, or black rat, usually live in the roofs of buildings, *R. norvegicus* usually live in the cellar or basement of buildings (Hanney, 1975; Jackson, 1982). *R. norvegicus* are excellent swimmers (Cottam, 1948; Dagg and Windsor, 1972; Jackson, 1982) even as neonates (Schapiro et al., 1970; Dagg and Windsor, 1972; Clarac et al., 1998), and can island-hop by swimming 400 m across open ocean (Russell et al., 2005). Feral rats will dive for food intended for fish, and prey on young fish, in a fish hatchery (Cottam, 1948). Field observations indicate that many members of some colonies of wild rats (*R. norvegicus*) dive for and feed on snails and mussels inhabiting the bottom of the Po River in Italy (Gandolfi and Parisi, 1973; Parisi and Gandolfi, 1974). Observational studies of wild rats in a semi-natural environment indicate that rats can dive underwater and exhibit intense predation on freshwater mollusks (Nieder et al., 1982; Nieder, 1985). Additionally, rats living in a Chilean intertidal zone prey upon 40 different types of intertidal species, including mobile organisms from the mid to very low intertidal zone, which suggests rats dive to capture prey (Navarrete and Castilla, 1993). Collectively these studies indicate that wild *R. norvegicus* exploit semi-aquatic

environments, and will often dive underwater while foraging for food. Currently there is sparse information available regarding physiological characteristics of the natural diving performance in small-bodied mammalian divers such as the rat. However, the dive performance, oxygen storage capacity, partitioning of body oxygen stores and ADL has been investigated in the star-nosed mole, one of the world's smallest mammalian divers (McIntyre et al., 2002). This suggests that similar information could soon be available for the rat.

SUITABILITY AS LABORATORY RESEARCH ANIMALS

Although rat fanciers in Japan bred rats for unique coat colors in the 1700s (Jacob, 2010), it is quite likely that the domestication of albino *R. norvegicus* involved the sport of "rat-baiting" (Robinson, 1965; Hanney, 1975; Lindsey et al., 2006). In Europe in the eighteenth century, a popular arena betting sport involved training dogs to kill as many rats as possible in as short a time as possible, and is probably the origin of the term "rat race." A champion dog, "Billy," was able to kill 100 rats in 5 min 30 s (Hanney, 1975)! To ensure availability of rats for this sport, promoters would collect and hoard hundreds of rats. Often, when naturally occurring albino rats were discovered, these rats would be retained and kept for show purposes and/or breeding. It is likely that these albino show rats, or their descendants, were the first to be used in scientific experiments, especially if they were semi-tamed by frequent handling from birth (Richter, 1954; Robinson, 1965). The first published paper using albino rats in the laboratory (by Philipeaux, 1856) was on the effects of adrenalectomy (Richter, 1954; Robinson, 1965).

Systematic development of breeding colonies of albino rats was started in the late 1800s and early 1900s (Lindsey et al., 2006), and Henry H. Donaldson of the Wistar Institute in Philadelphia is credited with popularizing the use of Norway rats for scientific purposes (Richter, 1954; Lindsey et al., 2006). Standardization of the albino rat at the Wistar Institute through selective breeding gave a broad foundation for the use of the rat in nutrition, biochemistry, endocrinology, genetic, behavioral, psychobiology and neuroscientific research (Lindsey et al., 2006). While working at the Wistar Institute, Eunice Chace Greene published the all-time classic "Anatomy of the Rat" (Greene, 1963), and Helen Dean King was instrumental in the inbreeding experiments that helped establish many different strains of albino rats (Lindsey et al., 2006). The Wistar Institute was also instrumental in developing modern rat husbandry standards, as clean and healthy rats are essential for accurate research (Lindsey et al., 2006). Most present-day domesticated *R. norvegicus* are albino, and their clean white appearance undoubtedly enhances their popularity (Richter, 1954). Additionally, defects in vision due to lack of pigmentation tends to make them less likely to escape and therefore easier to handle (Richter, 1954). There are many strains of albino and hooded *R. norvegicus*, including PA, Lewis, Buffalo, Long–Evans, Fischer, Sprague-Dawley, Holtzman, Albany, PAR/Lou, and germ-free (gnotobiotic), many of which can trace their origins to the original Wistar strain (Lindsey et al., 2006).

Scientific investigation with the albino rat often receives criticism about the perceived artificiality of the domestic rodent (Boice, 1971, 1981). Consistent with this doubt is the assumption that the albino rat is degenerate and dull compared with wild rats (Robinson, 1965). Albinism occurs naturally in *R. norvegicus*, and appears in wild Norway rats bred in captivity (Donaldson, 1912; Hatai, 1912; Richter, 1954). Albinism is due to a recessive gene, *c*, and rats homozygous for *c* are totally depigmented, having white pelage and pink eyes (Robinson, 1965). Although there are differences between wild and albino rats in terms of their endocrine system and behavior, these differences have not been attributed to the albino allele (Robinson, 1965). Albino rats are more docile and less nervous than wild rats, making them easier to handle (Richter, 1954; Hulin and Quinn, 2006). Additionally, albino rats have smaller adrenal glands, making them less capable of dealing with conditions of stress, and have larger gonads (Richter, 1954). Except for mutant forms of the laboratory rat specifically bred for biomedical investigations (Altman and Katz, 1979), the differences in the endocrine system appear to be the main physiological difference between laboratory rats and feral rats. Albino rats can thrive in feral conditions (Price and Huck, 1976; Boice and Adams, 1980), and a colony of wild albino rats estimated at 2000 individuals survived several winters (with temperatures reaching −25°F) at the dump in Missoula Montana (Minckler and Pease, 1938). Thus, although domestication has produced some anatomical, physiological, and behavioral changes in laboratory rats (Richter, 1954), research has demonstrated that behaviorally laboratory rats are representative of wild *R. norvegicus* (Boice, 1981) and that domestication is not necessarily equivalent to degeneracy (Boice, 1973). However, genotypically there are differences between wild *R. norvegicus* and various strains of inbred rats (van den Brandt et al., 2000; Kloting et al., 2003; Hulin and Quinn, 2006).

The considerable background work in physiology and biochemistry has made the rat an ideal experimental animal in many areas of biological research (Gill, 1985; Gill et al., 1989; Jacob, 2010). Indeed the rat has been used extensively in the fields of neuroscience, basic genetics, cancer, immunology, reproduction, behavior, aging, toxicology, and transplantation (Gill, 1985; Jacob, 2010). More is known about the rat than any non-human species (Richter, 1954; Gill, 1985; Gill et al., 1989; Jacob, 2010), and it is sometimes forgotten that the phenomenal progress of biomedical research would have been impossible without the use of rodents as experimental animals (Hanney, 1975; Gill, 1985). Among many of the advantages the rat has for its use in scientific research is its size: the rat is large enough to be handled easily and to allow surgical manipulations, and is small enough to be conveniently and economically housed in large numbers in animal facilities (Richter, 1954; Gill et al., 1989). The rat is a particularly useful experimental animal for cardiorespiratory research, including the diving response, because the physiology of the rat is well characterized across all major organ systems, its anatomy [both gross (Greene, 1963) and neuroanatomical (Paxinos and Watson, 1998; Paxinos et al., 1999; Swanson, 2004)] is well known, and the whole animal is ideal for work relating to systems biology (Jacob, 2010). Additionally, the recent publication of the rat genome database should facilitate an explosive growth in the use of the rat as a biomedical model in the near future (Jacob, 2010).

PHYSIOLOGICAL RESPONSES DURING SIMULATED DIVING IN THE LABORATORY

Voluntarily diving rats have a substantial diving response that is qualitatively similar to that of muskrats. Comparable results in rats are found whether heart rate and blood pressure are recorded using trailing arterial cannulae (McCulloch et al., 1997; Ollenberger and West, 1998b) or implanted transmitters (McCulloch et al., 2010; Panneton et al., 2010b). In rats trained to dive 3 m through an underwater maze, heart rate, and mean arterial blood pressure decrease immediately upon submersion, heart rate by 78% (from 453 ± 12 to 101 ± 8 BPM) and mean arterial blood pressure by 25% (from 143 ± 1 to 107 ± 5 mmHg; McCulloch et al., 2010; **Figure 5B**). After its initial decrease, mean arterial blood pressure then increases, reaching a maximum of 174 ± 3 mmHg 4–5 s after submersion (McCulloch et al., 2010). Pre-existing chemoreceptor drive, achieved by altering arterial PO_2 and/or PCO_2, does not have any effect on the cardiovascular responses to voluntary diving (McCulloch et al., 1997). Additionally, during long duration (approximately 100 s) forced dives, rats do not attempt to breathe even though there are radical changes in arterial PO_2, PCO_2, and pH (Panneton et al., 2010a). Together these studies suggest that the chemoreceptor reflex in rats is not important in initiating the cardiovascular response to diving and is actually suppressed during diving. However, bilateral section of the carotid sinus nerve or destruction of the carotid body chemoreceptors attenuates the bradycardia response during forced submersion in conscious rats (Huang and Peng, 1976). Pretreatment with the muscarinic antagonist atropine eliminates the bradycardia associated with voluntary diving, and, even with the decrease in cardiac output due to the bradycardia, mean arterial blood pressure increases to 202 ± 5 mmHg during the dive (McCulloch et al., 1997). These results suggest that during voluntary diving in the rat there is both a parasympathetically mediated bradycardia and a sympathetically mediated peripheral vasoconstriction (McCulloch et al., 1997, 2010). Blood corticosterone levels indicate that rats not trained in the diving protocol find voluntary diving stressful, whereas repetitive daily training in rats decreases the stressfulness associated with voluntary diving (McCulloch et al., 2010). Trained rats find diving no more stressful than being handled daily by a human (McCulloch et al., 2010). However dive training has no effect on diving heart rate or mean arterial blood pressure, as quantitatively similar heart rate and blood pressure responses are found in both trained and untrained rats during voluntary diving (McCulloch et al., 2010).

Forced submersion in conscious rats either trained to dive or naïve to diving also produces a substantial bradycardia, with heart rate decreasing from approximately 460 to 90 BPM when using implanted transmitters (McCulloch et al., 2010; Panneton et al., 2010a,b), from approximately 400 to 105 BPM when using trailing ECG leads (Fahlman et al., 2011), and from approximately 400 to 140 BPM when using trailing arterial cannulae (Lin, 1974; Lin and Baker, 1975; Huang and Peng, 1976). During forced diving in rats trained to dive, mean arterial pressure decreases immediately upon submersion, from 135 ± 2 to 119 ± 5 mmHg, but then increases to 189 ± 4 mmHg 4–5 s into the dive (McCulloch et al., 2010; **Figure 5C**). During forced submersion cardiac output decreases by approximately 70% (Lin, 1974; Lin and Baker, 1975), and blood flow is maintained to the coronary, bronchial, and cerebral circulations but decreases to the intestine, spleen, kidney, tail, and skin (Lin and Baker, 1975). There is a great reduction in blood flow to muscle during long duration forced dives in rats, as blood lactate levels due to muscle anaerobic glycolysis increase during recovery and not during the dive (Scholander, 1940). During forced submersion in conscious rats, pretreatment with the muscaric antagonist atropine greatly attenuates, but does not eliminate, the bradycardia (Lin, 1974). This surprising finding may be related to the intraperitoneal, rather than intravenous, route of atropine injection. Still, total peripheral resistance increases

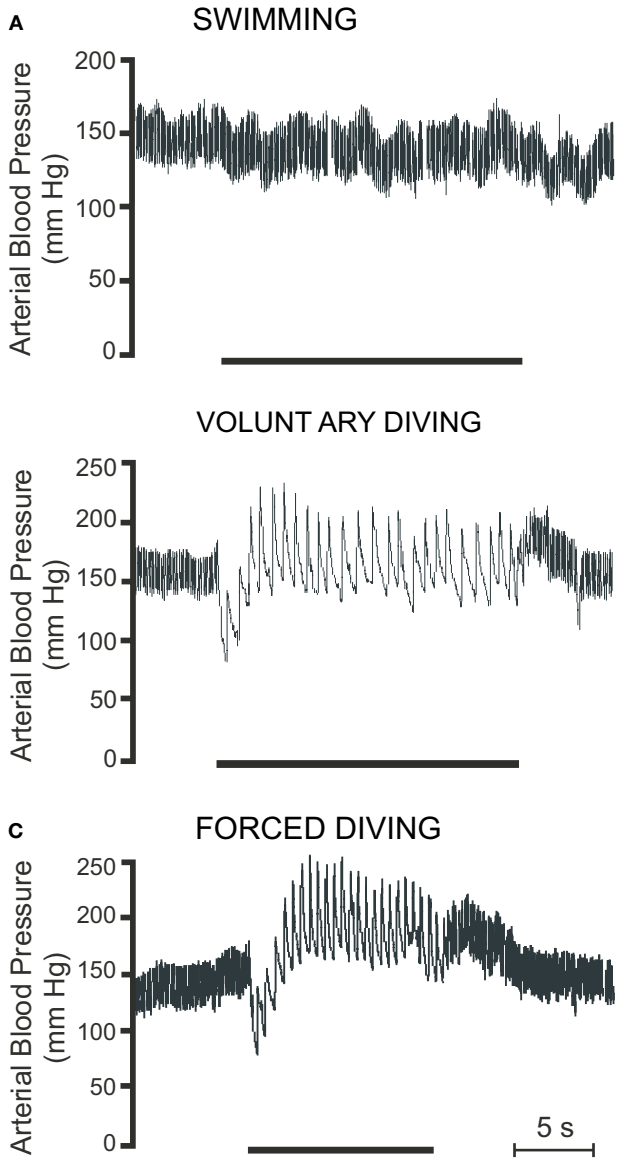

FIGURE 5 | Raw traces showing pulsatile arterial blood pressure during (A) swimming, (B) voluntary diving, and (C) forced diving in rats trained to dive. Period of swimming or diving is indicated by the bar underneath the trace. Breaks in trace indicate periods where the radiotelemetric signal was lost (Modified from McCulloch et al., 2010).

by a factor of 4 during forced submersion, and pretreatment with intraperitoneal atropine produces a 37% increase in arterial blood pressure (Lin, 1974). These results thus suggest that forced submersion, like voluntary submersion, also causes both parasympathetically mediated bradycardia and sympathetically mediated peripheral vasoconstriction. Changes in cardiac output distribution similar to those seen during forced diving were later found in voluntarily diving rats, as there is a 69% decrease in cardiac output and a 438% increase in peripheral resistance (Ollenberger and West, 1998b). Blood flow is largely restricted to the head and thorax (Ollenberger et al., 1998), and there is a 21% decrease in cerebrovascular resistance that results in a 168% increase in cerebrovascular blood flow (Ollenberger and West, 1998b). Blood corticosterone levels indicate that forced diving is stressful to both trained and untrained rats (McCulloch et al., 2010). The magnitude of bradycardia is similar during both voluntary and forced diving (McCulloch et al., 2010; Panneton et al., 2010b), while the increase in blood pressure is greater during forced diving (McCulloch et al., 2010). Therefore rats appear to be different from muskrats, as muskrats show an intensification of the bradycardia during forced dives compared with voluntary dives (MacArthur and Karpan, 1989; McCulloch and Jones, 1990). Also rats have a maximal forced dive underwater endurance of 2 min, which is much less than the 12 min of muskrats (Irving, 1939; Scholander, 1940). Rats, like muskrats, have an accentuated antagonism between the parasympathetic and sympathetic limbs of the ANS in regards to the control of heart rate, so that during diving parasympathetic activity overpowers sympathetic activity (McCulloch et al., 2010). Due to the variability of the responses and the differences of the responses compared with voluntary diving, it is suggested that the use of forced submergence in naïve rats not trained to dive should avoided when investigating the hemodynamic responses to diving (Panneton et al., 2010b).

There may also be a genetic component to the magnitude of the bradycardic response to forced diving in rats (Fahlman et al., 2011). The responses of two inbred strains of rats (Fischer and Buffalo) were compared to the responses from an outbred strain of rats (Wistar). Despite similar pre-dive heart rates (approximately 400 BPM), dive heart rate is 121 ± 14 BPM in Fischer rats, 103 ± 31 BPM in Wistar rats, and 93 ± 13 BPM in Buffalo rats (Fahlman et al., 2011). Thus genetically distinct populations of rats demonstrate divergent cardiac responses to diving, which suggests that a portion of the mammalian diving response may be a heritable trait.

In addition to the physiological investigation of the diving response in rats, psychologists have used diving-for-food situations in rats to show the influence of spatial environment on social organization (Grasmuck and Desor, 2002) and investigate the importance of social learning in the acquisition of behavior (Galef, 1980, 1982). Additionally, psychologists have found during the investigation of learned helplessness that rats will often dive underwater during the exploratory phase of the forced swim test (Binik et al., 1979; Hawkins, 1987; Abel, 1994; Kelliher et al., 2000; Linthorst et al., 2002; Campbell et al., 2003).

NEUROPHYSIOLOGICAL CONTROL OF DIVING RESPONSE

Submerging rats anesthetized with urethane causes a 51% decrease in heart rate and a 23% increase in mean arterial blood pressure (Huang et al., 1991). Nasal water flow plus apnea in rats anesthetized with Innovar (a combination of droperidol and fentanyl) produces a 77% decrease in HR and a 41% decrease in mean arterial blood pressure (McCulloch and West, 1992; McCulloch et al., 1995; Ollenberger and West, 1998a; **Figure 6A**). Nasal stimulation with ammonia vapors in rats anesthetized with urethane causes a 43% decrease in heart rate and an 11% increase in mean arterial blood pressure (Rybka and McCulloch, 2006; Hollandsworth et al., 2009; Panneton et al., 2010b; **Figure 6B**). Nasal stimulation with 100% CO_2 in rats anesthetized with a combination of α-chloralose and urethane causes a 47% decrease in heart rate and a 28% increase in mean arterial blood pressure (Yavari et al., 1996; **Figure 6C**). Collectively these studies indicate that anesthetized rats exhibit cardiovascular responses during nasal stimulation similar to those observed during conscious voluntary diving, although the magnitude of the responses are variable and dependent upon the anesthetic and method of nasal stimulation used in each preparation. However, although the physiological responses of nasopharyngeal stimulation in anesthetized animals resemble those seen in conscious diving animals, it is uncertain if the same neural circuits are used in eliciting this response (Panneton et al., 2010b). Conversely, these table preparations enable extremely detailed physiological investigations in a more controlled laboratory setting. In a comparison of various techniques that could be used to investigate the central control of the diving response, it was suggested that decerebrated, rather than anesthetized, rats receiving nasal stimulation with ammonia vapors be used, as decerebrated rats also show cardiovascular responses similar to those seen during voluntary diving (Panneton et al., 2010b). Additionally, because these responses are seen in decerebrated animals, this suggests that the neural circuits for the diving response are intrinsic to the brainstem (Panneton et al., 2010b).

Stimulation of the trigeminal receptors innervating the nose and nasal passages is thought to provide the most important afferent input for the initiation of the cardiovascular responses to diving. The cardiovascular changes associated with diving are initiated with immersion of the rats' nares in the water, as opposed to just swimming on the surface of the water (McCulloch et al., 2010; Panneton et al., 2010b; **Figure 5A**). Infusing the nasal passages of the rat with local anesthetic eliminates the cardiovascular responses to nasal stimulation (McCulloch et al., 1995; Yavari et al., 1996). Bilateral sectioning of the trigeminal nerve, especially the AEN, also virtually eliminates the heart rate response to nasal simulation (McCulloch et al., 1995; Rybka and McCulloch, 2006). However, after unilateral sectioning of one AEN, the remaining AEN can still provide sufficient afferent input to initiate the cardiorespiratory changes consistent with the nasopharyngeal response (McCulloch et al., 1995; Rybka and McCulloch, 2006). Electrical stimulation of the rat AEN produces bradycardia, a slight hypertension, and apnea, both in anesthetized whole animal preparations (Dutschmann and Herbert, 1996, 1997, 1998b), and unanesthetized decerebrate working heart brainstem preparations (Dutschmann and Paton, 2002a,b; Dutschmann et al., 2004; Rozloznik et al., 2009; **Figure 6D**). It is likely that small unmyelinated fibers within the AEN of the rat are responsible for carrying the afferent information that initiates the cardiorespiratory

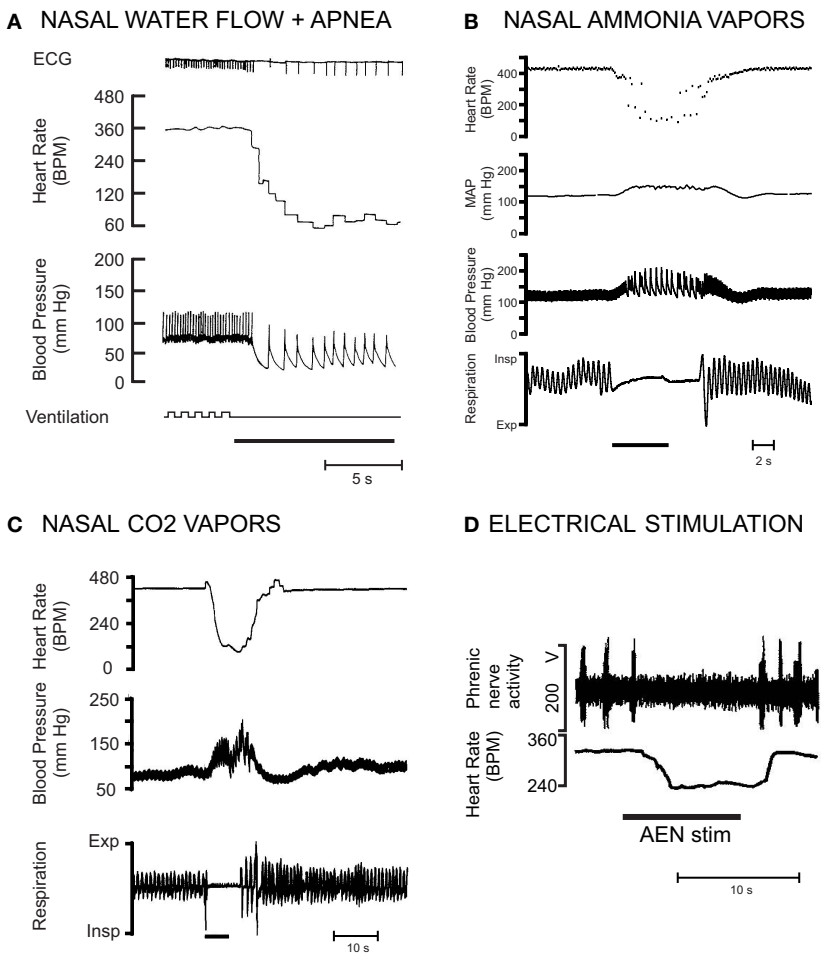

FIGURE 6 | Examples of different ways that the diving response has been in initiated in rat preparations. The magnitude of the responses are variable and dependent upon the anesthetic and method of nasal stimulation used. **(A)** Nasal water flow plus concurrent apnea in a paralyzed and artificially ventilated rat anesthetized with Innovar (From McCulloch and West, 1992). **(B)** Nasal stimulation with ammonia vapors in a rat anesthetized with urethane (From Hollandsworth et al., 2009). **(C)** Nasal stimulation with 100% carbon dioxide in a rat anesthetized with a mixture of α-chloralose and urethane (From Yavari et al., 1996). **(D)** Electrical stimulation of the anterior ethmoidal nerve in a neonatal rat *in situ* unanesthetized decerebrate arterially perfused working heart brainstem preparation (Modified from Dutschmann et al., 2004).

responses to nasal simulation to secondary neurons located within the MDH (Hollandsworth et al., 2009).

Although activation of receptors in the nasal passages is important for initiating the cardiovascular responses in the anesthetized rat, lung deflation during the nasal stimulation is necessary to sustain the cardiovascular changes (McCulloch and West, 1992). Nasal water flow plus expiratory apnea results in an immediate and sustained bradycardia and hypotension, and is more effective in inducing cardiovascular changes than when either of the two stimuli are applied individually (McCulloch and West, 1992). Stimulation of afferents within the recurrent laryngeal nerve or superior laryngeal nerve is not required for elicitation of the cardiovascular responses to nasal water flow, as the responses are unaltered after sectioning of these nerves (McCulloch and West, 1992). Chemoreceptor stimulation is unnecessary for the initiation of the cardiovascular responses to nasal water flow, as reduction of chemoreceptor stimulation, or chemoreceptor stimulation through pre-existing hypoxia or hypercapnia, does not alter the cardiovascular responses to nasal stimulation (McCulloch and West, 1992). However, a potent facilitation of bradycardia is elicited by simultaneous submaximal activation of AEN afferents and peripheral chemoreceptors in a rat working heart brainstem preparation, and this could be the basis of the intensification of bradycardia during the late states of a dive when the afferent drive from peripheral chemoreceptors is increased (Rozloznik et al., 2009). Also, during long duration (50 s) simulated dives in anesthetized rats, increasing arterial CO_2 produces a decrease in cerebrovascular resistance and an increase in cerebral blood flow (Ollenberger and West, 1998a).

As stated previously, a detailed review of the central pathway of the diving response is beyond the scope of this review, and again readers are instead referred to Panneton's contribution in this issue. However, a brief synopsis of studies in which rats have been used for this purpose will be presented. The central projections of

the AEN were followed using transganglionic tracers (Panneton et al., 2006; Hollandsworth et al., 2009). After injecting WGA-HRP into the AEN, terminal projections are seen throughout the trigeminal sensory complex, but primarily within laminae I and II of the ventral tip of the ipsilateral MDH at the level of the area postrema (Panneton et al., 2006; Hollandsworth et al., 2009). Most of the AEN projections to the MDH likely are small unmyelinated fibers (Hollandsworth et al., 2009). Other non-trigeminal AEN projections are to the ventrolateral medulla and parabrachial complex (Panneton et al., 2006; Hollandsworth et al., 2009). After repetitive voluntary diving (McCulloch, 2005) or nasal stimulation (Dutschmann et al., 1998; Rybka and McCulloch, 2006; Hollandsworth et al., 2009), neurons within the MDH of rats express the protein Fos, a marker of neuronal activation. Injection of anterograde tracers into the MDH identified the connections of these secondary neurons to important autonomic locations within the brainstem, including the nucleus tractus solitarius (NTS), ventrolateral medulla, A5, Kölliker-Fuse, and parabrachial complex (Feil and Herbert, 1995; Panneton et al., 2006). Injection of the retrograde tracer Fluorogold into these tertiary brainstem locations confirmed the projections of the MDH neurons (Feil and Herbert, 1995; Panneton et al., 2006). These anatomical connections may potentially identify the neural circuits of the diving response in the rat (Panneton et al., 2006).

The NTS may play a role in modulation of the diving response, as lesions of the NTS attenuates the diving bradycardia (Huang et al., 1991), and microinjection of a 5-HT_3 agonist into the commissural NTS potentiates the bradycardia (Rozloznik et al., 2009). Additionally, injection of the calcium channel blocker cobalt chloride into the medial NTS blocks the pressor response elicited by electrical stimulation of the AEN (Dutschmann and Herbert, 1998a). However these results may be debatable, as injection of the glutamate receptor antagonist kynurenate into the NTS does not reduce the increase in splanchnic sympathetic nerve discharge seen after nasal stimulation with ammonia vapors (McCulloch et al., 1999b). Selective groups of catecholaminergic neurons within the brainstem, specifically the A1, C1, A2, A5, sub-coeruleus areas (McCulloch and Panneton, 2003), and globosa neurons within the lateral A7 area (McCulloch, 2003), express Fos after repetitive voluntary diving. A majority of bulbospinal sympathoexcitatory neurons within the rostral C1 area of the rostral ventrolateral medulla are activated by nasal stimulation (McCulloch et al., 1999b), and may be responsible for the increase in sympathetic tone that occurs during diving. Nasal stimulation also affects the firing patterns of neurons within the ventrolateral medulla that have respiratory-related activity (McCulloch et al., 1999b; Dutschmann and Paton, 2002a,b). Nasotrigeminal stimulation causes inspiratory neurons to cease firing and hyperpolarize, and postinspiratory neurons to depolarize and discharged persistently (Dutschmann and Paton, 2002a). After long duration forced dives, Fos-positive neurons are found in brainstem areas that contain chemosensitive neurons, such as the ventral surface of the medulla, the midline raphe, the parapyramidal nucleus, and retrotrapezoid nucleus, as well as in the commissural NTS that receives primary afferent projections from peripheral chemoreceptors (Panneton et al., 2010a). Additionally, after injections of the anterograde tracer BDA into the MDH, labeled fibers are located along the ventral surface of the medulla where presumptive chemosensitive Fos-positive neurons are located (Panneton et al., 2010a). It may be these connections that inhibit the chemoreceptor reflex during diving (Panneton et al., 2010a). More rostrally within the brainstem, neurons within the Kölliker-Fuse nucleus may mediate the apnea that is induced after trigeminal stimulation in the rat (Dutschmann and Herbert, 1996, 1997, 1998b, 1999; Dutschmann et al., 1998, 2004).

SUMMARY

Investigation of the central nervous integration of the cardiorespiratory responses to diving is important for a number of reasons. The diving response enables animals to remain submerged underwater for extended periods of time, and an understanding of how this occurs is of inherent interest. The diving response demonstrates one of the most powerful patterns of autonomic reflexes observed in animals, and represents a radical functional reorganization of brainstem homeostatic control. This also is of inherent interest. The diving response may also be important clinically as part of the trigemino-cardiac reflex, nasopharyngeal reflex, and/or sudden infant death syndrome. Finally, an understanding of the neuronal circuitry that exists within the brainstem of animals like muskrats and rats will help determine how cortical afferent signals in marine animals can modify the basic autonomic reflex.

From preceding sections, it is obvious that both muskrats and rats have a very robust diving response similar to that of many marine species. In this regard, both these animals are ideal choices for investigation of the physiological responses to diving (**Table 1**). Additionally, if a species is to be used in neurophysiological or neuroanatomical studies, then the brains from adults of that species need to be of a relatively uniform size. This uniformity is necessary to enable stereotaxic targeting of brain and brainstem structures. Additionally, the organization of that species' brain should not deviate significantly from the typical mammalian scheme. This is necessary to facilitate comparisons of the functions and anatomical connections of homologous structures across species. The brains of muskrats and rats fulfill these criteria, and atlases of the brainstem of muskrats and rats are available. These atlases have facilitated the investigation of the central pathways of the diving response. In contrast, investigations elucidating the neural control of the diving response in marine mammals are much more difficult, because of their native aquatic environment, their relatively large and non-uniformly sized brains, and the paucity of information about those brains. Additionally, the cost associated with building and maintaining the facilities necessary to house marine animals may make such investigations prohibitive.

Both the muskrat and rat have advantages and disadvantages in their use as models for investigating the central control of the diving response. One advantage for using muskrats is that they have a true semi-aquatic lifestyle in their natural habitat. Many aspects of the behavior and physiology of muskrats in both field and simulated diving situations have been investigated and well characterized. In comparison, rats are primarily terrestrial animals, although feral rats can and do dive in their natural habitat. Other advantages for using muskrats are their size, availability, and modest housing requirements, at least in comparison with marine animals. Rats have the same advantage of size. Additionally, rats are very easily available from many commercial vendors,

Table 1 | Comparison of diving characteristics in muskrats and rats.

Parameter	Muskrat	Rat
WEIGHT		
Adult male (g)	1217 ± 124 (Doyle et al., 1988)	300–400 (Baker et al., 2006)
Adult female (g)	1008 ± 121 (Doyle et al., 1988)	250–300 (Baker et al., 2006)
HEART RATE		
Resting (BPM)	241 ± 16 (McCulloch and Jones, 1990)	405 ± 4 (McCulloch et al., 2010)
Disturbed (BPM)	259 ± 13 (McCulloch and Jones, 1990)	453 ± 5 (McCulloch et al., 2010)
Diving, voluntary		
Pre-dive (BPM)	297 ± 13 (McCulloch and Jones, 1990)	453 ± 12 (McCulloch et al., 2010)
Dive (BPM)	130 ± 9 (McCulloch and Jones, 1990)	101 ± 8 (McCulloch et al., 2010)
SWIMMING SPEED (in lab)		
Surface (m/s)	0.2–0.75† (Fish, 1984)	0.22 ± 0.01 (McCulloch, unpublished)
Underwater (m/s)	0.45 ± 0.04 (MacArthur, 1992)	0.36 ± 0.01 (McCulloch, unpublished)
LUNG		
Oxygen storage capacity (ml O_2/kg)	6.4* (MacArthur, 1990)	
BLOOD		
Hematocrit (%)	40.5 ± 1.7 (Snyder, 1983)	40.8 ± 1.1 (Snyder, 1983)
Hemoglobin (g/100 ml blood)	13.0 ± 0.4 (Snyder, 1983)	14.4 ± 0.4 (Snyder, 1983)
P_{50} (40 mmHg)	27.7 ± 1.1 (MacArthur, 1984c)	35.5 ± 0.9 (MacArthur, 1984c)
Volume (ml/100 g)	9.7 ± 0.3* (MacArthur et al., 2001)	6.4 (Baker et al., 2006)
Oxygen storage capacity (ml O_2/kg)	14.4* (MacArthur, 1990)	
MUSCLE MYOGLOBIN		
Heart (mg/g tissue)	7.4 ± 0.1 (Snyder, 1983)	5.4 ± 0.5 (Snyder, 1983)
Skeletal (gastrocnemius; mg/g tissue)	13.3 ± 0.5 (Snyder, 1983)	1.6 ± 0.3 (Snyder, 1983)
Oxygen storage capacity (ml O_2/kg)	4.4* (MacArthur, 1990)	
DIVING		
Resting oxygen consumption in air (ml O_2/g/h)	0.78 ± 0.01 (MacArthur and Krause, 1989)	0.87 (Schmidt-Nielsen, 1983)
Diving oxygen consumption (ml O_2/g/h)	2.22 (MacArthur and Krause, 1989)	
Total body oxygen storage capacity (ml O_2/kg)	25.2* (MacArthur, 1990)	
Aerobic dive limit (s)	40.9* (MacArthur, 1990)	
Maximum submersion duration (forced dive, min)	12 (Irving, 1939)	2 (Irving, 1939)

† *Against current in swim flume; *summer-acclimatized adult muskrats.*

and with genetic standardization, many inbred and outbred strains are available. Muskrats are not as freely available as rats, and the use of professional trappers and the cooperation of local Wildlife agencies may be necessary to secure a reliable source of muskrats. Rats have the advantage in that many existing animal facilities are designed for housing rodents such as rats and mice. Although muskrats can be easily housed in such facilities, regulatory hurdles to do so may be prohibitive. Legitimate concerns over the entry of zoonotic diseases and parasites, and the safety of facility personnel, may be raised. A big advantage for the use of rats is that the brains of rats are much more thoroughly characterized, both anatomically and functionally. Neuroscientific data obtained from rats are much more comparable with those from the literature, as rats are used extensively in many areas of biomedical research. Many of these other disciplines can also lay the groundwork for investigation of specific aspects of the rat diving response. In comparison, muskrats have only been used in a handful of neuroscientific studies.

Another potential disadvantage of using the rat is the "stigma" of using a domesticated animal. However, for every research question there is an appropriate animal model on which it can be most conveniently studied. Rats are often regarded as strictly terrestrial animals, and for laboratory animals this is usually true. However, if the central circuitry involved in integration of the diving response is to be characterized, it will have to be done in an animal in which the basic cardiovascular and respiratory circuitry is well characterized. The rat is such an animal. In the wild *R. norvegicus* is used to, and adept at, swimming and diving underwater. The laboratory rat is the domesticated albino version of the wild rat. This suggests that the laboratory rat should not be regarded merely as a terrestrial animal, but rather as an animal that rarely gets the opportunity to swim and dive because of how it is housed. The laboratory rat is descended from a semi-aquatic feral form, having been domesticated within the last 150 years, and in this sense is a good choice for studies of the diving response. Because so much is known about the physiology and central anatomy of the laboratory rat, it should make an excellent animal model for investigating the central control of the mammalian diving response.

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Decompression vs. decomposition: distribution, amount, and gas composition of bubbles in stranded marine mammals

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Gas embolic lesions linked to military sonar have been described in stranded cetaceans including beaked whales. These descriptions suggest that gas bubbles in marine mammal tissues may be more common than previously thought. In this study we have analyzed gas amount (by gas score) and gas composition within different decomposition codes using a standardized methodology. This broad study has allowed us to explore species-specific variability in bubble prevalence, amount, distribution, and composition, as well as masking of bubble content by putrefaction gases. Bubbles detected within the cardiovascular system and other tissues related to both pre- and port-mortem processes are a common finding on necropsy of stranded cetaceans. To minimize masking by putrefaction gases, necropsy, and gas sampling must be performed as soon as possible. Before 24 h *post mortem* is recommended but preferably within 12 h *post mortem*. At necropsy, amount of bubbles (gas score) in decomposition code 2 in stranded cetaceans was found to be more important than merely presence vs. absence of bubbles from a pathological point of view. Deep divers presented higher abundance of gas bubbles, mainly composed of 70% nitrogen and 30% CO_2, suggesting a higher predisposition of these species to suffer from decompression-related gas embolism.

Keywords: gas emboli, decompression, putrefaction, gas-off, marine mammals, nitrogen, strandings

INTRODUCTION

Marine mammals moved from land to water around 55–60 million years ago (Ponganis et al., 2003). Marked anatomical and physiological modifications were necessary to meet the physical demands of living in water instead of air (Williams and Worthy, 2002). These required variations include adaptations for breath-hold diving, temperature regulation in cold water, water, and salt balance, underwater navigation, and high pressure at great depth (Elsner, 1999).

Marine mammals have not been considered to suffer from decompression sickness (DCS) because of anatomical, physiological, and behavioral adaptations that help them to prevent gas bubble formation. More specifically compression of the respiratory system together with blood-flow changes during the dive would limit the amount of nitrogen absorbed on a dive (Scholander, 1940; Butler and Jones, 1997; Kooyman and Ponganis, 1998; Fahlman et al., 2006). Compression of the respiratory system was suggested to force the air into the upper airways where no gas exchange should take place, therefore it was assumed that the finite volume of gas available in the lung would not be sufficient to increase the tissue and blood inert gas tension. Finally, partial pressure of nitrogen in the blood would decrease as nitrogen is distributed among tissues (Piantadosi and Thalmann, 2004).

In human breath-hold divers, nitrogen accumulates in tissues as pulmonary nitrogen partial pressure (PN_2) increases with depth. If the surface interval between repeated dives is insufficient to remove the inert gas, nitrogen accumulation will occur (Ferrigno and Lundgren, 2003). A causal relationship between breath-hold diving in humans and DCS is only slowly being accepted despite the growing number of cases of DCS-like symptoms in breath-hold diving humans (Schipke et al., 2006). Some of these symptoms might include vertigo, visual disturbances, unconsciousness, and partial or complete paralysis of one or more extremity that could be temporary or permanent. When these symptoms respond to recompression, the most reasonable explanation is the presence of bubbles due to gas phase separation in the body (Paulev, 1965). It has been generally assumed that the risk of DCS is virtually zero during a single breath-hold dive in humans, however DCS has also been reported (although unusual) in 2 out of 192 deep single breath-hold dives (Fitz-Clarke, 2009). Diagnosis of both cases was based on DCS symptoms and recovery after treatment in a hyperbaric chamber.

Recent work has suggested that the depth of lung compression is not the same for all marine mammals species (Moore et al., 2011) and one suggested reason is because of anatomical differences of the thorax and even of the lung airways (Belanger, 1940;

Denison and Kooyman, 1973; Kooyman, 1973). Indirect measurements of nitrogen uptake and removal have suggested that alveolar collapse occurs at between 30 m (Falke et al., 1985) in the Weddell seals and 70 m in the bottlenose dolphins (Ridgway and Howard, 1979). However, the physiological assumptions used in these studies are suspect (Bostrom et al., 2008) and direct measurements in seals and sea lions suggest that alveolar collapse and cessation of gas exchange may not happen until depths greater than 100 m (Kooyman and Sinnett, 1982).

Moreover, intramuscular nitrogen levels as great as two to three times the normal surface levels have been measured in voluntary diving bottlenose dolphins after repeated short duration dives to 100 m depth (Ridgway and Howard, 1979). Moore et al. (2009) described a high prevalence of bubble lesions in bycaught seals and dolphins trapped at depth (15 out of 23) compared to stranded marine mammals (1 out of 41), probably due to off gassing from supersaturated tissues. In addition, several theoretical studies have predicted end-dive nitrogen levels for marine mammals that would cause a significant proportion of DCS cases in land mammals (Houser et al., 2001; Zimmer and Tyack, 2007; Hooker et al., 2009).

In the last 8 years, an increasing number of studies have reported lesions related to *in vivo* bubbles. Systemic venous gas emboli were first described in an atypical beaked whale (BW) mass stranding related to military maneuvers that occurred in the Canary Islands in 2002. BWs (8 out of 8) presented acute lesions consistent with acute trauma due to *in vivo* bubble formation (Jepson et al., 2003; Fernandez et al., 2005). Chronic gas bubble lesions were also reported in single strandings of Risso's dolphin (3 out of 24), common dolphins (3 out of 342), harbor porpoises (1 out of 1035), and in a Blainville's BW (1 out of 1) stranded in the United Kingdom (Jepson et al., 2003, 2005). Further gas analyses from one Cuvier's BW stranded along the Spanish coastline in temporal and spatial association with military (naval) exercises and one UK-stranded Risso's dolphin with chronic gas embolic lesions in the spleen, have confirmed high nitrogen content, of around 95% in the Risso's dolphin case (Bernaldo de Quirós et al., 2011). Additionally, dysbaric osteonecrosis (a chronic pathology of deep diving recognized in humans) has been described in sperm whales (Moore and Early, 2004). Finally, a recent publication showed the existence of intravascular bubbles and peri-renal subcapsular emphysema (gas found beneath the kidney capsule) in live stranded dolphins using a B-mode ultrasound (Dennison et al., 2012).

Given these recent observations, it is valuable to review the diving behavior of cetaceans. Some species of BWs have dive profiles not previously observed in other marine mammals such as very deep foraging dives (up to 2000 m and as long as 90 min), and relatively slow controlled ascents followed by a series of bounce dives of 100–400 m (Hooker and Baird, 1999; Tyack et al., 2006). These diving profiles are considered as extreme dives and alterations of these dive sequences by a behavioral response might induce excessive nitrogen supersaturation driving growth of bubbles in a manner similar to DCS in humans (Cox et al., 2006). Indeed, it was in BWs stranded in spatiotemporal concordance with military maneuvers when the "gas bubble lesions" were described for the first time (Jepson et al., 2003; Fernandez et al., 2005). Authors suggested a DCS-like disease as a plausible mechanism for explaining the observed lesions. These findings have been widely discussed since then and have become a scientific controversy. Further investigations, including analysis of gas in bubbles were recommended (Piantadosi and Thalmann, 2004).

Here we present a comprehensive study of prevalence, amount, distribution, and composition of intravascular bubbles and subcapsular emphysema found in cetaceans stranded on the Canary Islands coast, Spain, with different decomposition codes. The waters of the Canary Islands are one of the richest and most diverse areas in the Northeast Atlantic, with 28 different cetacean species reported, 21 of the Odontocete and 7 of the Mysticete group. Of these 28 species, at least 26 have been found stranded on the coasts of the Canary Islands (Martin et al., 2009). The high biodiversity, including both shallow and deep diving species, make the Canary Islands an excellent natural laboratory for the study of gas emboli in cetaceans with different diving behaviors.

MATERIALS AND METHODS

MATERIALS

Animals included in the study were stranded cetaceans in the Canary Islands between 2006 and 2010, mass stranded sperm whales in Italy in 2009, and two sea lions (*Otaria byronia*) from aquatic-parks of the Canary Islands submitted for necropsy to our institution. A total of 88 necropsies were performed on marine mammals belonging to 18 different species. Species were segregated into two broad groups: deep divers and non-deep divers, defining deep divers as those species known to dive deeper than 500 m for foraging (*Kogia*, *Physeter*, *Ziphius*, *Mesoplodon*, *Globicephala*, and *Grampus*; Gannier, 1998; Astruc and Beaubrun, 2005; Aguilar de Soto, 2006; Tyack et al., 2006; Watwood et al., 2006; West et al., 2009). These genera were further studied separately except for *Ziphius* and *Mesoplodon*, which were studied together as the family Ziphiidae. Twenty-nine out of 88 (33%) necropsied animals were deep divers.

Animals were identified by their stranding codes, represented by CET ("cetacean") followed by the stranding number. Animals not stranded in the Canary Islands were identified by their investigation numbers, represented by I ("investigation") followed by their corresponding numbers and the year when their necropsies were performed (**Table 1**). Cause(s) of death (defined as pathological entities) were determined by the Division of Histology and Animal Pathology of the Institute for Animal Health, University of Las Palmas de Gran Canaria (ULPGC; Unit of Cetaceans Research, 2006, 2007, 2008, 2009, 2010; Arbelo, 2007; **Table 1**).

METHODS

At necropsy, putrefaction of the animal was evaluated using a morphological decomposition code from one to five according to Kuiken and García-Hartmann (1991) where one is the animal alive (becomes code 2 at death), code 2 is when the animal is extremely fresh (no bloating), code 3 is moderate decomposition (bloating, skin peeling but organs still intact), code 4 is advanced decomposition (major bloating, organs beyond recognition), and finally code 5 when no organs are present. Dissection, gas sampling and analysis were performed following procedures described by Bernaldo de Quirós et al. (2011). A total of 429 samples were recovered and analyzed from the studied animals, 208 (48%) of which belong to deep diving animals.

Table 1 | Identification number of the marine mammals included in the study, biological information, stranding circumstances, storing conditions, decomposition code, and most likely (*) cause of dead established by individual pathological studies.

Identification number	Species	Deep diver	Gender	Age	Active stranding	Mass stranding	Frozen	Decomposition code	Cause of dead*
CET 339	*Globicephala macrorhynchus*	Yes	F	Adult	Yes	No	No	2	Septicemia
CET 360	*Globicephala macrorhynchus*	Yes	M	Calve	Yes	No	No	1	Infectious meningitis
CET 361	*Globicephala macrorhynchus*	Yes	ND	Adult	No	No	No	5	Trauma
CET 362	*Stenella frontalis*	No	F	Adult	Yes	No	No	1	Septicemia
CET 363	*Stenella frontalis*	No	F	Subadult	?	No	Yes	2	Obstruction by foreign body
CET 364	*Delphinus delphis*	No	M	Subadult	No	No	Yes	3	Infectious meningoencephalitis
CET 367	*Stenella coeruleoalba*	No	M	Calve	No	No	Yes	5	Abnormal development
CET 368	*Stenella frontalis*	No	M	Juvenile	?	No	Yes	3	Trauma
CET 369	*Balaenoptera physalus*	No	F	Adult	No	No	No	4	Trauma
CET 370	*Stenella coeruleoalba*	No	M	Adult	Yes	No	No	3	Septicemia
CET 371	*Stenella frontalis*	No	F	Adult	?	No	No	2	Trauma
CET 372	*Balaenoptera* borealis	No	F	Subadult	No	No	No	5	Not determined
CET 373	*Delphinus delphis*	No	F	Adult	Yes	No	No	2	Septicemia
CET 374	*Stenella coeruleoalba*	No	M	Adult	No	No	No	3	Trauma
CET 375	*Stenella coeruleoalba*	No	M	Neonate	No	No	Yes	3	Neonatal weakness
CET 376	*Stenella coeruleoalba*	No	F	Adult	No	No	Yes	2	Neoplasia
CET 379	*Mesoplodon bidens*	Yes	M	Adult	?	No	No	2	Trauma, Septicemia
CET 380	*Stenella coeruleoalba*	No	M	Subadult	?	No	No	2	Infectious encephalitis
CET 381	*Delphinus delphis*	No	M	Subadult	No	No	Yes	4	Infectious encephalitis
CET 382	*Delphinus delphis*	No	M	Adult	?	No	No	3	Sinusitis and meningoencefalitis by *Nasitrema* sp.
CET 384	*Stenella frontalis*	No	M	Adult	No	No	Yes	3	Toxoplasmosis
CET 390	*Globicephala macrorhynchus*	Yes	M	Calve	?	No	No	3	Septicemia
CET 393	*Stenella frontalis*	No	F	Adult	?	No	No	2	Neoplasia
CET 395	*Stenella frontalis*	No	M	Adult	No	No	Yes	2	Neoplasia
CET 397	*Kogia breviceps*	Yes	F	Adult	No	No	No	3	Trauma, septicemia
CET 399	*Globicephala macrorhynchus*	Yes	M	Neonate	No	No	No	5	Trauma
CET 400	*Stenella coeruleoalba*	No	M	Adult	No	No	No	2	Parasitoses, senile disease
CET 402	*Stenella coeruleoalba*	No	F	Adult	No	No	No	5	Septicemia, senile disease
CET 404	*Kogia breviceps*	Yes	M	Adult	Yes	No	No	1	Encephalopathy of unknown etiology
CET 409	*Stenella coeruleoalba*	No	F	Subadult	No	No	Yes	3	Infectious encephalitis, Parasitoses
CET 413	*Pseudorca crassidens*	No	M	Juvenile	Yes	No	No	2	Trauma, septicemia
CET 418	*Stenella frontalis*	No	F	Adult	?	No	Yes	2	Heart failure
CET 419	*Steno bredanensis*	No	F	Juvenile	No	No	Yes	2	Septicemia
CET 421	*Stenella frontalis*	No	M	Calve	No	No	Yes	2	Not determined
CET 425	*Delphinus delphis*	No	F	Juvenile	No	No	Yes	4	Parasitoses
CET 430	*Steno bredanensis*	No	F	Subadult	No	No	No	4	Not determined
CET 431	*Grampus griseus*	Yes	M	Juvenile	Yes	No	No	2	Infectious meningitis
CET 434	*Steno bredanensis*	No	M	Adult	No	Yes	No	5	Not determined

(Continued)

Table 1 | Continued

Identification number	Species	Deep diver	Gender	Age	Active stranding	Mass stranding	Frozen	Decomposition code	Cause of dead*
CET 435	*Stenella frontalis*	No	F	Adult	No	No	No	5	Trauma
CET 437	*Steno bredanensis*	No	F	Adult	No	Yes	No	5	Not determined
CET 438	*Steno bredanensis*	No	F	Subadult	No	No	No	5	Not determined
CET 456	*Grampus griseus*	Yes	F	Adult	Yes	No	Yes	2	Infectious meningoencephalitis
CET 459	*Kogia breviceps*	Yes	M	Adult		No	No	3	Trauma, bycatch?
CET 460	*Stenella coeruleoalba*	No	M	Calve	No	No	No	4	Trauma
CET 462	*Stenella frontalis*	No	F	Calve	No	No	Yes	5	Not determined
CET 463	*Physeter macrocephalus*	Yes	F	Neonate	Yes	No	No	1	Parental segregation
CET 464	*Globicephala macrorhynchus*	Yes	M	Juvenile	No	No	No	4	Enterotoxaemia septicemia
CET 469	*Stenella coeruleoalba*	No	F	Adult	?	No	No	3	Parasitoses, senile disease
CET 471	*Ziphius cavirostris*	Yes	F	Calve	?	No	No	2	Chronic renal failure
CET 472	*Grampus griseus*	Yes	F	Subadult	No	No	No	2	Trauma
CET 473	*Steno bredanensis*	No	F	Juvenile	Yes	No	No	2	Septicemia
CET 474	*Stenella coeruleoalba*	No	M	Adult	No	No	Yes	2	Septicemia
CET 476	*Stenella coeruleoalba*	No	F	Calve	No	No	No	2	Chronic renal failure
CET 482	*Delphinus delphis*	No	F	Adult	No	No	Yes	3	Bacterial bronchopneumonia
CET 483	*Grampus griseus*	Yes	M	Adult	Yes	No	No	2	Venous gas embolism
CET 487	*Stenella coeruleoalba*	No	F	Juvenile	No	No	No	5	Not determined
CET 504	*Globicephala macrorhynchus*	Yes	M	Adult	No	No	No	4	Meningitis, septicemia
CET 505	*Tursiops truncatus*	No	M	Juvenile	No	No	No	5	Not determined
CET 506	*Stenella coeruleoalba*	No	F	Neonate	No	No	Yes	4	Not determined
CET 509	*Tursiops truncatus*	No	M	Subadult	No	No	No	4	Not determined
CET 510	*Mesoplodon europaeus*	Yes	M	Adult	?	No	No	3	Trauma, possible viral disease
CET 512	*Globicephala macrorhynchus*	Yes	M	Adult	No	No	No	3	Septicemia
CET 515	*Stenella frontalis*	No	M	Adult	No	No	Yes	2	Toxoplasmosis
CET 517	*Delphinus delphis*	No	M	Adult	No	No	No	2	Chronic renal failure
CET 520	*Physeter macrocephalus*	Yes	F	Calve	No	No	No	4	Trauma
CET 521	*Delphinus delphis*	No	ND	Juvenile	No	No	Yes	5	Not determined
CET 522	*Stenella frontalis*	No	M	Adult	No	No	Yes	3	Toxoplasmosis
CET 523	*Balaenoptera acutorostrata*	No	M	Calve	No	No	No	2	Parental segregation
CET 526	*Tursiops truncatus*	No	F	Adult	?	No	No	2	Septicemia
CET 527	*Stenella coeruleoalba*	No	F	Adult	Yes	No	Yes	3	Parasitoses Bacterial infection
CET 530	*Stenella frontalis*	No	F	Adult	?	No	No	2	Trauma toxoplasmosis
CET 531	*Stenella frontalis*	No	M	Juvenile	?	No	No	2	Septicemia
CET 533	*Grampus griseus*	Yes	M	Adult	No	No	No	4	Not determined, Parasitoses
CET 534	*Grampus griseus*	Yes	M	Subadult	Yes	No	No	1	Viral disease, bacterial/mycotic infection
CET 537	*Stenella coeruleoalba*	No	F	Adult	No	No	No	2	Septicemia
CET 542	*Kogia sima*	Yes	ND	Adult	No	No	No	4	Septicemia
CET 543	*Tursiops truncatus*	No	M	Adult	No	No	No	5	Senile disease, parasitoses
CET 544	*Physeter macrocephalus*	Yes	M	Juvenile	No	No	No	5	Trauma, ship collision

(Continued)

Table 1 | Continued

Identification number	Species	Deep diver	Gender	Age	Active stranding	Mass stranding	Frozen	Decomposition code	Cause of dead*
CET 546	*Stenella coeruleoalba*	No	M	Adult	Yes	No	No	3	Trauma, stranding stress syndrome
CET 547	*Mesoplodon europaeus*	Yes	M	Adult	?	No	No	4	Not determined
CET 548	*Stenella frontalis*	No	M	Calve	No	No	No	2	Viral disease
CET 549	*Grampus griseus*	Yes	F	Adult	No	No	No	2	Venous gas embolism
CET 552	*Balaenoptera borealis*	No	M	Juvenile	No	No	No	5	Pending
I134/07	*Otaria byronia*	No	M	Adult	No	No	No	3	Not determined
I185/09	*Mesoplodon bidens*	Yes	F	Adult	No	Yes	No	5	Pending
I344/07	*Otaria byronia*	No	M	Juvenile	No	No	No	2	Iatrogenic pneumomediastinum
I298/09	*Physeter macrocephalus*	Yes	M	Juvenile	Yes	Yes	No	3	Not determined
I299/09	*Physeter macrocephalus*	Yes	M	Juvenile	Yes	Yes	No	2	Not determined
I300/09	*Physeter macrocephalus*	Yes	M	Juvenile	Yes	Yes	No	2	Not determined

? Indicates those animals that have been found dead, without signs of active stranding, but where passive stranding can not be either confirmed since they were found already dead.

Samples containing blood were considered contaminated, since putrefaction gases from the blood will alter original gas sample composition. When no gas compound had a chromatographic signal higher than the detection limit, the sample was considered empty. In addition, if only one compound was present in slightly higher quantities than the established detection limit, the sample was considered small and not representative of its original composition. Finally samples with an atmospheric-air-like composition (around 79% nitrogen and 21% oxygen) were considered as "atmospheric-air polluted." Thus, samples with blood, empty, or identified as small or atmospheric-air polluted were not included in the results. These samples represented 28% of the total. Most of them were emptied intestines or blood contaminated samples from the heart when only the vacutainer was applied. This problem was prevented later in the study by using an aspirometer (Bernaldo de Quirós et al., 2011).

The amount of gas present in veins and tissues was evaluated retrospectively using pictures and data in the necropsy reports. Gas amount was semi-quantified by giving a score to different vascular locations as well as to the presence of subcapsular gas (emphysema), defined as macroscopically visible gas found beneath the capsule of body organs (e.g., kidneys). Vascular locations studied for gas scoring were subcutaneous, mesenteric, and coronary veins as well as the lumbo-caudal venous plexus. We used the following score for these locations: grade 0 is no bubbles, grade I represented few bubbles and grade II represented abundant presence of bubbles. Prevalence of subcapsular gas, was evaluated with a similar grading system: grade 0 is no subcapsular gas, grade I represented the presence of subcapsular gas in one or two organs, and grade II represented wide distribution through the body organs. The summation of gas score (0–II) in the different vascular locations and tissues (subcapsular gas; $n = 5$), gave the total gas score for each animal that ranged from 0 to 10. However, because gas score was done retrospectively, some information was missing on occasion. Those cases on which only one localization could not be scored were marked with an asterisk (*), indicating that the total gas score might be up to two units higher according to the established gas score (e.g., 8*). If information from more than one localization was missing, the total gas score was not reported as it would not be a realistic estimate. Finally, there were some animals with advanced putrefaction where the veins could not be clearly distinguished from the rest of the tissues. Gas score was not undertaken in these cases.

The proportion of observations were analyzed for non-random associations between two categorical variables with the Fisher exact test. Hypothesis testing was considered significant when the corresponding P-value was less than 0.05. All data were analyzed using Sigma Stat software, version 3.5.

RESULTS

PREVALENCE OF BUBBLES

Intravascular bubbles were found in 51 out of 88 animals (58%). They were absent in 33 out of 88 (37%) and the observation was not correctly undertaken in 4 out of 88 animals (5%). Bubbles were statistically ($P = 0.006$) more frequently present in deep divers (76%) compared to non-deep divers (52%). Subcapsular emphysema (mostly peri-renal) was found in 57 out of 88 animals (65%). It was found in 66% of the non-deep diving animals and in 71% of the deep divers. This difference was statistically non-significant ($P = 0.474$).

A positive relationship between decomposition code and presence of intravascular bubbles and/or subcapsular emphysema was

found (**Figure 1**). Intravascular bubbles were present in all the studied animals with decomposition code 4 or higher. However, intravascular bubbles had a higher prevalence ($P = 0.006$) in deep divers compared to non-deep divers regardless of decomposition code (**Figures 2** and **3**); they were present in 57% of the fresh (decomposition code 2) deep diving animals compared to 20% of non-deep divers ($P = 0.001$), and in 100% of deep diving animals with decomposition code 3 compared to 50% of non-deep divers within the same decomposition code ($P = 0.019$). In addition, subcapsular emphysema was found in 64% of the deep divers vs. 44% with decomposition code 2, and in 66% of the deep divers vs. 61% of the non-deep divers with decomposition code 3, although these differences were not statistically significant ($P = 0.204$ and $P = 0.648$ respectively).

In 40% of the fresh animals with subcapsular emphysema, intravascular bubbles were additionally present, while 61% of the fresh animals with intravascular bubbles also had subcapsular emphysema.

In freshly dead animals, intravascular bubbles were seen in 7 out of 14 of the cetaceans that were known to strand actively (by swimming ashore) but only in 3 out of 15 that were found floating ashore dead or dead in the beach with no signs of active stranding. Animals found dead but suspected to have stranded alive were not included in the study. Differences were not statistically significant ($P = 0.128$).

AMOUNT OF BUBBLES (GAS SCORE)

Non-deep divers

Gas score from fresh non-deep diving animals was always lower than half of the scale (5 on a scale of 10). Forty-four percent of them (11 out of 25) did not present either intravascular bubbles or subcapsular emphysema (gas score 0). Gas score of dolphins with incipient autolysis (decomposition code 3) varied greatly (from 0 to 7). Dolphins with advanced autolysis (decomposition code 4) had gas score ranging from 5 to 8 and potentially 10, while all animals in a very advanced state of autolysis (decomposition code

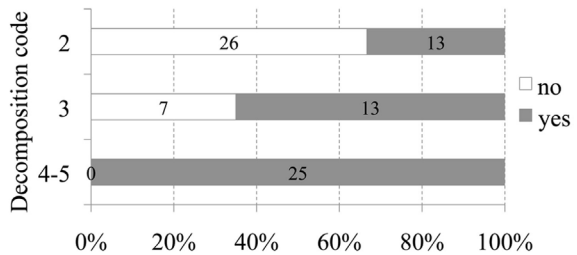

FIGURE 1 | Number and relative percentage of animals with intravascular bubbles (in dark gray) compared to those without bubbles (in white) regarding to decomposition code.

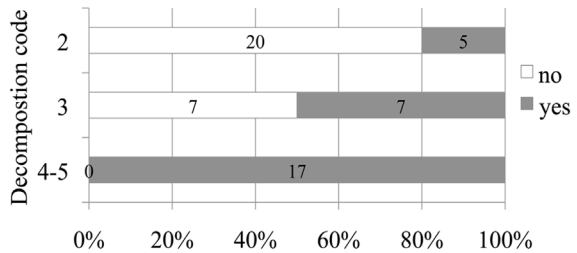

FIGURE 2 | Number and relative percentage of non-deep diving animals in which bubbles were observed (in dark gray) compare to those in which bubbles were absent (in white) attending to decomposition code.

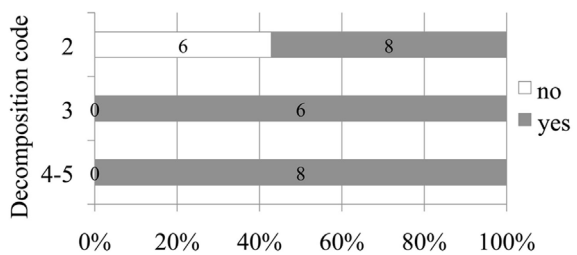

FIGURE 3 | Number and relative percentage of deep diving animals in which bubbles were observed (in dark gray) compare to those in which bubbles were absent (in white) attending to decomposition code.

5) had the maximum gas score until an advanced state of decay was reached and integrity of the tissues was lost (**Figure 4**).

Deep divers

Gas score from fresh deep divers was higher compared to non-deep divers. Thirty-five percent of the animals (5 out of 14) presented gas score 5 or higher. Indeed, two of these animals presented the highest gas score of all studied animals with decomposition code 2. They presented high (7*) and very high (9) gas score. All deep divers with decomposition code 3 presented bubbles, most of them with values around or slightly higher than gas score 5. Deep divers with decomposition code 4 had a gas score ranging from 7 to 10, while those with decomposition code 5 had a gas score of 9 or 10 (**Figure 5**).

GAS COMPOSITION

Intravascular bubbles of non-deep divers

The main gas compound in intravascular bubbles recovered from non-deep divers in a fresh status or with incipient autolysis (codes 2 and 3) was nitrogen. Hydrogen was found in some samples of these animals (in one animal with decomposition code 2 and in two animals with decomposition code 3), especially in the mesenteric veins. When hydrogen was not present, nitrogen concentrations were between 70 and 90%. On the other hand, samples from more decomposed animals (codes 4 and 5) consisted of high levels of CO_2 (>30%) together with some hydrogen and/or nitrogen (**Figure 6**). In some cases, the sample was 100% CO_2.

INTRAVASCULAR BUBBLES OF DEEP DIVERS

Kogiidae

Only four *Kogia* sp. individuals were studied. In addition they presented low gas scores, therefore few gas samples could be obtained. Gas samples were mainly composed of nitrogen (70–90%) although hydrogen appeared with decomposition code 4 (**Figure 7**).

Physeteridae

In sperm whales, gas composition from fresh animals had consistently high nitrogen content (around 70%). This gas composition changed dramatically with incipient autolysis. Gas composition from more decomposed animals consisted of a mixture of hydrogen, CO_2, and nitrogen. Oxygen was only present in the three animals that stranded alive in the coast of Italy (Bernaldo de Quirós et al., 2011; Mazzariol et al., 2011; **Figure 8**).

Globicephala

Fresh pilot whales presented none or few bubbles; therefore only one sample from a fresh animal could be obtained. This sample was composed of a high concentration of nitrogen (74%) together with oxygen and CO_2. Samples from animals with incipient autolysis varied in composition. There were some intravascular bubbles with high nitrogen content although hydrogen was already present. The rest of the bubbles were composed of a mixture of CO_2, hydrogen, and nitrogen in similar concentrations. In more decomposed animals, nitrogen levels decreased further, and the

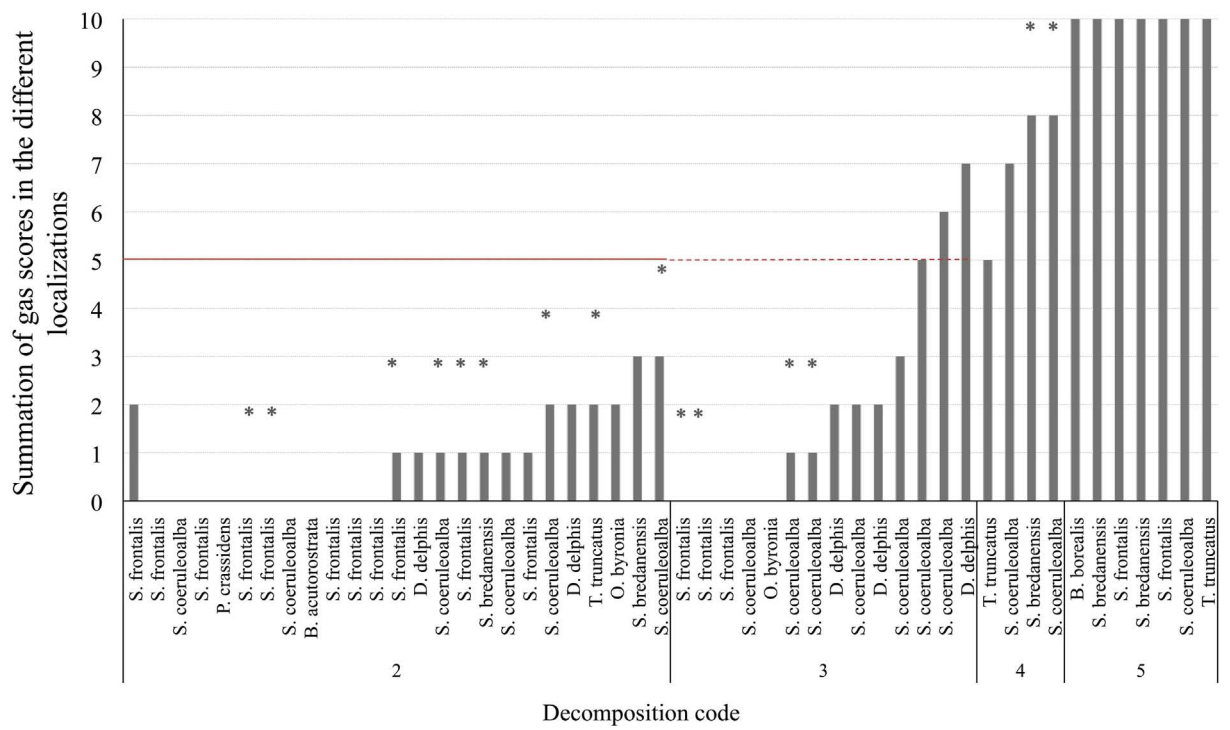

FIGURE 4 | Total gas score (summation of gas scores in the different localizations) for each animal (non-deep divers). Asterisks represent the maximum potential summation gas score for a given animal, on which one localization was not adequately observed for bubbles. The red line is presented at half of the total gas score (5 of 10) in order to better illustrate the total gas scores of animals that fall above and bellow the medium.

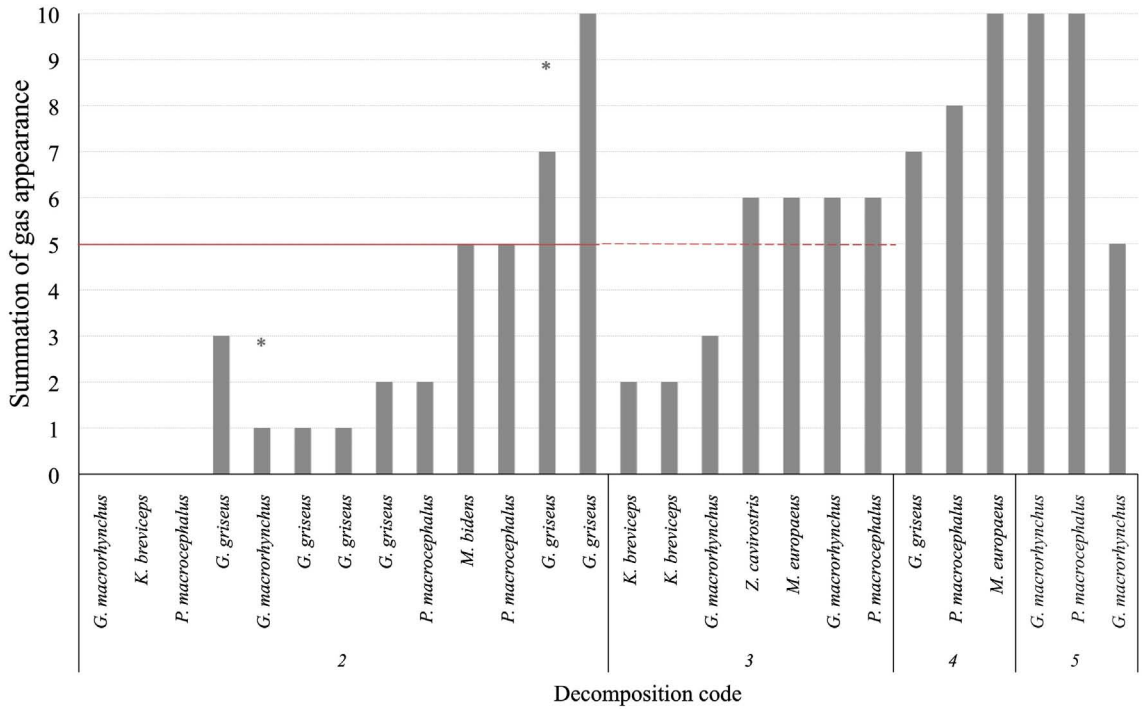

FIGURE 5 | Total gas score (summation of gas scores in the different localizations) for each animal (deep divers). Asterisks represent the maximum potential summation gas score for a given animal, on which one localization was not adequately observed for bubbles. The red line is presented at half of the total gas score (5 of 10) in order to better illustrate the total gas scores of animals that fall above and bellow the medium.

gas was composed of a mixture of CO_2 and hydrogen; CO_2 having the highest concentration with nitrogen having concentrations lower than 30%. In the samples from the most decomposed animals, nitrogen was no longer present. Intravascular bubbles were composed of a mixture of CO_2 and hydrogen with similar concentrations, although CO_2 content was always slightly higher than hydrogen. In summary, a clear difference was found in gas composition of bubbles in animals with different decomposition codes (**Figure 9**).

Grampus

Samples from fresh animals were composed of nitrogen, CO_2, and in most cases some oxygen. They were composed of $76 \pm 7\%$ of nitrogen and $15 \pm 9\%$ of CO_2, with the exception of "CET 483" where higher CO_2 concentrations were found (40–60%). All these samples were clearly different from samples obtained from a more decomposed animal (with decomposition code 4) whose samples were mainly composed of CO_2 and hydrogen (**Figure 10**).

Ziphiidae

Only one sample was successfully analyzed from a fresh animal. It was composed of high nitrogen content (81%) and CO_2. Gas composition from more decomposed animals was highly variable, with no clear trend along decomposition codes. Hydrogen was always present except for two samples from "CET 547" with decomposition code 4. In this same animal there were three more samples with low hydrogen concentration (below 15%). With the exception of these samples, nitrogen was always lower than 60% in the more decomposed animals. Samples were a mixture of CO_2, nitrogen, and hydrogen. In some samples nitrogen was absent and bubbles were composed of CO_2 and hydrogen exclusively (**Figure 11**).

INTESTINAL GAS

Gas composition from the intestines was highly variable with no trend with PM time. Nitrogen was found in high concentrations (higher than 60%) in 21% of the samples. However, nitrogen was found in very low concentrations in the rest of the samples. In 40% of the samples, nitrogen was not present. In most of the samples (67%), CO_2 was the major compound reaching values as high as 100%. Hydrogen was very frequently found in the intestine (in 64% of the samples). Therefore most of the samples were a mixture of high CO_2 levels with hydrogen and some, if any, nitrogen. Methane was only detected in Kogiidae and Physeteridae.

SUBCAPSULAR EMPHYSEMA

Subcapsular gas was composed of around 80% of nitrogen and 20% of CO_2 in the fresh animals studied (a Risso's dolphin and a sea lion), except from one sample recovered from the peri-renal area of a common dolphin (CET 517). Gas composition of subcapsular samples from the fresh animals was clearly different from those of more decomposed animals, which mainly consist on CO_2 and hydrogen. Nitrogen was lower than 30% when present. Nitrogen was found in high concentrations in only one sample from the most decomposed animals. This sample also presented the highest concentration of oxygen (15% compared to a maximum of 4% in the rest of the samples; **Figure 12**).

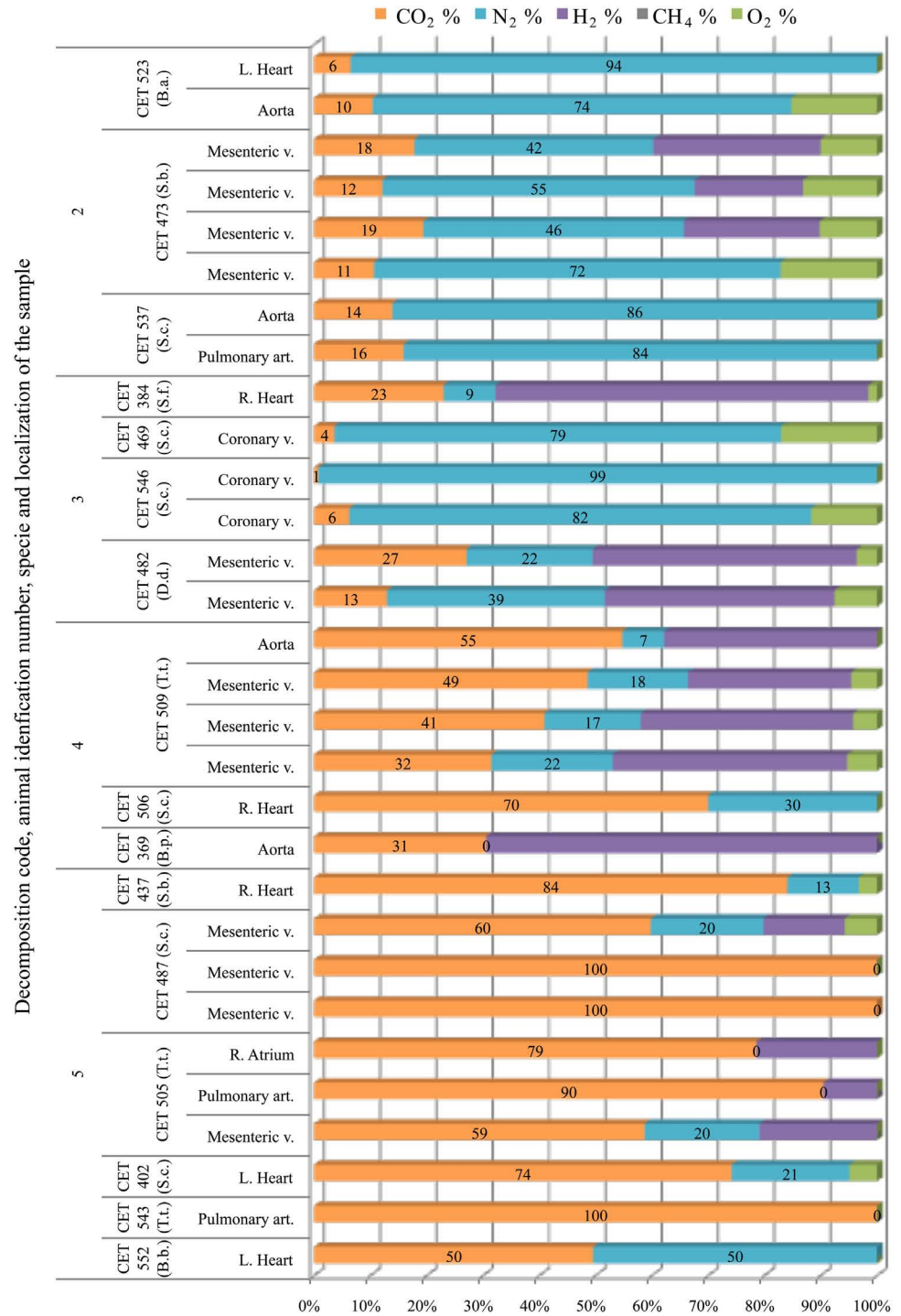

FIGURE 6 | Intravascular bubble's gas composition sampled form different non-deep diving species (B.a., *Balaenoptera acutorostrata*; S.b., *Steno bredanensis*; S.c., *Stenella coeruleoalba*; S.f., *Stenella frontalis*; D.d., *Delphinus delphis*; T.t., *Tursiops truncatus*; B.p., *Balaenoptera physalus*; B.b., *Balaenoptera borealis*), in different tissues and decomposition codes, illustrating the contribution of each gas to the total amount in percentage μmol.

GAS FROM THE PTERYGOIDAL SINUSES

Gas composition from the sinuses remained constant for longer PM time compared to gas recovered from other tissues. Nitrogen was always the major compound (58–87%) until decomposition code 4 was reached. Within these decomposition codes, CO_2 was present in concentrations ranging from 10 to 36%. After

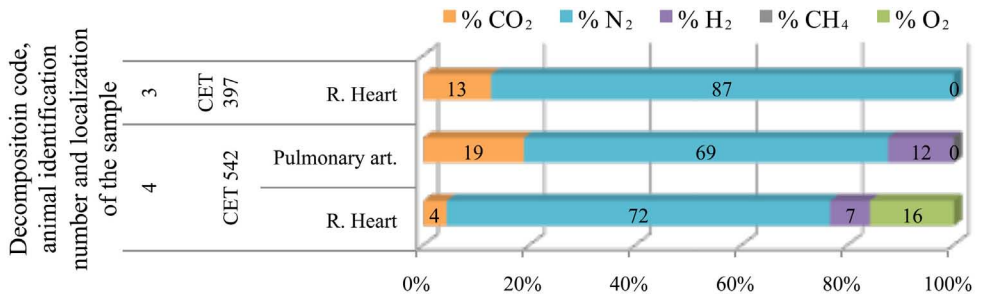

FIGURE 7 | Intravascular bubble's gas composition of deep diving animals belonging to Kogiidae family in different tissues and decomposition codes, illustrating the contribution of each gas to the total amount in percentage μmol.

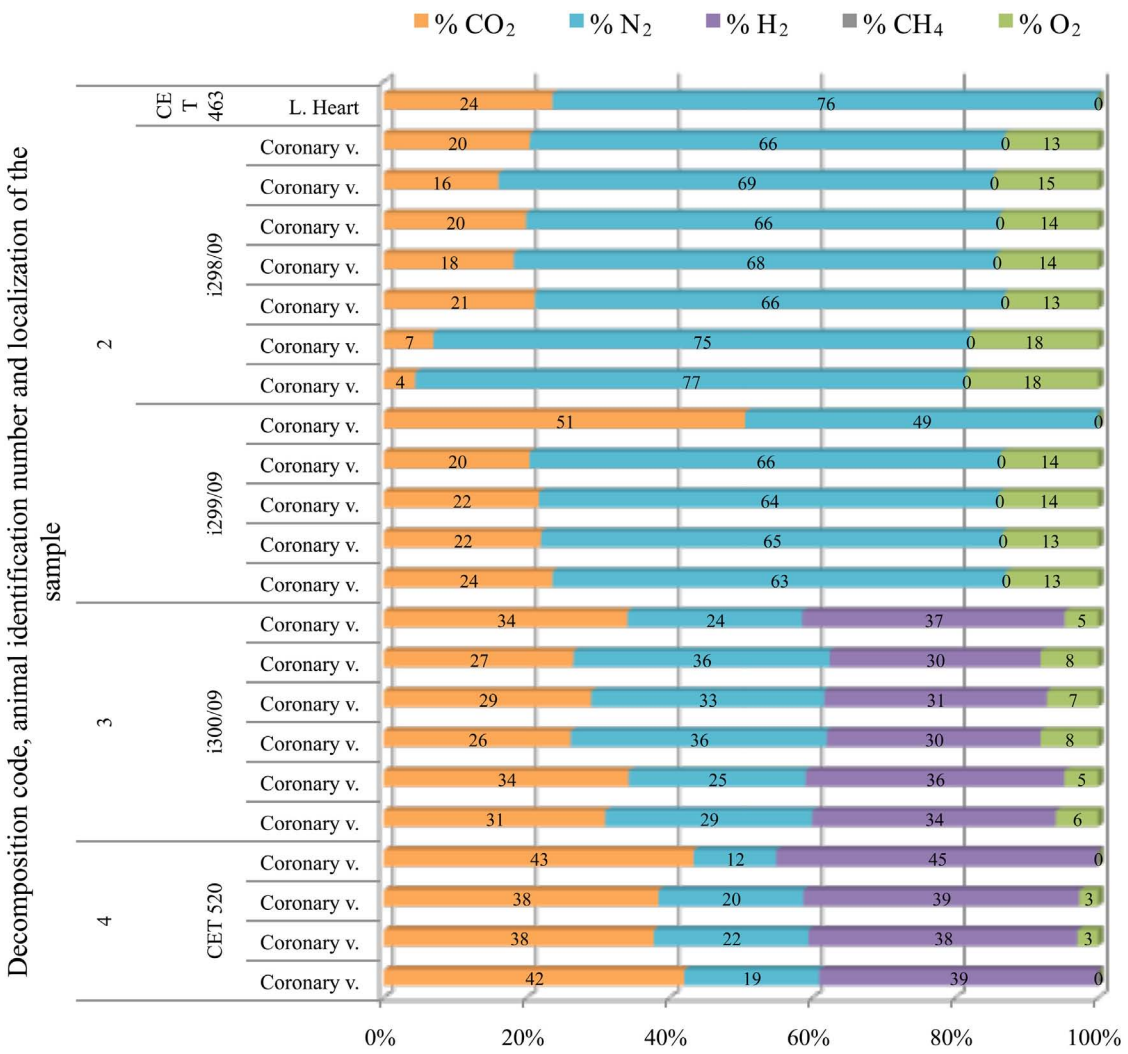

FIGURE 8 | Intravascular bubble's gas composition of deep diving animals belonging to Physeteridae family in different tissues and decomposition codes, illustrating the contribution of each gas to the total amount in percentage μmol.

decomposition code 4, CO_2 increased devaluating nitrogen presence. There were two samples with atmospheric-air like composition (CET 512 and CET 504) (**Figure 13**).

DISCUSSION

This is the first comprehensive study of prevalence, amount (gas score), distribution, and composition of bubbles found in stranded

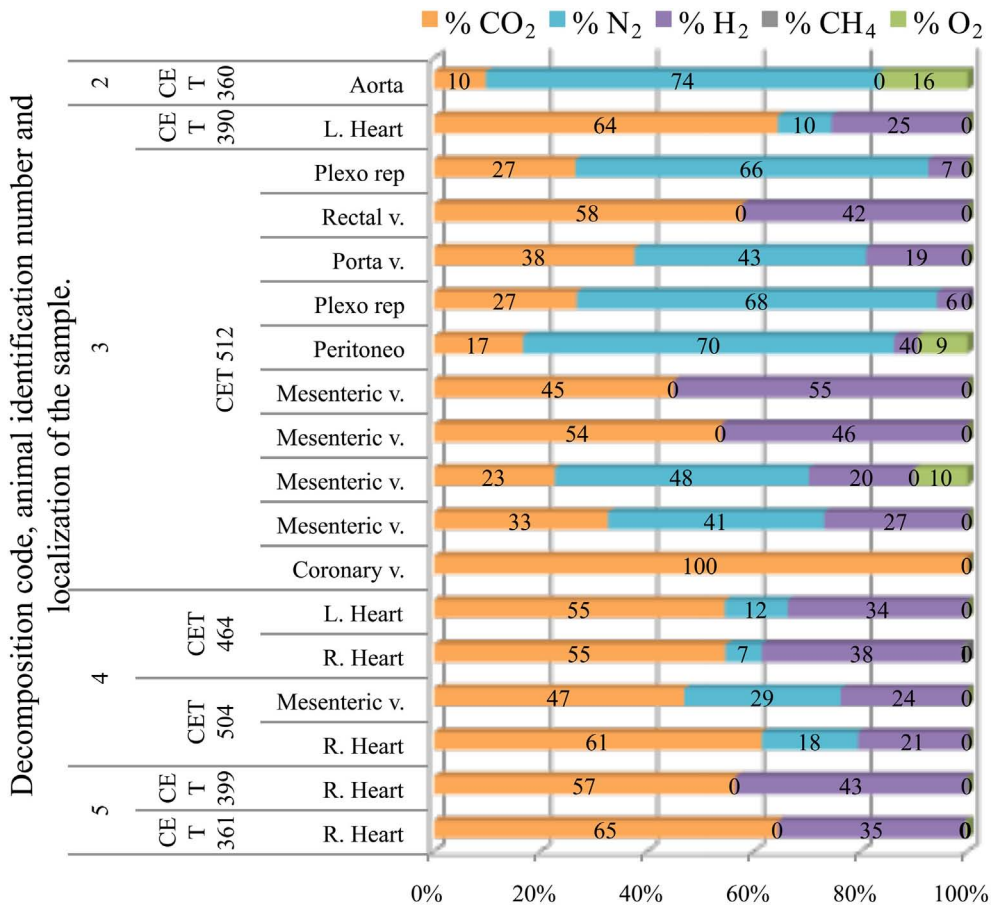

FIGURE 9 | Intravascular bubble's gas composition of deep diving animals belonging to *Globicephala macrorhynchus* specie in different tissues and decomposition codes, illustrating the contribution of each gas to the total amount in percentage μmol.

cetaceans. The animals included in the study presented different decomposition codes allowing us to study putrefaction gases that may mask a decompression-induced gas phase. In addition, the high number of animals (83) and species included in the study (18) enabled us to relate the results with the diving behavior of the animals. This study demonstrates the utility of scoring and analyzing the gas in stranded marine mammals, especially in fresh animals, encouraging the performance of the necropsy as soon after death as possible.

BUBBLE PREVALENCE AND ABUNDANCE

Fifty-one out of 88 stranded animals presented macroscopic bubbles during the necropsy. This represents 58% of the animals that were studied indicating that the presence of gas bubbles within the cardiovascular system in stranded cetaceans is a common gross finding during necropsy.

In forensic human pathology the presence of intravascular gas in carcasses decomposing under different environmental conditions is well known and widely reported (Knight, 1996). Indeed, bubbles were found in all the studied animals with decomposition code 4 and 5. Putrefaction is a continual process of gradual decay and disorganization of organic tissues and structures after death that results in the production of liquids, simple molecules, and gases (intravascular gas and/or putrefactive emphysema; Vass et al., 2002; Lerner and Lerner, 2006). However, it is very unusual to find gas bubbles within the cardiovascular system in fresh necropsied domestic animals or humans (King et al., 1989; Knight, 1996). Therefore, the most interesting data are those recorded from fresh animals. Our results demonstrated that it is not uncommon to find intravascular bubbles in fresh cetaceans. Thirty-three percent of our fresh animals presented intravascular bubbles to some extent, but only two animals presented high gas score. Recent studies have demonstrated that large amounts of intravascular bubbles found within a few hours PM are not due to putrefaction processes (Bernaldo de Quirós, 2011). Indeed, these animals were the only ones diagnosed with gas embolism according to the pathological studies carried out by the ULPGC. Therefore amount of intravascular bubbles is more important than the mere presence of bubbles from a pathological point of view in stranded cetaceans, and the gas score is also an efficient tool to distinguish between gas embolism and putrefaction gases in stranded cetaceans. Although we used a simple gas scoring technique with a scale from 0 to 10 because the study was done retrospectively, we encourage using a larger gas score scale (0–27) described by Bernaldo de Quirós (2011). This

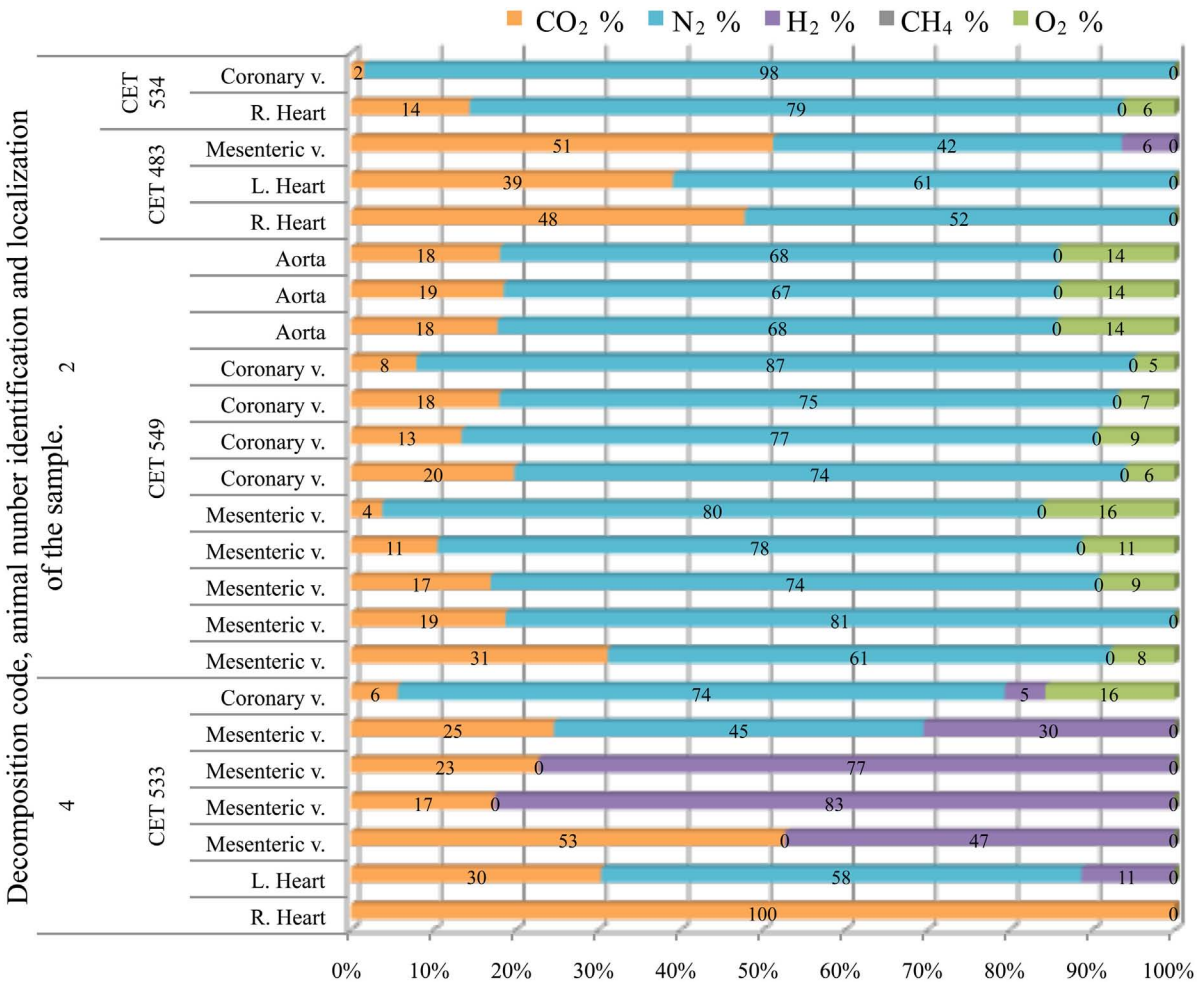

FIGURE 10 | Intravascular bubble's gas composition of deep diving animals belonging to *Grampus griseus* specie in different tissues and decomposition codes, illustrating the contribution of each gas to the total amount in percentage μmol.

gas score has a grading scoring of 0–6 for the different vascular localizations ($n = 4$) and a 0–3 grading scoring for subcapsular gas (0–3). Therefore, total gas score in each animal ranged from 0 to 27. A larger scale highlights better the possible differences in gas score along decomposition codes.

Subcapsular emphysema in the peri-renal area has been described in 22 of 22 alive stranded animals using B-mode ultrasound (Dennison et al., 2012). In our study, we found it in 20 out of 39 fresh animals. Although the prevalence is smaller in our study, it still suggests that subcapsular emphysema is a common finding in dead stranded cetaceans. However, we could not find a clear relationship between subcapsular emphysema and intravascular bubbles. There is a 60% chance that a fresh animal with intravascular bubbles presents subcapsular emphysema, but 60% of the fresh animals with subcapsular emphysema showed no intravascular bubbles. Therefore, intravascular bubbles can occur without simultaneous subcapsular emphysema and *vice versa*.

An interesting result from our data was that intravascular bubbles were more frequently found ($P = 0.006$) and in higher quantities in deep divers than in non-deep divers regardless of decomposition code. The higher prevalence of intravascular bubbles related to the diving behavior of the species, together with the fact that finding bubbles in necropsied domestic animals or humans is very unusual and mostly linked to iatrogenic air embolism or diving fatalities (Knight, 1996; Muth and Shank, 2000), suggest that the most parsimonious explanation for the presence of small quantities of intravascular bubbles in fresh stranded cetaceans would be diving physiology related (Tikuisis and Gerth, 2003). Thus our results further suggest that deep divers might be at a higher risk from decompression.

GAS COMPOSITION

Intravascular bubbles and subcapsular emphysema of fresh marine mammals (decomposition code 2) showed high concentrations of nitrogen (>70%) and values of CO_2 around 20%. Similar results were obtained in animal models exposed to air embolism or compression and decompression using the same methodology (Bernaldo de Quirós, 2011). Furthermore, these values are in accordance to what has been reported in cases of air embolism and/or DCS in humans and laboratory animals

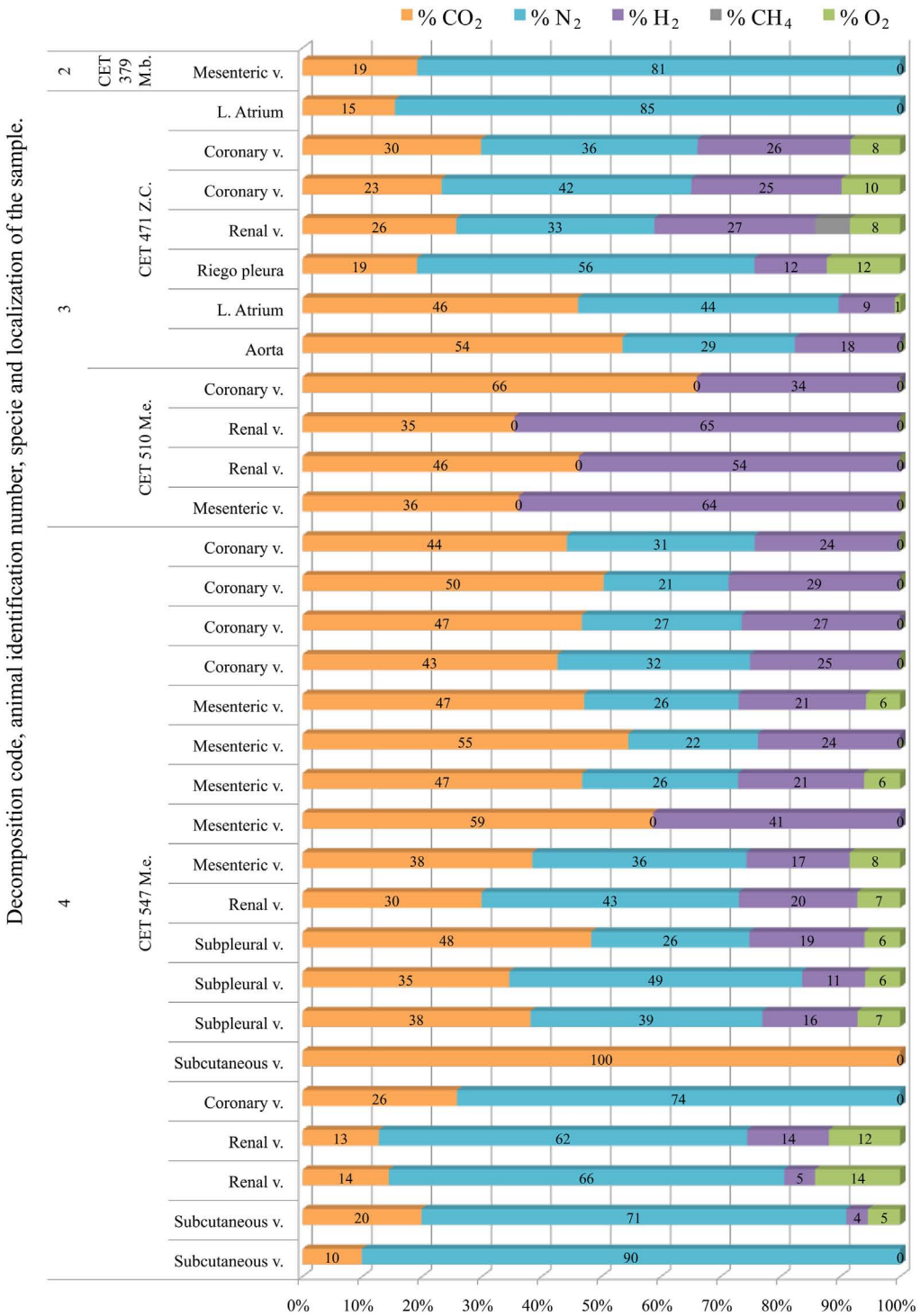

FIGURE 11 | Intravascular bubble's gas composition of deep diving animals belonging to *Ziphiidae* (M.b., *Mesoplodon bidens*; Z.c., *Ziphius cavirostris*; M.e., *Mesoplodon europaeus*) family in different tissues and decomposition codes, illustrating the contribution of each gas to the total amount in percentage μmol.

(Bert, 1878; Armstrong, 1939; Pierucci and Gherson, 1968; Smith-Sivertsen, 1976; Ishiyama, 1983; Bajanowski et al., 1998; Bernaldo de Quirós, 2011). In addition, gas from pterygoideal sinuses showed high concentrations of nitrogen in animals with decomposition codes 2 and 3. This was an additional finding that needs further investigation (**Figure 13**).

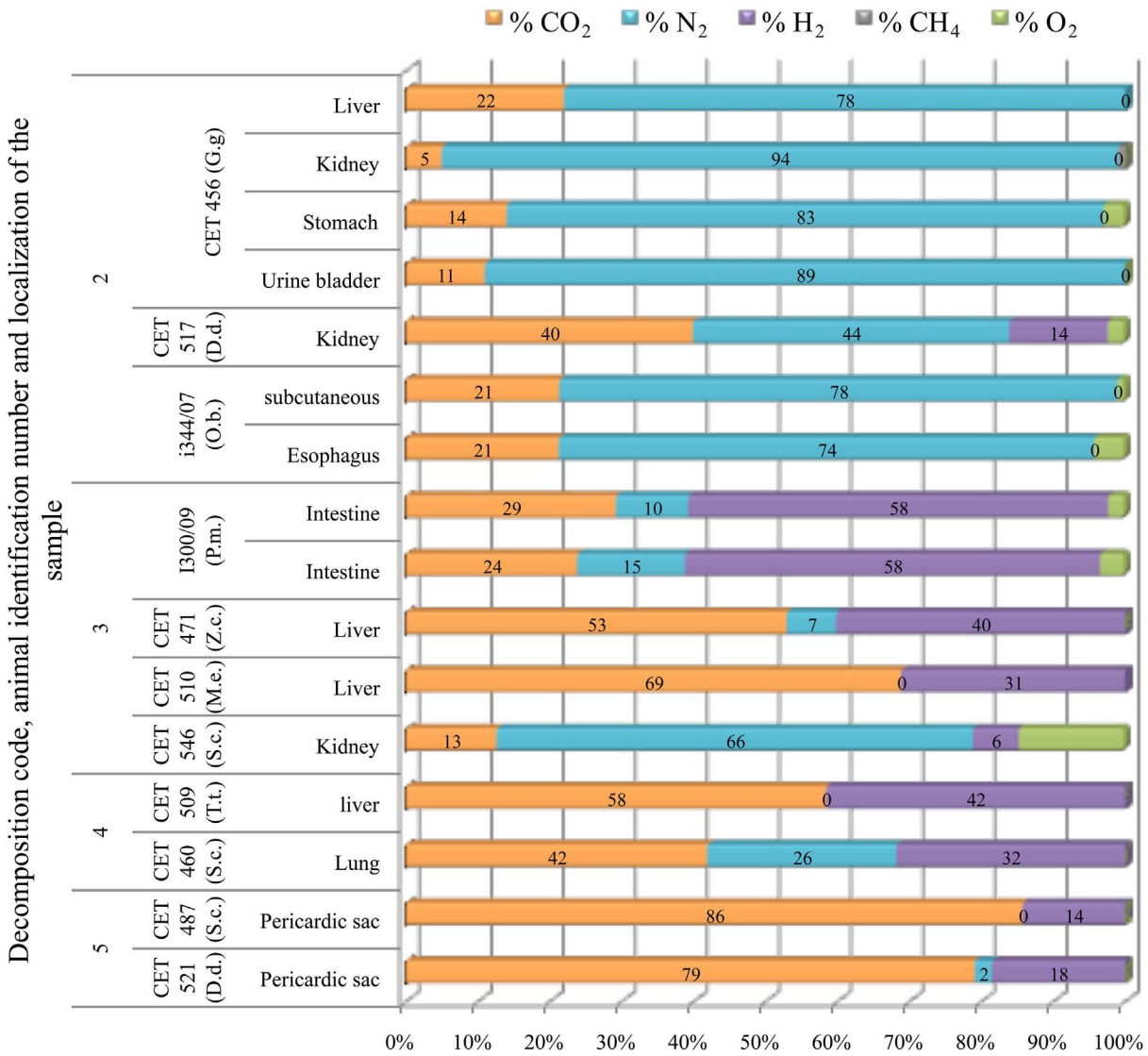

FIGURE 12 | Subcapsular gas (emphysema) composition found in different tissues from deep diving (G.g., *Grampus griseus*; D.d., P.m., *Physeter macrocephalus*; Z.c., *Ziphius cavirostris*; M.e., *Mesoplodon europaeus*) and shallow diving animals (D.d., *Delphinus delphis*; O.b., *Otaria byronia*; S.c., *Stenella coeruleoalba*; T.t., *Tursiops truncatus*) with different decomposition codes, illustrating the contribution of each gas to the total amount in percentage μmol.

Hydrogen, which is a putrefaction indicator (Pierucci and Gherson, 1969), was mostly found after decomposition code 3 in gas collected from the different localizations (intravascularly, subcapsularly, or in the pterygoideal sinuses). Animals with incipient autolysis (decomposition code 3), had gas samples that varied from typical gas embolism composition to putrefaction gases (Pierucci and Gherson, 1968, 1969). Samples from animals with decomposition code 4 and 5 always presented high CO_2 concentrations together with hydrogen in most of the cases and low concentration of nitrogen (mostly lower than 40%) if present. In a few decomposed samples, small quantities of oxygen were also detected. This gas composition was very similar to the gas composition of the subcapsular emphysema found in very decomposed animals and completely different from gas composition of intravascular bubbles and subcapsular emphysema in fresh animals.

These data show that detection of hydrogen and/or very high levels of CO_2 are "signal" gases indicative of putrefaction, while nitrogen decreases progressively until disappearing (not detected chromatographically) in carcasses with the highest decomposition code (code 5). Similar results have been described in humans and laboratory animals (Pierucci and Gherson, 1968, 1969; Keil et al., 1980; Bajanowski et al., 1998). In this sense, we can state that hydrogen in stranded cetaceans is also a key putrefaction marker with one exception that will be discussed later. In some cases, we have found one sample's composition to be clearly different from the rest of the samples, presenting significantly higher nitrogen and oxygen content. Thus, we have considered as a first option, a

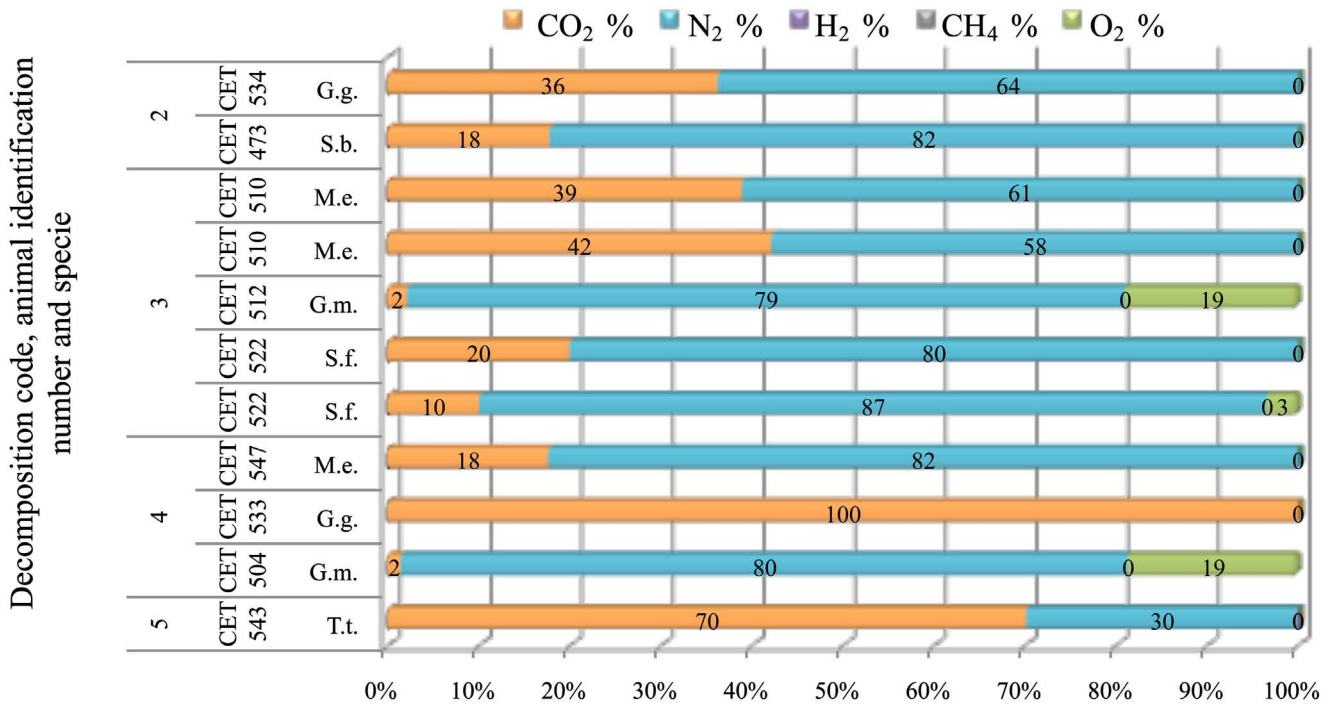

FIGURE 13 | Gas composition of pterygoid air sacs from animals (G.g., *Grampus griseus*; S.b., *Steno bredanensis*; M.e., *Mesoplodon europaeus*; S.f., *Stenella frontalis*; G.m., *Globicephala macrorhynchus*; T.t., *Tursiops truncatus*) with different decomposition codes, illustrating the contribution of each gas to the total amount in percentage μmol.

likely contamination of atmospheric-air during sampling (see as an example the sample from CET 546 in **Figure 12**). The exception previously mentioned, was a *Steno bredanensis* (CET 473) with decomposition code 2 that presented hydrogen in the mesenteric veins. Here, it is important to remember that these are stranded animals, and in most of the cases sick animals. Therefore these gas results should be considered together with the individual diagnostic analysis (including pathology, microbiology, toxicology, etc.). Speculatively, we have considered acute gastrointestinal disease as a primary source of hydrogen or due to overlapping with other pathogenic processes, although further research is needed in order to confirm this hypothesis.

One of the most interesting and unexpected results was CO_2 concentrations of around 40–50% in the animal with the second highest gas score for fresh animals and diagnosed with gas embolism by the ULPGC (CET 483). This gas composition is not in accordance with traditional DCS theories or some of the empirical values reported for DCS (Bert, 1878; Smith-Sivertsen, 1976; Ishiyama, 1983; Tikuisis and Gerth, 2003). However Armstrong (1939) reported values of 30% of CO_2 in decompressed goats, and Bernaldo de Quirós (2011) has recently reported very similar CO_2 concentrations to our results in rabbits.

In addition there are several empirical and physical models suggesting an important role of CO_2 in bubble formation: (i) there is a higher prevalence of elevated CO_2 in bends following dives using compressed air (Behnke, 1951), (ii) a statistically significant increase in DCS risk in rats breathing elevated levels of CO_2 in either He-O_2, or N_2-O_2 mixtures during the hyperbaric exposure has been reported (Berghage et al., 1978), (iii) bubbles moving vertically through different water layers alternately saturated with air or CO_2, increase in size in the CO_2 saturated water and decreasing in the air-saturated layer (Harvey, 1945), (iv) bubbles formed with less mechanical agitation and grew at a faster rate in decompressed tubes filled with water saturated of CO_2 rather than nitrogen (Harris et al., 1945). All these findings, suggest that CO_2 might play an important role in bubble formation due to the high diffusivity of this gas. If this is the case, prolonged dives where CO_2 will build up might pose an additional risk to animals with nitrogen saturation.

Theoretical studies have predicted BWs end-dive nitrogen levels that would cause a significant proportion of DCS cases in land mammals (Houser et al., 2001; Zimmer and Tyack, 2007; Hooker et al., 2009). In addition BWs have both the deepest (3120 m) and longest (137 min) dives ever recorded from an air-breathing mammal (Schorr et al., 2011). Furthermore, BWs exposed to mid-frequency sonar playback have reacted with unusual longer and slower ascent dives (Tyack et al., 2011). All BWs included in our study presented a minimum gas score of 5, this was not found in the other studied sub groups. Although more fresh animals should be studied in order to make conclusions, our results suggest that BWs might be the most sensitive species to bubble formation. Future theoretical studies should consider CO_2 accumulation in addition to nitrogen saturation, and its possible role in bubble formation.

We have shown that gas analysis may be a valid a technique to differentiate between gas embolism and putrefaction gases in stranded cetaceans. Therefore we encourage gas analysis. To try to avoid putrefaction-masking gases, necropsy, and gas sampling

must be performed as soon as possible, before 24 h PM is recommended but preferably within 12 h PM. However, if gas analyses are not possible due to logistics, we strongly recommend doing the gas scoring, which is of no additional cost.

SOURCE OF BUBBLES

In summary, intravascular bubbles and subcapsular emphysema are a common finding during necropsies of stranded marine mammals, although they are present in low quantities (low gas score) and with a gas composition of around 70–80% of nitrogen and 20–30% of CO_2. They are more frequently found ($P = 0.006$) and in higher quantities in deep divers vs. non-deep divers. They have higher prevalence in active stranded animals, although they are also present in passive stranded animals. Based on gas composition and in the highest prevalence of bubbles in deep divers, the most likely source of these bubbles is decompression-related.

In air-breathing animals, nitrogen can build up in tissues and formed bubbles. Although they only have a finite amount of air in their lungs available for nitrogen diffusion, PN_2 in the alveoli increases with compression of the thorax as the pressure increases. This results in a net exchange of nitrogen across the alveolar membrane and it is either taken up or removed by the blood and tissues depending on the partial pressure gradient. During ascent, nitrogen diffusion from tissues to blood and alveolus is slower, and if the surface interval is too short, not all of the nitrogen that has been taken up is removed. This may result in nitrogen build up in tissues during repeated dives (Ferrigno and Lundgren, 2003). When the sum of the dissolved gas tensions (oxygen, CO_2, nitrogen, helium) and water vapor exceeds the local absolute pressure, a state known as supersaturation, bubbles may form due to gas phase separation (Hamilton and Thalmann, 2003; Vann et al., 2011). Bubbles can form without negatively impacting a diving animal, but bubbles can have mechanical, embolic, and biochemical manifestations ranging from trivial to fatal (Vann et al., 2011). They are generally considered a pivotal event in the occurrence of DCS (Francis and Simon, 2003).

Decompression bubble formation may still occur even after death if the animal dies at depth and is later depressurized, a phenomenon known as *post mortem* off gassing (Brown et al., 1978; Lawrence, 1997; Cole et al., 2006; Lawrence and Cooke, 2006; Wheen and Williams, 2009). *Post mortem* off gassing has been proposed for explaining the existence of bubbles in marine mammals trapped in fishing nets (Moore et al., 2009). These animals died at depth and were later hauled out. Our studied animals are stranded marine mammals, which presumably have not died at depth. Thus, *post mortem* off gassing is ruled out as a plausible mechanism. The stated absence of bubbles in stranded animals in the Moore et al. study probably reflects a relative, rather than absolute absence, with bubble quantity being far more obvious in the animals drowned at depth that off-gassed *post mortem*. These authors probably failed to record the low prevalence of bubbles commonly seen in stranded animals in the current study (Michael Moore, personal communication). It is interesting that the Moore et al. findings likely reflect a routine supersaturation state in foraging, diving marine mammals. Analysis of the gas bubble composition of drowned bycatch would test this hypothesis.

The most likely explanation for the observed gas composition in the freshly dead animals is that bubbles were *in vivo* or *peri* mortem formed from supersaturated tissues by physiological off gassing. Indeed, a recent study has shown that intravascular bubbles and peri-renal subcapsular emphysema occur in live marine mammals and without clinical consequences (Dennison et al., 2012). The stranding itself has been proposed as a causal mechanism for bubble formation in marine mammals (Houser et al., 2010; Dennison et al., 2012). According to this hypothesis, a fast stranding will result in the inability to recompress resulting in problematic supersaturation of the tissues. In addition, as a consequence of the stranding, there would be tissues of reduced perfusion and blood pooling that would continue to off-gas, but because the vascular flow to the lung is supposed to be compromised, nitrogen elimination would not be as effective and autochthonous bubble formation would increase (Houser et al., 2010).

Another plausible stranding mechanism related would be tribonucleation. Tribonucleation occurs when two surfaces in intimate contact but separated by a viscous liquid film are suddenly separated in an abrupt way. Then, the viscosity will avoid sudden filling and cavities, which will be filled by diffusing gases, can occur (Banks and Mill, 1953; Hayward, 1967; Campbell, 1968; Ikels, 1970; Blatteau et al., 2006). This phenomenon has been proposed to happen with movements, exercise (McDonough and Hemmingsen, 1984a,b, 1985a,b), within the joints (Fick, 1911), or in the heart valves (Hennessy, 1989). The agonal actions of the beached animals have been proposed to promote bubble formation (Houser et al., 2010).

We have found intravascular bubbles in 3 out of 15 of the animals that stranded passively, where reduced perfusion and tribonucleation are not expected. Regardless of the low number of passive strandings studied, our results support the idea that bubbles can occur independently to the stranding, as reported in three BWs (one fresh, one partially autolytic, and an autolytic animal) which were recovered floating in the atypical mass stranding event of the Canary Islands in 2002 (Fernandez et al., 2005). However, since we have found a higher prevalence of bubbles in actively stranded animals (although the difference was non-statistically significant), we suggest that stranding is not a necessary factor but a contributing factor in bubble formation.

More recently, Houser et al. (2010) and Dennison et al. (2012) hypothesized that the abnormal behavior of moribund cetaceans that might spend a period of time at sea with reduced depth and repetitiveness, may allow them to wash out nitrogen prior to stranding. Few (Dennison et al., 2012) or no (Houser et al., 2010) empirical data were presented to support this suggestion. Our study animals are mostly single stranded specimens, affected by different pathologies (**Table 1**). According to the mentioned hypothesis, it is presumed that our case studies have washed out their nitrogen prior to stranding. Our results do not support this hypothesis; intravascular bubbles were found in 57% of the fresh deep diving animals and in 20% of the non-deep diving animals, and they were mainly composed of nitrogen (70–80%). Dennison et al. (2012) further hypothesize that the prevalence of bubbles will be less in most single stranded than mass stranded animals, if they were diving less over an extended period. Since our study cases are mostly single stranded animals, we could not test this

hypothesis. Further studies are needed to confirm whether mass stranded animals have higher prevalence of bubbles compared to single–stranded animals.

If bubbles are not a consequence of the stranding, and since no embolic related lesions were found in the pathological study, it is reasonable to consider as a hypothesis that this small amount of bubbles composed of nitrogen found in fresh stranded animals, were silent bubbles. The existence of potentially asymptomatically bubbles in marine mammals has been reported before (Dennison et al., 2012). However, we studied dead animals where symptoms could not be evaluated, therefore our results are indicative but not conclusive by itself of the existence of silent bubbles in marine mammals. A recent review of evidences for gas bubble incidence in marine mammals concluded that they may deal with bubbles on a more regular basis than previously thought (Hooker et al., 2012) and suggested that our view of marine mammal adaptations should therefore change from one of simply minimizing nitrogen loading to one of management of the nitrogen load. Our study further supports the hypothesis that decompression-induced bubbles are relatively common but that marine mammals have unknown adaptations allowing them to tolerate these under natural conditions. It is important to remember that our case studies have only few bubbles and died from numerous pathological reasons different from gas embolism. Only two animals, showing a lot of bubbles widely dispersed and with no other pathological signs, were diagnosed with massive gas embolism.

Further research is needed in order to clarify questions that remain unanswered from this study. The clinical impact of subcapsular emphysema remains unclear from this study since gas composition indicates a plausible physiological decompression-related off gassing origin, but no clear relationship with intravascular bubbles was found. More studies are needed in order to better understand the formation of this gas and the plausible impact that might have on the health status of the animals. Comparison between single-active, single-passive, mass stranded, and by caught animals, might contribute to a better understanding of the causative factor of intravascular bubble formation as well as nitrogen supersaturation levels on these animals, and different species sensibilities (like deep divers vs. non-deep divers). These will provide new data that should be considered in future diving physiology models and studies.

In conclusion, this study has demonstrated that bubbles are a common finding in stranded cetaceans. The amount and composition of these bubbles in fresh animals suggests that these bubbles have formed from nitrogen-supersaturated tissues, most likely formed *in vivo*. This study has also demonstrated that these bubbles were more frequently found in deep divers indicating a higher risk of decompression to these species. Further research is needed in order to determine if there are any species-specific sensibilities to bubble formation as preliminary results point out, as well as the implications that CO_2 accumulation along a dive might have for these animals from a diving physiology perspective. Finally, monitoring live non-deep diving and deep diving animals with an ultrasound might help to confirm the hypothesis deduced from our results.

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The use of diagnostic imaging for identifying abnormal gas accumulations in cetaceans and pinnipeds

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Recent dogma suggested that marine mammals are not at risk of decompression sickness due to a number of evolutionary adaptations. Several proposed adaptations exist. Lung compression and alveolar collapse that terminate gas-exchange before a depth is reached where supersaturation is significant and bradycardia with peripheral vasoconstriction affecting the distribution, and dynamics of blood and tissue nitrogen levels. Published accounts of gas and fat emboli and dysbaric osteonecrosis in marine mammals and theoretical modeling have challenged this view-point, suggesting that decompression-like symptoms may occur under certain circumstances, contrary to common belief. Diagnostic imaging modalities are invaluable tools for the non-invasive examination of animals for evidence of gas and have been used to demonstrate the presence of incidental decompression-related renal gas accumulations in some stranded cetaceans. Diagnostic imaging has also contributed to the recognition of clinically significant gas accumulations in live and dead cetaceans and pinnipeds. Understanding the appropriate application and limitations of the available imaging modalities is important for accurate interpretation of results. The presence of gas may be asymptomatic and must be interpreted cautiously alongside all other available data including clinical examination, clinical laboratory testing, gas analysis, necropsy examination, and histology results.

Keywords: computed tomography, ultrasound, magnetic resonance imaging, cetacean, decompression sickness, bends, pinniped, gas bubbles

BACKGROUND

Historically marine mammals have been considered protected from decompression sickness (DCS) due to anatomical, physiological, and behavioral evolutionary adaptations. The most important of these concepts was that lung compression induced pulmonary shunting and altered peripheral blood flow by a combination of dive-induced bradycardia with peripheral vasoconstriction, limited the amount of nitrogen that could cross from the pulmonary alveoli in to blood and from blood in to tissues (Scholander, 1940; Butler and Jones, 1997; Kooyman and Ponganis, 1998). Observations of DCS-like gas and fat embolic lesions in stranded beaked whales (Fernández et al., 2005) and acute and chronic gas embolic lesions in multiple cetacean species (Jepson et al., 2003, 2005) in addition to dysbaric osteonecrosis-like skeletal lesions in sperm whales (Moore and Early, 2004) have ignited a contentious debate with divergent opinions on how marine mammals manage gases during diving (Hooker et al., 2012).

In humans, *de novo* gas bubble formation is reported under hyperbaric and, with less frequency, hypobaric environmental conditions (Pilmanis et al., 2004; Barak and Katz, 2005). In both scenarios, gas bubbles have the opportunity to form when the environmental pressure of nitrogen reduces more rapidly than its tissue pressure, so that the dissolved tissue nitrogen transitions into its gaseous phase (Pilmanis et al., 2004). DCS is the term used when clinical symptoms of this transition manifest in humans. While bubbles are thought to be the precursor for the development of DCS, particularly if within the arterial circulation (Ljubkovic et al., 2011), the presence of gas bubbles alone does not indicate that DCS has, or will, develop (Brubakk and Eftedal, 2001; Barak and Katz, 2005; Ljubkovic et al., 2011). Symptoms experienced in human DCS cases are very varied and non-specific, and are dependent on where gas bubbles lodge and the degree of tissue damage caused. Tissue damage occurs due to primary or secondary mechanisms. The primary mechanisms are thought to be via vascular occlusion (embolism) resulting in tissue ischemia, and parenchymal damage or joint distension due to extravasated bubbles that expand during ascent, resulting in a space-occupying lesion that causes pain (Kayar et al., 1997; Thom et al., 2011). Secondary mechanisms include activation of the complement cascade or activation of inflammatory pathways (Kayar et al., 1997; Thom et al., 2011). These pathways are an area of great interest in human DCS studies and are triggered via endothelial damage and the release of microparticles (small vesicles or fragments that are released from stressed or damaged endothelium and circulate in the blood) in response to decompression stress (Barak and Katz, 2005; Brubakk et al., 2005; Ljubkovic et al., 2011; Thom et al., 2011). Multiple risk factors have been modeled to investigate what factors result in the development of DCS in humans subsequent to gas bubble formation, but the process remains poorly understood (Weathersby et al., 1984, 1992; Pilmanis et al., 2004; Barak and Katz, 2005).

APPLICATION OF DIAGNOSTIC IMAGING FOR ABNORMAL GAS ACCUMULATIONS

Available diagnostic imaging for use in cetaceans and pinnipeds includes radiography, ultrasound, and magnetic resonance imaging (MRI) and the use of these modalities in marine mammal medicine is not new. Published use of radiography in marine mammal research dates back to the 1960s (Felts and Spurrell, 1966; Sommer et al., 1968) and has been described in the clinical setting (Sweeney, 1990). The use of computer tomography (CT), MRI, and ultrasound in marine mammals has also been described (Brooks et al., 2001; Van Bonn et al., 2001). Radiography describes the use of x-rays to produce an image and digital radiography, by this definition, includes direct radiography (DR), computed radiography (CR) and CT. Each of these diagnostic imaging modalities produces an image via different methods, and a basic understanding of the physics including limitations and potential pitfalls, is needed so that the modalities are applied appropriately. Size and weight limitations exist and the use of diagnostic imaging in larger cetaceans or even large pinnipeds may be limited or even impossible. When considering imaging dead animals as part of a post mortem investigation, only fresh-dead, intact cadavers should be considered as appropriate for evaluation for gas. If disarticulation is needed to accommodate imaging, or if decomposition has begun, the presence of any gas observed on images becomes questionable. However other information may still be acquired from imaging such cases that could help determine the cause of death.

DIRECT RADIOGRAPHY (DR) AND COMPUTED RADIOGRAPHY (CR)

DR and CR have increased the sensitivity for detection of small volume gas accumulations compared to conventional screen-film radiography (Marolf et al., 2008). Despite this, superimposition of structures, the need for relatively large gas accumulations for detection, and practical aspects (maneuvering large marine mammals to position radiographic plates, production of not insignificant amounts of scatter radiation in larger animals, and difficulties in achieving adequate kVp for penetration) often limit the use of DR and CR. Appropriate applications do exist in smaller sized pinnipeds and cetaceans, and successful identification of abnormal gas has been published (Van Bonn et al., 2011). Because of the limitations, DR and CR will not be discussed further.

ULTRASOUND

Diagnostic ultrasound utilizes sound in the realm of 2–20 MHz. The sound beam produced by the piezoelectric transducer penetrates the patient and is reflected at interfaces created by different structures or substances. The proportion of the sound beam reflected is relative to the differences in acoustic impedance of the substances at interfaces (Zagzebski, 1996; Drost, 2007). The greatest difference in acoustic impedance is created at gas-fluid and gas-tissue interfaces and results in near perfect reflection of the sound beam. This causes specific artifacts to be created and displayed on the gray-scale image (Zagzebski, 1996). In the case of large accumulations of gas, reverberation artifacts with equally spaced repetitive bright lines are observed, **Figure 1**. Furthermore, gas bubbles present as a foam produce a specific subtype of reverberation artifact referred to as ring-down artifact, **Figure 2**, where sound bounces between the small gas bubbles before finally being reflected back to the transducer. This artifact is identified by a lack of tapering toward the bottom of the image. Larger gas bubbles present as individuals or in small clusters are often observed as bright foci on the image and may or may not produce reverberation artifact (Kirberger, 1995; Feldman et al., 2009). B-mode ultrasound can be used to detect both stationary and moving gas bubbles.

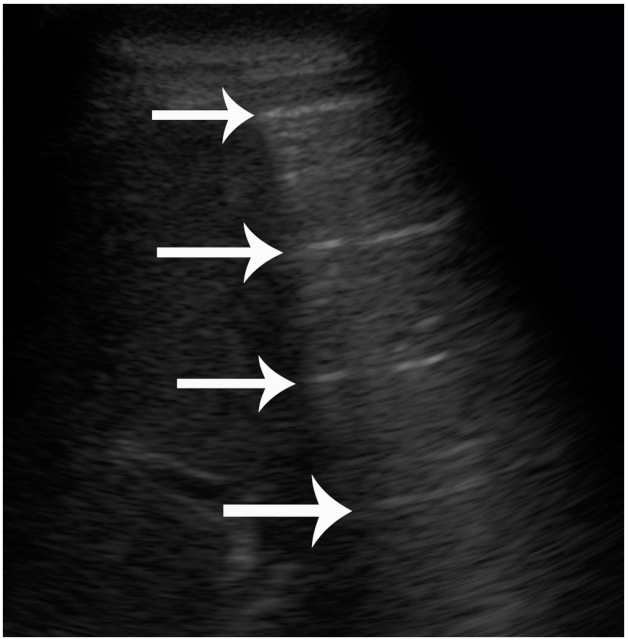

FIGURE 1 | B-mode ultrasound image from a live-stranded common dolphin (*Delphinus delphis*). Reverberation artifact of normal lung. Some of the liver is seen on the left side of the images. The gas within the periphery of the lung causes near perfect reflection of the sound beam. The result is a repeated, equally spaced hyperechoic line (bright) being displayed on the image (arrows).

Doppler ultrasound measures the frequency shift of reflectors as they pass through the sound beam by application of the Nyquist equation (Zagzebski, 1996; Drost, 2007). The frequency shift is within the audible range and can be assessed qualitatively as sound or observed quantitatively as a tracing on the computer display. Most modern ultrasound machines can provide both data simultaneously to improve sensitivity. Pulse-wave Doppler is typically used to evaluate intravascular blood flow. When intravascular gas bubbles traveling with the blood pass through the sound beam, the high difference in acoustic impedance between the gas bubble and the surrounding blood results in a high frequency peak on the pulse-wave tracing and an audible "chirp" that is distinguishable from the background sound of the moving red blood cells (Gillis et al., 1968; Kirberger, 1995; Feldman et al., 2009). Pulse-wave Doppler is only applicable in the case of abnormal gas bubble formation, in the detection of circulating gas bubbles.

B-mode and pulse-wave Doppler ultrasound have been used successfully in the research setting to evaluate for the presence of bubbles in human divers (Gillis et al., 1968; Ljubkovic et al.,

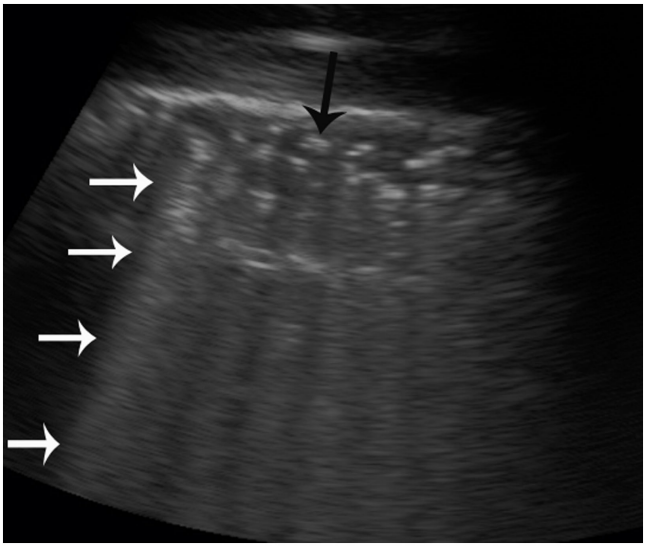

FIGURE 2 | B-mode ultrasound image of a live-stranded common dolphin (*Delphinus delphis*) kidney. Multiple hyperechoic (bright) foci are observed at the top of the image (black arrow). Ring-down artifact is seen (white arrows) as repeating hyperechoic (bright) lines that do not taper or diminish toward the bottom of the image, confirming that gas is present.

2011), in the clinical setting for cases of suspected air embolism of any etiology including DCS, and in contrast echocardiography studies where stabilized microbubbles are injected intravenously (Bonagura and Pipers, 1983; Stewart, 2003; Miller et al., 2008) B-mode, pulse-wave Doppler and combined B-mode and pulse-wave Doppler methodologies are comparable for intravascular gas bubble detection and quantification (Brubakk and Eftedal, 2001).

Highly portable ultrasound machines that can run from battery power and be easily transported are fairly widely available. These units are typically the size of a laptop computer, but hand-held devices are also being developed. This makes ultrasound highly desirable particularly in the case of live-stranded cetaceans where delays in relocation and release may be detrimental and units can be taken directly to the side of the animal. Ultrasound also has a place in the recently dead animal pre-necropsy when advanced cross-sectional imaging modalities (CT or MRI) are not available to help determine if abnormal gas is present prior to dissection.

Several limitations exist when using ultrasound. Depth of penetration is limited and is dependent on the transducer being used. For example, a transducer operating at 4 MHz typically has a maximal depth of penetration of approximately 25 cm, allowing evaluation of the kidneys in pinnipeds, bottlenose dolphins, and beluga and pilot whales, while for a 12-MHz transducer the maximal depth may be as low as 6 cm, allowing evaluation of the eye. Even ignoring the depth limitations, it is not possible for the entire animal to be thoroughly evaluated via ultrasound for gas as regions that normally contain gas, such as the gastrointestinal tract and lungs, prevent evaluation of structures that lie deep to them. When evaluating for abnormal gas accumulations, only regions that do not normally contain gas and lie fairly superficially can be assessed. The most easily accessible and identifiable organs that lie peripherally are the blubber, superficial musculature, eyes, liver, and kidneys (Dennison et al., 2012).

COMPUTED TOMOGRAPHY

Like DR and CR, CT utilizes x-rays to produce an image with tomography referring to the fact that images are acquired as slices or sections. CT is superior to conventional screen-film CR and DR as superimposition of structures is almost eliminated, depending on operator-selected parameters. CT has greater sensitivity for small differences in attenuation that allows distinction of structures that are indistinguishable on DR, CR, or conventional screen-film radiography (Gore et al., 2000; Bushberg et al., 2002). Different generations of CT scanner exist that utilize varying technologies but the typical scanners currently in use have a CT tube and detector(s) that circle the patient whom is positioned within the gantry. Beams of x-rays pass through the patient through 360°. Different tissues attenuate the x-ray beam to varying degrees and therefore the number of photons (x-rays) that reach the detector at each scanning point around the patient will be different, determined by the tissues passed through. Once all data for an image have been acquired, reconstruction algorithm selections are applied to produce a recognizable displayed image. Usually reconstruction algorithms for both bone and soft tissue optimization are applied, resulting in the production of two series for the same area. Each pixel in the image has a designated shade of gray that represents an attenuation value. Attenuation is quantitative and is measured in Hounsfield units (HU). Average attenuations for fat are −100 HU, soft tissues are +30 to +50, and cortical bone are +1000 to +3500 HU (Bushberg et al., 2002). Essentially gas does not affect the x-ray beam as it passes through, resulting in a very negative HU value and a pixel coloration that is very dark gray to black, **Figure 3**.

Computer tomography is able to distinguish between adjacent soft tissue structures better than conventional screen-film radiography, DR, and CR, but contrast between soft tissue structures remains limited using this x-ray based modality. Careful examination and knowledge of CT anatomy is needed to determine the location of gas observed. Determining the exact location of individual accumulations as vascular, parenchymal, or subcapsular, may be difficult, but distinction between normal and abnormal gas accumulations is usually possible. Patterns of distribution can be useful, for example, branching linear accumulations are suggestive of an intravascular location.

The biggest limitations for the use of CT in marine mammals are non-portability that requires the animal be taken to the scanner, and gantry size and table weight limitations that limit the size of animal that can be scanned. Typical CT gantry internal diameter measurements are around 90 cm but the available scanning field of view is usually less than this. Table weight limitations are around 250–300 kg (Saunders and Ohlerth, 2011) although modified tables designed to support the weight of adult horses and other large animals have been developed. CT tables can usually move approximately 1 m through the gantry but for longer animals, repositioning mid scan may be necessary if whole body evaluation is desirable. In live cetaceans the dorsal fin can be a limiting size factor and may prevent the caudal thorax/cranial abdomen from passing through the gantry.

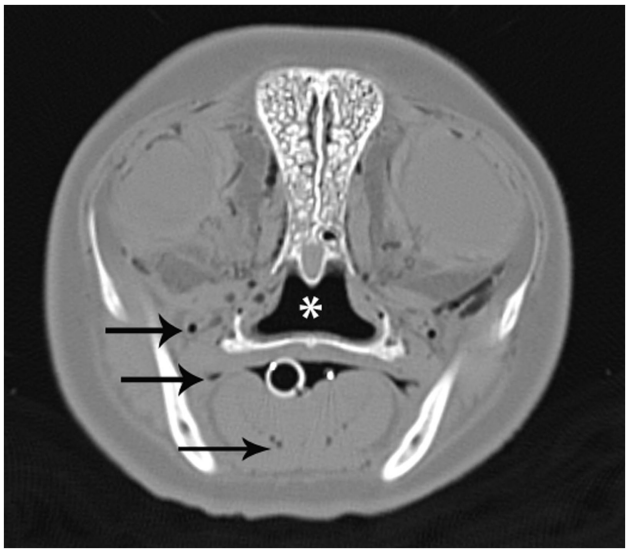

FIGURE 3 | A CT image of the head at the level of the eyes from a bycatch gray seal (*Halichoerus grypus*). The image was acquired using a bone reconstruction algorithm and displayed on a wide window that results in only gas being displayed as dark gray or black. Normal gas accumulations in the nasopharynx (*) and oral cavity (white circle) are observed. Abnormal gas accumulations within tissues and vasculature, some demonstrated by black arrows, are also present.

Computer tomography and MRI have both been used to evaluate victims of human diving fatalities prior to autopsy in order to document the extent of gas accumulations and have proven to be invaluable for focusing autopsy efforts (Ozdoba et al., 2005). In clinical medicine, the fast scan time, and high sensitivity for gas makes CT the preferred modality when gas is suspected or its presence needs to be ruled out (Gore et al., 2000; Sebastia et al., 2000; Schindera et al., 2006). For example, CT of the head might take 3–5 min and abdomen 5–10 min depending on the type of scanner. Abnormal gas can be definitively diagnosed from a single scan, unlike in MRI, by measuring the HU of a suspicious area.

MAGNETIC RESONANCE IMAGING

The greatest advantage of MRI is the contrast this imaging modality affords between and within soft tissue structures, although the use of non-ionizing radiation is also a consideration in live animals. MRI detects signals or energy released from hydrogen within water molecules (Bushberg et al., 2002; Tidwell, 2007). Retrospective manipulation of raw data into alternative sequences is not possible with MRI and therefore appropriate sequence selection prior to scanning is vitally important. Furthermore, multiple sequences in multiple planes are needed for a complete evaluation. Different MRI sequences are used to exploit different properties of the water contained within tissues, which means that the same tissue will often have different signal intensities or shade of gray applied, among different sequences (Tidwell, 2007). Gas contains very few protons and produces a signal void among all MRI sequences represented by black regions on the final images (Tidwell, 2007). Magnetic field inhomogeneities exist at air-tissue interfaces on some sequences, particularly gradient echo sequences. This results in the production of a susceptibility artifact (Rabushka and Kuhlman, 1994; Bushberg et al., 2002) and theoretically allows small gas accumulations to be identified when susceptibility-sensitive sequences are selected.

Care in interpretation of susceptibility artifact is needed, as its presence is not specific to gas. Any substance that results in local inhomogeneities in the magnetic field such as gas, metal, or deoxygenated blood can produce susceptibility artifact on gradient echo sequences (Bushberg et al., 2002; Zhuo and Gullapalli, 2006). This is particularly important to consider in cadaver imaging where deoxygenated blood is to be expected. Blood and gas can be distinguished by comparing different sequences.

Similar to CT, MRI gantry size limitations exist, and units are static requiring that the animal be transported to the scanner. Higher Tesla magnets (1–3 T) that produce high quality images are closed gantry magnets and diameter is typically similar to CT gantries at up to 90 cm. Large bore magnets are becoming available and are particularly geared toward facilitating equine imaging. Open magnets are inherently low Tesla magnets compared to closed magnet configuration (Bushberg et al., 2002) and that affects scan time (making it longer), image quality (lowered), and sequences available (affecting full characterization of pathology observed). However, open magnets may permit imaging in, for example, live cetaceans where the dorsal fin would otherwise be size-prohibitive.

In order to maximize information gained from an MRI study, different sequences in different planes are needed. For MRI a separate scan is required for each sequence and each plane of acquisition, unlike CT. Thus when using MRI each region may require a scan time of 30 min depending on the sequences acquired and the desired amount of coverage. This may increase in live animals if contrast medium is administered. Dedicated animal MRI scanners are available in academic institutions and commercially, and some MRI availability through human imaging centers may be negotiable. However the availability of MRI is generally less than CT in veterinary medicine at this time.

Normal accumulations of gas may obscure adjacent abnormal gas on MRI due to susceptibility artifact ad resolution limitations, **Figure 4**. Cases have been published, however, where MRI has been successful at identifying intestinal wall gas (*pneumatosis intestinalis*) in intestines that contain normal luminal gas (Rabushka and Kuhlman, 1994). Cerebellar gas has been successfully diagnosed using MRI in a California sea lion (Van Bonn et al., 2011).

APPLICATION OF DIAGNOSTIC IMAGING IN DETERMINING THE MECHANISM OF SUPERSATURATION

The suggestion that supersaturation is occurring in marine mammals under normal circumstances is contrary to historical understanding of marine mammal physiology. Early studies had concluded that lung compression and alveolar collapse, and associated pulmonary shunting terminating gas-exchange, occurred between 25 and 30 m in pinnipeds (Falke et al., 1985) and at around 70 m in cetaceans (Ridgway and Howard, 1979). However if supersaturation is occurring it suggests that our understanding of how marine mammals manage gases during diving is poor. Previously it has been shown that compression of the respiratory system and

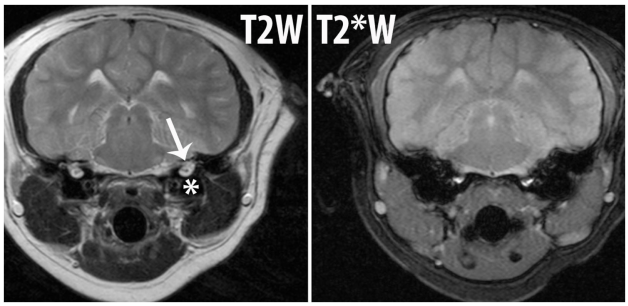

FIGURE 4 | Magnetic resonance imaging images from a California sea lion (*Zalophus californianus*) to demonstrate the appearance of gas and the associated susceptibility artifact. Both images are at the same level on the same patient. T2W is a regular MRI sequence and T2*W is sensitive to susceptibility artifact. The air within the bulla (*) on the T2W image can be seen and the inner ear structures (arrow) are also present. The same image is shown after T2*W acquisition and the amount of gas within and overall size of the bulla looks larger and the "blooming" effect of the susceptibility artifact due to an air-tissue interface results in loss of conspicuity of the inner ear structures. If abnormal gas were present in the inner ear, it would not be identifiable from this image.

pulmonary shunt that develops with depth (Kooyman and Sinnett, 1982) results in tissue and blood gas tensions that are higher than expected. A recent study using a custom-built hyperbaric CT chamber large enough to hold a small marine mammal, was performed to assess the effect of pressure on the respiratory system (Moore et al., 2011). Results estimated that the depth for complete alveolar collapse, or pulmonary shunting, in the gray seal to be 58 m when the dive began with a diving lung volume of 50% of total lung capacity. In the harbor porpoise, when the lungs were inflated to total lung capacity prior to the start of the dive, the collapse depth was estimated at 133 m. While the use of cadavers does have limitations, the results concurred with empirical (Kooyman and Sinnett, 1982; Fahlman et al., 2011) and theoretical (Fahlman et al., 2009) data, suggesting that the depth at which pulmonary shunting occurs and gas-exchange terminates is affected by lung volume and is likely more varied and often deeper than previously accepted. Importantly, this suggests that in animals diving with full or near full lung volume, a portion of the dive may occur where the pressure allows a significant amount of nitrogen to be taken up before gas-exchange terminates. This scenario would accommodate development of a supersaturated state and could explain the underlying mechanism permitting *de novo* gas bubble formation.

APPLICATION OF DIAGNOSTIC IMAGING TO IDENTIFY *IN VIVO* GAS BUBBLES IN MARINE MAMMALS

Several studies have been performed in marine mammals to determine if gas bubbles exist using a variety of diagnostic imaging modalities (Houser et al., 2001; Moore et al., 2009; Dennison et al., 2012). Pulse-wave Doppler, B-mode ultrasound, and CT have all been utilized and results among groups of animals, particularly free-ranging vs. captive-maintained animals, have been confounding.

A study of bycaught phocids and cetaceans that died at depth and were hauled to the surface in gill nets demonstrated generalized, diffuse, and severe gas bubble formation within soft tissues, lipid-rich tissues, vasculature, and synovial, cerebrospinal, ocular and lymphatic fluids using CT, necropsy, and histological examinations (Moore et al., 2009). A pathological cause for the vast gas accumulations observed was not identified during necropsy or histopathology. Post mortem off-gassing of supersaturated tissues and fluids during ascent to sea level was hypothesized as the most likely etiology of the gas bubble accumulations. It is certainly possible that some of the bubbles observed were a result of putrefaction, but the extent of bubbling substantially exceeded that of stranded animals of similar time post mortem when examined, and histology did not demonstrate significant autolysis.

Following on from the bycatch study results, stranded free-ranging cetaceans were identified as a study cohort that, because of the animals' disrupted dive pattern with loss of the opportunity for recompression, would provide an optimized environment for *in vivo*, *de novo* gas bubble formation in live animals, if gas bubble formation due to supersaturation occurs (Dennison et al., 2012). Evaluation of the kidneys, selected as easily accessible and identifiable organs using B-mode diagnostic ultrasound that should not normally contain gas, demonstrated bilateral subcapsular and intrarenal gas accumulations unanimously among the stranded cetaceans evaluated. The exact location of the gas bubbles (subcapsular vs. parenchymal vs. vascular) could not be determined using ultrasound in those cases. CT and necropsy examinations of animals that died or were euthanized within that cohort not only confirmed the renal gas as subcapsular and intravascular, but also identified abnormal gas accumulations in non-renal intravascular locations and within the subdermal sheath. It is unknown if the multifocal, multicompartmental accumulations in those cadavers represented underestimation of gas bubble load via renal ultrasound, that the greater bubble load was related to the ultimate demise of those individuals, or that gas production in those cases progressed in the time between ultrasound, CT, and necropsy. Regardless, the data demonstrated that gas bubble formation does occur in cetaceans under certain circumstances. Several stranded cetaceans with renal gas identified by ultrasound in that study were released uneventfully and some were monitored in the short term using satellite telemetry. Despite the presence of renal gas while stranded, the successful outcome of those free-ranging dolphins suggests that the gas bubble accumulations observed were asymptomatic.

Comparison groups were utilized by Dennison et al. (2012) in an attempt to determine if gas bubble formation only occurred in animals with the ability to undertake significant dives: a fact that would add credence to the hypothesis that gas bubble formation occurs in supersaturated individuals. Shallow water dwelling, free-ranging cetaceans, captive-maintained cetaceans, and stranded pinnipeds also underwent renal ultrasound, but no evidence of renal gas was identified in these groups. These negative data concurred with a separate study performed in a single captive-maintained dolphin that underwent forced repetitive dives over a short period of time (Houser et al., 2010). In that study, pulse-wave Doppler did not demonstrate gas within intrahepatic portal veins or brachiocephalic veins. The differing "life-styles" of the captive-maintained and shallow-dwelling free-ranging cetaceans compared to the free-ranging, live-stranded

dolphins may provide a valid explanation for the negative gas results: insufficient opportunity for chronic supersaturation of tissues to develop in shallow-dwelling cetaceans.

Barotrauma occurs when pressure alterations result in damage to the tissues surrounding air-filled cavities. This is because the air is compressible while the surrounding tissue is not. If the damage permits entrance of compressed air into the vascular system while still at depth, air can travel to an abnormal location, then expand to form a "space-occupying" lesion resulting in tissue damage as decompression occurs. Imaging such animals prior to necropsy provides invaluable information regarding distribution and degree of gas bubble formation, much of which may not be directly observed during necropsy without disruption or dissection of tissues.

Barotrauma is a valid differential diagnosis when gas bubbles are identified in an individual particularly if there is evidence of concurrent damage to a gas filled cavity or lung tissue. Cases of diagnosed marine mammal barotrauma evaluated with diagnostic imaging have been published. Pneumocerebellum developed in a neurologically abnormal, stranded, free-ranging California sea lion with additional evidence of rib fracture and pneumothorax (Van Bonn et al., 2011). Here cerebellar gas accumulations were speculated to have developed when circulating gas bubbles introduced into the vascular system from the damaged pulmonary tract traveled to and became entrapped within the cerebellar parenchyma while still at depth. The bubbles then expanded during resurfacing. Gas was recognized within the caudal fossa using standard radiography but further characterization (intraparenchymal, subarachnoid, subdural, epidural) was not possible. The large intraparenchymal cerebellar gas cavitations were clearly evident on all MRI sequences as signal voids with susceptibility artifact on susceptibility – sensitive sequences.

Abnormal gas accumulations are non-specific for the type of gas present and as such, imaging alone cannot distinguish decomposition from disarticulation/dissection from *de novo* formation. It is possible that as more cases are fully evaluated, gas bubble distributions associated with the different etiologies may be distinguishable. Even then, confirmation of the likely etiology based on the type of gas present and its likely significance will need a combined approach including imaging, gas analysis, and histology. Of course, this will not be possible in the live animal, but barotrauma victims would not be expected to present as otherwise healthy individuals with normal behavior unlike the dolphins with presumed asymptomatic renal gas described (Dennison et al., 2012). Evidence of pneumothorax, pneumoperitoneum or damage to normally gas-filled structures may explain abnormal gas accumulations in some cases but distinguishing between trauma or barotrauma is difficult from imaging alone. Crossover may exist and in supersaturated animals that sustain barotrauma or other trauma to gas-filled structures, abnormal gas accumulations due to concurrent processes is quite feasible.

THE SIGNIFICANCE OF ABNORMAL GAS ACCUMULATIONS DEMONSTRATED VIA DIAGNOSTIC IMAGING IN MARINE MAMMALS

The formation of gas bubbles in marine mammals may in many cases be asymptomatic and as such the presence of gas alone must be interpreted with caution. The live-stranded, free-ranging cetacean renal gas accumulations observed with B-mode ultrasound were considered entrapped or "safe" with presumption that they were interstitial in location, or more specifically were not arterial in location which is maximally detrimental with respect to the likelihood of the development of DCS and resultant damage due to vascular occlusion (Brubakk and Eftedal, 2001; Barak and Katz, 2005; Dennison et al., 2012). However vascular gas bubbles have been observed on CT images and during necropsy examinations in dead stranded animals that were identified as positive for renal gas while still alive (Dennison et al., 2012), suggesting that some gas may be circulating. Vascular studies in this group of animals to confirm this have not yet been performed.

Intravascular circulating gas bubbles can theoretically lodge anywhere in the body, limited only by their size. While there is the temptation to say that very small bubbles are therefore innocuous, in the biological setting it is possible that small bubbles could pass through protective capillary beds, particularly if the bubbles are partially compressed, only to occlude larger vessels if they coalesce to form larger bubbles or expand during final ascent. Bubbles entering the arterial side of circulation in humans have been associated with development of DCS (Ljubkovic et al., 2011).

Circulating gas bubbles of any size that come into contact with endothelium during their travels can activate inflammatory or complement pathways via release of microparticles or directly damage endothelial cells as they pass (Barak and Katz, 2005; Thom et al., 2011). Studies have shown that endothelial damage is related to bubble load in humans (Brubakk and Eftedal, 2001). The probability of contact between bubbles and endothelium is theoretically greater in smaller gage blood vessels such as capillaries than in larger gage vessels where the volume of blood and laminar blood flow may help distance bubbles from the endothelial cells. Typically in humans, the lungs act as a large filter, removing many bubbles from the circulation but when high bubble load is present, arterial gas bubbles are seen with some frequency even in the absence of congenital heart defects such as patent foramen ovale (Ljubkovic et al., 2011). Given the identification of renal gas bubbles in marine mammal, the kidneys, or its adjacent vasculature may be functioning as a filter or possibly as a method of detection. It is likely that other anatomical, physiological, or behavioral mechanisms also play a role in protection against gas bubble formation or the prevention of deleterious effects.

Given the temporal and spatial association between beaked whale strandings and anthropogenic noise (Jepson et al., 2003; Fernández et al., 2005; D'Amico et al., 2009), the potential effects of anthropogenic noise on marine mammals deserve special consideration. The concern for auditory effects of anthropogenic noise have been voiced following CT and necropsy findings of aural, periaural, and intracranial hemorrhage in beaked whales and have led to imaging recommendations in similar stranding events (Ketten and Montie, 2008). However non-auditory effects also warrant consideration as anthropogenic sound may alter behavior increasing the risk of bubble formation by decompression, or directly initiating or advancing bubble formation in supersaturated tissues through rectified diffusion (Houser et al., 2001). Exposure of bubbles to sound could result in bubble rupture and subsequent tissue damage if sufficient numbers of bubbles are present,

as has been shown in *in vivo* studies of isolated rabbit hearts perfused with intravenous stabilized microbubbles (Miller et al., 2008) creating a very high bubble load. Reported tissue damage observed in that study associated with gas bubble rupture included microvascular leakage, petechiae formation, cardiomyocyte death, and premature ventricular arrhythmias. Extrapolating these data, prolonged sound exposure in marine mammals that have formed gas bubbles may, therefore, have serious consequences. Thus, auditory and non-auditory effects of anthropogenic sound should be considered as potential causes of marine mammal strandings particularly when evidence of gas bubbling is present. Furthermore, if utilizing B-mode ultrasound to evaluate stationary bubbles, when high volumes of gas bubbles are identified, prolonged examination of tissue containing gas as small bubbles using a high mechanical index without movement of the sound beam should be avoided to prevent potentially iatrogenic deleterious effects.

CONCLUSION

The evidence suggests that supersaturation and gas bubble formation occur in marine mammals. Under normal circumstances anatomical, physiological, and behavioral adaptations must exist to help protect susceptible animals from detrimental effects, and in many cases gas bubble formation is asymptomatic. Diagnostic imaging can be useful to determine if gas bubbles are present. Ultrasound is a highly portable and easily accessible imaging modality that can be applied to live-stranded animals on the beach, but the use of ultrasound in the live animal may underestimate abnormal gas distribution and bubble load. CT or MRI studies of dead marine mammals pre-necropsy where possible will allow better documentation of the distribution and bubble load present than can be achieved with necropsy observations alone, but the application of these modalities in the live-stranded animal is likely to remain very limited. Comparisons between distributions of gas accumulations identified via different imaging modalities from cases determined as most likely due to or know to be barotrauma, decomposition, or bycatch from unknown causes presumed to be *de novo* gas formation may help separate out etiologies based on imaging characteristics. This would require correlation with histopathology and gas analyses. Future studies should develop deployable field imaging techniques to determine if spontaneous gas bubble formation occurs in marine mammals that are not entering a disturbed or stranded state. In addition, further imaging studies in live-stranded cetaceans are needed to determine if gas bubbles exist both in tissues and the vasculature incidentally, what bubble load or distribution may have detrimental effects and under what circumstances such detrimental accumulations occur.

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Fatty acid use in diving mammals: more than merely fuel

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Diving mammals, are under extreme pressure to conserve oxygen as well as produce adequate energy through aerobic pathways during breath-hold diving. Typically a major source of energy, lipids participate in structural and regulatory roles and have an important influence on the physiological functions of an organism. At the stoichiometric level, the metabolism of polyunsaturated fatty acids (PUFAs) utilizes less oxygen than metabolizing either monounsaturated fatty acids or saturated fatty acids (SFAs) and yields fewer ATP per same length fatty acid. However, there is evidence that indicates the cellular metabolic rate is directly correlated to the lipid composition of the membranes such that the greater the PUFA concentration in the membranes the greater the metabolic rate. These findings appear to be incompatible with diving mammals that ingest and metabolize high levels of unsaturated fatty acids while relying on stored oxygen. Growing evidence from birds to mammals including recent evidence in Weddell seals also indicates that at the whole animal level the utilization of PUFAs to fuel their metabolism actually conserves oxygen. In this paper, we make an initial attempt to ascertain the beneficial adaptations or limitations of lipids constituents and potential trade-offs in diving mammals. We discuss how changes in Antarctic climate are predicted to have numerous different environmental effects; such potential shifts in the availability of certain prey species or even changes in the lipid composition (increased SFA) of numerous fish species with increasing water temperatures and how this may impact the diving ability of Weddell seals.

Keywords: fatty acids, diving mammals, PUFA, ontogeny, lipids

INTRODUCTION

Diving mammals, such as the Weddell seal (*Leptonychotes weddellii*), are under extreme pressure to conserve oxygen as well as produce adequate energy through aerobic pathways during breath-hold diving. It is recognized that diving mammals undergo a dive response involving apnea (cessation of breathing), bradycardia, and subsequent decreased cardiac output, as well as peripheral vasoconstriction (reduced blood flow or ischemic conditions in the working skeletal muscles) causing the animal to depend solely on a finite supply of oxygen stored internally. In other words, when diving mammals are active (foraging), they are holding their breath, their working muscles receive little to no blood flow, and they rely solely on internal stores of oxygen, to fuel, and sustain a lipid based aerobic metabolism. Therefore, dive duration can be directly correlated to the rate of oxygen utilization and any factors that affect the rate of oxygen consumption could alter dive times (Fahlman et al., 2008; du Dot et al., 2009; Williams et al., 2011; Shiomi et al., 2012).

While dependant on lipid and protein from a typically piscivorous prey source, lipids provide double the energy per mass when compared to protein and are important as a stored energy source as well as for thermoregulation and hydrodynamics (Castellini et al., 2009). More detailed use of lipids reveal a possible developmental role as well as preferential mobilization of specific fatty acids during lactation (Wheatley et al., 2008; Trumble et al., 2010). Trumble et al. (2010) reported that the primarily ice-based Weddell seal pup had increased polyunsaturated fatty acids (PUFAs) recovered from lipids associated with their skeletal muscle whereas saturated fatty acids (SFAs) were decreased when compared with adults. Assuming similar lipid consumption between age classes, these findings led to speculation that pups were metabolizing SFAs in greater amounts than the adult age class. While consuming a milk high in unsaturated fatty acids (UFA; Wheatley et al., 2008), it was hypothesized that these young seals increased their metabolic and energetic advantage (increased oxygen to metabolize SFA when compared to UFA and more energy per mass) to combat their high mass-specific heat loss and metabolic limitations of being an endothermic mammal in Antarctica. As these seals continued to mature to adults, the fatty acids recovered were primarily monounsaturated fatty acids (MUFAs), however, there appeared to be an exchange between SFA and PUFA between these age classes (Trumble et al., 2010). In this paper, we explain the stoichiometry associated with fatty acid metabolism and hypothesize on the advantages and disadvantages of these fatty acid groups, primarily PUFAs, in diving mammals inhabiting polar waters. Finally, we offer some speculation on potential disadvantages of metabolizing certain groups of fatty acids in a changing environment.

OXYGEN AND DIVING

The important role for the rate of oxygen consumption and diving was recently elucidated in emperor penguins (*Aptenodytes forsteri*; Shiomi et al., 2012). The results of this study indicated that once

a certain amounts of work or numbers of flipper strokes were reached, the animals initiated their return to the surface regardless of the amount of time spent diving. To date, this is the strongest evidence that alteration in oxygen consumption or work effort significantly alter dive times. Along with the aforementioned emperor penguins, Weddell seals represent extreme examples of this breath-hold model. These divers routinely dive under an ice ceiling, which offers less than 5% access to breathable surface area (**Figure 1**). This is in sharp contrast to open water divers such as elephant seals (*Mirounga angustirostris*) or other non-polar pinnieds. These constraints demand that the Antarctic divers be more tightly regulated with respect to their oxygen utilization; they must complete a dive cycle and find a breathing hole before their oxygen stores are depleted. Previous research has illustrated how these and open water divers are uniquely adapted to their environment and how energy is conserved even at the molecular level (Burns, 1996; Burns et al., 1998; Kanatous et al., 1999, 2001, 2002, 2008a,b; Burns et al., 2005, 2007; Clark et al., 2006, 2007; Noren et al., 2008b; Ptitsyn et al., 2010; Trumble et al., 2010; Ponganis et al., 2011; Williams et al., 2011). For example, studies of adult Weddell seals, harbor seals (*Phoca vitulina*) and Steller sea lions (*Euematopias jubatus*) have revealed that their muscle adaptations to maintain a lipid based aerobic metabolism under the hypoxic conditions associated with breath-hold diving include: (1) an increased aerobic capacity (or one that is matched to routine levels of exertion), (2) a reliance on fatty acid catabolism for aerobic ATP production, (3) enhanced oxygen storage and diffusion capacity, and (4) a reduced dependency on blood-borne oxygen and metabolites (e.g., decreased capillary density) compared to terrestrial mammals (Davis et al., 1991; Davis and Kanatous, 1999; Kanatous et al., 1999, 2001, 2002). Recently, physiological studies have begun to describe the development of diving capacity and oxygen stores in diving mammals (Noren et al., 2004, 2005, 2006, 2008a; Clark et al., 2006, 2007; Kanatous et al., 2008a). Recent data have reported

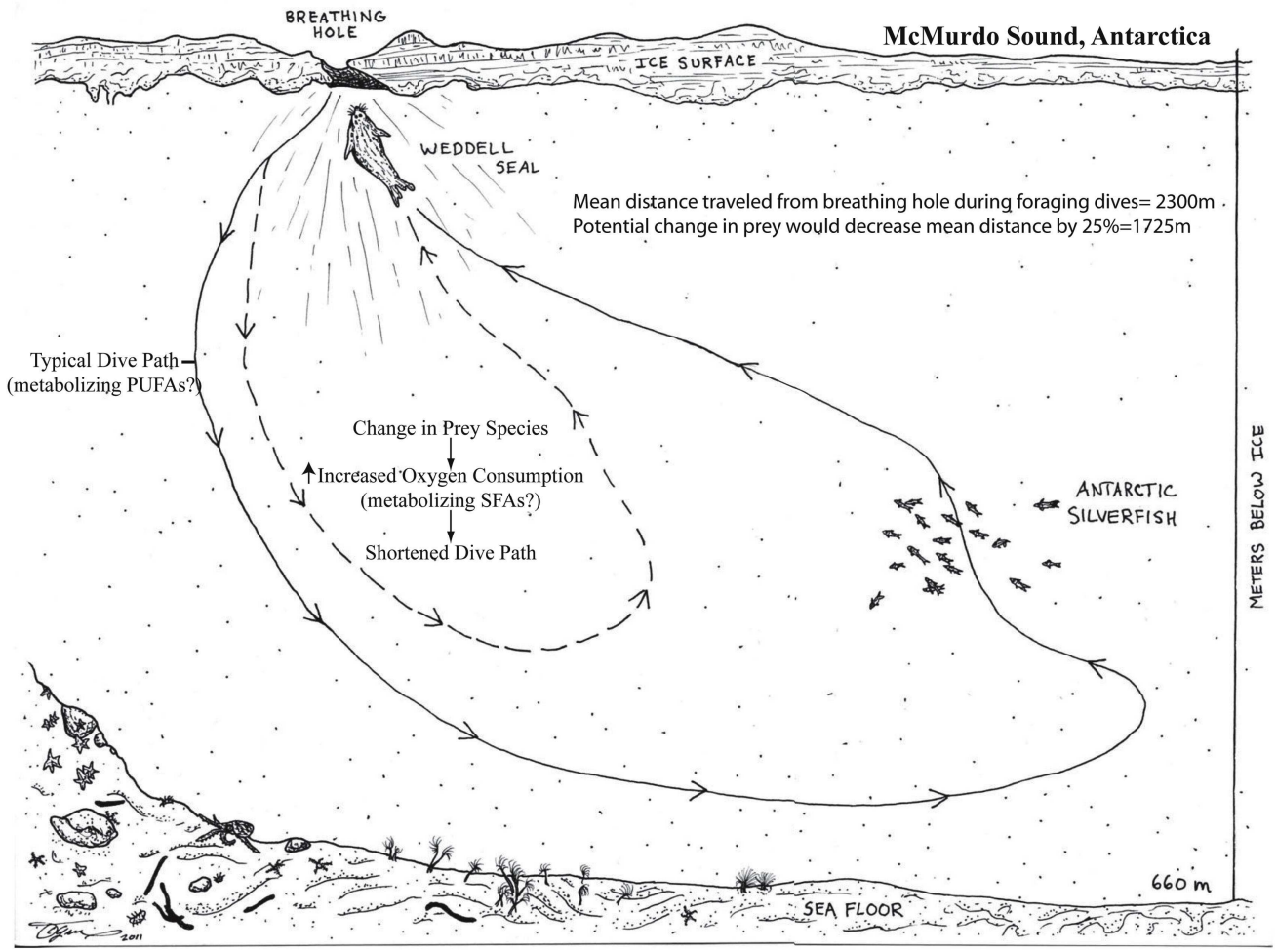

FIGURE 1 | Weddell seals routinely feed on a diet of Antarctic silverfish (*Pleuragramma antarcticum*) that is rich in unsaturated fatty acids. Recent data indicates that similar to long distance migratory birds, adult Weddell seals preferentially metabolize PUFAs in contrast to either monounsaturated or saturated fatty acids (Trumble et al., 2010).

numerous physiological changes such as an increase in the concentration of myoglobin to levels ∼60% of those found in adults. These adaptations occur during weaning periods and are necessary for these animals to initiate independent foraging (Burns, 1996; Kanatous et al., 2008a).

In terms of skeletal muscle development, the cessation of weaning and commencement of diving in Weddell seals induces developmental changes in muscle physiology that parallel changes in activity. Preliminary microarray results indicate differential expression of RNA transcripts associated with various ontogenic signaling pathways, in addition to differences in transcripts associated with lipid metabolism, among age classes (Kanatous et al., 2008b; Ptitsyn et al., 2010). These previous findings mirrored results describing significant changes in the lipid composition and potential utilization of fatty acids in skeletal muscle during these developmental stages (Trumble et al., 2010). In addition, where this developmental trend of increasing exercise capacity occurs from birth in terrestrial mammals, we found that the skeletal muscle physiology of Weddell seal pups suggests a higher capacity for exercise than juveniles or adults. This is indicated by pups having the highest percentage of type I slow oxidative fibers as well as the highest mitochondrial volume densities of any age class. A relative lack of an increase in aerobic enzymes, however, indicates that these results may be due to an adaptation for enhanced non-shivering thermogenesis (Kanatous et al., 2008a). Furthermore, juveniles were recently shown to have the highest expression of myoglobin (72.4 ± 7 vs. 55.9 ± 2.5 and $35.5 \pm 3\,mg\,g^{-1}$ wet mass muscle in adults and pups, respectively) supporting the hypothesis that myoglobin concentrations differ with behavioral differences in diving activity (Kanatous et al., 2008a).

FATTY ACIDS AND STOICHIOMETRY

Typically known as a major source of energy, lipids also participate in structural and regulatory roles that have an important influence on the physiological functions of an organism. As essential fatty acids, it is well known that PUFAs of $n-3$ and $n-6$ groups are considered the most powerful intracellular and intercellular mediators and modulators of the cell signaling network and cell membrane fluidity (important in polar habitats). The action of PUFAs has been shown to have various effects on immune and inflammatory processes (Puertollano et al., 2004). While the processes above are certainly important for a developing diving mammal, fatty acids play a large role in fueling metabolism and may provide insight into diving adaptations. For mammalian diver's it is imperative to conserve oxygen to maximize success of an intake of primarily unsaturated fats and proteins. This intake of UFAs and subsequent transport and oxidation within skeletal muscles (assuming skeletal muscle activity during diving) may to be an advantage to the diver. Specifically, the moles of oxygen needed to metabolize any particular SFA are slightly greater than that for a similar length UFA (MUFA and PUFA). This stoichiometry of oxygen consumption is based on the number of double bonds and thus hydrogen involved (see below for example). If extrapolated to the fatty acid groups (not the individual fatty acids) identified in Weddell seal blubber (14–24 carbon; Trumble et al., 2010), the mean moles of oxygen needed to oxidize PUFAs (29 mol O_2/FA) is greater than MUFAs (26 mol O_2/FA) and SFAs (23 mol O_2/FA). Upon closer examination, a majority of saturated and unsaturated fatty acids recovered (percent of total) from the blubber (Wheatley et al., 2008) and muscle of Weddell seals are 18 carbons (18C) in length (>50%, Trumble et al., 2010). Specifically, an 18C SFA containing 36 hydrogen atoms requires $(36/4) + 18 - 1 = 26$ mol of oxygen for complete oxidation whereas the most commonly recovered MUFA, 18:1, requires 25.5 mol and the most commonly recovered PUFA, 18:3 requires 25 mol of oxygen for complete oxidation. Based on mean percent fatty acid composition from Weddell seal pups (SFA, 28%; UFA, 72%), juveniles (SFA, 31%; UFA, 69%), and adults (SFA, 30%; UFA 70%; Trumble et al., 2010) and an estimated mean total percent blubber mass for each age class (Gales and Burton, 1987; Noren et al., 2008b; pups, 32 ± 0.8%; juveniles, 21.5 ± 0.7%; and adults, 23.9 ± 0.7%) estimates of moles of oxygen required to oxidize saturated and unsaturated fatty acids can be calculated (**Figure 2**). This may equate to UFA-oxygen conservation when compared with metabolizing similar amounts of SFAs in Weddell seal adults. While this alteration of oxygen consumption appears insignificant in terms of oxygen moles oxidized between these 18C fatty acids, when extrapolating to a 400-kg adult (35% muscle mass; Ponganis et al., 1993) and differences in mass-specific metabolic rates ($0.676\,M_b^{0.75}$/kg; Kleiber, 1975) metabolizing an unsaturated 18C fatty acid offers an increase of ∼30 s of dive time (not taking activity budget into account) during a 30-min dive. A dive time shortened by 30 s (assuming dive locomotion at 1–3 m/s), would shortened the distance covered by 30–90 m, which could significantly alter foraging or breathing hole location success.

The stoichiometric relationship between saturated and unsaturated fatty acids from an energy production (ATP) perspective also results in differences, which may also influence diving. Using the most common fatty acid recovered (18:1, Trumble et al., 2010), we surmise that the oxidation of unsaturated fatty acids requires additional auxiliary enzymes (isomerases and a reductase). This becomes important in that the double bonds formed in beta-oxidation are *trans* as opposed to *cis* in naturally occurring unsaturated fatty acids. Specifically, an isomerase is required for breaking the double bond in MUFAs, which are typically between carbons 9 and 10. Thus, oleate (18:1) requires three cycles of beta-oxidation with each cycle removing two carbons, replacing the double bond at the number 3 position. The hydratase of beta-oxidation requires the double bond to be in the *trans* configuration and at second carbon location. Therefore, the ATP count for MUFAs (>50% of total percent of FAs recovered) is two less than the fully saturated analog because one fewer $FADH_2$ is generated (for 18:0, there are 146 ATP produced; for 18:1 there would be 144 ATP produced). Further, to process the second double bond in a PUFA, for example 18:2, another reductase and isomerase are required. Since the double bonds are typically in the 9 and 12 positions, the three cycles of beta-oxidation can proceed as usual, resulting in the double bonds at the 3 and 6 positions. Beta-oxidation will then proceed to form a *trans*-2, *cis*-4 product. The mammalian version of the second auxiliary enzyme, another reductase, reduces this to a singly unsaturated product with a *trans* double bond in the 3 position. With this additional reductase involved, the ATP count will be three fewer than for a fully saturated version because, in addition to the loss of $FADH_2$, one NADPH is used for this additional

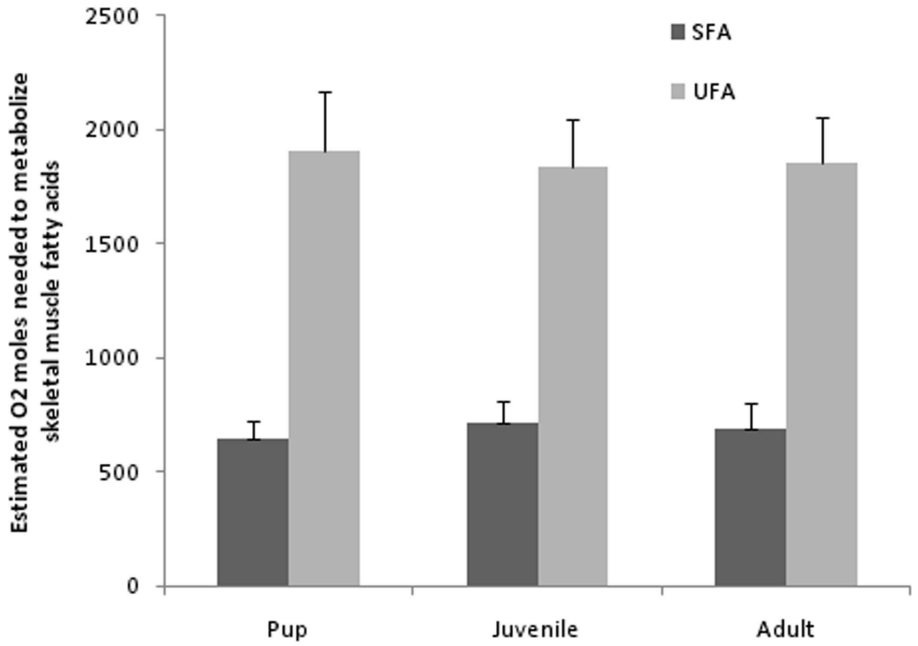

FIGURE 2 | Estimated moles of oxygen needed to completely oxidize saturated (SFA), and unsaturated (UFA) fatty acids recovered from Weddell seal skeletal muscles ($n = 20$) in 2006. Calculations derived from mean moles of oxygen (see text) and mean lipid amounts in skeletal muscle (Trumble et al., 2010) among age groups and mean mass of age groups (estimates for blubber mass derived from Noren et al., 2008b) and mean total percent of fatty acids recovered. note: fatty acids include (SFA; C14:0–24:0), monounsaturated (MUFA; 14:1*n*−5, 16:1*n*−9, 16:1*n*−7, 18:1*n*−9, 18:1*n*−7, 18:1*n*−11, 20:1*n*−9, 20:1*n*−7, 22:1*n*−11, 22:1*n*−9, 24:1*n*−9), and polyunsaturated (PUFA; 18:2*n*−6, 18:3*n*−6, 18:3*n*−3, 18:4*n*−3, 20:2*n*−9, 20:2*n*−6, 20:3*n*−6, 20:4*n*−6, 20:3*n*−3, 20:4*n*−3, 20:5*n*−3, 22:4*n*−6, 22:5*n*−6, 22:5*n*−3, 22:6*n*−3).

reductase. Thus, the ATP count for a fatty acid configured as 18:2 would only be 141 as compare to 146 from a saturated 18C fatty acid. While there may be a loss in ATP production by preferentially metabolizing UFAs as compared to SFAs, dives are more limited by oxygen than ATP thus the oxygen conservation may dramatically outweigh the differences in ATP production for these animals.

THE FATTY ACID TRADE-OFF: MEMBRANE COMPOSITION

The consumption of a high fat diet produces a surplus of NADH and $FADH_2$, which subsequently increases the generation of reactive oxygen species (ROS). Indeed, a high fat diet has been shown to increase the rate of H_2O_2 (ROS species) production in skeletal muscle mitochondria (Nadal-Casellas et al., 2010). This production of ROS has been reported to contribute to the processes of aging as well as progression of numerous disorders such as Type II diabetes and Parkinson disease in mammals (Kang and Hamasaki, 2003). Ultimately, the increased rate of ROS production by the mitochondria results in mitochondrial dysfunction in skeletal muscle. However, recent studies have begun to elucidate the differential role of cellular membrane lipid composition in the susceptibility to ROS damage (Hulbert, 2008a,b, 2010; Montgomery et al., 2011). These studies have shown that membranes composed of higher concentrations of PUFAs have an increased susceptibility to ROS damage by lipid peroxidation, which ultimately shortens lifespan in animals. However, it has been speculated that membranes higher in MUFAs protect against ROS damage (Hulbert, 2010). Trumble et al. (2010) reported that greater than half of all the recovered fatty acids associated with the skeletal muscle of Weddell seals were MUFAs. We speculate this increase in MUFA levels supports in ameliorating damage by ROS. New research also found that membranes of the relatively long-lived short-beaked echidna (*Tachyglossus aculeatus*) are more monounsaturated and less polyunsaturated than would be predicted from their body size which were hypothesized to ameliorate ROS damage and promote long life spans (Hulbert, 2008a,b, 2010). This association between PUFA membrane levels appears to be related to life span (Hulbert, 2010) as well as Basal metabolic rate (BMR) and appears to hold true for most terrestrial mammals and birds studied (Hulbert, 2008a,b, 2010).

Basal metabolic rate represents cellular living at a minimal cost within certain assumptions such that the mass-specific BMR has been shown to vary among species inversely related to body size. An idea proposed and developed by Hulbert and Else (1999) suggests that this variation in BMR is not only a function of body size but also directly related to cellular metabolic rate which is directly correlated to the lipid composition of the membranes (Hulbert and Else, 1999). Further, and mentioned above, there appears to be a direct positive correlation between PUFA levels in the membrane and a higher BMR. We report here for the first time, that Weddell seals appear to follow the concept proposed by Hulbert and Else (1999); PUFAs are increased in the skeletal muscle membrane of the smaller pup and decline with the larger animals (**Figure 3**). While unknown whether this switch in membrane fatty acids occurs during development in terrestrial mammals, we report that this metabolic switch in membrane composition occurs after pupping or lactation and seems to be transitioned

in juveniles. In terrestrial mammals, higher levels of PUFAs have been reported in the membranes of relatively smaller mammals and have been associated with leakier membranes and more active pumps (Hulbert, 2010). This "PUFA-permeable prefigure" may add evidence to our recent findings that Weddell seal pups offset their thermal challenge of being an endothermic animals born into an extremely harsh environment of Antarctica through the use of increased and potentially uncoupled metabolism. In short, it appears that non-diving pups adopt the metabolic and cellular "benefits" of the relatively small terrestrial mammal PUFA rich membrane composition. At this time, we do not know the mechanisms involved with this membrane switch. However, it appears to coincide with development, or the physiological shift to adult divers. Further, as membrane PUFA levels change, MUFA and SFA levels increase in adult membrane composition for possible additional protection from ROS damage from proportionally high rates of sustained lipid metabolism and from the reperfusion of the muscles associated with the end of dives (**Figure 3**).

Interestingly, an additional "benefit" or adaptation of ingesting and metabolizing PUFAs, especially in adult diving mammals, may be its influence on heart rate. A report by Gudbjarnason et al. (1978) reported that the heart rate of animals, ranging from mice to whales, was directly related to the levels of 22:6*n*−3 in their cardiac phospholipids (Gudbjarnason et al., 1978). Subsequently, it was determined that this increase in 22:6*n*−3 was also positively correlated with increased Ca^{2+}ATPase activity in the cell (Infante, 1987) which led to the "membrane pacemaker theory" mentioned above (Hulbert and Else, 1999). This notion that heart rate is influenced by *n*−3 fatty acids has been pursued by several investigators with the general consensus that ingestion of *n*−3 fatty acids tend to lower resting heart rates (Christensen et al., 1999; Billman and Harris, 2011). Assuming that the relatively lower PUFA levels associated in the skeletal muscle lipids is a function of increased metabolizing of PUFAs and not a function of decreased PUFA intake, then our findings may indicate a preferential fatty acid adaptation to lower heart rate during diving. It should be mentioned that PUFAs cannot be formed *de novo* by mammalian cells and therefore must be obtained from the diet. Assuming a PUFA rich prey equal in proportion to what is found in milk (Wheatley et al., 2008) then our findings suggest PUFAs were preferentially mobilized by the muscle for energy and, more importantly, switch in levels in the skeletal muscle membranes. At this time, we can only speculate that this switch may suggest a regulation of desaturases or elongases associated with membrane lipid constituents. Further research is needed to confirm this hypothesis.

It has been reported that long distance migratory birds (*Calidris pusilla*) ingest a diet high in *n*−3 PUFA prior to migration, potentially acting as a molecular signal to prime flight muscles and increase oxygen efficiency while using less energy (Maillet and Weber, 2006; Piersma, 2011). Several studies contribute to this notion and suggest that PUFAs may affect the performance of skeletal muscles and locomotion in a variety of vertebrates (McKenzie et al., 1998; Helge et al., 2001; Infante et al., 2001; Valencak et al., 2003). We speculate PUFAs, especially *n*−3's, play an important role in the development of foraging from pup to adults and that these fatty acids may be functionally related to oxygen conservation, locomotor performance, life stage specific energetic demands and diving ability. In a recent study involving rats switched from a SFA diet to fish oil (high *n*−3 PUFAs) myocardial oxygen consumption, coronary flow and percent oxygen extraction was significantly reduced while maintaining integrity of contractile function in the heart muscle (Pepe and McLennan, 2007). The metabolic costs of foraging are critical components of the energy budget of animals and will have direct affect on the amount of prey they must obtain to survive. This is especially true for breath-hold deep divers; where any increases in the metabolic costs will significantly alter their dive times and foraging success. As stated above, our recent data indicates that similar

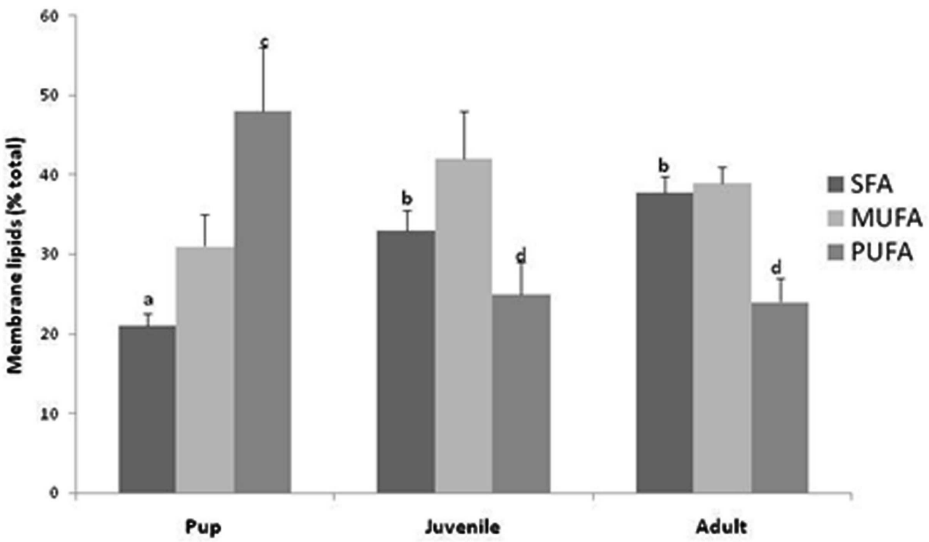

FIGURE 3 | Membrane (phospholipids) fatty acids (saturated, SFA; monounsaturated, MUFA; and polyunsaturated, PUFA) in Weddell seal skeletal muscle (pup, *n* = 5; juvenile, *n* = 5; adult, *n* = 5). Differences in lower case letters denotes statistical differences among age classes ($p < 0.05$).

to long distance migratory birds, adult Weddell seals preferentially metabolize oxygen-conserving PUFAs in contrast to either MUFAs or SFAs, which enable the seals to preserve their limited oxygen resources during diving. An important area of future research will be elucidating the molecular controls that regulate this switch in metabolic fuel during ontogeny.

FATTY ACIDS AS REGULATORY SIGNALS

Because myoglobin has been shown to increase during the weaning period of both penguins and seals before they initiate diving (Burns et al., 2005, 2007; Clark et al., 2007) the potential for myoglobins regulation to be dependent on its interplay with specific fatty acids seems plausible. Indeed, recent evidence has shown that the amount and type of lipids act as important initial regulators of myoglobin in diving mammals (De Miranda Jr. et al., 2012) with some indication of secondary regulation by activity level and calcium signaling (Kanatous et al., 2008b, 2009; Ptitsyn et al., 2010). In comparative animal studies, an increase of *n*−3 PUFAs or a high *n*−3 to *n*−6 ratio of fatty acids was shown to prompt increases in calcium transport and calcium absorption in PUFA-deficient rats (Kruger and Horrobin, 1997; Weiss et al., 2005). It has been established in terrestrial animals and diving mammals that calcium signaling, as well as its downstream targets of calcineurin and NFAT, plays an important role in determining fiber type distribution, aerobic capacity and myoglobin concentrations in skeletal muscles (Chin et al., 1998; Spangenburg and Booth, 2003). Recent evidence from our labs indicates this increase of myoglobin expression in weaning animals before they start diving may be initiated through an activity and calcium independent pathway. While further studies in cell culture may yield a more specific response to certain fatty acids, our current research strongly suggests that the pre-dive increases in myoglobin of divers during weaning may be associated with changes in the lipid composition of the milk associated with nursing (De Miranda Jr. et al., 2012). Additional results from our labs suggests that the when compared to mouse samples (*Mus musculus*), marine mammal species demonstrated increased levels of sphingolipids and ceramides in the cell membrane. Specifically, sphingomyelin, phosphatidylcholine, ceramide, and sphingosine were elevated in the cellular membranes. While preliminary, these differences may result in changes in total cellular metabolism since these lipid constituents are known to be involved in cell signaling pathways.

CONCLUSION

While certainly incomplete, we have pointed out some potential advantages/disadvantages of metabolizing fatty acids in diving mammals. While it has been described that fatty acids in polar marine mammals are elevated in C16–C24 UFAs, we make an initial attempt to ascertain the beneficial adaptations or limitations of these lipids constituents. At the stoichiometric level, the metabolism of UFAs may be correlated to oxygen conservation (Suarez, 2011; Weber, 2011) while producing fewer ATP. There is evidence that indicates cellular metabolic rate is directly correlated to the PUFA composition of the membranes: birds to terrestrial mammals utilize PUFAs to conserve oxygen, possibly through lowering cardiac rate. Additionally, we speculate that ontogenetic tradeoffs of fatty acids in the skeletal muscle and cellular membrane may play a vital role in the regulating changes in metabolism and establishing certain skeletal muscle adaptations (i.e., myoglobin) to diving in Weddell seals. As a current point of discussion, changes in Antarctic climate are predicted to have numerous different environmental effects; such potential shifts in the availability of certain prey species or even changes in the lipid composition (increased SFA) of numerous fish species with increasing water temperatures (Phleger et al., 1999). As we have calculated above, a slight change in fatty acid saturation could alter the diving capacity of Weddell seals. In order to clearly understand the physiology of these divers and predict how these animals will react to physiological challenges, we must understand the full suite of adaptations; from potential shifts in metabolic fuels that could affect dive times and foraging ability to understanding the molecular switches to become an efficient diver. Further research is required on the role specific lipids have on regulating homeostatic pathways. Our understanding of the role of lipids in regulating the homeostasis of the animals is in its initial stages.

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Size matters: spleen and lung volumes predict performance in human apneic divers

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Humans share with seals the ability to contract the spleen and increase circulating hematocrit, which may improve apneic performance by enhancing gas storage. Seals have large spleens and while human spleen size is small in comparison, it shows great individual variation. Unlike many marine mammals, human divers rely to a great extent on lung oxygen stores, but the impact of lung volume on competitive apnea performance has never been determined. We studied if spleen- and lung size correlated with performance in elite apnea divers. Volunteers were 14 male apnea world championship participants, with a mean (SE) of 5.8 (1.2) years of previous apnea training. Spleen volume was calculated from spleen length, width, and thickness measured via ultrasound during rest, and vital capacity via spirometry. Accumulated competition scores from dives of maximal depth, time, and distance were compared to anthropometric measurements and training data. Mean (SE) diving performance was 75 (4) m for constant weight depth, 5 min 53 (39) s for static apnea and 139 (13) m for dynamic apnea distance. Subjects' mean height was 184 (2) cm, weight 82 (3) kg, vital capacity (VC) 7.3 (0.3) L and spleen volume 336 (32) mL. Spleen volume did not correlate with subject height or weight, but was positively correlated with competition score ($r = 0.57$; $P < 0.05$). Total competition score was also positively correlated with VC ($r = 0.54$; $P < 0.05$). The three highest scoring divers had the greatest spleen volumes, averaging 538 (53) mL, while the three lowest-scoring divers had a volume of 270 (71) mL ($P < 0.01$). VC was also greater in the high-scorers, at 7.9 (0.36) L as compared to 6.7 (0.19) L in the low scorers ($P < 0.01$). Spleen volume was reduced to half after 2 min of apnea in the highest scoring divers, and the estimated resting apnea time gain from the difference between high and low scorers was 15 s for spleen volume and 60 s for VC. We conclude that both spleen- and lung volume predict apnea performance in elite divers.

Keywords: spleen contraction, hematocrit, vital capacity, diving response, breath-hold, mammalian, diving capacity, training

INTRODUCTION

Humans have the ability to contract the spleen during diving and increase circulating hematocrit (Hurford et al., 1990) which seems to prolong apneas when performed in series (Schagatay et al., 2001). This response is well known in seals, where spleen size varies greatly between species and in some deep divers like the Weddell seal it is exceptionally large with the ability to store 2/3 of the body's erythrocytes during rest (Qvist et al., 1986). In highly aerobic species like the horse and the dog, the spleen contracts and elevates hematocrit during exercise (Barcroft and Stevens, 1927). The human spleen also contracts, increasing circulating Hb and Hct during both apnea (Schagatay et al., 2001) and exercise (Laub et al., 1993; Stewart and McKenzie, 2002), which will increase blood gas storage capacity. The spleen contraction is probably active, i.e., not due to reduced blood flow to the organ (Bakovic et al., 2003) and the reversible elevations of Hct and Hb are not due to hemoconcentration by extravasation of plasma (Schagatay et al., 2001). Spleen size shows great individual variation in humans (Prassopoulos et al., 1997), and also its ability to contract appears to vary between individuals (Schagatay et al., 2005) but it has never been determined whether these factors correlate with individual apneic diving ability.

The sport Apnea has developed during the past 15 years, and involves disciplines with the aim to reach the greatest possible depth, immersion time and horizontal distance under water, and serves as a good model for studying maximal apneic diving performance in humans. The ability to perform extended apnea depends mainly on three factors: (1) the total body gas storage capacity in lungs, blood, and tissues; (2) asphyxia tolerance, or the tolerable levels of hypoxia and hypercapnia as determined by, e.g., brain hypoxia tolerance and the CO_2 buffering capacity; and (3) metabolic rate, which is determined mainly by work economy and the ability to restrict metabolism via the diving response. A large spleen with the ability to eject a great quantity of stored erythrocytes into circulation would be beneficial for apneic duration both by increasing blood oxygen storage and enhancing buffering capacity, and an important part of oxygen storage in humans is the lung supply.

A way to increase gas storage capacity would be to maximize lung volume, thereby increasing oxygen stores and also allowing

more CO_2 to be transferred to the lungs during apnea via dilution effect. In addition, large lungs with small residual volume (RV) also help the diver to attain greater depth without risking "squeeze," as the depth at which compression to RV is reached would be greater. Increased lung volume furthermore facilitates equalization of middle ears and sinuses. Breath-hold divers are known to overfill their lungs before diving via buccal pumping or "lung packing" (Örnhagen et al., 1998), i.e., by pumping air into the lungs via a swallowing motion. However, there are also negative effects of large lung volume including increased buoyancy as well as a high intra-thoracic pressure, with a negative effect on venous return and increased risk of syncope (Andersson et al., 1998). The diving response, known to limit metabolism during diving, may also be decreased by a large inspired lung volume (Andersson and Schagatay, 1998a,b), and over filling of the lungs could, by counteracting the diving response, elevate metabolism during diving, at least while the diver is not at depth. Trained apnea divers have previously been reported to have large lungs (Carey et al., 1956) suggesting that the positive effects may outweigh the disadvantages. However, the lungs of typical marine mammals are no larger than those of terrestrial, and, e.g., the deep diving Weddell seal exhales before diving in order to avoid excess nitrogen loading, and relies mainly on blood- and tissue (mainly muscle myoglobin) oxygen storage during diving (Butler, 2001). Conversely, competitive apneists are known to train to increase lung volume, by stretching and lung packing maneuvers (Örnhagen et al., 1998), but to our knowledge no systematic study of the effect of lung volume on competitive apnea diving performance in elite apneists has been performed.

We therefore aimed to compare spleen and lung volume, two possible dive-prolonging characteristics, to the individual scores in an apnea world championship competition. We hypothesized that scores in the competition would be positively correlated with both spleen and lung volume.

MATERIALS AND METHODS

PARTICIPANTS AND SETTING

This study complied with the Declaration of Helsinki and the methods used had been approved by the local human ethics board. Information about the study was posted in the competition area of the championship. Fourteen male subjects from 10 different nations volunteered for the study and, after receiving more detailed information about the study, signed an informed consent form. Their mean (SE) age was 29 (2) years, and several were individual world record holders. The participants had an average of 5.8 (1.2) years of experience in apnea training. Their total apnea training load averaged 6.2 (0.6) h/week and other physical training 6.3 (0.7) h/week in the month leading up to the competition. Apnea training varied between individuals, but always included serial and maximal static dry and immersed apneas, walking apnea and dynamic apneas in a pool, and, for the weeks leading up to the competition, dynamic deep diving on a line in the sea or a lake. Physical training mainly included endurance training like swimming, running and cycling, which some divers combined with strength training of major swimming muscles. Anthropometric and training data is presented in **Table 1**.

Table 1 | Correlation values of anthropometric- and training data to scored total points.

$n = 14$	Mean (SE)	Max–min	Total points r
Height (cm)	184 (1.6)	196–177	0.31
Weight (kg)	82 (3.2)	118–72	−0.11
Body mass index (units)	24.2 (0.8)	33.7–21.6	−0.29
Spleen vol. (mL)	333 (33)	598–198	0.57 ($P < 0.05$)
Spleen vol./height	1.81 (0.18)	3.20–1.08	0.55 ($P < 0.05$)
Spleen vol./weight	4.14 (0.45)	7.73–3.28	0.57 ($P < 0.05$)
Vital cap. (L)	7.3 (0.25)	8.9–5.5	0.54 ($P < 0.05$)
Vital cap./height	39.5 (1.3)	47.2–30.1	0.49 ($P < 0.1$)
Vital cap./weight	90.3 (4.1)	113.5–55.4	0.45
Apnea training (h/w)	6.2 (0.6)	10.5–2.0	0.43
Physical training (h/w)	6.3 (0.7)	12–0	−0.48 ($P < 0.1$)

The apnea competition involved accumulation of points from dives of maximal depth, time and horizontal distance for a total score (**Table 2**). In competitive apnea, points are gained in direct relation to depth, time, and distance in the respective disciplines, producing an overall score that renders the winner by adding up the scores from three disciplines. The three disciplines were Constant weight deep diving with fins, where 1 m depth generates 1 point, Static apnea, where 5 s generates 1 point, and Dynamic apnea with fins, where 2 m yields 1 point. The total score was used as a representation of individual overall diving performance.

MEASUREMENTS

Subjects arrived at the laboratory after at least 45 min without apnea, physical activity, or eating. Ambient temperature was 23–25°C. Vital capacity (VC) in the standing position, without lung packing, was measured three times (Microlab spirometer, Micro Medical Ltd., Kent, UK). Subjects' height and weight were also measured and training data collected. Spleen measurements were obtained via ultrasonic imaging (Mindray DP-6600, Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China) using the following protocol: Divers were semi-seated vertically on an elevated stool for approximately 3 min while the site for spleen measurements was identified from the dorsal side, followed by measurement of spleen maximal length, width, and thickness every minute for 7 min. From these triaxial measurements of length (L), width (W), and thickness (T), spleen volume was calculated using the Pilström formula: $L\pi\ (WT - T^2)/3$ (Schagatay et al., 2005).

In order to estimate the contribution of the expelled blood volume to resting apneic duration, the three highest scoring divers were asked to perform a time limited apnea of 2 min, to determine their magnitude of spleen contraction, in addition to the baseline measurements. This short apnea was performed in the sitting position without prior hyperventilation. Triaxial spleen measurements were obtained every minute before the apnea and after the apnea to obtain a recovery profile.

ANALYSIS

To obtain an individual baseline measurement of spleen volume, the mean of the two largest subsequent volume measurements recorded during the 7 min resting period was used, this was done

Table 2 | Disciplines and explanation of some special terms used in sports apnea (see also Schagatay, 2009, 2010, 2011).

Constant weight	Competition discipline with the goal to swim the deepest vertically and back to the surface, using fins and the same weights, on one breath
Static apnea	Competition discipline with the goal to remain maximal duration resting under water in a pool on one breath
Dynamic apnea	Competition discipline with the goal to swim the longest distance horizontally under water in a pool using fins, on one breath
Squeeze	Barotrauma of e.g. ears, sinuses or lungs when gas volumes change due to changes in hydrostatic pressure with depth
Blood shift	When blood pools in the vessels of the thorax, replacing the air volume lost during lung compression at depth, which will counteract lung squeeze
Free-fall	Method used by deep divers to limit energy expenditure; they stop swimming when reaching negative boyancy at depth
Lung packing	Overfilling the lungs beyond TLC by using the glossopharynx as a pump

to minimize the influence of measurement error due to slight changes in probe placement, and to limit the influence of the pulsatile changes in spleen volume. With measurements of VC the largest of the three measurements was used in the analysis, which is the standard procedure to limit the influence of the unaccustomed subject's failure to fill or empty lungs completely in each test. Spleen and lung volume, height, weight and training data from the divers were compared with the individual total scores in the competition via Pearson product-moment correlation. For an estimation of the relative contribution to apneic duration of spleen- and lung volumes, the mean values of spleen and lung volumes from the three subjects with the highest competition scores were compared to those of the three lowest-scoring divers. Between-group comparisons were completed using unpaired Student's t-tests and within-group tests using paired t-tests. The accepted level for differences was $P < 0.05$.

A statistical multivariable regression partial least squares model, with cross-validation, was established using the software SIMCA-P+ v.12. The degree of fit (R^2) of the model was 63% and the model validity (Q^2) 55%, where the variables spleen volume, lung volume, height, number of years of apnea training and the amount of physical training per week were described by the model. A permutation test was conducted to validate the results.

RESULTS

DIVING PERFORMANCE

Mean (SE) competition dive performances of participating subjects were 75 (4) m for constant weight deep diving, 5 min 53 (39) s for static apnea and 139 (13) m for dynamic apnea, which are to our knowledge the highest mean performances reported in any subject group studied in apnea physiology. The mean total competition score for the tested divers was 211 (8) points.

CORRELATIONS BETWEEN ANTHROPOMETRIC DATA AND PERFORMANCE

Spleen volume

Mean (SE) spleen volume was 336 (32) mL with range 215–598 mL. Spleen volume was not correlated with individual height or weight, but was positively correlated with total competition score ($r = 0.57$; $P < 0.05$; **Figure 1A**), and also after correction for height ($r = 0.55$; $P < 0.05$) and weight ($r = 0.57$; $P < 0.05$; **Table 1**).

Vital capacity

Mean (SE) VC was 7.3 (0.25) L with a range from 5.5 to 8.9 L. No correlation between VC and subjects height or weight was observed. There was a positive correlation between VC and total competition score ($r = 0.54$; $P < 0.05$; **Figure 1B**).

Other factors

Subject height, weight, and BMI were not correlated with performance (**Table 1**). There was no correlation between performance and hours of apnea training during the month prior to the competition, but there was a trend toward negative correlation between physical training and performance ($r = -0.48$; $P < 0.1$; **Table 1**).

Multivariable analysis

Spleen- and lung volume were significantly (99%) related to the total sum of scores the divers accomplished from the three disciplines. The variables height, number of years of apnea training and the amount of apnea training or physical training per week were not significantly related to the total sum of scores. The result was confirmed by the permutation test.

ESTIMATED CONTRIBUTIONS FROM SPLEEN AND LUNG VOLUMES TO APNEIC PERFORMANCE

Effect of spleen volume

The three highest scoring divers (mean 242 pts) had a mean (SD) spleen volume of 538 (53) mL and the three lowest-scoring divers (mean 172 pts) a volume of 270 (71) mL; ($P < 0.01$; **Figure 2**) while height and weight of the two groups were similar, at 183 (4.7) and 183 (4.0) cm, and 77 (3) and 91 (23) kg, respectively (NS).

When the highest scoring subjects performed a 2 min apnea, their mean spleen volume decreased by 260 mL ($P < 0.05$; **Figure 3**). Based on the spleen volume reduction observed, the splenic blood contribution to apneic duration in high performing divers was estimated. Spleen blood contains at least double the normal hematocrit (Laub et al., 1993; Stewart and McKenzie, 2002) and with 260 mL of spleen blood being added to the circulation, equivalent in Hb content to at least 500 mL of whole blood, it could bind another 100 mL of O_2. Estimating $\dot{V}O_2$ during resting apnea, based on RMR measurements via indirect calorimetry (Byrne et al., 2005), 2.67 mL O_2/kg/min, as approximately 200 mL O_2/min, the extra O_2 stored in the three top-scoring divers would last for 30 s. The smaller spleens in the three lowest-scoring divers would, assuming a similar 50% spleen volume reduction, contribute extra O_2 lasting approximately 15 s. Thus, the three

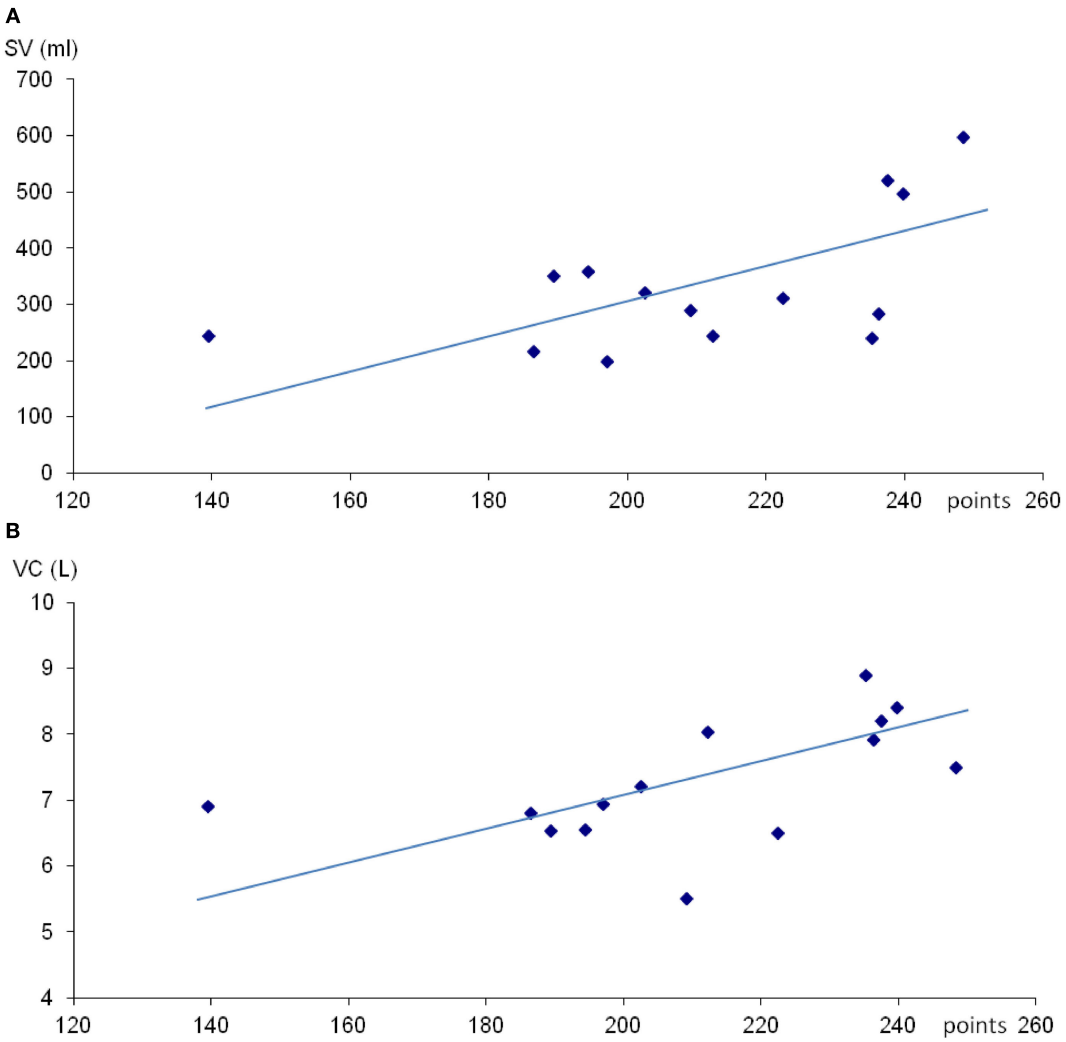

FIGURE 1 | Scatter diagram of spleen volume (SV; A) and vital capacity (VC; B) against total points in apnea competition. For **(A)**, $r = 0.57$ and for **(B)**, $r = 0.54$ (both $P < 0.05$).

best divers could extend apneic duration by 15 s in comparison to the three lowest-scoring divers, due to the extra O_2 storage capacity resulting from the Hb expelled by their larger spleens.

Effect of vital capacity

Mean (SD) VC was 7.9 (0.36) L in the three top-scorers and 6.7 (0.19; $P < 0.01$) in the three lowest-scoring divers (**Figure 2**). The mean VC in the three best divers was thus more than 1 L greater than in the three lowest-scoring divers, equivalent to approximately 200 mL of extra O_2 storage, allowing for an extension of apneic duration by approximately 1 min.

DISCUSSION

A significant correlation was found between accumulated competition score and both spleen volume and lung volume, despite the relatively small number of subjects investigated in this study, suggesting that both these factors may predict competitive apneic performance. There was a large span of individual variation in spleen- and lung volume among divers, showing that specific values of such traits are not necessarily prerequisite for participation at the elite level. However, from the group correlation analysis it is clear that both these factors were associated with the production of top results. This is a novel finding, which may be useful in predicting performance in apneic divers. An interesting question arising from these findings is whether apnea-specific training can increase spleen and lung size, or if successful divers self-recruit to the sport by virtue of this physiological predisposition.

SPLEEN VOLUME

Previously reported spleen volumes in the general population vary greatly, and the average in 140 healthy Caucasians of both genders was found to be 215 mL, spanning from 107 to 342 mL (Prassopoulos et al., 1997). These values appear to be lower than the mean of 336 mL observed in our study's elite apneists. The span 215–598 mL in our studied divers starts with the normal value reported by Prassopoulos et al. (1997). In accordance

with that study, we found no correlation between subject height or weight and spleen volume, and they also did not find any difference between genders or age groups. Geraghty and associates reported a mean value of 238 mL in 47 mid-aged males, spanning 76–400 mL, but a volume of only 180 mL in females (Geraghty et al., 2004). They had corrected values for height and weight using a standardized body size. When standardized for height and weight, our divers' mean spleen volume was in the 90th percentile of Geraghty and associates' observed range and the values of our three best divers more than 100 mL above their recorded maximum. Our lowest-scoring divers were in the 60th percentile, thus near the median presented by Geraghty and associates. It cannot be ruled out that differences in methods between the studies cited above and the present study could yield different results. However, in a study on 10 non-divers of both genders, using the same method for spleen measurements as in the present study but with subjects in a prone position, we found a mean spleen volume of 275 mL, with a range of 185–391 mL (Schagatay et al., 2005). This is similar to the other values reported for non-divers, but clearly less than the 336 mL found in the elite divers in the present study. Bakovic et al. (2003) reported a mean spleen volume in 10 elite divers of 344 mL, which corresponds well to the divers in the present study, but spleen volume in their control group was only slightly smaller.

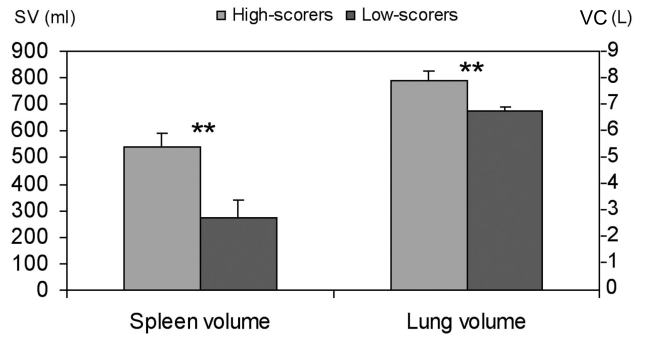

FIGURE 2 | Mean (SD) spleen volume (SV) and lung volumes vital capacity (VC) in highest ($n = 3$) and lowest ($n = 3$) scoring divers. Significance at $P < 0.01$ is indicated by **.

This is the first report of competitive apneists' spleen volumes in relation to their performance. The finding supports a previous indication that trained apneists may have larger spleens or more pronounced spleen contraction, or possibly a higher spleen hematocrit than non-divers; a greater elevation of Hb was observed after three maximal apneas in trained apneic divers compared to both non-diving athletes and sedentary non-divers (Richardson et al., 2005). Studies by Hurford et al. (1990), Bakovic et al. (2003) and Prommer et al. (2007) also suggest that spleen contraction could be at least somewhat augmented in apneic divers. However, in previous studies the apneas performed by the divers have been longer than for non-divers, which could possibly account for their more powerful spleen contraction.

Seal species performing deep and long during dives appear to have larger spleens that average suggesting that benefits of such traits are present in diving mammals (Qvist et al., 1986). It is not known if training can increase mammalian spleen volume, but the spleen appears to have a high regenerative ability. After the experimental removal of 2/3 of the spleen, the remaining tissue showed compensatory growth in baboons (van Rensburg et al., 1991). In humans, growth of a small, accessory spleen after removal of the main spleen has been demonstrated (Hong et al., 1963) and the intact human spleen increased in volume to 179% its original size within a month in parallel with liver regeneration after part of the liver had been surgically removed (Kotton and Fine, 2008), suggesting that the same growth factors are involved. Whether our findings indicate training-stimulated growth or reflects individual predisposition can not be determined in this study, but the spleen volume of the three most successful divers is clearly in the upper

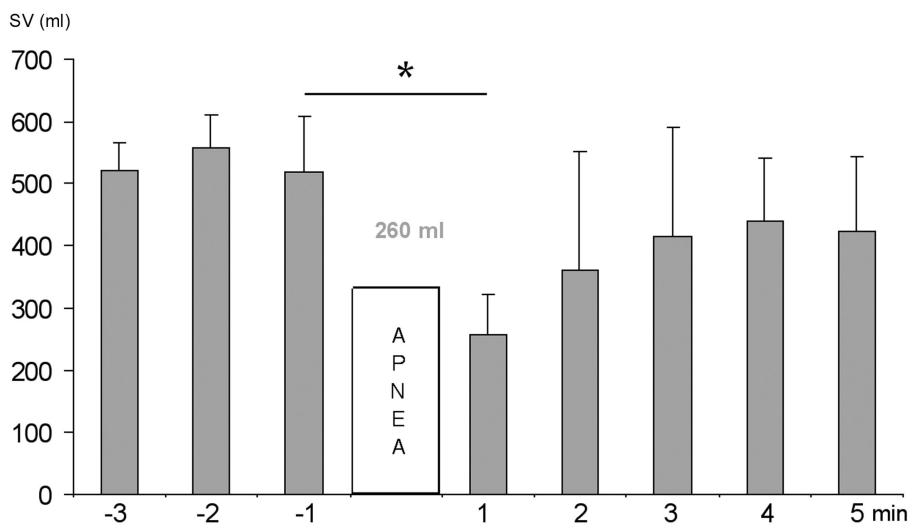

FIGURE 3 | Minute to minute mean (SD) spleen volume (SV) before and after 2 min apnea in the three highest scoring divers. Significance at $P < 0.05$ is indicated by *.

range of that observed in healthy humans, which may indicate this possibility.

LUNG VOLUME

Previously reported mean VC in the general adult male population was 4.1 L for 225 Caucasian subjects with a mean age of 19 years and height of 162 cm (Chiba et al., 1984; Laub et al., 1993) and after correction for differences in height, our subjects' mean VC was 158% of this reference group. Thus, the 7.3 L mean lung volume, with a span from 5.5 to 8.9 L in our study's elite divers, was well above normal. Previously reported mean VC in eight male divers was 6.1 L (span 5.6–6.7 L; Andersson et al., 2002) and 6.3 L in another group of 15 male divers (span 5.0–7.1 L; Andersson et al., 2004), where both groups were of similar age, height, and weight as in the present study but also included recreational apnea divers. No comparison between competition results and VC among divers has been published previously, but the results are in line with studies comparing groups of non-divers with trained divers and reporting greater lung volumes in the divers (see review by Ferretti and Costa, 2003). There is also a report of similar lung volumes between apnea divers and non-divers (Bakovic et al., 2003).

Large lungs in skilled divers could be due to individual predisposition, increased respiratory muscle strength or chest flexibility, or it could be due to training-stimulated lung growth. A direct increase in lung volume as a response to training is suggested by the longitudinal changes in divers observed by Carey et al. (1956). Other research suggests an enlarging effect on the lungs by high altitude exposure and by swimming, but most likely not by other sports (Gaultier and Crapo, 1997). The presence of stem cells in lung tissue (Kotton and Fine, 2008), as well as the observation that lobular removal of lung lobes in children may lead to regeneration to normal size within 2 years (Nakajima et al., 1998) suggests that lung growth may be induced in man, at least at a young age. It is also suggested by statements from elite divers that lung volume has been increased substantially since they started training in adult age by specific lung training protocols (personal communication), and while only minor (3%) increases in VC were observed in a study using lung packing alone in previously untrained subjects (Nygren-Bonnier et al., 2007) we have seen a larger increase in lung volume after training, using the complex training programs developed by elite divers (unpublished results).

While many mammalian divers have lung volumes comparable to terrestrial mammals, there are also semiaquatic species with large lungs, e.g., the sea otter (Lenfant et al., 1970). With a diving pattern resembling the natural diving in humans (Bodkin et al., 2004; Schagatay et al., 2011) it is likely that sea otters also benefit from maximizing lung oxygen storage, while deep diving species may on the contrary exhale before submersing and allow lung collapse at depth.

CONTRIBUTIONS FROM SPLEEN AND LUNG TO APNEIC DURATION IN TOP ATHLETES

Extended apneic duration is required for good performance in all disciplines of competitive apnea. Previous research has shown an apnea-prolonging effect by repeated apneas in intact but not in splenectomized subjects, ascribed to splenic contraction (Schagatay et al., 2001; Bakovic et al., 2003). A larger spleen with the ability to eject more stored red blood cells would be beneficial for the apnea diver, allowing enhancement of both body oxygen stores and asphyxia tolerance via increased buffering of CO_2, and subsequent prolonged apneic duration. The estimated contribution of the spleen blood to apneic duration in the most successful divers, compared to the least successful divers was 15 s, based on the estimated difference in spleen volume reduction between these groups. Reported spleen volume reductions of between 18 and 35% in other studies (Hurford et al., 1990; Bakovic et al., 2003; Schagatay et al., 2005; Richardson et al., 2006) shows that the spleen contractions seen in our top three divers was indeed high, and suggests that the low scorers would probably not display a more powerful contraction. With less relative spleen contraction in low scorers the time gain in high-scorers would be even greater. It should further be noted that the single, non-maximal apnea used in this study would, perhaps not have elicited the full spleen response (Schagatay et al., 2001, 2005). Metabolic rate during apnea performance is most likely lower than in any other sport, and apnea performance is often preceded by meditation-like stages. The VO_2 during resting apnea in 78 healthy males was found to be 2.67 mL O_2/kg/min via indirect calorimetry (Byrne et al., 2005) which is lower than most RMR values reported. As divers have low metabolic rate, we estimated that our divers would use about 200 mL O_2/min. It is even possible that apneic $\dot{V}O_2$ is lower, as >20% reduction of the resting aerobic metabolism during apnea on account of the diving response has been demonstrated (Andersson et al., 2008). In addition to the increased oxygen storage, the increased Hb during apnea would enhance the CO_2 buffering capacity and delay the urge to breathe and the individual breaking point. In competitive apnea, the differences in splenic contribution may thus play a decisive role for competition results, and spleen contraction may be initiated during the warm up procedures often seen (Schagatay, 2010).

The mean VC in the three most successful divers studied was more than 1 L greater than in the three lowest-scoring divers, likely affecting apneic duration by adding about 200 mL of O_2 to the total body stores corresponding an additional 60 s of apnea, which shows that lung volume is a major factor for determining human apneic duration. Aside from enhanced O_2 storage, the CO_2 storage capacity would be increased by the larger VC by a dilution effect which could prolong apneas further. An enlarged VC could furthermore contribute to faster recovery after apnea by reducing relative dead space and increasing alveolar gas exchange by enlarging minute ventilation at a given respiratory frequency. This could provide additional benefits to competitive apneists, and also be important in repeated diving in for example fishing divers. However, lung volume is not known to be large among diving mammals, and some seals dive after exhalation, likely reducing buoyancy and avoiding decompression illness by limiting alveolar gas exchange when the lungs are allowed to collapse during diving (Butler, 2001). This may be compared to professional Ama divers, diving with only 85% of VC for repeated sea harvesting at moderate depths (Hong et al., 1963). Thus not only positive effects result from using large lung volumes and for sub-maximal dives the optimal volume may be below total lung capacity (TLC). Competitive apneists instead fill their lungs beyond TLC by "lung packing" (Örnhagen et al., 1998) which has been shown to prolong apneas

compared to diving at TLC, which in turn yielded longer apneas than using 85% of TLC (Overgaard et al., 2006). This demonstrates the great importance of lung gas storage capacity for extended human diving. The TLC to RV ratio is also important for reaching great depths and increasing TLC without increasing RV would thus be beneficial for deep diving (Schaefer et al., 1968).

OTHER FACTORS

Training

The tendency for a negative correlation between time spent with general physical training and apneic performance could possibly reflect a time limitation when divers spent more time with apnea related training during the weeks before the competition, but it is also in line with the previous findings that there is no increase of the diving response with increasing physical fitness (Strømme and Ingjer, 1978; Schagatay et al., 2000). The lack of correlation between time invested in apnea training and competition result was somewhat surprising (**Table 1**), as previous findings have found that apnea training enhanced diving bradycardia and prolonged apneic duration (Schagatay et al., 2000). It is clear, however, that specific apnea training, with effects on several systems essential for apneic diving, is necessary at this level of performance, as the average time spent on apnea training in the tested group was >6 h/week.

Physiological responses and techniques

Many physiological factors aside from spleen- and lung volume are likely involved in setting the limits in competitive apnea diving (reviewed in Schagatay, 2009, 2010, 2011). In the swimming disciplines, in addition to the tolerance to hypoxia, technique, and work economy are important (Schagatay, 2010). Also the magnitude of diving response (Andersson and Schagatay, 1998a; Schagatay and Andersson, 1998) anaerobic capacity and tolerance of accumulation of hypoxia induced factors are likely factors involved in determining individual performance (Schagatay, 2010). Baseline hematocrit is known to be high in divers (Richardson et al., 2005), a condition which may be induced by apnea training (de Bruijn et al., 2008). Depth disciplines are dependent on advanced training and use of specific equalization techniques to avoid squeeze when the lung is compressed below RV, and factors such as the capacity for "blood shift" (Ferrigno et al., 1987) and the ability to use free-fall (**Table 2**) in order to conserve energy will affect the depth reached (Schagatay, 2011). For depth, also the neuropsychological tolerance to nitrogen narcosis (Ridgway and McFarland, 2006) will be increasingly important, and with depths increasing beyond 100 m there is also an increasing risk of developing decompression sickness on ascent (Schagatay, 2011) and a negative effect of a large lung air supply may become evident; divers may eventually have to allow lung collapse to reach further (Fitz-Clarke, 2007).

Not only physiological features and individual results determine who participates in the world championship in a young sport like apnea. There is little financial incentive in apneic diving, and the sport is generally not supported by national talent development programs. Therefore, socioeconomic factors may also determine the circumstances for training and participation in competitions. Despite limited recruitment, there have been dramatic and continual improvements in elite apneic performance in recent years, showing that human diving capacity may approach that of some semiaquatic species, e.g., the sea otter, once the full capacity is explored. Individual spleen- and lung volume measurement may be an effective method of identifying "hidden" talent on performance. Longitudinal study is necessary to determine whether these features are inherent or acquired by apnea-specific training.

CONCLUSION

This study suggests that spleen volume as well as lung volume may contribute to successful apnea performance in humans. A correlation of spleen volume with apneic performance has never been described before but is functionally logical, as ejection of additional red blood cells to circulation will both enhance oxygen storage and CO_2 buffering capacity. Lung volume has previously been reported to be greater in divers than in non-divers, but this is the first report connecting VC to apnea competition results on the individual level. Studies of performance-predicting factors in competitive apnea will provide a functional evaluation of human apneic diving ability in a mammalian context and, from a sports perspective, it may help to identify talent and aid divers in improving training techniques and performance.

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Vascularization of air sinuses and fat bodies in the head of the Bottlenose dolphin (*Tursiops truncatus*): morphological implications on physiology

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Cetaceans have long been considered capable of limiting diving-induced nitrogen absorption and subsequent decompression sickness through a series of behavioral, anatomical, and physiological adaptations. Recent studies however suggest that in some situations these adaptive mechanisms might be overcome, resulting in lethal and sublethal injuries. Perhaps most relevant to this discussion is the finding of intravascular gas and fat emboli in mass-stranded beaked whales. Although the source of the gas emboli has as yet to been ascertained, preliminary findings suggest nitrogen is the primary component. Since nitrogen gas embolus formation in divers is linked to nitrogen saturation, it seems premature to dismiss similar pathogenic mechanisms in breath-hold diving cetaceans. Due to the various anatomical adaptations in cetacean lungs, the pulmonary system is thought of as an unlikely site of significant nitrogen absorption. The accessory sinus system on the ventral head of odontocete cetaceans contains a sizeable volume of air that is exposed to the changing hydrostatic pressures during a dive, and is intimately associated with vasculature potentially capable of absorbing nitrogen through its walls. The source of the fat emboli has also remained elusive. Most mammalian fat deposits are considered poorly vascularized and therefore unlikely sites of intravascular introduction of lipid, although cetacean blubber may not be as poorly vascularized as previously thought. We present new data on the vasculature of air sinuses and acoustic fat bodies in the head of bottlenose dolphins and compare it to published accounts. We show that the mandibular fat bodies and accessory sinus system are associated with extensive venous plexuses and suggest potential physiological and pathological implications.

Keywords: delphinid, plexus, pterygoid, sinus, veins, fat, decompression sickness

INTRODUCTION

Since the 1980s, numerous beaked whale mass strandings have been temporally and/or spatially associated with deployment of naval mid-frequency active sonar (Simmonds and Lopez-Jurado, 1991; Frantzis, 1998; Evans and England, 2001; Fernandez et al., 2004, 2005). Research into potential causal mechanisms underlying these events is logistically difficult, in part because of constraints such as insufficient funding and the cryptic nature of beaked whales and because of legal and public constraints surrounding live-animal physiological experimentation (reviewed in Cox et al., 2006). Most of the progress that has been made on this topic has been made either through tagging of live beaked whales (Tyack et al., 2006) or from post-mortem morphological and pathological studies (Fernandez et al., 2004, 2005; Hooker et al., 2009). Hypotheses regarding the etiology of the strandings range from physical acoustic trauma to systemic gas and fat embolic syndrome caused by behavioral alterations in dive profiles (Cox et al., 2006; Rommel et al., 2006; Hooker et al., 2011). The tagging studies have generated invaluable insights into the diving behavior of certain beaked whale species, and these data have provided a platform for modeling of gas kinetics such as nitrogen uptake and elimination (Houser et al., 2001; Fahlman et al., 2006; Tyack et al., 2006; Zimmer and Tyack, 2007; Hooker et al., 2009).

Post-mortem examinations of beaked whales stranded in the Canary Islands have identified decompression sickness (DCS)-type sequelae, suggesting that gas bubble formation may be at the root of some of the observed strandings (Fernandez et al., 2004, 2005). Additional findings of apparent acute and chronic embolization in other odontocete species suggest that gas bubble disease in cetaceans may be more common than initially thought (Jepson et al., 2003, 2005). Much debate has focused on individual physiological, behavioral, or anatomical traits that may predispose beaked whales to nitrogen saturation and subsequent DCS-like lesions (Cox et al., 2006; Fahlman et al., 2006, 2009; Rommel et al., 2006; Hooker et al., 2009). Following Scholander's legacy of comprehensive consideration of all aspects of diving biology, we suggest that a combination of physiological, anatomical, and behavioral characteristics contribute to this predisposition and that research should focus on integrating all the aforementioned characteristics (Scholander, 1940).

Currently, our only insights into the functional implications of the anatomy are to be inferred from pathological findings. To begin

this discussion, we chose to focus on the lesions that were observed in the beaked whales that stranded in the Canary Islands in September of 2002 (Fernandez et al., 2005). The most conspicuous lesions discovered on post-mortem examination were intravascular emboli widely disseminated throughout the kidneys, lungs, liver, and central nervous system. Interestingly, these emboli were found to sometimes be composed of gas, other times of fat; however both were often present simultaneously within a single tissue. These findings suggest that either different body areas or types of tissue were compromised separately or a single region containing intimate gas and fat associations was damaged. In either situation, in addition to the involvement of gas-filled spaces and fatty tissues, the other requirement for such dissemination of the emboli is vascular introduction and transportation. Therefore, unless there was intravascular introduction of gas and fat throughout the body or generalized autochthonous embolus formation, we suggest that the most likely location for vascular introduction of gas and fat emboli would be anatomical regions where gas, fat, and blood vessels are intimately associated (**Figure 1**; see also Movie S1 in Supplementary Material). We therefore agree with Jepson et al. (2005) who alluded to the fatty tissues of the odontocete head as a reasonable source of fat emboli due to the extensive vascular structures associated with both cranial air-filled sinuses and acoustic fat bodies and the frequent observation of hemorrhages within those tissues in DCS-like cases of strandings (Boenninghaus, 1904; Slijper, 1936; Fraser and Purves, 1960; Ridgway et al., 1974; McFarland et al., 1979; Fernandez et al., 2004, 2005).

There is a relative paucity of published information concerning beaked whale anatomy, especially the anatomy relating to diving physiology or issues of potential susceptibility to mid-frequency active sonar (reviewed in Rommel et al., 2006). Gas kinetic modeling has shown that knowledge of the vascular anatomy is integral to understanding the dynamics of nitrogen gas uptake and elimination (Fahlman et al., 2006, 2009; Hooker et al., 2009). Some of the most relevant beaked whale anatomy relating to diving physiology is therefore, likely to be the morphology and function of blood vessels, about which nothing has been published. Although the arterial system of cetaceans has received considerable attention, the venous system has remained largely undescribed (Breschet, 1836; Murie, 1873; Wilson, 1879; Ommanney, 1932; Slijper, 1936; Walmsley, 1938; Harrison and Tomlinson, 1956; Fraser and Purves, 1960; Viamonte et al., 1968; Du Boulay and Verity, 1973; McFarland et al., 1979; Vogl and Fisher, 1981, 1982; Vogl et al., 1981). Given the lack of information on beaked whale vascular anatomy, this work will explore the vasculature associated with air spaces and fat bodies of the bottlenose dolphin head as it might relate to the formation of gas and fat emboli and the absorption and elimination of nitrogen gas. We hope that the information contained herein acts like a springboard from which our knowledge of the vascular anatomy of deep diving cetaceans can evolve.

Before discussing the vascular anatomy of the region, a presentation of anatomical details of the accessory sinus system seems prudent. Cetaceans have lost the air-filled paranasal sinus system found in terrestrial mammals (Mead, 1975; Mead and Fordyce, 2009). However, cetaceans do have gas-filled sinuses (the gas is derived from respiratory air but may vary in composition) that are similar to those of the paranasal system. The cetacean accessory sinus system (**Figures 2** and **3**) is unique (Fraser and Purves, 1960; Mead and Fordyce, 2009); these un-pigmented mucosa-lined structures, which are located on the ventral aspect of the skull, are typically associated with hearing and acoustic isolation of the ears (Houser et al., 2004). The ventral sinus system is distinguished from the dorsal air sacs by appearance and function; the lining of the dorsal sacs is composed of pigmented epithelium (Reidenberg and Laitman, 2008) and these sacs are associated with sound production. Both sets of air-filled structures are confluent with the respiratory system.

In their stunning monograph, Fraser and Purves (1960) outlined much of the anatomy of the pterygoid and peribullar sinuses and their surrounding auditory structures by piecing together information from corrosion casts and dissections of various delphinid species and compiling published literature. Their anatomical descriptions included the bones, sinuses, and vascular structures of the region and have acted as the foundation of our review and much of our research on this topic as a whole. Fraser and Purves (1960) noted the extensive vascular investment of the lining of the accessory sinus system present in delphinid species. The vascular nature of the pterygoid and peribullar tissues surrounding and investing these sinuses has been highlighted in other cetaceans, including mysticetes such as fin whales (Murie, 1873; Boenninghaus, 1904; Ommanney, 1932; Walmsley, 1938).

The accessory sinus system is therefore composed of a set of interconnected and physiologically dynamic structures (**Figure 3**) that have been described as both gas-filled and blood-filled (Fraser and Purves, 1960). This apparent contradiction can be explained because the spaces contain a series of gas-filled sinuses, the walls of which possess extensive vascular structures (**Figure 4**). Within this system the relative volumes of gas and blood can apparently be dynamically altered by hydraulic pressures of the respiratory system and the environment and by the amount of blood in the surrounding vascular structures (Fraser and Purves, 1960). Changes in volume of the gas-filled sinuses can be compensated for by corresponding changes in the adjacent soft tissues and with an incursion of blood if the sinus geometry is constrained by bone [Fraser and Purves, 1960; as in the hamular lobe of the pterygoid sinus (Pty sinus) – see below]. The sinuses occasionally contain a considerable amount of viscous, stable foam whose source and composition remain elusive.

The gas-filled part of the accessory sinus system is an extension of the middle ear cavity (Mead and Fordyce, 2009) that is connected to the upper respiratory system via the Eustachian tube (ET, auditory tube; **Figure 2**). The blood-filled portion (individual elements are often referred to as fibro venous plexuses) is an extensive network of veins and venous blood sinuses (Fraser and Purves, 1960) that we describe in detail for the first time. Fraser and Purves (1960) describe a long history of anatomical interest in the accessory sinus system, starting with descriptions of the peribullary sinus by Major in 1672 and Tyson in 1680. This system has been referred to by a variety of names such as: the pterygoid sinus system (Fraser and Purves, 1960; Mead and Fordyce, 2009); pneumatic cavities (Boenninghaus, 1904); Eustachian system (Anderson, 1978); Eustachian sacs (Fraser and Purves, 1960); postpalatine sinus (Flower, 1867); sinus cavities (Houser et al., 2004); air-filled sinuses (Cranford et al., 2008a);

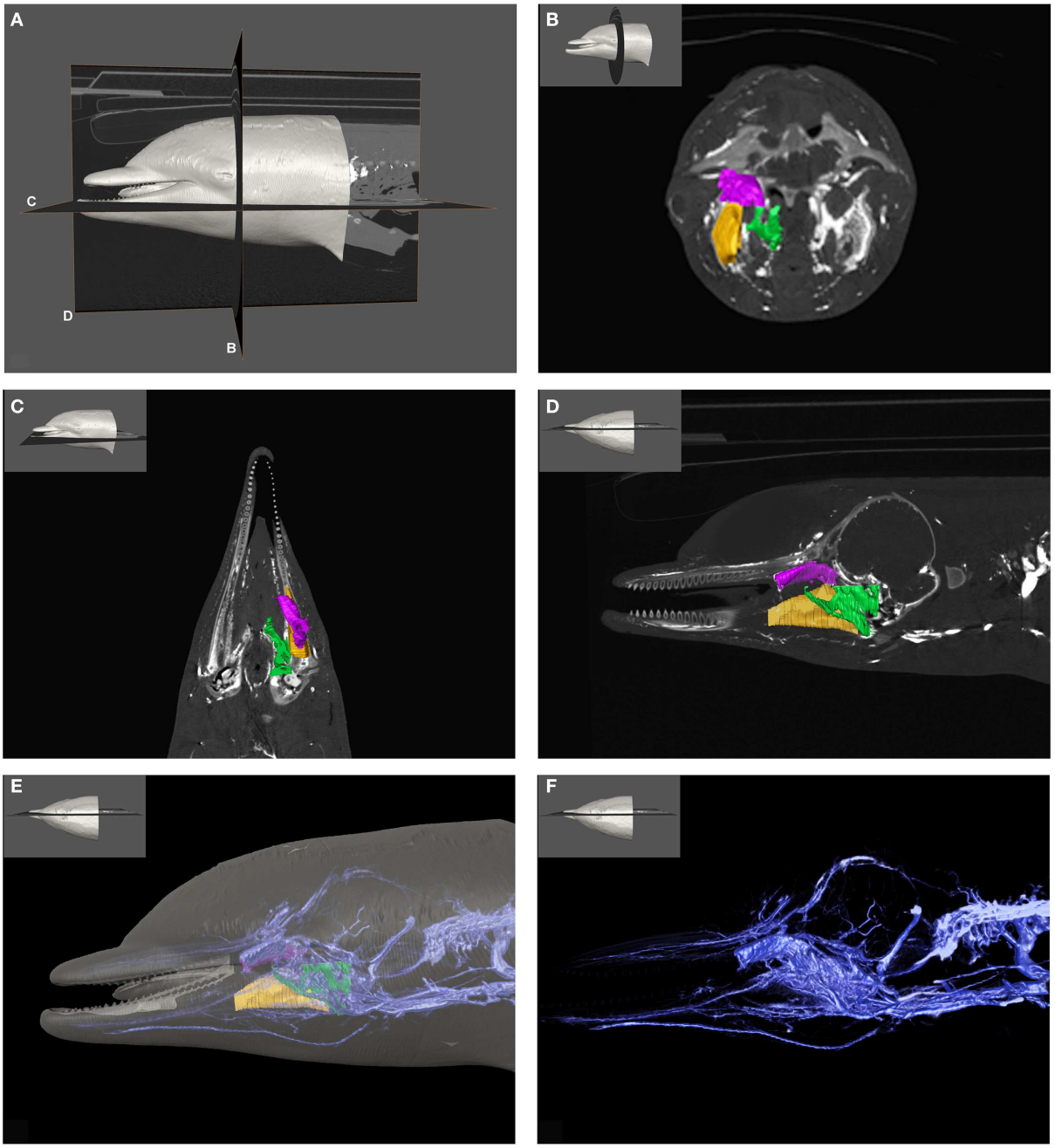

FIGURE 1 | Plate combining computed tomographic (CT) slices with volume renderings of the pterygoid and peribullar plexus (green), intramandibular fat body plexus (yellow), and anterior lobe (purple) to illustrate overall location of the structures. Bright white areas in CT slices, and dark blue structures in volume renderings represent contrast enhanced venous structures. **(A)** Shows the plane of section for **(B–D)**. **(B)** Shows a transverse view, **(C)** shows a coronal view, and **(D)** shows a sagittal view. **(D)** Shows a reference outline of the external surface of the dolphin overlaid on a three-dimensional reconstruction of the venous system (blue) and structures of interest. **(E)** Shows only the venous system so the more detailed structures can be observed. **(F)** Shows a medial view of the veins of the right side of the head, as seen from a mid-sagittal plane of section. Note that the small inset panels within each larger panel show the orientation of the plane of section for each panel.

air sac system (Reidenberg and Laitman, 2008). Recently, the accessory sinus system and the nasal cavity were imaged in two live bottlenose dolphins (*Tursiops truncatus)* by Houser et al. (2004). The nomenclature that we use for the accessory sinus system originated with Beauregard (1894); it was refined by Fraser and Purves (1960) and clarified by Mead and Fordyce (2009). Although they are an invaluable resource, Fraser and Purves (1960) create considerable confusion with their "interchangeable use of cavity, lobe, sac, and sinus" (Mead and Fordyce, 2009).

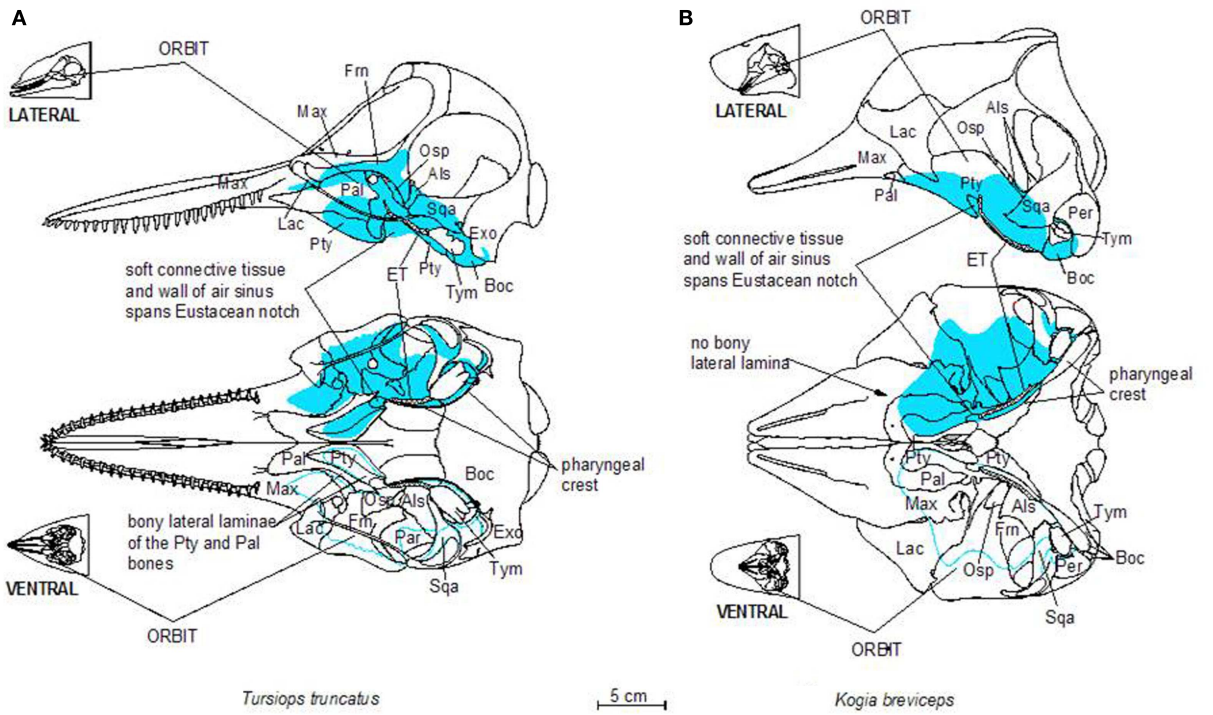

FIGURE 2 | Schematic illustration of dorsal and ventral skeletal associations to the accessory sinus system in a bottlenose dolphin (A) and a pygmy sperm whale (B). Three letter abbreviations refer to bone names as follows: Als, alisphenoid; Boc, basioccipital; Exo, exoccipital; Frn, frontal; Lac, lacrimal; Mas, mastoid (mastoid process of the periotic bone); Max, maxilla; Osp, orbitosphenoid; Pal, palatine; Pty, pterygoid; Sqa, squamosal; Tym, tympanic bulla; Vom, vomer.

MATERIALS AND METHODS

Specimens for this study were obtained from 24 deceased, stranded cetaceans recovered along the east coast of the United States and west coast of Florida (**Table 1**). Eleven bottlenose dolphins and 13 deep diving odontocetes were examined. Beach-cast carcasses were recovered by marine mammal stranding networks authorized by stranding agreements from the National Marine Fisheries Service U.S. Marine Mammal Health and Stranding Program. All work was conducted under a parts authorization from National Marine Fisheries Service, pursuant to 50 CFR 216.22 and 216.37, and with prior approval from University of Florida's IACUC (Permit #: 200801345) and University of North Carolina Wilmington's IACUC (Permit #: A0809-019).

Specimens used for gross dissection of the accessory sinus system were either dissected fresh, or frozen, thawed, and dissected when logistics allowed. Schematic illustrations of the accessory sinus system and the simplified venous connections were made using EasyCAD (Evolution Computing, Phoenix Arizona 85020). Specimens used for describing the vascular anatomy were injected with contrast medium into either the venous system or both the venous and arterial systems, following the procedural methodology outlined by Holliday et al. (2006). Prior to injection of contrast media, all specimens obtained a vascular flush using 0.9% phosphate buffered saline solution and some received a subsequent 5% neutral buffered formalin vascular perfusion to enable a protracted dissecting period. Most specimens were targeted for examination of the venous system, and were therefore flushed through the arteries and out of the veins. Once the effluent ran clear, the flush was stopped and the specimens were refrigerated while being allowed to drain for several hours.

Prior to injecting the vascular contrast material, 5 mL balloon catheters were placed in the vessels to be injected and inflated until a good seal was formed. In specimens that were to be imaged via computed tomography (CT), the vascular system of interest received a mixture of liquid latex and barium sulfate suspension (Liquid Polibar Plus, Bracco Diagnostics Inc.), while vessels only destined for dissection received pure latex (Carolina Biological Inc.). All specimens were refrigerated for 2 days following injections, to allow the latex cast to cure, and if unpreserved with formalin, where subsequently frozen at −20°C. Specimens that were imaged via CT were scanned at the thinnest slice thickness possible based on the specimen length, and whenever possible the volumes were reconstructed to 0.5 mm thickness to allow high resolution imaging of fine caliber vasculature. The resultant DICOM data was post-processed using Amira® software (Visage Imaging Inc., San Diego, CA, USA) on a Gateway desktop with memory and processor upgrades. Following imaging and post-processing, all specimens were thawed and dissected to validate and/or clarify imaged structures. Although the focus of this study was to elucidate the venous morphology in regions of interest in the head of *Tursiops*, the authors felt that a comparative examination of deep diving odontocete cetaceans would

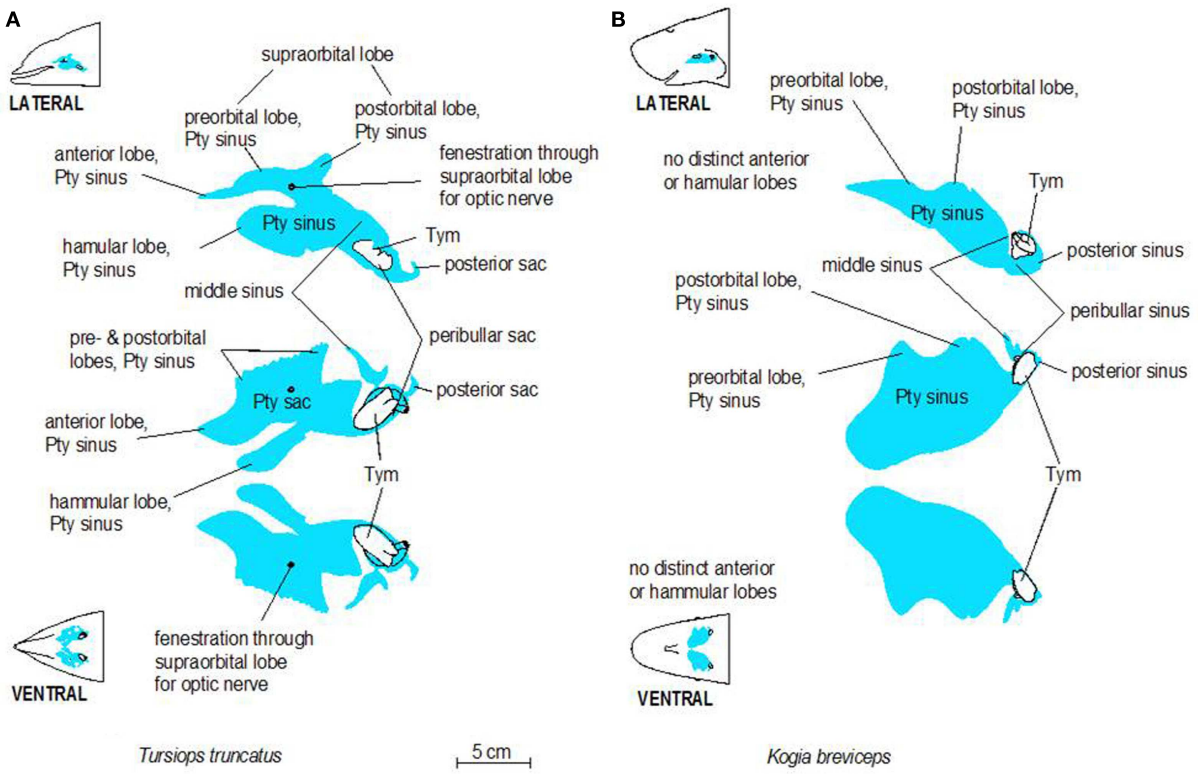

FIGURE 3 | Schematic illustrations of lateral and ventral views of the pterygoid sinus system in bottlenose dolphins (A) and pygmy sperm whales (B). Note the more complex lobes observed in dolphins, relative to the simple geometry of the system in pygmy sperm whales.

be valuable given the association of deep divers and sonar-related strandings. The authors therefore opportunistically obtained specimens from pygmy and dwarf sperm whales (*Kogia, breviceps,* and *sima*), sperm whales (*Physeter macrocephalus*), and Gervais' beaked whales (*Mesoplodon europaeus*). Some of the sperm whale and pygmy sperm whale specimens were of sufficient quality and from young enough animals that could fit into the CT gantry. Prior to vascular dissection, those specimens were imaged according to the aforementioned angiographic protocol and the data obtained was used to guide the dissections. As the focus of this study was on *Tursiops*, only cursory mention is made with respect to findings from the other species.

RESULTS[1]

AIR SINUS AND FAT BODY MORPHOLOGY

Sinuses

What is known about the accessory sinus system of the cetacean head varies considerably by species, however delphinid and phocoenid species remain the best described. It is however clear that in all odontocete species there are extensive gas-filled sinuses on the ventral side of the skull. Interestingly, our preliminary research shows that the accessory air sinuses of deep diving odontocetes such as beaked whales, sperm whales, and pygmy and dwarf sperm whales are much larger, relatively, than those of even large delphinid species like pilot whales, and are invested with intricate and seemingly more voluminous venous arrangements. Much of the internal surface of these sinuses is lined with copious masses of convoluted, intercommunicating, and valve-less veins separated from the air spaces by walls so thin that they are translucent (**Figures 4** and **5**).

AIR SINUS EXTENT

The accessory sinus system is mucosa-lined and juxtaposed (on at least one side) to bones of the basicranium. The sinus system exists as a bilaterally paired system of blind structures. These sinuses (**Figure 3**) may extend rostrally to cover the ventral aspect of the palate, parts of the orbit, and pharynx and caudally to surround the tympanic bulla of the ear (Tym, **Figure 2**), the temporomandibular joint (TMJ), and the joint between the skull and the hyoid apparatus. The accessory sinus system is dominated by the Pty sinus. This sinus extends from just rostral to the orbit back to the region of the Tym. The Pty sinus has two more or less distinct lateral lobes[2] that may extend dorsad around the eyestalk (preorbital and postorbital lobes). In *Tursiops*, these lobes are expanded and may meet dorsal to the eye as a supraorbital lobe. The presence of an air-filled sinus

[1] In-text references to vascular structures that are labeled in figures will be followed by a number representing the label assigned in the figures. Important tributaries of structures maintain the parent vessel's number followed by apostrophes. Non-vascular structures are abbreviated with three letters.

[2] Lobes are partitions or outgrowths of a sinus.

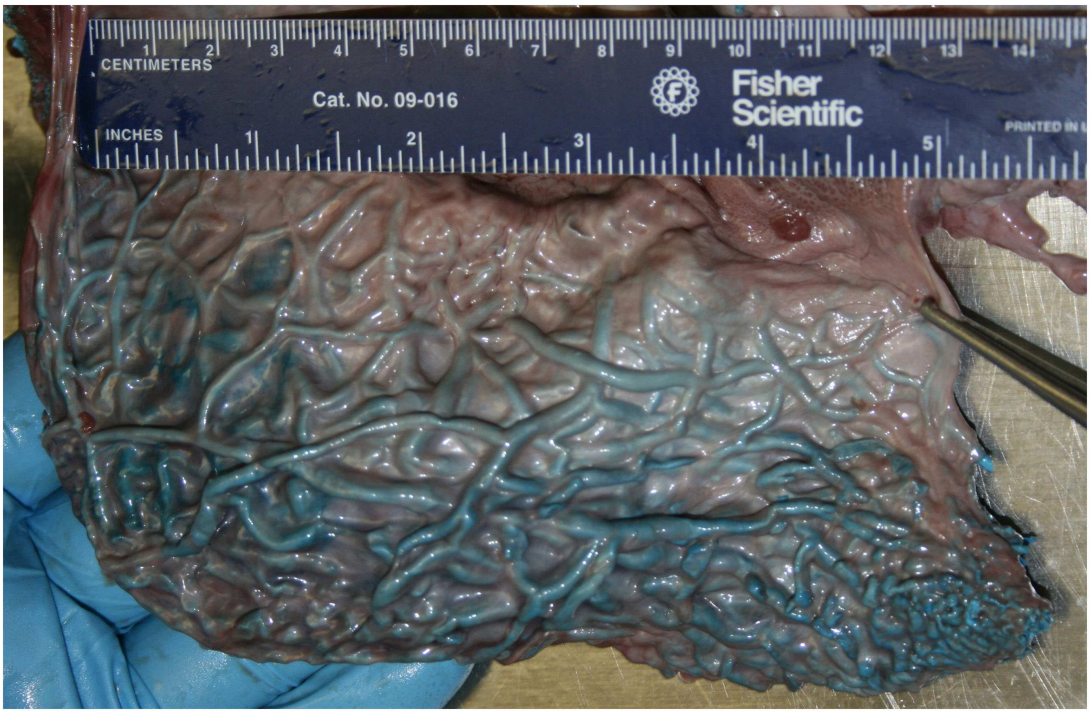

FIGURE 4 | Medial view of the pterygoid venous plexus that lines the lateral wall of the pterygoid sinus of a neonatal sperm whale (*Physeter macrocephalus*). Note the considerable volume and complexity, and the very thin wall separating the venous blood from the sinus air.

on the ventral aspect of the supraorbital process of the frontal bone dorsal to the eye necessitates a fenestration (optic infundibulum; Fraser and Purves, 1960) through the sinus to accommodate passage of the optic nerve, because the sinus extends over the region where the nerve exits the braincase.

The Pty sinus in *Tursiops* has a hamular lobe – within the hollowed hamulus of the pterygoid bone – and an anterior lobe. These two lobes are formed by an indentation of the pterygoid sinus, caused by the lateral laminae of the pterygoid bone and palatine bones. This bony structure is lacking in the pygmy sperm whale (*Kogia breviceps*) and thus, distinction of those lobes is meaningless. At its caudal end, the Pty sinus connects to three smaller sinuses, all of which are relatively close to the region occupied by the ear, the TMJ, and the attachment of the hyoid apparatus via the tympanohyal cartilage: the most lateral of these three sinuses (the middle sinus) is associated with the TMJ; a slightly more caudomedial sinus (posterior sinus) is associated with the tympanohyal joint and is bordered by the paroccipital crest; and a caudomedial sinus (peribullar sinus) that helps separate the tympanic bulla from the adjacent bones.

BONY WALLS

Despite the variable geometry of the recesses and fossae of the sinus system, the bony associations of the sinuses follow some similar patterns among all odontocetes studied thus far. Delphinid species show marked similarity among the different species, while kogiids, physeteriids, and ziphiids all show similarity between themselves. In delphinids, the lateral wall of the pterygoid sinus is partly encased by the bony lateral laminae of the pterygoid and palatine bones. Conversely, in deep diving species like ziphiids, kogiids, and physeteriids, the lateral wall of the Pty sinus is composed entirely of soft tissue capable of collapsing onto itself. When manually manipulated, the Pty sinus is easily closed under the weight of the surrounding tissues, suggesting that maintaining it in an expanded state may require a degree of pressurization. Although the significance of these features is unknown, it is difficult to ignore the common threads among the non-delphinid deep diving cetaceans. Shallow divers such as *Tursiops*, have a deep indentation in the Pty sinus. In contrast, deep divers such as *Kogia* lack or have less distinct anterior and hamular lobes.

BONY RECESSES

The sinuses of the accessory sinus system follow the contours of slight depressions (fossae; Mead and Fordyce, 2009) in the skull bones on the ventral basicranium (**Figure 2**); these depressions typically have a smoother surface than the other regions of these skull bones. The relatively large Pty sinus extends from the palatine and maxillary bones rostral to the orbit and caudally to the region of the Tym of the middle ear. The Tym sits in a deep recess bordered medially by the basioccipital bone. The pharyngeal crest (Mead and Fordyce, 2009), a ridge of bone that delineates the bony lateral margins of the pharynx, is formed by the ventrally projecting crests of the Pty and Boc bones. Note how the lobes of the pterygoid sinus in *Tursiops* extend much farther into the orbit than those of *Kogia*; these lobes join distally to form the supraorbital lobe of the Pty sinus, on the ventral aspect of the supraorbital crest of the frontal bone.

Table 1 | List of specimens used for this study, including species, common name, total body length (TBL), date of stranding, and type of use for this research.

Specimen ID	Species	Common name	TBL (cm)	Gender	Date stranded	Research use
CMA1109	*T. truncatus*	Bottlenose dolphin	191	F	17-May-11	A, V
Hubbs0909	*T. truncatus*	Bottlenose dolphin	249		27-Feb-09	A, V
ECW-005	*T. truncatus*	Bottlenose dolphin	284	M	16-Dec-10	A, V
MMC-Tt-0708	*T. truncatus*	Bottlenose dolphin	256	M	25-Jul-08	V
MMC-Tt-0107	*T. truncatus*	Bottlenose dolphin	271	F	12-Nov-06	I
BRF 164	*T. truncatus*	Bottlenose dolphin	262	F	23-Jul-07	V, S
RJM 003	*T. truncatus*	Bottlenose dolphin	188	F	28-Jun-08	V, S
VMSM 20031104	*T. truncatus*	Bottlenose dolphin	204	M	26-Dec-03	S
VAQS 20061067	*T. truncatus*	Bottlenose dolphin	174.2	M	6-Aug-06	V,S
PBN 003	*T. truncatus*	Bottlenose dolphin	246	F	14-Feb-08	V,S
VAQS 20051086	*T. truncatus*	Bottlenose dolphin	195.4	M	17-Jul-05	S
MML0802	*F. attenuata*	Pigmy killer whale	208	M	30-Jun-05	A, V
MMC-Pm-0908	*P. macrocephalus*	Sperm whale	324	F	29-Sep-08	A, V
VAQS 2008 1002	*K. sima*	Dwarf sperm whale	160	M	28-Jan-08	S
FMMSN0906	*K. breviceps*	Pygmy sperm whale	167	M	10-Jul-09	A, V
VAQS 20071006	*K. breviceps*	Pygmy sperm whale	263	F	27-Feb-07	V,S
CLP 001	*K. breviceps*	Pygmy sperm whale	225	M	22-Nov-07	V,S
KLC 059	*K. breviceps*	Pygmy sperm whale	223.5	M	16-Nov-09	S
KLC 025	*K. breviceps*	Pygmy sperm whale	213	F	22-Dec-08	V,S
MDB 056	*K. breviceps*	Pygmy sperm whale	263.5	M	15-Dec-09	S
MARS0903	*M. europaeus*	Gervais' beaked whale	229	M	2-Aug-09	A, V
WAM 593	*M. densirostris*	Blaineville's beaked whale	423	M	28-Jan-04	S
MDB 023	*M. densirostris*	Blaineville's beaked whale	434	F	15-Sep-08	S
VAQS 20091107	*M. bidens*	Sowerby's beaked whale	397	M	8-Nov-09	S

Abbreviations for genus name are as follows: T., Tursiops; F., Feresa; P., Physeter ; K., Kogia; M., Mesoplodon. Abbreviations for research use are as follows: A, angiography; I, MRI/CT imaging; V, vascular dissection; S, sinus dissection.

EUSTACHIAN TUBE

The ET connects one half of the accessory sinus system and the ipsilateral middle ear to the respiratory system (**Figure 2**). The ET is a distinct, soft tissue, mucosa-lined, hollow tube located mostly within the lumen of the Pty sinus, attached to its ventral mucosa. The dorsal opening of the ET is within the essentially vertical nasal cavity just above the palatopharyngeal muscle, which acts as a sphincter to isolate the nasal cavity from the rest of the upper respiratory system during a dive. The ET extends ventrally within the nasal cavity to the Eustachian notch (Mead and Fordyce, 2009; pterygoid notch – Fraser and Purves, 1960; tubal notch – Schulte, 1917), which is a distinct cleft in the pterygoid bone on the ventral aspect of the skull (**Figure 2**). Near the apex of the Eustacean notch, the ET enters the accessory air sac system by passing through the wall of the Pty sac and a layer of connective tissue (which spans the Eustacean notch) to enter the internal bony nares of the respiratory system. The ET enters (but does not open to) the accessory sac system at the lateral aspect of the Eustachian notch. Within the Pty sinus the ET extends caudally, roughly parallel to the pharyngeal crest, to open just rostral to the opening of the middle ear cavity. In *Tursiops*, we have observed that the ET is surrounded by part of the adjacent pterygoid and peribullar venous plexus, however the luminal surface of the ET is also trabecular but has never injected with casting latex, suggesting it is a non-vascular structure. The caudal end of the ET is open to the lumen of the Pty sac rostral to the bony opening of the Tym – proximally in *Tursiops*, distally in *Kogia*.

BLOOD-FILLED PART (OF THE ACCESSORY SINUS SYSTEM)

All of the aforementioned lobes of the accessory sinus system are, in some form, associated with a venous plexus. The Pty sinus proper is associated with the most extensive venous plexus; however, all of the lobes are surrounded by or associated with a sizeable venous plexuses. Interestingly, the portions of the accessory sinus system along the basicranium are associated with venous plexuses directly connected via sizable emissary veins to the intracranial veins draining the brain.

FAT BODIES

We have observed extensive vascularization in three of the largest acoustic fat bodies of dolphins, namely the melon, intramandibular fat body (IMFB), and extra-mandibular fat body (EMFB) (**Figures 1** and **6–8**) in agreement with the observations of Fraser and Purves (1960), Maxia et al. (2007), and Slijper (1936) (for more complete descriptions of the fat bodies see Norris, 1969; Norris and Harvey, 1974; Cranford et al., 1996, 2008a; Koopman et al., 2003; Scano et al., 2005; Harper et al., 2008; McKenna et al., 2011). The melon fat is located on the dorsorostral aspect of the skull, extending from the rostral border of the nasal passage to the apex of the melon. Ventrally, the melon fat is separated from the

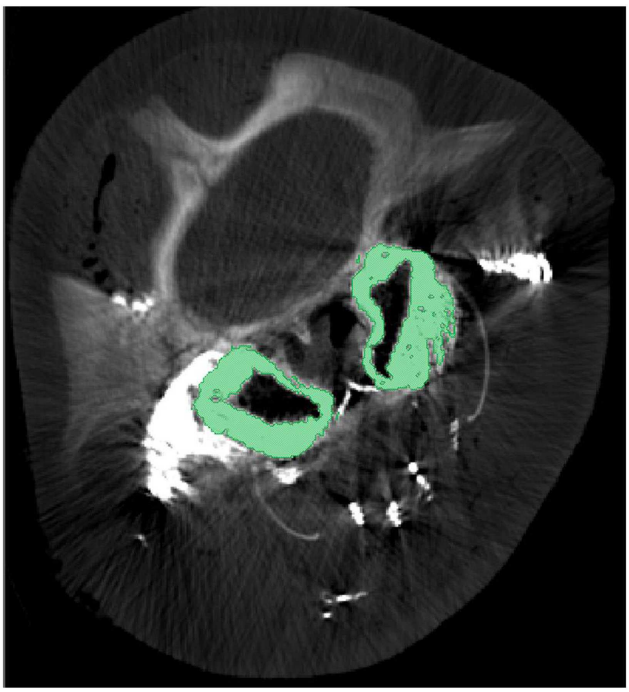

FIGURE 5 | Cross-sectional computed tomographic image at the level of the eyes of a pygmy sperm whale with contrast enhanced veins. The green regions represent the pterygoid venous plexus that surrounds much of the pterygoid sinus. Note that during post-mortem examination the pterygoid venous plexus is usually empty and the green-colored space being occupied by it in this image is occupied by air in the expanded air sinus.

ventral structures of the head by the vomer, maxillary, premaxillary, pterygoid, and palatine bones and the mesorostral cartilage. Each IMFB is located along the medial margin of the ipsilateral dentary bone. The dentary is hollow medially, along the caudal two-thirds of its length, and this cavity is filled with the IMFB which extends caudally to attach to the tympanoperiotic complex by way of dorsal and ventral branches of the fat body (Norris and Harvey, 1974; Ridgway, 1999; Cranford et al., 2008a). Medially, the lateral pterygoid muscles flank the IMFB, while its lateral border is defined by the dentary (Fraser and Purves, 1960). Each EMFB borders the lateral surface of each dentary. Along their ventral aspects, which extend ventral to the dentary, the EMFB and IMFB merge without any grossly visible distinction. On its lateral aspect the EMFB integrates seamlessly with the blubber surrounding the lower jaw. Although the individual veins draining each of these fat bodies are different, all three drainage fields eventually converge into the external jugular (#2, **Figures 7–10**) and facial veins (#3, **Figures 7–9**) and via anastomoses (#1′, **Figures 9** and **10**) into the internal jugular veins.

The melon fat is primarily drained by a multitude of veins that converge with the veins draining the rest of the tissues of the region (#9, **Figures 7**, **13** and **14**) such as the nasal apparatus, maxillae, and maxillary (dorsal) lips. These veins converge, somewhat, as they travel through the dorsal infraorbital foramina of the maxillary and premaxillary bones, emerging on the ventral aspect of the skull as part of the plexus of the accessory sinus system and determined herein to be the dorsal continuation of the maxillary vein component of the pterygoid venous plexus (#6‴, **Figures 14** and **15E**). From there, the plexus receives input from the nasal (#6″), palatine veins (#6″), cavernous sinus (#16), ventral petrosal sinus (#27), intramandibular plexus (#10), pterygoid venous plexus (#11), before coalescing into a single maxillary vein (#6) that follows the pterygoid crest to become the external jugular vein (**Figures 7**, **12** and **13**). Additional but less voluminous drainage of the melon also likely occurs through various terminal branches of the facial vein that wraps around the antorbital notch (#3, **Figures 7** and **9**).

The IMFB is invested with an intricate, voluminous plexus of anastomosing small caliber veins (#10, **Figure 8**). On cross section, the plexus can be seen throughout virtually the entire substance of the IMFB (#10, **Figure 11**), except for a small roughly circular region near its ventral margin (red asterisks, **Figure 11**). Histological quantification of vessel density has not yet been performed, however numerous specimens with latex-injected vasculature have consistently shown this well-defined region of reduced vascularization. Throughout its length, this un-vascularized region of fat forms a circular tube extending along much of the length of the IMFB. Interestingly, its position changes caudally as the tube approaches the ear, where it bends dorsad and attaches directly on the tympanoperiotic complex. Although the venous plexus appears undifferentiated in the way it invests the IMFB, the dorsal aspect of the plexus appears to have an increased density of longitudinally oriented veins that extend rostrally. A portion of the increased dorsal venous density contributes to the formation of a peri-arterial venous rete (PAVR), a rosette of veins that surrounds and follows the mandibular alveolar artery rostrad and has therefore been termed the mandibular alveolar plexus (#10′, **Figures 11** and **13**). The venous investment of the EMFB (#20, **Figure 9**) appears more diffuse and the drainage field less singular than that of the IMFB. Unlike the IMFB, the venous investment of the EMFB does not appear as dense, however despite its more diffuse nature, the veins of the EMFB still maintain a plexiform arrangement of notable extent.

VASCULAR ANATOMY

As is common to most domestic mammals (e.g., cow, horse, pig, cat), the external jugular vein of the bottlenose dolphin is the main drainage route of the pterygoid vasculature although the internal jugular vein can be the primary drainage (e.g., dog; (Ghoshal et al., 1981; Nickel et al., 1981; Evans, 1993; Schaller, 2007). What is different in the dolphin, however, is that the linguofacial vein branches off of the brachiocephalic trunk instead of the external jugular vein, and in some cases as a common branch with the internal jugular vein. Additionally, the facial vein gives off the mandibular vein that in domestic mammals originates from the maxillary vein – the main continuation of the external jugular vein – or one if its tributaries. As we are just now beginning to elucidate the vascular anatomy of deep diving cetacean species, the following description of venous branching patterns is based solely on that observed in *Tursiops*. Additional clarification can be obtained in the (Movie S1 Supplementary Material) provided. **Table 2** shows the structures labeled in **Figures 7–15** and their

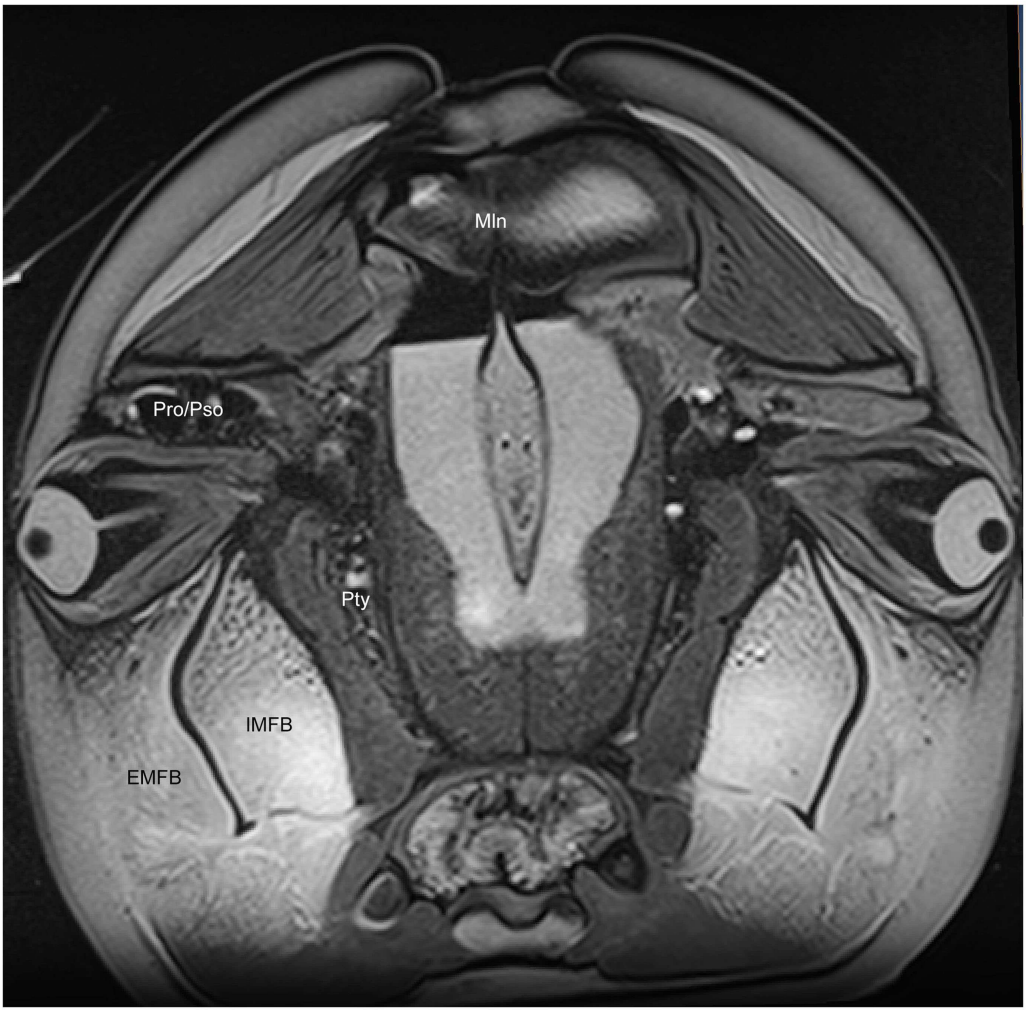

FIGURE 6 | Magnetic resonance imaging cross-sectional view of the head of a bottlenose dolphin at the level of the eyes, showing association of the intra- (IMFB) and extramandibular fat bodies (EMFB). Also labeled are portions of the melon (MIn), pterygoid sinus (Pty) and connection between the preorbital (Pro) and postorbital (Pso) lobes of the pterygoid sinus system.

corresponding names. The plexuses associated with the mandibular fat bodies and accessory sinus system are drained primarily by three parent veins, namely the facial, external jugular, and internal jugular veins.

FACIAL VEIN

At about the level of the paroccipital crest and just lateral to the skull attachment of the tympanohyal element, the facial vein trifurcates into large lateral and ventromedial veins and a midline coalescing plexiform mass of small veins[3] (#3, 5 and 6, **Figures 7–9** and **13**).

(1) The lateral branch of the facial vein (#3, **Figures 7–9** and **13**) extends dorsolaterally around the dentary, passing at an oblique angle under the mandibular condyle and crossing the condyloid crest on its path to the external ophthalmic plexus (#7) and the antorbital notch of the maxillary bone. In addition to its medial connection through the orbit to the external ophthalmic plexus, the vein continues around the antorbital notch to the dorsal aspect of maxillary and premaxillary bones to drain the tissues of the melon, nasal plugs, and dorsal lips (#9, **Figures 7** and **13**). This vein is consistent in location and drainage field to the facial vein of terrestrial mammals.

(2) The middle branch of the facial vein (#5, **Figures 7–9** and **13**) can arise as either a single branch or a brush-like spray of small veins that extend from the rostral margins of the of the facial vein trifurcation. Whether single or plexiform, the structure eventually becomes a spray of numerous small veins that enter the dense connective tissues associated with the ear and lower jaw attachment to the skull. Upon entering the connective tissue, the veins contribute – along with the pterygoid plexus described below–to the formation of

[3]Traditionally venous branching descriptions follow a distal to proximal path representing the retrograde flow of venous blood; however for conceptual simplicity we have chosen to describe branching patterns starting at proximal main branches and progressing out to more distal branches.

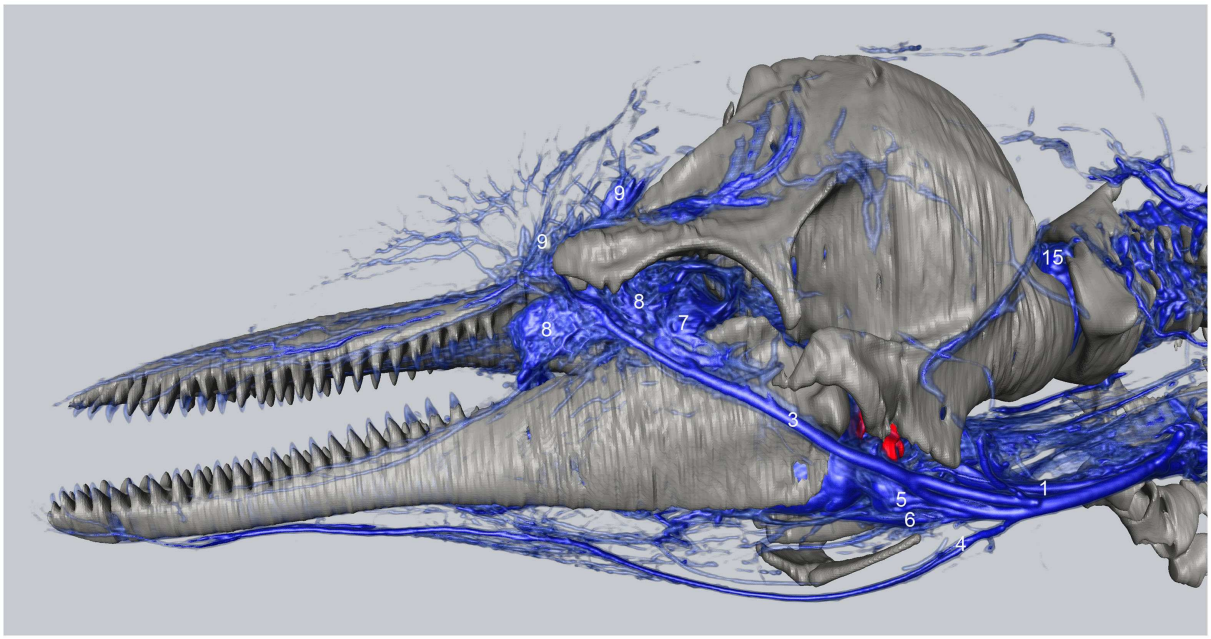

FIGURE 7 | Three-dimensional angiographic reconstruction of the left lateral aspect of the head of the bottlenose dolphin, showing associations of superficial veins and bony elements. Veins were assigned names as follows: (1) internal jugular, (3) facial vein, (4) submental vein, (5) mandibular vein, (6) maxillary vein, (7) external ophthalmic plexus, (8) anterior lobe plexus, (9) melon veins. The red structure represents the tympanoperiotic complex. The external jugular is located medial to the facial vein and can therefore not be seen.

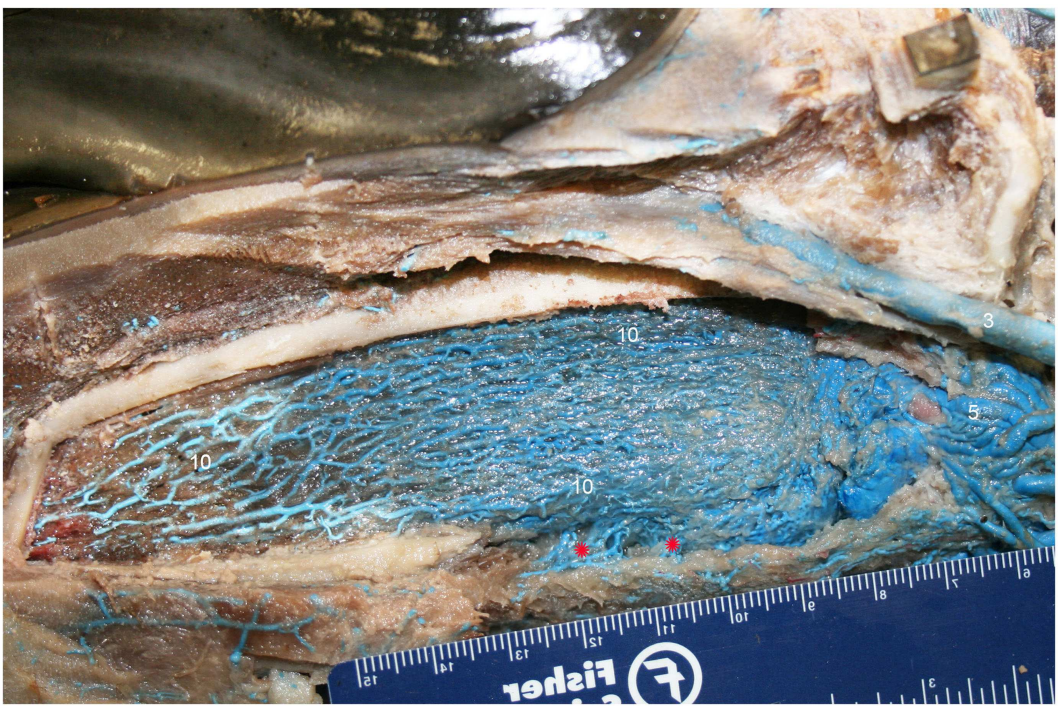

FIGURE 8 | Lateral view of right dentary (image has been flipped to simulate left lateral orientation for consistency between images) with a window cut out of the lateral wall in order to visualize the intramandibular fat body plexus. Veins were assigned names as follows: (3) facial, (5) mandibular, (10) intramandibular fat body plexus. Note that red asterisks show the main connection between the intramandibular and extramandibular fat body veins.

FIGURE 9 | Ventrolateral view of the right side of the bottlenose dolphin neck showing the jugular and facial veins, and the complex anastomoses between the structures (image has been flipped to simulate left lateral orientation for consistency between images). Structures were assigned names as follows: (1) internal jugular, (1′) anastomotic branches to the external jugular and to plexus surrounding external carotid artery, (2) external jugular, (3) facial, (5) mandibular, (6) maxillary, (17) external carotid artery, (18) internal carotid artery with surrounding venous plexus, (19) venous plexus surrounding the external carotid artery (*vena plexi commitans arteria carotidis externa*), (20) extramandibular fat body plexus, (21) vagus nerve, (22) hypoglossal nerve.

the fibrovenous plexus described by Fraser and Purves. This fibrovenous plexus is composed of an intricate network of small (∼1–5 mm diameter) caliber anastomosing veins that invest much of the connective tissue and wrap around part of the tympanic bulla of the ear bone complex. The fibrovenous plexus[4] extends rostrally, gradually losing its connective tissue component as it transitions into the soft fatty tissues of the IMFB. As the ventrolateral portion of the fibrovenous plexus extends into the IMFB to become the mandibular plexus, the veins appear to distribute throughout most of the fatty substance (#10, **Figures 8**, **11**, **13** and **14**). Along its dorsal margins the IMFB plexus coalesces to form a PAVR (#10′, **Figures 11** and **13**) that surrounds the mandibular artery and courses rostrad toward the mental foramina of the distal dentary and mandibular symphysis, occasionally giving off branches to the teeth. This PAVR is consistent with the mandibular (inferior) alveolar vein described in terrestrial mammals and is therefore responsible for draining blood from the teeth and rostral portions of the lips of the lower jaw.

(3) The third and most medial branch of the facial vein (#6, **Figures 9**, **10**, **14** and **15**) is similar in size to the lateral branch. Approximately 5 cm after the ventromedial branch emerges from the trifurcation it is joined by the maxillary vein, the main terminus of the large external jugular vein (#2, **Figures 9**, **10**, **14** and **15**). This medial branch of the facial vein might, therefore, be considered an anastomotic branch to the maxillary vein. The two veins join to form a larger vein that travels roughly horizontally to the level of the ceratohyal cartilages of the hyoid apparatus. At that level, the vein gives off smaller lingual and pharyngeal branches before curving dorsad along the pterygoid crest. It should be noted that the lingual vein – usually arising from the maxillary vein further rostrad – can occasionally branch off of the facial vein as a common trunk with the aforementioned ventromedial anastomotic branch to the maxillary vein.

INTERNAL JUGULAR VEIN

The internal jugular vein (#1, **Figures 7**, **9**, **10**, **13** and **14**) can arise either singularly or as a common trunk with the linguofacial vein, directly from the brachiocephalic vein. Once the internal jugular vein has traversed the short neck and reached the head, it contributes to the drainage of three regions.

[4]Fibrovenous plexus – a complex system of venous channels and connective tissue forming a plexus that extends from the connective tissue of the ear to the pterygoid region; it is mostly associated with the lining of the sinuses.

FIGURE 10 | Ventromedial view of the right half of a mid-sagittally sectioned bottlenose dolphin showing the jugular branching patterns. Veins were injected with blue latex and arteries with red. Structures were assigned names as follows: (1) internal jugular, (1′) anastomotic branches to the external jugular and to plexus surrounding external carotid artery, (2) external jugular, (6) maxillary, (11) pterygoid plexus, (12) peribullar plexus, (13) temporal sinus, (15) epidural veins, (16) cavernous sinus, (18) internal carotid artery with surrounding venous plexus, (18′) regressed terminus of internal carotid artery, (19) external carotid artery with surrounding venous plexus, (24) emissary vein of foramen ovale, (25) emissary vein of jugular foramen.

(1) The first and most proximal (caudal) contribution is formed by numerous anastomoses (#1′, **Figures 9**, **10**, **14** and **15**) between the internal and external jugular veins. These anastomoses arise from the ventral aspect of the internal jugular vein and travel obliquely ventrorostrad to fuse with the external jugular plexus surrounding the external carotid artery (#19, **Figures 9** and **10**), in agreement with the findings of Ridgway et al. (1974). Like the mandibular alveolar vein, the plexus surrounding the external jugular vein forms a PAVR. From this point rostrad, the external jugular vein (#2) becomes the maxillary vein (#6) and drains the majority of the pterygoid plexus as well as parts of the nasopharyngeal, palatine, and dorsal nasal regions. Due to the anastomoses, the internal jugular vein might therefore be considered to facilitate this drainage.
(2) The second and third branches arise in common from a bifurcation of the distal internal jugular vein. The proximal of the two branches curves sharply rostrad to become the ventral petrosal sinus (#27, **Figure 15D**) that lines the dorsal aspect of the peribullar plexus (#12, **Figures 14** and **15**).
(3) The third branch extends vertically from the bifurcation of the internal jugular vein, to enter the jugular foramen as the terminus of the internal jugular vein (#25, **Figures 15A–D**). As it enters the caudal portion of the calvarium, it fuses with the temporal/sigmoid/transverse dural sinus, to form one of the main drainage paths for blood from the brain case.

EXTERNAL JUGULAR VEIN (#2, FIGURES 9, 10, 14 AND 15)

The external jugular vein arises directly from the brachiocephalic trunk as a plexiform structure composed of small and large veins that surround the external carotid artery. The ventral portion of this plexus is highlighted by a distinct vein of considerably larger caliber – the external jugular vein – than the rest of the plexus veins. As the external jugular vein traverses the neck and approaches the head, it receives the anastomotic branches (#1′, **Figures 14** and **15**) of the internal jugular (#1) and facial veins (#3). It finally receives an anastomotic branch from the facial vein and then curves medially to follow the basioccipital and pterygoid crests as the maxillary vein (#6). Along its course through the pterygoid region, the external jugular vein forms numerous branches and countless small anastomoses with other venous tributaries. The main branches invest the palatine, nasopharyngeal, pterygoid, and dorsal head tissues as follows:

(1) On its course to the rostroventral aspect of the brain case, the maxillary vein sends dorsorostrad oriented veins of a

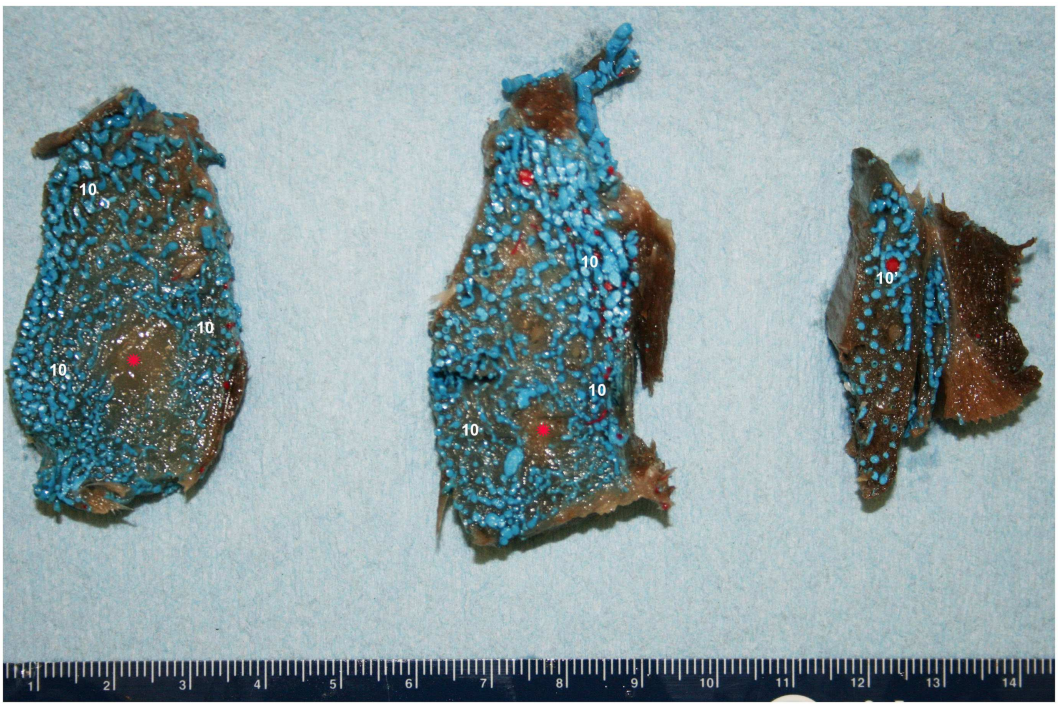

FIGURE 11 | Cross-sectional view of the intramandibular fat body (IMFB) of a bottlenose dolphin, progressing from caudal (left) to rostral (right). Note the extensive investment of the fat with a venous plexus (10). Also note the peri-arterial venous rete (10′-PAVR) surrounding the red mandibular alveolar artery in the dorsal region of the fat body, and the regions of un-injected fat highlighted with red asterisks near the ventral aspect. Due to the location and association with the concomitant artery, the PAVR has been termed the mandibular alveolar plexus.

plexiform nature (#6′, **Figures 12B–D**) that invest the tissues on the roof of the oral cavity. These veins were considered to be the palatine veins which are shown to form a palatine plexus.

(2) Before it breaks up into the countless veins that compose the pterygoid plexus (#11, **Figures 12C–E**, **14** and **15**) that invests the lining of the pterygoid sinus and drain the substance of the pterygoid muscles, the maxillary vein sends rostrodorsad oriented branches (#6″, **Figures 12B–F**) into the palatopharyngeal muscles where they anastomose with other palatopharyngeal veins supplied by the external jugular vein and a large pharyngeal plexus that surrounds the rostral esophagus and laryngeal cartilages (red asterisks, **Figure 12A**). At this level the maxillary vein loses its singular identity as it breaks up to become part of the pterygoid plexus associated with the accessory sinus system (#11, **Figures 12C–F**, **14** and **15**). Nonetheless, the lateral region of the plexus extends laterally under the external pterygoid muscle – such that is forms a cradle for the muscle – and fuses with dorsal extensions of the intramandibular plexus. The combined plexuses extend dorsad toward the maxillary bone and narrow considerably as they approach the ventral aspect of the maxillary and premaxillary bones. The plexus surrounds all but the medial aspect of the maxillary artery as both structures travel to the infraorbital foramina (#6‴, **Figures 14** and **15E**). This dorsal extension of the venous plexus maintains its course to the ventral infraorbital foramina, in juxtaposition with the corresponding maxillary artery, and is therefore considered herein as the terminus of the maxillary vein. Just before the plexus reaches the infraorbital foramina, its rostral aspect sends a large mass of highly convoluted veins rostrad. These veins form a robust venous plexus (preorbital and anterior lobe plexus) that travels along the ventral lining of the anterior lobe of the accessory sinus system (#8, **Figures 7** and **13–15**).

DISCUSSION

SIGNIFICANCE OF THE ANATOMY

Despite the significant reorganization of the skull bones to accommodate dorsally located nares and a large melon, many of the major veins in the heads of odontocetes find analogs in domestic mammals. Similarly, although we have no developmental data on these venous structures, the striking similarity in distribution and location of many of those structures suggests that they are also homologs of the veins seen in domestic mammals. Nonetheless, a few significant elaborations on structures were observed, especially in regions associated with acoustic fat bodies and air sinuses. Specifically, given the novel evolutionary nature of the cetacean mandibular fat bodies, the venous plexuses of the intra- and EMFBs find no notable homolog in domestic mammals. Although it is unknown whether or not the venous plexuses of the accessory sinus system might find homologs in the pterygoid plexuses of various domestic mammals such as horses, cows, and dogs, the complexity and volume of the plexus system of cetaceans appears unparalleled.

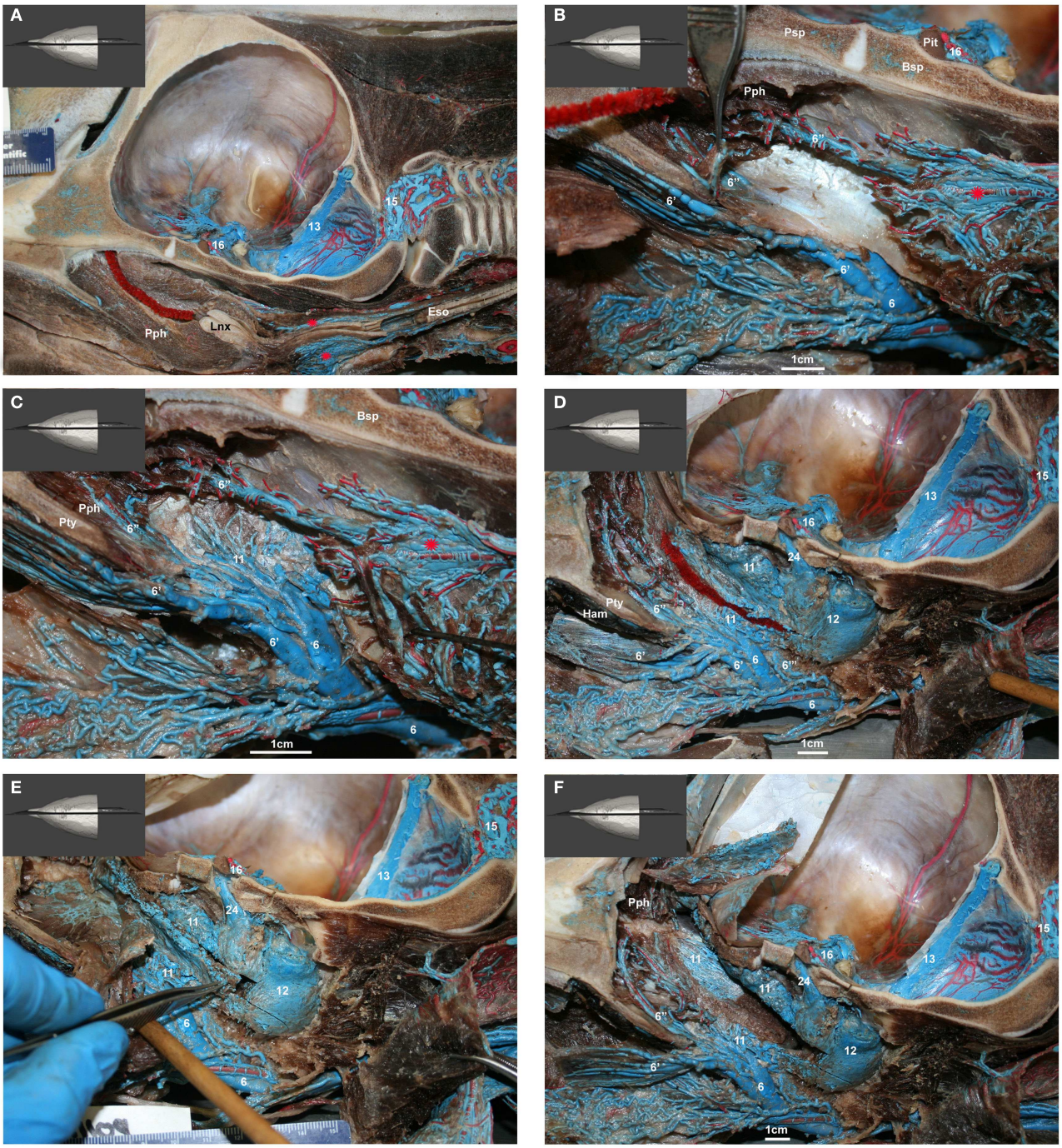

FIGURE 12 | (A–F) Medial view of gross dissection of a bottlenose dolphin with latex-injected vessels. Approximate scale bars have been added whenever a ruler was not present in the photograph. **(A)** Shows the location of the pharyngeal plexus (red asterisks) in relation to the esophagus (Eso) and laryngeal cartilages (Lnx). The red structure represents the nasal passage surrounded by the palatopharyngeal muscle (Pph). **(B)** Shows the pharyngeal plexus (red asterisk), the maxillary vein (6) sending a palatine plexus (6′) that invests the roof of the oral cavity, and contributing to the nasopharyngeal veins (6″) that drain the palatopharyngeal muscles (Pph). Also visible are the cavernous sinus (16), pituitary gland (Pit), and presphenoid (Psp) and basisphenoid (Bsp) bones that form part of the floor of the brain case. **(C)** Shows the contribution of the maxillary vein (6) to the formation of the lateral wall of the pterygoid plexus (11). Also visible is the pharyngeal plexus (red asterisk). **(D)** Shows the medial aspect of the peribullar plexus (12) and dorsal pterygoid plexus (11) with the bone of the pterygoid crest removed. Also visible are the emissary vein (24) traveling through the oval foramen, the temporal sinus (13) and epidural venous plexus (15). The red structure represents the Eustachian tube which extends from the tympanic bulla to the pharynx along the pharyngeal crest. Note the visible blue latex within the hamular (Ham) lobe of the Pty. This portion of the plexus can displace the entire volume of air within the hamular lobe. **(E)** Shows the dissection of the dorsal pterygoid and peribullar plexuses. **(F)** Shows the opened pterygoid and peribullar sinus, exposing part of the internal sinus lining and the connection between the lateral portion of the pterygoid venous plexus (11) that connects to the IMFB plexus. Note that the small inset panels within each larger panel show the orientation of the plane of section for each panel.

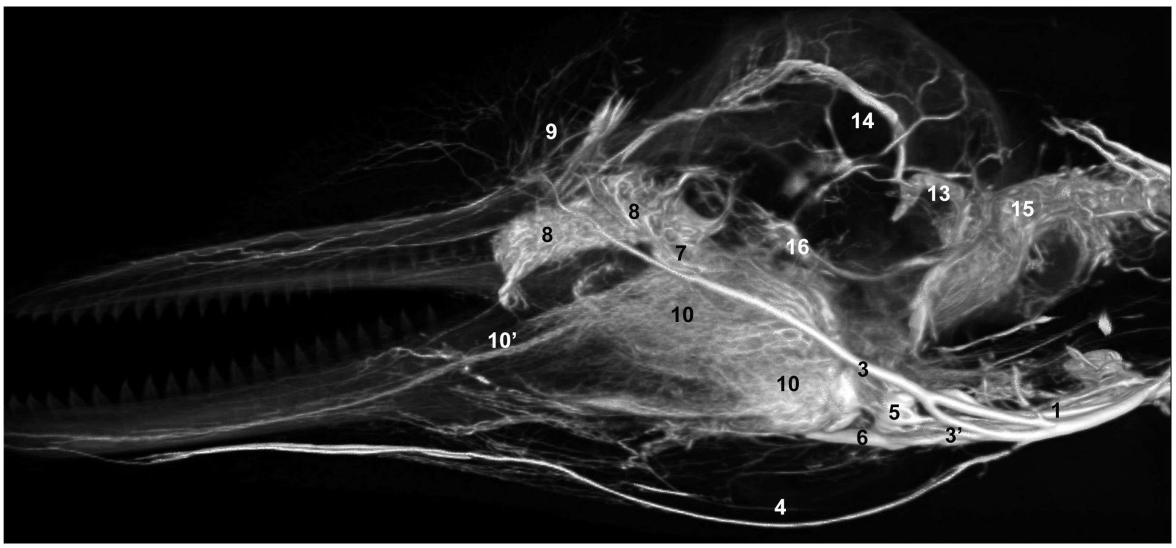

FIGURE 13 | Lateral view of a volume rendering of computed tomographical angiography of a bottlenose dolphin head showing complexity of venous investment. The bone has been removed in the rendering to allow viewing of the spatial relationships between the superficial and deep venous structures. Veins were assigned names as follows: (1) internal jugular, (3) facial, (3″) anastomotic branch to the maxillary vein, (4) submental, (5) mandibular, (6) maxillary, (7) external ophthalmic plexus, (8) anterior lobe plexus, (9) melon veins, (10) intramandibular fat body plexus, (10′) mandibular alveolar plexus, (13) temporal sinus, (14) dorsal sagittal sinus, (15) epidural venous plexus, (16) cavernous sinus.

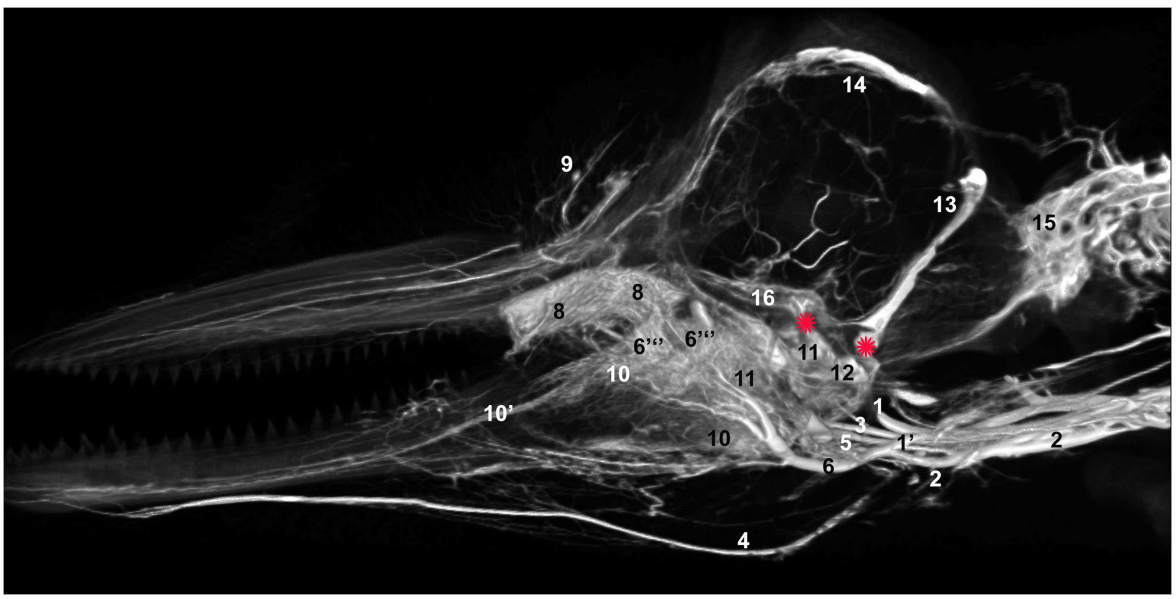

FIGURE 14 | Mid-sagittal view of a volume rendering of computed tomographical angiography of a bottlenose dolphin head showing complexity of venous investment along medial aspect. The bone has been removed from the rendering to allow viewing of all venous structures. Veins were assigned names as follows: (1) internal jugular, (1′) internal jugular anastomosis with external jugular, (2) external jugular, (3) facial, (4) submental, (5) mandibular, (6) maxillary, (6‴) dorsal continuation of the maxillary vein, (8) anterior lobe plexus, (9) melon veins, (10) intramandibular fat body plexus, (11) pterygoid sinus plexus, (12) peribullar sinus plexus, (13) temporal sinus, (14) dorsal sagittal sinus, (15) epidural venous plexus, (16) cavernous sinus. Note the two red asterisks showing the two basicranial emissary connections between intracranial and extracranial veins. These emissaries form robust connections between the plexuses of the pterygoid sinus system and the dural sinus system.

ACCESSORY SINUS SYSTEM

Since the accessory sinus system is not completely encased in robust, rigid bony compartments, it is presumably exposed to the effects of changing barometric pressures encountered during a dive. In order to avoid pathologies associated with physical injury of tissues exposed to dysbaric changes it seems safe to assume that

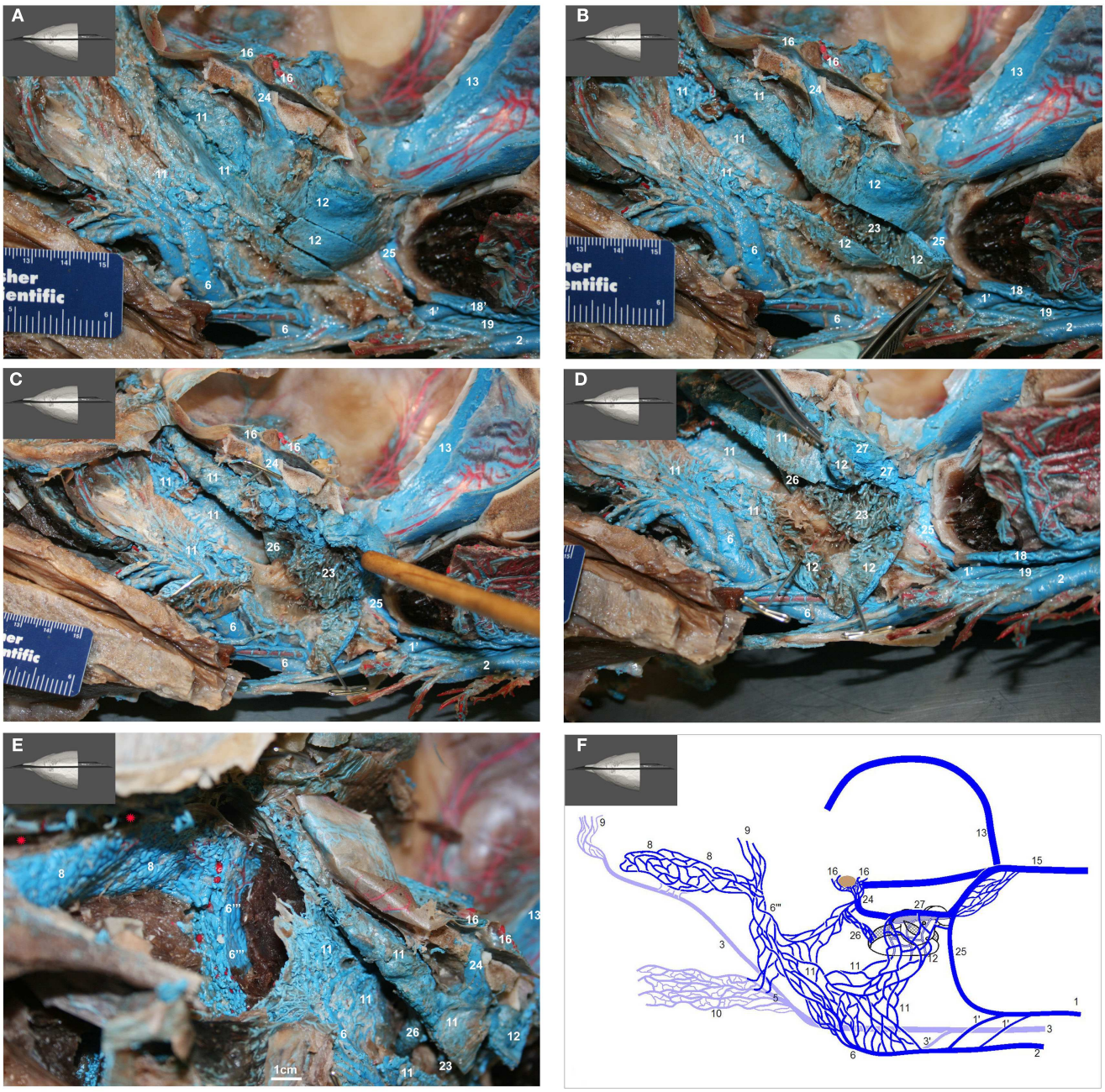

FIGURE 15 | (A–E) show medial view of gross dissection of the pterygoid and basicranial regions, identifying some of the key venous structures outlined in the text (structure numbers correspond to those in previous images). **(A)** Shows the medial wall of the plexuses associated with the accessory sinus system and their emissary connections (24 and 25) to the intracranial dural sinuses. **(B)** Shows the pterygoid (11) and peribullar (12) plexuses being opened, to see the lateral wall of the pterygoid sinus plexus and the *corpus cavernosum* of Boenninghaus (23). **(C)** is similar to **(B)** but with the peribullar sinus opened further to expose the *bulbous venosus epibularis* of Boenninghaus (26) emerging from within the tympanic bulla (red asterisk) and merging with the dorsal part of the pterygoid and peribullar plexuses. **(D)** is similar to **(C)** but with the dorsal aspect of the peribullar plexus opened further to expose the ventral petrosal sinus (27) and its caudal connection to the internal jugular emissary vein of the jugular foramen (25). The tympanic bulla is marked with a red asterisk. **(E)** Shows an oblique rostral view of the left dorsolateral extension of the pterygoid plexus (11) around the external pterygoid muscle (Pte) as it forms the dorsal continuation of the maxillary vein (6‴) that passes through the infraorbital foramina. Also shown is the extensive and complex nature of the anterior lobe plexus (8) and the anterior lobe (red asterisks) it is associated with. **(F)** Shows a simplified schematic illustration of the medial view based on a modification of Boenninghaus' (1904) illustration of the venous connections in the pterygoid and basicranial regions. Shown are the main patterns of venous connections of the plexuses associated with the intramandibular fat body, accessory sinus system and the intracranial dural system. Lighter blue vessels are more lateral (behind) darker structures. The brown structure represents the pituitary gland. Note that the *corpus cavernosum* of Boenninghaus (23) is not illustrated here due to its location lateral (behind) the peribullar plexus (12).

Table 2 | List of soft tissue (blood vessels and nerves) structure labels used in the figures and their corresponding names.

Structure label	Structure name
1	Internal jugular vein
1′	Anastomotic branch to external jugular vein
2	External jugular vein
3	Facial vein
4	Submental vein
5	Mandibular vein
6	Maxillary vein
6′	Palatine plexus
6″	Nasopharyngeal veins
6‴	Dorsal maxillary vein continuation
7	External ophthalmic plexus
8	Anterior lobe plexus
9	Melon veins
10	Intramandibular venous plexus
10′	Mandibular peri-arterial venous rete/mandibular alveolar veins
11	Pterygoid plexus
12	Peribullar plexus
13	Temporal sinus
14	Dorsal sagittal sinus
15	Epidural veins
16	Cavernous sinus
17	External carotid artery
18	Internal carotid artery
18′	Regressed terminus of internal carotid artery
19	Venous plexus surrounding external carotid artery
20	Extramandibular venous plexus
21	Vagus nerve
22	Hypoglossal nerve
23	*Corpus cavernosum* of Boenninghaus
24	Emissary vein of oval foramen
25	Emissary vein of jugular foramen
26	*Bulbous venosus epibularis* of Boenninghaus
27	Ventral petrosal sinus

there exists a significant amount of physiological flexibility within those structures. The apparent susceptibility of the accessory sinus system to external pressures may have significant implications on diving gas kinetics. The intimate anatomical association between large venous masses with thin linings and pockets of air exposed to changing pressures begs a question regarding absorption and clearance of gases during a dive (Reidenberg and Laitman, 2008). With increasing hydrostatic pressures, gases in air are progressively driven into solution. This phenomenon is responsible for increased absorption of nitrogen in the lungs of a scuba diver. Marine mammals had long been thought to be exempt from significant accumulation of nitrogen during dives because they do not breathe compressed air at depth, and because their pulmonary alveoli are believed to progressively collapse with depth, thereby isolating pulmonary gases away from absorptive respiratory surfaces (Scholander, 1940). Since the pressurized pulmonary air is thought to be segregated from the pulmonary blood circulation, nitrogen absorption is believed to be limited (Kooyman, 1973; Ridgway and Howard, 1979). Yet these facts seem contradictory to the findings of gas emboli in beaked whales, as diving-related gas emboli in humans are typically associated with pulmonary injury or autochthonous bubble growth due to improper management of nitrogen gases and/or air absorbed at depth (Brubakk et al., 1999; Neuman, 1999). Indeed, recent studies have begun to show that gas emboli within stranded deep diving cetaceans are composed primarily of nitrogen gas, adding further support to the notion that under certain circumstances, nitrogen saturation may be possible (Bernaldo de Quirós et al., 2011, 2012). Additionally, recent gas modeling in cetaceans has shown that the risk for nitrogen saturation may in fact be a concern (Houser et al., 2001; Fahlman et al., 2006, 2009; Hooker et al., 2009, 2011).

Fraser and Purves (1960) stated that the elaborate plexus investing the air sac system was "apparently entirely subservient to the proper functioning of the latter." Given the intimate association of the accessory sinus system with the aforementioned venous plexuses, this seems like a reasonable conclusion. We suggest that the large venous investment of the air sinuses may provide an alternate mode of nitrogen absorption or elimination during the course of a dive. Such gas kinetics may be beneficial during normal diving, but may pose a threat of DCS or venous gas embolism when diving conditions are artificially or inappropriately altered. During descent, the increasing pressure could drive nitrogen out of the sinus and into the surrounding venous plexus, as happens in the lungs. Admittedly, whether such diffusion across the venous wall is possible will likely depend on the thickness of the venous wall and the nitrogen partial pressure differential between the venous blood and sinus air.

Although Fraser and Purves (1960) conducted some histological examinations of the lining of these sinuses, we suggest that future studies conduct histomorphometry in order to facilitate evaluation of the likelihood of gas exchange across those surfaces. Since the venous plexuses are not composed of capillary beds but rather veins of variable size, diffusion across their walls at normal pressures is likely to be minimal or non-existent, but would instead require considerable pressure differentials to drive gas across. Modeling of such a function may provide insights in the absence of physiological data. Although a reasonable assumption may be that only as much nitrogen as is present in the sinus could ultimately be absorbed, the intranarial connection between the pulmonary and accessory sinus system should not be ignored, since decreasing gas volume in the sinuses could be supplemented by pulmonary air in order to enable equalization of the middle ears. Interestingly, if indeed nitrogen gas can be exchanged at the lining of the accessory sinuses, a reduction in pressure – as happens during ascent – could result in reversal of nitrogen flow from the blood to the sinuses, providing a non-pulmonary mechanism for elimination of nitrogen from the blood. During ascent, reducing pressure in the sinuses may be able to draw nitrogen out of the venous blood lining the sinuses. Nitrogen could then be cleared through the ET and out of the nasal passages. A recent analysis of the gas found in the pterygoid sinuses of stranded cetaceans showed that the sinuses have consistently high nitrogen gas levels (Bernaldo de Quirós et al., 2012). Additionally, elevated levels of CO_2 were also found, suggesting that diffusion of CO_2

across the sinus membranes should also be examined. Given the complexities of working with and interpreting results from post-mortem specimens, more research on this topic is needed, however it may provide support for our suggestions.

Another possible function of the accessory sinus vasculature that was postulated by Fraser and Purves (1960) may involve the redistribution of blood into the venous plexuses to accommodate the reduction of air volume during descent. As suggested by Fraser and Purves (1960), this could allow the lost volume of air to be replaced with blood to avoid dysbaric trauma to the sinus tissues as well as facilitating hydrostatic equilibrium of the ears which are surrounded by the sinus system. Such a pressure-related redistribution of blood to the sinus plexuses may help explain the common presence of foam in the accessory sinus system, conceivably created by venous transudate that could help generate and stabilize the foam. Although such a blood-redistribution function may seem plausible in delphinids that have rigid bony lateral pterygoid laminae (**Figure 2**), it may not explain the presence of an intricate plexus in deep diving odontocetes that have a flexible lateral wall that can likely deform medially to accommodate the reduction in air volume consequent to compression. There is little doubt that deep diving marine mammals have evolved mechanisms for limiting nitrogen gas absorption and mitigating or managing bubble formation, whether *in situ* or intravascular. Nonetheless, given the extreme nature of the diving life styles of some of the deep diving cetaceans, it is possible that they live at the limits of physiological tolerance, with small margins for error regarding gas management. Retention of these vascular plexuses despite the modified accessory sinus system anatomy may reflect their need for greater control over gas management.

FAT BODIES

The acoustic fat bodies of the lower jaw are interesting on many levels and have been a topic of considerable research and debate. At the forefront is their presumed function as analogs to the external pinnae of other mammals, receiving and channeling sound to the ears (Norris, 1969; Norris and Harvey, 1974; Cranford et al., 1996, 2008a,b). This suggested function naturally implicates these structures in any discussion concerning auditory impairment resulting from intense ensonification. Perhaps less obvious is the possible role of the acoustic jaw fats as a source of fat emboli or as a nitrogen sink (Kooyman, 1973; Jepson et al., 2005). Koopman (2007) suggested that deep diving odontocetes such as beaked whales and sperm whales may fill their blubber with lipids that provide some type of physiological or mechanical advantage to diving (e.g., nitrogen sink). Mammalian fats have traditionally been considered poorly vascularized structures, and given the general paucity of cetacean vascular information, this paradigm has understandably been propagated in the field of cetacean biology and discussions of vascular investment of fats (Houser et al., 2001; Fahlman et al., 2006, 2009; Hooker et al., 2009). In contrast, our recent vascular research has shown that some cetacean fatty tissues are very well vascularized. As noted by Fraser and Purves (1960), extra- and intra-mandibular jaw fats are proving to be extensively vascularized by veins (see **Figures 8**, **9** and **11**) and often well vascularized by arteries. Jepson et al. (2005) alluded to the possibility that damage to the tissue barrier between the acoustic lipid and the venous lumen could introduce fat into the circulation and lead to the formation of fat emboli. Such damage might result from physical trauma such as intense ensonification blunt or sharp force trauma, or from traumatic expansion of gas bubbles within nitrogen-saturated adipose tissue.

Two factors make the acoustic jaw fat interesting to us from this perspective. Firstly, fat is known to absorb nitrogen well and therefore can act as a sink for nitrogen that can expand once hydrostatic pressure is reduced (Lango et al., 1996). Secondly, the close anatomical association of these fat bodies and their venous plexuses to the gas-filled sinuses and extensive pterygoid vascular networks may place them at increased risk of receiving either (1) elevated nitrogen levels absorbed and accumulated through the sinus lining, or (2) gas emboli generated in the veins lining of the sinuses. Nitrogen absorbed through the sinus lining could travel to the fat bodies via the robust connections described, being absorbed by the fat body and expanding within the adipose tissue, or expanding within the IMFB veins and disrupting the vascular barrier between the blood and lipid. Any of the aforementioned situations could conceivably result in physical damage of the fat with subsequent release of lipid into circulation.

The high solubility of nitrogen in fat tissue is well-documented and results in high levels of nitrogen absorption in adipose tissue (Lango et al., 1996). Traditionally however it has been thought that nitrogen loading and unloading of fatty tissues is limited by the poor perfusion of the tissue (Fahlman et al., 2006, 2009). Since the acoustic jaw fats are indeed well vascularized, it is conceivable that they may exchange nitrogen at higher rates than are typically associated with fatty tissues. Interestingly, there appears to be an exceptionally disproportionate number of veins relative to arteries relative to most other tissues. Though no functional studies exist from which to draw any conclusions, we suggest that an elevated venous density would allow for faster nitrogen elimination and may provide a mechanism for rapid nitrogen clearance in a vital tissue that when damaged can significantly reduce an individual's chances of survival. We also cannot ignore the fact that the large volume and high surface area construction of the IMFB venous plexus may facilitate regional heterothermy of the intramandibular fats that the veins invest. Altering the temperature of the fat could affect the degree of solubility of nitrogen and therefore not only affect nitrogen loading and unloading but also influence the ease with which lipid can be mobilized and introduced into the vasculature, resulting in fat embolization.

Another possibility is that by modulating the volume of venous blood in the mandibular acoustic fats, cetaceans may be able to alter the density of the fats either due to increased blood density or temperature change, thereby effecting a change in acoustic properties of the fat. This could allow cetaceans to either fine tune their hearing to specific frequencies or adjust the density of the fat to maintain their hearing in altered ambient temperature environments, as may happen during a transition from warm to cold water. Such functions may be supported by the finding that false killer whales are able to actively control their hearing based on the received echo from a target they are echolocating on (Nachtigall and Supin, 2008). Finally, we cannot ignore the possible effect that regional mandibular heterothermy may have on modulating other sensory components. A large portion of the inferior alveolar nerve of the mandibular branch of the trigeminal nerve passes through the intramandibular plexus on its course to

the brain. If the intramandibular plexus is capable of producing temperature changes within the mandible, those changes may be able to modulate the sensory input from the nerve by affecting the amplitude, speed, and/or duration of the action potentials (Inman and Peruzzi, 1961; Ishiko and Loewenstein, 1961).

Numerous researchers have shown that temperatures of the blubber and extremities of cetaceans are often near ambient water temperatures, and since the mandible is a peripheral structure with high surface area it may frequently reach temperatures well-below normal mammalian core body temperature (Noren et al., 1999; Meagher et al., 2002, 2008; Barbieri et al., 2010). Temperatures below 20°C have been shown to cause sharp decreases in the amplitude of the action potential of rapidly adapting Pacinian corpuscles, while the action potential often disappears at temperatures below 15°C (Inman and Peruzzi, 1961; Ishiko and Loewenstein, 1961). Therefore, it may be possible for the mandibular plexuses to modulate the temperature of the surrounding tissues in order to overcome the adverse affects suboptimal temperatures may have on proper functioning of peripheral sensory nerves. Although this kind of temperature modulation of peripheral nerves might serve no apparent benefit in terrestrial mammals, the role of the inferior alveolar nerve is not understood in cetaceans and this implication should perhaps not be completely discounted given the conductive heat loss that occurs in water and the highly derived adaptations of cetaceans.

Interestingly, the melon fat appears to be drained by veins that are concentrated along the periphery of the fat body, with large veins avoiding a direct course through the main substance of the melon fat. Conversely, the IMFB is densely invested with veins. As we must assume that anatomical structures are not the result of evolutionary processes resulting in meaningless manifestations, we are compelled to believe that this distinct difference may be reflective of a functional role. It is possible that this reflects different functional needs to modulate physical characteristics of the fat bodies.

CLARIFICATIONS/INCONSISTENCIES

Interestingly, Fraser and Purves (1960) noted the presence of distinct pterygoid and maxillary veins, with the maxillary vein traveling lateral to the paroccipital process and tympanohyal, and merging with the IMFB plexus. They then noted that it merges with the deep temporal vein as it travels caudad. They made no mention of the maxillary vein's proximal connection to the external jugular vein as is common in domestic mammals, and noted that the pterygoid vein eventually joined with the mandibular and internal jugular veins. They described the pterygoid vein as running along the pharyngeal crest for a considerable length, eventually terminating as countless ramifications of the fibrovenous plexus associated with the accessory sinus system. We found this description to be confusing for a number of reasons. First, in most domestic mammals (except dogs) the pterygoid vein is a branch of the maxillary vein, which like the linguofacial vein is one of the terminal branches of the external not internal jugular vein. Indeed, in our delphinid specimens the intramandibular plexus and subsequent mandibular alveolar veins were seen consistently arising as a branch of the facial vein. Secondly, our findings in *Tursiops* suggest that the structure described by Fraser and Purves (1960) as the pterygoid vein is in fact consistent in location and course with the maxillary vein – traditionally considered the terminal branch of the external jugular vein – and the pterygoid venous plexus is instead formed by numerous small pterygoid veins that branch off of the maxillary vein and by the subdivision of the maxillary vein itself. Thirdly, since the internal jugular vein enters the jugular foramen, the only large veins we have observed traveling lateral to the tympanohyal cartilage and proximal stylohyal bone are the continuation of the external jugular vein that wraps around the tympanohyal cartilage, and the facial vein (#3, **Figure 7**) which is far removed from this location as it courses just deep to the blubber layer and subcutaneous fat. Our findings are consistent with the patterns observed by Ommanney (1932) in fin whales (*Balaenoptera physalus*) and by Walmsley (1938) who noted that the maxillary vein of the fetal fin whale is formed by veins accompanying the external carotid artery and tributaries from the pterygoid venous plexus. Additionally, the vein described by Fraser and Purves (1960) as the pterygoid vein formed in our specimens the terminal branch of the external jugular vein and passed dorsad through the infraorbital foramina, a pattern consistent with that of the maxillary vein of domestic mammals. Finally, we were not able to identify any substantial veins in the location identified by Fraser and Purves (1960) as the maxillary vein, unless they were referring to one of the veins of the external ophthalmic plexus (#7; rete vena ophthalmica externa of Slijper, 1936) or one of the many sizable veins of the pterygoid venous plexus.

The aforementioned differences could be due to the fact that the specimens used by Fraser and Purves (1960) were decapitated specimens that may have been missing the more proximal branches of the jugular veins. Nonetheless, the illustrations presented by Fraser and Purves (1960) show the vascular and skull morphology from the occipital condyles rostrad, including the internal jugular vein, however the external jugular vein has been completely omitted, as has the origin of the maxillary vein. It may be that the proximal most trunk labeled as the internal jugular vein is in fact the common jugular, from which the internal and external jugular veins branch. The ventral branch (external jugular) would then give off the maxillary vein as its primary branch. It is also possible that due to the use of decapitated specimens, Fraser and Purves (1960) considered the anastomotic branch of the internal jugular vein – seen in all of our specimens – as the main drainage path of the mandibular and maxillary veins, since they neither mention nor illustrate the external jugular vein.

Another source of confusion for us was the reference made by McFarland et al. (1979) to the *rete vena ophthalmica externa* first noted by Slijper (1936). McFarland et al. (1979) showed a venous vascular cast of a large extracranial retial structure adjacent to the cranium and connecting to the spinal veins via the first three intervertebral foramina. McFarland et al. state that this rete lies in the lower jaw and is "probably the *rete vena ophthalmica externa*" described by Slijper (1936). In none of our specimens did the intramandibular plexus connect directly to the epidural veins. Although difficult to discern from the photographic perspective provided, the positional references seem inappropriate and we therefore respectfully suggest that the vascular cast may be of the plexus investing the parieto-frontal region that in our specimens had anastomoses with the superficial cervical and occipital veins

and ultimately the epidural circulation. Our findings suggest that the rete pictured by McFarland et al. (1979) may in fact be composed of numerous structurally and regionally distinct retia that warrant separate nomenclature. Interestingly, despite the numerous fine caliber veins and plexuses that injected in our specimens, the only direct connections we observed between the spinal veins and the plexuses on ventral aspect of the skull were via the internal jugular emissary vein of the jugular foramen and the emissary vein of the foramen ovale. It is unclear at this time if this was an artifact of the injection medium, an individual variation, or a misidentification of structures; however it appears to be a significant difference. Given the degree of plasticity inherent in venous connections, it is possible that these connections manifest differently between individuals of a given species.

At the level of the medial aspect of the orbit, the lateral portion of the plexus associated with the Pty sinus fuses with dorsal offshoots of the intramandibular venous plexus and progresses dorsad to become the anterior lobe venous plexus. The largest portion of the anterior lobe venous plexus is located ventral and rostral to the eyestalk, and just ventral to the maxillary and premaxillary bones, in close association to the ventral lining of the preorbital and anterior lobes of the accessory sinus system. The anterior lobe venous plexus acts as a crossroads for numerous veins that converge on it. It has connections to intracranial blood via the ophthalmic plexuses that exit the orbital fissure, the melon via the facial vein and the infraorbital veins, and the ventral skull via the plexus of the accessory sinus system and maxillary vein. These collateral connections may be important for understanding compartmentalization of gases and observed distribution of gas and fat emboli.

Upon initial examination of the anatomy of the vasculature in the region of the Pty and peribullar sinuses, it becomes apparent that although there are homologies that may be drawn, the general pattern of venous investment seems relatively random. However, upon closer examination, it becomes evident that the locations and connections of the plexuses are relatively consistent between individuals, with only the localized branching patterns within the plexuses showing much variability. Nonetheless, it appears that in addition to those structures with likely homologies to generic mammalian structures, almost every available space between the jugular veins and distal head structures is filled with plexuses of anastomosing veins, forming what can only be considered a complex of countless collateral pathways. Although deciphering the exact anatomy of the venous structures themselves is important, the connections between those structures may be equally important from a functional perspective, as they form collateral pathways and alternate routes for transport of emboli and compartmentalization of gases.

The paired cavernous sinuses of domestic mammals form a ring-like venous structure around the pituitary gland (#16, **Figures 12** and **15**), just dorsal to the basisphenoid bone (Ghoshal et al., 1981). Rostrally the sinuses connect to the ophthalmic veins via the orbital fissure, while caudally they run confluent with the ventral petrosal and basilar sinuses. The ventral petrosal sinuses connect the caudolateral aspect of the cavernous sinuses to the ventral margin of the sigmoid sinuses (Evans, 1993). In *Tursiops*, these sinuses – located at the floor of the brain – connect the cerebral venous circulation to the plexuses of the accessory sinus system. Fraser and Purves (1960) noted that "the petrosal and cavernous sinuses of cetaceans are divested of the bony cranial protection found in terrestrial mammals, due to the displacement of the tympanoperiotic from participation in the wall of the cranium". We found this statement confusing since the cavernous sinus of most domestic mammals lies in a similar position – relative to the basicranial bones – as what we observed in dolphins (Ghoshal et al., 1981; Evans, 1993; Schaller, 2007), namely on the dorsal aspect of the pre- and basisphenoid bones. Fraser and Purves (1960) then stated that in cetaceans the cavernous tissue body or spongy mass of Beauregard is the homolog of the cavernous sinus of terrestrial mammals. This too seems problematic since the spongy mass of Beauregard was illustrated by Boenninghaus (1904) as surrounding the tympanic bulla of the ear (if it is the same as the structure labeled *corpus cavernosum*), ventral to the ventral petrosal sinus. This is an unusual location for the cavernous sinus.

We suggest that the cavernous sinus of the bottlenose dolphin is in fact in the expected intracranial location just rostral to the clivus, surrounding the *sela turcica* and pituitary gland, and that the spongy mass of Beauregard is in fact a novel cetacean structure that represents a complex of interconnected veins between the ventral petrosal sinus, peribullar venous plexus, and pterygoid venous plexus. Although our search was not exhaustive, we have found no homologous structure in domestic mammals. The cavernous sinuses of *Tursiops* are directly connected to the middle meningeal veins, the ophthalmic plexuses, the ventral petrosal sinuses, pterygoid plexuses, and the emissary veins of the foramina ovalia (emissary vein of foramen lacerum medium of Boenninghaus). The two lateral components are connected across the midline via structures much like the intercarvernous sinuses of domestic mammals. This venous loop is therefore consistent in location and drainage with the cavernous sinus of terrestrial mammals, and seems contradictory to the statements of Fraser and Purves (1960), as it is located well within the calvarium.

An additional source of confusion arising from the descriptions of Fraser and Purves (1960) was found in their statement that "under the hydrostatic pressures available, the *corpus* (*cavernosum*) could be erected by way of the internal carotid. . ." This appears to conflict with our findings as well as Boenninghaus' illustration that the *corpus cavernosum* is venous in nature, connected by ventral tributaries to the pterygoid and maxillary veins (#6, **Figures 15A–D**) and dorsal tributaries to the ventral petrosal sinus (#27, **Figure 15D**), rather than the carotid arterial system. Although an arterial component was observed, it appears modest and paled in comparison to the venous component. Additionally, the *corpus cavernosum* appeared grossly identical to the surrounding peribullar and pterygoid venous plexuses that connect directly to it. Therefore, it seems reasonable to assume that if the *corpus cavernosum* does indeed function as erectile tissue, this function can likely also be attributed to the venous plexuses surrounding it.

SYNOPSIS

Intriguing features of the veins in the head of the dolphin are the sheer volume and complexity they display. Indeed, the veins show similarly complex patterns throughout the dolphin body, often times occupying every available space within cavities and between tissues. In the head and neck, venous plexuses are seen investing the IMFBs and EMFBs, the cranial sinuses, ophthalmic

regions, nasal passages, tracheal mucosa, surrounding the esophagus, within the epidural spaces, and inside the brain case. It is possible that the venous system has simply formed a network of collateral drainage pathways throughout the body (Harrison and Tomlinson, 1956), that can facilitate adequate drainage of blood from the central nervous system during periods of elevated pressure (e.g., Valsalva phenomenon), in order to avoid damaging pressure-sensitive nervous tissue (Monro–Kellie doctrine). Alternatively, it may be that despite the plexiform similarity, different portions of the venous system serve different purposes, whether collateral drainage, regional heterothermy, gas exchange, or some other function. For instance, the venous plexus found within the tracheal mucosa has been hypothesized to act as either a compensating mechanism for the reducing air volume during diving-related compression or as erectile tissue that modifies the deformation properties of the tracheal wall (Cozzi et al., 2005). Similarly, Fraser and Purves (1960) suggested that the role of the pterygoid plexuses was tied to the proper functioning of the pterygoid sinuses.

The predominant piece of information missing from this anatomical picture is the lack of functional physiological data on blood flow and gas characteristics in the aforementioned structures. We strongly suggest that further studies of these structures may shed light on important physiological processes previously discounted or neglected. We believe that a considerable amount of information can be garnered from further post-mortem studies, however we feel that relatively non-invasive, ethical, live cetacean experiments on functional morphology can be conducted and may be the only way to clarify some of the large data gaps that currently exist.

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SUPPLEMENTARY MATERIAL

The Supplementary material for this article can be found online at: http://www.frontiersin.org/Aquatic_Physiology/10.3389/fphys.2012.00243/abstract

Movie S1 | Three-dimensional reconstruction of a bottlenose dolphin with a contrast-enhanced venous system, showing spatial relationships of the structures discussed. As the left lateral structures are clipped away, the medial aspect of the pterygoid region comes into view. Note that the colors that appear correspond to the same colors presented in Figure 1: pterygoid and peribullar venous plexus (green), anterior lobe venous plexus (purple), and IMFB plexus (yellow).

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Constraint lines and performance envelopes in behavioral physiology: the case of the aerobic dive limit

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Constraint lines—the boundaries that delimit point clouds in bivariate scattergrams—have been applied in macro-ecology to quantify the effects of limiting factors on response variables, but have not been applied to the behavioral performance and physiological ecology of individual vertebrates. I propose that behavioral scattergrams of air-breathing, diving vertebrates contain informative edges that convey insights into physiological constraints that shape the performance envelopes of divers. In the classic example of repeated cycles of apnea and eupnea in diving, air-breathing vertebrates, the need to balance oxygen consumption, and intake should differentially constrain recovery for dives within or exceeding the aerobic dive limit (ADL). However, the bulk of variance observed in recovery versus dive duration scattergrams originates from undetermined behavioral variables, and deviations from overall stasis may become increasingly apparent at progressively smaller scales of observation. As shown on dive records from 79 Galápagos fur seals, the selection of appropriate time scales of integration yields two distinct recovery boundaries for dive series within and beyond the estimated ADL. An analysis of the corresponding constraint lines is independent of central tendencies in data and avoids violating parametric assumptions for large data sets where variables of interest account for only a small portion of observed variance. I hypothesize that the intercept between these constraint lines represents the effective ADL, and present physiological and ecological considerations to support this hypothesis.

Keywords: aerobic dive limit, dive response, oxygen debt, constraint line, distribution boundary, performance envelope, edge effect, quantile regression

INTRODUCTION

Physiology investigates mechanisms for maintaining homeostasis over a range of temporal and spatial scales (Costa and Sinervo, 2004). The scale at which an organism and the surrounding environment are sampled is particularly important: within the scope of behavioral and physiological plasticity, deviations from stasis may increasingly occur at progressively smaller spatial and temporal scales. To reduce the impact of variation secondary to plasticity, physiological experiments in the laboratory often attempt to create steady state conditions to reduce residual variation and to analyze constraints and limiting factors (Costa and Sinervo, 2004). However, physiological data collected in the field rarely reflect steady state conditions and significant deviations from homeostasis are readily observed. A classic example is the repeated cycle of apnea and eupnea in diving, air-breathing vertebrates. On a larger scale, oxygen consumption and intake need to be balanced. On a small scale, significant deviations occur during every dive as animals deplete stored oxygen to differing degrees and may even increase oxygen debt by temporarily switching to less efficient anaerobic metabolism (Kooyman et al., 1981; Kooyman, 1989). This subsequently requires additional oxygen to remove metabolites that would otherwise threaten homeostasis. The need to balance the use of oxygen stored and used in different tissue may be further modulated through the temporal partitioning of some activities that are obligate only on a longer scale, such as digestion, detoxification, mobilization of stored energy, or even the replenishment of oxygen stores (Davis et al., 1983; Kooyman, 1989; Ponganis et al., 1993).

THE AEROBIC DIVE LIMIT

The aerobic dive limit (ADL) was originally defined by G. L. Kooyman as the dive duration associated with the onset of blood lactate accumulation, presumably resulting from anaerobic energy production following oxygen depletion in some tissue (Kooyman et al., 1980). Available oxygen stores and consumption rates are key determinants of the onset of lactate accumulation, in conjunction with multiple fine scale regulatory adjustments commonly referred to as the "dive response" (Kooyman et al., 1981; Butler and Jones, 1997; Kooyman and Ponganis, 1998; Davis and Kanatous, 1999). As a result of the central and integrative positioning within energy metabolism, the ADL has been proposed as a useful concept in comparative diving physiology in a number of variants that include calculated and behavioral threshold estimates if empirical lactate measurements are impossible (Kooyman, 1989; Kooyman and Ponganis, 1998; Butler, 2006; Ponganis et al., 2010). The ADL may be considered an intrinsic trait subject to natural selection (Butler and Jones, 1997; Davis et al., 2004), and has been used for interspecific comparisons (Kooyman, 1989; Boyd and Croxall, 1996; Kooyman and Ponganis, 1998) and intraspecific studies

on ecology, ontogeny and aging (Kooyman et al., 1983; Gentry and Kooyman, 1986; Thorson and Le Boeuf, 1994; Burns and Castellini, 1996; Horning and Trillmich, 1997a; Ponganis et al., 1997; Kooyman and Ponganis, 1998; Watanuki and Burger, 1999; Pitcher et al., 2005; Richmond et al., 2006; Weise and Costa, 2007; Cook et al., 2008; Hindle and Horning, 2010; Hindle et al., 2011; Shero et al., 2012). These studies suggest that some species and age classes remain predominantly aerobic for most dives, while others—birds in particular—frequently dive well in excess of the estimated ADL. However, the extent to which the ADL represents an actual threshold that constrains foraging behavior, energy acquisition, and ultimately individual fitness remains unclear (Davis et al., 2004; Ponganis et al., 2010, 2011).

To understand the limiting effects of intrinsic traits such as the ADL on individual behavior and ultimately fitness, we may analyze variance in behavioral responses such as dive ratios (dive duration in relation to post-dive recovery time) with respect to extrinsic factors such as prey depth or distribution (Gilmour et al., 2005). The prediction of an increase in post-dive recovery times (response variable) with increasing dive duration (predictor) is directly derived from the need to maintain long-term oxygen balance. For dives exceeding the ADL a disproportionate increase in such recovery times is predicted to accommodate less efficient (partially) anaerobic metabolism. Analyses of dive records for most species consistently yield highly significant positive correlations between dive durations and recovery times, largely as a result of the application of conventional measures of central tendencies to very large data sets (>>1000 dives, i.e., **Figure 1**). Such analyses account for little of the observed variation and violate parametric assumptions of homogeneity of variance. The few extant attempts at deriving the ADL from recovery time inflection estimates applied qualitative visual determination (Cook et al., 2008) subject to much interpretation. For example, applying a "bottom contour plot" visual approach to the data set shown in **Figure 1** yields four inflection points at 1.92, 2.75, 3.5 and 3.67 min. However, these inflection points may be defined by single dives for which recovery times may be uncoupled from any immediate need to process any accumulated lactate if that is used as substrate in subsequent dives or accumulated over several dives. Furthermore, extremely large variance in recovery times for given dive durations tend to blur any effects and yield broad uncertainties in ADL estimates (i.e., Walton et al., 1998; Cook et al., 2008).

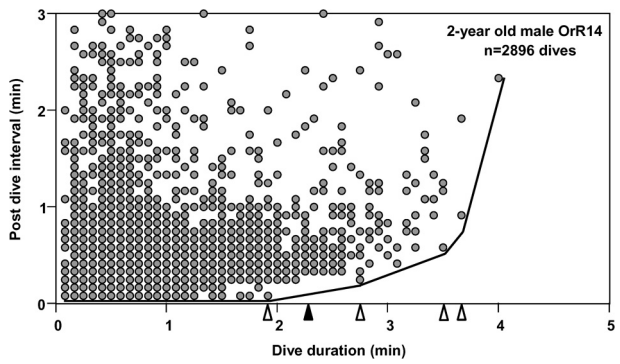

FIGURE 1 | Post-dive recovery intervals are plotted as a function of dive duration, for 2-year old male Galápagos fur seal OrR14. Data pairs from 2896 dives recorded during 18 deployment days are depicted. Due to sampling at a discrete rate of 5 s, many data points are plotted on top of each other. Open triangles identify four possible inflection points in a bottom contour line (see text). The solid black triangle identifies the dive duration associated with the Surface Time Inflection point (see text).

This lack of a consistently quantifiable behavioral manifestation of the ADL may be a result of several phenomena: (1) Individuals could choose to carry or accrue oxygen debts over several dives, only to pay off incurred debts after several dives. This would mean that any increase in recovery times resulting from lactate production could occur at a later time, not immediately following a dive exceeding the ADL. If accrual and payoff are flexible, our ability to analyze their association may be compromised. (2) The oxygen balance constraint is unidirectional in imposing minimum recovery times, while extended recovery times may be less constrained, for example by forces acting on foraging trip efficiency or individual fitness on much longer temporal scales, if at all. In this case, extended recovery times may appear "random" and could be influenced by variables not monitored by classic experimental designs, resulting in insufficient explanation of residual variation.

CONSTRAINT LINES

Constraint lines are best described as the boundaries that delimit point clouds in bivariate scattergrams. In a seminal paper, Thomson et al. (1996) highlighted that fundamental processes often constrain patterns of statistical variation. In the editor's note Carol Augspurger added that "Bivariate scattergrams convey ecological information not only when they show linear or curvilinear relationships, but also when they fall into diffuse clouds, as long as the clouds have informative edges." These informative edges are called constraint lines, under the assumption that they represent the effect of limiting factors on a response variable with a lesser degree of unexplained residual variation than in the core of a diffuse cloud. Constraint lines have been applied in macroecology to describe and quantify the effects of limiting factors on response variables (Thomson et al., 1996; Guo et al., 1998; Kelt and Van Vuren, 2001). More recently, Anderson and Jetz (2005) used multiple constraint lines to characterize the field metabolic rate of endotherms within a broad latitudinal cline of environmental conditions. Their "metabolic constraint space" combined intrinsic and extrinsic factors to link the behavior and physiology of individuals to ecology and biogeography at a global scale. However, constraint lines have not been used to analyze intrinsic traits and the performance of individual animals. Here, I propose to use constraint lines—a technique that has gained increasing attention in the ecological community—to characterize physiological limits on performance, and specifically the ADL of diving, air-breathing vertebrates.

MATERIALS AND METHODS

I examined existing data on dive behavior from Galápagos fur seals (*Arctocephalus galápagoensis*) obtained in 1990 and 1991 at the Cabo Hammond rookery on Fernandina Island,

Galápagos, Ecuador, as previously described (Horning and Trillmich, 1997a). The original research was conducted under a research permit issued by the Galápagos National Park Service, and followed the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, 1996). In brief, Wildlife Computers Mk4 Timed-Depth Recorders (0–250 m × 1 m resolution, sampling at 5 s—except for two records sampled at 10 s) were deployed on 96 fur seals ranging in age from 6 months to adults. 194,361 dives were recorded during 1199 observation days. Mean deployment duration was 12.5 days (range 3–25 d). Depth records were processed as previously described (Horning and Trillmich, 1997a) to provide baseline-drift corrected sequential listings of dive durations and post-dive surface times.

DIVE DATA ANALYSIS

To consider a hypothetical disproportionate increase in recovery times for dives exceeding the ADL, I first plotted post-dive recovery times against all dive durations for each individual, as shown in the example of **Figure 1**. While some increase in recovery times for longer dive durations was observed, no simple and obvious determination of the ADL was possible for any of the records. To compensate for a possible delayed payoff of an oxygen debt incurred during a (partially) anaerobic dive, I estimated the number of dives exceeding the ADL after which an accumulated, incremental oxygen debt would have to be dealt with for adult females as illustrated in **Figure 2A**. In a first step, I estimated the likely contribution to oxygen debt accrual by only those dives that exceed the calculated ADL. Gentry et al. calculated the ADL of a hypothetical otariid from oxygen stores and utilization rates by pooling available data from multiple otariid species. For a body mass range of 30–100 kg, their estimates were best described by the equation $\text{ADL} = 1.57 \times \text{body mass}^{0.22}$ (Gentry et al., 1986). The mean body mass of all 32 adult fur seal females in the data set was 28.8 kg (± 3.1 SD).

Using the above equation from Gentry et al., I estimated an ADL of 3.29 min for a 29 kg adult Galápagos fur seal female. The median duration of only those dives exceeding this estimated ADL was 3.44 min.

Thus, most dives by adult females in this study that exceeded the calculated ADL did so by only 0.15 min on average, or about 4.5%. This short apneustic duration in excess of the calculated ADL could be called the median anaerobic increment. Given that such a short anaerobic increment increases the oxygen debt only by a small amount, it is possible that such debt is accrued and payoff (in the form of increased recovery times) is deferred. Next, I estimated the possible number of typical anaerobic increments in a series of dives after which payoff can no longer be deferred. The maximum dive duration observed in each of these animals represents a rare performance extreme and as such an overall physiological limit which apparently cannot easily be exceeded. The average maximum dive duration of all 32 adult females in the study was 4.47 min, suggesting that the maximum anaerobic increment is 1.18 min (4.47–3.29). Under this assumption, after eight typical dives exceeding the estimated ADL by the median anaerobic increment, the maximum anaerobic increment is reached and payoff can no longer be deferred (**Figure 2A**). Thus, after eight of the most common, presumably partially anaerobic dives an increase in recovery times can no longer be avoided. To relate recovery times to dive durations under this scenario of multiple shorter anaerobic increments with payoff deferred over up to eight dives, the combined recovery times for eight sequential dives has to be related to the combined duration of eight sequential dives. To that extent, I calculated the moving-sum of all possible sequential combinations of eight successive Post-Dive-Intervals (PDI) and the corresponding sum of eight successive dives for all dives recorded for a particular animal, as illustrated in **Figure 2B**. The scatterplots resulting from the moving-sum transformation show all possible pairings of dive durations with recovery times for series of eight dives, including those with shortest observed recoveries. In the resulting moving-sum plots, linear increases in minimum post-dive recovery times for sets of eight sequential dives were observable as lower distribution boundaries in many of the dive records, as shown in **Figure 3A**. Beyond a distinct surface time inflection point (STI), minimum observed post dive surface recovery times increased at a disproportionate rate, as characterized by a second boundary of steeper slope. If larger or smaller integration values are used for the moving sum, the inflection point becomes progressively less pronounced.

DETERMINATION OF SURFACE TIME INFLECTION POINTS

I used quantile regression (Cade et al., 1999) to separately quantify the lower boundaries for all combinations of two split segments (Yaeger and Ultsch, 1989; Granato, 2006) of all moving-sum data pairs for individual dive records, programmed in Turbo-Pascal (Borland). Since the original dive duration versus PDI data and the corresponding moving-sum data were strongly heteroscedastic (i.e., **Figures 1** and **3A**), I used the non-parametric Kendall-Theil robust line estimator (Sen, 1968; Granato, 2006) to calculate the τ-th regression quantiles for heterogeneous distributions where the τ-th proportion of data located below the regression line is weighted by 1-τ, and the 1-τ th proportion located above the regression line is weighted by τ (Cade et al., 1999). Calculations were simplified by using only cross-median paired slopes to determine the Kendall-Theil estimator (De Muth, 2006). Since individual dive records covered a broad range of dives from 511 to 6727 dives and were typically heavily skewed toward shorter duration dives and PDIs, I progressively decreased τ from an initial value of 0.5 in steps of 0.01 until the τ-th proportion contained between 5 and 20 samples, and while successive slopes or elevations differed significantly. I determined the significance of slope differences via a rank-sum test of slopes and elevations used to calculate the Kendall-Theil medians for $\alpha = 0.05$. I performed this procedure iteratively for all combinations of split segments for values from 25 to 95% of the abscissal range (see Yaeger and Ultsch, 1989) and calculated the STI point as the intersection between the two quantile regression lines that yielded the lowest combined root mean square error (Granato, 2006), as illustrated in **Figure 3B**.

Since a split, two-segmented linear model always provides an equal or better fit (equal or lower combined root mean square error) than a single fit (Yaeger and Ultsch, 1989), I established two criteria to qualify an STI as reflecting a "disproportionate increase" in minimal post-dive recovery times: (1) the slope of

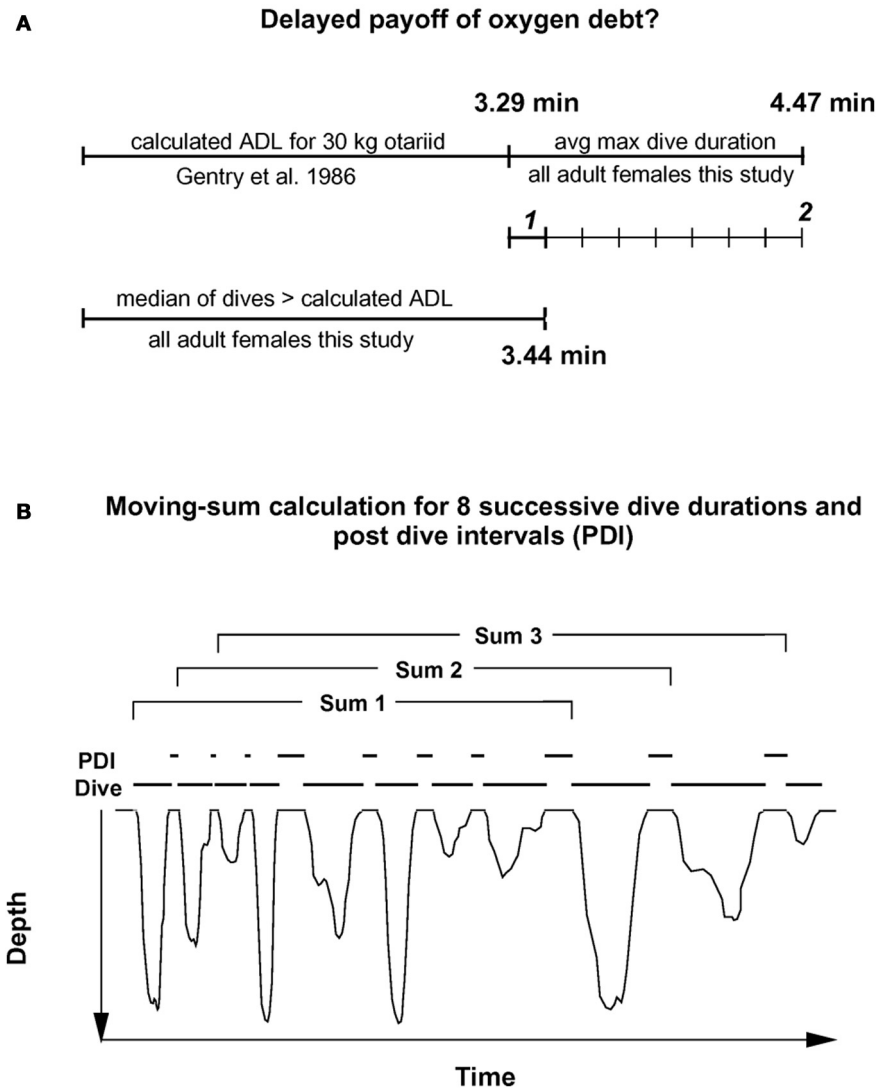

FIGURE 2 | Moving sum compensation of oxygen debt accrual. **(A)** Estimating the number of dives after which the maximum anaerobic increment (see text) has been accrued. ***1*** represents the median anaerobic increment by a typical dive exceeding the calculated ADL. ***2*** represents the maximum anaerobic increment based on the difference between calculated ADL, and mean maximum dive duration. **(B)** The moving sum calculation. Dive durations for eight sequential dives are summed, and the corresponding eight post-dive recovery intervals (PDI) are summed (see text for details). For each successive sum, one new dive—PDI pair is added, and the oldest pair is dropped. This moving sum is performed for all dives recorded for a particular animal. Resulting plots are shown in **Figures 3**, **4**, and **5**.

the fit to the right of the STI has to be significantly greater than that to the left; (2) the STI has to meet a continuity restriction: the abscissa of the intercept has to fall within 10% of the split between segments. Dividing the STI value by eight yielded equivalent durations for single dives, beyond which minimal recovery times increased at a disproportionate rate.

RESULTS

Of the 96 depth records examined, I excluded two records from subsequent analyses because of differing sampling rates (10″ versus 5″), and 12 records from ages <220 days since animals in this age class play in the water without diving and do not yet forage independently (Horning and Trillmich, 1997a). Of the remaining 82 records, three contained fewer than 400 dives (60, 167, and 371 dives respectively) and exhibited no apparent edges to the diffuse point clouds. 79 records allowed the computation of segmented regression quantiles. 51 of these records yielded two constraint lines with distinct STIs (i.e., **Figure 4**). Twenty-eight records violated previously set criteria of continuity (i.e., **Figure 5A**) or slope differences (i.e., **Figures 5B,C**). Records with slope violations exhibited a single lower constraint line with few or no points beyond the upper line limit ($n = 24$). These animals likely did not dive for durations beyond the STI. Records with a continuity violation exhibited few points or diffuse point clouds beyond the upper limit of the lower constraint line ($n = 4$). These animals did perform some dives beyond the STI, but were apparently

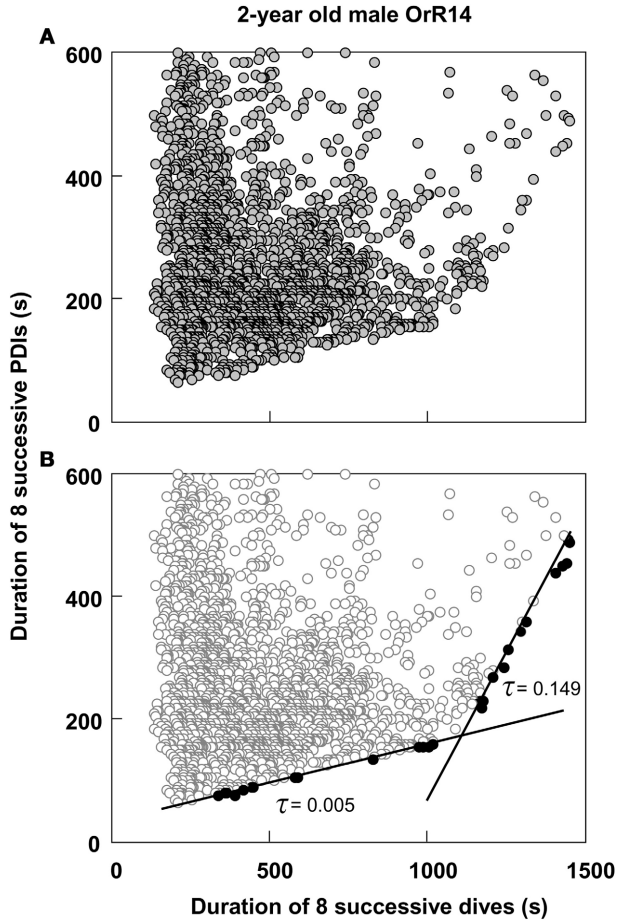

FIGURE 3 | Moving sum plot for 2-year old male Galápagos fur seal OrR14. (A) The plot resulting from the moving sum calculation for eight successive dive—PDI pairs as explained in **Figure 2B**, for 2-year old male fur seal OrR14. **(B)** The best split Kendall-Theil quantile regressions yielding $n \leq 20$ for the τ-th proportion (solid circles, $n = 13$ for each regression) with the lowest combined root mean square errors (see "Materials and Methods"). Dividing the abscissal value of the regression intersect by eight yields the equivalent duration for a single dive. The intersect at 1100 s corresponds to a single dive duration of 137.5 s or 2.29 min. This value is shown as a solid black triangle in **Figure 1**.

spending more time at the surface than minimally necessary for such dives. For these records, I used the largest value on the abscissa for the lower regression (continuity violation) or for a single, combined regression (slope difference violation) to characterize the maximum observed dive duration *not* resulting in increased PDI.

DISCUSSION

The moving sum calculation uncouples the distribution boundaries from small scale (dive-by-dive) deviations from homeostasis enabled by the plasticity of the dive response. The resulting constraint lines yield consistent intercepts that are defined by a much larger number of dives than, and distinct from visually placed inflections in "bottom contour plots" (see **Figure 1**). The efficacy of the moving-sum plots in visualizing distinct and disproportionate increases in minimum observed recovery times for sets of dives does suggest that (1) Galápagos fur seals do at times exceed their ADL, (2) apparently do so by comparably small amounts on individual dives, and (3) may incrementally accrue an oxygen debt which is "paid off" when a threshold is reached after a series of contributing dives. The linear increase in minimum recovery times for dives sets < the STI (**Figure 4**) is contrary to earlier hypotheses on "optimal breathing" that suggest an asymptotic decrease in oxygen transfer rates with increasing PDI (Kramer, 1988). However, estimates of blood flow and PO_2 measurements on diving seals (see Ponganis et al., 2011) and models of oxygen transport between tissues (Davis and Kanatous, 1999) suggest such a decrease as highly unlikely for surface intervals typically much shorter than 30 s for Galápagos fur seals.

Constraint-related data collected under steady-state, controlled laboratory conditions can often be adequately described by measures of central tendencies, but field data may rarely be, since the introduction of other factors that are less-, or not at all limiting tend to blur the point cloud in a skewed direction. Quantile regression appears a usable technique for the characterization of the lower boundary line resulting from the moving-sum transformation, and it avoids violating parametric assumptions that are challenging for large data sets typical in diving animal telemetry. This approach is particularly applicable under the a priori assumption of a functional, mechanistic relationship unilaterally constraining a specific response variable irrespective of its central tendencies influenced by many other variables that are rarely recorded, even if the predictor contributes only a small amount to the overall variance observed. As we move from the center of a distribution toward a constrained boundary, the contribution to variance shifts from unmeasured factors toward the measured predictor (Cade and Noon, 2003).

Despite the postulated mechanistic relationship, my interpretation that the disproportionate increase in minimum surface times for dives exceeding the STI is driven by at least partially anaerobic metabolism, and thus is a manifestation of the ADL, has to be considered a hypothesis that remains to be tested. In the absence of empirical data on PO_2 or blood lactate, we can presently only consider the physiological and ecological context to assess the validity of this hypothesis. As expected for the ADL, the STI does indeed exhibit a strong positive relationship to body mass (**Figure 6**). This relationship is best described by the equation STI (min) = a + b × ln [body mass(kg)] where $a = -2.76$, $b = 1.77$. Mean values (±S.D.) for age classes are: adult females 3.16 min (±0.37, $n = 14$), 1.5–2 years 1.98 min (±0.27, $n = 23$), yearlings 1.28 min (±0.23, $n = 14$). For 14 adult females the mean STI is only slightly below the calculated ADL of 3.28 min (calculated after Gentry et al., 1986 from the power equation ADL = 1.57 × body mass$^{0.22}$). For younger animals with a lower body mass the STI falls increasingly below the calculated ADL (**Figure 6**). We previously showed that select parameters (blood hemoglobin concentration and hematocrit) related to oxygen storage and transport capacity of young fur seals reach levels of adult females by the age of one year (Horning and Trillmich, 1997b). However, muscle parameters such as muscle mass and myoglobin concentrations were not assessed and may mature at slower rates. Furthermore, Gentry and collaborators derived

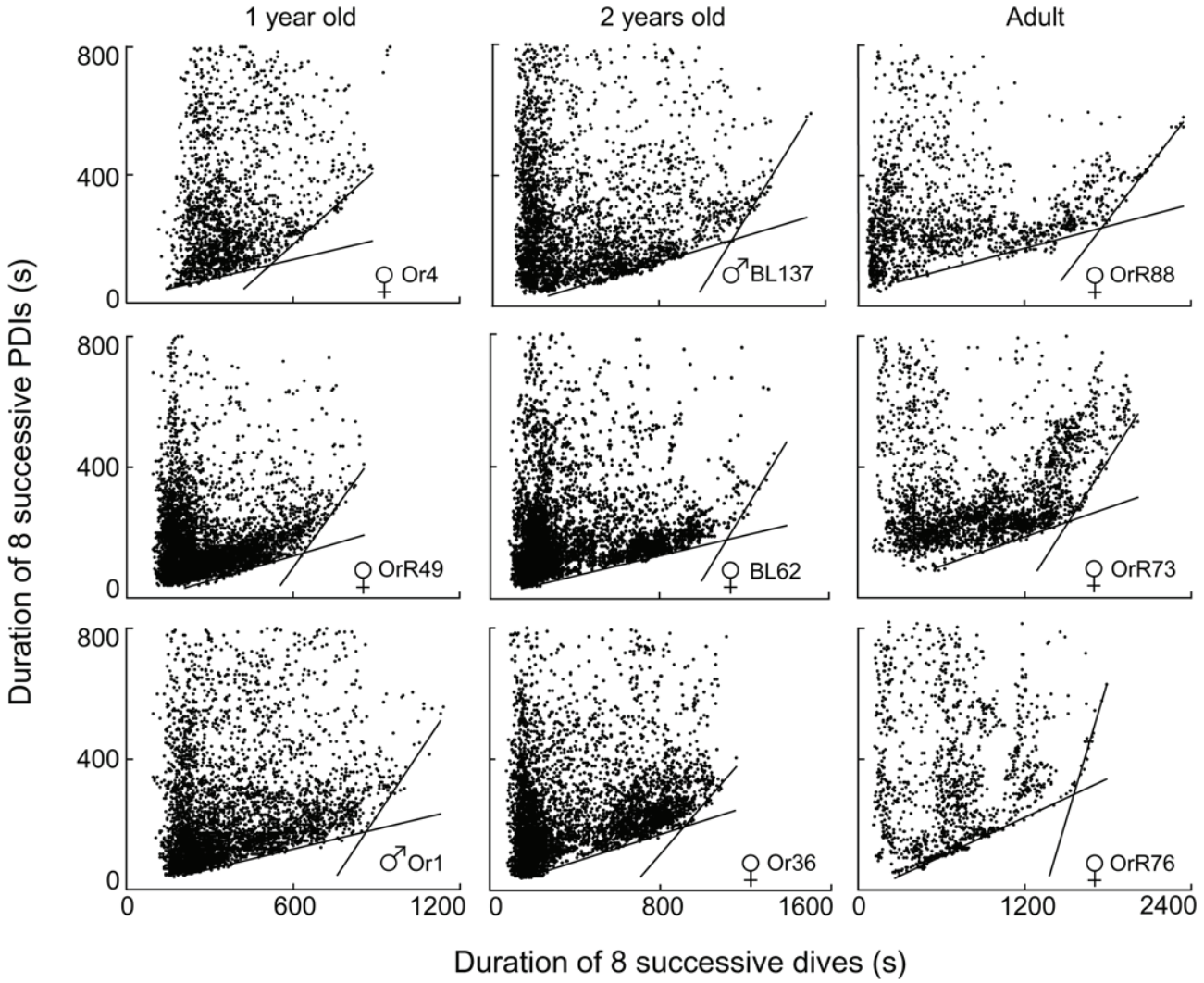

FIGURE 4 | Best split τ-th regression quantiles for nine different Galápagos fur seals of three different age classes. Dividing the abscissal values of the intersection of the two regression lines by eight yields the equivalent durations for single dives.

their equation describing the calculated ADL of a hypothetical otariid from parameter estimates combined from adults of multiple species (Gentry et al., 1986). Maximum and median dive performances (dive depths and durations, swim speeds, vertical travel distances per trip to sea) of younger fur seals ages 1–2 years range from 50 to 75% of values reached by adult females (Horning and Trillmich, 1997a), matching the pattern shown in **Figure 6**. The notion that juveniles are more constrained in their independent diving and foraging ability than adult females is further supported by their greater mass loss during lunar periods of reduced foraging efficiency resulting from periodically reduced prey accessibility (Horning and Trillmich, 1999). The percentage of dives exceeding the STI is only slightly and insignificantly higher for all juveniles combined (10.1%, ±5.7 S.D., $n = 37$) than adult females (7.99%, ±6.08 S.D., $n = 14$), but for yearlings (14.1%, ±5.4, $n = 14$) the difference to adult females is significant (Student's t, $p < 0.012$) (**Figure 7**). This likely explains the greater proportion of inflection points successfully determined in juvenile records (37 in 47) than adult females (14 in 32).

Thus, it appears that the performance envelope of the Galápagos fur seal is shaped by two constraint lines on either side of the STI. From the ADL interpretation of the STI, we can derive the expectation that *the proximity of recovery times to the "aerobic" boundary* for dives shorter than the STI should be influenced more by the intrinsic need to optimize foraging efficiency—for example when resources are scarce—rather than by limits imposed by physiological maturation, age or mass. This leads to the prediction that the Kendall-Theil Median Residual (KTMR) of the aerobic portion of the quantile regression should be lower during the equatorial warm season (February–April) when cold-water upwelling is suppressed, reducing nutrient influx and primary productivity (Barber and Chavez, 1983; Kogelschatz et al., 1985, and see Horning and Trillmich, 1997a) than during the more productive cold season (June–November). Indeed KTMR

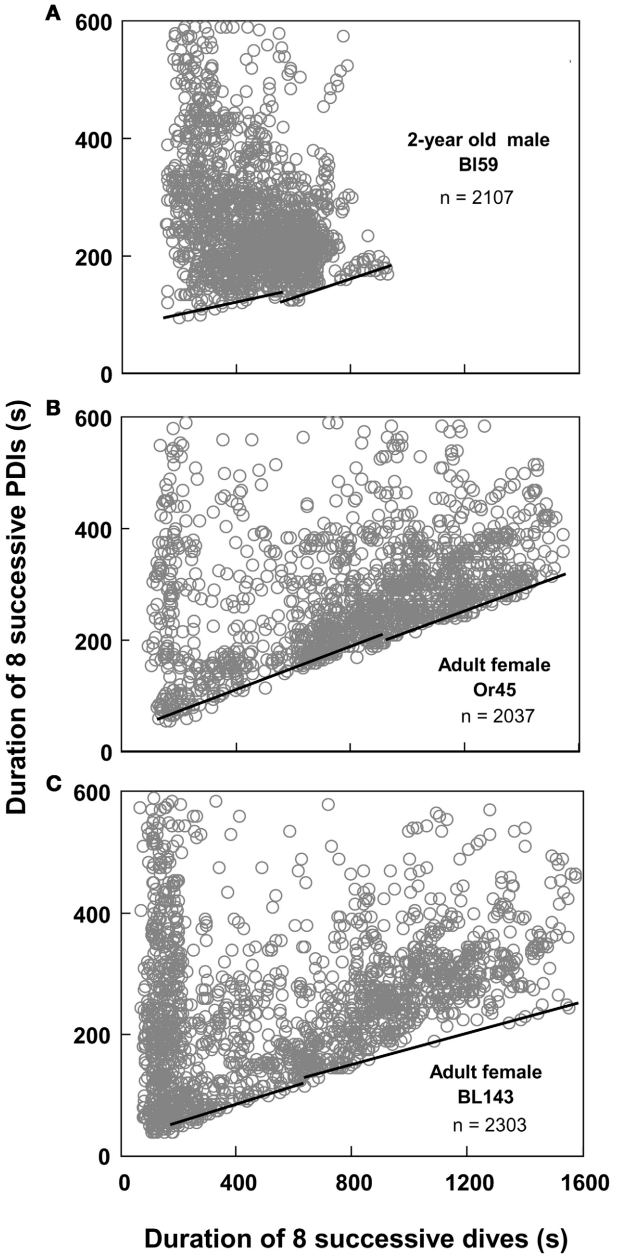

FIGURE 5 | Continuity and slope violations. (A) Best split τ-th regression quantiles with a continuity violation: the abscissal value of the intersection between the two regression lines does not fall within 10% of the split. **(B,C)** Best split τ-th regression quantiles with a non-significant slope difference. Dividing the abscissal values of the regression intersects by eight yields the equivalent durations for single dives.

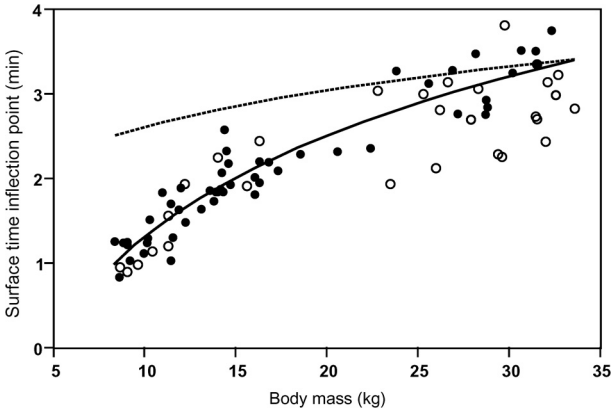

FIGURE 6 | The Surface Time Inflection point (STI) as a function of body mass. The Surface Time Inflection points computed for single-dive durations from the intersections of best split quantile regressions are plotted against body mass (solid circles). The points are best described by the equation (solid line): $STI = -2.76 + 1.77 \times \ln(\text{body mass})$. The ADL for a hypothetical otariid calculated from body mass (calculated after Gentry et al., 1986 from the power equation $ADL = 1.57 \times \text{body mass}^{0.22}$) is shown (dashed line). Maximum observed dive durations ≤ STI for records with slope or continuity violations (see text and **Figure 5**) are shown as open circles.

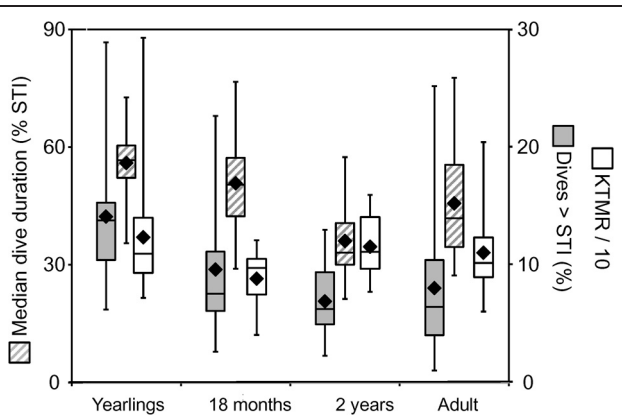

FIGURE 7 | Constraint metrics for juvenile Galápagos fur seals. Three constraint metrics derived from the STI (see text) are shown for three juvenile age classes and adult female Galápagos fur seals. Yearlings and 2-year-old animals were observed during the cold season, 18-month-old juveniles were observed during the resource limited warm season, adult females during both seasons. Solid black rhombes are means, center box lines are medians, box limits and whiskers are inner and outer quartiles, respectively. The median dive duration of individuals is shown in percent of the STI (left y-axis). The percent of dives exceeding the STI and the Kendall-Theil Median Residual (KTMR, see text) are shown on the right y-axis.

values dropped from a mean of 143.9 (±104.5 S.D.) for 20 yearlings observed during the cold season to a mean of 87.8 (±24.9 S.D.) for ten 18-month-old animals observed during the warm season (the difference was not significant with Student's $p = 0.117$) and increased again to 114.7 (±26.3 S.D.) for seventeen 2-year-old animals observed during the cold season (this second difference was significant with Student's $p = 0.0186$) (**Figure 7**). For adult females the effect was reversed, with mean KTMR values of 100.6 (±28.4) for 20 females observed during the cold season, and 124.6 (±32.5) for 12 females observed during the warm season ($p = 0.042$). However, this is likely driven by a much greater energetic demand on lactating females during the peak of the (cold) breeding season, when they are more likely to have a

dependent pup unable to feed itself, possibly in addition to an older still dependent yearling.

From the ADL interpretation of the STI we can also derive the notion that *the proximity of typical dive durations to the ADL* should be indicative of the extent to which individuals exploit the full extent of behavioral plasticity as constrained by physiological maturation and body mass. This leads to the specific prediction that the median dive duration expressed as a percentage of the STI should be highest for yearlings, and decline for older juveniles following weaning, followed by an increase for adult females. Indeed, this value declines from a mean of 55.9 ($\pm$10.1) for fourteen yearlings past 50.8 ($\pm$14.2) for seven 18-month-olds (n.s. $p = 0.37$) to 36.1 ($\pm$9.6) for nineteen 2-year old animals ($p = 0.012$) (**Figure 7**), and then increases again to 45.66 ($\pm$14.8) for fourteen adult females ($p = 0.0495$).

The variance observed in these three measures of individual constraint (KTMR, the median dive duration expressed in percent of STI, and the percent of dives exceeding the STI) in the Galápagos fur seals is fully consistent with our prior findings of constraints across a range of temporal and spatial scales (Horning and Trillmich, 1999). Adult females appear most challenged to optimize diving behavior during the (cold) reproductive season, whereas juvenile fur seals appear most challenged during the warm, resource limited season, and after weaning. As expected, younger juveniles are more constrained and need to push their limits to a greater extent than older juveniles. Adult females however appear to be additionally constrained by the energetic demands of lactation and are diving closer to their physiological limits than 2-year old juveniles.

Irrespective of an empirical validation of the STI = ADL hypothesis, I propose that these three measures of the proximity of central tendencies to the constraint boundaries in the dive record scatterplots may be useful metrics to quantify the need to optimize foraging behavior, and how close to the limits of their plasticity diving animals may operate at several temporal scales.

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The effects of experimentally induced hyperthyroidism on the diving physiology of harbor seals (*Phoca vitulina*)

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Many phocid seals are expert divers that remain submerged longer than expected based on estimates of oxygen storage and utilization. This discrepancy is most likely due to an overestimation of diving metabolic rate. During diving, a selective redistribution of blood flow occurs, which may result in reduced metabolism in the hypoperfused tissues and a possible decline in whole-body metabolism to below the resting level (hypometabolism). Thyroid hormones are crucial in regulation of energy metabolism in vertebrates and therefore their control might be an important part of achieving a hypometabolic state during diving. To investigate the effect of thyroid hormones on diving physiology of phocid seals, we measured oxygen consumption, heart rate, and post-dive lactate concentrations in five harbor seals (*Phoca vitulina*) conducting 5 min dives on command, in both euthyroid and experimentally induced hyperthyroid states. Oxygen consumption during diving was significantly reduced (by 25%) in both euthyroid and hyperthyroid states, confirming that metabolic rate during diving falls below resting levels. Hyperthyroidism increased oxygen consumption (by 7–8%) when resting in water and during diving, compared with the euthyroid state, illustrating the marked effect of thyroid hormones on metabolic rate. Consequently, post-dive lactate concentrations were significantly increased in the hyperthyroid state, suggesting that the greater oxygen consumption rates forced seals to make increased use of anaerobic metabolic pathways. During diving, hyperthyroid seals also exhibited a more profound decline in heart rate than seals in the euthyroid state, indicating that these seals were pushed toward their aerobic limit and required a more pronounced cardiovascular response. Our results demonstrate the powerful role of thyroid hormones in metabolic regulation and support the hypothesis that thyroid hormones play a role in modulating the at-sea metabolism of phocid seals.

Keywords: thyroid hormones, metabolism, hypometabolism, diving, hyperthyroidism, harbor seal, heart rate, lactate

INTRODUCTION

Diving mammals spend extended periods underwater, periodically returning to the surface to breathe. Under water, adjustments to their metabolic rate must occur in order to manage a limited supply of oxygen, while the accumulation of carbon dioxide and possibly lactate has to be tolerated. In the 1930's and 1940's Scholander and Irving described the most striking physiological alterations in avian and mammalian divers during forced submergence experiments: apnea, a profound slowing of heart rate (bradycardia), peripheral vasoconstriction, and accumulation of lactic acid (Irving, 1939; Scholander, 1940). Scholander referred to these responses as the "master switch of life," because they were found not only in divers, but also in most vertebrates faced with asphyxia. These metabolic adjustments allowed for the reduction in whole-body aerobic metabolism and served as a mechanism to conserve the limited oxygen for hypoxia-sensitive tissues, such as the heart and brain (Scholander, 1963). To this day, these observations are accepted as the main components of the so-called diving response.

In the last few decades, however, technological development has enabled us to record behavioral and physiological variables in many species when voluntarily diving in their natural environment. From these studies, it has become apparent that the physiological responses during natural diving are much less pronounced than during forced submergence (for review see: Kooyman, 1985; Butler and Jones, 1997). In fact, most observations of freely diving animals indicate that the majority of dives are well within their aerobic dive limit (ADL), defined as the longest possible dive duration without an increase in blood lactate concentration (Kooyman, 1985). Hence, during routine diving, most divers might not require the extreme cardiovascular responses observed in forced submergence experiments. Some species, however (e.g., northern and southern elephant seals, *Mirounga angustirostris* and *Mirounga leonina*) remain submerged for periods longer than would be expected based on the estimates of available oxygen stores and metabolic rate (Le Boeuf et al., 1988; Hindell et al., 1992). A reduction in metabolism during diving to a level below resting ("hypometabolism"), or the

partial reliance on anaerobic metabolism, or a combination of both have been proposed as physiological mechanisms to extend voluntary dive duration (Castellini, 1985; Fedak and Thompson, 1993). The latter authors investigated the behavioral and physiological consequences for a seal when using anaerobic metabolism to extend dive duration. The poor energy yield associated with anaerobic metabolism, combined with the accumulation of lactate make this metabolic process a costly endeavor. Consequently a seal would have to drastically increase the time spent at the surface following an anaerobic dive and/or be forced to conduct a series of short dives, well within its calculated ADL (Fedak and Thompson, 1993). While this might occur in some divers on rare occasions, anaerobic metabolism seems to contribute little energy during routine diving in endotherms (Butler, 2004). An alternative concept that could explain dive durations beyond the calculated ADL is hypometabolism. One central aspect of the diving response is the selective redistribution of blood flow during diving, possibly leading to hypometabolism in under-perfused tissues and a decline in whole-body metabolism. Although the mechanisms are still being investigated, regional hypothermia could be one potential way of achieving a reduced metabolic state.

Physiological control during diving is complex. In addition to neural control, a role for hormonal contribution in diving was suggested, based on the observation that adrenal glands continue to be perfused during forced submergence in Weddell seals (Zapol et al., 1979). In freely diving Weddell seals (*Leptonychotes weddellii*), Hochachka and colleagues found that adrenal hormone concentrations (both epinephrine and norepinephrine) increased as a function of dive duration and decreased rapidly during recovery (Hochachka et al., 1995). Besides catecholamines, other hormones, such as thyroid hormones, may be important regulators of the dive response.

Thyroid hormones have widespread physiological and metabolic activity which affects the function of virtually every organ. Thyroid hormones profoundly influence oxidative metabolism in all vertebrate classes and hence their involvement in the setting of metabolic rates in endotherms was proposed (Hulbert, 1986). Hulbert and Else (1981) suggested that the fivefold greater metabolic rate of mammalian tissues compared with reptile tissues was due to a threefold greater thyroxine (T_4) level in mammals. In humans, basal metabolic rate (BMR) measurements were used historically as a clinical test for evaluating thyroid function (Klein, 2001). The crucial role that thyroid hormones play in regulating metabolism can be seen in humans with abnormal thyroid function; e.g., thyrotoxicosis is associated with an increase in endogenous glucose production, hepatic insulin resistance, and concomitant hyperglycemia (for review see Chidakel et al., 2005). In addition to the profound effects on energy metabolism, thyroid hormones also strongly affect the cardiovascular system. Various mammalian studies demonstrated a strong positive correlation between elevated circulating thyroid hormone levels and resting heart rate (Rutherford et al., 1979; Breisch et al., 1989; Karaus et al., 1989; Hoey et al., 1991; Sernia et al., 1993).

Given the crucial role of thyroid hormones in the regulation of metabolism, hypometabolism during diving may be associated with a reduced thyroid hormone level in divers. In fact, Hochachka speculated that at-sea hypometabolism and metabolic efficiency in seals is likely influenced by thyroid hormone concentrations (Hochachka, 1991). While thyroid hormone concentrations have never been measured during diving, an approach used in other systems is to examine the consequences of hormone excess. A common strategy in thyroid hormone research has been to address specific questions by inducing hyperthyroidism (Hulbert, 2000). The majority of these studies considered the effect of the hormone on the resting state of organisms rather than specific periods of metabolic demand, when energy turnover is likely to be affected by thyroid hormones. Diving is such an example, where animals undergo a variety of metabolic and cardiovascular adjustments. The hyperthyroid state is associated with elevated metabolic rate and elevated heart rate; physiological responses that are opposite to those commonly observed during diving. Hence, if metabolism is increased in hyperthyroid seals, their diving ability would likely be negatively affected. Furthermore, we hypothesized that if hyperthyroidism has functionally the opposite effects on seals as hypothyroidism, then a reduced metabolic rate during diving might be indicative of a hypothyroid state during normal diving.

The present study aimed to investigate the functional importance of hypometabolism during diving in harbor seals. By administering thyroid hormone (T_4), we intended to offset the hypometabolism in a diving seal. If metabolism in the hyperthyroid state is increased during diving, then oxygen consumption would increase and a decrease in the seal's aerobic dive capacity would likely result. We therefore expected that in order to perform a similar dive pattern, hyperthyroid seals would require a stronger cardiovascular response (i.e., increased bradycardia and peripheral vasoconstriction) when compared to a euthyroid seal and would possibly incur a greater contribution from anaerobic metabolism. Alternatively, seals might end dives prematurely. To investigate these mechanisms, we monitored metabolic rate, heart rate, and post-dive lactate concentrations in euthyroid and hyperthyroid harbor seals performing 5-min dives on command.

MATERIALS AND METHODS

STUDY ANIMALS AND TRAINING

Five captive juvenile harbor seals (*Phoca vitulina richardii*, two males and three females) with a mean body mass of 30 ± 3.5 kg (range: 26–35 kg) were used in this study. Animals were captured at a maximum age of one month (seals 1 and 2 in August 1996 and seals 3–5 in September 1997) and maintained in captivity for about 1 year before being used in the experiments. We designed a carefully directed training regime, based on positive reinforcement, to shape the animal's behavior for the experiments. During diving experiments, seals were never restrained in any way and were free to cooperate or to refuse to perform the dives. By the time the experiments were conducted, the seals had been trained for more than 6 months to perform dives in which they remained stationary underwater for exactly 5 min on command. This procedure, in combination with the young age of the seals and the extended time spent with them, resulted in tame study animals and highly controlled conditions. Experiments started with

seals in the euthyroid state and upon completion of these trials, hyperthyroidism was induced and experiments in the hyperthyroid state followed. Experiments for seals 1 and 2 in both euthryroid and hyperthyroid condition were conducted in winter (December 1997 to March 1998), while experiments for seals 3–5 took place during summer (June to September 1998). During this time, air/water temperatures ranged from 5.0/6.1°C in winter to 25.5/17.5°C in summer. All seals gained body mass (M_b) during the study (mean overall increase: 4.0 ± 1.6 kg) and mass gain rate (kg month^{-1}) was not different between thyroid states ($F = 0.79$, $p = 0.43$). All experimental procedures were approved by the UBC Animal Care Committee and were in compliance with the principles promulgated by the Canadian Council on Animal Care.

THYROID HORMONE ADMINISTRATION PROTOCOL

Hyperthyroidism was induced by administration of the levothyroxine *Synthroid* (Abbott Laboratories, Abbott Park, IL, USA), a synthetic form of Thyroxine (T_4). The seals were maintained at a dose of 50 $\mu g \cdot kg^{-1} \cdot day^{-1}$, which was administered in tablet form twice daily for up to 6 weeks. To monitor the resulting thyroid hormone concentrations and determine the degree of hyperthyroidism, blood samples were taken twice weekly, 3–5 h after the first levothyroxine dose of the day, using an indwelling extradural vein catheter. Serum concentration of free T_4 was chosen as the basis for assessment of the thyroid status for the following reasons: (1) T_3 is primarily an intracellular hormone and its serum concentration fluctuates randomly, making it a less reliable indicator of thyroid status than T_4 (Feldman and Nelson, 1996); (2) total thyroid hormone levels in plasma (T_3, T_4) reflect the levels of thyroid binding plasma proteins rather than the concentration of the biologically active form (free T_3, free T_4) (Hulbert and Else, 1999). Hence, free T_4 was chosen as the physiologically most relevant indicator of thyroid status and was determined using the ACCESS Immunoassay System with the Access free T4 Reagent Pack (Beckman Coulter, Fullerton, CA, USA). The validity of the assay was confirmed by the results of parallelism and recovery of added hormone. The radioimmunoassay results can be affected by species-specific differences in the affinity of binding protein relative to the affinity of the antibody employed and most assays were developed for humans. However, while this could have affected the absolute values determined for the seals, it would have not affected the relative differences observed between treatment groups.

RESPIROMETRY

Metabolic rates of seals were determined measuring oxygen consumption rates (ml $O_2 \cdot min^{-1} \cdot kg^{-1}$) using an open flow-through respirometry system. An acrylic dome (volume: ~50 l), floating on the water surface, served as metabolic chamber. Airflow was measured using an electronic mass flowmeter (Omega Engineering, Inc., Stamford, CT, USA). A variable vacuum pump, connected in series with the mass flowmeter, pulled air through the system. A flow of 10 $l \cdot min^{-1}$ kept the fraction of oxygen within the dome above 18.5%. Air was continuously subsampled from the main flow and drawn through two columns filled with a drying agent (Drierite) and a CO_2 scrubbing agent (Ascarite), prior to determination of oxygen content. The oxygen content was determined by use of a paramagnetic oxygen analyzer (Beckman, Schiller Park, IL, USA). The oxygen analyzer was calibrated before each trial using 99.995% pure N_2 (PraxAir, Richmond, BC, Canada) and outside air (set to 20.95% O_2). Ambient temperature and humidity were monitored throughout the trials. Voltage output from the oxygen analyzer and flowmeter were fed into an A/D-converter (DATAQ Instruments, Inc. Akron, OH, USA), and average values were recorded and stored every 5 s onto a desktop computer using WinDaq (DATAQ Instruments Inc.). The system was calibrated using the nitrogen dilution technique and oxygen consumption was calculated with equation 11b in Fedak et al. (1981).

To measure resting metabolic rate (RMR) in water, a seal was directed to place its head into the respirometry dome and oxygen consumption was measured for a minimum of 6 min. A platform 1 m below the dome allowed the seal to remain stationary, without having to spend additional energy for maintaining position when breathing within the dome. During the dive trials a seal submerged on command and remained stationary at the tank bottom, approximately 1.5 m below the surface. Upon completion of a dive, the seal immediately surfaced to breathe into the respirometry dome. Post-dive oxygen uptake was measured while the seal's head remained within the dome for at least 6 min, after which pre-dive levels had been reached. The seal was then instructed to leave the dome, while dome air was continuously sampled until the oxygen concentration reached ambient values.

Oxygen consumption rate was calculated over individual dive cycles (dive and subsequent post-dive surface interval). To this end, the total amount of oxygen removed from the ambient air during the post-dive surface interval was divided by the duration of the dive cycle (Castellini et al., 1992). Dive cycles in our study consisted of a 5 min dive followed by a 6 min surface interval.

LACTATE

To check for possible lactate washout after a dive, blood samples were taken in a separate round of dive trials conducted during the summer months. Three seals (seals 3–5) were trained to station on deck (adjacent to the dive tank) for 15 min immediately after completion of a dive. Using an indwelling catheter positioned in the extradural vein, the first blood sample was obtained within 2 min after a dive. Subsequent samples were taken at 5, 9, 12, and 15 min after surfacing. Blood samples were transferred into vacutainers and kept on ice. The catheter was flushed with saline between each sampling. Without delay the blood was centrifuged and the plasma was removed and kept frozen at −80°C until analysis. Lactate concentration was determined using an assay (modified after Bergmeyer, 1970) for analysis in a photometer (UV/VIS Spectrometer, Model Lambda 2, PerkinElmer, Wellesley, MA, USA). Two replicate measurements were taken from each sample to ensure reproducibility and were compared with standards of known lactate concentration.

HEART RATE

To investigate the effect of thyroid hormone level on heart rate, we recorded the electrocardiogram (ECG) of seals during

the dive trials and when resting on land, using a purpose built data-logging system. This system consisted of a modified Tattletale Lite data-logger (Onset Computer Corp., Pocasset, MA, USA) and incorporated a modified Polar OEM ECG amplifier [Polar Electro Inc., Lake Success, NY, USA; for details see: Andrews (1998)]. The entire data-logger assembly was cast in epoxy (Sealtronic Ltd., Burnaby, BC, Canada). Two skin-surface electrodes were attached to each seal. They were positioned anterior (above scapula) and posterior (above hip) to the heart, forming a diagonal line across the dorsal midline. This position resulted in a clean ECG signal even during movement and diving. To fasten the data-logger, two plastic webbing buckles were glued onto the seal (10-min epoxy) which fitted to counterparts attached to the data-logger. This set-up allowed for easy instrumentation of the seals before a trial. We sampled the ECG at a frequency of 100 Hz. At the end of each trial the data-logger could easily be removed and data were downloaded onto a desktop computer.

For analysis data were displayed in AcqKnowledge (BIOPAC Systems Inc., Goleta, CA, USA) to detect the QRS complex, and the mean inter-beat interval (r–r interval) was determined before, during, and after a dive and then converted to heart rate (beats·min^{-1}) for surface and submersion periods. Two seconds were omitted at the beginning and end of a dive to avoid rounding errors. To compute a heart rate profile for the dive trials, we averaged heart rate over a 20 s period before a dive (pre-dive), over each 1 min period during a dive, and over 30 s after completion of a dive (post-dive). We furthermore calculated mean dive heart rate for the entire period of submersion, which was also expressed as a percentage of resting heart rate.

DATA COLLECTION

Metabolic rate, heart rate, and post-dive plasma lactate concentrations were determined from separate trials. For metabolic rate and heart rate measurements, a minimum of 10 trials per seal were conducted for both the euthyroid and hyperthyroid state. Post-dive lactate concentrations were determined after five dives per seal for both thyroid conditions. Only one successful 5-min dive per seal was conducted each day.

cADL AND MAXIMUM OBSERVED DIVE DURATION

To investigate the effect that a changed thyroid status might have on the aerobic diving capacity of seals, we determined the calculated aerobic dive limit (cADL) for euthyroid and hyperthyroid seals. Total body O_2 stores for seals were estimated according to Davis et al. (1991, Appendix 2). The cADL was then calculated for each seal and thyroid hormone condition by dividing estimated O_2 stores by the mean RMR measured for each seal as an approximation of metabolic rate during stationary diving (i.e., without muscular work) (Castellini et al., 1992). In separate trials, four seals were equipped with a purpose-built submergence sensor, mounted to the head, that enabled us to record the duration of spontaneous dives, which they conducted undisturbed in their communal holding tank (16.5 m long × 2 m wide × 1.5 m deep). All four of these seals were monitored during 3–5 trials (each lasting 24–48 h) of spontaneous diving in each thyroid condition. For each trial the single longest dive duration was recorded.

STATISTICAL ANALYSIS

All statistical analyses were conducted using JMP (v.8.0.2.2, SAS Institute Inc.). Differences in free T_4 levels, oxygen consumption rates ($s\dot{V}o_2$), lactate concentrations, heart rate, cADL, and maximum observed dive duration during different thyroid states (euthyroid vs. hyperthyroid) and, where applicable, during different activities (resting vs. diving) were tested using a linear mixed-effects model (standard least squares regression fitted by REML). Thyroid status and activity were included as fixed effects, while seal ID was included as a random effect. To test for significance of the correlation between diving heart rate and post-dive blood lactate concentration, the Pearson Product Moment Correlation test was used. Significance was accepted at the level of $p < 0.05$. Data are presented as means ± 1 standard deviation (mean ± SD).

RESULTS

EXPERIMENTALLY INDUCED HYPERTHYROIDISM

Administration of levothyroxine (50 µg·kg^{-1}·day^{-1}) resulted in a sustained and significant 2.5–4.5-fold increase in serum free T_4 levels in all harbor seals (31 ± 11.1 pmol·l^{-1}) when compared with the euthyroid control state (9 ± 2.7 pmol·l^{-1}; $F = 77.40$, $p < 0.0001$; **Figure 1**). Euthyroid free T_4 levels were greater in seals 1 and 2, which were measured during the winter months and the relative increase in free T_4 after administration of levothyroxine was lower than in seals 3–5 measured during summer (**Figure 1**).

METABOLIC RATE

Oxygen consumption rate ($s\dot{V}o_2$) was significantly affected by both thyroid status ($F = 18.79$, $p < 0.0001$) and activity ($F = 229.78$, $p < 0.0001$). During diving, $s\dot{V}o_2$ was significantly reduced by about 25% in both the euthyroid ($F = 162.60$, $p < 0.0001$) and hyperthyroid state ($F = 86.38$, $p < 0.0001$) when compared with resting at the surface of the water (**Table 1**). In the

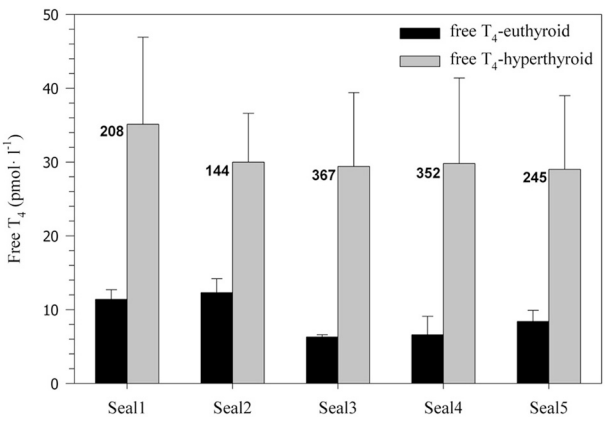

FIGURE 1 | Free serum T_4 concentration (pmol·l^{-1}) before and after experimentally induced hyperthyroidism in five harbor seals (values are means ± SD; $n = 6$ samples per seal and condition). Numbers beside the bars indicate the percent increase in free T_4 levels associated with levothyroxine treatment.

Table 1 | Oxygen consumption rates ($s\dot{V}o_2$) in the euthyroid and hyperthyroid state.

Activity	Oxygen consumption rates		
	Euthyroid ($ml \cdot min^{-1} \cdot kg^{-1}$)	Hyperthyroid ($ml \cdot min^{-1} \cdot kg^{-1}$)	% increase in hyperthyroid state
Resting	8.83 ± 1.05	$9.42 \pm 1.16^{\dagger}$	$6.7^{\dagger}$
Diving	$6.61 \pm 0.90^{*}$	$7.16 \pm 1.16^{*\dagger}$	$8.3^{\dagger}$

*Values are grand means which were established from individual seal means ($N = 5$ seals, $n = 10$ trials per seal and condition). *Indicates a significant difference from the resting situation and †indicates a significant difference from the euthyroid state.*

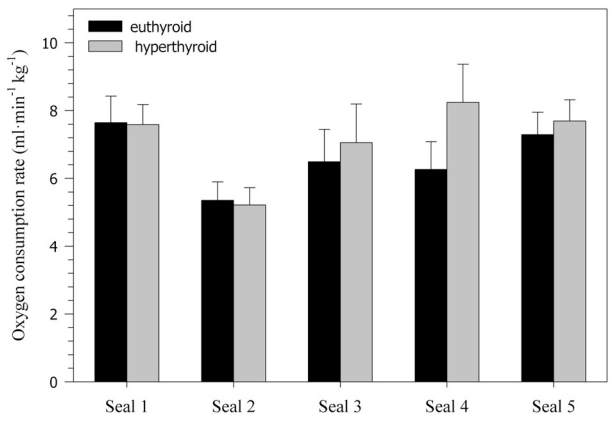

FIGURE 2 | Oxygen consumption rates ($ml \cdot min^{-1} \cdot kg^{-1}$) during diving in five euthyroid and hyperthyroid seals (values are means ± SD; $n = 10$ trials per seal and condition).

hyperthyroid state, seals had a significantly greater $s\dot{V}o_2$ (~7–8%) during both resting ($F = 7.11$, $p < 0.01$) and diving ($F = 13.21$, $p < 0.001$) than in the euthyroid state (**Table 1**, **Figure 2**). As with most variables investigated, the response by seals varied between individuals. For example $s\dot{V}o_2$ during diving in seals 1 and 2, measured during winter, did not differ with thyroid state, while it did vary in seals 3–5 (**Figure 2**).

LACTATE

There was no significant difference in resting blood lactate concentration between the euthyroid and hyperthyroid condition (1.68 ± 0.25 vs. 2.12 ± 0.53 mM; $F = 4.17$, $p = 0.05$; **Figure 3**), but in two of the three harbor seals, resting lactate levels were elevated in the hyperthyroid condition. After a 5 min dive in the euthyroid state, blood lactate concentration was no different from resting [La]. However, in two out of three seals, post-dive lactate concentrations were distinctly elevated in the hyperthyroid state. This response was consistent throughout the five trials conducted with each seal (**Figure 3**). Accordingly, mean post-dive lactate concentrations in the hyperthyroid condition were significantly greater than in the euthyroid condition over the time-course observed ($F = 121.40$, $p < 0.0001$).

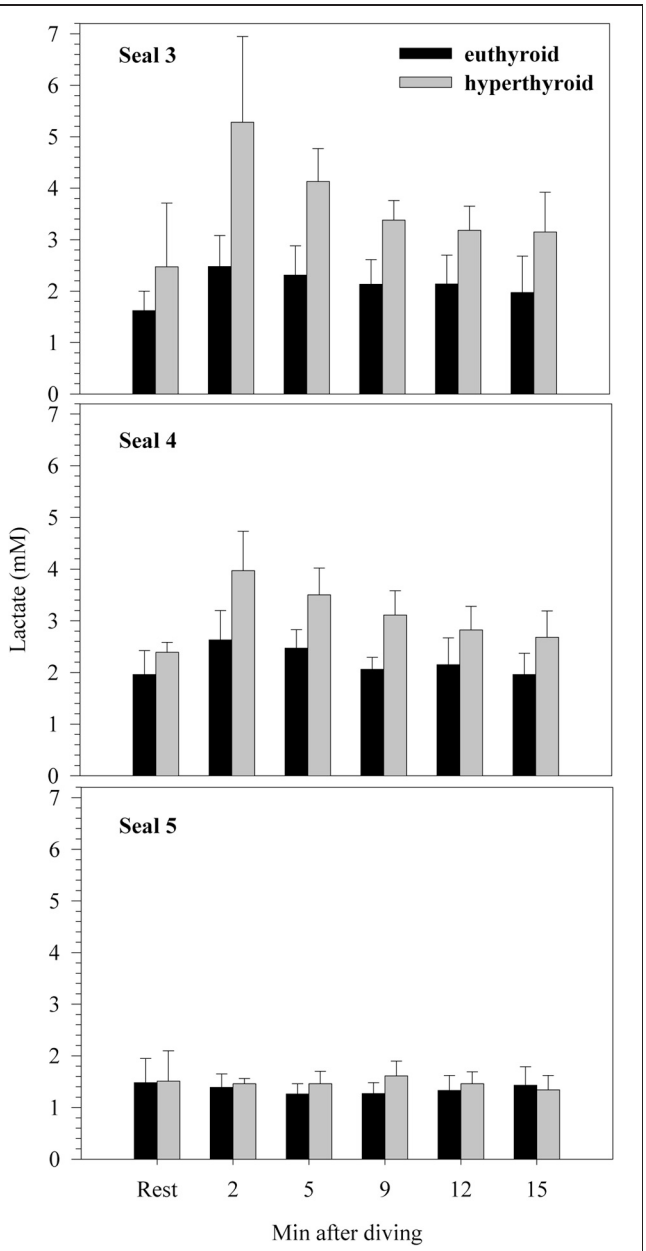

FIGURE 3 | Resting and post-dive lactate concentrations (mM) for three individual harbor seals (values are means ± SD; $n = 5$ trials per seal and condition).

HEART RATE

Heart rates of resting harbor seals in the euthyroid state (hauled out on deck) averaged 114.3 ± 4.0 beats·min^{-1}. In the hyperthyroid state resting heart rates were significantly elevated by about 5% (119.6 ± 4.2 beats·min^{-1}; $F = 7.15$, $p < 0.01$), when compared with the euthyroid state. During the dive trials the following general heart rate pattern was observed in both euthyroid and hyperthyroid states (**Table 2**): Heart rate was elevated before submergence (~10–20% above resting) and dropped immediately upon submergence to less than 40 beats·min^{-1} (~30% of

Table 2 | Heart rates before, during, and after submergence in the euthyroid and hyperthyroid state.

Phase of dive cycle	Euthyroid (beats·min^{-1})	Hyperthyroid (beats·min^{-1})	Hyperthyroid (% of euthyroid)
Pre-dive	133.35 ± 5.09	130.76 ± 7.38	98.1
Dive: min 1	31.76 ± 4.18	29.23 ± 6.01†	92.0†
Dive: min 2	27.61 ± 3.38	25.39 ± 4.71†	91.9†
Dive: min 3	22.39 ± 2.13	20.43 ± 5.29†	91.2†
Dive: min 4	18.23 ± 3.46	17.41 ± 4.21	95.5
Dive: min 5	16.60 ± 3.86	15.62 ± 3.06†	94.1†
Post-dive	144.72 ± 8.00	145.76 ± 5.12	100.7

Values are grand means which were established from individual seal means (N = 5 seals, n = 10 trials per seal and condition. †Indicates a significant difference from the euthyroid state.

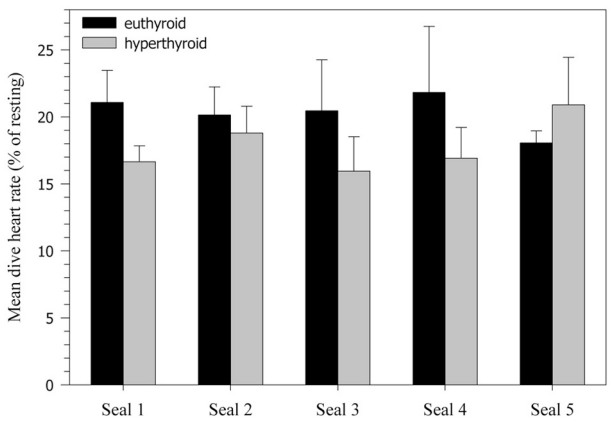

FIGURE 4 | Heart rate decline during 5 min dives (% of resting heart rate). Values are means ± SD ($N = 5$ seals, $n = 10$ trials per seal and condition).

resting value). Heart rate then continued to fall throughout the dive to a minimum of 16 beats·min^{-1}. Upon emergence heart rate increased to a level of ∼20–25% above resting. Mean dive heart rate was significantly lower in the hyperthyroid state and, hence, diving bradycardia was more pronounced during experimental hyperthyroidism ($F = 13.98$, $p < 0.001$; **Figure 4**), when compared with the euthyroid state. Throughout the various phases of submergence (min 1–5 of submergence), diving heart rates in the hyperthyroid state were significantly lower than in the euthyroid state, while heart rates before and after submergence did not differ significantly (**Table 2**). Furthermore, mean dive heart rate was significantly negatively correlated with post-dive lactate concentration in three seals in the hyperthyroid state (Pearson's correlation coefficient $= -1$, $p = 0.003$). A lower heart rate during diving in these seals was associated with a greater post-dive lactate concentration.

cADL AND MAXIMUM OBSERVED DIVE DURATION

As each animal continued to grow during the experimental period, the M_b of seals was greater during the hyperthyroid trials when compared with the euthyroid trials (**Table 3**).

Table 3 | Estimated total body O_2 stores, cADL, and maximum observed dive duration for seals in the euthyroid and hyperthyroid state.

Seal	M_b(kg)	O_2 store (ml)	cADL (min)	Maximum dive duration (min)
EUTHYROID				
Seal 1	32.2	1831.1	6.52	4.68
Seal 2	35.9	2037.5	7.86	7.49
Seal 3	30.0	1702.2	6.20	5.03
Seal 4	26.3	1495.9	6.35	7.35
Seal 5	30.9	1753.8	5.61	
Grand mean	31.1 ± 3.5	1764.1 ± 196.9	6.51 ± 0.83	6.14 ± 1.49
HYPERTHYROID				
Seal 1	33.6	1908.5	5.69	2.42
Seal 2	36.8	2089.0	7.54	4.72
Seal 3	30.9	1753.8	6.07	2.32
Seal 4	28.6	1624.8	5.92	3.25
Seal 5	30.9	1753.8	5.35	
Grand mean	32.1 ± 3.1†	1826.0 ± 178.1†	6.11 ± 0.85†	3.18 ± 1.11†

O_2 stores were calculated according to Davis et al. (1991).
cADL was calculated by dividing estimated O_2 stores by the mean RMR measured for each seal as an approximation of metabolic rate during stationary diving. Values for max. dive duration are means established from 3–5 trials (lasting 24–48 h), during which the single longest spontaneous dive was recorded. †Indicates a significant difference from the euthyroid state.

Accordingly, estimated body O_2 stores for seals were significantly greater during hyperthyroid trials (**Table 3**; $F = 8.73$, $p = 0.04$). However, because RMR increased in hyperthyroid seals (**Table 1**), their cADL was significantly reduced when compared with euthyroid seals (**Table 3**; $F = 10.91$, $p = 0.03$). Maximum dive duration observed during spontaneous diving activity was considerably and significantly shorter in hyperthyroid seals (**Table 3**; $F = 55.68$, $p < 0.01$).

DISCUSSION

EFFECTS OF ELEVATED THYROID HORMONE CONCENTRATION ON RMR

We found a significant increase in RMR of harbor seals after administration of T_4. However, the increase in metabolic rate observed in our study was surprisingly low when compared with the large response to experimental hyperthyroidism typically observed in terrestrial animals (Hoch, 1974; Dauncey, 1990). An insufficient degree of hyperthyroidism can be ruled out as an explanation for the relatively small changes in metabolic rate and heart rate in the hyperthyroid state as levothyroxine administration significantly elevated free T_4 levels (to 2.5–4.5 times the euthyroid level; **Figure 1**) for a prolonged period. The effects of experimentally elevated thyroid hormone levels on metabolic rate have never been investigated in pinnipeds, but a number of pinniped studies suggest a strong correlation between RMR and thyroid hormone levels. RMR in immature, growing pinnipeds is elevated when compared with adult animals and this is generally attributed to greater energetic requirements associated with the formation of new tissue and higher thermoregulatory costs (Hart and Irving, 1959; Miller and Irving, 1975; Rea and Costa, 1992). In those studies on harbor seals and northern elephant

seals, RMR slowly decreased as the animals approached maturity. Other studies reported increased thyroid activity in young southern elephant seals (Little, 1991; Bryden, 1994), harp seals, (*Phoca groenlandica;* Leatherland and Roland, 1979), gray seals (*Halichoerus grypus*; Engelhardt and Ferguson, 1980), and harbor seals compared with adults (Stokkan et al., 1995; Woldstad and Jensen, 1999), which coincides with the elevated RMR observed in juvenile pinnipeds. Natural variations in the amount of plasma thyroxine were reported to be correlated with changes in the RMR of adult harbor seals (Ashwell-Erickson and Elsner, 1981) and gray seals (Boily, 1996). Ashwell-Erickson et al. (1986) investigated the relationship between metabolism and thyroid hormone concentration during molt in harbor and spotted (*P. largha*) seals. They observed a concurrent decrease in RMR and thyroid hormone concentration at the onset of molt, which would serve to decrease food requirements at a time when seals have to stay out of the cold water. Once hair regeneration was well underway, thyroid hormone concentrations increased to their maximum, and RMR rose to its pre-molt level. In juvenile seals, metabolic rates during the annual molt fluctuated by ±15%, concurrent with free T_4 fluctuations (Ashwell-Erickson et al., 1986). Although the observed increase in metabolic rate during hyperthyroidism was modest, these results support the claims that fluctuations in RMR in seals are at least in part driven by changes in thyroid hormone levels.

There are several possible explanations for the limited magnitude of the increase in metabolic rate during hyperthyroidism in our harbor seals. (1) Seals might be able to down-regulate their sensitivity to thyroid hormones. Hochachka et al. (1991) suggested that high-altitude adapted humans did have a decreased sensitivity to thyroid hormones, which could increase their muscle efficiency in the face of hypoxia. Similarly, a down-regulated sensitivity to thyroid hormones could explain the small increase in harbor seal RMR we observed, even at substantially elevated plasma concentrations of free T_4. (2) The limited effect of thyroid hormones on harbor seal RMR could be due to potential differences in cell membranes between terrestrial and diving mammals. In general, thyroid hormones increase the permeability of cell membranes through various mechanisms. The resulting leakier membranes require more energy for ion pumping to maintain the ionic integrity of cells (Hulbert and Else, 1981). In diving animals, however, the permeability of cell membranes also determines hypoxia sensitivity. Hochachka and co-workers compared hypoxia resistance and diving ability and found that mitochondria from seal livers were more successful in surviving hypoxia when compared with liver mitochondria of terrestrial animals (Hochachka et al., 1988). In addition, cell membranes of hypoxia-tolerant liver tissue in seals were less permeable than those in the hypoxia-sensitive brain (Hochachka et al., 1988). This reduction in cell membrane permeability would reduce metabolic rate and might therefore be an important mechanism for extending hypoxia tolerance in diving animals (Hochachka and Guppy, 1987). A reduction in cell membrane permeability, by a yet unknown mechanism, could reduce the sensitivity of cell membranes to thyroid hormones in seals and could explain the relatively small increase in RMR observed in our harbor seals after T_4 administration.

HYPOMETABOLISM DURING DIVING

Oxygen consumption and heart rate in both the euthyroid and hyperthyroid state were significantly reduced during diving when compared with resting (**Tables 1** and **2**), indicating that metabolic suppression occurred during diving. Metabolic rate was decreased by about 25% during diving, when seals lay motionless on the bottom of the tank. This is similar to what was reported for northern elephant seals diving under comparable conditions (Webb et al., 1998). The presence of hypometabolism during natural diving has been suggested in other pinniped studies (Guppy et al., 1986; Le Boeuf et al., 1988; Hindell et al., 1992). The degree of hypometabolism typically observed in these seals varies according to their dive behavior (i.e., resting underwater *vs.* exercise). Castellini et al. (1992) measured oxygen consumption in freely diving Weddell seals conducting dives of up to 82 min and found that metabolic rate during active diving under the ice declined with increasing dive duration, but was not significantly different from resting values. Our seals were not exercising, but rather simply laying on the bottom of the tank, which may have allowed them to reduce metabolic rate below the resting level.

HYPERTHYROID DIVING METABOLIC RATE

Oxygen consumption while diving when the seals were hyperthyroid was significantly greater (∼7–8%) than when they were euthyroid (**Table 1**, **Figure 2**). Hence, the ability to reduce metabolism during diving was impaired in the hyperthyroid state. Hyperthyroid post-dive plasma lactate concentrations were significantly elevated in two of the three seals when compared with euthyroid post-dive levels (**Figure 3**). This illustrates that at least in these two seals there was a net contribution from anaerobic pathways to overall diving metabolism. If lactate accumulated during a dive is not completely metabolized during the following surface period, post-dive oxygen consumption will not accurately reflect the metabolic energy expended during that dive. Kooyman et al. (1980) showed that Weddell seals need up to 1 h to process the lactate produced during extended dives. Consequently, hyperthyroid seals in our study might not have processed all the lactate produced during a dive within the 6 min spent post-dive inside the dome. This is supported by lactate levels that were still elevated 15 min after completion of a dive in two of the three hyperthyroid seals (**Figure 3**). Hence, diving metabolic rate in the hyperthyroid state might have been even higher than measured in our study.

In the present study we attempted to experimentally shorten the ADL of seals by increasing diving metabolic rate via an induced hyperthyroid state. Maximum dive duration during spontaneous diving activity in hyperthyroid seals was about half of that observed in euthyroid seals (**Table 3**), suggesting that aerobic dive capacity was significantly altered during hyperthyroidism. Accordingly, we estimated the cADL of our harbor seals to be 6.5 min in the euthyroid state and 6.1 min in the hyperthyroid state (**Table 3**). In agreement with the estimated cADL of 6.5 min for euthyroid seals, we found no evidence of a net anaerobic contribution to their diving metabolism. By contrast, post-dive lactate concentrations were significantly elevated in two hyperthyroid seals (**Figure 3**), in which the experimental dive

duration (5 min) was closer to their cADL (mean cADL for seals 3–5 was 5.8 min). Hence, the increased oxygen consumption during diving as a consequence of their hyperthyroidism might have pushed seals toward their aerobic limit and triggered an increase in anaerobic metabolism. However, the lactate response was not consistent in all individuals. This could suggest that the experimental dive duration of 5 min is within a transition zone where seals increasingly rely on anaerobic metabolism, or that diving metabolic rate, even when resting on the bottom, is not fixed but can vary both between and within individuals.

Numerous studies demonstrated a correlation between the amount of lactate accumulated in the blood and the level of exercise during a dive. For example, in bottlenose dolphins (*Tursiops truncatus*), lactate concentration after a 5 min stationary breath-hold at the surface was elevated but consistently lower than after active dives of comparable duration (Williams et al., 1999). Similarly, lactate concentration in beluga whales (*Delphinapterus leucas*) was elevated to about three times the resting value after both active swim trials and deep dives, while there was little increase after sedentary breath-holds near the surface (Shaffer et al., 1997). This suggests that locomotor effort during active dives in the above studies increased metabolic costs beyond what could be supplied by aerobic means and necessitated a net contribution from anaerobic pathways.

The increased lactate concentrations we observed in two hyperthyroid seals after 5 min dives is remarkable because it did not result from increased muscular activity. During all dives seals remained stationary and motionless at the bottom of the tank. Hence, the increase in metabolic rate caused by hyperthyroidism was sufficient to require energy production beyond the aerobic capacity during these dives. Alternatively, the increased post-dive lactate levels during hyperthyroidism could have been due to the effect of increased thyroid hormones on glucose turnover. Hyperthyroidism is known to be associated with increased gluconeogenesis and insulin resistance (Chidakel et al., 2005). However, steady-state lactate concentrations are not usually elevated during hyperthyroidism, at least in fed animals (c.f. Sugden et al., 1990), and although two of our three seals did have increased resting lactate, the difference was not statistically significant, whereas post-dive lactate was significantly increased. Furthermore, our supposition that the increase in post-dive lactate was due to an increased rate of oxygen store depletion during hyperthyroid diving is supported by the lower diving heart rate and negative correlation between diving heart rate and post-dive lactate, as explained below.

HYPERTHYROID HEART RATE

Perhaps the most interesting finding in the present study was the stronger bradycardic response during diving in the hyperthyroid condition, when compared with the euthyroid condition (**Table 2**, **Figure 4**). Based on studies in terrestrial mammals, in which thyroid hormone stimulation caused elevated heart rates (tachycardia) (Palacios et al., 1979; Rutherford et al., 1979; Williams et al., 1980; Karaus et al., 1989), one might have expected that heart rates would also be elevated in diving hyperthyroid harbor seals. However, as we predicted, seals frequently displayed significantly lower diving heart rates in the hyperthyroid condition when compared to euthyroid diving heart rates. The seals did appear to have two different, albeit persistent, strategies to cope with hyperthyroidism. One individual did display an increased heart rate during diving in the hyperthyroid state. In contrast, the other four seals exhibited a more profound bradycardia during diving in the hyperthyroid state (**Figure 4**). This suggests that in the hyperthyroid condition, at least some of our seals might have been pushed toward their aerobic limit, so that a more pronounced diving response became necessary to conserve oxygen. Similar cardiovascular responses to increased metabolic requirements during diving, as a consequence of stress or prolonged dive duration, have been shown in seals (Fedak, 1986) and are of crucial importance for the conservation of oxygen for the hypoxia-intolerant tissues of the heart and brain.

In our study, in both the euthyroid and hyperthyroid condition, seals developed a pronounced bradycardia during 5 min dives and the time course of this heart rate decline was comparable, despite differences in thyroid hormone concentrations (**Table 2**, **Figure 4**). The absence of elevated diving heart rates in our hyperthyroid seals, despite an increased level of thyroid hormones, clearly suggests that the dive response was able to override any direct hormonal effect on heart rate.

We found a strong correlation between the degree of diving bradycardia and post-dive lactate production during 5 min dives during hyperthyroidism. The highest post-dive blood lactate values occurred after dives during which heart rate was lowest. Assuming that blood pressure is maintained, a heart rate decline during diving is indicative of increased vasoconstriction. The stronger the vasoconstriction, the more likely contributions from anaerobic metabolism become in under-perfused tissues, resulting in lactate production and, hence, increased post-dive lactate concentrations.

INDIVIDUAL VARIABILITY AND SEASONAL EFFECTS

Responses in all parameters often varied between individuals, suggesting that there is no standard response of a harbor seal to any given experimental condition. Each seal responded somewhat differently to the challenge of diving and elevated thyroid hormones. Some of the variation in the observed responses might be related to differences in environmental conditions (i.e., summer vs. winter) and the corresponding seasonal changes in natural hormone levels and ambient temperatures. A number of studies demonstrated seasonal variations in thyroid hormone concentrations of seals, often in association with molt (Ashwell-Erickson et al., 1986; Renouf and Brotea, 1991; Boily, 1996). The scope of seasonal variation in free T_4 levels is typically smaller in adult seals than in juveniles. For example, free T_4 during molt increased on average between 14 and 46% in sub-adult and adult harbor seals (Renouf and Brotea, 1991) and by ∼12% in adult gray seals (Boily, 1996). By contrast, in juvenile harbor and gray seals free T_4 levels doubled during molt (Renouf and Brotea, 1991; Boily, 1996). In our study, free T_4 levels in euthyroid seals 1–2 (measured during the winter) were ∼67% greater than in euthyroid seals 3–5 (measured during the summer), indicating similar seasonal variation in these juvenile seals (**Figure 1**). However, after levothyroxine administration free T_4 levels in all seals were greatly increased (on average by ∼263%; **Figure 1**) and well beyond the

scope of any natural seasonal fluctuation. Hence, the contribution of seasonal changes in free T_4 levels to the observed variation in physiological responses was most likely limited. There may have been a slight seasonal difference in sensitivity to hyperthyroidism as only the three summer seals (Seals 3–5) showed an increase in diving oxygen consumption in the hyperthyroid state (**Figure 2**), but the mean percent increase for these 3 seals (15.2%) was still surprisingly small given their very large (321%) increase in free T_4 levels.

Differences in individual behavior and cooperation during experimentation but also in diving capacity (defined as the maximum observed dive duration) may have further contributed to the observed variability in physiological responses, which was most evident during the diving experiments. This illustrates the importance of taking into account the physiological diversity that exists within animal populations when investigating the physiological mechanisms associated with diving. The variability observed in our seals as they dealt with the increased diving metabolism due to induced hyperthyroidism emphasizes the plasticity of physiological responses. It furthermore underlines the ability of air breathing, diving vertebrates to successfully adapt to a variety of challenging situations.

CONCLUSIONS

Perhaps most surprising was the observation that hyperthyroidism did not induce a more pronounced change in the physiology of resting harbor seals. Although both metabolic rate and heart rate were increased in the hyperthyroid state, this increase was small in comparison to the increase reported during experimental hyperthyroidism in terrestrial vertebrates. A possible explanation for the weak response to elevated T_4 might be a decreased sensitivity of diving animals to the action of thyroid hormones. Another striking result is the occurrence of elevated lactate concentrations after a 5 min dive in some seals, when diving in the hyperthyroid state. We measured a significant elevation in oxygen consumption during diving in the hyperthyroid condition (∼7–8%), when compared with the euthyroid control condition. Overall metabolic rate (aerobic plus anaerobic) during diving might have been even higher in the hyperthyroid condition, as indicated by the elevated post-dive lactate concentration. Most interestingly, increased metabolic rate during diving in the hyperthyroid state was accompanied by a decreased heart rate, suggesting that the higher metabolic rate required a stronger cardiovascular response to enable seals to conserve oxygen and stay submerged for the requested duration. To unequivocally illustrate this chain of events, a larger sample size than was possible in our study would be advantageous. Similarly, for a clear determination of the critical dive time, beyond which metabolic adjustments during hyperthyroidism are required, it would be desirable to conduct trials with multiple dive durations, covering a greater range than our study. Nevertheless, our results demonstrate the powerful role of thyroid hormones in metabolic regulation and support the notion that they might be instrumental in modulating the at-sea metabolism of phocid seals.

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Changes in dive behavior during naval sonar exposure in killer whales, long-finned pilot whales, and sperm whales

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Anthropogenic underwater sound in the environment might potentially affect the behavior of marine mammals enough to have an impact on their reproduction and survival. Diving behavior of four killer whales (*Orcinus orca*), seven long-finned pilot whales (*Globicephala melas*), and four sperm whales (*Physeter macrocephalus*) was studied during controlled exposures to naval sonar [low frequency active sonar (LFAS): 1–2 kHz and mid frequency active sonar (MFAS): 6–7 kHz] during three field seasons (2006–2009). Diving behavior was monitored before, during and after sonar exposure using an archival tag placed on the animal with suction cups. The tag recorded the animal's vertical movement, and additional data on horizontal movement and vocalizations were used to determine behavioral modes. Killer whales that were conducting deep dives at sonar onset changed abruptly to shallow diving (ShD) during LFAS, while killer whales conducting deep dives at the onset of MFAS did not alter dive mode. When in ShD mode at sonar onset, killer whales did not change their diving behavior. Pilot and sperm whales performed normal deep dives (NDD) during MFAS exposure. During LFAS exposures, long-finned pilot whales mostly performed fewer deep dives and some sperm whales performed shallower and shorter dives. Acoustic recording data presented previously indicates that deep diving (DD) is associated with feeding. Therefore, the observed changes in dive behavior of the three species could potentially reduce the foraging efficiency of the affected animals.

Keywords: marine mammal, dive, sonar

INTRODUCTION

Our understanding of the effects of military sonars on marine mammals has increased in the past decades (Richardson et al., 1995; Nowacek et al., 2007), but large gaps of knowledge still exist (Nowacek et al., 2007). Attention has focused on beaked whales due to several stranding events coinciding in time and space with military sonar operations (e.g., Simmonds and Lopez-jurado, 1991; Frantzis, 1998; Jepson et al., 2003; Cox et al., 2006; D'Amico et al., 2009). It has been suggested that these strandings are associated with a change in dive behavior which leads to development of tissue nitrogen gas bubbles and symptoms related to decompression sickness (DCS) (Jepson et al., 2003). Cetaceans often are reported to respond to anthropogenic noise with avoidance (e.g., Morton and Symonds, 2002; Olesiuk et al., 2002; Kastelein et al., 2008a,b; Tyack, 2008), either by horizontally swimming away from the sound source (Nowacek et al., 2004; Lusseau, 2009; Miller et al., 2011) or vertically, by a change in diving behavior (Miller et al., 2009). Such behavioral responses may protect the animals from direct physical injuries such as hearing impairment, but are likely to involve costs of leaving preferred habitat, costs of increased energy of locomotion as well as reduced feeding or higher risks of predation, etc. (Lusseau, 2009). Since cetaceans spend a significant amount of their time submerged, almost all behavioral responses are expected to result in a change in dive pattern. Cetaceans find their prey at depth, but need to return to the surface to breathe. An optimal foraging dive should minimize the energetic cost of traveling to the depth of the prey and maximize the energetic intake (Kramer, 1988). A change in diving behavior may hence have potential consequences involving ecological effects such as reduced foraging efficiency as well as potential physiological consequences such as DCS (Kvadsheim et al., 2012).

In this paper, we investigate whether and how whales changed their diving behavior during exposures to naval sonar sounds. The diving behavior before and during sonar exposure was studied here for three odontocete species with very different natural diving behavior; the deep diving (DD) sperm whale (*Physeter macrocephalus*), the intermediate diving pilot whale (*Globicephala melas*) and the shallow diving (ShD) killer whale (*Orcinus orca*).

MATERIALS AND METHODS

Data were collected during three field periods; November 2006, May/June 2008 and May/June 2009, in the Northern Norwegian Sea. The November season comprised two experiments with killer whales, the rest of the experiments were all conducted in May/June. All field experiments were conducted with the FFI

research vessel "R/V H.U. Sverdrup II," and with a smaller vessel, an outboard workboat (2006) or "M/S Strønstad" (2008, 2009) as an observation vessel. Whales were tagged with archival sensor packages recording their diving behavior (time versus depth) as well as received sound before, during and after sonar transmissions. Animal experiments were conducted under permits issued by the Norwegian Animal Research Authority (permits no 2004/20607 and S-2007/61201), and in compliance with ethical use of animals in experimentation. The research protocols were also approved by the University of St. Andrews Animal Welfare and Ethics Committee as well as the Woods Hole Oceanographic Institutional Animal Care and Use Committee.

TAG AND SONAR EQUIPMENT

Data on dive behavior were collected by dtags (digital tags, Johnson and Tyack, 2003) attached to the back of the whales by suction cups. These multi sensor tags include stereo hydrophones, a pressure sensor, 3-axis accelerometers, 3-axis magnetometer and a VHF radio transmitter. In this study we have focused on dive behavior. Data on vocal behavior and horizontal movements are also collected by the tag, and such data are reported in Miller et al. (2011).

After a period of 1–7 h collecting baseline behavioral data, sonar signals were transmitted to the whales using the Royal Netherlands Navy's multipurpose acoustic source SOCRATES (Sonar CalibRAtion and TESting, Netherlands Organization for Applied Scientific Research (TNO), The Hague, The Netherlands). Two different signal frequency bands were used; low frequency active sonar (LFAS, 1–2 kHz) and mid frequency active sonar (MFAS, 6–7 kHz) either as frequency modulated hyperbolic upsweep or downsweep signals. Maximum source levels varied from 197–214 dB re 1 μPa at 1 m with 1 s pulse duration and 20 s inter pulse intervals (duty cycle of 5%). A 10 min ramp up was conducted before full power transmission. Further details of the experimental procedures can be found in Miller et al. (2011) and Kvadsheim et al. (2009).

EXPERIMENTAL PROCEDURE

Whales were located by visual observers or towed hydrophone arrays, and tagged from a small boat. The experimental procedure was as follows: (1) tag 1 or 2 whales in a group, (2) post-tagging observation period to collect data on baseline behavior after recovery from tagging, (3) conduct a Controlled Exposure Experiment (CEE) which consisted of 1–4 source vessel approach exposure sessions, (4) collect post-exposure data, and (5) recover tags upon release for analysis. The tag was attached to the whale using a long carbon fiber pole or a pneumatic tag launching system (Aerial Rocket Tagging System, ARTS) (Kvadsheim et al., 2009). The whales were tracked by VHF and visually during the entire period. After a baseline period of 1–7 h, the source vessel "Sverdrup," towing the sonar source, started moving towards the whales from a distance of 5–8 km and at a speed of $\sim$ 4 m/s while sequentially transmitting a series of LFAS, MFAS, or no sonar signals (Silent control). Only one single sonar sound type was presented during each vessel approach session, and the order of the sound types presented was changed between each whale subjects. "Sverdrup" adjusted its course to approach the whale until 1 km distant, at which point its course was fixed. The distant ramp-up and the approaching source ship resulted in an escalation of the received levels of sonar sound.

During each exposure session, visual observers ensured that a safety limit of 100 m was kept between any animal and the source, and a shut-down of the system would occur if any animal moved closer than this.

DATA ANALYSIS

Pressure recordings were converted to depth using calibration values for each tag device. A dive was defined as any submergence for longer than 10 seconds to a depth >1 m, and dive duration as the time period between two surfacings. Duration and maximum depth were identified for all dives.

Analysis of changes in dive behavior

Cetaceans conduct different types of dives during different activities such as feeding, socializing, and traveling. Deep dives likely represent foraging periods, while shallow dives may be either resting dives between deep dives, or relate to traveling or socializing. To separate deep and shallow dives, a log-frequency analysis was used (Sibley et al., 1990) for all three species. The log-frequency analysis followed the two-process model of Sibley et al. (1990), and the dive mode criteria were calculated by the formula given in Slater and Lester (1982) to minimize the number of misclassified dives (Miller et al., 2010).

To examine whether diving behavior changed during sonar exposure sessions, we compared diving behavior before and during sonar exposure sessions. Exposure sessions commenced at random with respect to the animals' behavioral mode and therefore dive behavior of each individual whale was compared with its own pre exposure (PRE) behavior (baseline) in the time period just before sonar onset. For each exposure (LFAS/MFAS/Silent) a period of equal duration as the exposure period was defined as PRE.

Changes in diving behavior were examined in three steps. The first step was to determine the dive mode of the animal in the PRE and Exposure periods using pre-defined criteria: (1) ShD, if the period contains only dives shallower than the log frequency criterion for that species. This ShD mode was usually associated with traveling. (2) DD, when the animal conducted a series of dives deeper than the log frequency criterion with only a few shallow dives between each deep dive. This DD mode was usually associated with foraging. (3) ShD with occasional deep dives (SoD), when the animal was diving shallow as for mode ShD, but then conducted one deep dive and returned to ShD. Such dives may be exploratory dives, e.g., to search for prey. Sperm whales are known to continuously conduct deep foraging dives with a few shallow dives between. However, during several of the exposures, deep dives (according to the log frequency criteria) appeared to be shallower than the regular foraging dives. DD for sperm whales were hence divided into normal deep dives (NDD) if they were within the average deep dive depth during baseline $\pm$s.d, and unusually shallow deep dives (UsDD) if they were shallower than this range. Dives modes were categorized for the two periods (PRE and Exposure) to examine whether the animal changed its overall dive behavior during sonar exposure.

The second step was to measure three dive variables; dive duration, dive depth, and dive rate (number of dives/duration of period), for deep and shallow dives separately, and compare those between the PRE and Exposure period for each species and exposure type. The average and 95% confidence interval (1.96 standard error) were calculated and plotted for all these parameters to enable a comparison of the range of each of these parameters between the PRE and Exposure for each individual and exposure session.

Changes in depth and duration may reveal whether the nature of the dives changed during sonar exposure, while dive rate indicates whether the animal spent more or less time diving and is therefore an indication of changes in dive mode.

The third step involved an overall evaluation of each individual exposure, considering the dive mode as well as the range of depth, duration, and rate of the dives in the PRE and Exposure period, to define whether a change in dive behavior had occurred or not. Additionally, vocal records of echolocation and tailslaps during a dive (reported in Miller et al., 2011) were used to identify foraging dives. Each individual exposure was thus categorized as either a change in dive behavior in response to sonar or not.

RESULTS

NORMAL DIVING BEHAVIOR

Killer whales conducted most dives in the upper 50 m of the water column (**Figure 1A**), with periods of DD to a maximum depth of 140 m, alternating with periods of diving close to the surface (**Figure 2**). Long-finned pilot whales also spent the majority of their time in the upper 50 m (**Figure 1B**), separated by bouts of multiple deep foraging dives to 300–600 m (**Figure 3**). Sperm whales on the other hand spend more than 80% of their time deeper than 10 m (**Figure 1**), conducting long, deep foraging dives to 150–1500 m, each followed by a short surface period with a few shallow dives (**Figure 4**).

KILLER WHALES

The log frequency analysis estimated 21 m as the depth separating deep and shallow dives for killer whales. Deep dives tended to occur in bouts, with periods of ShD with only occasional single deep exploratory dives in between. An example of a killer whale dive record is shown in **Figure 2**.

LFAS

A total of six LFAS exposures were conducted, with four different killer whales (**Figure 5**). Animals 144a and 144b were in DD mode at the onset of the first LFAS session. Visual inspection of the dive record indicates that they responded to the sonar by a clear change in behavior involving a switch to ShD (**Figures 2**, **5**). For both animals in this exposure, depth, and duration of shallow dives increased compared to the PRE period (**Figure 5**). For the remaining four exposures, the animals (including 144a and 144b) were in ShD mode or SoD at the start of the exposure, and did not change their diving mode in response to the sonar (**Figure 5**). During these exposures there were no apparent differences in depth and duration of dives in the PRE and Exposure period (**Figure 5**).

MFAS

A total of 5 MFAS exposures were conducted with four different killer whales. None of these whales shifted dive mode during MFAS exposure (**Figure 5**). One whale (327 s) were conducting deep dives at sonar onset. This whale continued DD, but with a reduced rate for deep dives (**Figure 5**).

Silent control

Only one silent exposure was conducted. During this period there was no change in dive mode or any of the dive variables (**Figure 5**).

LONG-FINNED PILOT WHALES

The log frequency analysis indicated 34 m as the separation depth for shallow vs. deep dives of long-finned pilot whales. Long-finned pilot whales had a distinct separation of periods of deep dives and periods of shallow dives. During DD periods, lasting typically 2–3 h, long-finned pilot whales conducted 5–10 dives to 300–600 m, each dive lasting 7–9 min. An example of a pilot whale dive record is shown in **Figure 3**.

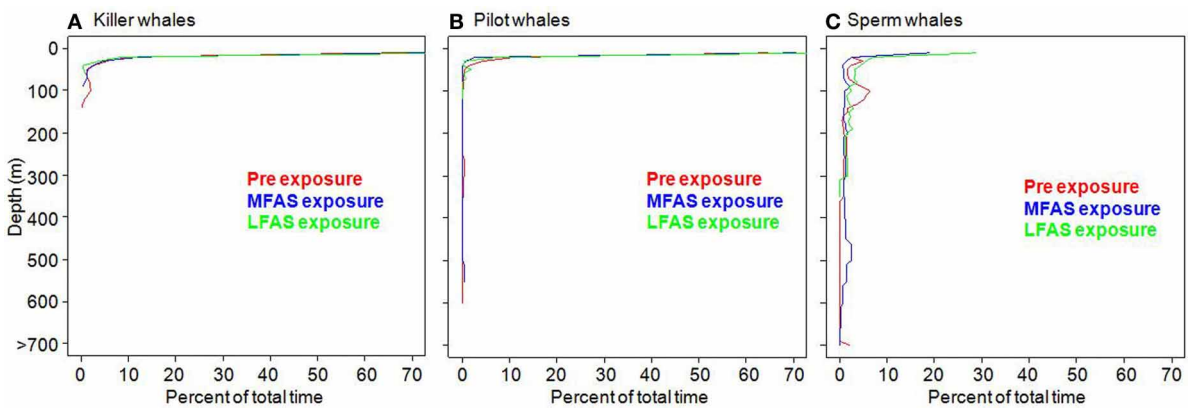

FIGURE 1 | Percent of total time spent at different depths in the water column during the different experimental conditions (LFAS, MFAS, PRE) for (A) killer whales, (B) long-finned pilot whales and (C) sperm whales.

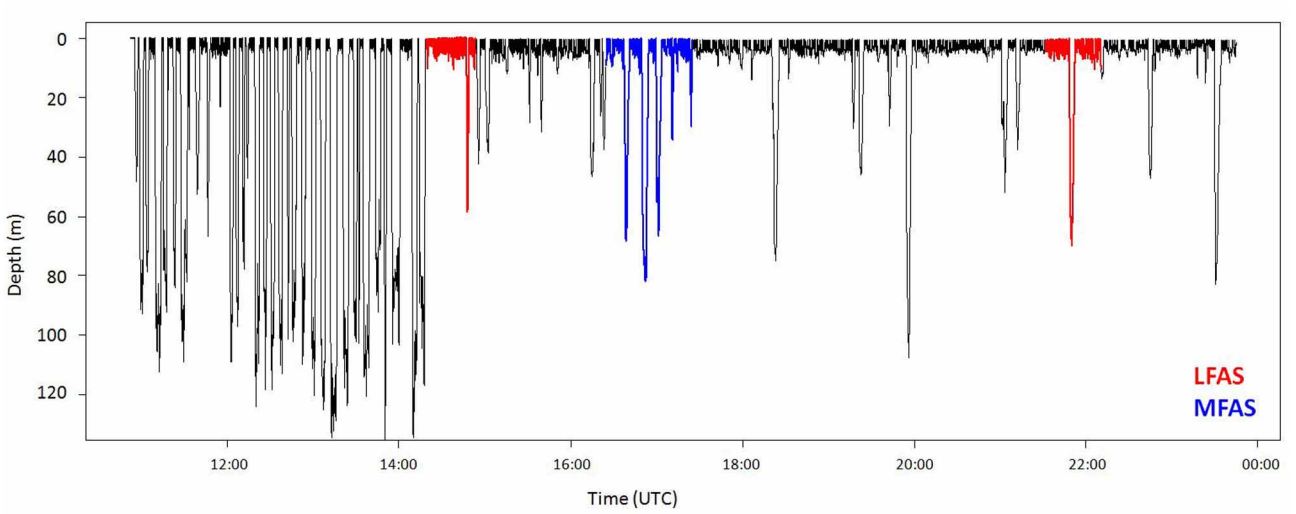

FIGURE 2 | Full dive record of killer whale oo09_144b. The whale made deep dives prior to the first LFAS exposure, and stopped deep diving at onset (red). Both prior to and during MFAS exposure (blue), the killer whale conducted both shallow and deep dives. During the second LFAS exposure, the whale was shallow diving before sonar onset, and continued shallow diving with one occasional deep dive.

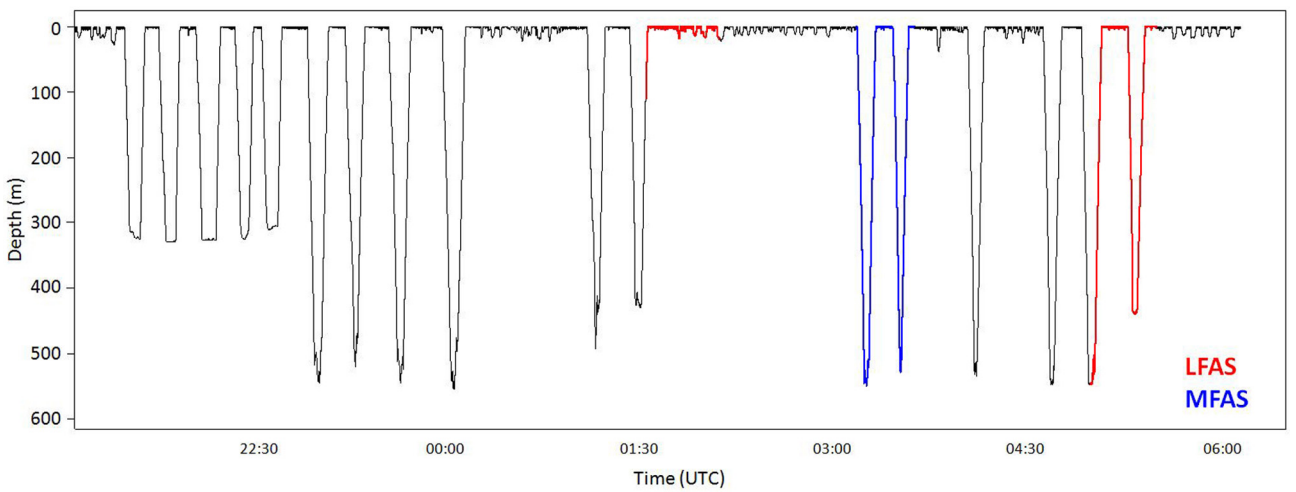

FIGURE 3 | Full dive record of pilot whale gm09_156b. This record show typical pilot whale diving behavior, with periods of shallow diving, followed by bouts of deep dives. Before onset of the first LFAS exposure, the whale conducted two deep dives, but turned to shallow diving at LFAS exposure onset (red). The unusually low number of dives in this deep diving bout indicates that this may be an effect of the exposure. During MFAS exposure (blue), the whale conducted regular deep dives. During the second LFAS exposure, the whale continued deep diving.

LFAS

A total of 11 LFAS exposures were conducted with seven different long-finned pilot whales. During 6 of the 11 exposures, the whales were in shallow dive mode at sonar onset, with all but one remaining in shallow mode throughout the exposure period. One whale (154d) switched to ShD with occasional dives greater than the 34 m criterion, but still shallower than 100 m depth. In the remaining five LFAS-exposures, the animals were in DD mode at sonar onset, with all but one (156b) shifting to shallow mode during LFAS exposure (**Figure 6**). These shifts were accompanied by increased dive rate for shallow dives and decreased rate for deep dives, with the shallow dives generally being deeper and of longer duration (**Figure 6**).

MFAS

A total of six MFAS exposures were conducted with six different long-finned pilot whales. Four of the six whales were in shallow dive mode at sonar onset, of which three stayed in shallow dive mode, and one initiated deep dive mode during MFAS exposure (156b). The remaining two whales were in deep dive mode at sonar onset, of which one continued DD (159a). The other (154d) had been conducting dives greater than 34 m though

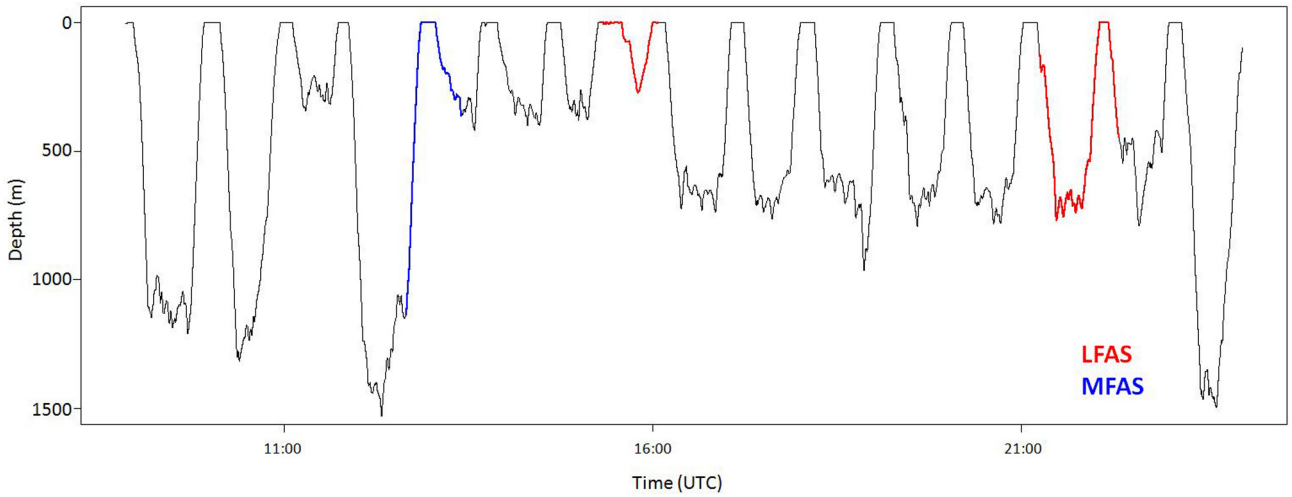

FIGURE 4 | Full dive record of sperm whale sw09_160a. Record show typical sperm whale dive behavior (Teloni et al., 2008) with deep dives followed by a surface period. During MFAS exposure (blue), the animal switched from normal deep dives to shallower but still deep diving. During the first LFAS exposure (red), the animal conducted an unusually short and shallow dive after a long surface period. During the second LFAS exposure the whale conducted what appears as a normal deep dive.

shallower than 100 m, and shifted to ShD during MFAS exposure (**Figure 6**).

Silent control

Four silent exposures were conducted with four different long-finned pilot whales. In three of four exposures, whales were in DD mode at onset and continued DD throughout the exposure period. One whale (159a) was in ShD mode at the onset, and initiated DD during exposure (**Figure 6**).

SPERM WHALES

Sperm whales showed stereotyped dive behavior with regular DD to depths of 200–1500 m, with an average dive duration of 25 min followed by a period of 5–15 min of ShD close to the surface. The log-frequency analysis demonstrated 13 m as the separation depth for shallow vs. deep dives of sperm whales. The only dive mode observed for sperm whales was continuous deep dives with short inter-deep-dive intervals. An example of a sperm whale dive record is shown in **Figure 4**.

LFAS

A total of six LFAS exposures were conducted with four different whales. Prior to all six exposures whales did normal deep diving (NDD). During four of the six LFAS exposures, whales shifted to UsDD, with all of these exposure deep dives being on average shallower and of shorter duration then deep dives in the PRE period (**Figure 7**). One of the whales subjected to a second exposure continued NDD during LFAS.

MFAS

A total of four MFAS exposures were conducted to four different whales. Three conducted NDD during MFAS exposure. The other (sw141a) was conducting relatively shallow foraging dives in the PRE period, and initiated a normal deep dive during MFAS (**Figure 7**).

Silent control

Only two silent exposures were conducted with two different sperm whales in two different sessions, both with NDD in the PRE period. One of the whales (sw141a) made an unusually-shallow deep dive during Silent Exposure, while the other continued NDD (**Figure 7**).

DISCUSSION

In this study, we investigated three species with very different natural dive patterns. These differences indicate spatial niche separation in their foraging strategies. Killer whales spend 90% of their time in the upper 20 m, and hardly ever dive deeper than 100 m (**Figures 1, 2** and **5**), while sperm whales spend 80% of their time diving deeper than 20 m, with foraging dives ranging from 150 to 1500 m (**Figures 1, 4** and **7**). Long-finned pilot whales also spend most of their time close to the surface but typically conducted bouts of foraging dives to intermediate depths of 300–600 m (**Figures 1, 3** and **6**).

When in DD mode at sonar onset, both killer and long-finned pilot whales apparently reacted to LFAS transmission by switching to ShD. Based on recordings of echolocation clicks and tail slap sounds on the tag record (Miller et al., 2011), such deep dives are likely to be foraging dives. Shallow dives on the other hand did not have any such vocalizations associated (Miller et al., 2011) and these dives are therefore likely not associated with feeding. A change from deep to ShD during LFAS exposure are thus likely to involve cessation of feeding. Shifts from deep to ShD for both killer- and pilot whales were associated with a general increase in maximum depth and duration of shallow dives. This indicates that shallow dives have different purposes in different behavioral modes. During periods of deep foraging dives, shallow dives may be driven by respiratory needs. When the animal is in ShD mode, as when traveling, the depth and duration of the dives are likely optimized for energy-efficient swimming and there might be an

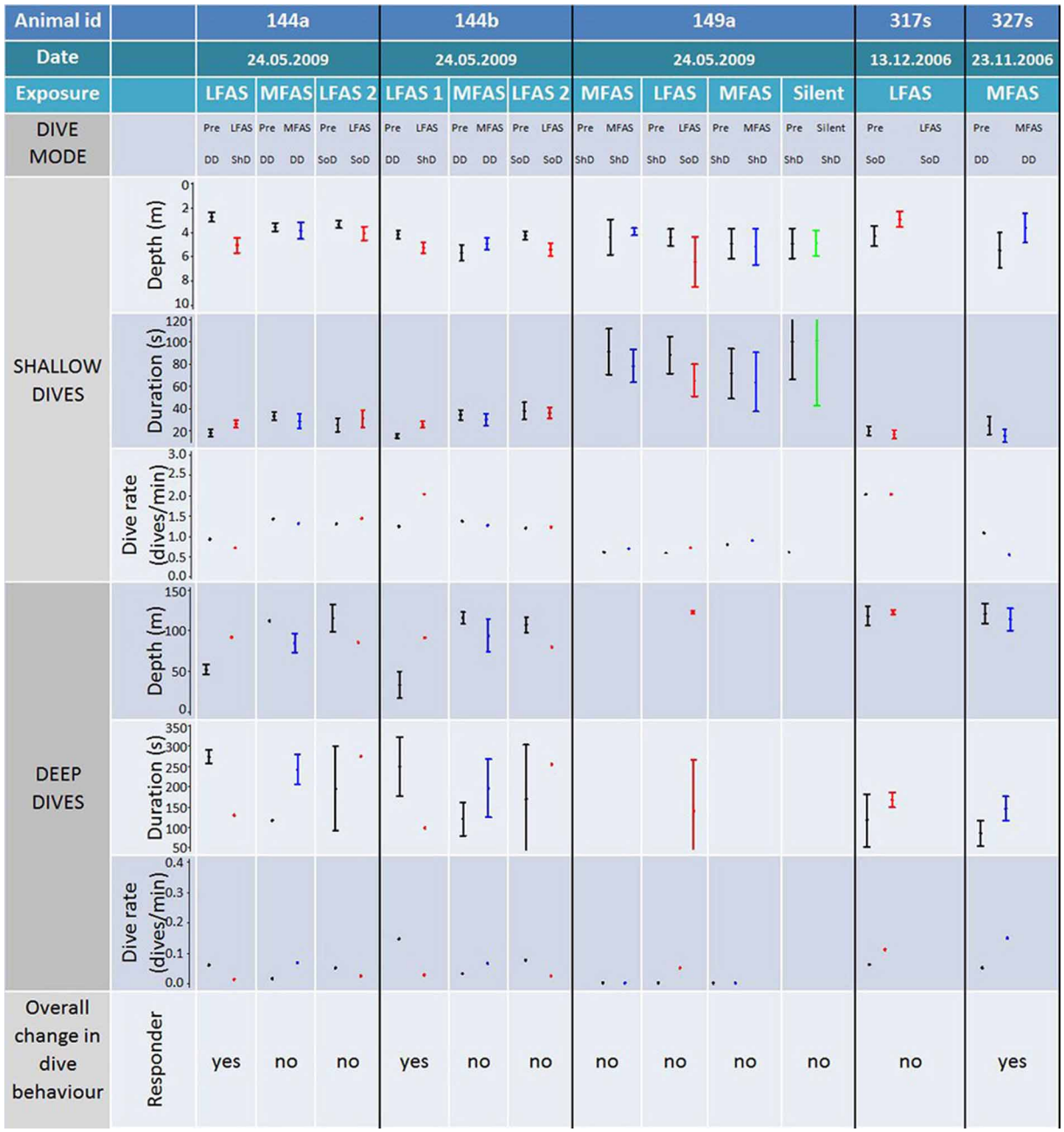

FIGURE 5 | Comparison of individual exposures to killer whales. Figure shows animal id, exposure type, dive mode (during PRE and Exposure), as well as dive depth, duration, and rate for both shallow and deep dives. Log frequency analysis defined deep and shallow dives to be deeper and shallower than 21 m respectively for killer whales. Codes for dive modes are: deep diving (DD), shallow diving (ShD) and shallow diving with occasional deep dive (SoD). Bars show the 95% confidence interval. Dive rate for deep and shallow dives are number of dives in the PRE/Exposure period divided on the duration of the period, given as dives/min. All individual exposure sessions are classified either as a "responder" or not, based on the combined comparison of the depth, duration rate, and mode of the PRE and Exposure period.

energetic (e.g., hydrodynamic) benefit of diving somewhat deeper to avoid the surface drag.

Individuals of both species that were in ShD mode at LFAS onset generally continued ShD without any change in dive behavior. Tag recordings from these shallow dives did not record echolocation or tail slaps (Miller et al., 2011), indicating shallow mode to be associated with travel or resting rather than feeding. However, if the response of the animals to sonar is to travel away from the sonar source, as described by Miller et al. (submitted) for several of these whales, continuing to travel could also be considered a response, especially if directed away from the source (Miller et al., 2011).

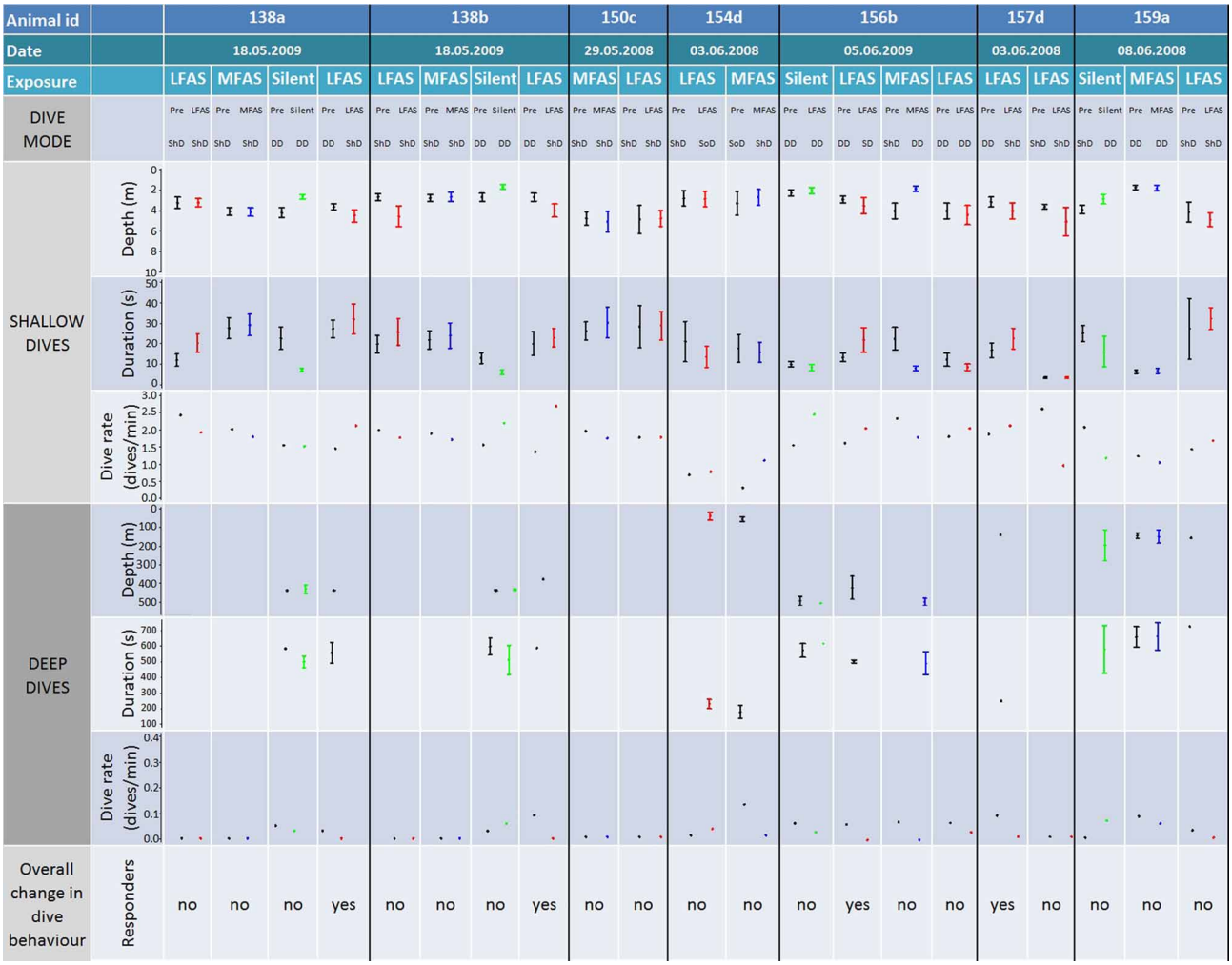

FIGURE 6 | Comparison of individual exposures to long-finned pilot whales. Figure shows animal id, exposure type, dive mode (during PRE and Exposure), as well as dive depth, duration, and rate for both shallow and deep dives. Log frequency analysis defined deep and shallow dives to be deeper and shallower than 34 m respectively for killer whales. Codes for dive modes are: deep diving (DD), shallow diving (ShD) and shallow diving with occasional deep dive (SoD). Bars show the 95% confidence interval. Dive rate for deep and shallow dives are number of dives in the PRE/Exposure period divided on the duration of the period, given as dives pr min. All individual exposure sessions are classified either as a "responder" or not, based on the combined comparison of the depth, duration rate, and mode of the PRE and Exposure period.

The reaction to MFAS was somewhat different for killer and long-finned pilot whales. Long-finned pilot whales conducted normal, deep dives during MFAS exposure (**Figure 6**) with recordings of foraging vocalization (Miller et al., 2011), indicating them to be foraging dives. For killer whales, one individual (327s) was in DD mode at onset of MFAS sonar, and these dives were confirmed as foraging dives by tag recording of echolocation and tail slaps in the PRE period as well as visual observations of feeding at the surface (Miller et al., 2011). The whale kept making deep dives until halfway into the exposure, before changing to ShD. The deep dives performed in the first half of the exposure lacked foraging sounds on the tag record (Miller et al., 2011), indicating cessation of feeding.

For killer and long-finned pilot whales, changes in diving behavior were most likely to occur if the animals were conducting deep foraging dives at exposure onset. Those whales that changed their dive behavior were not always those subject to the highest received sound pressure levels (Miller et al., 2011), indicating that behavioral mode may be as important as sonar exposure level in determining whether the animal will change its diving behavior or not. These findings agree with some previous studies of other cetaceans. Wartzok et al. (2004) showed that whether belugas (*Delphinapterus leucas*) responded to anthropogenic noise depended more on their activity and motivation rather than sound exposure level. Right whales (*Eubalaena glacialis)* exposed to alerting stimuli interrupted foraging dives (Nowacek et al., 2004), and killer whales changed from feeding to traveling in response to ship noise (Lusseau, 2009). Bowhead whales (*Balaena mysticetus*) differed in response to seismic shooting depending on whether the whales were

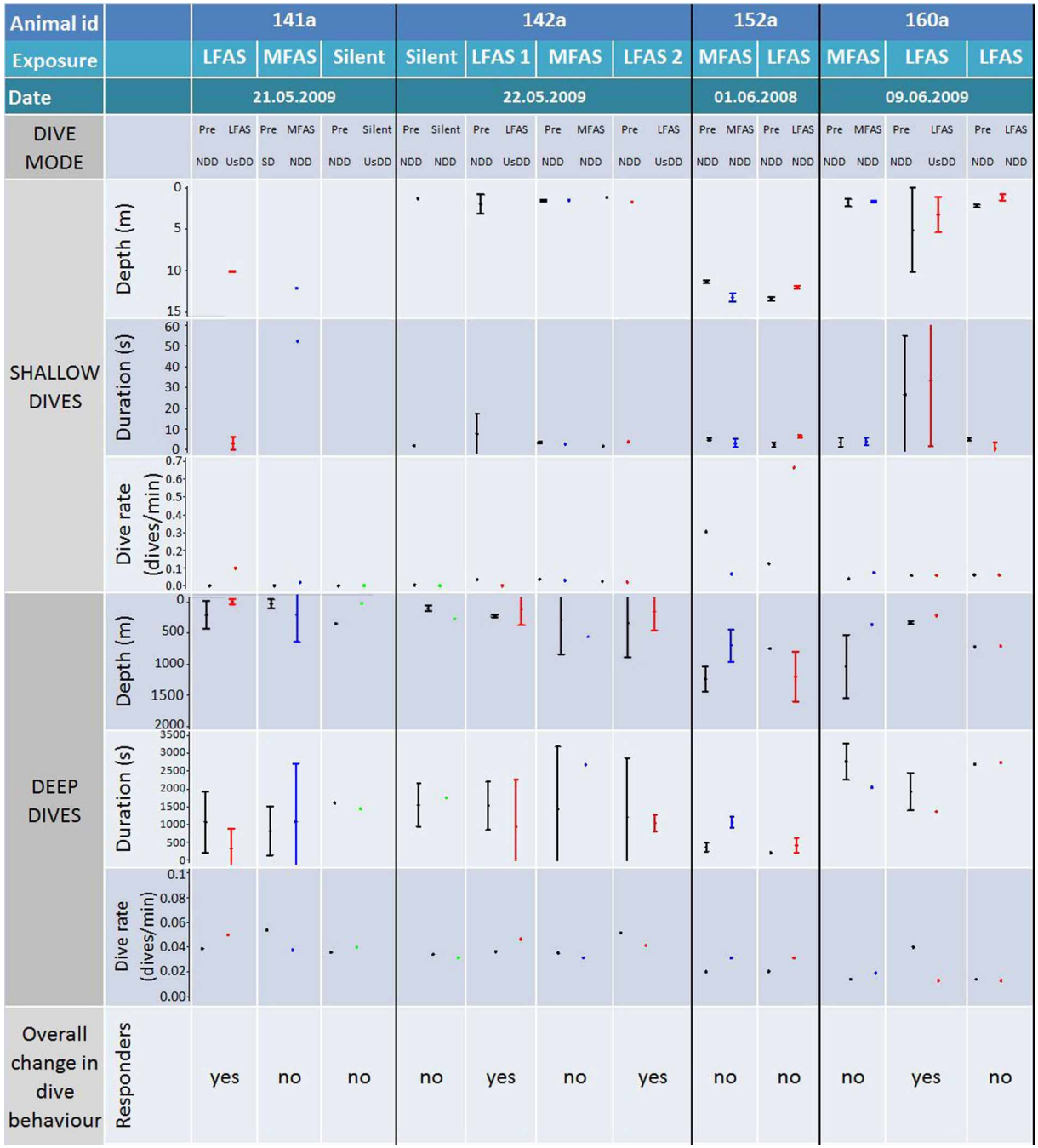

FIGURE 7 | Comparison of individual exposures to sperm whales. Figure shows animal id, exposure type, dive mode (during PRE and Exposure), as well as dive depth, duration, and rate for both shallow and deep dives. Log frequency analysis defined deep and shallow dives to be deeper and shallower than 13 m respectively for killer whales. Codes for dive modes are: shallow diving (ShD), normal deep diving (NDD), and unusual shallow deep diving (UsDD). Bars show the 95% confidence interval. Dive rate for deep and shallow dives are number of dives in the PRE/Exposure period divided on the duration of the period, given as dives pr min. All individual exposure sessions are classified either as a "responder" or not, based on the combined comparison of the depth, duration rate, and mode of the PRE and Exposure period.

feeding (Miller et al., 2005) or migrating (Richardson et al., 1999).

Sperm whales continued to dive deep during LFAS, but these dives appeared to be unusually shallow compared to dives in the PRE period (**Figure 7**). Deep dives during LFAS exposure were also reported to rarely contain any vocal activity (Miller et al., 2011), indicating they were not foraging dives. During MFAS exposure, sperm whales made NDD for animals in this

area (Teloni et al., 2008) with vocalizations associated with feeding, indicating normal foraging activity (Miller et al., 2011). The changes in dive behavior of sperm whales during LFAS transmission agree with previous studies showing sperm whales to silence during exposure of continuous low frequency (∼50 Hz) transmission (Bowles et al., 1994), as well as pingers of higher frequency (6–13 kHz) (Watkins and Schevill, 1977), while Madsen et al. (2002a) found no effect on vocal behavior of sperm whales in response to seismic surveys. Studies of sperm whale diving behavior in response to seismic air guns showed one case of abnormally long resting behavior near the sea surface during exposure at high SPL (Miller et al., 2009), and Madsen et al. (2002c) suggested variations in received levels as explanation for variations in vocal responses to anthropogenic noise.

BIOLOGICAL IMPLICATIONS OF CHANGES IN DIVE BEHAVIOR

All three species showed examples of changes in dive behavior during LFAS exposure and for killer whales also during MFAS exposure, which imply disruption of feeding activity. The change in diving was often associated with reduced echolocation vocalizations or complete silencing (Miller et al., 2011), confirming feeding cessation. Lost feeding opportunities could have significant biological effects depending on food availability and the duration of the exposure. This might be particularly severe for killer whales which feed on distinct food patches such as herring schools (Simila et al., 1996; Nøttestad et al., 2002), which might be lost if feeding is disrupted. Pilot (Weilgart and Whitehead, 1990) and sperm whales (Watwood et al., 2006) depend on sound production for prey localization and capture as they forage at depth. Both the LFAS and MFAS signals overlap in frequency with some of the foraging sounds of killer whales (Hoezel and Osborne, 1986; Ford, 1989), long-finned pilot whales (Taruski, 1979; Weilgart and Whitehead, 1990) and sperm whales (Madsen et al., 2002b,c). Therefore, sonar signals might disturb important communication between individuals during feeding, but masking of the calls will be limited because of the low duty cycle (5%) of the sonar. All three species showed stronger changes during LFAS than MFAS signals. This may be due to the difference in frequency, or it may be explained by the higher source level of the LFAS signals.

It has been proposed that a change in dive behavior in response to sonar could increase the end dive N_2 levels and risk of tissue bubble formation in cetaceans (Jepson et al., 2003). How the observed changes in dive behavior modify this risk is not obvious. Using a previously published model (Fahlman et al., 2006) on the same dataset as described here, Kvadsheim et al. (2012) investigated how the observed changes in behavior alter the end-dive N_2 levels, and thereby the risk of DCS. They found that the shallower DD seen in sperm whales implied an increased risk, but the change in risk was still within the normal risk range for this species. This agrees with the results in stranded animals that indicate a higher prevalence of bubbles with a gas composition associated with decompression stress in deep divers such as sperm whales and beaked whales (Bernaldo de Quiros, submitted). The changes from deep to ShD mode and the shallow dives becoming deeper seen in killer whales and long-finned pilot whales resulted in reduced risk of DCS (Kvadsheim et al., 2012). Other theoretical studies have suggested that repetitive ShD might involve an increased risk (e.g., Zimmer and Tyack, 2007). Even if the dives were deeper they were still quite shallow (>30 m) and therefore probably still within the depth zone were N_2 is removed from the body.

Killer whales and long-finned pilot whales appear to show less changes in diving behavior during LFAS and MFAS exposure sessions during traveling mode compared to feeding mode. However, if the response of the animals to sonar is to travel away from the sonar source, as described by Miller et al. (2011), continuing to travel could also be considered a response, especially if directed away from the source (Miller et al., 2011). However, a change in travel direction during a continuous traveling state may not lead to any changes in diving behavior, the behavior examined here.

For all three species in DD mode, initial exposure to LFAS altered diving mode, resulting in likely feeding cessation or lack of conducting foraging dives. Whales subjected to a second LFAS exposure even in deep dive mode did not consistently alter their behavior, implying that there might be some habituation to the sonar in some cases.

The observed change in behavior would not result in any detrimental effects for the exposure experiments presented here, lasting only ∼30 min. However, an authentic naval exercise may involve much longer periods of sonar exposure, e.g., 24 h of continuous sonar transmission (Friedman, 2006; Ainslie, 2010) or several days in the case of large international fleet exercises, and if foraging dives are not performed throughout this period, and such events are frequent, the effect will be much more severe.

METHODOLOGICAL CONSIDERATIONS

The costly and complex logistical requirements for conducting experiments like those in the current study highly limit the number of replicates. The sample size for each species, experimental condition and behavioral mode, therefore will be low. Small sample sizes are prone to risk of conducting type II statistical errors; i.e., accepting a null hypothesis when it should be rejected. Additionally, the need for testing each species, exposure type behavioral mode will need many similar tests to be conducted. Such multiple testing results in a high risk of conducting type I statistical error, i.e., obtaining a significant change when there in fact is none due to the multiple testing. Such statistic would hence not be very reliable. Therefore, a descriptive approach have been taken instead, by presenting our results more as a case-by-case interpretation for each individual exposure, by taking into account the different parameters dive mode, depth, duration, and dive rate and comparing data during each exposure session to the animal's own baseline (the PRE period).

Two of the 12 experiments with killer whales were conducted in winter [327 s (MFAS) and 317 s (LFAS)], the reminder in summer. Dive depth may vary between seasons due to variations in distribution depth of prey, and feeding dives recorded in this study were deeper in summer compared to in winter. Feeding killer whales were exposed once in winter to MFAS and once in summer to LFAS, with the LFAS summer experiment resulting in an abrupt cessation of feeding and change to ShD early in the sonar exposure session,

while in the winter MFAS experiment the whales continued DD and feeding at least halfway into the exposure. However, it cannot be determined whether it was the difference in transmission types, seasonal differences, or other sources of variability of responsiveness that cause the difference in response onset.

Some animals were subject to two exposures of the same type (**Figures 5–7**). The response to the second exposure may thus be influenced by the animals prior experience with this signal, causing either a sensitization or habituation, depending on whether the signal was perceived as a real threat or not. Killer whales 144a and 144b did not resume deep foraging dives after the first LFAS exposure, and the observed lack of response to the second exposure may therefore be influenced by the animals already being in travel mode. In contrast, both pilot and sperm whales tended to resume deep foraging dives between exposure sessions. Long-finned pilot whales 138a and 138b were conducting shallow travel dives at onset of the first LFAS exposure, continuing this behavior, while in the second exposure they were conducting deep foraging dives at LFAS onset, but then switched to shallow travel dives. Pilot whale 157d was conducting deep foraging dives at onset of the first LFAS exposure, switching to shallow travel dives, while during the second LFAS exposure the whale was conducting ShD, and continued this. However, pilot whale 156b was conducting deep foraging dives prior to both LFAS exposures, but switched to shallow travel dives only for the first exposure. Both of the sperm whales subject to multiple LFAS exposures were conducting normal deep foraging dives prior to all LFAS exposures. They both responded by switching to unusual shallow deep dives without foraging during the first LFAS, and this was also the case for the second exposure for sperm whale 142a for the second exposure. Sperm whale 160a did however continue NDD in the second exposure. The present result does not show any clear signs of habituation or sensation, but one should however always treat the second exposure with care.

CONCLUSIONS

The present study have shown that killer whales that are feeding at onset of exposure may change their diving behavior by switching from deep feeding dives to shallow travel dives when exposed to LFAS (1–2 kHz) and MFAS (6–7 kHz) naval sonar signals. When traveling at sonar onset however, no changes in dive behavior were found. Long-finned pilot whales and sperm whales performed normal deep foraging dives during MFAS exposure, while during LFAS exposures, long-finned pilot whales performed fewer deep foraging dives and some sperm whales performed shallower and shorter dives without foraging.

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