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RESEARCH ARTICLE

Solubility of nitrogen in marine mammal blubber depends on its lipid composition

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SUMMARY

Understanding the solubility of nitrogen gas in tissues is a crucial aspect of diving physiology, especially for air-breathing tetrapods. Adipose tissue is of particular interest because of the high solubility of nitrogen in lipids. Surprisingly, nothing is known about nitrogen solubility in the blubber of any marine mammal. We tested the hypothesis that N_2 solubility is dependent on the lipid composition of blubber; most blubber is composed of triacylglycerols, but some toothed whales deposit large amounts of waxes in blubber instead. The solubility of N_2 in the blubber of 13 toothed whale species ranged from 0.062 to 0.107 ml N_2 ml^{-1} oil. Blubber with high wax ester content had higher N_2 solubility, observed in the beaked (Ziphiidae) and small sperm (Kogiidae) whales, animals that routinely make long, deep dives. We also measured nitrogen solubility in the specialized cranial acoustic fat bodies associated with echolocation in a Risso's dolphin; values (0.087 ml N_2 ml^{-1} oil) were 16% higher here than in its blubber (0.074 ml N_2 ml^{-1} oil). As the acoustic fats of all Odontocetes contain waxes, even if the blubber does not, these tissues may experience greater interaction with N_2. These data have implications for our understanding and future modeling of diving physiology in Odontocetes, as our empirically derived values for nitrogen solubility in toothed whale adipose were up to 40% higher than the numbers traditionally assumed in marine mammal diving models.

Key words: Odontocetes, decompression sickness, wax ester.

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INTRODUCTION

The interactions between nitrogen gas (N_2) and biological tissues during diving play major roles in constraining diving behavior. Adipose tissue is of particular concern, because N_2 gas is five times more soluble in lipids than it is in water (Langø et al., 1996; Weathersby and Homer, 1980), and thus at any blood/fat interface gas will diffuse out of circulation and into adipocytes, a relationship that can lead to increased risk of decompression sickness (DCS). DCS occurs when N_2 dissolved in tissues comes out of solution too rapidly during decompression and forms bubbles (emboli), causing cellular disruption and dysfunction in multiple organs and potentially death (see Doolette and Mitchell, 2001). Not surprisingly, increased DCS risk has been linked to obesity in humans and other animals (Carturan et al., 2002; Dembert et al., 1984; Douglas, 1985; Lam and Yau, 1989; Rattner et al., 1979) as adipose can act as a 'sponge' for nitrogen gas – absorbing it in large quantities during dives, but releasing it, potentially too quickly, during decompression.

Marine mammals make an interesting model for studying nitrogen gas dynamics because they are superlative divers with enormous fat deposits. These animals can make dives more than 1800 m deep with durations exceeding 90 min (Kooyman et al., 1981; Tyack et al., 2006; Watwood et al., 2006). The thick blubber layer that surrounds the body can comprise more than 30% of body mass (see Koopman, 2007; Pond, 1998), which for a human diver would be considered high risk. Despite their enormous fat deposits, marine mammals are thought to be protected from DCS risk because of a combination of the mammalian dive response and alveolar collapse, both of which act to limit circulation and exchange of nitrogen gas during diving (Kooyman and Ponganis, 1998). In addition, breath-hold divers such as Odontocetes only take down one lungful of air, which will also limit nitrogen intake. However, recent observations of emboli in tissues of stranded marine mammals have raised questions about the exact nature of nitrogen gas dynamics in these animals. Some authors (Jepson et al., 2003; Jepson et al., 2005) have offered post-mortem evidence that toothed whales do experience DCS, and others (Tyack et al., 2006; Zimmer and Tyack, 2007) have proposed through modeling of dive data that a disruption of normal diving patterns may interfere with normal nitrogen gas dynamics. One of the difficulties in considering how the adipose tissues of diving mammals interact with nitrogen gas, and the degree to which bubble formation is possible, is the complete lack of empirical data on the solubility of nitrogen in these tissues. Indeed, models of N_2 gas compartmentalization and saturation in diving mammals (e.g. Fahlman et al., 2006) assume fat solubility values for nitrogen obtained from biological oils (plant based) or dog and pig tissues (Weathersby and Homer, 1980), which we think may underestimate the saturation potential in toothed whales (see below).

The chemical composition of odontocete blubber shows a high degree of interspecific variation (Litchfield et al., 1975; Koopman, 2007). In most vertebrates, including many odontocetes, triacylglycerols (TAG; three fatty acids esterified to a glycerol backbone) are the most common, and usually the only, form of lipid storage molecule in adipose tissue (Pond, 1998). However, in the deepest diving whales, the blubber is dominated by wax esters (WE; a fatty acid esterified to a fatty alcohol), rather than TAG (Koopman, 2007; Litchfield et al., 1975). WE have very different physical and biological properties from TAG and are also difficult to synthesize and metabolize, and thus do not represent an optimal way to store excess lipid energy (see Koopman, 2007). The presence of high concentrations of WE (62–100% of all lipids) in the blubber of sperm

(families *Physeteridae* and *Kogiidae*) and beaked (family *Ziphiidae*) whales (Koopman, 2007) has puzzled biologists for decades and currently the function of these odd lipids, and why they occur in the blubber of these families, remains unknown. We were interested in examining whether WE might exhibit different nitrogen gas dynamics to TAG, which could have implications for diving physiology in the very deep divers. Unfortunately, nitrogen solubility data for wax esters do not exist.

There are surprisingly few studies of N_2 gas solubility in any lipid compounds, with most of the literature having emerged before the 1970s. This is largely a consequence of methodology: solubility of gases in fluids was traditionally quantified using a modified version of a van Slyke apparatus, an intricate instrument developed in 1912 composed of blown glass tubes filled with volumes of mercury (van Slyke and Neill, 1924). These instruments yield very reliable solubility measurements and are still in use today (e.g. Houser et al., 2010); however, their use is somewhat limited as so few remain in existence. The empirical studies that have been carried out on lipids (for reviews, see Langø et al., 1996; Weathersby and Homer, 1980) indicate that manufactured oils of different biochemical composition have different N_2 solubility coefficients (e.g. N_2 solubility in butter and olive oil are 0.10 and 0.07 ml gas ml^{-1} medium, respectively – butter contains more saturated fatty acids, while olive oil has more monounsaturated fatty acids). All of the tested materials in the literature contain TAG; there are no data describing nitrogen solubility in any other class of lipids (thus no data for WE) and very few measures for fatty tissues from any animal. Our main goal was to measure nitrogen solubility in oils extracted from marine mammal blubber from a wide range of species, contrasting blubber composed mainly of TAG with that containing WE, and to compare these values with published data on commercial oils and animal tissues. Investigation of the relationships between tissue characteristics and diving physiology in animals such as beaked and sperm whales is particularly timely in the light of mass stranding events, some of which have been associated with naval sonar activities, in which evidence of emboli has been reported (e.g. Fernández et al., 2005). As part of our study, we developed a new method for measuring nitrogen solubility in oils, which is described in the Appendix.

MATERIALS AND METHODS

Specimens

Blubber from a wide variety of toothed whales was tested, and included the following species (listed by family for ease of interpretation in Table 1, and with species short-forms used in Fig. 2 in parentheses): Phocoenidae (porpoises) – Dall's porpoise *Phocoenoides dalli* (True) (*Pd*), harbour porpoise *Phocoena phocoena* (Linneaus) (*Pp*); Delphinidae (dolphins) – common dolphin *Delphinus delphis* Linnaeus (*Dd*), short-finned pilot whale *Globicephala macrorhynchus* Gray (*Gm*), Risso's dolphin *Grampus griseus* (Cuvier) (*Gg*), northern right whale dolphin *Lissodelphis borealis* Peale (*Lb*), Pacific white-sided dolphin *Lagenorhynchus obliquidens* (Gill) (*Lo*), bottlenose dolphin *Tursiops truncatus* (Montagu) (*Tt*); Kogiidae (small sperm whales) – pygmy sperm whale *Kogia breviceps* Blainville (*Kb*), dwarf sperm whale *K. sima* Owen (*Ks*); and Ziphiidae (beaked whales) – Gervais' beaked whale *Mesoplodon europaeus* (Gervais) (*Me*), True's beaked whale *M. mirus* True (*Mm*), Cuvier's beaked whale *Ziphius cavirostris* Cuvier (*Zc*). We used one adult specimen per species as this was meant to be an initial survey of blubber containing differing amounts of TAG and WE. Blubber samples were obtained from stranded animals through marine mammal stranding networks across the USA. Only animals deemed to be in good body condition (i.e. not emaciated), and specimens of Smithsonian Code 2 or lower (Geraci and Lounsbury, 1993), were included to ensure tissue quality was not compromised. All samples had been frozen at –20°C prior to analysis.

Lipid composition analysis

Lipids were extracted from blubber using a modified Folch procedure (Folch et al., 1957; Koopman et al., 1996). Blubber samples were first trimmed to remove any desiccated tissues and all epidermis and muscle/connective tissue. The bulk of the extracted oil was used for the nitrogen solubility measurements as described above, and a small aliquot (~0.5 ml) was reserved for analyses of lipid profiles. Lipid classes were separated and quantified *via* thin-layer chromatography-flame ionization detection (TLC-FID). Samples were spotted on chromarods (Chromarod-SIII, Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) and developed in 94/6/1 hexane/ethyl acetate/formic acid. Classes were then quantified as % of total lipid mass by TLC-FID using an Iatroscan MK-6 (Mitsubishi Kagaku Iatron Inc.). Identification was confirmed through the use of lipid class standards (Nu Chek Prep, Elysian, MN, USA). Peaks were integrated with Peaksimple software (Peaksimple 3.29, SRI Instruments, Torrance, CA, USA). For gas chromatography (GC) analysis, fatty acid butyl esters (FABE) were prepared from total lipid extracts (see Koopman et al., 1996). Butyl esters were used as opposed to methyl esters (more commonly used) because short-chain fatty acids are volatile (Koopman, 2007). Fatty acids/alcohols were analyzed by GC using a Varian capillary gas chromatograph (3800; Varian Inc., Walnut Creek, CA, USA) with a flame ionization detector (FID) in a 30 m × 0.25 mm column coated with nitroterephthalic acid modified with polyethylene glycol (Zebron, Phenomenex, ZB-FFAP column, Torrance, CA, USA) with He as the carrier gas. The following temperature program was used: 65°C for 2 min, hold at 165°C for 0.40 min after ramping at 20°C min^{-1}, hold at 215°C for 6.6 min after ramping at 2°C min^{-1}, and hold at 250°C for 5 min after ramping at 5°C min^{-1}. Up to 80 different fatty acids/alcohols were identified (see Koopman, 2007; Koopman and Zahorodny, 2008). Peak identification was based on comparisons of retention time to standards (Nu Chek Prep) and known samples. Peaks were integrated using appropriate response factors (Ackman, 1991) with a Galaxie Chromatography Data System (Version 1.8.501.1, Varian Inc.), and peak identification was manually confirmed for each run.

Nitrogen solubility

The solubility of N_2 gas in each oil was measured using a method described previously (Snedden et al., 1996) but with significant alterations for use on a standard gas chromatograph with a thermal conductivity detector (TCD). Oils were gently perfused with N_2 gas inside a sealed glass syringe, which was located within an argon environment (sealed glove box) to avoid atmospheric contamination. N_2-saturated oil was transferred to an argon-filled headspace vial and incubated at 37°C. The resulting concentrations of N_2 and Ar in a headspace vial post-incubation were quantified by GC with TCD, and solubility was calculated as Ostwald coefficients (ml gas dissolved ml^{-1} oil) using mass balance and standard curves derived from injections of pure N_2 gas. Prior to investigating marine mammal oils, we carried out rigorous QA/QC on our system using olive oil as the test material. Specific details on the apparatus and the methodology can be found in the Appendix.

The relationship between WE content and N_2 gas solubility values was tested using linear regression. To explore broad relationships

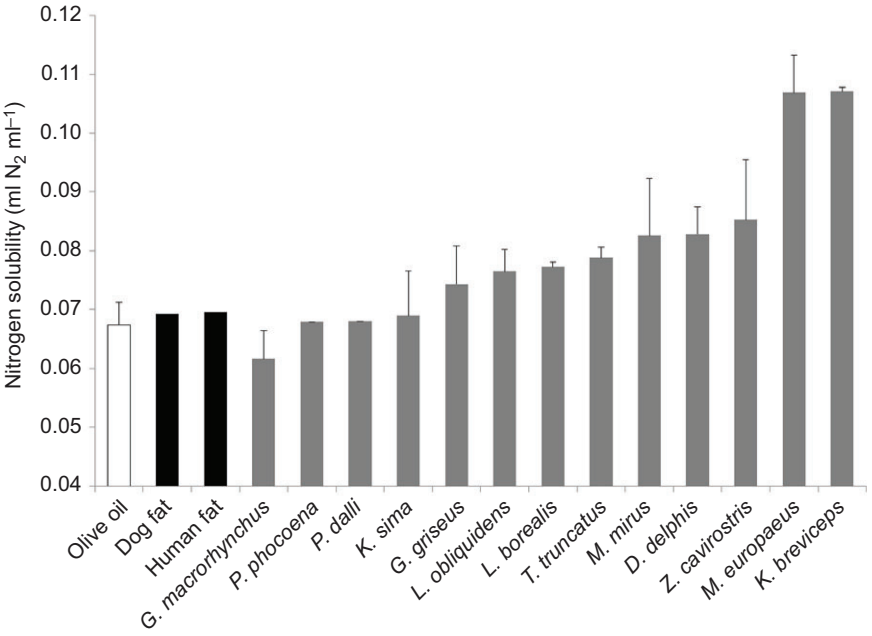

Fig. 1. Mean nitrogen solubility values in blubber from 13 species of marine mammals. Error bars represent 1 s.d. Also shown are the mean values for olive oil (white bar) and for dog and human fat (from Ikels, 1964) (black bars) for comparison.

between nitrogen solubility and lipid chemistry, fatty acid and fatty alcohol components were grouped into saturated (no double bonds), monounsaturated (one double bond) and polyunsaturated (two or more double bonds) categories; these categories were regressed against solubility values using α=0.05 as the significance level.

RESULTS

Nitrogen solubility of marine mammal blubber

The mean (±s.d.) N_2 solubility value for the olive oil we tested was 0.0674±0.0038 ml N_2 ml^{-1} oil. The mean N_2 solubility value for all blubber samples was 0.079±0.0136 ml N_2 ml^{-1} oil. However, there was considerable variation across species (Fig. 1). The lowest solubility value of 0.0616±0.0048 ml N_2 ml^{-1} oil was obtained from the pilot whale (*G. macrorhynchus*), while both Gervais' beaked whale (*M. europaeus*, 0.1069±0.0063 ml N_2 ml^{-1} oil) and the pygmy sperm whale (*K. breviceps*, 0.1071±0.0007 ml N_2 ml^{-1} oil) had values more than 40% higher. Only one of the 13 species (*G. macrorhynchus*) had N_2 solubility values lower than the value obtained for olive oil in this study. Three species had values close to, and only slightly higher than, olive oil (*P. phocoena*, *P. dalli* and *K. sima*, range 0.0679–0.0690 ml N_2 ml^{-1} oil). The other nine species tested all had mean estimated solubility values >0.07 ml N_2 ml^{-1} oil (Fig. 1).

Nitrogen solubility and lipid composition

Overall, there was a positive relationship between WE content and N_2 solubility. At the family level there was a trend of increasing solubility with higher amounts of WE (Table 1). The beaked whales (Ziphiidae) exhibited the greatest mean solubility values overall (0.092 ml N_2 ml^{-1} oil) and blubber composed almost entirely of WE (99.4% of total lipid mass), in contrast to the porpoises (Phocoenidae), which had no WE (0%) and the lowest mean solubility value (0.068 ml N_2 ml^{-1} oil). Differences among families were not significant for solubility (P=0.355), but WE content was significantly higher (P<0.0001) in the Ziphiidae and Kogiidae than it was in Delphinidae and Phocoenidae (Table 1). On the individual species level, those with higher amounts of WE in their blubber had some of the higher solubility values (Fig. 2). Species with little or no WE in their blubber exhibited a smaller range of solubility values than did the wax-containing species. Linear regression analysis indicated that the relationship between these two variables was positive and significant (P=0.003), but that only about one-half of the variation in solubility for each species could be explained by WE content alone (R^2=0.52). There were no significant relationships between fatty acid/alcohol bond number and nitrogen solubility (all P>0.5; data not shown), meaning that N_2 gas solubility was independent of fatty acid/alcohol composition.

DISCUSSION

We present here the first empirical measurements of nitrogen gas solubility in marine mammal tissues. Our data indicate that the solubility of N_2 gas in the blubber of some toothed whale species is much higher than that of olive oil (often a 'model' oil for biological systems), higher than values reported for adipose tissues of other mammals and, most importantly, higher than values used in physiological models designed to explain and predict the behavior of nitrogen gas in the bodies of marine mammals during diving (e.g. Fahlman et al., 2006; Zimmer and Tyack, 2007). We also show that the chemical composition of the lipids in blubber – namely, the presence of wax esters – has a strong influence on its N_2 solubility. The overall observation from our dataset is that blubber containing WE tended to have higher nitrogen solubility values than blubber without WE; all of our higher solubility values were obtained from species with blubber dominated by WE. These data can shed light on the physiological adaptations possessed by some toothed whales, and have implications for future modeling efforts in diving physiology. Our study also emphasizes that a single reference value for the solubility of nitrogen in mammalian 'fat' is not appropriate for all species. We anticipate that this study will provide a foundation for future work aimed at examining nitrogen solubility in the tissues of marine mammals and in other diving animals, such as penguins, seals and turtles, and could also have applications for studies of human diving physiology.

Nitrogen solubility in toothed whale blubber

Given the fact that the olive oil solubility values we obtained with our new methodological approach (mean 0.0674 ml N_2 ml^{-1} oil; see Appendix for details on apparatus design and testing) were within

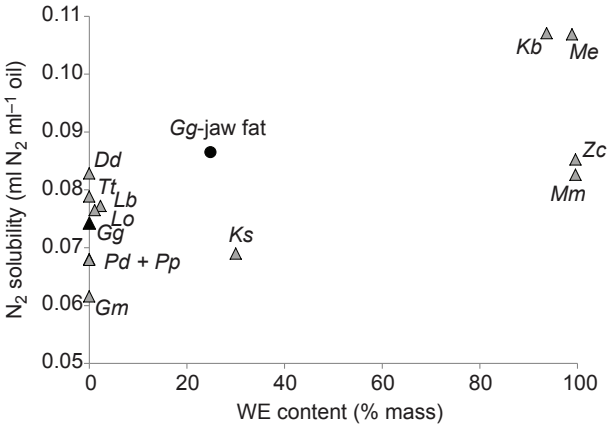

Fig. 2. N_2 solubility as a function of wax content in the blubber of toothed whales. The overall linear relationship between N_2 solubility and wax ester (WE) content was significant (P=0.003; R^2=0.52). Species names are coded as *Gs* (*Genus species*); see Materials and methods for full genus and species names. Also included on the figure are data from a sample of acoustic fat (jaw fat; black circle) from the same *Grampus griseus* (*Gg*) specimen that the blubber was analyzed from for this species (black triangle).

the range of Ostwald values previously reported in the literature (0.067–0.075 ml N_2 ml^{-1} oil) for this material (Abraham and Ibrahim, 2006; Battino et al., 1968; Ikels, 1964; Langø et al., 1996; Snedden et al., 1996; Vernon, 1907; Weathersby and Homer, 1980), we are confident that the data we report here do accurately represent the solubility of nitrogen gas in blubber oils. Unfortunately, there is little to compare our values with, as methodological constraints have severely limited the amount of nitrogen solubility information available in the literature. Data for commercial biological oils are few and fairly similar (cod liver oil 0.073, corn oil 0.07, cottonseed oil 0.071 and lard 0.067 ml N_2 ml^{-1} oil) (Langø et al., 1996). These values all hover around 0.07 ml N_2 ml^{-1} oil, likely explaining why this is the value commonly used for 'fat' in models of gas dynamics (Davidson et al., 1952; Fahlman et al., 2006; Zimmer and Tyack, 2007).

It is important to note that solubility values for pure oils do not represent values on a tissue basis, as fatty tissues are not 100% lipid. Blubber lipid content varies intra- and inter-specifically, and also within an individual according to season, age, reproductive status, body condition or the specific sample location on the body (Koopman et al., 2002; Dunkin et al., 2005; Koopman, 2007; McClelland et al., 2012). Within our dataset, blubber lipid content (% wet mass) varied from a low value of 60% in *T. truncatus* to 91.4% in *P. phocoena*; with a larger comparative dataset of 28 species (N=233 specimens), Koopman reported lipid content values ranging from <40% in some of the Delphinids to >90% in narwhals (Koopman, 2007). Using our empirically derived solubility data for pure oils in combination with measures of lipid content and oil density from the same samples, we calculated nitrogen solubility values for blubber on a tissue basis (ml N_2 g^{-1} blubber). This calculation assumes that the influence of non-lipid components on overall tissue solubility will be low. Not surprisingly, our calculations yielded a large range of values, from 0.049–0.051 ml N_2 g^{-1} blubber in *G. griseus*, *G. macrorhynchus* and *T. truncatus* to 0.067 ml N_2 g^{-1} in *P. phocoena* and 0.097 ml N_2 g^{-1} in *M. europeaus* – this last value being 98% higher than that of *G. griseus*. The only comparable values that exist for animal tissues are for marrow lipids (horse 0.0602, ox 0.065, pig 0.069 and sheep 0.073 ml N_2 g^{-1}) (Langø et al., 1996) and for dog (0.0693 ml N_2 g^{-1}) and human (0.0695 ml N_2 g^{-1}) adipose (Ikels, 1964). Clearly, both the nitrogen solubility of pure lipid and the lipid content of a tissue will influence its N_2 solubility; however, for toothed whales lipid class may have a stronger effect than lipid content. The four highest values (>0.076 ml N_2 g^{-1} blubber) were all from species with high wax content in the blubber (*K. breviceps*, *M. mirus*, *Z. cavirostris* and *M. europaeus*; all the species on the right side of Fig. 2), even though lipid content ranged from 62.7% in *K. breviceps* to 82.9% in *Z. cavirostris*. In two species with similar lipid contents (79%), the tissue N_2 solubility value for the animal with a high blubber wax content (*M. europaeus*, 0.097 ml N_2 g^{-1}) was 47% higher than that of the species with no wax in its blubber (*L. obliquidens*, 0.066 ml N_2 g^{-1}). We have deliberately not presented blubber nitrogen solubility data on a tissue basis for all of the species we examined because we do not intend these numbers to be used as definitive, species-specific tissue solubility. Instead, our purpose was to point out the importance of accounting for tissue lipid content in addition to potential variation in the solubility of its pure lipids when modeling nitrogen absorption in an animal; thus, the incorporation of solubility measurements based on pure fats/oils in models of nitrogen uptake by adipose tissues will actually overestimate absorption, as some fraction of adipose tissue is composed of elements with solubilities lower than that of pure lipid.

Given our results, the fact that the nitrogen solubility values reported in the literature for commercial oils of plant and fish origin are all ~0.07 ml N_2 ml^{-1} oil is not that surprising as none of these oils contain any WE. WE are not a normal feature of mammalian adipose; even the closest living relatives to the whales, the ungulates (Agnarsson and May-Collado, 2008; Heyning and Lento, 2002), have adipose consisting entirely of TAG (Pond, 1998); all mysticetes (baleen whales) and some of the odontocetes follow this general trend, as do all pinnipeds (Budge et al., 2006; Koopman, 2007; Litchfield et al., 1975; Lockyer et al., 1984). Specific families of toothed whales are unique in this regard.

Clearly, the presence of wax alone does not fully explain N_2 gas solubility in blubber; WE content explained only 52% of the variation in solubility (Fig. 2). Yet, it does appear that something about lipid class composition constrains nitrogen solubility, as species with blubber containing only TAG had a lower range of solubility values (0.062–0.083 ml N_2 ml^{-1} oil) than did blubber containing almost all WE (range 0.083–0.107 ml N_2 ml^{-1} oil). WE have lower density, are more hydrophobic and resistant to oxidation, and are less sensitive to phase change with changing temperature than TAG (Hadley, 1985; Lee and Patton, 1989; Nevenzel, 1970). Whether and how these properties might be associated with the solubility of N_2 gas are unknown and warrant further investigation. We should note here that the physiological functions of, and potential selective pressures leading to, the presence of WE in the tissues of toothed whales are completely unknown, despite decades of interest in this question (see above).

Although the types of neutral storage lipids present (i.e. WE) influenced the nitrogen solubility of whale blubber, fatty acid bond number did not. The only other study to compare fatty acid composition against gas solubility also reported a lack of connection between N_2 solubility and fatty acid composition for olive oil, cod liver oil and lard (Vernon, 1907). This is somewhat surprising as the introduction of double bonds significantly alters many of the physical properties of fatty acids, including melting point and density, relationships that ectothermic animals use in their adaptations to thermal regimes by modifying cellular features such as membrane structure. Apparently, N_2 solubility in lipids is not impacted by fatty acid composition, at least on the scale we and previous authors have

Table 1. Mean (±s.d.) estimated N_2 solubility values and WE content for oil extracted from blubber in the four families of toothed whales investigated in this study

Family	N_2 solubility (ml N_2 ml^{-1} oil)	WE content (% total lipid mass)
Phocoenidae, porpoises (*Pd*, *Pp*)	0.068±0.0001	0.0
Delphinidae, dolphins (*Dd*, *Gg*, *Gm*, *Lb*, *Lo*, *Tt*)	0.075±0.007	0.6±1.0
Kogiidae, small sperm whales (*Kb*, *Ks*)	0.088±0.027	61.9±45.0
Ziphiidae, beaked whales (*Me*, *Mm*, *Zc*)	0.092±0.013	99.4±0.4

WE, wax ester.
Letters in parentheses indicate species included in each family, coded as *Gs* (*Genus species*); e.g. *Dd* is *Delphinus delphis* (see Materials and methods for full genus and species names).

measured – perhaps the effect of lipid class is so strong that subtle fatty acid influences are lost in these broad comparisons.

Physiological and biological implications of the data

With these new data on nitrogen solubility in marine mammal tissues, the obvious question is whether this has any significance for the diving physiology of these animals. During a dive, peripheral vasoconstriction is assumed to direct blood flow only to crucial organs such as the brain (e.g. Kooyman et al., 1981), thus restricting blood flow through the blubber and severely limiting gas exchange between blood and blubber. As tissue perfusion is one of the main factors affecting rates of nitrogen saturation, this aspect of the dive response is one of the reasons that blubber is deemed to be a 'slow' tissue in models of marine mammal diving physiology; as such, it is not considered to have a major influence on the body's overall nitrogen flux during the average dive bout (Fahlman et al., 2006). This assumption is at least partially contradicted, however, by differential metabolism through the depth of the blubber (Koopman et al., 2002; Koopman, 2007; Krahn et al., 2004; Montie et al., 2008), and the presence of dietary fatty acid markers (indicating turnover) in the blubber of bottlenose and beaked whales (Hooker et al., 2001; Koopman, 2007), animals that spend >95% of their lives submerged. Both lines of evidence demonstrate that there is exchange between the vascular and blubber compartments, some of which must occur when the animal is underwater. In addition, the microvascular density in toothed whale blubber is equal to, or greater than, that of terrestrial mammals (McClelland et al., 2012), meaning that the degree of vascularization does not constrain N_2 uptake/release in blubber. From measurements of nitrogen tension in the muscles of diving *Tursiops*, Ridgway and Howard showed that these animals may be vulnerable to decompression sickness at depths less than 70 m, as they are not protected from circulating nitrogen *via* lung collapse (Ridgway and Howard, 1979). Measurements of lung collapse using computed tomography (CT) of carcasses (Moore et al., 2011) suggest that alveolar collapse in marine mammals may occur much deeper, such that diving mammals could be vulnerable to circulating nitrogen in a greater fraction of dives, especially in shallower parts of the water column (less than 100 m). From these studies it is clear that there is considerable debate about the depth of lung collapse in diving Odontocetes, and comparisons of models of living animals with those of carcasses should be made with caution. However, the combination of blubber perfusion, circulating N_2 at depths above lung collapse, and a higher N_2 solubility value in the large blubber compartment means that there is the potential for blubber to absorb, and subsequently release, greater amounts of N_2 than previously thought. This may not be as much of an issue for smaller species that make shorter dives and have blubber composed only of TAG. Fahlman and colleagues assumed a 5-fold increase in the nitrogen solubility of the blubber of the bottlenose dolphin *T. truncatus* (Fahlman et al., 2006) compared with the blood [based on blood and oil values from Weathersby and Homer, 1980 (Weathersby and Homer, 1980)], which translates into an Ostwald coefficient of 0.072 – slightly higher than our empirically derived value of 0.051 ml N_2 g^{-1} blubber for this species. But what if the blubber has the solubility value of a Gervais' beaked whale (0.097 ml N_2 g^{-1}), which represents a 35% increase?

To put this solubility difference in perspective, we estimated the difference in volumes of N_2 that could be absorbed, based on a standard *Mesoplodon* adult weighing 1200 kg (Reidenberg and Laitman, 2009) with a blubber mass of 21.2% of body mass (Mead, 1989). The maximum possible N_2 absorption (such that the blubber becomes saturated with N_2 at surface pressures), employing the often-used coefficient of 0.07 ml g^{-1}, was equal to 17.8 l of N_2. Using our empirically derived value of 0.097 ml g^{-1}, the volume of nitrogen absorbed increases to 24.7 l, or 39% more. Clearly, this represents one extreme of the nitrogen absorption spectrum, which is not likely to occur under natural circumstances, but it does serve to demonstrate the potential nitrogen volume involved with these different N_2 solubility values in animals with such massive adipose depots.

The toothed whales with large amounts of WE in their blubber are characterized by making deep (>400 m) dives that can last over an hour (Beatson, 2007; Tyack et al., 2006). Having fat with higher nitrogen solubility might seem inherently dangerous as it could lead to greater storage of nitrogen while submerged, and a corresponding increased release of nitrogen upon surfacing. Models of DCS risk in diving mammals indicate that the probability of bubble formation is highest when animals make repetitive shallow dives and when they are in water shallower than the depth of alveolar collapse (Ridgway and Howard, 1979; Zimmer and Tyack, 2007). In theory, the beaked and sperm whales spend little time at such depths, minimizing the potential risk associated with a more absorptive nitrogen 'sponge' in the blubber. Animals routinely making much shorter, shallower dives (porpoises, dolphins) spend a greater fraction of time in this 'risk zone' of the water column, and perhaps the added nitrogen burden that might result from waxy blubber would increase DCS risk. According to Fahlman and colleagues, a small reduction in inert gas load (5%) can reduce DCS risk by 50% (Fahlman et al., 2006). Conversely, one assumes that an increase in inert gas load increases DCS risk. From an evolutionary perspective, it is possible that the deeper divers can afford to have WE and hence high nitrogen solubility in their blubber (for an as yet unknown physiological function) but the shallower divers cannot and this has contributed to the lack of WE in their blubber.

How then do we reconcile the observations of fat and gas emboli in the tissues of beaked whales stranded in association with tactical sonar events (Fernández et al., 2005)? An unexpected observation that emerged from our study is the potentially higher risk of bubble formation in the fatty tissues associated with echolocation in the toothed whales. Toothed whales have two main fat depots: blubber and the specialized cranial acoustic fats used for the transmission

(melon) and reception (mandibular fat bodies) of high frequency sound. One of the characteristic features of the acoustic fats is that they all contain some amount of WE (varying from ~5–10% in porpoises to >90% in beaked whales) (Koopman et al., 2006; Koopman and Zahorodny, 2008), even if the blubber of the species in question does not. Necropsies of stranded whales have suggested that the acoustic fat bodies might be vulnerable to the formation of gas bubbles under certain conditions (Fernández et al., 2005; Jepson et al., 2003). In particular, animals stranding in association with Navy sonar events have shown evidence of gas bubble-associated lesions and fat emboli. For example, Fernández and colleagues reported hemorrhage and petechiae in the melons and mandibular fat bodies, and fat emboli distributed widely in the lungs and other organs of nine beaked whales that stranded along the coast of the Canary Islands within 4 h of the onset of a naval exercise in 2002 (Fernández et al., 2005). The authors concluded that their observations were similar to cliniocopathologic findings of human DCS patients, and animals experimentally exposed to decompression. This led them to suggest that bubbles could have formed in nitrogen-saturated adipocytes, disrupting them and potentially introducing both fat and gas into the venous system. Fernández and colleagues pointed to adipose as having a tendency for bubble growth; specifically, the acoustic tissues are a likely location of bubble formation and a possible point source of fat emboli (Fernández et al., 2005). The acoustic fats are highly perfused, with blood flow in dolphin melon and jaw fats being up to four times higher than that in the blubber (Houser et al., 2004), a process believed to be important for thermoregulatory control of the density of these tissues, so that sound speed gradients and thus wave guide actions of the sound projection and reception pathways are preserved (Houser et al., 2004). Because deep divers such as beaked whales limit their use of echolocation to depths below ~500 m (Tyack et al., 2011), their acoustic fat bodies may well be experiencing considerable perfusion under high pressure conditions – and if these wax-rich fat bodies have different nitrogen solubilities from other body compartments, increased nitrogen loading could result.

To explore this possibility we compared nitrogen solubility of blubber with that of cranial acoustic tissues using samples from a Risso's dolphin. The blubber of this species lacks WE but the mandibular fat bodies contain 24% WE; in accordance with our hypothesis, this increase in wax content was accompanied by a higher N_2 gas solubility value (by 16%; see *Gg* in Fig. 2). This observation, although preliminary, has interesting implications for diving in toothed whales and interpretations of post-mortem findings of stranded animals. It would seem that the combination of a high perfusion rate, even at depth (where they are actively foraging using echolocation), and potentially higher solubility for nitrogen gas could mean that the acoustic fat bodies (i) are 'faster' tissues than blubber, and (ii) might store appreciable quantities of N_2 during a dive, making them a potential hot spot for bubble formation. Clearly, further testing of N_2 solubility in the acoustic fat bodies, in comparison with the blubber of a greater number of species, will shed light on diving physiology in the echolocating whales. In addition, empirical data on vasculature and perfusion rates of these tissues during and between dives is imperative for realistic modeling of gas dynamics in these animals.

APPENDIX

Supplemental methods

Apparatus design

To minimize exposure to atmospheric nitrogen, all experiments were conducted within a sealed glove box (Plas Labs 818-GB, Lansing, MI, USA) that contained an atmosphere of argon. To create the argon atmosphere the glove box was repeatedly evacuated using a vacuum pump (Fisher Scientific, Pittsburgh, PA, USA) and then refilled with 99.9995% ultra carrier grade argon (Airgas Specialty Gases, Riverton, NJ, USA). Before each experiment the internal gas composition was checked (see below) and additional evacuation cycles were performed to maintain internal nitrogen levels below 0.5%. Argon and vacuum hoses were thick-walled Tygon and were kept as short as possible.

Approximately 4 ml of sample oil was drawn up into a modified (see Snedden et al., 1996) 10 ml gas syringe (Hamilton, Reno, NV, USA). To allow the introduction of nitrogen gas, a 3 mm hole was drilled down the length of the plunger handle, into which was fitted a 1.6 mm o.d.×0.25 mm i.d. 316 stainless steel tube (New England Small Tubes, Litchfield, NH, USA). This tube passed through a hole (1.5 mm) that had been drilled through the Teflon plunger cap and its threaded end was sealed with a silicon o-ring, a Teflon washer and a small (no.72) stainless steel nut. The external end of the tube was attached using a compression fitting to a stainless steel 3-way valve (Swagelok, Solon, OH, USA). High purity (99.999%) experimental nitrogen gas was routed from a compressed air cylinder through the wall of the glove box *via* a through hull compression fitting (Blue Sea Systems, Bellingham, WA, USA) into the test syringe. All tubing was 3 mm stainless steel. Before introduction into the syringe, nitrogen gas was passed through an inline gas filter (Varian). Gas flow was regulated using a low-flow metering valve (Swagelok) and monitored with a 16-series digital gas flow meter (Alicat Scientific, Tucson, AZ, USA). In order to maintain the integrity of the argon atmosphere, all waste gas was directly vented outside the glove box *via* 3 mm stainless steel tubing.

Prior to each solubility run, background levels of nitrogen were monitored by injecting samples of glove box gas phase onto the GC column as described below for experimental samples. Nitrogen was bubbled into the oil at flow rates between 5 and 7 ml min^{-1} for 30 min. Following this, the gas was shut off and the oil was allowed to sit until all visible bubbles had been eliminated and the oil was clear (5 min). To facilitate transfer to headspace vials, the oil was moved from the incubation plunger to another 10 ml gas syringe through a 3-way low deadspace T-valve (Hamilton). Thus this valve could be positioned to vent nitrogen outside the glove box (during perfusion), or switched to allow transfer of oil to the other syringe. After filling, the transfer syringe was unscrewed from the T-valve and its contents were gently transferred into 2×10 or 20 ml pre-weighted headspace vials taking care not to completely empty the syringe to avoid introducing nitrogen to the headspace vials. The temperature of the headspace vials and of both syringes was maintained at 37°C throughout the experiment with hollow metal heating coils that were connected to a waterbath (Fisher Scientific). Temperature was monitored using a multi-channel temperature meter (Fluke, Everett, WA, USA). The initial headspace in the test vials was composed of the atmosphere within the glove box. Over the 3 h incubation period, dissolved nitrogen equilibrates with the argon headspace within the vial. Subsequently, 25 µl of the headspace was extracted from each vial through a septum sampling port (Hamilton) in the side of the glove box using an air-tight syringe equipped with a Chaney adapter (Hamilton). Headspace vials were then removed from the glovebox (*via* an entry air lock) and weighted to determine the actual oil volume (oil density and vial mass were recorded prior to this).

The headspace sample was immediately hand-injected onto a 25 m Molsieve 5A column (Varian) running isothermal at 50°C with helium as the carrier gas on a Varian GC (3800). Peaks were

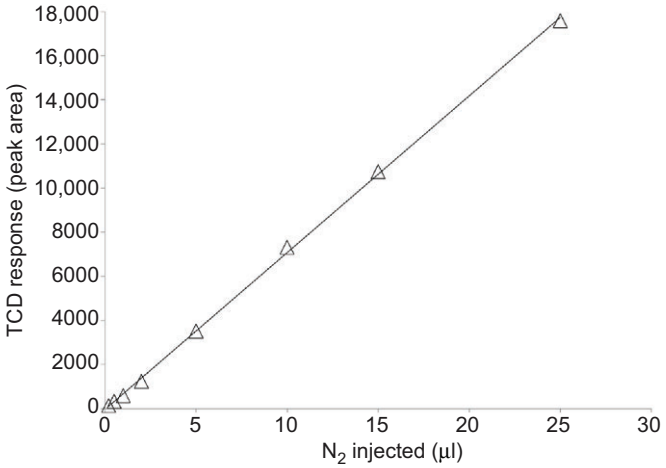

Fig. A1. Standard curve for injections of known amounts of N_2. Points show the linear response of the thermal conductivity detector as a function of the amount of pure nitrogen gas injected into the gas chromatograph; the variables were highly correlated (R^2>0.99).

quantified using a TCD and identified and integrated using Galaxie GC software (version 1.8, Varian). Peak areas were converted to nitrogen volume using the standard curve generated by injecting known volumes of nitrogen (see below). Nitrogen solubility values were calculated as Ostwald coefficients (ml gas dissolved ml^{-1} oil) using mass balance from oil volume and amount of nitrogen in the headspace of each vial after any background nitrogen in the chamber had been accounted for.

Testing of the apparatus

The sensitivity and accuracy of the TCD was tested by injecting a range of volumes (0.2–25.0 µl) of pure nitrogen gas into the column and plotting peak area against gas volume. This procedure resulted in a standard curve that was used to determine nitrogen gas volume from test peaks during the solubility experiments. The standard curve was repeated every few months to ensure TCD sensitivity and to update the standard curve equation for the nitrogen volume calculations. We optimized our procedure through numerous trials with olive oil (Pompeian Extra Virgin), testing different flow rates for bubbling, duration of bubbling and incubation periods, and

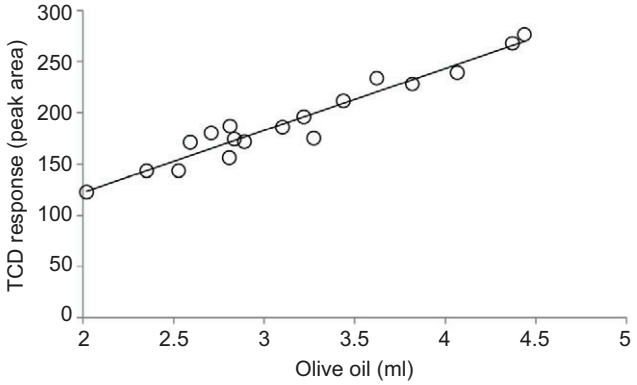

Fig. A2. Linear relationship between the volume of olive oil and amount of N_2 gas released. Points show the area of liberated N_2 peak from varying amounts of olive oil incubated with nitrogen gas; linear regression between the variables was significant (P<0.0001; R^2=0.94).

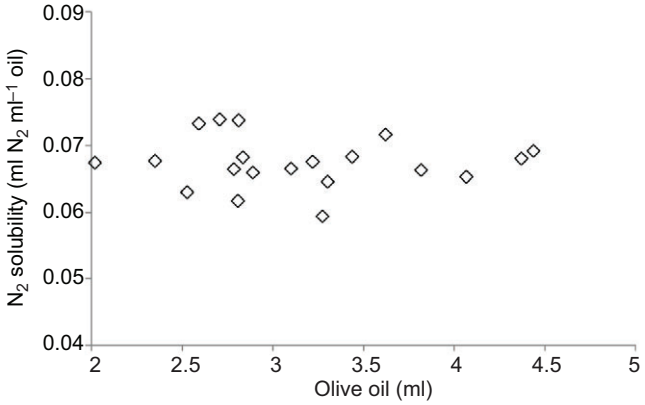

Fig. A3. Independence of calculated N_2 solubility values from amount of olive oil incubated with N_2 gas. There was no relationship between these variables (P=0.85; R^2=0.002).

volumes of oil to use. To determine reproducibility, peak area from the TCD was regressed against oil volume (a greater volume of oil should produce a larger peak). We also regressed nitrogen solubility values against oil volume to show independence of these two variables (solubility should not depend on how much oil was tested). Finally, the olive oil data were compared with literature values of Ostwald coefficients for olive oil at 37°C.

Supplemental results – apparatus testing

The response of the TCD detector to varying amounts of pure nitrogen gas was both reliable and linear (see example standard curve, Fig. A1), with strong correlations between the variables (in all cases R^2>0.99). The slope of this standard curve over the gas injection range of 0.2 to 25 µl is 710.7 with an R^2 value of 0.9995. We feel confident that the response of the TCD is linear throughout our experimental range because when the curve is calculated using only the points from 0.2 to 5 µl, the slope is 707.9 with an R^2 value of 0.9968. The amount of nitrogen gas (measured as TCD peak response area) liberated from 18 incubations with varying amounts of olive oil (range 2.0–4.5 ml) was strongly correlated with oil volume (P<0.0001; R^2=0.938), indicating that the system was reproducible (Fig. A2). Nitrogen solubility values calculated from peak areas and standard curves were not correlated with oil volume (P=0.851), confirming independence between the amount of oil tested and the calculated solubility values on a per volume basis (Fig. A3). The overall mean (±s.d.) estimate of nitrogen solubility in olive oil at 37°C using our apparatus was 0.0674±0.0038 ml N_2 ml^{-1} oil, yielding a coefficient of variation of 5.6%.

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