© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 2620-2630 doi:10.1242/jeb.122606

RESEARCH ARTICLE

Nitrogen solubility in odontocete blubber and mandibular fats in relation to lipid composition

Gina L. Lonati*, Andrew J. Westgate, D. Ann Pabst and Heather N. Koopman

ABSTRACT

Understanding toothed whale (odontocete) diving gas dynamics is important given the recent atypical mass strandings of odontocetes (particularly beaked whales) associated with mid-frequency naval sonar. Some stranded whales have exhibited gas emboli (pathologies resembling decompression sickness) in their specialized intramandibular and extramandibular fat bodies used for echolocation and hearing. These tissues have phylogenetically unique, endogenous lipid profiles with poorly understood biochemical properties. Current diving gas dynamics models assume an Ostwald nitrogen (N_2) solubility of 0.07 ml N_2 ml^{-1} oil in odontocete fats, although solubility in blubber from many odontocetes exceeds this value. The present study examined N_2 solubility in the blubber and mandibular fats of seven species across five families, relating it to lipid composition. Across all species, N_2 solubility increased with wax ester content and was generally higher in mandibular fats (0.083±0.002 ml N_2 ml^{-1} oil) than in blubber (0.069±0.007 ml N_2 ml^{-1} oil). This effect was more pronounced in mandibular fats with higher concentrations of shorter, branched fatty acids/alcohols. Mandibular fats of short-finned pilot whales, Atlantic spotted dolphins and *Mesoplodon* beaked whales had the highest N_2 solubility values (0.097±0.005, 0.081±0.007 and 0.080±0.003 ml N_2 ml^{-1} oil, respectively). Pilot and beaked whales may experience high N_2 loads during their relatively deeper dives, although more information is needed about *in vivo* blood circulation to mandibular fats. Future diving models should incorporate empirically measured N_2 solubility of odontocete mandibular fats to better understand N_2 dynamics and potential pathologies from gas/fat embolism.

KEY WORDS: Diving physiology, Toothed whale, Acoustic fats, Decompression sickness, Embolism

INTRODUCTION

Marine mammals are superlative divers, and it has been suggested that they avoid diving-related injuries, such as decompression sickness, because of physiological adaptations for a life underwater (e.g. Butler and Jones, 1997; Fahlman et al., 2006; Hooker et al., 2012). Decompression sickness results from changes in pressure that can lead to the formation and expansion of gas bubbles ('gas embolism'). In tissues, these bubbles can produce mechanical and biochemical problems, which can be fatal (reviewed in Vann et al., 2011). The occurrence of decompression sickness in air-breathing divers depends on the animal's diving regime (Fahlman et al., 2014). During a dive, tissues can become saturated or supersaturated with inert gases, such as nitrogen (N_2), and if ambient pressure decreases faster than the tissues can release the gas, decompression sickness can result (Boyle, 1670; Vann et al., 2011; Hooker et al., 2012).

Currently, loggerhead sea turtles (*Caretta caretta*) are the only diving non-human marine vertebrate to have been clinically diagnosed with decompression sickness (García-Párraga et al., 2014). However, in the last two decades there have been several atypical mass stranding events, particularly of beaked whales, that have been spatio-temporally associated with the use of naval mid-frequency sonar (Frantzis, 1998; Cox et al., 2006); some of these stranded whales exhibited pathologies resembling decompression sickness (Jepson et al., 2003; Fernández et al., 2005). Gas bubbles have also been found in other stranded and by-caught marine mammals (Jepson et al., 2005; Moore et al., 2009; Van Bonn et al., 2011; Dennison et al., 2012). These gas bubbles appear to be dominated by N_2 (Bernaldo de Quirós et al., 2012, 2013), suggesting that deviations from normal diving behaviors may interrupt gas dynamics and cause decompression-related injuries in marine mammals.

Mathematical models have been used to simulate N_2 dynamics in marine mammals by dividing the body into compartments: blood, central circulation, brain, muscle and fat (e.g. Zimmer and Tyack, 2007; Kvadsheim et al., 2012; Fahlman et al., 2014). The first four compartments are all assumed to have N_2 solubility values equivalent to the value derived for blood: ~0.014 ml N_2 ml^{-1} medium (Weathersby and Homer, 1980; Langø et al., 1996). In comparison, the fat compartment is typically assigned a N_2 solubility of 0.07 ml N_2 ml^{-1} medium, based on measurements of olive oil and bone marrow from oxen and sheep (Weathersby and Homer, 1980; Langø et al., 1996).

Recently, Koopman and Westgate (2012) measured N_2 solubility in blubber from a variety of odontocete families, including oceanic dolphins, porpoises, pygmy and dwarf sperm whales, sperm whales and beaked whales. Blubber is a specialized hypodermal fat depot in marine mammals with thermal, metabolic, hydrodynamic and structural roles (reviewed in Pabst et al., 1999). Solubility ranged from 0.062 to 0.107 ml N_2 ml^{-1} oil, with the four highest values (>0.076 ml N_2 ml^{-1} oil) from deeper divers (sperm and beaked whales). Therefore, N_2 dynamics in odontocete fats may differ from those predicted by mathematical models. Interestingly, there was also a relationship between N_2 solubility and lipid class composition in the blubber (Koopman and Westgate, 2012). Odontocetes are unique amongst mammals in their ability to synthesize wax esters [WEs – a fatty acid (FA) esterified to a fatty alcohol (FAlc)] in addition to the typical triacylglycerol (TAG – three FAs esterified to a glycerol backbone) lipid class found in mammalian fats (Pond, 1998). Higher WE content was correlated with higher N_2 solubility in odontocete blubber (R^2=0.52) (Koopman and Westgate, 2012).

Department of Biology and Marine Biology, University of North Carolina Wilmington, 601 S. College Road, Wilmington, NC 28403, USA.

*Author for correspondence (gina.lonati@myfwc.com)

Received 20 March 2015; Accepted 17 June 2015

The Journal of Experimental Biology

The Journal of Experimental Biology (2015) 218, 2620-2630 doi:10.1242/jeb.122606

List of symbols and abbreviations

EMFB	extramandibular fat body
FA	fatty acid
FABE	fatty acid butyl ester
FAlc	fatty alcohol
GC	gas chromatograph
Gm	*Globicephala macrorhynchus* – short-finned pilot whale
IMFB	intramandibular fat body
Kb	*Kogia breviceps* – pygmy sperm whale
Md	*Mesoplodon densirostris* – Blainville's beaked whale
Me	*Mesoplodon europaeus* – Gervais' beaked whale
Mm	*Monodon monoceros* – narwhal
Pm	*Physeter macrocephalus* – sperm whale
Sf	*Stenella frontalis* – Atlantic spotted dolphin
Ss	*Sus scrofa domesticus* – domestic pig
TAG	triacylglycerol
TLC	thin layer chromatography
WE	wax ester

Not all odontocetes contain WEs in their blubber, but every species examined to date contains WEs in its acoustic fats (Ackman et al., 1973; Litchfield and Greenberg, 1974; Litchfield et al., 1975; Koopman et al., 2006; Koopman, 2007), which are specialized cranial adipose depots involved in echolocation and hearing (Norris, 1968). These fats include the melon, which collimates outgoing sounds dorsal to the skull, and the intramandibular and extramandibular fat bodies (IMFBs and EMFBs, respectively), which surround the mandible and channel incoming sounds toward the ears (Norris, 1968; Varanasi et al., 1975; Cranford et al., 1996; Koopman et al., 2006). The acoustic fats consist of unique, endogenously synthesized lipid components (i.e. FAs and FAlcs) that vary across odontocete families (Litchfield et al., 1975; Koopman et al., 2006). More specifically, the families of oceanic dolphins (Delphinidae), narwhal and beluga (Monodontidae) and porpoises (Phocoenidae) possess high concentrations of short, branched isovaleric acid (*i*-5:0; five carbons); beaked whales (Ziphiidae) have high concentrations of medium-length, branched isolauric acid (*i*-12:0; 12 carbons); and the acoustic fats of sperm whales (Physeteridae), pygmy/dwarf sperm whales (Kogiidae) and river dolphins that have been studied (Platanistidae and Iniidae) contain high concentrations of saturated straight FAs with 10, 12, 14 and 16 carbons (10:0, 12:0, 14:0 and 16:0) in their acoustic fats (Mori et al., 1965; Ackman et al., 1975a; Litchfield et al., 1975, 1976, 1978; Koopman et al., 2006). These unique lipid components are scarce in the blubber of these animals, which is instead dominated by longer, saturated and monounsaturated components (e.g. 16:0, 16:1n-7, 18:1n-9, 20:1n-9) and some polyunsaturated components (e.g. 18:2n-6, 20:5n-3 and 22:6n-3), many of which are of dietary origin (Litchfield et al., 1975; Koopman, 2007). Regardless of dominant lipids, there appears to be a common topographical distribution in acoustic fats; WEs and shorter chain components are concentrated in the center of the tissue, surrounded by TAGs and longer chain components (Koopman et al., 2006). As sound speed decreases through lipids with shorter chains and higher WE content (Gouw and Vlugter, 1967; Varanasi et al., 1975), this arrangement likely focuses outgoing and incoming sound (Malins and Varanasi, 1975; Blomberg and Jensen, 1976; Blomberg and Lindholm, 1976; Koopman et al., 2006), although the specific mechanisms and pathways have not yet been established.

In terms of diving physiology, it is uncertain how these unusual lipids influence the gas properties of the acoustic fats, especially pertaining to N_2 dynamics. Koopman and Westgate (2012) found that N_2 solubility in the mandibular fat of a Risso's dolphin (*Grampus griseus*) was 16% greater than in its blubber (0.087 ml N_2 ml^{-1} oil, ~25 wt% WE versus 0.074 ml N_2 ml^{-1} oil, 0 wt% WE). These authors hypothesized that the difference in WE content could be responsible for the difference in N_2 solubility but recommended that additional studies were needed to relate lipid chemistry to gas properties.

The goals of this study were to (1) measure N_2 solubility in the blubber and mandibular fat bodies from odontocetes that represent five different families, including seven different species (supplementary material Table S1), and (2) relate N_2 solubility to the distinctive lipid profiles of these fat depots. As a comparison, fat from a terrestrial mammal (*Sus scrofa domesticus*, the domestic pig) was also analyzed, as it lacks the unique, endogenous lipids observed in odontocetes. This study compared potential N_2 loading capacities in different fat depots and species, which could allow for better evaluation of decompression-related risks in odontocetes.

RESULTS

Nitrogen solubility

The N_2 solubility of odontocete fats ranged from 0.062±0.007 ml N_2 ml^{-1} oil in Atlantic spotted dolphin blubber to 0.101±0.004 ml N_2 ml^{-1} oil in short-finned pilot whale EMFBs. Generally, values in mandibular fats were higher than those in blubber. Combining all species, the mean Ostwald N_2 solubility values were: blubber 0.069±0.007 ml N_2 ml^{-1} oil, IMFB 0.078±0.011 ml N_2 ml^{-1} oil and EMFB 0.088±0.008 ml N_2 ml^{-1} oil. The N_2 solubility in pig back fat was 0.066±0.005 ml N_2 ml^{-1} oil. Out of the 37 odontocete samples, 28 (76%) had Ostwald values higher than 0.07 ml N_2 ml^{-1} oil, 17 (46%) were higher than 0.08 ml N_2 ml^{-1} oil and one of the pilot whale EMFBs exceeded 0.10 ml N_2 ml^{-1} oil (Table 1, Fig. 1).

Lipid composition

Lipid content was variable across species and tissues, ranging from 40.4 wt% in sperm whale blubber to 93.1 wt% in Gervais' beaked whale EMFB (Table 1). The mean lipid content across all species for each tissue was: blubber 53.8±10.2 wt%, IMFBs 79.5±6.7 wt% and EMFBs 80.8±7.4 wt%. The lipid content of pig back fat was 66.6 wt%.

TAGs and WEs together comprised the majority (>94.0 wt%) of lipid classes in the blubber and mandibular fats. Other lipid classes that constituted the remainder were free FAs, cholesterol and phospholipids, which are not reported. The ratios of TAGs to WEs varied greatly between species and tissues (Table 1). Delphinids (Atlantic spotted dolphins and short-finned pilot whales) did not contain any WEs in their blubber, but sperm whale, pygmy sperm whale and beaked whale blubber contained >81.4 wt% WEs. Pig back fat did not contain any WEs. In the mandibular fats, WEs were most prevalent in the sperm whale EMFB (98.7 wt%) and IMFB (75.6 wt%), followed by the Blainville's beaked whale IMFB (74.7 wt%). Pygmy sperm whale mandibular fats contained variable WE content (IMFB: 7.3–17.7 wt%, EMFB: 24.5–92.1 wt%). Delphinid mandibular fats had between 5.3 and 49.4 wt% WEs, and narwhal EMFB had the lowest WE content (0.8 wt%).

The relative abundance of FA and FAlc components varied by tissue type and phylogeny (Fig. 2). The blubber of all species was dominated (>61.2 mol%) by saturated and monounsaturated FAs and FAlcs of at least 16 carbons, with low concentrations of branched-chain components (<7.8 mol%). When comparing IMFBs and EMFBs within each species, absolute quantities of FAs and FAlcs changed with TAG:WE ratios, but the identities of dominant components remained similar within each family.

The Journal of Experimental Biology (2015) 218, 2620-2630 doi:10.1242/jeb.122606

Table 1. Summary of nitrogen solubility values and lipid composition data for tissues from each species

Tissue	Species	N_2 solubility (ml N_2 ml^{-1} oil)	% Lipid content	% TAG	% WE	Average carbon no.	% Saturated branched chains	% Saturated straight chains	% Mono-unsaturated straight chains	% Poly-unsaturated straight chains
Back fat	Ss	0.066±0.005	66.6	100.0	0.0	17.4	0.0	35.4	**44.7**	19.9
Blubber	Pm	0.072±0.007	40.4	1.3	98.5	17.4	1.7	22.4	**75.4**	0.5
	Kb	0.075±0.001	44.9±8.3	3.5±2.4	94.3±1.1	17.3±0.4	1.7±0.3	29.0±3.9	**68.2±3.6**	0.5±0.2
	Md	0.072±0.005	64.7	0.0	98.9	17.4	2.5	28.1	**68.3**	1.1
	Me	0.073±0.006	72.9	13.4	81.5	17.3	6.0	22.9	**70.5**	0.6
	Sf	0.062±0.007	53.1±3.0	97.1±2.9	0.0	17.1±0.2	7.0±0.7	24.4±0.8	**50.4±2.4**	18.2±2.7
	Gm	0.066±0.006	57.8±4.1	96.0±1.7	0.0	17.4±0.03	1.1±0.2	26.0±0.6	**67.4±2.2**	5.5±2.4
IMFB	Pm	0.075±0.006	75.0	23.4	75.6	16.1	3.5	44.7	**51.5**	0.3
	Kb	0.066±0.003	73.4±2.7	86.3±5.9*	13.0±5.3	15.7±0.8	3.7±2.1	45.9±9.9	**49.4±11.2**	1.1±0.7
	Md	0.079±0.001	90.7	25.1	74.7	13.2	**71.7**	23.7	4.6	<0.1
	Me	0.076±0.006	93.1	65.6	34.0	13.4	**49.9**	34.3	15.5	0.3
	Sf	0.076±0.007	77.3±0.7	91.1±2.7	7.9±2.2‡	9.4±0.7	**68.2±1.4**	14.9±1.2	10.5±1.0	0.4±0.2
	Gm	0.093±0.004	81.0±2.5	83.1±3.3	15.9±2.8	9.4±0.2	**79.1±0.9**	8.8±0.3	11.8±1.3	0.3±0.2
EMFB	Pm	0.081±0.003	86.2	1.3	98.7	16.2	2.5	43.9	**53.6**	<0.1
	Kb	0.085±0.001	82.1±7.4	41.3±33.9	58.2±33.8	14.3±0.2	14.4±8.5	**59.7±5.6**	25.5±4.9	0.3±0.2
	Md	0.081±0.003	87.2	53.5	46.1	12.9	**61.5**	31.6	6.9	<0.1
	Me	0.083±0.005	93.1	72.2	27.4	12.3	**68.0**	29.3	2.7	<0.1
	Mm	0.082±0.003	82.1	99.0	0.8‡	10.2	**65.9**	13.5	19.3	0.7
	Sf	0.086±0.003	70.7±3.1	77.3±7.3	21.4±6.6	9.2±0.2	**81.3±2.4**	12.1±0.9	6.3±1.4	0.3±0.3
	Gm	0.101±0.004	81.0±0.9	56.4±5.7	43.1±5.5	9.7±0.3	**84.6±1.9**	11.1±1.9	4.3±0.1	<0.1

Data include percentage lipid content (wt%), percentage lipid classes (TAG, triacylglycerol; WE, wax ester; % of all lipid classes), average carbon number and percentages of lipid component structure categories (mol% of total lipid profile). Percentage TAG and WE may not total 100% because phospholipids, cholesterol and free fatty acids were present in some specimens. Dominant lipid component structure categories are in bold for each sample. Lipid data where $N>1$ are expressed as means±s.d. for the species. Nitrogen solubility values are expressed as means of replicates ±s.d. For species abbreviations, see List of symbols and abbreviations.

*The TAG components of one *K. breviceps* blubber sample could not be quantified.

‡The WE components of the three *S. frontalis* IMFBs and *M. monoceros* EMFB could not be quantified, so lipid structure percentages only represent TAG FAs.

Branched chains constituted the majority (>49.9 mol%) of delphinid, narwhal and beaked whale mandibular fats, with *i*-5:0 acid most prevalent in the former two and *i*-12:0 most prevalent in the latter. Sperm whale and pygmy sperm whale mandibular fats featured mostly saturated and monounsaturated components (>75.7 mol%), including medium-length FAs (10 and 12 carbons). In contrast, pig back fat contained exclusively straight TAG FAs: 35.4 mol% saturated, 44.7 mol% monounsaturated and 19.9 mol% polyunsaturated; the majority of these (>98.1 mol%) were at least 16 carbons. Polyunsaturated components did not comprise more than 7.3 mol% of any individual odontocete lipid profile, except for Atlantic spotted dolphin blubber (15.8–21.2 mol%). For a more detailed summary of FA and FAlc concentrations in these tissues, see supplementary material Tables S2–S4.

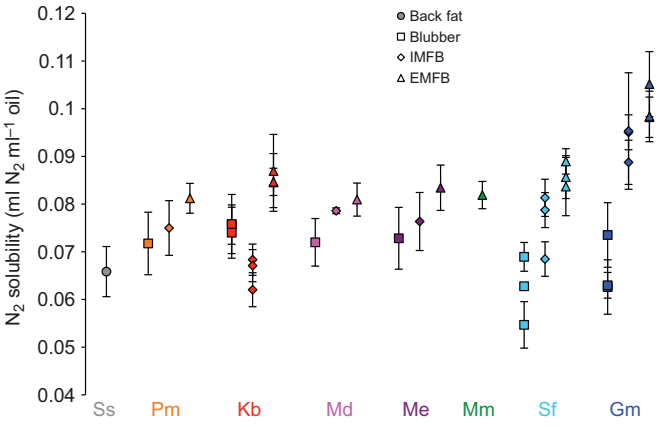

Fig. 1. N_2 solubility averages for each individual tissue analyzed. Blubber, intramandibular fat bodies (IMFBs) and extramandibular fat bodies (EMFBs) are color coded by species. Species where $N>1$ will have overlapping data for individuals. Pig back fat is also shown. Error bars represent ±s.d. If error bars are not visible, s.d. were small. Ss, *Sus scrofa domesticus*; Pm, *Physeter macrocephalus*, Kb, *Kogia breviceps*; Md, *Mesoplodon densirostris*; Me, *Mesoplodon europaeus*; Mm, *Monodon monoceros*; Sf, *Stenella frontalis*; Gm, *Globicephala macrorhynchus*.

Non-parametric ANOSIM analyses in PRIMER demonstrated significant differences in lipid profiles across species and across tissue (P=0.01 for both, global R=0.42 and 0.24, respectively). The CLUSTER analysis yielded four groups of samples in which lipid profiles were >40% similar on the multidimensional scaling (MDS) plot (2D stress=0.11): (1) sperm whale mandibular fats and all blubber samples except those from delphinids, (2) beaked whale mandibular fats and two pygmy sperm whale EMFBs, (3) delphinid and narwhal mandibular fats, and (4) delphinid blubber, pig back fat, pygmy sperm whale IMFBs and one pygmy sperm whale EMFB (Fig. 3A).

Nitrogen solubility versus lipid composition

When comparing the clusters identified by the MDS analysis (Fig. 3B), delphinid and narwhal mandibular fats had the highest average N_2 solubility (cluster 3: 0.088±0.010 ml N_2 ml^{-1} oil), followed by beaked whale mandibular fats and two pygmy sperm whale EMFBs (cluster 2: 0.082±0.004 ml N_2 ml^{-1} oil). These two clusters had average N_2 solubility values significantly higher (P<0.03) than those of the other two clusters (cluster 1: 0.075±0.003 ml N_2 ml^{-1} oil, cluster 4: 0.067±0.008 ml N_2 ml^{-1} oil). Clusters 2 and 3 were not significantly different from each other (P=0.28), but the average N_2 solubility of cluster 1 was significantly greater than that of cluster 4 (P=0.04).

There was no linear correlation between N_2 solubility and WE content when all tissues were combined (P=0.29; Fig. 4A). When individual regressions were tested for each odontocete tissue type (Fig. 4B), N_2 solubility in the blubber significantly increased with WE content (P<0.01, R^2=0.54), although this regression did not include any samples with WE content between 0 and 81 wt%. Therefore, blubber solubility should be interpreted based on either high or low WE content, rather than a continuous distribution of WEs. Regressions of N_2 solubility against WE content were not significant

The Journal of Experimental Biology (2015) 218, 2620-2630 doi:10.1242/jeb.122606

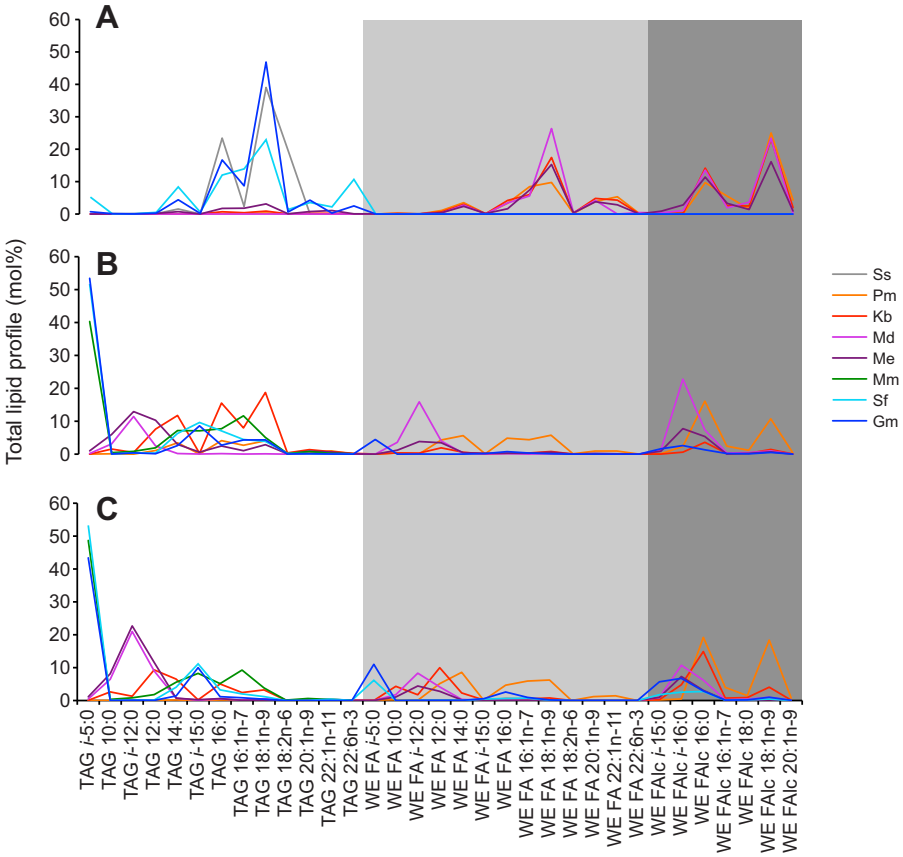

Fig. 2. Lipid profiles for the different tissues. Profiles are illustrated as line graphs for (A) blubber, (B) IMFBs and (C) EMFBs. Major lipid components are listed along the *x*-axis and peaks represent the mol% of the corresponding triacylglycerol fatty acid (TAG FA, within the white boxes), wax ester fatty acid (WE FA, within the light gray boxes) or wax ester fatty alcohol (WE FAlc, within the dark gray boxes). Each color represents a different species. For species where $N>1$, the species mean is presented.

for either IMFBs or EMFBs (P=0.98 and 0.91, respectively). When the data were separated by dominant lipid structure category, there was a significant positive correlation between WE content and N_2 solubility for only the monounsaturated straight category (P<0.01, R^2=0.59; Fig. 4C). However, samples dominated by saturated branched components had a significantly higher mean N_2 solubility than those dominated by monounsaturated straight components (P<0.01). Samples dominated by saturated straight components did not differ significantly from those dominated by saturated branched (P=0.46) or monounsaturated straight components (P=0.06). N_2 solubility was negatively correlated with average carbon number (P<0.01, R^2=0.54; Fig. 4D).

Multivariate regression (P<0.01, adjusted R^2=0.69) predicted N_2 solubility based on average carbon chain length (CL) and WE content (WE) following:

$$N_2 \text{ solubility} = (-0.0029 \times \text{CL}) + (0.00014 \times \text{WE}) + 0.113. \quad (1)$$

The standard error of the N_2 solubility estimate is ±0.006, and the standard errors of the model parameters are: CL ±0.0003, WE ±0.00003 and intercept ±0.004.

DISCUSSION

N_2 solubility related to lipid composition

This study is the first to investigate N_2 solubility in the blubber, IMFBs and EMFBs from a variety of odontocetes and relate it to biochemical composition. N_2 solubility varied with lipid composition; increased WE content was associated with increased N_2 solubility, as demonstrated in odontocete blubber previously (Koopman and Westgate, 2012), but this pattern was confounded by FA and FAlc branching, chain length and the presence of double bonds in the mandibular fats. Samples dominated by shorter, branched components exhibited the highest N_2 solubility values, which increased with increasing WE content. In contrast, lipids with longer hydrocarbon chains and more double bonds had lower N_2 solubility values for given WE content values (Fig. 4C,D).

Koopman and Westgate (2012) found no influence of FAs or FAlcs on N_2 solubility in blubber; WE content alone explained 52% of the variation in solubility values, which were obtained from 13 odontocete species (families Phocoenidae, Delphinidae, Kogiidae and Ziphiidae). However, blubber does not contain the high concentrations of unique, family-specific components (e.g. *i*-5:0, *i*-12:0 and medium-/long-chain saturated components) found in acoustic fats. The present study reveals that these unique components, or, more importantly, combinations of these components with WEs, may elevate N_2 solubility when present in large concentrations. This observation supports the hypothesis that N_2 dynamics differ across the species and tissues examined here, and it suggests that N_2 dynamics of other odontocete tissues may be predicted by their lipid composition (see below for caveats associated with blood flow).

The biochemical mechanisms that alter gas properties in lipids with certain structures or chain lengths are still unclear. It is possible that intermolecular interaction strength between FAs and FAlcs (Fedors, 1974) is affected by hydrocarbon chain saturation, length and branching in odontocete fats, thereby altering their N_2 solubility. The same may be true for WE molecular interactions relative to those of TAGs. No study has examined the effect of lipid branching on gas solubility, but oxygen solubility has been shown to increase as alcohol solvents decrease in chain length (Kutsche et al., 1984), and alkanes (only single hydrocarbon bonds) have

The Journal of Experimental Biology (2015) 218, 2620-2630 doi:10.1242/jeb.122606

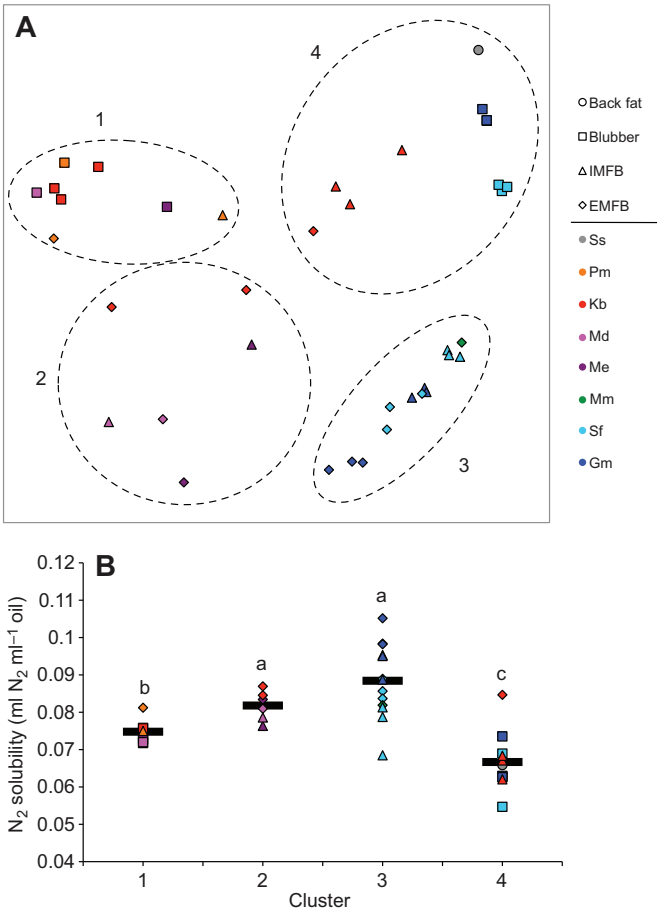

Fig. 3. Multidimensional scaling comparison of sample lipid profiles in the context of N_2 solubility. (A) A multidimensional scaling (MDS) plot comparing lipid profiles of pig back fat, blubber, IMFBs and EMFBs. Data points that are closer together in the two-dimensional space have more similar lipid profiles than points that are farther apart. The 2D stress value (0.11) indicates confidence in the placement of samples relative to each other. The CLUSTER analysis revealed four clusters of samples that are more than 40% similar. The observed global *R* statistic (0.42) indicates deviation from the null hypothesis that no differences exist. The clusters are as follows: (1) sperm whale mandibular fats and all blubber samples except those from delphinids, (2) beaked whale mandibular fats and two pygmy sperm whale EMFBs, (3) delphinid and narwhal mandibular fats, and (4) delphinid blubber, pig back fat, pygmy sperm whale IMFBs and one pygmy sperm whale EMFB. Data points are color coded by species. (B) N_2 solubility values in each cluster. Means are denoted with black bars and data points are color coded by species. Clusters with different letters had significantly different N_2 solubility values (ANOVA with Tamhane's *post hoc* test, $P<0.04$).

greater hydrogen gas solubility than alkenes (at least one double hydrocarbon bond) of fewer than nine carbons (Ghosh et al., 2003).

Short-chain WEs are believed to be important constituents of odontocete acoustic tissues because of their role in sound transmission (Malins and Varanasi, 1975; Varanasi et al., 1975). However, these components also have relatively high N_2 solubility potential, possibly putting animals at a higher risk of decompression-related injuries under certain conditions. Given these trade-offs, it is possible that the lipid profiles observed in odontocete acoustic fats might have evolved under the constraints of both the acoustic and gas solubility properties of these fats. Interestingly, delphinids and narwhals with high concentrations of branched components (>65.9 mol%) have lower WE content (<49.4 wt%), and sperm and pygmy sperm whales with high WE content (13.0–98.7 wt%) have low concentrations (<14.4 mol%) of branched components in their acoustic fats. Beaked whales have both high concentrations of WEs and branched-chain molecules in their acoustic fats, but perhaps lengthening the branched components (*i*-12:0 versus *i*-5:0) buffers increases in N_2 solubility. This hypothesis requires further testing in conjunction with acoustic studies, but it would clarify the interactions between lipid profiles, sound transmission, diving physiology and the evolution of these unusual tissues.

Implications for dive models

Diving models rely on assumptions about gas properties and perfusion rates of tissues to predict whole-body N_2 loads during dives (e.g. Fahlman et al., 2006, 2009; Zimmer and Tyack, 2007; Hooker et al., 2009). A value of 0.07 ml N_2 ml^{-1} medium has been used to calculate N_2 saturation rates in fat compartments, based on measurements of olive oil and oxen and sheep bone marrow (Weathersby and Homer, 1980; Langø et al., 1996). Koopman and Westgate (2012) demonstrated that N_2 solubility in the blubber of most odontocetes, particularly from deeper-diving species with high WE content, was higher than 0.07 ml N_2 ml^{-1} oil. In the present study, N_2 solubility values for 21 of the 25 mandibular fats measured (84%) were greater than 0.07 ml N_2 ml^{-1} medium. Pilot whale EMFBs and IMFBs had N_2 solubility values that were 43.7% and 32.9% higher, respectively, than this value. The EMFBs of all other odontocete species tested had mean N_2 solubility values exceeding 0.08 ml N_2 ml^{-1} oil, representing a >14% increase. Therefore, current diving models may underestimate N_2 loading in odontocete fats and particularly overlook the risk for developing decompression-related injuries in the acoustic fat tissue compartment. The amount of N_2 stored in these acoustic fats may not influence N_2 dynamics in the rest of the body, because these tissues are relatively small in size, but high N_2 saturation in these tissues could have important physiological consequences for this essential sensory system (see below). The N_2 solubility parameters of dive models should be revised based on phylogeny, or mandibular fats may warrant their own compartment in these models. Eqn 1 may be useful for informing models and tuning them to different species if lipid profiles are known.

Although this study does not address perfusion rates of odontocete tissues, accurate blood flow parameters are essential to modeling and understanding the implications of gas properties in diving marine mammal tissues. Traditionally, it has been thought that blood is shunted from non-essential body compartments to essential organs such as the brain and heart during a dive (reviewed by Kooyman et al., 1981). Thus, fats have been considered 'slow' loading tissues because of assumed low perfusion and/or generally modest vascularization relative to 'essential' tissues (e.g. Zimmer and Tyack, 2007; Fahlman et al., 2009, 2014). However, recent studies have demonstrated that blood flow in diving marine animals may be more variable during different dive phases and conditions, such as elevated exercise and foraging/feeding (Zapol et al., 1979; Butler, 1982; Noren et al., 2012). Some odontocetes (e.g. bottlenose dolphins, pygmy sperm whales and Blainville's beaked whales) have similar, if not more extensive, microvascularization in their blubber compared with terrestrial mammals (pigs) (McClelland et al., 2012). In addition to having complex macrovasculature (i.e. veins and arteries) (Costidis and Rommel, 2012), the acoustic fats of some beaked whales, pilot whales, pygmy sperm whales and bottlenose dolphins have recently been shown to contain modest densities of microvessels (M. Gabler, personal communication), which may enable gas exchange between blood and fat. Houser et al.

The Journal of Experimental Biology (2015) 218, 2620-2630 doi:10.1242/jeb.122606

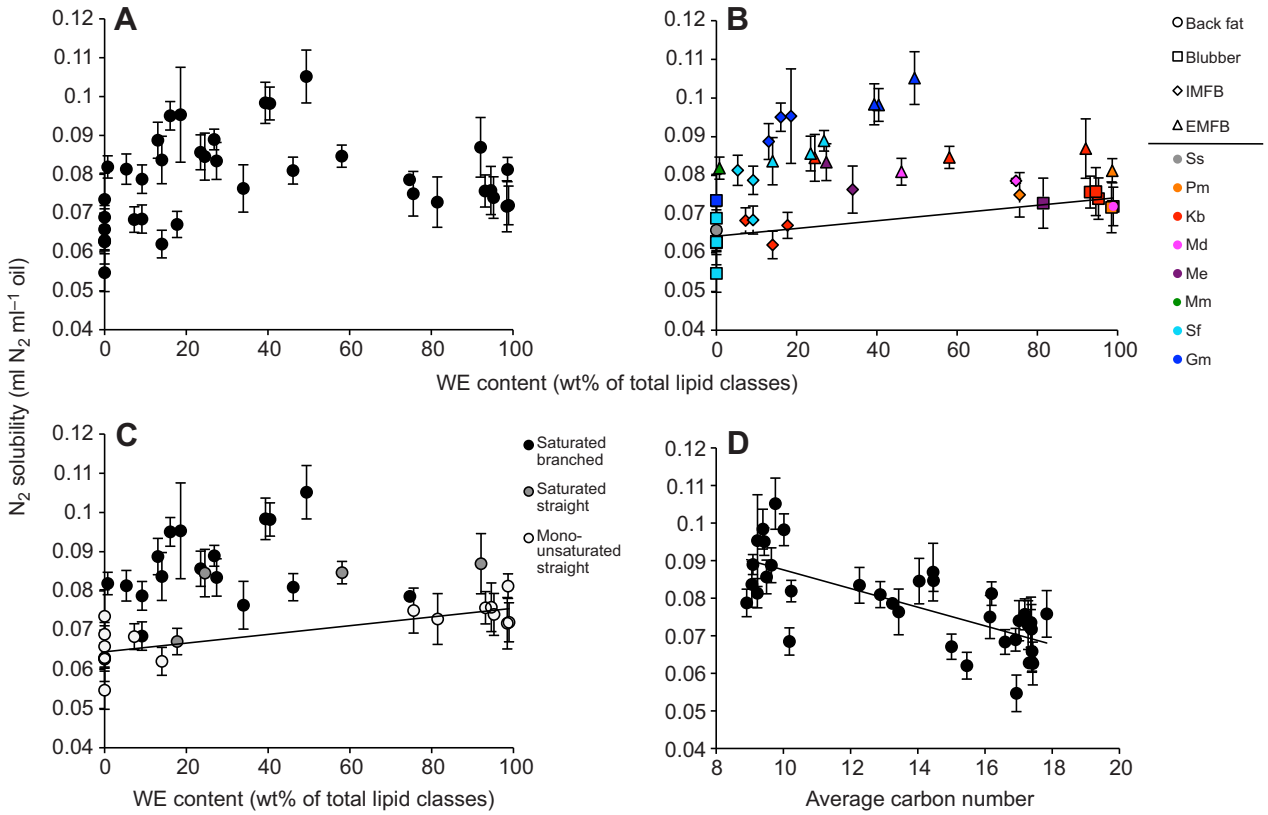

Fig. 4. N_2 solubility in relation to lipid composition in odontocete tissues. (A) N_2 solubility versus WE content for all samples examined. The linear regression was not significant (P=0.29). (B) N_2 solubility versus WE content, separating samples by blubber, IMFBs and EMFBs. Data points are color coded by species (see Fig. 1), and pig back fat is shown for reference. Only the linear regression for the blubber samples (black line) was significant (P<0.01, y=0.0001x+0.064, R^2=0.54). (C) N_2 solubility versus WE content separating samples by dominant structure category: saturated branched, saturated straight and monounsaturated straight. Only the linear regression for samples dominated by monounsaturated straight chains was significant (P<0.01, y=0.0001x+0.064, R^2=0.59). An ANOVA and Tukey's *post hoc* test demonstrated that samples dominated by saturated branched chains had a significantly higher average N_2 solubility than those dominated by monounsaturated straight chains (P<0.01). (D) N_2 solubility versus average carbon number. The linear regression was significant (P<0.01, y=−0.002x+0.112, R^2=0.54), such that N_2 solubility was negatively correlated with average carbon number.

(2004) examined blood flow in a live bottlenose dolphin and showed that the acoustic fats were indeed perfused when the animal was at the surface. These authors hypothesized that perfusion continues during dives to stabilize thermal gradients and maintain auditory function, although gas exchange rates are still uncertain because low substrate metabolism was observed in these tissues (Houser et al., 2004). In summary, perfusion and gas exchange between blood and fat at depth are still poorly understood, but re-evaluating such parameters will increase the reliability of models predicting diving marine mammal gas dynamics.

While this study presents new information about the N_2 properties of odontocete fats, the concept of emboli originating in fat depots is not a novel idea (Jepson et al., 2005; Fernández et al., 2005; Kitano and Hayashi, 1981). N_2 saturation may have consequences *in situ* for the relatively small acoustic fat depots when the animal decompresses, especially if normal diving behavior is interrupted and these tissues cannot equilibrate. Not only could this disrupt auditory function but also damaged fat cells could introduce both gas and fat emboli into the vascular system (as suggested by Costidis and Rommel, 2012; see also Hulman, 1995; Kitano and Hayashi, 1981). This could explain the cranial hemorrhaging and gas/fat embolism observed in some beaked whales that mass stranded in the Canary Islands in 2002 following naval exercises (Fernández et al., 2005). With the finding that decompression emboli are composed predominately of N_2 gas (Bernaldo de Quirós et al., 2012, 2013) and the increased concern about anthropogenic noise disrupting normal cetacean diving behavior (Nowacek et al., 2007; Weilgart, 2007), studies on N_2 dynamics in various tissues, particularly the acoustic fats, are important for understanding cetacean diving physiology, observed pathologies and the impacts of anthropogenic sounds underwater.

Interspecific comparison of decompression injury risk

The information from this study may be used to predict differences in N_2 loading and subsequent risk of decompression injuries across cetacean species. Dolphins, such as Atlantic spotted dolphins, that are shallow divers (Davis et al., 1996) may be at relatively low risk of decompression injuries, despite high N_2 solubility in their acoustic fats. Limited amounts of N_2 will dissolve into the blood and tissues before they decompress because they do not spend much time at high ambient pressures (Fahlman et al., 2014). Pilot whales, which have been classified as intermediate-depth divers (Kvadsheim et al., 2012; Fahlman et al., 2014), had the highest N_2 solubility values measured in this study, potentially resulting in a high risk of decompression injuries. Intermediate-depth divers spend more time above the depth of lung collapse where N_2 will dissolve from the lungs into the blood and circulate to perfused tissues (Fahlman et al., 2014). Pilot whales are also very active, fast divers at depth, known for their underwater foraging 'sprints' (Aguilar Soto et al., 2008), possibly requiring enhanced circulation

The Journal of Experimental Biology (2015) 218, 2620-2630 doi:10.1242/jeb.122606

that could result in tissue supersaturation during repetitive dives (Fahlman et al., 2014). The narwhal is another intermediate-depth diver, though it can dive for longer than 25 min and to depths greater than 1000 m (Heide-Jørgensen and Dietz, 1995). As narwhals lack high concentrations of WEs, their acoustic fats may not absorb as much N_2 compared with pilot whales, and their risk for decompression injuries may be lower. Beaked whales are known to be some of the deepest and longest divers of all air-breathing vertebrates (Tyack et al., 2006; Schorr et al., 2014). The N_2 that dissolves in the blood before lung collapse has a long time to saturate even poorly perfused, 'slow' tissues. This can result in the continuous accumulation of N_2 over subsequent dives, as the release of N_2 will also be slow. Thus, even moderate N_2 solubility may result in high N_2 loads in beaked whale tissues, particularly fats (Rommel et al., 2006). Sperm and pygmy sperm whales may experience relatively low N_2 loads in their fats because of low N_2 solubility, despite being deep, long-duration divers (Watwood et al., 2006; Beatson, 2007). Finally, pig back fat lacks the short-chain components and WEs that elevated N_2 solubility in some odontocete tissues. Mysticete (baleen whale) fats also lack these components and WEs (Ackman et al., 1975b; Yamato et al., 2014), so perhaps they have relatively low N_2 solubility and experience lower N_2 loads as well. To date, no data exist on N_2 solubility in mysticete or river dolphin fats. The above predictions assume adequate microvascularization of these fats for proper gas exchange and are contingent on perfusion at depth as well as lung/alveolar collapse, gas diffusion in various tissues and other aspects of diving physiology.

Lipid composition

Lipid composition data largely reflected phylogeny and/or the function of the fat depot and agreed with data reported in other studies on blubber and acoustic fats (Litchfield et al., 1975, 1976, 1978; Malins and Varanasi, 1975; Koopman et al., 2006; Koopman, 2007). In all species, the blubber contained dietary (e.g. 22:6n-3) and endogenous and exogenous monounsaturated components (e.g. 16:1n-7, 18:1n-9, 20:1n-9 and 22:1n-11) and had high average carbon numbers (>16.9). The most remarkable difference in blubber across species was the dominance by either TAG (delphinids and narwhal) or WE (sperm, pygmy sperm and beaked whales), rather than a proportionate mixture of the two. The longer chain, diet-derived components and presence of TAGs or WEs may benefit certain species differently, based on the buoyant, thermal, structural or metabolic properties of these lipids (Pabst et al., 1999; Koopman et al., 2002; Koopman, 2007; Dunkin et al., 2010; Bagge et al., 2012) or perhaps some other physiological purpose. No study has investigated the mobilization of WE-rich blubber in deep-diving sperm, pygmy sperm and beaked whales, which might elucidate why only some species accumulate WEs in their blubber.

The phylogenetic signal was strong in the mandibular fats, which exhibited large quantities of endogenous lipids associated with sound transmission (Malins and Varanasi, 1975; Varanasi et al., 1975). Specifically, delphinids contained mostly *i*-5:0 and *i*-15:0 acids with moderate WE content (5.3–49.4 wt%), the narwhal had *i*-5:0, *i*-15:0 and 16:1n-7 acids with low WE content (0.8 wt%), and beaked whales produced *i*-12:0 and 12:0 acids and *i*-16:0 alcohol with high WE content (27.4–74.7 wt%). Sperm and pygmy sperm whales both featured straight, saturated and monounsaturated components (e.g. 14:0, 16:0, 16:1n-7 and 18:1n-9 acids and 16:0 and 18:1n-9 alcohols), but sperm whales had high WE content (98.7 wt%), while the WE content of pygmy sperm whale mandibular fats was more variable (7.3–92.1 wt%). Finally, pig back fat only contained TAGs and was dominated by monounsaturated and polyunsaturated FAs longer than 16 carbons (i.e. 16:1, 18:1 and 18:2), which is more typical of terrestrial mammals (Wood et al., 1978; Pond, 1998).

The compositional data presented here are not representative of entire fat bodies and must be interpreted within the limitations of the study. Again, work on the topology of odontocete fats has demonstrated that these tissues are highly heterogeneous (Wedmid et al., 1973; Karol et al., 1978; Koopman et al., 2006; Maxia et al., 2007; Zahorodny Duggan et al., 2009). Consistency of sampling locations was the goal in this study, but mandibular fats were not sampled on a fine spatial scale for lipid composition analyses, because the N_2 experiments required relatively large amounts of lipid. Therefore, it is important to note sampling locations and report tissue collection techniques when comparing lipid composition in these fats and to consider the data presented here accordingly (e.g. supplementary material Tables S2–S4). The higher degree of variation in pygmy sperm whale fats (see above) may have resulted from the heterogeneity of mandibular fat composition combined with the challenges associated with sampling mandibular fats in this particular genus. Pygmy sperm whale heads have very different morphologies from those of other odontocetes (Barroso et al., 2012; Thornton et al., 2015), with exceptionally bulky fat bodies, thin mandibles and high degrees of spatial heterogeneity in lipid composition (Karol et al., 1978; Koopman et al., 2006), potentially making it more difficult to sample homologous sites in these animals. To better understand how these biochemical differences affect sound transmission interspecifically, finer-scale lipid analyses need to be conducted in conjunction with acoustic experiments. In addition, certain lipid constituents may affect other biochemical properties, such as melting point and thus phase of the fat bodies, that may also influence sound transmission or gas properties of these tissues; unfortunately, such data are currently not available.

Conclusions

This study is the first to link N_2 solubility with the FA and FAlc composition of lipids in marine mammals. It specifically demonstrates that N_2 solubility in odontocete blubber and mandibular fats differs and is influenced by the unique lipids these animals synthesize. As composition varies with both phylogeny and fat depot, different species may experience varying N_2 loads in different fatty tissues. Larger quantities of shorter, saturated branched-chain FAs and FAlcs, combined with increased WE content, appear to increase N_2 solubility above the value typically used for modeling gas dynamics in diving whales. Short-finned pilot whale fats had the highest N_2 solubility values observed in this study and were associated with high *i*-5:0 combined with moderate WE concentrations. As pilot whales are intermediate divers (Fahlman et al., 2014) that may spend more time above the depth of lung collapse, large amounts of N_2 could circulate to and dissolve in their tissues. In addition, the beaked whales examined had both high concentrations of *i*-12:0 and high WE content. Beaked whales are particularly deep and protracted divers, potentially leading to accumulation of N_2 in tissues during repetitive dives. Thus, if normal diving behavior is interrupted, pilot and beaked whales may be at elevated risk of developing decompression-related injuries in tissues such as acoustic fats relative to other species. These observations could explain the injuries resembling decompression sickness observed in recent mass strandings of some beaked whales following naval mid-frequency sonar exercises. More work on the perfusion of tissues

The Journal of Experimental Biology (2015) 218, 2620-2630 doi:10.1242/jeb.122606

and gas exchange rates during dives will be necessary to better understand odontocete N_2 dynamics, but this study provides a foundation for predicting N_2 solubility in odontocete fats based on their distinctive lipid profiles.

MATERIALS AND METHODS

Fat samples

Blubber and mandibular fats were obtained from euthanized or freshly dead animals in good body condition (i.e. no thin or emaciated specimens). Dissections were performed either directly following death or after the animal had been frozen post-mortem and subsequently thawed. Supplementary material Table S1 summarizes the animals from which tissues were obtained. Briefly, fats were collected from one sperm whale (*Physeter macrocephalus*), three pygmy sperm whales (*Kogia breviceps*), one Blainville's beaked whale (*Mesoplodon densirostris*), one Gervais' beaked whale (*Mesoplodon europaeus*), one narwhal (*Monodon monoceros*), three Atlantic spotted dolphins (*Stenella frontalis*) and three short-finned pilot whales (*Globicephala macrorhynchus*). For the narwhal, only the EMFB was examined. As a terrestrial mammal comparison, back fat from a domestic pig (*Sus scrofa domesticus*) was obtained courtesy of Harris Teeter (Wilmington, NC, USA).

Tissues were stored at −20°C prior to subsampling. Approximately 20 g of tissue was sampled from each fat depot. The whole blubber thickness was sampled, excluding the epidermis and underlying subcutaneous fat, connective tissue and muscle. For the IMFBs and EMFBs, samples were taken caudal to the mandibular hiatus, near the 'acoustic window' described by Norris (1968). Efforts were made to maintain homologous sampling locations across species. Only fats from one side of the jaw were analyzed as prior work has demonstrated consistent lipid composition between right and left mandibular fats (Koopman et al., 2006; Z. T. Swaim, unpublished).

Nitrogen solubility

N_2 solubility measurements followed a protocol described by Koopman and Westgate (2012), involving a sealed, argon-filled glove-box chamber (Plas Labs 818-GB, Lansing, MI, USA). N_2 gas was bubbled at a rate of 5 ml min^{-1} through a temperature-controlled syringe (Hamilton syringe, Reno, NV, USA) containing approximately 3–5 ml of lipid at 35°C for 30 min. This temperature was chosen so that data in this study could be directly compared with data from Koopman and Westgate (2012). Saturated lipid was transferred to 10 ml screwcap headspace vials (Supelco, Bellefonte, PA, USA) and allowed to equilibrate with the argon headspace of the vials for 3 h. After this period, headspace gas was sampled with a 25 µl airtight syringe (Hamilton syringe) and injected into a gas chromatograph (GC) with a thermal conductivity detector (Varian Inc., Walnut Creek, CA, USA). The 25 m Molsieve 5A column (Varian Inc.) was maintained at 50°C with helium as the carrier gas. Samples of the chamber atmosphere were collected at the start of the incubation periods to assess background levels of N_2 in the chamber. These values were subtracted from the N_2 peak measured in each headspace vial, yielding numbers used to calculate the N_2 solubility of the lipid as an Ostwald coefficient (ml N_2 dissolved in a given volume of oil). The mass of the oil was determined gravimetrically, and volume was determined using the density of the pure extracted lipid (measured prior to N_2 solubility experiments). Ostwald coefficients were calculated from the amount of N_2 in the headspace and the resulting volume of lipid tested. The N_2 response factor for the GC was also tested throughout the study. For more details regarding methods, calibration and chamber set-up, consult the appendix of Koopman and Westgate (2012).

Lipid analysis

Lipids were extracted and lipid content was determined using a modified Folch method (Folch et al., 1957; Koopman et al., 1996) utilizing 2:1 chloroform:methanol with 0.01% butylated hydroxytoluene. Lipid class composition was determined via thin layer chromatography (TLC) with a flame ionization detector (Iatroscan MK-6s, Iatron Laboratories, Inc., Tokyo, Japan) in a 94:6:1 hexane:ethyl acetate:formic acid solvent system (adapted from Ackman et al., 1973) and quantified using Peak Simple, Version 3.29 (SRI Instruments, Torrance, CA, USA) with standards from Nu Chek Prep, Inc. (Elysian, MN, USA) (see Ackman et al., 1973, for details). TLC silica plates (TLC Uniplates 5×20 cm, Analtech, Inc., Newark, DE, USA) were used to separate TAGs from WEs with the same solvent system as above. For GC analysis, TAG FAs and WE FAs were esterifed to butyl esters using BF_3 in butanol (Supelco); butyl esters are necessary to avoid loss of short-chain components (Budge et al., 2006). TAGs and WEs were butylated separately, forming TAG fatty acid butyl esters (FABEs), WE FABEs and WE FAlcs. WE FABEs and WE FAlcs were subsequently separated using TLC silica plates and the same solvent system as above; separation was necessary because of co-elution of branched-chain FABE and straight-chain FAlc during GC analysis. Some butylated WEs components were sampled prior to TLC to account for the loss of volatile, short-chain FAs. FAs and FAlcs were then identified and quantified with a capillary GC (Varian Inc., see above) with a flame ionization detector in a 30 m×0.24 mm column coated with nitroterephthalic acid modified with polyethylene glycol (Zebron ZB-FFAP column, Phenomenex, Torrance, CA, USA), with helium as the carrier gas. The following temperature program was used: 65°C for 2.0 min, ramp up by 20°C min^{-1} to 165°C and then hold for 0.4 min; ramp up by 2° C min^{-1} to 215°C and then hold for 6.6 min; ramp up by 5°C min^{-1} to 250° C and then hold for 5 min (Koopman and Westgate, 2012). Nu Chek Prep standards were used to identify peaks, which were then integrated using appropriate response factors (Ackman, 1991) with the Galaxie Chromatography Data System (Version 1.8.501.1, Varian Inc.). Peak identification was manually confirmed for each run.

After correcting for the loss of volatile, short-chain FAs, percentages of TAG FAs, WE FAs and WE FAlcs were normalized to 100 wt% and then converted to mol% so WE FA and WE FAlc profiles could be adjusted to a 1:1 ratio. These percentages were then combined into a total lipid profile based on the lipid class composition of each sample (i.e. TAG FABEs, WE FABEs and WE FAlcs together totaled 100 mol%). Statistical analyses (see below) were conducted on mol% lipid data, which are summarized for comparison with previous literature in supplementary material Tables S2–S4.

Statistics

Lipid composition

A total of 97 FAs and 34 FAlcs were identified in lipid samples across fat depots and species, although not all components were present in each specimen. As a result, profiles were reconstructed using a subset of 39 FAs and 28 FAlcs shown to constitute the majority (>90.7 wt%) of all lipid profiles before normalization. There were five samples that did not contain either enough TAGs (one pygmy sperm whale blubber sample) or WEs (all three Atlantic spotted dolphin IMFBs and narwhal EMFB) for recovery, requiring the insertion of random numbers below the level of detection (0.05 wt%) into these profiles to distinguish them during the statistical analyses below (see Cheng and Church, 2000). The mol% values of saturated branched-chain, saturated straight-chain, monounsaturated straight-chain and polyunsaturated straight-chain lipid components were summed for each sample and are reported as 'structure categories'. Each sample's dominant structure category was defined as the structure category with the greatest mol% for that sample.

Average carbon number is defined as the average number of carbons that comprise the hydrocarbon chains of FAs and FAlcs within an individual's particular fat body. This value was obtained by weighting each component's mol% by its carbon chain length and averaging these values across the entire lipid profile.

Lipid profile data are not independent or normally distributed, preventing the use of parametric analyses such as multivariate regressions. Commonly, such datasets can be transformed by selecting one component (e.g. 18:0) and scaling all other components proportionately (Budge et al., 2006). However, because some samples in the present study lacked either TAGs or WEs, this transformation was not possible. Thus, the integrated mol% lipid composition profiles were analyzed using a non-parametric statistical software package (Plymouth Routines in Multivariate Ecological Research – PRIMER Version 6.1.6.0, PRIMER-E, Ltd, Ivybridge, UK). First, a resemblance matrix was generated based on Bray–Curtis dissimilarity. A MDS plot, which places samples within a two-dimensional

space based on the resemblance matrix, was produced to compare overall lipid profiles of samples. Samples that were closer together in the two-dimensional space exhibited more similar lipid profiles. A 2D stress value <0.2 indicated confidence in the placement of samples relative to each other. The resemblance matrix was analyzed with a CLUSTER analysis, which reveals groups of samples on the MDS plot with a given percentage of similarity among samples. An ANOSIM test, which is a non-parametric approximate analog of a one-way ANOVA, compared overall profiles grouped separately by species and by tissue. The null hypothesis is that no differences exist, and the histogram of the permutation distribution of R (the test statistic) is centered at zero. The observed global R statistic ranges from 0 to 1, with higher values indicating greater deviation from the null hypothesis. When the null hypothesis was rejected, a SIMPER (Similarity Percentages) analysis was used to reveal which individual FAs or FAlcs were the most important in terms of differences observed in the MDS plot and calculated by the ANOSIM test (see Clarke and Gorley, 2006; Lane et al., 2011; Yamato et al., 2014).

N_2 solubility versus lipid composition

Mean N_2 solubility values of each cluster identified by the MDS analysis were compared with a one-way ANOVA. The error variance was not equal across groups (Levene's test of equality of error variances, P=0.04), so a Tamhane's *post hoc* test was used (SPSS).

N_2 solubility data were then related to specific elements of the lipid profiles through a series of regressions that incorporated subsets of the lipid data. N_2 solubility and WE content were initially compared with one linear regression for all samples (SPSS), as lipid class was shown to strongly influence N_2 solubility in other experiments with blubber (Koopman and Westgate, 2012). Because this overall relationship was not significant (see Results), these data were then segregated by tissue and three separate regression analyses were performed (SPSS). Pig back fat was omitted from all but the initial regression analysis.

To explore the phylogenetic effect of specific FA/FAlc components on N_2 solubility, samples were separated into groups based on their dominant structure category: (a) saturated branched chains, (b) saturated straight chains and (c) monounsaturated straight chains. Again, N_2 solubility data were plotted against WE content and regressions were performed for each dominant structure category (SPSS). Additionally, an ANOVA was performed, comparing the N_2 solubility values of these three dominant structure categories, followed by a Tukey's *post hoc* test (SPSS). Next, N_2 solubility values were compared directly with average carbon numbers of tissues using linear regression (SPSS).

Finally, as both average carbon number and WE content may influence N_2 solubility, and they are considered independent variables, data were combined into a multivariate linear regression model to predict N_2 solubility (Excel). The assumptions of this model are that the data are distributed normally, there is a linear relationship between N_2 solubility and both independent variables or 'partial regression coefficients' (carbon number and WE content), data are measured with negligible uncertainty and the variance of error is homogeneous across both independent variables. For all statistical tests, a significance level of α=0.05 was used. All means are presented ±s.d.

Acknowledgements

We gratefully acknowledge the generous contribution of tissues and volunteer assistance from the UNCW Marine Mammal Stranding Program (with a Stranding Agreement from NOAA Southeast Region), National Park Service – Cape Hatteras (especially Paul Doshkov), Virginia Aquarium & Marine Science Center, the Smithsonian Institution National Museum of Natural History, NOAA Southeast Fisheries Science Center Beaufort, North Carolina lab, Cape Cod Stranding Network, Karen Clark with the North Carolina Wildlife Resources Commission and Christian Legner with the North Carolina Aquarium on Roanoke Island. We would like to thank Molly Gabler of UNCW for her microvascular data. We also thank Bill McLellan, Alex Costidis, Butch Rommel, Wayne Gerth, Sue Budge, Patty Rosel and Andreas Fahlman for helpful advice, insight and ideas. Tissues were held under a Marine Mammal Tissue permit issued by the National Marine Fisheries Service Southeast office issued to H.N.K.

Competing interests

The authors declare no competing or financial interests.

Author contributions

H.N.K. and A.J.W. initially conceived this project, designed the study and secured funding. D.A.P. and H.N.K. provided resources for tissue collection, and G.L.L. sampled tissues. G.L.L. acquired lipid composition data and A.J.W. acquired nitrogen solubility data with assistance from H.N.K. and G.L.L. G.L.L. performed statistical analyses to interpret data and composed the manuscript, with input from D.A.P., H.N.K. and A.J.W. All authors reviewed the article, approved it for submission and can be held responsible for all aspects of the work.

Funding

Support for this project was provided by the Office of Naval Research, Undersea Medicine, grant no. N000141210442, and by the UNCW Graduate School, UNCW Biology Graduate Student Association and UNCW Graduate Student Association. Stranding response was carried out under UNCW IACUC Protocol A1112-013 and supported in part by NOAA Prescott Stranding Grants to UNCW.

Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.122606/-/DC1

References

Ackman, R. G. (1991). Application of gas-liquid chromatography to lipid separation and analysis: qualitative and quantitative analysis. In *Analysis of Fats, Oils and Lipoproteins* (ed. E. G. Perkins), pp. 270-300. Chicago, IL: American Oil Chemists Society.

Ackman, R. G., Sipos, J. C., Eaton, C. A., Hilaman, B. L. and Litchfield, C. (1973). Molecular species of wax esters in jaw fat of Atlantic bottlenose dolphin, *Tursiops truncatus*. *Lipids* **8**, 661-667.

Ackman, R. G., Eaton, C. A., Kinneman, J. and Litchfield, C. (1975a). Lipids of freshwater dolphin *Sotalia fluviatilis*: comparison of odontocete bioacoustic lipids and habitat. *Lipids* **10**, 44-49.

Ackman, R. G., Hingley, J. H., Eaton, C. A., Sipos, J. C. and Mitchell, E. D. (1975b). Blubber fat deposition in mysticeti whales. *Can. J. Zool.* **53**, 1332-1339.

Aguilar Soto, N., Johnson, M. P., Madsen, P. T., Díaz, F., Domínguez, I., Brito, A. and Tyack, P. (2008). Cheetahs of the deep sea: deep foraging sprints in short-finned pilot whales off Tenerife (Canary Islands). *J. Anim. Ecol.* **77**, 936-947.

Bagge, L. E., Koopman, H. N., Rommel, S. A., McLellan, W. A. and Pabst, D. A. (2012). Lipid class and depth-specific thermal properties in the blubber of the short-finned pilot whale and the pygmy sperm whale. *J. Exp. Biol.* **215**, 4330-4339.

Barroso, C., Cranford, T. W. and Berta, A. (2012). Shape analysis of odontocete mandibles: functional and evolutionary implications. *J. Morphol.* **273**, 1021-1030.

Beatson, E. (2007). The diet of pygmy sperm whales, *Kogia breviceps*, stranded in New Zealand: implications for conservation. *Rev. Fish. Biol. Fisheries* **17**, 295-303.

Bernaldo de Quirós, Y., González-Díaz, O., Arbelo, M., Sierra, E., Sacchini, S. and Fernández, A. (2012). Decompression vs. decomposition: distribution, amount, and gas composition of bubbles in stranded marine mammals. *Front. Physiol.* **3**, 177.

Bernaldo de Quirós, Y., Seewald, J. S., Sylva, S. P., Greer, B., Niemeyer, M., Bogomolni, A. L. and Moore, M. J. (2013). Compositional discrimination of decompression and decomposition gas bubbles in bycaught seals and dolphins. *PLoS ONE* **8**, e83994.

Blomberg, J. and Jensen, B. N. (1976). Ultrasonic studies on the head oil of the North Atlantic pilot whale (*Globicephala melaena melaena*). *J. Acoust. Soc. Am.* **60**, 755-758.

Blomberg, J. and Lindholm, L.-E. (1976). Variations in lipid composition and sound velocity in melon from the North Atlantic pilot whale, *Globicephala malaena malaena*. *Lipids* **11**, 153-156.

Boyle, R. (1670). New pneumatical experiments about respiration. *Philos. Trans. R. Soc. Lond.* **5**, 2011-2031.

Budge, S. M., Iverson, S. J. and Koopman, H. N. (2006). Studying trophic ecology in marine ecosystems using fatty acids: a primer on analysis and interpretation. *Mar. Mamm. Sci.* **22**, 759-801.

Butler, P. J. (1982). Respiratory and cardiovascular control during diving in birds and mammals. *J. Exp. Biol.* **100**, 195-221.

Butler, P. J. and Jones, D. R. (1997). Physiology of diving of birds and mammals. *Physiol. Rev.* **77**, 837-899.

Cheng, Y. and Church, G. M. (2000). Biclustering of expression data. In *Proceedings of the Eighth International Conference on Intelligent Systems for Molecular Biology (ISMB)* (ed. R. Altman), pp. 93-103. Menlo Park, CA: AAAI Press.

Clarke, K. R. and Gorley, R. N. (2006). *PRIMER v6: User Manual/Tutorial*. Plymouth, UK: PRIMER-E Ltd.

Costidis, A. and Rommel, S. A. (2012). Vascularization of air sinuses and fat bodies in the head of the bottlenose dolphin (*Tursiops truncatus*): morphological implications on physiology. *Front. Physiol.* **3**, 243.

Cox, T. M., Ragen, T. J., Read, A. J., Vos, E., Baird, R. W., Balcomb, K., Barlow, J., Caldwell, J., Cranford, T., Crum, L. et al. (2006). Understanding the impacts of anthropogenic sound on beaked whales. *J. Cetacean Res. Manag.* **7**, 177-187.

Cranford, T. W., Amundin, M. and Norris, K. S. (1996). Functional morphology and homology in the odontocete nasal complex: implications for sound generation. *J. Morphol.* **228**, 223-285.

Davis, R. W., Worthy, G. A. J., Würsig, B., Lynn, S. K. and Townsend, F. I. (1996). Diving behavior and at-sea movements of an Atlantic spotted dolphin in the Gulf of Mexico. *Mar. Mamm. Sci.* **12**, 569-581.

Dennison, S., Moore, M. J., Fahlman, A., Moore, K., Sharp, S., Harry, C. T., Hoppe, J., Niemeyer, M., Lentell, B. and Wells, R. S. (2012). Bubbles in live-stranded dolphins. *Proc. R. Soc. B. Biol. Sci.* **279**, 1396-1404.

Dunkin, R. C., McLellan, W. A., Blum, J. E. and Pabst, D. A. (2010). The buoyancy of the integument of Atlantic bottlenose dolphins (*Tursiops truncatus*): effects of growth, reproduction, and nutritional state. *Mar. Mamm. Sci.* **26**, 573-587.

Fahlman, A., Olszowka, A., Bostrom, B. and Jones, D. R. (2006). Deep diving mammals: dive behavior and circulatory adjustments contribute to bends avoidance. *Respir. Physiol. Neurobiol.* **153**, 66-77.

Fahlman, A., Hooker, S. K., Olszowka, A., Bostrom, B. L. and Jones, D. R. (2009). Estimating the effect of lung collapse and pulmonary shunt on gas exchange during breath-hold diving: the Scholander and Kooyman legacy. *Respir. Physiol. Neurobiol.* **165**, 28-39.

Fahlman, A., Tyack, P. L., Miller, P. J. O. and Kvadsheim, P. H. (2014). How man-made interference might cause gas bubble emboli in deep diving whales. *Front. Physiol.* **5**, 13.

Fedors, R. F. (1974). A method for estimating both the solubility parameters and molar volumes of liquids. *Polym. Eng. Sci.* **14**, 472.

Fernández, A., Edwards, J. F., Rodríguez, F., de los Monteros, A. E., Herráez, P., Castro, P., Jaber, J. R., Martín, V. and Arbelo, M. (2005). "Gas and fat embolic syndrome" involving a mass stranding of beaked whales (family Ziphiidae) exposed to anthropogenic sonar signals. *Vet. Pathol.* **42**, 446-457.

Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509.

Frantzis, A. (1998). Does acoustic testing strand whales? *Nature* **392**, 29.

García-Párraga, D., Crespo-Picazo, J. L., Bernaldo de Quirós, Y., Cervera, V., Martí-Bonmati, L., Díaz-Delgado, J., Arbelo, M., Moore, M. J., Jepson, P. D. and Fernández, A. (2014). Decompression sickness ('the bends') in sea turtles. *Dis. Aquat. Org.* **111**, 191-205.

Ghosh, A., Chapman, W. G. and French, R. N. (2003). Gas solubility in hydrocarbons – a SAFT-based approach. *Fluid Phase Equilib.* **209**, 229-243.

Gouw, T. H. and Vlugter, J. C. (1967). Physical properties of triglycerides III: ultrasonic sound velocity. *Fette Seifen Anstrichm.* **69**, 159-164.

Heide-Jørgensen, M. P. and Dietz, R. (1995). Some characteristics of narwhal, *Monodon monoceros*, diving behaviour in Baffin Bay. *Can. J. Zool.* **73**, 2120-2132.

Hooker, S. K., Baird, R. W. and Fahlman, A. (2009). Could beaked whales get the bends? Effect of diving behaviour and physiology on modelled gas exchange for three species: *Ziphius cavirostris, Mesoplodon densirostris* and *Hyperoodon ampullatus*. *Respir. Physiol. Neurobiol.* **167**, 235-246.

Hooker, S. K., Fahlman, A., Moore, M. J., Aguilar de Soto, N., Bernaldo de Quirós, Y., Brubakk, A. O., Costa, D. P., Costidis, A. M., Dennison, S., Falke, K. J. et al. (2012). Deadly diving? Physiological and behavioural management of decompression stress in diving mammals. *Proc. R. Soc. B. Biol. Sci.* **279**, 1041-1050.

Houser, D. S., Finneran, J., Carder, D., Van Bonn, W., Smith, C., Hoh, C., Mattrey, R. and Ridgway, S. (2004). Structural and functional imaging of bottlenose dolphin (*Tursiops truncatus*) cranial anatomy. *J. Exp. Biol.* **207**, 3657-3665.

Hulman, G. (1995). The pathogenesis of fat embolism. *J. Pathol.* **176**, 3-9.

Jepson, P. D., Arbelo, M., Deaville, R., Patterson, I. A. P., Castro, P., Baker, J. R., Degollada, E., Ross, H. M., Herráez, P., Pocknell, A. M. et al. (2003). Gas-bubble lesions in stranded cetaceans. *Nature* **425**, 575-576.

Jepson, P. D., Deaville, R., Patterson, I. A. P., Pocknell, A. M., Ross, H. M., Baker, J. R., Howie, F. E., Reid, R. J., Colloff, A. and Cunningham, A. A. (2005). Acute and chronic gas bubble lesions in cetaceans stranded in the United Kingdom. *Vet. Pathol.* **42**, 291-305.

Karol, R., Litchfield, C., Caldwell, D. K. and Caldwell, M. C. (1978). Compositional topography of melon and spermaceti organ lipids in the pygmy sperm whale *Kogia breviceps*: implications for echolocation. *Mar. Biol.* **47**, 115-123.

Kitano, M. and Hayashi, K. (1981). Acute decompression sickness. Report of an autopsy case with widespread fat embolism. *Acta Pathol. Jpn.* **31**, 269-276.

Koopman, H. N. (2007). Phylogenetic, ecological, and ontogenetic factors influencing the biochemical structure of the blubber of odontocetes. *Mar. Biol.* **151**, 277-291.

Koopman, H. N. and Westgate, A. J. (2012). Solubility of nitrogen in marine mammal blubber depends on its lipid composition. *J. Exp. Biol.* **215**, 3856-3863.

Koopman, H. N., Iverson, S. J. and Gaskin, D. E. (1996). Stratification and age-related differences in blubber fatty acids of the male harbour porpoise (*Phocoena phocoena*). *J. Comp. Physiol. B.* **165**, 628-639.

Koopman, H. N., Pabst, D. A., McLellan, W. A., Dillaman, R. M. and Read, A. J. (2002). Changes in blubber distribution and morphology associated with starvation in the harbor porpoise (*Phocoena phocoena*): evidence for regional differences in blubber structure and function. *Physiol. Biochem. Zool.* **75**, 498-512.

Koopman, H. N., Budge, S. M., Ketten, D. R. and Iverson, S. J. (2006). Topographical distribution of lipids inside the mandibular fat bodies of odontocetes: remarkable complexity and consistency. *IEEE J. Oceanic Eng.* **31**, 95-106.

Kooyman, G. L., Castellini, M. A. and Davis, R. W. (1981). Physiology of diving in marine mammals. *Annu. Rev. Physiol.* **43**, 343-356.

Kutsche, I., Gildehaus, G., Schuller, D. and Schumpe, A. (1984). Oxygen solubilities in aqueous alcohol solutions. *J. Chem. Eng. Data.* **29**, 286-287.

Kvadsheim, P. H., Miller, P. J. O., Tyack, P. L., Sivle, L. D., Lam, F. P. A. and Fahlman, A. (2012). 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. *Front. Physiol.* **3**, 125.

Lane, H. A., Westgate, A. J. and Koopman, H. N. (2011). Ontogenetic and temporal variability in the fat content and fatty acid composition of Atlantic herring (*Clupea harengus*) from the Bay of Fundy, Canada. *Fish. Bull.* **109**, 113-122.

Langø, T., Mørland, T. and Brubakk, A. O. (1996). Diffusion coefficients and solubility coefficients for gases in biological fluids: a review. *Undersea Hyperb. Med.* **23**, 247-272.

Litchfield, C. and Greenberg, A. J. (1974). Comparative lipid patterns in the melon fats of dolphins, porpoises and toothed whales. *Comp. Biochem. Physiol. B* **47**, 401-407.

Litchfield, C., Greenberg, A. J., Caldwell, D. K., Caldwell, M. C., Sipos, J. C. and Ackman, R. G. (1975). Comparative lipid patterns in acoustical and nonacoustical fatty tissues of dolphins, porpoises and toothed whales. *Comp. Biochem. Physiol. B* **50**, 591-597.

Litchfield, C., Greenberg, A. J. and Mead, J. G. (1976). The distinctive character of Ziphiidae head and blubber fats. *Cetology* **23**, 1-10.

Litchfield, C., Greenberg, A. J., Ackman, R. G. and Eaton, C. A. (1978). Distinctive medium chain wax esters, triglycerides, and diacyl glyceryl ethers in the head fats of the pacific beaked whale, *Berardius bairdi*. *Lipids* **13**, 860-866.

Malins, D. C. and Varanasi, U. (1975). Cetacean biosonar, Part II: the biochemistry of lipids in acoustic tissues. In *Biochemical and Biophysical Perspectives in Marine Biology*, Vol. 2 (ed. D. C. Malins and J. R. Sargent), pp. 237-290. London, UK: Academic Press.

Maxia, C., Scano, P., Maggiani, F., Murtas, D., Piras, F., Crnjar, R., Lai, A. and Sirigu, P. (2007). A morphological and ^{13}C NMR study of the extramandibular fat bodies of the striped dolphin (*Stenella coeruleoalba*). *Anat. Rec.* **290**, 913-919.

McClelland, S. J., Gay, M., Pabst, D. A., Dillaman, R., Westgate, A. J. and Koopman, H. N. (2012). Microvascular patterns in the blubber of shallow and deep diving odontocetes. *J. Morphol.* **273**, 932-942.

Moore, M. J., Bogomolni, A. L., Dennison, S. E., Early, G., Garner, M. M., Hayward, B. A., Lentell, B. J. and Rotstein, D. S. (2009). Gas bubbles in seals, dolphins, and porpoises entangled and drowned at depth in gillnets. *Vet. Pathol.* **46**, 536-547.

Mori, M., Saito, T., Watanabe, Y. and Nakanishi, Y. (1965). Composition of lipids in the head cavity, blubber and meat of a sperm whale. *Bull. Jpn. Soc. Sci. Fish.* **31**, 638-643.

Noren, S. R., Kendall, T., Cuccurullo, V. and Williams, T. M. (2012). The dive response redefined: underwater behavior influences cardiac variability in freely diving dolphins. *J. Exp. Biol.* **215**, 2735-2741.

Norris, K. S. (1968). The evolution of acoustic mechanisms in odontocete cetaceans. In *Evolution and Environment* (ed. E. T. Drake), pp. 297-324. New Haven, CT: Yale University Press.

Nowacek, D. P., Thorne, L. H., Johnston, D. W. and Tyack, P. L. (2007). Responses of cetaceans to anthropogenic noise. *Mamm. Rev.* **37**, 81-115.

Pabst, D. A., Rommel, S. A. and McLellan, W. A. (1999). The functional morphology of marine mammals. In *Biology of Marine Mammals* (ed. J. E. Reynolds, III and S. A. Rommel), pp. 15-72. Washington, DC: Smithsonian Institution Press.

Pond, C. M. (1998). *The Fats of Life*. Cambridge, UK: Cambridge University Press.

Rommel, S. A., Costidis, A. M., Fernández, A., Jepson, P. D., Pabst, D. A., McLellan, W. A., Houser, D. S., Cranford, T. W., van Helden, A. L., Allen, D. M. et al. (2006). Elements of beaked whale anatomy and diving physiology and some hypothetical causes of sonar-related stranding. *J. Cetacean Res. Manag.* **7**, 189-209.

Schorr, G. S., Falcone, E. A., Moretti, D. J. and Andrews, R. D. (2014). First long-term behavioral records from Cuvier's beaked whales (*Ziphius cavirostris*) reveal record-breaking dives. *PLoS ONE* **9**, e92633.

Thornton, S. W., McLellan, W. A., Rommel, S. A., Dillaman, R. M., Nowacek, D. P., Koopman, H. N. and Pabst, D. A. (2015). Functional morphology of dorsal acoustic structures in pygmy (*Kogia breviceps*) and dwarf (*K. sima*) sperm whales. *Anat. Rec.* **298**, 1301-1326.

Tyack, P. L., Johnson, M., Aguilar Soto, N., Sturlese, A. and Madsen, P. T. (2006). Extreme diving of beaked whales. *J. Exp. Biol.* **209**, 4238-4253.

Van Bonn, W., Montie, E., Dennison, S., Pussini, N., Cook, P., Greig, D., Barakos, J., Colegrove, K. and Gulland, F. (2011). Evidence of injury caused by gas bubbles in a live marine mammal: barotrauma in a California sea lion *Zalophus californianus*. *Dis. Aquat. Org.* **96**, 89-96.

Vann, R. D., Butler, F. K., Mitchell, S. J. and Moon, R. E. (2011). Decompression illness. *Lancet* **377**, 153-164.

Varanasi, U., Feldman, H. R. and Malins, D. C. (1975). Molecular basis for formation of lipid sound lens in echolocating cetaceans. *Nature* **255**, 340-343.

Watwood, S. L., Miller, P. J. O., Johnson, M., Madsen, P. T. and Tyack, P. L. (2006). Deep-diving foraging behaviour of sperm whales (*Physeter macrocephalus*). *J. Anim. Ecol.* **75**, 814-825.

Weathersby, P. K. and Homer, L. D. (1980). Solubility of inert gases in biological fluids and tissues: a review. *Undersea Biomed. Res.* **7**, 277-296.

Wedmid, Y., Litchfield, C., Ackman, R. G., Sipos, J. C., Eaton, C. A. and Mitchell, E. D. (1973). Heterogeneity of lipid composition within the cephalic melon tissue of the pilot whale (*Globicephala melaena*). *Biochim. Biophys. Acta* **326**, 439-447.

Weilgart, L. S. (2007). The impacts of anthropogenic ocean noise on cetaceans and implications for management. *Can. J. Zool.* **85**, 1091-1116.

Wood, J. D., Enser, M. B., MacFie, H. J. H., Smith, W. C., Chadwick, J. P., Ellis, M. and Laird, R. (1978). Fatty acid composition of backfat in large white pigs selected for low backfat thickness. *Meat Sci.* **2**, 289-300.

Yamato, M., Koopman, H., Niemeyer, M. and Ketten, D. (2014). Characterization of lipids in adipose depots associated with minke and fin whale ears: comparison with “acoustic fats” of toothed whales. *Mar. Mamm. Sci.* **30**, 1549-1563.

Zahorodny Duggan, Z. P., Koopman, H. N. and Budge, S. M. (2009). Distribution and development of the highly specialized lipids in the sound reception systems of dolphins. *J. Comp. Physiol. B.* **179**, 783-798.

Zapol, W. M., Liggins, G. C., Schneider, R. C., Qvist, J., Snider, M. T., Creasy, R. K. and Hochachka, P. W. (1979). Regional blood flow during simulated diving in the conscious Weddell seal. *J. Appl. Physiol.* **47**, 968-973.

Zimmer, W. M. X. and Tyack, P. L. (2007). Repetitive shallow dives pose decompression risk in deep-diving beaked whales. *Mar. Mamm. Sci.* **23**, 888-925.