

## RESEARCH ARTICLE

# Fatty acid composition and N<sub>2</sub> solubility in triacylglycerol-rich adipose tissue: the likely importance of intact molecular structure

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## ABSTRACT

Diving tetrapods (sea turtles, seabirds and marine mammals) are a biologically diverse group, yet all are under similar constraints: oxygen limitation and increased hydrostatic pressure at depth. Adipose tissue is important in the context of diving because nitrogen gas (N<sub>2</sub>) is five times more soluble in fat than in blood, creating a potential N<sub>2</sub> sink in diving animals. Previous research demonstrates that unusual lipid composition [waxes and short-chained fatty acids (FA)] in adipose tissue of some whales leads to increased N<sub>2</sub> solubility. We evaluated the N<sub>2</sub> solubility of adipose tissue from 12 species of diving tetrapods lacking these unusual lipids to explore whether solubility in this tissue can be linked to lipid structure. Across all taxonomic groups, the same eight FA accounted for 70–80% of the entire lipid profile; almost all adipose tissues were dominated by monounsaturated FA (40.2–67.4 mol%). However, even with consistent FA profiles, there was considerable variability in N<sub>2</sub> solubility, ranging from 0.051±0.003 to 0.073±0.004 ml N<sub>2</sub> ml<sup>-1</sup> oil. Interestingly, differences in N<sub>2</sub> solubility could not be attributed to taxonomic group ( $P=0.06$ ) or FA composition ( $P>0.10$ ). These results lead to two main conclusions: (1) in triacylglycerol-only adipose tissues, the FA pool itself may not have a strong influence on N<sub>2</sub> solubility; and (2) samples with similar FA profiles can have different N<sub>2</sub> solubility values, suggesting that 3D arrangement of individual FA within a triacylglycerol molecule may have important roles in determining N<sub>2</sub> solubility.

**KEY WORDS:** N<sub>2</sub> solubility, Adipose tissue, Diving tetrapods, Decompression sickness, Diving physiology

## INTRODUCTION

Diving tetrapods (e.g. sea turtles, seabirds and marine mammals) possess unique physiological adaptations for a life underwater (Davis, 2014; reviewed by Ponganis, 2011). Collectively, these adaptations encompass the ‘dive response’ (apnea, bradycardia and peripheral vasoconstriction), as well as other anatomical (e.g. compressible lungs, reinforced tracheas and a fusiform body shape to reduce drag) and biochemical adaptations (such as increased levels of oxygen-binding respiratory proteins and increased activity of enzymes in skeletal muscle to increase aerobic diving capacity; Berta et al., 2015; Davis, 2014; Fish, 2000; Moore et al., 2014; Ponganis, 2011; Ridgway and Howard, 1979; Scholander, 1940). It has been suggested that through these adaptations, diving animals

are able to conserve onboard oxygen stores and can avoid diving-related injuries such as decompression sickness (DCS; Davis, 2014; Fahlman et al., 2006; Hooker et al., 2012). However, recent evidence suggests that some diving tetrapods may experience DCS-like symptoms during diving. To date, loggerhead sea turtles (*Caretta caretta*) are the only air-breathing non-human diving vertebrates that have been clinically diagnosed with gas embolism resulting in DCS (Fahlman et al., 2017; García-Párraga et al., 2014). These animals were entrapped at depth in trawls and gillnets and presented with intravascular emboli in both blood vessels and tissue (García-Párraga et al., 2014). Pathological symptoms similar to those observed in humans experiencing DCS have also been demonstrated in the jaw fats (i.e. the tissues these animals use for echolocation) of deep-diving toothed whales, in individuals whose dives were disrupted by mid-frequency (between 6.8 and 8.2 kHz) sonar activity (Cox et al., 2006; Dennison et al., 2012; Fernández et al., 2005; Jepson et al., 2003, 2005), as well as in sperm whale bones (Moore and Early, 2004). Additionally, there is an increased incidence of intravascular bubbles observed in the heart, lymph nodes and kidneys in bycaught seals, including grey (*Halichoerus grypus*), harp (*Pagophilus groenlandicus*) and harbor seals (*Phoca vitulina*), and whales, including the harbour porpoise (*Phocoena phocoena*), common dolphin (*Delphinus delphis*) and Pacific white-sided dolphin (*Lagenorhynchus obliquidens*; Bernaldo de Quirós et al., 2012, 2013; Moore et al., 2009). Ultimately, it appears that diving air-breathing tetrapods are able to avoid decompression symptoms under natural diving conditions; however, if their diving behavior is disrupted (i.e. owing to either sonar or bycatch), they have an increased susceptibility for developing DCS.

During a dive, differential partial pressure between the breathed gas and the dissolved gas tension in the body results in the diffusion of nitrogen gas (N<sub>2</sub>) from the lungs into the blood, and then from the blood into the tissues (Vann et al., 2011). During the ascent, the impetus for gas diffusion out of the tissues and into the blood is a gradient of higher gas partial pressure in the tissue compared with the blood. While at depth, N<sub>2</sub> will accumulate in the tissues, as the total tension of dissolved gases exceeds the ambient pressure, resulting in supersaturation. The N<sub>2</sub> supersaturation predisposes the tissue to bubble (emboli) formation and the development of DCS when sufficiently extensive and rapid decompression occurs (Dennison et al., 2012; Hooker et al., 2012; Vann et al., 2011). The formation of gas emboli can cause mechanical and biochemical problems in multiple tissues (e.g. blood vessels, brain, adipose tissue and muscle) and can potentially cause death (Dennison et al., 2012; Doolette and Mitchell, 2001). Therefore, to understand the potential for DCS in diving tetrapods, it is imperative to understand the extent to which N<sub>2</sub> can dissolve into tissues.

Subcutaneous white adipose tissue is present in all diving tetrapods and has many functions, including metabolic energy storage, endocrine functions involved in regulating energy balance, thermoregulation and as structural support (Berry et al., 2013;

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Crandall et al., 1997; Mohsen-Kanson et al., 2013; Pond, 1998). Adipose tissue is of particular concern for DCS in diving animals because  $N_2$  is five times more soluble in fat than it is in blood and it can become a potential  $N_2$  'sink' in animals consistently diving to depth (Fahlman et al., 2007; Langø et al., 1996; Weathersby and Homer, 1980). At any blood–adipose tissue interface, gas will diffuse out of circulation and into the tissue, potentially increasing the risk of DCS. All adipocytes within an adipose depot must be in close contact with at least one microvessel in order for the tissue to exhibit many of the functions listed above (Gersh and Still, 1945). Studies on terrestrial animals (e.g. mice, humans and pigs) suggest that mammals possess highly vascularized subcutaneous adipose tissue depots (ranging from 1 to 3% microvessel volume densities); however, the extent of blood–adipose tissue interfaces was unknown in any diving mammal until recently (Gabler et al., 2018; Hemmeryckx et al., 2008; Lijnen et al., 2006; McClelland et al., 2012). Current evidence suggests such interfaces exist in the blubber and adipose tissue of diving cetaceans, with microvessel volume densities (consisting of capillaries, microvenules and microarterioles) ranging from ~0.8% in the blubber of deep-diving beaked whales to ~10.0% in the blubber of shallow-diving bottlenose dolphins (Gabler et al., 2018; McClelland et al., 2012).

Despite the increased risk for the occurrence of gas emboli that adipose tissue imposes, there are surprisingly few studies on  $N_2$  solubility in biological lipids. Manufactured oils have been investigated, including butter, cod liver oil, corn oil, lard and olive oil, each having a different  $N_2$  solubility value (ranging from 0.067 ml  $N_2$  ml<sup>-1</sup> oil in lard to 0.10 ml  $N_2$  ml<sup>-1</sup> oil in butter; Langø et al., 1996; Weathersby and Homer, 1980). Most of the data available for  $N_2$  solubility in biological lipids are from the adipose tissues of pigs (0.054 ml  $N_2$  ml<sup>-1</sup> oil), humans (0.061 ml  $N_2$  ml<sup>-1</sup> oil), dogs (0.069 ml  $N_2$  ml<sup>-1</sup> oil) and sheep bone marrow (0.073 ml  $N_2$  ml<sup>-1</sup> oil; Gabler et al., in press; Ikels, 1964; Langø et al., 1996; Weathersby and Homer, 1980). In general, lipid is assigned a  $N_2$  solubility of 0.07 ml  $N_2$  ml<sup>-1</sup> medium in breath-hold diving animal models, which is based on measurements from olive oil, adipose tissue from dogs and sheep bone marrow (Fahlman et al., 2006; Langø et al., 1996; Weathersby and Homer, 1980). In the tissues of toothed whales, however,  $N_2$  solubility shows considerably greater and more variable values (up to 0.10 ml  $N_2$  ml<sup>-1</sup> oil; Koopman and Westgate, 2012; Lonati et al., 2015). Two important aspects of lipid composition have been linked to the higher values observed in these animals: lipid class composition and fatty acid profiles. The storage lipids in the adipose tissue of most diving tetrapods and all terrestrial mammals are triacylglycerols (TAG; three fatty acids attached to a glycerol backbone; Pond, 1998). However, in the blubber of some families of toothed whales, the presence of wax esters (WE; fatty acid esterified to a fatty alcohol) has been shown to increase  $N_2$  solubility by ~35%, compared with blubber containing only TAG (Koopman and Westgate, 2012; Lonati et al., 2015). Second, the specialized cranial adipose tissue depots (referred to as the acoustic fats) used for echolocation and hearing in odontocete (toothed) whales contain a mixture of WE and TAG composed of unusual short and branched-chain fatty acids/alcohols (BCFA/BCFAlc; Koopman et al., 2006; Litchfield et al., 1975; Lonati et al., 2015). These acoustic fats have higher  $N_2$  solubility values than blubber from the same animal (Lonati et al., 2015). Clearly, the lipids of some of the toothed whales are very unusual. With this study, we sought to expand the knowledge of the relationships between lipid composition and  $N_2$  solubility in the adipose tissue across a wider range of diving tetrapod taxa lacking these unusual lipids.

In sea turtles, seabirds, baleen whales and pinnipeds (seals and sea lions), adipose tissue is composed almost entirely of TAG, containing straight-chain FA that are 12–24 carbons in length (Budge et al., 2006; Pond, 1998). As diving tetrapods, these animals have similar oxygen limitation constraints; however, owing to the differences in habitat, diet, thermal requirements and metabolic physiology, they exhibit a range of diving depths and durations. For example, on average, Adélie penguins dive to 26 m for 1 min, Weddell seals dive to 150–400 m for 10–12 min, sperm whales dive to >600 m for >50 min and some beaked whales reach depths of almost 3000 m and dive for over 2 h (Chappell et al., 1993; reviewed in Ponganis, 2011; Schorr et al., 2014). Currently, there are no data on the  $N_2$  solubility values in adipose tissues of any of these species. Therefore, there were two main goals of this study. The first was to provide new data on  $N_2$  solubility values in adipose tissue from a different group of diving tetrapods, including 12 different species representing five broad diving taxonomic groups (sea turtles, seabirds, baleen whales, toothed whales and pinnipeds), with two terrestrial mammals (pigs and humans) for comparison. The second goal was to determine whether the FA composition of adipose tissues containing only TAG could be related to  $N_2$  solubility data, as has been previously shown with other lipid components in toothed whale blubber and acoustic tissues (Koopman and Westgate, 2012; Lonati et al., 2015).

## MATERIALS AND METHODS

### Tissue collection

Subcutaneous adipose tissue was collected opportunistically from 18 individuals representing 10 species of diving tetrapods; only adult individuals that were deemed to be in 'good body condition' (not emaciated and no evidence of decomposition) were included (e.g. Geraci and Lounsbury, 1993), except for the sei whale, where the tissue was moderately decomposed (refer to Table S1 for data on the individuals sampled). Additional data for four other species were obtained from the literature (see below). Species were divided into four groups for comparisons: (1) sea turtles, (2) seabirds, (3) marine mammals (baleen whales, toothed whales and pinnipeds) and (4) terrestrial mammals (pigs and humans).

Sea turtle samples (leatherback, *Dermochelys coriacea*,  $n=3$ , and loggerhead, *Caretta caretta*,  $n=2$ ) were collected from inside the carapace. Seabird samples consisted of thoracic adipose tissue from the common eider duck (*Somateria mollissima*,  $n=2$ ), Adélie penguin (*Phygoscelis adeliae*,  $n=2$ ) and emperor penguin (*Aptenodytes forsteri*,  $n=1$ ). Three groups of marine mammals were investigated: (1) baleen whales (mysticetes), (2) pinnipeds and (3) toothed whales (odontocetes). Baleen whales included the minke whale (*Balaenoptera acutorostrata*,  $n=2$ ) and sei whale (*B. borealis*,  $n=1$ ). The pinnipeds included the grey seal (*Halichoerus grypus*,  $n=1$ ), harbor seal (*Phoca vitulina*,  $n=2$ ) and California sea lion (*Zalophus californianus*,  $n=2$ ). The toothed whales included the Atlantic spotted dolphin (*Stenella frontalis*,  $n=3$ ) and short-finned pilot whale (*Globicephala macrorhynchus*,  $n=3$ ) from Lonati et al. (2015); although their blubber contains small quantities of odd short and BCFA (much less than the acoustic tissues), there are no WE present. We wanted to compare all diving tetrapods with adipose tissue composed of only TAG. Terrestrial mammal data from the subcutaneous adipose tissue of domestic pigs (*Sus scrofa domesticus*,  $n=3$ ) and humans (*Homo sapiens*,  $n=5$ ) were also included (data from Gabler-Smith et al., 2020). All adipose tissue samples were held under appropriate permits: all marine mammal samples used in this study were collected and held under a Letter of Authorization from the National Marine Fisheries

Service, Southeast Region to H.N.K., sea turtle samples were collected under permit 14ST77 to H.N.K., eider duck samples were collected under permit 2012-433 to M.K.G. and H.N.K., and penguin samples were collected under an Antarctic Conservation Act Permit 2013-003 to Steve Emslie. Once adipose tissue samples were obtained, they were wrapped in plastic wrap (to prevent the tissue from oxidizing) and kept at  $-20^{\circ}\text{C}$  until further analysis.

### Nitrogen solubility

Lipids were extracted from adipose tissue samples using a modified Folch method to determine lipid content (percent wet weight, wt%; Folch et al., 1957; Koopman et al., 1996). To obtain enough lipid for the  $\text{N}_2$  measurements, approximately 20 g of tissue was sampled. For marine mammal samples, the entire blubber depth was used. Each sample was trimmed of any skin, muscle and connective tissue, and lipids were extracted in a solvent of 2:1 chloroform/methanol with BHT. Pure lipid extractions were used in measuring  $\text{N}_2$  solubility for each animal.  $\text{N}_2$  solubility determinations were made for each sample (two to eight times) using gas chromatography after each lipid sample was saturated with pure  $\text{N}_2$  gas at  $37^{\circ}\text{C}$  in an argon-filled modified glove-box as described in Koopman and Westgate (2012) and Lonati et al. (2015); see those publications for detailed methods. Over the course of the solubility measurements,  $\text{N}_2$  solubility was measured for olive oil ( $n=41$ ) to serve as quality control for the instrument ( $0.068\pm 0.001$  ml  $\text{N}_2$  ml $^{-1}$  oil, mean $\pm$ 1 s.d. and CV $<$ 2%).

### Lipid and fatty acid analysis

A small aliquot ( $\sim 0.5$  g) of the pure lipid extract (see above) was used to determine lipid class and FA components. Lipid class was quantified using automated thin layer chromatography with flame ionization detection (TLC/FID with an Iatroscan MK-6s; Iatron Laboratories, Inc., Otkyo, Japan). Samples were spotted onto chromarods and developed in a solvent system of 94:6:1 hexane:ethyl acetate:formic acid. Peak Simple 329 software (SRI Instruments, Torrance, CA, USA) was used to quantify peaks, which were identified using known standards (NuChek Prep, Elysian, MN, USA). Lipid class percentages were measured as wt% of total lipids.

FA components were analyzed using temperature-programmed capillary gas-liquid chromatography (GC) on a Varian 3800 GC (Varian, Palo Alto, CA, USA) as described in Lonati et al. (2015). As most samples contained  $>98\%$  triacylglycerols, the entire lipid extract was esterified to form fatty acid butyl esters (FABEs) using  $\text{BF}_3$  in butanol (Supelco, Bellefonte, PA, USA; Budge et al., 2006). Butyl esters were used rather than the more often used methyl esters in case any short chain (and thus volatile) FA were present. Each FA was described using the nomenclature  $A:Bn-X$ , where  $A$  is the number of carbon atoms,  $B$  is the number of double bonds and  $X$  is the position of the double bond closest to the terminal methyl group.

### Statistical analyses

#### Nitrogen solubility

A one-way ANOVA (SPSS, version 24, IBM Corp., Armonk, NY, USA) was used to determine whether there were any differences in  $\text{N}_2$  solubility based on taxonomic groups. The ANOVA included the following groups: sea turtles, seabirds, baleen whales, pinnipeds, toothed whales and terrestrial mammals.

#### Lipid composition

A total of 72 FA were identified using gas chromatography; however, our analysis included only the FA found in concentrations  $>1.0$  mol% (Glandon et al., 2016). The mean concentrations by

taxonomic group of these 30 FA can be found in Tables S2 (present study) and S3 (comparative data from the literature). The total mol% values of saturated straight-chain (SFA), monounsaturated straight-chain (MUFA) and polyunsaturated straight-chain fatty acid (PUFA) lipid components were summed for each sample and reported as structure categories. Average carbon chain number was determined by weighting each component's mol% by its carbon chain length and averaging these values across the entire lipid profile (consisting of all 72 FA identified; Lonati et al., 2015).

Lipid FA profile data are not independent or normally distributed, preventing the use of parametric analyses, so the integrated mol% lipid composition profiles (see Tables S2, S3) were analyzed using a non-parametric statistical software package (PRIMER, version 6.1.6.0, PRIMER-E, Ltd, Ivybridge, UK). If a particular FA was present in some samples and not detected in other samples (i.e. trans-16:1n-10 in leatherback sea turtles), the concentration of that FA was changed to a random percentage from 0 to 0.05%. This value was chosen because it is below the minimum detectable level of the gas chromatograph (0.05%) but is not so small that it would result in extreme outliers (Cheng and Church, 2000; see Lonati et al., 2015). A resemblance matrix was generated using the Bray-Curtis dissimilarity coefficient calculation (the most commonly used coefficient for biological community analysis). Multi-dimensional scaling (MDS) plots, which place samples within a two-dimensional space based on the values from the resemblance matrix, were produced to compare overall lipid FA profiles across broad taxonomic groups and for different  $\text{N}_2$  solubility groups. Samples closer together in the two-dimensional space exhibited similar lipid FA profiles. A two-dimensional stress value  $<0.2$  indicated confidence in the placement of samples relative to each other (Clarke and Gorley, 2006). Lipid profiles were compared across taxonomic groups using an analysis of similarities (ANOSIM; one-way, maximum permutations=9999), which is a non-parametric approximate analog of a one-way ANOVA. The null hypothesis was that no differences in overall FA profiles exist across the groups 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. If the null hypothesis was rejected, a similarity percentages (SIMPER) analysis was used to reveal which individual FA were the most important in terms of differences calculated by the ANOSIM test (see Clarke and Gorley, 2006).

#### Nitrogen solubility and lipid composition

$\text{N}_2$  solubility was related to the specific FA components by using separate linear regressions to determine relationships between solubility and the lipid structure categories: (1) SFA, (2) MUFA, (3) PUFA and (4) BCFA (SPSS). A linear regression was also used to determine whether there was a relationship between carbon chain length and  $\text{N}_2$  solubility. An independent samples  $t$ -test was used to determine whether there was a difference in  $\text{N}_2$  solubility between diving (sea turtles, seabirds and marine mammals all grouped together) and non-diving animals (pigs and humans).

Because the diving groups were  $\sim 70\%$  similar to each other based on FA composition (see Results), but some of the  $\text{N}_2$  solubility values (even within the same species) were different, individuals were classified into five  $\text{N}_2$  solubility groups by dividing the range of the solubility values by five (e.g. high= $>0.065$ , medium-high= $0.061-0.065$ , medium= $0.057-0.061$ , medium-low= $0.054-0.057$  and low= $<0.054$ ) to analyze the variability in FA composition across the solubility groups. Across these groups, the 13 dominant FA were ranked according to total



mol% to determine whether any patterns existed between the dominant FA and  $N_2$  solubility. Separating the data into these groups allowed us to not only investigate the spread of  $N_2$  solubility values without considering phylogeny (taxonomic groups), but also determine whether there were any obvious patterns in FA composition that might be obscured by considering taxonomic groups only, rather than individual samples (because  $N_2$  solubility varied within taxonomic groups and species). To quantify these differences, lipid profiles were compared across the five  $N_2$  solubility groups using an ANOSIM (one-way, maximum permutations=9999). As described above, the null hypothesis was that no differences in overall FA profiles exist across the groups and the histogram of the permutation distribution of  $R$  (the test statistic) is centered at zero. If the null hypothesis was rejected, a SIMPER analysis was used to reveal which individual FA were the most important in terms of differences calculated by the ANOSIM test (see Clarke and Gorley, 2006). To qualitatively analyze whether the FA profiles were similar within high and low  $N_2$  solubility groupings, the four samples with the highest  $N_2$  solubility and the four samples with the lowest  $N_2$  solubility were separated. All data (unless noted otherwise) are presented as means  $\pm$  1 s.d. Statistical analyses were considered significant for values of  $P \leq 0.05$ .

## RESULTS

### Nitrogen solubility

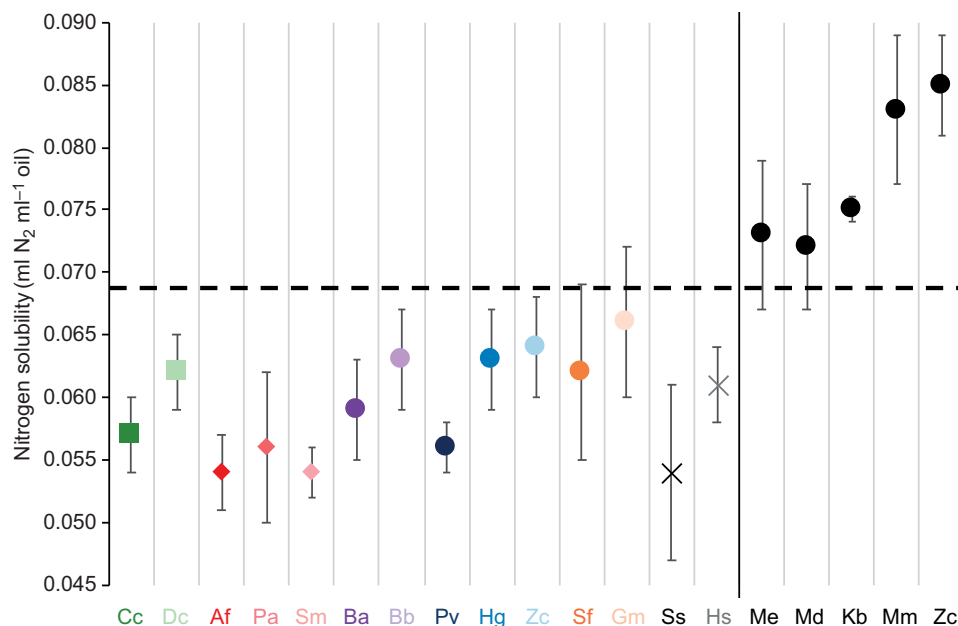
The mean  $N_2$  solubility value for all adipose tissue examined here was  $0.060 \pm 0.005$  ml  $N_2$  ml $^{-1}$  oil. However, there was variation across individuals (Fig. 1, Table 1), ranging from  $0.051 \pm 0.003$  ml  $N_2$  ml $^{-1}$  oil (emperor penguin) to  $0.073 \pm 0.004$  ml  $N_2$  ml $^{-1}$  oil (short-finned

pilot whale). There were no significant differences ( $P=0.06$ ) in the mean  $N_2$  solubility values for the different taxonomic groups (mean value for sea turtles= $0.060 \pm 0.003$ , seabirds= $0.055 \pm 0.004$ , baleen whales= $0.060 \pm 0.002$ , pinnipeds= $0.060 \pm 0.004$ , toothed whales= $0.064 \pm 0.006$  and terrestrial mammals= $0.058 \pm 0.004$  ml  $N_2$  ml $^{-1}$  oil). Similarly,  $N_2$  solubility did not differ significantly between diving (mean  $0.060 \pm 0.005$  ml  $N_2$  ml $^{-1}$  oil) and non-diving animals (mean  $0.058 \pm 0.004$  ml  $N_2$  ml $^{-1}$  oil,  $P=0.39$ ) in this study.

### Lipid and fatty acid composition

Lipid content varied across species, ranging from 30.4 wt% in the leatherback to 86.9 wt% in the harbor seal. TAG comprised the majority of the lipid classes in all samples (>95 wt% except for the eiders; Table 1). The remainder of most samples contained small amounts of free FA and cholesterol; however, the adipose tissue from eider duck samples contained 16.0 wt% phospholipids. None of the samples contained any WE.

Adipose lipids from all tetrapod groups exhibited similar FA profiles. All samples were dominated by MUFA (ranging from 40.2 mol% in the California sea lion to 67.4 mol% in the short-finned pilot whale), except for the leatherback sea turtles, where SFA dominated ( $53.4 \pm 2.4$  mol%; Table 1). The non-parametric analysis (SIMPER) of the FA profiles of the taxonomic groups indicated that all groups were 40 to 70% similar to each other. The most common FA across groups was 18:1n-9 (Fig. 2). This FA was dominant in all species (ranging from 13.6 to 46.8 mol%; Tables S2, S3), except for the sei whale and the eider ducks. The highest FA in the sei whale was 20:1n-9 (14.4 mol%; Table S2).



**Fig. 1. Mean nitrogen ( $N_2$ ) solubility values for the adipose tissue and blubber from a variety of diving and terrestrial tetrapod species.** Squares are sea turtles, diamonds are seabirds, circles are marine mammals and crosses are terrestrial mammals. Black circles are sperm and beaked whales. Values are means  $\pm$  1 s.d. Sea turtles: Cc, *Caretta caretta* (loggerhead,  $n=2$ ) and Dc, *Dermodochelys coriacea* (leatherback,  $n=3$ ); seabirds: Af, *Aptenodytes forsteri* (emperor penguin,  $n=1$ ), Pa, *Phygoscelis adeliae* (Adélie penguin,  $n=2$ ) and Sm, *Somateria mollissima* (common eider duck,  $n=2$ ); baleen whales: Ba, *Balaenoptera acutorostrata* (minke whale,  $n=2$ ) and Bb, *B. borealis* (sei whale,  $n=1$ ); pinnipeds: Pv, *Phoca vitulina* (harbor seal,  $n=2$ ), Hg, *Halichoerus grypus* (grey seal,  $n=1$ ) and Zc, *Zalophus californianus* (California sea lion,  $n=2$ ); toothed whales: Sf, *Stenella frontalis* (Atlantic spotted dolphin,  $n=3$ ) and Gm, *Globicephala macrorhynchus* (short-finned pilot whale,  $n=3$ ); terrestrial mammals: Ss, *Sus scrofa domestica* (pig,  $n=3$ ) and Hs, *Homo sapiens* (human,  $n=5$ ). Data for Sf and Gm are from Lonati et al. (2015) and data for Ss and Hs are from Gabler et al. (in press). The sperm whales include Kb, *Kogia breviceps* (pygmy sperm whale); beaked whales include Me, *Mesoplodon europaeus* (Gervais' beaked whale), Md, *Mesoplodon densirostris* (Blainville's beaked whale), Mm, *Mesoplodon mirus* (Sowerby's beaked whale) and Zc, *Ziphius cavirostris* (Cuvier's beaked whale). The data for the sperm and beaked whales are from Koopman and Westgate (2012) and Lonati et al. (2015). The dashed line is the mean value reported for olive oil at 37°C (from Gabler-Smith et al., 2020).

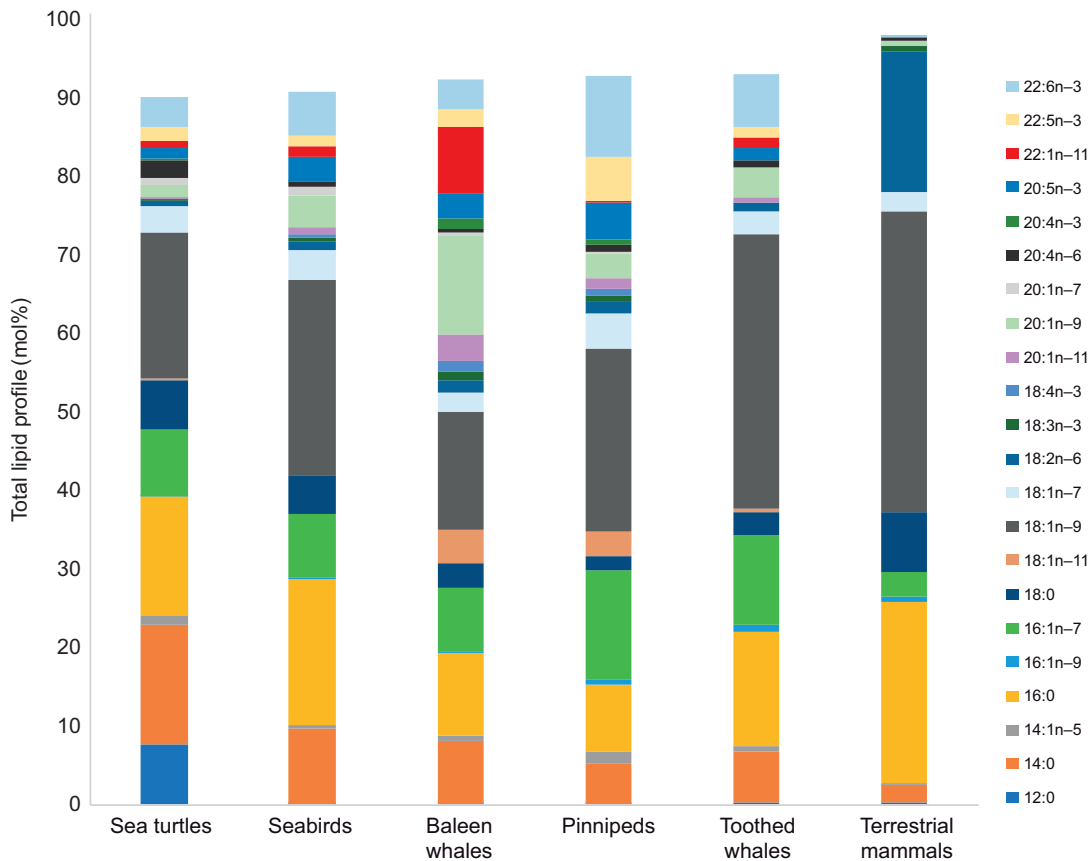
**Table 1. Summary of nitrogen (N<sub>2</sub>) solubility values and lipid composition (including all fatty acids) data for adipose tissue from a variety of diving and non-diving tetrapods**

Taxonomic group	Species	N <sub>2</sub> solubility (ml N <sub>2</sub> ml <sup>-1</sup> oil)	Lipid content (wt%)	% TAG	Average carbon no.	% Saturated branched chains	% Saturated straight chains	% Mono-unsaturated straight chains	% Poly-unsaturated straight chains
Sea turtles	Dc (3)	0.062±0.003	54.6±17.1	98.2±1.3	16.3±0.1	1.0±0.2	<b>53.4±2.4</b>	34.8±4.0	10.7±0.6
	Cc (2)	0.057±0.003	71.2±10.5	98.7±0.7	17.2±0.1	0.83±0.2	41.1±1.6	<b>41.7±0.8</b>	16.3±0.6
Seabirds	Sm (2)	0.054±0.002	73.2±7.5	83.0±9.20	17.4±0.1	0.41±0.01	37.7±0.3	<b>49.5±0.6</b>	12.4±0.3
	Pa (2)	0.056±0.006	62.3±3.2	99.3±0.5	17.4±0.1	0.65±0.03	36.1±3.5	<b>45.2±1.7</b>	18.0±1.8
	Af	0.054±0.003	50.5	95.3	17.7	0.91	32.1	<b>51.1</b>	15.9
Baleen whales	Ba (2)	0.059±0.004	45.9±11.7	99.7±0.3	18.3±0.2	0.74±0.1	21.6±0.3	<b>58.2±0.1</b>	19.4±0.1
	Bb	0.063±0.004	52.3	99.8	18.2	0.79	25.3	<b>56.7</b>	17.1
Pinnipeds	Pv (2)	0.056±0.002	83.6±4.7	99.8±0.05	17.9±0.1	1.57±0.07	17.6±6.3	<b>54.7±9.5</b>	26.1±3.3
	Hg	0.063±0.004	66.4	99.2	18.0	0.79	8.9	<b>66.0</b>	24.3
	Zc (2)	0.064±0.004	69.6±6.9	99.2±0.6	18.3±0.2	0.65±0.05	23.9±2.7	<b>40.2±1.1</b>	35.2±1.7
Toothed whales	Sf (3)	0.062±0.007	53.1±3.0	97.1±2.9	17.1±0.2	7.0±0.7	24.4±0.8	<b>50.4±2.4</b>	18.2±2.7
	Gm (3)	0.066±0.006	57.8±4.1	96.0±1.7	17.4±0.03	1.1±0.2	26.0±0.6	<b>67.4±2.2</b>	5.5±2.4
Terrestrial mammals	Ss (3)	0.054±0.007	77.9±4.0	99.5±0.2	17.4±0.0	0.0±0.0	39.2±2.4	<b>41.1±3.6</b>	19.6±1.8
	Hs (5)	0.061±0.003	64.6±5.7	99.8±0.1	17.3±0.1	0.36±0.2	28.6±4.5	<b>50.4±2.1</b>	20.6±3.3

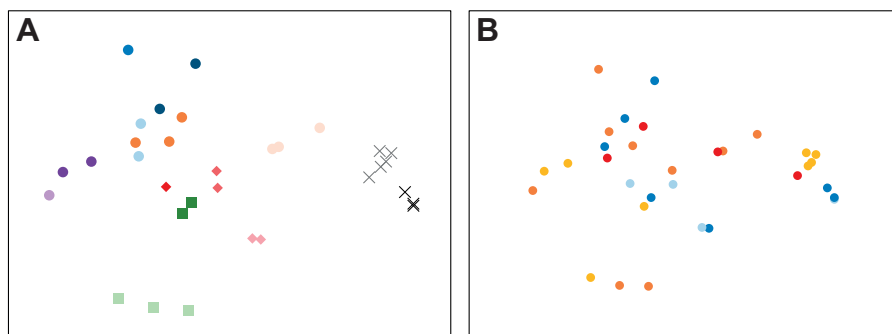
For species abbreviations, see Fig. 1. Percent triacylglycerol (TAG) values do not total 100% because phospholipids, cholesterol and free fatty acids were present in some species in small amounts. Dominant lipid component structure categories are in bold for each species. For species where  $n > 1$ , values are expressed as means±s.d.; numbers within the parentheses indicate sample size. Nitrogen solubility values are expressed as means of replicates ±s.d. Data from toothed whales were published in Lonati et al. (2015) and data from terrestrial mammals were published in Gabler-Smith et al. (2020).

This FA was found in modest amounts in the blubber of all baleen whales (ranging from 10.6 to 14.4 mol%). The FA with the highest levels in the eider ducks was 16:0 (24.0±2.7 mol%), which was also high in the penguins (14.9–24.0 mol%).

Although the overall lipid profiles of the taxonomic groups exhibited less than a 30% difference in overall FA profile, non-parametric ANOSIM analyses in PRIMER did reveal some significant differences ( $P=0.001$ , global  $R=0.84$ , MDS plot; Fig. 3A). Most of the



**Fig. 2. Generalized fatty acid (FA) profiles of the adipose tissue from different taxonomic groups of tetrapods.** Profiles are illustrated as bar graphs. Major components (>1.0 mol%) are presented in different colors within the bars and represent the mol% of the corresponding triacylglycerol (TAG) FA for each group. Numbers do not all total to 100%; the remainder are small or unknown components. Data for toothed whales are from Lonati et al. (2015) and for terrestrial mammals are from Gabler-Smith et al. (2020).



**Fig. 3. Two-dimensional multidimensional scaling (MDS) plots comparing FA profiles of the individual samples.** Individual samples with similar FA profiles are plotted closer together, compared with samples with dissimilar FA profiles, which are plotted farther apart. (A) MDS of the overall FA profiles of the adipose tissues of diving and terrestrial tetrapods, grouped by broad taxonomic category; 2D stress=0.10,  $P=0.001$ . Squares are sea turtles, diamonds are seabirds, circles are marine mammals and crosses are terrestrial mammals. For species identification, colors are noted in Fig. 1. (B) Same MDS plot, this time coded according to relative  $N_2$  solubility (2D stress=0.01,  $P=0.80$ ): red circles are high  $N_2$  samples ( $>0.065$ ), orange circles are medium-high (0.061–0.065), yellow circles are medium (0.057–0.061), dark blue circles are medium-low (0.054–0.057) and light blue circles are low ( $<0.054$ ).

differences across groups were attributable, not surprisingly, to concentrations of the most abundant FA, 18:1n-9 (Fig. 2, Table 2). The four other FA that were attributed to differences between the groups were 12:0 and 14:0 (found in high concentrations within the sea turtles,  $P=0.008$ ; Table 3), 16:0 in seabirds ( $P<0.02$ ) and 20:1n-9 in baleen whales ( $P<0.02$ ). Terrestrial mammals differed from the diving animals in concentrations of 18:2n-6 ( $P<0.01$ ; Table 3).

#### Nitrogen solubility and lipid composition

There were no significant relationships between SFA, MUFA, PUFA, branched chain or average carbon number and  $N_2$  solubility (all  $P>0.10$ ). Surprisingly, the FA profiles of the samples with the four highest (short-finned pilot whale, human, Atlantic spotted dolphin and California sea lion; values ranging from 0.065 to 0.073 ml  $N_2$  ml<sup>-1</sup> oil) and four lowest (eider duck, Adélie penguin, emperor penguin and pig; values from 0.051 to 0.053 ml  $N_2$  ml<sup>-1</sup> oil)  $N_2$  solubility values were quite similar (Fig. 4). Further, ANOSIM revealed that there were no significant differences in overall FA profiles in the five  $N_2$  solubility value groups (global  $R=-0.054$ ,  $P=0.80$ , MDS plot; Fig. 3B). All groups were dominated by 18:1n-9 (range of 26.5 to 31.8 mol%) followed by 16:0 (range of

13.1 to 19.6 mol%; Table 3). There were 8 FA (14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 18:2n-6 and 20:1n-9) that accounted for 70–80% of all FA in all  $N_2$  binned groups, further suggesting that the fatty acid composition across all of these  $N_2$  solubility groups was very similar.

#### DISCUSSION

##### $N_2$ solubility

This study is the first to investigate  $N_2$  solubility in the adipose tissue of a variety of diving tetrapods in which lipids were composed of only TAG, without any of the WE and unusual FA previously shown to affect  $N_2$  solubility. Although lipid class and FA data in the adipose of many of the species examined here have been previously reported by other authors, the key to our study is that  $N_2$  solubility was measured in the same lipid samples in which we obtained our FA data, thereby eliminating any interspecific variation in FA composition that would have been introduced by using only previously published data. Though the solubility values varied across individuals [0.051±0.003 (penguin) to 0.073±0.004 ml  $N_2$  ml<sup>-1</sup> oil (pilot whale)], the overall values obtained in the present study are similar to most solubility values previously

**Table 2. Results of the ANOSIM (analysis of similarities) by taxonomic group to determine differences in fatty acid (FA) composition**

Group 1	Group 2	R	P	% Difference	FA	Contributing %
<b>Baleen whale</b>	Pinniped	0.86	0.02	34.0	20:1n-9	14.2
<b>Baleen whale</b>	Sea turtle	0.98	0.02	38.7	20:1n-9	13.5
Baleen whale	<b>Seabird</b>	0.78	0.02	35.1	18:1n-9	14.2
Baleen whale	<b>Toothed whale</b>	0.69	0.02	37.5	18:1n-9	26.3
Baleen whale	<b>Terrestrial mammal</b>	1.00	0.001	57.2	18:1n-9	21.1
Pinniped	<b>Sea turtle</b>	0.77	0.01	38.3	14:0	13.8
Pinniped	<b>Seabird</b>	0.54	0.01	31.2	16:0	16.7
Pinniped	<b>Toothed whale</b>	0.43	0.02	31.2	18:1n-9	22.6
Pinniped	<b>Terrestrial mammal</b>	0.43	<0.001	51.4	18:2n-6	16.4
<b>Sea turtle</b>	Seabird	0.48	0.01	30.5	12:0	14.9
Sea turtle	<b>Toothed whale</b>	0.67	0.002	36.3	18:1n-9	23.5
Sea turtle	<b>Terrestrial mammal</b>	1.00	<0.001	49.7	18:1n-9	21.5
Seabird	<b>Toothed whale</b>	0.27	0.05	30.0	18:1n-9	21.5
Seabird	<b>Terrestrial mammal</b>	1.00	0.01	39.4	18:2n-6	21.6
Toothed whale	<b>Terrestrial mammal</b>	0.84	0.003	38.9	18:2n-6	21.9

The  $R$ -values are the ANOSIM test statistic (range 0–1). If significant differences exist,  $R$  will be greater than or equal to 0; an  $R$ -value of 0 indicates no difference in FA profiles. The  $P$ -values are from the pairwise analyses of the ANOSIM. The % difference column represents the percentage of group 1's total lipid composition that differs from that of group 2. The FA presented are the top FA responsible for the differences between the groups with contributing % as the difference between groups 1 and 2. The groups that are bolded indicate the group that contains the higher concentration of the major FA contributing to the difference. All  $P$ -values  $\leq 0.05$  are considered significant.

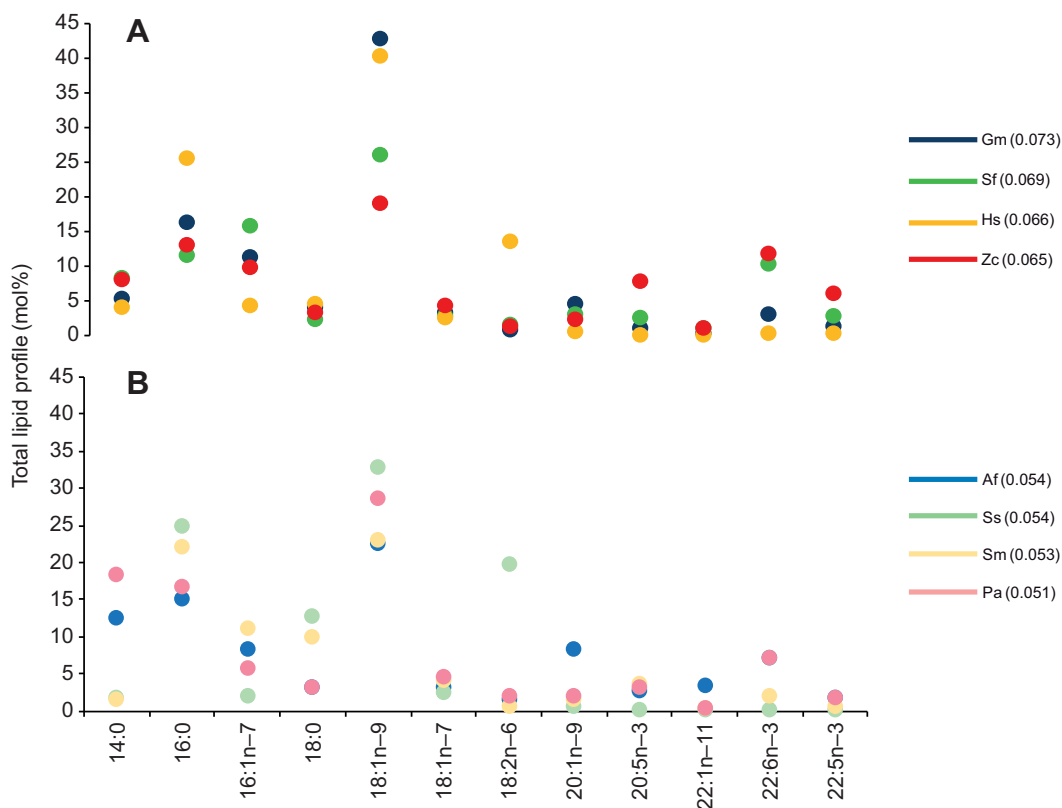
**Table 3. FA concentrations (mol%) for the 13 most abundant components for each of the binned  $N_2$  solubility value groups ( $ml N_2 ml^{-1}$  oil); individuals were assigned to one of five different  $N_2$  solubility groups, in descending order of  $N_2$  solubility for that sample (high, medium-high, medium, medium-low and low)**

FA	High (>0.065)	Medium-high (0.061–0.065)	Medium (0.057–0.061)	Medium-low (0.054–0.057)	Low (<0.054)
18:1n-9	31.83	27.27	29.42	26.55	26.59
16:0	16.45	13.13	16.05	17.95	19.57
16:1n-7	10.16	8.17	6.67	10.61	6.65
14:0	6.23	9.09	7.01	4.67	8.35
22:6n-3	6.17	5.95	2.05	5.58	3.95
18:2n-6	4.13	1.20	10.06	5.47	5.86
18:0	3.45	3.51	3.49	6.20	7.18
18:1n-7	3.06	3.25	2.64	3.74	3.48
20:5n-3	2.72	2.20	1.14	2.69	2.27
20:1n-9	2.50	4.44	3.56	1.61	2.98
22:5n-3	2.37	2.28	1.15	2.32	0.99
12:0	0.40	3.38	2.29	0.25	0.14
22:1n-11	0.53	1.34	2.88	0.49	0.93

FA are generally arranged in order of highest to lowest concentration. The shaded rows represent the same eight FA that account for 70–80 mol% of the lipid profile for all  $N_2$  solubility groups.

measured from other lipid and adipose tissues composed of only TAG molecules. Manufactured lipids such as lard and olive oil have  $N_2$  solubility values similar to those obtained for samples used in this study (0.067 and 0.070  $ml N_2 ml^{-1}$  oil, respectively; Langø et al., 1996; Weathersby and Homer, 1980), whereas butter has a much higher value (0.10  $ml N_2 ml^{-1}$  oil; Langø et al., 1996). Solubility values obtained previously from the adipose tissues of terrestrial mammals are also similar to those in the present study, including adipose tissue from dog (0.069  $ml N_2 ml^{-1}$  oil) and bone

marrow of ox and sheep (0.065 and 0.073  $ml N_2 ml^{-1}$  oil, respectively) (Ikels, 1964; Langø et al., 1996; Weathersby and Homer, 1980). Comparatively, the  $N_2$  solubility in other body tissues is much lower than the values typically reported for adipose tissues: 0.015  $ml N_2 ml^{-1}$  oil in human blood, 0.015  $ml N_2 ml^{-1}$  oil in calf brain and 0.016  $ml N_2 ml^{-1}$  oil in goat liver (Langø et al., 1996). The only other  $N_2$  data come from toothed whales, in which blubber solubility ranges from 0.062  $ml N_2 ml^{-1}$  oil (short-finned pilot whale and Atlantic spotted dolphin) to 0.107  $ml N_2 ml^{-1}$  oil



**Fig. 4. The 12 most abundant FA (mol%) in the lipid profiles and  $N_2$  solubility ( $ml N_2 ml^{-1}$  oil).** (A) The samples with the four highest  $N_2$  solubility values (Gm, short-finned pilot whale; Sf, Atlantic spotted dolphin; Hs, human; Zc, California sea lion). (B) The samples with the four lowest  $N_2$  solubility values (Af, emperor penguin; Ss, pig; Sm, eider duck; Pa, Adélie penguin). Data for the short-finned pilot whale and the Atlantic spotted dolphin are from Lonati et al. (2015) and data for the human and pig are from Gabler-Smith et al. (2020).

(pygmy sperm whale; Koopman and Westgate, 2012; Lonati et al., 2015). The acoustic tissues of toothed whales also show a similar range, from 0.066 ml N<sub>2</sub> ml<sup>-1</sup> oil (inner jaw fat, pygmy sperm whale) to 0.101 ml N<sub>2</sub> ml<sup>-1</sup> oil (outer jaw fat, short-finned pilot whale; Lonati et al., 2015). As demonstrated in the present study, diving tetrapods with adipose tissue containing only TAG have lower N<sub>2</sub> solubility values compared with diving toothed whales with adipose tissue containing unusual lipids (i.e. WE and short-chain FA).

### Lipid composition

Lipid composition was quite similar between all taxonomic groups studied. In almost all cases, the individual FA present and their general concentrations were similar across the species examined. In all samples, the adipose tissue contained both endogenous and exogenous monounsaturated components (e.g. 14:0, 16:0, 18:0, 16:1n-7, 18:1n-9, 22:1n-11 and 22:6n-3) and had high carbon numbers (>16.3; range of 16.3-18.3; Table 1; Tables S1 and S2). The FA in the highest concentration across all groups of animals was 18:1n-9, which is a common lipid found in marine vertebrate adipose tissues (Budge et al., 2006). Previous research has demonstrated that diet has a strong influence on the FA composition in adipose tissue of marine vertebrates (Ackman et al., 1971b); however, some studies also indicate that marine animals may have some control on specific FA metabolism, indicated by the discrepancy between the FA of prey items and the FA composition of the predator's adipose tissue (e.g. Grahl-Nielsen and Mjaavatten, 1991). Most FA in vertebrates are even-numbered and straight, between 14 and 24 carbons in length with zero to six double bonds (Budge et al., 2006; Iverson, 2009). FA that are greater than 14 carbons in length are often deposited in animal tissue with minimal modification from the diet (Iverson, 2009). The presence of the long-chain PUFA in top predators is from direct deposition of FA acquired from prey items that have consumed algae (phytoplankton and zooplankton; Iverson, 2009), as algae are the only organisms with enzymes capable of producing long-chain PUFA (e.g. 20:5n-3 and 22:5n-6; Iverson et al., 1997; Iverson, 2009). *De novo* synthesis occurs from two-carbon precursors that are elongated by sequential additions of two-carbon units; these chains can grow between 14 and 18 carbons (Iverson, 2009). Vertebrates restrict *de novo* FA synthesis to 14:0, 16:0 and 18:0, and their MUFA counterparts 14:1n-5, 16:1n-7 and 18:1n-9 (Budge et al., 2006; Iverson, 2009). The lipid composition data obtained in this study also generally agree with what has previously been documented sea turtles, seabirds, cetaceans, pinnipeds and terrestrial mammals (sea turtles: Ackman and Burgher, 1965; Ackman et al., 1971a; Davenport et al., 1990; Guitart et al., 1999; Holland et al., 1990; seabirds: Groscolas, 1990; Dahl et al., 2003; Johnston, 1973; Johnson and West, 1973; Speake et al., 1999; baleen whales: Bottino, 1978; Meier et al., 2016; toothed whales: Koopman, 2007; Koopman et al., 2003; Lonati et al., 2015; pinnipeds: Ackman and Hooper, 1974; Beck et al., 2005; Iverson et al., 1997; Liwanag et al., 2012; pigs and humans: Budge et al., 2006; Calder et al., 2015; Duran-Montgé et al., 2010; Gabler et al., in press; Hodson et al., 2008; Kingsbury et al., 1961; Wood et al., 2008).

### N<sub>2</sub> solubility related to lipid composition

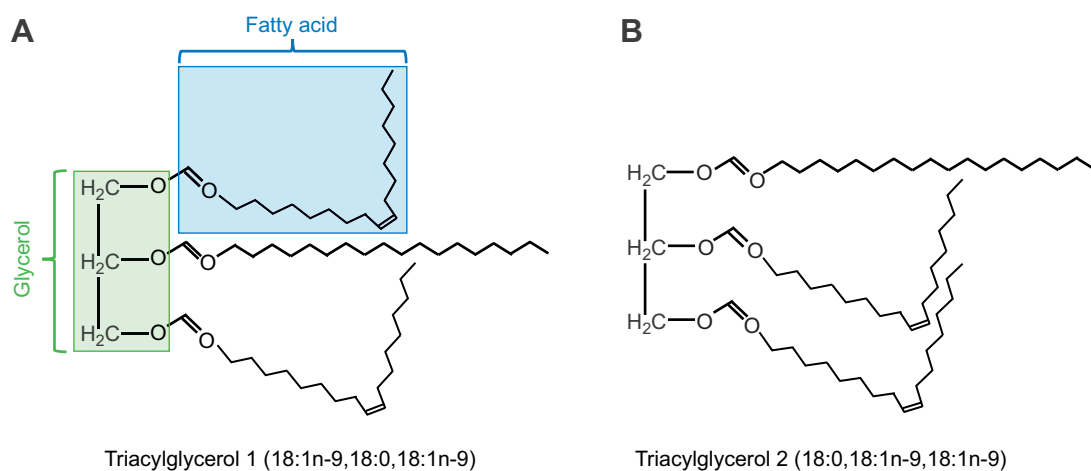
N<sub>2</sub> solubility values of the adipose tissue in the present study did not show any clear or specific relationships with lipid composition (Fig. 3B). Solubility values varied across taxonomic groups but also within a species (Figs 1 and 2, Table 1). Previous studies have

demonstrated that N<sub>2</sub> solubility is related to lipid composition in the blubber and jaw fats of toothed whales (Koopman and Westgate, 2012; Lonati et al., 2015). Koopman and Westgate (2012) showed that WE content alone explained 52% of the variation observed in N<sub>2</sub> solubility of the blubber of toothed whales; however, these authors found no relationships between N<sub>2</sub> solubility and FA or FAlc composition. Later, Lonati et al. (2015) demonstrated that adipose tissue from the acoustic fats (jaw fats and melon) of toothed whales, which contain high concentrations of unique lipid components (e.g. short branched chain *i*-5:0, *i*-12:0 FA and WE), have higher N<sub>2</sub> solubility values compared with the blubber of the same individual. These two studies showed that both increased concentrations of WE and the presence of the unique BCFA are associated with significantly increased N<sub>2</sub> solubility in adipose tissues (Lonati et al., 2015).

Therefore, as lipid composition has been shown to be related to N<sub>2</sub> solubility, the assumption was that any variation we observed in N<sub>2</sub> solubility in adipose tissue containing only TAG would be associated with the chemistry of these molecules – namely, their FA composition. However, this was not the case, as can easily be seen in Fig. 4, which shows the FA profiles from the samples with the four highest N<sub>2</sub> solubility values (Fig. 4A) and the four lowest N<sub>2</sub> solubility values (Fig. 4B). The adipose tissues from the human sample and the pig sample had similar FA profiles; however, they had very different N<sub>2</sub> solubility values (0.066 and 0.054 ml N<sub>2</sub> ml<sup>-1</sup> oil, respectively, differing by ~20%; Fig. 4). Similarly, the Atlantic spotted dolphin sample and the eider duck sample had similar FA profiles, but very different N<sub>2</sub> solubility values (0.069 and 0.053 ml N<sub>2</sub> ml<sup>-1</sup> oil, respectively, differing >20%; Fig. 4). It is also clear that within the samples with four highest and four lowest N<sub>2</sub> solubility values (Fig. 4), there is some variation in FA composition, meaning that there are multiple combinations of different FAs that lead to the same high or low N<sub>2</sub> solubility value. Additionally, the ANOSIM analysis comparing the lipid profiles across the different groups of N<sub>2</sub> solubility values did not reveal any significant differences (Fig. 3B). These results indicate that differences in the individual FA pool of a sample do not have a large influence on N<sub>2</sub> solubility in adipose tissues composed of only TAG.

Therefore, we propose that the main variable that determines N<sub>2</sub> solubility must be the chemistry of the intact TAG molecules themselves, namely the 3D structure of the arrangement of individual FA on a TAG molecule (i.e. positional specificity). Positional specificity in TAG molecules (not measured in this study) describes the location of each component FA on the glycerol portion of the molecule, with notations sn-1 and sn-3 for the FA linked to the outer carbons of the glycerol backbone and sn-2 for the FA located on the internal position (Fig. 5A, present study; Breckenridge, 1978; Brockerhoff et al., 1966). To illustrate this, Fig. 5A and B show two TAG molecules, each with the same FA composition; however, the FA constituents are located on different positions between TAG 1 and TAG 2. These configurations will result in different 3D conformations, as any double bonds introduce bends in the FA tails that, in turn, displace the other two FA on the TAG. The 3D structure of the intact TAG molecules is very important in terms of function, both physically and biologically. The positions of the FA on the TAG molecules influence the physical properties of oils, such as melting point and crystallization properties as well as their metabolism (Hagemann, 1988; reviewed in Motoyama, 2012; Redgrave et al., 1988). Given these physical characteristics, it is therefore reasonable to propose that the 3D structure of a TAG molecule might also influence its inert gas solubility.





**Fig. 5. Positional stereospecificity of FA in TAG molecules.** TAG composition: three FA, shown in blue, esterified to a glycerol backbone, shown in green. FA are positioned on certain carbons, noted by sn-1, sn-2 and sn-3. Two TAG molecules demonstrating the potential for differences in the three-dimensional conformation with varying FA composition. (A) TAG with two different FA (18:1n-9 on sn-1, 18:0 on sn-2 and 18:1n-9 on sn-3); (B) TAG with the same two FA, but these FA are on different positions of the glycerol backbone (18:0 on sn-1, 18:1n-9 on sn-2 and sn-3).

There are limited data on the specific composition of the component FA in TAG molecules for marine species (and other related species) in the literature; however, it is known that the positional distribution of FA in TAG of animal fat is not random (Brockerhoff et al., 1968). Studies on the stereospecific positional distribution of the FA in terrestrial mammal TAG lipids suggest there may be some general phylogenetic differences in the structures of TAG molecules. In the adipose tissue of domestic pigs, 16:0 is exclusively found on position sn-2 (~80 mol%; Karupaiah and Sundram, 2007; Mattson et al., 1964). A similar pattern is observed in wild, non-domesticated swine relatives such as the wild boar and peccary, in which high concentrations of 16:0 are also found on position sn-2 (Mattson et al., 1964). In the adipose tissue of domestic pigs, 18:2n-6 can also predominate at position sn-2 (Reiser and Reddy, 1959). Additionally, unsaturated FA appear to be positioned at sn-3, and 18:1n-9 occurs on both positions sn-1 and sn-3 (Mattson et al., 1964; Reiser and Reddy, 1959). Interestingly, other artiodactyls (i.e. hippopotamus, camel, white-tailed deer, sheep and cow) seem to have a different pattern, with <19 mol% of 16:0 occurring in position sn-2 (Mattson et al., 1964). With the exception of the pig, unsaturated FA seem to be concentrated at position sn-2 (Brockerhoff, 1966; Mattson et al., 1964). The adipose tissues of humans appear to be similar to most artiodactyls (excluding the pig), with MUFA and 18:2 appearing at position sn-2. Additionally, 16:0 accumulates at sn-1 and sn-3 and 14:0 at sn-2 in humans (Brockerhoff, 1965; Brockerhoff, 1966). Therefore, the general trends for positional specificity in terrestrial mammals are: (1) in position sn-1, saturated FA of endogenous origin predominate, (2) in position sn-2, unsaturated and short-chain FA of exogenous origin predominate and (3) at position sn-3, long-chain FA are present, but this is also the position where the most randomness occurs (Brockerhoff, 1966). For instance, FA 14:0 would occur in the highest concentrations at sn-2, owing to the FA being short in length; however, because 14:0 is also a SFA, it would also occur in high concentrations at position sn-1.

The structures of intact TAG in the lipids of marine mammals appear to show some similarity to the patterns observed for terrestrial mammals, but some differences also exist. In the blubber of baleen whales (e.g. sei whale), SFA (14:0, 16:0 and 18:0) and long-chain PUFA ( $C_{20}$  and  $C_{22}$ ) appear on positions sn-1 and sn-3, similar to the arrangement in terrestrial mammals (Bottino, 1978;

Brockerhoff et al., 1968). MUFA (16:1 and 18:1) dominate on position sn-2, which is different than terrestrial mammals and PUFA can sometimes appear on this position (Bottino, 1978). There even appears to be some specificity to the positioning of the unique branched, short-chain FA *i*-5:0 in three families of toothed whales: delphinids, phocoenids and monodontids, with *i*-5:0 predominant at the sn-1 and sn-3 positions (Wedmid and Litchfield, 1976). In the TAG molecules of the lipids of pinnipeds, the position of the FA seems to be determined by chain length (Brockerhoff, 1966). In harbor and harp seal oil, shorter and SFA accumulate on position sn-2 (i.e. 14:0, 15:0, 16:0 and 17:0), but can sometimes be observed on positions sn-1 and sn-3 (Brockerhoff et al., 1968; Wanasundara and Shahidi, 1997; Yoshida et al., 1996). In both pinnipeds, PUFA (i.e. 20:5n-3, 22:5n-3 and 22:6n-3) occupy primarily position sn-3, followed by sn-1 (Ando et al., 2018; Brockerhoff et al., 1968; Litchfield, 1968; Wanasundara and Shahidi, 1997). In leatherbacks, SFA (e.g. 12:0, 14:0, 16:0 and 18:0) are found mostly on position sn-1 in the highest amounts and secondarily on position sn-3 (Brockerhoff et al., 1968). 16:1 and 18:1 are found on position sn-2, as well as most PUFA (e.g. 22:6; Brockerhoff et al., 1968; Litchfield, 1968). Long-chain FA predominant on position sn-3 (Brockerhoff et al., 1968). A single seabird species (cormorants) was investigated and it was determined that 16:0 and 18:0 are found mostly on position sn-1 and MUFA are on either position sn-2 or sn-3 (Brockerhoff et al., 1968). With the data presented above, different patterns of FA positional stereospecificity exist across different groups of animals, and as our study suggests, likely across individuals within a species. This suggests that there could be differences in the intact TAG structures, which might explain the similar FA compositions in samples with different  $N_2$  solubilities observed in the present study. It is important to note that the studies describing intact TAG structure above provide very general information on positional specificity for each species, without reporting variation across individuals (considering sex, life stage, season and habitat) or tissues within species.

There is also surprisingly little information about how the stereospecificity of FA on a larger lipid molecule (TAG, or in the case of membranes, phospholipids) will affect gas solubility. The few data that do exist describe solubility and FA structure across lipid membranes, composed of a phospholipid bilayer. Phospholipids are made up of two FA esterified to a glycerol molecule that also

contains a polar head group (derived from phosphatidic acid; Budge et al., 2006). Lower solubility and diffusivity of oxygen occurs from decreased membrane fluidity arising from a decrease in unsaturated FA; unsaturated FA contain double bonds that create 'bends' within the structure (Pace and Chan, 1982; Subczynski et al., 1992; Träuble, 1971). Experiments on oxygen diffusion through vesicles made of lecithin (mixtures of glycerophospholipids including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidic acid) suggest that gas diffuses more readily through vesicles composed of dimyristoyl (2, 14:0 FA) than dipalmitoyl (2, 16:0 FA), with diffusion rates of  $4.7 \times 10^{-5}$  and  $1.56 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ , respectively (Fischkoff and Vanderkooi, 1975). These results indicate that the conformation of entire intact molecules, determined by the types of FA present in the TAG molecules and their positions, likely influences gas solubility. Future work is needed to consider how different intact TAG molecules influence gas solubility and diffusion. This is a promising area of research for both TAG and phospholipid gas dynamics, and of potential importance in the context of global climate change as atmospheric and aquatic levels of oxygen and carbon dioxide undergo significant shifts on both small and large scales.

### Implications for diving models

Owing to the difficulties involved with collecting data on the partial pressures of  $\text{N}_2$  within the blood and tissues of freely diving air-breathing animals, mathematical models have become pivotal to simulating  $\text{N}_2$  dynamics in marine vertebrates to assess their risk of DCS (Fahlman et al., 2007; Fahlman et al., 2014; Hooker et al., 2012; Houser et al., 2001; Kvadsheim et al., 2012; Zimmer and Tyack, 2007). Diving models rely on assumptions about physiological responses collected from terrestrial mammals, which differ from marine mammals (Fahlman et al., 2006; 2009). Perfusion rates of many marine mammals are also unknown; therefore, models often assume changes in cardiac output (product of heart rate and stroke volume) are related to heart rate, which is an easily measured variable in captive animals (Fahlman et al., 2006; 2009; Hooker et al., 2009; Zimmer and Tyack, 2007).  $\text{N}_2$  solubility is another important component in these models, but the value most often used in these models is obtained from experiments on terrestrial mammals. The current value used to calculate  $\text{N}_2$  saturation rates in fat compartments of breath-hold diving animals is  $0.070 \text{ ml N}_2 \text{ ml}^{-1}$  medium, and is based on values obtained for olive oil and oxen and sheep bone marrow (Fahlman et al., 2006; Langø et al., 1996; Weathersby and Homer, 1980). However, it has been shown that this value is much lower than the actual solubility value measured in some toothed whale (e.g. odontocete) blubber and acoustic fats. Koopman and Westgate (2012) demonstrated that  $\text{N}_2$  solubility values in the blubber of most odontocetes was higher than  $0.070 \text{ ml N}_2 \text{ ml}^{-1}$  lipid. Lonati et al. (2015) further demonstrated that  $\text{N}_2$  solubility values in the jaw fats are even higher, with 84% of the fats measured having higher values than  $0.070 \text{ ml N}_2 \text{ ml}^{-1}$  lipid. The values measured in the present study demonstrate that the adipose tissues of most diving tetrapods, composed of mostly TAG lipids, have  $\text{N}_2$  solubility values lower than  $0.070 \text{ ml N}_2 \text{ ml}^{-1}$  lipid (Table 1). We had hoped that knowing the FA composition (analytically relatively easy to measure) of fat composed of only TAG might be used to predict  $\text{N}_2$  solubility without having to physically measure the solubility (analytically very difficult to measure). However, we have demonstrated that knowledge of the FA pool alone does not permit such predictions, which is why we think the intact TAG structure will be a more important factor influencing  $\text{N}_2$  solubility in these tissues.

We recognize that our values were obtained at a set temperature and at standard pressure under experimental conditions, which will not be the case for diving animals in the wild. Diving tetrapods exhibit a range of diving depths (from 26 m reached by the Adélie penguin and up to 3000 m by some beaked whales; Chappell et al., 1993; Schorr et al., 2014), which means that the subcutaneous adipose tissue of these animals will experience a range of temperatures and pressures during the different phases of a dive; variation in these conditions will likely affect solubility properties. However, there are few data on  $\text{N}_2$  solubility in biological lipids at different pressures and conflicting data for different temperatures. Gerth (1985) showed that  $\text{N}_2$  solubility in olive oil at a set temperature of  $37^\circ\text{C}$  increased with pressure under experimental conditions, but this author also pointed out that olive oil is a non-ideal solution and showed that its solubility patterns deviate from Henry's law, resulting in higher solubility increases with pressure than linearly predicted. Blubber and other subcutaneous adipose tissues are composed of even more complex lipid mixtures than olive oil; therefore, we assume that these adipose tissue lipids will also deviate from what is predicted by Henry's law. As an animal dives to depth, not only will the pressure increase, but temperature will also decrease. Data from the literature give no clear indication as to the relationship between temperature and  $\text{N}_2$  in various biological lipids: under some experimental conditions, decreasing temperature leads to a decrease in solubility while others show an opposite trend (reviewed in Langø et al., 1996). Preliminary data using olive oil (and the apparatus from the present study to measure solubility) suggest that as temperature decreases,  $\text{N}_2$  solubility also decreases (A. Westgate, unpublished data). However, this conclusion is based on only a few data points. Additionally, most lipids will become solid or pseudo-solid as they cool (i.e. phase change), which may also affect  $\text{N}_2$  solubility. Because these biological lipids are complex and contain many components (as we have demonstrated), each with different melting points, this phase change will occur over a wide temperature range. For example, lipids in the blubber of *Mesoplodon densirostris* begin to melt at  $1.7^\circ\text{C}$  and are fully melted at  $27.1^\circ\text{C}$  (T. Ernst, unpublished data), suggesting that blubber in a beaked whale will be in the phase-change temperature zone while the animal is diving; unfortunately, few other data on phase change temperatures of marine lipids are available. Clearly, more information on the effects of pressure and temperature on  $\text{N}_2$  solubility in the complex lipid mixtures of adipose tissue, and how this might be impacted by blood flow, is needed in order to fully understand solubility patterns in actively diving tetrapods.

### Conclusions

In conclusion, this study presents the first measurements of  $\text{N}_2$  solubility in the adipose tissue and blubber of a variety of diving air-breathing tetrapods from four broad taxonomic groups (sea turtles, seabirds, cetaceans and pinnipeds), yielding novel data on  $\text{N}_2$  solubility, and providing the FA composition of the same samples. In adipose tissue composed mainly of TAG molecules, there appears to be no predictable relationship between the composition of the FA pool in the TAG and  $\text{N}_2$  solubility, contrary to what has been previously demonstrated in adipose tissues with WE and short- and branched-chain FA (Koopman and Westgate, 2012; Lonati et al., 2015). Further, the  $\text{N}_2$  solubility across and within taxonomic groups (and even species) was highly variable, suggesting that solubility values cannot be predicted based on phylogenetic relationships, even broadly. We posit that it is the specific positioning of the FA within the TAG molecules themselves that

is influencing the solubility of N<sub>2</sub> in these tissues. Based on the variability in N<sub>2</sub> solubility within and among taxa, further research on the stereospecific positions of the FA on TAG molecules may allow for a more accurate prediction of solubility patterns in the adipose tissues of diving animals.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: M.K.G.-S., A.J.W., H.N.K.; Methodology: A.J.W.; Validation: A.J.W.; Formal analysis: M.K.G.-S., A.J.W., H.N.K.; Investigation: M.K.G.-S., A.J.W.; Resources: M.K.G.-S., A.J.W., H.N.K.; Writing - original draft: M.K.G.-S.; Writing - review & editing: M.K.G.-S., A.J.W., H.N.K.; Visualization: M.K.G.-S., H.N.K.; Supervision: A.J.W., H.N.K.; Funding acquisition: A.J.W., H.N.K., M.K.G.-S.

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#### Supplementary information

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