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REVIEW

Function and evolution of specialized endogenous lipids in toothed whales

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ABSTRACT

The Odontocetes (toothed whales) possess two types of specialized fat and, therefore, represent an interesting group when considering the evolution and function of adipose tissue. All whales have a layer of superficial blubber, which insulates and streamlines, provides buoyancy and acts as an energy reserve. Some toothed whales deposit large amounts of wax esters, rather than triacylglycerols, in blubber, which is unusual. Waxes have very different physical and physiological properties, which may impact blubber function. The cranial acoustic fat depots serve to focus sound during echolocation and hearing. The acoustic fats have unique morphologies; however, they are even more specialized biochemically because they are composed of a mix of endogenous waxes and triacylglycerols with unusual branched elements (derived from amino acids) that are not present in other mammals. Both waxes and branched elements alter how sound travels through a fat body; they are arranged in a 3D topographical pattern to focus sound. Furthermore, the specific branched-chain acid/alcohol synthesis mechanisms and products vary phylogenetically (e.g. dolphins synthesize lipids from leucine whereas beaked whales use valine). I propose that these specialized lipids evolved first in the head: wax synthesis first emerged to serve an acoustic function in toothed whales, with branched-chain synthesis adding additional acoustic focusing power, and some species secondarily retained wax synthesis pathways for blubber. Further research is necessary to elucidate specific molecular mechanisms controlling the synthesis and deposition of wax esters and branched-chain fatty acids, as well as their spatial deposition within tissues and within adipocytes.

KEY WORDS: Wax ester, Blubber, Acoustic fat, Isovaleric acid, Branched-chain fatty acid, Melon

Introduction

Lipids are crucial for all animals, playing roles ranging from compartmentalization to cell signaling, energy storage, insulation and buoyancy, among other functions. In marine mammals [specifically pinnipeds (seals, sea lions and walruses) and cetaceans (whales)], the most easily recognized lipid feature is the thick layer of blubber covering the body. The morphology, distribution and composition of the blubber have been the subject of many studies focusing on thermoregulation, hydrodynamic streaming, buoyancy and energy reserves (e.g. Iverson et al., 1995; Pabst et al., 1999; Worthy and Edwards, 1990; Biuw et al., 2003; Singleton et al., 2017). Blubber thickness varies with many factors, including species, habitat, growth, health, reproduction and season, but ranges from <0.5 cm in tropical dolphins to >50 cm in bowhead whales (Iverson and Koopman, 2018). Similarly, the lipid content of blubber will vary according to these factors. The lipid composition of blubber reflects contributions from the diet as well as *de novo* synthesis. In general, the adipose tissues of marine mammals are similar to those of other mammals in terms of storing mainly triacylglycerols containing fatty acids with ∼14–24 carbons (Pond, 1998). However, a subset of the marine mammals, the toothed whales (suborder Odontoceti), have evolved unique lipid synthesis pathways that allow them to deposit an unusual suite of endogenous lipids [including wax esters and branched-chain fatty acids (BCFA)] into their adipose tissues. Here, I review these unusual lipids in toothed whales, describe in which tissues and species they are found, suggest potential functions of these lipids and provide some ideas about how these unusual physiological adaptations might have evolved.

The relevant adipose tissues: blubber and the acoustic fat bodies

Blubber is a specialized form of adipose tissue, located in the hypodermis. Blubber is composed of adipocytes, connective tissue and vasculature (see Iverson and Koopman, 2018). In toothed whales, blubber is stratified through its depth, in terms of lipid composition, adipocyte morphology, and in some cases, the distribution of the microvasculature (i.e. capillaries, microarterioles and microvenules) (e.g. Montie et al., 2008; Koopman, 2007; McClelland et al., 2012). The fatty acid composition of the blubber of many toothed whales shows a gradient from the epidermis to the muscle–blubber fascial interface, with higher concentrations of dietary and unsaturated fatty acids in the innermost layers, and more saturated, endogenous lipids in the outer layers (Ackman et al., 1971; Krahn et al., 2004; Koopman, 2007). It has been suggested that this reflects the deposition (and potential for mobilization) of dietary-derived lipids during periods of fattening and fasting, respectively, because adipocytes closer to the body core will be (i) warmer, and likely in the fluid state, and (ii) more vascularized for nutrient exchange between blood and fat. Generally, the inner layers of the blubber of toothed whales are thought to be more metabolically active, with the outer layers playing a larger role in body shaping and hydrodynamics (Koopman et al., 2002; Struntz et al., 2004). The structure, metabolic properties and specific functions of blubber are known to vary across species, life-history stages and habitats; however, species-specific data on the characteristics of blubber are only available for less than half of the 75 species of odontocetes recognized today.

The second important adipose depot is the specialized cranial fat bodies (the ‘acoustic fats’), which play important roles in the transmission and reception of sound during echolocation and hearing (see Fig. 1). All toothed whales possess a fatty ‘melon’ that sits in the skull basin in the ‘forehead’ region; high-frequency sound

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Journal of Experimental Biology (2018) 221, jeb161471. doi:10.1242/jeb.161471

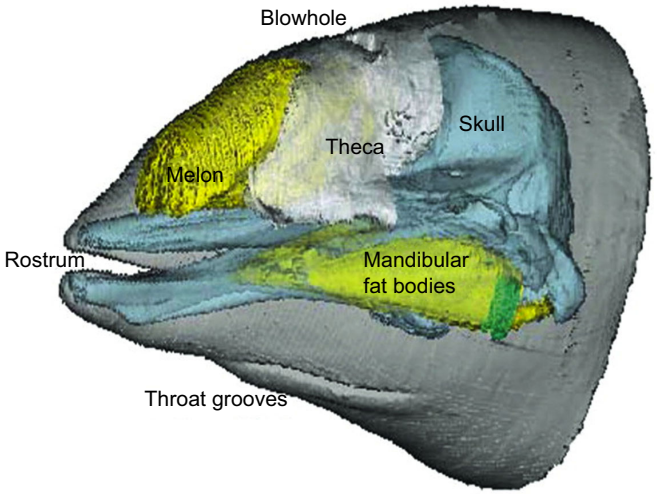

Fig. 1. Acoustic fats (melon and mandibular fats, in yellow) in a Cuvier's beaked whale, *Ziphius cavirostris*. This computer-generated image was constructed from CT scans of soft tissues. Image courtesy of Soldevilla et al. (2005).

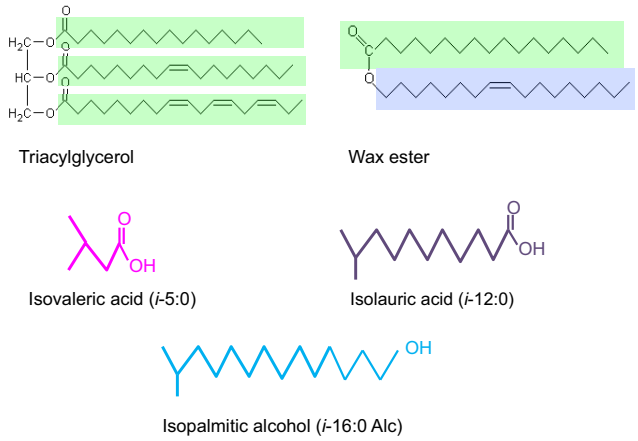

Fig. 2. Unusual endogenous lipids in toothed whales. (A) Triacylglycerol, which comprises three fatty acids, shown in green, esterified to glycerol. (B) Wax ester, which comprises a fatty acid, shown in green, esterified to a fatty alcohol (shown in blue). Triacylglycerols and wax esters are lipid classes. (C) Isovaleric acid and (D) isolauric acid are fatty acids derived from leucine (*i*-5:0) and valine (*i*-12:0), respectively, and (E) isopalmitic alcohol is a fatty alcohol (*i*-16:0alc) derived via the valine pathway, common to most acoustic fat bodies.

is generated in the nasal apparatus and focused out through the melon. Odontocetes also have fatty deposits surrounding their lower jaws; these are the intra-mandibular (within the enlarged mandibular fossa) and extra-mandibular (on the outside of the fossa, under the blubber) fat bodies, which focus incoming sound directly to the ear bones. The melon and jaw fats have very high lipid contents (typically >80% wet weight; Koopman et al., 2006; Lonati et al., 2015) and are believed to be synthesized entirely endogenously, *in situ* (Malins and Varanasi, 1975; Morii and Kaneda, 1982; Koopman et al., 2003, 2006). The acoustic fats were first proposed to function in the collimation, or focusing, of outgoing and incoming sound by Norris (1968), and have since been demonstrated to do so, both empirically and via modeling (e.g. Blomberg and Lindholm, 1976; Au, 1993; Aroyan, 2001; Soldevilla et al., 2005; Cranford et al., 2008). Despite the melon and jaw fats being common to all toothed whales, these tissues show considerable phylogenetic variation in composition (as will be discussed below) and in morphology (Cranford et al., 1996; McKenna et al., 2012). Unfortunately, data on the acoustic fats are lacking for many species, with lipid composition information available for less than a third of all toothed whales.

Lipid classes: the unusual wax esters

In most animals (including mammals), excess lipid energy is stored as triacylglycerols (Pond, 1998; Fig. 2). However, some animals (mostly marine species) synthesize and deposit large quantities of wax esters (Fig. 2) as storage lipids (in muscle, liver and adipose tissues). Sea anemones, calanoid copepods, lanternfishes and coelacanths are among the groups with a lipid store dominated by wax esters, along with the toothed whales (Nevenzel, 1970). Many fish that store wax esters inhabit deep waters (from 300 to >1000 m) and use these lipids in bones and muscles for buoyancy because they lack a gas-filled swim bladder (Ling et al., 2009). Wax esters are present in smaller quantities in a variety of species: for example, the wax blooms on the surface of desert insects, where they serve to limit water loss and reflect solar radiation, and in the earwax (9% wax esters) of humans, where it serves to protect the eardrum from debris particles (Hadley, 1985; Bortz et al., 1990; Okuda et al., 1991). Baleen whales have plugs of wax in their ear canals (Trumble et al., 2013), the functions of which are unknown. However, the toothed whales are the only mammals to store large amounts of wax esters within adipose tissues. Although the exact mechanisms of wax ester synthesis in toothed whales are not known, it is generally assumed (from *in vivo* experiments in fish) that the precursors for wax esters (fatty acids and alcohols) all arise from the same fatty acid pool; that is, the fatty alcohol is derived from the fatty acid of the corresponding chain length (Nevenzel, 1970; Kayama and Nevenzel, 1974). In addition, wax ester deposition is decoupled from wax ester ingestion. For example, North Atlantic right whales (*Eubalaena glacialis*), which feed selectively on wax-rich *Calanus* copepods, do not contain any wax esters in their blubber (see Swaim et al., 2009). As Kayama and Nevenzel (1974) stated, 'wax esters are not persistent dietary survivors in the food web', but rather are biosynthesized by the animals that store them.

Fatty acids and alcohols: the unusual lipid class constituents

Normally, mammalian fatty acid synthesis involves the sequential addition of two-carbon units (from malonyl-CoA) to an acetyl-CoA precursor (often from a carbohydrate source; see Gurr and James, 1971; Hadley, 1985). This mainly occurs in the liver, although adipose and mammary gland cells can also make fatty acids *de novo* (Hadley, 1985). The usual endpoint of mammalian fatty acid synthesis is palmitic acid (16:0), with some subsequent elongation and desaturation steps to produce longer 18–22 carbon fatty acids; mammals cannot synthesize polyunsaturated fatty acids because they lack the appropriate desaturase enzymes. The amount of fatty acid synthesis that occurs depends on an individual's health, body condition and diet (Pond, 1998; Budge et al., 2006). Marine mammals typically consume high-fat, low-carbohydrate diets and exhibit lower rates of fatty acid synthesis than many other groups (Budge et al., 2006).

In a novel metabolic adaptation, toothed whales produce BCFA in addition to normal straight-chain biosynthesis. The acoustic fats of some species contain high concentrations of shorter BCFA (Fig. 2), such as isovaleric acid (*i*-5:0; 3-methylbutanoic acid) and isolauric acid (*i*-12:0; 10-methyl undecanoic acid). These fatty acids arise from a completely different origin. BCFA are derived from the catabolism of branched-chain amino acids (BCAA) (Morii and

Kaneda, 1982; Brosnan and Brosnan, 2006; Crown et al., 2015). Most amino acids are degraded in the liver; however, BCAA (i.e. leucine, valine and isoleucine) are poorly metabolized by the liver and instead are degraded in muscle and adipose tissue (Rosenthal et al., 1974; Crown et al., 2015). According to Brosnan and Brosnan (2006), the three BCAA are catabolized simultaneously because the first two degradation enzymes are common, especially the one that is rate-limiting (Shimomura et al., 2001; see Fig. 3A). The first step is the conversion of each BCAA to its corresponding α-keto acid by mitochondrial branched-chain aminotransferase. The second, and flux-limiting, step is via the branch chain keto-acid dehydrogenase complex, which converts each α-keto acid into its corresponding acyl-CoA: isobutyryl-CoA (valine), α-methylbutyryl-CoA (isoleucine) and isovaleryl-CoA (leucine). These three acyl-CoAs are then processed by their respective dehydrogenase enzymes to another set of intermediates, and after additional enzyme reactions the final products are propionyl-CoA (valine and isoleucine), acetyl-CoA (isoleucine and leucine) and acetoacetate (leucine) (see Brosnan and Brosnan, 2006; Newgard, 2012; Crown et al., 2015). In adipose tissue, propionyl-CoA is then used for the synthesis of odd-numbered fatty acid chains and acetyl-CoA is the precursor for even-numbered fatty acid chains, both via elongation (Crown et al., 2015).

Toothed whales have modified these pathways, particularly in their cranial acoustic fats, to deposit large quantities of isobutyl-CoA and isovaleryl-CoA-derived fatty acids (Fig. 3B). The most common BCFA present in toothed whales are *i*-5:0, *i*-10:0, *i*-12:0, *i*-15:0, *i*-16:0 and *i*-16:0 alcohol (see Fig. 2). To determine their sources, Morii and Kaneda (1982) exposed very fresh tissues (melon, blubber, muscle and liver) from a striped dolphin (*Stenella coeruleoalba*) to radiolabeled (^{14}C) leucine, isoleucine and valine using tissue culture. The incorporation of these labeled amino acids into BCFA was negligible in muscle and liver, slightly higher in blubber and highest in the melon tissue. Furthermore, all the odd-chained BCFA (*i*-5:0, *i*-13:0 and *i*-15:0) were derived from leucine, all the even-chained BCFA (*i*-4:0, *i*-12:0, *i*-14:0 and *i*-16:0) were derived from valine, and isoleucine yielded the anteiso acids (*ai*-5:0 and *ai*-15:0). One of the fascinating aspects of this pathway in toothed whales is the strong phylogenetic component to the specific identity of the deposited BCFA; some families favor valine-derived lipids whereas others use the leucine pathway (see below).

Interestingly, the only mammals in which *i*-5:0 accumulates are humans with isovaleric acidemia, a genetic deficiency in isovaleryl-CoA dehydrogenase, the enzyme that converts isovaleryl-CoA to β-methylcrotonyl-CoA (Fig. 3A; Tanaka et al., 1966, 1988; Budd et al., 1967). The accumulation of *i*-5:0 in the blood of patients with isovaleric acidemia causes a suite of neurological abnormalities (severe mental deficiencies, convulsions and coma), and is fatal in 50% of patients if not treated within two weeks of birth (Nyhan, 1984). Wretlind (1957) demonstrated that *i*-5:0 was highly toxic to mice, having a lower LD_{50} compared with that of other short chains. The specific mechanisms of its toxicity are not well known, but *i*-5:0 is known to produce coma and EEG changes, and to inhibit mitochondrial respiration (Haas and Stumpf, 1981). Nevertheless, some toothed whales synthesize and deposit high concentrations of this and other BCFA in their adipose depots without accumulating them in the blood (H.N.K., unpublished data).

Waxes in the blubber of toothed whales

Within the toothed whales, a number of species store wax esters in blubber, either in addition to, or in complete replacement of, the triacylglycerols. Fig. 4 summarizes the lipid class composition of blubber for the 31 species for which data are available, representing beaked whales, sperm whales, dolphins, porpoises and one river dolphin (Litchfield et al., 1975, 1976; Koopman, 2007). The beaked and sperm whales have very high levels of wax (~60–99%) in their blubber (i.e. the first eight species listed along the *x*-axis in Fig. 4). Most of these waxes contain saturated and monounsaturated longer-chain (18–22 carbon) fatty acids and alcohols (e.g. Litchfield et al., 1976). There are only trace levels of wax (≤5% of total lipid content) in the blubber of some dolphins and porpoises.

We have known about wax esters in the blubber of beaked whales for centuries. According to the Speculum Regale – Konungs skuggsjá (1250) (King's Mirror), a Norwegian document written in approximately 1250, 'The beaked whale[s] are not fit to be eaten, for the fat that is drawn from them cannot be digested either by man or by any beast that may partake of it. For it runs through them…'. This statement reflects that most mammals are incapable of digesting wax esters and, if consumed, these lipids pass through the digestive system unprocessed, leading to keriorrhea, a term derived from the Greek words *keri* and *diarroia*, which mean 'wax' and 'to flow through', respectively (Berman et al., 1981; Ling et al., 2009). Even though mammals appear unable to digest waxes, other predators are capable of digesting these lipids in important prey species (e.g. copepods, lanternfish and oilfish). Place (1992) noted that seabirds such as petrels and albatrosses consume and digest wax routinely, assimilating these lipids with high efficiency, whereas mammals such as dogs are unable to digest these lipids. Place attributed the ability of birds to digest wax to several factors, including elevated bile salt levels, extended food reprocessing in the gizzard, and the possible use of a bile-dependent carboxyl ester lipase from the pancreas.

Although most mammals cannot digest wax esters, there are a few exceptions. North Atlantic right whales feed exclusively on wax ester-rich (90% of all lipids) copepods. An analysis of fecal material collected from free-swimming whales indicated that they are able to digest and assimilate >99% of ingested wax esters (Swaim et al., 2009). Similar observations have been made for minke whales (*Balaenoptera acutorostrata*) consuming wax-rich krill; these whales can digest ~94% of consumed waxes (Nordøy, 1995). Furthermore, the wax-rich lanternfish (myctophids), which represent >65% of all deep-sea fish biomass worldwide and the majority of the deep scattering layer (Irigoien et al., 2014), are important prey items for many marine mammals. Given that mammals do not express a specific wax ester lipase, the most parsimonious explanation is a reliance on symbiotic gut microbes, similar to the requirement for cellulase-containing microorganisms in fermenting herbivores to digest cellulose; however, this has yet to be confirmed. Furthermore, the presence of gut microbes (or another mechanism) allowing digestion of wax esters does not necessarily mean that the necessary enzymes are present to permit mobilization of stored waxes from adipose tissue.

The dominant presence of wax esters in high quantities in blubber is limited to three families of toothed whales (Fig. 4): Physeteridae (giant sperm whale), Kogiidae (pygmy and dwarf sperm whales) and Ziphiidae (beaked whales). These three families also represent the species that undertake the longest and deepest dives. Sperm whales (*Physeter*) frequently dive deeper than 600–800 m, and have been recorded at depths exceeding 2000 m; beaked whales also dive to depths of >1000 m, and the current record dive (2992 m) is held by a Cuvier's beaked whale (*Ziphius cavirostris*; reviewed in Pabst et al., 2016). This poses a conundrum when interpreting the evolutionary patterns and functions of these lipids – are waxes an adaptation for deep and long dives, or is the presence of waxes in the

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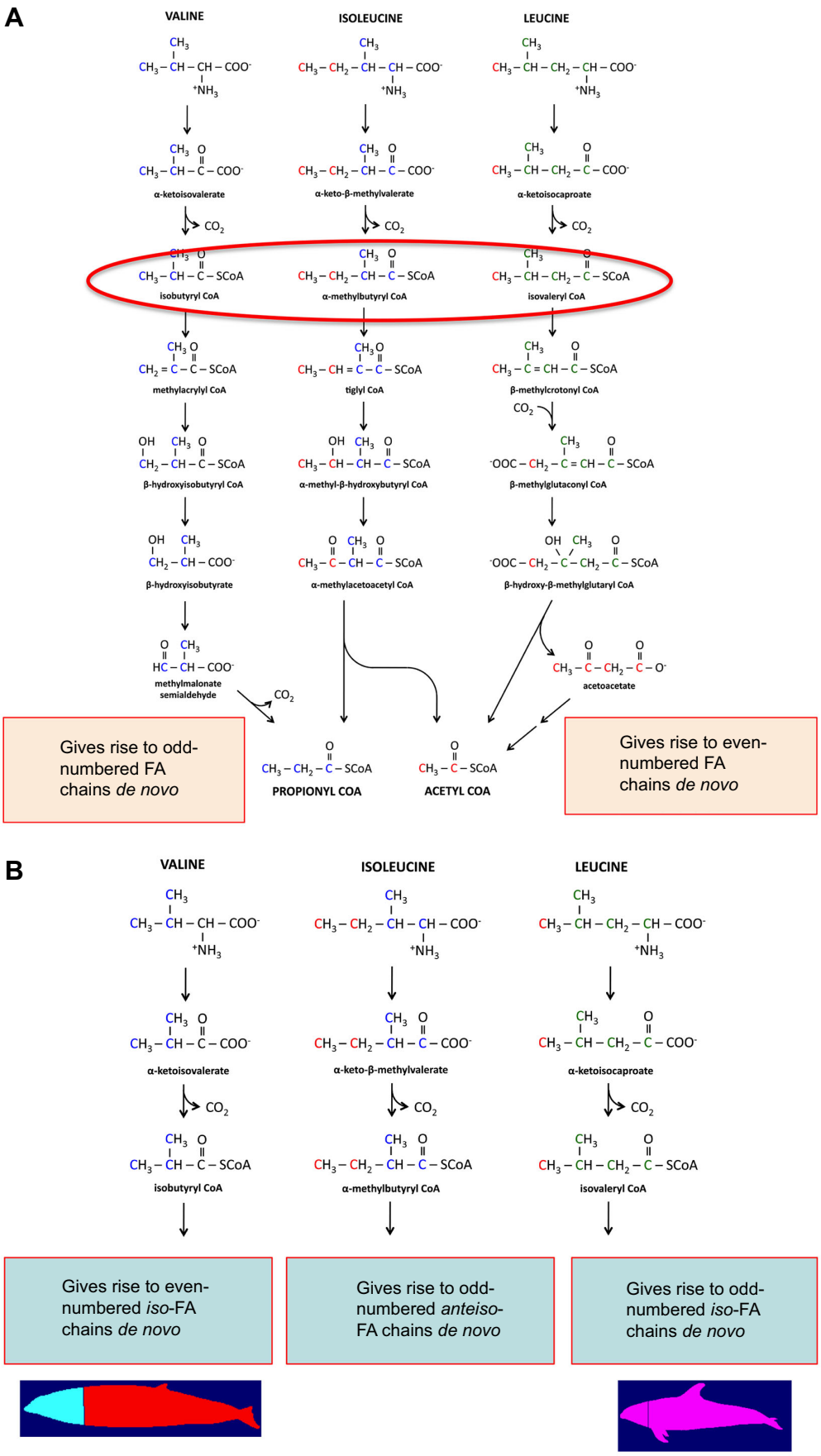

Fig. 3. Normal branched-chain amino acid (BCAA) degradation and fatty acid (FA) precursor formation pathways. (A) Usually, propionyl-CoA (from valine and isoleucine) leads to the formation of odd-numbered fatty acid chains *de novo*, and acetyl-CoA (from isoleucine and leucine) yields even-numbered fatty acid chains. Toothed whales use disruptions in this pathway (circled in red) to synthesize branched-chain fatty acid (BCFA) precursors. The dehydrogenases required to convert isobutyryl-CoA, methylbutyryl-CoA and isovaleryl-CoA to the next intermediates have been lost or are underexpressed. Figure modified from Crown et al. (2015). (B) BCAA pathway modifications in toothed whales for the synthesis of BCFA. Isobutyryl-CoA (from valine) ultimately leads to *i*-12:0, which is found in beaked whales. Isovaleryl-CoA (from leucine) ultimately leads to *i*-5:0, which is found in dolphins. The unusual fatty acids in the waxes and triacylglycerols of the acoustic fats are denoted by teal (*i*-12:0) or pink (*i*-5:0) in the head regions of the toothed whale shapes. Pink blubber indicates the presence of the leucine-*i*-5:0 pathway in the blubber. Red blubber indicates 'mammalian' fatty acids. Although the exact mechanisms of formation are unknown, the process has been confirmed via radiolabeling studies (Morii and Kaneda, 1982). Figure modified from Crown et al. (2015).

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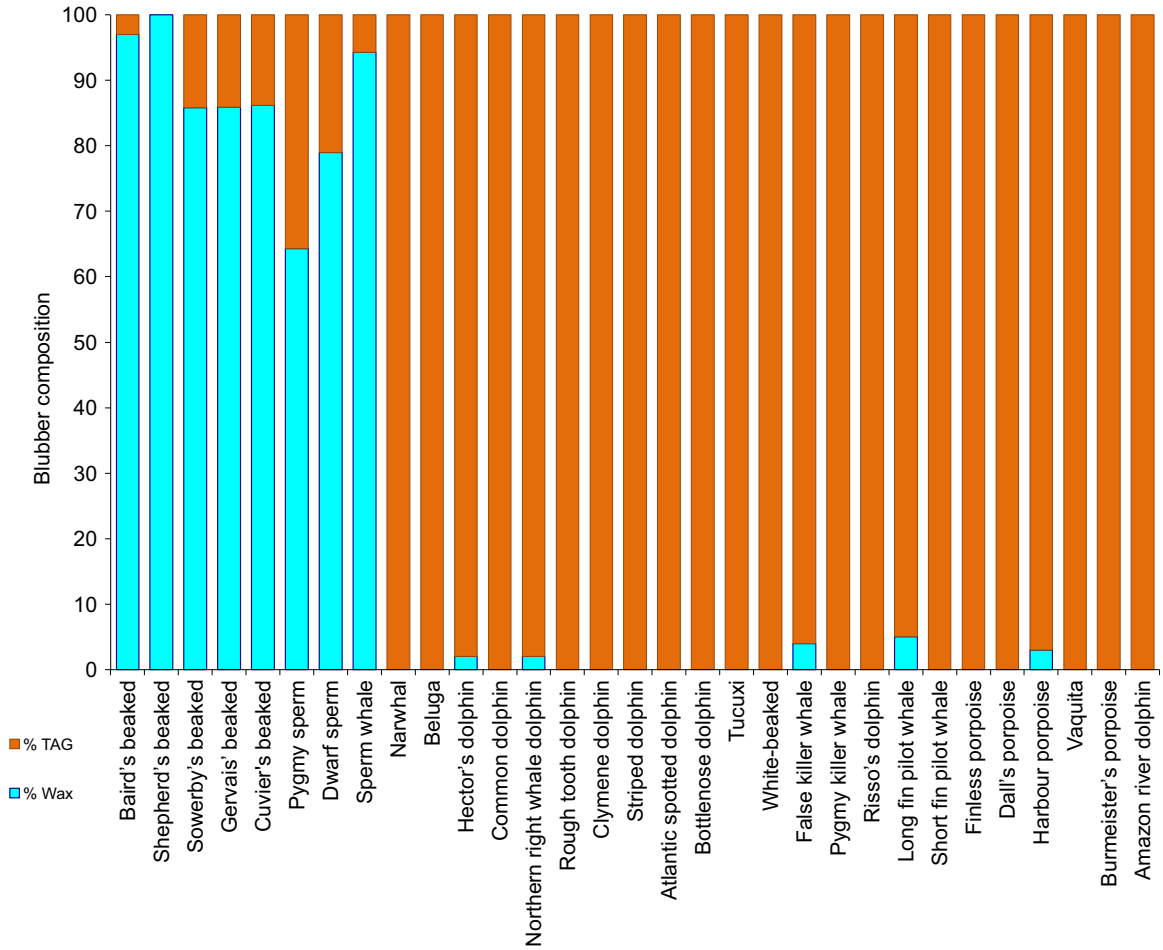

Fig. 4. Wax ester content of toothed whale blubber (% total lipids). The first five species (Baird's beaked whale, Shepherd's beaked whale, Sowerby's beaked whale, Gervais' beaked whale and Cuvier's beaked whale) are members of the Ziphiidae; the pygmy sperm whale and dwarf sperm whale belong to the Kogiidae family and the sperm whale is a member of the Physeteridae. TAG, triacylglycerol. Data are from Litchfield et al. (1975, 1976) and Koopman (2007).

blubber a consequence of phylogenetic lineage? Below I describe some characteristics of waxes that may offer some potential adaptive advantages, or at least possible reasons for the presence of high concentrations of wax esters in the blubber of adult toothed whales.

Wax esters offer more buoyant force than other lipids, with typical density values of 0.86 g cm^{-3}, whereas triacylglycerols have density values of 0.93 g cm^{-3} (Sargent, 1976). Recent work indicates that deep diving toothed whales devote a greater fraction of their body mass to bone and muscle, rather than to brain and viscera (Pabst et al., 2016). Bone and muscle have lower metabolic rates than brain and visceral organs and are also denser. The incorporation of wax esters into blubber would provide greater positive buoyancy, possibly offsetting the additional mass of muscle and bone to achieve neutral buoyancy when diving to below the depths of lung collapse; however, this idea requires further investigation.

Blubber containing wax esters also has a higher energy density than blubber with only triacylglycerols. Pure 'fat' is generally assumed to yield ~39 kJ g^{-1}; however, in reality, this depends on its lipid composition. Oil extracted from the wax-rich blubber of beaked and sperm whales contains ~42–43 kJ g^{-1}, representing an additional 10% of potential stored energy (A. J. Westgate and H.N.K., unpublished data). The key term here is 'potential' because we know neither the specific enzymatic steps used in wax ester synthesis and deposition by toothed whales nor the metabolic cost [i.e. adenosine triphosphate (ATP)] for these processes. Perhaps more importantly, we do not know whether toothed whales mobilize stored waxes during periods of energy deficiency or during increased energy expenditure; therefore, it is possible that the increased potential energy yield afforded by wax-rich blubber is not utilized as an energy reserve by toothed whales. Blubber containing wax also has a higher nitrogen solubility than blubber with only triacylglycerols (Koopman and Westgate, 2012). However, how this affects gas dynamics while diving is not yet known.

The thermal properties of blubber are influenced by both quality and quantity. Bagge et al. (2012) were the first to demonstrate that blubber with wax provided better insulation than blubber without wax by comparing the thermal properties of the blubber of short-finned pilot whales (*Globicephala macrorhynchus*; 0% wax) with that of pygmy sperm whales (*Kogia breviceps*; ~82% wax). Although the total amount of lipid present in these species was similar (~60% wet weight), the blubber of *Kogia* provided better insulation. Furthermore, a recent examination of Gervais' beaked whales (*Mesoplodon europaeus*; 99% wax) by Singleton et al. (2017) revealed that the blubber of this species was a superior insulative material, with conductivity values ~33% lower than that of *Kogia* blubber (largely because of the high lipid content in *Mesoplodon*). This does not mean that wax esters are required for blubber with good insulative properties. Despite containing only triacylglycerols, the blubber of harbor porpoises (*Phocoena phocoena*) has very low conductivity values because its lipid

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content is very high (Worthy and Edwards, 1990). Another interesting observation from this thermal work is that blubber may function as a phase change material (Dunkin et al., 2005; Bagge et al., 2012; Singleton et al., 2017). Both blubber with only triacylglycerols and blubber with high wax ester content have been shown to potentially function in this way, possibly serving as a thermal buffer capable of storing and releasing heat.

Finally, wax ester-rich blubber develops in an ontogenetic fashion. The ratio of wax esters to triacylglycerols in the blubber of pygmy sperm whales and Sowerby's beaked whales (*Mesoplodon bidens*) gradually increases with increasing body size (Koopman, 2007). These data suggest that wax ester-rich blubber is not formed *in utero*, but requires deposition and accumulation over time. Typically, the blubber layer of toothed whale fetuses contains very little lipid (Struntz et al., 2004); a heavy insulating layer during development would cause the embryo's temperature to rise to unsafe levels. In harbor porpoises, calves that are only a few months old have thick (~2–3 cm), lipid-rich (80–90% lipid) blubber, whereas in fetuses and newborns this layer is much thinner (0.5–1.0 cm) and contains less lipid (~50%) (Koopman, 1998; H.N.K., unpublished data). Some lipid must be deposited prior to parturition to protect the newborn from heat loss after birth. In animals with wax-rich blubber, this is in the form of triacylglycerols rather than waxes. If waxes are not mobilized for energy (see above), then it might be advantageous for younger toothed whales to build up a usable 'energy buffer' in their blubber in the form of triacylglycerols. Another possibility is that the enzymes for wax ester synthesis are not expressed until later in life. Regardless, it appears that animals must reach the sub-adult stage before waxes dominate their blubber lipids.

Lipids in the acoustic fats

The melon and jaw fats of all toothed whales contain a mix of triacylglycerols and wax esters – with the possible exception of the Ganges river dolphin (*Platanista gangetica*); see below. The wax ester content of the acoustic tissues ranges from ~1% in the narwhal (*Monodon monoceros*) to >90% in pygmy sperm whales; however, it is difficult to generalize about species-specific wax content because the topographical distributions of the lipids within the acoustic fat bodies are complex, heterogeneous, non-random and related to acoustic function (Wedmid et al., 1973; Blomberg and Lindholm, 1976; Flewellen and Morris, 1978; Karol et al., 1978; Koopman et al., 2006). Within an acoustic fat body, subsamples collected less than a cm apart can vary in lipid composition: for example, the composition of a single lipid parameter can vary 2- to 3-fold (e.g. Koopman et al., 2006). However, within an animal, the lipid composition (in terms of the suite of lipids present) and the relative distribution of the melon and the mandibular fats are exceptionally similar. Thus, for species in which only one fat body has been described (e.g. melon), it is fair to assume that the same array of lipids will exist in the other acoustic fat body (jaw fats).

Unusual short-chain fatty acids were first observed by Chevreul in 1817, who observed a volatile, strong-smelling acid in the tissues of a pilot whale that he termed 'acide delphinique' (reviewed in Blomberg, 1974). Gill and Tucker (1930) confirmed the structure as isovaleric acid in *Tursiops*, and Lovern (1934) noticed that levels of *i*-5:0 varied tremendously between tissues, with high levels in the acoustic fats, detectable levels in the blubber, and none in the heart, lungs and liver of *Phocoena*. A flurry of activity in the 1970s (see Ackman et al., 1971, 1973, 1975; Litchfield et al., 1975, 1976; Varanasi et al., 1975) led to the characterization of acoustic lipids in a wider range of species, and the phylogenetic patterns of unusual lipids recognized today.

It is easiest to consider the synthesis and deposition of these varied fatty acids (found in both triacylglycerols and waxes) in a taxonomic fashion, organized by family [see Fig. 5 for the modified phylogeny (based on Gatesy et al., 2013) used below to map suggested steps in the evolution of acoustic fats; species-specific composition data are summarized with their respective sources in Table 1]. At the base of the odontocete tree, the sperm whales (Physeteridae and Kogiidae) have acoustic fats dominated by straight-chain fatty acids (12:0, 14:0, 16:0) and a monounsaturated fatty acid (14:1). Physeteridae lipids, with longer and monounsaturated fatty acids, appear less derived than those of the *Kogia* species, which contain more 10:0 and 12:0. These families do have some branched fatty acids, occurring in trace amounts in Physeteridae and ~10–15% in Kogiidae.

The next recognized family (Platanistidae) is represented by a single genus of river dolphin, *Platanista*. This family is problematic because data on acoustic fat composition are exceptionally limited as specimens are very difficult to obtain. The two reports on these tissues indicate an apparent lack of wax esters but high levels of 16:1, with 'shorter' fatty acids (<17 carbons, not identified) accounting for 76% of all fatty acids. Trace amounts of *i*-5:0, *i*-14:0 and *i*-16:0 have been found in the blubber of this species, so although not reported in the acoustic fats, it seems as though those pathways exist. It may be that wax esters are absent, or present at such low levels that methods used decades ago did not detect them.

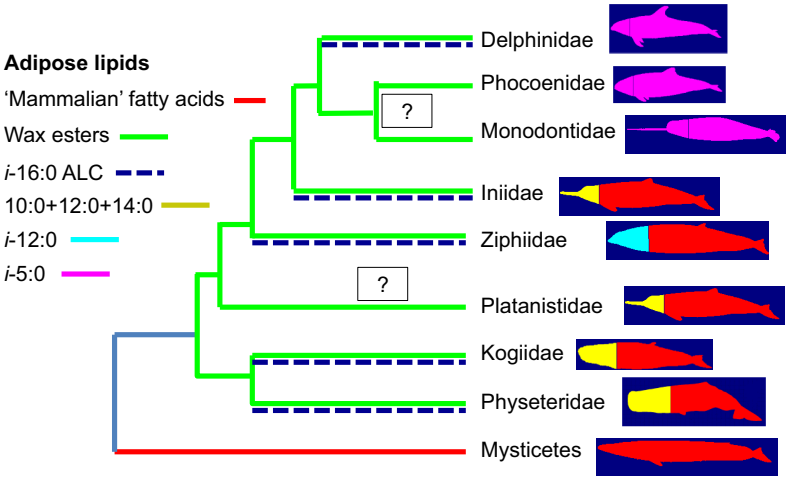

Fig. 5. Simplified odontocete phylogeny (Gatesy et al., 2013) showing the steps proposed in the evolution of acoustic lipid synthesis. Green lines indicate groups in which wax esters are found in acoustic fats. Dark-blue dashed lines indicate families in which the presence of *i*-16:0alc has been confirmed in acoustic fats. The unusual fatty acids in the waxes and triacylglycerols of the acoustic fats are denoted by yellow, teal or pink in the head regions of the toothed whale shapes. The two question marks indicate that it is not known whether the Platanistidae are able to synthesize waxes, and that the composition of waxes found in the acoustic tissues of Monodontidae and Phocoenidae remain undetermined.

Table 1. Dominant lipids in the acoustic fats (melon and mandibular fats) of toothed whales

Family and species	Common name	Wax esters	Fatty acids (% of total FA)	Fatty alcohols (% of total FAlc)	Source
			Delphinidae		
Globicephala macrorhynchus	Short-finned pilot whale	16–43%	*i*-5:0 70–82%	*i*-15:0 16–23%, *i*-16:0 33%, 16:0 18–20%	Lonati et al., 2015; Yanes, 2016
Globicephala melas	Long-finned pilot whale	33–45%	*i*-5:0 46–96%, *i*-15:0 1–14%, *i*-16:0 3–4%	*i*-16:0 36–55%, 16:0 10–21%	Wedmid et al., 1973; Litchfield and Greenberg, 1974;
Grampus griseus	Risso's dolphin	6–24%	*i*-5:0 1–48%, *i*-14:0 10%, *i*-15:0 16%	–	Litchfield and Greenberg, 1974; Litchfield et al., 1975; Koopman and Westgate, 2012; H.N.K., unpublished data
Pseudorca crassidens	False killer whale	19–22%	*i*-5:0 49%	–	Litchfield and Greenberg, 1974; Litchfield et al., 1975
Delphinus delphis	Short-beaked common dolphin	10%	*i*-5:0 2–23%,14:0 10%, *i*-15:0 13%	–	Litchfield and Greenberg, 1974; Koopman et al., 2003; H.N.K., unpublished data
Stenella attenuata	Pantropical spotted dolphin	10–58%	*i*-5:0 22–26%, *i*-15:0 9–20%	*i*-16:0 9–40%, 16:0 14–35%	Koopman et al., 2006; H.N.K., unpublished data
Stenella frontalis	Atlantic spotted dolphin		*i*-5:0 26–35%, 14:0 2–7%, *i*-15:0 13–25%	–	H.N.K., unpublished data
Tursiops truncatus	Bottlenose dolphin	10–50%	*i*-5:0 25–95%, *i*-15:0 1–4%	*i*-15:0 21–24%, *i*-16:0 21–31%, 16:0 24–34%	Varanasi and Malins, 1971; Zahorodny Duggan et al., 2009
Lissodelphis borealis	Northern right whale dolphin	3%	*i*-5:0 25–48%	–	Litchfield et al., 1975; Koopman et al., 2003
Sotalia fluviatilis	Tucuxi	22–49%	*i*-5:0 32–51%	*i*-15:0 17–23%, *i*-16:0 38–46%, 16:0 21%	Litchfield and Greenberg, 1974; Ackman et al., 1975; Litchfield et al., 1975
			Phocoenidae		
Phocoena dalli	Dall's porpoise	Absent?	*i*-5:0 33–35%	–	Litchfield and Greenberg, 1974; Litchfield et al., 1975
Phocoena phocoena	Harbor porpoise	1–6%	*i*-5:0 20–35%, *i*-14:0 2%, 14:0 9%, *i*-15:0 5%	–	Koopman et al., 2003, 2006;
			Monodontidae		
Delphinapterus leucas	Beluga	Absent?	*i*-5:0 40–60%, *i*-15:0 10–15%	–	Litchfield et al., 1971
Monodon monoceros	Narwhal	1–2%	*i*-5:0 48%, *i*-15:0 11%	–	Robisch et al., 1972; R. Pelletier, unpublished data
			Iniidae		
Inia geoffrensis	Amazon river dolphin	53–66%	12:0 18–25%, 14:0 17–28%, 16:0 4–7%, 16:1 3–5%, *iso*-acids present 6–8% (*i*-12:0, *i*-14:0, *i*-16:0)	*i*-16:0 9%, 16:0 64%	Ackman et al., 1971; Litchfield and Greenberg, 1974; Litchfield et al., 1975
			Ziphiidae		
Berardius bairdii	Baird's beaked whale	26–40%	*i*-10 2–17%, *i*-11:0 3–14%, *i*-12:0 19–29%, 12:0 10–20%, *i*-14:0 2–6%, 16:0 5–8%	*i*-15:0 10%, *i*-16:0 46–58%, 16:0 22–33%	Litchfield et al., 1976, 1978;
Mesoplodon densirostris	Blainville's beaked whale	46–75%	*i*-10:0 7–8%, *i*-12:0 10–20%, 12:0 10%		Litchfield et al., 1976; Lonati et al., 2015
Mesoplodon bidens	Sowerby's beaked whale	39–65%	*i*-10:0 3–14%, 10:0 1–10%, *i*-11:0 1–6%, *i*-12:0 4–50%, 12:0 9–26%, *i*-14:0 1–8%	*i*-16:0 32%, 16:0 47%	Koopman et al., 2006; H.N.K., unpublished data
Mesoplodon europaeus	Gervais' beaked whale	15–35%	*i*-10: 1–12%, 10:0 1–12%, *i*-11:0 1–16%, *i*-12:0 1–40%, 12:0 4–20%, *i*-14:0 1–8%	*i*-16:0 5–65%, 16:0 25–60%	Koopman et al., 2006; Lonati et al., 2015; H.N.K., unpublished data
Mesoplodon mirus	True's beaked whale	25–61%	*i*-10:0 6%, *i*-11:0 11%, *i*-12:0 33%, 12:0 11%	*i*-16:0 28%, 16:0 41%	Yanes, 2016
Ziphius cavirostris	Cuvier's beaked whale	3–24%	*i*-10:0 16%, *i*-11:0 22%, *i*-12:0 23–30%	*i*-16:0 36%, 16:0 27%	Litchfield et al., 1976; Yanes, 2016; H.N.K., unpublished data
			Platanistidae		
Platanista gangetica	Ganges river dolphin	Absent?	16:0 17%, 16:1 39%, 18:1 21%; 76% of all FA shorter than 16 C	–	Tsuyuki and Itoh, 1972; Litchfield and Greenberg, 1974
			Kogiidae		
Kogia breviceps	Pygmy sperm whale	30–92%	11:0 4–5%, 12:0 10–24%, 14:0 6–9%, 16:1 8–20%; *iso*-acids present ~10% (*i*-10:0, *i*-12:0, *i*-14:0); absence of *i*-5:0?	*i*-16:0 12–18%, 16:0 54–57%	Karol et al., 1978; Koopman et al., 2006; Yanes, 2016.
Kogia sima	Dwarf sperm whale	18–41%	Absence of *i*-5:0?	–	Litchfield and Greenberg, 1974; Litchfield et al., 1975
			Physeteridae		
Physeter macrocephalus	Sperm whale	60–90%	12:0 10–30%, 14:0 11–17%, 16:0 6–18%, 16:1 8–22%; *iso*-acids trace only	–	Morris, 1973, 1975; Litchfield and Greenberg, 1974

The presence of wax esters, and the dominant fatty acids and alcohols are summarized by family; only species for which data exist have been included. Dashes indicate an absence of data.

Journal of Experimental Biology (2018) 221, jeb161471. doi:10.1242/jeb.161471

Given that the entire dataset for this family is based on two specimens, with inconsistent analytical approaches, the conclusions for this group are tenuous at best, which is unfortunate given its placement in the toothed whale family tree.

The beaked whales (Ziphiidae) are the second group of large, deep-diving toothed whales; they contain relatively large amounts of waxes in their acoustic tissues (up to 75%), with the lowest values occurring in *Ziphius*. This group has adopted the 'valine' pathway to deposit large amounts of even BCFA in their melons and jaw fats (mostly *i*-12:0, with some *i*-10:0 and *i*-14:0). *Ziphius* is unusual within this group in having relatively large amounts of *i*-11:0 in its acoustic fats, which likely originates from the leucine pathway (an oddity given that this is used by the Phocoenidae, Monodontidae and Delphinidae).

The rest of the river dolphins (aside from *Platanista*) cluster together, but acoustic fat composition data are only available for Iniidae – to my knowledge there is no information for *Pontoporia* or *Lipotes*. *Inia* can be characterized by substantial amounts of wax esters (53–66% of all lipids present) with high concentrations of 12:0, 14:0 and 16:0 acids. Even-numbered BCFA are also present (2–5%).

The last three families, Monodontidae (beluga and narwhal), Phocoenidae (porpoises) and Delphinidae (dolphins) are united in their strong expression of the leucine-*i*-5:0 synthesis pathway. All species in these groups have acoustic tissues dominated by isovaleric acid, which accounts for the vast majority of all fatty acids present; they also have significant amounts of *i*-5:0 in their blubber (see below). These three families also have elongated the *i*-5:0 precursor, which enables them to deposit high concentrations of *i*-15:0. The valine pathway appears to be still viable here; however, the even BCFA are not synthesized to any great degree. Interestingly, all dolphins have significant amounts of wax esters in their acoustic fats, whereas porpoises, beluga and narwhal do not – the reasons for this potential character loss are unknown.

Despite the incredible and strongly phylogenetic diversity in the fatty acids of the acoustic fat wax esters and triacylglycerols, the fatty alcohols present in the waxes show surprising conservation across species (Table 1). Yanes (2016) found several consistent fatty alcohols in the mandibular fats of short-finned pilot whales (Delphinidae), pygmy sperm whales (Kogiidae) and several species of beaked whales (Ziphiidae). The fatty acids of the waxes were, as expected, dominated by *i*-5:0 in pilot whales, *i*-12:0 in beaked whales and 12:0 in pygmy sperm whales. However, 16:0alc and *i*-16:0alc were present in all the waxes, together comprising 50–75% of all fatty alcohols. This observation agrees with previous studies suggesting that the array of fatty alcohols in acoustic waxes is much smaller across the suborder (Ackman et al., 1973; Wedmid et al., 1973; Litchfield et al., 1978), indicating some common synthesis pathways and pointing to some evolutionary patterns.

In all species in which individuals of different ages have been studied, concentrations of wax esters and unusual fatty acids accumulate in an ontogenetic fashion. This pattern has been observed in dolphins (*Tursiops*), porpoises (*P. phocoena*), beaked whales (*M. europaeus*) and sperm whales (*Physeter*) (Morris, 1973, 1975; Gardner and Varanasi, 2003; Koopman et al., 2006; Zahorodny Duggan et al., 2009). Studies on species with different life-history timelines indicate that the attainment of an 'adult-like' composition of acoustic lipids coincides with the timing of nutritional independence (i.e. the duration of maternal care; Koopman and Zahorodny, 2008).

There is a general understanding that the acoustic tissues, regardless of composition, are somehow metabolically isolated or encapsulated because they are not mobilized during starvation (e.g. Koopman et al., 2003). However, these fat bodies are definitely not 'cut off' from the rest of the body because imaging and tracer studies of a live bottlenose dolphin by Houser et al. (2004) have demonstrated significant blood flow in these tissues. The acoustic tissues have significant macrovasculature (e.g. Costidis and Rommel, 2016), and it is hypothesized that these large vessels allow controlled flow of warmed blood to the acoustic fats to keep them in a constant phase for echolocation. Houser et al. (2004) detected no metabolic activity in the acoustic tissues (a glucose analog was not taken up), which either means acoustic fat cells lack glucose transporters, or there is little direct exchange with the blood. The latter idea is supported by the very low density of microvessels in these tissues compared with blubber from the same individual (Gabler et al., 2017).

In terms of function, the acoustic fats are important for focusing outgoing (through the melon) and incoming (through the jaw fats) sound. For animals using high-frequency sound in an aquatic medium, fat provides a good impedance-matching material compared with air (see McKenna et al., 2012). However, it is the arrangement of these lipids within the fat bodies that further serves to fine-tune sound-beam focusing. In all species in which the spatial distribution of lipids in the acoustic fats has been studied, there is a consistent overall pattern of higher concentrations of wax esters and shorter or branched chains in the middle of fat bodies, with less wax and more triacylglycerols with longer fatty acids in the exterior portions of fat bodies. This patterns holds regardless of which specific lipids are present – it is the spatial difference in relative chain length and wax ester content that is conserved (Litchfield et al., 1973; Wedmid et al., 1973; Karol et al., 1978; Koopman et al., 2006; Yanes, 2016).

What is the purpose of this arrangement? Wax esters and short-chain and branched fatty acids will decrease the speed at which sound travels through lipid, as demonstrated with extracted oils by Varanasi et al. (1975). In long-chain triacylglycerols, sound travels at ∼1460 m s^{-1}. When long-chain waxes are added, this value drops to ∼1440 m s^{-1}. However, in dolphin triacylglycerols containing high levels of *i*-5:0, the sound speed drops to ∼1380 m s^{-1}, and further declines to ∼1360 m s^{-1} when shorter-chain waxes are added. Together with the spatial distribution outlined above, this results in a high-velocity 'shell' surrounding a 'slow' core, regardless of which lipids are present. Such an arrangement can collimate, or focus, sound, either outwards from the melon, or inwards through the jaw fats to the ear bones. Fine-tuning of the acoustic beam could be achieved by changes in temperature (adjusting blood flow) or by muscles and connective tissues actively changing the shape of the acoustic fats (e.g. Houser et al., 2004; Harper et al., 2008). Thus, it is clear that the odontocetes have not only evolved new specialized adipose depots for acoustic function, but also different patterns of lipid synthesis and preferential spatial distributions of these lipids to optimize sound transmission and reception.

Evolution of the system

My favorite quote on this subject is from Morris (1986): 'it should really come as no surprise that an animal's biochemical composition is as good an indicator of its evolutionary origins as its skeleton'. Given that blubber is a shared character of both baleen whales and toothed whales, and that baleen whales do not deposit wax esters or BCFA in their blubber, it is probably fair to assume that the presence of these lipids in the blubber is not an ancestral whale condition. Such an assertion is supported by data indicating that the

accumulation of wax esters and BCFA requires time, with neonatal odontocete tissues being largely dominated by triacylglycerols containing 'more typical' endogenous fatty acids (Koopman et al., 2003; Zahorodny Duggan et al., 2009).

Recent evidence suggests that the ability to echolocate and for high-frequency hearing arose ∼28 million years ago on the odontocete stem lineage, shortly after the divergence from the mysticetes (Geisler et al., 2014; Park et al., 2016). Because all toothed whales examined possess some wax esters, and some type of shorter-chain (<16 carbons) or BCFA in the acoustic fat bodies, I propose that the synthesis of these unusual lipids originated in the cranial adipose tissues, as an adaptation to enhance echolocation and hearing. Furthermore, I suggest that the evolution of this system might have occurred in the following series of steps, the first two steps being common to all toothed whales (see Fig. 5). Step 1: synthesis and deposition of wax esters in the acoustic tissues. Nevenzel (1970) noted that the tissues of rats and pigs were capable of converting long-chain alcohols into wax esters, and suggested that the enzyme responsible for synthesizing waxes is 'widespread' in animal tissues with active fat metabolism. One missing piece of this puzzle is an understanding of the tissues from which the melon and mandibular fats are derived, which might help to elucidate how the synthesis and storage of wax esters first occurred – and these may have different origins. For example, Fraser and Purves (1960) proposed that the intra-mandibular fat body may be a highly modified form of bone marrow, whereas Costidis and Rommel (2016) suggested that the extra-mandibular fat body may be modified blubber. This is an interesting question given that the suites of lipids present in all acoustic depots within an individual are very similar. Presumably the initial deposition of wax esters in these tissues was highly localized, from which the selective advantage of being better able to focus sound can easily be imagined. Whether the acoustic tissues in the earliest Odontocetes were initially filled with 'typical' mammalian triacylglycerols, or whether the capacity for wax ester synthesis was present from the beginning, will remain unknown. Step 2: the first modification of the BCAA degradation pathway (for valine) to synthesize *i*-16:0 alcohol for waxes. Along with straight-chain 16:0 fatty alcohol, this is the most conserved fatty alcohol element across the toothed whales, regardless of which fatty acid pathway is favored. The synthesis of this fatty alcohol would require the catabolism of valine to isobutyryl-CoA, the elongation of isobutyryl acid to isopalmitic (*i*-16:0) acid and the subsequent conversion of *i*-16:0 to its fatty alcohol form. Thus, this physiological adaptation was of pivotal importance because all these processes would need to take place for *i*-16:0 alcohol deposition. At this point, there was some divergence across families, in terms of the specific fatty acid synthesis pathways that emerged as 'dominant'. Step 3: deposition of shorter, but straight-chain fatty acids (<16 carbons, particularly 10:0–14:0) in the waxes and triacylglycerols of the acoustic fats. This trait is shared by several of the more basal families: Physeteridae, Kogiidae, Platanistidae (see caveats for Platanistidae above) and Iniidae (yellow heads in Fig. 5). Some of these animals deposit small amounts of even-chained BCFA (see Table 1), although they are not the main fatty acids present. This finding could be interpreted as evidence of further modifications to the valine–isopalmitic alcohol pathway outlined above – if this sequence of events is correct, then the ability to produce branched-chain components was already present. Step 4: further modification of the valine degradation pathway to deposit large amounts of *i*-12:0 in the acoustic tissues, to the point at which it is the dominant fatty acid present; this pattern is characterized by the beaked whales (Family Ziphiidae; teal head in Fig. 5). Step 5: a new alteration of BCAA catabolism, this time for leucine, to yield *i*-5:0 and *i*-15:0 components. These dominate the acoustic tissues of the Monodontidae, Delphinidae and Phocoenidae (pink heads in Fig. 5). This is perhaps the most puzzling development because it required very different adjustments to metabolism, when (again, if this inferred evolutionary sequence is correct) a physiological path to the formation of short BCFA already existed (valine to *i*-4:0); however, it would be a lipid with exceptionally low sound speed compared with those deposited by other families. For Phocoenidae and especially for Monodontidae, the emergence of *i*-5:0 as the dominant fatty acid appears to be coupled with a major reduction in the deposition of wax esters. The functional significance of this is unknown.

In addition, many members of the Monodontidae (dolphins, porpoises and the monodontids) have adapted the leucine-*i*-5:0 pathway in their blubber (pink blubber in Fig. 5). In the blubber of harbor porpoises (*P. phocoena*), belugas, northern right whale dolphins (*Lissodelphis borealis*) and Hector's dolphins (*Cephalorhynchus hectori*), *i*-5:0 can contribute 20–50% of the total fatty acids present (Koopman et al., 2003). In all species in which *i*-5:0 forms a significant portion of the blubber, concentrations of this fatty acid are much higher in the outermost layers, and *i*-5:0 accumulates over time, being positively correlated with age (Koopman et al., 1996). Litchfield et al. (1971) initially suggested that the accumulation of *i*-5:0 in blubber simply represents a 'spillover' from synthesis in the acoustic fats, which is unlikely. First, this mechanism would require *i*-5:0 to travel through the bloodstream from its point of synthesis to the blubber; however, *i*-5:0 is thought to be formed in tissues *in situ* (e.g. Malins and Varanasi, 1975) and has not been detected in blood (H.N.K., unpublished data). Second, higher concentrations of *i*-5:0 are found in the blubber of animals from cooler/colder habitats than in animals from warmer/hotter habitats (Koopman et al., 2003). Isovaleric acid has a very low melting point (−37.6°C; Fasman, 1975), which could enable tissue fluidity to be maintained in the outer layers of blubber of animals inhabiting cooler/polar waters (Koopman, 2007), providing another role for this unusual fatty acid.

Future directions

Clearly, much remains to be learned about the synthesis, evolution and function of the endogenous lipids in the toothed whales. Some of the questions that still need to be addressed are as follows. (1) Why are there different endpoints for the conversion of dominant BCAA–BCFA (*i*-4:0 versus *i*-5:0)? According to Brosnan and Brosnan (2006), BCAA are catabolized 'in lockstep' because the first two enzyme steps are shared by all three amino acids. Simultaneous conversion of BCAA–BCFA does not occur in the acoustic fats of toothed whales. (2) Why have the monodontids (beluga and narwhal), and possibly the porpoises, virtually eliminated wax esters from their acoustic tissues? (3) How is the ontogenetic development of the synthesis and deposition of unusual lipids in acoustic fats and blubber controlled? (4) Why have waxes been 'retained' in the blubber of some species? (5) Can wax esters be mobilized from adipocytes by toothed whales? Further research is required to better understand the patterns of lipid synthesis in river dolphins and deposition in the context of the entire sub-order of toothed whales.

Metabolomics and genomics have a range of potential applications that could further our understanding of lipid metabolism. Although the prospects of obtaining exceptionally fresh tissue (e.g. Khudyakov et al., 2017) to determine messenger RNA levels are slim, it might be possible to use proteomics to determine which

enzymes are being synthesized in different tissues, in different regions of the same tissue, and at different life-history stages. It would also be interesting to compare the genes of toothed whales with those of baleen whales, to identify which aspects of metabolism are altered for echolocation. Furthermore, there are exciting options for examining the fine-scale distributions of metabolites, for example, using matrix-assisted laser desorption/ionization imaging mass-spectrometry (Aichler and Walch, 2015), which might further our understanding of the evolution and molecular physiology of these unusual lipids.

Acknowledgements

This work is the result of many specimens, collaborations and discussions with colleagues, and the continued quest to understand the evolution of lipid metabolism in the toothed whales. Funding for much of the work has come from UNCW, the Office of Naval Research and the Grand Manan Whale & Seabird Research Station, among others. Samples were generously provided by many stranding organizations and research groups, including the UNCW Marine Mammal Stranding Program, Virginia Aquarium Stranding Program, Smithsonian Institution and National Marine Fisheries Service; much appreciation is owed to those who collect specimens. Much of this work would not have been possible without the curiosity and analytical expertise of Sue Budge. I also owe deep gratitude to Jim Ballantyne for helping me take the first steps on this research path, and to Andy Read, Sara Iverson and Ann Pabst for guidance. I wish to thank my fellow JEB Biology of Fat symposium participants for making me appreciate the potential applications of metabolomics and genomics. This paper is dedicated to Jim Mead, a dear friend and colleague who is fond of ziphiids and all things evolutionary, and to the memory of R. G. Ackman, eccentric biochemist and pioneer in the studies of unusual odontocete lipids.

Competing interests

The author declares no competing or financial interests.

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