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Distribution and development of the highly specialized lipids in the sound reception systems of dolphins

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Abstract Fat bodies in the heads of toothed whales, which serve to transmit and receive sound, represent extraordinary examples of physiological specialization in adipose tissues among mammals, yet we know surprisingly little about their biochemical composition. We describe the spatial distributions and development of unusual endogenous lipids (branched-chain ["iso"] molecules and wax esters) in the mandibular fat bodies of bottlenose dolphins (*Tursiops truncatus*) using an ontogenetic series (fetus to adult; $n = 10$). Although concentrations of iso-acids, iso-alcohols and waxes were lower in younger dolphins than in adults, the same relative spatial arrangement was present in all age classes, implying a set "pattern" of acoustic lipid distribution that is established very early in life. In all age classes, a small region of blubber overlying the lateral region contained unusually high concentrations of iso-acids, exhibiting a tenfold increase over "normal" adjacent blubber. Being chemically more similar to the acoustic fat bodies, this region may serve as an entry point for sound into the head. Developmental accumulations of some iso-acids and iso-alcohols occurred more rapidly than others, implying that not only are the spatial distributions of branched-chain molecules under extremely fine-scale control, but the regulatory mechanisms controlling acoustic lipid synthesis are also highly complex.

Keywords Bottlenose dolphin · Ontogeny · *Tursiops* · Echolocation · Iso-acids

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Introduction

Odontocete cetaceans have undergone several adaptations from the original terrestrial mammal body plan to thrive in their aquatic environment. Some of the most drastic changes include modifications of the head (in both bony features and soft tissues) for the specialized adaptation of echolocation. Fatty tissues found in the forehead (the melon) are considered to be of importance in projecting and focusing high-frequency sound produced for echolocation (Norris 1968). The melon of odontocetes has been shown to have a non-homogeneous lipid structure, consisting of fatty acids (FA) which conduct sound at varying rates of speed (Gouw and Vlugter 1967; Hustad et al. 1971). The manner in which these FA are spatially arranged results in a low velocity melon core which is surrounded by higher velocity tissue, resulting in refraction and collimation of a beam of sound. Comparable in function to the melon, it is hypothesized that mandibular fats found in and around the lower jaws also act as waveguides focusing incoming sound toward the ear (Norris 1968). This area of the lower jaw is termed the "acoustic window" (Norris 1968), and has shown to be a region of high

hearing sensitivity in several odontocete species (Bullock et al. 1968; Møhl et al. 1999).

Not only is the presence of these fat bodies a departure from typical mammalian morphology, but the biochemical composition of these fats is also unusual, as the acoustic fatty tissues (melon and jaw fats) contain unusual FA that are rare in mammalian systems. Typically adipose tissue is composed of dietary and/or endogenous FA that are 14 carbons or longer (Pond 1998). However, acoustic tissues are dominated by shorter length endogenous iso-acids (branched chained FAs) (Koopman et al. 2006; Litchfield and Greenberg 1974; Litchfield et al. 1975) found in the form of triacylglycerols (TAG; 3 FA bound to a glycerol backbone) and/or wax esters (WE; a single FA esterified to a single fatty alcohol—FAlc). Normally, endogenous FA are synthesized in the cell by the sequential addition of two-carbon subunits. Iso-acids, however, are formed as intermediates of amino acid catabolism (Malins et al. 1972; Morii and Kaneda 1982; Tanaka et al. 1966). Some of these molecules are toxic to mammals; in humans, the accumulation of isovaleric acid (5 carbon, branched chain FA) is attributed to a chronic and often fatal metabolic disease state (Tanaka et al. 1966). Likely as a function of this toxicity, iso-acids are not mobilized from odontocete acoustic tissues and their biochemical composition is seemingly not influenced by poor health or nutritional status (Cranford 1999; Koopman et al. 2003).

The lipid classes and their FA and FAlc constituents that dominate acoustic tissues are not uniform across the various families of odontocetes. Dolphins, porpoises, narwhals and belugas all possess high levels of isovaleric acid (*i*-5:0) in their acoustic fat, mostly in the form of TAG, although WE are also an important component in dolphin tissues (Litchfield and Greenberg 1974; Litchfield et al. 1975). The acoustic tissues of beaked whales and sperm whales tend to be dominated by WE that contain other branched chain FA such as *i*-10:0, *i*-11:0, *i*-12:0 and *i*-13:0 (Koopman et al. 2006; Litchfield et al. 1978). It is apparent from this phylogenetic diversity of acoustic lipid constituents that no single lipid class or FA is unique in its ability to facilitate the transmission of high frequency sound through tissue since all of these animals are believed to be capable of echolocation. It has been suggested that the key factor is not the absolute chain length of FA present, but rather the spatial layout and *relative* length of these shorter-chained (and often branched) FA in comparison to the longer FA found in surrounding tissue that influences the path of sound through the head (Ackman et al. 1971; Koopman et al. 2006; Litchfield et al. 1975).

Although most studies have focused on the acoustic tissues of adults, there is some indication that younger conspecifics lack the full adult complement of acoustic FA, implying an important developmental aspect to this tissue (Gardner and Varanasi 2003; Koopman and Zahorodny 2008; Koopman et al. 2006; Morris 1975). The melon has been studied in greater detail than jaw fat, but few studies have aimed to explore the spatial heterogeneity in detail, including all lipid components, of either of the acoustic tissues across multiple age classes. Thus, information is lacking in how sound propagation might be influenced in the immature animal. In our previous study (Koopman and Zahorodny 2008), we evaluated a subset of acoustic lipids in dolphins, considering only the TAG portion. Here, we examined the full acoustic lipid complement in the jaw fats of an ontogenetic series of bottlenose dolphins (*Tursiops truncatus)*, a well-studied marine mammal often used as a model for odontocete physiology and bioacoustics. The objectives were to (1) determine the lipid composition of mandibular fats in a three-dimensional context to resolve whether the lipids exhibit specific spatial arrangements, and (2) to examine ontogenetic patterns of the distribution and accumulation of the FA and FAlc constituents of TAG and WE.

Methods

Samples were collected from ten bottlenose dolphins (*T. truncatus*) that either stranded or died incidentally in fisheries along the coastlines of North Carolina and Virginia. Necropsies were performed using standard protocols (McLellan et al. 2002; Geraci and Lounsbury 1993) and samples were either collected immediately, or tissues were frozen for subsequent dissection. Only tissues that were considered fresh and showed little sign of decomposition were used (Geraci and Lounsbury 1993). Samples (see Fig. 1) were collected from three tissue types: fat from inside the mandibular fossa (inner jaw fat), fat adhered to the outer region of the mandible (outer jaw fat) and cranial blubber (the blubber superficial to the outer jaw fat) from adults ($n = 4$; total lengths 259, 259.5, 274, 281 cm), subadults ($n = 3$; total lengths 171, 180, 194 cm), calves ($n = 2$; total lengths 109, 124.5 cm) and a fetus (total length 63.5 cm). Assignment to age category was based on total standard length (Read et al. 1993), and the presence/absence of neonatal characteristics (Dearolf et al. 2000).

To evaluate the spatial layout of lipids, approximately 55 subsamples (0.5 g each) were taken from the three tissue types of each animal. Only the right side was evaluated since there is evidence for bilateral symmetry in other odontocetes (Koopman et al. 2006). Tissues collected were sliced into transverse sections (see Fig. 1), and subsamples taken from the rostral face of each. Three of the sections contained inner jaw fat, outer jaw fat and blubber (slices 1–3), the fourth section (slice 4) contained samples from blubber and inner jaw fat, and the final section contained

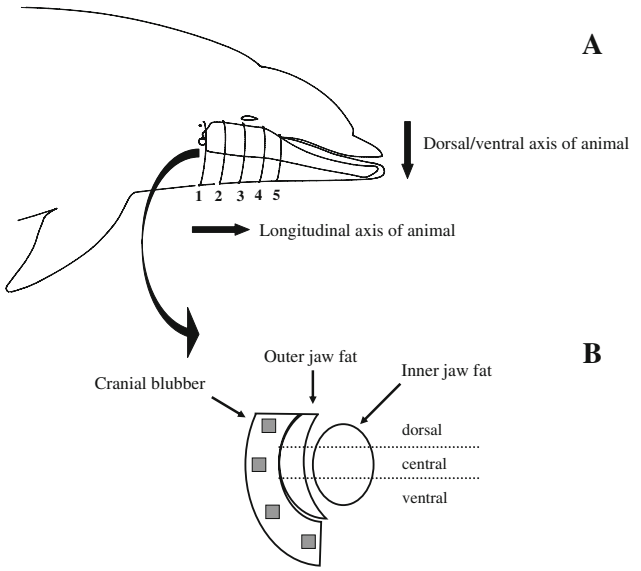

Fig. 1 Schematic of bottlenose dolphin (**a**), *black lines* along the mandible indicate the location of transverse sections. The resultant slices 1–5 were then subsampled as shown in the cross-section (**b**). Cranial blubber was subsampled at the *four shaded areas*. Inner and outer jaw fat were subsampled and classified as dorsal, central or ventral as according to their locations as indicated by the *dashed lines*. Schematic courtesy of S.A. Rommel

only cranial blubber (slice 5). In order to compare among age classes, and since the size of the fat bodies varied with the size of the animal, sections for subsampling were made referencing landmarks (the rostral and caudal ends of the mandibular fossa, and the auditory bulla). Homologous sites were sampled in each head, and thus the sizes of sampled sections were scaled with the size of each individual. Thickness of slices ranged from 10 to 40 mm between heads (depending on the size of the dolphin), but was always of consistent thickness within a head.

Lipids were extracted from each subsample using a modified Folch procedure (Folch et al. 1957; Koopman et al. 1996). Lipid was resuspended in hexane and stored under nitrogen at −20°C until further processing. Overall lipid content values, for all subsamples combined, were compared across age classes using ANOVA with $\alpha = 0.05$.

Lipid classes were separated and quantified for each subsampling site through thin-layer chromatography-flame ionization detection (TLC-FID). Samples were spotted on chromarods (Chromarod-SIII, Mitsubishi Kagaku Iatron Inc, Tokyo, Japan) and developed in 94/6/1 hexane/ethyl acetate/formic acid. Classes were then quantified by TLC-FID using an Iatroscan MK-6 (Mitsubishi Kagaku Iatron Inc, Tokyo, Japan). Identification was confirmed through use of lipid class standards (Nu Chek Prep, Elysian, MN, USA). Peaks were integrated with Peaksimple software (Peaksimple 3.29, SRI Instruments, Torrance, CA, USA).

Thin-layer chromatography-flame ionization detection is a destructive method so standard TLC was used to separate and recover TAG and WE in order to determine FA and FAlc constituents of each lipid class. Because the absolute minimum amount of any given lipid class we required for this analysis was 15 mg, not all lipid classes were recovered from each sample. In the instances where WE represented <20% of the sample, only the TAG data are presented, although TAG and WE were separated by TLC for all samples to ensure purification of the TAG fraction. For separation, whole lipid was spotted onto TLC plates (Adsorbosil-Plus 1 TLC plates, Alltech, Columbia, MD, USA; conventional 250 µm layer thickness, 20 cm × 20 cm) which were developed in 94/6/1 hexane/ethyl acetate/formic acid. Lipid class bands were visualized with dichlorofluoroscein under UV light, then TAG and WE bands were recovered separately and extracted with chloroform.

The FA components of triacylglycerols were converted to butyl esters (FABE-TAG, fatty acid butyl esters from TAG) for GC analysis (see Koopman et al. 1996). Butyl esters were used as opposed to methyl esters (more commonly used) because short-chained FA are volatile (Koopman 2007). Due to coelution of components, it was necessary to separately analyze the fatty acid butyl esters from WE (FABE-WE) and free fatty alcohol (FAlc) portions of the WE. FABE-WE and FAlc were prepared using a similar protocol with equivalent amounts of 23:0 acid and 19:0 alcohol (Nu Chek Prep, Elysian, MN, USA) added as internal standards. These internal standards allowed us to join the two separate analyses so that results could be reported as total proportions of FABE and FAlc combined. A small aliquot of esterified WE sample was reserved for GC analysis to account for any loss of short chain acids during the TLC step. FABE-WE and FAlc in the remaining sample were separated through conventional TLC using 70/30/1 hexane/ethyl ether/glacial acetic acid. FABE-WE was extracted from silica with hexane while the FAlc was extracted from silica with 1:1 hexane:diethyl ether.

Fatty acid butyl esters from TAG and FABE-WE were analyzed by a Varian (CP-3800) gas chromatograph (Varian Inc., Palo Alto, CA, USA) fitted with a flame ionization detector (FID) and a 30 m × 0.25 mm column coated with 50% cyanopropyl polysiloxane (J&W Scientific DB-23 column). Injector and detector temperatures were held at 250 and 270°C, respectively. The temperature program was as follows: initial column oven temperature was 65°C held for 2 min, ramped at 20°C/min to 165°C, held for 0.4 min, ramped at 2°C/min to 215°C and held for 6.6 min, ramped at 5°C/min to 240°C and held for 1 min. FAlc were analyzed on the same GC/FID using a 30 m × 0.25 mm column coated with nitroterephthalic acid modified with polyethylene glycol (Zebron, ZB-FFAP column). Injector and detector temperatures were both held at 250°C and the following temperature program was used;

initial column oven temperature was 100°C, held for 5 min, ramped at 10°C/min to 250°C and held for 15 min.

Peak identification was based on comparisons of retention time to standards (Nu Chek Prep, Elysian, MN, USA) and known samples. Peak areas were integrated using appropriate response factors (Ackman 1991) with Galaxie Chromatography Data System (Version 1.8.501.1, Varian Inc., Palo Alto, CA, USA), and are reported as mole percent (mole %) of total FA present. WE data were reconstructed through the amalgamation of data from multiple chromatograms generated from fractions (FA and FAlc) that were separated during processing (as described above), using the internal standards. Peak identification was confirmed using a Thermo Polaris Q GC/MS with the same GC conditions described above. Electron impact ionization was used with spectral matching against the NIST library. Acetate derivatives of FAlc were formed following Christie (1989) to create more stable compounds for analysis.

To test for spatial heterogeneity and patterns of acoustically important FA, the data were analyzed using non-parametric tests. Analyses were done separately on each slice for each of the tissue types (cranial blubber, inner jaw fat and outer jaw fat). Cranial blubber was compared through direct site to site comparison, however inner and outer jaw fat were categorized as coming from the dorsal-most, central or caudal-most region (see Fig. 1) and compared by pooled region. To determine if there were specific spatial patterns of high acoustic FA, each subsample site was ranked within each slice in each head according to its total branched-chain FA content. Analysis of variance (ANOVA) was then performed on ranked data, producing a test similar to the Kruskal–Wallis test (Kruskal and Wallis 1952; Siegel and Castellan 1988; Steel and Torrie 1980). For these tests, animals were divided into three age categories; adult, subadult and calves/fetus; factors tested were age class and sample site ($\alpha = 0.05$). Sample site differences were evaluated further using either Bonferonni (variances equal according to Levene's test) or Tamhane's T2 (variances unequal) tests. Statistical comparisons were carried out only on TAG-FA due to the low number of samples that contained adequate levels of WE for analysis. All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA) and all means are presented ± standard error. For ease of presentation, we classified FA and FAlc into several groups reflecting structure (branched components, chain lengths) and primary origin. For our purposes, we considered "Dietary" components to be those that can only be of dietary origin: all polyunsaturated FA (PUFA), and all monounsaturated FA (MUFA) ≥ 20 carbons in length. The unusual endogenous components (*i*-5:0, *i*-15:0, *i*-16:0) are presented separately. The "endogenous" category therefore refers to more common products of biosynthesis: 14:0, 14:1*n*-5, 16:0, 16:1*n*-7, 18:0 and 18:1*n*-9. The latter two FA could also be categorized as originating from the diet, but we have placed them in the endogenous category as animals are capable of synthesizing these (Pond 1998); in reality, they are present at such low levels in acoustic fats (see below) that placing them in either category does not alter our overall results.

Results

Approximately 70 FAs were routinely identified from both TAG and WE in tissues examined. The dominant lipid class (as % of total lipid), and FA (as mole % of total FAs) varied between different tissue types and age classes. Despite the large number of FA identified most samples were dominated by iso-acids, except in the case of cranial blubber where levels of dietary and other endogenous FA were high in certain spatial regions.

Composition of adult tissues

In adults, TAG and WE were the major lipid classes detected in samples from the three tissues. Cranial blubber was comprised mainly of TAG (mean 98.49% ± 0.28 SE), and WE content was so low in this tissue it could not be recovered for FA or FAlc analysis. Usually blubber is comprised of polyunsaturated dietary FA and other saturated and MUFA of dietary or endogenous origin (Koopman 2007). Some areas of the cranial blubber did follow this characteristic trend; however, unlike typical blubber, samples of this tissue exhibited localized areas of high iso-acid concentrations. Furthermore, cranial blubber did not show stratification, another typical feature of cetacean blubber.

Iso-acids in TAG exhibited a high degree of spatial heterogeneity in this tissue and the pattern was remarkably consistent between animals (Fig. 2a). The magnitude of iso-acid concentration varied as much as tenfold between samples that were <50 mm apart. ANOVA confirmed a significant spatial pattern to iso-acid content; each slice was analyzed independently to evaluate this pattern. The caudal most section of the blubber (slice 1) did not have high iso-acid content at any of the sample sites and did not show any spatial structure (no sample site effect; $P > 0.13$). Concentrations of iso-acids were highest at sample site 2 for subsequently sampled slices (all $P < 0.001$), except for the last slice (slice 5), in which sample site 1 exhibited the highest levels. Samples that were taken from the ventral side of the animals (sample site 4) always contained the lowest levels of iso-acids across all slices.

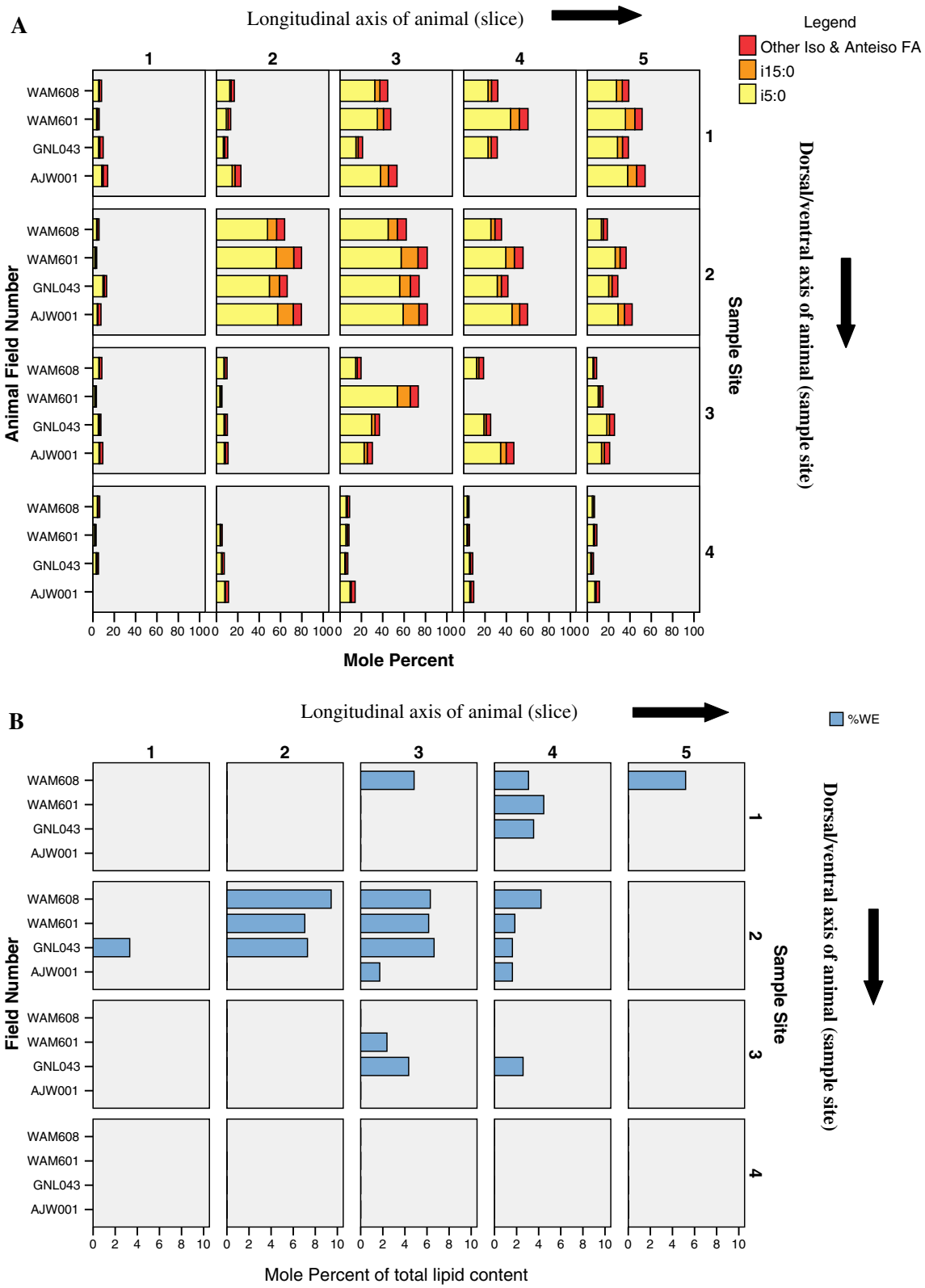

Fig. 2 **a** Spatial variation of iso-acid composition (as mole % of total FA present) in the cranial blubber of adult ($n = 4$) bottlenose dolphins. Each individual animal sampled is shown to depict the consistency of the FA "blueprint." *Columns* represent the transverse slice taken; *rows* represent the location of the sample site (1–4; dorsal to ventral). Three of the sections contained inner jaw fat, outer jaw fat and blubber (*slices 1–3*), the fourth section (*slice 4*) contained samples from blubber and inner jaw fat and the final section contained only cranial blubber (*slice 5*). **b** Location of WE found in the cranial blubber of adults. Layout is the same as above

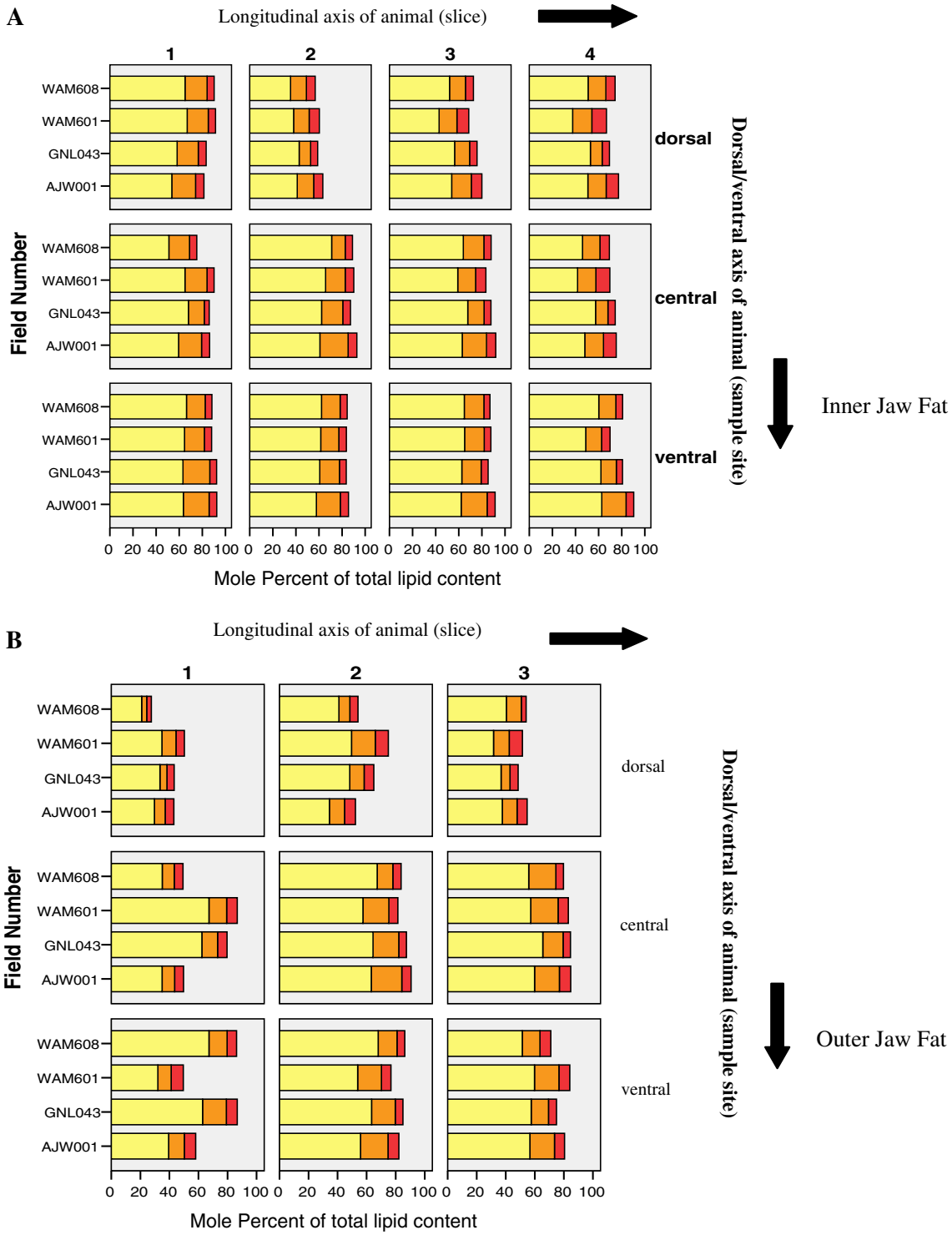

Fig. 3 **a** Spatial variation of iso-acid composition (as mole % of total FA present) in the inner jaw fats of adult ($n = 4$) bottlenose dolphins. Each individual animal sampled is shown to depict the consistency of the FA pattern. *Columns* represent the transverse slice taken; whereas *rows* represent the location of the sample site as it is classified as coming from the dorsal, central or ventral location of the fat body. **b** Spatial variation of iso-acid composition (as mole % of total FA present) in the outer jaw fats of adult ($n = 4$) bottlenose dolphins. Legend is the same as for Fig. 2a. Layout is the same as above

Although the mean WE content was low (1.51% ± 0.28 SE) in cranial blubber, it was also present in discrete spatial locations which coincided with the areas of highest iso-acid concentration (Fig. 2b). WE content was highest at sample site 2 in slices 2–4 (up to 9.44%), but never present in samples taken from the ventral side (sample site 4). This

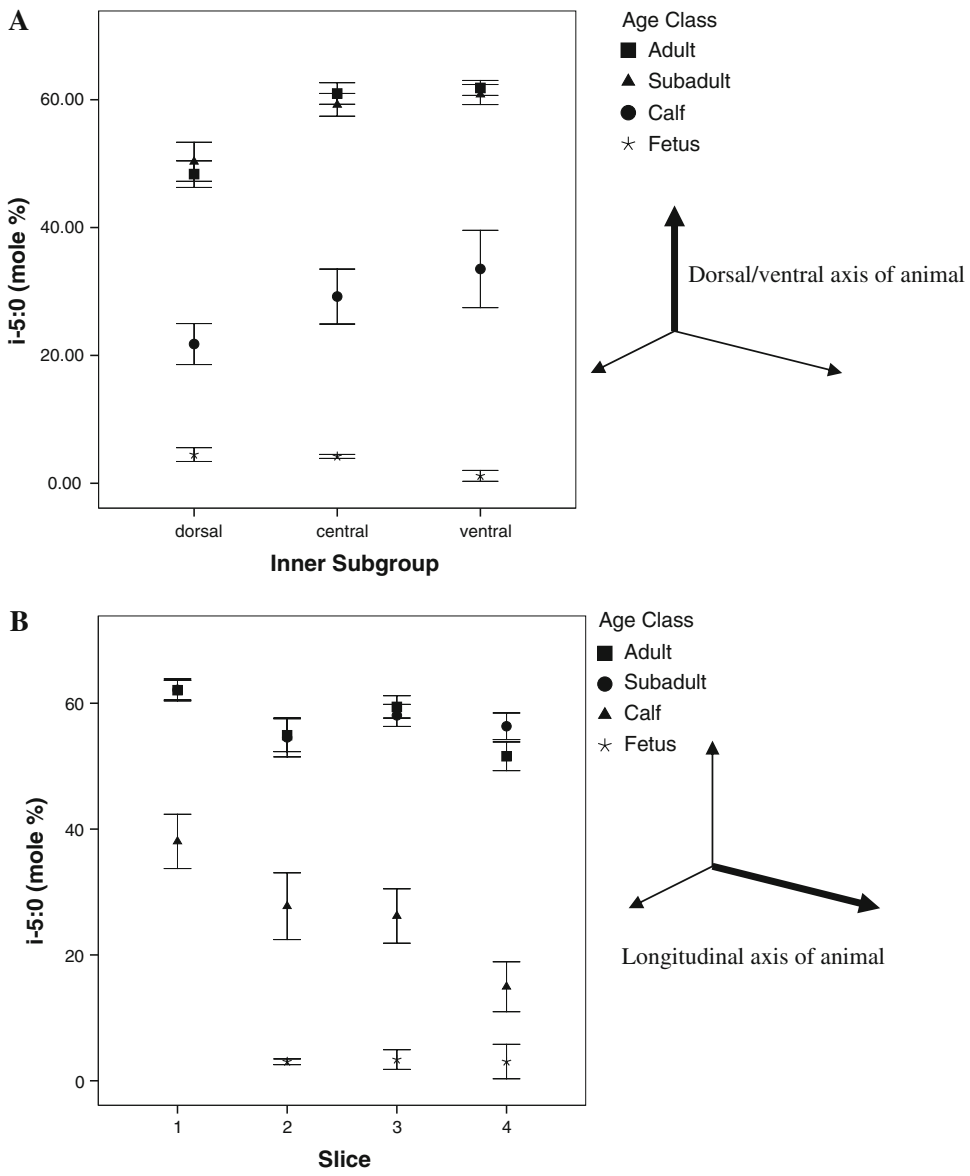

Fig. 4 Scatterplot of isovaleric acid (as mole % of TAG) of inner jaw fat **a** along the dorsal/ventral axis of the animal for all age classes (adult $n = 4$, subadult $n = 3$, calf $n = 2$, fetus $n = 1$), **b** as averaged across all transverse sections. See Fig. 1 for sampling orientation. *Error bars* represent 1 standard error

corresponds with the spatial location of iso-acids from TAG; the highest levels of WE and iso-acids coincide, and the lowest proportions of iso-acids also coincides with the absence of WE.

Both the inner and outer jaw fat of adults were comprised of a mix of TAG (mean inner; 72.11% ± 2.03 and outer 87.52% ± 1.70) and WE (mean inner 27.89% ± 2.03 and outer 11.18% ± 1.60). TAG components consisted of high iso-acids (mostly *i*-5:0) in both inner and outer jaw fat, but with considerably less spatial heterogeneity (Fig. 3) than that found in the cranial blubber. The dorsal components of the inner jaw fat in slices 2 and 3 contained significantly lower iso-acid content ($P < 0.004$), however the trend was not significant for the first or last slices ($P > 0.368$). Outer jaw fat had significantly lower iso-acid content in the dorsal section of the first slice ($P < 0.002$) but showed no other site specific effects. Despite the fact that the pattern is poorly defined there did appear to be a compositional gradient of fewer iso-acids in the dorsal most section of both the inner and outer jaw fat, and along the entire length of the fat body, with decreasing amounts in progressively rostral samples (Fig. 4). The WE present in inner jaw fat followed a similar, but more pronounced spatial pattern (Fig. 5). Less WE was present in all dorsal regions sampled, with an increasing WE content toward the ventral part of the jaw fat (Fig. 5a). Similarly there was a compositional gradient of WE which was greatest near the tympanoperiotic complex (as high as 68.34%) and decreased in samples taken from successive rostral sections (Fig. 5b).

There was sufficient WE to allow recovery for compositional analysis from a number of samples from the inner

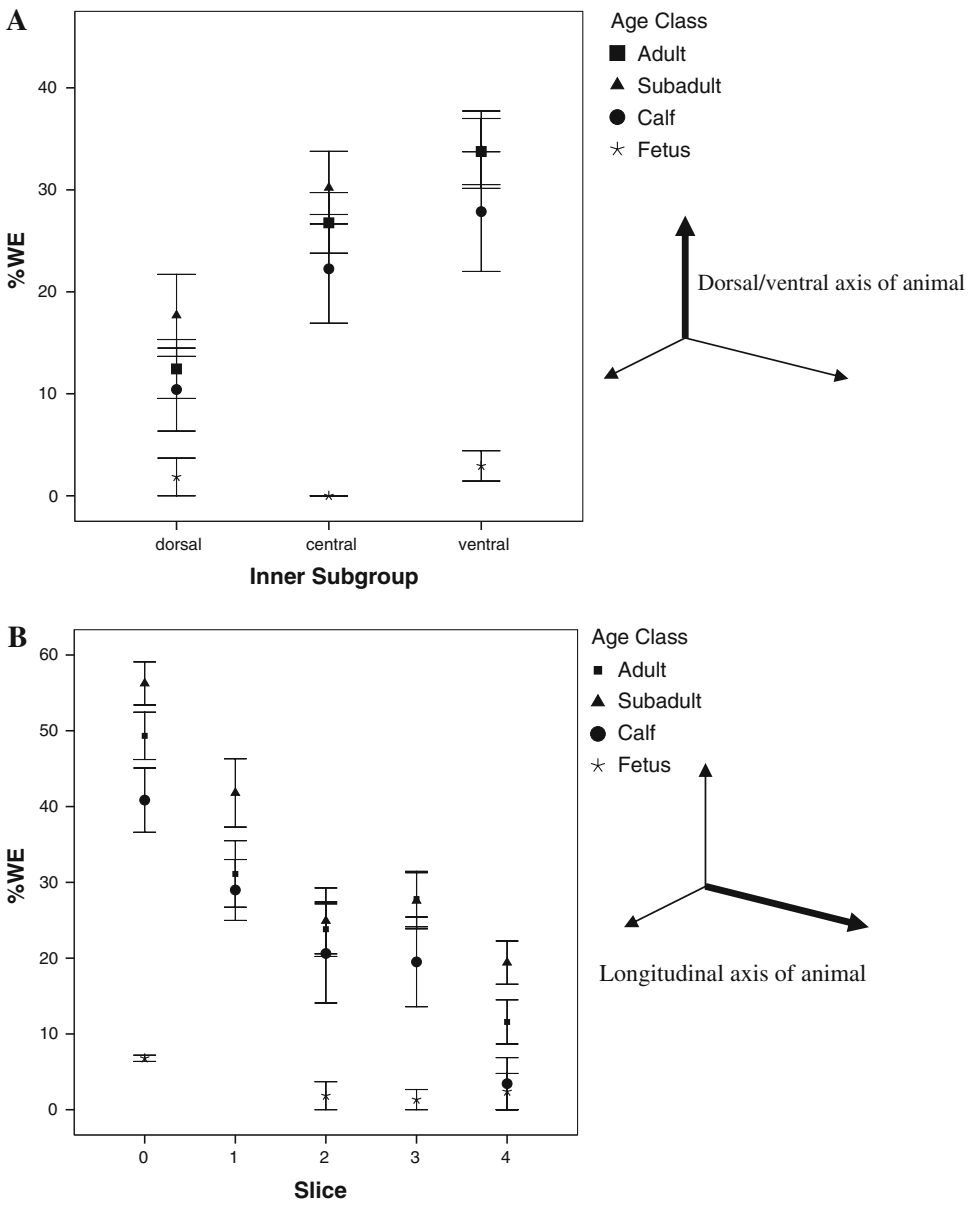

Fig. 5 Scatterplot of mean WE content (as mole % of total lipid) of inner jaw fat **a** along the dorsal/ventral axis of the animal for all age classes (adult $n = 4$, subadult $n = 3$, calf $n = 2$, fetus $n = 1$), **b** as averaged across all transverse sections. See Fig. 1 for sampling orientation. Slice 0 refers to the fats that are directly attached to the earbones (see text for discussion). *Error bars* represent 1 standard error

and outer jaw fats of all adults and subadults, and one of the calves. A total of 80 and 28 samples, from inner and outer jaw fat, respectively, were analyzed for FA and FAlc composition (with only 7 and 4, respectively, coming from the calf). In adults, the FA portion of WE contained high levels of *i*-5:0, with means of 94.6 and 92.2 mole % in inner and outer jaw fat, respectively (Table 1). The balance of the FA portion of WE in adults was mostly composed of *i*-15:0 (2.2–3.5 mole %) and other branched chain alcohols (Table 1). In contrast, the FAlc fraction had three major components: *i*-15:0, *i*-16:0 and 16:0 alcohol moieties each comprising ~24–31 mole % of the FAlc in adults (Table 1). The remaining FAlc portion was composed primarily of *i*-17:0, 18:1*n*-9, and 15:0 alcohols. PUFA and MUFA of dietary origin (e.g., 20:1*n*-9) were absent in the WE fraction of both the inner and outer jaw fats of adults.

Although sample size was insufficient to repeat the spatial analysis we carried out with the TAG (e.g., as in Figs. 1a, 3), we were able to demonstrate patterns of variation in FAlc levels. For example, there was a significant positive correlation between levels of *i*-15:0 FA in TAG and *i*-15:0 FAlc in WE ($P < 0.001$, adjusted $R^2 = 0.82$; Fig. 6) in all samples of inner jaw fat for which both TAG and WE composition were determined. In other words, sample sites containing high levels of *i*-15:0 FA in the TAG fraction were also characterized by high concentrations of *i*-15:0 fatty alcohol in the WE portion, suggesting some co-variation amongst components with similar structures.

Table 1 Mean and standard error of all WE components (mole % of total FA and FAlc) for all age classes separated by tissue types

Tissue type	Age class		*i*-5:0 FA	*i*-15:0 FA	Other branched FA	Endogenous FA	*i*-15:0 FAlc	*i*-16:0 FAlc	16:0 FAlc	Other branched FAlc	Non-16:0 endogenous FAlc
Inner	A	$\bar{x}$	94.63	1.24	2.23	1.73	30.58	30.72	25.10	5.90	3.52
		SE	0.43	0.08	0.17	0.20	1.01	0.76	1.10	0.10	0.28
	S	$\bar{x}$	91.90	0.79	4.02	3.00	11.54	30.76	34.12	4.21	10.27
		SE	1.07	0.06	0.48	0.51	0.78	1.65	1.28	0.14	0.65
	C	$\bar{x}$	25.89	3.81	25.17	40.09	5.04	11.87	36.32	8.89	21.38
		SE	2.90	0.18	0.96	3.43	0.52	1.54	1.06	1.41	0.83
Outer	A	$\bar{x}$	92.23	1.35	3.50	2.60	26.43	23.51	30.22	5.89	7.66
		SE	1.17	0.21	0.55	0.49	2.49	1.32	2.02	0.22	0.84
	S	$\bar{x}$	86.53	0.87	7.89	4.20	10.31	23.07	36.67	3.63	14.88
		SE	3.79	0.09	2.02	1.55	1.25	1.83	1.47	0.33	0.89
	C	$\bar{x}$	18.94	3.39	26.90	44.25	5.16	11.66	35.07	9.02	23.61
		SE	4.56	0.18	1.60	6.00	1.04	1.48	1.19	0.22	1.49

A adult (38 inner and 10 outer subsamples), *S* subadult (35 inner and 14 outer subsamples), *C* calf (7 inner and 4 outer subsamples)

See Sect. "Results" for numbers of individuals sampled, as they differ from Table 2. "Non-16:0 endogenous FAlc" refers to total alcohols in the endogenous category in Table 2, except 16:0 as it is presented separately. Not all fatty acids and alcohols are presented here so classes do not sum to 100%

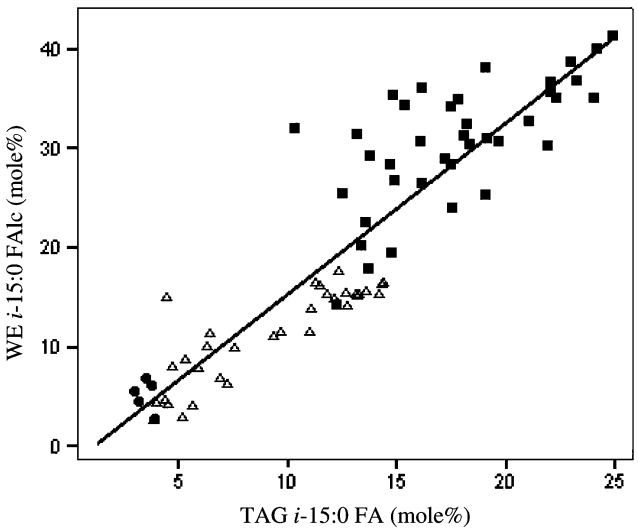

Fig. 6 Scatterplot of *i*-15:0 acid components of TAG vs. those of WE alcohols (all as total mole % for either TAG or WE) in inner mandibular fat bodies of bottlenose dolphins. These points represent all samples for which both TAG and WE composition were available in this tissue; there are multiple samples from individuals. Age classes are represented by *different symbols*: calf (*circles*), subadults (*triangles*), adults (*squares*). Linear regression between the two components (all samples combined) was significant ($P < 0.0001$, in the form $y = 1.727x$, no significant intercept) with an adjusted R^2 value of 0.82

Ontogeny of acoustic tissues

Animals from three other age classes (subadult, calf and fetus) were examined and compared to the adult form described above. The lipid content of younger animals differed such that lipid wet weight percent of adult and subadults > calves > fetus (ANOVA, Tukey Adjustment; $P < 0.0001$), indicating that calves and the fetus were still accumulating lipid in their tissues, while subadults had generally attained adult lipid levels. Within the three categories of tissue sampled (inner jaw fat, outer jaw fat and cranial blubber), inner jaw fat consistently yielded the highest wet weight percent lipid, except in the case of the fetus (Table 2). Mean lipid content of cranial blubber and outer jaw fat were more highly variable with larger ranges (Table 2). No discernable patterns of spatial lipid content were detected outside these tissue type and age difference trends.

As was the case with the adult samples, TAG was the dominant lipid class in all tissues. WE was also detected, but in varying degrees according to tissue type and age (Table 2). Samples from the cranial blubber of all ages were comprised largely of TAG, with small areas of localized WE. Inner and outer jaw fat contained higher levels of WE within the three oldest age classes, but fetal tissues contained very little WE.

The same suite of FA was identified in the juvenile samples as was found in the adults, however the proportions varied (Fig. 7). In the absence of high levels of iso-acids in younger animals there were correspondingly higher levels of endogenous FA (14:0, 14:1*n*-5, 16:0, 16:1*n*-7, 18:0, 18:1*n*-9; Table 3). Iso-acid accumulation increased with age (Fig. 8). Although the magnitude of iso-acid content varied with age, the pattern and spatial layout of regions with high amounts of iso-acids remained the

Table 2 Mean lipid content (% wet weight) and lipid class content (as percent of total lipids) from three tissue types (inner jaw fat, outer jaw fat and associated cranial blubber) ± standard error

Age class	Inner jaw fat			Outer jaw fat			Blubber		
	Lipid content	TAG	WE	Lipid content	TAG	WE	Lipid content	TAG	WE
Adult	80.39 ± 0.83	72.11 ± 2.03	27.89 ± 2.03	61.51 ± 3.41	87.52 ± 1.70	11.18 ± 1.60	57.41 ± 1.57	98.49 ± 0.28	1.51 ± 0.28
Subadult	80.13 ± 1.70	67.55 ± 2.44	32.44 ± 2.44	70.16 ± 3.78	78.64 ± 2.31	20.58 ± 2.29	59.25 ± 2.25	95.77 ± 0.61	4.23 ± 0.61
Calf	61.01 ± 3.57	74.48 ± 3.04	24.91 ± 3.16	51.11 ± 4.87	85.27 ± 3.22	13.65 ± 3.36	55.07 ± 2.69	95.81 ± 1.11	3.75 ± 1.13
Fetus	11.71 ± 2.69	77.37 ± 8.47	2.79 ± 0.97	18.24 ± 2.99	85.46 ± 5.68	1.72 ± 0.67	18.37 ± 1.69	91.99 ± 1.61	0.50 ± 0.24

For tissues with lipid classes that do not sum to 100%, the remaining lipid classes consisted of phospholipids and free fatty acids

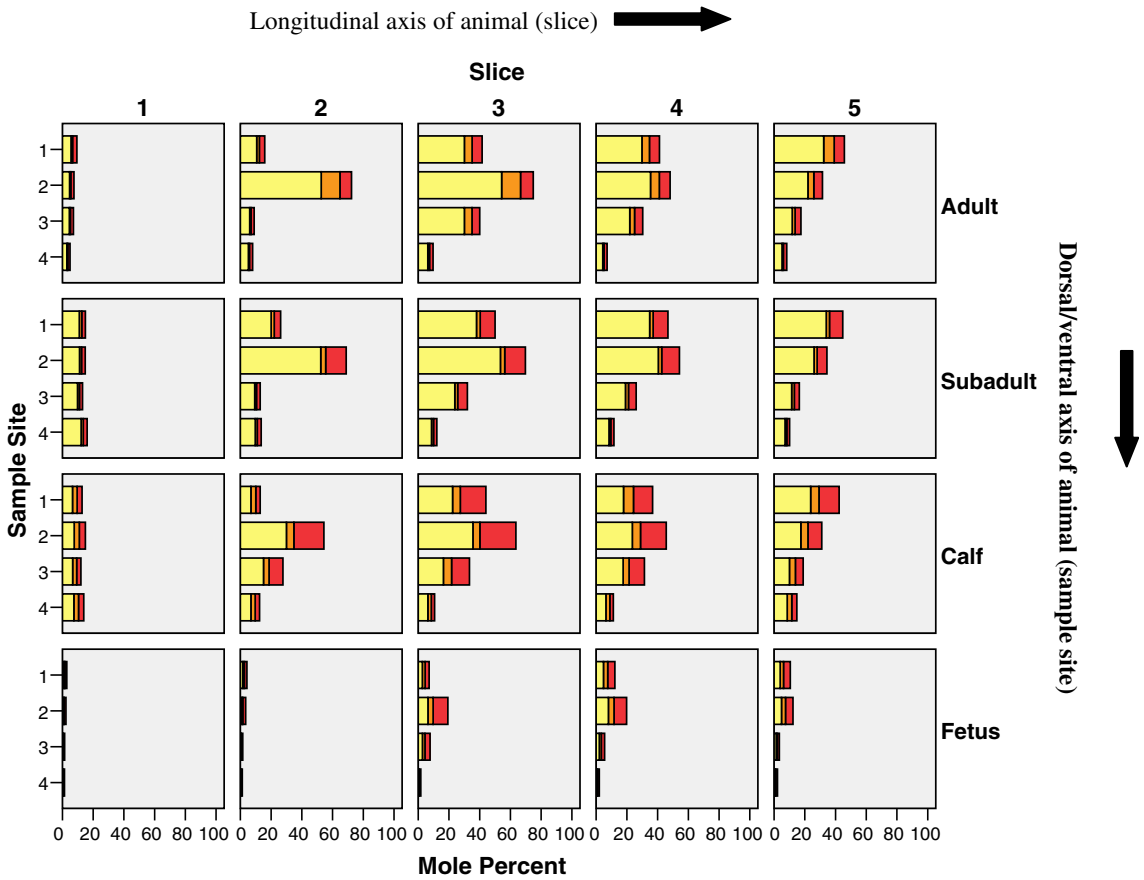

Fig. 7 Spatial variation of iso-acid composition (as mean mole % of total FA present) in the cranial blubber of all age classes (adult $n = 4$, subadult $n = 3$, calf $n = 2$, fetus $n = 1$). *Columns* represent the transverse slice taken; *rows* represent the location of the sample site (1–4; dorsal to ventral). Colors of iso-acids are the same as in Fig. 2a

same across all ages in cranial blubber (no age effect of ANOVA; all $P > 0.39$). There was little spatial heterogeneity in adult inner and outer jaw fat, and there was also no age effect (ANOVA, all $P > 0.29$) seen in outer jaw fat; however in the inner jaw fat there was a slight age effect. In slices 2 and 3, the calf tissues followed the same pattern as those of adults, with dorsal levels of branched-chain FA being low, but in these youngest animals, ventral tissues also had low iso-acid levels ($P < 0.04$ for slice 2 and $P < 0.038$ for slice 3). However, as the same general pattern existed across all age classes, and because there was such a small amount of spatial organization in this tissue to begin with, we did not consider this to be a significant deviation from the general trends observed in all other tissues.

As with TAG-FA, the identities of the WE-FA and FAlc present in the jaw fats of younger animals were the same as those of adults, but in different proportions (Table 1). Levels of *i*-5:0 WE-FA in the inner and outer jaw fats of subadults (mean 91.9 ± 1.1 and 86.5 ± 3.8 mole %,

Table 3 Mean and standard error of all TAG components (mole % of total FA) for all age classes separated by tissue types

Tissue type	Age class		*i*-5:0	*i*-15:0	Other branched acids	Endogenous	Omega 3	Omega 6	Dietary MUFA	Other PUFA	Other FA
Inner	A	$\bar{x}$	58.15	16.91	7.01	15.06	0.04	0.26	0.03	0.09	2.45
		SE	0.51	0.40	0.18	0.99	0.01	0.02	0.01	0.01	0.19
	S	$\bar{x}$	58.60	8.57	10.05	19.39	0.05	0.36	0.02	0.15	2.81
		SE	1.21	0.56	0.29	1.54	0.03	0.04	0.02	0.03	0.22
	C	$\bar{x}$	32.27	4.81	15.45	39.09	0.86	0.84	0.05	0.36	6.16
		SE	2.71	0.29	0.61	2.23	0.19	0.09	0.02	0.04	0.42
	F	$\bar{x}$	3.61	1.62	5.88	64.76	4.76	2.59	0.19	1.05	15.53
		SE	0.66	0.30	1.00	1.67	1.15	0.74	0.05	0.14	1.18
Outer	A	$\bar{x}$	49.01	11.74	6.46	26.38	0.80	0.66	0.54	0.30	4.11
		SE	1.96	0.65	0.19	1.87	0.16	0.08	0.13	0.04	0.33
	S	$\bar{x}$	52.84	4.69	9.31	27.98	0.56	0.67	0.12	0.29	3.56
		SE	2.45	0.45	0.42	2.43	0.23	0.11	0.05	0.05	0.30
	C	$\bar{x}$	21.16	4.69	12.17	50.37	1.90	1.12	0.06	0.51	8.01
		SE	2.42	0.36	1.09	2.59	0.28	0.13	0.02	0.06	0.48
	F	$\bar{x}$	2.48	1.31	3.50	71.90	3.52	1.55	0.13	1.00	14.61
		SE	0.53	0.26	0.66	1.44	0.70	0.33	0.04	0.14	0.87
Blubber	A	$\bar{x}$	18.96	3.56	4.26	53.04	5.63	2.12	2.64	1.02	8.77
		SE	1.98	0.46	0.27	1.78	0.42	0.12	0.28	0.06	0.30
	S	$\bar{x}$	22.02	1.81	5.59	55.14	4.29	2.18	0.63	0.97	7.38
		SE	1.98	0.08	0.55	1.82	0.37	0.13	0.07	0.05	0.26
	C	$\bar{x}$	14.48	3.84	8.55	58.89	3.20	1.32	0.15	0.70	8.88
		SE	1.60	0.21	1.12	2.29	0.31	0.11	0.02	0.05	0.23
	F	$\bar{x}$	2.16	1.22	2.72	76.52	2.23	1.01	0.15	0.80	13.12
		SE	0.51	0.25	0.56	1.41	0.21	0.09	0.01	0.05	0.69

A adult, *S* subadult, *C* calf, *F* fetus

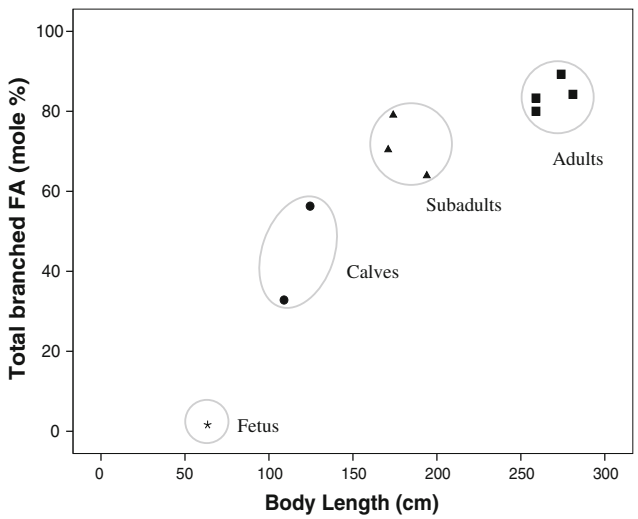

Fig. 8 All branched chain FA as a function of body length sampled from inner jaw fat TAG at homologous sample sites. Similar allometric relationships were seen in other sampling locations of acoustic FA (outer jaw fat and cranial blubber at the acoustic window)

respectively) were similar to those of adults (see above), but those of the calf were lower (mean 25.9 ± 2.9 and 18.9 ± 4.6 mole % for inner and outer, respectively). In the calf, the most prevalent FA present in WE was 16:0 (mean inner 25.9 ± 1.9, outer 26.4 ± 1.6 mole %), with the balance of the WE-FA portion consisting largely of 14:0, *i*-12:0 and 12:0. Concentrations of *i*-16:0 and 16:0 FAlc in the subadults were close to adult values (in the 25–34 mole % range see Table 1) but values for *i*-15:0 FAlc (mean inner 11.5 ± 0.8, outer 10.3 ± 1.2 mole %) were less than half those of adults. Interestingly, the calf had comparable 16:0 FAlc levels to the older animals, but both *i*-15:0 (inner 5.0 ± 0.5, outer 5.2 ± 1.0 mole %) and *i*-16:0 (inner 11.8 ± 1.5, outer 11.7 ± 1.5) FAlc values were lower than those of subadults and adults. In the calf, the most prevalent FAlc present (besides 16:0 and *i*-16:0) were 18:1*n*-9 (~12 mole %), *i*-17:0 (~8 mole %), 18:1*n*-7 (~8 mole %) and 18:0 (~7 mole %). As with adults, PUFA and MUFA of primarily dietary origin (≥20 carbons) were not detected.

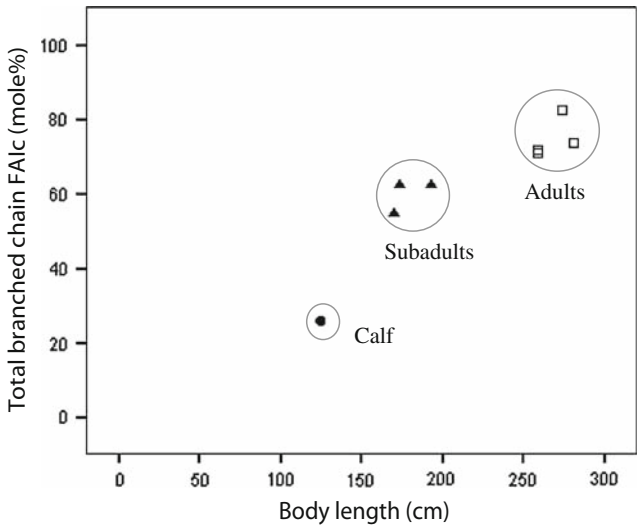

Fig. 9 All branched chain FAlc as a function of body length sampled from inner jaw fat WE at homologous sample sites. As WE analysis could not be carried out at all sites, there are fewer individuals represented here than in Fig. 8

As fewer samples could be analyzed for WE content, we were unable to perform any statistical comparisons of the distributions of FA components across different age classes. However, it does appear that the accumulation of iso-acids and iso-alcohols in WE does increase with age (Table 1; Fig. 9). Comparison of the accumulation of total branched TAG-FA and branched WE-FAlc with increasing body size showed remarkably similar patterns in both components (Figs. 8, 9).

Discussion

The importance of the acoustic fat found in and around the lower jaw and its role in transmission of sound to the ears is generally accepted although the specific mechanisms and pathways by which this system works still need to be elucidated (Brill 1988; Brill and Harder 1991; Bullock et al. 1968; McCormick et al. 1970). However little information exists about the ontogenetic variability and development of these tissues. Furthermore, the composition of the superficial tissue (cranial blubber) has been virtually ignored which is problematic when speculating on the manner in which sound is influenced by these tissues. We addressed these data shortfalls by examining the fine scale lipid content of cranial blubber, along with the inner and outer jaw fats of several dolphin age classes.

As we have shown here the acoustic tissues of young animals do not possess the same biochemical composition as adults. Previous work by Gardner and Varanasi (2003) showed that levels of *i*-5:0 lipids in melon tissue increased as a function of body length in bottlenose dolphins, indicating a biochemical change in this structure as a function of age. Our evidence shows that not only does *i*-5:0 content increase with age in the jaw fats of bottlenose dolphins, but that spatial patterns for lipid class and FA accumulation are established as early as the fetal stage. Subadults and younger animals had FA content in their tissues that did not represent adult concentrations, even though the pattern of lipid accumulation appeared to be set. The presence of FA patterns so early in life is not surprising, as other authors have noted early lipid pattern development in various acoustic tissues (the spermaceti organ and jaw fats) of calves (Koopman et al. 2006; Koopman and Zahorodny 2008; Morris 1975). However, this is the first time such a pattern has been described at such a fine-scale molecular level in an ontogenetic series or in an animal as young as a fetus.

Surprisingly little spatial variation was found in both the inner and outer jaw fats of adults. Previous studies have reported a channel of high concentrations of short-chained, often branched FA in the middle of the inner jaw fat along the length of the fat body in other odontocete species (*Kogia, Phocoena phocoena*, *Stenella attenuata* and some beaked whales) (Koopman et al. 2006), however this was not the case here. The bottlenose dolphin appears to be different from other odontocetes in its more uniform spatial distribution of FAs in inner jaw fats. Levels of *i*-5:0, *i*-15:0 and other iso-acids were uniformly high (combined iso-acid concentrations were generally greater than 60 mole % and often closer to 80 mole %) throughout the adult inner mandibular fat. The only notable exception is that the lowest concentrations of iso-acids were located near the dorsal, or thickest, portion of the mandible. This evidence suggests some preferential deposition of acoustic FAs around the pan bone.

The cranial blubber examined here exhibited a more complex FA composition and arrangement than either the inner or outer jaw fat. Blubber generally performs several functions, one of which is to act as a metabolic store (e.g., Koopman et al. 2002). Typically, bottlenose dolphin body blubber contains ~ 20% dietary PUFA and about 10% dietary MUFA, and FA are stratified within the blubber layer, with concentrations of specific FA varying throughout blubber's depth (Koopman 2007; Samuel and Worthy 2004). However, the cranial blubber at the acoustic window (superficial to the outer jaw fat) had low values of dietary FA, almost to the point of exclusion (Table 3). In contrast, all of the sample sites that had low iso-acid content had much greater (up to 20%) content of dietary FA, and these areas more closely represent typical metabolic storage blubber found elsewhere on the body. The lack of stratification and decreased presence of dietary FA suggests that the regions of cranial blubber overlying outer jaw fat may have an acoustic function rather than a metabolic role.

It appears that two margins of this specialized cranial blubber were captured in our sampling. The first transverse section of blubber (slice 1) contained low iso-acid values (which were similar to body blubber) along the entire dorso-ventral height, delineating what is likely the caudal margin of the acoustic window. The ventral midline also appeared more similar to body blubber in FA composition than to acoustic fat, delineating the ventral margin of the acoustic window in the blubber. Blubber at the acoustic window, as described by Norris (1968), appears to be more specialized than previously assumed, as other authors have regarded cranial blubber as having no acoustic function (Robisch et al. 1972; Varanasi and Malins 1971).

Prior to our work only one study had included cranial blubber overlying the mandibular fat in analysis of acoustic tissues (Varanasi et al. 1973). In that study all the fatty tissues lying external to the mandible were excised together and divided into large transverse sections. Samples close to the acoustic window were found to have larger amounts of isovaleroyl lipids (> 80% of TAG had *i*-5:0 acid as components); however blubber and the outer jaw fat were not separated for analysis. Hence the higher proportions may very well be attributed to the presence of the outer jaw fat itself as it tends to be most pronounced over the pan bone and diminishes further rostral. In an earlier study (Koopman and Zahorodny 2008) we detected spatial differences in the composition of the cranial blubber, but this was done on a wt% (rather than mole %) basis, an approach that results in an underestimate of concentrations of short-chain components. Thus no other study has shown fine scale layout of iso-acid accumulation, in terms of individual molecules (moles) present, in the cranial blubber surrounding the acoustic window.

In addition to isovaleroyl acids we also report the presence of WE in the acoustic tissues sampled. Synthesis of wax esters is not common among animals and is not a feature of the adipose of mammals other than cetaceans. Although fewer samples were analyzed for WE composition, there was still an ontogenetic signal in accumulation of both WE-FA and WE-FAlc. By the time dolphins reach the subadult age class they had accumulated "adult-like" concentrations of *i*-5:0 FA and *i*-16:0 FAlc; however concentrations of *i*-15:0 FAlc were much lower than adult values (Table 1; Fig. 6). It appears, therefore, that the rates of biosynthesis and accumulation of the different FA and FAlc constituents of the acoustic fats are not necessarily uniform. In bottlenose dolphins, for example, *i*-16:0 FAlc levels increase more rapidly than do *i*-15:0 FAlc levels in WE; yet WE *i*-15:0 FAlc and TAG *i*-15:0 FA show somewhat similar patterns of synthesis (Fig. 6) while WE *i*-15:0 FA remains comparatively low (Table 1). The differential rate of conversion/accumulation of *i*-15:0 and *i*-16:0 is notable, as these molecules arise from different amino acid precursors. FAlc are produced via the reduction of the corresponding FA (e.g., the precursor for *i*-15:0 FALc is *i*-15:0 FA), and then are incorporated with FA into WE molecules (Sargent 1976). Therefore in theory all FA and FAlc components of TAG and WE arise from a single pool of FAs. However, the conversion of FA to FAlc is not random, as the enzymes responsible for this process seem to be specific for saturated and MUFA, with PUFA rarely being converted to alcohol form (see Budge et al. 2006). Here we see high (but not uniform) levels of conversion of saturated/branched components (particularly *i*-15:0, *i*-16:0 and 16:0) from FA form to FAlc.

The results of this study imply that in the adult dolphins examined here almost every wax molecule located in a region of high WE concentration had *i*-5:0 as its FA moiety, by virtue of the > 90 mole % levels of this FA (Table 1). This is much higher than the 48 mole % value reported for the mandibular canal fat of a single specimen of *T. truncatus* by Ackman et al. (1973). However, the Ackman et al. (1973) dolphin was only 1.6 m long, making it intermediate in body size between the calves and subadults in our study; thus it is not surprising that the *i*-5:0 FA levels in this smaller animal were lower. Interestingly, we did not detect any *i*-5:0 FAlc in the WE of the dolphins we examined, nor did Ackman et al. (1973). Small quantities (2.6 mole %) of this alcohol were found in the mandibular fat body of a *T. gilli* specimen examined by Varanasi and Malins (1971); it is possible there is some individual variation in FAlc synthesis among dolphins, but all available data indicate that *i*-5:0 FAlc is not a major component of dolphin acoustic tissues, even though *i*-5:0 FA clearly is.

The fetus and calves in this study had low levels of lipid content in all tissues, indicating that the deposition of lipid is an ongoing process until the animals matured to the stage of subadults/adults. However, this result is not surprising since developing adipocytes generally need time to hypertrophy (Pond 1998). Fetal tissues were dominated by TAG, indicating that WE had yet to be deposited in any great amount en utero. By the time dolphins reached the calf stage, more WE had been deposited into the tissues. Subadults tended to have higher mean percent of wax esters present in their tissues than adults in the inner and outer jaw fat, as well as the blubber. However, this difference is proportional, and not an absolute measure of the WE content. Since WE are not generally metabolized by mammals (Pond 1998) it is likely that the proportionally lower WE content in adults can be explained as a more rapid accumulation of WE in subadults and subsequent continual accumulation of TAG into adulthood.

Adult dolphins had high concentrations of iso-acids in their tissues; however younger age classes did not have such high values and as a consequence tended to have

higher mole % values of other FA. The ontogenetic accumulation of iso-acids appeared to occur quickly in all the tissue types examined here and increases as a function of body size (a proxy for age). This is similar to the results reported by Gardner and Varanasi (2003) who examined the accumulation of melon isovalerate lipids in young and mature bottlenose dolphins and harbour porpoises. Iso-acids are synthesized by young bottlenose dolphins themselves, and are not passed from mother to calf via milk. Studies of bottlenose dolphin milk have shown that there is no *i*-5:0 present (Ackman and Eaton 1971), thus the varying degrees of accumulation of iso-acids of different age classes observed in this study are a reflection of the time needed to synthesize and deposit these FA.

The composition and spatial distribution along with the ontogenetic disparity of lipid classes and their components have functional implications for hearing in these animals (e.g., Malins and Varanasi 1975; Morris 1986; Wedmid et al. 1973), and clearly the fine scale spatial accumulation of lipid classes suggests a functional purpose. There is an inverse relationship between the length of a carbon chain and sound speed (Gouw and Vlugter 1967; Hustad et al. 1971), thus sound passes more slowly through shorter FA, and more rapidly through long molecules. Similar to the inverse relationship between carbon chain length of FA and sound speed, the proportion of WE (rich in branched structure), has been shown to cause a marked decrease in ultrasonic velocity (Varanasi et al. 1975). Our study found that there was a higher instance of longer chained FA over the dorsal section of the mandible and a higher instance of short and branched FA over the pan bone. This particular pattern of lipid distribution may serve to refract incoming sound waves down toward the thinnest region of the pan bone. Differences in sound velocity between two media or tissue types translate into the refraction of sound waves at the interface where the media meet (e.g., Litchfield et al. 1973; Wedmid et al. 1973). This theoretical interpretation of our lipid data makes sense, as the region over the pan bone has been shown to be one of the most sensitive parts of the head for sound reception in dolphins (Brill and Harder 1991). It is also likely that the acoustic window we described in the cranial blubber serves to impedance-match sound traveling from the environment toward the auditory bulla of the dolphin, creating a more consistent FA medium from which sound can pass through the blubber, the outer jaw fat and into the inner jaw fat.

We determined that almost every WE molecule (in high WE density regions) must contain *i*-5:0 as its acid portion. A similar trend was has been noted in the WE fraction of the innermost (deepest) region of the melon of the pilot whale (*Globicephala melas*); this molecular arrangement is believed to lower the speed of sound waves moving through the inner portion of the melon, relative to the outer melon regions, resulting in the formation of a beam aimed in front of the animal during echolocation (Wedmid et al. 1973). Whether an inverse effect of channeling incoming sound is created by the high levels of *i*-5:0 WE in the mandibular fat bodies of bottlenose dolphins has not yet been empirically tested, as very little work has been done on the speed of sound transmission through the jaw fats of odontocetes. It should be noted here that the exact pathway(s) by which sound can enter the head and be directed to the ears still need to be elucidated (e.g., see Cranford et al. 2008); however we feel that given the unusual and organized nature of the lipids in the mandibular fat bodies it is certainly reasonable to suggest that these fats are involved in sound transmission.

The ontogeny of lipid accumulation shown here could imply that young animals may not have the same ability to receive sound as adults. While subadults do have similar biochemical makeup of their acoustic tissues to adults, they are still accumulating specific iso-acids and iso-alcohols (e.g. *i*-15:0). The disparity between the biochemical content of adult tissues and calves is even more apparent, leading to the suggestion that while subadults may be in the final stages of biochemical acoustic development, calves are in the early developmental stages, which may influence their ability to receive sounds. There is evidence suggesting ontogenetic development of echolocation in bottlenose dolphins. Bottlenose dolphins do not echolocate immediately after birth; the youngest bottlenose dolphin recorded to have made sounds resembling adult echolocation clicks was 14 days old (Lindhard 1988; Reiss 1988). Furthermore, there is evidence of developmental patterns in echolocation of young bottlenose dolphins, suggesting maturation towards a functional echolocation system (Hendry et al. 2005; Tranel and Kuczaj 2005). Lipids are associated with two components of the echolocation system; the sound generating unit and the sound receiving unit (Norris 1968). It can be inferred that there may be some developmental aspect of the sound receiving unit from evidence of development in the sound generation unit. Evidence is provided here for the first time of development in the complete biochemical composition of the sound-receiving acoustic fats in the bottlenose dolphins. In the future, pairing analyses of the biochemical compositions and spatial arrangements of acoustic fat bodies with acoustic behavior studies would allow us to greatly improve our understanding of exactly how these remarkably specialized adipose tissues develop and function.

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