ONTOGENY AND ORGANIZATION OF ACOUSTIC LIPIDS IN JAW FATS OF THE BOTTLENOSE DOLPHIN (*Tursiops truncatus)*

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ABSTRACT

Specialized acoustic fat bodies located around the mandibles of odontocetes have the proposed role of focusing received sound towards the ear. Previous studies have suggested that the distribution of lipids in these fat bodies may form a waveguide for incoming sound. In bottlenose dolphins (*Tursiops truncatus*) these fat bodies are comprised of triacylglycerols (TAG) and wax esters (WE). Fine-scale topographic distribution and rapid ontogenetic accumulation of branched-chain (iso) fatty acids (FA) in jaw fat and cranial blubber of bottlenose dolphins (n=10) are described here. Iso-acids are unusual endogenous lipids formed as byproducts of amino acid breakdown and have a hypothesized role in sound transmission. Isovaleric acid (*i*-5:0) is toxic to most mammals, so the considerable presence of this FA in dolphin acoustic tissues represents an unusual physiological trait. Iso-acids were the dominant FA constituents in jaw fat, often comprising up to 80 wt%. Fetal concentration of *i*-5:0 was extremely low (<3 wt%) compared to adults (up to 52 wt%), and calves and subadults exhibited intermediate values. Little FA variation was found in adult inner jaw fat except for reduced values of iso-acids at the dorsal-most region along the length of the fat body. Adult outer jaw fat had highest iso-acid accumulation over the thinnest region (pan bone) of the mandible, with low iso-acid values at the dorsal-most regions sampled. In all animals, blubber contained very low levels of iso-acids. The notable exception was a small area of blubber on the lateral mandibular region overlying Norris' "acoustic window". Blubber here exhibited up to a 10-fold increase in iso-acids compared to adjacent blubber. This acoustic

window blubber is thus more chemically similar to acoustic mandibular fat, than to body blubber, and likely confers less impedance to sound waves. Young animals showed rapid accumulation of *i*-5:0 and *i*-15:0 in acoustic tissues and variation in iso-acid accumulation across age classes was described by logarithmic curves which accounted for upwards of 68% of variation. Patterns of lipid distribution of inner and outer jaw fat were apparent in animals as young as calves, while patterns were apparent in cranial tissues even earlier; at the fetal stage.

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INTRODUCTION

Cetaceans evolved from a terrestrial ancestor more then 50 million years ago and have since undergone exceptional evolutionary adaptation to their aquatic environment (reviewed in Fordyce, 2002). Many adaptations to terrestrial life became less useful when the ancestral cetacean recolonized the water. Structures such as those used for production and reception of sound, which were highly specialized for life in air, were maladapted to a secondarily aquatic life (Norris, 1968). Once submerged in water, the ancestral cetacean would have received sound through a large number of loci on the body rather than through the single mode of reception (the ear), and it would have traveled through the body in pathways that were never intended for acoustic reception (Norris, 1968). Despite these functional hurdles, odontocete cetaceans (the toothed whales) have evolved adaptations to life in the oceans and have developed the ability to echolocate in their aquatic environment. Although sound plays an important role in socialization and communication for virtually all marine mammals, neither the mysticetes nor pinnipeds are known to echolocate (Norris, 1968; Au *et al.*, 2000; Schusterman *et al.*, 2000). Echolocation is widespread and probably universal among the odontocete cetaceans (Norris, 1968). Odontocetes have specialized fatty tissues involved in the delivery and reception of sound, but in many cases the specific morphology and function of these structures has not yet been determined. The goal of this study is to elucidate the biochemical composition of the sound-receiving fatty tissues in an ontogenetic series of bottlenose dolphins (*Tursiops truncatus*).

Evolutionary Adaptations Related to Sound Production and Reception

Almost all odontocetes possess large specialized adipose tissue deposits in their foreheads (referred to as the melon), and in and around their lower jaws (the inner and outer jaw fats) (Norris, 1968). These depots contain unusual endogenous lipids referred to as acoustic lipids; the specific lipid composition of these tissues varies significantly between odontocete families (Litchfield & Greenberg, 1974; Litchfield *et al.*, 1975). In 1968, Norris hypothesized that the melon projects and focuses high-frequency sound, which itself is produced somewhere in the head above the region of the superior bony nares (Ridgway *et al.*, 1980). Cranford *et al.* (1996) later determined that the biosonar sound is generated by the monkey lips/dorsal bursae complex (MLDB) located caudal to the melon. Comparable in function to the melon, Norris (1968) also postulated that the mandibular fats act as waveguides focusing incoming sound to the ear; he referred to the region superficial to these fat bodies as the acoustic window. These fats were presumed to serve the same function as the pinnae (which are absent in odontocetes) and external auditory meatus, funneling sound to the middle and inner ears. Norris (1968) initially presumed that these fatty structures were homogeneous in composition, acting as a passive channel through which sound travels. It has however been demonstrated that there is topographic variation in lipid composition within acoustic tissues (melon and jaw fats) (Litchfield *et al.*, 1973; Wedmid *et al.*, 1973; Karol *et al.*, 1978; Koopman *et al.*, 2006) of at least some odontocete species. It is now generally accepted by most scientists that the mandibular fats act as acoustic receivers (Norris, 1968),

although historically it has been the cause of some debate (Purves & Pilleri, 1983).

The Acoustic Window

After Norris (1968) first suggested that the lower jaw may serve as an acoustic window, attempts were made to validate or disprove the theory. Much evidence now exists in the literature to support Norris' theory. A range of electrophysiological techniques and experiments (Bullock *et al.*, 1968; McCormick *et al.*, 1970) have been employed to investigate the hearing systems of odontocetes and to determine where on the body peak hearing sensitivity occurs. From a number of these studies it was determined that the highest hearing sensitivity was over the lower jaw (in the area described by Norris as the acoustic window) (Bullock *et al.*, 1968; Mohl *et al.*, 1999), and that the external auditory meatus is most likely vestigial (McCormick *et al.*, 1970). Definitive confirmation that bottlenose dolphins use their jaws to receive echolocation information came when Brill (Brill, 1988; Brill *et al.*, 1988; Brill & Harder, 1991) performed behavioural experiments on *T. truncatus*. His experiments showed that bottlenose dolphins performed poorly at echolocation tasks while wearing sound-attenuating neoprene rubber hoods over their lower jaws.

Acoustic Lipids

The melon and mandibular jaw fats have similar roles in sound conduction (albeit in opposite directions) and are both considered acoustic tissues (Morris,

1986). Within an individual, both types of these acoustic tissues are biochemically similar to each other but biochemically distinct from other adipose tissues within the body (Varanasi & Malins, 1972; Litchfield & Greenberg, 1974). The primary lipid constituents of interest in the acoustic tissues of odontocetes are triacylglycerols (three fatty acids bound to a glycerol molecule) and wax esters (a fatty acid linked by an ester bond to an alcohol) (Malins & Varanasi, 1975; Pond, 1998). Investigation into the lipid composition of the acoustic tissues in a range of odontocetes has centered on distinguishing the unique biochemical properties underlying their acoustic function. Odd-chained and unusual endogenous fatty acids have been found to be common constituents of many odontocetes' acoustic tissues (Litchfield & Greenberg, 1974; Litchfield *et al.*, 1975; Koopman *et al.*, 2006).

Lipid classes and fatty acids that dominate the acoustic tissues are not uniform across various families of odontocetes. Delphinidae (dolphins), Phocoenidae (porpoises) and Monodontidae (narwhals and belugas) all possess very high levels of isovaleric acid (*i*-5:0) in their acoustic fat, mostly in the form of triacylglycerols, with wax esters also being an important component in delphinid tissues (phocoenids and monodontids have trace amounts of wax esters) (Litchfield & Greenberg, 1974; Litchfield *et al.*, 1975). The acoustic tissues of Ziphiidae (beaked whales), Physeteridae (sperm whales) and Kogiidae (dwarf and pygmy sperm whales) tend to be dominated by wax esters. Isovaleric acid is not present in these animals, although some species contain other branched chain fatty acids such as *i*-10:0, *i*-11:0, *i*-12:0 and *i*-13:0 (Litchfield & Greenberg,

1978; Koopman *et al.*, 2006). It is apparent from this phylogenetic diversity of acoustic lipid constituents that no one type of lipid class or fatty acid is unique in its ability to facilitate the transmission of high frequency sound, since all of these animals are believed to be able to echolocate. It has been suggested that it is not necessarily the presence of a particular short-chained branched fatty acid (such as isovaleric acid) that confers advantageous properties to the acoustic tissues, but rather the existence of compartments of *relatively* shorter-chained, and often branched, fatty acids compared to those found in surrounding tissue (Ackman *et al.*, 1971; Litchfield *et al.*, 1975; Koopman *et al.*, 2006).

Isovaleric Acid

Isovaleric acid is found in relatively high concentrations in delphinid acoustic tissues (Litchfield & Greenburg, 1974, Litchfield *et al.* 1975). The accumulation of *i*-5:0 in marine mammal acoustic tissues is a notable occurrence since this fatty acid is commonly considered toxic to most mammals. In humans its accumulation, which occurs as the result of a genetic disease called isovaleric acidemia, can be fatal (Tanaka *et al.*, 1966). All iso-acid lipids, including *i*-5:0, are endogenous and do not appear to originate from the diet (Malins & Varanasi, 1975). Typically, endogenous fatty acids are synthesized in the cytoplasm by the sequential addition of 2-carbon subunits (Voet *et al.*, 2002). Iso-acids however are formed as a by-product of amino acid catabolism. Isovaleric acid, for example, is produced in the mitochondria as an intermediate during the normal catabolism of leucine, during conversion of α-keto isocaproic acid to 3-methyl

crotonyl coA. Isovaleroyl dehydrogenase catalyzes this reaction, and under normal conditions isovaleroyl coA is rapidly converted to 3-methyl crotonyl coA. If the activity of this enzyme is blocked, isovaleric acid will accumulate (Tanaka *et al.*, 1966; Malins *et al.*, 1972; Morii & Kaneda, 1982).

Isovaleric acid is found in high concentrations in the acoustic tissues of some odontocetes, but not necessarily in other parts of the body (Litchfield *et al.*, 1971; Robisch *et al.*, 1972; Koopman *et al.*, 2003). This dichotomy of fatty acid storage between blubber and the acoustic tissue of the melon and jaw fats strengthens the theory that the fatty acids found in the head have a specific function. Although a small number odontocetes do have *i*-5:0 in their blubber, the historical explanation for it has been that it was a result of excess production of the acid synthesized for acoustic fats (Litchfield *et al.*, 1971). It has however since been suggested that *i*-5:0 is found in higher concentrations in the blubber of odontocetes from cold environments and may serve to maintain blubber flexibility (Koopman *et al.*, 2003). This suggests fine-scale control of placement of iso-acids in tissue for a specific function, whether it be in acoustic tissue, or blubber.

Lipids in Acoustic Tissues of *Tursiops truncatus*

The melon of bottlenose dolphins has been shown to be a heterogeneous lipid structure in which fatty acids appear to be arranged in a three-dimensional framework (Litchfield *et al.*, 1973). A study on compositional topography of the melon in the bottlenose dolphin (Litchfield *et al.*, 1973) revealed regional differences in the tissues that appeared to be a gradient change in fatty acid

distribution. Since sound travels at different velocities through different fatty acids, the authors speculated the inner melon served as a low velocity core, surrounded by higher-velocity tissue, resulting in refraction and collimation of a beam of sound. Similar results of a lower velocity melon core, rich in isovaleroyl lipids, have been found in pilot whales (Blomberg & Lindholm, 1976).

The fatty acids that are found in the mandibular fats have not been as well characterized as those in the melon. This may be a function of the relative difficulty in obtaining these structures as they are located on the inner and outer jaw and attached to the ear bones. Disarticulation of the jaw must be done with care so as not to macerate or dislocate these structures. The jaw fats of a bottlenose dolphin (*Tursiops truncatus*) have been shown to consist of triacylglycerols (60 wt%) and wax esters (40 wt%) (Ackman *et al.*, 1973; Litchfield *et al.*, 1975). These tissues are also known to contain high amounts of isovaleric acid (Litchfield *et al.*, 1975); for example the wax esters contain as much as 43.6 mol% isovaleric acid in *Tursiops gilli,* the Pacific coastal bottlenose dolphin (Varanasi & Malins, 1970). However much that is known about these tissues has come from reports of single specimens {one adult (Varanasi & Malins, 1971) and one subadult (Ackman *et al.*, 1973)} and there are no descriptions of any regional or ontogenetic differences within the jaw fats.

Acoustic tissues such as the melon are not homogeneous in their biochemical composition. Recently the three-dimensional spatial distribution of the fatty acids in the jaw fat of some odontocetes has been examined. A central channel of low-

velocity fatty acids was identified through the length of the mandibular fat in a number of odontocete species (Koopman *et al.*, 2006).

Lipid Ontogeny

Lipid content in acoustic tissues differs between adults and juveniles. For instance, lipid class composition in the spermaceti organ of a sperm whale calf (*Physeter catadon*) differed from that of adults, implying an important developmental aspect to lipid accumulation of this organ (Morris, 1975). Gardener and Varanasi (2003) demonstrated that the content of isovalerate lipids in the melon increased significantly with body length (a proxy of age) in bottlenose dolphins, suggesting that there is an ontogenetic change to the biochemical structure of the melon. Similar ontogenetic evidence was found in Gervais' beaked whale (*Mesoplodon bidens*) jaw fats (Koopman *et al.*, 2006) and in harbor porpoise (*Phocoena phocoena*) melon (Koopman *et al.*, 2003). Within an individual, there are many similarities between the melon and jaw fats and it is likely that the mandibular fats may also undergo an ontogenetic change in biochemical composition; however this aspect of acoustic development has yet to be explored in most species of odontocetes.

Stability of Acoustic Lipids

The lipids in the acoustic tissues are not mobilized during times of nutritional stress or poor health. Koopman *et al.* (2003) found that starvation affects the composition of blubber lipid content in harbour porpoises (*Phocoena phocoena*)

but not the lipid content of the melon, suggesting melon lipids are conserved for a specific function. Cranford (1999) also noted that death could occur due to starvation in neonate sperm whales, yet these animals retained robust spermaceti organs. Since both melon and mandibular jaw fat have an acoustical function rather than metabolic function it is likely that the content of the mandibular fat will not change with nutritional status either.

Goals and Significance

It is apparent that there are deficits with regards to what is known about the sonar apparatus of odontocetes, including the development of acoustic tissues and their biochemical spatial distribution. This study is the first to examine the characteristics of the mandibular fats in an ontogenetic series of bottlenose dolphins. The specific objectives are to (1) determine the lipid composition of mandibular fats in a three-dimensional context to resolve whether (like the melon and the jaw fats of other some other odontocetes) these lipids exhibit specific spatial arrangements and, (2) investigate the ontogenetic patterns in lipids; that is, to determine whether the lipid composition of mandibular fats of young animals are similar to those of adults.

The bottlenose dolphin is a well-studied marine mammal, and is often used as a model for odontocete physiology and bioacoustics, likely due at some level to its success in captivity and its proximity to the coastline (resulting in stranded animals being accessible for study). Ontogenetic development of lipid composition in acoustic tissues may be significant in determining whether a

young bottlenose dolphin can perceive sound as well or in the same way as do its adult counterparts. With increasing ocean exploration and exploitation causing escalations in the ambient sound level in the ocean, the impact of sound on marine life is a pressing environmental concern (Committee on Potential Impacts of Ambient Noise in the Ocean on Marine Mammals, 2003). Understanding the mechanisms of hearing in marine animals, both for adult and juvenile animals is imperative in assessing any potential consequences of sound in their environment.

METHODS

Specimen Collection

Samples of bottlenose dolphins were obtained through the University of North Carolina Wilmington Marine Mammal Stranding Program (Wilmington, NC, UNCW IACUC #2000-001, #2003-013, #2006-015), the Cetacean and Sea Turtle Team at the NOAA Beaufort Laboratory (Beaufort, NC), and the Virginia Aquarium and Marine Science Center (Virginia Beach, VA). All animals had died either as a result of natural causes, due to fisheries interaction, or were euthanized for humane reasons. Specimens were collected between November 2000 and October 2006 from the coasts of North Carolina and Virginia and only those considered to be fresh were used. Each dolphin was carefully examined using standard dissection protocols (outlined in McLellan *et al.*, 2002). From each animal, a suite of morphometric and meristic measurements were taken, as well as tissue samples collected for a variety of other studies on dolphin

anatomy, physiology and health. Seven specimens (those with lower tissue quality) were used in the preliminary portions of the study to perfect dissection techniques and subsampling protocols; and to develop/perfect sample preparation techniques (Table 1). Data from these 7 animals are not presented here as they were not sampled consistently and represent only preliminary results.

A total of 10 specimens of *T. truncatus* were used for analysis representing four different life history stages (see Table 2); fetus (n=1), calf (n=2), subadult (n=3) and adult (n=4). Determination of life history stage was based on total standard length (Read *et al.*, 1993), and the presence/absence of neonatal characteristics (Dearolf *et al.*, 2000).

Sample Collection

During routine necropsy procedures, heads were removed by a transverse cut at the atlanto-occipital joint and were either sampled immediately or frozen and then thawed for later dissection. The objective was to collect two main mandibular fat bodies: 1) the inner mandibular (jaw) fat, defined as the fat found within the deep mandibular fossa and including fat attached directly to the earbones; and 2) the outer mandibular (jaw) fat, which is adhered to the outer surface of the mandible and lies just deep to the blubber (Figure 1). The overlying blubber was also collected. Tissue collection was accomplished by first removing the lower jaw from the skull. Horizontal incisions were made from the orbit rostral to the gape, and from the orbit caudal to the limit of the tissue

Table 1. Field identification number, sex, and Smithsonian Institution (SI) condition code of bottlenose dolphins (*Tursiops truncatus*) used for technique and sampling development and preliminary analysis.

Field Number	***Sex***	***Length (cm)***	***Smithsonian Code***	***Date of Collection***
WAM 607	M	173	2	Nov 20, 2004
WAM 595	M	180	3	Apr 1, 2004
VMSM 2003 1043	M	190.5	2	May 29, 2004
VMSM 2004 1079	F	195	2	Jul 16, 2004
MMB 008	M	204	3	Nov 14, 2004
CJH 003	F	229.5	2	Nov 6, 2004
MMB 004	M	275	2	Jul 13, 2003

Table 2. Field identification number, sex, length, life history stage, Smithsonian Institution (SI) condition code, number of subsamples and date of collection; taken from the right side of the head of bottlenose dolphins (*Tursiops truncatus*) for mandibular fat composition analysis.

Field Number	***Sex***	***Length (cm)***	***SI Code***	***Life History Stage***	***No. of Sub-samples***	***Date of Collection***
WAM 560F	F	63.5	2	Fetus	41	Nov 21, 2000
WAM 606	M	109	2	Calf	44	Nov 19, 2004
WAM 610	M	124.5	3	Calf	43	Aug 14, 2005
BCB 002	M	171	2	Subadult	58	Jan 11, 2006
WAM 627	M	174	2	Subadult	57	Oct 5, 2006
AJW 002	M	194	2	Subadult	51	Apr 21, 2006
GNL 043	F	259	2	Adult	57	Nov 4, 2005
WAM 608	F	259	2	Adult	57	Dec 4, 2004
AJW 001	M	274	2	Adult	56	Mar 5, 2005
WAM 601	M	281	2	Adult	57	Jul 30, 2004

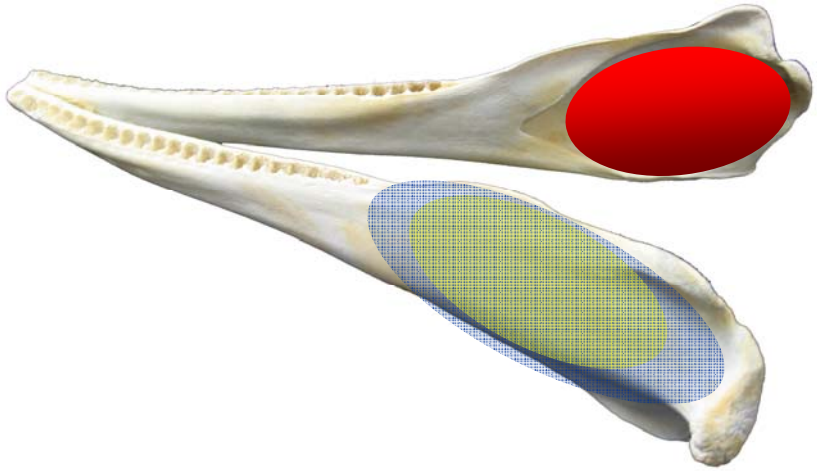


Figure 1. The mandibles of a bottlenose dolphin. The shaded areas represent the various tissues collected and sampled; red represents the inner jaw fat, green represents the outer jaw fat and blue represents the blubber lateral to the outer jaw fat.

collected (which was delineated by the incision just caudal to the nuchal crest during decapitation) at which time the temporomandibular joint was severed. Next a ventral midline incision was made to remove the tongue and associated musculature and finally the tympano-periotic complex was then exposed by carefully peeling back the fats that were adhered to the ear bones. All remaining musculature and tissue that held the mandible to the skull were carefully cut through until the lower jaw (including the delicate ear fats) was separated from the skull.

From the preliminary dissections carried out on the first 7 heads (for which data are not presented) it was clear that the best way to preserve tissue orientation was to maintain a tissue attachment between the inner and outer jaw fats. Inner and outer jaw fats were therefore removed together as a single tissue unit along with the associated blubber, for both the right and left sides of the head to ensure that all three components remained in accurate relative position to one another for subsampling. This was done by carefully peeling the tissue from both sides (inside the fossa and along the outer side of the pan bone) of the mandible. Tissue collection was from 10cm (5cm in the case of the fetus) caudal of the tip of the mandible to the limit of the tissue (line of decapitation). Jaw fats were then wrapped in plastic and refrozen so that the tissue would be solid for the subsampling phase. Acoustic lipids have very low melting points (CRC Press, 1975) and thus to limit the loss of fatty acids, the tissue needed to be chilled between the head dissection and subsampling steps.

A series of transverse sections were made while the tissue was in a semi-frozen state using tissue from the right side of the head only, since there is evidence for bilateral symmetry (Koopman *et al.*, 2006). Since the size of the mandibular fat bodies varied with the size of the individual animal, sections were made referencing two landmarks (see below). A total of four sections were made that were each comprised of inner jaw fat, outer jaw fat, and blubber, while a fifth and final section was only comprised of outer jaw fat and blubber (Figure 2). The objective was to sample homologous sites in each head, and thus the sizes of sampled sections scaled with the size of each individual. The initial incision was made using the caudal edge of the mandibular fossa as a guideline so the resultant first slice (Slice 1) contained the fat that was adhered to the tympano-periotic complex, a small amount of inner jaw fat caudal to the caudal edge of the mandible, as well as the corresponding blubber and outer jaw fat (Figure 3). Subsequent slices (Slices 2, 3 and 4) were made so the total inner jaw fat was divided evenly into three sections and a final section was made that contained only blubber and a residual amount of outer jaw fat (Slice 5). Thickness of slices ranged from 1–4 cm between heads, but was always of consistent thickness per specimen.

Between 10 and 15 small subsamples (each approximately 0.5g and 0.5cm^3) were taken from the rostral face of each slice (Figure 3) for an approximate total of 58 samples per head. Three samples were also taken from the area where the inner jaw fat adhered to the periotic complex (see Figure 4 for location of these three sample sites). These three sample sites from the periotic complex

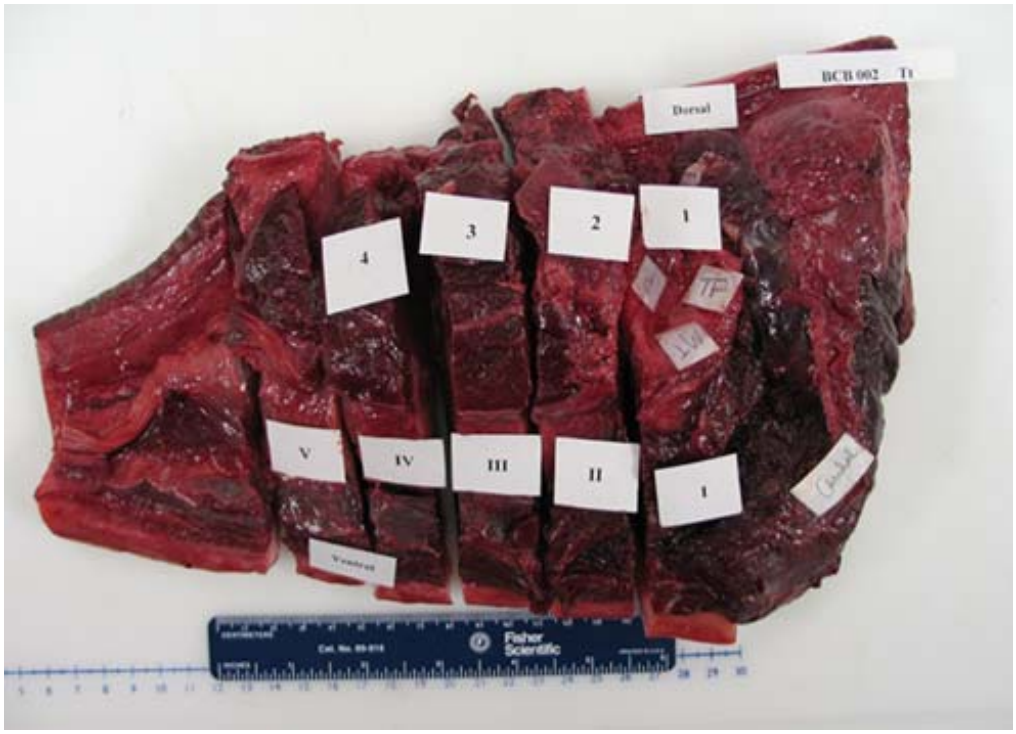


Figure 2. Representative photo of the three tissue types sampled and the transverse sections made through the fat bodies and blubber. Numbers 1-4 represent the 4 slices that represent the inner jaw fat, while I-V represent the outer jaw fat and blubber.

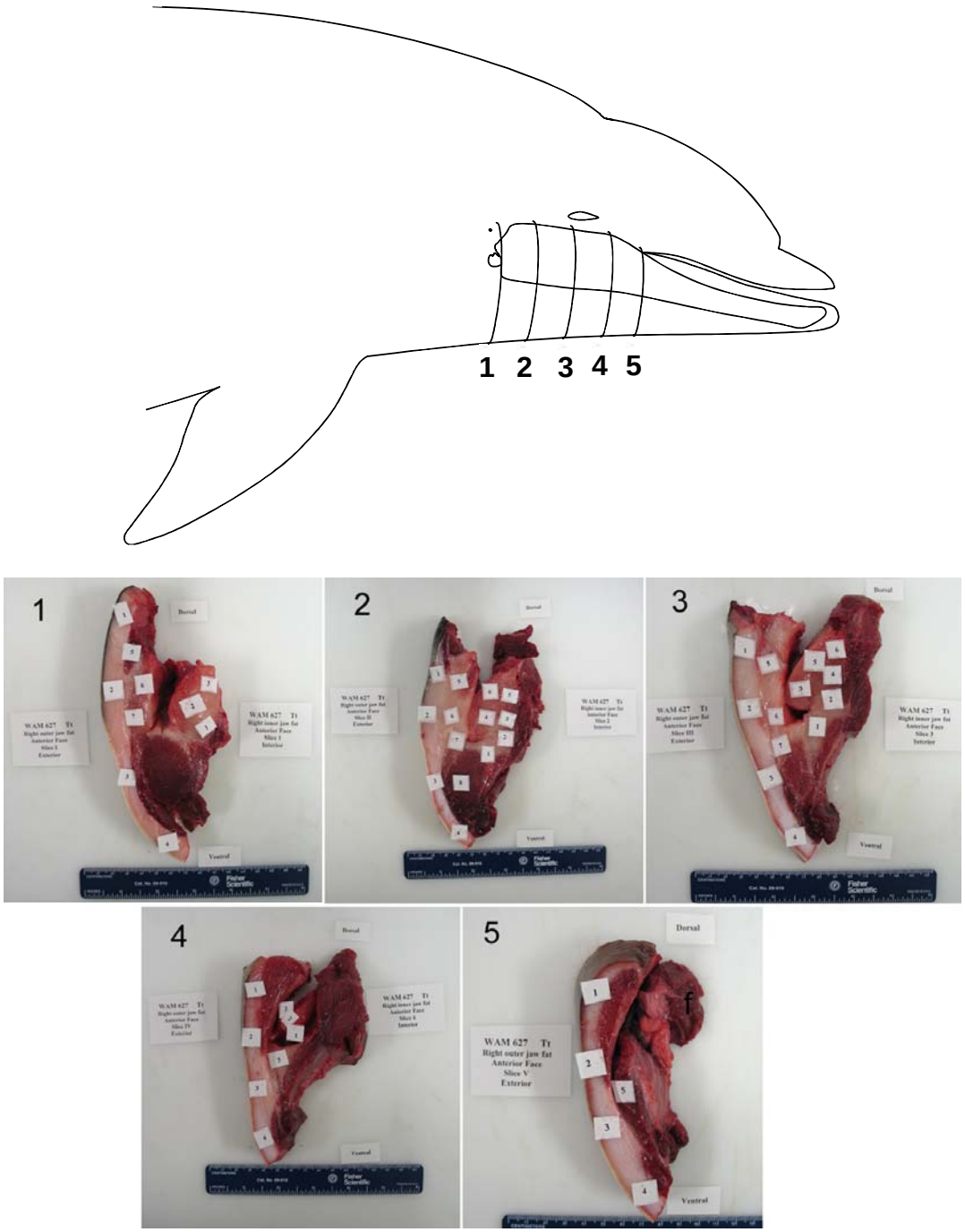


Figure 3. Schematic of a bottlenose dolphin and sampling sites. The black lines along the mandible indicate the location of the transverse sections taken, numbers 1-5 indicate the slice numbers and correspond to the numbers on the representative photos below. Photos are the rostral face of all sections, and locations of subsamples are represented by the small squares on each section. Schematic courtesy of S.A. Rommel.

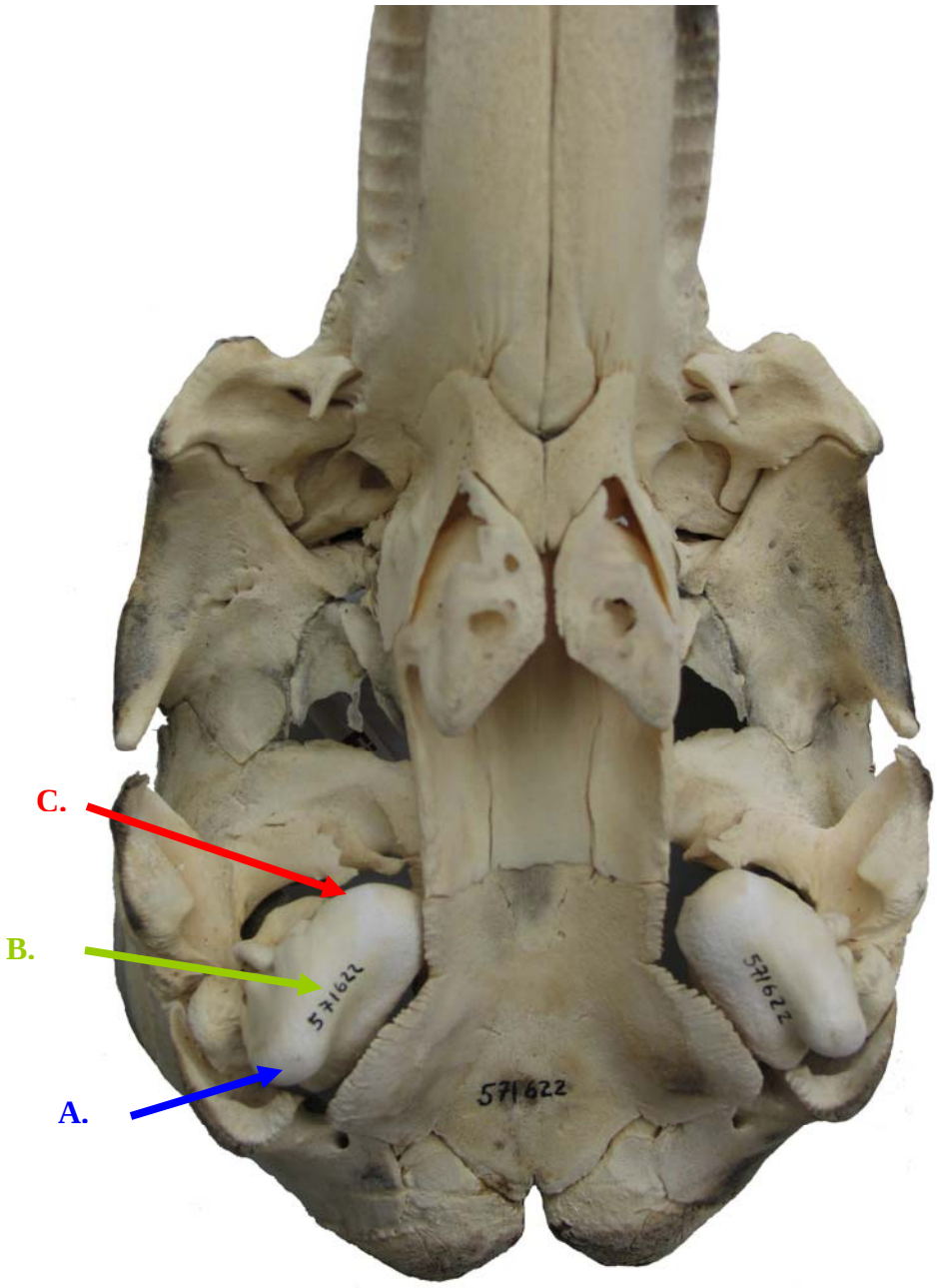


Figure 4. Ventral view of a bottlenose dolphin skull, mandible removed, with the periotic complex visible. The three sampling locations (A, B, C) taken from the fat adhered to the periotic complex are indicated with arrows. Access to skull courtesy of Smithsonian Institute, National Museum of Natural History.

were chosen to reflect a range over this structure, as well as an attempt to sample as close to the periotic bone itself. Overall, fewer samples were taken for calves and fetus heads (see Table 2 for the number of subsamples taken per animal examined). Every effort was made to maintain consistent homologous sampling between heads, through the use of digital photography, to standardize sampling so that comparisons between animals could be made.

Lipid Extraction and Preparation

Lipids from each subsample were extracted using a modified Folch procedure (Folch *et al.*, 1957; Koopman *et al.*, 1996). Each subsample was carefully weighed and placed in 9 ml of 2:1 chloroform methanol with 0.01% BHT (butylated hydroxytoluene) to prevent oxidation. The sample was left to soak in the solvent for approximately three days and then manually crushed with a glass rod to ensure complete extraction of lipids from adipocytes. Methanol was removed by adding 3 ml of 0.7% NaCl, vortexing to ensure thorough mixing of the solution and centrifuging at 2000 rpm for 5 minutes to separate layers. The methanol-water layer was discarded and the chloroform-lipid layer retained. Complete removal of water from solution was achieved through use of anhydrous Na_2SO_4. The solvent was then evaporated under a stream of N_2 gas to obtain percent lipid content (wet weight) of the tissue sampled. Lipid was resuspended in hexane and stored under nitrogen at -20 ˚C until further processing.

Small aliquots of lipid from each subsampling site were used to determine and quantify lipid classes. Samples (1 µl of 15 mg/ml) were spotted on chromarods

(Chromarod-SIII, Mitsubishi Kagaku Iatron Inc, Tokyo, Japan) and developed in 94/6/1 hexane/ethyl acetate/formic acid for a period of 40 minutes. Classes were then quantified by thin-layer chromatography-flame ionization detection (TLC-FID) (Iatroscan, MK-6s, Mitsubishi Kagaku Iatron Inc,, Tokyo, Japan). Identification was confirmed through use of lipid class standards (Nu Chek Prep, Elysian, MN). Peaks were integrated with Peaksimple software (Peaksimple 3.29, SRI Instruments, Torrance, CA).

The goal of the lipid analysis was to determine the individual fatty acid (FA) and fatty alcohol (FAlc) constituents of the primary neutral lipid classes (wax esters –WE; and triacylglycerols -TAG) separately for each class. As TLC-FID is a rapid and quantitative but destructive method, physical separation and recovery of classes for further analysis was carried out using standard TLC. However, because the absolute minimum amount of any given lipid class required for further analysis was 15 mg, not all lipid classes were recovered from each sample. In all cases, there was sufficient TAG for FA analysis, but not all subsamples contained enough WE to be able to quantify FA and FAlc. From early analysis (data not shown) it appeared that a lower threshold of 20% WE was the best cutoff to determine when there was enough WE to determine composition and this cutoff was used for all specimens. When WE represented less then 20% for these samples, only the TAG data are presented, even though TAG and WE were separated by TLC for all samples to ensure purification of the TAG fraction.

TLC plates were pretreated to extract any impurities by developing each plate (Adsorbosil-Plus 1 TLC plates, Alltech, Columbia, MD; conventional 250μm layer thickness, 20x20cm) in petroleum ether and discarding the top 1 cm of silica. For analysis, 50 mg of whole lipid was spotted onto TLC plates and 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 to reclaim lipids. Silica was filtered out of suspension with chloroform using glass wool (Fisherbrand). Reclaimed lipid was then dried down to evaporate solvent under a stream of N_2 gas, weighed and resuspended in hexane.

The fatty acid components of triacylglycerols were esterified as butyl esters (FABE-TAG, fatty acid butyl esters from TAG) by placing 50 mg of TAG in 1 ml of hexane and 1 ml of BF_3 in butanol in a test tube and heating to 100 °C for 1 hour. After cooling, samples were washed twice with 3 ml of water to remove butanol and dried with Na_2SO_4 and stored under N_2. Butyl esters were used as opposed to methyl esters (more commonly used) because the short-chained fatty acids are volatile (Koopman, 2007).

Wax ester processing was more complicated. Due to coelution problems on the GC columns, the FABE-WE and free fatty alcohol (FAlc) portions of the WE had to be split and run separately on the GC. The bonds between the FA and FAlc in each WE were broken when the FA were esterified as butyl esters (FABE-WE, fatty acid butyl esters from WE) using a similar protocol as TAG preparations. FABE-WE preparation differed from that of FABE-TAG as two

internal standards were introduced into the sample by adding a hexane/standard mix instead of hexane alone. The use of these internal standards was necessary to be able to "reconstruct" the correct proportions of FA and FAlc in each WE post-processing. The internal standard suspension was made by adding 50mg of 23:0 acid (Nu Chek Prep, Elysian, MN) and 50 mg of 19:0 alcohol (Nu Chek Prep, Elysian, MN) to 50 ml of hexane. A small aliquot of esterified WE sample was reserved for GC analysis to account for any loss of short chain acids during subsequent preparation. The remaining sample (comprised of FABE-WE and FAlc) was separated through conventional TLC using 70/30/1 hexane/ethyl ether/glacial acetic acid. Bands were again visualized with dichlorofluoroscein under UV light. FABE-WE was extracted from silica with hexane while the FAlc was extracted from silica with 1:1 hexane:ethyl ether. Ethyl ether was evaporated under N_2, both FABE-WE and FAlc were evaporated to small volumes (approximately 50 μl) and stored under N_2.

Fatty Acid and Fatty Alcohol Analysis

FABE-TAG, FABE-WE were analyzed by a Varian (CP-3800) gas chromatograph (Varian Inc., Palo Alto, CA) fitted with a flame ionization detector (FID) and a 30m x 0.25 mm column coated with 50% cyanopropyl polysiloxane (J&W Scientific DB-23 column). Injector and detector temperatures were held at 250°C and 270°C respectively. The temperature program used (developed by (Koopman *et al.*, 1996) took 45 minutes for elution of each injection. 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 minutes, ramped at 5°C/min. to 240°C and held for 1 minute.

FAlc were analyzed on the same GC/FID using a 30m x 0.25mm 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. This temperature program was developed by (Koopman *et al.*, 2006) and took 35 minutes to complete each run.

Data Processing

Peak identification was based on comparisons of retention time to standards and known samples. Peaks were integrated using appropriate response factors (Ackman, 1991) with Galaxie Chromatography Data System (Version 1.8.501.1, Varian Inc., Palo Alto, CA), and peak identification was manually confirmed for each run. The values of identified FA and FAlc were summarized and exported into a Microsoft Excel spreadsheet.

Wax Ester Component Reconstruction

Wax ester data was reconstructed through the amalgamation of data from multiple chromatograms generated from fractions (FA and FAlc) that were separated during processing (as described above in Lipid Extraction and

Preparation). To facilitate the reconstruction of the WE components, internal standards were added (23:0 acid and 19:0 alcohol) during processing to both the FA and FAlc fractions, and a small aliquot was sampled prior to separation to account for loss. Thus, three chromatograms were generated for each WE processed: 1.) unseparated WE to account for loss of short-chained FA during processing; 2.) FABE fraction of the WE; 3.) FAlc fraction of the WE. Multiple corrections were used to generate a final spreadsheet of WE constituents present in each sample which are outlined in Appendix A.

Statistical Analyses

For analysis of variation within tissues, FA from TAG were binned into 6 categories: 1.) *i*-5:0; 2.) *i*-15:0; 3.) all other iso- and anteiso-acids; 4.) dietary FA; 5.) endogenous FA; and 6.) other FA (see Table 3 for a listing of individual FA components of each group). Similarly, FA and FAlc from WE were binned into categories to compare trends in types of FA in different tissues (see Table 4 for a list of FA groupings and Table 5 for FAlc groups and their constituents). Logit categorical analyses with Bonferonni adjustments ($\alpha = 0.0005$) were used for examining the statistical differences between sampling sites of FA from TAG (SAS 9.1, SAS Institute Inc., Cary, North Carolina). Significant differences were assessed using 95% global confidence intervals (CI) for each analysis where significant differences existed when CI did not overlap. No statistical analyses were performed on WE components due to the low number of samples that yielded adequate WE.

Table 3. Groupings of FA from TAG. These FA groupings (along the top of the table) were used to compare trends in types of FA in different tissues.

Endogenous	*Dietary*	*Other*	*All other iso- and anteiso-acids*	*iso-5:0*	*iso-15:0*
14:0	16:2n-4	4:0	iso-4:0	iso-5:0	iso-15:0
14:1n-5	16:2n-6	5:0	iso-10:0		
16:0	16:3n-1	6:0	iso-11:0		
16:1n-7	16:3n-4	7:0	ante-11:0		
18:0	16:3n-6	8:0	iso-12:0		
18:1n-9	16:4n-1	9:0	iso-13:0		
	16:4n-3	10:0	ante-13:0		
	18:2n-7	11:0	iso-14:0		
	18:2D5,7	11:1	ante-15:0		
	18:2n-4	12:0	iso-16:0		
	18:2n-6	12:1a	iso-17:0		
	18:3n-1	12:1b			
	18:3n-3	13:0			
	18:4n-3	13:1			
	18:3n-4	14:1n-9			
	18:3n-6	14:1n-7			
	18:4n-1	15:0			
	20:1n-11	unk15a			
	20:1n-9	unk15b			
	20:1n-7	15:1a			
	20:2n-9	15:1b			
	20:2n-6	15:1n-8			
	20:3n-3	15:1n-6			
	20:4n-3	unk16a			
	20:5n-3	unk16b			
	20:3n-6	16:1n-9			
	20:4n-6	16:1n-11			
	21:5n-3	7Me16:0			
	22:1n-11	16:1n-5			
	22:1n-9	18:1n-5			
	22:1n-7	unk1			
	22:2n-6	unk2			
	22:3n-3	17:0			
	22:4n-3	17:1			
	22:4n-6	18:1n-7			
	22:5n-6	18:1n-11			
	22:5n-3	19:0			
	22:6n-3	19:1			
	24:1n-11	20:0			
	24:1n-9				

Table 4. Groupings of FA from WE. These FA groupings (along the top of the table) were used to compare trends in types of FA in different tissues.

Endogenous	*Dietary*	*Other*	*All other iso- and anteiso-acids*	*iso-5:0*	15:0
14:0	16:2n-4	4:0	iso-4:0	iso-5:0	15:0
14:1n-5	16:2n-6	5:0	iso-10:0		
16:0	16:3n-1	6:0	iso-11:0		
16:1n-7	16:3n-4	7:0	ante-11:0		
18:0	16:3n-6	8:0	iso-12:0		
18:1n-9	16:4n-1	9:0	iso-13:0		
	16:4n-3	10:0	ante-13:0		
	18:2n-7	11:0	iso-14:0		
	18:2D5,7	11:1	iso-15:0		
	18:2n-4	12:0	ante-15:0		
	18:2n-6	12:1a	iso-16:0		
	18:3n-1	12:1b	iso-17:0		
	18:3n-3	13:0			
	18:4n-3	13:1			
	18:3n-4	14:1n-9			
	18:3n-6	14:1n-7			
	18:4n-1	15:0			
	20:1n-11	unk15a			
	20:1n-9	unk15b			
	20:1n-7	15:1a			
	20:2n-9	15:1b			
	20:2n-6	15:1n-8			
	20:3n-3	15:1n-6			
	20:4n-3	unk16a			
	20:5n-3	unk16b			
	20:3n-6	16:1n-9			
	20:4n-6	16:1n-11			
	21:5n-3	7Me16:0			
	22:1n-11	16:1n-5			
	22:1n-9	18:1n-5			
	22:1n-7	unk1			
	22:2n-6	unk2			
	22:3n-3	17:0			
	22:4n-3	17:1			
	22:4n-6	18:1n-7			
	22:5n-6	18:1n-11			
	22:5n-3	19:0			
	22:6n-3	19:1			
	24:1n-11	20:0			
	24:1n-9				

Table 5. Groupings of FAlc from WE. These FAlc groupings (along the top of the table) were used to compare trends in types of FAlc in different tissues.

Saturated, Mono-unsaturated, and Unknown Alcohols	*All other iso and anteiso Alcohols*	*iso-15:0 Alcohol*	*iso-16:0 Alcohol*	*16:0 Alcohol*
16:1n-7 Alc	iso-12:0 Alc	*i*-15:0 Alc	*i*-16:0 Alc	16:0 Alc
16:1 Alc	iso-14:0 Alc			
18:1n-9 Alc	ante-15:0 Alc			
18:1n-7 Alc	ante-16:0 Alc			
18:1n-5 Alc	iso-17:0 Alc			
10:0 Alc	ante-17:0 Alc			
11:0 Alc	iso-18:0 Alc			
12:0 Alc				
13:0 Alc				
14:0 Alc				
15:0 Alc				
17:0 Alc				
18:0 Alc				
Unk Alc A				
Unk Alc B				
Unk Alc 1				
Unk Alc 2				
Unk Alc 3				
Unk Alc 4				
Unk Alc 5				

Site-specific, and FA-specific regression analyses were performed using SPSS (SPSS Inc., Chicago, Il.).

RESULTS

To explore the overall lipid content and lipid class of these tissues, samples were pooled together into the appropriate tissue category (inner jaw fat, outer jaw fat and blubber). Samples of lipid from tissue adhered to the tympano-periotic complex were pooled together with the inner jaw fat, unless otherwise indicated. Site-specific distribution of FA are also described. Both the adult patterns of lipid distribution, and patterns of ontogenetic change, are described below under spatial variation for each tissue type (inner jaw fat, outer jaw fat and blubber).

Lipid Content

Lipid components were extracted and processed to completion for a total of 519 of the 529 sample sites from the 10 individuals outlined in Table 2. Inner jaw fat always yielded the highest wet weight percent lipid within the three categories of tissue sampled (all samples pooled into inner jaw fat, outer jaw fat and associated cranial blubber) for both adult and subadult age classes (>80 wt%, Figure 5; Table 6). The mean lipid content of blubber and outer jaw fat were highly variable among adults and subadults but were not different from each other either across age class or tissue type. Fetal mean lipid content values were consistently low (<33 wt%) for all tissue types examined. Calves exhibited intermediate values that followed a similar trend as the older animals (ie. mean

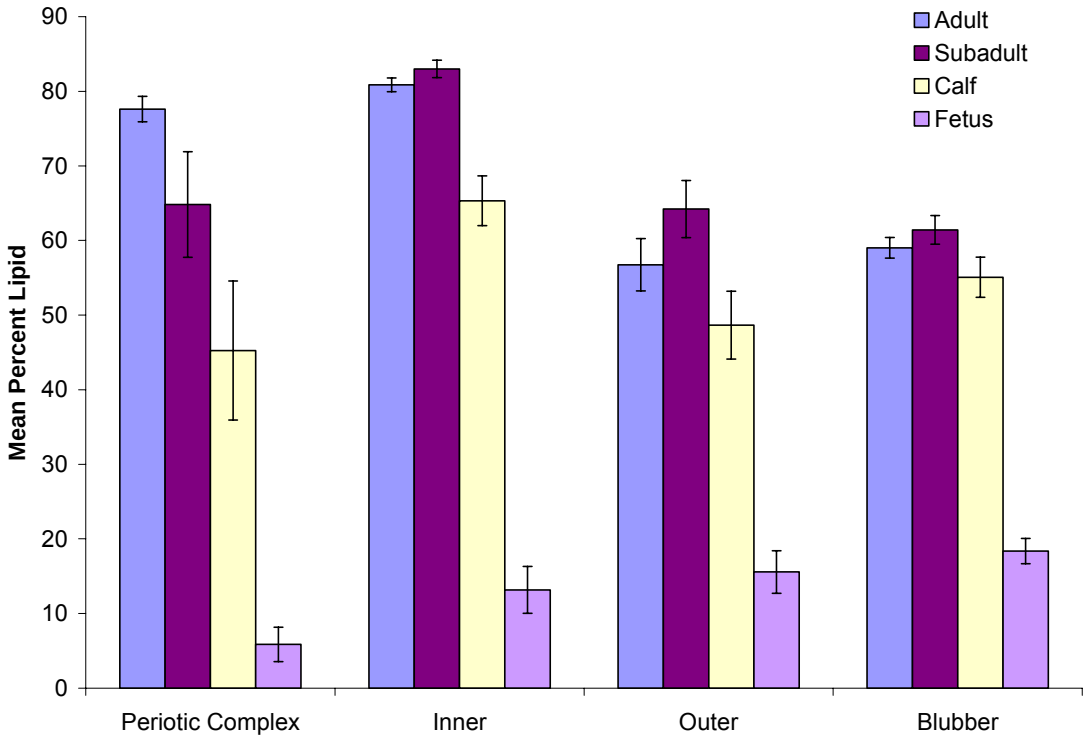


Figure 5. Mean wet weight percent of lipid extracted from tissue types (samples at periotic complex, inner jaw fat, outer jaw fat and associated cranial blubber) ± standard error of mean. Sample sizes are as in Table 6.

Table 6. Mean percent wet weight of lipid extracted from three tissue types (inner jaw fat, outer jaw fat and associated cranial blubber) ± standard error of mean.

Age Class	*No. of Indiv.*	*Inner Jaw Fat*			*Outer Jaw Fat*			*Blubber*		
		No. of Samples	*Mean ± SEM*	*Range*	*No. of Samples*	*Mean ± SEM*	*Range*	*No. of Samples*	*Mean ± SEM*	*Range*
Adult	4	84	80.39 ± 0.83	46.04-89.88	46	56.74 ± 3.50	0.10-82.13	96	59.02 ± 1.38	18.28-80.12
Subadult	3	57	80.13 ± 1.70	27.15-92.42	34	64.22 ± 3.83	6.57-87.22	75	61.41 ± 1.93	12.61-82.84
Calf	2	28	61.01 ± 3.57	16.70-89.42	20	48.65 ± 4.54	9.12-76.44	38	55.07 ± 2.69	25.00-82.50
Fetus	1	10	11.71 ± 2.69	2.13-24.36	11	15.56 ± 2.86	2.17-33.81	20	18.37 ± 1.69	9.29-33.16

lipid content was highest for the inner jaw fat, and the values of lipid content were lower for both outer jaw fat and cranial blubber; Figure 5). Overall the lipid content of the adults and subadults were highest for all tissues but were not different from each other (ANOVA, Tukey Adjustment; P=0.546). The lipid content of younger animals differed such that 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 generally had attained adult lipid levels.

Lipid Class Composition

Lipid classes were determined for all 519 samples. All three tissue types examined had both TAG and WE components in such high concentrations that the presence of other lipid classes was undetectable, except in the case of the fetus where some phospholipids were also detected. Most samples from the inner and outer jaw fat were comprised of mainly TAG and WE, while samples taken from blubber consisted largely of TAG (Table 7).

Inner jaw fat had the highest proportion and cranial blubber had the smallest proportion of WE (Figure 6). Inner jaw fat contained on average 30% WE, however higher proportion of WE were found around the periotic complex comprising almost 50% of the sample at these sites. Fetal tissue had significantly lower values of WE than all other animals for all tissue types. Subadults exhibited slightly higher WE composition then the adults in all tissues types (Figure 6). A total of 142 of the 519 subsamples had high enough WE

Table 7. Mean lipid class content (as percent of total lipids) from three tissue types (inner jaw fat, outer jaw fat and associated cranial blubber) ± standard error of mean. Sample sizes are as in Table 6. For tissues that do not add up to 100% the remaining lipid classes consisted of phospholipids and free fatty acids.

Age Class	*Inner Jaw Fat*		*Outer Jaw Fat*		*Blubber*	
	TAG	WE	TAG	WE	TAG	WE
Adult	72.11 ± 2.03	27.89 ± 2.03	87.52 ± 1.70	11.18 ± 1.60	98.49 ± 0.28	1.51 ± 0.28
Subadult	67.55 ± 2.44	32.44 ± 2.44	78.64 ± 2.31	20.58 ± 2.29	95.77 ± 0.61	4.23 ± 0.61
Calf	74.48 ± 3.04	24.91 ± 3.16	85.27 ± 3.22	13.65 ± 3.36	95.81 ± 1.11	3.75 ± 1.13
Fetus	77.37 ± 8.47	2.79 ± 0.97	85.46 ± 5.68	1.72 ± 0.67	91.99 ± 1.61	0.50 ± 0.24

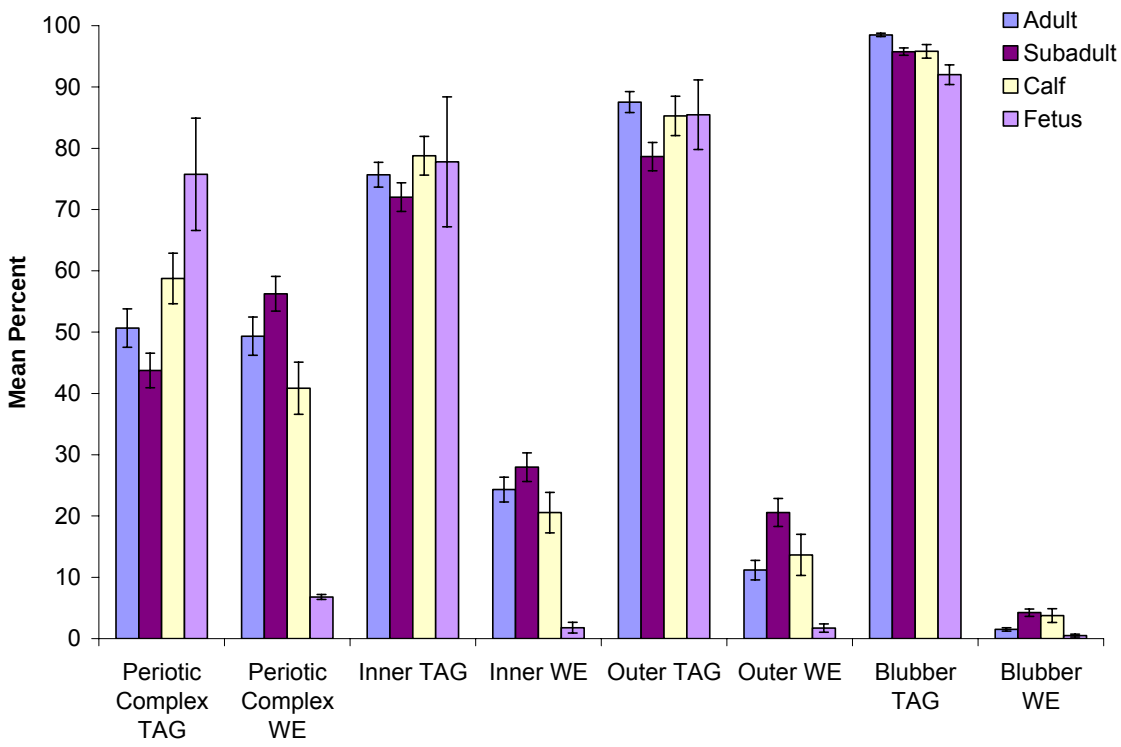


Figure 6. Mean percent of TAG and WE in each tissue type sampled (inner jaw fat, outer jaw fat and associated cranial blubber) for all age classes ± standard error of mean. Sample sizes as in Table 6.

content (>20%) to be recovered for analysis of composition (FA and FAlc analysis).

Dominant Fatty Acid Components of Triacylglycerols

Since TAGs were the dominant lipid class, fatty acids from TAG data were available for all subsamples (519). However, WE were only recovered from 27% of the samples, thus less emphasis can be placed upon the comparisons of WE components across tissue type and age class. TAG and WE components are reported separately here, and the FA from TAG are analyzed in greater detail. Approximately 70 fatty acids were routinely identified from the TAG component of tissues, and the dominant fatty acids (as wt% of total fatty acids) varied between different tissue types and age classes. All means below are presented ± the standard error of the mean.

Inner Jaw Fat

Adult inner jaw fat was dominated by *i*-5:0 (43.08 ± 1.05 wt%) and *i*-15:0 (23.50 ± 0.63 wt%) FA (Table 8). Subadult inner jaw fat appeared similar in composition to that of adults except for a lower proportion of *i*-15:0 (12.18 ± 0.84 wt%) corresponding to an increased proportion of 16:1n-7 (9.58 ± 0.72 compared to adult 4.63 ± 0.41 wt%) and other iso-acids (11.85 ± 0.44 compared to adult 8.07 ± 0.16 wt%). Calf inner jaw fat also had high *i*-5:0 content (21.81 ± 2.17 wt%), representing the most abundant fatty acid, but was also high in other iso-acids as well as 16:1n-7, 16:0, 14:0 and 18:1n-9. The fetal inner jaw fat was

Table 8. Mean and standard error of all TAG components (wt% of total FA) for all age classes separated by tissue types. See Table 3 for listings of the fatty acids in each category. Sample sizes as in Table 6. (MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids).

Tissue Type	Age Class		*i*-5:0	*i*-15:0	All other *i*-acids	All antei-acids	14:0	14:1n-5	16:0	16:1n-7	18:0	18:1n-9
Inner	Adult	Mean	43.08	23.50	8.07	0.92	5.37	0.49	7.14	4.62	0.15	2.86
		Std.error	1.05	0.63	0.16	0.04	0.20	0.07	0.35	0.41	0.02	0.32
	Subadult	Mean	43.73	12.18	11.85	0.98	5.77	0.46	7.49	9.58	0.27	3.35
		Std.error	1.19	0.84	0.44	0.03	0.17	0.03	0.58	0.72	0.05	0.50
	Calf	Mean	21.81	5.78	14.45	0.60	9.63	1.86	10.96	16.85	0.96	7.59
		Std.error	2.17	0.30	0.67	0.04	0.37	0.09	0.85	0.51	0.15	0.51
	Fetus	Average	1.95	1.65	5.39	0.19	11.41	2.75	18.77	23.29	3.02	7.63
		St error	0.36	0.29	0.99	0.03	1.36	0.43	0.71	2.91	1.00	1.19
Outer	Adult	Mean	34.45	15.55	6.55	1.19	6.04	1.06	7.70	10.73	0.45	8.06
		Std.error	1.80	0.99	0.22	0.04	0.18	0.18	0.39	1.00	0.07	0.88
	Subadult	Mean	38.63	6.52	10.10	1.03	6.35	0.97	6.85	16.26	0.42	6.41
		Std.error	2.20	0.69	0.59	0.06	0.14	0.08	0.51	1.06	0.08	1.06
	Calf	Mean	13.20	5.22	10.92	0.44	10.91	3.21	10.89	22.75	0.92	8.80
		Std.error	1.87	0.37	1.07	0.05	0.49	0.19	0.54	1.14	0.12	0.75
	Fetus	Mean	1.34	1.33	3.13	0.12	12.83	4.18	17.69	30.48	1.48	7.17
		Std.error	0.29	0.27	0.65	0.03	0.98	0.44	0.82	2.13	0.49	0.96
Blubber	Adult	Mean	14.06	5.24	3.93	0.98	4.30	2.23	6.64	20.50	0.93	20.16
		Std.error	1.40	0.64	0.27	0.06	0.11	0.09	0.22	0.74	0.06	0.86
	Subadult	Mean	16.04	2.22	5.69	0.44	5.39	2.44	7.55	27.04	1.03	15.76
		Std.error	1.47	0.13	0.55	0.04	0.09	0.08	0.23	0.50	0.07	0.67
	Calf	Mean	8.72	4.18	7.79	0.30	9.27	5.35	8.37	30.33	0.70	10.05
		Std.error	1.14	0.26	1.05	0.04	0.22	0.18	0.22	1.20	0.05	0.54
	Fetus	Mean	1.18	1.26	2.43	0.07	11.86	7.46	11.53	41.06	0.89	6.72
		Std.error	0.28	0.27	0.54	0.02	0.45	0.24	0.27	1.19	0.10	0.38

Table 8 Continued. Mean and standard error of all TAG components (wt% of total FA) for all age classes separated by tissue types. See Table 3 for listings of the fatty acids in each category. Sample sizes as in Table 6. (MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids).

Tissue Type	Age Class		Total Omega 3	Total Omega 6	Dietary MUFA	Other PUFA	Other FA
Inner	Adult	Mean	0.06	0.36	0.05	0.12	3.18
		Std.error	0.02	0.03	0.02	0.02	0.21
	Subadult	Mean	0.08	0.51	0.02	0.21	3.51
		Std.error	0.03	0.06	0.02	0.03	0.27
	Calf	Mean	1.18	1.10	0.06	0.45	6.71
		Std.error	0.25	0.11	0.03	0.05	0.35
	Fetus	Average	5.82	2.95	0.23	1.14	13.83
		St error	1.29	0.81	0.06	0.13	1.36
Outer	Adult	Mean	1.17	0.88	0.77	0.38	5.02
		Std.error	0.23	0.10	0.18	0.04	0.37
	Subadult	Mean	0.77	0.90	0.16	0.37	4.27
		Std.error	0.30	0.14	0.07	0.05	0.32
	Calf	Mean	2.59	1.41	0.08	0.60	8.08
		Std.error	0.38	0.15	0.03	0.06	0.46
	Fetus	Mean	4.37	1.80	0.16	1.10	12.83
		Std.error	0.81	0.36	0.04	0.15	1.05
Blubber	Adult	Mean	6.24	2.25	2.83	1.03	8.70
		Std.error	0.45	0.12	0.29	0.05	0.27
	Subadult	Mean	4.85	2.41	0.70	1.01	7.43
		Std.error	0.42	0.14	0.07	0.05	0.24
	Calf	Mean	4.18	1.60	0.18	0.78	8.19
		Std.error	0.38	0.12	0.03	0.05	0.17
	Fetus	Mean	2.84	1.20	0.19	0.88	10.44
		Std.error	0.25	0.10	0.01	0.05	0.22

dominated by 16:0 and 16:1n-7, which together comprised a mean of approximately 42 wt%. Other saturated and monounsaturated endogenous fatty acids, such as 14:0 and 18:1n-9, were present in the fetal tissue in high proportions. Although the proportion of *i*-5:0 was relatively low (1.95 ± 0.36) all iso-acids together accounted for approximately 9 wt% in the fetus. Virtually no dietary fatty acids (see Table 3 for FA in this category) were detected in this tissue for any age class.

Outer Jaw Fat

Outer jaw fat consisted of the same suite of FA as the inner jaw fats (Table 8), however the values of both *i*-5:0 (ANOVA, Tukey Adjustment; $P<0.0001$) and *i*-15:0 (ANOVA, Tukey Adjustment; $P<0.0001$) were less than that of the inner jaw fats. *I*-5:0 (34.45 ± 1.80 wt%) and *i*-15:0 (15.55 ± 0.99 wt%) were the primary constituents of the outer jaw fat among adults. *I*-5:0 (38.63 ± 2.20 wt%) was also the most abundant fatty acid in the outer jaw fat of subadults, however *i*-15:0 (6.52 ± 0.69 wt%) accounted for a much lower proportion of FA, with an apparent corresponding increase in 16:1n-7 (subadults; 16.26 ± 1.06, adults 10.73 ± 1.00 wt%) and other iso-acids (subadults; 10.10 ± 0.59, adults 6.55 ± 0.22 wt%). In both calves (22.75 ± 1.14 wt%) and the fetus (30.48 ± 2.13 wt%) 16:1n-7 was the dominant FA. Although *i*-5:0 (13.20 ± 1.87 wt%) and other iso-acids (10.92 ± 1.07 wt%) were among the three most dominant FA groups in calves, 14:0, 16:0 and 18:1n-9 were also present in high proportions. Fetal tissue had generally low proportions of *i*-5:0 (1.34 ± 0.29 wt%), *i*-15:0 (1.33 ± 0.27 wt%) and other iso-

acids (3.13 ± 0.65 wt%), and were dominated mainly by endogenous FA such as 14:0, 16:0 and 18:1n-9. Very few dietary FA were found in the outer jaw fat, however omega-3 FA were present in higher proportions in younger animals, and were highest in the fetus (5.82 ± 1.29 wt%).

Cranial Blubber

The cranial blubber of animals in all age classes was dominated by 16:1n-7 (Table 8). 18:1n-9 (adults; 20.16 ± 0.86, subadults; 15.75 ± 0.67 wt% and *i*-5:0 (adults; 14.06 ± 1.40, subadults; 16.04 ± 1.47 wt%) were the second and third most abundant FA in this tissue in adults and subadults. The calves (8.72 ± 1.14 wt%) and the fetus (1.18 ± 0.28 wt%) had much lower proportions of *i*-5:0 and higher proportions of other endogenous FA such as 14:0, 16:0 and 18:1n-9. Omega-3 and other dietary FA were also seen in the blubber in larger proportions than in any of the other two tissue types.

I-5:0 and *i*-15:0

Both *i*-5:0 and *i*-15:0 are unusual, endogenous fatty acids that are presumed to have acoustic functions (see Introduction), hence their distribution and concentration are of great interest. They were both abundant in the inner and outer jaw fats, and their proportions appeared to positively co-vary in adults. Young animals however, tended to have smaller values of *i*-15:0 while the values of *i*-5:0 of subadults were at, or near adult levels, indicating differential accumulation of these iso-acids. This trend was most obvious in outer jaw fat.

Plots were made to examine the relationship between age classes and FA values of *i*-5:0 and *i*-15:0 in adults and developing animals (subadults and calves), for both the inner and outer jaw fat (Figures 7-8). A linear line was forced between these variables for each of the age groups so comparisons of slopes could be made for both inner (Figure 7) and outer jaw fat (Figure 8).

In the outer jaw fat, the slope of the line for the developing animals (slope=0.107) was only about one quarter of that in adults (slope=0.426). The slope of the line for developing animals (slope=0.280) in the inner jaw fat was much closer to that of adults (slope=0.329), however there was still an approximate 15% difference in accumulation rate between *i*-5:0 and *i*-15:0. Fetal tissues were excluded from this analysis since there was only a single sample of this age class.

Inner Jaw Fat and Spatial Variation of Triacylglycerol Components

Adults

The location and content of the FA groupings were analyzed with a logit categorical data analysis model (Agresti, 1996). Individual comparisons were made using a Bonferroni correction of 0.0005. Sample sites were delineated as dorsal, central or ventral representing their relative positions to each other (not that the samples were from the dorsal or ventral midline of the animal).

Very few differences were found in the FA composition between sample locations in the inner jaw fat. All samples were consistently high in total iso-acid content (generally > 80 wt%). Only two sampling locations (at the dorsal regions

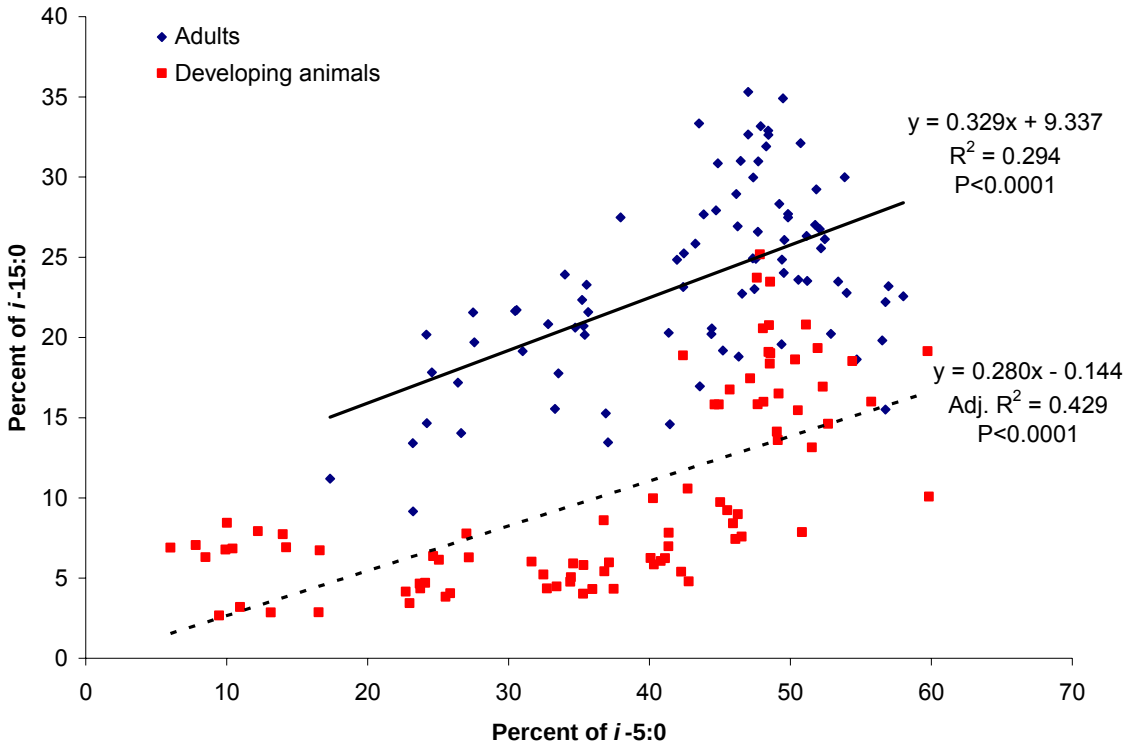


Figure 7. Scatterplot of *i*-5:0 and *i*-15:0 proportions of the inner jaw fat for adults (blue) and developing animals (subadults and calves in red).

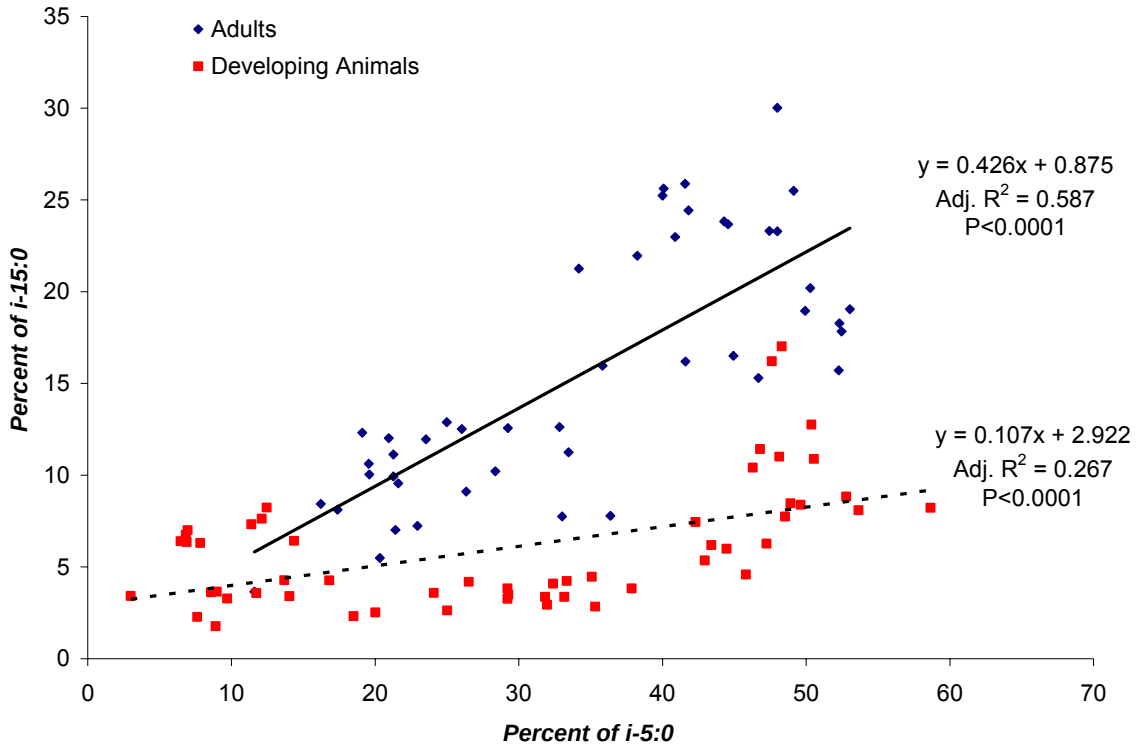


Figure 8. Scatterplot of *i*-5:0 and *i*-15:0 proportions of the outer jaw fat for adults (blue) and developing animals (subadults and calves in red).

in slices 2 and 3) within the inner jaw fat were found to have significantly lower values of *i*-5:0 and *i*-15:0 acids from other locations (Figure 9). At these sampling sites there was a corresponding increase in the endogenous fatty acid content (see Table 3). Although graphically there appeared to be a trend of decreasing *i*-5:0 and *i*-15:0 in subsequent rostral sections, there was no significant difference between slice 1 and slice 5.

Samples taken from fat adhered to the periotic complex (Figure 4) were comprised predominantly of iso-acids (> 80 wt%) and the three ear sites were not significantly different from each other in FA composition or proportion (Figure 10). These samples taken from the ear bones were also not different in composition or proportion from the adjacent inner jaw fat (slice 1).

Ontogenetic Change in Inner Jaw Fat

The inner jaw fat of subadults (Figure 11) was very similar in FA composition to that of adults, however it displayed a slightly lower overall mean wt% of iso-acids, specifically *i*-15:0 (Table 8). Values of *i*-5:0 content were not different between these two age classes (Table 8) and the proportions of *i*-5:0 remained consistent (showing no significant differences according to the logit categorical model) between similar spatial locations. The spatial distribution was the same for the subadults as was described above for adults showing only one significant difference within the inner jaw fat. Just like the adults, there were no significant differences found in FA composition between sample sites in the initial transverse section (slice 1). The dorsal section of the second transverse section

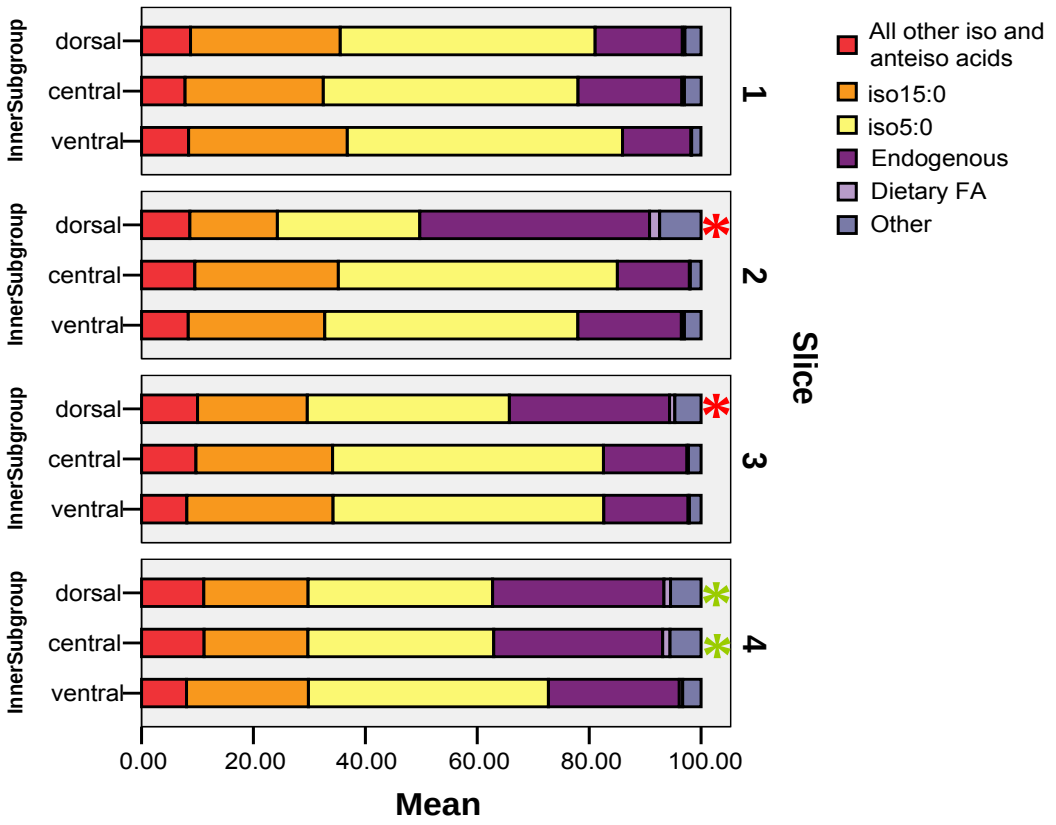


Figure 9. Mean percentage of fatty acid groupings in adult inner jaw fat. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). Red asterisks denote locations where the values of *i*-5:0 are significantly lower compared to other sample areas within the same slice. Sample sites indicated by the green asterisks denote areas not significantly different then others in the same slice, however they are more similar to the values of *i*-5:0 at the dorsal location in slices 2 and 3 indicated by the red asterisk.

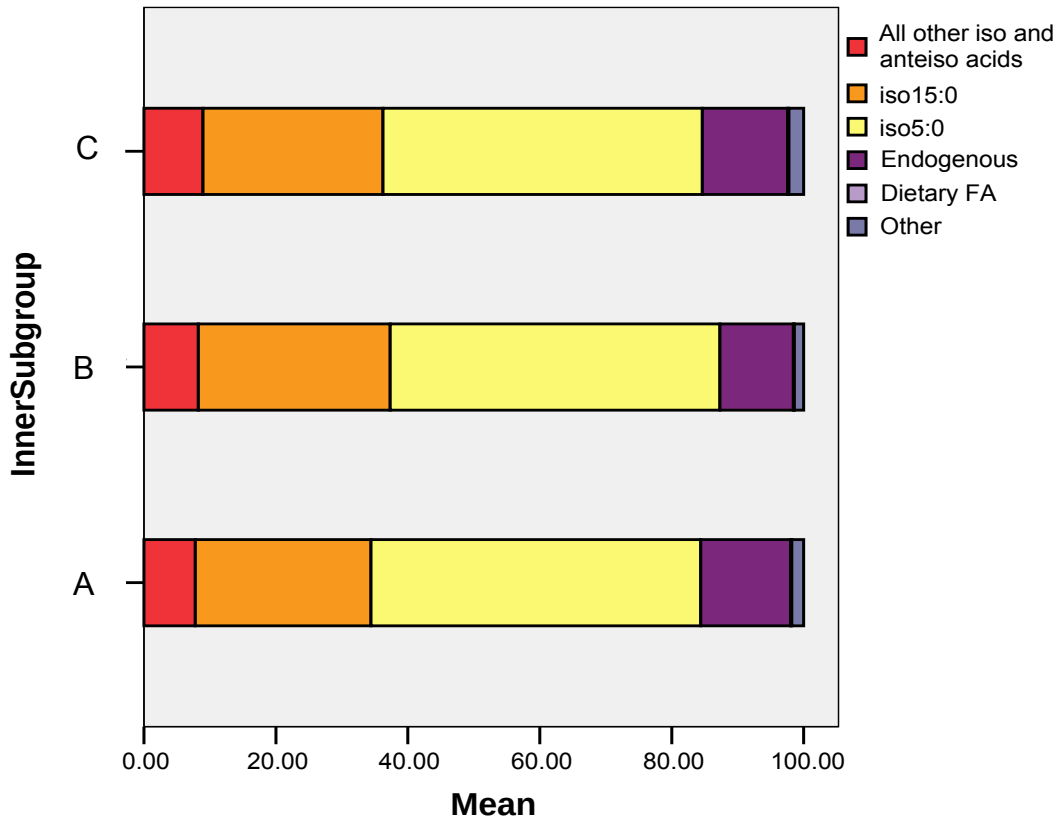


Figure 10. Mean percentage of fatty acid groupings for adults at the three sample sites at the tympano-periotic complex. Constituents of fatty acid groupings are outlined in Table 3, see Figure 4 for a diagram of these ear fat locations.

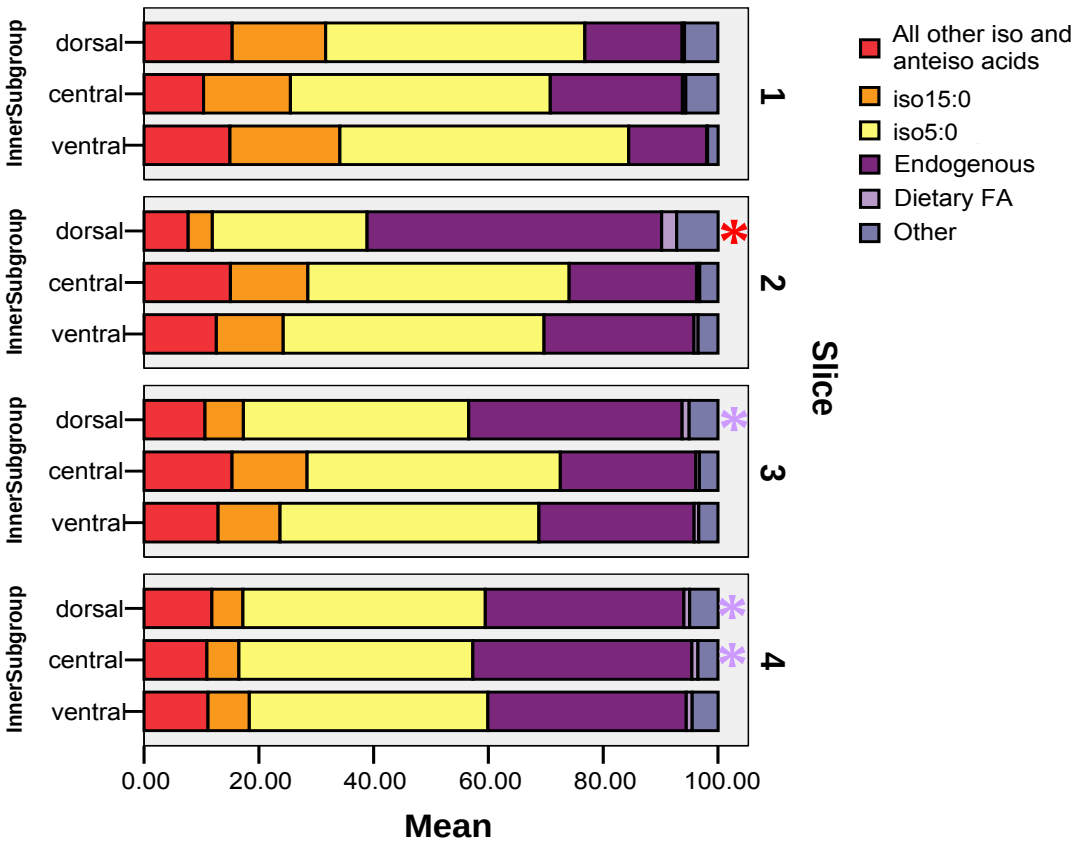


Figure 11. Mean percentage of fatty acid groupings of the inner jaw fat in subadults. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). The red asterisk denotes the location where the value of *i*-5:0 is significantly lower compared to other sample areas within the same slice. Light purple asterisks indicate non-significant trends that are similar to adult inner jaw fat.

(slice 2) was significantly lower in *i*-5:0 content then either the central or ventral portions of the same section. Although no other statistically significant differences were noted between sampling locations, a similar trend of lower *i*-5:0 content in the dorsal part of slice 3 of this tissue is apparent in Figure 11.

Calves also exhibited similar spatial distribution to that of both adult and subadult inner jaw fat (Figure 12). However, calves exhibited lower proportions of *i*-5:0 and *i*-15:0, and higher wt% of other iso-acids. The spatial distribution of iso-acids was again remarkably consistent in calves, showing decreased iso-acid (*i* -5:0 and *i*-15:0) components in samples from the dorsal-most region across all transverse sections (although this difference was not statistically significant). No statistical comparisons could be made on fetal inner jaw fat since samples were collected from a single animal, however some patterns were apparent (Figure 13). There are no data for samples from the initial transverse section (slice 1), however the spatial distribution of the second transverse section (slice 2) mirrored all the other age classes with relatively fewer iso-acids in the dorsal section compared to either central or ventral components (Figure 13). Subsequent sections however did not yet exhibit the same spatial pattern; general iso-acid concentration in this tissue is low and the tissue is dominated by endogenous FA.

Samples taken from the three sites adjacent to the tympano-periotic complex were similar in FA composition to each other, and to the first transverse section for each of the sampled adults, subadults and calves (Figure 14). Fetal samples from the area of the ear bones appeared consistent to each other as well (Figure

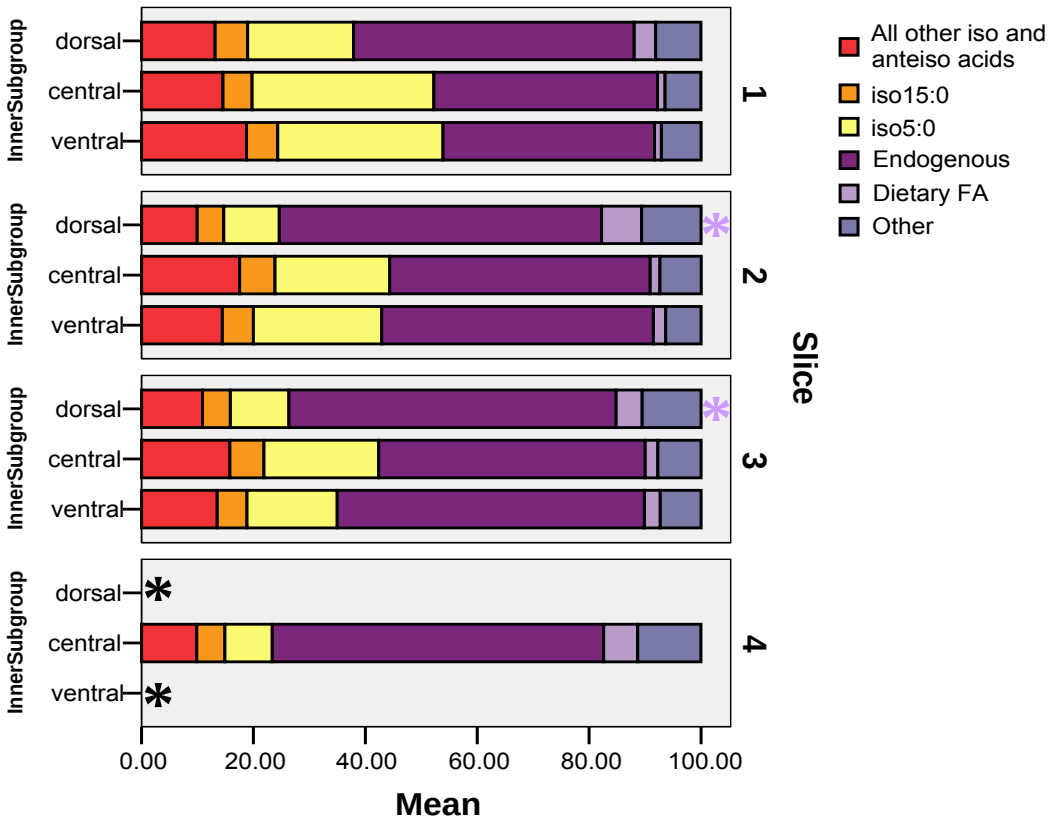


Figure 12. Mean percentage of fatty acid groupings of the inner jaw fat in calves. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). Black asterisk denotes area where no data exist. Light purple asterisks indicate non-significant trends that are similar to adult inner jaw fat.

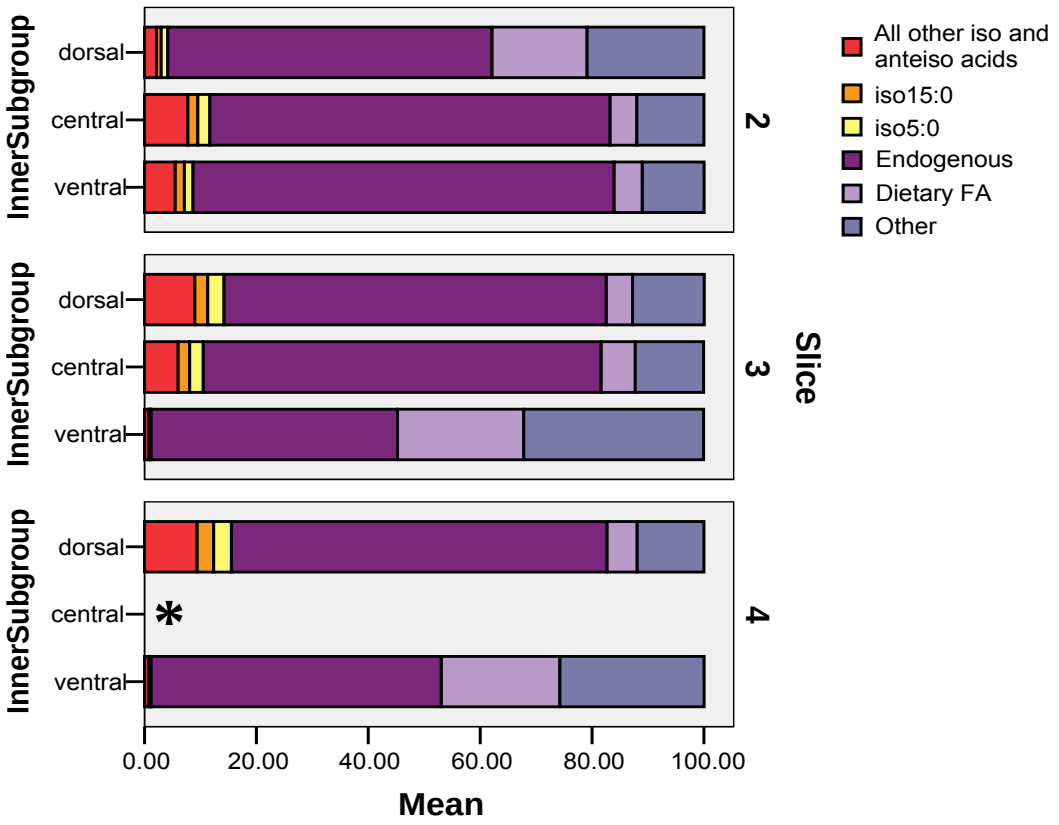


Figure 13. Mean percentage of fatty acid groupings in fetal inner jaw fat. Constituents of fatty acid groupings are outlined in Table 3. No data exist for slice 1; slice 2 is the caudal-most section for this animal, however it is comparable to slice 2 of older animals, subsequent slices are further rostral (see Figure 3 for schematic). Black asterisk denotes area where no data exist.

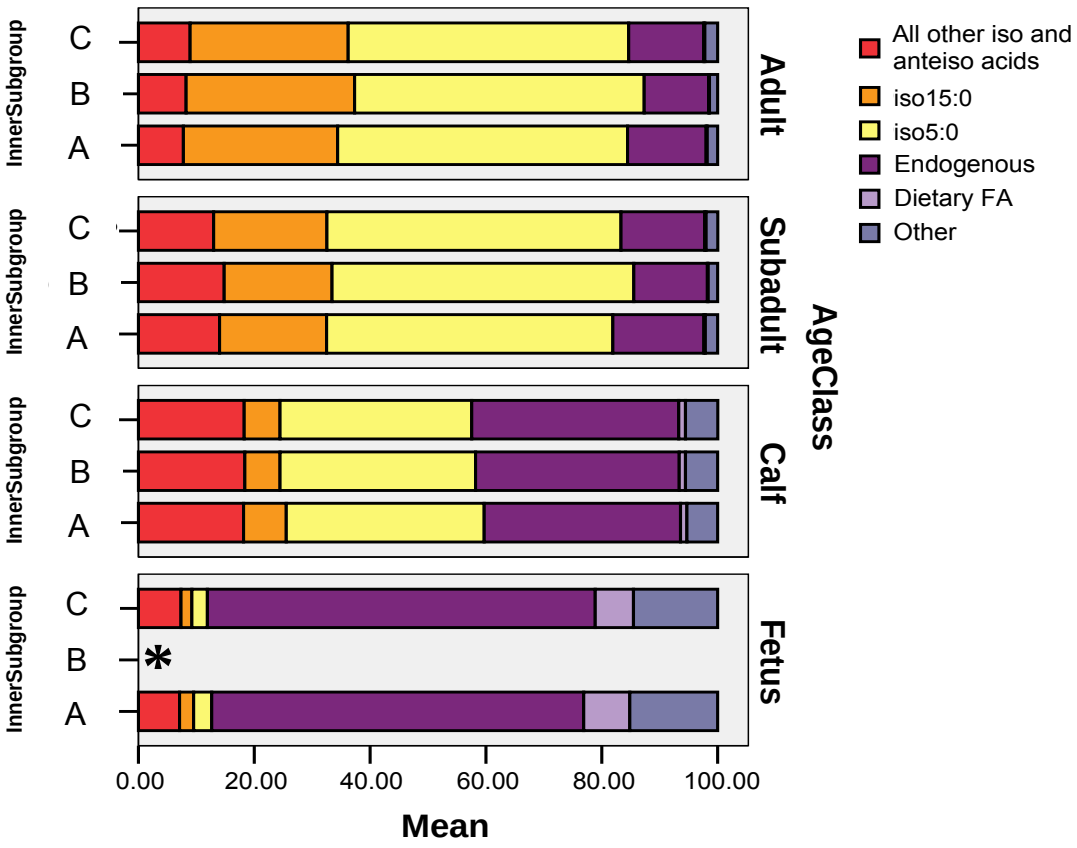


Figure 14. Mean percentage of fatty acid groupings at the three sample site locations that adhere to the periotic complex. Constituents of fatty acid groupings are outlined in Table 3. See Figure 4 for a diagram of these ear fat locations. Black asterisk denotes area where no data exist.

14), however statistical comparisons could not be made on fetal tissues (since n=1). Although values of iso-acid contents were consistent across samples within an age class, the values of *i*-5:0 were significantly lower for calves, but not for subadults. This trend also held true with *i*-15:0 being lower in calves then adults and subadults at all three ear sampling locations.

Outer Jaw Fat and Spatial Variation of the Triacylglycerol Components

Adults

Outer jaw fat contained high proportions of *i*-5:0 and *i*-15:0 although at lower mean percent values than the inner jaw fat (Table 8, ANOVA, Tukey Adjustment; $P<0.0001$). The dorsal component of the outer jaw fat consistently exhibited significantly lower proportions (logit categorical analysis; $P<0.0005$) of *i*-5:0 then the central or ventral regions across all transverse sections (Figure 15). Corresponding to this decrease was a significant increase in endogenous FA.

Ontogenetic Change in Outer Jaw Fat

Subadult spatial variation of *i*-5:0 mirrored that of adults showing significantly lower proportions in the dorsal section of the first transverse section (slices 1) (Figure 16). Although the second and third transverse sections (slices 2 and 3) did not exhibit a significantly lower value of *i*-5:0 compared to the central and ventral portions, a similar trend could be seen between adults and subadults. Subadults had mean weight percents of *i*-5:0 (subadults; 7.6–58.7 wt%) that

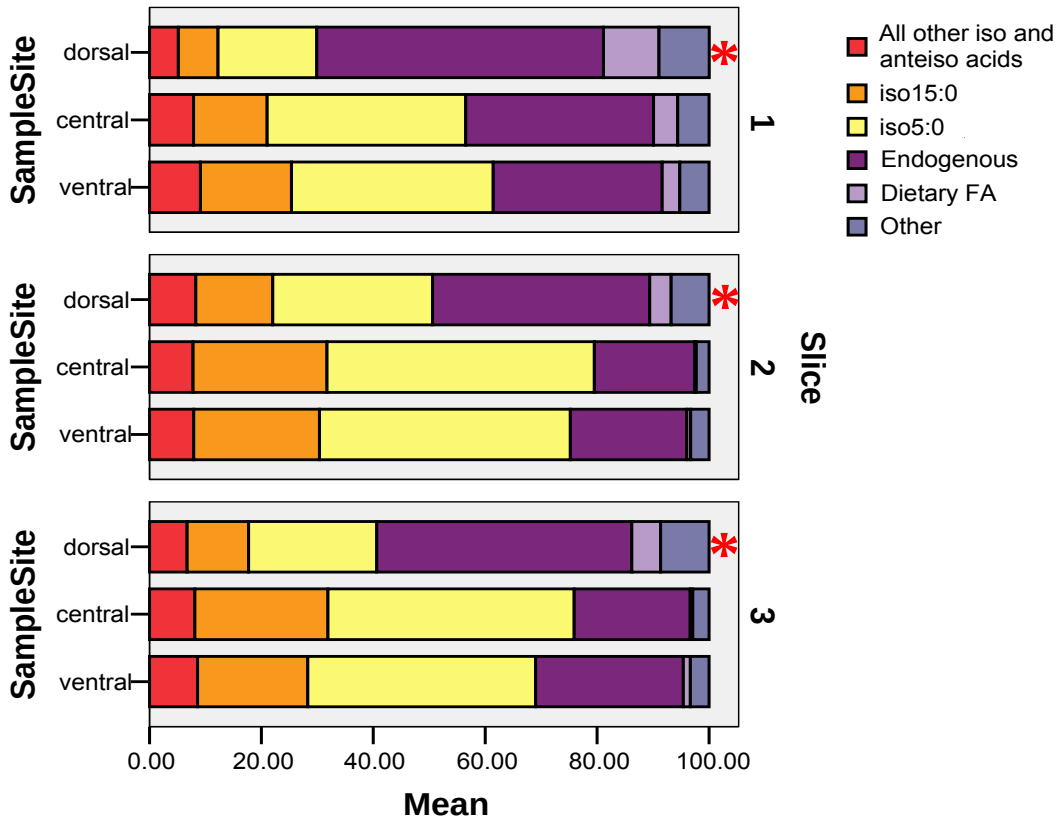


Figure 15. Mean percentage of fatty acid grouping in outer jaw fats of adults. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral. The red asterisk denotes the location where the value of *i*-5:0 is significantly lower compared to other sample areas within the same slice.

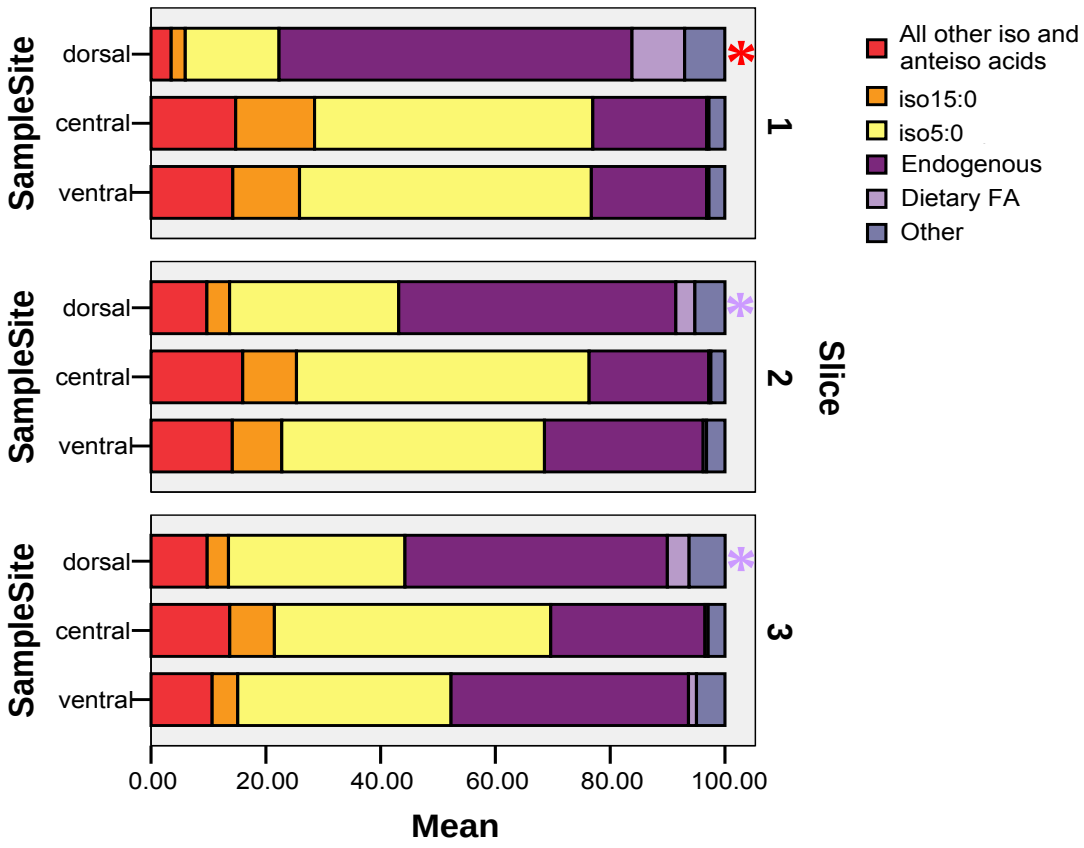


Figure 16. Mean percentage of fatty acid grouping in outer jaw fats of subadults. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral. The red asterisk denotes the location where the value of *i*-5:0 is significantly lower compared to other sample areas within the same slice. Light purple asterisks indicate non-significant trends that are similar to adult inner jaw fat.

were not different from adults (adults; 11.6-53.0 wt%) at each spatial site, however the mean weight percent of *i*-15:0 was generally lower in subadults.

Calves and fetal outer fat exhibited much lower weight percent values of *i*-5:0 and *i*-15:0. In calves, there were significant differences between sample sites in one of the transverse sections (slice 2, dorsal site being lowest in *i*-5:0) that mirrored the trend in adults and subadults, and although this was the only statistically significant difference (Figure 17), there was an apparent pattern that mirrored the two older age classes. Fetal tissue contained low values of all iso-acids, and the trends observed in all the other age classes were not yet apparent (Figure 18).

Cranial Blubber and Spatial Variation of the Triacylglycerol Components

Adults

Iso- and anteiso-acids were located in high concentrations at discrete locations within the cranial blubber. Samples along the first transverse section (Slice 1), which was the most caudal section sampled, exhibited extremely low values of all iso-acids (Figure 19) and were dominated by the endogenous lipid grouping (Table 3). The fatty acid composition of this slice was consistent, with none of the fatty acid groupings showing significant variation across sample sites within this slice. The subsequent two sections (Slices 2 and 3) showed an increased level of *i*-5:0 and *i*-15:0 at specific sampling site locations. Sampling sites 2.1 and 2.2 (divided into the medial and lateral blubber at sampling site 2) contained significantly higher mean percent of *i*-5:0 and *i*-15:0 when compared to

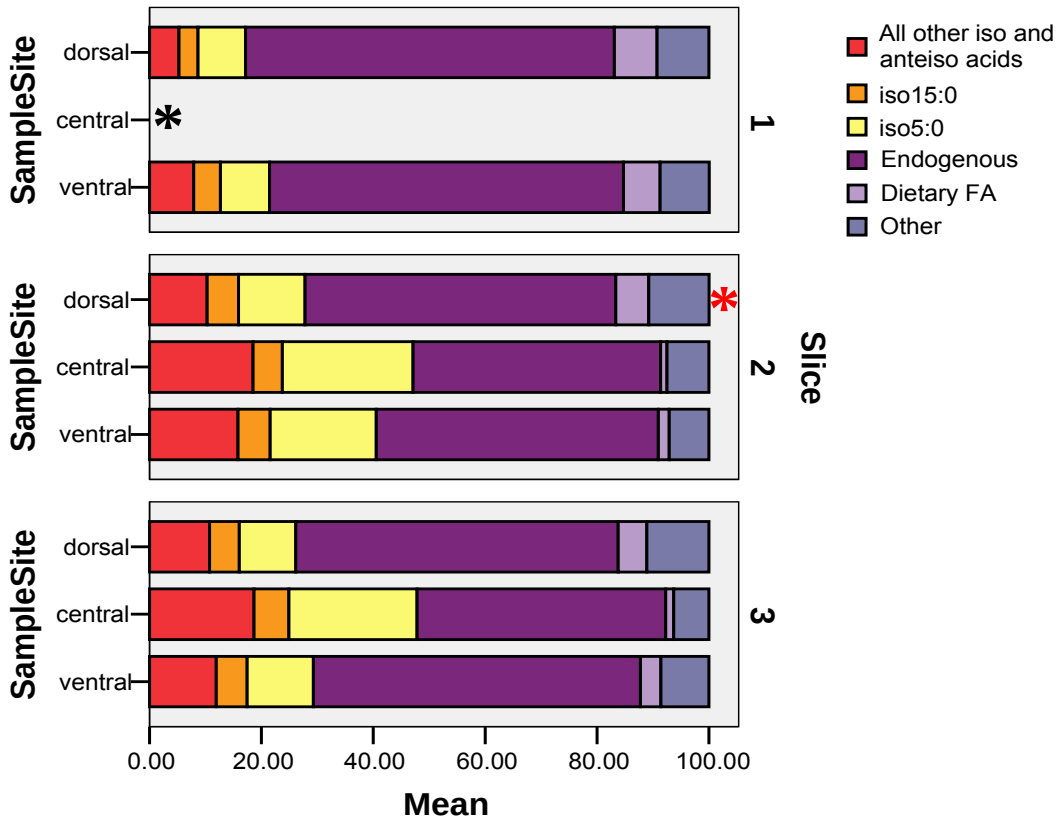


Figure 17. Mean percentage of fatty acid groupings in the outer jaw fat of calves. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral. The red asterisk denotes the location where the value of *i*-5:0 is significantly lower compared to other sample areas within the same slice. Black asterisk denotes area where no data exist.

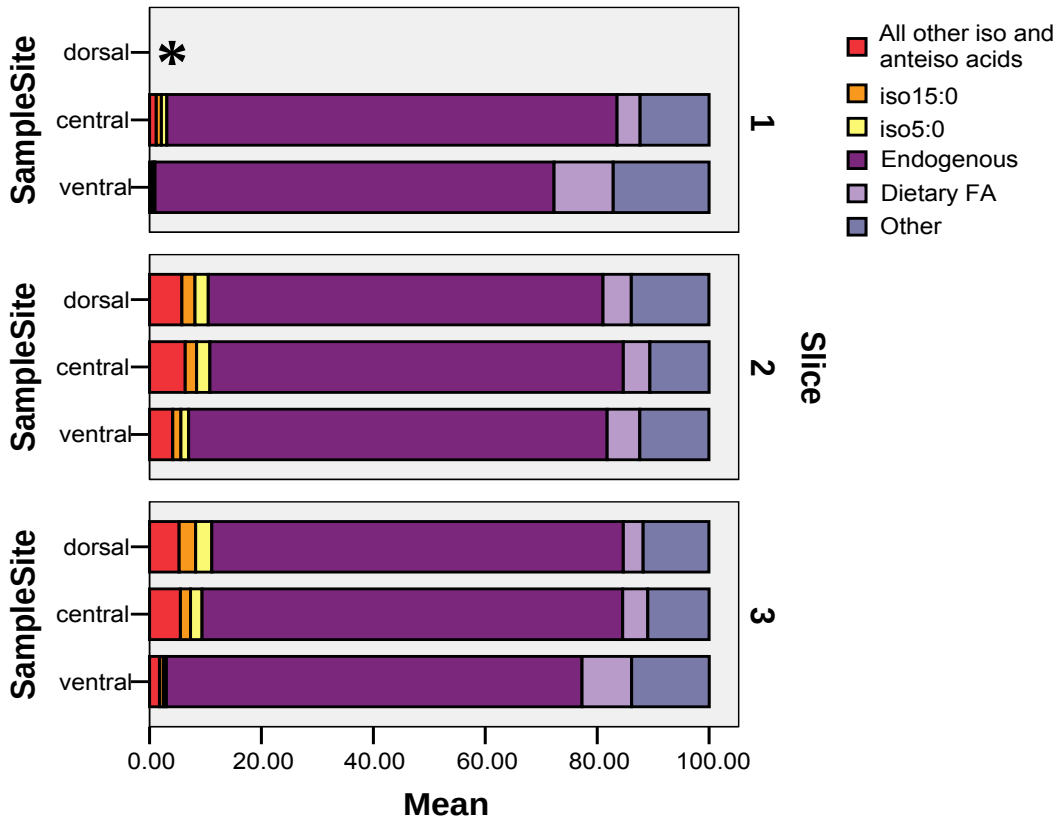


Figure 18. Mean percentage of fatty acid groupings in fetal outer jaw fat. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral. Black asterisk denotes area where no data exist.

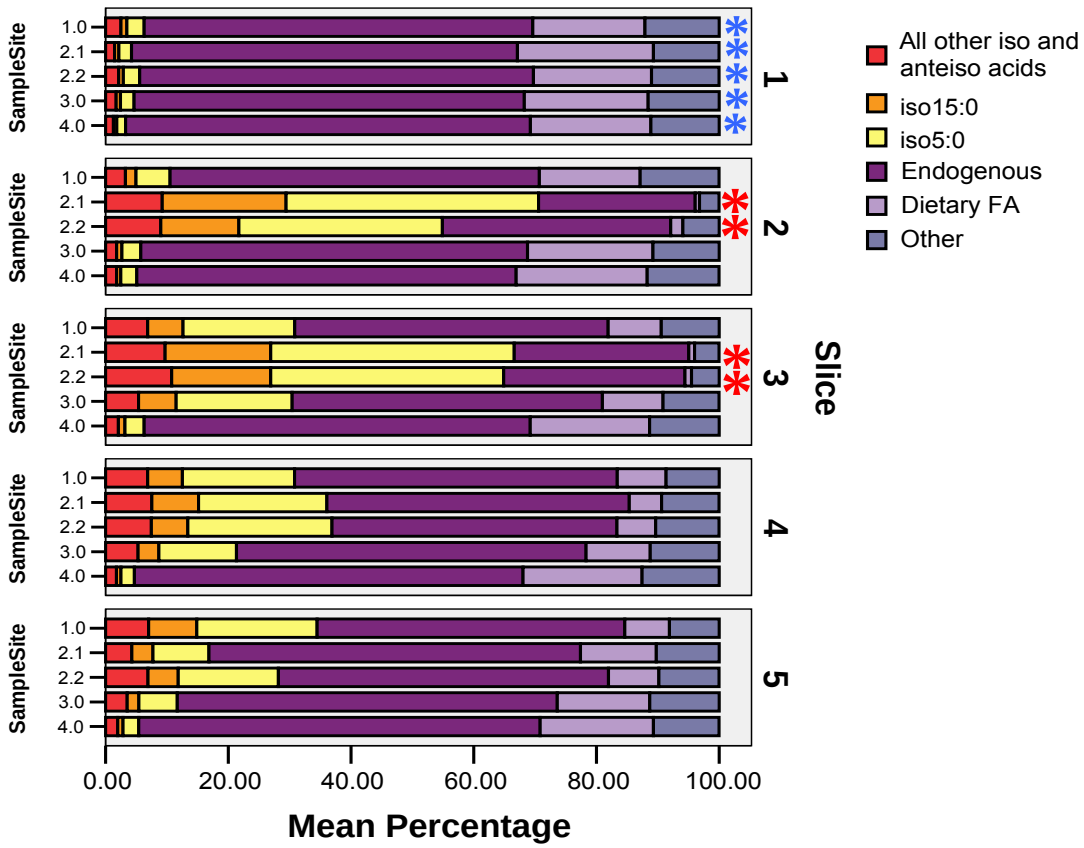


Figure 19. Mean percentage of fatty acid groupings found in the cranial blubber of adults. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). Sample sites 1 - 4 represent sampling along each transverse section where sample site 1 is most dorsal and sample site 4 is along the ventral midline. Sample site 2 was divided into inner and outer blubber depths; where 2.1 represents the deep (inner blubber) and 2.2 represents the superficial blubber (outer blubber). Sample sites with significantly high values of iso acids are represented by red asterisks, while sites with almost no iso acids along the caudal margin are represented by blue asterisks.

other sample sites in each slice (logit categorical analysis; P<0.0005). *I*-5:0 was always significantly higher in concentration than *i*-15:0 in both inner and outer blubber. With the increase in the mean weight percent of these iso-acids there was a corresponding significant decrease in both the dietary fatty and endogenous fatty acid groupings, but not of the fatty acids designated within the category 'other'. The dietary acids in these sample sites (2.1 and 2.2) were virtually absent, which was a noticeable difference compared to all other sample sites in the cranial blubber. Neighboring sample sites (1 and 3) showed intermediate iso-acid values that were significantly less then those observed at sample site 2, but were nonetheless higher than regular cranial blubber. These observations can be visualized clearly in Figure 19. The successive and final two sections (slices 4 and 5) showed decreasing amounts of *i*-5:0 and *i*-15:0 content that remained generally consistent among sample sites, except for the ones found along the ventral midline (sample site 4) which were significantly lower in *i*-5:0 and *i*-15:0 content.

Comparisons of these sites can also be made along the longitudinal axis of the animal (Figure 20). *I*-5:0 and *i*-15:0 percentages generally increased along the longitudinal axis of the animal at the dorsal-most sampling site (site 1). Inner and outer blubber at sample site 2 (2.1 medial, 2.2 lateral) showed a peak in iso-acid values at the second and third transverse sections with a steady decline in iso-acids in sections further rostral. Samples taken at sample site 3 exhibited generally low iso-acid composition with a peak at the third transverse section, decreasing in slices located further rostral. Samples along the ventral midline

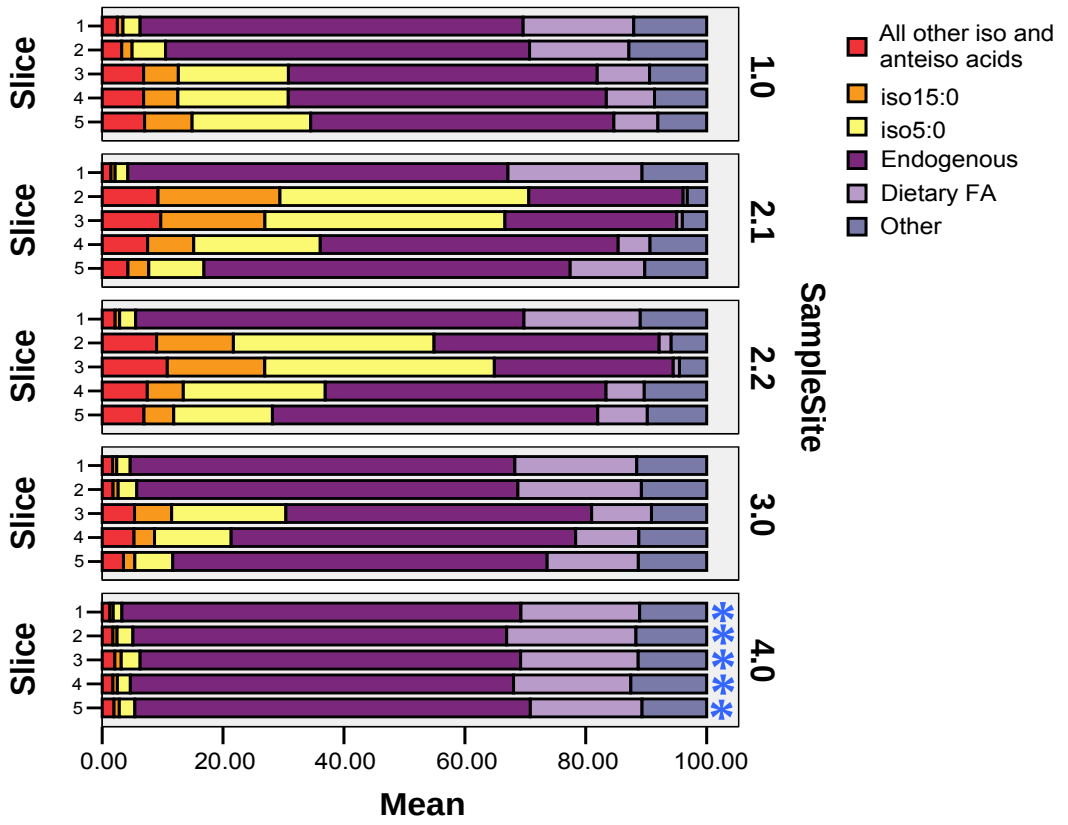


Figure 20. Mean percentage of fatty acid groupings found in the cranial blubber of adults (along the longitudinal axis). Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). Sample sites 1 - 4 represent sampling along each transverse section where sample site 1 is most dorsal and sample site 4 is along the ventral midline. Sample site 2 was divided into inner and outer blubber depths; where 2.1 represents the deep (inner blubber) and 2.2 represents the superficial blubber (outer blubber). The layout of this graph is so that comparisons can be made along the longitudinal axis of the animal. Sample sites are grouped together (along the right axis), and each slice is represented along the left axis, for example all sites along the ventral midline (sample site 4) are represented in the bottom panel. Most notable is the near absence of iso acids in the sites at the dorsal midline (represented by blue asterisks).

consistently had very few iso-acids and were dominated by the endogenous fatty acids.

Ontogenetic Change in Cranial Blubber

The spatial complexity of iso- and anteiso-acid accumulation in cranial blubber was reflected in the spatial FA distribution in the subadults (Figure 21), the calves (Figure 22) and even the fetus (Figure 23). The areas of significantly high *i*-5:0 accumulation present in the adults were mirrored in the subadults (generally highest at the #2 sampling location for slices 2 and 3) and the actual weight percent did not vary significantly between the adult and subadults at any spatial sampling scale. As with adults, subadults sites with high levels of *i*-5:0 corresponded to a decrease in the dietary FA components of the sampling sites. The proportion of *i*-15:0 in the FA of each spatial location in subadults was generally lower than in adults (Figure 21). All samples along the first transverse section of and along the ventral midline of the animals had the lowest values of iso-acids for the cranial blubber sampled.

Although calves exhibited a lower mean weight percent of iso-acids than the older two age classes, the same spatial trend was still apparent. Although not statistically significant (except at the second transverse section), it was apparent that the tendency for the highest iso-acid accumulation in the blubber was at the second sampling site at all transverse sections (Figure 22). The trend of low iso-acids persisted for the whole of the first transverse section and the sampling sites along the ventral midline (Site 4).

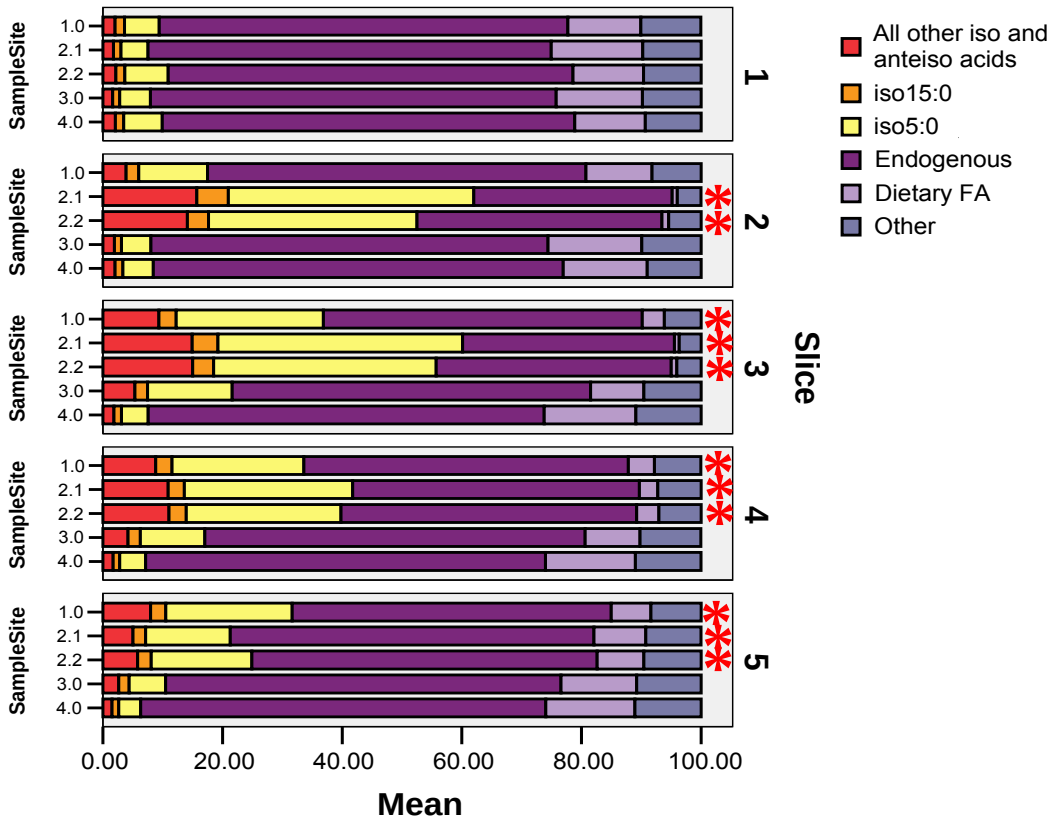


Figure 21. Mean percentage of fatty acid groupings found in the cranial blubber of subadults. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). Sample sites 1 - 4 represent sampling along each transverse section where sample site 1 is most dorsal and sample site 4 is along the ventral midline. Sample site 2 was divided into inner and outer blubber depths; where 2.1 represents the deep (inner blubber) and 2.2 represents the superficial blubber (outer blubber). Sample sites with high values of iso acids are represented by red asterisks; these samples have significantly higher values of *i*-5:0 than sites within the same section.

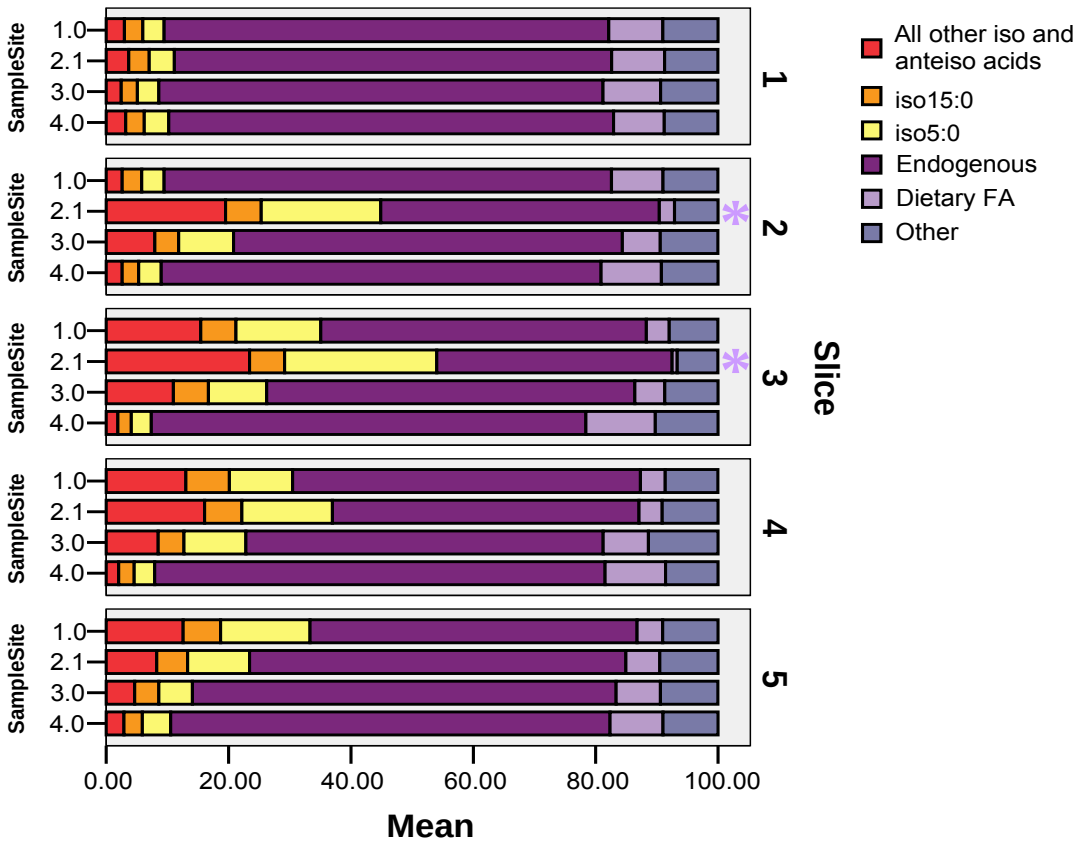


Figure 22. Mean percentage of FA groupings found in the cranial blubber of calves. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). Sample sites 1 - 4 represent sampling along each transverse section where sample site 1 is most dorsal and sample site 4 is along the ventral midline. Samples were too small to allow for sample site 2 to be divided into inner (2.1) and outer (2.2) blubber (as were done for adults and subadults) thus sample site 2 was assigned a value of 2.1. The purple asterisks indicate sample sites that show areas of high *i*-5:0 and *i-15*:0 in the same locations as adults.

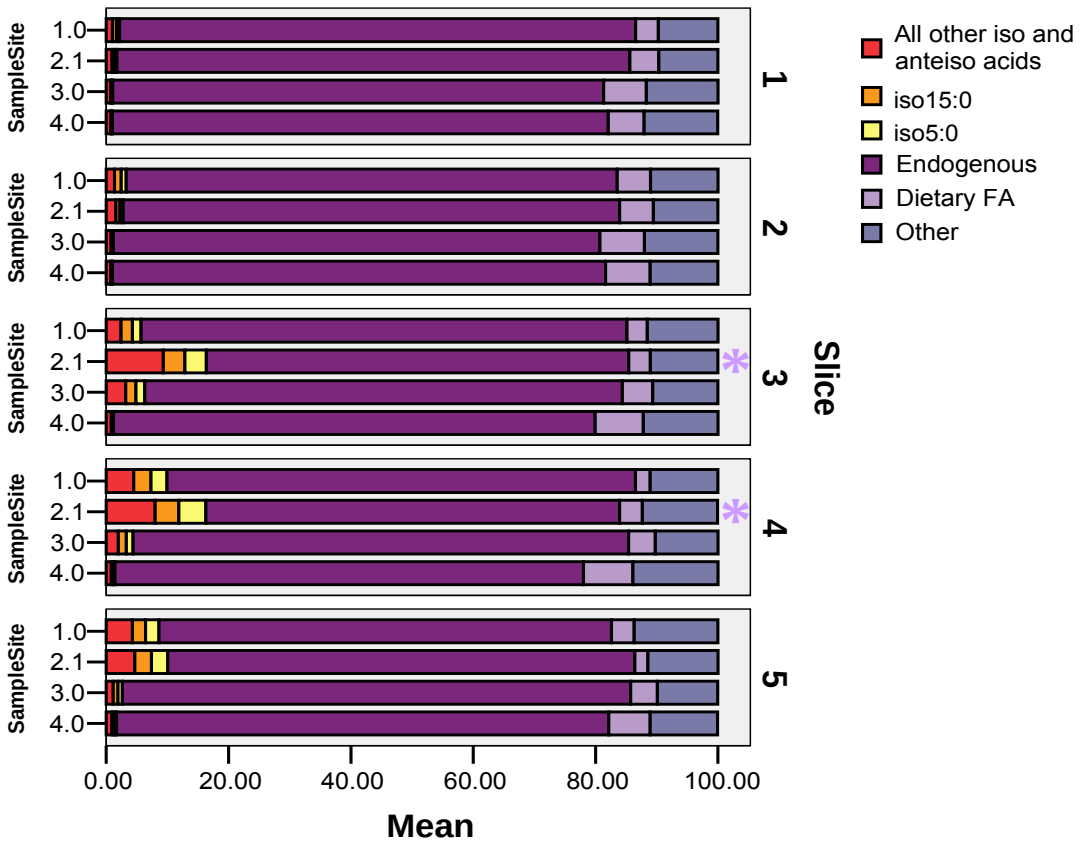
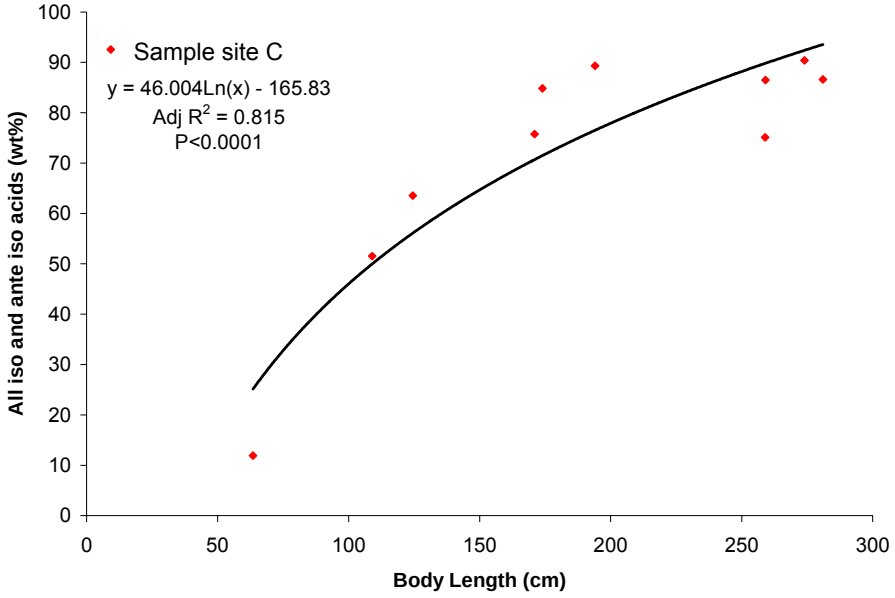


Figure 23. Mean percentage of FA groupings found in the fetal cranial blubber by sample and slice. Constituents of fatty acid groupings are outlined in Table 3. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). Sample sites 1 - 4 represent sampling along each transverse section where sample site 1 is most dorsal and sample site 4 is along the ventral midline. Samples were too small to allow for sample site 2 to be divided into inner (2.1) and outer (2.2) blubber (as were done for adults and subadults) thus sample site 2 was assigned a value of 2.1. The purple asterisks indicate sample sites that show areas of high *i*-5:0 and *i-15*:0 in the same locations as adults.

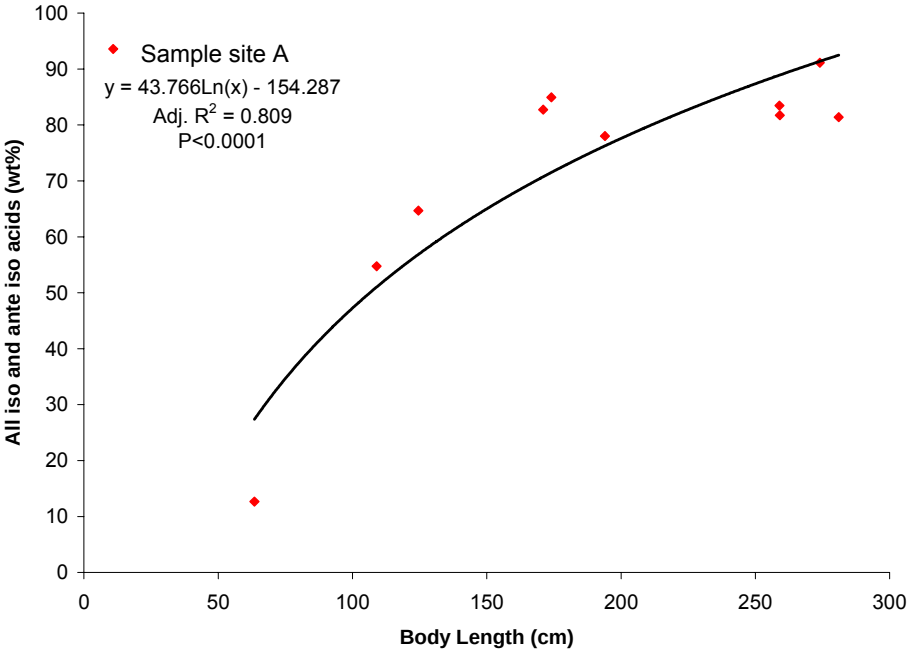
Although fetal tissue could not be compared to others using the logit categorical model, the framework for the same patterns exhibited by all other age classes was apparent (Figure 23) with the second sampling site already exhibiting the highest (albeit comparatively lower weight percent) iso-acid accumulation in the blubber.

Ontogenetic Accumulation of Iso-Acids

Representative sampling sites for each tissue type examined were chosen to identify the ontogenetic iso-acid pattern of accumulation. These sites were chosen as they had homologous samples for each of the ten animals. Two sites from each of the ear fats (Figure 24), the inner jaw fat (Figure 25), the outer jaw fat (Figure 26) and cranial blubber at the acoustic window (Figure 27), for which there were corresponding samples for all animals, were examined. Body length and weight percent of all iso-acids were plotted, and lines of best fit were chosen based on the highest adjusted R^2 value (adjusted R^2 accounts for negative fluctuations around the population value whereas R does not) (Tabachnick & Fidell, 2007) from linear, logarithmic, exponential, power and logistic models. Logarithmic models were the best fit for all. Adjusted R^2 values were generally very high for all sample sites examined (0.68-0.91) and all showed significant ($P<0.002$) relationships (see Figures 24-27 for adjusted R^2 values and P values for individual sample sites). It is important to note that sampling sites from tissues represent different regions within each tissue (ie. inner jaw fat sampling

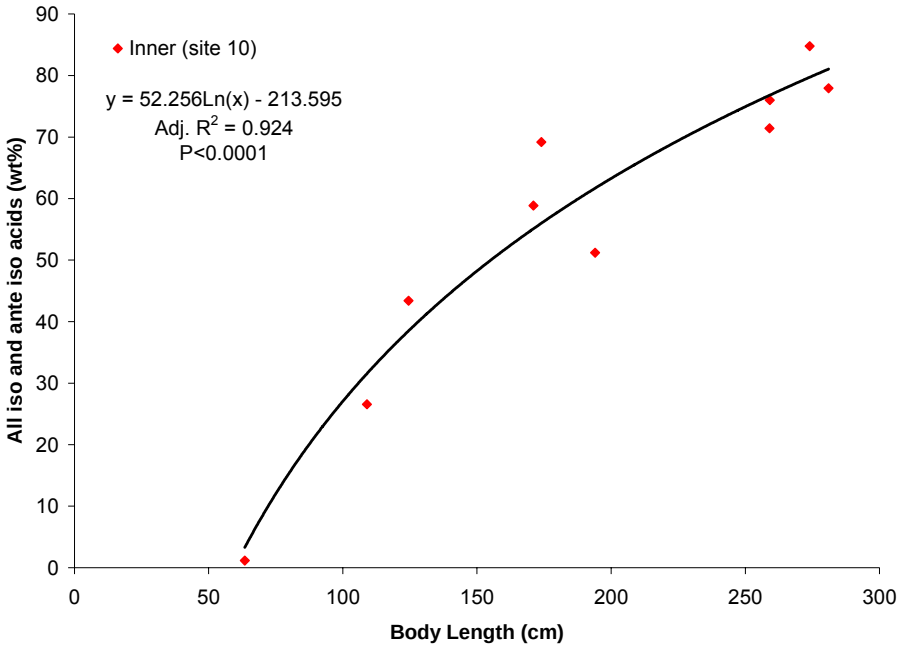


A.

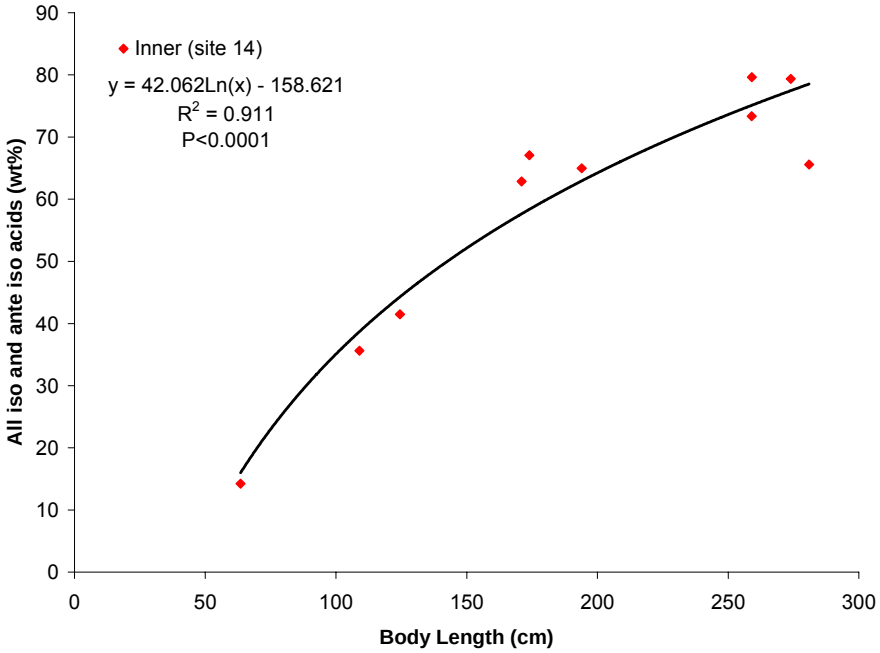


B.

Figure 24. Relationship between iso-acid accumulation and body length at sites sampled at the periotic complex. A. fat adhered to the sample site C; B. fat adhered at the sample site A, see Figure 4 for location of these sampling sites in relation the periotic complex.

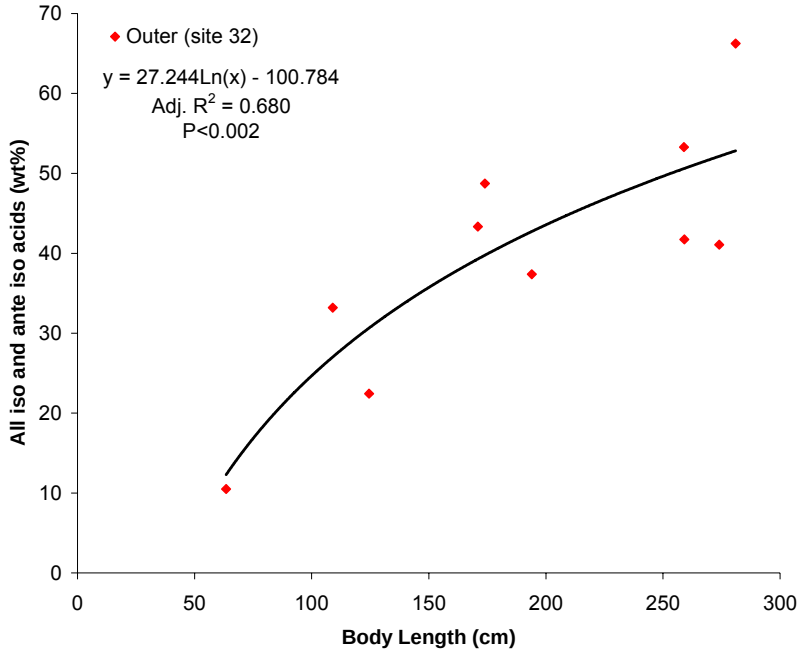


A.

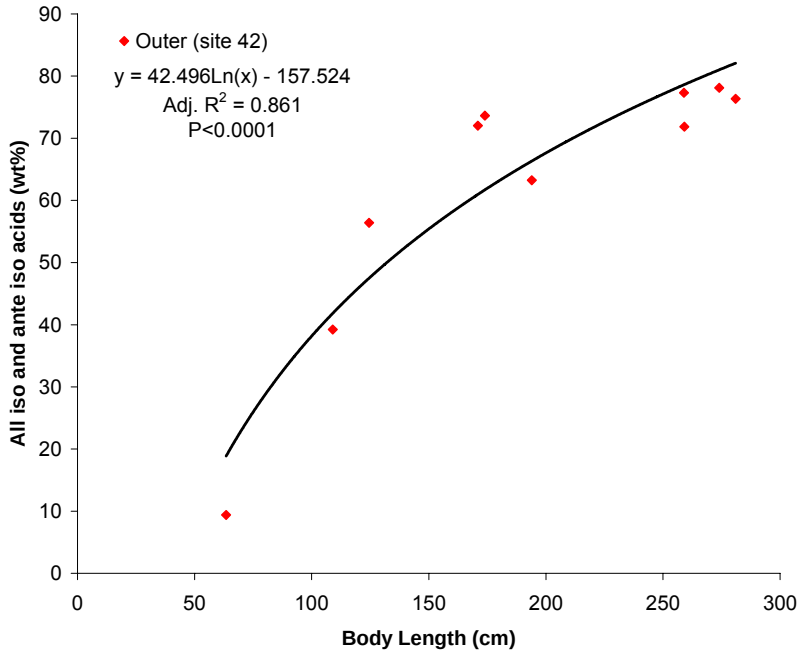


B.

Figure 25. Relationship between iso-acid accumulation and body length at representative sites sampled from the inner jaw fat. A. from the ventral subsection; B. from the dorsal subsection.

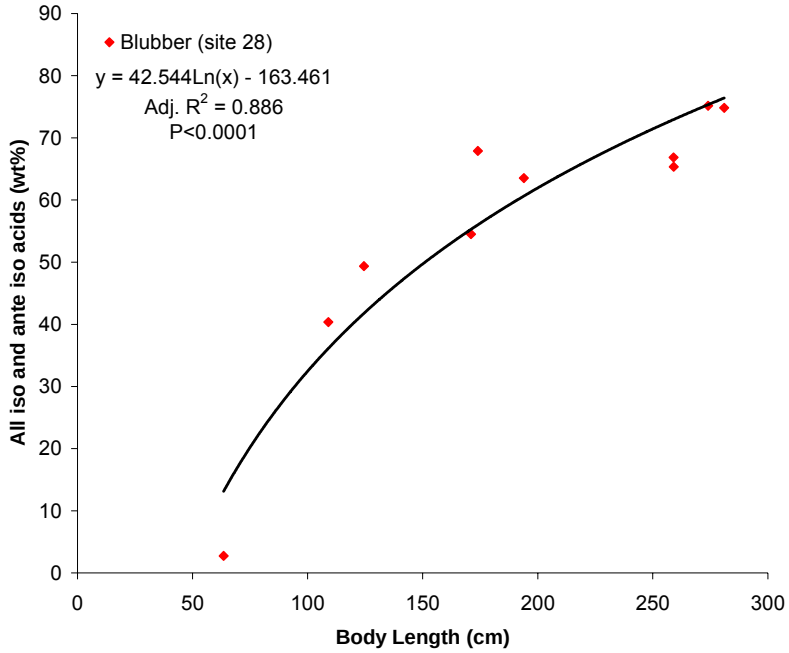


A.

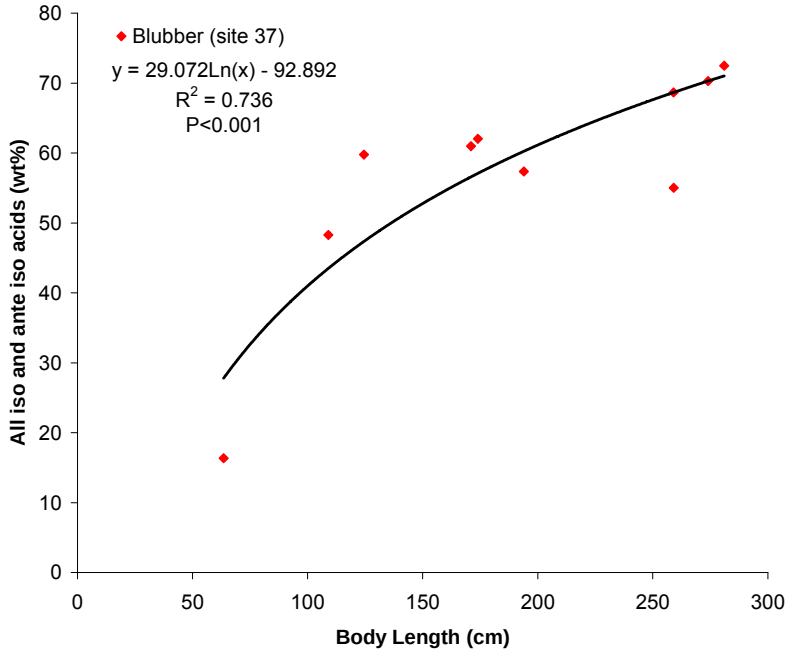


B.

Figure 26. Relationship between iso-acid accumulation and body length at representative sites sampled from the outer jaw fat. A. from the dorsal subsection; B. from the central subsection.



A.



B.

Figure 27. Relationship between iso-acid accumulation and body length at representative sites sampled from the cranial blubber. A. sample site 2 medial blubber (inner blubber), section 2; B. sample site 2 medial blubber (inner blubber), section 3.

sites are from the dorsal and central region of that tissue), thus there are differences in slope values (Figures 24-27) even within a tissue.

Dominant Fatty Acids and Fatty Alcohols of Wax Esters

A total of 142 samples yielded enough WE for further processing of this lipid class, most of which came from the inner jaw fat (n=108) and fewer from the outer jaw fat (n=32). Only two samples from the cranial blubber yielded enough WE to quantify. No samples of fetal WE were analyzed as the fetus did not have enough WE to reclaim and process.

Generally, 34 different fatty acids were routinely identified in WE, as well as 26 different fatty alcohols. The proportion of the FA and FAlc varied through the age classes (Table 9). *I*-5:0 was the dominant FA in adult inner jaw fat, endogenous FA were the second largest constituents, and 15:0 FA, iso15:0 Alc, 16:0 Alc and *i*-16:0 Alc were also large components of this tissue (Figure 28). *I*-15:0 FA was present in WE, but only in small amounts (categorized within the all other iso/ante-iso FA grouping in Table 4). *I*-15:0 FA was found in much larger amounts in the FA from TAG, however, 15:0 FA was found as a similarly large component of FA from WE. Another notable occurrence was the lack of *i*-5:0 Alc in any of these tissues.

The inner jaw fat of subadults (Figure 29) followed the same general trend as adults, however with lower proportions of 15:0 FA. The proportion of 16:0 Alc in this tissue appeared to be higher in subadults (and calves) compared to adults.

Table 9. Mean and standard error of all WE components (wt%) for all age classes separated by tissue types. The number of samples varies due to WE content and recovery. No fetal samples were included here as the WE content was too low for recovery. nd = no data. The sum of all fatty acid and fatty alcohol constituents are equal to 100%.

Tissue Type	Ageclass		*No. of Indiv.*	*No. of samples*	*i*-5:0 FA	15:0 FA	Endogenous FA	Dietary FA	Other FA	All other iso/anteiso FA	iso15:0 Alc	16:0 Alc
Inner	Adult	Average	4	50	19.48	10.84	18.88	2.33	2.84	1.88	9.12	7.27
		Std.error			0.37	0.55	0.61	0.08	0.30	0.11	0.27	0.39
	SubAdult	Average	3	42	17.49	4.66	21.46	2.96	7.43	2.91	3.58	10.56
		Std.error			0.51	0.35	0.77	0.10	0.46	0.21	0.23	0.63
	Calf	Average	2	16	1.39	0.94	19.84	1.38	8.38	14.08	2.57	15.71
		Std.error			0.29	0.17	1.12	0.25	0.62	0.95	0.17	0.54
Outer	Adult	Average	4	10	19.34	7.53	17.45	2.71	5.06	2.82	8.22	9.05
		Std.error			1.51	0.93	1.76	0.28	0.88	0.29	1.02	0.73
	SubAdult	Average	3	17	14.79	3.78	20.48	4.13	11.20	5.09	2.72	11.25
		Std.error			1.02	0.56	1.19	0.54	0.72	0.51	0.23	0.95
	Calf	Average	2	5	0.60	0.73	23.63	1.23	8.32	12.18	2.34	15.25
		Std.error			0.19	0.20	2.39	0.33	1.01	0.78	0.55	1.11
Blubber	Adult	Average	0	0	nd	nd	nd	nd	nd	nd	nd	nd
		Std.error			nd	nd	nd	nd	nd	nd	nd	nd
	Subadult	Average	1	1	10.86	1.93	15.88	2.41	14.63	8.27	3.37	14.52
		Std.error			nd	nd	nd	nd	nd	nd	nd	nd
	Calf	Average	1	1	1.05	1.85	13.67	0.81	9.98	16.58	3.28	14.94
		Std.error			nd	nd	nd	nd	nd	nd	nd	nd

Table 9 Continued. Means and standard error of all WE components (wt%) for all age classes separated by tissue types. The number of samples varies due to WE content and recovery. No fetal samples were included here as the WE content was too low for recovery. nd = no data. The sum of all fatty acid and fatty alcohol constituents are equal to 100%.

Tissue Type	Ageclass		*No. of Indiv.*	*no. of samples*	iso16:0 Alc	All Sat., Mono-Unsat. And Unk. Alcs	All other iso/anteiso Alc
Inner	Adult	Average	4	50	9.02	15.31	2.89
		Std.error			0.23	1.28	0.08
	SubAdult	Average	3	42	9.84	15.83	3.13
		Std.error			0.38	1.16	0.13
	Calf	Average	2	16	10.09	20.14	5.34
		Std.error			1.37	1.53	0.61
Outer	Adult	Average	4	10	6.96	17.23	3.36
		Std.error			0.59	2.05	0.19
	SubAdult	Average	3	17	6.69	16.14	3.22
		Std.error			0.52	1.39	0.53
	Calf	Average	2	5	7.67	21.93	5.92
		Std.error			2.30	2.55	0.72
Blubber	Adult	Average	0	0	nd	nd	nd
		Std.error			nd	nd	nd
	Subadult	Average	1	1	12.30	13.44	2.41
		Std.error			nd	nd	nd
	Calf	Average	1	1	13.78	18.31	5.45
		Std.error			nd	nd	nd

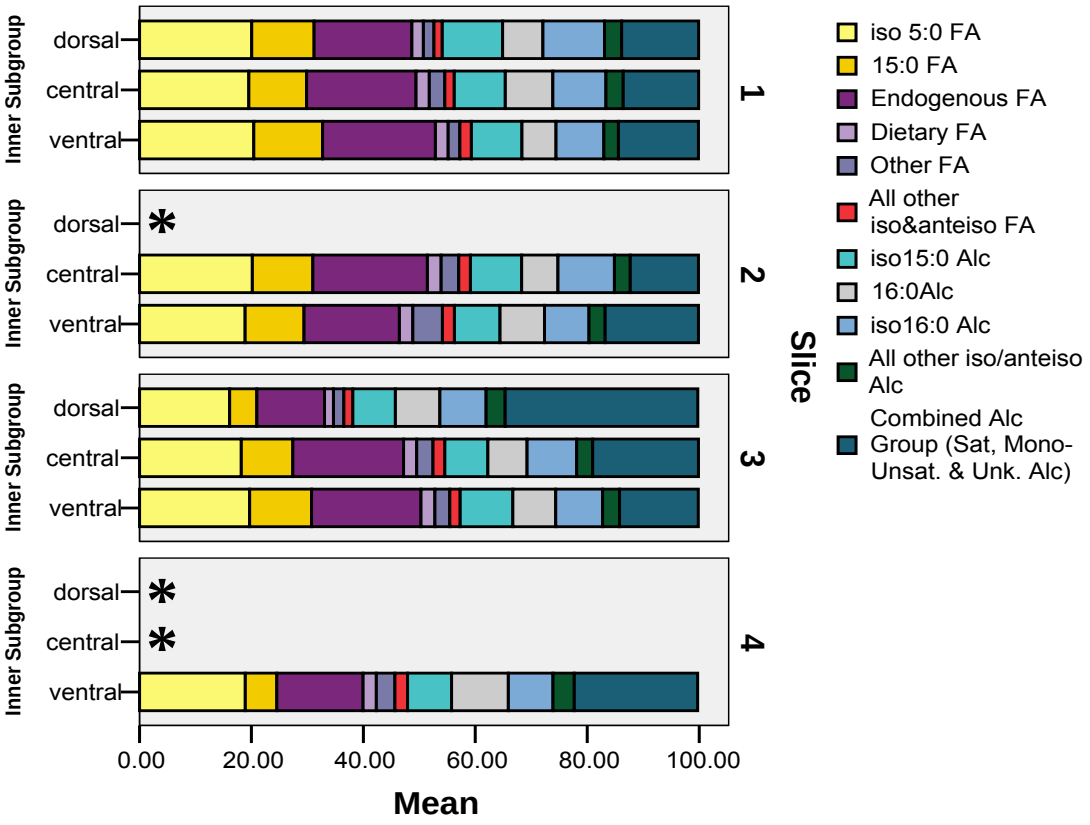


Figure 28. Mean percentage of fatty acid and fatty alcohol groupings from WE in adult inner jaw fat. Constituents of these groupings are outlined in Tables 4 and 5. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). A black asterisk denotes areas where data are missing because WE content was < 20%.

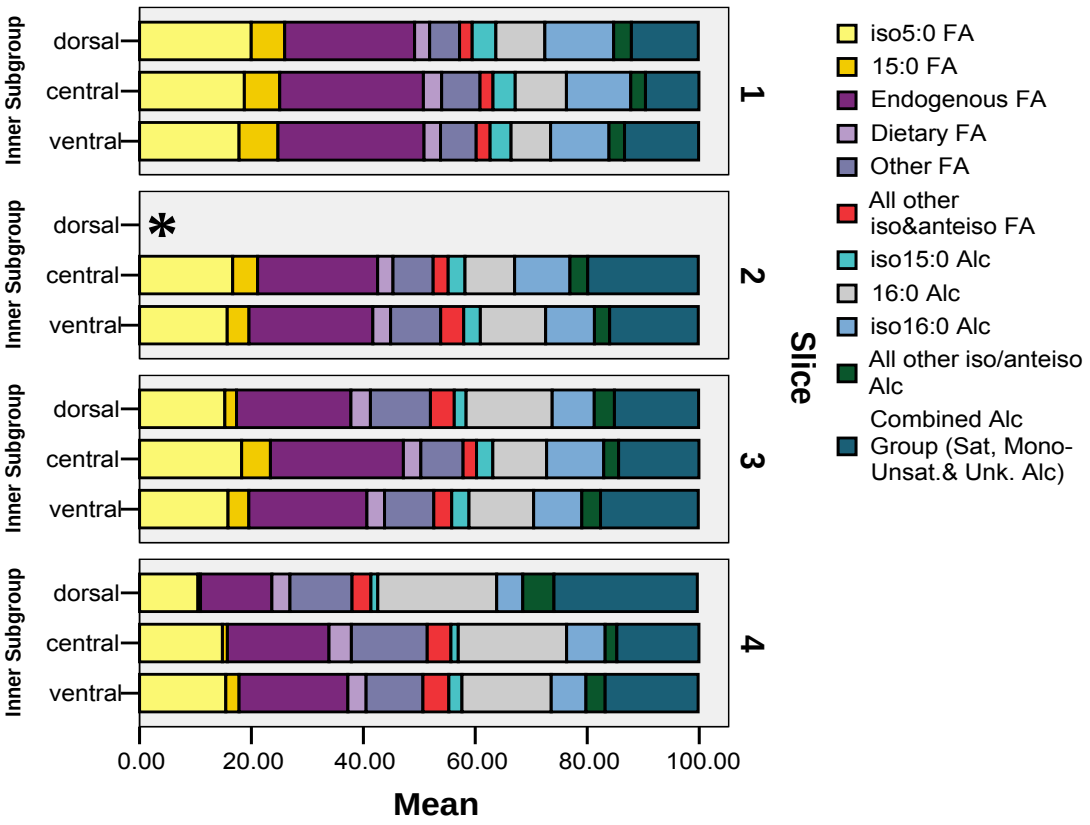


Figure 29. Mean percentage of fatty acid and fatty alcohol groupings from WE in subadult inner jaw fat. Constituents of these groupings are outlined in Tables 4 and 5. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). A black asterisk denotes an area where data are missing because WE content was < 20%.

It was apparent that the FA and FAlc proportions of calf tissue components were very different from subadult and adult inner jaw fat. Endogenous fatty acids, 16:0 Alc, other iso- and anteiso-acids (not *i*-5:0) and *i*-16:0 Alc were the main constituents in this tissue. Calves also tended to have higher proportions of other saturated and monounsaturated alcohols compared to the older age classes. There was also a noticeable lack of *i*-5:0 and 15:0 FA that were present in much higher proportions in older animals (Figure 30), but oddly a higher mean weight percent of other iso- and anteiso-FA (mainly *i*-12:0, *i*-14:0, *i*-15:0, *i*-16:0).

The trends of FA and FAlc proportions held true for the outer jaw fat as well and the proportions across age classes mirrored those of the inner jaw fat. *I*-5:0 was the dominant FA (higher in adults than calves), followed by high proportion of endogenous FA (higher in calves than adults). Again, the proportion of 16:0 Alc in this tissue was higher in younger animals, while the reverse trend was true for the combined group of alcohols (representing saturates, mono-unsaturates and unknown alcohols, see Table 5 for a list of alcohols in this group), which were highest in the adults.

Spatial Distribution of Fatty Acids and Alcohols in Wax Esters

The smaller number of WE samples recovered precluded any logit categorical analyses for spatial analysis (similar to that done on the TAG portions of the same tissue). However, mean proportions of FA and FAlc were graphed in a similar manner to explore possible trends in the spatial distribution in both the inner (Figures 28-30) and outer (Figures 31-33) jaw fats. Since WE were not

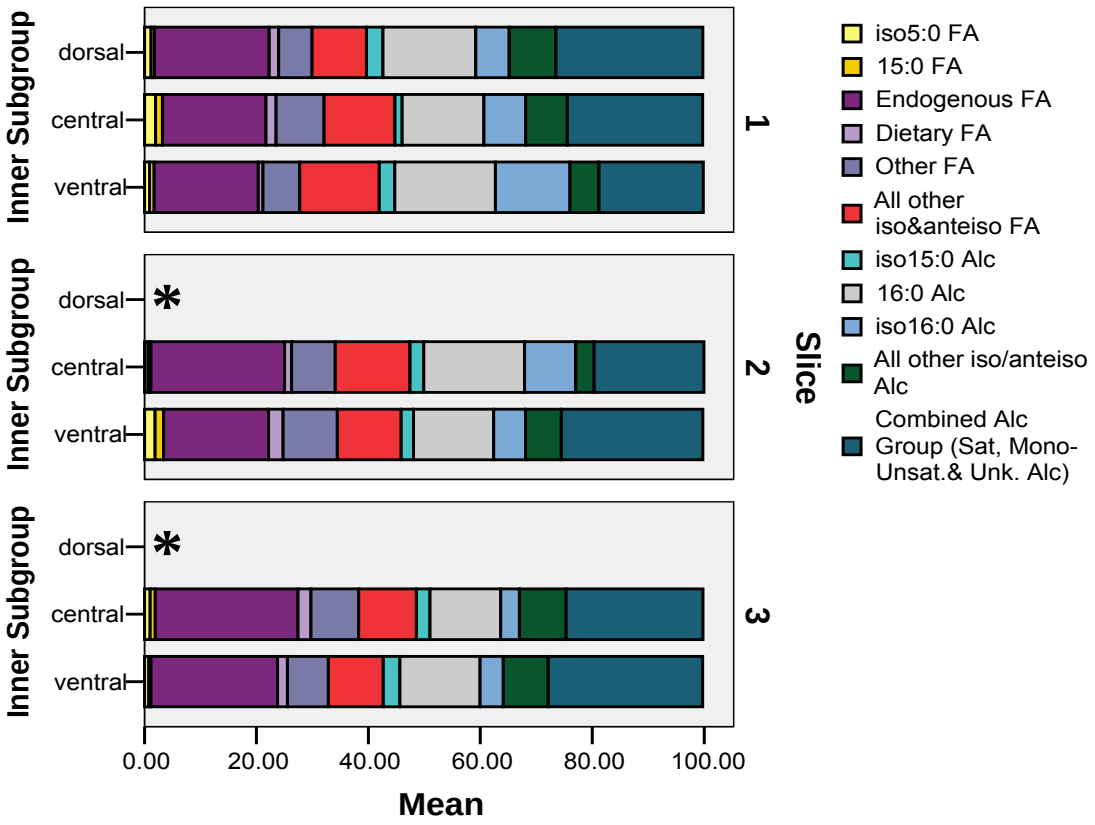


Figure 30. Mean percentage of fatty acid and fatty alcohol groupings from WE in calf inner jaw fat. Constituents of these groupings are outlined in Tables 4 and 5. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). A black asterisk denotes areas where data are missing because WE content was < 20%. No WE data exist for slice 4 (WE content was < 20%).

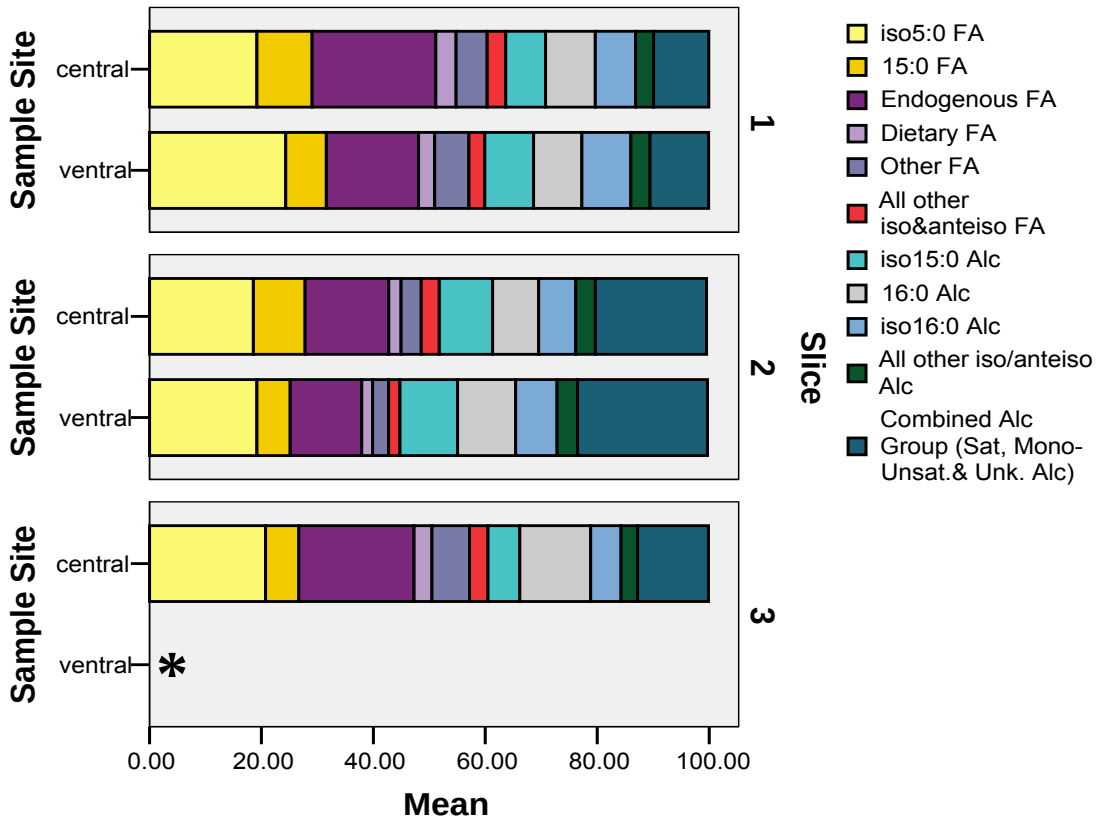


Figure 31. Mean percentage of fatty acid and fatty alcohol groupings from WE in adult outer jaw fat. Constituents of these groupings are outlined in Tables 4 and 5. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). A black asterisk denotes an area where data are missing because WE content was < 20%. No WE data are available for any dorsal subgroup of the adult outer jaw fat.

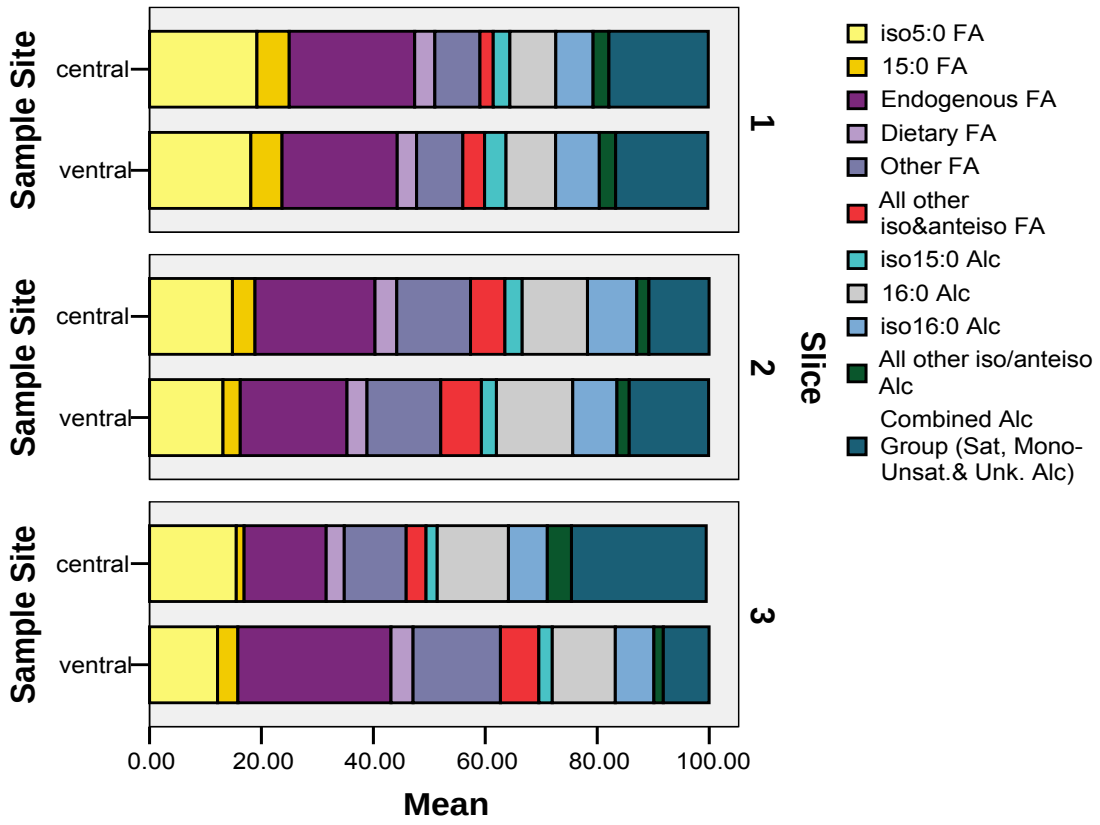


Figure 32. Mean percentage of fatty acid and fatty alcohol groupings from WE in subadult outer jaw fat. Constituents of these groupings are outlined in Tables 4 and 5. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). A black asterisk denotes an area where data are missing because WE content was < 20%. No WE data are available for any dorsal subgroup of the subadult outer jaw fat.

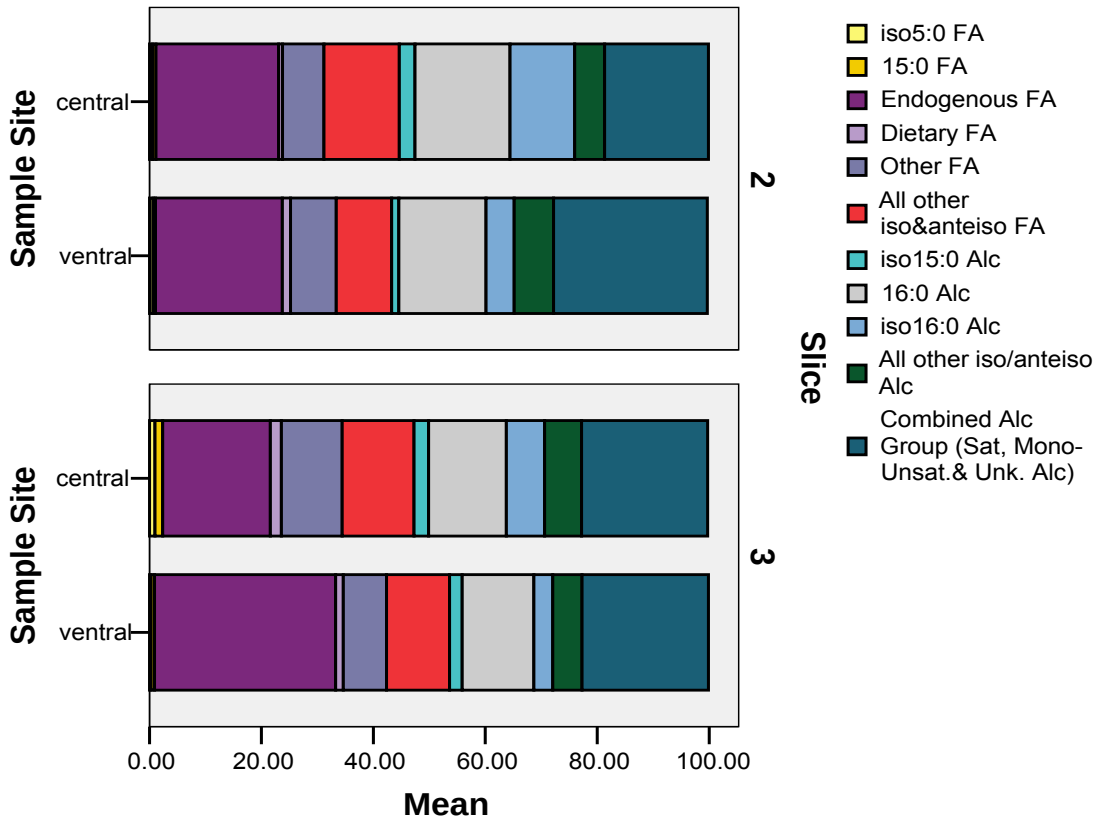


Figure 33. Mean percentage of fatty acid and fatty alcohol groupings from WE in calf outer jaw fat. Constituents of these groupings are outlined in Tables 4 and 5. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections). A black asterisk denotes an area where data are missing because WE content was < 20%. No WE data are available for slice 1 or any dorsal subgroup of the calf outer jaw fat.

recovered for all sample sites, there are some missing sample sites in each of the graphs. The sites where data do not exist are denoted with a black asterisk. Only two samples of WE were recovered from blubber; these values are reported in Table 9.

Very few changes in the proportions of FA and FAlc were noted in the spatial layout in the inner jaw fat of adults. The outer jaw fat of adults did exhibit some spatial complexity (Figure 31) with some variation in the content of *i*-5:0 and *i*-16:0 Alc, which varied inversely with the proportion of the endogenous group of FA (when either *i*-5:0 and *i*-16:0 Alc decreased, the endogenous FA increased). However, despite any variation, no consistent trend could be discerned across sections or at different sampling locations (central or ventral; no WE were recovered from any dorsal location of adult inner jaw fat).

The most obvious difference is the FA and FAlc composition was across age classes. Adults and subadults differ only slightly in the proportions of certain components (subadults having higher amounts of other FA such as 18:1n-5, 16:1n-9 and 15:1n-8). Calves, however, have almost no *i*-5:0 or 15:0 FA (which are found in large amounts of adult tissue), but an increased proportion of other iso and ante iso-acids (namely *i*-12:0, *i*-14:0, *i*-15:0, *i*-16:0, although together these still only accounted for approximately 10% of the total WE) (see Figure 30).

Although there was little variability in the components within each age class it is useful to consider the spatial layout of the WE in the tissue. The WE content of the inner jaw fat was generally around 30%, and samples with less then 20% WE content could not be reclaimed (Figure 34). Nearly 50% of the lipid class

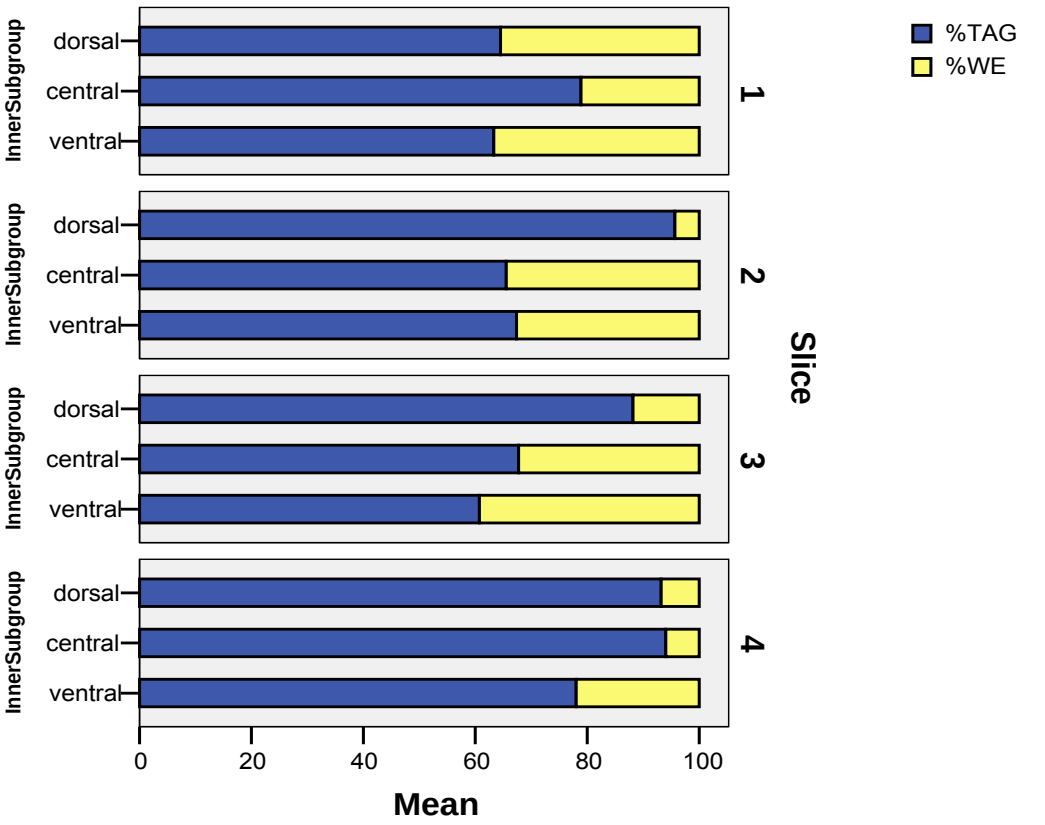


Figure 34. Mean percent of triacylglycerols and wax esters in the adult inner jaw fat. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections).

components sampled at the periotic complex consisted of WE (Figure 35). The FA and FAlc constituents at this sampling area in adults generally consisted of approximately 20 wt% *i*-5:0 and 10 wt% 15:0 acid (Figure 36). Although young animals again differed from older ones in the FA and FAlc content, the sample sites within an age class were not different from each other.

Fatty Acids Present in Triacylglycerols Versus Those in Wax Esters

The major components of FA from WE and TAG differed slightly. Although not always the largest contributors, the endogenous FA (18:0, 18:1n-9, 16:0, 16:1n-7, 14:0 and 14:1n-5) tended to be large components of both lipid classes, even in some cases being a larger component (as a group) than the iso-acids. However *i*-5:0 tended to be the single most dominant fatty acid in both lipid classes in adult tissues. The next most common FA differed between the two classes: *i*-15:0 was the second-most commonly found in the FABE-TAG, and the-second most common FA identified in FABE-WE was 15:0 FA.

Lipid Class Separation in the Fetus Head

The small amount of lipid recovered from most of the sampling sites for the fetus head (Table 6) precluded lipid class separation by TLC (as was done for all other heads). Since there was a low WE content (Table 7) it was presumed that the majority of FA identified were from TAG components of this tissue. To test this assumption, and to determine whether the FA composition of the fetus could indeed be compared to the other animals, any sample that had enough lipid to

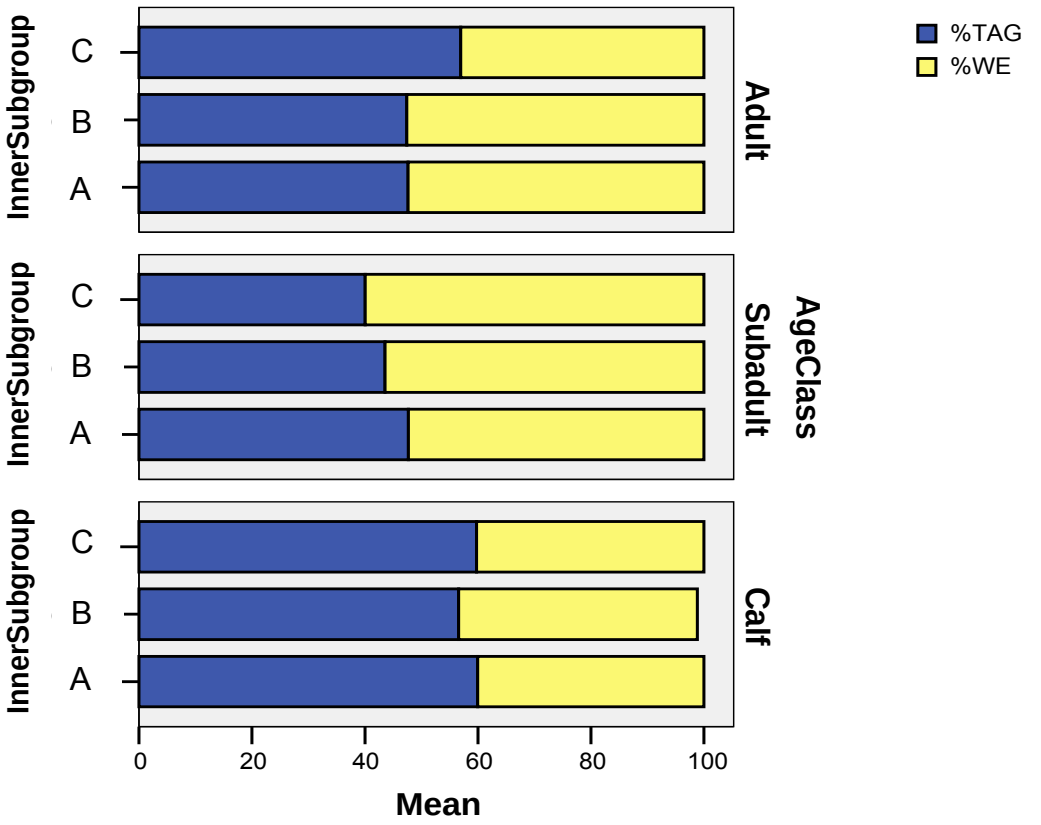


Figure 35. Mean percent of triacylglycerols and wax esters at the three sample site locations that adhere to the periotic complex for the adult, subadult and calves examined. See Figure 4 for a diagram of these ear fat locations.

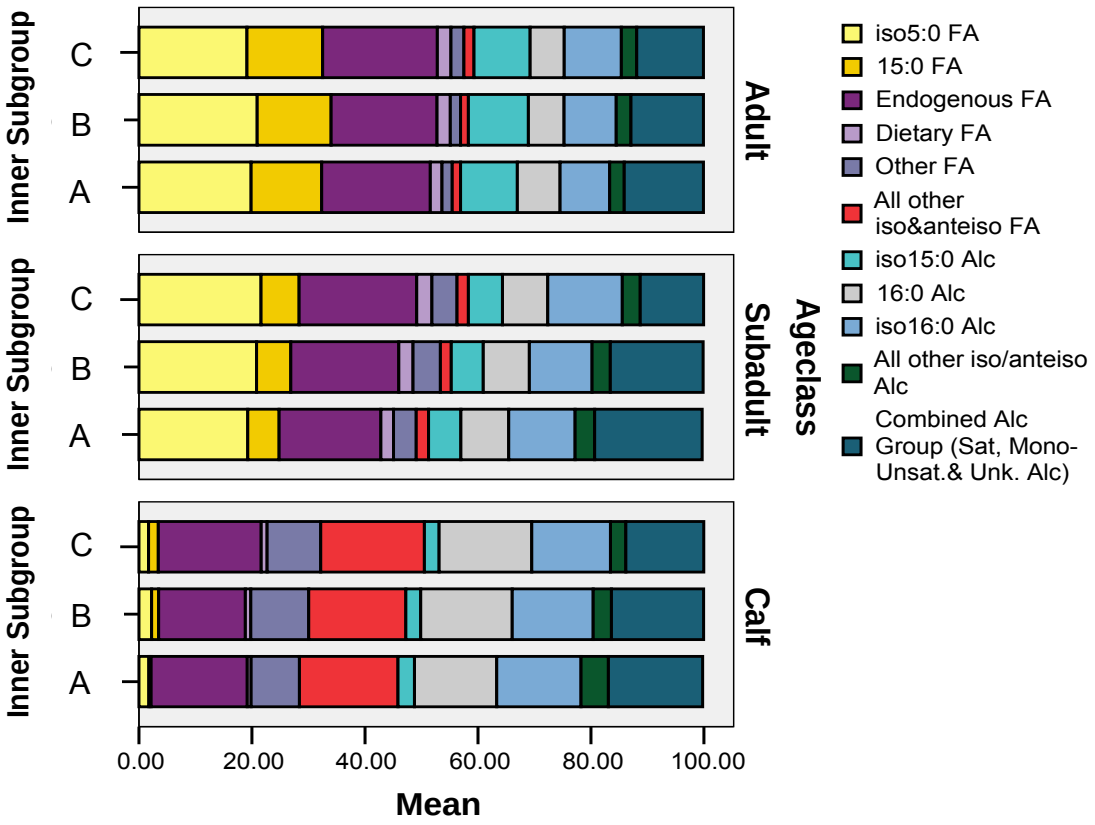


Figure 36. Mean percentage of fatty acid and fatty alcohol groupings from WE at the three sample site locations that adhere to the periotic complex for the adult, subadult and calves examined. See Figure 4 for a diagram of these ear fat locations. Constituents of these groupings are outlined in Tables 4 and 5.

separate into lipid classes, as well for processing a parallel sample without separation, were analyzed. A total of five sample TAG-only and total lipid pairs were examined and compared (data not shown). No sample showed any major difference in FA constituents, and major components (such as 14:1n-5, 16:0, 16:1n-7, 18:1n-9) did not generally differ by more than 2% (for example values of 14:1n-5 were 8.73 wt% and 8.9 wt% in the separated vs. unseparated sample; in the same sample, the values of 16:1n-7 were 47.72 wt% and 50.20 wt% in the separated vs. unseparated sample).

DISCUSSION

Distribution of Lipids in Adult Cranial Acoustic Tissue

Lipid Content and Lipid Class Composition

The acoustic tissues of the adult bottlenose dolphin are high in lipid content, and values from this study (inner: 80.4 wt%, and outer: 56.7 wt% mandibular jaw fat) were consistent with those reported by other researchers (melon 60-87 wt%; and jaw fat 70 wt%) (Varanasi & Malins, 1971; Litchfield & Greenberg, 1974). Both the cranial blubber (mean: 59 wt%; range: 18-80.1 wt%) and outer jaw fat (mean: 56 wt%: range: 0.1-82.0 wt%) had lower mean wet wt% compared to inner jaw fat. The wide range of blubber lipid content is not uncharacteristic of this tissue in bottlenose dolphins, as it has been previously recorded as low as 18.4 wet wt% lipid (Varanasi & Malins, 1971) and up to 68 wet wt% lipid (Struntz *et al.*, 2004). Variability of these values is likely due to sample site location and individual variation.

All three tissue types examined were comprised mainly of TAG, the most common storage lipid in mammals (Pond, 1998). Similar to other studies of bottlenose dolphins (Varanasi & Malins, 1971; Ackman *et al.*, 1973), considerable proportions of waxes were found in the inner (0-58%) and outer (0-37%) jaw fat. Interestingly, the inner mandibular fat that was adhered to the periotic complex had the highest concentration of WE present (up to 58%) (Figure 6). This high WE concentration at sites near the earbones has been noted in other odontocete species and has been hypothesized to have acoustic function in collimating sound towards the ear (Koopman *et al.*, 2006). WE have been found in the mandibular fat of the bottlenose dolphin (Varanasi & Malins, 1971; Ackman *et al.*, 1973) and other species (Litchfield & Greenberg, 1974; Litchfield *et al.*, 1975; Koopman *et al.*, 2006) and WE are also the main storage lipid in blubber of the sperm whale and beaked whales (Litchfield *et al.*, 1975). However, WE are generally considered to be uncommon in mammalian adipose tissue (Pond, 1998).

Small amounts of WE were also found in discrete locations of the cranial blubber; these areas coincide with the acoustic window over the pan bone (Figure 37). The occurrence of WE found here in the blubber is especially unusual since bottlenose dolphin body blubber does not generally contain WE (Koopman, 2001; Koopman, 2007) even though acoustic fats (melon and jaw fats) do. The WE deposited in these tissues do not come from WE in the diet

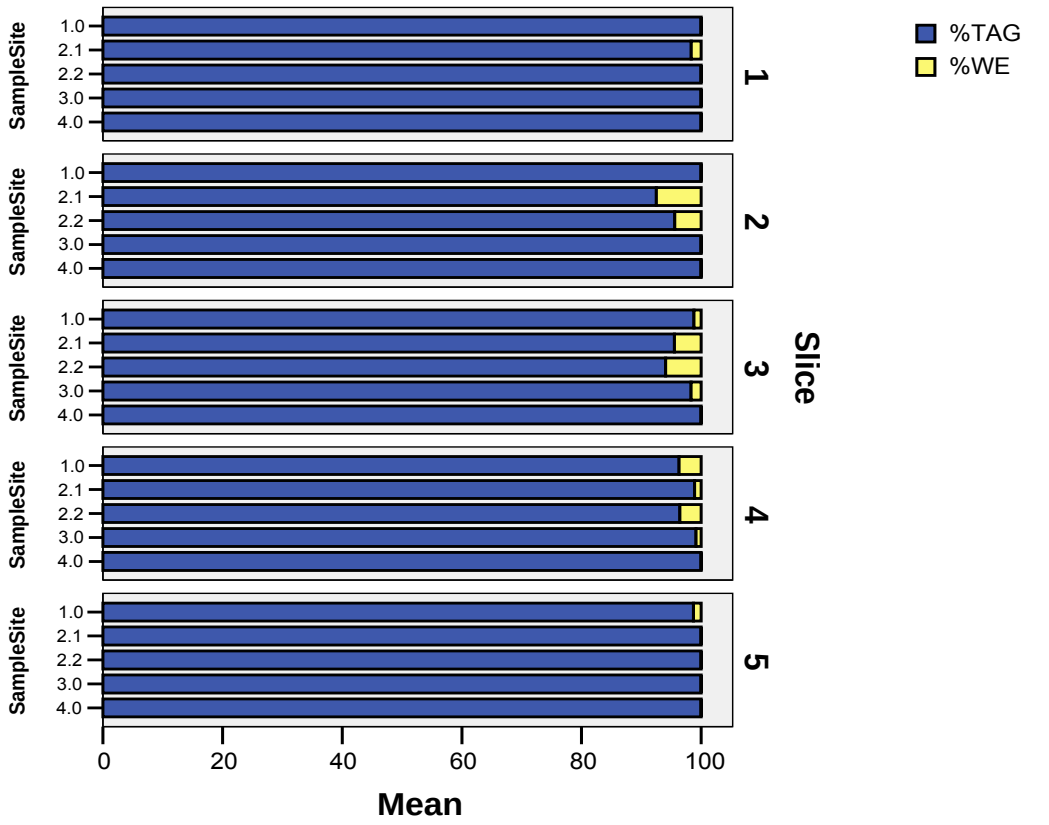


Figure 37. Mean percent of triacylglycerols and wax esters in the adult cranial blubber. Slice 1 is the caudal-most section and subsequent slices are further rostral (see Figure 3 for schematic of sections).

since the consumption and storage of wax are decoupled (Budge *et al.*, 2006). Although the components of WE (FA and FAlc) may be endogenous or dietary, they are specifically and selectively converted to WE at the site of deposition (Budge *et al.*, 2006). Thus it seems likely that WE found in the blubber at the area of the acoustic window serves some purpose other than metabolic storage. The FA composition of the cranial blubber also tends to support this assertion and will be discussed in more detail below.

Dominant Fatty Acids in Triacylglycerols

Since the predominant lipid class found in the inner and outer jaw fat and cranial blubber was TAG, the description of spatial distribution will focus on this lipid class. The single most dominant fatty acid in inner and outer jaw fat was *i*-5:0 (>40 wt% in adult inner jaw fat), which has been commonly found in high levels in the acoustic fat of delphinids (Ackman *et al.*, 1973; Litchfield *et al.*, 1975). *I*-15:0 was also found in notable proportions (>20 wt% in adult inner jaw fat). Although *i*-15:0 has been reported in previous studies on acoustic fat of bottlenose dolphins (greater than 10 mol%) (Varanasi & Malins, 1971), its contribution to the FA makeup has been overlooked in favor of studying the isomeric composition of isovaleroyl triacylglycerols or wax esters (Varanasi *et al.*, 1973; Litchfield & Greenberg, 1974; Litchfield *et al.*, 1975). That is to say, because of the unusually high concentration of *i*-5:0, most early papers studied the overall structure and positional location of *i*-5:0 within TAG and WE rather than looking at other, longer-chained components, or their distributions. This

study, however, provides a closer examination of the spatial distribution, and ontogenetic changes in both *i*-5:0 and *i*-15:0. It is apparent that both *i*-5:0 and *i*-15:0 co-vary, and the value of *i*-15:0 turns out to be an indicator of ontogenetic change, as the concentration of this acid was the only component that differed between subadult and adult jaw fats.

Spatial Variation of Fatty Acid Composition in Inner Jaw Fat

Little spatial variation were found in the inner jaw fats of adults and the content and proportion of FA was consistent within this tissue. 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 for other odontocete species (*Kogia, Phocoena phocoena*, *Stenella attenuata* and some beaked whales) (Koopman *et al.*, 2006), however this was not the case in bottlenose dolphins. Almost all FA present in the inner jaw fat were of endogenous origin (iso-acids and 14:0, 14:1n-5, 16:0, 16:1n-7, 18:0 and 18:1n-9) and there was a virtual lack of dietary acids in this tissue (Figure 9). The bottlenose dolphin appears to be unique in its more uniform spatial distribution of fatty acids compared to the more complex arrangement of fatty acids in other odontocetes. Levels of *i*-5:0, *i*-15:0 and other iso-acids were uniformly high (combined iso-acid concentrations were generally greater than 60 wt%, often closer to 80 wt%) throughout the adult inner mandibular fat except at the dorsal-most portions of the fat body, where there were significantly lower values of these iso-acids. This dorsal part of the fat body is also the location of increased

vascularity in this tissue, including increased presence of small arteries, arterioles and veins (Zahorodny *et al.*, 2005). There was a trend of decreasing levels (although not statistically significant) of iso-acids, and lipid content, towards the rostral end of the inner mandibular fat, along with a trend of the fat body becoming more uniformly vascularized towards the rostral end (Zahorodny *et al.*, 2005). So it appears that areas of higher vasculature in the inner jaw fat of bottlenose dolphins tend to have marginally lower amounts of iso-acids.

Spatial Variation of Fatty Acid Composition in Outer Jaw Fat

Similar to studies of the outer jaw fat of other odontocete species (Koopman *et al.*, 2006), the highest concentration of *i*-5:0 was found in the area overlying the thinnest region of the pan bone (see red asterisks in Figure 15, and schematic in Figure 38). The central and ventral regions of the outer jaw fat were significantly higher in both *i*-5:0 and *i*-15:0 than the area along the dorsal edge of the mandible. This pattern is evidence of fine-scale variation and preferential deposition of acoustic fatty acids around the pan bone. Similar to the inner jaw fat, virtually no dietary FA were found in this tissue; only small amounts were present in the dorsal sections, corresponding to the lower concentrations of iso-acids. This pattern was also remarkably consistent in younger animals (subadults and calves) and the ontogenetic consistency of the spatial distribution of both the inner and outer jaw fats will be discussed in further detail below.

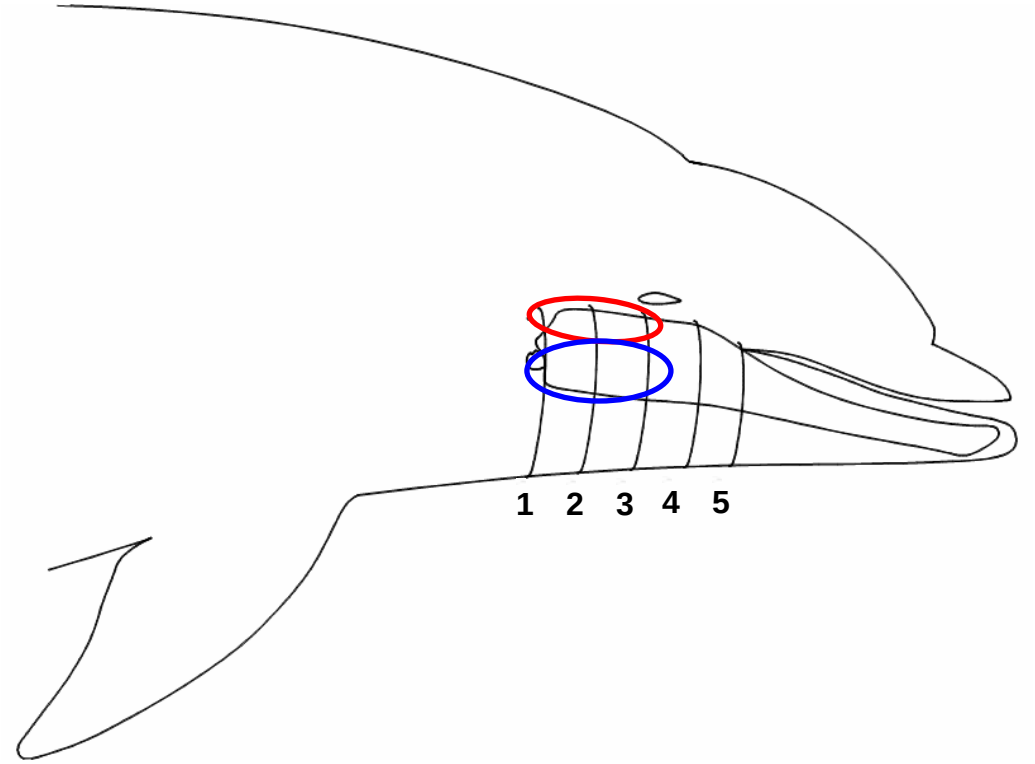


Figure 38. Regions of high and low iso-acids in the cranial blubber. Schematic represents the location of sampling sections taken from the bottlenose dolphin. Area circled in blue represents the region of higher *i*-5:0 and *i*-15:0 in the adult outer jaw fat which also corresponds to the area of the acoustic window. The area circled in red corresponds to outer jaw fat that exhibits significantly lower values of *i*-5:0 and *i*-15:0. Schematic courtesy of S.A. Rommel.

Spatial Variation of Fatty Acid Composition of Cranial Blubber

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 (Bullock *et al.*, 1968; McCormick *et al.*, 1970; Brill, 1988; Brill & Harder, 1991). However, an obvious question is: how do these specialized acoustic fats function with a blubber layer overlying them. This question was addressed by examining the FA content of cranial blubber along with the inner and outer jaw fats. Previous studies of the acoustic fat of cetaceans have primarily focused on the lipid class content and layout of FA and FAlc in the inner and outer jaw fats (Varanasi & Malins, 1971; Litchfield & Greenberg, 1974; Litchfield *et al.*, 1975; Koopman *et al.*, 2006). Only one study has included the cranial blubber overlying the mandibular fat in analysis of acoustic tissues (Varanasi *et al.*, 1973). However, in that study all the fatty tissues lying external to the mandible were excised together and divided into six large, 6 cm transverse sections. Samples close to the acoustic window were found to have larger amounts of isovaleroyl lipids (> 80% of TAG had isovaleric acid as components). The Varanasi *et al.*, (1973) study however did not distinguish between the blubber and the outer jaw fat, so the higher proportions may very well be attributed to the presence of the outer jaw fat as it tends to be most pronounced over the pan bone, but diminishes further rostral. No other studies have shown fine scale layout of iso-acid accumulation in the cranial blubber surrounding the acoustic window.

Body blubber of the bottlenose dolphin does not usually contain high amounts of iso-acids (Samuel & Worthy, 2004; Walton *et al.*, 2007), particularly *i*-5:0 which

is generally present in low quantities (inner blubber 0.8 ± 0.1 wt%; outer blubber 2.7 ± 0.2 wt%) (Koopman *et al.*, 2003). In my study, however, the cranial blubber was found to have localized areas of high concentrations of the short-chained and iso-acids more typically found in acoustic tissues such as the jaw fats and melon. Furthermore, these areas of high iso-acid accumulation also tended to coincide with the uncommon occurrence of WE in the blubber. Thus, the region of blubber overlying the outer jaw fat and pan bone had unusually high amounts of *i*-5:0 and the presence of WE; both of these traits are indicative of acoustic fat.

Blubber performs several functions, one of which is to act as a metabolic store (eg. Koopman *et al.*, 2002). Typically, dietary fatty acids (see Table 3 for a list of dietary FA) are found in the blubber, and in the bottlenose dolphin, FA are stratified within the blubber layer (Samuel & Worthy, 2004; Koopman, 2007). However, the cranial blubber at the acoustic window has extremely low values of dietary FA, almost to the point of exclusion (see red asterisks in Figure 19). In contrast, all of the sample sites that have low iso-acid content (see blue asterisks in Figures 19 and 20) have much greater (up to 20%) contribution of dietary FA, and these areas more closely represent typical metabolic storage blubber found elsewhere on the body. Additionally, stratification of FA within the cranial blubber was examined, and found to be lacking (sample site 2; where 2.1 was inner blubber and 2.2 was outer blubber) across all transverse sections. The lack of stratification and decreased role of dietary FA further supports the idea that the

regions of cranial blubber overlying the acoustic window have an acoustic function rather than a metabolic role.

These localized areas of accumulation of WE, and short-chained and iso-acid components in the blubber correspond to the area of the acoustic window. The region of highest iso-acid accumulation in blubber overlies the pan bone (areas circled in blue in Figure 39), which also corresponds to the region of outer jaw fat with the highest iso-acid accumulation (Figure 38). It appears that two margins of this acoustic window were captured in the sampling of the blubber. 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 (Figure 39). 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 (Figure 39). This compartmentalization of acoustically important FA and WE in the blubber likely allows sound to travel inwards towards the outer and inner mandibular fats with less impedance than regular blubber would confer. Blubber at the acoustic window appears to be much more specialized than previously assumed, as other authors have regarded cranial blubber as having no acoustic function (Varanasi & Malins, 1971; Robisch *et al.*, 1972). It seems likely however that these studies did not test blubber that directly overlies the pan bone; in fact they analyzed cranial blubber from the 'anterior cranial area' and the 'posterior cranial blubber', both non-descriptive sites that may have missed the acoustic window. In addition to this biochemical evidence of specialized blubber at the

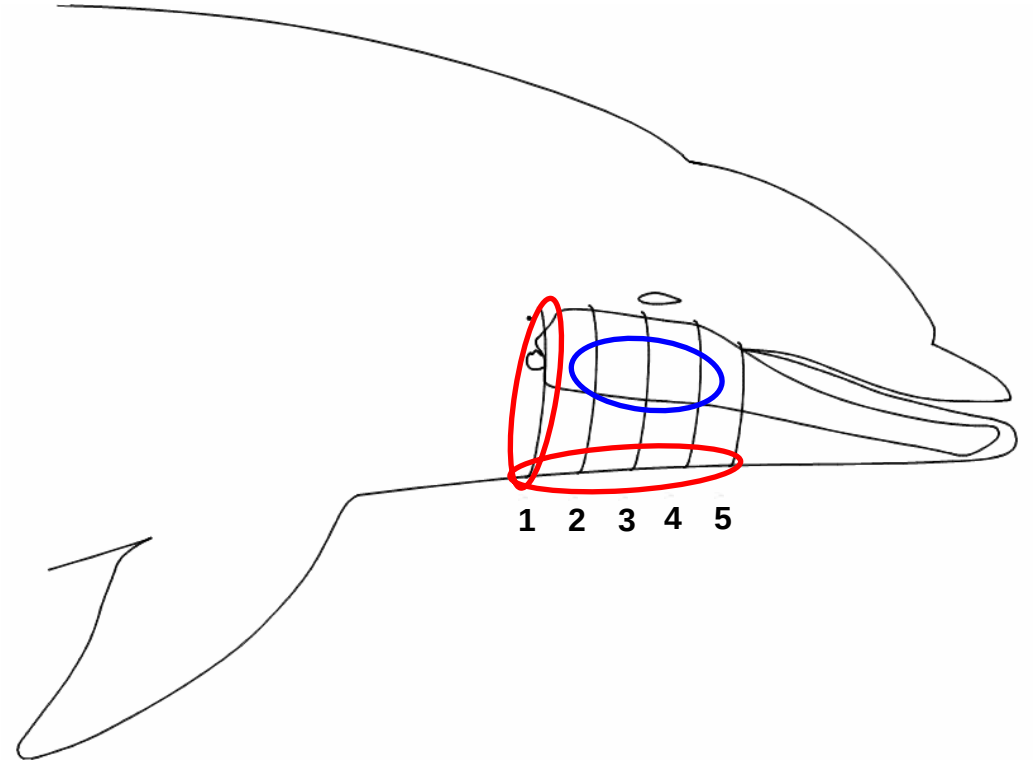


Figure 39. Regions of high and low iso-acids in the outer jaw fat. Schematic represents the location of sampling sections taken from the bottlenose dolphin. Areas circled in red represent the regions of lowest *i*-5:0 and *i*-15:0 in the adult cranial blubber. The area circled in blue represents the area of highest *i*-5:0 and *i*-15:0 accumulation in the adult cranial blubber. Schematic courtesy of S.A. Rommel.

acoustic window, there is also anecdotal evidence to support this view in CT images of bottlenose dolphin heads. Computed tomography (CT) scans taken of a bottlenose dolphin (Figure 40) reveal an area of less dense tissue around the mandibles which can be identified as the inner and outer jaw fat. However, the density of the blubber adjacent to the mandibular fat bodies appears to remain consistent, suggesting similar FA composition. This anecdotal evidence however does need to be considered cautiously since no direct density measurements have been made of this CT image. Similar variations in apparent density are also visible in other published CT images (McKenna, 2005).

Dominant Fatty Acids and Fatty Alcohols of Wax Esters

The FA and FAlc components of the bottlenose dolphin jaw fat have been described previously (Varanasi & Malins, 1971; Ackman *et al.*, 1973), however these studies reported values from single animals (one adult, one subadult respectively) and did not consider any spatial layout or preferential deposition of WE. WE found here are primarily made of saturated and monounsaturated FA and FAlc, which is typical of marine waxes (Sargent, 1976; Sargent *et al.*, 1976). Surprisingly neither of the previous studies on *Tursiops* mandibular fat found significant values of 15:0 acid as part of the WE, as was shown in this study (Table 9). Those studies reported instead, similar concentrations of *i*-15:0 FA to my 15:0 values. The presence of *i*-15:0 FA would not be surprising since it is a large constituent of the TAG-FA, however the identification of the 15:0 peak found here in WE was reconfirmed against standards using gas chromatography.

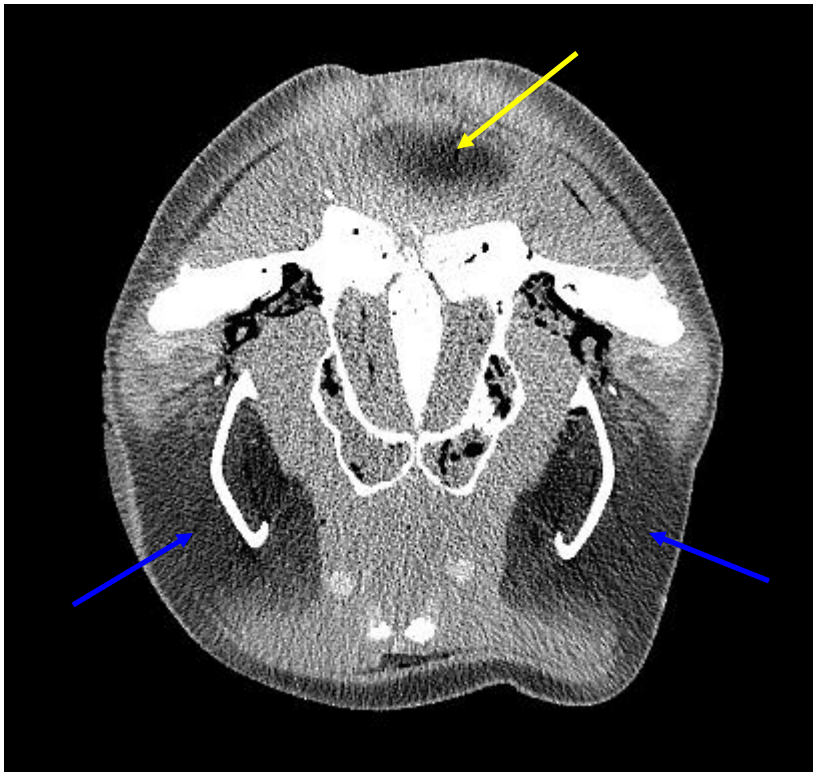


Figure 40. Transverse X-ray CT image of a bottlenose dolphin head (AJW001). The melon is indicated by the yellow arrow while the blue arrows indicate the jaw fats. The mandibular fat bodies appear darker (less dense) as does the blubber that lies adjacent to the inner and outer fat fats. Scan courtesy of Smithsonian Institute, National Museum of Natural History.

Also of note is the complete absence of *i*-5:0 Alc from samples in this study, an observation supported by previous research on WE composition of bottlenose dolphin jaw fat (Ackman *et al.*, 1973) where no *i*-5:0 Alc was recovered.

Very little spatial variation was noted in the FA and FAlc components of WE. The most noticeable trend was the difference in the FA and FAlc composition between age classes (discussed below).

Ontogeny

Ontogeny of Lipid Content and Lipid Classes

Very few studies have looked at the development of FA in acoustic tissues. One study showed that the melon of the bottlenose dolphin had increasing concentrations of isovaleroyl lipids as a function of body length, indicating a biochemical change in this structure as a function of age (Gardner & Varanasi, 2003). Thus, young animals do not possess the same biochemical makeup as adults with regards to their acoustic tissues. Evidence here shows that patterns for lipid class and FA accumulation are set up early in life. Young animals exhibited intermediate values for most lipid components of the acoustic tissues studied (from percent lipid, to FA/FAlc composition of the lipid classes), but exhibited similar spatial patterns to those of adults (ie. see Figures 19, 21, 22 and 23).

The fetus and calves examined had low levels of lipid content in all tissues (Figure 5), 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 to time hypertrophy (Pond, 1998) and a low mean wet weight percent of lipid has been previously reported in the blubber of bottlenose dolphin fetuses and calves (Struntz *et al.*, 2004). Fetal tissues were dominated by TAGs, indicating that WE had yet to be deposited in any great amount *en utero* (max. value was 3.4% WE). By the time dolphins reached the calf stage, much more WE had been deposited into the tissues (inner jaw fat 0-47% WE; outer jaw fat 0-43% WE). 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 (Figure 6). However, this difference was proportional, 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 TAGs into adulthood.

Ontogeny of Iso-Acid Accumulation

The results of this study suggest that the pattern of lipid accumulation in acoustically important tissues is set up as early as the fetal stage. However in young animals, the FA and FAlc compositions in the tissues were not yet fully developed and did not represent adult values, even though the blueprint, or pattern, appeared to be set. This pattern is evident when reviewing the FA composition of the inner jaw fat across age classes. While subadults appeared to be almost identical to adults in their FA composition (Figure 9 adult; Figure 11

subadult), the quantity of *i*-15:0 in this tissue continued to accumulate into adulthood. When calves and subadults were grouped into a category as developing animals, and the *i*-5:0 and *i*-15:0 content examined and compared to adults (Figure 7), it became clear that that there was a significant difference in the rate of accumulation of *i*-15:0. In outer jaw fat (Figure 8), the trend is even more obvious. The slope of the line for the developing animals was approximately one quarter of that in adults. This means that developing animals had, on average, about a quarter of the values of *i*-15:0 in their tissues compared to adults. That is to say that although younger animals appear to have similar ranges of *i*-5:0, they have not yet accumulated *i*-15:0. This evidence suggests that *i*-5:0 is preferentially accumulated first in the younger animals and that animals continue to deposit *i*-15:0 into adulthood.

The ontogenetic accumulation of iso-acids appeared to occur quickly in all the tissue types examined here. The accumulation of iso-acids was best described by a logarithmic relationship (Figures 24-27) for all tissues. All representative sampling sites had high R^2 values, suggesting that much variation could be explained by body size, providing evidence that most of the observed differences in age classes were likely due to growth of the animals. The rates of accumulation of iso-acids between representative samples from the periotic complex were remarkably similar, shown by similar coefficients of growth (Figure 24; sample site C: 46.0, and sample site A: 43.8). The coefficients of growth for two representative regions of the inner jaw fats were dissimilar (Figure 25; dorsal: 42.1, and ventral: 52.3), however the difference may be due to the described

lower values of iso-acids found in the dorsal region of the inner jaw fat (which is more highly vascularized). A similar trend was also seen in the two representative regions of the outer jaw fat examined (Figure 26; dorsal: 27.2, and central: 42.5). The dorsal section of the outer jaw fat accumulated lower concentrations of iso-acids, thus the coefficients of growth were not the same in all sections. The iso-acid accumulation of cranial blubber at the two acoustic window sites also exhibited disparate coefficients of growth (Figure 27; inner blubber section 2: 42.5, and inner blubber section 3: 29.1); which is surprising since both sites represented the same sampling areas of neighboring sections. This difference in rate of accumulation may be due to outliers in Figure 27B. Regardless of some differences of accumulation rates, and the concentration of iso-acids achieved, it is obvious that there is a strong ontogenetic component to the composition of the acoustic fats studied here. 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 & Eaton, 1971), thus the varying degrees of accumulation of iso-acids of different age classes observed in this study are likely a reflection of the time needed to synthesize and deposit these FA.

The spatial patterns of FA distribution described in adult tissues within this study were mirrored in the younger animals for all the tissue types examined (Figures 11, 12 and 13 for inner jaw fat; Figures 16, 17 and 18 for outer jaw fat; Figures 21, 22 and 23 for blubber). The ability to discern 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 (Morris, 1975; Koopman *et al.*, 2006). However, this is the first time such a pattern has been described in an ontogenetic series (adults, subadults, calves and fetus) or in an animal as young as a fetus.

Early Development and Other Fatty Acid Components

Adults exhibited extremely high concentrations of iso-acids, however as shown in this study, younger age classes did not always have such high values and as a consequence tended to have higher weight percent values of other FA. Fetal tissue in particular had higher concentrations of saturated and monounsaturated FA than tissues of adults. Palmitic acid (16:0) was always found in higher concentration in fetal tissues than adults, which is not surprising since it is the primary product of lipogenesis (Pond, 1998). The fetus also exhibited higher amounts of 14:1n-5 (mean 5.4 wt%), and 16:1n-7 (mean 33.9 wt%) than the adults (means 1.4 wt% and 12.6 wt% respectively). The trend of high saturated and monounsaturated FA (of endogenous origin) and elevated 14:1n-5 and 16:1n-7 has been reported previously in newborn hooded seals and beluga whale calves, and has been interpreted as high Δ-9 desaturase activity (Iverson *et al.*, 1995; Birkeland *et al.*, 2005). Levels of these endogenous FA were likely derived from a combination of fetal synthesis and maternal transfer (Iverson *et al.*, 1995).

Despite the near exclusion of dietary FA from the inner and outer jaw fat, omega 3 and omega 6 fatty acids are found in the blubber of adults (although

generally not at the acoustic window) and in all the tissues examined in the fetus (Table 8). The presence of these polyunsaturated (classified as dietary FA in this study – see Table 3) fatty acids {important as precursors to messengers within and between cells (Pond, 1998)} in the fetus must also be a result of maternal transfer through the placenta since these cannot be synthesized *de novo* by mammals (Iverson, 1993).

Synthesis of Lipid Components

Synthesis of Acoustically Important Fatty Acids

As seen in this study, iso-acids are a large component of acoustic tissues, especially *i*-5:0 and *i*-15:0. These are endogenous FA and do not originate from the diet (Malins & Varanasi, 1975). *I*-5:0 and *i*-15:0 are synthesized as iso5:0-coA during the catabolism of leucine in the mitochondria. In dolphins, *i*-5:0 is directly incorporated into lipid structures rather than being degraded into acetate or acetoacetate as is the mammalian norm (Tanaka *et al.*, 1966; Malins *et al.*, 1972; Morii & Kaneda, 1982). Other iso-acids such as *i*-4:0*, i*-12:0, *i*-14:0 and *i*-16:0 are synthesized during valine catabolism (Morii & Kaneda, 1982). The pathway for iso-acid production is highly conserved, as even bacteria synthesize them from amino acid precursors (Allison, 1977). All of these other iso-acids were found in at least trace amounts in all tissue types examined. In the case of young animals these other iso-acids can account for a relatively large component of the FA present {for instance other iso-acid component of calf blubber TAG

(Figure 22) averaged 7.8 wt%; and WE components of calf inner jaw fat (Figure 30) averaged 14.1 wt%}.

Iso-acids can be produced from amino acids by certain anaerobic bacteria (Allison, 1977). Although samples here were taken from post-mortem tissue, there was no evidence of putrefaction. The fine scale layout of iso-acids described here is consistent across all heads examined and this level of specificity precludes the possibility of bacterial sources of these FA.

Synthesis of Wax Ester Fractions and Components

The WE fractions of samples examined in this study appeared to exhibit preferential incorporation of certain components. The FAlc components of the WE should, in theory, be similar to the FA since they are reduced from FA before incorporation into WE (Budge *et al.*, 2006). The exception to this rule is that saturated and monounsaturated FA are preferentially converted to FAlc (meaning there is an exclusion of polyunsaturated fatty acids from being converted to FAlc). However, in this study, there was a distinct absence of *i*-5:0 Alc in WE. Presumably there is a large pool of *i*-5:0 from which to reduce, into an alcohol, for the inclusion into WE since the *i*-5:0 values in FA from TAG content were upwards of 40 wt%. Thus it appears that bottlenose dolphins preferentially include *i*-5:0 FA in WE, but do not convert this FA to FAlc. Similarly, *i*-15:0 Alc is a considerable component (up to 9 wt%) of the alcohol fraction of WE, yet it is not found in high concentrations as a FA moiety in WE, instead 15:0 FA is incorporated into WE. Thus it is apparent that there is preferential incorporation

of certain FA into WE, otherwise the FA profile from WE would be identical of that from TAG. The mechanism by which preferential incorporation could occur is unknown, since the pool of FA for the anabolism of both TAG and WE are presumably the same, being endogenously synthesized at the location site. Furthermore, the biochemical advantage that these FA and FAlc might confer to WE remains unknown. What is apparent is that although there is an ontogenetic change in the concentration of FA and FAlc in WE, adults have very little spatial variation in both the FA and FAlc content of WE. Thus, the presence of WE itself and not its FA or FAlc components may be important since it has been shown that WE, rich in branched structure, causes a decrease in ultrasonic velocity (Varanasi *et al.*, 1975).

Bottlenose Dolphins and Phylogenetic Diversity of Acoustic Lipids

The findings presented here on the content and spatial distribution of FA and FAlc in bottlenose dolphins appear to be fairly unusual. One previous study has shown there to be a channel of high content of branched, or low carbon chained, FA in the center of the mandibular canal in several species, including some beaked whales, a harbor porpoise and a delphinid (*Stenella attenuata*), forming a channel towards the ear (Koopman *et al.*, 2006). A similar pattern was not seen in the bottlenose dolphins studied here. Instead there appears to be a more uniform distribution of iso-acids in the inner mandibular canal in *T. truncatus*. It is unclear why there are disparate results between this study and that of Koopman *et al.* (2006). Although the Koopman study did include only a single delphinid

specimen, it showed consistent patterns with other animals in that study. It seems likely that difference in topographic FA distribution between bottlenose dolphins and spotted dolphins is due to a difference at the species level of delphinids. This could be due to the ecological niche that these odontocetes occupy since all animals in the Koopman study were pelagic, whereas bottlenose dolphins tend to be coastal animals (Leatherwood & Reeves, 1983). Further comparative studies of coastal and pelagic species are necessary to validate this hypothesis.

Delphinidae in general, along with Phocoenidae and Monodontidae, all contain isovaleric acid in their acoustic tissues, mostly in the form of triacylglycerols, with a smaller amount of isovalerate wax esters also being found in these families (Litchfield & Greenberg, 1974; Litchfield *et al.*, 1975). No isovaleric acid is found in the acoustic tissues of Ziphiidae (beaked whales), Physeteridae (sperm whale) or Kogiidae (dwarf and pygmy sperm whales) (Litchfield & Greenberg, 1974; Litchfield *et al.*, 1975). Delphinid fat consists mainly of triacylglycerols and a significant amount (greater then 3 wt%) of wax esters, whereas the Phocoenidae and Monodontidae are almost exclusively composed of triacylglycerols with little or no wax esters present. In contrast to the delphinids, all five genera of the family Ziphiidae have been found to contain higher amounts of wax esters and a complete absence of isovaleric acid both in blubber and in acoustic head tissues. However these tissues do contain other branched chain fatty acids such as *i*-10:0, *i*-11:0, *i*-12:0 and *i*-13:0 (Litchfield & Greenberg, 1978). *I*-12:0 is the dominant FA of these iso-acids in ziphiids

(Koopman *et al.*, 2006), and is a by-product of valine catabolism. This evidence suggests that although each phylogenetic group exhibits a dominant amino acid biosynthetic pathway in adults, young animals may not be as strongly constrained in the types of pathways used in the making of unusual endogenous lipids.

Functional Significance

The composition and spatial distribution of lipid classes and their FA and FAlc components have functional implications for hearing in these animals. There is an inverse relationship between the length of a carbon chain and sound speed (Gouw & Vlugter, 1967; Hustad *et al.*, 1971), thus sound passes more slowly through shorter FA, and more rapidly through long molecules. Unlike the previous study (Koopman *et al.*, 2006) in which an internal channel of low carbon weight FA was found in the inner jaw fat directing sound towards the earbones, the inner jaw fat of bottlenose dolphins exhibited a consistently high and relatively uniform layout of the short chained FA *i*-5:0. The exception to this observation was the lower values of *i*-5:0 at the dorsal-most sampling regions of this fat body. These areas tended to have higher proportions of longer chained FA. Presumably, the dorsal region of the inner jaw fat could serve in a similar manner as the fat surrounding the sound channel described by Koopman *et al.* (2006), refracting sound waves down towards the middle and ventral regions of the inner jaw fat and towards the earbones, which are found close to the ventral region of the mandible. Alternatively, these regions within the inner jaw fat may

have reduced iso-acids because the dorsal section of the inner jaw fat also contains more blood vessels including small arteries, arterioles and veins (Zahorodny *et al.*, 2005). Given the fact that isovaleric acid is toxic to most mammals (Wretlind, 1957) it would seem beneficial to reduce the possibility of mobilizing these toxic FA and limit the possibility of their circulation in the blood.

Similar to the inner jaw, the outer jaw fat exhibited lower values of short chained FA and iso-acids at the dorsal-most regions sampled. Again with the increased composition of longer chained FA in the dorsal region of the outer jaw fat, this may serve to refract incoming sound waves down towards the thinnest region of the pan bone. The dorsal-most region of the outer jaw fat is found along the dorsal edge of the mandible, which is not the thinnest region of this bone. Thus refracting sound waves down over the pan section of the mandible (the thinnest part of the mandible) would facilitate sound waves traveling from the outer jaw fat inwards towards the inner jaw fat and earbones.

WE content also performs a functional role in the transmission of sound from the environment to the ears. 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). WE content in this study was found to be highest in the inner jaw fat, especially at the sites where the fat adheres to the earbones. WE content here is also rich in branched-chain acids (*i*-5:0) and branched-chain alcohols (*i*-15:0Alc and *i*-16:0Alc) (Figure 36). The manner in which this conveys

acoustic advantage remains elusive, but clearly the fine scale spatial accumulation of lipid classes suggests a functional purpose.

I describe here, for the first time, a window in the blubber that is biochemically more similar to the acoustic fat found in inner and outer jaw fat than regular body blubber. The means by which dolphins can deposit specific FA on such a fine scale, within an organ as large as blubber, remain unknown. It is however likely that this biochemical window in the cranial blubber serves two roles. The first is to impedance-match sound traveling from the environment towards the ears 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. The second role may be to focus incoming sound over the pan bone, the thinnest part of the mandible, to facilitate the passage of sound waves through the bone. The highest levels of short and branched-chain FA were directly over the pan, with intermediate values of these FA found just dorsal and ventral to this area. The presence of some longer-chain FA in these areas just dorsal and ventral may serve to slightly refract sound waves so they can pass through the pan.

The ontogeny of lipid accumulation shown here suggests that young animals may not have the same ability to receive returning echolocation clicks as adults. While subadults do have very similar biochemical makeup of their acoustic tissues to adults, they are still accumulating some specific iso-acids (*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 of biochemical acoustic development. This may affect their ability to receive echolocation 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 & 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 biochemical composition of the sound receiving acoustic fats in the bottlenose dolphins.

Conclusions

Several significant new findings are reported here on the cranial adipose tissues found in bottlenose dolphins.

1. The inner jaw fat and the fatty tissue adhered to the periotic complex is more homogeneous than expected, with consistently high accumulation of iso-acids.
2. The outer jaw fat varies in a manner that iso-acids are highest in regions overlying the pan bone.

3. There is a window of high iso-acid accumulation in the blubber overlying the acoustic window and this blubber is more similar to fat found in the inner and outer jaw fats than typical body blubber.
4. The pattern for lipid accumulations is set up at an early age for all tissue components (inner and outer jaw fat and cranial blubber).

Future Directions

This study has revealed several new aspects about the acoustic fat of the bottlenose dolphin. It has also created new avenues of investigation. Clearly the biochemical mechanisms by which dolphins can preferentially incorporate certain FA and FAlc into lipids, and then the means by which they can deposit these lipids into discrete regions, needs to be investigated.

This study has also described a new biochemical window in the blubber of the bottlenose dolphin. Further investigation in other odontocetes seems prudent to determine if this is unique to bottlenose dolphins or common among all odontocetes. Additional studies are also needed to elucidate the physiological or metabolic method by which this biochemical difference in blubber arises. It is unknown how these unusual endogenous FA are deposited into specific sites within the blubber; and the physiological/biochemical means by which cells signal the initiation of this accumulation is unknown.

Accumulation of unusual endogenous FA have been described here in an animal as young as a fetus, however it is unknown if the fetus is able to biosynthesize these FA, or if they are transferred to the fetus via the placenta.

Many new questions exist about the manner in which these unusual FA and FAlc are synthesized and are so consistently deposited, and this offers new avenues for research in this field.

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APPENDIX

Appendix A. Procedure used to reconstruct WE data files.

Correcting FABE-WE

Correction for loss of the short chained FA *i*-5:0 was accomplished first by calculating the ratio of *i*-5:0 to 23:0 in the unseparated fraction of the sample. This ratio was then used to calculate the corrected value of *i*-5:0 in the separated fraction of FABE by multiplying it by the value of 23:0 acid, thus using the equation below:

Equation 1: $R = (U i\text{-}5{:}0 / U23{:}0) * S23{:}0$

where: R is the corrected value of *i*-5:0

U*i*-5:0 is the value of *i*-5:0 from the unseparated fraction of WE

U23:0 is the value of 23:0 acid from the unseparated fraction of WE

S23:0 is the value of 23:0 from the separated fraction of WE

To account for the proportional change in *i*-5:0 concentration in the separated fraction due to the above calculation, and due to the exclusion of the 23:0 standard from further inclusion in the analysis, all other FA values were corrected using the following equation:

Equation 2: $\text{Correction Factor A} = (100-R)/(100-O i\text{-}5{:}0-S23{:}0)$

where: R is the corrected value of *i*-5:0 (see Eqn. 1 above for calculation)

O*i*-5:0 is the original value of *i*-5:0 from the separated faction of WE

S23:0 is the value of 23:0 from the separated fraction of WE

Thus the remaining FA (except *i*-5:0) in each unseparated fraction was multiplied by its own Correction Factor A.

Correcting FAlc-WE

Fatty alcohol files needed to be corrected since the standard 19:0 alcohol was excluded from any further analysis. A correction factor (Correction Factor B) was calculated for each FAlc with the following equation:

Equation 3: Correction Factor B = (100)/(100-19:0Alc)

where: 19:0Alc is the value of 19:0 alcohol in the fatty alcohol fraction in each sample.

Thus the remaining FAlc in each fatty alcohol fraction was multiplied by its own Correction Factor B so that each FAlc sample totaled 100%.

Combining FA and FAlc

To combine both the FABE and FAlc into a final spreadsheet a final correction was made to each the FABE and FAlc so that the total values of all FA and FAlc equaled 100% and that they were proportionally accurate

Equation 4:

Proportional Correction Factor for FAlc = ((100-19:0Alc)/19:0Alc)/(((100-19:0Alc)/19:0Alc)+((100-23:0)/23:0))

Equation 5:

Proportional Correction Factor for FA = ((100-23:0)/23:0)/(((100-19:0Alc)/19:0Alc)+((100-23:0)/23:0))

The values of each FAlc were multiplied by the proportional correction factor for FAlc (Eqn. 4) and the values of each FA were multiplied by the proportional correction factor for FA (Eqn. 5). This provided the final reconstructed WE

sample information. These corrections were performed on every WE sample individually because all these correction factors varied for each sample.