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Seasonal and Ontogenetic Variation in Subcutaneous Adipose of the Bowhead Whale (*Balaena mysticetus*)

BALL HOPE C.,[1,2]* STAVARZ MADELINE,[1] OLDAKER JONATHAN,[1] USIP SHARON,[2] LONDRAVILLE RICHARD L.,[1] GEORGE JOHN C.,[3] THEWISSEN JOHNANNES G.M.,[2] AND DUFF ROBERT JOEL[1]

[1]Department of Biology, The University of Akron, Akron, Ohio
[2]Department of Anatomy and Neurobiology, Northeast Ohio Medical University, Rootstown, Ohio
[3]North Slope Borough Department of Wildlife Management, Barrow, Alaska

ABSTRACT

Cetacean evolution was shaped by an extraordinary land-to-sea transition in which the ancestors of whales became fully aquatic. As part of this transition, these mammals evolved unusually thick blubber which acts as a metabolic reservoir as well as an insulator and provides buoyancy and streamlining. This study describes blubber stratification and correlates it to seasonal variation, feeding patterns, and ontogeny in an arctic-adapted mysticete, the bowhead whale (*Balaena mysticetus*). Bowheads are unique among mammals for possessing the largest known blubber stores. We found that adipocyte numbers in bowheads, like other mammals, do not vary with season or feeding pattern but that adipocyte size and structural fiber densities do vary with blubber depth. Anat Rec, 00:000–000, 2015. © 2015 Wiley Periodicals, Inc.

Key words: blubber; adipose; seasonal variation; ontogeny; bowhead

Blubber of marine mammals serves vital physiological functions. Blubber is mostly adipose tissue that is located in the subdermal matrix and is laced with connective tissue fibers. It cloaks the body of marine mammals and has allowed cetaceans to colonize even very cold habitats (Parry, 1949; Koopman, 1998; Pabst et al., 1999; Todet, 2001; Hamilton et al., 2004). Blubber displays great variability in thickness, biochemical composition, stratification, and cell density in all marine mammals: pinnipeds, cetaceans, and sirenians (Iverson, 2002; Best et al, 2003; Struntz et al., 2004; Koopman, 2007; Rosa et al., 2007; Castellini et al., 2009). Composed primarily of low-melting temperature unsaturated fatty acids, cetacean blubber is an effective, low-maintenance insulator in cold water and may be so effective that bowheads have specialized organs that cool them down (Käkelä and Hyvärinen, 1996; Pabst, 1996; Iverson, 2002; Dunkin et al., 2005; Ford, 2013). Biomechanically, blubber reduces energetic costs of locomotion via streamlining cetacean baupläne and contributing to the maintenance of buoyancy (Pabst, 1996; Dearolf et al., 2000; Iverson, 2002; Kipps *et al*., 2002; McLellan et al., 2002).

Anatomically, we define "blubber" as the reticular layer of the dermis and it thus excludes the epidermis, papillary layer of the dermis, and the hypodermis, by following similar definitions by Haldiman et al. (1985) and Haldiman and Tarpley (1993). The hypodermis of bowheads, especially nursing and recently weaned individuals, exhibits additional adipose reserves which are structurally weaker than the blubber and a hypodermis that is morphologically very different from the blubber

*Correspondence to: Hope C. Ball, Department of Anatomy and Neurobiology, Northeast Ohio Medical University, 4209 State Route 44, PO Box 95, Rootstown, OH 44272.
E-mail: hcball08@gmail.com

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(Rosa, 2006). Alaskan native subsistence hunters use the word "maktak" to describe the external adipose layers, and whales that have significant hypodermal reservoirs are described as having "double-maktak." A similar fatty hypodermis layer has also been observed in fin whales (Lockyer et al., 1984).

Our study focuses on the blubber, not the hypodermis. Blubber in most cetaceans is vertically stratified in both structure and lipid composition with variations occurring with depth within the tissue (Ackman et al., 1975a,b; Aguilar and Borrell, 1990; Koopman et al., 1996, Krahn et al., 2004; Struntz et al., 2004; Montie et al., 2008). In regard to structure, little variation in connective tissue, adipocyte size, and adipocyte number amounts were detected in more external blubber layers, whereas greater variations in these traits were seen in more inner layers of fin whale (*Balaenoptera physalus*), harbor porpoise (*Phocoena phocoena*), and bottlenose dolphin (*Tursiops truncatus*) blubber (Aguilar and Borrell, 1990; Koopman et al., 1996; Struntz et al., 2004). Biochemical stratification in lipid composition occurs in the blubber of harbor porpoises between external and internal blubber layers (Koopman et al., 1996).Taken together, these studies have identified that more internal blubber layers are closely associated with lipid utilization, whereas more external layers are less dynamic and fulfill a primarily insulative and biomechanical function (Ackman et al., 1965; Koopman et al., 2002; Mau, 2004; Struntz et al., 2004; Montie et al., 2008). Histological and biochemical blubber parameters are related to environmental conditions to formulate hypotheses about developmental and environmental correlates of morphology. Blubber fatty acid composition and structure has also been used to infer population distribution, phylogeny, and feeding ecology in a variety of species including bowheads (Lockyer et al., 1984; Hoekstra et al., 2002; Budge et al., 2004; Samuel and Worthy, 2004; Budge et al., 2008; Cooper et al., 2009; Loseto et al., 2009).

This study quantifies adipocyte morphological variation in bowheads to examine how these characteristics respond to seasonal fluctuations in feeding patterns. Bowhead whales (*Balaena mysticetus*) are an ideal species for the study of blubber architecture and composition. Their epidermis is thickened and has the thickest blubber layer in the animal kingdom (up to 35 cm), and this layer increases with age (Haldiman and Tarpley, 1993; Rosa, 2006; George et al., 2007). Bowheads undertake biannual migrations between Beaufort Sea summer feeding grounds north of Alaska and Bering Sea wintering grounds west of Alaska, and feeding may only be intermittent in winter, and hence whales would rely on stored lipid resources to meet energetic demands (George et al., 1989; Lowry, 1993; Moore and Reeves, 1993; Stromberg, 2004 [Observations on the distribution of lipid in the epidermis and dermis of the skin of the bowhead whale (Balaena mysticetus). Final Report of the Period Submitted to the North Slope Borough Department of Wildlife Management. Unpublished]; COSEWIC, 2005). There are also ontogenetic differences, bowhead juveniles feed less efficiently because their baleen is not full grown and may rely on stored lipids during this period (Koski et al., 1992; George et al., 1999; Best et al., 2003; COSEWIC, 2005; Rosa, 2006). The variations in blubber over bowhead life history are of obvious importance in understanding the maintenance and regulation of lipids.

This study investigated blubber morphology of postmortem tissue samples from bowhead whales near Barrow, Alaska. We measured adipocyte cell count, mean adipocyte cell size, and area of structural fiber. The aims of our study were to: 1) quantify these blubber characteristics in regards to stratification in bowheads; 2) test if bowheads exhibited seasonal (Fall/Spring) differences in blubber architecture; and 3) investigate the influence of ontogeny on blubber structural morphology.

MATERIALS AND METHODS

Specimens

Bowhead (*B. mysticetus*) full-depth blubber samples were collected under NOAA-NMFS permit # 814-1899-03. The collection of all specimens followed the provisions of the Marine Mammal Protection Act of 1972 (amended MMPA: 16 U.S.C. *et seq*) and the Endangered Species Act of 1973 (amended ESA 16 U.S.C. 1531 *et seq*). All bowheads sampled were from the Bering–Chukchi–Beaufort population and age classifications (either adult or juvenile) were based on the examinations of baleen length at the time of sampling (COSEWIC, 2005; Lubetkin et al., 2008).

Postmortem blubber samples included all tissue from the base of the epidermis into lower-blubber layers but excluding the hypodermis. All samples were acquired on site in Barrow, Alaska during native subsistence harvests with the approval from the Iñupiat captains. These harvests occur twice a year, and the tissues were sampled from 10 individuals taken during both seasons: Fall or Spring (Table 1). The samples were stored in 4% T1
of paraformaldehyde solution at 4°C. All samples were taken at dorsal midline location on the animal, a location standard for most field, and biopsy sampling (Aguilar and Borrell, 1991; Samuel and Worthy, 2004; Rosa, 2006). An additional ventral midline location sample was also acquired for two individuals (2011B3 and 2012B7) to examine if bowhead whales demonstrated selective mobilization of lipids from this region observed in porpoises (Koopman, 1998).

The samples were 16–24 cm in depth, too large to allow for whole mount histological tissue preparations. Variation throughout the depth of the blubber was therefore assessed based on the six subsamples at different depths. Groups of two samples are referred to as superficial, intermediate, or deep blubber (Fig. 1). Superficial F1
samples were taken at 4 cm and 7 cm from the base of the epidermis. Deep samples were taken at 4 cm and 7 cm above the hypodermis. Two intermediate blubber samples were taken (2 cm apart) from the middle between the superficial and the deep samples (Haldiman et al., 1985; Haldiman and Tarpley, 1993; Rosa, 2006). At each sampling location, 10 small, noncontiguous blubber samples (2 × 2 mm) were excised from surrounding adipose. These were oriented parallel to the tissue-sectioning face and paraffin embedded.

Paraffin Embedding

Tissues were dehydrated and paraffin infiltrated overnight using Surgipath® Infiltrating Medium (Leica) in a Tissue-TEK® processor (Miles Scientific) under constant

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TABLE 1. Bowhead whales sampled for histological analysis

Sample number	Season	Age classification	Sex	Depth of blubber (cm)	Location of sampling
2009B7	Fall	Adult	Female	24	Dorsal midline
2010B15	Fall	Adult	Female	NS	Dorsal midline
2011B8	Fall	Adult	Female	20	Dorsal midline
2009B9	Fall	Adult	Male	19.5	Dorsal midline
2010B16	Fall	Adult	Male	16	Dorsal midline
2012B6	Spring	Adult	Male	NS	Dorsal midline
2012B8	Spring	Adult	Male	16	Dorsal midline
2011B3	Spring	Adult	Female	NS	Dorsal midline
2011B3	Spring	Adult	Female	NS	Ventral midline
2012B7	Spring	Adult	Female	18	Dorsal midline
2012B7	Spring	Adult	Female	18	Ventral midline
2009B11	Fall	Juvenile	Female	NS	Dorsal midline
2012B15	Fall	Juvenile	Male	18	Dorsal midline

NS, not sampled.

pressure and vacuum at room temperature (unless otherwise noted). We completed alcoholic formalin treatment for 180 min, 70% alcohol for 50 min, 80% alcohol for 60 min, 95% alcohol for 90 min, 100% alcohol for 110 min, Xylene (Fisher Scientific) for 90 min followed by paraffin infiltration for 120 min at 58°C. Once infiltrated, the tissues were then placed into embedding molds (Surgipath® Medical Industries), oriented in a parallel plane to the sectioning face, and embedded in Surgipath® Blue Ribbon embedding media (Leica). Embedded tissues were cut into 8-μm sections on a Surgipath® microtome (Surgipath® Medical Industries) with blades oriented perpendicular to blubber depth for all tissue samples to further reduce shrinkage (Struntz et al., 2004). The sections were mounted on microscope slides rehydrated and stained with eosin and hematoxylin (H&E) and cover-slipped with Permount® (Fisher Scientific). The slides were visualized on an Olympus, BX60 microscope, and digital images taken with an Olympus, DP71 digital camera with a resolution of 1.06 pixels/μm^2 and Olympus, DP controller 3.3.1.292 supplied software package (Fig. 2). F2

Cell Measurements and Statistical Analyses

Images were analyzed using ImageJ software (National Institutes of Health, MD) and the cell measurements were completed using the previously published protocols (Struntz et al., 2004; Montie et al., 2008). For each of the six blubber depths examined, a 1 mm × 1 mm box was positioned over the center of a 2 mm × 2 mm image. The images were converted to standard-value gray scale images first, allowing for the estimations of percent structural fiber for each layer of the blubber. Fibers were black after conversion which permitted the application of threshold functions for the estimates of structural fiber area per square millimeter analyzed. The counts of adipocytes, the measurements of structural fiber area, and the average adipocyte size were analyzed for the six blubber sample locations and are reported as superficial, intermediate, or deep blubber. As there were 10 samples for each layer, the numbers of adipocytes intersecting the diagonals of the 1 mm × 1 mm grid per sample were counted. Average adipocyte sizes (cross-sectional areas) were calculated by averaging the areas of the cells intersecting the diagonal (from upper left to lower right) of the 1 mm × 1 mm center-positioned grid. The samples were analyzed using two-way ANOVAs to investigate the effects of location within the blubber (among the six sampled locations), season of sampling (Fall vs. Spring), and ontogeny (adults vs. juveniles).

RESULTS

Stratification of Bowhead Whale Blubber

Histological analyses of samples taken from Fall adult, Spring adult, and Fall juvenile whale blubber samples show that superficial, intermediate, and deep blubber have distinct characteristics based on a variety of morphological characters (Fig. 2). Adipocyte size and fibrous density varied depending on blubber depth. Although the comparisons of adipocyte cell counts among individuals demonstrated no significant variation between samples from superficial or intermediate blubber ($P = 0.06$), significant variation was detected ($P = 0.02$) between these and the deep blubber samples (Fig. 3). Mean adipocyte area (μm^2) of the superficial samples was significantly lower than in intermediate blubber layer ($P = 0.04$). Adipocyte average area of cells in the intermediate blubber layer was found to be significantly larger ($P = 0.03$) than deep-layer adipocytes (Fig. 3). Fiber density varied across these layers of blubber (Fig. 3). Fiber densities were significantly highest in the superficial blubber ($P = 0.03$), with significantly lower densities detected in the intermediate and deep blubber ($P = 0.03$ and $P = 0.04$, respectively). The comparisons of ventral midline samples, acquired from two whales (2011B3 and 2012B7), show that there is no significant difference between dorsal and ventral values in adipocyte cell count ($P = 0.12$), average adipocyte cell area ($P = 0.13$), or structural fiber area ($P = 0.14$) (Fig. 3). F3

It is thus clear that blubber displays different histological characteristics depending on depth in the blubber column. The changes appear to be gradual and the six depths sampled (Fig. 3) appear to show a gradual trend and not distinct layers.

Seasonal Differences in Blubber Morphology

The second objective of this study was to assess whether the characteristics of blubber morphology in

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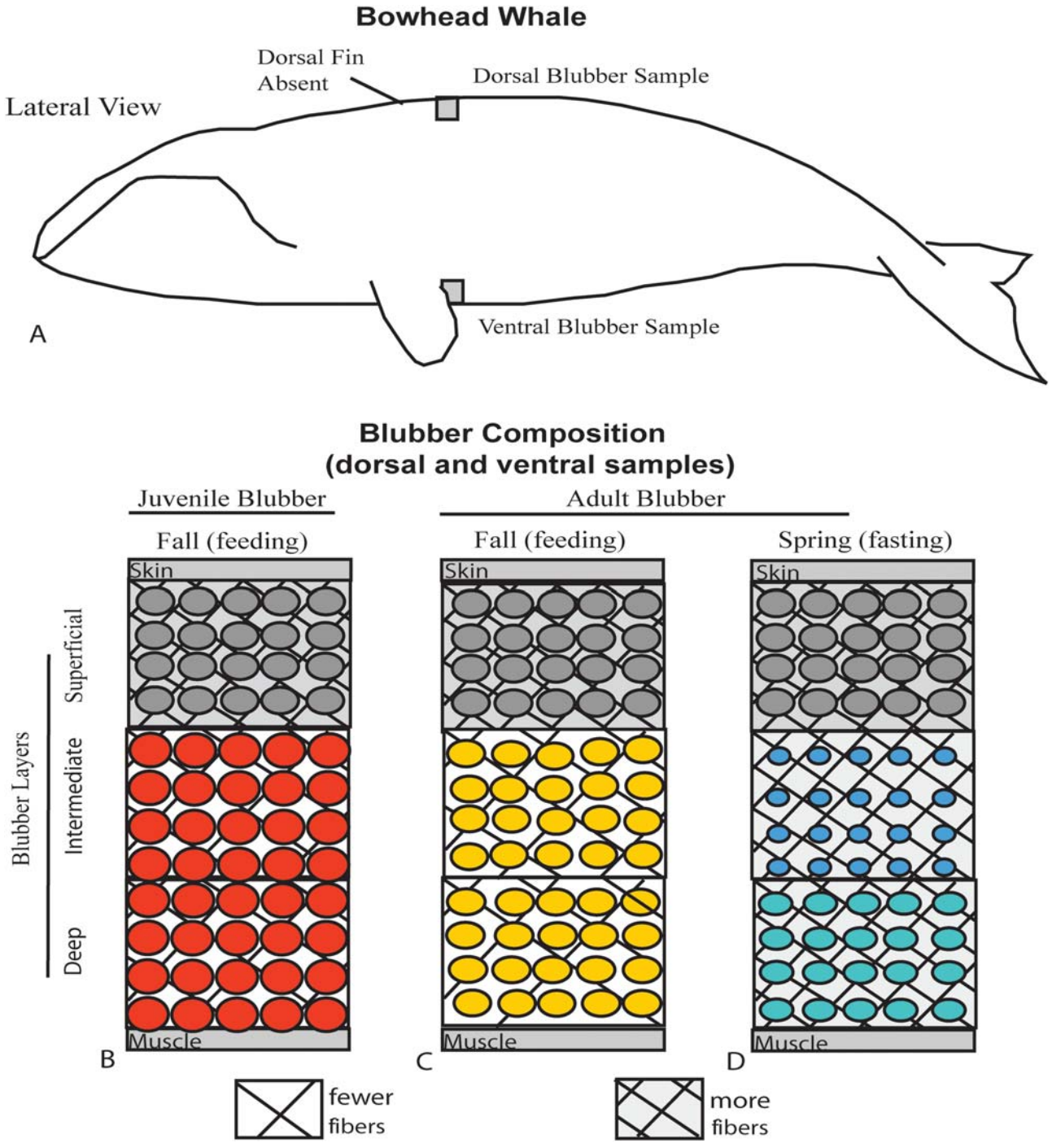

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Fig. 1. Location of bowhead whale sampling (**A**) and schematic representation of major morphological characteristics of seasonal and age variations in blubber among Fall juveniles (**B**), Fall adults (**C**), and Spring-harvested adults (**D**).

adult bowhead whales sampled during the Fall and Spring seasons differed. We examined the variations in average adipocyte cell counts, average adipocyte area (per μm^2), and structural fiber densities in superficial, intermediate, and deep blubber. The comparisons of adipocyte cell counts between Fall and Spring whales shows no significant difference in cell counts among superficial ($P = 0.15$), intermediate ($P = 0.08$), or deep ($P = 0.10$) blubber (Fig. 3). Adipocyte size between Fall and Spring adults demonstrated significant variation with blubber depth (Fig. 3), with larger adipocytes in Fall adults in the superficial ($P = 0.03$), intermediate ($P = 0.04$), and deep ($P = 0.03$) blubber layers (Fig. 3). Although there was no significant difference in structural fiber density

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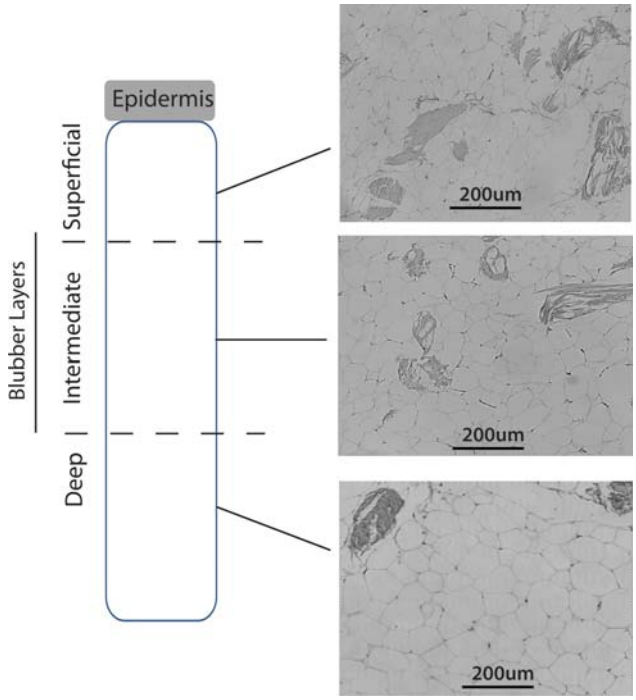

Fig. 2. Representative H&E-stained sections of (**A**) superficial and (**B**) deep blubber of bowhead whale 2009B7.

detected in the intermediate ($P = 0.07$) blubber, there were significant variations in fiber densities between Fall and Spring adults detected between the superficial ($P = 0.04$) and the deep ($P = 0.03$) blubber (Fig. 3). For both Fall and Spring adults, the largest difference in both adipocyte area and structural fiber densities was detected between the intermediate and the deep blubber.

The Effects of Ontogeny on Blubber Morphology

We also assessed the effects of ontogeny on blubber morphology through histological examinations of blubber samples from Fall-sampled adults and Fall-sampled juveniles. The examinations of adipocyte cell counts detected no significant variation in adipocyte number in superficial ($P = 0.14$), intermediate ($P = 0.13$), or deep ($P = 0.15$) blubber (Fig. 3). When adipocyte size was examined, significant differences were detected only in the intermediate ($P = 0.01$) and deep ($P = 0.04$) layers of blubber; not the superficial layer ($P = 0.38$; Fig. 3). THe differences in structural fiber densities were detected only in intermediate blubber ($P = 0.02$) between Fall-sampled adults and juveniles. There were no significant differences in fiber densities among superficial ($P = 0.30$) or deep ($P = 0.40$) blubber (Fig. 3).

DISCUSSION

This study examined several characteristics of blubber morphology (structural fiber density, adipocyte cell counts, and adipocyte size) in postmortem samples from bowhead whales taken during two seasonal native subsistence hunts (Fall and Spring) near Barrow, Alaska, and assess factors, such as age and season of sampling, that may affect these characteristics.

Stratification of Bowhead Blubber

Bowhead blubber samples were divided into superficial, intermediate, and deep samples. Our study detected the transitions from superficial to deep blubber that appeared gradual. Owing to sampling differences, however, we cannot rule out the possibility that true distinct layers do exist in bowhead subcutaneous adipose consistent with the findings of Rosa (2006). In all samples, the superficial samples are composed of small-sized adipocytes of intermediate number and the highest levels of fiber density (Figs. 1 and 3). Intermediate blubber was composed of greater numbers of large-sized adipocytes and a lower structural fiber density (Figs. 1 and 3). These patterns are similar to those observed in other nonarctic cetaceans; notably, bottlenose dolphins from the southern United States (Struntz et al., 2004; Montie et al., 2008). Deep blubber samples consist of the smallest adipocyte populations. Adipocytes of these samples were larger in size, and embedded in a meshwork of fibers with moderate densities (Figs. 1 and 3). Biological variations in fatty acid composition and deposition and histological stratification of blubber into distinct layers have been demonstrated in several cetacean species including bottlenose and common dolphins (*Delphinus* sp.), Atlantic sei (*Ba. borealis*), and fin (*Ba. physalus*) whales (Ackman et al., 1975b; Aguilar and Borrell, 1990; Koopman et al., 1996, 2002; Samuel and Worthy, 2004; Smith and Worthy, 2006). Where visibly distinct layering patterns have been detected in cross-sections of blubber in some species, layering in bowhead blubber has not been found to be visibly distinct, but this study shows that there is a quantifiable difference from superficial to deep blubber in bowhead whales (Hansen and Cheah, 1969; Bottino, 1978; Lockyer et al., 1984; Koopman, 1996).

Seasonal Changes in Bowhead Blubber Composition

The examinations of blubber morphology from adults taken during Fall and Spring revealed seasonal variation in bowhead blubber. Within season, the analyses of adipocyte size and number between dorsal and ventral midline samples detected no significant differences (Fig. 3), demonstrating that adipocyte size is uniform within thoracic blubber. Furthermore, we detected no significant differences in overall blubber depth owing to season supporting previous observations of bowhead blubber (Table 1) (George, 2009). Possibly, owing to the sparcity of depth measurements available for Spring adults, this difference also suggests that bowheads maintain overall thickness by modulating water or fluid content with lipid loss as we were unable to detect the significant variation in fiber density at our sampling locations (Fig. 3).

Spring-sampled adults demonstrated smaller adipocytes than Fall-sampled adults with the most pronounced differences detected in the intermediate and deep blubber layers (Figs. 1 and 3). Spring-sampled adults also demonstrated higher structural fiber densities at all three levels than Fall-sampled adults (Fig. 3). This contrasts the finding by Rosa (2006) that there

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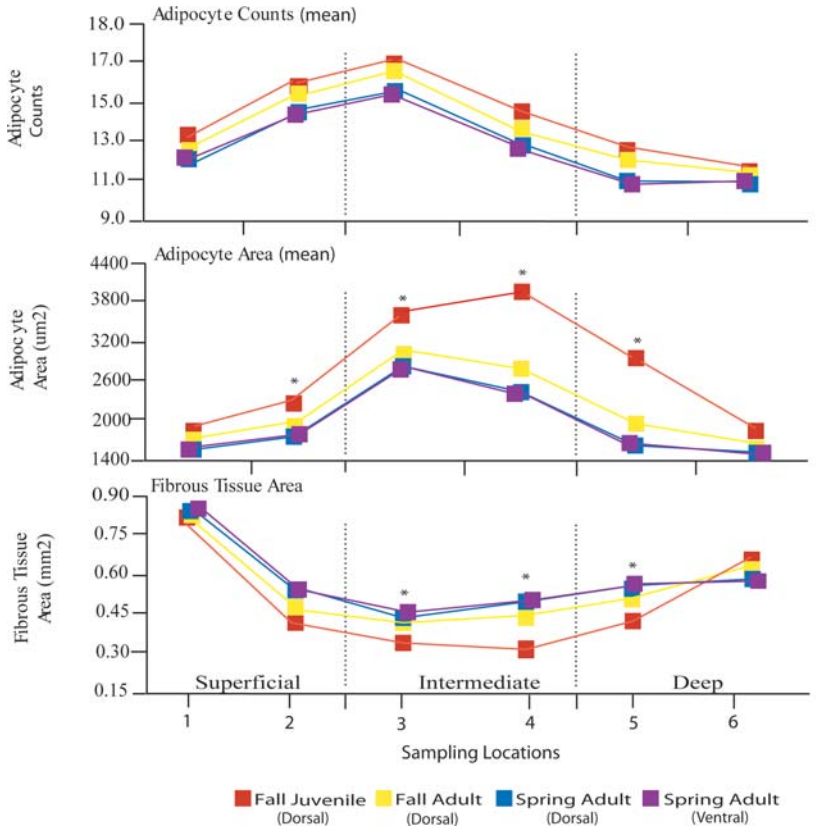

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Fig. 3. The measurements of adipocyte cell number, average adipocyte size, and fibrous tissue area density in Fall adult, Spring adult, and Fall Juvenile bowhead whales. Sampling locations denoted with asterisk signify the significant differences in mean adipocyte number, structural fiber density, and/or average adipocyte size, detected by two-way ANOVA, between individuals and are described in detail in the text.

is no difference in the percentage of structural fibers between spring and fall caught whales. Adipocyte and structural fiber content fluctuations over the year can be explained by several factors. There is marked seasonal variation in feeding patterns in bowhead with prolonged winter fasts and heavy feeding in summer (Schell et al., 1989; Moore and DeMaster, 1998; Dehn et al., 2006). These seasonal feeding patterns necessitate the changes in lipid utilization—a switch from primarily exogenous food consumption to stored lipid reserves to supply energetic demands. Variations in nutrient availability affect mammalian adipocytes through the changes in cell size but not number of cells (Young, 1975; Pond, 1998; Singh et al., 2012). In aquatic mammals, these effects of nutrient deprivation appear most pronounced within intermediate blubber demonstrated by alterations in structural fiber densities which increase with adipocyte shrinkage to maintain overall blubber depth (Koopman et al., 2002). When combined with the lower thermal conductivity of cold water, blubber prevents heat loss during winter months in bowheads (Worthy and Edwards, 1990). Variations detected among blubber levels reflect the differences in blubber function. Small variations in superficial blubber may be owing to the function of this blubber in insulation, buoyancy, and streamlining, whereas the function of deeper blubber may be more related to energy storage. In contrast, the warmer body temperatures, increased microvasculature, biochemical composition, and biochemical activity in intermediate and deep blubber of many cetacean species suggest that these layers are more metabolically active and important for lipid storage and lipolysis (Lockyer et al., 1984; Käkelä and Hyvärinen, 1996; Pond, 1998; Koopman et al., 2002; McClelland et al., 2012).

Ontogenetic Differences in Blubber Morphology

Reproductively immature, juvenile whales sampled during Fall hunts differ in blubber morphology from Fall-sampled adults. Juvenile whales had higher numbers of larger adipocytes and lower structural fiber densities at each layer examined (Figs. 1 and 3). Consistent with this, Rosa (2006) noted that a greater proportion of blubber consists of collagen in older whales. Juvenile bowhead whales inhabit cold arctic waters and, owing to their smaller body size, possess a higher surface area to volume ratio. Larger, lipid-rich adipocytes (especially in intermediate and deep blubber layers where the most significant variations were detected) assist in providing adequate insulation (McLellan et al., 2002; Montie et al., 2008). Furthermore, juvenile bowheads feed less efficiently owing to shorter baleen plates after weaning

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(Schell et al., 1989; Koski et al., 1992; George et al., 1999; Rosa, 2006). Less effective feeding capabilities for this prolonged period coupled with the energetic demands of migration and development subject juvenile bowheads to greater demand for energy stores in the form of lipids to support growth.

Bowhead blubber morphology is influenced by the effects of ontogeny and season, which may influence prey availability as well as lipid utilization. Seasonal changes in blubber morphology occur are correlated with seasonal feeding activities and winter fasts in other migrating mysticetes (Mackintosh and Wheeler, 1929; Lockyer et al., 1984,1985). Prolonged fasting during the winter likely results in smaller-sized adipocytes as lipid is slowly utilized to provide energy for energetic demands. Juvenile Fall whales have adipocytes significantly larger than Fall-sampled adult whales. These increased adipose stores are important for both insulation and energy storage as the young enter a long period of inefficient feeding prior to baleen maturation.

Seasonal and developmental demands influence how bowheads regulate and alter their physiology. Little is currently known regarding metabolic regulation in bowheads or, indeed, any cetacean species. Understanding the patterns and pathways of regulation involved in lipid mobilization and utilization will provide vital insight into bowhead physiology, nutrition, ecology, and health assessment.

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