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Beyond Thermoregulation: Metabolic Function of Cetacean Blubber in Migrating Bowhead and Beluga Whales

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Abstract

The processes of lipid deposition and utilization, via the gene *leptin* (*Lep*), are poorly understood in taxa with varying degrees of adipose storage. This study examines how these systems may have adapted in marine aquatic environments inhabited by cetaceans. Bowhead (*Balaena mysticetus*) and beluga whales (*Delphinapterus leucas*) are ideal study animals- they possess large subcutaneous adipose stores (blubber) and undergo bi-annual migrations concurrent with variations in food availability. To answer long-standing questions regarding how (or if) energy and lipid utilization adapted to aquatic stressors, we quantified variations in gene transcripts critical to lipid metabolism related to season, age and blubber depth. We predicted Leptin tertiary structure conservation and assessed inter-specific variations in *Lep* transcript numbers between bowheads and other mammals. Our study is the first to identify seasonal and age-related variations in *Lep* and lipolysis in these cetaceans. While *Lep* transcripts and protein oscillate with season in adult bowheads reminiscent of hibernating mammals, transcript levels reach up to 10-times higher in bowheads than any other mammal. Data from immature bowheads are consistent with the hypothesis that short baleen inhibits efficient feeding. Lipolysis transcripts also indicate young Fall bowheads and those sampled during Spring months limit energy utilization. These novel data from rarely examined species expand existing knowledge and offer unique insight into how the regulation of *Lep* and lipolysis has adapted to permit seasonal deposition and maintain vital blubber stores.

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Author Contributions

All experiments were conceived and designed by: HCB, JGMT, JWP, RLL, and RJD. Experiments were performed by: HCB and JWP. The data was analyzed by: HCB, JWP, RLL, CV and RJD. Authors who contributed reagents, materials and or analysis tools were: JGMT, RS and JCG. Manuscript was written by: HCB, RJD and RLL.

Competing Interests

Conflict of Interest: The authors declare that they have no conflict of interest.

Keywords

leptin; metabolic activity; bowhead whale; development; blubber

Introduction

Mammalian model organisms play a critical role in elucidating developmental patterns, biological functions, and ecological or environmental significance of complex physiological systems. However, contrasting these model systems to species exhibiting extreme phenotypes can advance a field of study by elucidating novel mechanisms of physiology (e.g. Schmidt-Nielsen's camels; Schmidt-Nielsen et al 1981). Examinations of physiological pathways in these non-model systems are crucial in this regard- they augment existing knowledge with novel information about evolutionary and developmental adaptations. Here, we examined two non-model species, bowhead (*Balaena mysticetus*) and beluga (*Delphinapterus leucas*) whales. These species have large adipose stores required for survival in Arctic conditions and have adapted unique processes governing adipose deposition and lipid utilization.

Rodent model systems helped characterize the biological effects and signaling mechanisms of the adipose-derived peptide hormone *leptin* (*Lep*), best known for its roles in weight regulation, appetite control and energy metabolism. The protein inhibits appetite and stimulates metabolic rate to match energetic demands (Zhang et al. 1994; Considine et al. 1996 and Friedman 2009). However, the pleiotropic effects of *Lep* extend beyond weight regulation and include reproductive function, angiogenesis, immune function and aspects of skeletal remodeling (Sierra-Honigmann et al. 1998; Steppan et al. 2000; Friedman 2009; Carlton et al. 2012 and Motyl and Rosen 2012). Effects are induced via binding and oligomerization with the leptin receptor (*LEPR*) (Tartaglia et al. 1995; Bates and Myers 2003 and Cammisotto and Bendayan 2007). While six receptor isoforms are known in mammals, only one (*LEPR-b*) possesses all signaling domains necessary to affect metabolic activity (Fong et al. 1998 and Munzberg et al. 2005).

Circulating *Lep* mRNA, protein and total adipose tissue are positively correlated in mammals (Considine et al. 1996; Cammisotto and Bendayan 2007; Townsend et al. 2007; Yang et al. 2008 and Kelesidis et al. 2010). Species that undergo migration or hibernation require large seasonal changes in total adipose reserves to assist with survival during energetically demanding and non-feeding periods. Hibernating ground-dwelling rodents and migratory bats build adipose reserves through variations in foraging habits and consumption rates , but another contributing factor may be adaptive temporal *leptin resistance* (Kronfeld-Schor et al. 2000; Bates and Myers 2003; Cammisotto and Bendayan, 2007 and Florant and Healy 2012). *Leptin resistance* refers to transient or long-term loss-of-function in Leptin signaling feedback, such that an equivalent level of Leptin produces less biological response due to modification in its signaling pathway. Possible modifications include changes in *Lep* expression, leptin-receptor isoforms compliments or alterations in downstream intracellular signaling (Townsend et al. 2008 and Yang et al. 2008). In this regard, *leptin resistance* may be an adaptive response resulting in increases in adipose volume, *without* expected increases

in *Lep* mRNA or circulating Leptin protein levels (Lee et al. 1997 and Fong et al. 1998). Given the 'dogma' of *Lep* (more adipose equates to higher *Lep* mRNA and protein titers) we expect that animals maintaining very large adipose reserves, such as bowhead and beluga whales, would display constantly high levels of *Lep* transcripts, but this has not been investigated in cetaceans until now. Our study bridges a crucial knowledge gap by exploring, for the first time, seasonal and developmental variations in *Lep* and *LEPR* in these mammals with extreme naturally occurring lipid reserves.

Lipolysis is the fundamental process of lipid utilization, fueling development, reproduction, lactation and migration. Triacylglycerols (TAGs) are the most abundant energy storage molecule in eukaryotic organisms. Three enzymes catalyze sequential steps leading to their degradation: adipose triglyceride lipase (*PNPLA2*), hormone sensitive lipase (*LIPE*) and monoglyceride lipase (*MGLL*) (Yeaman 2004; Zimmermann et al. 2004; Zechner et al. 2005; Smirnova et al. 2006 and Lass et al. 2011). Although it is well known that cetaceans possess large adipose stores in the form of blubber, the metabolic function and physiology of blubber is still poorly understood. Body size, longevity and population status prohibit traditional lab investigation and thus studies have focused upon examination of fatty acid composition and detectable changes with location, age, season and/or food availability; primarily from biopsies of a limited number of species (Aguilar and Borrell 1990; Hooker et al. 2001; Koopman et al. 1996; Koopman 2007; Montie et al. 2008; Rosa et al. 2007 and Marcoux et al. 2015).

Cetaceans possess specialized subcutaneous matrix of adipose and connective tissue (blubber) that functions as an energetic reserve while assisting in buoyancy and minimizing conductive heat loss (Haldiman and Tarpley 1993; Käkelä and Hyvärinen 1993; Pabst 1996; Iverson 2002; Dunkin et al. 2005 and Rosa 2006). The evolution of blubber contributed to cetaceans' ability to colonize a wide range of aquatic habitats, ranging from tropical to polar, and its anatomy is highly variable across species (Aguilar and Borrell 1990; Crandall et al. 1997; Koopman 1998; Webb et al. 1998; Koopman 2007; Montie et al. 2008; Bagge et al. 2012; McClelland et al. 2012; Ball et al. 2015). Exclusively Arctic species, such as bowhead and beluga whales, require and utilize extensive blubber reserves that cannot be substantially altered without negatively affecting homeostasis, locomotion, and buoyancy (Iverson 2002; George et al. 2007; George et al. 2016). Our work is the first to examine seasonal and developmental transcript patterns of lipolytic genes in cetaceans. Here, we expand on existing knowledge in order to better understand how the regulation of lipolysis has adapted to permit seasonal deposition and maintenance of extensive blubber stores.

The bowhead whale (*Balaena mysticetus*) is an ideal study taxon for questions of lipid deposition and utilization for it possesses the most extreme adipose reserves of any animal. Subcutaneous adipose (blubber) can reach up to 50cm in depth with blubber composing 40–50% of the approximately 90,000 kg total body mass in mature adults (Lowry 1993; Jefferson et al. 1993 and George et al. 2007). Mysticete whales, such as bowheads, use baleen (a specialized form of keratin) to filter and concentrate planktonic and benthic zooplankton (Dehn et al. 2006; Mead and Brownell 2005; Moore and DeMaster 1998; Hazard and Lowry 1984 and Lowry and Burns 1980). Seasonal waxing and waning of sea ice-cover affects primary and secondary productivity and result in bi-annual migrations and

concurrent seasonal shifts in feeding patterns (Fig 1). Periods of intense feeding during short, summer months contrast with what is likely only intermittent feeding in during Winter and Spring migrations (Lowry et al. 2004; Hoekstra et al. 2002; Dehn et al. 2006; Moore and Reeves 1993 and COSEWIC 2005). When born, the baleen of bowhead calves is less than 20cm in length (Schell and Saupe 1993; Schell et al. 1989; Lowry 1993 and Lambertsen et al. 1989; George, 2009). After weaning, bowhead youths experience a unique 4–6 year maturation period, not observed in other mysticetes (Nerini, et al., 1984; Koshi, et al., 1993; Schell et al, 1993; George et al. 2016). During this time, individual growth is almost entirely limited to the head region and the baleen lengthens and frays increasing feeding efficiency (Schell and Saupe 1993; Schell et al. 1989; Lowry 1993 and Lambertsen et al. 1989; Lubetkin et al. 2008; George, 2009; Fortune et al., 2012; George et al. 2016). As a result, immature youth whales are less efficient feeders and thought to rely primarily on lipid reserves for energetic demands (Lowry and Burns, 1980; Koski et al. 1993; Moore and DeMaster 1998; George et al. 1999; Dehn et al. 2006 and Rosa 2006). Extreme alterations in feeding patterns and prolonged developmental life history necessitate tight seasonal and ontogenetic regulation of metabolism and lipolysis.

Beluga whales (*Delphinapterus leucas*) are another exclusively Arctic/subarctic cetacean that provides an interesting contrast to bowhead whales. While smaller in size (averaging approximately 1,600 kg in weight), belugas also possess large adipose stores that can reach up to 12cm in depth and they undertake bi-annual migrations similar to those of the bowhead whales (Doidge 1990; Jefferson et al. 1993; Martin and Smith 1999; Suydam et al. 2001 and Suydam 2009; Fig 1). Unlike bowheads, belugas are odontocetes (toothed whales) which actively hunt benthic and pelagic prey throughout the year with diets varying with season and location (Frost and Lowry 1984; Welch et al. 1993, Watts and Draper 1986; Byers and Roberts 1995; Moore and DeMaster 1998 and Quakenbush et al. 2015). Differences in feeding strategy and prey availability may have lessened selection pressures for tight seasonal and developmental regulation of metabolism and lipid utilization.

We investigated quantitative and qualitative patterns of transcripts related to cellular metabolism (*Lep*, its receptors, LEPR and long isoform *LEPR–b*) and lipolysis (*LIPE* and *PNPLA2*) within blubber of bowheads and belugas varying in age and/or season. Our aims were to: 1) quantify and compare variations in metabolic and lipolytic activity with season, age and blubber depth, 2) test the predicted Leptin tertiary structure conservation of cetacean leptin compared to that of terrestrial mammals and 3) to assess cross-species variation of *Lep* transcript levels between bowhead and terrestrial mammals (rat, mouse, and human) the only mammals for which such data are currently available. Our findings provide valuable insight into the adapted regulation of *Lep* function and lipolysis specifically in bowheads and how these modifications affect whole organism physiology, providing valuable insights into how mammalian species can adapt to novel environments through physiological changes.

MATERIALS AND METHODS

Sample Acquisition

Post mortem, full-integument subcutaneous adipose (blubber) samples were obtained from bowhead whales (*Balaena mysticetus*) sampled during either Fall or Spring Inupiat subsistence hunts near Barrow Alaska under NOAA-NMFS permit 814–1899-03. Serum was also obtained from 15 individuals differing in season and age of sampling. An unusually large sample size (totaling 20 individual bowheads) were examined: three Fall mature adults, five Fall sub-adults, eight Fall youths (aged 1–4 years) as well as one Spring mature adult and three Spring youths (Table 1). Age-classifications of mature adult (12 meters and above in length), sub-adult (9–12 meters in length) and youth (less than 9 meters in length) were determined through measurements of body and baleen length and were based on bowhead-specific age estimations described by Lubetkin et al. (2008). Fall subsistence hunts and sampling occurred during the Fall migration from Beaufort Sea summering grounds to wintering grounds in the Bering Sea (Moore and Reeves 1993 and COSEWIC 2005; Fig 1). Spring subsistence hunts and sampling took place during Spring migrations from the Bering to the Beaufort Sea (Fig 1). Samples of subcutaneous adipose from five beluga whales (*Delphinapterus leucas*) were also acquired from subsistence hunts in Point Lay, Alaska; three mature adults and two youths. Age classifications were established based on observation of dermal coloring and body length observations (adults are pure white and over 2.5m in length; juveniles were grey in color and less than 2.5 meters) (Sergeant 1973; Brodie, 1989; Doige, 1990; Heide-Jørgensen and Teilmann 1994 and Suydam, 2009).

Beluga whales sampling was limited to summer, negating the ability to assess seasonal variations in this species. All samples from bowheads and belugas were acquired with the full cooperation of Inupiat captains and hunters. Blood sera was processed and immediately frozen; tissue samples were immediately preserved in RNAlater® (Ambion, Grand Island, NY, USA) prior to storage at −80°C.

Rodent adipose samples were acquired, post-mortem, from three 6-week old C57BL/6J female mice and three 12-week old Long-Evans female rats under published guidelines of animal care and use (NIH Publication No. 85-23, revised 1996). Samples were collected and immediately preserved in RNAlater® (Ambion, Grand Island, NY, USA) prior to storage at −80°C. Human total subcutaneous adipose RNAs, pooled from 20 individuals, were purchased for different body mass indices (BMIs): randomized BMI's (Clontech, Mountain View, CA, USA), individuals with BMI lower than 24.99% (ZenBio, Research Triangle Park, NC, USA) and those with BMI exceeding 30% (ZenBio, Research Triangle Park, NC, USA).

RNA isolation/cDNA synthesis

Cetacean subcutaneous adipose (blubber) is extensive in depth. Blubber samples utilized in this study when measured from the base of the epidermis to the top of hypodermis averaged between 15–20 cm in bowheads and approximately 10cm in belugas. To assess variation in gene expression with depth, blubber was subsampled at three different depths. For bowheads, superficial samples were isolated 6cm from the base of the epidermis, deep

blubber was sampled 6cm above the connected hypodermis and intermediate blubber was sampled midway between the sites of superficial and deep sampling. Beluga blubber was sampled in a similar fashion: superficial samples were isolated 2cm from the base of the epidermis, deep blubber was sampled 2cm from the connected hypodermis and intermediate samples were taken midway between these two sampling locations. Total RNA was isolated from all tissues under RNAse-free conditions (RNAse OUT™, GBiosciences, St. Louis, MO, USA) following recommended TRI-Reagent® (Ambion, Grand Island, NY, USA) protocols for high lipid samples. RNA was quantified with triplicate reads using a Nanodrop® ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) and gDNA contamination was assessed via PCR (**Online Resource 1**) in an Eppendorf Personal minicycler (Eppendorf, Hauppauge, NY, USA). cDNAs and no reverse transcriptase controls were synthesized from extracted mRNAs and purchased human RNAs following manufacturer recommended protocols for the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Grand Island, NY, USA), quantified based on triplicate reads on a Nanodrop® ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) and normalized to 50ng total RNA.

QPCR Assays

Lep, LEPR, LEPR-b, PNPLA2, and *LIPE*, were amplified from cetacean, rodent and human cDNAs using specifically designed gene and species-specific primers (**Online Resource 1**). All gene and species-specific qPCR products were sequence verified with BigDye® Terminator v3.1 Cycle Sequencing kit on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA) before continuing analyses (**Online Resource 2**). Quantitative PCR reactions were run in triplicate with no reverse transcriptase and primer controls on an ABI 7300 (Applied Biosystems, Grand Island, NY, USA) using iTaq™ SYBR® green Supermix with ROX (Biorad, Hercules, California, USA). Efficiency was assessed using previously published protocols (Yuan et al. 2007; **Online Resource 2**). To avoid known complications resulting from variable control/housekeeping gene expressions, which demonstrated wide disparity in expression among and across animals of the same age and sampling season, we used a previously validated form of absolute qPCR which allowed for comparisons of absolute transcript copy number, in equivalent amounts of starting RNA, among whales of different ages and harvest season and between species in comparative analyses (Ball et al. 2013).

ELISA

Enzyme-linked immunosorbent assay (ELISA) was conducted with a heterologous rat Leptin kit (RayBiotech, Norcross, GA, USA) following recommended manufacturer's protocols. Bowhead whale sera were processed at the time of sampling for each individual (Table 1). Comparisons of titer were calculated using analysis of variance calculations (ANOVA) and Wald and Wolfowitz tests (Wald and Wolfowitz 1940).

Data Analysis

Gene and species-specific dilution curves and linear regression equations were generated for each target gene and utilized in analyses of target transcript copy numbers from cetacean,

rodent and human cDNAs (**Online Resource 3**; Ball et al. 2013). qPCR efficiency was calculated following previously published methods for all assays (**Online Resource 3**; Yuan et al. 2007; Ball et al. 2013). We calculated average expression levels for each gene at superficial, intermediate and deep adipose levels as well as an overall gene average. We conducted a two-way ANOVA to compare variation in metabolic and lipolytic activity with season and age in bowheads. We examined both overall expression levels and deep blubber expression levels as previous work has primarily analyzed deeper adipose levels. When appropriate we applied log values for gene expression to improve variance equality based on Levene's test for equality of variances. Effect sizes for factors and their interaction were estimated using partial η^2 (e.g., Cohen, 1988). Given the lack of seasonal variation in our Beluga sample (i.e., only summer samples were available), we compared gene expression between adult and young Belugas using a t-test. Finally, we considered variation in transcript numbers across blubber depths in Bowheads using a repeated measures ANOVA. For significant results, pairwise comparisons were conducted between depths based on the least significant difference (LSD) criterion. Effect sizes were estimated using partial η^2. While we acknowledge that sex is likely an additional factor potentially influencing gene activity, we lacked enough males and females for each age and season to effectively consider this variable. An initial analysis including sex demonstrated no significant sex effects and low power leading us to exclude sex as a factor in the current analysis. All tests were evaluated at α=0.05.

Homology Modeling of Whale Leptin

Complete Leptin amino nucleic acid sequences were determined for both bowhead and beluga whales (**Online Resource 3**) and predicted amino acid sequences determined. Homology models for bowhead and beluga Leptins were created using YASARA. The top model was aligned to known human leptin using the MUSTANG algorithm (Konagurthu et al. 2006). Molecular dynamic simulations were performed using the AMBER03 force field in YASARA with 0.997 g/mL water. Interactions with the leptin receptor (LEPR-b) are based on our previous docking work from human Leptin and LEPR-b (Prokop et al. 2012).

Results

Variation in metabolic and lipolytic activity across blubber depth and season

Seasonal effects or age and season interactions were detected in all genes assayed for metabolic and lipolytic activity in bowhead whales. Fall mature and sub-adult bowheads exhibited higher number of *Lep* transcripts, a minimum average of 16,000 *Lep* transcripts per 50 ng total RNA, than Spring counterparts (Fig 2A). Two-way ANOVA identified a significant effect of season on average *Lep* transcripts with Fall mature and sub-adult bowheads exhibiting higher transcripts in blubber than adult Spring sampled bowheads ($p = < 0.001$; Table 2; Fig 2A). *Lep* transcripts in deep blubber were also significantly different with season ($p= < 0.001$; Table 3). Fall mature and sub-adults demonstrated more *Lep* transcripts in deep blubber than the Spring adult counterparts (Fig 2A).

Two-way ANOVA were utilized to examine the effects of season on transcript levels of *LEPR* and *LEPR-b* from Fall mature and sub-adults compared to the Spring adult. Analyses

found significant seasonal effects in *LEPR* transcript levels only when transcript numbers from all three blubber layers (superficial, intermediate and deep) were averaged (p= < 0.001; Table 2; Fig 2B); but not when the metabolically active deep blubber values alone were considered (p = 0.097; Table 3). Two-way ANOVA examining seasonal effects on *LEPR-b* isoform detected significant differences in deep blubber transcript levels (p = < 0.001; Table 3; Fig 2C) but no significant difference when layers were averaged (p = 0.093; Table 2).

Lipolytic gene expression also demonstrated seasonal differences in bowhead whales. For both Fall and Spring animals, the highest transcript concentrations were detected in the deep blubber location (Fig 3). Two-way ANOVA detected significant seasonal differences in transcript numbers for *PNPLA1* and *LIPE* (Tables 2, 3). Spring sampled mature and youths demonstrated higher average transcripts of *LIPE* and *PNPLA2* than Fall mature and sub-adults (Fig 3). Likewise, *PNPLA2* and *LIPE* transcripts in deep blubber were in the Spring adult and youths than the Fall sampled mature and sub-adults (Fig 3). For belugas, sampling was conducted only during summer and seasonal affects could not be addressed.

Variation in metabolic and lipolytic activity across blubber depth with ontogeny

Ontogenetic effects were also detected in metabolic and lipolytic activity. Differences were more pronounced in bowheads than in beluga whales. Two-way ANOVA examining the effects of age on average *Lep* transcript numbers detected significant differences across ages (p = < 0.001; Table 2). Fall and Spring youth bowheads tended to exhibit reduced expression compared to mature and sub-adult same-season counterparts (Fig 2A). These significant differences in *Lep* transcripts were still apparent when only deep blubber transcript levels were utilized for two-way ANOVA analyses (p= < 0.0001; Table 3; Fig 2A). Interestingly, these youths expressed higher *Lep* levels, not in deep blubber as might be expected, but in the most superficial layer, which was a similar pattern to spring adult whale (Fig 2A). However, repeated measure ANOVA examining variations across blubber layers for all individuals of all seasons detected no significant difference in *Lep* transcript number (p = 0.149; Table 4).

Significant ontogenetic differences were also detected in two-way ANOVA of age on average *LEPR* and *LEPR-b* expression (Fig 2B, C; Table 2). The effects of age on transcript levels was found to be significant for both *LEPR* and *LEPR-b* when both average (p = < 0.001 for both genes; Table 2) and deep blubber (p = < 0.001 for both genes; Table 3) transcripts were considered, suggestive of age-related differences in *Lep* receptor complements. The significant interaction between age and season for *LEPR-b* likely reflects that Fall youth exhibit the lowest expression levels while Spring youth exhibit the highest average levels of expression (Fig 2C).

Genes involved with lipolytic capacity also demonstrated ontogenetic differences in bowhead whales. Both *PNPLA2* ((p = < 0.001) and *LIPE* ((p = < 0.001) demonstrated significant differences in transcript numbers with age than same season adult counterparts (Fig 3; Table 2). Bowhead youths showed lower average transcript levels in the Fall, but youth transcript levels in the Spring were either higher (*PNPLA2*) or similar (*LIPE*) to those of adults (Fig 3). Similarly, two-way ANOVA conducted to examine the effects of age on

transcript levels from deep blubber also showed significant differences in both *PNPLA2* ((p = < 0.001) and *LIPE* (p = < 0.001) (Fig 3; Table 3).

Variation in metabolic and lipolytic activity with ontogeny in belugas

Two-sample T-tests of transcript levels between summer adults and youths found significant differences with ontogeny in beluga *Lep* transcript levels when averaged (p= 0.046) and within the deep blubber layer (p= 0.002; (Fig 2D; Table 5). Significant differences were also detected in average (p =0.016) and deep blubber transcript levels of *LEPR*, but not in average or deep *LEPR-b* transcript levels (p= 0.606 and p = 0.053, respectively; Fig 2E, F; Table 5).

Summer youth belugas also demonstrated ontogenetic differences in lipolytic gene expression compared to summer adults, although these differences were more pronounced in bowhead whales. Two-sample T-tests detected a significant difference in average transcript number in *PNPLA2* (p = 0.013) but not average *LIPE* (p = 0.063; Table 5). When analyses examine transcripts from deep blubber, transcripts of *LIPE* (p = 0.044) were significantly different, but not those of *PNPLA2* (p = 0.052; Fig 3; Table 5).

Seasonal and Ontogenetic Differences in Leptin Titer

Examinations of seasonal differences in plasma Leptin protein detected significantly higher levels in mature and sub-adults sampled during the Fall season compared to those from the Spring (p= 0.034; Fig 4). Leptin plasma level comparisons among Fall age categories detected significantly lower Leptin titer levels (p= 0.042) from sera of sampled youths compared to samples from mature and sub-adult whales (Fig 4).

Leptin Homology and Function is Conserved

The amino acid sequence of bowhead Leptin demonstrates a high degree of similarity and phylogenetic association with other cetaceans and terrestrial artiodactyl species. Homology modeling techniques, applied to the novel bowhead Leptin sequence, demonstrate that Leptin tertiary structure is highly conserved when compared to the known human Leptin crystal structure (Fig 5A). The protein models provide similar molecular dynamic simulations suggesting no changes in structural packing or dynamics among species (Fig 5B). Two variants (blue) are found between beluga and bowhead Leptin; however the variants are outside of the binding pocket with LEPR (Fig 5C) suggesting that these variants do not account for alteration in Leptin signaling. Several amino acids differ between human and whale Leptin proteins with only a single functionally conserved hydrophobic variant (L110V) found at the interface between leptin and LEPR (Fig 5D). Together, these data suggest that Leptin function in bowhead and beluga whales is not affected by structural modifications to the protein or to LEPR binding interaction (Fig 5).

Cross-Species Comparative Differences in Leptin Expression

Lep transcript levels were compared among cetaceans (bowhead and beluga whales), humans of varying BMIs and two rodent species (Long-Evans rats and C57BL/6 mice) all taxa for which data are currently available (see reviews by Friedman and Mantzoros, 2015; Münzberg and Morrison, 2015; Park and Ahima, 2015). Equivalent amounts of starting RNA

material were examined across species, and bowhead blubber produced, on average, between 1,000–42,000 copies per 50 ng total RNA. Furthermore, we detected variation in *Lep* transcript expression of more than *50 fold difference* within a year. This is significantly higher expression than that found in subcutaneous adipose of mice ($p = 0.04$), rats ($p = 0.03$), or human total adipose ($p = 0.046$) of any studied BMI (Fig 6). Bowheads produce *Lep* transcripts with greater abundance than other mammalian taxa studied to date (Fig 6).

DISCUSSION

Model animal systems have been vital to determining the functional and mechanistic regulation of lipid deposition and utilization. Against this backdrop, it is possible to measure variations in lipid metabolism necessary to facilitate life in extreme habitats. Cetaceans have acquired extensive evolutionary adaptations to contend with colonization of fully Arctic habitats, including the acquisition of extensive subcutaneous adipose stores (Tsagkogeorga et al. 2015). Here, we examined how metabolic and lipolytic activity varied with age, depth and season in two Arctic adapted cetaceans and then examined how those patterns compared to those of several well-characterized mammal systems.

Bowhead and belugas express transcripts of *Lep, LEPR, LEPR-b, PNPLA2* and *LIPE* which demonstrate high sequence homology with artiodactyls (**Online Resource 2**; Ball et al. 2013). Furthermore, tertiary structure of Leptin, a four-helix bundle similar to other class-1cytokines, is remarkably conserved and the interaction of this protein with its receptor appears to be unchanged through mammalian evolution (Zhang et al. 1997, Crespi and Denver 2006; Prokop et al. 2012 and Niv-Spector et al. 2005). Tertiary structure models of bowhead Leptin further support conservation with the majority of amino acid substitutions in bowhead Leptin or leptin-receptor (LEPR) on external locations away from known leptin-receptor binding sites (Zhang et al. 1997; Crespi and Denver 2006; Niv-Spector et al. 2005; Hammond et al. 2012 and Prokop et al. 2012; Fig 5). Given the physiological and developmental importance of Leptin, high conservation of binding interaction is not surprising and suggests modifications in Leptin function stem not from differences in ligand-receptor binding capabilities but from seasonal, temporal or age-related alterations in gene expression or regulatory pathways. This would also suggest that future characterizations of genomic changes within the promoter of *Lep* of bowhead and beluga whales would provide valuable insights into functional regions of *Lep* gene transcription.

Studies of non-model taxa have traditionally been hindered by the inability to compare expression among species. To generate these data, we used absolute real-time quantitative PCR (qPCR), which allows for sensitive, reproducible measurements and inter-species comparisons (Ball et al. 2013). Our methods permitted the quantification of seasonal and age-related variations in expression in two families of cetaceans. Further, only absolute quantification allows comparison among species (the comparisons to mouse and human data are not possible with relative qPCR). Traditional qPCR assays compare “target” gene expression to that of an endogenous control, but these “controls” are often unreliable, especially in non-model organisms such as cetaceans, where sample rarity limits validation of controls (**Online Resource 4**; Ball et al. 2013). Studies of marine mammal physiology are commonly hindered by the physical size of the organism, aspects of life history, access to

viable samples and regulatory considerations. However, recent studies have begun to employ qPCR to examine immune function and pollution response in various taxa (Sitt et al. 2008; Kakuschke et al. 2006 and Mollenhauer et al. 2009). Here, we utilized absolute qPCR to identify novel seasonal and ontogenetic differences in genes associated with metabolic and lipolytic activity. The unique size and extent of cetacean adipose depots also permitted unique examination of expression differences at various depths within the blubber providing insight into the dynamics of blubber metabolism.

Leptin (*Lep*) is well-established as the primary hormone regulating mammalian adipose stores, and examinations of sex-specific, developmental and seasonal differences in humans and other mammals led to the development a simple model: that a positive correlation exists between total adipose stores and Leptin titer/*Lep* expression (Zhang et al. 1994; Dussere et al. 2000; Hube et al. 1996; Montague et al. 1997; Havel et al. 1996; Kelesidis et al. 2010 and Considine et al. 1996). Traditionally considered simply a barometer of total adipose storage, *Lep* is now considered an active participant in the liberation of lipids in response to changing energetic demands. For some taxa, particularly those undergoing seasonal hibernation or migration, a temporary *leptin resistance* must be conferred to reduce the anorexigenic effects of *Lep* and permit deposition of sufficient adipose to survive an extended period of fasting (Kronfeld-Schor et al. 2000; Cammisotto and Bendayan 2007 and Bates and Myers 2003). Arctic adapted cetaceans, such as the bowhead and beluga whales, maintain enormous adipose depots suggesting possible modifications of *Lep* or *LEPR* function.

Bowhead whales experience a period of intense summer feeding during which adipose stores are renewed, at least to a large extent, prior to Fall migration and presumed Winter fasting. Tissues collected from Fall mature and sub-adults showed very deep blubber, with an average depth of ~18cm. In accordance with *Lep* dogma, bowhead whales, with their large adipose stores, expressed high concentrations of Leptin protein. Mature adult Fall hunted whales produced extreme levels of *Lep* transcripts: assays indicated individuals produced averages of 20,000 to 50,000 transcripts per 50ng total RNA in their blubber (Fig 2). The highest concentrations were exhibited in the deep, most metabolically active, blubber layer (Thellin et al. 1999; Bustin 2002 and Jorgensen et al. 2006). Fall sub-adults also demonstrated elevated *Lep* mRNA transcripts although lower than those detected in mature Fall adults (Fig 2). Spring sampled bowheads contrast starkly with Fall whales. Unlike recently well-fed Fall mature and sub-adults, the Spring mature and youths were sampled returning to summer feeding grounds after a presumed period of prolonged Winter fasting. Despite this, both age classifications still possess and require large quantities of blubber (average depth of sampled whales was ~16cm) for buoyancy and thermal regulation (Iverson 2002 and George et al. 2007). Another explanation for this occurrence is that, blubber may be a structural organ, where thickness changes little across season or life stages, to maintain a streamlined body shape and for storage of lipids obtained in the future (Ball et al. 2015; J. C. George, personal observation). Indeed, histological analyses of blubber from bowhead whales detected seasonal reductions in adipocyte cell size (not adipocyte numbers) which were offset by increases in structural fiber density to maintain total blubber depth and streamlining with season (Ball et al. 2015). *Lep* dogma would predict that depletion in lipid stores would lead to reduced transcript levels and that again appears to be the case: Spring mature adult and youths both demonstrated severely reduced *Lep* mRNA production (with

averages of only 1500–4300 copies per 50ng total RNA (Fig 2). We sought to confirm our mRNA findings through examinations of circulating Leptin protein from Fall and Spring sampled bowheads. The lack of a bowhead-specific assay required the utilization of a commercially available heterologous assay, but despite these shortcomings, results appeared to confirm transcript results: titer detected from sera of Fall sampled mature and sub-adults were significantly higher than those from Spring sampled adult and sub-adults, suggesting circulating protein titer in bowheads varies in sync with variations seen in *Lep* transcript expression (if not in magnitude; Fig 4). It is well established that hibernating and migrating mammals maintain high *Lep* levels throughout hibernation/migration and experience a sudden, significant reduction in transcripts prior to awakening/migratory return, and it appears bowheads may also experience this phenomenon (Ormseth et al 1996; Hissa et al 1998; Kronfeld-Schor et al. 2000; Rousseau et al. 2003; Townsend et al. 2008 and Florant and Healy 2012). From a physiological perspective, low *Lep* transcript concentrations relay a signal of adipose depletion to the animals. This sense of “starvation”, combined with other migratory cues (such as ice cover, water temperature and photoperiod changes) may assist in driving migrations to and from summer feeding grounds.

Leptin exerts its physiological influence via interactions with the leptin receptor (*LEPR*). Only *LEPR-b* possesses all intracellular and membrane spanning domains necessary to mediate the full physiological effects of *Lep* (Fong et al. 1998 and Munzberg et al. 2005). Regardless of season, significantly higher *LEPR-b* transcript concentrations were detected in deep blubber compared to superficial blubber samples, which might be expected due to the increased vasculature of deep blubber (McClelland et al. 2012; Fig 2). What was not predicted were the significantly higher concentrations of *LEPR-b* transcripts encountered in deep blubber in Spring mature and youth whales (Fig 2). Increased expression of *LEPR-b* transcripts, coupled with greatly decreased *leptin* mRNA expression, suggests an additional mechanism of increased *Lep* sensitivity in these animals. They not only produce significantly less *Lep* transcript (thereby triggering physiological responses towards increased feeding and metabolic conservation), but produce significantly higher transcript levels of *LEPR-b* in accessible deep blubber layers with which to “read” this signal. Seasonal shifts in receptor complement have been documented in other mammals, and are now confirmed in mature and sub-adult bowheads (Baskin et al. 1998 and Townsend et al. 2008).

Marine mammals blubber is a highly organized connective tissue matrix containing adipocytes, which store lipids in the form of either triacylglycerols (mysticetes) or wax esters (odontocetes) and function in energy storage as well as in signaling and homeostatic activities (Brown 2001; Haemmerle et al. 2011 and Smirnova et al. 2006). Here, we examined seasonal variation in transcript levels of two enzymes catalyzing the breakdown of triacylglycerols (TAGs) to free fatty acids (FFAs): adipose triglyceride lipase (*PNPLA2*) and hormone-sensitive lipase (*LIPE*). While work to correlate increases in *PNPLA2* and *LIPE* mRNA with protein expression was limited due to sampling constraints, we predicted differential expression with blubber depth as metabolic stratification is known to occur within blubber of several species (Ackman et al. 1975; Samuel and Worthy 2004; Meagher et al. 2008 and McClelland et al. 2012). Seasonal and age-specific variations in expression were detected with elevated concentrations of both *PNPLA2* and *LIPE* transcripts

demonstrated in mature adult and youth Spring whales (Fig 3). One explanation for this relates to the seasonal changes in prey availability experienced by bowheads, which require mobilization of lipids for energetic demands unlike other cetacean taxa (Samuel and Worthy 2004; Meagher et al. 2008 and McClelland et al. 2012). Elevated mRNA transcripts suggest they are liberating lipids, at least partially, via up-regulation of these lipolytic enzymes. Also, environmental and physiological stressors may influence *LIPE* expression, which is known to be hormonally regulated and influenced by such stressors (Slavin et al. 1994; Fruhbeck et al. 1997 and Kurpad et al. 1994). Lower concentrations in Fall mature and sub-adult whales may reflect the fact these animals are well-fed at the time of sampling and likely feeding during migration. Thus, they do not need to rely solely on stored adipose for energetic requirements and do not require the upregulation of lipolytic enzymes (Fig 3).

Bowhead whales, like all Mysticeti, are filter feeders utilizing plates of keratin (baleen) to filter zooplankton from surrounding waters. Development of fully functional baleen plate takes years to complete, during which time young whales are unable to feed efficiently, subjecting them to unique life history constraints (Lowry 1993; Lambertsen et al. 1989; Lambertsen et al. 2005; Reeves and Leatherwood 1985; George, 2009; George et al., 2016). The young feed heavily on lipid-rich milk, and as a result, possess high total fat content (George et al. 1999; George et al. 2007; Rosa 2006 and COSEWIC 2005). It was predicted that young bowheads would have high *Lep* transcript and titer levels due to the large volume of adipose deposits (Considine et al. 1996; Kelesidis et al. 2010). However, *leptin* transcript levels in Fall and Spring youths were extremely low and were most similar to levels detected in the Spring mature adult (Fig 2). Interestingly, the physiological repercussions of these low levels are appetite stimulation to encourage feeding and a reduction in metabolic rate. Youths of both bowhead and beluga whales demonstrated a pattern variant in their *Lep* expression which is consistent with this interpretation (Fig 2). Furthermore, the fact that the highest transcript numbers were detected in the superficial, not the deep (metabolically active) layer as was the case in all mature and sub-adults surveyed, seems to support a reduced metabolic rate during development. Once again, we sought to confirm our transcript findings with measurements of Leptin titer. The heterologous assay detected significantly lower Leptin titers in sera of Fall and Spring youths than samples from Fall mature and sub-adults (Fig 4). Ontogenetic differences were most strikingly apparent in bowheads, suggesting tighter developmental regulation of *Lep* in this species. Together, decreased *Lep* transcripts and differential expression suggest metabolic processes have evolved to drive feeding and minimize energy utilization for survival during baleen maturation.

LEPR-b transcript levels were reduced in Fall youth bowheads and belugas compared to same season adult age classes which may reduce *Lep* sensitivity and encourage adipose deposition in these whales (Fig 2). The effects were most pronounced in bowhead whales. However, both species might be further augmented by yet undetected downstream signaling differences. The slight increase in *LEPR-b* transcripts in more metabolically active deep blubber may also allow for detection of low *Lep* titer levels vital for other non-metabolic developmental pathways such as bone maintenance and immune response (Steppan et al. 2000; Carlton, et al. 2012; Motyl and Rosen, 2012).

Balancing maintenance of adipose stores with energetic demands is of particular importance to developing young whales. Youth bowhead and beluga whales demonstrated reduced expression of both *PNPLA2* and *LIPE* transcripts compared to same season adults (Fig 3). From a developmental perspective, an animal of this age strives to build and maintain extreme adipose stores and reduction in *PNPLA2* and *LIPE* expression likely assists in minimizing hydrolysis of existing fat stores and maintains necessary adipose deposits.

Knowing bowheads produce extreme transcript levels of *Lep* is of itself novel, but placing those expression levels into the context of well-characterized mammalian systems allowed us to determine if these extreme levels were indeed indicative of *Lep* hyper-production. We were able to compare the novel *Lep* transcript levels from bowheads and belugas to those detected in two rodent models (C57BL/6 mice and Long-Evans rats) and pooled RNA of humans differing in BMI (randomized, lower than 24.99% and exceeding 30%); both for whom *Lep* signaling pathways and function are well characterized. Here, the true novelty of the seasonal and ontogenetic variations in bowhead whales is fully appreciated. Human, rat and mouse *Lep* transcript concentrations were significantly lower than those detected in belugas and ~50–100 fold less than levels detected in Fall sampled adult bowheads (Fig 6). Within bowheads, seasonal and ontogenetic differences in *Lep* expression show a ~50 fold change in expression between Spring mature and sub-adults, Fall youths and Fall mature and sub-adults (Fig 6). It appears that in bowheads, *Lep* transcript levels are only similar to those of other mammalian adipose after extended periods of intermittent feeding or during tight developmental regulation of lipid utilization. In whale adipose, it is not simply a case of more adipose producing more signal; rather with equivalent amounts of total RNA, a significantly greater *Lep* transcript frequency is seen in whale when compared to similar tissues in mice, rats or humans.

Finally, why are there such extreme seasonal and ontogenetic variations in gene expression in bowhead whales? We hypothesize that bowheads, and to a lesser extent belugas, require *dramatic* differences in total *Lep* signal to induce significant physiological and behavioral changes affecting their adipose stores, feeding habits or initiation of migration. It is not simply the amount of signal arriving at the hypothalamus which elicits a response, but rather the overall *change* in signal tone that is registered and stimulates the physiological response (Townsend et al. 2008 and Florant and Healy 2012). This is a notable departure from the conclusions of typical mammalian obesity models. Whales appear to express extremely high constitutive expression due to their large adipose stores, and may have evolved to over-express *Lep* as a means of creating greater seasonal or developmental differences. Furthermore, the high degree of variation seasonally and developmentally in *Lep* transcript levels, well outside of the values of other mammals, do not appear to be offset by correlating increases in *LEPR*, suggestive of temporal *leptin resistance* in these cetaceans. Together results from this study imply that, in cetaceans, *Lep* functions more as an appetite control mechanism than a barometer of total lipid stores.

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Abbreviations

LEPR	leptin receptor
***LEPR*-b**	leptin-receptor b isoform
PNPLA2	adipose triglyceride lipase
Lep	leptin
LIPE	hormone sensitive lipase
MGLL	monoglyceride lipase
TAG	triacylglycerol
18S	18S ribosomal RNA
Rs9	ribosomal protein 9
Rs15	ribosomal protein 15
ANOVA	analysis of variance

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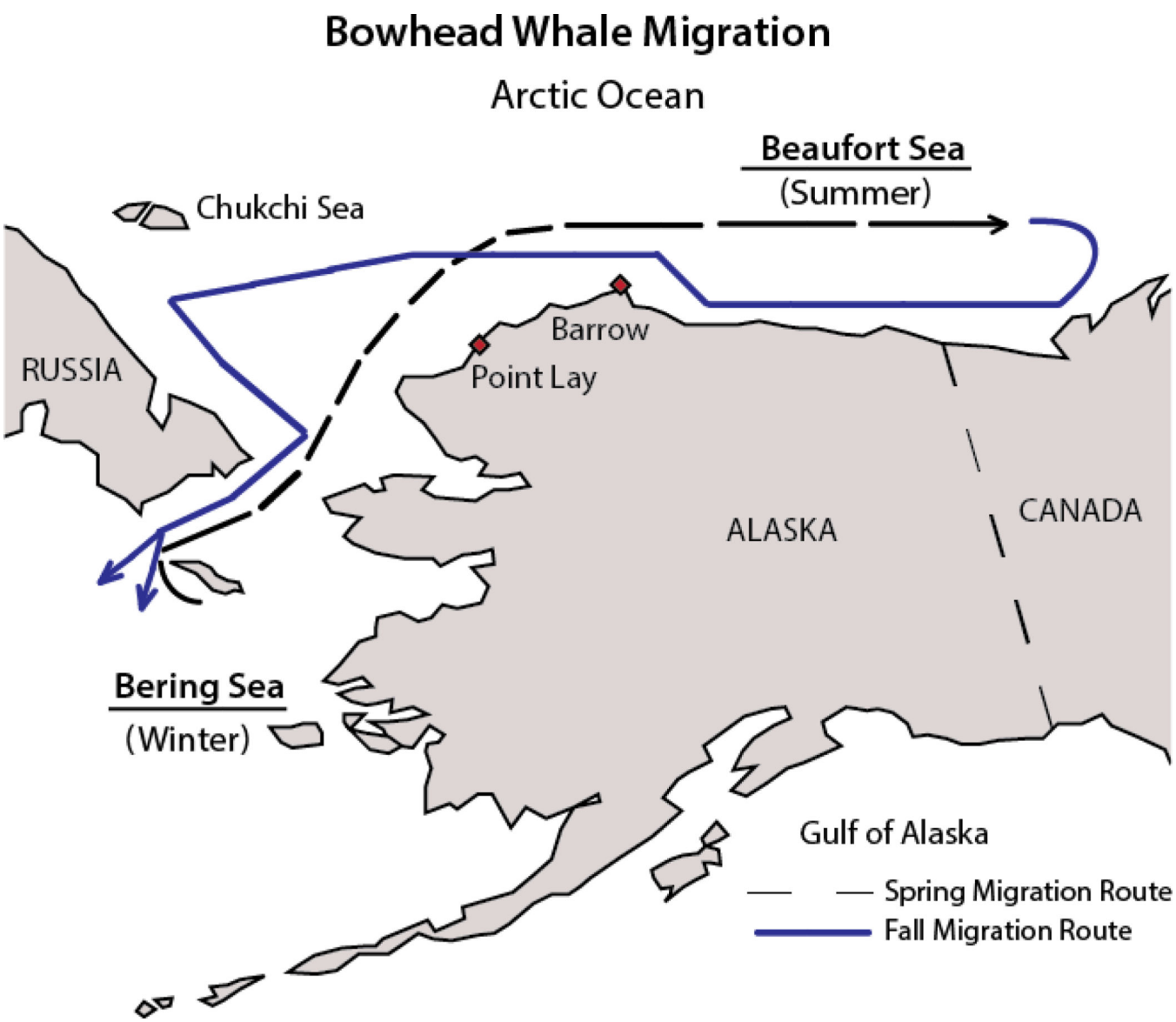

Fig 1. Bowhead and belugas share bi-annual migratory routes but bowheads likely experience more dramatic seasonal shifts in feeding patterns

Migration in both species occurs as a result of increasing sea ice cover and decreasing daylight in the Autumn. While beluga whales are pelagic and benthic feeders with probably more frequent feeding bouts, bowheads experience caloric restriction and dramatic, seasonal shifts in feeding due to differences in Arctic productivity (Quakenbush et al. 2010 and Citta et al. 2012)

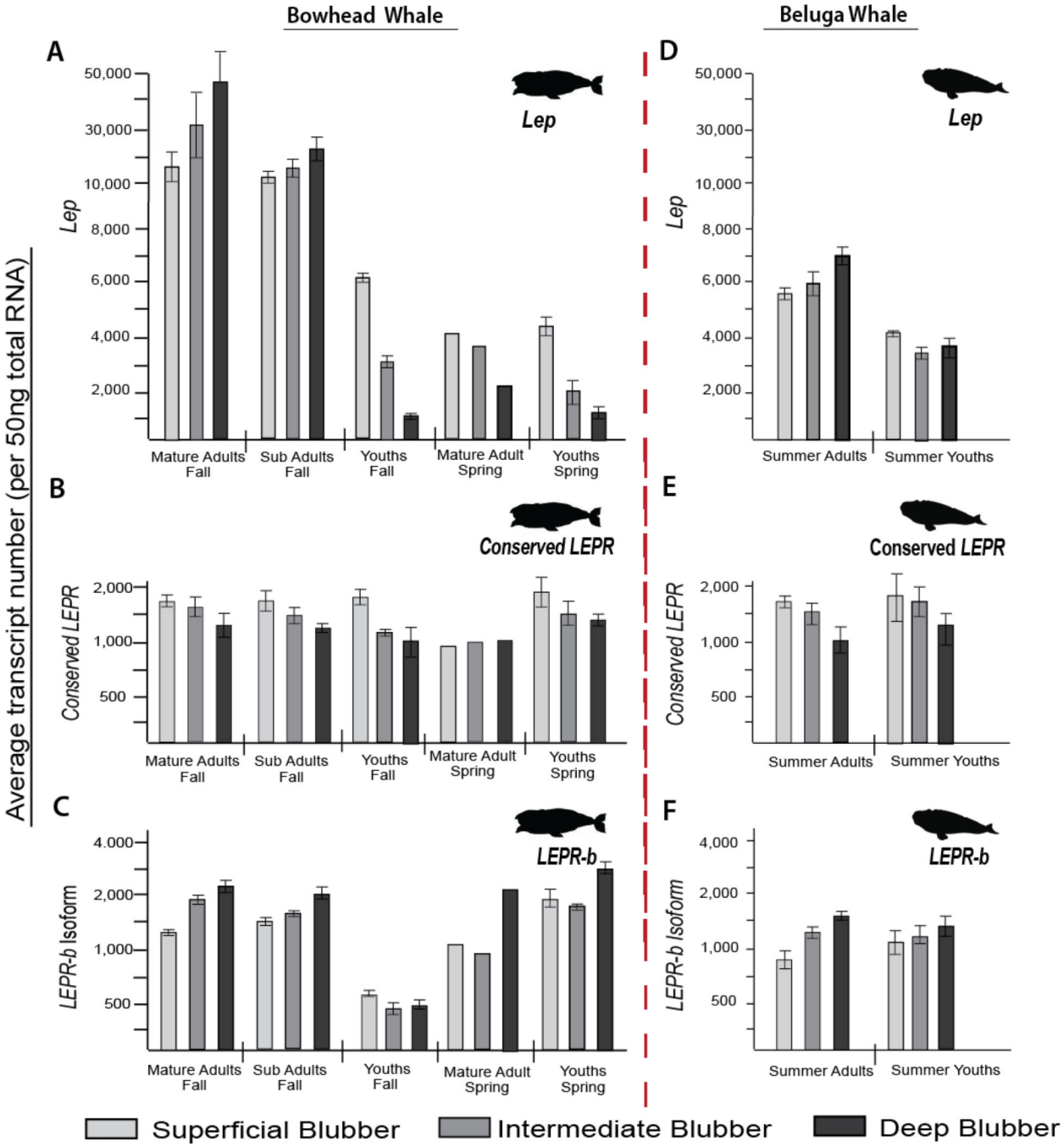

Fig 2. Seasonal and Ontogenetic Differences in transcripts of *Lep* and *LEPR-b*, but not conserved *LEPR*, in bowhead and beluga whales

Leptin, conserved *LEPR* and *LEPR-b* transcript levels differ significantly with age in bowhead (A–C) and beluga (D–F) whales and also with season in bowheads. Transcripts levels were measured per equivalent amounts of starting RNA sampled from subcutaneous adipose. Error bars depict 95% confidence intervals

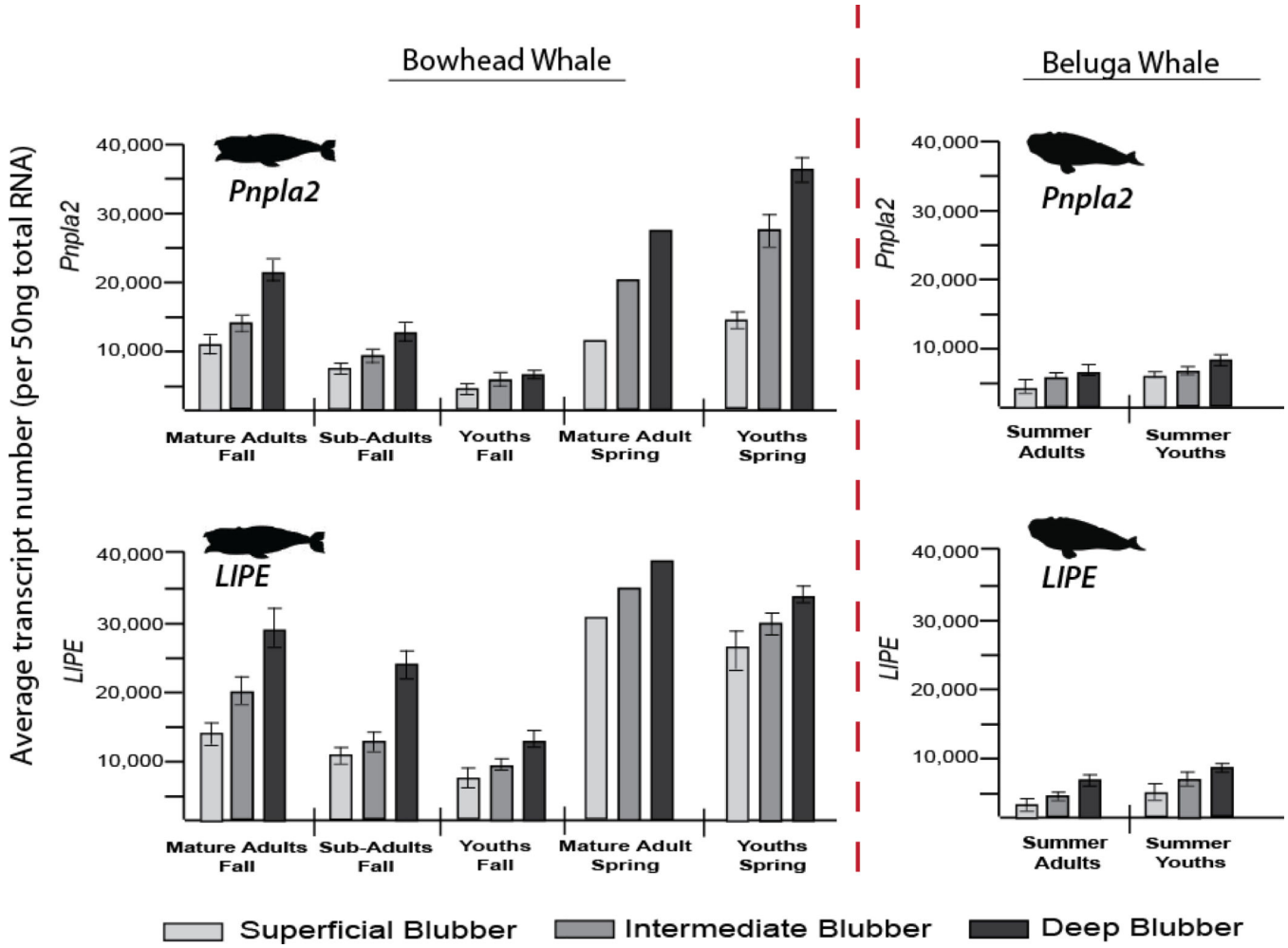

Fig 3. Seasonal and Ontogenetic Differences in Lipolytic Activity of bowhead and beluga whales

Analyses detected significant differences in *PNPLA2* and *LIPE* transcript levels with season and age in bowhead, but not beluga, whales. Transcripts levels were measured per equivalent amounts of starting RNA sampled from subcutaneous adipose. Error bars depict 95% confidence intervals.

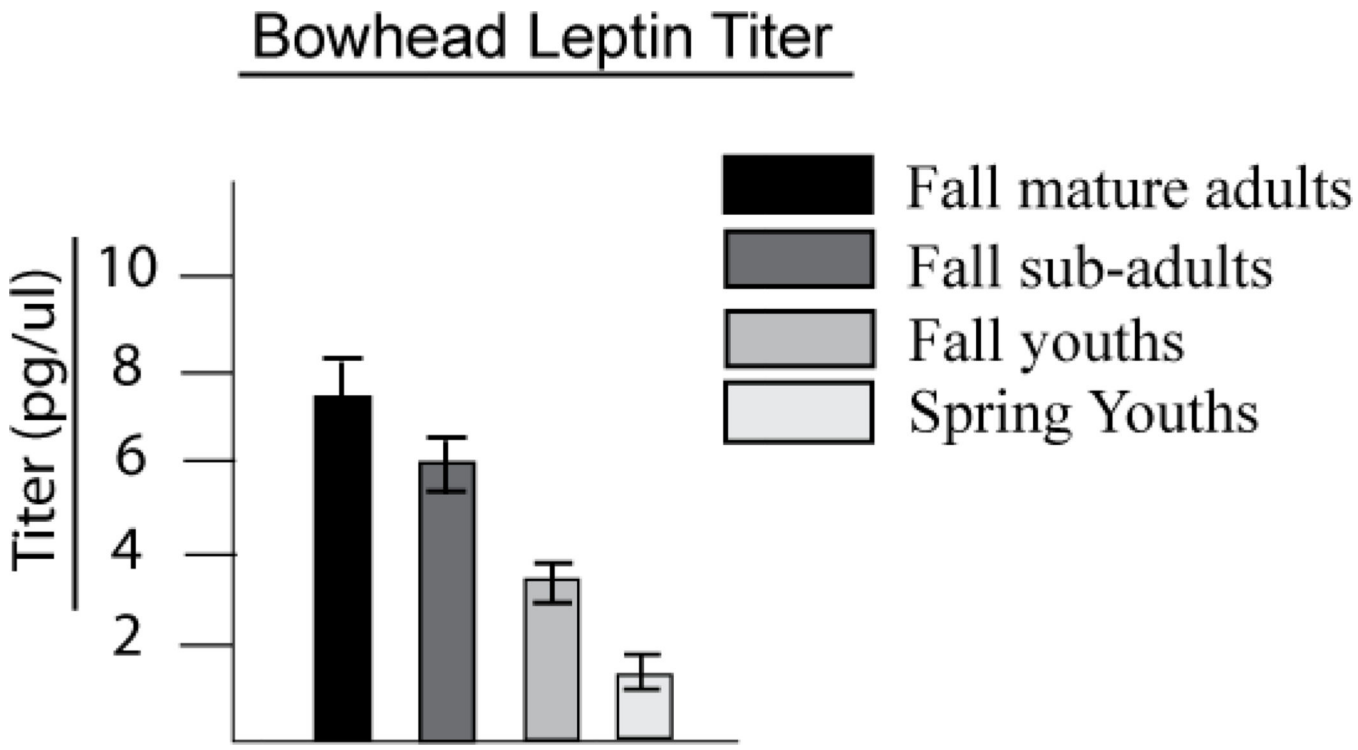

Fig 4. Measurements of Leptin titer (pg/μl) from bowhead sera correlated with seasonal and ontogenetic changes in mRNA expression of bowhead whales

Seasonal and ontogenetic variations in Leptin titer were measured from sera of mature and sub-adult Fall bowheads and three Spring sub-adults. Assays were conducted using a heterologous assay. Error bars depict 95% confidence intervals

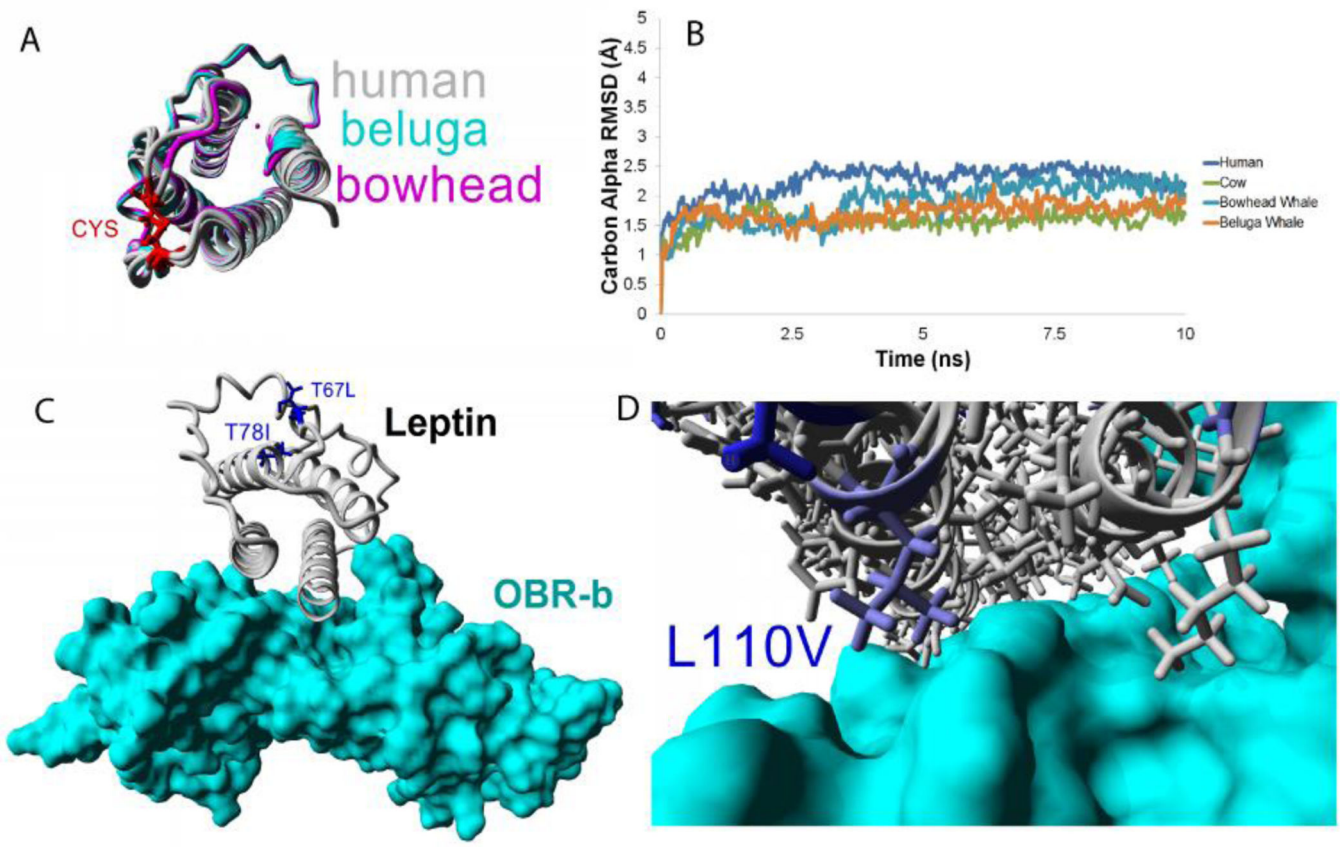

Fig 5. Dynamic model simulation comparisons of human, beluga and bowhead Leptins

(A) Homology models for human (gray), beluga whale (cyan), and bowhead whale (magenta) aligned to the solved NMR structure of human Leptin (pdb file 1ax8). (B) Molecular dynamic simulations of each model and also that of the closely related cow Leptin. (C) Variants (blue) between beluga and bowhead Leptin proteins (gray) shown relative to the binding pocket with LEPR-b (cyan). (D) Amino acids differences between human and the whale Leptin proteins shown for the interface between Leptin (gray) and LEPR-b (cyan) interactions

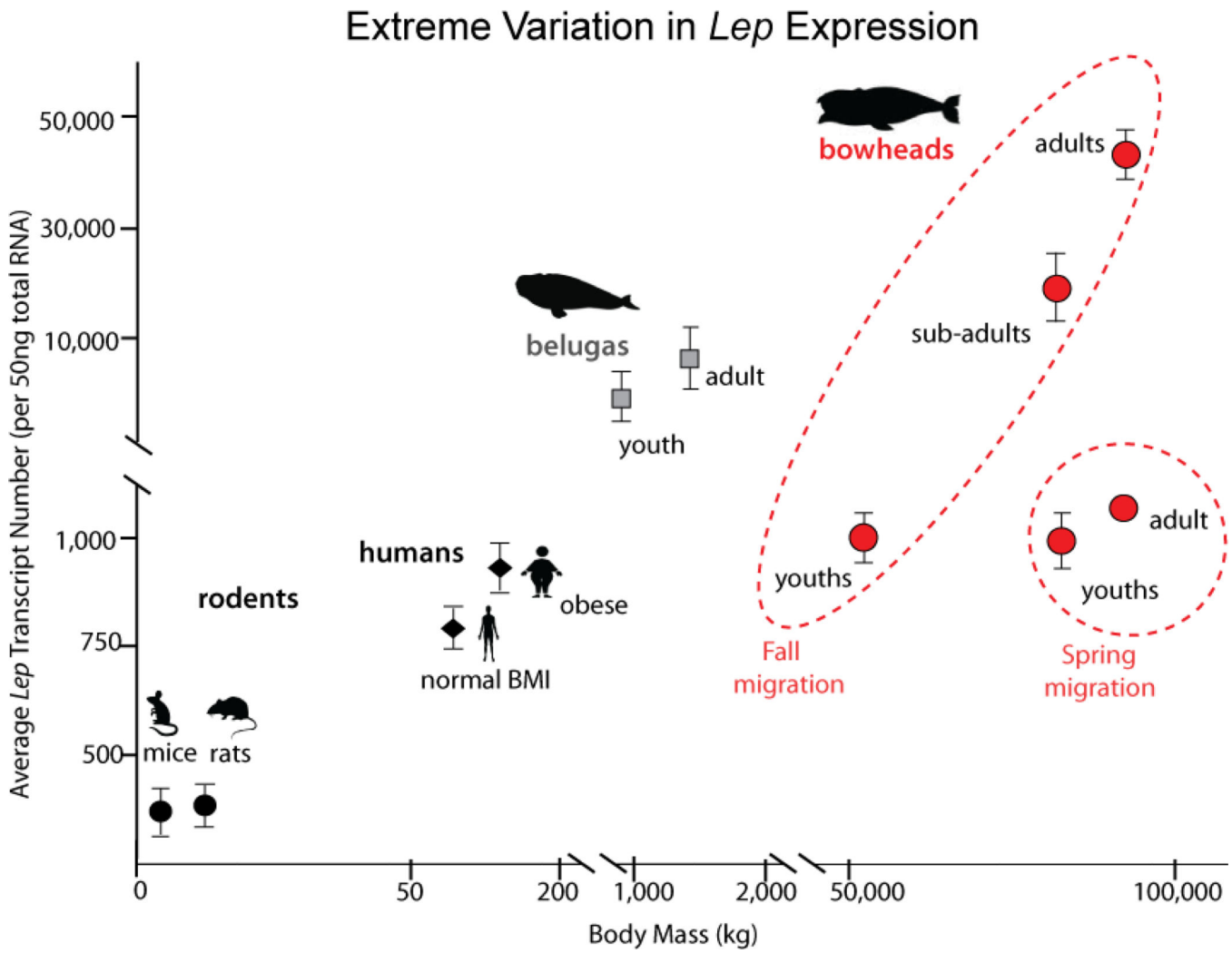

Fig 6. Bowheads demonstrate extreme *Lep* transcript variation with age and season

This multispecies comparison illustrates beluga whales, demonstrate slight variation in *Lep* transcripts with age and physiology. Bowheads are *unique* among mammals: adults in the Fall display a greater than two-fold higher expression than same season youths and Spring mature and sub-adults. This is unique because all cetaceans possess large adipose reserves regardless of age or season. Measurements of *Lep* transcripts number were from equivalent amounts of starting total RNA and error bars depict 95% confidence intervals

Table 1

Demographic information for sampled whales

Species	Sample Number	Season	Age Classification	Length (M)	Sex	Location of Sampling
Balaena mysticetus	2011B6	Fall	Mature adult	16.9	Male	Dorsal midline
	2011B9	Fall	Mature adult	12.5	Female	Dorsal midline
	2010B15	Fall	Mature adult	12.5	Female	Dorsal midline
	2012B18	Fall	Sub-adult	9.4	Female	Dorsal midline
	2012B19	Fall	Sub-adult	9.4	Male	Dorsal midline
	2009B7	Fall	Sub-adult	11.3	Female	Dorsal midline
	2009B14	Fall	Sub-adult	10.2	Female	Serum only
	2012B16	Fall	Sub-adult	10.3	Male	Dorsal midline
	2009B9	Fall	Youth	8.7	Male	Dorsal midline
	2009B11	Fall	Youth	7.2	Female	Dorsal midline
	2009B12	Fall	Youth	8.7	Female	Serum only
	2009B13	Fall	Youth	7.95	Male	Serum only
	2010B16	Fall	Youth	7.9	Male	Dorsal midline
	2011B8	Fall	Youth	8.4	Female	Dorsal midline
	2012B15	Fall	Youth	8.4	Male	Dorsal midline
	2012B20	Fall	Youth	8.9	Male	Dorsal midline
	2011B3	Spring	Mature adult	17.5	Female	Dorsal midline
	2012B6	Spring	Youth	8.2	Male	Dorsal midline
	2012B7	Spring	Youth	9.0	Female	Dorsal midline
	2012B8	Spring	Youth	8.3	Male	Dorsal midline
Delphinapterus leucas	2009LDL26	Summer	Juvenile	< 2.5	Male	Dorsal midline
	2009LDL25	Summer	Juvenile	< 2.5	Male	Dorsal midline
	2012LDL2	Summer	Adult	> 2.5	Female	Dorsal midline
	2012LDL8	Summer	Adult	> 2.5	Male	Dorsal midline
	2009LDL20	Summer	Adult	> 2.5	Male	Dorsal midline

Table 2

Two-Way ANOVA examining the effects of season and age on average transcript number in Bowheads. Bold type signifies statistical significance ($p \leq 0.05$).

Dependent Variable*	Age			Season			Age × Season Interaction		
	F, df	p-value	Partial Eta^2	F, df	p-value	Partial Eta^2	F, df	p-value	Partial Eta^2
Average Leptin	115.1, 2	< **0.001**	0.950	155.64, 1	< **0.001**	0.928	3.274, 1	0.095	0.214
Average conserved LEPR	1049.76, 2	< **0.001**	0.994	723.89, 1	< **0.001**	0.984	1388.08, 1	< **0.001**	0.991
Average LEPR-b	8696.05, 2	< **0.001**	0.999	1.23, 1	0.289	0.093	563.87, 1	< **0.001**	0.979
Average PNPLA2	1368.6, 2	< **0.001**	0.996	226.39, 1	< **0.001**	0.950	2615.93, 1	< **0.001**	0.995
Average LIPE	247.02, 2	< **0.001**	0.976	296.77, 1	< **0.001**	0.961	2.167, 1	0.167	0.153
Average 18S	13877.3, 2	< **0.001**	1.000	15.09, 1	**0.002**	0.557	3.026, 1	0.107	0.201
Average Rs9	2598.36, 2	< **0.001**	0.998	643.04, 1	< **0.001**	0.982	588.68, 1	< **0.001**	0.980
Average Rs15	4748.16, 2	< **0.001**	0.999	6.813, 1	**0.023**	0.362	335.23, 1	< **0.001**	0.965

*Log values were utilized when results of Levene tests for equality of variances were significant.

Table 3

Two-Way ANOVA examining the effects of season and age on transcript number in deep blubber of Bowhead whales. Bold type signifies statistical significance ($p \leq 0.05$).

Dependent Variable*	Age			Season			Age × Season Interaction		
	F, df	p-value	Partial Eta^2	F, df	p-value	Partial Eta^2	F, df	p-value	Partial Eta^2
Leptin Deep Blubber	335.89, 2	< **0.001**	0.982	278.56, 1	< **0.001**	0.959	1.814, 1	0.203	0.131
Conserved LEPR Deep Blubber	238.49, 2	< **0.001**	0.975	1.294, 1	0.278	0.097	84.22, 1	< **0.001**	0.875
LEPR-b Deep Blubber	17586.5, 2	< **0.001**	1.000	2292.0, 1	< **0.001**	0.995	3289.03, 1	< **0.001**	0.996
PNPLA2 Deep Blubber	419.67, 2	< **0.001**	0.986	31.466, 1	< **0.001**	0.724	1008.98, 1	< **0.001**	0.988
LIPE Deep Blubber	198.86, 2	< **0.001**	0.971	41.941, 1	< **0.001**	0.778	0.058, 1	0.813	0.005
18S Deep Blubber	3284.7, 2	< **0.001**	0.998	29.95, 1	< **0.001**	0.714	35.35, 1	< **0.001**	0.747
Rs9 Deep Blubber	686.48, 2	< **0.001**	0.991	0.097, 1	0.760	0.008	433.84, 1	< **0.001**	0.973
Rs15 Deep Blubber	1577.64, 2	< **0.001**	0.996	257.64, 1	< **0.001**	0.955	40.67, 1	< **0.001**	0.772

*Log values were utilized when results of Levene tests for equality of variances were significant.

Table 4

Repeated measure ANOVA examining variations in target transcript copy number with depth in blubber of Bowhead whales. Comparisons among blubber layers are results of pairwise comparisons.

Dependent Variable	F, df	p-value*	Partial Eta^2	Comparison Among Blubber Layers
Leptin	1.889, 2	0.149	0.106	
Conserved LEPR	63.914, 2	**< 0.001**	0.800	Superficial > Intermediate > Deep
LEPR-b	13.574, 2	**< 0.001**	0.459	Deep > Intermediate = Superficial
PNPLA2	15.921, 2	**< 0.001**	0.499	Deep > Intermediate > Superficial
LIPE	33.056, 2	**< 0.001**	0.674	Deep > Intermediate > Superficial
18S	123.425, 2	**< 0.001**	0.885	Deep > Superficial > Intermediate
Rs9	215.407, 2	**< 0.001**	0.931	Deep > Intermediate > Superficial
Rs15	195.745, 2	**< 0.001**	0.924	Deep > Intermediate > Superficial

* Bold values are statistically significant $p \leq 0.05$).

Table 5

Two-sample t-test comparing gene transcript number in youth versus adult belugas.

Variable	t, df	p-value*	Comparison Between Ages
Leptin Ave	13.561, 1.01	**0.046**	Adult > Youth
Leptin Deep Ave	11.874, 2.863	**0.002**	Adult > Youth
LEPR Ave conserved	−20.808, 1.239	**0.016**	Youth > Adult
LEPR Deep conserved	−19.595, 2.973	**<0.001**	Youth > Adult
LEPRb Ave	−0.603, 2.081	0.606	Youth = Adult
LEPRb Deep	4.022, 2.080	0.053	Adult = Youth
PNPLA2 Ave	−26.695, 1.191	**0.013**	Youth > Adult
PNPLA2 Deep	−11.313, 1.038	0.052	Youth = Adult
LIPE Ave	−6.001, 1.337	0.063	Youth = Adult
LIPE Deep	−3.628, 2.650	**0.044**	Youth > Adult

*Bold values are statistically significant $p \leq 0.05$).