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Seasonal variation in testes size and density detected in belugas (*Delphinapterus leucas*) using ultrasonography

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Belugas are thought to exhibit seasonal variation in testes size, but a temporal gap in postmortem sampling of wild belugas has precluded a description of the occurrence or the extent of this seasonal variation. This study aimed to utilize longitudinal monitoring of belugas in aquaria with known siring histories to assess seasonal variation in testes size, and its association with circulating testosterone concentration and testicular tissue density. Testes volume was estimated using linear measurements obtained via ultrasonography. Testicular tissue density was assessed by measuring the pixel intensity (PI) of ultrasound images of the testis. Five adult males, including 4 proven sires, were monitored for at least 1 continuous year; 2 of the males were monitored for > 2 years. A total of 154 ultrasound examinations (including 71 suitable for PI measurements) and 119 blood samples were available for analysis. Significant seasonal variation in testes volume, circulating testosterone concentration, and testicular PI were observed, with peak activity occurring between January and April. Seasonality of testicular volume was best described by a cubic function, while seasonal variations in testosterone and PI were best described by quadratic functions. Individuals differed significantly in both testes size and rate of change. On average, testes size increased by 60% from minimum to maximum values. These results are consistent with observations of reproductive seasonality both in the wild and in aquaria, and suggest a relatively low demand for sperm in this species that is consistent with their classification as induced ovulators.

Key words: beluga, *Delphinapterus leucas*, reproductive seasonality, testes size, testosterone, ultrasonography

In seasonally breeding mammals, males often demonstrate seasonal variation in the energetic investment for sperm production, conserving energy by reducing testes size or function when conceptions are unlikely to occur (Kenagy and Trombulak 1986). Among seasonally breeding odontocetes, seasonal variation has been detected in testosterone concentrations, testes size, sperm production, or seminiferous tubule diameter (reviewed in Plön and Bernard 2007). Testes mass can increase dramatically in odontocetes, with harbor porpoises (*Phocoena phocoena*) and common dolphins (*Delphinus delphis*) undergoing 5-fold increases in testes mass in the breeding season (Sørensen and Kinze 1994; Westgate and Read 2007).

Belugas (*Delphinapterus leucas*), an Arctic and subarctic species of odontocete, breed in the late winter or early spring in both the wild (Sergeant 1973; Burns and Seaman 1988) and in aquaria (Robeck et al. 2005) so that births occur in the summer approximately 15.5 months later (Robeck et al. 2015). With this seasonal reproductive pattern, male belugas would be expected to undergo changes in testes morphology. Male belugas in aquaria have a seasonal pattern of testosterone production, with peak concentrations occurring from January through April (Robeck et al. 2005). Conceptions also occur seasonally in aquaria, with 80% occurring in March through May, a range that agrees with estimates of the breeding season in wild belugas (Brodie 1971; Burns and Seaman 1988; Robeck et al. 2005). Postmortem evaluations of testes from wild belugas demonstrate that most males reduce spermatogenesis outside of the breeding season, evidenced by degeneration of germ cells and extensive debris within the seminiferous tubules or epididymides devoid of sperm in mature adults (Burns and

Seaman 1988; Heide-Jørgensen and Teilmann 1994). Seasonal variation in testes size also appears to occur in wild belugas (Heide-Jørgensen and Teilmann 1994; Kelley et al. 2014).

Wild belugas are primarily sampled during the summer, several months after the presumed peak in breeding. Therefore, insufficient data are available to definitively describe the extent of seasonal variation in testes morphology. Collectively, postmortem studies have reported testes size measurements from more than 300 adult male belugas, yet only 1 observation is available for the months of December through March, and relatively few observations are available for April, May, and November relative to June through October (Kleinenberg et al. 1969; Brodie 1971; Sergeant 1973; Finley et al. 1982; Burns and Seaman 1988; Heide-Jørgensen and Teilmann 1994; Kelley et al. 2014). Based on the current understanding of beluga breeding seasons, this gap in data occurs at a crucial time when testicular recrudescence is predicted to occur.

Longitudinal studies of live males with known maturity status and siring histories would fill the existing temporal sampling gap and help describe the seasonal variation in testes size and function in belugas. The testes of belugas are located within the abdominal cavity, ventral and caudal to the kidneys, necessitating the use of ultrasonography to monitor live animals (De Guise et al. 1994). Ultrasonography is commonly used to assess testicular function in domestic species and has been applied to the study of reproductive function in male odontocetes in aquaria and in the wild (Brook et al. 2000; Robeck et al. 2009; Alves et al. 2012; Kastelic and Brito 2012). Estimates of testicular volume from ultrasound images correlate well with actual testicular volume measurements (Gouletsou et al. 2008). In addition to size, ultrasonography can also be used to determine the density of the testis tissue via measurements of the image brightness; relatively brighter (hyperechoic) areas of an ultrasound image correspond to denser tissue. The density of testicular tissue measured using ultrasound is correlated with seminiferous tubule area and sperm production in bulls (*Bos taurus*—Brito et al. 2012). These measurements are made by determining the pixel intensity (PI) of the image using computer software, resulting in an objective measure that allows longitudinal comparisons. Although not as effective as testes size measurements for monitoring testes function, PI can be a good indicator of the attainment of sexual maturity or the cessation of sperm production in males undergoing contraceptive treatment (Ülker et al. 2005; Brito et al. 2012). Relative echogenicity has been used to assess seasonal variation in testicular function in white-sided dolphins (*Lagenorhynchus obliquidens*—Robeck et al. 2009), and measurements of testicular PI have demonstrated seasonal variation and variation with reproductive status in finless porpoises (*Neophocaena* spp.—Wu et al. 2010a, 2010b; Yu et al. 2016). Despite the value of these tools, very few individuals of any odontocete species have been monitored longitudinally to assess seasonal variation in testes function (Desportes et al. 2003; Robeck et al. 2009; Wu et al. 2010b).

Given the seasonal variation known to exist in testosterone concentrations in male belugas and the common pattern of seasonal variation in testes size in seasonally breeding odontocetes, it is likely that belugas also undergo seasonal changes in testes size. However, the degree of this change is unknown. The aim of this project was to determine if seasonal variation in testes size in belugas can be detected via the longitudinal monitoring of males with known siring histories. Circulating testosterone concentrations were monitored for comparison, and the effectiveness of measurements of testicular PI in evaluating seasonality was assessed.

Materials and Methods

Animals.—The testicular volumes and circulating testosterone concentrations of 5 adult male belugas were monitored longitudinally for at least 1 calendar year at 3 different aquaria (Mystic Aquarium, Mystic, Connecticut; SeaWorld San Diego, San Diego, California; and Vancouver Aquarium, Vancouver, British Columbia, Canada; Table 1). DL1 was monitored for 29 consecutive months from August 2007 through December 2009. DL2 was monitored for 5 months from December 2008 to April 2009, then again for 29 months from March 2012 through July 2014. Measurements of PI (described below) were made on ultrasound images from DL1 and DL2. DL3, DL4, and DL5 were all monitored for 12 consecutive months from January to December 2008. All 5 males were housed in outdoor exhibits with chilled and filtered synthetic or natural sea water and had access to at least 1 mature female throughout the study period. Four of the 5 males had previously sired at least 1 calf via natural breeding. This project was approved by the Institutional Animal Care and Use Committees of Mystic Aquarium (Projects #07002 and #12001) and the University of Rhode Island (Project #AN12-02-016) and followed ASM guidelines (Sikes et al. 2016).

Table 1.—Study animal characteristics and available data for belugas (*Delphinapterus leucas*) monitored longitudinally for seasonal variation in testes size and circulating testosterone concentration.

Animal	Age[a] (year)	Length[b] (m)	Weight[b] (kg)	Location	Temp. range (°C)	Sire	Ultrasound exams	Blood samples	Years studied
DL1	27	4.4	1,042	Mystic Aquarium	5.6–16.7	Yes	56	46	2.4
DL2	26	4.0	945	Mystic Aquarium	5.6–16.7	Yes	54	22	2.8
DL3	20	4.0	1,300	Vancouver Aquarium	8.3–12.5	Yes	16	18	1
DL4	26	4.0	1,009	SeaWorld San Diego	11.1–15.0	Yes	15	15	1
DL5	39	4.0	892	SeaWorld San Diego	11.1–15.0	No	13	18	1

[a]"Age" refers to age at the beginning of the study period.
[b]Length and weight measurements were taken once during the study period.

Calculating testicular volume via ultrasonography.—Ultrasound exams were performed on belugas trained to lie unrestrained in lateral recumbency in a straight line at the water's surface. Exams were attempted once or twice per month in July through December and twice per month in the months of January through June by a single operator at each aquarium. Specific ultrasound equipment varied by location (Mystic Aquarium: GE Logiq Book with 3C-RS transducer, GE Medical Systems, Wuxi, Jiangsu, China; SeaWorld: GE Logiq E and GE Logiq E Vet with 4C-RS transducer, GE Medical Systems, Wuxi, Jiangsu, China; Vancouver Aquarium: SonoSite 180 with C60 transducer, Fujifilm SonoSite, Bothell, Washington).

To ensure that measurements were being made at the appropriate angle, the observer first visualized the testicular mediastinum, the thin hyperechoic band passing through the center of the testis (Brook et al. 2000). Two still digital images of the longitudinal view of the testis were saved for analysis. If the length of the testis exceeded the footprint of the probe, then as much of the testis as possible was visualized with the caudal border contained within the image. Two still images of the transverse view at the midpoint of the organ were taken for each testis, for a total of 4 images per testis per exam. This allowed for replicates of each linear measurement in an effort to increase precision.

Using these still images, digital measurements to the nearest hundredth of a centimeter were performed by the ultrasound operator using the analysis software available on the ultrasound machine. Dorsoventral diameter (depth) and lateral diameter (width) were measured on the transverse images. Length was measured in longitudinal view. If the testis did not fit on the screen, then the length to the midpoint was calculated by measuring from the caudal border of the testis to the widest point of the testis. This measurement was then doubled to calculate the total length. The indirect measurement of testis length using a ruler placed on the abdomen (described for bottlenose dolphins, *Tursiops aduncas*, by Brook et al. 2000) was unreliable in pilot observations, perhaps due to the fat pads present along the ventrolateral surface of belugas, necessitating a direct approach with ultrasound for this study. Both testes were measured within the same day. Each measurement was taken on both images of the same view, and the average of these 2 measures was calculated and used for analyses. While operators varied by facility, the same operator performed all of the exams and measurements for an individual animal throughout the study. Operators were veterinarians or trained specifically for this project by veterinarians who routinely use ultrasonography as a diagnostic tool.

Total testicular volume (TTV), or the sum of the volumes of the right and left testes, was then calculated using Lambert's formula for the volume of an ellipsoid applied to each testis (Brook et al. 2000):

$$V = (LWD)(0.71).$$

Calculating testicular PI.—The PIs of the testicular parenchyma relative to the PI of the blubber layer from testicular ultrasounds from DL1 and DL2 were determined using Image J (http://imagej.nih.gov/ij/) as an indicator of tissue density. Because many factors can influence the PI of an ultrasound image, images were utilized for this analysis from a single ultrasound machine (GE Logiq Book ultrasound machine at Mystic Aquarium) when the gain was equal to 50 and the scan depth was the same for both testes examined on the same day (DL1 = 34 observations, DL2 = 37 observations). Three points of analysis per image were averaged to determine blubber PI while 6 points of analysis were averaged to determine PI of the testicular parenchyma. Points were selected from homogenous regions of the image, avoiding areas that would artificially increase or decrease the PI, including the relatively brighter mediastinum and relatively darker shadowed areas. Blubber PI was subtracted from the PI of the testicular parenchyma to normalize for differences in the pressure applied to the probe by the operator.

Various scanning depths were used to adjust for seasonal changes in blubber thickness (range: 17–25 cm). Altering the scanning depth will affect the PI measurements, which inhibits its use as an indicator of tissue density. To correct for variation in depth between images, a correction factor was developed. Ultrasound images of the testes were taken at 1-cm intervals from 17 to 25 cm depth from DL2 in the same ultrasound session on 4 separate occasions. The testis PI normalized to blubber PI was calculated for each depth and linear regression was used to determine the relationship between image depth and PI. PI was significantly correlated with scan depth ($P < 0.0001$). The equation of the line was determined to be PI = −1.8686(Depth) + 56.033 ($r^2 = 0.69$). Each blubber-normalized PI measurement was corrected for depth through the use of this equation for both individuals. To improve precision, the resulting PI value for the right and left testes were then averaged together for the final PI value used in further analyses.

Testosterone assay.—When possible, blood samples were collected into serum separator or sodium-heparinized vacutainer tubes within 24 h of the ultrasound exam from a ventral fluke vein collected via trained behavior as a part of routine veterinary monitoring. Blood samples were collected in the morning hours, typically between 0900 and 1000 h. After centrifugation (2,000 × *g* for 10 min at 10°C), 1 ml of serum or sodium heparin plasma was obtained and placed in cryogenic vials and stored at −80°C. Testosterone measurements in human serum and plasma are similar and either type of sample can be used in medical research (Tworoger and Hankinson 2006).

Blood samples were assayed for testosterone using an EIA (Cayman Chemical, Ann Arbor, Michigan; Item #582701) previously validated for use with beluga plasma and serum (Richard et al., in press). Blood samples were extracted prior to the assay with diethyl ether (Sigma-Aldrich, St. Louis, Missouri; Catalog #346136) according to the EIA kit manufacturer's instructions. This kit has 100% reactivity with testosterone. Cross-reactivities reported by the manufacturer were 140% for 19-nortestosterone, 27% for 5α-dihydrotestosterone, 18.9% for 5β-dihydrotestosterone, 4.7% for methyl testosterone, 3.7% for androstenedione, and 2.2% for 11-keto testosterone; all other cross-reactivities were below 1%.

Extracted blood samples were assayed primarily at 1:40, but ranged between 1:10 and 1:80 depending on the expected

concentration of testosterone (Robeck et al. 2005). All samples were assayed in duplicate and the means were used in calculations. Individual samples with a $\%B/B_o$ between 20% and 80% and a coefficient of variation (*CV*) < 15% were accepted. Samples with *CV* > 15% were re-assayed, and blood samples outside of the range of the kit were re-assayed at a different dilution.

Blood samples were assayed at 2 different times; samples collected between 2008 and 2009 were assayed in one group (A, 6 assays), while samples collected between 2012 and 2014 (from DL2 only) were assayed in another (B, 4 assays). For group A, inter-assay variation was not rigorously assessed. For group B, 2 standard controls were run in each assay (testosterone: 100 and 25 pg/ml, $n = 4$). Inter-assay variation was calculated by determining the *CV* for the 2 standard controls on each plate. Inter-assay variation for group B was 10.0% for the 100 pg control and 17.7% for the 25 pg control. Intra-assay variation was calculated for both groups by averaging the *CV* for all of the samples with 20–80% binding on each plate. Intra-assay variation for groups A and B were 17.6 and 10.3%, respectively.

Data analysis.—Statistical analysis was performed using R (R Development Core Team 2015). Seasonal variations of TTV, PI, and testosterone concentrations were assessed separately with mixed effects regression models developed using the {lme4} package in R (Bates et al. 2015). To account for observations being clustered by individual, a random intercept term was incorporated into the model. Variation in each variable by 2 different definitions of breeding season was tested: high testosterone season (January through April versus all other months) and high conception season (March through May versus all other months) (Robeck et al. 2005). To describe the seasonal variation, an additive modeling approach was used to identify the polynomial regression model that best described the data using a centered time variable (month). Random intercept and random slope terms were tested to account for observations being clustered by individual. Model fits were compared using analysis of variance (ANOVA), log likelihood, and Akaike information criterion (AIC). Prior to seasonality analysis, TTV measurements were normalized to body length, as body mass fluctuates seasonally in belugas, whereas length will remain constant in adults. Differences in the rates of change of TTV in DL1 were assessed using analysis of covariance (ANCOVA). Box plots, created in R, show the interquartile range (box), the median (bold line), and the maximum (Q3) or minimum (Q1) value ≤ 1.5 times the interquartile range (whiskers). Intra-observer reliability for ultrasound measurements was assessed by comparing TTV calculated separately from the replicate measures taken from 2 sets of still images and plotting the replicate measures in a Bland–Altman plot with the {BlandAltmanLeh} package in R (Bland and Altman 1986; Lehnert 2015). Significance was set at $P < 0.05$.

Results

Seasonality of TTV.—A total of 154 ultrasound examinations were conducted. Due to variation in the animals' behavior, weather, or faulty equipment, 6 scheduled ultrasound examinations were not conducted. As a result, DL5 was not measured in the months of June, July, or September, and DL2 was not measured in September or November of 2012 or March of 2013.

The appearance of beluga testes on ultrasound was as described in studies of other odontocetes (Brook et al. 2000; Fig. 1). The length, width, and depth of the testes varied within and among individuals (Table 2). This variation occurred seasonally, with a seasonal pattern apparent for 4 of the 5 individuals (Fig. 2; Supplementary Data SD1). TTV was significantly higher from January through April ($P < 0.0001$) and from March through May ($P < 0.0001$) compared to all other months, but the effect was stronger using the predictor season of January through April. There were significant differences among individuals in both tests of seasonality (random intercept term, $P < 0.001$ for January through April and $P < 0.01$ for March through May). Seasonality of TTV was best described by a cubic fixed effect model with a random intercept term and random linear and quadratic slope effects (Supplementary Data SD2). TTV was generally highest in winter-spring, and lowest in late summer-fall, with individual TTV increasing by 60% from the minimum measurement to the maximum measurement on average (Table 3). The difference between replicate measures of TTV was 68 ± 59 cm^3; the mean difference of replicate measures is 6% of the mean of all TTV measurements made in this study (Fig. 3).

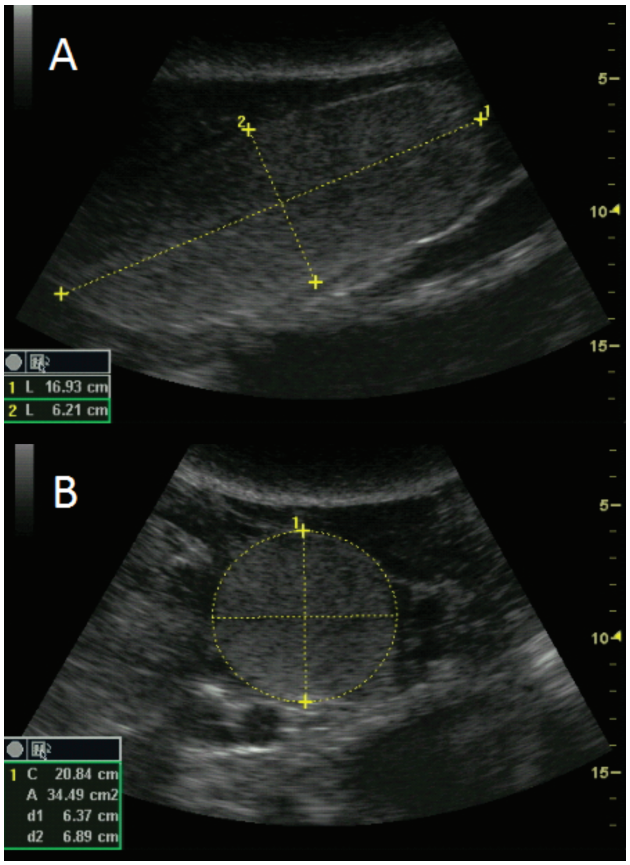

Fig. 1.—Appearance of a beluga (*Delphinapterus leucas*) testis on ultrasound, showing digital measurements of the organ in both longitudinal (A) and cross-sectional view (B).

Seasonality of circulating testosterone concentration.—Testosterone concentrations in blood were significantly higher between January through April than all other months ($P < 0.0001$), but did not vary between March through May and all other months ($P > 0.05$). The effect of individual (random intercept term) was not significant for the comparison between January through April and all other months ($P > 0.05$), but was significant for the comparison between March through May and all other months ($P < 0.01$). Seasonal variation in testosterone occurred in 4 of the 5 whales (Fig. 4). Seasonality of testosterone was best described by a quadratic fixed effects model with a random intercept and random linear slope term. The relationship between seasonal variation in testosterone and TTV is shown in Fig. 5.

Table 2.—Variation in component measures (in cm) of testicular volume by individual beluga (*Delphinapterus leucas*) monitored longitudinally for at least 1 year using ultrasound.

ID	Length		Dorsoventral diameter		Lateral diameter	
	Min	Max	Min	Max	Min	Max
DL1	14.02	20.16	5.47	8.41	6.02	7.74
DL2	15.08	18.75	5.85	7.58	5.40	7.20
DL3	11.00	21.80	3.89	7.17	4.74	7.09
DL4	14.88	22.48	6.49	8.22	6.47	8.06
DL5	12.18	19.00	5.90	7.07	5.38	8.29

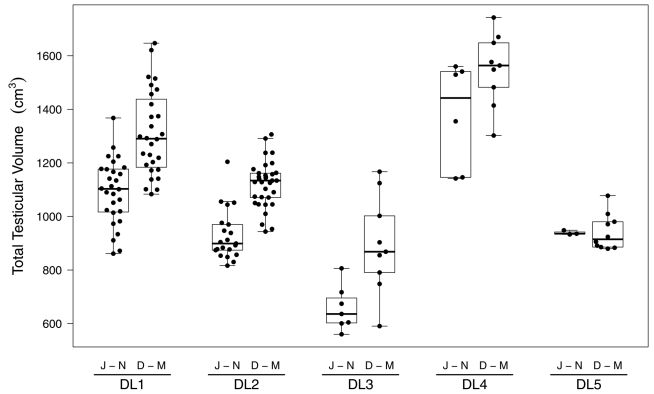

Fig. 2.—Variation in total testicular volume (cm^3) by individual and season (J–N = June through November, D–M = December through May) for belugas (*Delphinapterus leucas*) monitored longitudinally for at least 1 year using ultrasound. Circles represent individual observations.

Seasonality of PI.—A seasonal variation in the PI of testicular ultrasound images was apparent in both DL1 and DL2 (Fig. 6). PI was significantly higher from January through April compared to all other months ($P < 0.05$), but was not different from March through May compared to all other months ($P > 0.05$). Seasonality of PI was best described by a quadratic fixed effects model with a random intercept and random linear slope term. An increase in echogenicity of testicular tissue preceded the increase in testes size in DL1, while it was coincident with the increase in testes size in DL2. In both animals, echogenicity decreased prior to the decrease in testes size (Fig. 6).

Year-to-year variation within individuals.—Both DL1 and DL2 showed similar patterns of seasonal change in TTV, testosterone, and testicular PI (Fig. 6). While DL2 reached similar peak TTV measurements from year to year, DL1 reached a higher TTV in the 2nd year relative to the first (Fig. 7). This year-to-year variation was also reflected in a different rate of increase in TTV (September 2007 through March 2008, September 2008 through March 2009, September through December 2009) between years ($P < 0.0001$), with a faster rate of change occurring from September 2008 through March 2009 (slope = 3.32) than in September 2007 through March 2008 (slope = 2.13) or September through December 2009 (slope = 1.83) (Fig. 8). The highest rate of growth corresponds to a growth of 1.66 cm^3 of testicular tissue per testis per day.

Discussion

Through the use of longitudinal monitoring of individual belugas, this study provides important information on reproductive seasonality in male belugas that could not realistically be collected from wild belugas. Additionally, by assessing 3 measures of reproductive activity simultaneously in live animals, this study provides greater detail than has previously been presented for belugas. Seasonal variation in testes size in adult male belugas was observed, supporting hypotheses developed from postmortem studies of wild belugas and addressing gaps in these data caused by sampling logistics (Kelley et al. 2014). The pattern of seasonality was consistent with other studies of reproductive seasonality in belugas, with peak testes size and testosterone occurring in the late winter-early spring, when breeding occurs in both the wild and in aquaria (Burns and Seaman 1988; Robeck et al. 2005). Ultrasonography was a sufficiently sensitive method to detect seasonal variation in testes

Table 3.—Seasonal variation in total testicular volume (cm^3) of belugas (*Delphinapterus leucas*) measured longitudinally using ultrasound by individual and year studied. DL3, DL4, and DL5 were only monitored in 2008.

ID	Minimum volume	Maximum volume	Minimum month	Maximum month	Difference	Increased by a factor of:
DL1 2007–2008	861.21	1,336.56	October	March	475.35	1.6
DL1 2008–2009	1,014.17	1,647.03	August	March	632.86	1.6
DL2 2012–2013	830.04	1,157.69	October	February	327.65	1.4
DL2 2013–2014	816.37	1,306.38	August	February	490.01	1.6
DL3	560.15	1,167.21	October	March	607.06	2.1
DL4	1,141.89	1,742.87	July	December	600.98	1.5
DL5[a]	883.26	1,077.46	May	January	194.19	1.2

[a]No observations available from June, July, or September.

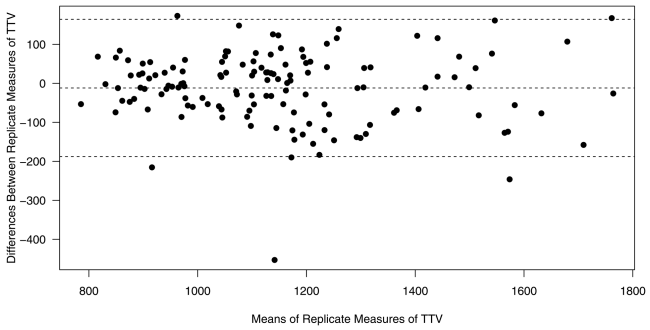

Fig. 3.—Bland–Altman plot of replicate measures of beluga (*Delphinapterus leucas*, $n = 5$) total testicular volume (cm^3) calculated from separate images taken during the same exam to assess intra-observer variation in measurements made using ultrasound.

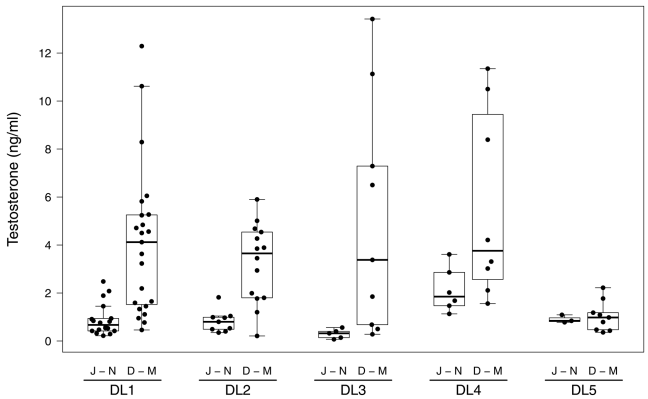

Fig. 4.—Variation in circulating testosterone concentration by individual and season (J–N = June through November, D–M = December through May) for belugas (*Delphinapterus leucas*) monitored longitudinally for at least 1 year. Circles represent individual observations.

size and density in belugas, as in other studies of odontocetes (Robeck et al. 2009; Wu et al. 2010b).

The testes sizes measured in this study are similar to those found in postmortem studies of belugas (linear measurements—Heide-Jørgensen and Teilmann 1994; volume measurements—Kleinenberg et al. 1969; Brodie 1971). As testes mass is the most commonly reported measure of testes size, determining the relationship between testes volume measured via ultrasound and testes mass would be helpful in expanding the utility of this method for assessing reproductive function in wild belugas. To our knowledge, only 1 report is available where mass and volume of the same testes were reported (Kleinenberg et al. 1969). Using the small data set ($n = 5$ adults) reported by Kleinenberg et al. (1969), a relationship between mass and volume can tentatively be obtained (M (g) = $1.13V$ (cm^3) − 27.5, $r^2 = 0.997$). Using this equation and the minimum and maximum volumes observed in this study (Table 3), the belugas studied had combined testes masses ranging from 605 to 1,941 g. These values are very similar to published values, although with a higher peak in this study, perhaps resulting from differences in how volume was measured (water displacement versus ultrasound) or the lack of data from peak season in postmortem studies.

Significant individual variation was found in testes size and in the rate of change in testes size with season. In postmortem studies of belugas, wide variation in testes size has been

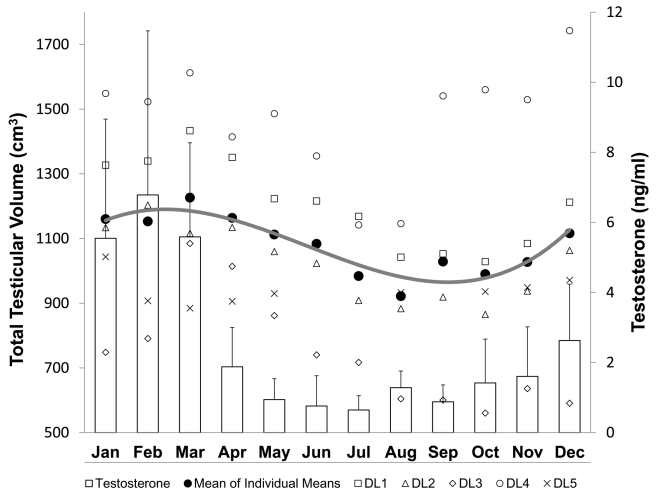

Fig. 5.—Seasonal variation in total testicular volume (TTV) (points representing individual monthly means separated by individual on primary y-axis) and testosterone (bars on secondary y-axis; mean of individual means ± *SD*). Filled circles represent the mean of the individual means of TTV by month, with the cubic fixed effects regression model plotted (gray line). TTV is plotted in raw form in cm^3 (not normalized to body length, as in statistical analyses). The mean of the individual means was not used in statistical analyses but was presented here for illustrative purposes.

found for belugas of similar body length (e.g., Sergeant 1973) or age (e.g., Heide-Jørgensen and Teilmann 1994). Individual differences in testicular activity have also been found, with males sampled at the same time found to be in various stages of regression (Burns and Seaman 1988). In 34 males sampled in Alaska between April and July, only 2 were found to be in breeding condition, with sperm absent from the epididymis in 22 of the animals (Burns and Seaman 1988). In 1 male trained in an aquarium to provide semen samples, O'Brien et al. (2008) noted that sperm was produced year-round, but sperm concentration varied seasonally. Testosterone concentrations of 1 ng/ml are thought to be sufficient to maintain spermatogenesis (O'Brien et al. 2008), but only 1 beluga in this study (DL4, the same animal studied in O'Brien et al. 2008) maintained this concentration of testosterone throughout the year. Although differences in hormone assays between studies could explain some of the variation, it is also possible that some males do not maintain sufficiently high testosterone throughout the year, resulting in the individual variation in spermatogenic activity found in Burns and Seaman (1988). However, without additional information on spermatogenesis to complement testes size or testosterone data, the relationship between these measures of reproductive activity are currently unknown.

Significant differences in TTV measurements between individuals could be due to individual variation in testes size or systematic biases within observers at each site, but our data do not allow for this discrimination to be made. Although variation between measures can be expected when using ultrasound on an unrestrained live animal, the degree of intra-observer variation was relatively small and did not obscure the seasonal pattern of testicular growth and regression. Intra-observer variation for this study was similar to that found in a study of dolphin testes

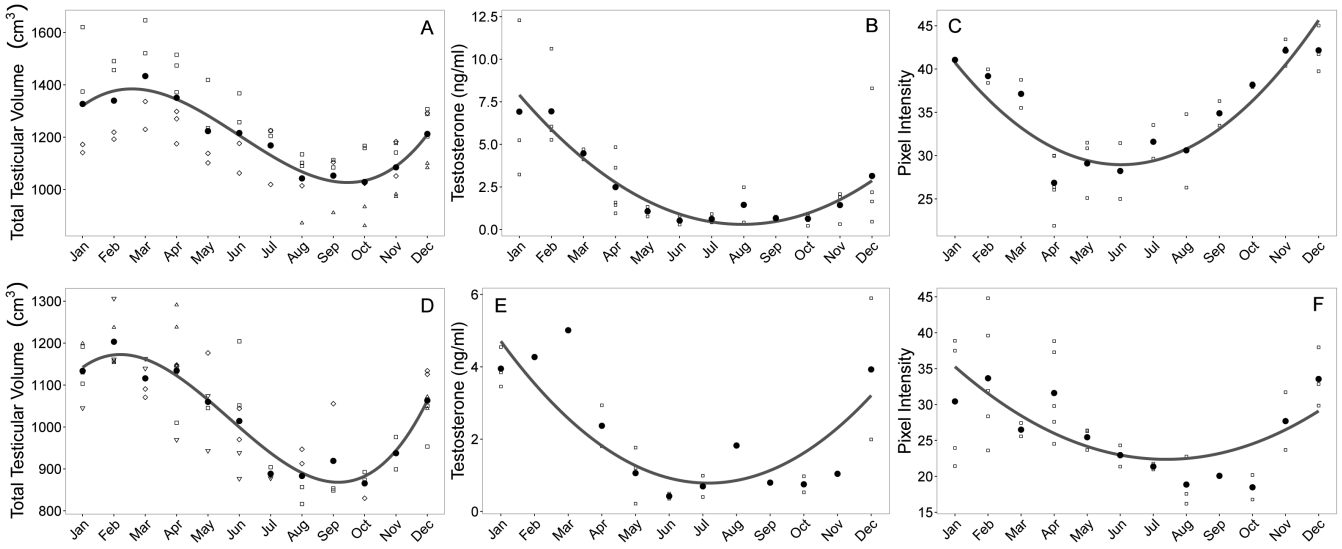

Fig. 6.—Seasonal variation in total testicular volume (TTV) measured using ultrasound, circulating testosterone, and the pixel intensity of testicular ultrasound images for belugas (*Delphinapterus leucas*) DL1 (A, B, and C, respectively) and DL2 (D, E, and F, respectively). Scales on the y-axis differ by individual. Open symbols represent individual observations, with different years represented by different marker shapes, while closed circles represent the monthly mean. Lines represent the fitted fixed effects curves determined from statistical analyses (TTV: cubic; testosterone and pixel intensity: quadratic).

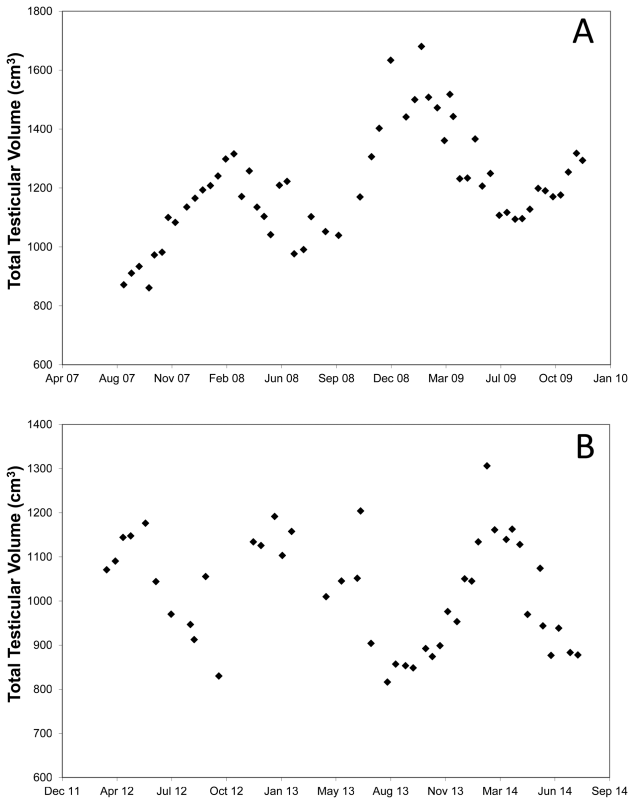

Fig. 7.—Longitudinal variation in total testicular volume (cm^3) measured using ultrasound across years for belugas (*Delphinapterus leucas*) DL1 (A) and DL2 (B). Scales on the y-axis vary by individual.

via ultrasound, where replicate measures generally varied by less than 0.5 cm between observers and 0.4 cm within observers (Yuen et al. 2009). Due to the multiplicative nature of the volume measurement, small variations in component linear measurements from one image to the next will result in relatively large variations in volume measurements. For example, if each linear measurement of average size testes in this studied varied by +0.4 cm, this would result in a difference of 159 cm^3, which would be greater than 1 *SD* above the mean difference in replicate measures of TTV found in this study. The inability to measure length directly in some cases likely contributed to some of the variation found. For longitudinal studies, 2 measurements per month are sufficient for the detection of seasonal patterns of growth and regression, as seasonality was apparent in this study with a sampling frequency of 1–2 times per month.

Testicular PI of ultrasound images was higher during the breeding season for both males studied, suggesting that this may be a useful measure of testicular activity in belugas. The expected pattern was observed, with lower echogenicity outside of the breeding season, which may indicate a decline in spermatogenesis (Brito et al. 2012). The timing of PI changes in these belugas suggests that changes in testicular activity occurred prior to changes in testes size. The degree of seasonal change in PI was lower than that found in finless porpoise testes (Wu et al. 2010b), but due to differences in equipment and methodology, direct comparisons of PI measurements between studies are likely inappropriate. Variation in scan depth and gain (necessitated by varying blubber thickness found in these belugas) dramatically affected PI measures. Although a correction factor was applied successfully in this study, future studies utilizing PI measures should be as consistent as possible to improve the ability to compare individuals. The greatest value of PI measurements is likely in the longitudinal monitoring of an individual. Although histology is unlikely to be available for comparison to PI measurements in belugas as it has been in bulls, future studies could further validate this technique by focusing on juveniles to monitor for expected changes in echogenicity with the attainment of sexual maturity.

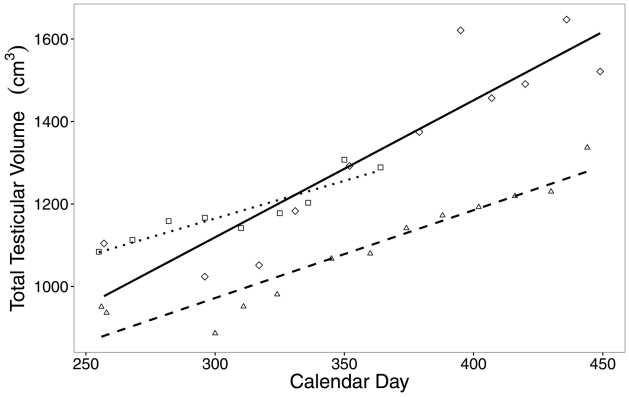

Fig. 8.—Variation in rate of total testicular volume increases by year for DL1. Calendar day is calculated from Julian dates so that the time series are uninterrupted from the fall of year 1 into the spring of year 2. September 2007–March 2008 (triangles, dashed line); September 2008–March 2009 (diamonds, solid line); September–December 2009 (squares, dotted line).

In DL1, the seasonal variation in testes size and testosterone concentration was not consistent from year to year, with TTV reaching a higher peak at a faster rate in the 2008–2009 season than in the other years studied. The 2008–2009 breeding season was markedly different from other seasons for DL1. Seven novel belugas, including a mature male and 4 mature females, were temporarily housed with DL1 from August of 2008 until April of 2009. This social change was associated with a temporary increase in circulating catecholamines in this animal (Spoon and Romano 2012). The difference in testicular growth that season relative to the others may have been a response to this change in social grouping. One possible mechanism for this difference could be the "challenge hypothesis," where a new social challenge causes an increase in reproductive activity in a male that previously was not challenged for breeding opportunities (Wingfield et al. 1990). Alternatively or in addition, the "Coolidge effect" may have stimulated higher reproductive activity via the introduction of novel females to the social group (Dewsbury 1981). In contrast, DL2 did not experience changes to social grouping during the study and the degree of seasonal variation was consistent from year to year. Social influences on sperm production have been suggested for managed groups of bottlenose dolphins (*Tursiops truncatus*), but the effect was hypothesized to be inhibitory (Robeck and O'Brien 2004). In the wild, adult male belugas travel together (Smith et al. 1994), and thus maintaining multi-male social groupings has been a goal of the cooperative managing belugas in aquaria in the United States and Canada. Reproductive rate is also presumed to be higher in multi-male groups, and the enhanced reproductive activity in DL1 coincident with a social change may provide a mechanistic explanation for this observation.

DL5 apparently did not undergo seasonal variation in either testes size or testosterone concentration during the study period, although both testes size and testosterone concentration were within the range of concentrations found in the other belugas studied. DL4 shared the same environment and displayed seasonal variation in both testes size and testosterone, indicating that environmental cues were not a significant factor. DL5 has not sired a calf in his lifetime, suggesting this pattern may be abnormal, but other factors such as access to females in breeding condition can contribute to breeding history. Disease can cause senescence in adult male bottlenose dolphins (Kemper et al. 2014), but this animal had no signs of illness and continues to be healthy 7 years later. As the oldest animal in the study, it is also possible that the lack of seasonal variation may be due to age-related senescence. In humans, a decline in free testosterone is associated with aging (Lamberts et al. 1997); some degree of age-related senescence in testicular morphology has also been observed in other male odontocetes, including pilot whales (Desportes et al. 1993) and finless porpoises (Wu et al. 2010a). Detecting senescence in wild male belugas by assessing testicular morphology would be difficult because sampling typically occurs out of breeding season, when most adult males are in the regression phase (Burns and Seaman 1988). With an understanding of typical seasonal changes, further longitudinal study of belugas in aquaria could be performed to assess age-related changes to testosterone secretion and testicular morphology.

These data suggest that the spring and autumn equinoxes may carry important photoperiod information for belugas. Testicular regression appeared to begin following the spring equinox, and recrudescence appeared to begin following the autumn equinox. Many physiological changes associated with the change in season are driven by photoperiod, including changes in circulating testosterone concentrations and spermatogenesis in some mammals (Goldman 1999). Arctic species, such as the beluga, receive less photoperiod information than species in lower latitudes, necessitating different regulatory mechanisms. In reindeer (*Rangifer tarandus*), photoperiod cues around the equinox appear to entrain seasonal rhythms, as opposed to circadian rhythms, as occurs in most other species (Lu et al. 2010). The potential importance of the equinox for belugas is further supported by the finding of seasonal variation in this group despite the variation in duration of photoperiod experienced by belugas at the different study sites. The regulatory cues for belugas are unknown, but longitudinal monitoring of belugas in aquaria may enable further investigation, as it has in Yangtze finless porpoises (*Neophocaena asiaeorientalis asiaeorientalis*—Yu et al. 2016).

The apparent time lag between peak testosterone concentration and peak testes size is consistent with the time lag observed between peak testosterone and conceptions in Robeck et al. (2005). This time lag would be expected to occur, as high testosterone is required to support spermatogenesis and thus recrudescence of testicular tissue. Due to the cycle of the seminiferous epithelium, there is also a lag between the initiation of testicular growth and peak spermatogenesis, or even between the initiation of testicular growth and the initiation of spermatogenesis (Martinet et al. 1984). The total length of the cycle of spermatogenesis ranges from 30 to 75 days in mammals, with cycle duration of approximately 60 days estimated for white-sided dolphins (Robeck et al. 2009). If belugas are consistent

with other mammals, an additional transit time through the epididymis of 10 days would follow. Therefore, belugas would be expected to initiate spermatogenesis at least 70 days prior to when females become fertile. Testicular growth and increasing echogenicity began in November, and this timing would allow male belugas to reach breeding condition in January, when females begin to exhibit estrous cycles (Robeck et al. 2005). Peak testes size, and thus presumably peak sperm production, occurred in February–March. This is in line with the peak season for conceptions in belugas in aquaria up to 60 days later (March through May—Robeck et al. 2005).

Testosterone concentrations declined dramatically after March, and testes size began to regress in April. If conceptions are still occurring through May, then this suggests that reproductive behavior occurs independently of high testosterone concentrations, as testosterone concentrations in April and May were similar to concentrations found in summer and fall months, outside of the breeding season. The regression in testes size and loss of echogenicity of testicular tissue at this time suggests that spermatogenesis is slowing as well. It is possible that spermatogenesis is suspended by this time, as has been found in wild belugas sampled as early as April that lacked sperm in the epididymis (Burns and Seaman 1988). This contrasts with harbor porpoises, which maintain elevated testes size for 1 month beyond when females are typically receptive (Neimanis et al. 2000). This apparent decline in demand for sperm prior to the termination (or perhaps even the peak) of breeding season suggests that belugas are able to establish sufficient sperm stores by April to allow for conceptions later in the season. This further supports a spermatogenic cycle of about 60 days, as it would be energetically beneficial to slow sperm production 2 months in advance of the end of the breeding season.

The degree of seasonal variation found in these belugas was less than the degrees of change found in some other species of seasonally breeding odontocetes, which may experience 4- to 5-fold increases in testes size. However, the degree of change was similar to the seasonal increase found in seasonally breeding sheep (*Ovis aries*, ~67% increase—Ortavant et al. 1988) and long-finned pilot whales (*Globicephala melas*, ~50% increase—Desportes et al. 1993). Even at maximum size, belugas have small testes relative to other cetaceans. The linear measurements of maximum testes size in this study were similar to those made on a harbor porpoise that weighed 37.5 kg, only 4.2% of the weight of the smallest beluga in this study (Desportes et al. 2003).

Testes size is often used to infer mating systems in mammals (Kenagy and Trombulak 1986). For other odontocetes, relatively large testes are thought to be in response to high copulation rates during very short breeding seasons (approximately 2 weeks in the harbor porpoise—Read 1990) or protracted breeding seasons with a high level of sperm competition (common dolphin—Murphy et al. 2005). Either situation creates high demand for sperm to increase reproductive success. In addition to small relative testes size, the small seasonal change in testes size and regression of the testes prior to the end of the breeding season observed in this study all imply relatively low demand for sperm in the beluga. However, little is known about the mating system of belugas, with several disparate strategies proposed with varying degrees of pre- and post-copulatory competition between males based on morphological characteristics such as sexual dimorphism and relative testes size (O'Corry-Crowe et al. 1997; Schaeff 2007; Kelley et al. 2014; Dines et al. 2015). However, this apparent low demand for sperm is consistent with the recent discovery that belugas are facultative induced ovulators (Steinman et al. 2012). In induced ovulators, the 1st male to mate with a female has a much higher chance to sire offspring than successive males, and these species tend to have smaller testes than males in spontaneously ovulating species (Iossa et al. 2008; Soulsbury 2010). The low testosterone concentrations during peak breeding season in this study suggest that male–male agonistic competition also is low in this species, as high concentrations are associated with aggressive behavior in other mammals (Trainor et al. 2009). The relatively low sperm concentration found by O'Brien et al. (2008), thought to perhaps be an artifact of the semen collection training process, may indeed be representative of belugas, as relatively low sperm concentrations are found in species that are induced ovulators (Soulsbury and Iossa 2010). Ovulation mode in female belugas may thus partially explain the relatively small testes in this species, suggesting that factors other than sperm competition are more important in determining individual reproductive success. Studies of beluga breeding behavior and paternity are needed to determine the mating rate and perhaps degree of promiscuity to improve our understanding of beluga mating strategies.

The improved understanding of the seasonality of reproduction will aid in the management of individual belugas in aquaria, allowing managers to identify the best time of year to train voluntary semen collection for use with artificial insemination, establish maturity, assess reproductive capabilities, and diagnose reproductive abnormalities. The methods employed here can also be used for noninvasive assessments of reproductive activity in wild belugas that are temporarily restrained for satellite tagging (e.g., Norman et al. 2012). Longitudinal sampling throughout the year, made possible by the study of belugas in aquaria, will continue to help fill gaps in our understanding of beluga reproduction. Given the logistical impediments to making direct observations of this Arctic-dwelling odontocete and the resulting difficulty in identifying the timing of key reproductive events, these longitudinal studies in aquaria will have an impact on conservation and management of wild belugas.

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Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Total testicular volume measured using ultrasound, in cubic centimeters (mean, standard deviation, and number of observations), by month for each individual beluga (*Delphinapterus leucas*) monitored longitudinally. The "Mean" column represents the mean of the individual means, with standard deviation and the total number of observations per month.

Supplementary Data SD2.—Model selection summary for describing seasonal variation of total testicular volume in belugas (*Delphinapterus leucas*) monitored longitudinally using ultrasound. The best model is shown in bold.

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