7-1-2014

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Journal of Zoo and Wildlife Medicine 46(3): 456–467, 2015

CIRCULATING CONCENTRATIONS OF THYROID HORMONE IN BELUGA WHALES (*DELPHINAPTERUS LEUCAS*): INFLUENCE OF AGE, SEX, AND SEASON

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Abstract: Thyroid hormones play a critical physiologic role in regulating protein synthesis, growth, and metabolism. To date, because no published compilation of baseline values for thyroid hormones in beluga whales (*Delphinapterus leucas*) exists, assessment of thyroid hormone concentrations in this species has been underused in clinical settings. The purpose of this study was to document the concentrations of total thyroxine (tT4) and total triiodothyronine (tT3) in healthy aquarium-maintained and free-ranging beluga whales and to determine the influence of age, sex, and season on the thyroid hormone concentrations. Archived serum samples were collected from healthy aquarium-maintained ($n = 43$) and free-ranging ($n = 39$) belugas, and serum tT4 and tT3 were measured using chemiluminescence immunoassay. The mean tT4 concentration in aquarium-maintained belugas was 5.67 ± 1.43 μg/dl and the mean tT3 concentration was 70.72 ± 2.37 ng/dl. Sex comparisons showed that aquarium-maintained males had significantly greater tT4 and tT3 (9.70 ± 4.48 μg/dl and 92.65 ± 30.55 ng/dl, respectively) than females (7.18 ± 2.82 μg/dl and 77.95 ± 20.37 ng/dl) ($P = 0.004$ and $P = 0.013$). Age comparisons showed that aquarium-maintained whales aged 1–5 yr had the highest concentrations of tT4 and tT3 (8.17 ± 0.17 μg/dl and 105.46 ± 1.98 ng/dl, respectively) ($P = 0.002$ and $P < 0.001$). tT4 concentrations differed significantly between seasons, with concentrations in winter (4.59 ± 1.09 μg/dl) being significantly decreased compared with spring ($P = 0.009$), summer ($P < 0.0001$), and fall ($P < 0.0001$) concentrations. There was a significant difference in tT4 and tT3 concentrations between aquarium-maintained whales (5.67 ± 1.43 μg/dl and 70.72 ± 15.57 ng/dl, respectively) and free-ranging whales (11.71 ± 3.36 μg/dl and 103.38 ± 26.45 ng/dl) ($P < 0.0001$ and $P < 0.001$). Clinicians should consider biologic and environmental influences (age, sex, and season) for a more accurate interpretation of thyroid hormone concentrations in belugas. The findings of this study provide a baseline for thyroid health monitoring and comprehensive health assessments in both aquarium-maintained and free-ranging beluga whales.

Key words: endocrine, *Delphinapterus leucas*, marine mammal, thyroid, thyroxine, triiodothyronine.

INTRODUCTION

Thyroid hormones play a critical physiologic role in regulating protein synthesis, growth, and metabolism.[1,5,12,16,28–32,34] Secretion is primarily regulated via negative-feedback control through the coordinated response of the hypothalamic-pituitary-thyroid axis; dysfunction along this axis may result in thyroid hormone concentration abnormalities that could have profound consequences on proper physiologic function and animal health.[1] Total thyroxine (tT4) generally represents >95% of the thyroid hormone output into circulation, whereas the free state of T4 makes up a negligible amount of the total measured thyroid hormone.[8] tT4 is typically greater than total triiodothronine (tT3), as thyroxine (T4) serves as a pool of prohormone that can be converted to triiodothronine (T3) by deiodinases in the target tissues.[8] Increased or decreased concentrations of

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T4 and T3 in circulation have been associated with the clinical syndromes of hyperthyroidism and hypothyroidism, respectively.[13,24] Because thyroid hormones affect numerous metabolic processes, their deficiency or excess can cause a variety of clinical signs, none of which are pathognomonic for the condition.[13,24] The diagnosis of a thyroid-related illness is ideally based on a combination of signalment and history as well as results of a physical examination and clinicopathologic assays.[13] Serum concentrations of thyroid hormones can be affected by many extrathyroidal factors, and environmental and biologic influences must be taken into account when seeking to accurately interpret thyroid hormone concentrations.[3,6–9,12,13,17,19,22–24,27–32,34–38]

There have been numerous thyroid-related maladies associated with alterations in T3 and T4 concentrations reported in odontocetes such as beluga whales (*Delphinapterus leucas*),[16] bottlenose dolphins (*Tursiops truncatus*),[5,10] and harbor porpoises (*Phocoena phocoena*).[22] Some of these abnormalities include follicular cysts, adenomatous hyperplasia, thyroiditis, goiter, and interfollicular fibrosis. Changes in serum hormone concentrations may be indicative of primary endocrine disorders, may occur as secondary effects of a primary disease elsewhere, or may reflect normal response to environmental or biologic change.[34] Endocrine disruption has been extensively considered in cetaceans,[6,7,9,14,16,17,22,23,35,36] and thyroid gland function and pathologic changes are key in determining the extent of the damage caused by endocrine-disrupting contaminants. Proper interpretation of these hormone concentrations must include an appreciation of the influence of environmental and extrathyroidal biologic factors on endocrine cycles.[31,32,34] Considering the previous reports of thyroid gland pathology in cetaceans, and the physiologic changes associated with such dysfunction in mammalian species, accurate monitoring and interpretation of thyroid hormone concentrations are vital for comprehensive health assessments.

Cetacean thyroid glands are large in proportion to their body weight, and circulating concentrations of tT4 are greater compared with those of most terrestrial mammals.[20,28,32] These characteristics suggest that cetaceans are able to maintain a large reserve of thyroid hormones that assist during periods of heightened need for increased thyroid activity.[28] Thyroid gland activity changes to accommodate growth phases, stressful stimuli, seasonal changes, and other hormonal cycles.[30,32] Seasonal cycles in thyroid activity are apparent in many species, and previous studies have shown evidence of thyroid hyperactivity in a population of wild belugas occupying a warm river estuary in the summer.[32] These dynamic changes suggest alterations in thyroid function based on external environmental temperature, water temperature, and other seasonal characteristics. All of these variables are important to consider when using thyroid hormone concentrations as part of a health assessment in both aquarium-maintained and free-ranging cetaceans.

To date, because no published compilation of baseline values for thyroid hormones in belugas exists, assessment of thyroid hormone concentrations in this species has been underused in clinical settings. Although clinicians may observe clinical signs and clinicopathologic abnormalities consistent with thyroid disease, this differential may not be highly considered due to the previous lack of research, limited supportive evidence, and lack of published baseline values surrounding thyroid hormones in this species. The data gained from this study represent an important first step in our understanding of thyroid hormone dynamics in belugas by providing baseline tT4 and tT3 concentrations for comprehensive health assessments in both aquarium-maintained and free-ranging belugas.

The purpose of this study was to determine the influence of age, sex, and season on circulating concentrations of tT4 and tT3 in aquarium-maintained belugas and to examine any significant differences in tT4 and tT3 concentrations in free-ranging belugas. We hypothesized that age, sex, and seasonal differences in thyroid hormones would be detectable in aquarium-maintained belugas. In addition, we hypothesized that free-ranging belugas would maintain greater concentrations of thyroid hormones compared with aquarium-maintained belugas. Finally, the hypothesis that comparisons between two free-ranging beluga populations would show significantly different thyroid hormone concentrations due to differences in latitude and ocean temperatures in their respective environments was proposed.

MATERIALS AND METHODS

There were a total of 407 samples analyzed from the 43 aquarium-maintained belugas ($n = 43$ belugas; $n = 368$ samples) and 39 samples analyzed from 39 free-ranging belugas ($n = 39$ belugas; $n = 20$ live captured, $n = 19$ subsistence hunted). Thus, there was an unbalanced design as

multiple samples were collected from some individuals and as few as one sample collected from each of the free-ranging animals. Samples in aquarium-maintained belugas were analyzed for tT4 in winter ($n = 57$), spring ($n = 104$), summer ($n = 117$), and fall ($n = 90$). There were 57 samples from 19 animals sampled described as a neonate, 20 individuals described as a juvenile, 259 samples from 29 individuals described as an adult, and 24 samples from 4 individuals described as geriatric. There were 18 males sampled, of which 43 samples were from spring, 46 from summer, 38 from fall, and 26 from winter. There were 25 females sampled, of which 61 samples were from spring, 71 from summer, 52 from fall, and 31 from winter.

Aquarium-maintained beluga whale sample collection

Serum samples were collected from apparently healthy belugas of both sexes, aged 1–41 yr, housed at North American zoos and aquariums. Apparent health of each individual was determined based on veterinary assessment including visual or physical examination; animal behavior, including normal training compliance and normal appetite; and results of routine diagnostic testing, including complete blood count and chemistry analysis. Each animal had between zero and four samples analyzed from any given year. There were 18 males and 25 females that composed the aquarium-maintained group. Belugas were grouped by age as follows: group 1, neonate to 5 yr; group 2, 6–10 yr; group 3, 11–30 yr; and group 4, >31 yr. For aquarium-maintained animals, there were 13 belugas in age group 1, 9 in group 2, 19 in group 3, and 3 in group 4. There were 9 adults, 9 juveniles, and 21 unknown age-class, free-ranging animals. Samples were collected from the periarterial venous rete on the dorsal or ventral aspect of the flukes in aquarium-maintained belugas during routine health screenings. Samples were generally obtained under behavioral control, although samples obtained under restraint were not excluded from this study. Archived serum samples were obtained from healthy belugas of both sexes, during various life stages, and throughout multiple seasons. Although most archived samples had been collected in the 5 yr before this study, samples were accepted up to 10 yr archived to obtain a larger sample size. Due to the sensitivity of thyroid-hormone binding globulin to temperature changes, samples were considered unacceptable and were not used if a freezer failure event had occurred. Archived samples had been stored in cryovials at –62.2°C (–80°F) and shipped on dry ice overnight before analysis. Seasons were divided up as follows: spring (1 March–31 May), summer (1 June–31 August), fall (1 September–30 November), and winter (1 December–28 February). For aquarium-maintained animals, there were 17 belugas with samples from spring, 11 with samples from summer, 5 with samples from fall, and 10 with samples from winter. For free-ranging animals, there were 10 samples from spring, 21 from summer, 8 from fall, and no winter samples. Every animal did not have a sample collected from each age category, season, or both.

Free-ranging beluga whale sample collection

Serum samples were collected from two wild Alaskan populations of belugas in Bristol Bay, Alaska, and the Chukchi Sea. Beluga whales in Bristol Bay ($n = 18$) were captured and restrained using methods described by Norman et al.[38] In brief, several small boats were used to direct the target animal parallel to shore. Once the animal was in shallow enough water for the net to reach the bottom, the net was deployed. The whale was then guided into shallower water for handling and sampling purposes.[38] The free-ranging sample population consisted of 21 males, 15 females, and 3 belugas of unknown sex. Similar methods were used for live-capture release studies on the Chukchi Sea belugas ($n = 2$), except a net was set from shore. Blood samples were collected from the periarterial venous rete on the dorsal aspect of the flukes as soon after capture and restraint as possible or as soon as possible from additional subsistence-hunted Chukchi Sea belugas ($n = 19$) in Point Lay, Alaska. Blood sampling occurred approximately 19 min (range 11–30 min) after the net was set, and it took 1–2 min to obtain the required sample volume.

We were unable to accurately categorize free-ranging belugas by age class (as was done for aquarium-maintained belugas) due to the difficulty of determining age based solely on length and coloration, which varies among beluga populations. Skin color may provide some indication of age, as white whales are often considered mature. However, gray females have been observed with calves, indicating they may reach sexual maturity before turning white.[30] Therefore, standard length (i.e., straight linear length from tip of rostrum to fluke notch) was used as a proxy for age. In the field, age class was assigned by the researchers based on overall appearance, with an emphasis on length and color. Nominally, animals

<300 cm in length were presumed to be juvenile, animals >320 cm were presumed to adult, and animals between 300 and 320 cm were qualitatively assessed based on other parameters, including color.

This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Mystic Aquarium, a division of Sea Research Foundation and assigned IACUC approval 10009. The study protocol was reviewed and approved by all participating facilities before submission of archived serum samples.

Serum sample analysis

tT4 and tT3 were analyzed from undiluted serum samples by a diagnostic laboratory (Pfizer Research and Development, Groton, Connecticut 06340, USA). All archived serum samples were shipped overnight on dry ice and were stored in a −80°C freezer before thawing for thyroid hormone analysis. Less than 4% of samples contained hemolysis or lipemia, and all samples that were grossly hemolyzed, lipemic, or both were excluded from the study. All samples were checked and rimmed, and all fibrin material was removed before analysis. Serum tT4 and tT3 were then measured using chemiluminescence immunoassay (ADVIA Centaur system, Siemens Healthcare, Malvern, Pennsylvania 19355, USA).[25,26] Approximately 75 µl of serum (25 µl for tT4, 50 µl for tT3) per sample was required to complete both thyroid hormone measurements. Adequate volume of retrospective samples was available for repeat assays of analytes if deemed necessary during the testing process.

The diagnostic laboratory used in this study (Pfizer Research and Development) is a good laboratory practices–regulated laboratory that is accredited by the College of American Pathologists (CAP) and is enrolled in the proficiency testing administered by CAP. Instrument requalification of the chemiluminescence immunoassay occurred before tT4 and tT3 analysis. Quantitative serum analytes were assessed for intra- and interassay accuracy and precision by comparing the means and CVs obtained against the stated manufacturer's range according to the guidelines established by the Clinical and Laboratory Standards Institute. All samples were analyzed per vendor package insert and were processed in the same manner as proficiency samples. Intra-assay accuracy and precision were considered to be acceptable in all analytes used in this study. Interassay accuracy and precision were assessed by comparing the obtained grand mean and CVs against the manufacturer's recommended range. The calculated interassay means using assayed controls were within the recommended range. Imprecision was assessed by comparing the calculated %CV to the manufacturer's recommended %CV for all levels of control material. Imprecision was considered acceptable if the observed %CV was less than or equal to the %CV recommended by the manufacturer. The calculated %CV was acceptable for all analytes measured in this study.[25,26]

Samples were assayed in singlicate based on the historical instrument validation; standards were assayed in triplicate and quality control was assayed in singlicate. Two standards were included in the assay kit, and Trilevel Lyphochek Immunoassay Plus (Bio-Rad Laboratories, Hercules, California 94547, USA) was used for quality control. The assay was re-run if the calibration failed or if quality control was outside of established limits (±2 SD). All samples were intermixed randomly within the same assay.

Intra-assay precision was determined using 20 replicates of pooled beluga serum within one analytical run on the Advia Centaur platform. Manufacturer-stated %CV for tT3 for a concentration of 56.7 ng/dl was 4.29%, and at a concentration of 132.8 ng/dl was 5.17%. Intra-assay precision with $n = 20$ replicates for tT3 mean was 75.8 ± 2.5 ng/dl, with a %CV of 3.3%. Manufacturers-stated %CV for tT4 for a concentration of 3.84 was 4.29% and at a concentration of 7.70 was 1.19%. Intra-assay precision with $n = 20$ replicates for tT4 mean was 5.86 ± 0.17 µg/dl, a %CV of 2.8%.

Interassay precision was determined using two pooled beluga serum samples that were prepared and analyzed twice a day for 5 days on the Advia Centaur platform. Manufacturer-stated %CV for interassay precision for tT3 (using three samples over 8 days on six systems assayed three times per day) for a concentration of 3.21 was 1.13%. For tT3 ($n = 10$), the mean was 89.5 ± 8.1 ng/dl and %CV was 9.1. For the second measurement of tT3 ($n = 10$), the mean was 74.1 ± 7.4 ng/dl and %CV was 10.0. The concentrations analyzed (one system twice per day over 5 days) were 75.8 and 74.1, with a %CV of 9.1 and 10.0%, which is comparable to the manufacturer's stated range. Manufacturer-stated %CV for interassay precision for tT4 (using three samples over 8 days on six systems assayed three times per day) for a concentration of 3.44 and 7.70 was 4.57 and 1.47%, respectively. For tT4 ($n = 10$), the mean was 6.11 ± 0.39 µg/dl, with %CV of 6.3. For the second measurement of tT4 ($n = 10$),

the mean was 6.12 ± 0.41 μg/dl and %CV was 6.7. The concentrations analyzed (one system twice per day over 5 days) were 6.11 and 6.12, with a %CV of 6.3 and 6.7, values comparable to the manufacturer-stated range. tT4 linearity experiment was conducted using a prepared pool of beluga serum with a tT4 concentration of 5.90 μg/dl. The pooled serum sample was analyzed and compared to the master curve standard curve. This pool was serially diluted using the tT4 assay diluent (reference 672202) to achieve concentrations ranging from 5.90 to 1.40 μg/dl. A master standard curve was prepared as per package insert using the manufacturer's tT4 master curve material (reference 672420). This master curve is a multipoint calibration curve in which the calibrator is run in triplicate, and the mean concentration of each calibrator was plotted versus the relative light units (RLU). The measured versus assigned concentrations were plotted and a slope of 1.288 for tT4 was calculated with an intercept of −1.50 using D. Rhoads EP Evaluator 7.0 version software (Kennett Square, Pennsylvania 19348, USA).

tT3 linearity experiment was conducted using a prepared pool of beluga serum that had a tT3 concentration of 76.0 ng/dl. The pooled serum sample was analyzed and compared to the master curve standard curve. This pooled serum sample was serially diluted using the T3 assay diluent (reference 672207) to achieve concentrations of 76–19.0 ng/dl. A master standard curve was prepared using the manufacturer's tT3 master curve material (reference 04848169). This master curve is a multipoint calibration curve in which the calibrator is run in triplicate, and the mean concentration of each calibrator was plotted versus the relative light units (RLUs). The measured versus assigned concentrations were plotted, and a slope of 0.904 for tT3 was calculated with an intercept of 11.0 by using D. Rhoads EP Evaluator 7.0 version software.

The replicate means for both tT4 and tT3 were compared against the assigned concentrations by using linear regression. Each slope was statistically compared with the value of 1 (i.e., parallelism) at the 95% confidence level. The *P*-values of tT3 and tT4 were calculated to be 0.37 and 0.07, respectively. So, the hypothesis that the in-study beluga whale data are parallel to the standard curves cannot be rejected. The parallelism hypothesis was accepted.

tT4 matrix evaluation was also performed in the pooled serum sample. The serum sample was diluted with assay diluent as stated in the tT4 assay package insert. The baseline sample concentration of 5.90 μg/dl was diluted to yield a 75, 50, and 25% concentration. The 75% concentration of 4.43 μg/dl was prepared by adding 3 parts pooled serum to 4 parts diluent, the 2.95 μg/dl concentration (50%) was prepared using a 1:2 dilution and the 1.48 μg/dl concentration (25%) was prepared using a 1:4 dilution. The acceptable percent recovery was 75–125%. The 4.43 μg/dl recovered at 99.4%, and the remaining dilutions recovered below 75%. There is a matrix effect at both the 1:2 and 1:4 dilutions. Samples analyzed for tT4 were analyzed undiluted.

tT3 matrix evaluation was also performed in the pooled serum sample. The serum sample was diluted with assay diluent as stated in the tT3 assay package insert. The baseline sample concentration of 76.0 ng/dl was diluted to yield a 75, 50, and 25% concentration. The 75% concentration of 57.0 ng/dl was prepared by adding 3 parts pooled serum to 4 parts diluent, the 38.0 ng/dl concentration (50%) was prepared using a 1:2 dilution, and the 19.0 ng/dl concentration (25%) was prepared using a 1:4 dilution. The acceptable percent recovery was 75–125%. The 57.0 ng/dl recovered at 109.6% and the remaining dilutions recovered above 125%. There is a matrix effect at both the 1:2 and 1:4 dilutions. Samples analyzed for tT3 were analyzed undiluted.

STATISTICAL ANALYSIS

Descriptive statistics that were tabulated for tT4 and tT3 concentrations included mean, median, 95% confidence interval, and minimum–maximum for tT4 and tT3 by sex, age class, season, and location (aquarium-maintained vs. free-ranging belugas). Normality of continuous variables was assessed using the Shapiro–Wilk test. Multiple linear regression models were built for tT4 and tT3 to account for multiple samples from a particular individual across other categorical variables, such as age class and season. Age class, season, and sex were dummy coded with males, geriatric animals, and winter being the reference category, whereas individuals were coded using effects coding, and all were included in the original model. Variables that did not contribute to the overall model were removed from the final model. The final model included age class, season, and individual variables, but not sex.

To create baseline values for tT4 and tT3 separately for age class, season, and sex, categorical variables were coded for the analysis, including sex (0 = male, 1 = female), facility (1 = aquarium maintained, 2 = free ranging), and wild

Table 1. Mean circulating levels of thyroid hormones (total thyroxine and total triiodothyronine) in aquarium-maintained ($n = 45$) and free-ranging ($n = 39$) beluga whales. Significance, $P < 0.05$.

Hormone,[a] population	Mean ± SE	Min.–Max.	Significant difference
tT3 (ng/dl), aquarium	70.72 ± 2.37	27–155	Age, sex
tT3 (ng/dl), free ranging	103.38 ± 4.23	59.75–161.2	Sex
tT4 (μg/dl), aquarium	5.67 ± 1.43	1.2–11	Age, sex, season
tT4 (μg/dl), free ranging	11.71 ± 3.36	4.7–16.3	Sex

[a] tT3 indicates total triiodothyronine; tT4, total thyroxine.

origin (1 = Bristol Bay, 2 = Chukchi Sea). Ordinal categories were assigned for age (1–4) and season (1 = spring, 2 = summer, 3 = fall, 4 = winter). To account for an unbalanced sample set, each individual with multiple samples from a particular category was compared over time using a non-parametric Friedman two-way analysis of variance (ANOVA) by rank. If no differences were observed, values for that individual were averaged and a single value was included in further analysis. Animals with missing samples were removed from the analysis. A Greenhouse–Geisser repeated measures ANOVA was then used to evaluate changes in tT4 and tT3 concentrations individually by season and age class. Independent sample *t*-tests were used to evaluate differences between sexes, facilities, age classes in free-ranging animals, and location of free-ranging animals. Sampling limitations prevented analysis of free-ranging belugas by age or season. Statistical significance was established using a *P*-value of <0.05. All statistical analyses were performed using SPSS 21.0 software (IBM Statistics, Chicago, Illinois 60606, USA).

RESULTS

Mean and minimum–maximum serum thyroid hormone concentrations for aquarium-maintained and free-ranging belugas are shown in Table 1. For tT4 and tT3 in aquarium-maintained belugas, significant differences were noted as a function of age, sex, and season. For tT4 and tT3 in free-ranging belugas, significant differences were noted as a function of sex in the Chukchi Sea population.

Aquarium-maintained belugas

tT4: Mean tT4 serum concentration differed significantly among seasons ($F_{2.326,65.128} = 7.223$, $P = 0.001$). tT4 concentrations in winter (4.95 ± 1.31 μg/dl) were significantly decreased compared with concentrations in spring (6.06 ± 2.39 μg/dl, $P = 0.009$), summer (8.63 ± 4.29 μg/dl, $P < 0.0001$), and fall (6.22 ± 2.06 μg/dl, $P < 0.0001$) (Table 2). However, tT4 was not significantly different between spring and summer ($P = 0.516$), spring and fall ($P = 0.555$), or summer and fall ($P = 0.917$). Sex comparisons showed that in animals that were sampled in the spring, summer, and fall, tT4 was significantly greater in males (9.70 ± 4.48 μg/dl) than in females (7.18 ± 2.82 μg/dl) ($P = 0.004$) (Table 3; Fig. 1). There was no difference in tT4 serum concentration between sexes in animals sampled in winter ($P = 0.273$). Mean tT4 concentration differed significantly between age classes ($F_{1.662,11.632} = 12.827$, $P = 0.002$). The concentration of tT4 in neonates (6.76 ± 0.98 μg/dl) was significantly greater than that in juveniles (5.08 ± 0.82 μg/dl, $P = 0.003$) and adults (4.65 ± 1.03 μg/dl, $P = 0.001$) (Fig. 2). Although sample size was limited, concentrations of tT4 did not differ significantly for females when they were pregnant versus when they were not pregnant ($F = 0.01$, df = 1, $P = 0.94$).

tT3: Mean tT3 serum concentration did not differ significantly between seasons ($F_{2.571,71.979} = 1.820$, $P = 0.159$). Males had greater tT3 (92.65 ± 30.55 ng/dl) than females (77.95 ± 20.37 ng/dl) ($P = 0.013$) (Table 2; Fig. 1). Mean tT3 concentration differed significantly between age classes ($F_{1.318,9.226} = 27.27$, $P < 0.001$) (Fig. 2). The concentration of tT3 in neonates (99.18 ± 18.69 ng/dl) was found to be significantly greater than in that in juveniles (82.79 ± 10.95 ng/dl, $P = 0.003$) and adults (63.96 ± 7.15 ng/dl, $P = 0.001$). Although sample size was limited, tT3 serum concentration did not differ significantly for females when they were pregnant versus when they were not pregnant ($F = 0.14$, df = 1, $P = 0.74$).

Free-ranging belugas

Juvenile free-ranging animals (10.83 ± 1.88 ng/dl) had significantly greater tT4 serum concentration than adult free-ranging animals (6.59 ± 1.83 μg/dl) ($P < 0.0001$). Similarly, juvenile free-ranging belugas (108.85 ± 24.54 μg/dl) had significantly greater tT3 than adult free-ranging animals (67.59 ± 11.59 μg/dl) ($P = 0.001$). tT4

Table 2. Seasonal variations in total thyroxine in aquarium-maintained belugas.

	Winter			Spring			Summer			Fall			
Hormone	*n*	Mean ± SE	Range	*n*	Mean ± SE	Range	*n*	Mean ± SE	Range	*n*	Mean ± SE	Range	Significance
tT4[a]	35	4.95 ± 1.31	2.4–8.2	49	6.06 ± 2.39	2.9–12.4	59	8.63 ± 4.29	3.3–16.3	45	6.22 ± 2.06	3.2–13.2	<0.05

[a] tT4 indicates total thyroxine.

Table 3. Sex-related differences in thyroid hormone concentrations in aquarium-maintained belugas across seasons.

	Male			Female				
Hormone[a]	*n*	Mean ± SE	Min.–Max.	*n*	Mean + SE	Min.–Max.	Significance	Season
tT4	160	9.70 ± 4.48	3.28–16.3	240	7.18 ± 2.82	3.48–13.4	<0.05	Spring, summer, fall
tT3	162	92.65 ± 30.55	48.2–161.2	240	77.95 ± 20.37	49.2–131.4	<0.05	Spring, summer, fall, winter

[a] tT3 indicates total triiodothyronine; tT4, total thyroxine.

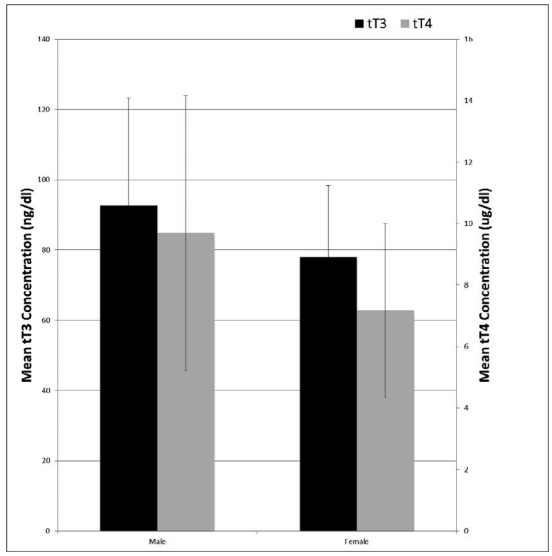

Figure 1. Sex-related differences in mean total thyroxine and mean total triiodothyronine concentrations in aquarium-maintained belugas.

concentrations were decreased in Bristol Bay belugas (8.88 ± 2.62 μg/dl) compared with Chukchi Sea belugas (14.13 ± 1.53 μg/dl) ($P < 0.0001$). Similarly, Bristol Bay belugas had decreased tT3 concentrations (89.49 ± 26.80 ng/dl) compared with Chukchi Sea belugas (115.30 ± 19.89 ng/dl) ($P = 0.001$). The lack of samples of known-age animals and the bias toward summertime sample collection did not allow a comparison of differences between seasons and ages of free-ranging belugas.

Aquarium-maintained vs. free-ranging belugas

Comparisons between free-ranging and aquarium-maintained belugas were restricted to non-winter samples from aquarium-maintained animals 6–30 yr old and all free-ranging animals that were sampled. Within this subset of data, there was a significant difference in tT4 concentration between aquarium-maintained (5.67 ± 1.43 μg/dl) and free-ranging (11.71 ± 3.36 μg/dl) ($P < 0.0001$) belugas, with free-ranging belugas exhibiting significantly greater concentrations of tT4 (Fig. 3). Similarly, there was a significant difference in tT3 concentration between aquarium-maintained (70.72 ± 15.57 ng/dl) and free-ranging (103.38 ± 26.45 ng/dl) ($P < 0.001$) belugas, with the free-ranging belugas exhibiting significantly greater tT3 concentrations (Fig. 3).

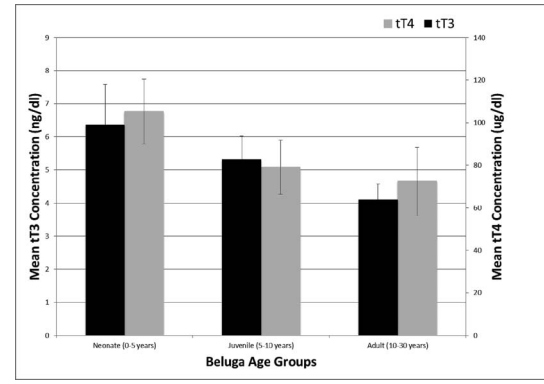

Figure 2. Age-related differences in mean total thyroxine and mean total triiodothyronine concentrations in aquarium-maintained belugas.

DISCUSSION

This study sought to determine whether age, sex, or season influenced thyroid hormones in aquarium-maintained belugas, and whether serum concentrations of tT4 and tT3 significantly differed for aquarium-maintained and free-ranging belugas. All of the variables examined in aquarium-maintained belugas (age, sex, and season) influenced thyroid hormone concentrations, and free-ranging belugas were found to exhibit significantly greater concentrations of circulating thyroid hormones compared with the aquarium-maintained belugas.

Aquarium-maintained belugas

Within the aquarium-maintained population, male belugas had significantly greater serum concentrations of tT4 and tT3 compared with

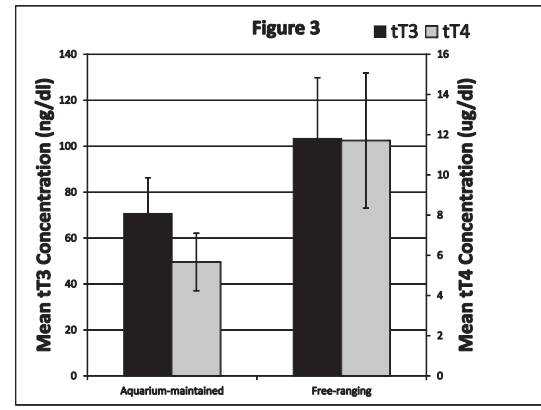

Figure 3. Mean total thyroxine and mean total triiodothyronine concentrations in aquarium-maintained vs. free-ranging belugas.

females. The reason for these sex-related differences in this study is unclear, and the biologic significance is unknown, but the variation agrees with a previous study where mature male belugas in the Hudson Bay population maintained significantly greater thyroid hormone concentrations than did the mature females in that population.[30] Hormonal changes associated with pregnancy and lactation in females can indirectly lead to elevations in tT4 and tT3 by increasing hormone binding capacity in the plasma.[34] Considering that reproduction is more closely regulated in an aquarium and that the number of pregnant belugas included in this study was limited, these factors may have influenced the pattern of difference between male and female beluga tT4 and tT3 concentrations. Although this study did not include an analysis of reproductive hormones in conjunction with thyroid hormones and the pregnant beluga sample size was limited ($n = 5$), the mean concentrations of tT4 and tT3 measured in pregnant belugas were not statistically different from those of nonpregnant belugas. Our sample size of lactating belugas was not large enough ($n = 3$) for accurate statistical comparison, but previous studies have noted a slight increase in tT4 concentrations in lactating wild dolphins.[34]

In aquarium-maintained belugas, tT4 concentrations were highest in fall, summer, and spring and lowest in winter. These seasonal trends are the first reported for aquarium-maintained odontocetes, as it was previously thought that the necessary environmental cues are lacking in the relatively consistent environment of aquarium-maintained animals.[15,32,34] Although seasonal trends in thyroid analytes have not been previously reported in aquarium-maintained belugas, these trends are consistent with earlier studies in free-ranging belugas that identified a period of thyroid hyperactivity when belugas migrated from the cold oceanic waters to the warm estuaries in the summer that coincided with biochemical and microscopic evidence of enhanced thyroid activity.[28,32] It was hypothesized that this trend was attributable to the changing environmental conditions, principally salinity and temperature in the estuaries, as well as the significant changes in day length at these high latitudes.[30,32] It has been suggested that a plausible role for seasonal adjustments in thyroid activity is to promote growth, and warm estuaries provide a favorable setting for the expression of such a cycle.[32] It is also likely that the movement of adult animals into warm and brackish estuaries may promote molting of the dermis, which is a necessary physiologic process in belugas. Because a portion of the zoologic institutions housing the animals used in this study were either outdoors, or the belugas were exposed to direct natural sunlight from large windows surrounding their exhibit, it is possible that their thyroid cycles were also responsive to photoperiod changes and seasonal fluctuations in pool temperatures. Detailed information on facility latitude, exhibit design, and pool temperature fluctuations were beyond the scope of the current study, but this would be an interesting avenue for future research. It was previously believed that aquarium-maintained belugas held under relatively constant environmental conditions did not show marked seasonal variations in thyroid hormones,[2,4,32] but the results herein clearly demonstrate seasonal patterns.

Interpretation of thyroid hormone concentrations in marine mammals must account for the dynamic changes that occur in association with significant life-history events.[2] Total T4 and tT3 concentrations were significantly greater in young belugas compared with older age groups. These belugas exhibited a pattern similar to that in humans, domestic mammals, and other marine mammals.[12,34] This period of maternal dependence can be energetically and physiologically demanding, as neonates attempt to balance the demands of body growth and lipid storage with energy expenditure during behavioral development.[3] Young marine mammals can face additional thermoregulatory challenges in the cold aquatic environments they inhabit,[3,12] and metabolically derived heat may be critical until an insulative blubber layer is established.[12,28] The calorigenic effects of thyroid hormones could explain the need for elevations during this time.[12,28] Although these concentrations reportedly decline during the first few weeks of life in most species,[12,28] the belugas sampled in this study appeared to maintain increased thyroid hormone concentrations for the first few years of life. This may be attributable to the longer period of codependence that exists between adult female belugas and their calves, as well as the continued growth and development that occurs during this time.

Free-ranging belugas

We found that free-ranging belugas maintained significantly greater concentrations of tT4 and tT3 compared with aquarium-maintained belugas. Greater tT4 concentrations have also been observed in free-ranging pinnipeds, manatees, and cetaceans compared with the same species in an aquarium.[8,18,34] Previous studies have suggested

that adaptation to an aquarium environment, including stable environmental conditions, consistent food sources, decreased temperature fluctuations, and decreased energetic demands, affects the sensitivity of the thyroid gland to stimulation by thyroid-stimulating hormone, thereby decreasing the amount of tT4 and tT3 in circulation.[31] Although thyroid hormone concentrations appear to stabilize at decreased levels in belugas fully acclimated to an aquarium environment,[31] it has been suggested that an increased metabolic rate in wild cetaceans allows them to be better suited to meet the energetic and thermal demands of the open ocean.[31,32]

Prey availability and vessel disturbances have been previously studied to evaluate the roles these factors may play in thyroid hormone balance in free-ranging cetaceans.[4] A previous study on an endangered population of killer whales (*Orcinus orca*) found that the combination of fecal glucocorticoids and T3 hormone measures is well suited to distinguish the relative contributions of psychologic and nutritional stress on the physiologic health of a population.[4] That study found that T3 concentrations decrease in response to nutritional stress but are largely unaffected by the psychologic stress caused by vessel disturbance.[4] Sustained food deprivation causes a decrease in T3 concentrations, slowing metabolism to conserve energy stores. In the Chukchi beluga population in this study, these trends appear to differ slightly, as this population tends to undergo a seasonal fasting period before capture or subsistence hunting, yet still maintains greater tT3 concentrations compared with aquarium-maintained belugas. The complex interplay of these factors means that free-ranging population baseline data, such as that described herein, is critically important for future comparisons.

The capture and temporary restraint of free-ranging animals, including beluga whales, can produce temporary physiologic changes mediated by the stress response.[33] Previous studies on thyroid hormone dynamics after capture in belugas showed that concentrations of thyroid hormones were markedly reduced after capture.[33] Belugas in that study were sampled at 6- to 7-hr intervals, and tT4 showed a slow and steady decline from the time of capture and placement in holding and remained this way for the study period of 100 hr.[33] tT3 showed an initially rapid decline from time of capture and then stabilized at this decreased concentration throughout the duration of the study.[33] This was suggested to be mediated by cortisol released during the acute phase of the stress response, as cortisol inhibits the conversion of T4 to T3.[33] This conversion occurs in peripheral tissues and reduces T4 secretion from the thyroid gland by suppressing the release of thyrotropin.[33] These findings suggest that concentrations of tT4 and tT3 in free-ranging belugas should perhaps be decreased compared with aquarium-maintained belugas due to cortisol inhibition of thyroid hormone release. For the Bristol Bay and Chukchi Sea populations, the archived serum samples used in this study were originally collected from free-ranging belugas after chase, capture, and restraint or collected immediately postmortem. Despite collection methods in the free-ranging belugas in this study, the findings herein suggest that either capture or restraint techniques were quick enough that this cortisol inhibition did not occur, or perhaps that these populations of free-ranging belugas under nonrestraint conditions would maintain even greater serum concentrations of thyroid hormones. Although blood samples were collected as soon after capture and restraint as possible, it is currently unknown how soon after capture thyroid hormones may show alterations or whether immediate postmortem sampling would affect thyroid hormone results. It is also unknown whether similar alterations may exist in aquarium-maintained animals during behavioral blood collection vs. restraint events. Additional research would be required in both free-ranging and aquarium-maintained animals to definitively determine how collection techniques might impact the values of thyroid hormones measured.

Limited information exists on the thyroid hormone system of odontocetes, especially in free-ranging populations. The noted differences in thyroid hormone concentrations between the two free-ranging beluga populations could be related to the northerly location and colder year-round water temperatures that the Chukchi Sea belugas inhabit compared with the Bristol Bay beluga population. The increased serum concentrations of tT4 and tT3 noted in the Chukchi Sea belugas and could constitute an adaptive response to their colder environment in attempts to increase endogenous heat production.[8] These results are consistent with a previous study in free-ranging bottlenose dolphins that found increased thyroid hormone concentrations in dolphins inhabiting an environment with lower annual mean water temperatures.[12] In addition to ocean temperature, previous studies have suggested that prey availability, nutritional quality, growth differences, and the presence of endocrine-disrupt-

ing chemicals can affect thyroid hormone concentrations in free-ranging cetaceans.[8] Documenting thyroid hormone concentrations in free-ranging belugas will provide a baseline for comparison that will allow researchers to monitor the effects of habitat disruption and changes due to increased human activity and pollutants, and varying climate in the Arctic.[19]

Clinicians should consider biologic and environmental influences (age, sex, and season) for a more accurate interpretation of thyroid hormone concentrations in belugas. In aquarium-maintained belugas, thyroid hormone concentrations within the expected range may be greater in fall, spring, and summer compared with those in winter. These concentrations should not be considered abnormal. Younger aquarium belugas will have greater thyroid hormone concentrations that may decline with age. Free-ranging and aquarium-maintained animals require separate baselines for "normal" thyroid hormone values as free-ranging animals may exhibit significantly greater concentrations of tT4 and tT3.

Acknowledgments: This paper is dedicated to Dr. Gerard N. Burrow (endocrinologist and former Chief Executive Officer of Mystic Aquarium) who enthusiastically encouraged and supported the study of the beluga thyroid. The authors thank the following people and organizations for providing samples: Dr. Karen Wolfe and Julie Lemon (Point Defiance Zoo and Aquarium); Dr. Marty Haulena and Chelsea DeColle (Vancouver Aquarium); Dr. Tonya Clauss, Dr. Al Dove, Harry Webb, and Nicole LaBove (Georgia Aquarium); Dr. Paul Calle, Kate McClave, and Dee McAloose (Wildlife Conservation Society); Drs. Les Dalton and Steve Osborn (SeaWorld San Antonio); and Dr. Caryn Poll and Mayela Alsina-Guerrero (John G. Shedd Aquarium). The authors acknowledge National Oceanic and Atmospheric Administration permits 782-1719-07 and 932-1489-10, Alaska Department of Fish & Game Animal Care and Use Committee (06-16), and thank the Bristol Bay Marine Mammal Council for approval and assistance in obtaining boat drivers and local logistical support. The authors also thank Dr. Robert Suydam (Department of Wildlife Management, North Slope Borough, Barrow, Alaska) and the community of Point Lay, Alaska. The authors are also grateful to Amy Delmonaco and Gayle Sirpenski (Mystic Aquarium) for assistance. This manuscript serves as publication 217 for Sea Research Foundation, Inc.

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Received for publication 1 July 2014