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Correlating Behavior with Non-Invasive Physiological Measures to Evaluate Mating Strategies in Belugas

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CORRELATING BEHAVIOR WITH NON-INVASIVE
PHYSIOLOGICAL MEASURES TO EVALUATE MATING
STRATEGIES IN BELUGAS

BY

JUSTIN THOMAS RICHARD

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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IN
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UNIVERSITY OF RHODE ISLAND

2016

DOCTOR OF PHILOSOPHY DISSERTATION
OF
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ABSTRACT

With conservation and ethical concerns facing cetaceans, minimally invasive research on reproduction is important for population management. Belugas (*Delphinapterus leucas*) are endangered in parts of their range, yet little is known about their breeding behavior and much of the existing research depends on post-mortem sampling. To date, there have been no directed studies of social interactions between belugas during the breeding season, and few studies have attempted to correlate reproductive physiology with behavior in any species of cetacean. Improved understanding of reproductive strategies in this species would facilitate management. This work describes the development of minimally invasive methods for determining sex, maturity, and reproductive cycle stage in belugas, and the utilization of these methods to assess relationships between reproductive physiology and behavior in a social group of belugas in an aquarium. The results of this work are interpreted in the context of the current understanding of beluga reproductive physiology and ecology.

Chapter 1 describes the physiological validation of blow (exhale) sampling for measuring testosterone and progesterone in belugas. Concentrations of both progesterone and testosterone in blow are correlated with circulating concentrations and reflect variation in the reproductive status of individuals.

Chapter 2 presents the development of DNA isolation from beluga blow samples and determines the relationship between various sample characteristics and DNA yield and performance in polymerase chain reactions (PCR). Although yield and quality varied greatly among samples, single exhale samples from wild and

aquarium belugas enabled PCR amplification of genes used in sex determination or population genetics.

Chapter 3 describes the seasonal variation in testicular volume and testosterone in male belugas studied longitudinally. This work revealed a seasonal increase in testes size in belugas of approximately 50%, filling a significant gap in knowledge for wild belugas.

Chapter 4 utilizes methods developed and validated in Chapters 1 and 3 to evaluate social behavior in a group of aquarium belugas in the context of reproductive physiology. Reproductive seasonality and the occurrence of reproductive events were detected using non-invasive techniques and used to contextualize patterns of association and the frequency of social behaviors of interest, including courtship.

Appendix 1 presents a general review of the literature regarding beluga mating strategies and reproductive biology; specific literature reviews are included with each chapter. Appendix 2 provides a synthesis of the dissertation and discusses the implications of these findings on the current understanding of beluga mating strategies, and by extension, the management and conservation of this species.

Appendix 3 presents laboratory protocols utilized in this dissertation, and provides supplementary data that was not included in Chapter 1.

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Finally, I wish to sincerely thank my wife, Carey, to the moon and back.

DEDICATION

To my parents, for instilling and encouraging my love of science,

and

To Owen, for whom I hope I can do the same.

PREFACE

This dissertation is in manuscript format. Chapters 1-4 of this dissertation have been prepared for submission to peer-reviewed journals. Appendix 1 provides a general introduction and review of the problem; more specific literature reviews are found within each chapter. Appendix 2 provides a synthesis and speculative discussion. Appendix 3 contains laboratory protocols utilized in the completion of this work, as well as supplementary data that were relevant to, but not included in Chapter 1.

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CHAPTER 1

“Testosterone and progesterone concentrations in blow samples are biologically relevant in belugas (*Delphinapterus leucas*)”

by

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Abstract

Steroid hormone analysis in blow (respiratory vapor) may provide a minimally invasive way to assess the reproductive status of free-swimming cetaceans, but biological validation of the method is needed to allow for the interpretation of hormone measurements in blow samples. Utilizing samples collected from trained belugas (*Delphinapterus leucas*) ($n = 20$), enzyme immunoassays for testosterone and progesterone were validated for use with beluga blow samples. Testosterone concentrations in 40 matched blood and blow samples collected from 4 male belugas demonstrated a significant positive correlation ($F_{1,38} = 41.7$, $p < 0.0001$). Progesterone concentrations in 64 matching blood and blow samples from 11 females were also significantly positively correlated ($F_{1,62} = 94.0$, $p < 0.0001$). Testosterone concentrations in blow varied seasonally in males monitored longitudinally, with the peak occurring during the breeding season (February – April). Testosterone concentrations (mean $\pm$ SD) in blow samples collected from adult males during the breeding season (February – April, 136.95 ± 33.8 pg/ml) were significantly higher than in those collected outside of the breeding season (May – January, 99.4 ± 39.5 pg/ml). Both adult male groups had blow testosterone concentrations that were higher than that of a juvenile male (<8 years) (59.4 ± 6.5 pg/ml) or female belugas (54.1 ± 25.7 pg/ml). Although there is a high degree of overlap between out of season adult males, juvenile males, and females at low testosterone concentrations, high testosterone concentrations in blow can be used to identify adult males. Matching blood and blow samples collected from wild belugas in Alaska (8 males and 2 females) were not positively correlated, but only males had blow testosterone

concentrations >65 pg/ml (6/8 males). Progesterone concentrations in blow also varied by reproductive status; pregnant females (410.6 ± 87.8 pg/ml) and females in the luteal phase of the estrous cycle (339.5 ± 51.0 pg/ml) had higher blow progesterone concentrations than non-pregnant females without a corpus luteum (242.5 ± 27.3 pg/ml). In three females that were pregnant and one female with a non-conceptive estrous cycle during the study period, a longitudinal change in blow progesterone was observed that corresponded with a change in pregnancy status or ovarian function. Testosterone concentrations in blow samples were also shown to be stable with collection under field conditions and during storage at -20° and -80° C. Blow sampling can be used to detect maturity, seasonality, pregnancy status, or ovulation in belugas in aquaria, or wild belugas that are temporarily stranded in areas such as the Cook Inlet, Alaska, providing a minimally invasive way to identify the reproductive status of an individual. Given the value of hormone analyses in blow samples demonstrated here, further method development to allow sampling of free-swimming wild belugas is warranted.

Introduction

Given the fundamental relationship between reproduction and endocrinology, reproductive steroid hormone determinations are frequently used to assess the reproductive status of individual animals, yielding information such as the sex, maturity status, and reproductive cycle stage. At the population level, this information is necessary to assess demographics and viability and is crucial to the development and long term monitoring of conservation plans (Labrada-Martagón et al. 2014;

Kersey and Dehnhard 2014). Cetaceans (whales, dolphins, and porpoises) particularly benefit from hormone monitoring due to the management concerns facing many populations.

The utility of reproductive steroid determination in cetaceans has been established through the use of blood samples. The measurement of progesterone in female blood samples can be used to diagnose pregnancy (Stewart 1994; Kellar et al. 2013a) and detect the luteal phase of the estrous cycle (Sawyer-Steffan et al. 1983). Testosterone determination in male blood samples has been used to assess sexual maturity (Daoquan et al. 2006; Desportes et al. 1994; Robeck and Monfort 2006) and identify the breeding season in seasonally breeding species (Robeck et al. 2005; Hao et al. 2007). While collecting samples for this purpose is complicated by the aquatic environment, advances in minimally invasive sampling methodologies and alternative matrices are replacing the need for blood sampling and enabling hormone determination research in wild, free-swimming cetaceans (reviewed in Amaral 2010).

The application of hormone determination in alternative matrices to cetacean research has allowed the assessment of sex, maturity status and reproductive seasonality, as well as population-level assessments of pregnancy rate that can inform management and conservation efforts (Kellar et al. 2009; Vu et al. 2015; Kellar et al. 2013b). To date, the most commonly used matrices for hormone determination in wild cetaceans are blubber samples obtained via remote biopsy sampling (Trego et al. 2013; Perez et al. 2010) and fecal samples collected from the water's surface (Rolland et al. 2005). Despite the value of these methodologies, they may not be appropriate for all species or all populations. Biopsy sampling may not be ideal if repeated

sampling is required, or if conservation concerns make even small risks to the animal's health undesirable. These risks may limit the sampling population available if investigators are required to avoid sampling mothers with young calves or the calves themselves (Kellar et al. 2014). Defecation may be infrequently observed, and collecting sufficient feces from smaller species of cetaceans is more difficult, especially if the feces rapidly disperse upon excretion, precluding collection (Green et al. 2007). Additionally, assigning fecal samples to particular individuals can be difficult in highly social species. These and other limitations in existing methodologies have led to a recent effort to develop “blow” (exhale) sampling as a source of hormones for analysis in cetaceans.

Blow, which is also referred to as respiratory vapor or exhaled breath condensate, can be collected without contacting the animal when the whale surfaces to breathe. Blow samples contain steroid and thyroid hormones at detectable levels, as demonstrated in a variety of cetaceans (Hogg et al. 2005; Hogg et al. 2009; Thompson et al. 2014; Hunt et al. 2014). Hunt et al. (2014) and Thompson et al. (2014) have shown that relatively inexpensive enzyme immunoassays can be validated to measure steroid hormones and thyroid hormones in cetacean blow, improving the accessibility of this technique. Additionally, blow sampling can be used to gather genetic information on the individual cetacean (Frère et al. 2010) or the microorganisms associated with the respiratory tract (Acevedo-Whitehouse et al. 2010). Due to the minimally invasive nature of this sampling, coupled with its ability to provide a wide variety of information, blow sampling has the potential to be a very useful tool in

monitoring cetacean populations and assessing the health and reproductive status of individuals (Hunt et al. 2013).

In order for blow sampling to reach its potential as a diagnostic tool for use in population assessments, the measurement of the various hormones in this matrix must be shown to be physiologically relevant. Testosterone (3 species) and progesterone (5 species) have been detected in blow samples from both mysticete and odontocete cetaceans (Hogg et al. 2005; Hogg et al. 2009; Hunt et al. 2014; Dunstan et al. 2012; Tizzi et al. 2010). However, without knowledge of how blow hormone concentrations relate to blood concentrations or the reproductive status of an individual, the ability to interpret blow hormone concentrations has been limited to assessing presence or absence. The use of blow as a diagnostic tool would be greatly enhanced if the relationship between hormone concentrations in blow and sex, maturity status, pregnancy status, or breeding season was known.

Belugas are an ideal study species for advancing the use of blow sampling as a diagnostic tool. Their reproductive biology is well understood, due to extensive post-mortem studies from subsistence harvests (*e.g.* Burns and Seaman 1988) as well as in depth longitudinal studies of live animals conducted in aquaria (*e.g.* Robeck et al. 2005). Progesterone in blood or progesterone metabolites in urine can be used to detect pregnancy and luteal activity (Steinman et al. 2012; Stewart 1994). Testosterone levels in blood can be used to detect sexual maturity and reproductive seasonality in males, with peak secretion occurring between January and April (Høier and Heide-Jørgensen 1994; Robeck et al. 2005). Belugas are widely held under professional managed care, making it possible to collect sufficient blow samples from

known individuals to develop hormone assays and to sample the same individuals longitudinally to assess changes in reproductive status over time. Although reproductive steroids have not been measured in beluga blow samples, blow sampling has already been shown to be an effective way to assess cortisol secretion in this species (Thompson et al. 2014). There is also potential to apply the methodology to wild belugas (Thompson et al. 2014), as well as a need for additional information to improve management, especially in endangered populations (National Marine Fisheries Service 2015).

The aim of this study was to determine if testosterone and progesterone concentrations in beluga blow samples are biologically relevant, laying the foundation for the development of blow sampling as a diagnostic tool in this species. Commercially available enzyme immunoassays for testosterone and progesterone were validated, and a physiological validation was performed using samples collected from aquarium belugas. Specifically, testosterone and progesterone measurements in matched blood and blow samples were compared, and the relationship between the concentrations of these hormones in blow and the sex, age, and reproductive status of these belugas was explored. Further validation was performed by measuring testosterone in the blow samples of wild belugas that are temporarily restrained for a health assessment project in Bristol Bay, Alaska to determine the applicability of this methodology in the field.

Methods

Study animals and sample collection

Zoological facilities

All blow and blood samples were collected from belugas with the voluntary cooperation of the animals via trained behaviors. Blow samples were collected from a total of 20 belugas (8 male, 12 female) from four different zoological facilities, ranging in age from 3-33 years (Table 1). Males less than 8 years old and females less than 6 years old were considered juveniles (Robeck et al. 2005). All belugas were housed in mixed sex groups with at least 2 males. Three of the four adult males sampled were proven sires. To assess seasonality of testosterone secretion, two males were sampled in all 12 calendar months, and all males were sampled between February and April, representing much of the period of peak testosterone secretion in belugas (January – April, Robeck et al. 2005). This project was approved by the Institutional Animal Care and Use Committees of Mystic Aquarium (Project #12001) and the University of Rhode Island (Project #AN12-02-016).

Ultrasonography and Characterization of Reproductive Status in Adult Females

To monitor for pregnancy, ultrasound exams were conducted on female belugas approximately twice per month at irregular intervals that varied by individual. Ultrasound exams were performed with the voluntary cooperation of the animal while the animal lied unrestrained in lateral recumbency at the water's surface. For the purposes of this study, a female later observed with a viable fetus was considered pregnant starting on the date that fluid was first visible in the uterus. The presence of a corpus luteum (CL) was detected via ovarian ultrasound or inferred by high

progesterone levels in blood (>3 ng/ml) (Robeck et al. 2010). In the absence of ultrasound or blood progesterone data, a CL was presumed to be present 30 days prior to a pregnancy diagnosis and to persist for 14 days from detection in non-conceptive cycles (Steinman et al. 2012; Robeck et al. 2010).

Blow sample collection

Blow samples were collected onto a nylon mesh (110 μ m, Elko Filtering Co., Miami, FL) stretched over a petri dish (100 mm diameter) and secured with a rubber band (Thompson et al. 2014). The nylon mesh and rubber bands were cleaned prior to use by soaking in 70% ethanol for 15 minutes, rinsed with Nanopure water, and air dried. For sample collection, the whales were trained to lift their head so that the blowhole was above the water's surface. The whale would then exhale once to clear any excess water from the blowhole surface. Then, 2-8 successive exhales were collected onto the mesh. The nylon was used to soak up any fluid that may have passed through onto the petri dish, and was immediately put into a 15 ml conical tube pre-loaded with half of a plastic syringe stopper in the bottom. The syringe stopper would allow for the later centrifugation of the nylon to retrieve the fluid blow sample. This tube was then frozen within 10 minutes at -20° C for up to 2 weeks until processing, or at -80° C until shipment on dry ice to the laboratory.

To retrieve the respiratory vapor, the tubes were thawed for 15 minutes and then centrifuged at 10° C for 30 minutes at $2600 \times g$. The volume of each sample was recorded and the fluid was frozen in 1.5 ml cryovials at -80° C until analysis.

Table 1. Sample availability for hormone analysis from aquarium belugas.

ID #	Sex	Maturity and Reproductive Status	Blow Samples Assayed for Testosterone	Blood Samples Assayed for Testosterone	Months Sampled for Testosterone	Blow Samples Assayed for Progesterone	Blood Samples Assayed for Progesterone
DL1	Male	Adult, proven sire	55	10	Jan - Dec	4	-
DL2	Male	Adult	28	20	Jan - Dec	3	-
DL3	Male	Adult, proven sire	7	7	Feb - Aug	4	-
DL4	Male	Adult, proven sire	4	-	Feb - Jun	0	-
DL5	Male	Juvenile	3	3	Feb - Apr	3	-
DL6	Male	Juvenile	2	-	Jan - Feb	1	-
DL7	Male	Juvenile	2	-	Mar, Dec	1	-
DL8	Male	Juvenile	5	-	Feb – Jun	3	-
DL9	Female	Juvenile	2	-	Feb, May	5	1
DL10	Female	Adult	1	-	Feb	3	0
DL11	Female	Adult, became pregnant	1	-	May	12	2
DL12	Female	Adult, became pregnant	2	-	May, Jul	67	14
DL13	Female	Adult	1	-	May	7	4
DL14	Female	Adult	4	-	Jun-Jul	22	14
DL15	Female	Adult	4	-	Jun - Oct	10	2
DL16	Female	Adult, became pregnant	1	-	Jun	22	14
DL17	Female	Adult	2	-	Feb, Apr	3	2
DL18	Female	Adult	2	-	Feb, Apr	6	4
DL19	Female	Adult	-	-	-	4	1
DL20	Female	Juvenile	-	-	-	6	6
Total			126	40		186	64

* Belugas were housed at: Georgia Aquarium, Atlanta; Mystic Aquarium, Mystic, CT; Shedd Aquarium, Chicago, IL; SeaWorld, San Antonio, TX

When possible, blood samples were collected into serum separator or sodium heparinized vacutainer tubes within one hour of blow sampling from a ventral fluke vein with the voluntary cooperation of the animal as a part of routine veterinary monitoring. Matching blood and blow samples were collected in the morning hours, typically between 0900 and 1000 hours. One ml of serum or sodium heparin plasma was obtained through centrifugation ($2000 \times g$ for 10 minutes at 10°C) and stored at -80°C .

Bristol Bay, Alaska

Blow samples were collected from 10 wild belugas (8 males and 2 females) in Bristol Bay, Alaska between August 25 and September 2, 2014 while they were being temporarily restrained for health assessment and satellite tagging (described in Norman et al. 2012). Samples were collected under National Marine Fisheries Service Marine Mammal Research Permit #14245. Animals were sexed and all were judged to be mature adults based on growth curves for this stock (Suydam 2009). Four exhales were collected per sample as described above, but due to field conditions it was not always possible to ensure that the blowhole was free of all water prior to collecting an exhale as it was with trained belugas. Once sealed inside a 15 ml conical tube, the samples were stored in a cooler on ice packs for 2-6 hours prior to centrifugation. The volume of each sample was measured and the fluid was transferred to 1.5 ml cryovials tubes for storage. The samples were frozen in liquid nitrogen within 8 hours of collection. Blood samples were also collected from these

animals from a dorsal or ventral fluke vein, centrifuged to obtain sodium heparin plasma, and stored in liquid nitrogen for transport.

Testosterone Enzyme Immunoassay Validation

A commercially available testosterone enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, Item #582701) was validated for use with beluga blow, serum, and plasma. This kit has 100% reactivity with testosterone. Cross-reactivities reported by the manufacturer were 140% for 19-nortestosterone, 27% for 5 α -dihydrotestosterone, 18.9% for 5 β -dihydrotestosterone, 4.7% for methyl testosterone, 3.7% for androstenedione, and 2.2% for 11-keto testosterone; all other cross-reactivities were below 1%. All blood samples were extracted with diethyl ether (Sigma-Aldrich, St. Louis, MO, Catalog #346136) according to the EIA kit manufacturer's instructions; blow samples were assayed without a sample preparation step. Parallelism to the standard curve was tested for blow, serum, and plasma using pooled male samples serially diluted from neat to 1:16 for blow and 1:10 to 1:100 for serum and plasma. Accuracy was tested for blow, serum, and plasma using pooled male samples spiked with an equal volume of known amounts of testosterone standard (125, 62.5, 31.3, 15.6, and 7.8 pg/ml). Accuracy was performed at 1:2 dilution for blow and 1:20 dilution for serum and plasma. Extraction efficiency in blood samples was tested by adding a known amount of testosterone (0, 250, 500, 1000 and 2000 pg) to subsamples of unextracted serum or plasma sample pools and performing the extraction protocol. Extraction efficiency was calculated as the observed amount of hormone quantified in the assay divided by the total amount of hormone expected

(native testosterone present in unspiked sample plus amount of spiked testosterone) multiplied by 100. To test for assay interference and recovery from the collection material, nylon mesh was spiked with assay buffer (negative control, n = 10) or known concentrations of testosterone (250, 125, 62.5, 31.3, 15.6, and 7.8 pg/ml) in three replicates. The spiked nylon was treated identically to a biological sample (placed in 15 ml conical tube, frozen >24 hours, thawed, centrifuged to recover spiked fluid, and refrozen until assay). Testosterone recovery (observed divided by expected multiplied by 100) was measured.

Progesterone Enzyme Immunoassay Validations

A commercially available progesterone enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, Item #582601) was validated for use with beluga blow, serum and plasma. This kit has 100% reactivity with progesterone. Cross-reactivities reported by the manufacturer were 14.0% for pregnenolone, 7.2% for 17 β -estradiol, 6.7% for 5 β -pregnan-3 α -ol-20-one, and 3.6% for 17 α -hydroxyprogesterone; all other cross-reactivities were below 1%. All blood samples were extracted with dichloromethane (Sigma-Aldrich, St. Louis, MO, Catalog #270997) according to the manufacturer's instructions. Parallelism to the standard curve was tested for serum and plasma using pooled female samples diluted 1:10, 1:20, 1:40, 1:60, and 1:80. Accuracy was performed for serum and plasma at 1:40 dilution using pooled female samples spiked with an equal volume of known amounts of progesterone standard (500, 250, 125, 62.5, and 31.25 pg/ml). Extraction efficiency in blood samples was tested by adding a known amount of progesterone (4000, 2000, 1000, and 500 pg) to

subsamples of unextracted serum or plasma sample pools and performing the extraction protocol; extraction efficiency was calculated as previously described for testosterone.

Untreated blow samples displayed parallelism to the standard curve, but failed the accuracy test, indicating matrix interference. Four extraction protocols for the extraction of progesterone from various matrices were tested (dichloromethane liquid-liquid microextraction and 3 variations of a solid phase extraction protocol) and all failed either parallelism or accuracy tests (See Appendix 3 for detailed methods). A diethyl ether liquid-liquid extraction was considered optimal and used for all samples. Samples (55 or 60 μ l) were placed in glass test tubes and 0.5 ml of diethyl ether was added. The samples were vortexed for 2 minutes, and then the aqueous layer was frozen in an ultralow freezer. The ether layer was poured off and the extraction procedure was repeated. The two ether layers were combined, dried under compressed air, and reconstituted in 110 or 120 μ l of assay buffer (for a final dilution of 1:2). Extraction efficiency in blow samples was tested by adding a known amount of progesterone standard (4000, 2000, 1000, and 500 pg) to subsamples of unextracted blow sample pools and performing the extraction protocol. Parallelism to the standard curve was tested for blow samples using pooled female samples serially diluted from neat to 1:8. Accuracy was performed for blow samples at 1:2 dilution using pooled female samples spiked with an equal volume of known amounts of progesterone standard (250, 125, 62.5, 31.25, and 15.625 pg/ml). To test for recovery from the collection material, nylon mesh was spiked with assay buffer (negative control, n = 4) or known amounts of progesterone (1000, 500, 250, and 125 pg) in two replicates.

The spiked nylon was treated identically to a biological sample (placed in 15 ml conical tube, frozen >24 hours, thawed, centrifuged to recover spiked fluid, and refrozen until assay). Progesterone recovery (observed divided by expected multiplied by 100) was measured.

Assay of biological samples

Blow samples were centrifuged at $8,000 \times g$ for 10 minutes to remove particulates and assayed at a 1:2 dilution for testosterone. Samples assayed for progesterone were centrifuged prior to extraction; extracted blow samples were assayed at 1:2 dilution. Extracted blood samples were assayed primarily at 1:20, but ranged between 1:2 and 1:80 depending on the expected concentration of testosterone or progesterone (Robeck et al. 2005). All samples were assayed in duplicate and the means were used in calculations. Individual samples with a %B/B₀ between 20 and 80% and a coefficient of variation (CV) < 20% were accepted. Samples with CV > 20% were re-assayed, and blood samples outside of the range of the kit were re-assayed at a different dilution. Two female blow samples assayed for testosterone had CV above the 20% cutoff (24.2 and 23.1), but were kept in the analysis due to lack of volume to re-run the assay for these samples. Blood samples with low progesterone (<300 pg/ml) were prone to higher CV; if re-assaying these samples did not result in a CV below the 20% threshold, the concentration measurement with the lowest CV was used for analysis (n=8). Two standard controls were run in each assay (testosterone: 100 and 25 pg/ml, n = 12; progesterone: 200 and 50 pg/ml, n = 14). Inter-assay variation was calculated by determining the CV for the two standard controls on each

plate. Intra-assay variation was calculated by averaging the CV for all of the samples with 20-80% binding on each plate.

Sample handling and storage

To test the effect of long term storage on testosterone concentration in blow, a pool of male blow samples was constructed and aliquoted into separate cryovial tubes and stored at -80° C. The pool was assayed 2 days after construction, then again 3, 17, 20, and 21 months later.

To determine if temporary chilled storage used during field work influenced testosterone concentration in blow, a separate pool of male blow samples was constructed and aliquoted in duplicate into separate cryovial tubes. Two samples were frozen immediately and replicate tubes were stored in a cooler on ice packs for 2, 4, 6, 8, and 10 hours before freezing at -80 °C to simulate field conditions. Both replicates from the 0 hour time point from this experiment had an unacceptable CV (>30%), so another experiment was conducted to examine the time between 0 and 2 hours. For this experiment, a third pool of male blow samples was aliquoted in duplicate into separate cryovial tubes; 2 samples were frozen at -80 °C immediately after construction, the others were stored in a cooler on ice packs for 30, 60, 90, and 120 minutes. The cooler remained at 4-7°C for the duration of these experiments. To test the effect of the freeze-thaw-freeze cycle that blow samples in this study were subjected to on testosterone concentration in blow, a pool of male blow samples was constructed and aliquoted in cryovial tubes. One sample was frozen at -80 °C immediately (no subsequent thaw-freeze), while the other tubes were stored in a -20°C

freezer for 1, 2, or 4 weeks. After the appropriate duration, they were thawed and refrozen at -80 °C until analysis.

To assess within sample variation, samples were collected on two separate collection devices; the first contained the blow from the first, third, and fifth exhale, while the second contained the blow from the second, fourth, and sixth exhale. A total of five samples collected in this manner from two different males were assayed for testosterone. To explore the influence that centrifugation might have on testosterone measurements, 4 samples from a male beluga were divided into two subsamples. One subsample was centrifuged prior to being assayed as described above, while the other was mixed thoroughly prior to being assayed.

Statistics

All statistical analyses were performed in R (R Core Team, 2015). Linear regression models (ANCOVA) were used to test for parallelism between serially diluted sample pools and the standard curve. Accuracy was tested by linear regression and considered acceptable if the slope of the line was not significantly different from 1. Linear regression was used to test for the effect of sample volume or sample handling regime on testosterone concentration. Assessing the correlation between hormone concentrations measured in blow and matching blood samples in aquarium belugas using linear regression required hormone concentrations to be log transformed to meet normality assumptions; Bristol Bay beluga blood and blow testosterone concentrations were correlated separately and were not transformed. For aquarium belugas with more than 5 matched observations, each individual's correlation was

examined separately to account for the uneven number of samples available from each beluga.

To describe seasonality of testosterone concentrations in blow collected from adult males, 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 using the {lme4} package in R (Bates et al. 2015). Model fits were compared using ANOVA, AIC, and Log-Likelihood.

To test for differences in log transformed blow testosterone concentrations in belugas with varying sex and reproductive status, mixed effects regression models were constructed using the predictors of sex, status (adult or juvenile), and season (Feb – Apr or May – Jan). To account for clustered observations within individual, a random intercept term was incorporated into the model. Significant interaction terms were evaluated graphically by constructing effects plots using the {effects} package in R (Fox 2003). The same method was used for log transformed progesterone concentrations in blow, using a predictor combining females with a corpus luteum or a pregnancy as well as a random intercept term. Males were left out of the model to avoid multicollinearity between sex and luteal activity and pregnancy, and thus sex differences will not be interpreted. Means are presented $\pm$ 1 SD and significance levels were set at $p < 0.05$.

Results

Males had larger blow sample volumes per exhale than females; blow sample characteristics are presented in Table 2.

Table 2. Blow sample characteristics (mean $\pm$ SD).

Source	# Exhales Collected	Volume per Exhale (μ l)
Aquarium Males	4.2 $\pm$ 0.9	78 $\pm$ 44
Aquarium Females	5.5 $\pm$ 2.0	57 $\pm$ 50
Bristol Bay	4	63 $\pm$ 40

Testosterone Assay Validation

The binding of a serially diluted pool of male blow samples was parallel to the standard curve ($F_{1,7} = 0.64$, $p = 0.45$) (Fig. 1). Testosterone was detectable in the sample pool for dilutions from neat to 1:16. The recovery of testosterone from spiked blow sample pools was $102 \pm 7\%$ ($y = 1.02x - 0.20$, $R^2 = .99$). The slope of the regression line was not significantly different from 1 (95% CI [0.94, 1.10]), demonstrating good accuracy (Fig. 2).

Fig. 1. Parallelism between a serially diluted male blow sample pool (triangles) and the linear portion of the standard curve (diamonds). Blow sample pool dilutions range from neat (1:1) to 1:16.

Fig. 2. Recovery of known concentrations of testosterone standard from spiked pooled blood samples after subtracting the concentration of an unspiked pool.

Fig. 3. Recovery ($\pm$ SE) of known concentrations of testosterone standard from spiked nylon ($n = 3$ per concentration) after subtracting the mean negative control value (45.02 pg/ml, $n = 10$) from each observation.

A low level of testosterone was recovered from negative control nylon spiked with assay buffer ($n = 10$, 45.02 ± 11.23 pg/ml). The nylon collection material did not interfere with the recovery of known amounts of testosterone, after subtracting the mean blank concentration of 45.02 from each observed concentration ($y = 0.9756x - 1.6594$, $R^2 = 0.99$) (Fig. 3). Results are reported uncorrected.

Serially diluted pools of extracted serum ($F_{1,11} = 1.26$, $p = 0.29$) and plasma ($F_{1,11} = 0.14$, $p = 0.72$) displayed binding parallel to the standard curve. Testosterone recoveries from spiked extracted serum ($y = 0.91x - 8.42$, $R^2 = 0.99$, 95% CI [0.68, 1.11]) and extracted plasma ($y = 0.88x + 6.68$, $R^2 = 0.97$, 95% CI [0.63, 1.10]) demonstrated good accuracy. The extraction efficiency for serum ranged from 88.3% to 109%, with a mean of 96.5%. The extraction efficiency for plasma ranged from 69.5% to 97.9%, with a mean of 79.2%.

The average lower limit of detection (80% B/B_0) was 10.7 pg/ml. Intra-assay variation was 8.37%; inter-assay variation was 6.3% for the 100 pg/ml control and 10.3% for the 25 pg/ml control.

Progesterone Assay Validation

The binding of a serially diluted pool of ether extracted female blow samples was parallel to the standard curve ($F_{1,6} = 0.05$, $p = 0.83$) (Fig. 4). Progesterone was detectable in the sample pool for dilutions from neat to 1:8. The recovery of progesterone from spiked blow sample pools was $102 \pm 17\%$ ($y = 1.06x - 5.62$, $R^2 = .97$). The slope of the regression line was not significantly different from 1 (95% CI [0.74, 1.37]), demonstrating good accuracy (Fig. 5). The extraction efficiency for

blow ranged from 100% to 117%, with a mean of 110% (Fig 6). For the results of extraction methods that were not effective, see Appendix 3.

Figure 4. Parallelism between a serially diluted female blow sample pool (triangles) and the progesterone standard curve (diamonds). Blow sample pool dilutions range from neat (1:1) to 1:8.

Fig. 5. Recovery of known concentrations of progesterone standard from spiked pooled blow samples after subtracting the concentration of an unspiked pool.

Fig. 6. Progesterone extraction efficiency from pooled female blow samples spiked with known amounts of progesterone standard.

Progesterone was recovered from negative control nylon spiked with assay buffer ($n = 4$, 118.80 ± 29.80 pg/ml). After subtracting the mean negative control concentration of 118.80 from each observation, the mean recovery of progesterone off of the nylon collection material was 77.68%, and was consistent across the concentrations tested ($y = 0.65x + 71.11$, $R^2 = 0.99$) (Fig. 7). Results are reported uncorrected.

Fig. 7. Recovery ($\pm$ SE) of known concentrations of progesterone standard from spiked nylon ($n = 2$ per concentration) after diethyl ether extraction and subtracting the mean negative control value (118.80 pg/ml, $n = 4$) from each observation.

Serially diluted pools of extracted serum ($F_{1,9} = 1.15$, $p = 0.31$) and plasma ($F_{1,7} = 0.83$, $p = 0.39$) displayed binding parallel to the standard curve. Progesterone recoveries from spiked extracted serum ($y = 0.95x - 26.94$, $R^2 = 0.998$, 95% CI [0.86, 1.03]) and extracted plasma ($y = 0.93x - 7.67$, $R^2 = 0.997$, 95% CI [0.83, 1.02]) demonstrated good accuracy. The extraction efficiency for serum ranged from 97.4% to 115.3%, with a mean of 105.7%. The extraction efficiency for plasma ranged from 119.2% to 130.3%, with a mean of 124.4%. The artificial inflation of plasma measurements will not affect the interpretation of the data in this study, as blood sample type was consistent throughout the study by individual (plasma was only collected from DL14, DL15, DL17, DL18, and DL19), most of the samples assayed were serum (44/64), and all of the samples from belugas that became pregnant during the study were serum samples.

The average lower limit of detection (80% B/B_o) was 32.6 pg/ml. Intra-assay variation was 10.9%; inter-assay variation was 6.6% for the 200 pg/ml control and 11.3% for the 50 pg/ml control.

Sample Handling Experiments

Storage of a pooled blow sample at -80°C for up to 21 months did not affect testosterone concentration beyond the inter-assay variation (CV = 7.6, $F_{1,3} = 1.82$, $p = 0.27$). The initial experiment testing the effects of storage in a cooler on testosterone concentration showed a significant effect of storage time on concentration ($F_{1,10} = 7.87$, $p = 0.02$). The CV for the 12 measurements of the same pool was 17%. However, both replicates of the 0 hours time point had high CV (34 and 36%). After dropping this time point, storage in a cooler for 2-10 hours before freezing did not significantly affect testosterone concentration ($F_{1,8} = 0.51$, $p = 0.50$); the CV for the 10 measurements of the same pool was 5.3%. A second experiment evaluated the effect of storage time on testosterone concentration from 0-2 hours more closely. Storage on ice packs for up to 2 hours did not affect testosterone concentration ($F_{1,8} = 2.14$, $p = 0.18$); the CV for the 10 measurements was 6.5%. Performing a thaw-freeze cycle after 1, 2, or 4 weeks at -20°C did not affect testosterone concentration ($F_{1,2} = 1.37$, $p = 0.36$), with the 8 measurements of the same pool having a CV of 5.8%.

For samples that were collected onto two separate collection devices, the average CV for the two samples for each divided sample was 9.0%. For samples that were divided and either centrifuged or mixed prior to assay, the average CV for the two samples for each divided sample was 6.6%. There was an insignificant negative

relationship between sample volume and testosterone concentration in male samples ($y = -0.04x + 121$, $F_{1, 104} = 2.69$, $p = 0.1$).

Assay of individual samples: Testosterone

Testosterone assay results are summarized in Table 3. Blow testosterone concentration was greater than the mean negative control concentration (45.0 pg/ml) for 90% (122/136) of the biological samples. Of those that were less than the mean negative control concentration, 7 were from females, two were from juvenile males, and 5 were from adult males. Two biological samples (both female) had testosterone concentrations lower than the lowest negative control sample (27.7 pg/ml).

Relationship between blood and blow

There was a significant positive correlation between matching log transformed blow and blood testosterone concentrations from aquarium belugas ($F_{1, 38} = 41.7$, $p < 0.0001$) (Fig. 8). Individual correlations for the 3 belugas sampled more than 5 times are shown in Table 4. After correcting for the mean negative control value and removing two observations with negative concentrations, blow testosterone concentration was $4.9 \pm 4.7\%$ of the matching blood concentration.

Table 3. Blood and blow testosterone concentrations (pg/ml) by sex, maturity status, season, and location.

Source	Blood Testosterone				Blow Testosterone			
	n	# Samples	Blood Testosterone (Mean $\pm$ SD)	Blood Testosterone Range	n	# Samples	Blow Testosterone (Mean $\pm$ SD)	Blow Testosterone Range
Aquarium Adult Male (all observations)	3	37	2462.4 $\pm$ 644.6	214.8 – 6853.5	4	94	119.3 $\pm$ 14.2	37.1 – 244.7
Aquarium Adult Male (Feb – Apr)	3	13	4024.4 $\pm$ 836.0	933.8 – 6853.5	4	31	136.95 $\pm$ 33.8	66.29 – 244.7
Aquarium Adult Male (May – Jan)	3	24	1607.0 $\pm$ 518.3	214.8 – 5897.0	4	63	99.4 $\pm$ 39.5	37.1 – 226.1
Aquarium Juvenile Male	1	3	114.3 $\pm$ 21.6	89.9 – 131.1	4	12	59.4 $\pm$ 6.5	30.28 – 92.3
Aquarium Female	0	0	--	--	10	20	54.1 $\pm$ 25.7	21.5 – 101.1
Bristol Bay Male	8	8	2195.9 $\pm$ 902.9	932.6 – 3560.3	8	8	90.8 $\pm$ 33.5	50.4 – 145.9
Bristol Bay Female	2	2		126.1; 175.3	2	2		62.5; 44.7

*For aquarium whales sampled more than once, means were first determined for each individual; the data displayed is the mean of the individual means.

Fig. 8. Correlation between log transformed blood and blow testosterone concentrations in aquarium belugas. Bold solid line: linear regression for all individuals combined ($y = 0.30x + 1.07$, $R^2 = 0.52$). Each beluga is plotted separately, with individual correlations plotted as thin lines for belugas with >5 observations.

Table 4. Individual correlations between blood and matching blow sample testosterone concentrations for belugas with >5 observations.

ID	Observations	Linear Equation	R^2	F	p
DL1	10	$y = 0.35x + 0.90$	0.58	10.83	0.01
DL2	20	$y = 0.39x + 0.79$	0.50	18.28	< 0.001
DL3	7	$y = -0.05x + 2.23$	0.01	0.07	0.80

Biological Relevance of Testosterone Concentrations in Blow

Seasonality of testosterone secretion was found in adult male blow samples.

Variation in log transformed testosterone concentrations by month was best described

by a quartic polynomial function with a random intercept term ($y = -.0843x + 0.0096x^2 + 0.0033x^3 - 0.0004x^4 + 1.9615$) (Fig 9). A model selection summary is shown in Table 5. A comparison between samples from belugas with varying sex and reproductive status showed a significant effect of individual (intercept, $p < 0.001$), as well as significant interactions between sex and status (adult or juvenile) ($p < 0.01$) and between sex and season (Feb – Apr or May – Jan) ($p < 0.001$). Effects plots demonstrated that adult males had higher testosterone in blow than juvenile males or females, and that adult males sampled during breeding season had higher testosterone concentrations in blow than all other groups (Figs. 10 and 11). Variation in blow testosterone concentrations by sex and reproductive status is shown in Fig. 12.

Fig. 9. Seasonal testosterone variation in adult male blow samples. Open circles: individual observations. Closed circles: monthly average. Regression line: quartic polynomial regression line.

Table 5. Model selection summary for describing seasonal variation of testosterone in adult male blow samples.

Model	AIC	Log Likelihood	ANOVA Results
Linear fixed effect, random intercept	-52.9	30.4	--
Quadratic fixed effects, random intercept	-54.0	32.0	Marginally better than previous ($p = 0.07$)
Cubic fixed effects, random intercept	-69.5	40.8	Significantly better than previous ($p < 0.001$)
Quartic fixed effects, random intercept	-72.4	43.2	Significantly better than previous ($p < 0.05$)
Quintic fixed effects, random intercept	-70.8	43.4	Not better than previous
Quartic fixed effects, random intercept and random linear slope	-68.7	43.4	Not better than previous

Fig. 10. Effect of maturity status on log transformed testosterone concentrations in blow by sex, with adult samples at $x = 1$ ("Adult") and juvenile samples at $x = 0$ ("Juvenile").

Fig 11. Effect of breeding season on log transformed testosterone concentrations in blow by sex, with male samples at $x = 1$ (“♂”) and female samples at $x = 0$ (“♀”).

Fig. 12. Untransformed blow testosterone concentrations by reproductive status. Points represent individual observations, with marker varied by individual.

Application to wild belugas

There was no significant relationship between blood and blow testosterone for belugas from Bristol Bay ($F_{1,8} = 0.79$, $p = 0.40$) (Fig. 13). Blow testosterone concentrations were $3.3 \pm 3.1\%$ of those in the matching blood sample. Three male samples exceeded 100 pg/ml; all six animals with blow testosterone concentrations >65 pg/ml were male.

Fig. 13. Correlation between blood and blow testosterone concentrations for Bristol Bay belugas ($y = 0.008x + 67.96$).

Assay of individual samples: Progesterone

Progesterone results are summarized in Table 6. All blow samples had concentrations higher than the mean negative control samples; two samples had concentrations lower than the highest negative control sample (151.7 pg/ml). Three belugas became pregnant during the study period. For one pregnant female (DL12), samples prior to pregnancy, during pregnancy, and after parturition were available. Samples from DL11 were available before and during pregnancy, while samples collected during pregnancy and after parturition were available from DL16. Luteal

activity was detected via ultrasound in 2 females (DL14 and DL16). For DL14, the CL persisted approximately 21 days. In DL16, the CL was visualized using ultrasound prior to pregnancy diagnosis. Two luteal phases were detected in DL12; a non-conceptive luteal phase inferred from a blood sample with a high progesterone concentration, as well as an inferred conceptive luteal phase approximately 10 weeks later, prior to the confirmation of pregnancy via ultrasound.

Relationship between Blood and Blow

There was a significant positive correlation between matching log transformed blow and blood progesterone concentrations from aquarium belugas ($F_{1, 62} = 94.0$, $p < 0.0001$) (Fig. 14). Individual correlations for the 4 belugas sampled are shown in Table 7. After correcting for the mean negative control value, blow progesterone concentration was $3.3 \pm 1.4\%$ of the matching blood concentration for pregnant females and $44.3 \pm 31.3\%$ of the matching blood concentration for non-pregnant females.

Biological Relevance of Progesterone Concentrations in Blow

Pregnant belugas and belugas with luteal activity had higher progesterone concentrations in blow than females without luteal activity or ongoing pregnancies ($p < 0.0001$) (Fig. 15). A blow concentration >330 pg/ml was a valuable diagnostic threshold: 6% of the blow samples collected from non-pregnant females and 80% of the blow samples collected from pregnant females or females in the luteal phase had progesterone concentrations that exceeded 330 pg/ml. Only pregnant belugas had

Table 6. Blood and blow progesterone concentrations (pg/ml) by sex and reproductive status.

Source	Blood Progesterone				Blow Progesterone			
	n	# Samples	Blood Progesterone (Mean $\pm$ SD)	Blood Progesterone Range	n	# Samples	Blow Progesterone (Mean $\pm$ SD)	Blow Progesterone Range
Female (all observations)	11	64	5477.7 $\pm$ 12451.1	92.9 – 19873.2	12	167	266.4 $\pm$ 70.8	143.8 – 1778.6
Adult Female (Pregnant)	2	19	10657.2 $\pm$ 1362.6	5004.3 – 19873.2	3	76	410.6 $\pm$ 87.8	181.0 – 1778.6
Adult Female (Non-pregnant, CL absent)	9	35	337.8 $\pm$ 159.3	99.3 – 1017.5	10	72	242.5 $\pm$ 27.3	143.8 – 415.3
Adult Female (Pregnancy unconfirmed, CL present)	2	3	6483.9 $\pm$ 2725.0	4557.1 – 9270.7	3	12	339.5 $\pm$ 51.0	240.0 – 417.3
Juvenile Female	2	7	127.8 $\pm$ 38.4	92.9 – 204.0	2	11	232.2 $\pm$ 14.2	150.7 – 385.8
Male	0	0	--	--	7	19	258.6 $\pm$ 42.2	167.0 – 366.7

*For whales sampled more than once, means were first determined for each individual; the data displayed is the mean of the individual means.

Fig. 14. Correlation between log transformed blood and blow progesterone concentrations in aquarium belugas. Bold solid line: linear regression for all individuals combined ($y = 0.16x + 1.97$, $R^2 = 0.60$). Each beluga with >5 observations is plotted separately, with individual correlations plotted as thin lines for the labeled individuals. Observations from all other individuals are plotted as circles.

Table 7. Individual correlations between blood and matching blow sample progesterone concentrations.

ID	Observations	Linear Equation	R^2	F	p
DL12	14	$y = 0.12x + 2.16$	0.51	14.64	0.002
DL14	14	$y = 0.44x + 1.22$	0.38	8.98	0.01
DL16	14	$y = 0.23x + 1.65$	0.74	38.8	<0.0001
DL20	6	$y = -0.07x + 2.49$	0.03	0.14	0.73

progesterone concentrations in blow >420 pg/ml. All three belugas that were pregnant during the study demonstrated temporal variation that was associated with changes in pregnancy status (Fig. 16). Although samples collected while a CL was active were rare (4 from DL12, 2 from DL16, and 6 from DL14), blow progesterone concentrations were higher than in females without luteal activity, and longitudinal variation in blow progesterone was observed with changes in luteal activity in DL14 (Fig 17).

Fig. 15. Untransformed blow progesterone concentrations by sex and reproductive status. Points represent individual observations, with marker varied by individual. To improve visualization, two outlying observations for pregnant females are not plotted in this graph (1088.7 and 1778.6 pg/ml).

Fig. 16. Longitudinal changes in blow progesterone concentrations in individual belugas that were pregnant during the study period. To improve visualization, two outlying observations for DL12 during pregnancy are not plotted in this graph (1088.7 and 1778.6 pg/ml).

Fig. 17. Progesterone concentrations in blow from DL14 for samples collected while a corpus luteum was present (326.5 ± 33.3) or absent (248.5 ± 62.5), as detected via ultrasound.

Discussion

This study has validated commercially available enzyme immunoassays for testosterone and progesterone in beluga blow samples and has demonstrated that the concentrations of these hormones in beluga blow samples are biologically relevant. Although both progesterone and testosterone have been detected in blow samples collected from other cetacean species, the ability to interpret hormone concentrations in blow samples has been limited due to a lack of this physiological validation. In this study, collecting relatively undiluted blow samples from belugas of known reproductive states allowed for comparisons between samples, establishing the value of progesterone and testosterone determination in beluga blow.

Comparison with Blood Samples

Both testosterone and progesterone concentrations in blow positively correlated with those in blood, demonstrating that blow sample analysis can serve as an indicator of the relative activity of these hormones in circulation. Blow testosterone concentrations reflected the expected variation in testosterone concentrations in blood due to reproductive status and seasonality that were observed both in this study and in previous studies on belugas (Robeck et al. 2005; Høier and Heide-Jørgensen 1994). Similarly, high progesterone levels in blood associated with pregnancy or luteal activity in belugas in this study and others (Robeck et al. 2005; Steinman et al. 2012; Stewart 1994) were also detected in blow samples. Most individuals sampled repetitively demonstrated positive correlations between the two matrices; those that did not either lacked high (DL20) or low (DL3) hormone

concentration observations to anchor the regression lines. Thus, the value of blow sampling is in the ability to make important distinctions between reproductive states (*e.g.* pregnant vs. non-pregnant) that are associated with relatively large differences in hormone concentrations, as opposed to fine-scale changes in hormone secretion.

The relative amount of testosterone and progesterone in blow compared to matching blood samples (approximately 3-5%, except for progesterone in non-pregnant females) is similar to the relative amount of steroid hormones in human saliva when compared to matching blood measurements (10%) (Gröschl 2008). As steroids likely enter saliva via passive diffusion, this similarity in relative concentration supports the hypothesis that steroids also enter the fluid lining the respiratory tract in cetaceans via passive diffusion (Hogg et al. 2009). The relatively high amount of progesterone in blow samples collected from non-pregnant females is unlikely to be due to a change in the mechanics of how the hormones enter blow samples in belugas of different reproductive states. Instead, the relatively high concentrations are more likely due to a matrix effect that artificially inflates progesterone concentrations in all biological samples and is amplified in samples collected from belugas with low blood progesterone concentrations.

Testosterone and progesterone levels in blow are much lower than those reported in the blubber or feces of other cetaceans. Unlike other matrices, there is little opportunity for the hormone to accumulate in the fluid lining the respiratory tract over time given the frequency with which belugas breathe (4-6 times per minute at rest). Fluid, and the hormones contained within it, is continually ejected with each exhale, while several hours may pass between subsequent defecations. All of the

belugas sampled in this study were breathing at baseline rates; it is possible that a long breath hold (as during a dive) could result in higher hormone concentrations in blow as more time is allowed for diffusion to occur. The comparison of samples consisting of the 1st, 3rd, and 5th or the 2nd, 4th, and 6th exhales suggest that the hormone concentrations vary little from breath to breath when there is not a long breath hold in between exhales. Any variation would depend on the relative rates of respiratory fluid production and steroid diffusion into this fluid. Further experiments on belugas in zoological facilities can be conducted to determine the effect of breath hold duration on hormone concentration in blow for improved application to free-swimming wild belugas.

Biological Relevance of Testosterone Determination in Blow

Despite the relatively low concentrations observed in this study, testosterone measurements in blow are diagnostically useful. While there is a high degree of overlap between out of season adult males, juvenile males, and females at low testosterone concentrations, high testosterone concentrations can be used to identify adult male belugas. Similar to testosterone determination in blood samples from seasonally breeding cetaceans, blow sampling is most diagnostically effective during the breeding season, when the overlap between adult males and other groups is dramatically reduced (Atkinson and Yoshioka 2007). However, testosterone concentrations in blow can still be informative outside of the breeding season, as evidenced by the analysis of wild beluga samples collected in late summer for this study. The seasonal variation in testosterone secretion in belugas reduces, but does

not eliminate, the value of blow sampling as a diagnostic tool for identifying adult males outside of the breeding season.

Given the low testosterone concentrations found in both immature males and females in this study and others (Robeck et al. 2005; Høier and Heide-Jørgensen 1994), as well as the low blow testosterone to blood testosterone ratio, this method is currently insufficiently sensitive to reliably differentiate juvenile males from females in this species. Many of the female and juvenile male blow samples contained very little testosterone, as the concentrations were within the range of the negative control samples. However, the three highest testosterone concentrations found in female samples were the only samples collected from pregnant females. In right whales, pregnant females have higher testosterone in fecal samples than non-pregnant females (Rolland et al. 2005). Testosterone has also been detected in presumed pregnant humpback whale blow samples (Hogg et al. 2009). However, the two female belugas sampled in Bristol Bay for this study were presumed pregnant based on blood progesterone values (data not shown), yet their blow testosterone concentrations were in the range of the negative controls. Because approximately 1/3 of female belugas are pregnant in wild populations (Burns and Seaman 1988), it will be important to sample additional pregnant females to improve the diagnostic capability of this technique.

Biological Relevance of Progesterone Determination in Blow

Progesterone measurements in blow reflected reproductive cycle stage in female belugas, with detectable increases in progesterone with the onset of pregnancy

or the presence of a corpus luteum in individual females monitored longitudinally. Although an increase in progesterone would be expected with these reproductive events, the relative increase in progesterone in blow with pregnancy state did not match those found in blood samples from this study or others (Stewart 1994; Robeck et al. 2005). While pregnancy resulted in more than a 30-fold increase in blood progesterone concentration, progesterone in blow only increases by a factor of 1.7, perhaps due to the inability of the steroid to accumulate in the fluid lining the respiratory tract. The lack of proportionality reduces the value of any one sample, especially when compared to other matrices, where distinctions between states are clearer (Rolland et al. 2005; Kellar et al. 2013a). Despite some ambiguity, most samples would be informative, as there was a diagnostically useful threshold (>330 pg/ml) that indicated pregnancy with reasonable certainty. Blow sampling may also be used to detect ovulation, although repeated sampling would be necessary to discriminate between a non-conceptive cycle and a pregnancy. The time of year that the samples are collected would further resolve this uncertainty, as estrous cycles are rare from July through December (Robeck et al. 2005). Additional sampling is required to fully develop the diagnostic value of this method.

Progesterone concentrations in blow were not valuable in determining the sex of the beluga in the absence of pregnancy or luteal activity. This was to be expected based on the available data on progesterone in male cetaceans; progesterone concentrations in beluga serum (Høier and Heide-Jørgensen 1994), bowhead blubber (Kellar et al. 2013a) and right whale feces (Rolland et al. 2005) have been similar for males and non-pregnant females. Given the low concentrations of progesterone in

blood for non-pregnant adults and juvenile females, this method is also not sensitive enough to detect maturity in females unless there is luteal activity or an ongoing pregnancy. This was also the case for progesterone measurements in the blubber of several other odontocete species (Trego et al. 2013).

Other factors influencing hormone concentrations in blow

In addition to reproductive status, other physiological factors may influence testosterone concentration in blow. In other species, including bottlenose dolphins in aquaria, testosterone secretion is affected by diurnal rhythm, with highest concentrations occurring in the morning (Funasaka et al. 2011). For this study, a majority of the samples were collected in the morning between 8:00 AM and 12:00 PM, including all of the matched blood and blow samples. However, the potential for a diurnal rhythm to affect these results cannot be dismissed. If the variation is significant, blow sampling would be an ideal method to use to study diurnal rhythm of testosterone secretion in belugas because blow samples can be collected with greater frequency than blood samples. Testosterone secretion may also be influenced by stress (Lynn et al. 2015) or contaminant load, which may be a particular problem for marine mammals that bioaccumulate endocrine disrupting toxins (Oskam et al. 2003; Subramanian et al. 1987). In future work it will be possible to measure both cortisol and testosterone in the same blow sample to investigate the influence of stress on testosterone in blow.

Hormone concentrations in blow samples are also affected by the sampling procedure. The belugas in this study exhaled once to clear away pooled water from their blowhole, but the volume of water remaining on or around the blowhole likely

varied from sample to sample, leading to some of the variation in blow hormone concentrations within groups found in this study. Increased risk for water contamination during sample collection in Bristol Bay under restraint conditions may have led to low testosterone concentrations in male blow samples when compared to matching blood samples and obscured the correlation between blood and blow that was found to occur in aquarium belugas. The probable water contamination is reflected in the higher sample volumes collected from wild belugas when compared to aquarium belugas. However, all of the wild male samples fell within the range of testosterone concentrations found in aquarium beluga samples collected in the summer or fall. Increasing sample size will aid in determining if testosterone in blow is correlated with testosterone in blood in wild belugas as it is in aquarium belugas.

Although it did not interfere with the assays for testosterone or progesterone, there is non-specific binding that likely results from the collection material used in this study. Collection material is known to affect salivary testosterone measurements in humans (Celec and Ostatníková 2012). This material was selected based on its performance with a Cayman Chemical cortisol EIA kit with beluga blow samples (Thompson et al. 2014). The collection material was cleaned following Thompson et al. (2014), who did not report negative controls, but spiked nylon with cortisol standard and observed recoveries similar to the recoveries in this study after correcting for the negative control. Perhaps the method used to clean the nylon for cortisol measurement is inappropriate for testosterone or progesterone measurements. Hunt et al. (2014) also tested for interference from negative control collection material spiked with testosterone and cortisol. They did not find detectable testosterone from negative

control samples, but their assay had a sensitivity of 50 pg/ml, which is higher than the mean negative control testosterone concentration found in this study (45 pg/ml) and thus a similar amount of interference would not have been detected using their assay. This non-specific binding is undesirable and future work should identify methods to eliminate it, either by different cleaning methods or through the use of a different collection material. However, all of the samples used in this study were collected in the same way, and validation experiments demonstrate that this effect was consistent across samples, allowing comparisons between samples for the purposes of this study. Eliminating this non-specific binding may also improve the diagnostic value of the method.

Application to unrestrained wild belugas

In order for hormone determination in blow to be applied to wild, free-swimming belugas, a single exhale should contain enough sample to perform the assay, as collecting multiple exhales from an unrestrained whale is unlikely. The hormone concentration should also not vary significantly from exhale to exhale, so that conclusions can be drawn from the analysis of the blow sample that is collected. In a majority of the cases in this study, a single exhale would have yielded a large enough fluid volume to perform the assay at a 1:2 dilution. With additional validation experiments, the sensitivity of this assay would allow for samples to be diluted even further prior to analysis, requiring as little as 10 µl of sample. Collecting samples on two different sample collection devices demonstrated that testosterone content is relatively consistent from one breath to the next during a sampling event. Hunt et al.

(2014) found large variation in hormone concentrations in successive breaths sampled in right whales. The findings in this study support their conclusion that this variability was most likely due to varying environmental water contamination and not physiological changes between exhailes, although there is potential for increased accumulation of hormone via diffusion over time during long duration breath holds.

Sampling in field conditions also requires that samples be stored appropriately until analysis. Several sample handling experiments demonstrated that testosterone in blow is stable both during temporary storage while chilled and long term storage while frozen. Thus, we can be confident that the storage protocols in this study do not affect the interpretation of testosterone concentrations. These findings are consistent with the stability of testosterone in serum (Stroud et al. 2007) and saliva (Durdiaková et. al 2013) in humans. They are also consistent with the stability of cortisol in right whale blow (Hunt et al. 2014). Hogg et al. (2005) found that testosterone was not stable in frozen bottlenose dolphin blow samples, which they attributed to the activity of bacteria that may metabolize steroids, as occurs in feces (Khan et al. 2002). Therefore an antibiotic was added to the dolphin blow samples to improve stability. The observed differences with this study may be due to species differences in the bacterial communities within the respiratory tract, the variation in spiked testosterone levels for stability tests, or the different analytical methods used. Under the field conditions in this study, there was no evidence to suggest the use of an antibiotic was necessary to preserve beluga blow samples. This allows for an accurate measurement of the volume of the blow sample and eliminates the need for an extraction step prior to immunoassay for testosterone. It was also unnecessary to perform the alcohol

extraction from the collection material described for right whale blow (Hunt et al. 2014) to acquire measurable testosterone or progesterone, although collection from free-swimming animals with high levels of environmental water contamination (as found in the right whale sampling) may ultimately necessitate an additional extraction step in belugas as well.

As recognized by others studying cetacean blow samples (Hogg et al. 2009; Hunt et al. 2014; Thompson et al. 2014), the most significant limitation to the application of this methodology to free-swimming cetaceans is dilution from environmental water. The use of exhaled breath condensate in human medicine faces similar challenges, and dilution markers may vary by analyte (Ahmadzai et al. 2013). The use of hormone ratios or other potential markers of dilution may partially alleviate this limitation and should be explored. Although insignificant, the negative relationship between blow sample volume and testosterone concentration in this study suggests that in addition to variation in environmental water contamination, variable fluid production within the respiratory tract may also have an effect on the interpretation of hormone concentrations. Therefore, it may be necessary to identify a dilution factor for fluid production within the animal as well as dilution by environmental water to fully standardize results and allow strict comparisons between samples.

In addition to the ability to accurately measure hormones in blow, the reproductive state of an unknown beluga should ideally be distinguished from a single sample. Sampling a larger number of belugas in various reproductive states would allow for the development of statistical models that could be used to determine the

probability that an unknown beluga is in a particular reproductive state based on hormone determinations in blow. Although the number of belugas in this study was small, this sample represents >60% of the belugas in US zoological facilities, including all of the living sires (Myers 2014). There is also the possibility that some samples were incorrectly classified by reproductive status due to the irregularity of blood sampling and ultrasound exams. The most likely misclassification was associated with the failure to detect luteal phases in non-pregnant females. Also, without an assessment of sperm production in the males studied, the maturity status may have been incorrectly assigned. The closely managed beluga breeding program in US zoological facilities will allow for increased sampling with greater frequency in the future.

Current Utility of Testosterone and Progesterone Determination in Beluga Blow Samples

An important advantage of blow sampling in zoological facilities is the relative ease with which samples can be collected, enabling frequent longitudinal monitoring of individuals. Blow sampling can be used to monitor the attainment of maturity in individuals, track reproductive cycles, and identify pregnancy in belugas that are not trained for blood or urine sampling or ultrasound examinations. The possibility of incorrectly identifying reproductive status would be reduced due to the ease of collecting multiple samples from the same animal. The ability to detect luteal activity, and thus ovulation, is important for belugas because they are facultative induced ovulators (Steinman et al. 2012). The detection of an active luteal phase in a female

with access to a male implies that the female likely copulated. Copulation is rarely observed in belugas, even when directed behavioral observations are conducted (Hill et al. 2015; Chapter 4, this dissertation). Therefore, hormone determination via blow sampling would improve reproductive management and also creates new research opportunities by allowing frequent, repetitive sampling of individuals.

For wild belugas, this method is immediately applicable to mass stranding events such as those that occur in Cook Inlet, Alaska, where groups of belugas may temporarily strand between high tides. This scenario improves the ability to interpret blow hormone concentrations by removing the variability that would be associated with environmental water contamination. One such event involved the live, temporary stranding of at least 76 belugas, representing nearly a quarter of the 315 belugas estimated to comprise that stock (National Marine Fisheries Service 2015). Significant portions of this endangered population could be sampled during a single event, providing important demographic information. Blow sampling would strike a balance between minimizing the stress of handling imposed on the animals while they are already under stress and maximizing the information that could be obtained during these events.

The ability to identify pregnant females non-invasively in this population would be a great benefit to population management. A significant effort is made in this population to identify individuals and to count calves during aerial censuses; having the ability to identify pregnant females and follow up on her success at birthing and rearing the calf would help identify potential causes for the lack of recovery in this population (National Marine Fisheries Service 2015). The ability to identify males in

breeding condition is also important, as reduced availability of breeding males can negatively impact population viability (Milner et al. 2007). Additionally, detecting maturity in belugas is difficult to perform through visual inspection alone; an improved understanding of the proportion of belugas that are mature through the use of blow sampling would aid in developing more accurate population models (Mosnier et al. 2015).

Taken together, the variation in blow testosterone and progesterone concentrations with reproductive status may also help to identify the breeding season in wild beluga populations. While there is a great deal of overlap, the peak of the breeding season appears to vary among stocks (Burns and Seaman et al. 1988; Heide-Jørgensen and Teilmann 1994), or is unknown or unreliably documented for some stocks due to the difficulties associated with determining the length of gestation in wild belugas (Suydam et al. 2009; Robeck et al. 2015). Determining when ovulations are occurring through progesterone determination and when males are in breeding condition through testosterone determination will help clarify when conceptions are actually occurring and thus identify periods when wild beluga populations may be more vulnerable to disturbance. In conjunction with photo identification of individuals and aerial surveys, blow sampling temporarily stranded individuals would allow for better management of endangered populations, such as the Cook Inlet stock.

Additional research is required for blow sampling to provide the same diagnostic capabilities as biopsy sampling in free-swimming cetaceans. However, the perceived risks associated with biopsy sampling may preclude research from being conducted or limit sampling capabilities. With further development, blow sampling

will provide a less invasive, yet informative alternative for monitoring population demographics and viability.

Conclusion

This study advances the use of cetacean blow samples for hormone determination by demonstrating that testosterone and progesterone concentrations in beluga blow samples are biologically relevant, varying by sex, maturity status, season, and reproductive cycle stage. The positive correlation between testosterone and progesterone in blow and blood found in this study is an important step in the development of cetacean blow sampling as a diagnostic tool for studying population demographics, and further justifies the continued study of other analytes that can be detected in blow samples. Continued validation experiments and method development using samples collected from cetaceans in zoological facilities will improve the application of blow sampling to wild cetaceans. This study provides a framework for interpreting testosterone or progesterone concentrations found in blow samples, which can be used to monitor breeding in zoological facilities, as well as to identify pregnant females or males in breeding condition in groups of wild belugas that are temporarily stranded.

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CHAPTER 2

“Minimally invasive molecular analyses in belugas (*Delphinapterus leucas*) using blow samples”

by

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Abstract

Blow (exhale) sampling in cetaceans may provide a minimally invasive alternative to biopsy sampling for genetic analyses that may be favored in vulnerable populations. However, the utility of single-exhale blow samples has not been evaluated, and the relationship between the number of exhales collected and DNA yield and its subsequent performance in polymerase chain reactions (PCRs) is unknown. DNA was extracted from a total of 98 blow samples collected from 11 aquarium-housed and 29 wild belugas, from Bristol Bay, Alaska. Blow samples consisted of 1, 2, or 4 successive exhales, with at least 9 samples per type from both aquarium-housed and wild belugas. DNA concentration and purity was assessed with a spectrophotometer, and PCR performance was assessed through the amplification of a fragment of the mitochondrial DNA control region or a nuclear marker of sex (ZF). Measurable DNA was recovered from 96 samples (98%), although DNA yield varied widely, both by sample (range: 0-4406 ng, mean = 701.5, SD = 1033.7), and by exhale (ng DNA/exhale) (range: 0-3723, mean = 427.1, SD = 721.8). The amount of DNA extracted per exhale was greater for aquarium samples than for Bristol Bay samples, but total yield was not proportional with the number of exhales for either group. Yields were similar for all aquarium sample types, while among Bristol Bay samples, four-exhale samples yielded 28x the amount of DNA as single-exhale samples. Successful beluga-specific PCR amplification occurred in 56/59 of the aquarium samples (23/25 single-exhale samples) and 28/39 of the Bristol Bay samples tested (7/10 of the single-exhale samples). The forcefulness of the breath and chance collection of large pieces of cellular debris likely shaped the relationship between the

number of exhales and the DNA yield. Using these methods, a single forceful exhale should yield enough DNA to perform multiple experiments, including the analysis of relatively large fragments of DNA. Although further development is required for the application of this technique to free-swimming belugas, it is immediately applicable to live-stranded belugas, such as the temporary mass strandings that occasionally occur in Cook Inlet, Alaska. Due to its minimally invasive nature, blow sampling has the potential to increase genetic sampling in protected populations, facilitating beluga research and management.

Introduction

Genetic sampling is fundamental to the management of cetaceans (whales, dolphins, and porpoises). In belugas (*Delphinapterus leucas*), the availability of tissue from subsistence harvests allows for relatively large sample sizes for genetic research (e.g. de March and Postma 2003). Genetic analysis of harvested belugas has elucidated stock structure, relationships between stocks, philopatric migratory behavior, and social structure of migratory groups (O’Corry-Crowe et al. 1997; Brown Gladden et al. 1997; Colbeck et al. 2012). Post-mortem genetic sampling has provided information that is critically important for population management in this species (Turgeon et al. 2012).

While post-mortem sampling is extremely valuable, new research opportunities are created when live animals can be sampled. Tissue collection from live animals can reduce sampling disparity between beluga stocks that are subject to varying harvest levels. This is especially important in stocks where hunting has been dramatically

reduced or eliminated due to conservation measures, as in the Cook Inlet of Alaska (National Marine Fisheries Service 2015). These stocks are also the populations most in need of genetic sampling to facilitate management. Biopsy sampling, achieved by firing a dart into a surfacing whale, is a common method employed in the study of cetaceans. Although effective for studying belugas (*e.g.* Meschersky et al. 2008), wildlife managers have at times avoided the use of biopsy sampling in some populations, in part due to concern for the behavioral responses or physical welfare of the sampled animal (McGuire and Stephens 2014). This creates a need for a less invasive method of acquiring DNA for analysis. Managers of wild populations may be more likely to support research programs that pose less risk to the welfare of the animal or are more compatible with other low impact research, such as the photo identification of individual belugas.

Minimally invasive tissue sampling methods have been developed in other species of cetaceans as alternatives to biopsy sampling. Feces can provide a source of DNA for analysis in cetaceans (Gillett et al. 2008; Parsons et al. 1999). However, beluga feces rarely float and are most often dissipated within the water column shortly after excretion, making sample collection logistically impossible under field conditions (personal observation). In dolphin species that voluntarily ride along the bow of a moving boat, skin swabs have been used to collect tissue (Harlin et al. 1999). However, belugas are not known to ride the bow of travelling boats, precluding the use of this technique in this species.

Recently, blow (exhale) sampling has been identified as a source of DNA for analysis in cetaceans (Frère et al. 2010). Due to the forcefulness with which

cetaceans exhale, cellular debris is commonly ejected along with the respiratory vapor that is also a source of steroid and thyroid hormones for analysis (Frère et al. 2010, Hunt et al. 2013). These cells, including epithelial cells and leukocytes, are commonly seen in cytological examinations of cetacean blow samples (Sweeney and Reddy 2001). Given the prevalence of microorganisms within the respiratory tract of cetaceans, genetic sampling of blow samples may also target microorganisms, as has been accomplished in a variety of cetaceans (Acevado-Whitehouse et al. 2010). Although similar to biopsy sampling in that a boat must approach the animal in order to collect the sample, blow sampling does not impose physical harm upon the animal and thus may provide a less invasive alternative for acquiring molecular data.

To date, cetacean DNA sampling in blow samples has been reported for bottlenose dolphins (Frère et al. 2010) and harbor porpoises (Borowska et al. 2014) in aquariums, and one wild bottlenose dolphin (Frère et al. 2010). Both studies found that DNA sequences isolated from blow samples matched those obtained from DNA isolated from blood from the same individual, validating the technique for use in these species. However, both of the studies utilized blow samples that consisted of more than one exhale (harbor porpoises: 5-6 breaths; dolphins: 4 breaths). While bow riding species may allow the collection of more than one exhale from the same animal, for many species, including the beluga, a single exhale is the most realistic sampling outcome. Therefore, while blow sampling for molecular analyses has been successful in these species, further investigation is required to determine if a single exhale would yield enough DNA to perform common analyses utilized in population management.

For blow sampling to be a reasonable alternative to biopsy sampling in cetaceans, DNA yield from a single exhale should be sufficient to allow for multiple experiments; for example, investigators may wish to identify the sex, mtDNA haplotype, and microsatellite genotype of an individual from the same sampling event. Therefore, an understanding of the relationship between DNA yield and the number of exhales collected is needed. Borowska et al. (2014) reported a mean yield of 1120 ng (range: 120-2480) from a total of 11 samples consisting of 5-6 exhales each (187-224 ng/exhale, assuming a 200 µl elution volume), while Frère et al. (2010) reported yields of approximately 2000 ng from a total of 6 samples consisting of 4 exhales each (500 ng/exhale, assuming a 200 ul elution volume). It is not clear if the collection of a single exhale would result in these predicted per-exhale yields. Using a different collection method, Acevedo-Whitehouse (2010) observed yields ranging from 2200-24000 ng from single exhales from a variety of free-swimming wild species, from smaller dolphins to large mysticete whales. If the yield can be reasonably predicted, the investigator can make informed decisions about the minimum number of exhales that should be collected.

Additionally, for blow sampling to be a realistic alternative to biopsy sampling, the DNA that is recovered from blow samples would not be excessively fragmented and allow for the amplification of a variety of target sequences, regardless of size. Currently, the largest nuclear DNA fragment that has been amplified from cetacean DNA extracted from blow samples is approximately 160 base pairs (Borowska et al. 2014; Frère et al. 2010). Amplifying longer sequences from blow would be useful for molecular sex determination, which is often necessary because sex is difficult to

determine in a free swimming beluga (Petersen et al. 2012). In belugas, molecular sex determination is commonly accomplished using a fragment of the *ZF* gene, which is approximately 1000 bp long (Shaw et al. 2003). The ability to amplify larger gene targets would also allow for research investigating evolutionary trends in immune function or the impact of anthropogenic effects on populations (O’Corry-Crowe et al. 2008). Therefore, validating the ability to amplify longer nuclear sequences from host DNA isolated from blow samples would be valuable.

Belugas may be a good candidate for single-exhale blow sampling for DNA analysis, given their large size relative to previously studied species. Perhaps with a larger exhale volume, more cells will be carried up with each exhalation, leading to higher DNA yields per exhale relative to smaller cetaceans. The availability of belugas in zoological facilities enables method development, and blow samples from wild animals can also readily be collected from wild belugas that are temporarily restrained for tagging purposes (Thompson et al. 2014).

Using blow samples collected from wild and aquarium belugas, this project aims to determine the relationship between number of exhales collected per sample and DNA yield, as well as the downstream performance of the extracted DNA in polymerase chain reactions (PCR). PCR performance with both mtDNA and large nuclear target sequences (>900 bp) will be assessed. To maximize the potential utility with wild belugas, additional factors that may influence DNA yield from blow samples will also be explored.

Methods

Study Animals

Blow sampling in aquarium animals (Mystic Aquarium, Mystic, CT and Shedd Aquarium, Chicago, IL) was performed with the voluntary cooperation of trained belugas of known sex. The belugas were trained to position their head so that their blowhole was above the water's surface, and to then exhale on cue. Blow samples were collected from wild belugas in Bristol Bay, Alaska in August or September of 2012, 2013, and 2014 while they were being temporarily restrained for health assessment and satellite tagging (as described in Norman et al. 2012). Wild beluga samples were collected under National Marine Fisheries Service Marine Mammal Research Permit #14245. This project was approved by the Institutional Animal Care and Use Committees of Mystic Aquarium (Project #12001) and the University of Rhode Island (Project #AN12-02-016).

Blow Sample Collection and Handling

Sample collection methods were similar to Frère et al. (2010). Blow samples consisting of one, two, or four successive exhales were collected into a polypropylene 50 ml conical tube (Fisher Scientific, Waltham, MA, #14-432-22) held inverted directly over the blowhole. The tube was tilted cranially by approximately 30-45° to maximize the collection of the fluid, which is angled cranially upon exhalation. No attempt was made to clear environmental water from the blowhole prior to sample collection to simulate sampling of a free-swimming beluga. The tubes were capped and placed on ice. For aquarium samples, 1 ml of 1X Tris-EDTA (TE) buffer was

added to the tube within 15 minutes of collection. For Bristol Bay samples, 1-1.5 ml of 1X TE buffer was added to the tube 30-90 minutes following collection. The tubes were rocked by hand to coat the inner surface of the tube with buffer. Samples were frozen in the 50 ml conical tubes at -20°C. For aquarium samples, the tubes were placed in the freezer within 15 minutes of sample collection. For wild samples, the tubes were held in coolers on ice packs for 4-6 hours before being placed in a freezer. Samples were shipped to the laboratory on dry ice or in liquid nitrogen.

A total of 11 aquarium belugas and 29 wild belugas were sampled (Table 1). Sample type varied by year for belugas sampled in Bristol Bay. In 2012, all samples were composed of 4 successive exhales. In 2013, all samples were composed of 2 successive exhales. In 2014, a single-exhale sample and a sample consisting of 2 successive exhales were collected from each beluga.

Table 1. Number of samples collected by sample type (number of successive exhales collected). The year that samples were collected from Bristol Bay is listed in parentheses.

Sample Source (Individuals)	One Exhale	Two Exhales	Four Exhales
Bristol Bay (n = 29)	10 (2014)	20 (2013 & 2014)	9 (2012)
Mystic Aquarium (n = 4)	25	10	10
Shedd Aquarium (n = 7)	14	0	0

DNA Extraction

After thawing, the 50 ml conical tubes were again rolled by hand to coat the inner surface of the tubes with buffer, and were then centrifuged for 10 minutes at 2060 x g. After pipetting up and down several times to dislodge material from the bottom of the tube, the fluid was pipetted from the conical tube into a 1.5 ml

microcentrifuge tube. This tube was then centrifuged in a microcentrifuge for 10 minutes at 13400 x *g*.

The presence or absence of a cell pellet was then recorded. If a pellet was visible, the supernatant was removed completely before performing the DNA extraction protocol. If a pellet was not visible, all but approximately 50 µl of supernatant was removed by micropipette from the tube without disturbing the lower layer of liquid that presumably would contain cellular material. For Bristol Bay samples, the presence of very fine sand in the samples made it difficult to determine if a cell pellet was present or not; thus approximately 50 µl of supernatant was left in all of the Bristol Bay samples.

DNA was isolated using the Qiagen DNEasy Blood and Tissue kit (Valencia, CA), following the manufacturer's tissue protocol with the following modifications. The addition of buffer ATL was reduced to account for any leftover volume of TE in the sample tube. The duration of the lysis step lasted one hour or in rare cases, until the pellet (if visible) was completely lysed, up to 3 hours. Samples were vortexed every 15-20 minutes during lysis. DNA was eluted in 50 µl of the provided buffer AE. This elution step was repeated into a separate tube. Two 50 µl elutions were preferred to a single 100 µl elution because the DNA was more likely to be concentrated enough in the first elution to then be used in PCR reactions without concentration.

Yield and Purity

DNA concentration (ng/μl) and purity (ratio of absorbance of 2 μl of sample at 260 and 280 nm) was then assessed using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham, MA). The total yield from each elution step was calculated (assuming 50 μl sample volume), and the yield from these two separate elutions were added together to calculate the total yield. Reported A_{260}/A_{280} ratios are from the first elution, when the DNA was most concentrated.

Blood Sampling

For comparison, blood samples were collected from trained belugas at Mystic Aquarium (n = 4) from the ventral fluke vein with the voluntary cooperation of the whale. DNA was isolated from 100 μl of whole blood using the Qiagen DNeasy Blood and Tissue kit (blood protocol) using the manufacturer's instructions. Blood DNA was eluted once into 200 μl of the provided buffer AE. DNA concentration and purity was assessed via NanoDrop. For each beluga's blood sample, 3 or 4 separate extractions were performed for a total of 14 extractions.

Molecular sex determination via Polymerase Chain Reaction

PCR performance was tested for Mystic Aquarium samples through a molecular sex determination test. The zinc finger gene (ZF), which has a sex-linked polymorphism, was amplified using primers LGL331 (5'-CAA-ATC-ATG-CAA-GGATAG-AC-3') and LGL335 (5'-AGA-CCT-GATTCC-AGA-CAG-TAC-CA-3') (Shaw et al. 2003). In belugas, the Y chromosome copy is 1006 bp long, while the X copy is 931 bp long. Thus, following electrophoresis, male samples will be indicated

by two bands, while females will only have a single band. PCR reactions were carried out in 50 µl (1X reaction buffer, 1.5 mM MgCl₂, 10 mM dNTPs, 2.5 µM forward and reverse primers, and 2.5 U Taq polymerase) using DNA from the first elution. For aquarium samples, a target of 30 ng of template was used for ZF or mtDNA PCRs although amplification was regularly achieved with much less.

The conditions for the reaction were 94°C for 3 min, then 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, followed by an extension step of 72°C for 10 min. The PCR product was loaded into a 2% agarose gel stained with ethidium bromide for electrophoresis. Bands were visualized under UV light and scoring was completed by eye. PCR performance was assessed through the presence or absence of the appropriate banding pattern.

mtDNA Amplification via Polymerase Chain Reaction

PCR performance was tested for Bristol Bay and Shedd Aquarium samples through the amplification of mitochondrial DNA (mtDNA) using primers L15926 (5'-ACA-CCA-GTC-TTG-TAA-ACC-3') and H00034 (5'-TAC-CAA-ATG-TAT-GAA-ACC-TCA-G-3') commonly used in the identification of mtDNA haplotypes in belugas (*e.g.* O'Corry-Crowe et al. 1997). For Bristol Bay belugas, a target of 60 ng of template was used in mtDNA PCRs to improve success. Low DNA yields from some samples precluded the use of target template DNA amounts in PCR reactions. Bristol Bay template DNA amounts ranged from 2 to 72 ng. PCR reactions were carried out in 50 µl (1X reaction buffer, 1.5 mM MgCl₂, 10 mM dNTPs, 2.5 µM forward and reverse primers, and 2.5 U Taq polymerase) with the following conditions: 35 cycles of 1.5

min at 94°C, 2 min at 48°C, and 3 min at 72°C, followed by a final extension step of 5 minutes at 72°C.

The PCR products were run through gel electrophoresis on 2% agarose gels stained with ethidium bromide. PCR performance was assessed by presence or absence of a band following electrophoresis. The bands were excised and DNA was extracted using a Gel Extraction kit (Qiagen, Valencia, CA). The DNA was submitted for sequencing by SANGER capillary electrophoresis at the University of Rhode Island Genomics and Sequencing Center and haplotypes were identified using BLAST (<http://blast.ncbi.nlm.nih.gov>). Haplotypes were named based on sequences published by Meschersky et al. (2008). To initially validate the technique, DNA isolated from blow and blood from three belugas was used in a mtDNA PCR; resulting bands were sequenced from one blood and one blow sample to ensure that results were replicated from the two DNA sources for each individual.

Sample Collection and Handling Effects on Yield

To determine if the strength of the breath influences DNA yield or PCR performance, 4 separate “calm breaths” were collected from 2 aquarium belugas (2 samples per beluga). Typically, the exhale collected from trained aquarium belugas is of similar force to the exhale used when the whale surfaces to breathe. The “calm breaths” were collected while the belugas were resting at the surface. The force of these breaths is much lower than the typically sampled breaths, and is similar to the force of the Bristol Bay beluga exhales collected under restraint conditions. DNA was

isolated using the protocol described above, and an mtDNA PCR was attempted using 30 ng of template DNA.

Wild beluga samples were kept on ice for up to 6 hours while in the field until they could be frozen. To test the effect of temporary chilled storage on DNA yield and PCR performance, 4 single-exhale blow samples were collected from 2 aquarium belugas (2 samples per beluga). Buffer TE was added to the samples, and they were stored in a 4°C refrigerator for 6 hours before being placed in a -20° freezer. DNA was isolated using the protocol described above, and an mtDNA PCR was attempted using 30 ng of template DNA.

To test the effect of long term storage on the ability to isolate DNA from blow samples, two single-exhale blow samples were stored at -80° C in 1.5 ml microcentrifuge tubes. One sample was stored for 22 months and the other was stored for 23 months before the DNA extraction protocol was performed. Yield and purity were assessed via NanoDrop. A ZF PCR was attempted using 30 ng of template DNA.

To test the influence of lysis time on DNA yield, two single exhale blow samples were collected from an aquarium beluga. After transferring the sample to a microcentrifuge tube, each sample was vortexed thoroughly and divided equally into two tubes. DNA was isolated from both tubes following the protocol described above, with one tube from each sample undergoing a 1 hour lysis time, and the matching tube undergoing a 6 hour lysis time. Yield and purity were assessed via NanoDrop. To estimate the contribution of microorganism DNA from environmental water in the blow sample, DNA was isolated from 1 ml of water from various locations in the

beluga whale exhibit at Mystic Aquarium ($n = 3$) or within Bristol Bay ($n = 3$). Yield and purity were assessed via NanoDrop.

Yields are expressed as the mean $\pm$ SD. Small sample sizes or samples clustered by individual that violated independence assumptions precluded rigorous statistical testing. The effects of variables of interest on DNA yield or PCR performance are shown using box plots created in R (R Core Team, 2015), where the box represents the interquartile range, the dark line represents the median, whiskers represent the minimum and maximum values, and outliers (>1.5 x the interquartile range away from the minimum or maximum values) are plotted as open circles unless specified otherwise by an accompanying legend.

Results

Measurable DNA was extracted from 96/98 (98%) blow samples. Results are summarized in Table 2. DNA yield varied widely, both by sample (range = 0-4406 ng, mean = 701.5, SD = 1033.7), and by exhale (ng/exhale) (range = 0-3723 ng, mean = 427.1, SD = 721.8). The amount of DNA extracted per exhale was greater for aquarium samples than for Bristol Bay samples (Fig. 1). Total yield was not proportional with the number of exhales for either group (Table 3). Among aquarium samples, total yield was influenced by individual (Fig. 2) as well as the presence or absence of a cell pellet following centrifugation prior to the extraction protocol (Fig. 3). The A_{260}/A_{280} ratios varied widely by sample and were occasionally outside of the normal range for nucleic acid samples (range: -7.9-34.1). Samples with a DNA yield >100 ng ($n = 62$) had a mean A_{260}/A_{280} of 1.8. Variation in A_{260}/A_{280} by sample type

for aquarium samples is shown in Fig. 4. DNA yield from 100 µl of whole blood was 2667 ± 843 ng (range: 1468-4558), with a mean A_{260}/A_{280} of 1.6.

The *ZF* PCR was successful for 42/45 of the Mystic Aquarium samples (23/25 of the single exhale samples, 9/10 of the two exhale samples, and 10/10 of the 4 exhale samples). The other three samples failed to amplify *ZF* via PCR. Of those samples that amplified, all yielded appropriate sex determination results. All 14 Shedd Aquarium single-exhale samples allowed amplification of mtDNA. Of the 39 Bristol Bay samples, 28 allowed amplification of mtDNA (Table 2). In general, samples that failed to amplify in a PCR reaction had lower yields than those that did. However, yield would not necessarily predict PCR performance, as samples with yields as low as 1.3 ng/µl (64.8 ng) did amplify *ZF*, while samples with yields as high as 59.3 ng/µl (2966 ng) did not (Fig. 5). Purity, as assessed by the A_{260}/A_{280} ratio, was not predictive of PCR success; samples that allowed amplification had A_{260}/A_{280} ratios ranging from -7.9 to 18.4, while those that did not had A_{260}/A_{280} ratios ranging from -0.5 to 34.1. The relationship between sample population, sample type, DNA yield and PCR performance is shown in Fig. 6.

Fig. 1. DNA yield per exhale for aquarium and Bristol Bay beluga samples.

Table 2. Results summary for DNA extraction from beluga blow samples.

Sample Source	# of Exhales	# of Samples	Total Yield, ng (mean $\pm$ SD)	Total Yield, ng (median)	Yield per exhale, ng (mean $\pm$ SD)	Yield per exhale, ng (median)	Mean A_{260}/A_{280}	% Samples with Visible Cell Pellet	% Samples with PCR Success
Mystic Aquarium	1	25	849 $\pm$ 1131	411	849 $\pm$ 1131	411	1.71	68	92
	2	10	1383 $\pm$ 1635	496	692 $\pm$ 817	248	1.84	90	90
	4	10	956 $\pm$ 892	674	238 $\pm$ 223	169	2.07	100	100
Shedd Aquarium	1	14	301 $\pm$ 404	125	301 $\pm$ 404	125	1.46	21	100
All Aquarium Single-Exhale Samples	1	39	648 $\pm$ 967	180	648 $\pm$ 967	180	1.61	57	95
Bristol Bay	1	10	47 $\pm$ 59	24	47 $\pm$ 59	24	4.52	-	70
	2	20	379 $\pm$ 657	98	189 $\pm$ 329	49	2.19	-	65
	4	9	1336 $\pm$ 1288	749	334 $\pm$ 322	187	1.39	-	89

Table 3. The fold increase in mean DNA yield relative to the mean DNA yield for single exhale samples from the same population (aquarium or Bristol Bay).

Number of Exhales	Fold Increase in Mean Yield	
	Aquarium	Bristol Bay
2	2.1	8.0
4	1.5	28.3

Fig. 2. Variation in DNA yield per exhale for 3 aquarium belugas sampled at least 12 times.

Fig. 3. Total DNA yield for aquarium samples with and without visible cell pellets following centrifugation prior to the DNA extraction protocol was performed.

Fig. 4. Variation in A260/A280 by sample type for aquarium belugas.

Fig. 5. Total DNA yield for blow samples by PCR performance.

Fig. 6. Effect of sample type and source population on DNA yield and PCR performance. All individual observations are plotted over the box plot.

Mitochondrial DNA sequences obtained from DNA isolated from blood and blow were identical for each beluga tested at Mystic Aquarium (gel electrophoresis results shown in Fig. 7, sequencing alignment for one beluga shown in Fig. 8). Each Mystic Aquarium beluga had a unique haplotype (S022, accession #DQ503433.2; S001, accession # DQ503430.2; S421, accession #JQ716354.1). Haplotypes were determined for 6 of the 7 Shedd Aquarium samples that amplified (S022 or S421). Sequencing was attempted for 24 Bristol Bay samples, and haplotypes could be assigned for 19 of these samples. Of those sequenced, 18 matched haplotype S022 and the other most closely matched S421, differing by a single base pair.

DNA yield from “calm” breaths (single-exhale) was $226 \text{ ng} \pm 83$. All four samples allowed amplification of mtDNA. DNA yield from aquarium samples that were chilled for 6 hours prior to freezing was $535 \text{ ng} \pm 518$. All four samples allowed amplification of mtDNA. For blow samples frozen for 22 and 23 months prior to DNA extraction, yield was 630 and 68 ng, respectively; both samples allowed amplification of mtDNA. Extending the length of the lysis step did not appreciably alter DNA yields (Table 4.). DNA yield from 1 ml of water from Bristol Bay ($399 \text{ ng} \pm 162$) was higher than 1 ml of water from the Mystic Aquarium beluga exhibit ($120 \text{ ng} \pm 30$).

Table 4. DNA yield (ng) for two samples that were divided evenly and subjected to 1 or 6 hours of lysis with proteinase K.

Sample #	Tube A: 1 hour lysis	Tube B: 6 hour lysis
1	1948	1775
2	257.4	344.8

Discussion

This study has shown that DNA can be reliably extracted from beluga blow samples, and that single exhale blow samples can yield sufficient DNA to perform common molecular analyses, as well as those that require larger fragments of DNA. The results of mtDNA haplotype sequencing from DNA extracted from single-exhale blow samples were identical to the results obtained from blood, validating the use of this method in belugas. This study also demonstrated that blow sampling for molecular analyses from temporarily restrained wild belugas can easily be performed while other tests or sampling are being conducted. The large number of blow samples studied revealed a wide variation in DNA yield, even within the same individual. Despite this

variability, most blow samples ultimately allowed for PCR amplification of a gene target that is commonly used in beluga research, and many would have allowed for the study of more than one genetic marker. Blow sampling is associated with greater risk for the investigator than biopsy sampling given the range of possible DNA yield outcomes; the amount of DNA that can be acquired from a biopsy sample would undoubtedly be higher, even when compared to the highest quality blow sample. However, the potential for blow sampling to serve as a less invasive alternative for acquiring DNA in belugas is clear, perhaps enabling research that would otherwise not be conducted.

DNA Yield from Blow Samples

The number of exhales collected per sampling event had a large effect on DNA yield, especially in Bristol Bay samples. Unexpectedly, yield was not proportional to the number of exhales collected, suggesting that the expulsion of cellular debris varied from exhale to exhale. The wide variation in yield from single exhale samples in aquarium belugas, even from the same individual, further supports this observation. Instead, the forcefulness of the breath and chance collection of large pieces of cellular debris likely shaped the relationship between the number of exhales and the DNA yield. Often, a forceful breath would result in a large piece of mucous-rich debris to be expelled; collecting multiple exhales would increase the odds of this occurring during the sampling event. This could explain the increase in DNA yield per exhale seen in Bristol Bay beluga samples, and is also reflected in the higher yields observed from samples that had a cell pellet following centrifugation compared to those that did not.

While flow rates were not measured, the lower DNA yields from Shedd Aquarium samples may be related to the force of the breath, as these samples were less likely than Mystic Aquarium samples to have pellets following centrifugation.

In aquarium samples, the declining median DNA yield per exhale with increasing number of exhales per sample could also be explained by an initial expulsion of cellular debris and a small volume of water pooled on top of the blowhole in the first breath leading to relatively high yields, followed by a declining amount of debris and environmental water in successive exhales that leads to lower yields. Submerging the blowhole and increasing the amount of time between exhales collected would more closely simulate the breathing pattern of a swimming beluga, and may have resulted in higher yields in successive exhales.

The forcefulness of the exhale that is collected likely resulted in the lower yields from Bristol Bay beluga samples relative to aquarium belugas. Aquarium belugas were trained to exhale forcefully to simulate the forceful exhale of a swimming beluga. By exhaling forcefully, a free-swimming whale ensures the clearing of water from the blowhole prior to inspiration and reduces the time spent at the surface. In contrast, the breaths of the Bristol Bay belugas were deep, yet calm and much less forceful than the breaths of trained or free-swimming belugas. The breaths observed under restraint conditions were similar to those seen in belugas under human care while sleeping or calmly lying at the surface. The breathing pattern of Bristol Bay belugas may be due to restraint conditions during temporary capture. In a study of pulmonary function with dolphins under human care, Brodsky et al. (2012) observed that dolphins that were voluntarily “beached” during testing had a 2-5 fold decrease in flow rates

when compared to dolphins that were fully supported in the water. While the belugas were not completely beached during restraint in Bristol Bay, the thorax was usually touching the bottom, which may have reduced their pulmonary flow rates as a result. Alternatively, the Bristol Bay belugas were exhibiting calm breathing patterns as an energy saving mechanism during capture. Either way, the resulting reduction in force or flow rates may have resulted in lower sample volumes, which would be expected to be related to the amount of cellular material present. In a study of dolphin exhaled breath condensate, less forceful breaths from one individual resulted in reduced sample volumes relative to other dolphins (Aksenov et al. 2014). In addition to reducing sample volume, perhaps reduced force led to the ejection of fewer cells with each exhale, and the chance ejection of larger pieces of cellular debris became less likely, ultimately leading to reduced yields. The 65% reduction in yield from aquarium samples consisting of “calm” breaths further supports this observation.

In the absence of large pieces of cellular debris, the volume of the blow sample (which would be influenced by both the number of exhales and the forcefulness of the breath) likely affects DNA yield. Blow sample volumes were not recorded in this study because the amount of buffer that was added to the sample could not be determined with sufficient accuracy in the field. In a separate study of more than 100 beluga blow samples, fluid volumes per exhale were routinely between 50 and 70 μl , ranging from less than 10 to several hundred μl (Ch. 1, this dissertation). While highly variable, this is considerably higher than blow sample volumes observed in harbor porpoises, which ranged from 15-50 μl from 5-6 breaths, or approximately 3-10 μl per exhale (Borowska et al. 2014). The larger size of the beluga likely results in larger

sample volumes relative to the much smaller harbor porpoise. Borowska et al. (2014) observed that DNA yield increased with sample volume, so a wide variation in blow sample volume could therefore account for some of the variation in per exhale yield observed in this study.

The mean DNA yield per exhale determined from single-exhale aquarium samples in this study compares favorably to those found in other odontocetes, being higher than either of the smaller species previously studied (Frère et al. 2010; Borowska et al. 2014). The higher yields per exhale found in the study by Acevedo-Whitehouse et al. (2010) may be due to the much larger size of the species studied and greater contribution from microorganisms in environmental water, as all of the species sampled were free-swimming and water content in the blow samples was likely high. The expected yield from a blow sample will be an important consideration when investigators are designing studies and considering tissue sources of DNA. Frère et al. (2010) reported that blow samples from bottlenose dolphins have DNA yields similar to those acquired from blood samples. Although there were several single exhale samples with yields that were greater than the yield from 100 µl of blood, this was not consistently the case in this study. Conservatively assuming 50 µl per exhale and using the mean DNA yield of all aquarium samples, DNA yields from blow samples are approximately half of the yield for an equal volume of blood. However, the wide variability in DNA yields observed in this study and the unknown contribution of microorganism DNA to the sample makes it difficult to predict the value of any given blow sample for molecular research, even in relatively controlled settings.

Unlike blood samples, a significant source of DNA in a blow sample will be microorganisms living in the respiratory tract or the surrounding water that pools on top of the blowhole prior to expiration (Acevedo-Whitehouse et al. 2010). The relative contribution of microorganism DNA to the total yield in blow samples from any species is unknown. A similar issue is observed in fecal samples, where DNA from prey items and bacteria can dilute the target host DNA upon extraction by a varied amount (Gillett et al. 2008). A PCR assay can be performed to estimate the amount of target DNA in a mixture of host and microorganism DNA (Ball et al. 2007). However, this assay would require a large amount of template to perform relative to the amount of DNA that is extracted from a blow sample, and may preclude the ability to perform other valuable tests on the sample. Therefore, despite its potential value, this test was not performed on the samples in this study.

Performance of Blow DNA in PCR

Ideally, PCR performance in wild and aquarium samples should have been compared using the same gene target, especially given the disparity in copy number of mtDNA compared to nuclear markers within a cell. After development of the protocol with aquarium samples however, the low yields in some of the Bristol Bay samples would have allowed for only one experiment, and the opportunity to obtain mtDNA sequences was considered more valuable than determining the animal's sex (which was known at the time of collection). Given the much greater number of mtDNA copies in a given cell compared to X or Y copies, the PCR performance of aquarium samples is more likely to have been underestimated than overestimated when

compared to the Bristol Bay samples. The reverse is likely for Bristol Bay samples; some Bristol Bay samples that allowed amplification of mtDNA may not have allowed for amplification of *ZF*. In general, DNA extracted from blow samples performed well in PCRs, although low yield from some samples may limit the number of genetic markers that can be examined in a given sample.

Given the importance of DNA template amount on PCR success, the lower DNA yields from some Bristol Bay samples likely led to the lower rate of PCR success compared to aquarium samples. The low yields at times created the inability to achieve target DNA template inputs into the PCR reaction. Template inputs are also influenced by the variation in the ratio of target (beluga) DNA to total (beluga and microorganism) DNA extracted from blow samples. Amplification can be reduced for samples with the same amount of target DNA when this ratio is low (Gillett et al. 2008). There was evidence that Bristol Bay samples had lower target to total DNA ratios than aquarium belugas, necessitating higher template inputs for PCRs and perhaps leading to a reduction in rate of PCR success. Without veterinary care or water filtration, wild whales may have had higher microorganism loads compared to aquarium belugas. Water from Bristol Bay also had higher DNA yields than aquarium exhibit water. While no effort was made to reduce contamination by environmental water, the amount of water contamination was probably higher in restraint conditions in Bristol Bay than in the more controlled setting found in aquariums, further reducing the ratio of target DNA to total DNA for Bristol Bay belugas. However, when the quality of the sample is high, very little template is required for mtDNA sequencing, leaving sufficient template to perform multiple experiments with the same sample.

The purity of DNA samples also influences PCR success (Boesenberg-Smith et al. 2012). Spectrophotometry was chosen to assess sample purity in this study due to its relative ease and the small sample size with which the assessment can be made. The highly variable A_{260}/A_{280} ratios observed in single-exhale samples were likely due to very low concentrations in these samples. At very low concentrations, the absorption at each wavelength will differ only slightly, if at all, resulting in readings that are outside of the normal A_{260}/A_{280} range for nucleic acid samples (Boesenberg-Smith et al. 2012). Because of this effect, this assessment of purity was not a good indicator of downstream performance for samples with low concentrations. The A_{260}/A_{280} ratio may also have been affected by environmental salt contamination or by protein present in samples with greater amounts of mucous. In samples that did not form a pellet following centrifugation, TE buffer was left in the tube prior to extraction; if not efficiently removed, buffer salts may also have falsely elevated the ratio. This factor could have disproportionately affected Bristol Bay samples due to the inability to determine if a pellet was present or not following centrifugation. Salt contamination was also suspected to influence A_{260}/A_{280} ratios of harbor porpoise DNA extracted from blow samples (Borowska et al. 2014). However, due to the unreliable nature of this assessment of purity at very low DNA concentrations, the A_{260}/A_{280} does not necessarily predict PCR success.

The different handling conditions the Bristol Bay samples were exposed to could also have led to a reduction in DNA quality and thus PCR success rate. However, there was no evidence that short term storage of the samples at cool temperatures prior to freezing had an effect the ability to extract or amplify DNA from

aquarium blow samples, with yields similar to those achieved from samples that had been frozen immediately. More experiments are needed to confirm that this is the case, but the reduced DNA yield and PCR success in wild beluga samples relative to aquarium samples is more likely a result of the differences in breathing pattern than sample collection or handling methods that varied in the two conditions.

Future Directions and Current Utility

Further method development should focus on collecting as much of the blow sample as possible from a given exhale. The collection method used in this study facilitates the collection of the sample through centrifugation, but does not collect the entire sample. Due to the diameter of the tube compared to the diameter of a beluga's blowhole, it is likely that less than half of the actual blow sample is collected in this method. For a free swimming animal, a collection device large enough to capture as much of the blow as possible should be devised, with the ability to efficiently transfer the sample from the collection device into a tube that can then be centrifuged.

Acevedo-Whitehouse (2010) utilized sterilized plastic sheets to create a large surface area for sample collection from wild whales and used swabs to retrieve the samples.

We are currently investigating the use of a sheet of parafilm for this purpose; the hydrophobic nature of the parafilm facilitates sample transfer via pipette into a tube that can then be centrifuged. Collecting a larger portion of the exhaled plume will likely lead to increased yields and therefore improve the utility of this technique. The forceful nature of the exhale of a swimming beluga will also increase the likelihood that sufficient DNA can be collected from single breath.

PCR success can be improved by optimizing PCR conditions for low template inputs, and DNA yield may be improved by using different extraction protocols. The relatively high level of mucous in blow samples may impact yield. For saliva samples, an overnight proteinase K digestion can be utilized to improve yields (Zakharkina et al. 2011). In the pilot experiment reported here, increasing the proteinase K lysis time by 5 hours had no effect on yield, although the relative mucous content may vary from sample to sample. DNA extraction kits that are more efficient for smaller amounts of starting tissue may also improve yield.

Experiments should also be performed to determine the relationship between DNA yield and the distance between the sampling device and the blowhole, as the proximity of the sampling device to the blowhole in this study is unlikely to be replicated with a free-swimming beluga. Studying belugas in zoological facilities will allow for further method development before wild whales are approached for sample collection, minimizing the impact to wild belugas while maximizing the likelihood that this method can be utilized effectively.

Although further development is required for the application of these techniques to free-swimming belugas, blow sampling is immediately applicable to live-stranded belugas, where sampling would be analogous to the sampling that has been accomplished in Bristol Bay. Belugas in the endangered Cook Inlet population occasionally temporarily mass strand between tidal cycles. These strandings may involve more than 50 whales at once, representing a significant portion of the entire stock that numbered 340 belugas in 2014 (National Marine Fisheries Service 2015). If the beluga is breathing calmly under these conditions, a minimum of two exhalations

should be collected, while collecting four would ensure that sufficient DNA is extracted to perform multiple analyses. Blow sampling presents an efficient sampling method that would require few materials or personnel in the field, enable the sampling of many individuals in a short period of time, and minimize disturbance to the whales during the stranding event without compromising the ability to collect important data about individuals. Ultimately, blow sampling for genetic analyses may not replace biopsy sampling, but would create sampling opportunities when more invasive sampling methods are unavailable or undesirable.

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CHAPTER 3

“Seasonal variation in testes size and density detected in belugas (*Delphinapterus leucas*) via ultrasonography”

by

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Abstract

Belugas are thought to exhibit seasonal variation in testes size, but a temporal gap in post-mortem 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 zoological facilities with known reproductive histories to assess seasonal variation in testes size, and its association with blood 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 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 141 ultrasound examinations (including 71 suitable for pixel intensity measurements) and 119 blood samples were available for analysis. Significant seasonal variation in testes volume, blood testosterone concentration, and testicular pixel intensity 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 pixel intensity 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 zoological facilities, and suggest a relatively low demand for sperm in this species that is consistent with their classification as induced ovulators.

Introduction

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 levels, testes size, sperm production, or seminiferous tubule diameter (reviewed in Plön and Bernard 2007). In particular, relative testes size has been used to indicate seasonal variation in odontocetes. 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 2006).

Belugas (*Delphinapterus leucas*), an Arctic and subarctic species of odontocete, breed in the late winter or early spring so that births occur in the summer, 15.5 months later (Suydam 2009; Robeck et al. 2015). With this seasonal reproductive pattern, male belugas would be expected to undergo changes in testes size or function. Male belugas in zoological facilities have a seasonal pattern of testosterone production, with peak concentrations occurring from January through April (Robeck et al. 2005). Conceptions also occur seasonally in zoological facilities, with 80% occurring in March – May, a range that agrees with estimates of breeding season in wild belugas (Robeck et al. 2005; Burns and Seaman 1988; Brodie 1971). Postmortem evaluations of wild beluga testes demonstrate that most males reduce spermatogenesis outside of the breeding season, evidenced by testicular histology 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 (Kelley et al. 2014; Heide-Jørgensen and Teilmann 1994).

However, wild belugas are primarily sampled during the summer, several months after the presumed peak in breeding. Therefore, insufficient data is available to definitively describe the extent of seasonal variation in testes size. 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 (Brodie 1971; Burns and Seaman 1988; Finley et al. 1982; Heide-Jørgensen and Teilmann 1994; Kelley et al. 2014; Kleinenberg et al. 1969; Sergeant 1973). 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 reproductive histories would fill the existing temporal sampling gap and help describe the seasonal variation in testes size and function in belugas. The testes of odontocetes are located within the abdominal cavity, necessitating the use of ultrasonography to monitor live animals. 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 zoological facilities and in the wild (Kastelic and Brito 2012; Robeck et al. 2009; Alves et al. 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 via ultrasound is correlated with seminiferous tubule area and sperm production in bulls (Brito et al. 2012). These measurements are made by determining the pixel intensity of the image using computer software, generating an objective measure that allows longitudinal comparisons. Although not as effective as testes size measurements for monitoring testes function, pixel intensity can be a good indicator of the attainment of sexual maturity or the cessation of sperm production in males undergoing contraceptive treatment, and could presumably be used to monitor seasonal variation in sperm production in odontocetes (Brito et al. 2012; Ulker et al. 2005). Relative echogenicity has been used to assess seasonal variation in testicular function in white sided dolphins (*Lagenorhynchus obliquidens*) (Robeck et al. 2009), and testicular pixel intensity measurements have demonstrated seasonal variation and variation with reproductive status in Yangtze finless porpoises (*Neophocaena phocaenoides asiaeorientalis*) (Wu et al. 2010a; Wu et al. 2010b). Despite the value of these tools, very few individuals of any odontocete species have been monitored longitudinally to assess seasonal variation in testes function (Robeck et al. 2009; Desportes et al. 2003; Yuen et al. 2006; Wu et al. 2010b).

Given the seasonal variation known to exist in male beluga testosterone concentrations 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 the suspected seasonal variation in testes size in

belugas can be detected via the longitudinal monitoring of males with known reproductive histories. Blood testosterone concentrations will also be monitored for comparison, and the effectiveness of testicular pixel intensity measurements in evaluating seasonality will also be assessed.

Methods

The testicular volumes and blood testosterone concentrations of 5 adult male belugas were monitored longitudinally for at least one calendar year at three different zoological facilities (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. DL3, DL4, and DL5 were all monitored for 12 consecutive months from January to December, 2008. All five males were housed in outdoor exhibits with chilled, manufactured salt water and had access to at least one mature female throughout the study period. Four of the five males had previously sired at least one calf. 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).

Table 1. Study animals.

Animal	Age^a (yr)	Length^b (m)	Weight^b (kg)	Location	Temp. Range (°C)	Sire	Ultrasound Exams	Blood Samples	Years Studied
DL1	27	4.4	1042	Mystic	5.6-16.7	Yes	55	46	2.4
DL2	26	4.0	945	Mystic	5.6-16.7	Yes	42	22	2.8
DL3	20	4.0	1300	Vancouver	8.3-12.5	Yes	16	18	1
DL4	26	4.0	1009	SWSD	11.1-15.0	Yes	15	15	1
DL5	39	4.0	892	SWSD	11.1-15.0	No	13	18	1

^a“Age” refers to age at the beginning of the study period.

^bLength and weight measurements were taken once during the study period.

Calculating testicular volume via ultrasonography

Ultrasound exams were performed with the voluntary cooperation of the animal while the animal lay unrestrained in lateral recumbency 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 zoological facility. Although the specific ultrasound equipment varied by facility, all exams were performed with a convex 3.5 MHz probe.

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.

Using these still images, measurements to the nearest hundredth of a cm were performed by the ultrasound operator using 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 fat pads present on the ventrolateral surface of belugas made an indirect measurement of length using a

ruler placed on the abdomen (as described Brook et al. 2000) unreliable in pilot observations, necessitating this direct approach. Both testes were measured within the same day. Each measurement was taken on both images of the same view, and the average of these two 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.

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: $V = (LWD)(0.71)$ (Brook et al. 2000).

Calculating Testicular Pixel Intensity

The pixel intensities (PI) of the testicular parenchyma relative to the pixel intensity 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 pixel intensity of an ultrasound image, images were utilized for this analysis from a single 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 six 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 may have been used to adjust for seasonal changes in blubber thickness in an individual (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. Pixel intensity was significantly correlated with scan depth ($p < 0.0001$). The equation of the line was determined to be $PI = -1.8686(\text{Depth}) + 56.033$ ($r^2 = 0.69$). Each blubber normalized PI measurement was corrected for depth through the use of this equation. 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 hours of the ultrasound exam from a ventral fluke vein with the voluntary cooperation of the animal as a part of routine veterinary monitoring. Blood samples were collected in the morning hours, typically between 0900 and 1000 hours. After centrifugation ($2000 \times g$ for 10 minutes at 10°C), one ml of serum or sodium heparin plasma was stored at -80°C .

Blood samples were assayed for testosterone using an EIA previously validated for use with beluga serum and plasma (Cayman Chemical, Ann Arbor, MI, Item #582701) (Richard et al., chapter 1 of this dissertation). Blood samples were extracted with diethyl ether (Sigma-Aldrich, St. Louis, MO, Catalog #346136) according to the EIA kit manufacturer's instructions prior to the assay. This kit has 100% reactivity with testosterone. Cross-reactivities reported by the manufacturer were 140% for 19-nortestosterone, 27% for 5 α -dihydrotestosterone, 18.9% for 5 β -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/Bo 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 two different times; samples collected between 2008 and 2009 were assayed in one group (A), while samples collected between 2012 and 2014 (from DL2 only) were assayed in another (B). For group A, interassay variation was not rigorously assessed. For group B, two 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 two standard controls on each plate. Intra-

assay variation was calculated for both groups by averaging the CV for all of the samples with 20-80% binding on each plate.

Data Analysis

Seasonal variation 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 two different definitions of breeding season was tested: high testosterone season (Jan – Apr vs. all other months) and high conception season (Mar – May vs. 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 ANOVA and AIC. Prior to seasonality analysis, TTV measurements were normalized to body length, as body mass fluctuates seasonally in belugas while length will remain constant in adults. Differences in the rates of change of TTV in DL1 were assessed using ANCOVA. Box plots displayed show the interquartile range (box), the median (bold line), and the whiskers show the maximum (Q3) or minimum (Q1) value ≤ 1.5 times the interquartile range. Intra-observer reliability for ultrasound measurements was assessed by comparing TTV calculated separately from the replicate measures taken from two sets of still images and plotting the replicate measures in a Bland-Altman plot (Bland and Altman 1986). Significance was set at $p < 0.05$.

Results

Seasonality of Total Testicular Volume

The appearance of beluga testes on ultrasound was as described in studies of other odontocetes (Brook et al. 2000) (Fig. 1). Due to variation in the animals' behavior, weather, or faulty equipment, some scheduled ultrasound examinations were not conducted. As a result, DL5 was not measured in the months of July or September, and DL2 was not measured in September or November of 2012 or March of 2013. TTV by month for each individual is shown in Table 2.

The length, width, and depth of the testes varied within and among individuals (Table 3). This variation occurred seasonally, with a seasonal pattern apparent for four of the five individuals (Fig. 2). TTV was significantly higher from Jan – Apr ($p < 0.0001$) and from Mar – May ($p < 0.0001$) compared to all other months, but the effect was stronger using the predictor season of Jan – Apr. There were significant differences between individuals in both tests of seasonality (random intercept term, $p < 0.001$ for Jan – Apr and $p < 0.01$ for Mar – 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 (Table 4). 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 5). The average difference between replicate measures of TTV was 68 cm^3 ; 85% differed by less than 125 cm^3 (Fig.3).

Seasonality of Blood Testosterone Concentration

Testosterone concentrations in blood varied significantly between Jan – Apr and all other months ($p < 0.0001$), but did not vary between Mar – May and all other months ($p > 0.05$). The effect of individual (random intercept term) was not significant for the comparison between Jan – Apr and all other months ($p > 0.05$), but was significant for the comparison between Mar – May and all other months ($p < 0.01$). Seasonal variation in testosterone occurred in four of the five whales (Fig 4).

Fig. 1. Appearance of beluga testis on ultrasound, showing digital measurements of the organ in both longitudinal (top) and cross sectional view (bottom).

Table 2. Monthly total testicular volume (mean, standard deviation, and number of observations) by month for each individual. The “Mean” column represents the mean of the individual means, with standard deviation and the total number of observations per month.

Month		DL1	DL2	DL3	DL4	DL5	Mean
January	Mean	1327	1134	748	1549	1043	1160
	SD	222	57	--	--	48	301
	# Obs	4	6	1	1	2	14
February	Mean	1340	1203	791	1523	908	1153
	SD	156	67	--	57	23	302
	# Obs	4	5	1	2	2	14
March	Mean	1434	1116	1085	1613	884	1226
	SD	186	43	117	51	2	292
	# Obs	4	4	2	2	2	14
April	Mean	1351	1135	1014	1414	906	1164
	SD	129	106	156	--	--	217
	# Obs	6	8	2	1	1	18
May	Mean	1223	1060	862	1486	930	1112
	SD	142	96	9	260	71	250
	# Obs	4	4	2	2	2	14
June	Mean	1216	1023	740	1355	1084	1084
	SD	129	125	93	--	--	231
	# Obs	4	5	2	1	1	13
July	Mean	1168	909	717	1142	--	984
	SD	100	42	--	--	--	213
	# Obs	4	4	1	1	0	10
August	Mean	1042	883	604	1146	933	922
	SD	105	58	--	--	--	204
	# Obs	5	4	1	1	1	12
September	Mean	1053	919	601	1541	--	1028
	SD	96	118	--	--	--	391
	# Obs	4	3	1	1	0	9
October	Mean	1029	866	560	1560	936	990
	SD	135	32	--	--	--	364
	# Obs	5	3	1	1	1	11
November	Mean	1085	938	636	1530	948	1027
	SD	96	55	--	--	--	325
	# Obs	6	2	1	1	1	11
December	Mean	1212	1063	591	1743	971	1116
	SD	101	66	--	--	--	419
	# Obs	6	6	1	1	1	15

Table 3. Variation in component measures of testicular volume by individual.

ID	Length		Dorso-ventral 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

Table 4. Model selection summary for describing seasonal variation of TTV. The best model is shown in bold.

Model	AIC	Log Likelihood	ANOVA Results
Linear fixed effect, random intercept	123.8	-57.9	--
Quadratic fixed effect, random intercept	113.3	-51.6	Significantly better than previous ($p < 0.001$)
Cubic fixed effect, random intercept	79.8	-33.9	Significantly better than previous ($p < 0.001$)
Quartic fixed effect, random intercept	81.5	-33.7	Not better than previous ($p > 0.05$)
Cubic fixed effect, random intercept, random linear slope	75.4	-29.7	Significantly better than previous ($p < 0.05$)
Cubic fixed effect, random intercept, random linear and quadratic slope	71.1	-24.5	Significantly better than previous ($p < 0.05$)
Cubic fixed effect, random intercept, random linear, quadratic, and cubic slope	73.7	-21.9	Not better than previous ($p > 0.05$)

Table 5. Seasonal variation in TTV by individual.

ID	Minimum Volume	Maximum Volume	Minimum Month	Maximum Month	Difference	Increased by a factor of:
DL1 2007-2008	861.21	1336.56	October	March	475.35	1.6
DL1 2008-2009	1014.17	1647.03	August	March	632.86	1.6
DL2 2012-2013	830.04	1157.69	October	February	327.65	1.4
DL2 2013-2014	816.37	1306.38	August	February	490.01	1.6
DL3	560.15	1167.21	October	March	607.06	2.1
DL4	1141.89	1742.87	July	December	600.98	1.5
DL5*	883.26	1077.46	May	January	194.19	1.2
Average	872.44	1347.89			475.44	1.6

*No observations available from July or September.

Fig. 2. Seasonal variation in TTV by individual. Circles represent individual observations.

Fig. 3. Bland-Altman plot of replicate measures of TTV to assess intra-observer variation.

Seasonality of testosterone was best described by a quadratic fixed effects model with a random intercept and random linear slope term. Intra-assay variation for groups A and B were 17.6 and 10.3%, respectively. Interassay variation for group B was 10.0% for the 100 pg control and 17.7% for the 25 pg control. The relationship between seasonal variation in testosterone and TTV is shown in Fig 5.

Fig. 4. Seasonal changes in blood testosterone concentration by season. Circles represent individual observations.

Seasonality of Pixel Intensity

A seasonal variation in the PI of testicular ultrasound images was apparent in both DL1 and DL2 (Fig. 6). Pixel intensity was significantly higher from Jan – Apr compared to all other months ($p < 0.05$), but was not different from Mar – May compared to all other months ($p > 0.05$). Seasonality of PI was best

described by a quadratic fixed model with a random intercept and random linear slope term. An increase in echodensity 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, echodensity decreased prior to the decrease in testes size (Fig. 6).

Fig. 5. Seasonal variation in TTV (points separated by individual on primary y axis) and testosterone (bars on secondary y axis; mean of individual means $\pm$ 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 (not normalized to body length, as in statistical analyses).

Fig. 6. Seasonal variation in TTV, blood testosterone, and the pixel intensity of testicular ultrasound images (from right to left) for DL1 (top row) and DL2 (bottom row). Open symbols represent individual observations, while closed circles represent the monthly mean. Lines represent the fitted curves determined from statistical analyses (TTV: cubic; testosterone and pixel intensity: quadratic).

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 second year relative to the first (Fig. 7). This was also reflected in a different rate of increase in TTV (Sep 2007 – Mar 2008, Sep 2008 – Mar 2009, Sep – Dec 2009) between years ($p < 0.0001$), with a faster rate of change occurring from Sep 2008 – Mar 2009 (slope = 3.32) than in Sep 2007 – Mar 2008 (slope = 2.13) or Sep – Dec 2009 (slope = 1.83) (Fig. 8). The highest rate of growth corresponds to a growth of 1.66 cm³ of testicular tissue per testis per day.

Discussion

Through the use of longitudinal monitoring of individual belugas, this study demonstrated seasonal variation in testes size in adult male belugas, supporting hypotheses developed from post-mortem studies of wild belugas (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 zoological facilities (Robeck et al. 2005; Burns and Seaman 1988). Ultrasonography was a sufficiently sensitive method to detect seasonal variation in testes size and density in belugas, as in other studies of odontocetes (Robeck et al. 2009; Wu et al. 2010b).

Fig. 7. Longitudinal variation in TTV across years for DL1 (top panel) and DL2 (bottom panel).

Fig. 8. Variation in rate of TTV increase by year for DL1. Calendar day is calculated from Julian dates so that the time series are uninterrupted from the fall of year one into the spring of year two. Sep 2007 – Mar 2008 (triangles, dashed line); Sep 2008 – Mar 2009 (diamonds, solid line); Sep – Dec 2009 (squares, dotted line).

The testes sizes measured in this study are similar to those found in post-mortem 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 one report is available where mass and volume of the same testes were reported (Kleinenberg et al. 1969). Using the small data set reported by Kleinenberg et al. (1969) ($n = 5$ adults), a relationship between mass and volume can tentatively be obtained ($M \text{ (g)} = 1.13V(\text{cm}^3) - 27.5$, $r^2 = 0.997$). Using this equation and the minimum and maximum volumes observed in this study (Table 4), the belugas studied had combined testes masses of 605-1941 g. These values are very

similar to published values, although with a higher peak in this study, likely due to the lack of data from peak season in post-mortem studies.

Significant individual variation was found in testes size and in the rate of change in testes size with season. In post-mortem studies of belugas, wide variation in testes size has been found for belugas of similar body length (*e.g.* Sergeant 1973) or age (*e.g.* Heide-Jørgensen and Teilmann 1994). Individual differences in testicular function 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 one 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, but only one beluga (DL4, the same animal studied in O'Brien et al. 2008) maintained this level 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 function are currently unknown.

Comparisons of testes size between individuals in this study should be made cautiously, as different observers were necessarily used at each zoological facility.

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 via ultrasound, where replicate linear measures generally varied by less than 0.5 cm (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. The inability to measure length directly in some cases likely contributed to some of the variation found. For longitudinal studies, two measurements per month are sufficient for the detection of seasonal patterns of growth and regression.

Testicular pixel intensity 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 echodensity 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 function 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 echodensity with the attainment of sexual maturity.

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 (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 zoological facilities in the US 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 values 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. Some degree of senescence has been observed in other odontocetes, including pilot whales (Desportes et al. 1993) and finless porpoises (Wu et al. 2010a). Detecting senescence in wild male belugas 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 zoological facilities could be performed to assess age-related changes to this pattern.

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 levels 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 regulatory cues for belugas are unknown, but longitudinal monitoring of belugas in zoological facilities may enable further investigation.

The apparent time lag between peak testosterone level 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 1984). The total length of the cycle of spermatogenesis ranges from 30-75 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. With testicular growth and increasing echodensity beginning in November, 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, occurs in February/March. This is in line with the peak season for conceptions in belugas in zoological facilities up to 60 days later (March – May) (Robeck et al. 2005).

Interestingly, testosterone concentrations decline dramatically after March, and testes size begins 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 levels in April and May were similar to levels found in summer and fall months, outside of the breeding season. The regression in testes size and loss of echodensity 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 stop or 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 4-5x increases in testes size. However, the degree of change was similar to the seasonal increase found in seasonally breeding sheep (~67% increase) and long-finned pilot whales (*Globicephala melas*) (~50% increase) (Ortavant et al. 1988; 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). In 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, next to nothing 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 et al. 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 first 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 (Soulsbury 2010; Iossa et al. 2008). The low testosterone levels during peak breeding season in this study suggests that male-male agonistic competition is also low in this species, as high levels are associated with aggressive behavior in other mammals (Trainor et al. 2009). The relatively low sperm concentration found in 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 zoological facilities, 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 could also be used for non-lethal assessments of reproductive function 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 zoological facilities, 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 studies will have an impact on wild beluga conservation and management.

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CHAPTER 4

“Minimally invasive physiological correlates of courtship behavior in belugas (*Delphinapterus leucas*)”

by

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Abstract

Simultaneous study of reproductive physiology and behavior can yield valuable insight into the reproductive biology of a species, yet these studies are rare in cetaceans due to logistical constraints. Recent advances in the understanding of beluga reproductive physiology can aid in interpreting behavior in these species. Minimally invasive measures (blow sampling, ultrasound exams, and behavioral observations) were utilized to describe reproductive seasonality in a group of four belugas under professional managed care. Seasonal variation in testosterone concentrations in blow was detected in both males, and seasonal variation in testes size was detected in the mature male. Three estrous cycles were inferred for an adult female. Intersexual associations were seasonal, with 85% of the interactions occurring between January and June, corresponding to the known sexual segregation in wild belugas in the summer and fall. Males initiated 95% of the intersexual interactions, yet terminated only 30%. Male-male associations were not seasonal in occurrence, in agreement with observations that male-male social relationships may be important in wild belugas. The “genital present” was considered an important courtship display behavior performed by males toward the female. This behavior was strongly seasonal, with 97% occurring between mid-February and early June. The frequency of genital present displays was not related to testosterone concentration in either male, primarily occurring after testosterone concentrations began to decline. Courtship primarily occurred while the male and female were cooperatively swimming in the same direction at the same pace. The female displayed variable responses to genital present displays. Female belugas may be able to employ pre-copulatory mate choice by

selecting which male to swim with as well as how to respond to courtship displays. Several measures indicated that the adult female in this study preferentially engaged in courtship with one of the males. As paired physiological and behavioral observations of individual wild belugas throughout the year are not feasible, these observations provide an important complement to the study of wild belugas.

Introduction

The inherent link between reproductive behavior and population dynamics creates a need for an understanding of mating strategies to facilitate population management. As some strategies may be more resilient to perturbation than others, understanding the strategy employed by a species is important information when faced with rapid environmental change (Quader 2005). Belugas (*Delphinapterus leucas*) are an Arctic and sub-Arctic species of cetacean that are well adapted for life among sea ice, using the ice edge for foraging (Asselin et al. 2012), a barrier for predation by killer whales (Sergeant and Brodie 1969), and as a driver of seasonal migration patterns and habitat use (Hornby et al. 2015). Sea ice loss or increasing water temperatures may thus be expected to alter beluga behavior. Variation in sea surface temperature can affect beluga migration patterns (Bailleul et al. 2012) and sea ice loss can affect their distribution (Heide-Jørgensen et al. 2010). These changes could also affect social behavior in belugas, as males and females are known to segregate outside of the breeding season and exploit differing habitats and ice conditions (Loseto et al. 2006). A reduction in habitat variability through the loss of sea ice may alter male-female association patterns, which could impact reproductive behavior.

Belugas breed in the late winter or early spring, when most populations are inaccessible to human observers (Burns and Seaman 1988). Thus, next to nothing is known about the breeding behavior of wild belugas. Observations of male-female associations are possible during the summer months in some areas (*e.g.* Alekseeva et al. 2013), but these interactions are unlikely to be associated with true breeding behavior given the timing of births and the gestation period of belugas (Robeck et al. 2015). Instead, these observations may reflect socio-sexual behavior, which is known to be a component of the social relationships of other cetaceans (Mann 2006). The availability of belugas in aquaria has allowed descriptive studies of reproductive behavior, but these observations have not yet been evaluated in the context of reproductive physiology and thus possible mating strategies in this species (Hill et al. 2015; Glabicky et al. 2010).

Due to the absence of direct observations, beluga mating strategies have been inferred primarily using morphological characteristics (O’Corry-Crowe et al. 1997; Dines et al. 2015; Kelley et al. 2014). However, the conclusions drawn from these inferences do not agree across studies. Belugas have alternatively been thought to have a polygynous mating system with competition between males for access to mates, or a more promiscuous system in which sperm competition may play an important role in belugas relative to the narwhal, a close relative. Dines et al. (2015) point out the lack of knowledge of the beluga mating system, but suggest that both pre- and post-copulatory selection are weak in this species. In their analysis, Dines et al. (2015) noted that belugas lacked obvious male weaponry or behavioral displays for

demonstrating male quality, and demonstrated a relatively small degree of sexual size dimorphism and investment in testes size.

Recent discoveries about beluga reproductive physiology will improve the ability to infer beluga mating strategies in the absence of direct observations. Seasonal variation in testes size and tissue density supports the finding that some males may suspend sperm production outside of the breeding season (Chapter 3, this dissertation; Burns and Seaman 1988). The relatively small maximum size of beluga testes as well as the small seasonal change relative to other seasonally breeding odontocetes indicates that demand for sperm is low in this species (Dines et al. 2015; Chapter 3, this dissertation). High circulating testosterone concentrations are associated with high levels of male-male competition or sperm competition (Dixson and Anderson 2004). However, both wild and aquarium belugas (Høier and Heide-Jørgensen 1994; Chapters 1 and 3, this dissertation) have lower peak circulating testosterone concentrations relative to other odontocetes (*e.g.* bottlenose dolphin, *Tursiops truncatus*, Schroeder and Keller 1989). With the observation that most conceptions occur after testosterone concentrations begin to fall and testes begin to regress (Robeck et al. 2005; Chapter 3, this dissertation), the frequency of courtship behavior may be unrelated to testosterone in this species, in contrast to convention in vertebrates (Wingfield et al. 1990). Although very few individuals have been studied, belugas also appear to have small ejaculate volumes relative to other cetaceans (O'Brien et al. 2008; Alexa McDermott, personal communication). Taken together, these observations suggest a reduced role for sperm competition or male contest competition relative to other species of cetaceans. These findings could all be related

to the recent discovery that belugas are facultative induced ovulators (Steinman et al. 2012).

Induced (vs. spontaneous) ovulation is associated with a different set of predictions of reproductive behavior, as the ability of an individual male to monopolize paternity is generally increased and male-male postcopulatory competition is reduced (Soulsbury 2010; Iossa et al. 2008). Copulatory behavior and association patterns may depend on the amount of time between copulation and ovulation (Gomendio et al. 1998). In belugas induced to ovulate with a GnRH analog, this time period is approximately 36 hours (Robeck et al. 2010). If subsequent mates have a chance to fertilize a female's ova, then the first male to copulate with the female might be expected to engage in longer courtship or mate guarding to thwart breeding attempts by other males to ensure paternity (Gomendio et al. 1998). Given the difficulty of monopolizing mates in a three dimensional marine environment, females may select to engage in lengthy courtship with a superior male as a way to ensure suboptimal mates do not copulate with her. In addition, if post-copulatory selection mechanisms are lessened with induced ovulation, then pre-copulatory selection by females would be of greater importance in ensuring high quality mates (Soulsbury and Iossa 2010). Therefore, female behavioral mechanisms for employing pre-copulatory selection of mates would be expected to occur.

To most accurately describe the mating strategies of a species, it is necessary to determine the reproductive condition of an individual at the time that reproductive behavior is observed, as individuals would be expected to behave differently depending on their reproductive condition (*e.g.* Muraco and Kucjaz 2015). Gonad

function, measured through testes size or follicular development, is a clear indicator of an individual's reproductive condition and is often used to assess maturity and reproductive condition in adults (Neimanis et al. 2000; Muraco and Kucjaz 2015). Reproductive steroid measurements can be used to detect changes in gonad function, and thus reproductive condition. Therefore, studies that correlate steroid hormone concentrations and behavior in wild animals are common across taxa, including several species of marine mammals (Bartsh et al. 1992; Burgess et al. 2012). Studies that incorporate measures of gonad function, hormone measures, and behavior are also feasible in some wild terrestrial mammals. For example, a study of free-ranging Soay sheep correlated scrotal circumference, testosterone concentrations in blood, and breeding behavior (Preston et al. 2012). However, longitudinal sampling of behavior and physiology in free-ranging cetaceans poses prohibitive logistical challenges.

Hormone sampling in wild cetaceans is limited by logistics, with blubber (via biopsy sampling) or feces presenting the most viable methods for obtaining reproductive steroids for analysis (Kellar et al. 2009; Rolland et al. 2005). However, fecal sampling can only be performed opportunistically, and biopsy sampling is inappropriate for repeated sampling of individuals. In zoological facilities, cetaceans can be trained for blood sampling or urine sampling as less invasive ways to monitor reproductive steroids (Robeck et al. 2005; Steinman et al. 2012). However, repeated blood sampling is often undesirable in an effort to limit inflammation, and urine sampling is trained less frequently than blood sampling in zoological facilities. As a result, paired studies of reproductive behavior and endocrinology in cetaceans are rare, but yield important insights on the reproductive biology of a species (Wells 1984;

Muraco and Kucjaz 2015; Wu et al. 2010). The physiological validation of blow (exhale) sampling for reproductive steroid hormone analysis in belugas (Chapter 1, this dissertation) facilitates repetitive sampling of individuals and thus improves the feasibility of paired studies of reproductive behavior and endocrinology in cetaceans.

For belugas, this type of study is most realistic in aquaria, as detailed subsurface observations in wild beluga habitat in March and April are logistically challenging, if not impossible. The study of cetacean behavior in aquaria has significant limitations, especially in space and social group composition. However, social behaviors can be similar across settings, and the benefits of performing observations in aquaria make them valuable complements to studies of wild cetaceans (Dudzinski et al. 2010; Dudzinski et al. 2012). Additionally, research on beluga reproductive physiology in aquaria has yielded results that are consistent with and often enhance the knowledge of wild beluga reproductive physiology, which is obtained primarily in the summer, outside of the breeding season (Robeck et al. 2005; Robeck et al. 2015; Burns and Seaman 1988; Heide-Jørgensen and Teilmann 1994). While a study of a small population of belugas in an aquarium cannot be used to infer the mating system or specific reproductive strategies of all wild belugas, the more intensive data collection that is possible in this setting can provide valuable information that can be used to inform studies of wild belugas.

In this study, non-invasive methods will be utilized to assess the relationship between reproductive physiology and behavior of a group of aquarium belugas. These results will be interpreted in the context of the current state of knowledge of their reproductive biology as well as their behavior in the wild. Intersexual interactions are

expected to show seasonality, as would intersexual socio-sexual behavior. Thus, courtship behavior is expected to be seasonal in nature and occur most frequently when females are most receptive (the follicular phase of the estrous cycle). Based on the understanding of conception timing relative to reproductive physiology, courtship is expected to be unrelated to testosterone concentration or testes size in males. The relative importance of male contest competition will be evaluated by quantifying male-male aggression and affiliative behavior. Following predictions based on ovulation mode and testes size in this species, a low copulation rate is expected, and opportunities for females to employ precopulatory mate choice are expected to occur.

Methods

This study was conducted on 4 belugas (F1, F2, M1, and M2) housed at Mystic Aquarium (Mystic, CT) in a 2.8 million liter outdoor exhibit that covers approximately 4000 m². The synthetic salt water was chilled in warmer months to temperatures <16° C; chilling was completed naturally in cold months to temperatures as low as 3°C. Most of the exhibit is visible from underwater through large acrylic windows; smaller satellite pools are only visible from the surface. These satellite pools can be isolated from the other portions of the exhibit through a gate system. Behavioral observations and physiological measurements were performed for one year (52 consecutive weeks, henceforth numbered consecutively from 1-52) from Aug 25 2013 – Aug 21 2014 on 2 male and 2 female belugas, although one female was only available for study for the first 21 weeks of the study (Table 1). This project was approved by the Institutional Animal Care and Use Committees of Mystic Aquarium (Project #12001) and the University of Rhode Island (Project #AN12-02-016).

Table 1. Study animals and data availability.

Individual (M = Male, F = Female)	Age^b	Length^a (cm)	Mass (kg)	Reproductive Status	Weeks Sampled	# Blow Samples	# Ultrasound Exams	# Blood Samples
F1	32	335	659	Nulliparous	52	51	--	14
F2	32	335	727	Nulliparous	21	14	--	--
M1	27	399	945	Proven Sire	52	96	23	--
M2	11	390	723	Never sired	52	104	15	--

^aLength and mass were measured once during the study period.

^bAge refers to the age of the animal at the beginning of the study period.

Physiological Assessments of Reproductive Condition

Reproductive Hormone Analysis in Blood and Blow

Blow (exhale) samples were collected twice per week from the males and once per week from the females, and analyzed as described in Richard et al. (Chapter 1, this dissertation). Briefly, blow samples were collected onto a pre-cleaned nylon mesh (110 μ m, Elko Filtering Co., Miami, FL) stretched over a petri dish and secured with a rubber band. For sample collection, the whales were trained to lift their head so that the blowhole was above the water's surface. The whale would then exhale once to clear any excess water from the blowhole. Then, 2-8 successive exhales were collected onto the same mesh. Fluid blow samples were retrieved from the nylon via centrifugation, and samples were stored at -80° C until analysis.

Blood sampling was attempted once per month with F1 with the voluntary cooperation of the animal as a part of routine veterinary monitoring. Blood samples were collected into sodium heparinized vacutainer tubes from the ventral fluke vein. One ml of serum or sodium heparin plasma was obtained through centrifugation (2000 $\times g$ for 10 minutes at 10° C) and stored at -80° C. Blood and blow samples were collected in the morning hours between 0900 and 1130, typically during the first training session of the day.

Male samples were assayed for testosterone using a commercially available enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, Item #582701) validated for use with beluga blow samples by Richard et al. (Chapter 1, this dissertation). A commercially available enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, Item #582601) validated for use with beluga blood and blow samples (Chapter 1, this

dissertation) was used to assay female samples for progesterone. For both testosterone and progesterone measurements, 55 μ l of sample was required; blow samples with volumes <55 μ l were unavailable for assay. A diethyl ether extraction step was performed on female blow samples assayed for progesterone (Chapter 1, this dissertation). Blood samples were extracted according to the manufacturer's instructions. All samples were assayed in duplicate and the means were used in calculations. Individual samples with a %B/B₀ between 20 and 80% and a coefficient of variation (CV) < 20% were accepted. Samples with CV >20% were re-assayed. Two standard controls were run in each assay (testosterone: 100 and 25 pg/ml, n = 12 assays; progesterone: 200 and 50 pg/ml, n = 14 assays). Inter-assay variation was calculated by determining the CV for the two standard controls on each plate. Intra-assay variation was calculated by averaging the CV for all of the samples with 20-80% binding on each plate. When available, multiple samples collected within the same week were averaged to obtain weekly testosterone values. Some of the hormone measurements in blow samples have been presented previously (Chapter 1, this dissertation), but are repeated here to contextualize behavioral observations.

Ultrasonographic assessments of reproductive condition

Total testicular volume (TTV) was determined for the males in this study as described previously (Chapter 3, this dissertation). Briefly, ultrasound exams were performed with the voluntary cooperation of the animal while the animal lied unrestrained in lateral recumbency at the water's surface. Exams were attempted twice per month by a single operator using a convex 3.5 MHz probe. Two still images

of the longitudinal view and 2 still images of the transverse view at the midpoint of the testis were taken for each testis, for a total of 4 images per testis per exam. Using these still images, measurements to the nearest hundredth of a cm were performed by the ultrasound operator using analysis software available on the ultrasound machine to the nearest hundredth of a cm. Dorsoventral diameter (depth) and lateral diameter (width) were measured on the transverse images. Length was measured in longitudinal view. Each measurement was taken on both images of the same view, and the average of these two measures was calculated and used for analyses. Total testicular volume, 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: $V = (LWD)(0.71)$ (Brook et al. 2000). Data on M1 has been reported previously (Chapter 3, this dissertation), but is repeated here for comparison to behavioral observations.

Inferring ovulation

The occurrence of ovulation in F1 was inferred through progesterone measurements in blow, using known estrous cycle stage lengths in belugas reported by Steinman et al. (2012). Previous work has shown that ovulation leads to an increase in progesterone concentrations in blow (Chapter 1, this dissertation). In F1, the presence of a corpus luteum (CL) was associated with an increase in blow progesterone that lasted approximately 20 days, from a baseline of 248.5 ± 62.5 pg/ml to 326.5 ± 33 pg/ml (mean $\pm$ SD). However, progesterone measurements in blow made when a CL was absent occasionally occurred within this elevated range, while two samples

collected when a CL was present had progesterone concentrations <300 pg/ml (Chapter 1, this dissertation). To minimize the chance of a false positive, ovulation was inferred to have occurred if two consecutive weekly samples exceed 326.5 pg/ml, indicative of the luteal phase lasting 29-32 days in non-conceptive cycles. The timing of ovulation was inferred by considering the lengths of the estrous cycle stages and all progesterone measurements available because the first elevated progesterone sample may not correspond to the start of the luteal phase given the time between samples. The follicular phase, which lasts 14-27 days, was conservatively presumed to occur in the week that ovulation was inferred to have occurred, as well as the two weeks preceding the week of ovulation. The follicular phase was allowed to overlap with the luteal phase of a previous cycle. Inferred ovulations must be separated by >30 days, given the inter-estrus interval of 33-34 days in two animals studied by Steinman et al. (2012).

If available, progesterone concentrations in blood samples were used to confirm or refute inferences. Progesterone data from blood were considered more clearly interpretable than those from blow, given the wider variation within a reproductive condition that can be found in blow relative to blood (Ch. 1, this dissertation). Progesterone concentrations in blood <1000 pg/ml was considered baseline (no pregnancy or luteal activity); pregnant females or females with an active CL have progesterone concentrations in blood >4000 pg/ml (Ch 1, this dissertation).

Behavioral observations

Ethogram

An ethogram was developed using published descriptions of beluga behavior (Hill et al. 2015; DiPaola et al. 2007), as well as pilot observations of the study group performed in the breeding season (Feb – Apr) of the previous year. A social interaction was defined as occurring when two or more whales are performing any of the social behaviors listed in the ethogram. The behaviors of interest for this study are listed in Table 2. Of particular interest was the “genital present” (Fig. 1). One whale length (~4 m) was used as a distance frame of reference for several behaviors.

Table 2. Ethogram of social behaviors of interest adapted from DiPaola et al. (2007), Hill et al. (2015), and pilot observations of the study group.

Behavior		Definition
States	Group Swim	Two or more whales swim in the same direction at approximately the same velocity; all whales are within 2 m of at least one other whale in the group; bodies can be aligned or staggered (one whale swims ahead of the other), but one whale may not be completely behind another; body orientation of individuals vary
	Social Milling	Two or more whales actively swim, drift passively, or lie still with no discernible pattern or in variable directions within 4 m of each other; may be associated with social displays
Events	Approach	A whale alters swim direction or speed to initiate interaction with another whale(s) while the other whale(s) does not alter swim speed or direction; resulting position is less than 4 m of recipient whale; interaction is initiated
	Separate	A whale alters swim direction or speed to terminate interaction with another whale(s) while the other whale(s) does not alter swim direction or speed; resulting position is > 4 m of previously interacting whale(s), terminating the interaction
	Open Mouth	With rostrum pointed in the direction of another whale, whale opens mouth wide enough so that the tongue is (or would be) visible
	Bite	A whale makes contact with another whale with an open mouth and partially closes jaws upon contact
	Bite Threat	With an open mouth, whale moves toward another whale to a distance of 1 m or less, but does not make physical contact
	Rake	A whale drags open mouth along the body of another whale so that either or both jaws make contact with the body of the other whale
	Jaw Clap	With rostrum pointed in the direction of another whale, whale rapidly and forcefully claps jaws together, once or several

		times in rapid succession; creates a percussive sound that may or may not be audible to the observer
	Chase	A whale swims rapidly at another, who flees in response
	Head Thrust	A whale sharply directs rostrum in the direction of another whale and rapidly returns head to its original orientation; depending on the location of the other whale, the direction of the thrust may vary; may be associated with corresponding body movement anterior to the dorsal ridge
	Melon Shake	A whale vigorously shakes head in dorsal/ventral plane, causing the melon to shake; behavior has a recipient if rostrum is directed at another whale
	Melon Flat	Anterior portion of the melon is compressed, flattening the melon along the maxilla, reducing or eliminating the normal rounded shape of the melon
	Melon Push	The anterior portion of the melon is pushed outward, moving the normal rounded shape of the melon toward the rostrum of the whale leaving a depression behind the melon
	Erection	Any part of the penis is visible during any social interaction; may occur simultaneously with any other social behavior in this ethogram
	Ventral Present	Whale rotates along long axis so that ventral surface is oriented toward another whale
	Genital Present	Male whale stops active forward progress by terminating fluke beating and drifts in the direction of another whale while arching their caudal peduncle so that the genital region is pushed closer to the recipient whale; caudal end of the caudal peduncle is correspondingly angled dorsally; rostrum is often directed toward the recipient whale for some portion of the presentation causing the body to assume an “S” shape; flukes and flippers may be held at various angles to control the drift towards the recipient whale; may result in contact of the genital region with the recipient or resumption of locomotion
	Genital Present Posture	Same as genital present except the acting whale does not drift in the direction of another whale; recipient is the whale closest to the presenting whale at the time of the present

Fig. 1. A sequential view of a genital present from M1 (bottom of frame) toward F1 (center of frame). Note the ventral orientation of F1 relative to M1 in frame 1 and the lateral orientation of F1 relative to M1 by frame 3. The “S” shape is clear in frame 4.

Observation Protocol

Continuous observations were conducted on the social group using a tripod-mounted digital video camera. Because the entire exhibit could not be filmed at one time, an event sampling rule was used in which the videographer focused filming on any social interaction that occurred during the filming period, regardless of participants, resulting in a continuous record of all social interactions. Filming was primarily conducted from the underwater viewing area of the Arctic Coast exhibit, in an area where visitors and staff members are regularly present so that the whales' behavior was not influenced by the presence of the observer. If social interactions >1 minute in duration occurred in one of the satellite pools that lack underwater viewing, observations were conducted from an above water vantage point that allowed for the detection of social interactions in the satellite pools. If two separate interactions were occurring in two locations that were not simultaneously visible, the most clearly visible interaction was filmed. If the whales were travelling between the main exhibit and satellite pools while interacting, the observer remained at the underwater viewing vantage point unless they remained out of sight for > 1 minute. Observations were only conducted outside of training sessions.

Four observation sessions were conducted per week: 2 in the morning hours between 700 and 1000 h, and 2 in the afternoon hours between 1500 and 1800 h, resulting in 2 hours of observations in each time block per week. Most observation sessions (80%) lasted 60 minutes, but sessions ranged from 30-90 minutes in duration. The days of the week where observations occurred were not consistent throughout the study, but observation effort each week was consistent; therefore behavioral data will

be grouped by week (#1-52) and will be compared to weekly assessments of reproductive physiology. The distribution of morning to afternoon filming was not equal in weeks 1 (3 PM, 1 AM), 29 (3 PM, 1 AM), and 52 (1 PM, 3 AM). In weeks 21, 32, and 40, 5 observation sessions were required to reach 4 h of observations in that week. A total of 211 observation sessions were conducted for a total of 208 h of observation.

Quantifying Behavior

Video was first screened for the presence of social interactions by one of two observers. The animals engaging in the interaction were identified, as was the duration of the interaction to the nearest 5 s (minimum 5 s), and the animal that initiated (“Approach”) and terminated (“Separate”) the interaction. Approaches and separations may not have been identified for interactions that either started or ended prior to or after the observation session, if the interaction was initiated or terminated out of view of the camera, if the whales interacted while swimming past each other, or if the whales appeared to initiate or terminate the interaction mutually. At this stage, the behavioral state was also identified. One behavioral state was assigned to each interaction. Some interactions were relatively long and included both milling and group swim behavioral states; these interactions were assigned group swim as the behavioral state. It was possible for the social grouping to change during a continuous interaction if a third whale joined 2 interacting whales. This observation was considered two separate interactions (the 2 whale interaction and the 3 whale interaction). Using these data, a weekly record of social interactions was created. The

total number of interactions, the average duration of interactions, and the total amount of time interacting was calculated for each social grouping and each behavioral state.

In the next stage of analysis, behavioral events performed during social interactions were coded and quantified using CowLog software (Hänninen and Pastell 2009) and continuous recording (Martin and Bateson 2007) by one of three observers. For each event, the actor, behavior, and recipient were recorded. In social groups of more than 2 whales, if the acting whale performed a behavior that was not directed toward a clear recipient, the recipient whale was considered to be the whale closest to the acting whale. Behavioral event coding was not possible for interactions that occurred in the satellite pools that lacked underwater viewing, although the occurrence, duration, and participants of the interactions in these pools were recorded. Weekly coefficients of association (COA) were calculated using the simple COA described in Cairns and Schwager (1987), with the amount of time spent interacting divided by the total amount of time observed per week, in minutes. Unless otherwise noted, the COAs between M1 and M2 were calculated using only interactions that occurred in the absence of a female. Weekly behavioral frequencies (events per minute of observation) were calculated separately for each individual, unless otherwise noted.

Genital presents

Every occurrence of a genital present between a male and female was reviewed by a single observer to describe the behavior in greater detail and to determine the recipient's response to the genital present display. The behavioral state and social

grouping at the time of the genital present was recorded. The general area of the recipient's body the actor directed the display toward (right lateral, left lateral, ventral, or dorsal surfaces), the relative body position of the actor during the display (horizontal, head angled toward bottom, head angled toward surface), and the position of the whales in the water column (completely submerged or any part of the body at the surface) were also recorded. A "receptiveness score" was developed for the recipient and assigned to each occurrence of a male toward female genital present (Table 3), with higher scores indicating increased receptivity.

Table 3. "Receptiveness score" scheme used to assign receptivity to genital presents. Each occurrence received one score from each of the three categories (swim speed, orientation, and contact), and the total score is the sum of these three component scores.

	Response	Score
Swim Speed	Recipient alters swim speed to increase distance from genital present	0
	Recipient continues to make forward progress during genital present (swim speed unchanged)	1
	Recipient stops active swimming and drifts during genital present	2
Orientation	Recipient rolls ventral surface away from the genital present	0
	Recipient rolls ventral surface toward the genital present	2
	Recipient remains stable along long axis so that genital present ends closer to dorsal surface than ventral surface	1
	Recipient remains stable along long axis so that genital present ends closer to ventral surface than dorsal surface	2
Contact	Contact does not occur	0
	Contact occurs on lateral or dorsal surfaces	1
	Contact occurs on ventral surface	2

Inter-observer reliability

Inter-observer reliability for the coding of behavioral events was assessed by having all three observers code the same 10 observation sessions selected from 10 different months. This video (10 hours total) included 133 minutes of social interaction (90 min of male/female interactions and 43 min of male/male interaction). The behavioral event coding for each observer was then aligned by the time each behavior occurred, allowing side by side comparison of each event. For each behavior, a pairwise kappa statistic was determined, using observer #1 as the reference observer. Behaviors coded by both observers #2 and #3 with “good” agreement or better ($\kappa > 0.6$) with observer #1 were considered in further analyses (Kaufman and Rosenthal 2009).

Statistical Analysis

Small sample sizes and dependent measures limited the ability to perform rigorous statistical analysis. Descriptive statistics (mean $\pm$ standard deviation) were used to assess seasonality of behaviors (January – June vs. July – December) and variation with the occurrence of follicular phases in F1. Box plots displayed show the interquartile range (box), the median (bold line), and the whiskers show the maximum (Q3) or minimum (Q1) value ≤ 1.5 times the interquartile range. Individual linear regressions were performed for each male to describe the relationship between weekly testosterone and the weekly frequency of genital presents or aggression in R (R Core Team 2015).

Results

Testosterone Assays

Two blow samples per week were available for all weeks for M1 except weeks 3, 25, 36, 39, 42, 42, 50, 51, where only a single sample was available for assay. Two samples per week were available for all weeks for M2 except weeks 35, 39, and 42. Three samples were available from M2 in weeks 9, 24, and 48. The average lower limit of detection (80% B/B₀) was 10.9 pg/ml. All biological samples assayed exceeded the lower limit of detection. Testosterone intra-assay variation was 7.7%; inter-assay variation was 5.7% for the 100 pg/ml control and 10.8% for the 25 pg/ml control.

Both males demonstrated seasonal variation in testosterone concentrations in blow (Table 4). M1 displayed a more gradual increase to approximately week 26, when testosterone began to gradually decline (Fig. 2). M1's testosterone remained elevated (greater than the mean value of all weekly observations, 107.0) from week 15 to week 36, with only two relatively low testosterone weeks during that time. M1's blow testosterone doubled between weeks 14 and 26. M2's blow testosterone concentration had a sharper peak that occurred between weeks 26 and 39 (greater than the mean value of all weekly observations, 97.0 pg/ml), with only one relatively low testosterone week occurring during that period (Fig. 3). M2's blow testosterone nearly tripled from week 22 to week 26.

Table 4. Blow testosterone concentrations (pg/ml). Weekly averages were calculated before calculating the mean, standard deviation, and range.

Animal	# Observations	Mean $\pm$ Standard Deviation	Range	Peak Week	Fold Change from Minimum
M1	96	107.0 $\pm$ 39.9	44.0 – 207.7	26 (Feb)	3.7
M2	104	97.0 $\pm$ 44.4	29.6 – 277.1	29 (Mar)	8.4

Testes Ultrasounds

M1 demonstrated seasonal variation in TTV, with peak TTV occurring in February and an apparent plateau in TTV between weeks 22-33 (January 21 – April 7) (Fig. 2). Total testicular volume remained above average (993.3 cm³) from week 18 – 38, with only one measurement below the average value during that period (April 21). M2's testes remained below 375 cm³ throughout the study period, and while the smaller TTV made a clear seasonal pattern less evident, in general the largest volumes were observed in March through May (Fig. 3). Measurements were unavailable for M2 prior to week 23. Total testicular volume data for each male is presented in Table 5.

Table 5. Total testicular volume (cm³).

Animal	# Observations	Mean $\pm$ SD	Range	Peak Month	Fold Change from Minimum
M1	23	993.3 $\pm$ 129.0	848.5 – 1306.4	February	1.54
M2	15	294.5 $\pm$ 32.7	247.5 – 366.0	March	1.49

Fig. 2. Blow testosterone concentration and total testicular volume for M1 (all observations).

Fig. 3. Blow testosterone concentration and total testicular volume for M2 (all observations).

Progesterone Assays

Once weekly blow samples were available for F1. Blood progesterone measurements were available from F1 for all months except for August 2013 and April 2014. Blow samples were commonly less than 55 μ l for F2, or had volumes that were too small to re-assay the samples if the CV was above 20%. Thus, only 13/21 weeks were sampled for F2 (Table 6). The average lower limit of detection (80% B/B₀) was 30.5 pg/ml. All biological samples exceeded the lower limit of detection. Progesterone intra-assay variation was 11.7%; inter-assay variation was 5.8% for the 200 pg/ml control and 15.5% for the 50 pg/ml control.

Table 6. Progesterone concentrations (pg/ml) from blood and blow.

Sample Type	# Observations	Mean $\pm$ SD	Range
F1 Blow	52	291.1 $\pm$ 90.8	156.5 – 763.8
F1 Blood	14	473.7 $\pm$ 111.1	293.1 – 710.2
F2 Blow	13	250.9 $\pm$ 49.8	143.76 – 312.1

Inferring ovulation in F1

Elevated progesterone was not observed in any blow samples collected from F2. A total of 12 F1 blow samples exceeded the elevated progesterone concentration of 326.5 pg/ml (Weeks 12, 20, 35-36, 38-39, 42, and 44-48) (Fig. 4). Three periods met the requirements for inferring ovulation: weeks 35-36 (April 20 – May 3), weeks 38-39 (May 11 – May 24), and weeks 44 – 48 (June 22 – July 26). None of the blood samples collected from F1 during the study had progesterone concentrations indicative of luteal activity or pregnancy. Blood samples relevant to interpreting estrous cycles were collected on May 8 (550.8 pg/ml), June 3 (465.6 pg/ml), July 3 (323.5 pg/ml) and August 6 (497.4 pg/ml).

For the first elevated progesterone period (weeks 35-36), a 10 day sampling gap occurred between the week 34 sample (April 15) and the week 35 sample (April 25). Based on the available information, ovulation is inferred to have occurred between April 7 and 9 (week 33), at least 29 d before the low progesterone blood sample on May 8. The corresponding follicular phase would have spanned weeks 31-33 (March 23 – April 12). The follicular phase was associated with low progesterone concentrations, as would be expected. A blood sample was not collected in April due to a lack of voluntary cooperation from F1. This inferred ovulatory event did not result in a conception.

The second period of elevated progesterone in blow occurred in weeks 38-39. Assuming the second ovulation occurred 33 days after the first (Steinman et al. 2012), the next ovulation would have occurred between May 10 and 12 (weeks 37/38), likely closer to May 10 than May 12, given the first elevated sample following this second inferred ovulation was collected on May 13 (week 38). The associated follicular phase is inferred to have spanned weeks 35 – 37 (April 20 – May 10), thus overlapping with the luteal phase of the previous estrous cycle. A low progesterone sample was collected in week 37, as might be expected for this stage. The blood sample collected on June 3 would have thus been collected in the latter stages of the luteal phase (day 24), which could account for the low observed progesterone concentration at this time. This inferred ovulation also did not result in conception.

The third period of elevated progesterone in blow was longer in duration than the previous two, occurring between weeks 44 and 48. If the second non-conceptive cycle was immediately followed by a third cycle, ovulation would have occurred

between June 12 – 14 at the earliest, with regression of the CL occurring by approximately July 11-16. Blow progesterone did indeed remain elevated during this time until July 22, with the first low progesterone sample collected on August 1. However, a low progesterone concentration was observed in the blood sample collected on July 3, close to when peak progesterone secretion should have been occurring from an ovulation occurring in mid June, decreasing the likelihood of an ovulation at that time. The low progesterone concentration measured in the blood sample on August 6 suggests that any subsequent luteal activity must have ceased by then. Using this date as an approximate cycle end date, ovulation could have occurred 29-32 days prior, between July 5 and 8, which is consistent with the low progesterone observed in blood on July 3, as well as the sustained elevated progesterone in blow through July 22. Thus, a third ovulation was inferred to have occurred between July 5 and 8 (weeks 45/46), with the associated follicular phase spanning weeks 43-45. This inferred ovulation also did not result in a pregnancy.

Fig. 4. Blood and blow progesterone concentrations for F1. Dotted line indicates 326.5 pg/ml, the threshold used for inferring ovulations (see Methods).

Behavioral Observations

Social interactions were observed in 197 of the 211 observation sessions and comprised 12% of the total time observed. A summary of the available data for analysis is presented in Table 7. A total of 45.2 minutes of interactions occurred in pools that lacked underwater viewing and thus lacked behavioral event data. Every possible social grouping was seen at least once. F2 was involved in just 7% of the recorded interactions, and there were no genital present behaviors presented toward her during the study. Therefore, any descriptions of courtship behavior will be in reference to F1. Interactions with F1 were comprised mostly of group swim interactions (by duration), while M1-M2 interactions were primarily milling interactions (Table 8). The approaching whale was identified for 2124 of the interactions (89%). The separating whale was identified for 2086 of the interactions (87%). Of all male-female interactions, males initiated 95% and terminated 30%. Of the male-male interactions, M2 initiated 80% and terminated 42%.

Table 7. Summary of social interactions observed.

Social Grouping	# Interactions Observed	Total Duration (min)
Male and Female	1052	951
Male only	1342	485
Female only	2	0.25
Total	2396	1437

Table 8. Amount of time in minutes spent interacting by group and behavioral state.

Social Group	Duration of Interactions	Milling		Group Swim	
		Proportion of Total Duration	Duration per Interaction	Proportion of Total Duration	Duration per Interaction
M1-F1	75.5	0.51	0.32	0.49	0.97
M2-F1	606.3	0.16	0.24	0.84	3.34
M1-M2-F1	175.2	0.07	0.24	0.93	2.15
M1-M2	485.3	0.89	0.35	0.11	0.77

Interobserver reliability

Observers #1-3 coded 57, 37, and 6% of the total interactions (by duration) that occurred in this study, respectively. The video used for determining interobserver reliability contained 9% of the total duration of interactions. The identity of the participants in an interaction had 100% agreement between all three observers.

Pairwise kappas calculated for each behavior are presented in Table 9. Melon push and melon flat had pairwise kappas less than 0.55, with a high rate of disagreement on whether the behavior occurred. These were also the most frequently coded behaviors, so pairwise kappas were calculated after these behaviors were removed from the matrix. Based on these results, ventral present, genital present posture, chase, head threat, and jaw clap were omitted from analysis. The extremely low frequency of chase and jaw clap contributed to the lack of agreement between observers. The aggressive behaviors bite, rake, and bite threat were condensed into a single aggressive category to improve agreement and allow further analysis. The following behaviors had “excellent” agreement ($\kappa \geq 0.80$) for both observer pairings: open mouth, melon shake, genital present, and erection (although there was only one observation of an erection in the video used for interobserver analysis).

Table 9. Pairwise kappas focused on specific behaviors in the ethogram. Behaviors selected for further analysis are identified by a “*”.

Behavior	Observer 1:Observer 2	Observer 1:Observer 3
Open Mouth*	0.86	0.86
Bite Threat	0.56	0.37
Bite	0.65	0.50
Rake	0.50	0.34
Jaw Clap	0	0
Head Threat	0.61	0.58
Chase	1.0	0
Erection*	1.0	1.0
Melon Shake*	0.82	0.83
Ventral Present	0.46	0.51
Genital Present*	0.88	0.84
Genital Present Posture	0	0.14
Bite, Rake, or Bite Threat*	0.69	0.65
Total # Observations	574	594

Seasonal Variation in COA

Interactions involving males and females occurred in every week of the year, and in 74% of the observation sessions. However, COA varied widely throughout the year, with 85% of the time of male-female interaction (811 minutes) and 96% of the total duration of group swims (682 minutes) occurring between January and June. There was less seasonal variability in interactions that only involved males; 59% of the time of male-male interaction (285 min) occurred between January and June (Fig. 5). There were not large differences in COA for the groupings of interest in relation to F1’s inferred follicular phases (Fig. 6).

Fig. 5. Box plot of seasonal variation in coefficients of association for social groupings of interest.

Fig. 6. Box plot of coefficients of associations for social groupings during (FP) and outside of (Non-FP) F1's presumed follicular phases.

Male interest in F1 generally occurred in short intervals, and appeared to be unrelated to testosterone concentrations, as much of the social interaction occurred after testosterone concentrations began to decline for both M1 and M2 (Fig. 7). Coefficients of association >0.05 were observed for M1 in weeks 21, 26-27, and 33-35, and for M2 in weeks 21, 26-27, 31, 33-36, 38-41, and 46. The COA of M1 and M2 was influenced by the occurrence of the triad interaction between M1, M2, and F1, where M1 was generally observed group swimming with F1 while M2 milled nearby without engaging either M1 or F1. To remove this potential bias, the COA between M1 and M2 was calculated after excluding interactions that included a female. The resulting M1-M2 COA remained relatively consistent throughout the year, with two periods of increased socialization (COA > 0.10) in weeks 10 and 31-32 (Fig. 8).

The proportion of interactions by week initiated by a male was generally >0.9 . There were 4 weeks with >10 male-female interactions where the proportion of male approaches was less than 0.9 (F1 more likely to approach males): weeks 37, 39, 42, 45. The proportion of interactions terminated by a female was generally >0.5 . There were 4 weeks with > 10 male-female interactions where the proportion of interactions terminated by a female was <0.5 (females less likely to end an interaction with a male): weeks 2, 22, 38, and 39.

Fig. 7. Seasonal variation in association patterns between the males and F1, in relation to blow testosterone concentrations and F1's inferred follicular phases.

Fig. 8. Patterns of association between M1 and M2 (in the absence of F1) in relation to blow testosterone concentrations and F1's inferred follicular phases.

Seasonal Variation in Genital Presents

A total of 541 genital presents were observed in the study, for an overall frequency of 0.04 per minute of observation, or .38 per minute of social interaction (once every 158 seconds of social interaction) (Table 10). Relatively few sessions contained a genital present, and 97% (270/278) of the male toward female genital presents occurred between weeks 26 – 41 (Fig. 9). The frequency of genital presents by males to F1 was unrelated to testosterone; 98% (111/113) of M1's genital presents to F1 occurred after peak testosterone (week 26) and 92% of M2's genital presents to F1 occurred after peak testosterone (week 29). Genital present frequency was not correlated with blood testosterone concentrations for either male (M1: $F_{1,50} = 2.75$, $p > 0.05$; M2: $F_{1,50} = 2.51$, $p > 0.05$) (Fig. 10). Genital present frequency was not related to testes size in M1, with 49% occurring in week 35 or later, when testes size fell below the mean value (993 cm^3) for the first time in 8 consecutive measurements (weeks 18-33). M1's genital presents to F1 were more likely to occur during F1's inferred follicular phases, with 70% (79/113) occurring in either the first or second inferred follicular phase. M2's genital presents to F2 were more likely to occur outside of F1's inferred follicular phases, with 37% (61/165) occurring in either the first or second inferred follicular phase. Neither male was observed performing a genital present toward F1 during her third inferred follicular phase in weeks 43-45. Only M1 was observed performing genital presents toward F1 during week 33 (April 6-12), closest to F1's first inferred ovulation. Both males were observed performing genital presents toward F1 during her second inferred follicular phase, but neither male was observed performing a genital present toward F1 in week 37, immediately

preceding the inferred ovulation. Of the 27 observation sessions that contained a male toward female genital present, 2 contained genital presents from both males toward F1, both in week 34, outside of an inferred follicular phase. M1 was not observed to perform a genital present toward F1 between April 26 and July 30, when he performed two genital presents. M2 performed 75% of his genital presents toward F1 during that period.

Table 10. Genital presents observed. The frequency per minute of interaction is the # of genital presents observed per minute of interaction between that pair of belugas.

Grouping	Actor-Recipient	# Genital Presents Observed	# Sessions with a Genital Present	Frequency of Genital Presents per Minute of Interaction
M1 and F1	M1-F1	113	11	0.45
	F1-M1	0	0	0
M2 and F1	M2-F1	165	18	0.21
	F1-M2	62	10	0.08
M1 and M2	M1-M2	70	14	0.14
	M2-M1	131	23	0.27

Fig. 9. Variation in the frequency of genital presents (GP) performed by the males toward F1 per minute of observation in relation to testosterone concentrations in blow and F1's inferred follicular phases.

Fig. 10. Frequency of genital presents (GP) in relation to testosterone concentrations. Lines represent linear regression models for each male.

Characterizing courtship

Observation sessions that contained a male toward female or female toward male genital present were investigated in greater detail in comparison to observation sessions that did not in an attempt to identify behavioral patterns indicative of courtship (Table 11). Genital presents tended to occur in sessions with high COAs between males and females, and 98% occurred during group swims. Of the genital presents from M1 to F1, 73% occurred within a social grouping of M1, M2, and F1, while only 3% of M2's genital presents occurred in this social grouping. No male toward male genital presents were observed in sessions when a male toward female genital present was also observed. All of F1's genital presents towards M2 occurred in sessions where M2 also displayed a genital present toward F1. Copulation was not observed in this study. Erections were rarely seen during male-female interactions (2 from M1, 1 from M2). Both of the erections observed from M1 occurred with a genital present, once on April 7, and once on April 25 (weeks 33 and 35).

Despite their small number relative to the total number of observation sessions, sessions that contained male toward female genital presents contained large proportions of the total occurrence of some behaviors, most notably group swim, open mouth, and melon shake (Fig. 11). For example, 80/117 (68%) of M1's open mouths toward F1 occurred during the 11 observation sessions that contained a M1-F1 genital present, while 373/595 (63%) of M2's open mouth behaviors toward F1 occurred during the 18 sessions with a M2-F1 genital present. Melon shakes by either sex were nearly exclusive to sessions that contained a genital present.

Table 11. Characteristics of observation sessions with interactions between M1 or M2 with F1 that either contain or do not contain genital presents from the male toward F1.

Proportion of Observations Sessions With:	M1		M2	
	With GP to F1 (11 sessions)	Without GP to F1 (68 sessions)	With GP to F1 (18 sessions)	Without GP to F1 (121 sessions)
Bite, Bite threat, or rake toward F1	0.36	0.07	0.33	0.18
Melon Shake to F1	0.36	0	0.89	0.16
Melon Shake from F1	0.09	0	0.56	0
Open Mouth to F1	0.64	0.09	0.83	0.5
Open Mouth from F1	0	0.01	0.67	0.13
Interactions with male	0.77	0.94	0.72	0.92
Open mouth, bite, bite threat, or rake toward male	0.64	0.71	0.61	0.77

Fig. 11. Proportion of all observations of each behavior (OM = open mouth, MSHK = melon shake) that occurred during an observation session with a genital present toward F1 for each male.

F1's Receptivity to Genital Present Displays

Genital presents were performed with approximately equal frequency toward F1's left or right side, and were rarely presented toward her ventral or dorsal surfaces, although M1 was more likely to present toward the ventral surface than M2 (Table 12). Males performed this behavior with their long axis parallel to the bottom or with their head angled down toward the bottom; there were no observations of this behavior with the male's head angled toward the surface. This behavior was also never performed by either male at the water's surface.

F1's receptivity toward genital present displays varied by male (Fig. 12). She was more likely to suspend active forward swimming during M1's displays, and was more likely to allow M1 to make contact during displays (Table 13). She was not observed to actively distance herself from any of M1's displays, but actively distanced herself from 21% of M2's displays. Receptivity during an inferred follicular phase was higher for genital presents by M1 (2.57 ± 1.00) than for M2 (1.45 ± 1.11), but receptivity with respect to inferred follicular phases did not vary within individual males (Fig 13). Mean receptivity for all genital present displays was higher for M1 when he performed a melon shake behavior in the same session (2.58, 4 sessions) than when he did not (2.08, 7 sessions). F1's receptivity scores for the genital presents from M1 with an erection were both 2. In both observation, F1 rolled her ventral surface away from M1 during the display, and contact did not occur with either of these presents.

Table 12. Detailed descriptions of genital present behaviors exhibited by the males toward F1.

Proportion of Genital Presents toward F1:	M1	M2
Toward F1's right side	.44	.36
Toward F1's left side	.42	.47
Toward F1's ventral surface	.11	.04
Toward F1's dorsal surface	.04	.13
While parallel to surface	.5	.59
With head angled toward bottom	.5	.41
With head angled toward surface	0	0
While at the surface	0	0

Fig. 12. F1's receptivity score by male performing the genital present.

Table 13. Receptivity scores (mean $\pm$ SD) for each male's genital presents toward F1.

Receptivity Score Component	M1	M2
Mean Speed Score	1.68 $\pm$ 0.47	1.06 $\pm$ 0.69
Mean Orientation Score	0.69 $\pm$ 0.60	0.52 $\pm$ 0.57
Mean Contact Score	0.21 $\pm$ 0.47	0.02 $\pm$ 0.13
Mean Receptivity Score	2.58 $\pm$ 1.02	1.60 $\pm$ 0.95

Fig. 13. Receptivity scores during (FP) or outside (Non-FP) of F1's inferred follicular phases by male.

Male Aggressive Behavior toward F1

Aggressive behavior (bite, bite threat or rake) was rare between M1 and F1, with only 27 events from M1 to F1 and 0 events from F1 to M1. All 27 events directed toward F1 occurred after week 28, by which point testosterone had already been in decline. More than half of the events directed toward F1 (14) occurred in one session (April 25) which was also the session in which genital presents from M1 to F1 were most frequent (Fig. 9). Aggression was more common between M2 and F1, with 51 events directed by M2 toward F1, and 8 events directed by F1 toward M2. Aggressive events between M2 and F1 were approximately evenly distributed throughout the year (Fig. 14). Aggression frequency toward F1 was not correlated with low testosterone concentration for either male (M1: $F_{1,50} = 0.32$, $p > 0.05$; M2: $F_{1,50} = 0.03$, $p > 0.05$) (Fig. 15).

Fig. 14. Variation in male aggression (bite, bite threat, and rake) directed toward F1 in relation to blow testosterone concentration and F1's inferred follicular phases.

Fig. 15. Frequency of male aggression toward F1 in relation to testosterone concentration. Lines represent linear regression models for each male.

Male-male social behavior

M1 performed 462 aggressive behavioral events (open mouth, bite, bite threat, or rake) toward M2 (0.04 events/min observations). M2 performed 1006 aggressive behavioral events (open mouth, bite, bite threat, or rake) toward M2 (0.08 events/min observations). There was not a clear seasonal pattern in the frequency of aggressive behaviors (Fig. 16). However, the rate of aggression from M1 to M2 per minute of observation was higher between January and June (0.048) than it was in July to December (0.026). M1's rate of aggression toward M2 was also higher when F1 was in an inferred follicular phase (0.052) than when she was not (0.033). M2's rate of aggression was higher in January through June (0.11) than it was in July through December (0.052), but there was not a clear difference when F1 was in an inferred follicular phase (0.084) or not (0.080).

Male toward male genital presents were more common in the fall and spring, but occurred in every month of the year except January (Fig. 16). A peak of male toward male genital presents occurred between weeks 28 and 33 (Mar 2 – Apr 12), when 59% of all male toward male genital presents were observed. Genital present rates (per minute of observations) were higher for both M1 (0.014) and M2 (0.027) when F1 was in an inferred follicular phase than when she was not (M1: 0.004, M2: 0.007). Male toward male genital presents also occurred at a higher rate between January and June (M1: 0.007, M2: 0.014) than July through December (M1: 0.005, M2: 0.007) for both males.

Fig. 16. Frequency of male-male aggression (open mouth, rake, bite, or bite threat) and male-male genital presents per minute of observation in relation to blow testosterone concentration and F1's inferred follicular phases.

Discussion

Physiological Assessments

The relationship between testes size and blow testosterone concentration in M1 in this study further validates the use of blow sampling as an indicator of reproductive function in belugas. Testes size remained elevated after testosterone began to decline in M1, consistent with previous observations in this species (Chapter 3, this dissertation). Although there also appeared to be a seasonal peak in M2's testicular volume, the sensitivity of ultrasound measurements may be insufficient to detect actual changes of those magnitudes, reducing the confidence with which seasonality

can be assessed (Chapter 3, this dissertation). The linear measurements of M2's testes were outside of the 95% confidence interval for measurements from both immature and mature belugas examined by Heide-Jørgensen and Teilmann (1994), and were approximately half the volume of the smallest adult male testes previously measured in aquaria (Chapter 3, this dissertation). Brodie (1971) found one animal with a testis volume of 130 cm³ with evidence of spermatogenesis, but sexual maturity was not evident for all belugas until testis volume exceeded 360 cm³ (TTV of approximately 720 cm³). In the same study, the youngest sexually mature male had 14 growth layer groups in the cross section a tooth, which previously corresponded to an age of 7 years, but is now thought to correspond to an age of 14 years (M2 was 11-12 years old during the study) (Stewart et al. 2006). In a study of belugas in aquaria, Robeck et al. (2005) found that the youngest male to sire a calf was 9 years old, but that the mean age of first reproduction in male belugas was approximately 13 years. The seasonal pattern of testosterone, while present, also differed from M1 and from other males studied previously, with a period of elevation shorter in duration (Chapter 3, this dissertation). Therefore, in the absence of a conception or a semen sample for evaluation, M2 was likely immature during the study period. This suggests that testosterone concentration measurements alone are insufficient for identifying maturity status in male belugas.

The identification of estrous cycle stages in F1 was critical to interpreting the behavior of all individuals in the group. Unfortunately, ultrasonographic or urinary hormone conjugate data, which can be effectively used to characterize the estrous cycle in belugas (Steinman et al. 2012), was unavailable for F1. Instead, progesterone

measurements in blow samples were used to identify luteal phases and to infer the occurrence and stages of the estrous cycle. While the utility of progesterone sampling in blow for this purpose has been demonstrated for belugas, there is a risk of falsely identifying a reproductive condition based on a single sample due to the variable nature of dilution in each sample (Chapter 1, this dissertation). This necessitated the conservative standard for identifying luteal phases in order to reduce the likelihood of falsely identifying an estrous cycle. The first two inferred estrous cycles identified fit with the estrous cycle stage durations described by Steinman et al. (2012), and occurred when estrous cycles frequently occur (Robeck et al. 2005). There also appeared to be behavioral correlates for these estrous cycles. The third estrous cycle identified was less clearly interpreted, and fell during a time when estrous cycles are less likely to occur (Robeck et al. 2005). Although aquarium belugas most often have two estrous cycles in a given breeding season, there are observations of up to seven estrous cycles in a single year for one female (Katsumata et al. 2006). Increased frequency of blow sampling, or ultrasound examinations at key times to confirm or refute findings from blow sampling would aid in identifying reproductive events with greater certainty.

The lack of a confirmed conception from three estrous cycles in the presence of a male has been documented in an aquarium beluga previously (Katsumata et al. 2006). Two possible explanations for this observation are linked to ovulation mode. While the mechanism for ovulation induction is unknown in belugas, in many species it is triggered by the physical act of copulation (Bakker and Baum 2000). If this is the case in belugas, then M2 could have induced ovulation without siring an offspring.

Alternatively, it is possible that F1 spontaneously ovulated in the absence of copulation. Belugas have been observed to ovulate spontaneously in the absence of a male, which Steinman et al. (2012) speculated may be related to self-stimulation by the female. F1 would also periodically engage in this behavior, perhaps triggering ovulation without copulation.

Behavioral Measures

The use of pair-wise behavior specific kappas revealed that several behaviors were subject to higher levels of disagreement. This emphasizes the importance of the use of these targeted evaluations of interobserver reliability in studies of behavior, so that high rates of agreement for more obvious behaviors do not mask poor agreement for others (Kaufman and Rosenthal 2009). Eliminating several behaviors in the intended ethogram did not affect the ability to assess seasonal variation in association, aggression, or courtship in this group.

Behavioral definitions should be altered in future studies. The open mouth display, which occurs in both aggressive and courtship contexts, should be accurately timed, as longer displays seemed to be associated with courtship. Bite threat is another behavior that might vary in different contexts, as evidenced by the reaction of the recipient. In aggressive contexts, this behavior appeared to be a threat of physical contact, or failed physical contact due to evasive action of the recipient. The movement toward the recipient in courtship contexts subjectively appeared slower and was associated with a less evasive response from the recipient relative to bite threats in

aggressive contexts. Evasive behaviors performed by the recipient in response to bite threats may aid in interpreting this behavior.

The nature and contexts of voluntary movements of the melon require additional attention. The belugas in this study frequently altered the shape of their melons, a behavior that has been documented previously for this species (Hill et al. 2015; DiPaola et al. 2007). Melon movements could possibly function as a behavioral modifier, altering the signal conveyed to the recipient by similar behaviors. For example, the belugas in this study frequently performed open mouth displays while altering the shape of their melon. Perhaps one shape is associated with an aggressive signal, while a different shape allows the open mouth display to convey an affiliative signal. The lack of agreement between observers in this study prevented such an analysis, but refined behavioral definitions could allow such a study to be performed with the existing video data.

Patterns of Intersexual Association

One pattern of behavior in this study that was similar to wild beluga behavior was sexual segregation, with intersexual association occurring almost exclusively between January and June. Several studies have documented sexual segregation of belugas in the summer and fall (Suydam et al. 2001; Loseto 2006). Observations of male-female interactions in July through Dec were short in duration and rarely appeared to be affiliative, with a very low occurrence of group swims and genital presents. In estuarine habitats, female belugas appear to actively avoid groups of male belugas (Smith et al. 1994). The observations in this study suggest a functional sexual

segregation despite continuous physical proximity. The period of high intersexual association observed in this study (January through June) corresponds with a time period that belugas are difficult to observe in the wild, although improving remote telemetry technology should enable further study of movement patterns during this time. Belugas typically do not enter more readily observable nearshore estuarine habitats until late June or July in most regions (Hornby et al. 2016; Richard et al. 2001), emphasizing the importance of behavioral observations in aquaria earlier in the year.

While relatively high levels of intersexual association occurred when estrous cycles are most likely to occur (Robeck et al. 2005), there did not appear to be differences in association patterns during F1's inferred follicular phases, when she would be expected to be most receptive. The uncertainty associated with identifying estrous cycle stages in this study could account for this observation. However, if female receptivity was the only factor influencing patterns of intersexual association, even shorter periods of relatively high intersexual association might be expected given the rarity of intersexual associations outside of the breeding season. Instead, the observed pattern of association might indicate a relatively long courtship period that lasts longer than the time of peak receptivity from the female. This may have occurred due to the lack of additional receptive females for the males to interact with, or in the case of M1, serious adult male competitors. However, if this is a consistent pattern of behavior in belugas, longer courtship could allow ovulation-inducing males to thwart breeding attempts by other males, while females prevent suboptimal males from breeding with her while she is most fertile by choosing to associate with a higher

quality mate. This may also provide the female with opportunity to evaluate potential mates, as induced ovulation likely reduces the opportunity for postcopulatory selection (Iossa et al. 2008). Relatively long periods of consortships have also been observed in humpback whales and bottlenose dolphins (Schaeff 2007).

Intersexual interactions were typically initiated by males and terminated by females in this group. This pattern was strong and consistent throughout the year. Two of the weeks where F1 was more likely than normal to approach the male occurred close to her inferred second and third ovulations. This suggests that periods with a reversal or reduced strength of the typical pattern may indicate important changes in behavior, and thus this behavior should be a component of future research.

Courtship Behavior

The strong seasonal nature of intersexual genital presents in this study that corresponds with the breeding season suggest a reproductive function for this behavior, as opposed to a more social function, although socio-sexual behavior for non-reproductive purposes is common in odontocetes (Schaeff 2007). Therefore, observation sessions containing male toward female genital presents were considered to be observations of courtship in this social group. Copulation was not observed to confirm this assumption, but copulation is rarely observed in belugas in aquaria (Glabicky et al. 2010; Hill et al. 2015). This contrasts with other species of odontocetes in both aquaria and the wild, where copulation rates can be high (Puente and Dewsbury 1976; Orbach et al. 2015). This could be a reflection of beluga mating

strategy, as lower copulation rates might be expected for induced ovulators depending on a male's ability to both induce ovulation and ensure paternity.

As proposed by Hill et al. (2015), group swimming served an important function in beluga social interactions observed in this study, particularly with intersexual associations. Group swimming was relatively rare, occurred almost exclusively during the breeding season, and was concentrated primarily in the observation sessions that also contained male toward female genital presents. Genital presents also almost exclusively occurred during group swim interactions. Group swimming was not necessarily synchronous, as described in other species (Connor et al. 2006), and it was clear that either the male or the female could lead the direction and pace of swimming during a group swim. Swimming in this manner requires cooperation from all participants, and could thus be used to assess mate choice in female belugas. In this study, both males were frequently simultaneously observed in a group swim with F1. However, courtship behavior during these triad interactions were almost exclusively between M1 and F1, with M2 apparently observing these interactions and rarely displaying toward F1 during them. With both males available for interaction at the same time, this is perhaps indicative of F1's choice to interact with M1 as opposed to M2 at those times. The close association between group swimming and genital presents further suggests that the female can select which males might be able to display toward her by choosing who to swim in close association with. This behavior is obviously constrained in aquaria, but its potential function in mate choice merits further study.

The genital present described in this study is similar to the pelvic thrust behavior described by Glabicky et al. (2010) and a combination of the horizontal “S” posture and pelvic thrust behaviors described by Hill et al. (2015). The behavior was described differently in this study in order to: more clearly define the body posture of the actor, remove any orientation restrictions on the occurrence of the behavior, identify the necessary presence of a recipient, clearly separate this behavior from copulation, account for the cessation of active swimming, and reduce the implication that the behavior is forcible by eliminating the use of the term “thrust,” because the genital present was generally slow and deliberate in the study group. The rarity of erections during this behavior suggests it has more of a display function in courtship as opposed to a direct relationship with copulation.

The overall frequency of genital present displays was lower than in Glabicky et al. (2010), which may be due to the differences in social group size or composition in these two studies. Glabicky et al. (2010) also found that the occurrence of this behavior had a strongly seasonal pattern, with a peak occurring in March. In this study, the frequency of genital presents peaked later, perhaps as a reflection of the estrous cycle timing in F1. Male belugas would likely adjust their courtship behavior to match the periods of receptivity of females. M2 performed more genital presents, but M1 performed a much larger proportion of the observed genital presents within F1’s inferred follicular phases. This could be the result of F1 preferentially choosing to swim with M1 during these times, enabling increased displays. As an adult and proven sire, M1 may also have been more able to detect F1’s receptiveness during these periods, and thus concentrated displays during this time. It is interesting that

M1's genital present behavior toward F1 varied in frequency with the occurrence of the follicular phase, but association did not; this further supports importance of prolonged courtship in this pair of belugas.

In addition to selecting which male to swim with and thus allow genital presents from, F1 could further control breeding opportunities by behaving differently in response to genital presents, and presumably copulation attempts as well. The slow, deliberate pace of the genital present, as well as the associated termination of active forward propulsion by the displaying male leaves clear opportunity for the female recipient to respond in a way that either allows contact to occur or not, as well as where on the body that contact can occur. In this study, F1 was observed to variably alter her pace or body orientation in response to genital presents, allowing the quantification of receptivity. The body position of belugas during copulation has not been formally described. Here, we assume that copulation must be performed in a ventral to ventral position, as in other species of odontocetes (Orbach et al. 2015), and thus locomotion and orientation patterns that would lead to contact on the ventral surface were considered to be associated with a higher level of receptivity. F1 displayed higher receptivity toward M1 than M2, allowing a far higher rate of contact and only displaying active avoidance in response to M2's displays. Some of F1's responses to genital presents can be considered analogous to the evasive behavior displayed by wild dusky dolphins (*Lagenorhynchus obscurus*) that variably roll their body away from males attempting to copulate (Orbach et al. 2015). On one occasion, F1 was observed to drift during a genital present from M1 with the flukes curled dorsally. Fluke posture could be explored as a further indication of receptivity.

The prevalence of open mouth and melon shake displays during observation sessions with male toward female genital presents suggests a role in courtship for these behaviors. Open mouth displays and mouthing have previously been suggested to have socio-sexual functions in addition to agonistic functions in belugas (Hill et al. 2015). The melon shake is especially interesting, as it almost exclusively occurred in courtship contexts and is clearly distinct from behaviors that occur in other contexts. There is also some evidence that melon shake displays from M1 were associated with higher levels of receptivity from F1, although this was not the case for M2. The melon of a beluga is remarkable in comparison to all other odontocetes due to its mobility, discussed above in the context of the difficulty of categorizing the range of movements possible. The melon is described as sexually dimorphic in belugas, with males having broader melons than females (Martin 1996). The primary function of the melon is to focus echolocation signals (Cranford et al. 1996), and focusing within the melon could aid in directing echolocation signals in this species (Penner et al. 1986). Altering the shape of the melon may also be associated with creating “facial expressions” (O’Corry-Crowe 2002). With the prominent role that melon movement behaviors appear to have in social interactions and especially courtship, perhaps this sexual dimorphism is a result of sexual selection by females for males with larger, broader, and thus more expressive melons. The differently shaped melons in males could also be related to sexually selected acoustic displays, as may be the case in sperm whales (*Physeter macrocephalus*) (Cranford 1999).

M2’s courtship behavior with F1 was qualitatively and quantitatively different from M1’s. M2 spent more time interacting with F1 than M1 did, and performed

display behaviors (open mouth, melon shake, genital present) with greater frequency. F1 would frequently perform open mouths, melon shakes, and genital presents toward M2, but was rarely, if ever, observed performing these behaviors toward M1. The periods of high levels of interaction between M2 and F1 tended to occur outside of inferred follicular phases. Juvenile male mammals may display ineffective courtship behavior or prolong the breeding season (Milner et al. 2007). It is possible that M2 observed courtship between M1 and F1 as part of a social learning process, and then continued to associate with F1 after M1's interest had waned. This is reflected in the shared peaks of association when courtship behavior from M1 was frequent and a lack of shared peaks of association when M2's courtship behavior was high. F1's choice to interact with M2 during these times is less clearly explained, although socio-sexual interactions have been observed between adults and juveniles in previous studies of belugas (Glabicky et al. 2010; Hill et al. 2015).

Perhaps the most striking observation through the paired study of physiological and behavioral measures is that the majority of presumed courtship behavior in this social group occurred after peak testosterone and for M1, at a time when testes size was about to decline. This finding agrees with the observation that most conceptions occur after peak testosterone has been reached, and testicular volume is declining in size (Robeck et al. 2005; Chapter 3, this dissertation). This is in contrast with many species of mammals, including spinner dolphins (*Stenella longirostris*), where testosterone is associated with breeding behavior in males (Wells 1984). This also contrasts with a study of finless porpoises (*Neophocaena phocaenoides asiaeorientalis*), where breeding behavior was correlated with testes size (Wu et al.

2010). Testosterone still likely played a role in regulating behavior, as both intersexual association and courtship behavior all but ceased for M1 after blow testosterone fell below an elevated concentration in week 37 for the first time in 14 consecutive weeks. Signaling by the female, perhaps via group swimming, may be important in eliciting appropriate courtship behavior from the male. However, if male reproductive physiology has returned to the baseline condition, perhaps this signaling would be ineffective. This could explain the observation that F1's inferred ovulation in July was not associated with courtship behavior from, or even elevated association with, either male.

Although F1 spent more time associating and engaged in more courtship behaviors with M2, there were several lines of evidence that suggest that F1 selected the mature, proven sire over the apparently immature male that had not yet sired any offspring. F1 chose to swim with M1 at key times during her estrous cycle, and was more receptive to M1's genital present displays. A higher proportion of M1's genital displays were also performed during inferred follicular phases, including a period of apparent exclusivity associated with the first inferred ovulation. In interactions with both males, F1 almost exclusively received displays from M1. The occurrence of genital presents from both males in the same observation session was rare, and only occurred outside of inferred follicular phases, when choices would have lesser consequences to F1's reproductive fitness. These observed patterns may be due to M2's juvenile status; females of many mammal species will preferentially choose mature mates over immature males (Clutton-Brock and McAuliffe 2009). Although

the traits preferred by F1 are unknown, F1 could have assessed M2's maturity status via size or behavior, as both differed from M1 during the study period.

Aggression was not an important component of courtship in these belugas, and in general, aggression was mild in this social group. In several species of odontocetes, including the beluga's closest living relative, the narwhal (*Monodon monoceros*), aggression that results in physical injury from teeth is common (MacLeod 1998). In this group, aggression did not result in a single rake mark throughout the study. There was no evidence that F1 was coerced into interacting with either male, as occurs in bottlenose dolphins (Scott et al. 2004). The relatively higher rates of bites, bite threats, and rakes that occurred during observation session with genital presents seemed to be associated with gentle mouthing that has previously been described as socio-sexual in belugas.

This study is not considered to be a comprehensive description of courtship behavior in this species, given the limited amount of time that observations could occur, the small number of animals involved and the possibility of individual variation in behaviors between individuals. Even in the small number of belugas studied thus far, individual differences have been observed. The ethogram developed by Hill et al. (2015) primarily focused on one mature male that preferentially performed genital presents (horizontal "S" postures) at the water's surface in a horizontal position with his left pectoral flipper pointed up. Neither male in this study was observed performing a genital present at the surface, nor did they display a clear orientation preference. The males in this study approached F1 from any side (right or left lateral, ventral, or dorsal) while either horizontal or with their head angled toward the bottom.

The study of additional males will allow for more comparisons, and perhaps even an exploration into the effect of social learning on courtship behavior, as males that are housed together may show similar behavior to each other that may be different from other social groupings.

Male-Male Social Interactions

Direct observations of wild belugas, as well as data from telemetry studies, suggest that male-male social relationships may be important in this species, at least for some parts of the year. In this social group, association between the males did not vary by season, in contrast to associations between males and females. Despite the extreme seasonality seen in genital presents toward F1, male toward male genital presents occurred throughout the year, indicating a potential social function of this behavior. Even though they were of different reproductive conditions, both males displayed aggressive and presumably affiliative behavior (genital presents) toward each other, often in succession. There was not a clear dominance/submission relationship between the two; in fact aggression was more commonly displayed from M2 toward M1. Some of the seemingly aggressive behavior may have been related to play or socio-sexual behavior, which is common between males in other odontocetes (Mann 2006). M2 was more likely to initiate interactions and M1 was more likely to terminate them, which may be related to play behavior in M2 and the reduced willingness of M1 to engage in such behavior given his age (Hill and Ramirez 2014). M2's apparent observation of M1's courtship behavior toward F1, along with the lack of M1's observation of M2's courtship behavior toward F1, suggests that M2 may

have been learning to engage in courtship. From currently available data, it is unclear if subadult male belugas would have the opportunity to observe this behavior in the wild, although in most cases a female would become pregnant while still accompanied by a nursing calf (Brodie 1971), implying that at least young calves would have an opportunity to observe courtship and mating.

Interestingly, male toward male genital presents were more common when F1 was in an inferred follicular phase. If male contest competition was an important feature of reproductive behavior, the opposite might be expected. In a highly competitive environment, a male engaged in courtship behavior would also be expected to be intolerant of the proximity of other males, yet M1 was very tolerant of M2's presence during courtship bouts with F1. Although rates of aggression between the males were higher during the breeding season, the relatively low level of aggression in both frequency and intensity between M1 and M2 suggests that competition for mates was minimal in this group. M1 may not have identified M2 as a threat due to M2's immaturity, and thus did not need to expend energy in competitive behavior. Further study of groups with multiple adult males is needed for comparison.

Conclusion

Despite the limitations of studying behavior in a small group of aquarium housed animals, several observed patterns were consistent with the limited knowledge of social behavior in wild belugas, including seasonal association patterns between males and females resulting in functional sexual segregation, and the relatively greater importance of male-male social relationships. The near absence of interaction

between males and females outside of the breeding season suggests that the observed pattern had a reproductive function, as opposed to socialization in general.

Periods of higher intersexual association were not necessarily confined to inferred follicular phases, suggesting relatively long courtship periods. These associations contained a variety of behaviors, including some that may have multiple functions depending on context, and required a high degree of female cooperation. These relatively long, complex interactions provided multiple opportunities for males to display toward the female, and for the female to actively respond to such displays by choosing whether or not to swim with the displaying male as well as choosing how to respond to behavioral displays. This observation is also consistent with predictions of reproductive behavior based on ovulation mode. The relative importance of these behavioral displays in pre-copulatory selection could account for the apparent low investment in pre- or post-copulatory traits in belugas found in an analysis of sexual selection in cetaceans (Dines et al. 2015).

The relatively low copulation rate is also consistent with the relatively small testes found in this species, as well as the relatively small seasonal variation in testes size (Chapter 3, this dissertation). Further study of groupings with multiple adults of both sexes will aid in assessing the relative importance of postcopulatory selection (sperm competition) in belugas. The apparent reduced association between measures of male reproductive physiology and courtship behavior is consistent with previous observations of male reproductive seasonality and the timing of conceptions (Robeck et al. 2005; Chapter 3, this dissertation). This suggests that signaling from the female

indicating receptivity plays a more important role in eliciting courtship behavior from the males than internal physiological cues.

Despite its limitations, blow sampling enabled a greater understanding of the reproductive physiology of the belugas in this study than would have otherwise been possible. Blow sampling is a comparatively easy, non-invasive way to assess reproductive physiology and it involves less intensive training than urine collection, blood sampling, or ultrasound examinations. This methodology will greatly facilitate paired studies of behavior and physiology of belugas in aquaria. With further development, blow sampling also has greater potential for application in wild belugas than more invasive forms of physiological assessments. Physiological validations of this methodology (Chapter 1, this dissertation) should also be pursued in other species of cetaceans.

While broad conclusions from this study are certainly limited by the social composition of this group of belugas, there were several findings that are consistent with predictions of reduced postcopulatory and increased precopulatory selection that follow from the recent discovery of induced ovulation in belugas. Further study using paired behavioral and physiological measures of belugas in aquaria with diverse social structures would allow for these hypotheses to be tested. The relative ease with which blow sampling can be used for this purpose, in addition to the high visibility of belugas in these settings makes this research feasible. In the absence of direct observations and physiological assessments of wild belugas during the breeding season, this knowledge could ultimately prove valuable for management purposes and predicting the adaptability of belugas to environmental perturbations.

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APPENDIX 1

GENERAL LITERATURE REVIEW

The Current Understanding of Beluga Mating Systems and Reproductive Strategies

The beluga (*Delphinapterus leucas*) is an Arctic and sub-Arctic species of whale that has received a great deal of attention recently given their association with sea ice and thus the potential impacts that climate change may have on their populations (Laidre et al. 2008; Moore and Huntington 2008). Effective beluga management requires sound information on their reproductive biology and the genetic structure of the population. However, very little information exists on beluga breeding behavior because they breed in the ice-covered spring in most locations, where they are unavailable to researchers (Burns and Seaman 1988). Additionally, due to the extensive regulations that protect fragile populations, such as the Cook Inlet stock in Alaska (Rugh et al. 2010), researchers have largely been unable to acquire tissues that would allow demographic or genetic questions to be answered. Therefore, advancing the knowledge of the poorly understood aspects of beluga reproductive biology and the development of non-invasive research tools would benefit their management in both zoological facilities and the wild.

To date, there have been no directed studies of social interactions between belugas during the breeding season, and there are few published descriptions of beluga courtship or copulatory behavior relative to other cetaceans (reviewed in Schaeff 2007). Existing studies are primarily descriptive in nature, and do not evaluate behavior in a larger physiological or ecological context (Hill et al. 2015; Glabicky et al. 2010). Instead, given the paucity of direct observations, the mating system and

strategies of belugas have been indirectly inferred by examining life history traits and comparing them to better known species. Through these analyses, belugas are presumed to alternatively have a polygamous mating system with high levels of pre-copulatory selection via male-male contest competition (Schaeff 2007; O’Corry-Crowe et al. 1997), high post-copulatory selection (sperm competition) relative to the narwhal, their closest living relative (Kelley et al. 2014), or neither high pre-copulatory or post-copulatory selection relative to other cetaceans (Dines et al. 2015).

Within a beluga population, more males are available to breed than females at any given time because females assume all of the costs associated with calf rearing and only breed in three year intervals (O’Corry-Crowe 2002). This skewed operational sex ratio results in male-male competition for breeding opportunities (Clutton-Brock 1989). In some mammals, this leads to sexual size dimorphism, with males reaching larger adult sizes than females (Lindenfors et al. 2002). Belugas are no exception; they are among the most sexually dimorphic odontocetes, with males reaching lengths approximately 15% longer than females (Luque and Ferguson 2010). Based on this observation, direct physical competition is predicted to play an important role in male reproductive success, as it does in other species of cetaceans with a high degree of sexual dimorphism (sperm whales, *Physeter macrocephalus*: Carrier et al. 2002; narwhals, *Monodon monoceros*: Silverman and Dunbar 1980). However, personal experience with multi-male social groups in an aquarium setting suggests that male-male aggression may occur less frequently than expected. Adult males in the wild associate closely with one another and often form their own same-sex pods (Suydam et al. 2001; Barber et al. 2001). Adult males in aquaria may form

social bonds that are readily apparent (pers. obs.). These associations could have a reproductive function, as in wild bottlenose dolphins (*Tursiops truncatus*), which may form male alliances to sequester and guard mates (Randic et al. 2012). Alternatively, the sexual dimorphism observed in belugas could be unrelated to mating strategies, stemming instead from phylogenetic relationships or ecological drivers (González-Suárez and Cassini 2014). Larger males are able to exploit different habitats than females, perhaps reducing intraspecific competition in a habitat that may be nutrient poor for part of the year (Loseto et al. 2006). Unraveling the selection mechanisms that drive sexual size dimorphism requires an understanding of the relative importance of body size in beluga mating strategies.

Sperm competition may also be a mechanism for males to compete or for females to employ mate choice. Relative testes size can be used to infer the importance of sperm competition in the mating strategy of mammalian species (Kenagy and Trombulak 1986). In species such as the harbor porpoise, relatively large testes (4% of the body mass) suggest the importance of sperm competition (Neimanis et al. 2000). In belugas, sperm competition is less likely to play a dominant role in male reproductive success; the testes account for ~0.1% of body mass (calculated from data in Kleinenberg et al. 1969), which places them among the smallest ratios in odontocetes studied thus far (Connor et al. 2000; Mesnick and Ralls 2002). However, testes size has primarily been assessed in the summer, when carcasses are readily available but breeding does not occur (Kelley et al. 2014). Testes size may change dramatically seasonally, as it does in many other species of

odontocetes (Plön and Bernard 2007), which could lead to an improved understanding of the importance of sperm competition in belugas.

Another aspect of a species' reproductive biology that may yield predictions about breeding behavior is their ovulation mode. Recently, beluga ovulation was discovered to be induced by copulation; all other odontocetes studied in sufficient detail ovulate spontaneously (Steinman et al. 2012). Induced ovulation is an effective strategy when females encounter males infrequently, but must conceive during the appropriate season, which encompasses a short time period, to ensure parturition during periods of favorable environmental conditions (Larivière and Ferguson 2003). This may be important for species that are often segregated sexually in highly seasonal environments, such as the beluga (Barber et al. 2001; Loseto et al. 2006). Induced ovulation is also expected to lead to some degree of mate guarding during the short period between copulation and fertilization (Iossa et al. 2008; Soulsbury 2010). In belugas induced to ovulate for artificial insemination, ovulation occurred approximately 36 hours after the artificially induced hormone surge (Robeck et al. 2010). Thus, beluga males might be expected to associate closely with a female for a day or two after copulation to thwart breeding attempts by other males. During this period of maximum fertility, females would have the opportunity to employ mate choice, copulating only with the guarding male or seeking copulations with competing males, enabling sperm competition (Clutton-Brock 1994). Currently, it is unknown if a female mates with multiple males during a single estrous cycle.

Monitoring Reproductive Function

In order to sufficiently test these predictions, it is necessary to accurately describe the reproductive condition of an individual at the time that behavior is observed. Gonad function, measured through testes size or follicular development, is a clear indicator of an individual's reproductive condition and is often used to assess maturity and reproductive condition in adults (Neimanis et al. 2000; Burns and Seaman 1988). However, hormonal correlates may enable more subtle physiological changes to be identified, because reproductive steroids regulate the breeding behavior of both sexes (Adkins-Regan 2005). Research on hormonal correlates of behavior among large mammals is logistically difficult and repeated blood sampling for hormone analysis influences the behavior of many species, so studies that employ blood sampling usually do so infrequently. Less invasive methods of assessing concentrations of reproductive hormones (using urine or feces) have improved research capabilities, but the time lag between when the hormone is exerting its effects in circulation to when they are excreted (often >24 hours in large mammals) limits the ability to resolve the relationship between behavior and hormone concentration (Anestis 2010). However, given the important role that reproductive hormones have on reproductive behavior, studies that correlate the two are important for explaining individual variation in reproductive behavior and the resulting variation in reproductive success.

Therefore, studies that correlate steroid hormone concentrations and behavior in wild animals are common across taxa, including several species of marine mammals (Bartsh et al. 1992; Burgess et al. 2012). Studies that incorporate measures of gonad

function, hormone concentration, and behavior are also feasible in some wild terrestrial mammals. For example, a study of free-ranging Soay sheep correlated scrotal circumference, testosterone concentrations in blood, and breeding behavior (Preston et al. 2012). However, research on hormonal correlates of behavior among cetaceans has been particularly sparse. Although methods are available for determining hormone concentrations in the feces and blubber of wild cetaceans (Hunt et al. 2013), opportunities have been limited because individuals are exceedingly difficult to monitor for long periods in their natural marine environment and behavioral observations are generally limited to surface observations.

Studying cetaceans in zoological facilities would alleviate many of these challenges. Belugas are already common research subjects; most of the available information about beluga reproductive physiology, parturition and calf rearing has been elucidated by studying trained belugas in zoological facilities (Robeck et al. 2005, Russell et al. 1997, Steinman et al. 2012). Despite the differences in environment, captive studies concur with what is known about beluga reproductive physiology in the wild, emphasizing the value of these studies (Robeck et al. 2005; O'Brien et al. 2008). Zoological facilities enable researchers to longitudinally monitor individuals trained to voluntarily participate in research procedures, providing unmatched access to biological information. The improved understanding of physiology gained in zoological facilities can then help form a baseline for improved management of wild populations (O'Brien and Robeck 2010).

Despite this potential, few studies attempting to correlate reproductive hormones with behavior have been completed in any species of captive cetacean. In a

study of a breeding group of captive spinner dolphins (*Stenella longirostris*), testosterone concentration could be correlated with an increase in the frequency of certain breeding behaviors (Wells 1984). Wu et al. (2010) were able to correlate the frequency of reproductive behavior (contacting erect penis to the genital region of another animal) to both testicular volume and blood testosterone concentration in a captive group of finless porpoises (*Neophocaena phocaenoides asiaeorientalis*). Muraco and Kuczaj (2015) were able to assess the estrous cycle stage of bottlenose dolphins (*Tursiops truncatus*) and identify behaviors associated with estrus in this species. However, the physiological measures used for these studies were collected infrequently and/or omitted samples from one of the sexes. Future studies in zoological facilities would benefit from sampling both sexes more frequently. Studies of wild cetaceans would become more feasible if a method of non-invasively monitoring steroid hormones became available.

Blow as a method of non-lethal sample collection from cetaceans

Due to the legal challenges facing cetacean researchers, many have worked to develop non-lethal methods for acquiring tissue from free-ranging cetaceans for genetic and hormone analyses, most of which require firing a biopsy dart into the animal (Noren and Mocklin 2012; Kellar et al. 2009). Recently, blow sampling has emerged as a less invasive alternative for studying wild cetaceans. Blow samples were first used to determine testosterone concentrations in trained dolphins by Hogg et al. (2005). Since then, testosterone, progesterone, or cortisol has been assayed in the blow of six different species of cetaceans, including cortisol in belugas (Hunt et al.

2014; Thompson et al. 2014; Hogg et al. 2009; Miller and Hall 2012). The presence of epithelial cells in a blow sample also allows for genetic analysis of the individual, a technique that has been developed for trained bottlenose dolphins and harbor porpoises (*Phocoena phocoena*) (Frère et al. 2010; Borowska et al. 2014). While these studies highlight the potential of blow sampling as a research tool, there are factors that currently limit the application of the technique. Genetic studies required 4-8 exhales from the same individual to accumulate sufficient cellular material, an unlikely scenario under field conditions (Frère et al. 2010; Borowska et al. 2014). Many individuals sampled for hormone analysis are of unknown gender or reproductive condition or belong to species with unknown endocrine cycles (Hogg et al. 2009). More importantly, reproductive hormone concentrations in blow have not been compared to those circulating in the blood for any species, preventing an understanding of the biological significance of hormone concentrations in blow.

The hormone cycles of belugas are well understood (Robeck et al. 2005; Steinman et al. 2012), and the availability of trained individuals of known gender and reproductive condition in zoological facilities offers an opportunity for the advancement of this methodology. Hormone concentrations measured in blow could be compared to those in matching blood samples to establish a correlation. Due to the variable amount of water vapor present in each blow sample, it is unlikely that the hormone concentration in blow will be an exact measure of the hormone concentration in circulation. However, given the 5-10 fold difference in circulating testosterone concentration in adults of both sexes in belugas (Robeck et al. 2005), it is reasonable to hypothesize that a biologically relevant threshold concentration of testosterone can

be established, above which all animals sampled are male. The dramatic difference in progesterone in pregnant and non-pregnant female belugas may allow for a similar technique to be used to separate pregnant from non-pregnant females (Calle et al. 1993; Stewart 1994). A threshold may also be identified for mature adults of both sexes, as explored by Høier and Heide-Jørgensen (1994) with reproductive hormones in beluga blood. These authors proposed a testosterone threshold of 3.30 nmol/l for identifying mature males; a similar predictive value may be identified for hormone concentrations in blow.

Previously, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been utilized to detect the presence and concentration of testosterone and progesterone in blow from other species, due to the small sample volume of a single exhale (Hogg et al. 2005). However, this technique requires prohibitively specialized equipment and may require advanced separation methods to provide the necessary sensitivity for measuring concentrations (Dunstan et al. 2012; Miller and Hall 2012). Validating enzyme immunoassays (EIAs) for the measurement of reproductive hormones in blow would greatly improve the applicability of the technique in aquarium managed, live-stranded, or temporarily restrained wild cetaceans, where multiple exhailes can be collected. Species with more voluminous exhailes may produce enough fluid for EIA analysis of a single exhale; as one of the largest odontocetes, belugas may be one such species. EIAs are already the standard for measuring steroid hormones in beluga blood, and have been validated for use with blow samples collected from right whales (*Eubalaena glacialis*) and for the

measurement of cortisol in beluga blow samples (Hunt et al. 2014; Robeck et al. 2010; Thompson et al. 2014).

Our understanding of wild beluga population dynamics would benefit from the development of this research tool. Hormone and genetic sampling would be simplified in endangered populations such as the Cook Inlet stock, where live strandings are common (Balsiger 2003). The care of belugas in zoological facilities would also be improved by reducing reliance on blood samples and enabling increased sampling frequency for research or medical purposes. If the utility of blow sampling is validated in belugas, method development would be fostered in other species whose conservation status necessitates non-invasive sampling.

The principal aims of this work are to develop methodologies that can be used to assess beluga reproductive condition in known individuals, and reproductive condition and gender in unknown individuals, and then utilize these methodologies to simultaneously assess physiology and behavior in a social group of belugas in an aquarium setting. The results of this study will be interpreted in the context of the current understanding of beluga reproductive biology in an effort to complement and inform studies of wild beluga behavioral ecology. Additionally, establishing the value of blow sampling as a minimally invasive tool to assess reproductive condition will allow this type of study to become more feasible for both wild and aquarium-managed belugas.

The aims of this dissertation are presented below.

Aim 1: Develop methodologies that can be used to assess beluga reproductive condition in known individuals, and reproductive condition and sex in unknown individuals.

Aim 1A: Assess the correlation between blood and blow hormone concentrations to develop threshold concentrations that are biologically informative.

Aim 1B: Improve applicability of blow sampling for wild populations by using the same sample for both hormone quantification and molecular sex determination.

Aim 1C: Assess seasonal variation in testes size in adults via ultrasonography.

Aim 2: Qualitatively describe beluga breeding behavior at the Mystic Aquarium.

Aim 3: Use methodologies developed in Aims 1 and 2 to determine the best physiological predictors of breeding behavior and association patterns in a group of aquarium belugas.

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APPENDIX 2

SYNTHESIS AND SPECULATIVE DISCUSSION

Improved understanding of the mating system or sex-specific mating strategies can improve species management and allow for more robust predictions of how a population might respond to perturbation. The mating strategies of belugas have not been determined with confidence because direct observations during the breeding season are not logistically feasible. Paternity studies, which can provide information on reproductive success in the absence of direct observation, are also lacking in this species. Despite the lack of data, several authors have attempted to infer beluga mating strategies from morphological or demographic information because this information provides context for the numerous studies of beluga population genetics and is important for the management of this species.

Among the features used to infer mating strategies in belugas are sexual size dimorphism, testes size, and the operational sex ratio. In this speculative discussion, I aim to evaluate these features in the context of the results of this dissertation as well as other recent observations relating to the reproductive biology of belugas, in order to develop new hypotheses that can be explored through studies in the wild and in zoological facilities to improve the understanding of beluga mating strategies.

Sexual size dimorphism and male contest competition

Sexual size dimorphism is considered to most often be associated with sexual selection (Shine 1989). In species in which males are the larger sex, this typically indicates a polygynous mating system with contest competition between males, where

larger body size confers a competitive advantage for males. As such, body size has been proposed to indicate contest competition in belugas (Schaeff 2007). However, the degree of sexual dimorphism varies among populations in belugas (Doidge 1990). Given the latitudinal cline in feeding ecology found in belugas (Yurkowski et al. 2016), it is possible that the observed pattern of sexual dimorphism may be driven by feeding ecology (Shine 1989; Gonzalez-Suarez and Cassini 2014; Suydam 2009). In marine mammals, large body size confers a thermoregulatory advantage as well as increased diving capacity due to increased oxygen storage ability, primarily in muscle (Berta and Sumich 2003). Larger male belugas are known to travel through areas that have greater ice coverage than smaller female or juvenile belugas (Suydam et al. 2001; Loseto et al. 2006). Increased diving capacity and thermoregulatory capacity due to larger body size may allow male belugas to reach feeding areas that are not attainable by other conspecifics, perhaps reducing intraspecific competition. These food sources would be expected to be high in quality given the degree of risk and energy invested in these movements (Suydam et al. 2001). If so, this high quality prey would support the observed dimorphism. In addition to limitations due to body size, female belugas are also limited in their ability to penetrate areas with heavy ice cover because they are accompanied by calves with limited dive capacity, forcing them to take fewer risks in their movements among heavy sea ice (Loseto et al. 2006). Females are also unable to invest as much energy to growth as males, as they mature at an earlier age and bear the high costs of lactation (Suydam 2009; Robeck et al. 2005). These factors could lead to the development of sexual size dimorphism in the absence of strong sexual selection (Shine 1989).

Variation in diet between the sexes, extent of sexual segregation, and sex-specific diving behavior could be compared to the degree of sexual size dimorphism observed within a group of belugas to determine the relative contribution of feeding ecology to sexual size dimorphism in belugas. Populations with a lesser degree of sexual size dimorphism might be expected to have less sex-specific variation in diet, less exaggerated sexual segregation in space and time, and fewer differences in sex-specific diving (i.e. foraging) behavior. Some observations are available that seem to lend support to this hypothesis; in lower latitudes, where food may be more readily available, females and males showing similar dietary patterns and apparently reduced levels of sexual segregation in space, but not time (Yurkowski et al. 2016; Colbeck et al. 2012). If sexual size dimorphism is primarily driven by natural selection, then it would be inappropriate to infer high levels of male contest competition in belugas because of the presence of sexual size dimorphism.

If contest competition is important in belugas, then males might be expected to travel separately from each other during the breeding season in search of receptive females, yet at least in the summer and fall, male belugas are typically found in groups (Smith et al. 1994; Colbeck et al. 2012). In sperm whales, roving males sexually segregate from females outside of the breeding season to feed in more productive areas, and then rove between pods of females, forming temporary consortships (Schaeff 2007). In the absence of coercion or herding behavior (Connor et al. 2000), there would be no fitness benefit to travelling in a group of adult males, as doing so would put an individual male in direct competition with its social partner(s). Male-male associations outside of the summer and fall are largely unknown for belugas.

Male movement patterns in relation to females might be explored using improved telemetry technology to determine if males travel more widely (presumably in search of females) during the breeding season. Interbreeding between stocks is known to occur in belugas while sharing wintering grounds, supporting the concept of males that rove between groups of females in search of mates (Turgeon et al. 2012; Clutton-Brock 1989). If possible, males found associating closely in summer would be fitted with telemetry devices simultaneously to further explore the persistence of these associations.

Observations of belugas in aquaria (Chapter 4, personal observations) suggest a minimal role of contest competition in male beluga mating strategies. Adult males are commonly housed together for years at a time without significant behavioral issues, unlike other species of mammals that require the separation of adult males during the breeding season to reduce risk of injury due to fighting. In the present study, there was a high degree of tolerance of the presence of a second male while the other male engaged in courtship behavior. While aggression was observed, it was relatively infrequent and mild, and did not result in injury as is commonly reported in other odontocetes (Chapter 4; MacLeod 1998). Certainly sexual size dimorphism may still be selected for under sexual selection if females actively choose larger males, but current evidence does not support an important role of sexual size dimorphism in contest competition. The degree of sexual dimorphism in belugas has also been interpreted differently by Dines et al. (2015), who considered the sexual dimorphism in belugas to be minimal compared to other cetaceans.

Therefore, the occurrence of sexual size dimorphism appears to be the only existing line of evidence suggesting that contest competition is an important male mating strategy in belugas. While telemetry studies of wild belugas would provide important information about male-male social relationships, these studies are expensive and logistically difficult to conduct. However, other more feasible studies can be employed to investigate the existence of male contest competition. Behavioral studies in multi-male groups in aquaria will be useful in testing the importance of this behavior, especially if males can respond physiologically to perceived competition (Chapter 3). A formal study of rake marks on wild belugas could aid in determining the rate of aggression (MacLeod 1998). This research could be performed either as a component of photo identification studies or in post-mortem research associated with subsistence harvests (*e.g.* Suydam 2009). Highly polygynous species with high rates of contest competition also tend to have males that live for shorter periods than females (Clutton-Brock and Isvaran 2007). Detailed age studies, perhaps using existing data (*e.g.* Harwood et al. 2002) may provide another way to assess male-male contest competition. If sex is not associated with varying longevity, then male contest competition is likely less important in this species. If males live longer than females, it may support the alternative hypothesis that sexual size dimorphism is driven by natural selection. Larger males may be able to store more blubber or have more plastic foraging behavior due to increased dive capacity, and thus be more likely to survive during times of nutritional unpredictability, improving longevity. Although age structures were not different between the sexes, the data presented by Harwood et al. (2002) from one beluga stock seems to suggest that males may reach older ages

more frequently than females despite selective hunting that preferentially removes males from the population.

Relative testes size and sperm competition

Measuring relative testes size offers great insight to the mating system of mammals, with larger testes conferring an advantage to males in multi-male systems (polyandry or polygynandry) where sperm competition is relatively high (Kenagy and Trombulak 1986; Connor et al. 2000; Gomendio et al. 1998). Two studies have attempted to use testes size in belugas to infer mating strategies. In Kelley et al. (2014), belugas were inferred to have a more promiscuous mating system in comparison to narwhals, given their larger relative testes size and rapid testicular growth at sexual maturity. Although sperm competition may be more prevalent in belugas than it is in narwhals, Dines et al. (2015) considered belugas to have low investment in postcopulatory traits, indicating relatively low levels of sperm competition compared to other cetaceans. However, as the work of Dines et al. (2015) used the data from Kelley et al. (2014), there was a lack of sampling during beluga breeding season, with only 2 individuals assessed between December and April. Considering testes size is approximately 50% larger during the breeding season (Chapter 3), both studies may have underestimated the relative importance of postcopulatory selection in belugas.

Comparing relative testes sizes between cetaceans is not always clear because testes size does not increase allometrically in this group (MacLeod 2010). Therefore, in the absence of additional statistical modeling, comparisons between species of

similar body size, as conducted by Kelley et al. (2014), would be most informative. The closest species included in the analysis conducted by MacLeod (2010) was the pilot whale, which has testes that account for 0.31% of the body mass, approximately 3 times the percentage in belugas (mean = 0.07% reported by Kelley et al. 2014; allowing for a 50% increase during the breeding season would increase the mean to approximately 0.11%). Using the regression developed from a study of a wide variety of mammals in Kenagy and Trombulak (1986), a 1000 kg beluga would be expected to have a testes mass that accounts for approximately 0.5% of their body mass (acknowledging that this is only a rough estimate given the lack of allometry in this trait among cetaceans). Therefore, even after accounting for a 50% increase in testes mass during the breeding season (Chapter 3), the observed testes size in belugas is still relatively small, perhaps for mammals in general. The degree of seasonal change is also small relative to other species of odontocetes (Chapter 3). These data suggest a lower demand for sperm in belugas when compared to other cetaceans, indicating that sperm competition is less important for belugas than other cetaceans, in concordance with the view presented by Dines et al. (2015). While Kelley et al. (2014) found that sperm competition was likely more important for belugas than it is for narwhals, it is possible that narwhals are an extreme case among cetaceans, with the characteristic tusk of male narwhals allowing for greater levels of polygyny than is generally possible among cetaceans, resulting in smaller testes sizes. The finding that the narwhal tusk may serve as an honest indicator of male quality in narwhals (Kelley et al. 2014) supports this suggestion and merits further study.

Other indicators of the relative importance of sperm competition in belugas

Sperm competition theory makes several predictions about male reproductive physiology in addition to selection for larger testes (Gomendio et al. 1998; Dixson and Anderson 2004). Therefore, the current knowledge of beluga anatomy, physiology and behavior, revealed through this study and others, can be evaluated to help determine the relative importance of sperm competition in belugas in the absence of direct observations. These suggestions are tentative, as other factors can influence these traits, and different groups of mammals can respond differently to similar pressures (Gomendio et al. 2011). Due to lack of data, sperm morphology and female reproductive anatomy (genital tract length and complexity, for example) will not be considered here, but are promising areas of future research in relation to mating strategies (Miller et al. 2002; Plön and Bernard 2006; Kelley et al. 2014).

Higher levels of sperm competition are associated with higher testosterone levels among primates (Dixson and Anderson 2004). Adult male belugas have relatively low circulating testosterone concentrations. In this study, belugas in aquaria had testosterone that ranged from 0.2 - 6.9 ng/ml, which was similar to the range reported by Robeck et al. (2005) for aquarium belugas and Høier and Heide-Jørgensen (1994) for wild belugas, although measurements are unavailable for wild belugas in the breeding season. This is considerably lower than maximum testosterone concentrations measured in species where sperm competition is thought to be more important: harbor porpoises (30 ng/ml, Desportes et al. 2003), Dall's porpoises (20 ng/ml, Temte 1991), and bottlenose dolphins (54 ng/ml, Schroeder and Keller 1989). The observed range is similar to the killer whale (Robeck and Monfort 2006), a

species where sperm competition is thought to be less important (Schaeff 2007). In addition to suggesting a reduced importance of sperm competition, relatively low testosterone concentrations also support the suggestion that male contest competition is not an important mating strategy in this species (Wingfield et al. 1990).

Ejaculatory frequencies are also higher in primates with more promiscuous mating systems (Dixson and Anderson 2004). This study and others (Hill et al. 2015; Glabicky et al. 2010) suggest that copulatory frequency, and presumably ejaculatory frequency, is low for belugas. Rates of copulation can be much higher among other odontocete species with larger relative testes sizes (Puente and Dewsbury 1976; Orbach et al. 2015). The low observed copulation rate in belugas also corresponds with the apparent low sperm demand for this species.

Sperm competition is also associated with greater numbers of motile sperm per ejaculate (Gomendio et al. 1998). Very little information is available on ejaculate size in belugas, but in two males trained to provide semen samples (O'Brien et al. 2008; Alexa McDermott, personal communication), ejaculate volume is small (approximately 2 ml) relative to smaller species of odontocete. For example, in bottlenose dolphins (*Tursiops truncatus*) trained to provide semen samples, mean ejaculate volume was 25.5 ml, more than 10 times the volume of a beluga ejaculate despite having a body mass less than 1/3 of that of a beluga (O'Brien and Robeck 2006). As a result of this small ejaculate volume, belugas have approximately 5 to 14-fold fewer spermatozoa per ejaculate compared to 3 species of delphinids (O'Brien et al. 2008). If the small observed ejaculate volumes observed in aquaria are representative, this would be consistent with the observation that there is a low

demand for sperm in this species, and would imply a reduced role of sperm competition.

Taken together, there are several lines of evidence that suggest that sperm competition is not an important reproductive strategy in belugas. Certainly these observations do not rule out the potential for sperm competition, they only suggest a reduced role of this strategy relative to other species. Additional insight would be gained through more detailed investigations of the female reproductive tract, particularly the vagina, where sperm competition would primarily be expected to occur (Gomendio et al. 1998; Kelley et al. 2014). Molecular investigations into paternity, such as those accomplished in the killer whale (Ford et al. 2011), would be particularly informative. Kinship studies are already common in belugas (e.g. Colbeck et al. 2012). This type of study may be facilitated in wild belugas, especially within small populations, through the use of minimally invasive blow sampling as a source of DNA for analysis (Chapter 2).

Operational sex ratio and competition for mates

One demographic feature that is predicted to have a strong influence on mating system is the operational sex ratio (OSR). This measure is different from the actual sex ratio (ASR) in that it quantifies the ratio of sexually available females to males (Reynolds 1996). Female belugas breed in two to three year intervals (Suydam 2009). Therefore, there would be two to three available females for each breeding age male beluga in a given population. Male belugas also have no role in parental care (O’Corry-Crowe et al. 1997), so remaining with any one female long term does not

carry a fitness advantage. This lack of male parental care and highly male skewed OSR would be expected to be associated with intense competition between males for mates (Schaeff 2007). However, the presumed competition need not be confined to contest competition. Sperm competition via multiple mating, scramble competition between males to find receptive females, and female mate choice are other mechanisms of male-male competition (Schaeff 2007). If contest competition and sperm competition are relatively unimportant male strategies, then perhaps scramble competition or female mate choice are of greater importance.

Male belugas have been observed to travel together outside of the breeding season in groups of 8-10, while groups of females with dependent young travel in much larger groups (Smith et al. 1994). If belugas maintain these group sizes during the breeding season, and belugas are widely dispersed during the breeding season (low population density), then conceivably these small groups of male belugas may rove between groups of females during the breeding season (Clutton-Brock 1989). The ability to find and consort with groups containing receptive females (scramble competition) would have a fitness advantage. Then, in any given encounter between such groups, there would be expected to be a greater number of receptive females relative to adult males, reducing the need for contest competition between these associating males to gain access to mates.

Once an encounter occurs, females may further ensure that a given male is a high quality mate by mating with multiple males and encouraging sperm competition and/or employing precopulatory mate choice. The apparently low copulation rate in the groups of belugas studied thus far, as well as the prolonged and complex courtship

behavior observed in this study (Chapter 4), suggest that precopulatory mate choice may be an important strategy in this species, perhaps in addition to some degree of scramble competition. These strategies would still allow for a polygamous mating system, with both females and males breeding with multiple mates over a given breeding season, given the relatively long duration of the breeding season and the fact that female belugas are seasonally polyestrous (Robeck et al. 2005). Observations of social behavior in wild belugas during the breeding season, perhaps inferred through the use of telemetry, are needed to evaluate these suggestions.

Theoretical effects of induced ovulation on mating strategies

The relative unimportance of contest competition or sperm competition as mating strategies in belugas are in concordance with theoretical predictions for the influence of ovulation mode on mating strategies. For female induced ovulators, copulation is required to trigger ovulation (Bakker and Baum 2000). Induced ovulation is thought to reduce post-copulatory competition in mammals because the first male to copulate is the most likely to induce ovulation and sire the resulting offspring (Soulsbury 2010, Iossa et al. 2008). Thus, demand for sperm would be reduced, in agreement with the findings presented in Chapter 3. Males might be expected to require fewer sperm per ejaculate, and to copulate less frequently, assuming one copulation is sufficient to induce ovulation (Soulsbury and Iossa 2010). These predictions are also consistent with the current understanding of beluga reproductive biology.

Induced ovulation is thought to be selected for when males and females are less likely to come into contact, as mature follicles persist for long periods in induced ovulators prior to copulation. This reduces the need of both sexes to be in the same place within a narrow fertile period. If this is the selection mechanism that resulted in induced ovulation in belugas, it might indirectly indicate the rarity with which groups of females encounter groups of males, perhaps further emphasizing the importance of scramble competition relative to other strategies in this species.

The lengthy estrus would also create sufficient time for females to employ precopulatory mate choice. The relatively long periods of association between males and the female, as well as frequent behavioral displays by males toward females in this study provided ample opportunity for the occurrence of mate choice. The variable response of the female to these association attempts and displays further supports the occurrence of mate choice (Chapter 4). Females may also employ postcopulatory mate choice in the absence of sperm competition through ovulation induction mechanisms that require threshold levels of stimulation for ovulation to occur (Lariviere and Ferguson 2003).

The ability of the first male to copulate with a female to monopolize paternity in induced ovulators also suggests that there would be a fitness advantage for males to be associating with a female when she first becomes receptive (Soulsbury 2010; Gomendio et al. 1998). In species with a relatively diffuse breeding season, like the beluga, males might be expected associate with females prior to estrus. The observations in this study provide some support for this suggestion (Chapter 4). A short period of postcopulatory association may also be expected, both for males to

thwart breeding attempts that might result in sperm competition, as well as for females to ensure only the selected male has an opportunity to copulate. This study also provided some evidence of this, with periods of exclusivity of particular male-female associations close to ovulation (Chapter 4).

Therefore, the combined data on reproductive physiology and behavior from this species are in accord with predictions that follow from the presence of induced ovulation in belugas, a condition that is thus far unique among odontocetes. These mating strategies would also coincide with the wealth of genetic information available for this species (e.g. O’Corry-Crowe et al. 2010). They also do not fundamentally challenge the inferred mating system of the species, which is generally considered to be some form of polygamous system (O’Corry-Crowe et al. 1997). Given the limited ability for direct observations, these suggestions are tentative but may provide further guidance in interpreting studies of wild belugas.

Future studies of belugas in aquaria

This discussion highlights the difficulty in trying to infer the mating system of the beluga in the absence of direct observations. Although the application of studies of behavior in zoological facilities to wild populations can be limited, there are some cases where behavioral observations of managed groups can inform wild studies (Dudzinski 2010). Evaluating mating strategies in belugas may provide one such example, especially because direct observations of belugas during the breeding season are not logistically feasible. Studies in aquaria could therefore fill knowledge gaps that are unlikely to be filled with direct observations of wild belugas. Indeed, several

observations in this study agree with the current understanding of beluga social behavior in the wild, namely sexual segregation outside of the breeding season, the strong seasonality of courtship behavior, and male-male association patterns (Chapter 4).

Studying existing groups with multi-male/multi-female social composition would allow for further exploration of the topics addressed here. Key issues to study in such groups would be: the timing of follicular phases within a group of females to determine the degree of synchrony, and thus degree of competition that would be expected for a given female; male-male aggression during receptive periods to further assess male-male contest competition; female responses to behavioral displays to males of various size and age as a measure of precopulatory mate choice; and ultimately the reproductive success of males of various age, size, and frequency or type of courtship behavior. Especially critical information would be to determine if females copulate with more than one male in a given period of receptivity. This is essential for determining the relative importance of sperm selection in belugas. A greater quantity of data, perhaps through remotely operated cameras, would be more likely to capture the act of copulation and allow for these analyses.

In addition to the knowledge that can be gained by studying behavior in aquarium belugas, the study of beluga reproductive physiology in aquaria has complemented or augmented the understanding gained from the study of wild belugas (Chapters 1 and 3, Steinman et al. 2012). Additionally, and perhaps critically, the access to abundant sample material from aquarium belugas allows the development

and validation of research tools that will improve the ability to study, and thus manage, wild belugas (Chapters 1 and 2).

Because of the ability to collect data throughout the year, longitudinally sample individuals, and observe behavior that is rare or difficult to see from the surface, studies in aquaria serve as important complements to studies of wild belugas. For example, the work by Kelley et al. (2014) provides an excellent complement to this work, as those authors had access to considerable amounts of material from wild belugas, but were restricted to morphological data collected post-mortem, primarily from the summer and fall. Although the current study had a vastly smaller sample size and includes few samples from wild belugas, it directly addresses the sampling gaps in the work of Kelley et al. (2014), leading to a more complete picture of beluga biology when considered in tandem than if either study were to be evaluated separately.

Management Implications of beluga mating strategies

Species with more promiscuous mating systems are thought to have lower extinction risks at small population sizes than other mating systems (Lee et al. 2011). The degree of polygyny can also influence extinction risk; if a small proportion of males are responsible for large proportion of offspring, any perturbation to a male's ability to sire offspring can have a large effect on female productivity. While few males are required to support a polygynous mating system, a reduction in quality males (adults, larger animals, animals with fully developed courtship behavior) could reduce fecundity in females that employ precopulatory mate choice (Lee et al. 2011; Quader 2005). In the absence of quality mates, females may delay breeding until a

quality mate is encountered, and as a result could miss the opportunity to breed in a given season. The apparent low level of postcopulatory selection, suggesting a lesser degree of promiscuity, as well as the potential for female mate choice in this species, make belugas theoretically more susceptible to extinction at small population sizes. The potential for sea ice loss in the Arctic to expand beluga distribution (Heide-Jørgensen et al. 2010), and thus decrease population density, could feasibly reduce encounter rates between the segregated sexes and enhance these extinction risks through reduced fecundity.

The sex ratio of a population can also affect how mating system is related to extinction risk. Female skewed sex ratios create greater degrees of stochasticity in polygynous populations, leading to higher extinction risk (Lee et al. 2011). This is an important consideration for small populations of belugas, as subsistence harvests generally target larger belugas. This can result in harvests composed primarily of adult males, due to their larger size (Suydam 2009; Harwood et al. 2002). The current evidence suggests that male belugas have relatively low sperm production capability, limiting the number of females a given male could inseminate over a short period of time. A relatively low capacity for increasing mating rate, in concert with prolonged periods of courtship that enable female precopulatory mate choice, means that if males in a beluga population were relatively rare, female fecundity could be greatly reduced. In saiga antelope, where males are preferentially hunted for their antlers, male limitation has been implicated in the collapse of wild populations (Milner-Gulland et al. 2003). A variety of effects of selective hunting have been documented in other mammals (reviewed by Milner et al. 2007).

While most subsistence hunts are sustainable (*e.g.* Harwood et al. 2002) and no ill effects would be expected from this selection, an unsustainable harvest occurred in Cook Inlet, Alaska in the last two decades of the 20th century. The population declined from approximately 1,300 belugas to fewer than 400 in a twenty year span, with as much as 20% of the population removed annually through hunting (NMFS 2015). This population is currently listed as Endangered because it has not increased in size, despite the absence of subsistence harvests in the last 10 years (NMFS 2015). The current factors that limit the recovery of this population are unknown. A number of possible threats have been identified for this population that could be impeding recovery, including nutritional limitation, noise pollution, and predation, all of which have the ability to limit fecundity and thus population growth (NMFS 2015). Most likely due to a lack of information on population demographics, or perhaps operating under the assumption that relatively few males are required for maximum productivity in belugas, mating strategies, OSR, and ASR were not identified as potential limitations to recovery. Additionally, directed efforts to evaluate these factors have not been specifically proposed in the recovery plan for this population (NMFS 2015).

The sex is unknown for most of the belugas that were harvested or struck and lost in Cook Inlet during the period of heavy exploitation (NMFS 2015). If the unsustainable harvest preferentially targeted adult males, as it does in other areas, it is possible that a very large proportion of the adult males in the population were removed during the period of heavy hunting pressure. If males are limited in their potential fecundity due to the combined effects of physiology and mating strategies, as suggested by the results of this study, then perhaps this population is male limited.

Given the long time to maturity in this species (Robeck et al. 2005), it could take an extended period of time for the sex ratio to balance sufficiently. Further, if learning is associated with courtship behavior, as suggested by this study, then perhaps removal of adult males through hunting also reduced behavioral diversity in this population. Inappropriate courtship behavior could then interfere with female precopulatory mate choice. If the threshold for accepting mates is not plastic, and fewer males meet threshold requirements for selection by females, either by size, behavior, or ability to induce ovulation, then female fecundity could be greatly reduced.

The methodologies developed in this work have their greatest potential application in the management of the endangered Cook Inlet belugas. The study of temporarily stranded belugas through minimally invasive blow sampling could yield important information on the sex ratio of the population, pregnancy rate, the structure of social groupings, and the relatedness (and perhaps paternity) of members of a social group. These temporarily stranded groups can consist of large proportions of the entire population at a given time (NMFS 2015) and therefore offer a tremendous opportunity to better understand the dynamics of this population. Blow sampling of free-swimming belugas could be employed in this population more feasibly than in others, given that a long-running photo ID project already exists, and a more extensive biopsy program is proposed (NMFS 2015). Both of these activities require close approaches to belugas, where blow sampling might be accomplished. While biopsy sampling might not be performed on very young belugas or females with attendant calves, blow sampling could be performed on these individuals with reduced welfare

risk. Thus blow sampling could complement ongoing or proposed studies and contribute to the improved management of this endangered population.

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APPENDIX 3

LABORATORY PROTOCOLS AND SUPPLEMENTARY DATA

CHAPTER 1:

Extraction protocol for blood samples to be assayed for testosterone

Adapted from protocol provided with Cayman Chemical testosterone EIA kit.

Perform all steps involving diethyl ether in the fume hood.

1. Aliquot a known amount of each sample (250 or 500 μ l) into a clean 10 ml glass test tube.
2. Add 5x the sample volume of diethyl ether (anhydrous ACS reagent, Sigma Aldrich #673811) with a glass pipette and mix for 2 minutes on a vortexer.
3. Allow the layers to separate. Cover tubes first with aluminum foil, and seal with Parafilm M wax (Sigma Aldrich #P7543) before removing from the fume hood.
4. Place tubes in a -80° freezer for at least 30 minutes to freeze the organic layer.
5. Move tubes to -20° freezer in lab space. Take tubes out one at a time, and pour the ether layer (not frozen, top layer) into a clean glass test tube. The organic layer will remain in the test tube.
6. Repeat extraction procedure (steps 2 – 5).
7. Evaporate combined ether extracts under compressed air.
8. Resuspend samples in original sample volume of EIA buffer (250 or 500 μ l).
9. Vortex 2 minutes.

Extraction protocol for blood samples to be assayed for progesterone

Adapted from protocol provided with Cayman Chemical progesterone EIA kit.

Perform all steps involving methylene chloride (dichloromethane) in the fume hood.

1. Aliquot a known amount of each sample into a clean glass test tube (250 or 500 μ l).
2. Add 4x the sample volume of methylene chloride (anhydrous, Sigma Aldrich #270997) and mix thoroughly with a glass Pasteur pipette by drawing up and down 20 times. Allow layers to separate.
3. Using Pasteur pipette, draw 90% of the bottom layer (methylene chloride layer) and transfer to a clean glass test tube.
4. Repeat this extraction procedure three times (steps 2 and 3).
5. Evaporate combined methylene chloride layers under compressed air.
6. Resuspend sample in original volume (250 or 500 μ l) of EIA buffer provided in the Cayman Chemical assay kit.
7. Vortex two minutes.

Extraction protocol for blow samples to be assayed for progesterone

1. Aliquot a known amount of each sample into a clean glass test tube (60 μ l).
2. Add 0.5 ml of diethyl ether with a glass pipette and mix for 2 minutes on a vortexer.
3. Allow the layers to separate. Cover the tubes with aluminum foil, and then seal them with Parafilm M wax before removing from the fume hood.
4. Place tubes in a -80° freezer for at least 30 minutes to freeze the organic layer.

5. Move tubes to -20° freezer in lab space. Take tubes out one at a time, and pour the ether layer (not frozen, top layer) into a clean glass test tube. Organic layer will remain frozen in the tube.
6. Repeat extraction procedure (steps 2 – 5).
7. Evaporate combined ether extracts under compressed air.
8. Resuspend samples in 120 ul of EIA buffer to achieve 1:2 dilution for assay.
9. Vortex 2 minutes.

Alternative extraction protocols for blow samples to be assayed for progesterone that were deemed inappropriate for use with this sample matrix

Methylene chloride liquid-liquid micro-extraction

Identical to extraction protocol for blood samples to be assayed for progesterone (see above), except with smaller sample volumes.

Solid phase extraction (SPE) protocols tested for progesterone in blow

Used solid phase extraction columns (Waters Corporation, Oasis HLB 6 cc vac cartridge, 200 mg sorbent per cartridge, 30 µm particle size, WAT106202). Fluid was pushed through the column using air forced through a 12 ml leur lock syringe fitted to the top of the extraction column using a syringe adapter (Waters Corporation, Sep-Pak reservoir adaptor, WAT054260). The pressure was controlled by hand so that the fluid would drip off of the column at the rate of approximately 1 drop per second.

Adapted protocols from Waters technical support.

SPE Protocol #1:

1. Condition column with 3.0 ml methanol (HPLC grade, Sigma Aldrich #34860).

2. Condition column with 3.0 ml nanopure water.
3. Load sample and nanopure water to a final volume of 1 ml. Add water to the column first.
4. Pass sample through column. Discard flow through.
5. Wash column with 5 ml nanopure water. Push two additional syringes full of air through the column.
6. Elute bound analytes with 2 ml of methanol. Repeat elution step and combine methanol fractions.
7. Dry methanol under compressed air.
8. Resuspend with EIA buffer provided with the Cayman Chemical assay kit to the original sample volume.

SPE Protocol #2:

1. Condition column with 3.0 ml methanol.
2. Condition column with 3.0 ml nanopure water. Repeat this conditioning step.
3. Load sample and nanopure water to a final volume of 1 ml. Add water to the column first.
4. Pass sample through column. Discard flow through.
5. Wash column with 5 ml 10% methanol in nanopure water. Push two syringes full of air through the column.
6. Elute bound analytes with 2.5 ml of methanol. Repeat elution step and combine methanol fractions.
7. Dry methanol under compressed air.

8. Resuspend with EIA buffer provided with the Cayman Chemical assay kit to the original sample volume.

SPE Protocol #3:

1. Condition column with 3.0 ml methanol.
2. Condition column with 3.0 ml nanopure water. Repeat this conditioning step.
3. Load sample and nanopure water to a final volume of 1 ml. Add water to the column first.
4. Pass sample through column. Discard flow through.
5. Wash column with 5 ml 5% methanol in nanopure water. Push two syringes full of air through the column.
6. Elute bound analytes with 3 ml of methanol. Repeat elution step and combine methanol fractions.
7. Dry methanol under compressed air.
8. Resuspend with EIA buffer provided with the Cayman Chemical assay kit to the original sample volume.

Table 1. Progesterone assay validation results for blow samples extracted using various methods.

Extraction Method	Parallelism Results	Accuracy Results	Extraction Efficiency Results	Recovery from Spiked Nylon	Recovery from Blank Nylon Mean $\pm$ SD	Biological Validation
Methylene Chloride	Pass $F_{1,8} = 0.01$, $p = 0.93$	Fail $y = 1.31x - 42.6$ $R^2 = 0.96$	-	-	-	-
SPE #1	Marginal $F_{1,8} = 4.15$ $p = 0.08$	-	-	-	-	-
SPE #2	Fail $F_{1,8} = 3.37$ $p = 0.01$	-	-	-	-	-
SPE #3	Pass $F_{1,13} = 0.09$ $p = 0.77$	Pass $y = 0.989x - 13.48$ $R^2 = 0.99$	$y = 1.12x - 36.16$ $R^2 = 0.99$	$y = 0.61x - 44$ $R^2 = 0.98$	Relative y High 395.1 ± 83.1	Pass: Pregnant 1.33X Non-pregnant
Diethyl Ether	Pass $F_{1,7} = 0.05$ $p = 0.83$	Pass $y = 1.06x - 5.6$ $R^2 = 0.97$	$y = 0.97x + 157.8$ $R^2 = 0.997$	$y = 0.65x + 71$ $R^2 = 0.99$	Relative y Low 118.8 ± 29.8	Pass: Pregnant 2X Non-pregnant

*Diethyl ether liquid-liquid extraction was selected for use in assaying blow samples for progesterone due to lower levels of matrix interference in control samples.

CHAPTER 2

DNA extraction protocol modified from Qiagen DNEasy Blood and Tissue Kit

(www.qiagen.com/handbooks)

1. Centrifuge 50 ml conical tube containing blow sample and 1 ml TE buffer for 7 min at 3000 RPM. Transfer all of the fluid into a microcentrifuge tube, pipetting up and down several times to ensure any pelleted material is retrieved. Contents may then be stored at -20°C until DNA extraction.

2. Centrifuge microcentrifuge tube at 10,000 RPM for 10 minutes in an effort to pellet cellular material.
3. If visible pellet is present, remove as much of the supernatant as possible using a pipette, being careful not to disturb the pellet. If a pellet is not visible, carefully remove TE buffer using a pipette from the top layer of fluid, leaving approximately 50 μ l of buffer TE in the tube.
4. Proceed with “Tissue” protocol. Adjust volume of buffer ATL added appropriately if TE was left in the tube in step 3.
5. Allow a minimum incubation for lysis of 1 hour at 56°C. Some samples with excess mucous may require longer lysis times.
6. After second wash step (Qiagen protocol step 6), centrifuge the tube for an additional 1.5 minutes at 13,000 RPM to ensure no ethanol carry over occurs.
7. After adding buffer AE (Qiagen protocol step 8), allow 5 minute incubation.
8. Elution is performed twice, into two separate tubes, allowing a 5 minute incubation with buffer AE each time.

General protocol for polymerase chain reaction

Prepare PCR reaction master mix:

10X STD Taq buffer (Mg free): 5 μ l per sample

10 mM dNTPs: 1 μ l per sample (0.2 μ M)

MgCl₂: 3 μ l per sample (1.5 mM)

Forward primer (2.5 μ M): 4 μ l per sample

Reverse primer (2.5 μ M): 4 μ l per sample

Taq: 0.5 μ l per sample

To each PCR reaction tube, add 17.5 µl of master mix, template DNA, and nanopure water to a final volume of 50 µl. One PCR reaction tube will be a negative control and lack template DNA.

Template DNA inputs may vary depending on the DNA concentration or quality of the sample.

Mix well and centrifuge, ensure no bubbles are present.

General PCR conditions: Cycle number and annealing temperature may vary depending on primers.

1 cycle: 94° C for 3 min

35 cycles: 94° C for 30 sec, 60° C for 30 sec, 72° C for 30 sec

1 cycle: 72° C for 10 min

Hold at 4° C

Store at -20° C until electrophoresis

Agarose gel electrophoresis

Running buffer:

50X TAE:

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA pH 8.0 (FW 372 = 18.6 g)

Distilled water to 1 liter

1X TAE:

20 ml 50X TAE

980 ml distilled water

2% Agarose gel:

1.0 g agarose in 50 ml 1X TAE buffer in a 250 ml flask

Cover flask with plastic wrap and heat for 1 minute. Swirl to mix, and heat for additional 30 seconds. Make sure agarose is dissolved.

Add 10 μ l of 2.5 μ g/ μ l ethidium bromide solution to final gel concentration of 0.5 μ g/ml

Pour into gel casting tray with comb, pop any air bubbles with a pipette tip, and let gel polymerize. Remove comb, and turn gel. Pour tank buffer 1X TAE to fill line.

Sample preparation and running the gel

Add 8.2 μ l of gel loading dye to 50 μ l PCR reaction tube.

Load 40 μ l to wells.

Load 10 μ l of DNA ladder to first well.

Plug in red and black leads to power supply and run at 80-96 V for approximately 45 minutes.

Turn off the power and unplug the electrophoresis box from the power supply.