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Assessing the Quantity and Downstream Performance of DNA Isolated from Beluga (*Delphinapterus leucas*) Blow Samples

Justin T. Richard,[1,2] Krystle Schultz,[1] Caroline Goertz,[3] Rod Hobbs,[4] Tracy A. Romano,[2] and Becky L. Sartini[1]

[1]*University of Rhode Island, 120 Flagg Road, Kingston, RI 02881, USA*
E-mail: jt_richard@uri.edu
[2]*Mystic Aquarium, a division of Sea Research Foundation, 55 Coogan Boulevard, Mystic, CT 06355, USA*
[3]*Alaska Sea Life Center, 301 Railway Avenue, Seward, AK 99664, USA*
[4]*National Marine Mammal Laboratory, 7600 Sand Point Way NE, F/AKC3, Seattle, WA 98115-6349, USA*

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 during polymerase chain reaction (PCR) is unknown. DNA was extracted from 98 blow samples collected from 11 aquarium-housed and 29 wild belugas in Bristol Bay, Alaska. Blow samples consisted of one, two, or four successive exhales, with at least nine 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. Measurable DNA was recovered from 96 samples (98%), although DNA yield varied widely, both by sample (range: 0 to 4,406 ng, mean = 701.5, SD = 1,033.7) and by number of exhalations (ng DNA/exhale) (range: 0 to 3,723, mean = 427.1, SD = 721.8). The amount of DNA extracted per exhale was greater for aquarium samples than for wild samples, but total yield was not proportional with the number of exhales for either group. Successful beluga-specific PCR amplification occurred in 56/59 of the aquarium samples (23/25 single-exhale samples) and 28/39 of the wild 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. This technique is immediately applicable to live-stranded belugas such as the temporary mass strandings that occasionally occur in Cook Inlet, Alaska, and has the potential to increase genetic sampling in protected populations with less disturbance than direct tissue sampling.

Key Words: blow, exhale, respiratory vapor, genetic sampling, beluga, *Delphinapterus leucas*

Introduction

Genetic sampling can play an important role in the management of cetaceans. In beluga whales (*Delphinapterus leucas*), the availability of tissue from subsistence harvests allows for relatively large sample sizes for genetic research that can provide important information for population management (e.g., O'Corry-Crowe et al., 1997; Turgeon et al., 2012). While postmortem sampling is extremely valuable, new research opportunities are created when live animals can be sampled. Tissue collection from live belugas could reduce the sampling disparity associated with variation in the size or composition of the harvest for different stocks. Remote biopsy sampling is an effective method for studying live belugas (Meschersky et al., 2008), but wildlife managers have at times avoided the use of biopsy sampling in some populations, in part due to concern for the behavioral responses or the physical welfare of the sampled animal (McGuire & Stephens, 2014). This creates a need for a less invasive method of acquiring DNA samples for analyses that is more compatible with other less invasive methods of data collection 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. Fecal sampling (Parsons et al., 1999; Gillett et al., 2008) and skin

swabs (Harlin et al., 1999) are two such methods that would be impractical for belugas as this species has diffuse feces and is not known to ride the bow of travelling boats. Recently, blow (exhale) sampling has been identified as an alternative source of DNA for analysis in cetaceans (Frère et al., 2010). Due to the forcefulness with which cetaceans exhale, cellular debris containing epithelial cells and leukocytes is commonly ejected along with the respiratory vapor (Sweeney & 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 to collect the sample, blow sampling does not require making contact with the animal and thus provides a less invasive alternative for acquiring molecular samples.

To date, cetacean DNA sampling in blow samples has been reported for bottlenose dolphins (*Tursiops truncatus*) (Frère et al., 2010) and harbor porpoises (*Phocoena phocoena*) (Borowska et al., 2014) in aquariums, and one wild bottlenose dolphin (Frère et al., 2010). However, both of the studies utilized blow samples that consisted of more than one exhale (harbor porpoises: five to six breaths; bottlenose dolphins: four breaths). While bow-riding species may allow the collection of more than one exhale from the same individual, for many species, including the beluga, a single exhale is the most realistic sampling outcome. Therefore, further investigation is required to determine if a single exhale would yield enough DNA to perform common analyses utilized for 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 analyses; for example, investigators may aim 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 1,120 ng from a total of 11 samples consisting of five to six exhales each (187 to 224 ng/exhale, assuming a 200-µl elution volume), while Frère et al. (2010) reported yields of approximately 2,000 ng from a total of six samples consisting of four exhales each (500 ng/exhale, assuming a 200-µl elution volume). However, it is not clear if the collection of a single exhale would result in these predicted per-exhale yields. If the actual relationship between number of exhales and yield can be reasonably predicted, the investigator can make informed decisions about the minimum number of exhales that would be necessary to obtain enough DNA to perform the desired analyses.

Additionally, for blow sampling to be a realistic alternative to biopsy sampling, the DNA that is recovered from blow samples must not be excessively fragmented and allow for the amplification of larger target sequences. Sex is very difficult to determine in a free-swimming beluga (Petersen et al., 2012), so the ability to amplify the relatively long fragment of the *ZF* gene used in sex determination (approx. 1,000 bp) would be beneficial (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, 2008). Therefore, validating the ability to amplify longer nuclear sequences from host DNA isolated from blow samples would be valuable.

Using blow samples collected from aquarium and wild 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 during polymerase chain reaction (PCR) amplifications. PCR performance with both mtDNA and large nuclear target sequences (> 900 bp) is assessed.

Methods

Study Animals

Blow sampling in aquarium animals (Mystic Aquarium [MA], Mystic, Connecticut, USA, and Shedd Aquarium [SA], Chicago, Illinois, USA) was performed with belugas of known sex. The belugas were trained to position their head so that their blowhole was above the water's surface and then to exhale on cue. Blow samples were collected from wild belugas in Bristol Bay (BB), 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 (NMFS) 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 sterile polypropylene 50-ml conical tube (#14-432-22; Fisher Scientific, Waltham, Massachusetts, USA) held inverted directly over the blowhole. The tube was tilted cranially by approximately 30 to 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. Tube caps were held away from the water in a gloved hand during collection; gloves were only used for one sampling event. The tubes were capped and placed on ice. For aquarium samples, 1 ml of 1X Tris-EDTA (TE) buffer (1% 1 M Tris-HCl, 0.2% 0.5 M EDTA) was added to the tube within 15 min of collection. For wild samples, 1 to 1.5 ml of 1X TE buffer was added within 30 to 90 min of collection. The tubes were rocked by hand to coat the inner surface of the tube with buffer and then frozen in the 50-ml conical tubes at -20° C. For aquarium samples, the tubes with 1X TE buffer were placed in the freezer within 15 min of sample collection. For wild samples, the tubes were held in coolers on ice packs for 4 to 6 h before being placed in a -20° C freezer. Samples were shipped to the laboratory on dry ice or in a liquid nitrogen dry shipper.

A total of 11 aquarium-housed and 29 wild belugas were sampled (Table 1). Sample type varied by year for belugas sampled in BB. In 2012, all samples were composed of four successive exhales; in 2013, all samples were composed of two successive exhales; and in 2014, a single-exhale sample and a sample consisting of two successive exhales were collected from each beluga. Sampling was typically completed in less than a minute, and the tube was held in place over the blowhole in between breaths to ensure sample collection as the interval between breaths was unpredictable.

DNA Extraction

DNA extraction was performed using methods similar to those in Frère et al. (2010) with some modifications. After thawing, the 50-ml conical tubes were again rolled by hand to coat the inner surface of the tubes with thawed buffer and were then centrifuged for 10 min at 2,060 × *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 min at 13,400 × *g*.

The presence or absence of an observed 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 BB 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 BB samples.

DNA was isolated using the Qiagen DNEasy® Blood and Tissue Kit (Valencia, California, USA), utilizing 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 1 hr or, in rare cases, until the pellet (if visible) was completely lysed, up to 3 h. Samples were vortexed every 15 to 20 min during lysis. DNA was eluted in 50 µl of the provided buffer AE. This elution step was repeated into a separate tube. Two separate 50-µl elutions were preferred to a single 100-µl elution because the DNA was more likely to be concentrated enough in the first 50-µl elution to then be used in PCR without evaporating the solvent to concentrate the DNA. Two elutions were performed to ensure that the majority of the DNA was recovered from the column.

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

Sample source (individuals)	One exhale	Two exhales	Four exhales
Bristol Bay (BB, *n* = 29)	10 (2014)	20 (2013 & 2014)	9 (2012)
Mystic Aquarium (MA, *n* = 4)	25	10	10
Shedd Aquarium (SA, *n* = 7)	14	0	0

Yield and Purity

DNA concentration (ng/µl) and purity (A_{260}/A_{280}, the ratio of absorbance of 2 µl of sample at 260 and 280 nm) was assessed using a NanoDrop™ 8000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The total yield from each 50-µl elution was measured separately, and the yield from these two separate elutions were added together to calculate the total yield (ng) for each sample. 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 MA ($n = 4$) from the ventral fluke vein. 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 AE buffer. DNA concentration and purity was assessed via NanoDrop™. For each blood sample, three or four separate extractions were performed for a total of 14 extractions.

Molecular Sex Determination via Polymerase Chain Reaction

PCR performance was tested for MA samples through a molecular sex determination test. The zinc finger (*ZF*) gene, which has a sex-linked polymorphism, was amplified using primers LGL331 (5'-CAA-ATC-ATG-CAA-GGA-TAG-AC-3') and LGL335 (5'-AGA-CCT-GAT-TCC-AGA-CAG-TAC-CA-3') (Shaw et al., 2003). In belugas, the Y chromosome copy is 1,006 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. PCRs were carried out in 50 µl (1X reaction buffer, 1.5 mM $MgCl_2$, 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.

An Eppendorf Mastercycler® EP (#5341) thermocycler was used for all PCRs. The conditions for the reaction were 94° C for 3 min, then 35 cycles of 94° C for 30 s, 55° C for 30 s, 72° C for 30 s, followed by an extension step of 72° C for 10 min. The PCR product (40 µl) was loaded into a 2% agarose gel stained with ethidium bromide for electrophoresis. Bands were visualized under UV light, and scoring was completed by visual examination. 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 BB and SA 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') that are commonly used in the identification of mtDNA haplotypes in belugas (e.g., O'Corry-Crowe et al., 1997). For BB 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 for PCRs. BB template DNA amounts ranged from 2 to 72 ng. PCRs were carried out in 50 µl (1X reaction buffer, 1.5 mM $MgCl_2$, 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 min at 72° C.

The PCR products were run through gel electrophoresis on 2% agarose gels stained with ethidium bromide. PCR performance was assessed by the presence or absence of a band following electrophoresis. The bands were excised, and DNA was extracted using a Gel Extraction kit (Qiagen). The DNA was submitted for Sanger sequencing using an Applied Biosystems 3500XL Genetic Analyzer (Foster City, California, USA) at the University of Rhode Island's Genomics and Sequencing Center. Haplotypes were identified using *BLAST* (http://blast.ncbi.nlm.nih.gov) and were named based on sequences published by Meschersky et al. (2008). To initially validate the technique, DNA isolated from blow and blood from three known belugas was amplified 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.

Effect of Exhale Strength

To determine if the strength of the breath influences DNA yield or PCR performance, four separate "calm breaths" were collected from two MA belugas (two 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 in aquarium animals and is similar to the force of the BB beluga exhales collected under restraint conditions (personal observations). DNA was isolated using the protocol described above, and a mtDNA PCR was attempted using 30 ng of template DNA.

Data Presentation
Yields were expressed as the mean ± 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 were shown using box plots created in *R* (R Core Team, 2015) in which the box represents the interquartile range, the dark line represents the median, whiskers represent the minimum and maximum values, and outliers (> 1.5× the interquartile range away from the minimum or maximum values) were plotted as open circles unless specified otherwise by an accompanying legend.

Results

Measurable DNA was extracted from 96/98 (98%) beluga blow samples. Results are summarized in Table 2. DNA yield varied widely, both by sample (range = 0 to 4,406 ng, mean = 701.5, SD = 1,033.7) and by exhale (ng/exhale) (range = 0 to 3,723 ng, mean = 427.1, SD = 721.8). The amount of DNA extracted per exhale (yield divided by the number of exhales) was greater for aquarium samples (586 ± 866 ng) than for BB samples (186 ± 295 ng). Total yield was not proportional with the number of exhales for either group (Table 3). Among aquarium samples, total yield was influenced by individual (Figure 1) as well as by the presence or absence of a cell pellet following centrifugation prior to the extraction protocol (Figure 2). 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 to 34.1) (Table 2). Samples with a DNA yield > 100 ng (n = 62) had a mean A_{260}/A_{280} of 1.78. DNA yield from 100 µl of whole blood was 2,667 ± 843 ng (range: 1,468 to 4,558), with a A_{260}/A_{280} of 1.59 ± 0.17.

The *ZF* PCR was successful for 42/45 of the MA samples (23/25 of the single-exhale samples, 9/10 of the two exhale samples, and 10/10 of the four exhale samples). The other three samples failed to amplify *ZF* via PCR. Of those samples that amplified, all yielded appropriate

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

Sample source	# of exhales	# of samples	Total yield, ng (mean ± SD)	Total yield, ng (median)	Yield per exhale, ng (mean ± SD)	Yield per exhale, ng (median)	A_{260}/A_{280} (mean ± SD)	% samples with visible cell pellet	% samples with PCR success
Mystic Aquarium	1	25	849 ± 1,131	411	849 ± 1,131	411	1.71 ± 0.64	68	92
	2	10	1,383 ± 1,635	496	692 ± 817	248	1.84 ± 0.28	90	90
	4	10	956 ± 892	674	238 ± 223	169	2.07 ± 0.41	100	100
Shedd Aquarium	1	14	301 ± 404	125	301 ± 404	125	1.44 ± 3.21	21	100
All aquarium single-exhale samples	1	39	648 ± 967	180	648 ± 967	180	1.61 ± 1.95	57	95
Bristol Bay	1	10	47 ± 59	24	47 ± 59	24	4.52 ± 10.45	--	70
	2	20	379 ± 657	98	189 ± 329	49	2.19 ± 3.88	--	65
	4	9	1,336 ± 1,288	749	334 ± 322	187	1.39 ± 0.14	--	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)

	Fold increase in mean DNA yield	
Number of exhales	Aquarium	Bristol Bay
2	2.1	8.0
4	1.5	28.3

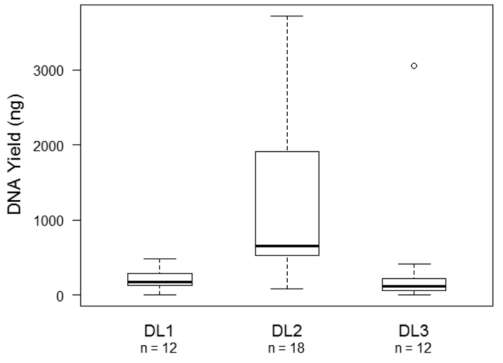

Figure 1. Variation in DNA yield per exhale for three aquarium belugas sampled at least 12 times

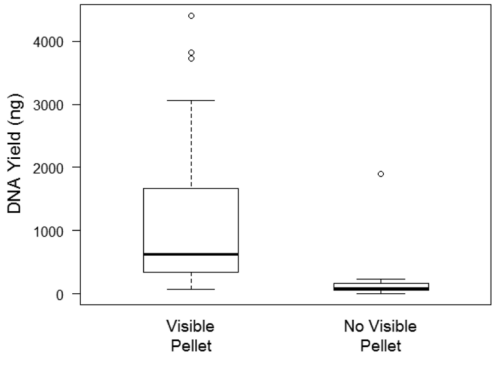

Figure 2. Total DNA yield for aquarium samples with and without visible cell pellets following centrifugation prior to the DNA extraction protocol being performed

sex determination results. All 14 SA single-exhale samples allowed amplification of mtDNA. Of the 39 BB samples, 28 allowed amplification of mtDNA (Table 2). In general, samples that failed to amplify in a PCR 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 (2,966 ng) did not. 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 Figure 3.

Mitochondrial DNA sequences obtained from DNA isolated from blood and blow were identical for the three MA beluga tested; each beluga had a unique haplotype (S022, Accession #DQ503433.2; S001, Accession #DQ503430.2; and S421, Accession #JQ716354.1). Haplotypes were determined for six of the seven SA samples that amplified (S022 or S421). Sequencing was attempted for 24 BB samples, and haplotypes could be assigned for 19 of these samples (8/8 from four exhales, 10/13 from two exhales, and 1/3 from one exhale). 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 ng ± 83. All four samples allowed amplification of mtDNA.

Discussion

This study has demonstrated that DNA can be reliably extracted from beluga blow samples consisting of one to four exhales. While the number of exhales collected had a large effect on DNA yield, single-exhale blow samples can yield sufficient DNA to perform common molecular analyses and allow the amplification of nuclear DNA fragments of at least 1,000 bp. The results of mtDNA haplotype sequencing from DNA extracted from single-exhale blow samples were identical to the results obtained from blood collected from the same individual, 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. Given the range of possible DNA yield outcomes, blow sampling is associated with a greater chance of acquiring insufficient material in a given sampling event than biopsy sampling; 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 possible, especially when the use of biopsy sampling is prohibited or restricted.

DNA Yield from Blow Samples

The number of exhales collected per sampling event had a large effect on DNA yield, especially in BB samples. However, this effect on 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

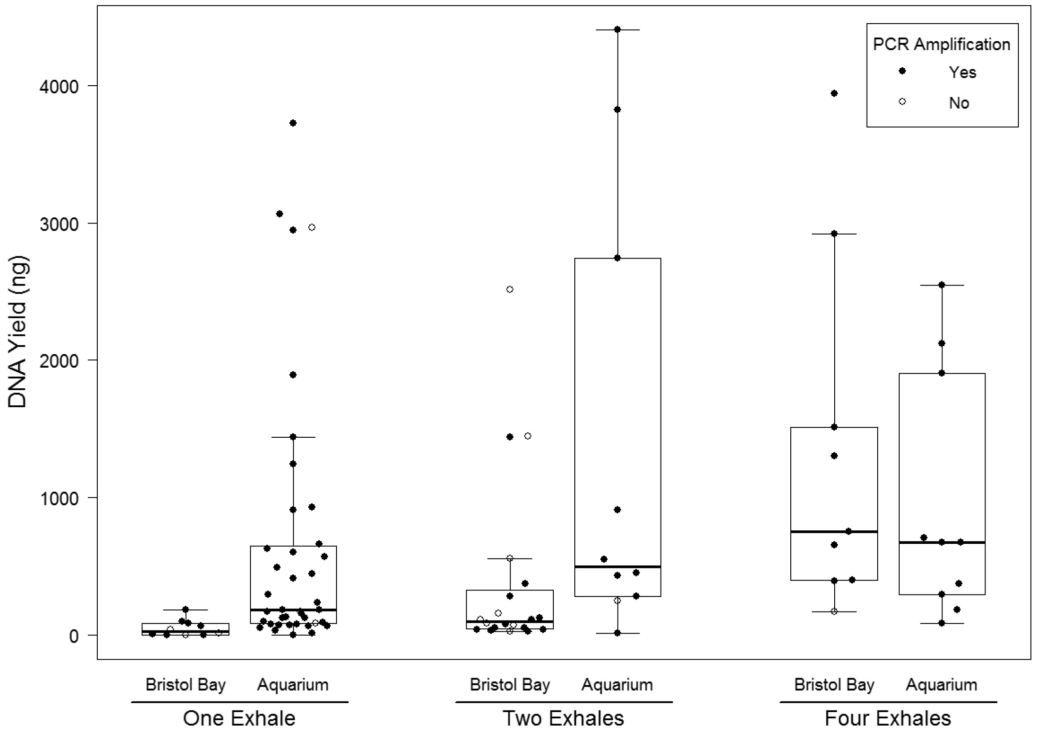

Figure 3. Effect of sample type and source population on DNA yield and PCR performance; all individual observations are plotted over the box plot.

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 a sampling event. This could explain the increase in DNA yield per exhale seen in BB 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 SA samples may be related to the force of the breath as these samples were less likely than MA samples to have pellets following centrifugation.

In aquarium samples, the declining median DNA yield per exhale with increasing number of exhales per sample could 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 that leads to relatively high yields. This is followed by a declining amount of debris and environmental water in successive exhales that leads to lower yields per exhale. 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 BB beluga samples relative to aquarium belugas. Aquarium belugas were trained to exhale forcefully to simulate the forceful exhale of a swimming beluga. In contrast, the breaths of the BB 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 BB belugas may be due to temporary restraint conditions during health assessments. 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 two- to five-fold decrease in air flow rates when compared to dolphins that were fully supported in

the water. While the belugas were not completely beached during restraint in BB, the thorax was usually touching the ground, which may have led to reduced pulmonary flow rates. Alternatively, the BB belugas were exhibiting calm breathing patterns as an energy saving mechanism during restraint. 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. Other differences between aquarium and BB samples (e.g., temporary storage on ice packs prior to freezing, duration of frozen storage prior to analysis, relative contribution of DNA from environmental water, and potential presence of beluga DNA in environmental water) were also explored and do not appear to be important factors (data not shown).

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 beluga blow samples, mean fluid volumes per exhale were 57 µl for females and 78 µl for males (Richard et al., 2017). This is considerably higher than blow sample volumes observed in harbor porpoises, which ranged from 15 to 50 µl from five to six breaths, or approximately 3 to 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; therefore, a wide variation in blow sample volume could account for some of the variation in per exhale yield observed in this study.

The expected yield from a blow sample will be an important consideration when investigators are designing studies and considering tissue sources of DNA. The mean DNA yield per exhale determined from single-exhale aquarium samples in this study compares favorably to those measured in other odontocetes, being higher than either of the smaller species previously studied (Frère et al., 2010; Borowska et al., 2014). 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 this 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.

With an improved understanding of the DNA yield and quality from blow samples, future studies can explore the use of this template source in multiplex reactions for nuclear microsatellite profiling, which would provide more robust information on population structure than mtDNA sequencing alone. Previous studies have shown that this is feasible using DNA isolated from blow (Frère et al., 2010; Borowska et al., 2014).

Performance of Blow DNA in PCR

Ideally, PCR performance in aquarium and wild 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 BB 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 BB samples. The reverse is likely for BB samples; some BB samples that allowed amplification of mtDNA may not have allowed for amplification of *ZF*.

In general, DNA extracted from blow samples performed well during PCRs, although low yield from some samples may limit the number of genetic markers that can be examined in a given sample. The low yields at times created the inability to achieve target DNA template inputs into the PCR, which may explain the lower rate of PCR success in BB samples compared to aquarium samples. 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, which likely varies from sample to sample. Amplification can be reduced for samples with the same amount of target DNA when this ratio is low (Gillett et al., 2008). PCR success can be improved by optimizing PCR conditions for low template inputs, and DNA yield may be improved by using different extraction protocols such as phenol-chloroform extraction. Given their higher cost and time investment, these methods may only prove beneficial when blow sampling creates research opportunities that are not otherwise possible.

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). Potentially 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 BB 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.

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 using this method. Collecting a larger portion of the exhaled plume will likely lead to increased DNA yields and, therefore, will improve the utility of this technique. With sufficient volume, steroid hormone quantification could also be performed using the same sample (Richard et al., 2017). The forceful nature of the exhale of a swimming beluga will also increase the likelihood that sufficient DNA can be collected from a single breath. Blow sampling methods in free-swimming cetaceans include the use of a long pole mounted to a boat and remote-controlled helicopters (drones) that can be flown into the exhaled plume (Acevedo-Whitehouse et al., 2010; Hunt et al., 2014). Either of these sampling platforms would support a broad sampling surface that could capture a larger percentage of the sample such as the plastic sheets employed by Acevedo-Whitehouse et al. (2010).

Although further development is required for the application of these techniques to free-swimming belugas, blow sampling is immediately applicable to live-stranded belugas for which sampling would be analogous to the sampling that has been accomplished in BB for this and other studies (Thompson et al., 2014). Belugas in the endangered Cook Inlet population occasionally temporarily mass strand between tidal cycles. These strandings may involve more than 50 whales at one time, representing a significant portion of the entire stock that numbered an estimated 340 belugas in 2014 (NMFS, 2015). This sampling scenario reduces the amount of environmental water in the sample and would enable the collection of multiple exhales, which will improve DNA yield and PCR performance. If the beluga is breathing calmly while stranded, a minimum of two exhales should be collected, while collecting four or more would ensure that sufficient DNA is extracted to perform multiple analyses. When compared to biopsy or blood sampling under these conditions, blow sampling presents an efficient sampling

method that requires less training, practice, and skill among personnel; reduces the number of personnel required to collect samples; and eliminates the need for bringing sharp instruments into hazardous field conditions. Blow sampling will also enable the sampling of many more individuals in a shorter period of time and will minimize disturbance to the whales during stranding events without compromising the ability to collect important data about individuals. Ultimately, blow sampling for genetic analyses may not replace biopsy sampling, but it will create sampling opportunities when more invasive sampling methods are unavailable or undesirable.

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