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Preliminary Analysis of the Proteome of Exhaled Breath Condensate in Bottlenose Dolphins (*Tursiops truncatus*)

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Abstract

The primary objective of this study was to conduct an initial analysis of the proteome of exhaled breath condensate or blow in aquarium-based bottlenose dolphins (*Tursiops truncatus*) and, secondarily, to determine the commonality of proteins identified in blow with those in plasma of the same animals as recently documented. Exhaled breath condensate was collected from four young (2 to 6 y old), male dolphins using a 50-mL Falcon tube held above the blowhole for ten sequential exhalations; total volume ranged from 60 to 122 µL. Subsequent to analysis of total protein, 20 µg of protein from each dolphin was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Of the four samples, three produced sufficient resolution of 15 bands that were excised from respective gels and subject to liquid chromatography-tandem mass spectrometry (LC-MS/MS) and used for protein identification. Mass spectra data were used to search the National Center for Biotechnology Information (NCBI) database restricted to all mammalian proteins. Based on proteins having ≥ 2 peptides and present in at least two of the three dolphins, a total of 220 blow proteins were identified. While a majority (38 to 51%) of proteins could not be categorized, gene ontology indicated protein binding (26%), cytoplasm constituents (16%), and immune response (16%) dominated the molecular function, cellular component location, and biological process domains, respectively. From noncontemporaneous samples, NCBI Accession numbers of 220 blow proteins described herein and 196 plasma proteins previously identified by LC-MS/MS in the same dolphins were matched. Results indicated a commonality of 21 proteins (5%), with ten (48%) related to the immune system (e.g., complement- and immunoglobulin-related proteins) and the remainder to other various biological systems. Although preliminary, the novelty of these results provides additional support that exhaled breath condensate can be a relevant, less invasive alternative to blood collection to assess or monitor physiological health and pathological states. More specifically, the commonality of several immune-related proteins between the circulatory and respiratory systems provides a foundation for future investigations to determine the potential of these blow proteins as biomarkers that may be diagnostic or prognostic of respiratory health in bottlenose dolphins and, perhaps, other cetaceans.

Key Words: blow, proteomics, exhaled breath condensate, cetacean, bottlenose dolphins, *Tursiops truncatus*

Introduction

Proteomic analysis provides fundamental information to evaluate the functional roles proteins have within and among various biological pathways to maintain homeostasis. Application of proteomics is most prominent in the identification of biomarkers, which are generally considered measurable characteristics that can be diagnostic or prognostic of physiological health and

pathological states related to homeostatic imbalance (Moore et al., 2007). While proteomics is well advanced in human medicine (Lippolis & De Angelis, 2016), its application for basic and applied purposes in veterinary medicine is still incipient but increasing (Ceciliani et al., 2014).

In cetaceans, blood-derived fluid proteomes have been characterized in a couple of studies involving bottlenose dolphins (*Tursiops truncatus*; Sobolesky et al., 2016; Miller et al., 2017). With the availability of the genome in bottlenose dolphins (Lindblad-Toh et al., 2011) and use of nontargeted proteomic analysis, a total of 58 serum proteins were identified in male and female dolphins and subsequently ranked in comparison with the human proteome (Sobolesky et al., 2016). In a more recent study (Miller et al., 2017), a total of 196 plasma proteins were identified in young, male dolphins and evaluated to determine the differential change in abundance encompassing performance-based physical activities; lysozyme, an immune-related protein, and flavin reductase, a metabolic-related protein, were higher before than after human–dolphin swim interactions.

Although blood is generally a medium that contains information on the physiological state of essentially all systems in the body, the sample collection process is considered relatively invasive, especially for wild or free-ranging animals that need restraint. As an alternative to the collection of blood samples, collection of exhaled breath condensate is considered a non-invasive process to assess physiological health and pathological states and is most advanced in human medicine for diagnostic and prognostic purposes (Harshman et al., 2014; Viglio et al., 2014; Hayes et al., 2016). In this regard, interest in collection of exhaled breath condensate for clinical application is being advanced in veterinary medicine (Zollinger et al., 2006; Reinhold & Knobloch, 2010) and, more specifically, in cetaceans (Hunt et al., 2013; Aksenov et al., 2014; Cumeras et al., 2014). In accord with management practices, aquarium-based cetaceans are conditioned to produce a forceful expiration or *chuff* from the blowhole for the collection of exhaled breath or blow to routinely assess general health. There are several distinct advantages for evaluating the physiological relevance of exhaled breath condensate in cetaceans: (1) collection is non-invasive compared to blood, urine, feces, or blubber/skin biopsy collections; (2) collections can be done in real time, frequently, and in a serial manner within an animal; (3) sample volumes can be unlimited; and (4) samples can be readily collected without the stress of capture-release in free-ranging animals.

In regard to omics-based analysis of exhaled breath in cetaceans, metabolomics has initially been used to characterize exhaled breath condensate from a free-ranging whale (Cumeras et al., 2014) and aquarium-based and free-ranging bottlenose dolphins (Aksenov et al., 2014; Zamuruyev et al., 2016). In addition to detection of various volatile compounds, various nonvolatile compounds were detected, including peptides and proteins. In regard to the latter, identification, quantification, and characterization of the proteins were apparently not documented.

The primary objective of the present study was to use proteomic analysis as a nontargeted approach to initially identify and classify the proteome of exhaled breath condensate or blow in aquarium-based bottlenose dolphins. A secondary objective was to determine the commonality of matched proteins identified in blow with those in plasma of the same animals as recently documented (Miller et al., 2017).

Methods

Dolphins and Dolphin Management

The study involved four young (2 to 6 y old), male bottlenose dolphins born under human care that were not subject to pharmacological treatment or therapy during the study and were considered healthy in accord with daily examinations by a resident veterinarian. Animals were housed at Dolphin Discovery in St Kitts (Federation of St. Kitts and Nevis: 17° 17' 05.4" N, 62° 42' 17.5" W), and study animals were maintained in an outdoor habitat or enclosure with two female bottlenose dolphins. The rock-piled enclosure contained natural sea water open to the Atlantic Ocean with an estimated volume of 8,710 m^3 and depth that ranged from 1.4 m at the submerged platforms to 5.0 m at maximum depth. Each dolphin was prescribed an individual diet of wild-caught fish and squid which was provided periodically throughout the day in association with training and performance-based activities. Animals were housed and managed in compliance with the U.S. Department of Agriculture (USDA) (2013), the standards and guidelines of the Alliance of Marine Mammal Parks and Aquariums (AMMPA) (2010), and the mission statement of the International Marine Animal Trainers' Association (IMATA) (2016). In addition, this study was approved by the Institutional Animal Care and Use Committee (IACUC No. 15-8-023) of Ross University School of Veterinary Medicine. Export and import of dolphin samples were in accord with the Convention on International Trade in Endangered Species (CITES) (Appendix II), the Marine Mammal Protection Act (MMPA), and the National Marine Fisheries Service (NMFS) (Permit No. 17305-00).

Collection of Exhaled Breath Condensate or Blow

The collection of exhaled breath condensate or blow from each of the four dolphins was accomplished by having the trainer cue the dolphin into position while the resident veterinarian swabbed sea water from atop the blowhole as shown in Figure 1. In addition, individuals collecting samples wore latex gloves and face masks to minimize cross-contamination between human, animal, and the environment. Thereafter, the veterinarian cued the dolphin to exhale or chuff while an assistant captured the condensate in an inverted 50-mL Falcon-type conical centrifuge tube (Fisher Scientific, Ottawa, ON, Canada) that was held approximately 10 cm above the blowhole for ten sequential exhalations (Figure 1). During the slight pause between chuffs, the collection tube was inverted and capped. Upon completion of the collections, each of the four tubes were stored on crushed ice and immediately transported to the laboratory (15 to 20 min). After centrifugation at 97 to 110 × g at 4°C for 1 min, total blow volume from each of the four dolphins was estimated with a calibrated pipet, transferred to labelled microcentrifuge tubes, and stored at -80°C until analysis. Total volume of blow was 60 µL for Dolphin A, 65 µL for Dolphin B, 110 µL for Dolphin C, and 122 µL for Dolphin D.

Protein Analysis and Separation

Total protein concentration was determined using 5 µL of blow from each of the four blow samples in the Bradford protein assay (Sigma, St. Louis, MO, USA) with bovine serum albumin (Sigma) as standard (Vandenplas et al., 2002). Total protein concentration for each sample was approximately 1 mg/mL. Blow proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970), with all materials and reagents purchased from BioRad (Hercules, CA, USA). Briefly, 20 µg (20 µL) of protein from each of the four blow samples were mixed with an equal volume of 2× Laemmli buffer with 5% β-mercaptoethanol as reducing agent. Thereafter, the samples were heated to 95°C for 5 min and allowed to cool to room temperature. For protein separation, 20 µL of the sample mixture from each dolphin was loaded on AnykD Criterion TGX Precast Protein Gels with BioRad broad range molecular weight standards from 6.5 to 200 kD in a separate lane. Electrophoresis was carried out at 45 mV until the bromophenol blue front reached the bottom of the gel. Bands of proteins were stained with Coomassie brilliant blue for 30 to 45 min and destained overnight on a plate rocker in 7% methanol. An image of the gel was captured using a Syngene G:BOX chemiluminescence imaging system (Frederick, MD, USA). As shown in Figure 2, bands of blow proteins were distinct for Dolphins A, C, and D but not Dolphin B. Although not known, total blow protein may have been underestimated for Dolphin B. Nonetheless, Dolphin B was not used in subsequent analysis. For Dolphins A, C, and D, 15 bands of proteins were sliced from the gel using a scalpel blade (Figure 2), placed in individual Eppendorf tubes with approximately 50 µL deionized water, labelled, and stored at 4°C until shipped for analysis. Gel slices with blow proteins

Figure 1. The collection of exhaled breath condensate or blow from each of the four dolphins was accomplished by having the trainer cue the dolphin into position while the resident veterinarian swabbed sea water from atop the blowhole (*left panel*). Thereafter, the veterinarian cued the dolphin to exhale or *chuff*, which was captured in an inverted 50-ml Falcon-type conical centrifuge tube that was held by the investigator approximately 10 cm above the blowhole for ten sequential exhalations (*right panel*).

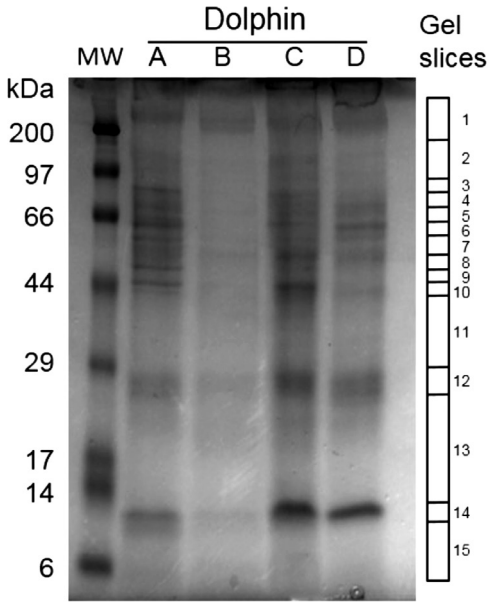


Figure 2. Polyacrylamide electrophoresis gel (SDS-PAGE) of exhaled breath condensate or blow from four young, male bottlenose dolphins. Approximately 20 µg of total protein from each dolphin blow sample was loaded into respective lanes. Excluding Dolphin B, 15 bands of proteins of different molecular weight (MW) were sliced from each lane for Dolphins A, C, and D.

were shipped with cold packs to the USDA/ National Animal Disease Center (Ames, IA, USA) for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Protein Digestion and Isolation

For each of the gel slices, proteins were digested with trypsin, and peptides were extracted using an In-Gel Tryptic Digestion Kit (No. 89871; Thermo Fisher Scientific, West Palm Beach, FL, USA). Thereafter, peptides were injected onto an HPLC column using a Proxeon Easy-nLC (Thermo Fisher Scientific) connected to the mass spectrometer. The chromatography used a trapping column (Proxeon Easy-Column, 2 cm, ID 100 µm, 5 um, 120A, C18) and an analytical column (Proxeon Easy-Column, 10 cm, ID 75 µm, 3um, 120A, C18). The gradient involved a Mobile Phase A (95% H_2O: 5% acetonitrile and 0.1% formic acid) and Mobile Phase B (5% H_2O: 95% acetonitrile and 0.1% formic acid). The gradient was as follows: 0% B for 3 min, 0 to 8% B from 3 to 5 min, 8 to 18% B from 5 to 85 min, 18 to 30% B from 85 to 100 min, 30 to 90% B from 100 to 105 min, and held at 90% B from 105 to 120 min at continuous flow rate throughout the gradient of 300 nl/min. The analytical column was connected to a PicoTip Emitter (FS360-75-15-N-20; New Objectives, Woburn, MA, USA) cut to size. The column and Emitter were attached to a LTQ OrbiTrap Velos Pro (Thermo Fisher Scientific) mass spectrometer using the Proxeon Nanospray Flex Ion Source. The capillary temperature was set at 275°C, and spray voltage was 2.8 kV. The mass spectrometer used a data dependent method. In MS mode, the instrument was set to scan 300 to 2,000 m/z with a resolution of 30,000 FWHM. A minimal signal of 20,000 could trigger MS/MS, and ten consecutive MS/MS were possible. The activation type used was CID, and repeat mass exclusion was set to 120 s.

Protein Search and Identification

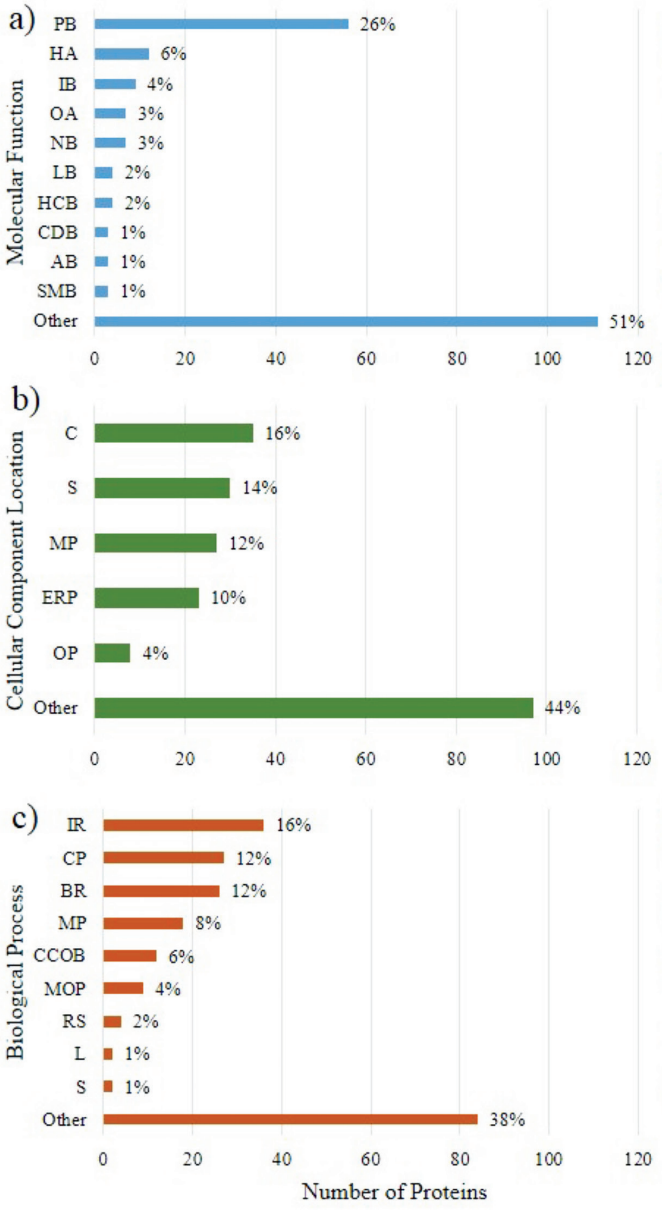
Mass spectrometry data from all slices from a single protein gel lane (i.e., dolphin) were combined, and MS/MS data were analyzed using *Protein Discoverer*, Version 1.4.1.14 (Thermo Fisher Scientific, San Jose, CA, USA). *Sequest* was set up to search the National Center for Biotechnology Information (NCBI) database restricted to all mammalian proteins downloaded 13 July 2016. *Sequest* was searched with a fragment ion mass tolerance of 1.2 Da and a parent ion tolerance of 10.0 ppm. Carbamidomethyl of cysteine and phosphorylation of serine, threonine, and tyrosine were specified in *Sequest* as variable modifications.

Scaffold, Version 4.6.1 (Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at > 95% confidence (or < 5% probability) by the Scaffold Local False Discovery Rate (FDR) algorithm. Initially, protein identifications were accepted if they could be established at > 99.9% confidence (or < 0.1% probability) and contained ≥ 1 peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing substantial peptide evidence were grouped into clusters. Among the three dolphins, identified proteins in the samples of blow were further defined as having ≥ 2 peptides and to be present in at least two of the three dolphins.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifiers PXD006260 and 10.6019/PXD006260.

Protein Classification

Gene ontology (GO) was used to classify the top physiological functions of the blow proteins into three domains—(1) molecular function, (2) cellular component location, and (3) biological process—based on information in the UniProt database. Using a manual approach, the NCBI Accession number for each of the blow proteins was referenced directly to corresponding entries in UniProt and assigned an ontology shorthand based on available information. If a protein did not have a corresponding entry or no GO information in UniProt, an attempt was made to



Molecular function	Key
Protein binding	PB
Hydrolase activity	HA
Ion binding	IB
Oxidoreductase activity	OA
Nucleotide binding	NB
Lipid binding	LB
Heterocyclic compound binding	HCB
Carbohydrate derivative binding	CDB
Antigen binding	AB
Small molecule binding	SMB
Other	Other

Cellular component	Key
Cytoplasm	C
Secreted	S
Membrane part	MP
Extracellular region part	ERP
Organelle part	OP
Other	Other

Biological process	Key
Immune response	IR
Cellular process	CP
Biological regulation	BR
Metabolic process	MP
Cellular component organization or biogenesis	CCOB
Multicellular organismal process	MOP
Response to stimulus	RS
Localization	L
Signalling	S
Other	Other

Figure 3. Among the three dolphins, a total of 220 proteins were uniquely identified in exhaled breath condensate using the NCBI database restricted to all mammalian proteins and subsequently categorized to a top molecular function (a), a top cellular component (b), and a top biological process (c) based on gene ontology (GO) using the UniProt database. The percentage of total proteins within each domain are indicated.

map the dolphin protein to an orthologous human protein (required to have ≥ 95% similarity) using NCBI BLAST or conduct a search of peer-reviewed publications in the public domain using the protein name. If there were multiple functions and no single top function could be objectively assigned or the protein had no UniProt entry, no human orthologue, and no scientific publications to clarify the functional role of the protein, it was assigned "other" within respective GO domains.

Commonality Between Blow and Plasma Proteomes

A recent study (Miller et al., 2017) was conducted that characterized the plasma proteome using a comparable approach in the same young, male bottlenose dolphins involved in the present study. Although blow and plasma samples were not collected simultaneously, NCBI protein Accession numbers of both proteomes were matched to determine the commonality of corresponding proteins in dolphin blow and plasma.

Results

Search of the Mammalia NCBI database with mass spectra data of exhaled breath condensate from three young, male bottlenose dolphins initially resulted in identification of 490 non-redundant or unique proteins with ≥ 1 peptide. Subsequent identification of proteins with ≥ 2 peptides and present in at least two of the three dolphins resulted in 220 proteins. Of those, 166 were predicted.

Protein classification with GO indicated various top functions within the molecular function, cellular component location, and biological process domains as shown in Figure 3. Despite the effort, many proteins (38 to 51% of all proteins) could not be assigned a top function within the molecular (approximately 112 proteins), cellular (approximately 97 proteins), and biological (approximately 84 proteins) domains and, therefore, were functionally classified as "other." Of those classified, 166 of 220 proteins (75%)

Table 1. Identification and gene ontology (GO) characterization of proteins in exhaled breath condensate or blow and blood plasma that were matched based on corresponding NCBI Accession numbers in young, male, aquarium-based bottlenose dolphins (*Tursiops truncatus*). Although samples were not contemporaneous, out of 220 blow proteins documented herein and 196 plasma proteins previously documented (Miller et al., 2017) in the same dolphins, 21 were common in both proteomes.

Protein	Accession no.	Biological process
Alpha-2-antiplasmin isoform 1	XP_004329591.1	Immune response
Complement C4-A	XP_004314433.1	Immune response
Complement factor D-like, partial	XP_004331670.1	Immune response
Complement factor H-like	XP_004322462.1	Immune response
Immunoglobulin IgA heavy chain constant region, partial	AAT65195.1	Immune response
Inter-alpha-trypsin inhibitor heavy chain H4	XP_004316605.1	Immune response
IgM heavy chain	AAG40853.1	Immune response
Lysozyme F1	CDM98806.1	Immune response
Polymeric immunoglobulin receptor (pIgR)	XP_004319136.1	Immune response
Pulmonary surfactant-associated protein B-like, partial	XP_004327549.1	Immune response
Ceruloplasmin (ferroxidase), partial	XP_004332161.1	Metabolic process
Deleted in malignant brain tumors 1 protein, partial	XP_004328181.1	Metabolic process
Antithrombin-III	XP_004321564.1	Coagulation
Factor XIIa inhibitor-like	XP_004315597.1	Coagulation
Fetuin-B-like, partial	XP_004318278.1	Biological regulation
Serum albumin isoform 1	XP_004322082.1	Biological regulation
Galectin-3-binding protein	XP_004323016.1	Cellular component organization or biogenesis
Leucine-rich alpha-2-glycoprotein	XP_004317495.1	Cellular component organization or biogenesis
Alpha-1-antitrypsin-like isoform 2	XP_004320746.1	Other
Kininogen-2-like	XP_004322387.1	Other
Vitamin D-binding protein-like	XP_004320663.1	Other

were listed as predicted in *T. truncatus* based on orthologous to other species. Nonetheless, major top functional roles for different classifications of proteins were indicated within each domain. For molecular function, binding dominated (approximately 57 proteins); for cellular component, cytoplasm constituents dominated (approximately 35 proteins); and for biological process, immune response dominated (approximately 35 proteins).

Of the total proteins identified in blow (220) and previously in plasma (196), 21 of the combined 416 proteins (5%) were identified as having matched NCBI Accession numbers in both proteomes (see Table 1).

Discussion

The novelty of the present study is highlighted by the use of proteomics as a nontargeted approach to identify, quantitate, and characterize the proteome of exhaled breath condensate or blow in three young, male, aquarium-based bottlenose dolphins. Although preliminary, 220 proteins (≥ 2 peptides and present in at least two of the three dolphins) were identified based on mass spectra data and search of the NCBI database restricted to Mammalia, inclusive of cetaceans. Subsequent characterization via GO and the UniProt database indicated the molecular function, cellular component location, and biological process domains were dominated by proteins with top functions associated with protein binding, cytoplasm constituents, and immune response, respectively. Matching the blow and plasma proteomes indicated a commonality of 21 proteins.

The metabolome of exhaled breath from aquarium-based and free-ranging male and female bottlenose dolphins was initially characterized during the development and validation of an intricate device to collect exhaled breath (Aksenov et al., 2014; Zamuruyev et al., 2016). Based on GC/MS and LC/MS analysis, respectively, constituents of the volatile organic fraction included various aliphatic and aromatic alcohols, hydrocarbons, and carbonyls, and that of the nonvolatile fraction included amino acids, peptides, lipid steroids, phospholipids, prostaglandins, carbohydrates, and smaller molecules (e.g., carbonic acids, amines, and pharmaceuticals). While these initial studies indicated the presence of various peptides or proteins in exhaled breath condensate in dolphins, the proteome apparently was not characterized.

In the present study, exhaled breath condensate from each bottlenose dolphin was collected in a 50-mL conical Falcon tube since it was readily available and practical and, thus, resulted in sufficient volume for protein analysis. The volume of condensate from Dolphins A, C, and D was more than adequate (range: 60 to 122 μL) for determination of total protein (5 μL) and separation and identification of proteins (20 μL) using the analytical methods described herein. Comparatively, collection of blow from aquarium-based beluga whales with a Falcon tube resulted in lesser volume compared to a nylon-meshed covered petri dish (Thompson et al., 2014). As reviewed in large whales (Hunt et al., 2013) and reported in bottlenose dolphins (Aksenov et al., 2014), there have been different methods of collection and subsequent analysis of exhaled breath condensate from multiple species of aquarium-based and free-ranging cetaceans. While many factors can influence the volume of blow collected (e.g., animal behaviour and size, force and number of chuffs, and individuals collecting samples), the method used in this study was considered successful and fit our purpose.

In addition to selecting or designing a method for collecting exhaled breath condensate from cetaceans that is most relevant for the intended purpose, there exist basic challenges associated with interpretation of the proteome of breath condensate that require acknowledgment. In humans, contaminant proteins in exhaled breath condensate have apparently originated from saliva or other portions of the gastrointestinal tract as well as ambient air (Fumagalli et al., 2012; Harshman et al., 2014). In cetaceans, it is not expected that blow would be contaminated by gastrointestinal tract proteins or microorganisms since the morphology of the respiratory tract is anatomically distinct from the digestive tract (Cozzi et al., 2017). In addition, individuals collecting samples in the present study wore gloves and face masks to minimize cross-contamination and, as another potential source of contamination, sea water was removed from atop the blowhole prior to sample collections or chuffs. Even though the structural and functional aspects of the blowhole are such that they prevent or minimize the entry of sea water in cetaceans (Berta et al., 2014; Cozzi et al., 2017), blowhole samples from aquarium-based and free-ranging whales (Hunt et al., 2013; Raverty et al., 2017) and dolphins (Morris et al., 2009; Bik et al., 2016) contain a wide variety of microbial species (bacteria and fungi) that are generally considered commensal or transient colonizers with minimal pathogenicity. In the present study, no attempt was made to analyze protein content of contemporaneous air and water samples or examine the respiratory tract or blow samples for microorganisms. While future studies are required to clarify potential sources of extraneous proteins in blow samples, the identification of 220 proteins in the exhaled breath condensate of dolphins was based on the NCBI database restricted to all mammalian

proteins (≥ 2 peptides), inclusive of *T. truncatus* and other cetaceans with a FDR of < 5%.

Herein, a total of 220 proteins were identified in exhaled breath condensate of bottlenose dolphins; however, many (38 to 51%) could not be functionally classified because of multiple top functions for which no one function could be objectively assigned; the protein had no UniProt entry, no human orthologue, or no substantial scientific publications. For those that were classified, the majority (75%) were listed as predicted in *T. truncatus* based on orthologous to other species. While UniProt provides a database that is essentially complete for the proteome of *Homo sapiens*, various levels of completeness exist for most other mammalian species, including cetaceans. Considering the genome of *T. truncatus* has been sequenced (Lindblad-Toh et al., 2011), a concerted effort is required to identify, verify, and characterize gene products for annotation to build more complete protein databases and ontologies for reference within and among cetaceans. Nonetheless and despite the limitations, the results with gene ontology for the molecular function, cellular component location, and biological process domains indicated the dolphin blow proteome was functionally dominated by protein binding (26%), cytoplasm constituents (16%), and immune response (16%), respectively. Correspondingly, in an earlier study of the plasma proteome in the same male bottlenose dolphins (Miller et al., 2017), the majority of proteins were functionally related to protein binding (26%), extracellular region part (38%), and metabolic process (13%) among the three domains, respectively. While the predominance of metabolic-related proteins in the plasma proteome in dolphins (Miller et al., 2017) corresponded to the physiological role of the circulatory system in many other mammalian species (Omenn, 2009), the basis for predominance of immune-related proteins of the blow proteome and functional relationship with the respiratory system in dolphins is less understood. Considering the limited availability of information of exhaled breath condensate in many animal species (Zollinger et al., 2006; Reinhold & Knobloch, 2010), including cetaceans, comprehension of the current results in dolphins requires inference to those in humans who have been most studied.

In humans, the proteome of exhaled breath condensate has been extensively evaluated using different methods of proteomic analysis in men, women, and children that have resulted in the identification of potential biomarkers diagnostic or prognostic of major respiratory diseases (Harshman et al., 2014). An apparent limitation for use of breath condensate in humans for proteomics, however, is the low abundance of total proteins (e.g., < 1 µg/mL); consequently, > 1 mL of condensate is required for analysis. Comparatively, total protein content of blow in bottlenose dolphins from a series of ten chuffs in the present study resulted in total protein content of approximately 1,000 µg/mL of which about 25 µL was required for proteomic analysis. Perhaps as a combination of increased vascular and lung tissue (Cozzi et al., 2017) and an enhanced, forceful respiratory cycle (Fahlman et al. 2015) compared to humans, cetaceans produce, dislodge, and expel an abundance of proteinaceous transudate from the lungs. Nonetheless, in a review (Harshman et al., 2014) covering multiple proteomic studies on exhaled breath condensate in healthy and diseased humans, the predominant proteins identified and characterized in healthy individuals were cytokeratins or keratins (e.g., Type I and II cytokeratins: CK-1, -5, -9, and -14 & -26), several inflammatory cytokines (e.g., interleukins, interferons, and tumor necrosis factor), and complement C protein (Fumagalli et al., 2012; Harshman et al., 2014). In general, keratins are a large family of cytoplasmic intermediate filament proteins primarily involved in structural support of epithelial cells of various tissues (Coulombe & Omary, 2002).

In the present study, some of the same keratins (Types I and II: CK-4, -9, -14, and -17) were identified in exhaled breath condensate of bottlenose dolphins in association with the top cytoplasmic function of the cellular component domain. By inference to the same keratins identified in the lungs of humans, CK-4 is typically expressed in the upper respiratory tract associated with the mucosal and oesophageal epithelia (Samen et al., 2007), CK-9 is typically expressed in association with the epidermis or skin (Langbein et al., 1993), and CK-14 and -17 are typically expressed in the lower respiratory tract in association with bronchii and alveoli epithelia (Hoffmann et al., 2008). It has been speculated that the source of some keratins in exhaled breath condensate of humans can be contaminants or extrinsic to the respiratory system (Kurova et al., 2011).

While some precautions were used to minimize or avoid human skin cell contamination (i.e., latex gloves and face masks) during the collection of blow samples in this study, it may be difficult to avoid dolphin skin cell contamination since the outermost epidermal layer is naturally shed up to 12 times per day (Hicks et al., 1985). In addition, human skin cell contamination could have potentially occurred during in-lab processing and analysis of the samples. In future, more comprehensive studies, appropriate negative controls are needed during the extraction and analysis processes to clarify the potential sources of keratins

in blow samples that may originate during collection or laboratory procedures.

As noted in humans, no apparent cytokines were identified via proteomics of exhaled breath condensate in bottlenose dolphins; however, several complement proteins (C4 and Factors D & H) and immunoglobulins (i.e., IgG Fc binding protein and polymeric Ig-receptor, IgA, IgG, and IgM) were identified such that they dominated the top function of the biological process domain. In contrast, proteomic analysis of human breath condensate of healthy individuals apparently did not detect complement and immunoglobulin proteins except for complement C3 and leukocyte-associated immunoglobulin-like receptor 1 (Harshman et al., 2014). Knowledge of the complement system and immunoglobulins in the lungs is most advanced in humans (Burnett, 1986). The primary function of all immunoglobulins is the recognition, binding, and neutralization of specific antigens such as environmental toxins, particulate matter, and pathogens (Schroeder & Cavacini, 2010) and, secondarily, to activate the complement system which is a series of complement proteins that are, in part, regulated by complement factors (Noris & Remuzzi, 2013). Without historical information of IgA, IgG, and IgM in exhaled breath condensate of dolphins, sources and functional roles of these immunoglobulins are based on those in the exhaled breath of humans (Burnett, 1986). Essentially, all immunoglobulins of exhaled breath condensate in humans appear to originate from both blood transudate and lung production (Burnett, 1986) such that IgA is predominant and plays a role in the neutralization of inhaled antigens or pathogens; IgG has various biological effects, which includes activation of the complement system; and IgM is the most effective complement fixing immunoglobulin. While there are many biological, analytical, and bioinformatic factors that may account for differences in the proteomes of exhaled breath condensate between humans and dolphins, the immune system of cetaceans has adapted to not only respond to potential contaminants and pathogens in ambient air but also to the aquatic environment.

The sea surface microlayer is the boundary interface between the atmosphere and ocean (1 to 1,000 μm thick) that is an aggregate-enriched biofilm with distinct microbial communities (Wurl et al., 2017). In cetaceans, with the structural and functional aspects of the respiratory tract having direct passage of air from the blowhole to lungs; little if any turbinates to filter air; enhanced and forceful nature of respiration; and despite a mechanism to limit inhalation of sea water (Berta et al., 2014; Cozzi et al., 2017), small quantities of environmental contaminants and pathogens of the sea surface microlayer may be aspirated into the tracheo-bronchial branches and deposited into alveolar spaces potentially leading to respiratory tract inflammation and infection. In a 30-y retrospective study (Venn-Watson et al., 2012) involving 42 dolphins housed in an open-ocean enclosure, 50% were diagnosed with pneumonia—about 43% with bacterial and 29% with fungal pneumonia. Therefore, considering pneumonia is one of the most common pathologies and causes of morbidity in bottlenose dolphins, accurate and reliable biological indicators are needed for regular assessment of respiratory health and early diagnosis of pulmonary disease.

Generally, blood is considered a medium that contains information on the physiological state of essentially all bodily systems since most cells communicate directly or indirectly through tissues or biological fluids with the circulatory system. In this regard, the plasma proteome as previously reported (Miller et al., 2017) and blow proteome as presented herein in the same young, male bottlenose dolphins were matched. While sampling and analysis of the plasma and blow were not contemporaneous, results indicated 21 proteins out of a total 416 proteins (5%) were common in both proteomes. About half (10 of 21, 48%) of the common proteins were representative of immune-related functions (e.g., several complement proteins and immunoglobulins), and the remainder were representative of other various biological processes. While future studies are required to clarify the functional relationship between the circulatory and respiratory systems, the commonality of approximately ten immune-related proteins in blow with those in plasma suggest, as in humans (Burnett, 1986), that these proteins of exhaled breath condensate likely originate systemically from blood transudate and locally from lung production and, therefore, may be top candidates for biomarker validation (Moore et al., 2007).

Conclusion

Nontargeted proteomic analysis of exhaled breath condensate or blow in young, male, aquarium-based bottlenose dolphins resulted in the identification and quantification of 220 proteins. Characterization of the proteome indicated protein binding (26%), cytoplasm constituents (16%), and immune response (16%) dominated the molecular function, cellular component location, and biological process domains, respectively. Matching of the blow and plasma proteomes indicated a commonality of 21 proteins such that 48% were related to the immune system. Although preliminary, the novelty of these results provides further support that exhaled breath condensate can be a relevant, less invasive alternative to blood collection to assess or monitor physiological health and pathological states. More specifically, the commonality of approximately ten immune-related

proteins between the circulatory and respiratory systems provides a foundation for future investigations to determine the potential of these blow proteins as biomarkers that can be diagnostic or prognostic of respiratory health in bottlenose dolphins and, perhaps, other cetaceans.

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