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Plasma Proteome and Clinical Biochemistry Associated with Performance-Based Physical Activity in Bottlenose Dolphins (*Tursiops truncatus*)

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Abstract

This study was designed to initially characterize and evaluate the proteome and other biochemical factors in plasma samples collected in association with performance-based physical activity involving four young male aquarium-based bottlenose dolphins (*Tursiops truncatus*). Blood samples were collected from the tail flukes approximately 15 min before ($N = 4$ samples) and 15 min after ($N = 4$ samples) the first public swim interaction of the day, which was reinforced with a daily scheduled feeding regimen and individually prescribed diets. Plasma extracted and trypsin-treated protein extracts were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) and conventional clinical biochemistry analysis. Mass spectra data were used to search the National Center for Biotechnology Information (NCBI) database restricted to *Tursiops truncatus*. The search resulted in the identification of 196 unique proteins with a broad range of functional roles based on gene ontology (GO) analysis. Differential regulation of proteins was based on log mean fold change (FC) and statistical probability such that the abundance of lysozyme (FC -1.2036; $p < 0.058$), an immune-related protein, and flavin reductase (FC -0.9702; $p < 0.004$), a metabolic-related protein, were highest before compared to after the swim interaction; both proteins decreased 58 and 52%, respectively. Correspondingly, 15 known proteins and other biochemical factors were highest before compared to after the swim interaction. Glucose, creatinine, alkaline phosphatase, blood urea nitrogen, calcium, and magnesium decreased 3 to 26% ($p < 0.004$ to 0.07). In conclusion, down regulation of immune- and metabolic-related proteins and multiple other biochemical factors after the swim interaction may represent a homeostatic response to high values in the absence of substantial food in-between the daily feeding regimen or in anticipation of food before the first swim interaction of the day. Thereafter, low values may represent a response to food consumption or satisfaction. The novelty of these initial results suggests that performance-based physical activity is not immunologically or metabolically challenging in bottlenose dolphins conditioned for swim interactive programs; however, future studies are required for clarification.

Key Words: cetacean, bottlenose dolphins, *Tursiops truncatus*, blood plasma, proteomics, clinical chemistry

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 processes and pathological states of an animal in response to various conditions or stimuli (Moore et al., 2007). Quantification of

increases or decreases in relative abundance of proteins associated with biological or physiological events have basic and applied implications in animal health, disease, and welfare.

Gel-based (2-dimensional electrophoresis or 2-DE) studies coupled to mass spectrometry (MS) as well as gel-free nontargeted proteomics approaches have been used to characterize the proteome for different reasons in various types of tissues of multiple animal species (Di Girolamo et al., 2014; Marco-Ramell et al., 2016); however, few studies are available on the basic characteristics of the blood proteome in association with physical activity, especially in wildlife species. In rats with induced osteoarthritis, proteomic analysis indicated a decrease in serum proteins associated with inflammation and the innate immune system in response to a 4-wk treadmill exercise project (Na et al., 2014). Correspondingly, in horses, the effects of endurance racing resulted in the differential increase and decrease in multiple plasma proteins that involved pathways associated with inflammation, coagulation, immune modulation, oxidant/antioxidant activity, and cellular and vascular damage (Scopetta et al., 2012). While limited, these proteomic studies provided novel information fundamental for subsequent validation and development of biomarkers that can potentially assess the therapeutic effects of exercise on disease and degree of athletic conditioning in terrestrial mammals. Comparatively, no published, peer-reviewed studies were found that have characterized or evaluated the effects of physical activity associated with public swim interactions on the blood proteome or clinical biochemistry in cetaceans.

The bottlenose dolphin (*Tursiops truncatus*) is the predominant cetacean bred, born, and housed under human care in a relatively controlled environment. They are readily accessible, highly social, amenable to conditioning, and abundant with historical data. In accord with the International Marine Animal Trainers' Association (IMATA) (2016) guidelines, dolphins are conditioned with positive reinforcement (e.g., food as part of a regular diet) to respond to cues from appointed handlers during daily veterinary health examinations, training sessions, and controlled, short-term performance or swim interactions with the general public.

With availability of the genome in bottlenose dolphins (Lindblad-Toh et al., 2011), the primary objective of the present study was to use proteomic analysis as a nontargeted approach to initially characterize the plasma proteome and evaluate the abundance of plasma proteins before vs after swim interactions with the public in aquarium-based bottlenose dolphins. Secondarily, conventional clinical chemistry analysis was used as a targeted approach to evaluate the response of known plasma constituents encompassing performance-based physical activity.

Methods

Dolphins and Dolphin Management

The study was conducted between October and November at Dolphin Discovery in St Kitts (Federation of St. Kitts and Nevis, Lat/Long: 17° 18' N, 62° 43' W) and involved four young (2 to 6 y) male bottlenose dolphins born under human care. The male dolphins were maintained in an outdoor habitat or dolphinarium with two female bottlenose dolphins, were considered healthy in accord with daily examinations by a resident veterinarian, and were not subject to pharmacological treatment or therapy during the study. The dolphinarium contained natural seawater 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 several times throughout the day by IMATA certified trainers in association with training or performance sessions. Animals were housed and managed in compliance with the U.S. Animal Welfare Act (AWA) (2013) and the *Standards & Guidelines* of the Alliance of Marine Mammal Parks and Aquariums (AMMPA) (2010). 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.

Performance-Based Physical Activity

Performance-based physical activity was defined on the basis of a dolphin swim interaction program with the general public. Briefly, dolphins have been behaviourally and physically conditioned in accord with IMATA (2016) guidelines to do various swim activities when cued by a trainer. Generally, swim interactive programs involved various degrees of physical activity with and without a human participant such as vocalizing, splashing, partial and complete breeching, and pulling and pushing human participants. During the program, dolphins were fed their respective daily diets, which also served as positive reinforcement for performance-based activities. For this study, the average duration of a swim interactive program was 44.2 min (range: 35 to 60 min). Within this time, dolphins interacted with 7 to 14 human participants, ranging from children (> 8 y) to adults.

Blood Collection
Blood sample collections were done within 15 min before the first swim interaction of the day, beginning at approximately 0900 h, which was concomitant with the first feeding of the day, and, again, within 15 min after the program ended. Due in part to the preliminary nature of the study and management constraints, samples collected before the swim interactive programs served as controls for comparison of results after the swim interactions. Preconditioned behavior facilitated the voluntary collection of blood samples which consisted of respective trainers cuing the dolphins to move into a dorsal-down or ventral-up position with the flukes presented to the veterinarian. Blood was collected (approximately 10 mL) via venous-puncture of vessels of the tail flukes using a 21-ga winged BD Vacutainer® Safety-Lok™ Blood Collection Set attached to a Vacutainer® tube containing sodium citrate (BD Company, Franklin Lakes, NJ, USA). Subsequent to collection, samples were placed on crushed ice, transported to the laboratory, and centrifuged at 110 × g for 10 min to generate platelet-rich plasma for possible future analysis of the platelet proteome. Thereafter, plasma was decanted into Eppendorf tubes, labelled, and stored at -80° C until protein extraction.

Protein Extraction and Digestion
Protein extraction involved 200 µL of plasma combined with 1,000 µL of cold acetone (-20° C) followed by vortex for 30 s and centrifugation at 16,000 × g for 15 min. Thereafter, samples were incubated for 3 h and centrifuged again as previously indicated. Supernatants were discarded, and protein extracts were air dried at room temperature and frozen at -80° C until analysis.

Protein extracts were re-solubilized in SDS buffer (4% SDS, 100 mM Tris/HCL pH 8.2, 0.1M DTT – dithiothreitol), boiled at 95° C for 5 min, and processed with high-intensity focused ultrasound (HIFU) for 10 min with ultrasonic amplitude set to 65%. Protein concentration was estimated using the Qubit® Protein Assay Kit (Life Technologies, Zurich, Switzerland). For each of the eight extracts, 50 µg of proteins were digested on-filter using an adaptation of the filter-aided sample preparation (FASP) protocol (Wiśniewski et al., 2009). Briefly, proteins were diluted in 200 µL of UT buffer (Urea 8M in 100 mM Tris/HCL pH 8.2), loaded on an Ultracel 3000 MWCO centrifugal unit (Amicon Ultra, Merck, Darmstadt, Germany), and centrifuged at 14,000 × g for 20 min. Reduced proteins were alkylated by incubation at room temperature with 100 µL iodoacetamide 0.05M in UT buffer for 5 min followed by three, 100 µL washing steps with UT buffer and three, 100 µL washing steps with NaCl 0.5M. On-filter digestion was done using 120 µL of 0.05 Triethylammonium bicarbonate buffer (pH 8) containing trypsin (Promega, Madison, WI, USA) in a ratio of 1:50 (w/w). Digestion was performed overnight in a wet chamber at room temperature. After elution, the solutions containing peptides were acidified to a final 0.1% TFA and 3% acetonitirile concentration. For each solution, peptides were desalted using self-packed C18 Stage-Tips (0.6 µL and binding up to 5 µg), dried, and re-solubilized in 30 µL of 3% acetonitrile and 0.1% formic acid for MS analysis.

Liquid Chromatography and Mass Spectrometry
Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on a QExactive mass spectrometer coupled to a nano EasyLC 1000 (Thermo Fisher Scientific, Bremen, Germany). The two-channel solvent composition was with 0.1% formic acid for channel A and 0.1% formic acid and 99.9% acetonitrile for channel B. For each of the peptide solutions, 2 µL was loaded on a self-made column (75 µm × 150 mm) packed with reverse-phase C18 material (ReproSil-Pur 120 C18-AQ, 1.9 µm, Dr. Maisch GmbH) and eluted at a flow rate of 300 nl/min by a gradient from 2 to 35% B in 80 min, 47% B in 4 min, and 98% B in 4 min. Samples were acquired in a randomized order. The mass spectrometer was operated in data-dependent mode (DDA), acquiring a full-scan MS spectra (300 to 1,700 m/z) at a resolution of 70,000 at 200 m/z after accumulation to a target value of 3×10^6 followed by higher-energy collision dissociation (HCD) fragmentation on the 12 most intense signals per cycle. HCD spectra were acquired at a resolution of 3.5×10^4 using normalized collision energy of 25 and maximum injection time of 120 ms. The automatic gain control (AGC) was set to 5×10^4 ions. Charge state screening was enabled, and single and unassigned charge states were rejected. Only precursors with intensity above 8.3×10^3 were selected for MS/MS (2% under-fill ratio). Precursor masses previously selected for MS/MS measurement were excluded from further selection for 30 s, and the exclusion window was set at 10 ppm. Samples were acquired using internal lock mass calibration on m/z 371.1010 and 445.1200.

Protein Identification and Quantification
The acquired raw LC-MS/MS data were processed by *MaxQuant*, Version 1.4.1.2, followed by protein identification using the integrated Andromeda search engine. Each file was kept separate in the experimental design to obtain individual quantitative values. Mass spectra data were searched against a forward National Center for Biotechnology

Information (NCBI) *Tursiops truncatus* database (release date: 15 January 2016) and concatenated to common protein contaminants and a reversed decoyed database to evaluate the false discovery rate (FDR). Carbamidomethylation of cysteine was set as fixed modification, whereas methionine oxidation and N-terminal protein acetylation were set as variable. Enzyme specificity was set to trypsin/P allowing minimal peptide length of seven amino acids and a maximum of two missed-cleavages. Precursor and fragment tolerances were set to 10 and 20 ppm, respectively, for the initial search. The maximum FDR was set to 0.01 (1%) for peptides and 0.05 (5%) for proteins. Label-free quantification was enabled, and a 2-min window for match between runs was applied with the requantify option selected. For protein abundance, intensities as expressed in the protein groups file were used, which corresponded to the sum of the precursor intensities of all identified peptides for the respective protein groups. Only quantifiable proteins defined as protein groups showing ≥ 2 razor peptides were considered for subsequent analysis. Data were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005435 (Vizcaíno et al., 2014, 2016).

Protein Classification

Identified proteins were classified for their functional roles using manual gene ontology (GO) analysis. Each protein name was entered into the Uniprot database and assigned a single top function for each of three domains—(1) molecular function, (2) cellular component, and (3) biological process—using *Tursiops truncatus* when available. If not available, top function of an identified protein was assigned based on orthologues of other cetaceans, artiodactyls, or other mammalian species. If the protein was not found through GO analysis or had no assigned function in Uniprot, then a BLAST search was conducted (www.ncbi.hlm.nih.gov/BLAST) using NCBI protein accession numbers. In addition, a literature search of the public domain using the protein name was conducted as needed. If a single top function could not be assigned or if functional information was lacking within the databases or scientific literature, then identified proteins were functionally classified as "other" within each domain.

Clinical Biochemistry

Conventional veterinary biochemistry analysis was used to evaluate concentrations of known physiological constituents of blood plasma samples collected from each of the four dolphins before and after performance-based physical activity (Thrall et al., 2012). Basic and comprehensive metabolic panels with respective automated methodologies (*VetScan*, Version S2; Abaxis North America, Union City, CA, USA; Catalyst Dx Analyzer, IDEXX Laboratories, Inc, Westbrook, ME, USA) were used to optically measure chemical reactions and quantitate respective biochemistries from reference or calibration data. Plasma concentrations were calculated for the following: (1) albumin (ALB, g/dL), (2) alkaline phosphatase (ALP, U/L), (3) alanine aminotransferase (ALT, U/L), (4) aspartate aminotransferase (AST, U/L), (5) blood urea nitrogen (BUN, mg/dL), (6) calcium (CA, mg/dL), (7) creatinine kinase (CK, U/L), (8) creatinine (CRE, mg/dL), (9) gamma-glutamy transpeptidase (GGT, U/L), (10) globulin (GLOB, g/dL), (11) glucose (GLU, mg/dL), (12) lactate dehydrogenase (LDH, U/L), (13) magnesium (MG, mg/dL), (14) phosphorus (PHOS, mg/dL), and (15) total protein (TP, g/dL).

Data Management and Statistical Analysis

Blood plasma samples collected before the swim interactions served as controls for comparison of results after the swim interactions. An increase or decrease in abundance of proteins before vs after the swim interactions were determined using both fold change and statistical significance. Fold changes encompassing physical activity were calculated using $\log_2$[mean intensity(after)/mean intensity(before)]. A decrease or increase in protein abundance was indicated by a mean fold change ≤ -0.585 or ≥ 0.585, respectively. Protein abundance or intensity values were normalized by scaling the median intensities in each of the extract samples to the same values and transformed (hyperbolic arcsine transformation) after missing values (zeros) were imputed using *missForest: Nonparametric Missing Value Imputation using Random Forest*, *R package*, Version 1.4 (Omasits et al., 2013). The transformed data were analyzed using a two-sided paired *t* test.

Prior to analysis of clinical chemistry results, the data were checked for homogeneity using Shapiro-Wilk's test and transformed if necessary. Thereafter, a two-sided paired *t* test was conducted for each of the 15 biochemical factors. In addition, the percent change before vs after the swim interaction was calculated for each of the factors among dolphins.

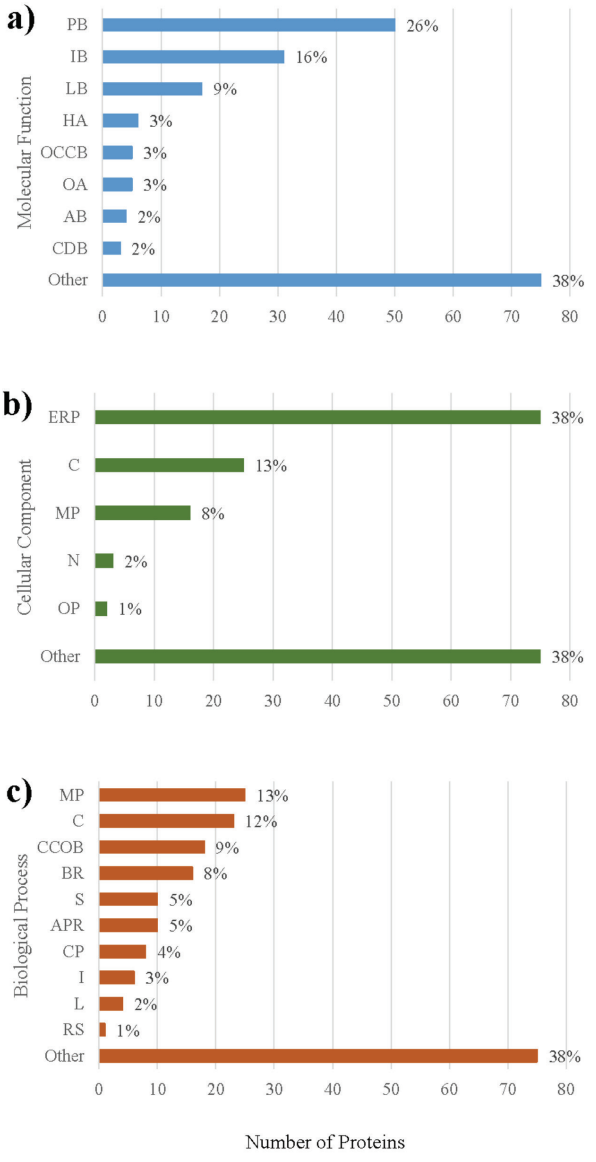
A statistically significant increase or decrease in protein abundances and other biochemical factors was indicated by a probability of $p \leq 0.05$. Probabilities between $p > 0.05$ and 0.1 indicated that a decrease or increase in values approached significance. The results are presented as the mean (± SD) of the untransformed data unless otherwise indicated.

Results

Proteome – Identification and Characterization

Species restricted (*Tursiops truncatus*) search of the NCBI database with mass spectra data resulted in the identification of 196 nonredundant or unique proteins in the plasma of young male bottlenose dolphins. No decoy protein was identified with more than one peptide; therefore, the protein FDR for all quantified proteins was estimated at or close to zero.

Protein characterization with GO analysis indicated various top functions within the molecular function, cellular component, and biological process domains (Figure 1). Although many plasma



Molecular function	Abrv.
Protein binding	PB
Ion binding	IB
Lipid binding	LB
Hydrolase activity	HA
Organic cyclic compound binding	OCCB
Oxidoreductase activity	OA
Antigen binding	AB
Carbohydrate derivative binding	CDB

Cellular component	Abrv.
Extracellular region part	ERP
Cytoplasm	C
Membrane part	MP
Nucleus	N
Organelle part	OP

Biological process	Abrv.
Metabolic process	MP
Coagulation	C
Cellular component biogenesis	CCB
Biological regulation	BR
Signalling	S
Acute Phase Response	APR
Cellular process	CP
Immune response	IR
Localization	L
Response to stimulus	RS

Figure 1. Among the four dolphins, a total of 196 proteins were uniquely identified through the National Center for Biotechnology Information (NCBI) database restricted to *Tursiops truncatus* and subsequently assigned to a top molecular function (a), top cellular component (b), and top biological process (c) based on gene ontology (GO). The percentage of total proteins for each top function, component, and process are indicated.

Table 1. Differentially regulated plasma proteins (2 of 196) that decreased in abundance based on a mean fold change (≤ -0.585) and *p* value ($p \leq 0.06$) in samples collected before ($N = 4$) and after ($N = 4$) performance-based physical activity in four young male bottlenose dolphins

Protein[1]	Accession no.	Biological process	Log fold change[2]	*p* value[3]
Lysozyme f1	CDM98806.1	Acute phase response	-1.20361	0.004
Flavin reductase (NADPH)	XP_004319421.1	Metabolic process	-0.97021	0.058

[1]Data are available via ProteomeXchange with identifier PXD005435.
[2]Defined as $\log_2$[mean intensity(after)/mean intensity(before)].
[3]Two-sided paired *t* test

proteins were assigned a single top function within each domain, a relatively large proportion of proteins (75 of 196) could not be assigned a top function regardless of domain and, therefore, were functionally classified as "other" within each domain (Figure 1).

Differential Regulation – LC-MS/MS Protein Quantification

Abundance of differentially regulated LC-MS/MS-detected plasma proteins lysozyme f1 and flavin reductase (NADPH) associated with performance-based physical activity are shown in Table 1 and Figure 2. While both proteins met the required fold change plus statistical significance indicative of a differential decrease in abundance, the remainder of the identified proteins (194) either lacked the fold change or statistical significance required for a differential increase or decrease.

Among Dolphins A, B, C, and D, abundance values for lysozyme and flavin reductase were higher among the four dolphins before the swim interactive program compared to after the program (Figure 2). Although there appeared to be more variability in abundance values of lysozyme and flavin reductase among dolphins before the program, especially Dolphin B, decreases in protein abundances thereafter appeared more consistent among animals. Based on fold changes and paired *t* tests, mean abundance values of both proteins were higher before compared to after the swim interaction (Table 1; Figure 2). Mean lysozyme and flavin reductase values decreased approximately 58 and 52%, respectively. Based on GO analysis, top biological processes for lysozyme and flavin reductase involved acute phase response and metabolism, respectively.

Differential Regulation – Clinical Biochemistry Quantification

Concentrations of known plasma constituents differentially changed in association with performance-based physical activity (Table 2). While mean circulating concentrations of all 15 proteins and other biochemical factors were higher before the swim interaction, they all decreased thereafter (range: 1.1 to 25.8%). The decline in mean concentrations was significant for CRE, GLU, and MG and tended to be significant for ALP, BUN, and CA.

Discussion

The novelty of the present study is highlighted by the use of gel- and label-free proteomics to aid in a nontargeted approach to identify and characterize the plasma proteome in young male bottlenose dolphins. Although preliminary, 196 proteins were identified based on mass spectra data and a search of the NCBI database with near zero FDR. Differentially regulated lysozyme f1, an immune-related protein, and flavin reductase (NADPH), a metabolic-related protein, were higher before compared to after performance-based physical activity associated with public swim interactions. Supportive of the differential change in abundance of proteins, clinical biochemistry analysis of known plasma constituents indicated higher concentrations of various metabolic-related proteins and other biochemical factors before compared to after the swim interactive programs.

A total of 196 nonredundant or unique plasma proteins were identified and quantified in male bottlenose dolphins after a search of the NCBI database restricted to *Tursiops truncatus,* which listed 37,468 total proteins for *T. truncatus* at the time of the search. Comparatively, as the most studied mammalian species, the plasma proteome of humans consisted of 1,929 nonredundant proteins of which the NCBI database recently listed 39,241,807 total proteins for *Homo sapiens* (Farrah et al., 2011). Homologous proteins among mammalian species do not always share 100% sequence similarity; consequently, potential analyzable tryptic peptides per protein can be limited depending on the degree of similarity and, thus, influence peptide match identification and ranking. Considering that the NCBI database is constantly being updated, future proteomic analysis

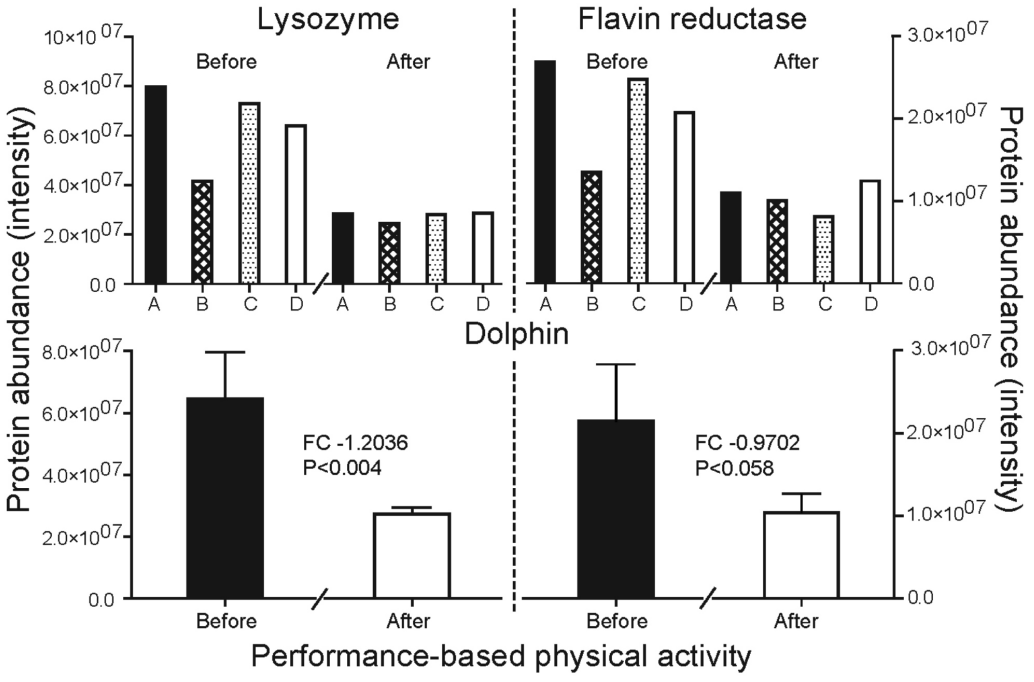


Figure 2. Individual dolphin (A, B, C, and D) and mean (± SD) abundance values (nontransformed) for lysozyme f1 and flavin reductase (NADPH) in plasma samples collected within 15 min before ($N = 4$) and 15 min after ($N = 4$) a public swim interaction are indicated. Based on mean fold change (FC, $\log_2$[mean intensity(after)/mean intensity(before)]) and paired *t* test, both proteins were higher before compared to after the swim interaction. Mean lysozyme and flavin reductase values decreased approximately 58 and 52%, respectively.

of bottlenose dolphin plasma will likely yield additional proteins.

Subsequent to identification, functional characterization indicated the plasma proteins in dolphins were associated with a broad range of top functions, which would be expected to be found within the circulatory system of mammalian species (Omenn, 2009). Among the molecular function, cellular component, and biological process domains, a majority of proteins were associated with protein binding (26%), extracellular region part (38%), and metabolic process (13%), respectively. Notably, a large proportion of proteins (38%) could not be assigned a top function despite using orthologues of other cetaceans, artiodactyls, and other mammalian species; BLAST; or searches of the scientific literature. 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, which was considered the main limitation for describing the characteristics or functional roles of many plasma proteins in bottlenose dolphins.

Recently, a total of 58 serum proteins were identified in male and female bottlenose dolphins and were subsequently ranked in comparison with the human proteome (Sobolesky et al., 2016). While the results indicated 11 proteins that were exclusive to dolphins, future analysis is required to clarify if the apparent discovery is cell- or species-specific, an artifact of the methodology, or incomplete or limited biological information in existing protein databases. Nonetheless, with the genome of *Tursiops truncatus* sequenced (Lindblad-Toh et al., 2011), a concerted effort is required to identify, characterize, and verify gene products for annotation to build more complete protein databases and ontologies for reference within and among cetaceans and other mammalian species. Thus, although protein identification and characterization for *Tursiops truncatus* was limited, in part, by incomplete annotation of protein databases and few orthologues, the novelty of this study established an initial basic plasma proteome profile for young adult male bottlenose dolphins with implications for identifying

Table 2. Mean (± SD) plasma concentrations of various clinically related biochemical factors in samples collected before ($N = 4$) and after ($N = 4$) performance-based physical activity in four young male bottlenose dolphins

	Performance-based physical activity			
Clinical biochemistry[1]	Before	After	% decrease	p value[2]
ALB (g/dL)	4.3 ± 0.4	4.1 ± 0.4	3.5	0.215
ALP (U/L)	429.2 ± 71.1	405.8 ± 55.9	5.1	0.055
ALT (U/L)	63.8 ± 43.3	61.5 ± 41.1	3.3	0.229
AST (U/L)	421.5 ± 238.5	407.8 ± 218.8	2.4	0.316
BUN (mg/dL)	48.5 ± 3.9	47.0 ± 4.2	3.1	0.058
CA (mg/dL)	5.8 ± 0.4	5.4 ± 0.1	6.7	0.073
CK (U/L)	186.8 ± 35.9	181.0 ± 21.5	2.0	0.517
CRE (mg/dL)	1.2 ± 0.1	0.9 ± 0.1	25.8	0.014
GGT (U/L)	28.5 ± 9.1	26.2 ± 7.1	6.5	0.135
GLOB (g/dL)	1.2 ± 0.5	1.1 ± 0.5	1.1	0.495
GLU (mg/dL)	99.8 ± 2.8	88.5 ± 4.2	11.3	0.004
LDH (U/L)	1,874.5 ± 498.4	1,780.0 ± 378.4	4.1	0.271
MG (mg/dL)	2.6 ± 0.1	2.2 ± 0.1	13.4	0.006
PHOS (mg/dL)	4.4 ± 0.3	4.0 ± 0.4	10.9	0.138
TP (g/dL)	5.5 ± 0.2	5.2 ± 0.1	4.4	0.126

[1]ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CA, calcium; CK, creatinine kinase; CRE, creatinine; GGT, gamma-glutamy transpeptidase; GLOB, globulin; GLU, glucose; LDH, lactate dehydrogenase; MG, magnesium; PHOS, phosphorus; and TP, total protein
[2]Two-sided paired t test; mean changes that were significant ($p \leq 0.05$) or approached significance ($p \leq 0.1$) are highlighted.

potential biomarkers to assess animal health and well-being.

Performance-based physical activity was associated with the differential regulation of two of 196 plasma proteins based on combined fold change and statistical significance of intensities or abundances in blood samples collected before compared to after public swim interactions with bottlenose dolphins. In each of the four dolphins, the abundances of lysozyme f1 and flavin reductase (NADPH) were consistently higher before and repeatedly lower after the interaction with mean decreases of approximately 58% for lysozyme and 52% for flavin reductase. In accord with GO analysis, lysozyme was assigned to acute phase response and flavin reductase to metabolic process as top functions in the biological process domain. Apart from lysozyme and flavin reductase, multiple other plasma proteins were identified within the same top biological processes (9 for acute phase response and 24 for metabolic process); however, they did not meet the criteria for differential increase or decrease associated with performance-based physical activity.

Lysozyme, also known as muramidase or N-acetylmuramide glycanhydrolase, is an inherent antibacterial protein widely distributed in various tissues and biological fluids (e.g., including spleen, lung, kidney, white blood cells, plasma, saliva, tears, milk, and respiratory secretions) and secreted by polymorphonuclear leukocytes (Dumoulin et al., 2007). It belongs to a class of enzymes that lyses the cell wall of certain bacteria cleaving the β-(1,4)-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine. The bacteriolytic role of lysozyme is characteristic of the innate immune system which is encompassed in the acute phase response that can be triggered by pathogenic invasion, infection, and inflammation (Cray et al., 2009). In this regard, lysozyme has been proposed as a *potential biomarker* for assessing responsiveness of the *immune system* to various pathological conditions (Dayala et al., 2012).

Compared to the extent of knowledge on lysozyme, less is known about flavin reductase. Nonetheless, it is as an enzyme that works with cofactor NADPH to catabolise flavin to riboflavin

(vitamin B_2), *flavin adenine dinucleotide* or flavin mononucleotide, biliverdins, methemoglobin, and pyrroloquinoline quinone in the circulatory system and liver (Stocker et al., 1987; Yamaguchi et al., 1994; Shalloe et al., 1996; Smith et al., 2008). Flavin reductase is also known as biliverdin-IXβ reductase for its enzymatic role in catabolism of biliverdin to bilirubin (Yamaguchi et al., 1994). Before lysozyme or flavin reductase can be considered biomarkers for assessing the physiological status of dolphins involved in performance-based physical activities, additional studies are needed to verify or validate (e.g., using commercially available bioassays or Western immunoblot analysis) these plasma proteins and to clarify their biological roles in this species.

A search of the scientific literature was conducted to find support for the decrease in abundance of these plasma proteins in association with performance-based physical activity in dolphins or other cetaceans, and in other mammalian species. Although no studies were located to directly account for the present results, a few studies were found that may be used for inference. Proteomic analysis of serum samples from rats with induced osteoarthritis resulted in a differential decrease in serum complementary component 9 in association with a 4-wk treadmill-based exercise program (Na et al., 2014). Correspondingly, proteomic analysis of plasma samples from horses resulted in a differential decrease in complement factor B, transthyretin, retinol binding protein 4, and apolipoprotein A1after an endurance race (Scoppetta et al., 2012). While complementary component 9 and complement factor B are encompassed in the acute phase response as characteristic of the innate immune system and inflammatory process, transthyretin, retinol binding protein 4, and apolipoprotein A1 are nutrient transport and cholesterol mobilizing proteins necessary for maintaining metabolic homeostasis (Frank & Marcel, 2000; Newcomer & Ong, 2000; Sarma & Ward, 2011).

In the present study, complement factor B and apolipoprotein AI were identified in dolphin plasma but were not differentially abundant since they did not meet the criteria for a differential increase or decrease associated with performance-based physical activity. In free-ranging bottlenose dolphins, gene expression of various immune- and metabolic-related factors were differentially abundant in peripheral blood samples collected at the time of capture and subsequent to release (Manica et al., 2008). While these studies conducted in rats, horses, and free-ranging dolphins do not necessarily provide direct support for the differential abundance of lysozyme and flavin reductase in the present study, together they support the concept of an integrated relationship among specific modes of action involving the immune and metabolic systems (i.e., immunometabolism) in maintaining homeostasis under different external and internal conditions in mammals (Mathis & Shoelson, 2011).

While nontargeted proteomics provided a novel approach to identify, characterize, and quantitate the differential regulation of plasma proteins in association with swim interactions in dolphins, conventional clinical biochemistry was used as a targeted approach to characterize and quantitate the differential regulation of known constituents of blood plasma (Thrall et al., 2012; Di Girolamo et al., 2014; Marco-Ramell et al., 2016). Of the basic and comprehensive metabolic panels, all 15 of the biochemical factors were higher before the swim interaction; thereafter, all decreased from 1 to 26%. While the decreases in glucose (GLU, 11%), creatinine (CRE, 26%), and magnesium (MG, 13%) were statistically significant, decreases in alkaline phosphatase (ALP, 4%), blood urea nitrogen (BUN, 3%), and calcium (CA, 7%) approached significance. Regardless of the results before and after the swim interaction, plasma concentrations of all the clinically related biochemical factors were generally within the range of reference values previously reported in the blood of healthy bottlenose dolphins of different ages and genders (Asper et al., 1990). Until plasma lysozyme and flavin reductase are validated in bottlenose dolphins, the relationship between these proteins and other known physiological constituents of blood during swim interactions remains unknown.

In the present study, dolphins were fed their daily diets, which were prepared on an individual basis in accord with standard management practices. In general, feeding began regularly in the morning (at approximately 0900 h) as the resident veterinarian and trainers of the dolphinarium started their daily routines involving health inspections and training. Thereafter, feeding continued throughout the day in association with training or performance sessions and ended by mid-afternoon (at approximately 1500 h). After the daily activities, the animals were free to roam and interact among themselves within the dolphinarium until the next morning when their daily routines, including feeding, began again.

Although various species of relatively small fish and other aquatic wildlife inhabit the open-ocean dolphinarium, it is not known if the dolphins preyed on them during or between feeding times. Reportedly (Venn-Watson & Ridgway, 2007), overnight fasting in aquarium-based bottlenose dolphins of various ages and genders resulted in a prolonged glucose tolerance curve and a transient hyperglycaemic or diabetes mellitus-like state. Of

the various clinically related biochemical factors analysed, serum concentrations of GLU, CRE, ALP, GGT, and CK were significantly higher in dolphins that fasted overnight for 10 to 14 h compared to nonfasted dolphins or those fed within 6 h. In an earlier study involving a limited number of bottlenose dolphins that fasted for 72 h, elevated serum glucose concentrations decreased 4 h after being fed (Ridgway et al., 1970). In the present study, there was an approximate 18-h interval from the last feeding of a day to the first feeding of a new day when blood samples were collected concurrently with the first pre-swim interaction. Subsequent to analysis, plasma concentrations of GLU, CRE, and ALP were or tended to be significantly higher before compared to after feeding, which was concurrent with the post-swim interaction. Considering the comparable results between the present and previous studies, the results herein may be supportive of a hyperglycaemic or diabetes mellitus-like state associated with overnight fasting in bottlenose dolphins (Ridgway et al., 1970; Venn-Wattson & Ridgway, 2007).

Apart from the necessity of providing proper and adequate nutrition in accord with standard management practices, daily feeding regimens also serve as positive reinforcement for training and performance of zoo- and aquarium-based animals (Laule, 2003; Brando, 2010). In this regard, increased activity and arousal in association with feeding are suggestive of anticipatory behaviour (Howell et al., 1993; Carlstead, 1996). Behavioural responses to predictable events are referred to as anticipatory (Spruijt et al., 2001; Bassett & Buchanan-Smith, 2007). Correspondingly, anticipatory behaviour is defined as responses elicited by rewarding stimuli that leads to and facilitates consummatory behaviour, which may be a positive experience for an animal that is expecting a reward (Spruijt et al., 2001).

In a preliminary study involving aquarium-based bottlenose dolphins, anticipatory behaviour, which included increased vigilance (i.e., looking or spy-hopping) and time on the surface, was observed beginning 15 to 20 min before training or public swim interactions (Jensen et al., 2013). After the training or performance sessions, vigilance decreased as dolphins spent more time submerged. The novelty of these results provided information that may be related to an entrained physiological response that is anticipatory as a result of regularly scheduled training or performance sessions supported by a concurrent feeding regimen (Spruijt et al., 2001; Bassett & Buchanan-Smith, 2007). Although behaviour related to anticipation of the first feeding of the day was not evaluated in the present study, the combined results herein and previously suggest that the differential abundance in immune- and metabolic-related proteins and other biochemical factors encompassing performance-based activity may be in part or wholly the result of overnight fasting or anticipation of a regularly scheduled feeding regimen. Nonetheless, considering the present study was designed as a preliminary evaluation of the physiology associated with performance-based physical activity, future studies with dolphins not involved in training or swim interaction programs as well as additional blood samples before and after food-reinforced swim interactions are required for clarification.

Conclusion

The results of the present study provided novel and fundamental information on the plasma proteome in aquarium-based young male bottlenose dolphins involved in public swim interactive programs, which were reinforced by a regularly scheduled feeding regimen. Use of nontargeted proteomics and a subsequent search of the NCBI database and GO analysis resulted in identification, characterization, and quantification of 196 proteins associated with various biological pathways from molecular, to cellular, to whole animal. Of the 196 proteins, lysozyme, an immune-related protein, and flavin reductase, a metabolic-related protein, were higher in plasma samples collected before compared to after the public swim interactions (58 and 52% decrease, respectively).

Correspondingly, targeted clinical chemistry analysis of known plasma constituents indicated all 15 proteins and other biochemical factors were higher before the swim interaction, including glucose, creatinine, alkaline phosphatase, blood urea nitrogen, calcium, and magnesium, and subsequently decreased (3 to 26%) after the interactive program. Although the results need to be interpreted cautiously due to the relatively small number of animals ($N = 4$ dolphins), down regulation of immune- and metabolic-related proteins and multiple other biochemical factors after the swim interaction may represent a homeostatic response to high values in the absence of substantial food in-between the daily feeding regimen or in anticipation of food before the first swim interaction of the day. Thereafter, low values may represent a response to food consumption or satisfaction. The novelty of these initial results suggest that performance-based physical activity is not immunologically or metabolically challenging in bottlenose dolphins conditioned for swim interactive programs; however, future studies are required for clarification.

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