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Effects of sex, age, and season on the variation of blood analytes in a clinically healthy *ex situ* population of bottlenose dolphins (*Tursiops* spp.)

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ABSTRACT

Background: A comprehensive evaluation of the effects of sex, age, and season on blood analytes in a robust population size of *ex situ* bottlenose dolphins (*Tursiops* spp.) has not been investigated to date.

Aim: To define the variation in hematological and biochemical analytes of dolphins due to sex, age, and season.

Methods: 1,426 blood samples collected from 156 clinically normal dolphins consisting of 59 males and 97 females in which 37 analytes were measured were retrospectively identified. The dolphins were categorized by age, sex, and season, and categories were compared.

Results: About 23 (64%) analytes differed by age. The number of differences between adjacent age groups decreased with advancing age. MPV, glucose, BUN, globulins, GGT and Cl progressively increased with age, whereas Abs lymphs, total protein, ALP, CK and Ca progressively decreased with age. Three (8%) of analytes differed between sex, whereas 16 (44%) analytes differed by season. Female dolphins had higher median iron (33 μmol/L) than male dolphins (25 μmol/L). Female dolphins also had higher Abs lymphs and MCHC, but Abs lymphs and MCHC also differed between age and season, respectively. Sex inconsistently and relatively infrequently influences analytes. Delphinids of advancing age experience immune senescence and decreasing renal perfusion or clearance.

Conclusions: These results demonstrate the importance of considering the influences of sex, age, and season on blood data, provide a baseline for accurate interpretation of clinicopathological analytes of delphinids in managed care, and will be useful for investigations into health, disease, and stressors of wild delphinids.

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1. Introduction

Hematology and serum biochemistry analysis is an important diagnostic and monitoring tool in an individual's health assessment. They are used to diagnose and evaluate physiological changes (e.g. reproductive activity) and pathological conditions (e.g. stress, disease) (Stockham and Scott, 2008). Ideally, blood analytes for an individual are compared against historical data from the same individual for observation of trends. Where this is not possible, blood data of an individual are compared against the distribution of data from a reference population and deviations from the reference population provides diagnostic information. Physiological conditions like aging, environmental factors, and various diseases can all cause alterations in inflammatory biomarkers, enzymes, metabolites, and mineral contents of an individual (Miller 1991; Bossart et al. 2001; Stockham and Scott, 2008; Nollens et al. 2019). It is important to know which of these factors drive such variations and how, so that deviations in an individual animal's test results can be correctly interpreted. Establishing reference intervals (RIs) for specific age categories can also provide insights into the physiology of aging (Lewis et al., 1998; Monke et al. 1998; Caldin et al. 2005; Sato et al. 2005).

Few studies have reported on the clinicopathological analytes of dolphins. Most of these studies reported on free-ranging dolphins with an unknown medical history and unconfirmed health status, which introduces a large amount of variability and limits their use as a diagnostic reference population (Fair et al., 2006; Goldstein et al. 2006; Hall et al. 2007; Schwacke et al. 2009). Additional challenges and limitations of these studies included low numbers of animals and samples, cortisol-induced artifacts as would be triggered by the capture of a free-ranging dolphin, different methods in sample

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handling, processing, and laboratory analysis, and no control measures to account for fasting status and serum quality. The diagnostic reference value of two other available datasets were limited by either small (Macchi et al., 2011) or focused (Venn-Watson et al., 2011) sampling. One study reported on the clinicopathological reference ranges of clinically healthy bottlenose dolphins, based on a comparable sample size to the dataset reported herein (Venn-Watson et al., 2007). However, these dolphins live in coastal netted enclosures, with access to live wild fish, whereas the dolphins reported herein live in land-based, consistently maintained systems and are fed frozen-thawed fish that is free of parasites. We can therefore expect the dataset reported herein to have even less inherent variability (Ruiz et al., 2009).

The *ex situ* bottlenose dolphin population housed at the SeaWorld facilities provides a unique window into understanding delphinid physiology. The results of managed population studies are particularly valuable because they allow for longer-term follow-up of individuals of known age, health status, and reproductive and nutritional statuses. The ability to control for many confounding variables when enrolling study subjects and samples has previously allowed for describing physiology of dolphins and whales (Robeck and Monfort 2006; Norman et al. 2013; O'Brien et al. 2017; Nollens et al., 2019; Robeck et al. 2017). Using data from 1426 blood samples collected as part of the routine preventive medicine program from 156 dolphins over a three-year period, the main objectives of this study were (1) to describe hematological and serum biochemical analyte variation in clinically healthy bottlenose dolphins; (2) to examine the effects of sex, age, and season on this variation; and (3) to generate insights into biological changes associated with aging in bottlenose dolphins.

2. Materials and methods

A total of 156 bottlenose dolphins (*Tursiops truncatus truncatus, Tursiops truncatus gillii,* and *Tursiops truncatus truncatus x gillii*) were group housed in natural daylight in one of eight pool systems containing natural or synthetic (Univar, Redmond, WA, USA) full-strength saltwater. The dolphins were fed a diet of frozen-thawed whole fish which contained some or all of the following fish: Atlantic herring (*Clupea harengus*), sardines (*Sardinops sagas*), Columbia River smelt (*Thaleichthys pacificus*), capelin (*Mallotus villosus*), squid (*Illex illecebrosus* or *Loligo opalescens*), pinfish (*Lagodon rhomboides*), or finger mullet (*Mugil* sp.) in varying amounts and percentages based on the caloric needs and dietary preferences of each dolphin. All food fish was suitable for human consumption. Animals were supplemented with Vita-Zu Marine Mammal multivitamin tablets (Mazuri, St. Louis, MO, USA).

Fasted blood samples were collected up to four times annually from all dolphins as part of SeaWorld's preventive medicine program between 10 September 2014 and 20 October 2017. Samples were restricted to those collected from clinically healthy dolphins, based on wellness examinations and medical history. Samples drawn from dolphins while on medication, other than multivitamin tablets, were excluded (Norman et al. 2013; Nollens et al. 2019). Based on the previously demonstrated effects of gestation and lactation on hematological analytes (Robeck and Nollens, 2013), all samples collected 18 months before and 6 months after parturition or while serum progesterone concentrations were elevated (>5000 pg/ml) for at least 5 weeks were excluded. Blood samples with any degree of hemolysis or lipemia were excluded (Howanitz et al. 2015).

For venipuncture, the dolphins were trained to present the ventral surface of their fluke to the attending veterinarian for sampling using a 21-gauge 1.5-inch needle. After cleaning and disinfection of the venipuncture site with 70% isopropyl alcohol (Henry Schein, Melville, NY, USA), blood samples were collected and analyzed as previously described for killer whales and beluga (Nollens et al., 2019; Norman et al. 2013). Blood was collected into BD Vacutainer tubes (Becton Dickenson, Franklin Lakes, NJ, USA) containing potassium-EDTA, sodium citrate, or thrombin. Hemoglobin concentration (Hb), red blood cell count (RBC), the mean corpuscular volume (MCV), red blood cell distribution width (RDW), platelet count (platelet), mean platelet volume (MPV), total white blood cell count (WBC), absolute segmented neutrophils (Abs segs), absolute lymphocytes (Abs lymphs), absolute monocytes (Abs monos), and absolute eosinophils (Abs eos), and erythrocyte sedimentation rates at 60 min (ESR 60) were measured using EDTA-anticoagulated blood. Samples were processed at the on-site veterinary diagnostic laboratories. The analytes Hb, RBC, MCV, RDW, platelet, MPV, and WBC were determined using either Abbott Cell Dyn 3500 R (Abbott Laboratories, Abbott Park, IL, USA) or Siemens Advia 2120i (Siemens Medical Solutions USA, Inc., Malvern, PA, USA) automated hematology analyzers using the manufacturer's reagents per standardized quality assurance and control protocols. From these analytes, hematocrit (HCT), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated. The differential cell counts were derived by manually counting 100 cells in EDTA-anticoagulated blood Wright-Giemsa stained blood films and then calculating the Abs segs, Abs lymphs, Abs

monos, and Abs eos from the percentages multiplied by the WBC. The biochemical analytes glucose, urea (BUN), creatinine (Creat), total bilirubin (Tbili), cholesterol, triglyceride, total protein (Tprotein), albumin, total globulins (globulin), calcium (Ca), phosphorus (P), sodium (Na), potassium (K), chloride (Cl), CO_2, iron as well as activities of alkaline phosphatase (ALP), alanine (ALT) and aspartate (AST) aminotransferases, gamma-glutamyl transpeptidase (GGT), creatinine kinase (CK), and lactate dehydrogenase (LDH) were measured using either the Ciba Corning Fast 4 (Ciba Corning, Cambridge, MA, USA) or the Olympus AU400E (Olympus Corporation, Center Valley, PA, USA) automated serum chemistry analyzer on thrombin-coagulated serum. All reagents used were designed for and purchased from the manufacturer. Fibrinogen (FIB) content was quantified using the Organon Teknika Coag-a-mate (Organon Teknika Corporation, Durham, NC, USA) or the Sysmex CA-500 (Sysmex America, Winter Springs, FL, USA) on citrate-anticoagulated blood. The estimated sedimentation rate (ESR) 60 was determined using a Sediplast Westergren ESR system (LP Italiana SPA, Milano, Italy). Correlation studies were conducted when equipment was transitioned as part of the best laboratory practices program, by analyzing 30 whole blood or serum samples on both pieces of equipment and calculating the linear correlation coefficient (R^2) for each analyte, resulting in strong agreement of R^2 (range 0.916–0.995). The on-site ASCP certified clinical laboratory scientists and field service engineers performed and documented analyzer calibrations and maintenance, and installed analyzer updates as prescribed by the manufacturer's recommended maintenance schedule to ensure instrument performance and reliability. On a regular basis, all clinical and analytical methods were tested with a multi-level, matched matrix, quality control material at a minimum frequency as recommended by the assay manufacturer. Quality control test results were compared to established RIs for validation of assay performance.

For analysis, age at the time of sampling (in years) was calculated by subtracting the date of sampling by the birth date, divided by 365. Each sample was assigned to one of four age categories (coded 0–4), defined as calf (C: 0–3 years), juvenile (J: 3.01–10 years), adult (10.01–30 years) and aged (>30.01 years). Samples were allocated season (coded 0 to 3) as follows: spring (1 March–31 May), summer (1 June–31 August), fall (1 September–30 November), and winter (1 December–29 February).

3. Statistical analyses

Statistical analyses were performed using Stata statistical software (version 14; StataCorp LP, College Station, TX, USA). A two-stage mixed effects maximum likelihood (ML) regression model (Nollens et al., 2019; Robeck et al. 2017; West et al. 2015) quantifying the relationship between the dependent variable (analyte) and fixed effect variables (stage 1, animal age or age category, sex, and season) and the random effect variable, animal ID (stage 2, $n = 11$, coded 1 to 32), was used to control for the variance associated with an unequal number of repeated measures per animal. The mixed effects model was used because it can incorporate the effect of the variance associated with the unbalanced design between and the correlated repeated measures from within each animal with the effects of any independent or fixed variables to predict their collective effect on the sample means (marginal means). The variation associated with the full model (all fixed and random effects) was initially determined, and then each fixed effect variable was removed iteratively in a backward direction. The two models were then compared (with and without the individual fixed effect variable that had been removed) using the LR test at $P < 0.05$. The variable was then retained or omitted depending on whether it contributed significantly toward the model explanation (final model) of the dependent variable. For brevity, only fixed variables with significant effects were discussed in the results. All final mixed models were checked for normality using quantile plots of the standard residuals. If quantile–quantile (qnorm) plots of standardized residuals exhibited non-normal distributions, then data were transformed as predicted by the Shapiro–Wilk test (Ladder command, STATA) until residuals were normalized. Finally, pairwise comparisons of the predicted marginal means between and within the fixed variables were made while applying the Šidák correction factor at a significance level of $p < 0.01$. Reference range data for each analyte was presented as the 50th, 5th, and 95th percentile. Analyte percentiles were partitioned into the appropriate categories (age group, sex, and/or season) based on the detection of significant differences in marginal mean values in during the post-hoc analysis.

4. Results

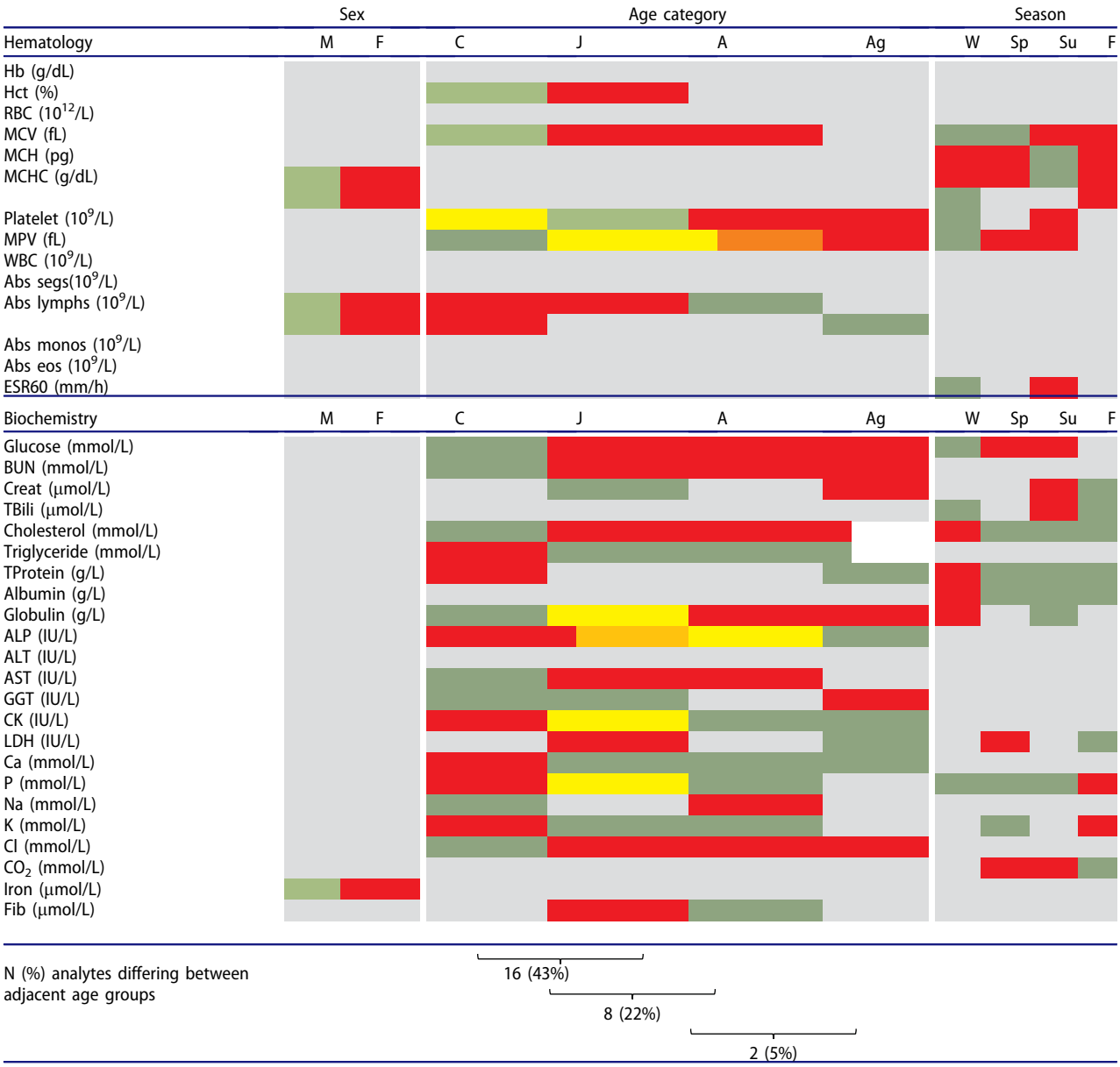
The 156 dolphins consisted of 59 males (M) and 97 females (F) (Table 1). Samples from female dolphins that were pseudo-pregnant, pregnant, or in early lactation were excluded. A total of 1426 blood samples met all inclusion criteria and were included in the study, resulting in 19,964 hematology and 32,798 biochemistry data points that were generated using consistent sample handling, processing techniques, and laboratory analytical methodology. Ages at

Table 1. Age and sex distribution of blood samples ($N = 1426$) and bottlenose dolphins (*Tursiops spp.*) sampled between 10 September 2014 and 20 October 2017.

Variable	Total samples ($n = 1426$) Sample number (%)	Dolphins in study ($n = 156$) Dolphin number (%)
Sex		
Females	880 (61.7)	97 (62.2)
Males	546 (38.3)	59 (37.8)
Age		
0–3 y	144 (10.1)	19 (12.2)
>3–10	318 (22.3)	46 (29.5)
>10–30	664 (46.6)	68 (43.6)
>30	300 (21.0)	23 (14.7)

which blood samples were drawn ranged from 4 days to 49.8 years with a respective mean and median of 18.9 and 17.0 years. The dataset consisted of 144 blood samples from 19 calves (7 M and 12 F), 318 blood samples from 46 juvenile dolphins (18 M and 28 F), 664 blood samples from 68 adults (26 M and 42 F), and 300 blood samples from 23 aged dolphins (8 M and 15 F). Three hundred and ninety-four blood samples (28%) were collected in spring, 336 (24%) were collected in summer, 402 (28%) were collected in fall, and 294 (21%) were collected in winter.

Table 2. List of 37 hematological and biochemical analytes in bottlenose dolphins (*Tursiops* spp.) and the effect of sex, age category (C: calf, J: juvenile, A: adult, Ag: aged)[a] or season (W: winter, Sp: spring, Su: summer, F: fall).[b] Gender, age or season categories between which a significant difference ($P \leq 0.01$, marginal means, sidak correction) was detected are highlighted in color, with green (lowest marginal mean), yellow (middle marginal mean) and red (highest marginal mean) within each group are listed. Analytes that did not differ are shaded in grey.



[a]C: 0 to 3 y, J: >3 to 10 y, A: >10 to 30 y, Ag: >30 y.
[b]W: Dec to < Mar, S: Mar to < Jun, Sm: Jun to < Sept, F: Sept to < Dec.
Abbreviations: Hb: hemoglobin concentration, Hct: hematocrit, RBC: red blood cell count, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, Platelet: platelet count, MPV: mean platelet volume, WBC: total white blood cell count, Abs segs: segmented neutrophil count, Abs lymphs: absolute lymphocyte count, Abs monos: monocyte count, Abs eos: eosinophil counts, ESR60: erythrocyte sedimentation rate at 60 minutes, BUN: blood urea nitrogen, Creat: creatinine, Tbili: total bilirubin, Tprotein: total protein, Globulin: total globulins, ALP: alkaline phosphatase, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma-glutamyl transpeptidase, CK: creatinine kinase, LD: lactate dehydrogenase, Ca: calcium, P: phosphorus, Na: sodium, K: potassium, Cl: chloride, Fib: fibrinogen.

Table 3. Reference ranges (50th, 5th, and 95th percentile) for bottlenose dolphin (*Tursiops* spp.) blood analytes by sex, age and season.

Blood analyte	Both sexes, all ages, all season
Hb (g/L)	141, 120–163
RBC (10^{12}/L)	3.3, 2.7–3.9
WBC (10^9/L)[a]	4930, 2960–8640
Abs segs(10^9/L)[a]	2995, 1537–5582
Abs monos (10^9/L)[a]	190, 44–518
Abs eos (10^9/L)[a]	610, 145–1702
ALT (IU/L)[a]	36, 19–67

	Female, all ages, all seasons	Male, all ages, all seasons
Iron (μmol/L)	33, 17 – 58	25, 15–53

	Both sexes, all seasons			
	0–3 years	>3–10 years	>10–30 years	>30 years
Hct (%)	41, 34–46	43, 38–47	42, 35–46	40, 34–48
BUN (mmol/L)[a]	15, 11–18	17, 14 – 21	17, 14–21	20, 14–32
Triglyceride (mmol/L)[a]	1.24, 0.55–2.52	0.67, 0.31–1.49	0.73, 0.36–1.89	0.85, 0.38–2.62
ALP (IU/L)[a]	1008, 321–3143	508, 267–929	324, 144–581	263, 144–559
AST (IU/L)[a]	153, 104–356	249, 149–443	228, 153–386	210, 139–421
GGT (IU/L)[a]	22, 13–33	25, 17–33	25, 15–43	33, 20–58
CK (IU/L)[a]	208, 110–417	144, 93–230	104, 64–191	96, 66–164
Ca (mmol/L)	2.38, 2.22–2.55	2.27, 2.12–2.45	2.25, 2.10–2.37	2.27, 2.07–2.45
Na (mmol/L)	152, 149–156	153, 148–156	154, 150–157	154, 150–157
Cl (mmol/L)	118, 113–122	120, 114–124	120, 115–125	121, 115–126
Fib (μmol/L)[a]	6.85, 4.82–9.11	6.82, 5.00–9.23	6.38, 4.70–8.85	6.59, 5.00–8.91

	Both sexes, all ages			
	Winter	Spring	Summer	Fall
MCH (pg)	43, 38–47	43, 38–47	42, 37–47	43, 38–48
ESR60 (mm/h)[a]	6, 0–22	5, 0–20	5, 0–18	5, 0–21
TBili (μmol/L)[a]	3.18, 3.08–3.28	3.44, 3.33–3.54	3.49, 3.37–3.63	3.23, 3.13– 3.35
Albumin (g/L)	45, 38–51	45, 38–51	44, 37–51	44, 38–49
$C0_2$ (mmol/L)	26, 22–33	27, 22–33	27, 21–33	26, 21 – 32

		All seasons	
	Age	Male	Female
Abs lymphs (10^9/L)[a]	Calf	1531, 558–2916	1594, 755–3262
	Juvenile	981, 280–1951	1212, 522–2419
	Adult	661, 217–1331	947, 354–1785
	Aged	624, 305–1218	953, 426–4816

	All ages		
	Season	Male	Female
MCHC (g/L)	Winter	335, 318–352	341, 322–363
	Spring	335, 320–355	340, 327–362
	Summer	331, 316–346	338, 321–365
	Fall	337, 320–360	343, 324–366

Analytes were partitioned into categories based on significant ($p < 0.01$) marginal mean differences.
[a]Analytes which were log transformed prior to analysis.
Abbreviations: Hb, hemoglobin concentration; Hct, hematocrit; RBC, red blood cell count; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, total white blood cell count; Abs segs, segmented neutrophil count; Abs lymphs, absolute lymphocyte count; Abs monos, monocyte count; Abs eos, eosinophil counts; ESR60, erythrocyte sedimentation rate at 60 minutes; BUN, blood urea nitrogen; Tbili, total bilirubin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; CK, creatinine kinase; Ca, calcium; Na, sodium; Cl, chloride; Fib, fibrinogen

Three (8%) of analytes differed between sex ($P < 0.01$; Table 2; Supplemental Tables 1 and 2). Female dolphins had higher median iron (33 μmol/L) than male dolphins (25 μmol/L). Female dolphins also had higher Abs lymphs and MCHC, but Abs lymphs and MCHC also differed between age and season, respectively (Tables 2 and 3, Supplemental Tables 1–3).

Differences ($p < 0.01$) were detected among at least some of the age categories for 23 (64%) analytes (Tables 2 and 3, Supplemental Tables 1–3). Five (36%) hematology and 18 (78%) of biochemistry values differed between age categories. A progressive upward or downward trend with age category was detected in 12 (32%) analytes (Table 2, Supplemental Table 2). MPV, glucose, BUN, globulins, GGT and Cl progressively increased with age. Abs lymphs, total protein, ALP, CK, and Ca progressively decreased with age. The number of differences detected between analytes of the adjacent age groups also decreased with age from 16 differences (43%) between calves and juvenile dolphins, to 8 (22%) between juvenile and adult dolphins, with the least differences 2 (5%) detected between the analytes of adult and aged dolphins (Table 2, Supplemental Table 2).

Table 4. Reference ranges (50th, 5th, and 95th percentile) for bottlenose dolphin (*Tursiops* spp.) blood analytes controlled for both sexes within age groups and seasons.

	0–3 years			
Variable	Winter	Spring	Summer	Fall
MCV (fL)	123, 112–132	118, 106–131	119, 110–133	118, 110–133
Platelet (10^9/L)	125, 89–234	149, 101–256	139, 106–233	135, 78–272
MPV (fL)[a]	11.0, 8.3 – 15.1	11.8, 10.1–15.5	12.2, 9.8 – 15.0	11.7, 9.3–13.8
Glucose (mmol/L)[a]	6.66, 4.55–8.66	6.88, 4.33–9.88	6.16, 4.44–9.71	6.83, 4.83–9.82
Creat (μmol/L)	124, 80–159	115, 80–168	115, 53–159	115, 80–167
Cholesterol (mmol/L)	5.67, 4.51–7.41	5.52- 3.70–8.13	5.41, 4.43–7.38	5.72, 4.17–7.69
TProtein (g/L)	66, 56–74	64, 57–73	67, 58–75	65, 55–75
Globulin (g/L)	22, 13–30	21, 15–28	21, 17–28	20, 15–27
LDH (IU/L)[a]	407, 316–642	442, 310–659	394, 298–520	432, 335–551
P (mmol/L)	1.84, 1.45–2.52	1.74, 1.00–2.26	1.81, 1.23–2.29	1.78, 1.32–2.36
K (mmol/L)	3.9, 3.4–4.6	4.0, 3.2–4.6	3.8, 3.4–4.7	4.0, 3.5–5.0
	>3–10 years			
	Winter	Spring	Summer	Fall
MCV (fL)	124, 114–135	124, 113–134	123, 113–136	126, 109–136
Platelet (10^9/L)	121, 72–174	118, 83–170	130, 89–198	120, 90–180
MPV (fL)[a]	12.1, 8.8–16.6	10.1, 12.5–17.6	11.5, 9.9–15.9	11.8, 8.8–15.2
Glucose (mmol/L)[a]	5.94, 4.61–8.21	5.49, 4.38–7.10	5.72, 4.16–7.16	5.99, 4.61–7.44
Creat (μmol/L)	114, 88–167	124, 88–186	115, 80–150	133, 97–168
Cholesterol (mmol/L)	5.23, 3.76–7.38	5.05, 3.52–6.97	4.82, 3.21–6.32	4.71, 3.08–6.89
TProtein (g/L)	71, 61–80	68, 59–78	69, 61–78	69, 61–78
Globulin (g/L)	25, 20–35	23, 17–32	24, 19–33	23, 18–32
LDH (IU/L)[a]	444, 342–607	461, 356–690	485, 380–775	449, 367–608
P (mmol/L)	1.61, 1.23–1.94	1.58, 1.29–1.84	1.61, 1.26–2.03	1.65, 1.29–2.00
K (mmol/L)	3.8, 3.4–4.3	3.7, 3.4–4.3	3.9, 3.5–4.2	3.8, 3.4–4.3
	>10–30 years			
	Winter	Spring	Summer	Fall
MCV (fL)	126, 109–141	125, 108–142	127, 116–140	132, 112–140
Platelet (10^9/L)	95, 52–142	99, 70–155	101, 63–148	82, 49–127
MPV (fL)[a]	13.2, 9.4–17.7	13.3, 10.5 – 16.8	13.6, 9.7–18.3	13.4, 9.4–19.0
Glucose (mmol/L)[a]	5.83, 4.11–7.94	5.44, 3.99–6.94	5.44, 4.00–6.94	5.49, 4.33–7.21
Creat (μmol/L)	141, 88–186	141, 97–186	142, 98–230	133, 97–185
Cholesterol (mmol/L)	201, 3.70–265	185, 123–265	183, 120–260	183, 135–251
TProtein (g/L)	71, 61–79	70, 62–78	71, 58–83	69, 61–79
Globulin (g/L)	26, 19–33	25, 19–33	27, 20–34	26, 19–32
LDH (IU/L)[a]	424, 328–565	446, 340–617	435, 308–716	426, 314–553
P (mmol/L)	1.45, 1.16–1.84	1.42, 1.10–1.81	1.49, 1.13–1.81	1.55, 1.23–1.91
K (mmol/L)	3.7, 3.4–4.1	3.7, 3.4–4.1	3.8, 3.4–4.4	3.8, 3.4–4.2
	>30 years			
	Winter	Spring	Summer	Fall
MCV (fL)	132, 112–140	132, 113–139	132, 113–141	129, 113–138
Platelet (10^9/L)	82, 49–127	88, 43–143	87, 37–130	85, 55–142
MPV (fL)[a]	14.2, 9.9–18.9	14.0, 11.9–19.4	14.3, 12.2–20.4	14.5, 9.7–20.5
Glucose (mmol/L)[a]	5.60, 4.38–7.05	5.60, 4.55–6.77	5.55, 4.33–7.16	5.61, 4.61–7.27
Creat (μmol/L)	177, 88–282	159, 97–283	177, 98–292	168, 97–274
Cholesterol (mmol/L)	5.41, 4.01–8.55	5.02, 3.68–7.30	5.15, 3.19–7.38	5.15, 3.73–7.9
TProtein (g/L)	74, 63–84	74, 57–85	71, 57–83	71, 59–82
Globulin (g/L)	28, 22–39	28, 21–37	27, 21–37	27, 21–36
LDH (IU/L)[a]	411, 310–645	404, 315–703	405, 306–602	428, 316–635
P (mmol/L)	1.52, 1.01–1.91	1.49, 1.10–1.94	1.52, 1.03–1.94	1.58, 1.20–2.00
K (mmol/L)	3.9, 3.4–4.5	3.8, 3.3–4.4	3.9, 3.5–4.3	4.0, 3.4–4.5

Abbreviations: MCV, mean corpuscular volume; Platelet, platelet count; MPV, mean platelet volume; Creat, creatinine; Tprotein, total protein; LDH, lactate dehydrogenase; Ca, calcium; P, phosphorus; Na, sodium; K, potassium

[a]Analytes were partitioned into categories based on significant ($p < 0.01$) marginal mean differences.

In total, seasonal difference was detected in 16 (44%) analytes ($p < 0.01$; Tables 2, 4, and5, Supplemental Tables 1, 2, and 4). Six (43%) hematology (MCV, MCH, MCHC, Platelet, MPV, and ESR 60) and 11 (48%) of biochemistry analytes (Glucose, TBili, Cholesterol, TProtein, Albumin, Globulin, LD, P, K, and CO_2) differed between season. The analytes most often peaked in summer ($N = 8$: MCV, Platelet, MPV, ESR60, Creat, TBili, and CO_2), followed by winter ($N = 6$: MCH, MCHC, Cholesterol, Tprotein, Albumin, and Globulin), spring ($N = 6$: MCH, MCHC, MPV, Glucose, LDH, and CO_2), and fall ($N = 5$: MCV, MCH, MCHC, P, and K). Conversely, the analytes most often dipped in winter ($N = 8$: MCV, MCHC, Platelet, MPV, ESR60, Glucose, TBili, and P), followed by summer ($N = 7$: MCH, MCHC, Cholesterol, TProtein, Albumin, Globulin, and P), fall ($N = 7$: Creat, TBili, Cholesterol, TProtein, Albumin, LD, and CO_2), and spring ($N = 6$): MCV, Cholesterol, TProtein, Albumin, P, and K). Analytes with seasonal differences differed

more frequently between the summer and winter season ($N = 12$, 32%) than between any other seasons.

5. Discussion

This study identified numerous effects of age, sex, and season on blood analytes of bottlenose dolphins under managed care, demonstrating the importance of considering the influence of intrinsic and extrinsic factors on blood data in a species of interest. Reference clinicopathological data, including baselines and RIs, are an essential tool for health assessments of individuals and populations, managed or free-ranging. References intervals should ideally be determined for each population to which these individual animals belong, thus reducing mainly extrinsic factor effects such as diet, sample storage and shipping, and analytical methodology (Stockham and Scott, 2008; Bossart et al. 2001). This is, however, impractical and at times impossible when working with non-domestic animal species. Prior to this study, RIs have been developed for only four populations of dolphins using different sample sizes, sampling designs, inclusion criteria, sample processing protocols, analytical methodology, and statistical analyses (Fair et al., 2006; Goldstein et al. 2006; Hall et al. 2007; Venn-Watson et al. 2007; Schwacke et al. 2009). Each of these can introduce biases in the values of blood analytes within a sampled population and in the factors that influence them. The reference ranges reported in this study are, in the strictest sense, only valid for the interpretation of deviations in blood analytes from the individual dolphins within this population. However, fluctuations in analytes that are consistently detected across the five differing clinicopathological datasets are more likely to represent fluctuations that result from true differences in bottlenose dolphin physiology (Table 5).

Only three (8%) analytes (MCHC, Abs lymphs and iron) differed between male and female dolphins in this population. Across hematological and biochemical surveys of bottlenose dolphins (Table 5), sex inconsistently and relatively infrequently influences analytes, with only between 8-28% of analytes affected by sex across studies of cetacean species. Of these, only serum iron was affected by sex in the majority (4 out of 5) of studies. In those populations, female dolphins, both managed and free-ranging, consistently had higher serum iron concentrations than dolphins. Female managed dolphins have higher absolute lymphocyte counts than male dolphins, but this sex difference was not present in free-ranging dolphins. Clinicopathological differences between male and female dolphins could be a product of social habits (Venn-Watson et al., 2007) or endocrine or reproductive differences (Robeck and Nollens, 2013). We excluded samples from female dolphins that were pseudo-pregnant, pregnant, or in early lactation, but did not eliminate any dolphins experiencing seasonal reproductive cycles.

A total of 23 (64%) analytes (Hct, MCV, Platelet, MPV, Abs lymphs, glucose, BUN, creatinine, cholesterol, triglyceride, TProtein, globulin, ALP, AST, GGT, CK, LD, Ca, P, Na, K, Cl, and fibrinogen) differed by age in this *ex situ* bottlenose dolphin study population. The effect of age was evaluated in all six *in situ* and *ex situ* delphinid study populations, and between 12% and 100% of blood analytes were found to change with age. Age was consistently a greater influence in managed than in free-ranging delphinids, with 64%, 83%, and 100% of analytes affected in managed delphinids compared to 12%, 18%, and 35% in free-ranging delphinids. This is likely because the age data of managed bottlenose dolphins is more accurate and reliable, and because more older managed care dolphins were available for study. The estimated age of the oldest enrolled free-ranging dolphin was 26 years old (Goldstein et al., 2006), whereas 23 bottlenose dolphins over the age of 30 were represented in this study alone. The age or age class of free-ranging dolphins had to be estimated from tooth growth layer groups after extracting a tooth (Goldstein et al., 2006; Hall et al. 2007) or from total body length (Schwacke et al., 2009). Because of this limitation, the adult (10–30 years) and aged (>30 years) groups had to be combined in one free-ranging population (Hall et al., 2007).

Presumably for these same reasons, no changes with age were previously identified in free-ranging delphinids. On the other hand, Hct, platelet counts, Tprotein, Ca and P were exclusively and consistently affected by age in all three delphinid populations in managed care. The trends in Hct and Na with age varied between populations. However, platelet counts progressively decreased. In other species, genetic factors, environmental factors, and various diseases have been proposed as causes for age-related decreases in platelets. However, megakaryocytes are the precursor cells of platelets, and dolphins, unlike most other mammals, have megakaryocytes in both the bone marrow and spleen, a proposed physiological adaptation of diving mammals (Cowan and Smith, 1999). The decrease in platelet counts with age could be a normal sequela to this unique adaption and/or may reflect senescence of the bone marrow microenvironment (McMahon and Kwaan, 2014). Total protein progressively increased with age in all three populations. This increase, paired with the increase in globulins, is likely the product of cumulative humoral immune challenge/antigenic stimulation over time. Serum Ca and P consistently decreased with age. These

Table 5. Reported effects of sex, age or season on 37 hematology and biochemical analytes in different delphinid species (bottlenose dolphin, *Tursiops truncatus* and killer whale, *Orcinus orca*) and populations are indicated (+).

Blood analyte	Bottlenose dolphin, managed, land-based[a]			Bottlenose dolphin, managed, coastal pen[b]			Bottlenose dolphins, free-ranging[c]			Bottlenose dolphins, free-ranging[d]			Bottlenose dolphins, free-ranging[e]			Killer whale, managed, land-based[f]			% Studies with effect detected		
N animals	156			52			255			143			59			32					
N samples	1426			1113			255			417			59			2823					
P cut-off	0.01			0.01			0.05			0.05			0.05			0.01					
Hematology	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season
Hb																	+	+		20	33
Hct		+			+		+						+				+	+	33	50	33
RBC							+			+	+	+					+	+	40	40	66
MCV		+	+														+	+		40	66
MCH			+							+	+	+		+			+		20	60	66
MCHC	+		+														+		20	20	33
Platelet		+			+												+			50	
MPV		+	+													+	+			33	33
WBC				+	+												+	+	16	33	33
Abs segs					+						+			+			+	+		66	33
Abs lymphs	+	+		+	+			+									+		33	66	
Abs monos																	+			16	
Abs eos				+	+												+		16	33	
ESR60			+		+												+			50	33

Biochemistry	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season
Glucose		+	+	+				+			+	+					+	+	16	66	100
BUN		+			+			+					+	+			+	+	16	83	33
Creat		+	+	+	+		+	+									+	+	33	66	66
TBili			+														+			16	33
Cholesterol		+	+		+			+					+				+	+	16	66	66
Triglyceride		+			+			+					+	+		+	+	+	33	83	33
TProtein		+	+		+												+	+		50	66
Albumin			+		+								+				+	+		33	100
Globulin		+	+		+			+									+	+		66	66
ALP		+			+			+									+	+		66	33
ALT					+												+	+		33	33
AST		+			+			+									+	+		66	33
GGT		+		+	+			+									+	+	16	66	33
CK		+			+									+	+		+		16	66	
LD		+	+		+			+									+			66	33
Ca		+			+								+				+			50	33
P		+	+		+											+	+	+	16	50	66
Na		+			+									+			+	+	16	50	33
K		+	+	+													+		16	33	33
Cl		+															+	+		33	33

(*continued*)

Table 5. Continued.

Biochemistry	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season	Sex	Age	Season
CO_2			+											+			33	33
Iron	+			+	+	+					+			+		66		
Fib		+												+			100	
N analytes affected	3	23	16	8	24		4	12		2	4	5	7	6		3	37	21
% affected of analytes assayed	8%	64%	44%	28%	83%		12%	35%		6%	12%	15%	21%	18%		8%	100%	57%

Effects that were not assayed are shaded in grey.
[a]This study.
[b]Venn-Watson et al. (2007).
[c]Schwacke et al. (2009).
[d]Hall et al. (2007).
[e]Goldstein et al. (2006).
[f]Nollens et al. (2019).

decreases in Ca and P were largest between the youngest age classes and likely reflect active deposition of bone. (Stockham and Scott 2008; Bossart et al. 2001; Nollens et al. 2019; Fair et al. 2006; Venn-Watson et al. 2007; Norman et al. 2013; Robeck and Nollens, 2013).

In addition, analytes that were recognized to be affected by age in at least some free-ranging populations in addition to all managed care population included Abs lymphs, glucose, BUN, Creat, Cholesterol, Triglyceride, ALP, AST, GGT, CK, LD. Of these, absolute lymphocyte counts, serum creatinine, ALP, CK, and LDH showed progressive upward or downward trends with age, as would be expected with the true physiological changes associated with aging. The progressive decrease in absolute lymphocytes supports that delphinids of advancing age naturally experience immune senescence (Nollens et al., 2019). Immune senescence develops as the thymus shrinks which results in a decline of naïve T cells (Boule and Kovacs, 2017; Isobe et al. 2017). With this decrease, T-cell receptor repertoire diversity narrows and B cell-induced antibody affinity declines with aging. Immune senescence leads to both decreased responses to acute infections and poor development of immunological memory (Boule and Kovacs, 2017; Isobe et al. 2017). Creatinine increased progressively with age. Creatinine is a breakdown product of creatine phosphate by creatinine kinase in muscle and is, in healthy animals, a reflection of muscle mass (Geraci and St. Aubin, 1979). The muscle mass of delphinids does not continually increase with advancing age. In fact, CK and LDH, both markers of muscle metabolism, decrease with age. Creatinine should therefore be interpreted here as a renal clearance assay. This finding suggests that renal perfusion or clearance decreases in aging delphinids. The progressive decrease in ALP activity with age, like Ca and P, likely reflects active bone growth or re-modeling in younger animals (Stockham and Scott, 2008; Bossart et al. 2001; Nollens et al. 2019).

Glucose decreased progressively with age in all four study populations in which an effect of age was detected. However, this effect of age was not detected in one managed bottlenose dolphin population (Table 5). Hypothesized reasons for the difference in this one population included older age, overnight fasting, larger meal sizes with higher purine loads, deviations from circadian rhythms, genetics, and/or insulin resistance (Venn-Watson et al. 2012; Venn-Watson et al. 2013).

A total of 16 (44%) analytes differed seasonally in this bottlenose dolphins study population. The effects of season have been assayed previously in only three delphinid (bottlenose dolphins and killer whales) study populations (Nollens et al., 2019;

Macchi et al. 2011), all of which are located in the southeastern and southwestern USA where only mild seasonal changes are expected. In these, between 15% and 57% of analytes were influenced by season. Even though, some of the annual circadian patterns, such as food availability, changes in diving patterns, body condition, and water temperature, to which seasonal variation was attributed in free-ranging cetaceans, are less pronounced for delphinids in managed care, seasonal fluctuations were more prevalent in managed populations (44% and 57%) than in the free-ranging population (15%) (Table 5). No seasonal variability was detected exclusively in managed cetacean populations and only one analyte (Ca) varied seasonally exclusively in the free-ranging delphinids (Table 5). Samples from female delphinids in managed care that were pseudo-pregnant or pregnant were removed, whereas these states cannot be detected, and therefore excluded, from the free-ranging delphinids. These reproductive events often occur seasonally (Robeck et al., 2004) and may have masked seasonal effects due to changes in photoperiod, water temperature, or food availability in the free-ranging population.

Only glucose and albumin varied seasonally in all three study populations in which the effect of season was assayed. Seasonal trends in serum glucose concentrations of dolphins were opposite to those in killer whales (Nollens et al., 2019). Bottlenose dolphins preferentially inhabit temperate and tropical waters, whereas killer whales are in effect cold water dolphins. The opposite seasonal trend in serum glucose could be a reflection of the opposite thermoregulatory demands in the presence of water temperature fluctuations. However, albumin peaked in winter in all three study populations. In healthy animals, fluctuations in albumin are most commonly a reflection of hydration and food protein intake (Allison, 2005). Cetaceans derive both their nutrition and hydration from their foodfish (Nollens et al. 2017). The consistently observed peak in albumin in winter may therefore reflect the seasonal fluctuations in food intake in response to varying thermoregulatory demand.

Establishing ranges for hematological and biochemical analytes in clinically healthy animals is in important step for the interpretation of these data during the care and diagnostics of managed and stranded animals. Such reference data are also used for population health investigations. Across studies, including this current study, many clinicopathological analytes were influenced by sex, age and season. From this, it is apparent that these influences need to be considered when interpreting blood analytes of delphinids as they drive physiological fluctuations in these species. Effects of sex, age, or season that are not consistently found in the majority of published studies are still important when interpreting blood analytes for individuals within each of those populations, as they may be an artefact of sampling, and laboratory or analytical method specific to that reference population. Wild delphinids are increasingly exposed to environmental stressors (Fair and Becker, 2000; Bossart 2011). Recognizing the true physiological trends that are consistently present across populations, such as those identified in this study, will aid and strengthen investigations into the impact of these stressors on wild delphinids, since it is impractical or impossible to establish normal ranges for each free-ranging delphinid population.

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