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VARIATION IN HEMATOLOGIC AND SERUM BIOCHEMICAL VALUES OF BELUGAS (*DELPHINAPTERUS LEUCAS*) UNDER MANAGED CARE

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Abstract: Blood analytes are critical for evaluating the general health of cetacean populations, so it is important to understand the intrinsic variability of hematology and serum chemistry values. Previous studies have reported data for follow-up periods of several years in managed and wild populations, but studies over long periods of time (>20 yr) have not been reported. The study objective was to identify the influences of partitioning characteristics on hematology and serum chemistry analytes of apparently healthy managed beluga (*Delphinapterus leucas*). Blood values from 31 managed belugas, at three facilities, collected over 22 yr, were assessed for seasonal variation and aging trends, and evaluated for biologic variation among and within individuals. Linear mixed effects models assessed the relationship between the analytes and sex, age, season, facility location, ambient air temperature, and photoperiod. Sex differences in analytes and associations with increasing age were observed. Seasonal variation was observed for hemoglobin, hematocrit, mean corpuscular volume, monocytes, alkaline phosphatase, total bilirubin, cholesterol, and triglycerides. Facilities were associated with larger effects on analyte values compared to other covariates, whereas age, sex, and ambient temperature had smaller effects compared to facility and season. Present findings provide important baseline information for future health monitoring efforts. Interpretation of blood analytes and animal health in managed and wild populations over time is aided by having available typical levels for the species and reference intervals for the degree to which individual animals vary from the species average and from their own baseline levels during long-term monitoring.

Key words: Beluga, blood chemistry, cetacean, *Delphinapterus leucas*, hematology, mixed effects models, variation.

INTRODUCTION

Measurements of hematologic and serum biochemical analytes are used to monitor organ function and detect diseases in marine mammals, allowing for characterization of changes in the health of a population and its members. Despite the common use of blood analytes to monitor the health of populations of marine mammals, relatively little has been published about variation in hematologic and serum biochemical values in cetaceans.[3,4,12,21,28] Differences in analyte values between and within individuals may indicate clinical disease or subclinical abnormalities, but may also be found in healthy individuals, reflecting differences in individual characteristics such as sex and age or differences in environmental factors such as seasonal changes, nutrients, and daylight length (photoperiod).[7,12,16,28]

A number of studies have examined circannual variation in blood analytes in terrestrial and aquatic species such as wild rodents and small mammals,[20,23] dogs,[26] wild ungulates,[8] managed pinnipeds,[29] humans,[15] and vertebrates.[9] Relatively few studies have addressed the influence of season, sex, and age in managed and wild cetacean populations.[12,28] Results of studies of managed populations are particularly valuable, despite limited sample sizes, because they allow for longer-term follow-up of individuals, compared to the logistical challenges associated with monitoring individuals in wild populations over time. Previous studies of managed cetacean populations have explored hematology and serum chemistry in small populations of animals over short time periods or without exploring variation patterns within the individual blood analytes.[4,25]

This study investigated variability in hematologic and serum chemical analytes at the level of the entire managed population and at that of the

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individual whale using data from samples collected over a 22-yr period between 1988 and 2010. The main study objectives were 1) to characterize the typical values of a wide panel of hematology and serum chemistry analyte values of apparently healthy belugas (*Delphinapterus leucas*) maintained in captivity; 2) to assess the impact of individual (age, sex) and environmental factors, such as seasonal variation, temperature, and photoperiod, on analyte values; and 3) to provide reference ranges for typical variation among and within belugas. The results may also have applications to free-ranging cetaceans, by providing reference levels for monitoring of clinical blood values from wild populations of marine mammals from individual observations and from repeated captures for health assessments and blood sampling.

MATERIALS AND METHODS

Study population

Thirty-one captive belugas (11 males, 20 females) underwent monthly blood sampling for varying periods of time over 22 yr (1988–2010), with additional sampling when warranted by the whale's medical or behavioral condition (Table 1). Twelve whales were housed in San Antonio (SA), Texas (29°26′N, 98°39′W); 9 in Orlando (OR), Florida (28°24′N, 81°27′W); and 10 in San Diego (SAD), California (81°45′N, 117°13′W). Mean length of follow-up from first to last sampling, and sex and age distributions at each of the facilities are summarized in Table 1. All whales were fed the same high-quality diet throughout the year, consisting of several species of fish caught at one point in time and subsequently deep-frozen until time of consumption. All animals were maintained in a marine environment in outdoor pools, in natural seawater at the San Diego facility, and artificial seawater in Orlando and San Antonio, at a constant water temperature of approximately 11–12°C. Animal management practices were consistent across all the facilities. All whales received vitamin and mineral supplementation.

Inclusion and exclusion criteria for blood analyte values were selected to restrict data to those representing normal, apparently healthy animals. First, analysis was restricted to blood values for each animal that fell within internal reference ranges established at each facility, plus those that were within ±3 SD on either side of their low and high reference range limits. Blood values were excluded from analyses if the reason for the blood draw was given as anything other than routine sampling or was suggestive of the presence of disease. Values were also excluded if they occurred within 30 days prior to a whale's death or if the animal presented with chronic illness defined by abnormal analyte values outside the ±3 SD, resulting in 1,379 hematologic and 1,391 serum chemistry data points. Lastly, 11 (0.8%) of these remaining hematologic data points were excluded from analyses due to moderate to severe sample hemolysis, as were 55 (4%) of the remaining serum chemistry data points.

Blood collection and processing

Most whales were trained to present their flukes above the water's surface so that blood could be drawn, cleanly and without restraint, from veins on the ventral surface. Blood was collected by venipuncture from the periarterial venous rete using a 19 gauge, 1/2-inch butterfly catheter or a 19 gauge, 1.5-inch needle (Becton, Dickinson, and Co., Franklin Lakes, New Jersey 07417, USA) after the skin was cleaned with an iodine solution, alcohol, or both. Whole blood was collected into a thrombin-supplemented serum separator tube and an ethylenediaminetetraacetic acid (EDTA) Vacutainer® tubes (BD Vacutainer, Becton, Dickinson), the latter being gently rocked for approximately 1 min. All tubes were immediately processed at an onsite laboratory.

Blood smears for differential white cell counts were made within a few minutes of sampling. Meanwhile, the serum separator tubes were allowed to sit for approximately 60–90 min until a clot had formed to minimize hemolysis, followed by centrifugation for approximately 5 min to separate the serum from the cells. Hematology tests (n 15 analytes), including hemoglobin (HB), hematocrit (HCT), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cells (WBC), were performed using a CellDyn analyzer (Abbott Laboratories, Abbott Park, Illinois 60064, USA). Manual hematologic techniques were used for WBC using the 1:100 dilution and RBC counts using a Unopette system (Becton-Dickinson). Duplicate blood smears were prepared using EDTA-treated blood and stained with the Wright-Giemsa method to perform manual leukocyte differentials based on counting 100 WBC per slide. Packed cell volume was quantified by centrifugation to confirm the automated HCT results at the San Antonio facility, and occasionally at the San Diego and Orlando facilities.

Table 1. Demographic features of belugas under managed care sampled for hematology and serum chemistry from 1988–2010. SA San Antonio, OR Orlando, SAD San Diego.

	No. of times sampled					
Whale ID	Hematology	Serum chemistry	Years sampled	Sex	Age sampled (yr)	Facility
1[a]	54	43	6	M	0–6	OR
2	47	47	5	F	22–26	SAD
3	33	33	3.3	M	3–7	OR
4[a]	93	86	14	F	2–16	OR
5	5	5	0.5	F	0.1–0.6	SA
6[a]	15	14	0.6	F	4–5	SAD
7	103	102	21	F	3–24	SA
8	36	33	6	M	34–40	SAD
9	29	29	2.5	M	0.5–3	SA
10[a]	2	2	1	M	1–2	OR
11	35	30	3.2	M	5–8	OR
12	33	33	3.3	M	4.5–8	OR
13[a]	23	18	2	F	0–2	OR
14[a]	16	15	0.6	M	0–1	SAD
15	54	53	8.5	F	0–9	SA
16	12	14	1.3	F	4–5	OR
17	144	145	22	F	3–24	SA
18[a]	110	109	20	F	2–22	SA
19[a]	31	28	6	F	34–40	SAD
20	99	93	13	M	14–27	SAD
21	6	6	0.3	F	31.0–31.3	SA
22	15	14	2.5	M	0.5–3.0	SA
23	2	3	0.1	F	0–0.1	SA
24	116	110	13	F	19–32	SAD
25[a]	4	4	0.1	F	0–0.1	SAD
26[a]	30	31	8	M	8–15	OR
27	117	116	17	F	8–25	SA
28[a]	26	21	1.2	F	0–1.2	SAD
29[a]	50	49	1	F	28–29	OR
30	44	48	10	F	0–11	SA
31[a]	2	2	0.1	F	0–0.1	SA
Total	1,386	1,336				

[a] Whale died during study period.

Clinical chemistry analytes (n 22) included glucose (GLU), creatinine (CRE), triglycerides (TRI), total protein (TP), albumin (ALB), globulin (GLB) calculated by difference, blood urea nitrogen (BUN), phosphorus (PHOS), alkaline phosphatase (AP), alanine aminotransferase (ALT), aspartate aminotransferase, total bilirubin (TBIL), cholesterol (CHOL), gammaglobulin transferase (GGT), creatinine phosphokinase (CPK), lactate dehydrogenase (LDH), calcium (CA), sodium (NA), potassium, chloride (CL), total iron (FE), and iron binding. Over the study period, standard analytical techniques were used to obtain serum chemistry values with analyzers that included the Gilford 203S (Ciba Corning Diagnostics, Oberlin, Ohio 44074, USA) with electrolytes determined on a Gilford Chemlyte (February 1988–January 1990), a Ciba-Corning 550 Express with electrolytes determined on a Ciba-Corning Fast 4 (Ciba Corning Diagnostics) (January 1990–December 2004), and the Olympus AU400E (Olympus America Corporate, Center Valley, Pennsylvania 18034, USA) (January 2004–present). At each update of analyzer model over the years, all facilities used the same analyzer during a given time period. Standardization of results was maintained using the same batch of control standards at all the facilities; however, results of control runs were not available for inclusion in the present analyses.

Statistical analyses

Totals of 1,386 hematology and 1,336 clinical chemistry data points were used in the analyses. Significant differences in mean analyte values

between the sexes were determined with the Student's *t*-test. Circannual patterns were assessed three ways. First, observations were grouped by month (season), (*n* number of hematology, serum chemistry data points) with winter (December–February; *n* 357, 339), spring (March–May; *n* 361, 349), summer (June–August; *n* 375, 366), and autumn (September–November; *n* 293, 282). Significant seasonal differences in mean analytes were evaluated with the Kruskal-Wallis test. Second, mean monthly temperatures for each of the four facility locations were obtained from historical climatologic data reports from the National Climatic Data Center.[18] Annual ambient temperature ranges were 0°C (winter) to the low to mid-30s°C (summer) in San Antonio, 23°C to 33°C in Orlando, and 19°C to 25°C in San Diego. Lastly, circannual daily daylight length (photoperiod) data at each facility was attained from duration of daylight–darkness tables from the United States Naval Observatory.[31]

A series of linear mixed effects models was used to assess the relationship between the analytes and six factors: sex, age at observation (continuous), season, facility location, ambient air temperature, and photoperiod.[30] The remaining, unexplained analyte variation was partitioned into that attributable to systematic differences among individual whales (interwhale variation) and that attributable to variations along the trajectory of measurements for an individual (intrawhale variation). All measurements on a given analyte meeting entry criteria were included, even animals having unequally spaced or differing numbers of observations. The intrawhale variation is meant to describe variation over time for an individual whale, not variation within samples at a single point in time. Estimation of partitioning factors (fixed effects of the six factors and interwhale and intrawhale variation) was by restricted maximum likelihood[33] using the Stata "xtmixed" command for mixed-effects models (Stata Corp., College Station, Texas 77845, USA).

Models were first fitted without predictors, to provide descriptive summaries of overall inter- and intrawhale variation. Demographic (sex and age) and environmental (facility, season, temperature, photoperiod) factors were next examined individually using univariable logistic regression. Sex (male or female), facility (three sites), season (four categories), and photoperiod (10–12.5 or 12.6–14 daylight hours) were coded as categorical variables, whereas age and temperature were continuous. Univariable associations with $P <$ 0.15 were further evaluated in multivariable mixed-effects models.[13] Secondary models were fitted with analyzer type as a covariate to assess the role of analyzer model as a confounder of the association between facility location and analyte value. The San Antonio facility was chosen as the referent group because of its large *n* compared to the other groups, which allowed for stable coefficients in the model results. To evaluate season as a proxy for ambient temperature and photoperiod, season was not included in the final multivariable models, but instead was replaced by ambient temperature and photoperiod at that facility for each data point. Interaction between season and facility location was tested to evaluate whether season effects differed across facilities, and residual analysis was used to assess model fit. Graphs were constructed using R (The R Project for Statistical Computing; http://cran.r-project.org/).

RESULTS

Unadjusted analyses

In unadjusted analyses, more than half the analytes (*n* 21/37; 57%) showed larger intrawhale, versus interwhale, variation over the course of the animal's life. This was most pronounced in hematologic, protein, and liver indices, whereas HB, MCV, MCH, reticulocytes (RETIC), lymphocytes, basophils, TP, ALB, GLB, AP, ALT, TBIL, CHOL, GGT, CPK, and CA showed smaller intrawhale variation versus interwhale (Tables 2, 3). As an example, the greater intrawhale variation (3.45) observed for HCT, compared to interwhale variation (3.34), indicates that out of the total variation observed for HCT levels, a greater proportion is attributable to variability in the animal's physiologic status (e.g., reproductive state, nutritional status, stress, underlying disease) versus variability between whales such as with sex and facility. The remaining analytes showed very similar inter- and intraindividual variation.

Effects of univariable predictors

Significant sex-specific differences were noted for most of the hematologic analytes (Table 4). HB, HCT, RBC, WBC, lymphocytes, and monocytes were greater in males than in females, whereas MCV, MCH, MCHC, nucleated red blood cells (NRBC), RETIC, bands, and segmented neutrophils were greater in females. Age was independently associated with all the analytes except segmented neutrophils, monocytes, eosinophils, basophils, and iron binding, with some

Table 2. Coefficients (SE) for associations of whale facility location, age, sex, and photoperiod with hematology analytes in belugas under managed care, 1988–2010, derived from linear mixed effects models. Coefficient *P*-value is indicated, with significant independent variables in bold (P 0.05).

Analyte[a]	Orlando[b]		San Diego[b]	
	Coefficient (SE)	*P*	Coefficient (SE)	*P*
HB (g/dl)	1.61 (0.64)[c]	**0.01**	1.08 (0.57)	0.06
HCT (%)	4.47 (1.52)	**0.003**	2.86 (1.36)	**0.04**
RBC (× 10^6/l l)	0.13 (0.06)	**0.04**	0.003 (0.06)	0.95
MCV (fl)	5.76 (3.20)	0.07	7.39 (2.85)	**0.01**
MCH (pg)	2.79 (1.71)	0.10	3.52 (1.52)	**0.02**
MCHC (g/dl)	0.09 (0.22)	0.67	0.10 (0.20)	0.62
NRBC (no./100 WBC)	0.22 (0.07)	**0.004**	0.02 (0.07)	0.79
RETIC (%)	0.70 (0.32)	**0.03**	0.79 (0.31)	**0.01**
WBC (× 10^3/l l)	1103.94 (589.57)	0.06	455.21 (532.52)	0.39
Bands (× 10^3/l l)	7.47 (26.10)	0.78	10.80 (23.91)	0.65
Segmented neutrophils (× 10^3/l l)	1185.30 (407.76)	**0.004**	558.31 (369.07)	0.13
Lymphocytes (× 10^3/l l)	228.45 (280.36)	0.42	464.88 (252.76)	0.07
Monocytes (× 10^3/l l)	33.36 (95.96)	0.73	109.44 (87.07)	0.21
Eosinophils (× 10^3/l l)	93.91 (59.48)	0.11	61.56 (53.90)	0.25
Basophils (× 10^3/l l)	10.89 (27.63)	0.69	46.58 (24.60)	0.06

[a] HB: hemoglobin; HCT: hematocrit; RBC: red blood cell count; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; NRBC: nucleated red blood cells; RETIC: reticulocytes; WBC: white blood cell count.

[b] Referent facility was San Antonio.

[c] All coefficients were adjusted for all of the variables listed.

analytes increasing (RETIC, bands, monocytes, GLB, BUN, PHOS, TBIL, LDH, CL), and the remaining decreasing with age.

Significant seasonal differences (P 0.05) were found for MCV, MCHC, and lymphocytes, but patterns varied considerably. Levels of MCV were lowest during winter, and peaked during early spring. Mean MCHC spiked in late summer, but remained fairly constant the rest of the year; however, corresponding changes in HB and HCT were not evident for either MCV or MCHC, nor were any seasonal changes noted in MCV and MCHC that might be explained by any changes in the values of HB and HCT (Fig. 1A).

Many of the serum analytes demonstrated significant seasonal differences: GLU, CRE, TRI, ALB, GLB, PHOS, AP, ALT, GGT, CPK, LDH, CA, NA, CL, FE, and iron binding. Those serum chemical analytes with highly significant (P 0.001) season differences are shown in Figure 1. Renal function analytes (CRE, CL) peaked during different seasons with maximum CRE in mid-summer (1.4 ± 0.04 mg/dl) (Fig. 1B), and CL in early spring (120.3 ± 0.5 mEq/L) (Fig. 1E). AP and ALT both peaked over the winter season, with AP's nadir in late spring and ALT's over late winter–early spring (Figs. 1C and D, respectively). Iron and iron binding displayed a relatively similar circannual pattern, but with iron binding peaking and reaching a nadir 2 mo before FE (Fig. 1F). Significant facility differences were noted for all analytes during univariable analyses with the exception of basophils, CHOL, and FE.

Multivariable models

Multivariable models included the individual demographic predictors (age, sex) and environmental factors (location, photoperiod); season and temperature were strongly collinear with photoperiod across all sites and were thus was omitted (Tables 2, 3). In addition, interaction terms were used to test for effect modification. Many analytes differed significantly across facilities (HB, HCT, RBC, MCV, MCH, NRBC, RETIC, segmented neutrophils, CRE, GLB, PHOS, ALT, TBIL, and LDH) with several analytes having more than one significant location (HCT, RETIC, and TBIL). Facility locations were associated with the largest effect size for hematology and serum chemistry compared to the remaining covariates (Tables 2, 3). Highly significant ($P < 0.001$) age effects persisted after adjusting for other factors for HB, HCT, RETIC, bands, lymphocytes, lymphocytes, and all of the serum chemistry analytes, except PHOS, CHOL, and iron binding, with many of these same analytes also determined to be significant in the univariable analyses. Sex effects were less prom-

Table 2. Extended.

Age		Sex		Photoperiod		Standard deviation	
Coefficient (SE)	*P*	Coefficient (SE)	*P*	Coefficient (SE)	*P*	Interwhale	Intrawhale
0.04 (0.01)	**<0.0001**	0.05 (0.54)	0.92	0.17 (0.07)	**0.01**	1.27	1.21
0.08 (0.02)	**<0.0001**	0.27 (1.29)	0.84	0.53 (0.18)	**<0.0001**	3.00	3.34
0.004 (0.001)	**<0.0001**	0.10 (0.05)	0.07	0.01 (0.009)	0.16	0.13	0.16
0.05 (0.03)	0.09	2.85 (2.72)	0.30	0.47 (0.26)	0.08	6.44	4.80
0.05 (0.02)	**0.003**	1.87 (1.45)	0.20	0.33 (0.13)	**0.01**	3.45	2.43
0.01 (0.004)	**0.004**	0.21 (0.19)	0.27	0.008 (0.05)	0.88	0.40	0.91
0.003 (0.002)	0.17	0.04 (0.06)	0.55	0.04 (0.04)	0.32	0.09	0.68
0.02 (0.004)	**<0.0001**	0.15 (0.28)	0.59	0.03 (0.03)	0.41	0.61	0.52
31.89 (9.83)	**0.001**	347.05 (499.51)	0.49	118.29 (90.42)	0.19	1,141.20	1,653.63
3.19 (0.57)	**<0.0001**	13.22 (22.08)	0.55	5.14 (5.86)	0.38	47.88	106.25
5.22 (7.09)	0.46	582.97 (345.37)	0.09	164.40 (66.09)	**0.01**	784.68	1,208.56
28.01 (4.43)	**<0.0001**	757.38 (237.66)	**0.001**	79.29 (40.13)	**0.05**	546.28	733.51
0.96 (1.74)	(0.55	96.48 (81.33)	0.24	45.46 (16.49)	**0.01**	183.46	300.27
0.35 (1.04)	0.74	33.50 (50.40)	0.51	16.10 (9.77)	0.10	114.40	175.72
0.45 (0.23)	**0.05**	29.35 (23.55)	0.21	1.45 (1.96)	0.46	55.88	35.64

inent in the multivariable analyses, with significant differences between sexes observed only for lymphocytes, TBIL, and iron binding. Several analytes were significantly associated with photoperiod, with HCT, TRI, AP, TBIL, and CPK highly significant.

DISCUSSION

This study, which examined the range of blood analyte variation that apparently healthy managed belugas are likely to track longitudinally over a multi-year time span, found multiple factors to be important predictors of analyte levels. Healthy individuals vary from each other, and even more within their trajectories over time, but it is possible to identify values occurring outside those ranges. When investigating health trends over time, partitioning in blood analytes due to facility, age, sex, and other biological factors must be taken into consideration. In this study, not only these factors, but also inter- and intrawhale random variation (that variation due to chance or uncontrollable factors that are inherent in the system used to sample and run the analytes), could be assessed using mixed effects models, because this large data set contained serial blood samples collected from the same animals over long periods of time, many years in some whales. Individual whale variability was controlled for with modeling of serial analyte measures collected from the same clinically normal animals over many years. Age, facility, season, and sex were all found to be important determinants for the majority of the analytes measured in belugas.

Animal-related factors

Evidence for physiologic developmental changes associated with maturation was evaluated by examining age-related differences. Age is known to affect a number of hematologic and serum chemistry analytes in wildlife species and thus was included in the multivariable models as an important predictor.[10,32] In this study, the beluga data set was more heavily populated by younger animals during the sampling period, potentially limiting the ability to characterize the true impact of older age.

Sex alone was associated with a number of differences in the circulating analytes examined, but these differences may not be biologically or clinically significant. Differences were pronounced in belugas for most of the hematologic analytes; however, almost all became insignificant once age and site were controlled for in the final multivariable models. Recognizing that reproductive status may influence the values of certain analytes such as RBC parameters, WBC, and segmented neutrophils, the significant differences noted between males and females (Table 4) may be partially attributed to pregnancy, seasonal shifts in reproductive hormones, environmental changes, other physiologic effects not specifically evaluated in this study, or confounded by other factors such as facility site.[6,27]

Table 3. Coefficients (SE) for associations of whale facility location, age, sex, and photoperiod with serum chemistry analytes in belugas under managed care, 1988–2010, derived from linear mixed effects models. Coefficient *P*-value is indicated, with significant independent variables in bold (P 0.05).

Analyte[a]	Orlando[b]		San Diego[b]		Age	
	Coefficient (SE)	*P*	Coefficient (SE)	*P*	Coefficient (SE)	*P*
GLU (mg/dl)	4.87 (5.34)[c]	0.36	0.51 (4.82)	0.92	0.53 (0.08)	**<0.0001**
CRE (mg/dl)	0.14 (0.11)	0.19	0.36 (0.10)	**<0.0001**	0.01 (0.002)	**<0.0001**
TRI (mg/dl)	18.34 (29.58)	0.54	29.37 (26.51)	0.27	4.15 (0.43)	**<0.0001**
TP (g/dl)	0.71 (0.28)	0.01	0.49 (0.25)	0.05	0.02 (0.003)	**<0.0001**
ALB (g/dl)	0.07 (0.25)	0.77	0.37 (0.23)	0.11	0.06 (0.002)	**<0.0001**
GLB (g/dl)	0.60 (0.18)	**0.001**	0.16 (0.16)	0.32	0.03 (0.002)	**<0.0001**
BUN (mg/dl)	1.59 (2.50)	0.53	3.80 (2.26)	0.09	0.21 (0.04)	**<0.0001**
PHOS (mg/dl)	0.30 (0.21)	0.15	0.52 (0.19)	**0.01**	0.01 (0.004)	**0.05**
AP (IU/L)	17.81 (26.01)	0.49	40.78 (23.74)	0.09	5.87 (0.30)	**<0.0001**
ALT (IU/L)	1.77 (1.71)	0.30	4.44 (1.53)	**0.004**	0.28 (0.02))	**<0.0001**
AST (IU/L)	2.43 (5.28)	0.65	0.30 (4.78)	0.95	0.38 (0.09)	**<0.0001**
TBIL (mg/dl)	0.10 (0.04)	**0.03**	0.12 (0.04)	**0.01**	0.004 (0.001)	**<0.0001**
CHOL (mg/dl)	3.75 (18.36)	0.84	12.25 (16.47)	0.46	0.54 (0.24)	**0.02**
GGT (IU/L)	1.22 (3.39)	0.72	5.10 (3.04)	0.09	0.20 (0.04)	**<0.0001**
CPK (IU/L)	11.93 (22.35)	0.59	23.44 (25.05)	0.24	2.97 (0.28)	**<0.0001**
LDH (IU/L)	20.97 (17.23)	0.22	36.46 (16.08)	**0.02**	2.45 (0.33)	**<0.0001**
CA (mg/dl)	0.19 (0.26)	0.46	0.15 (0.24)	0.53	0.06 (0.003)	**<0.0001**
NA (mmol/L)	0.17 (1.02)	0.87	0.65 (0.98)	0.51	0.09 (0.02)	**<0.0001**
K (mmol/L)	0.21 (0.16)	0.20	0.05 (0.15)	0.75	0.01 (0.002)	**<0.0001**
CL (mmol/L)	2.19 (1.48)	0.14	0.68 (1.34)	0.61	0.17 (0.02)	**<0.0001**
FE (l g/dl)	22.15 (26.78)	0.41	18.46 (24.56)	0.45	2.95 (0.56)	**<0.0001**
Iron binding (l g/dl)	2.89 (14.60)	0.84	6.69 (14.68)	0.65	0.16 (0.34)	0.63

[a] GLU: glucose; CRE: creatinine; TRI: triglycerides; TP: total protein; ALB: albumin; GLB: globulin; BUN: blood urea nitrogen; PHOS: phosphorus; AP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate aminotransferase; TBIL: total bilirubin; CHOL: cholesterol; GGT: gammaglutamyltransferase; CPK: creatinine phosphokinase; LDH: lactate dehydrogenase; CA: calcium; NA: sodium; K: potassium; CL: chloride; FE: total iron.

[b] Referent facility was San Antonio.

[c] All coefficients were adjusted for all of the variables listed.

The influence of reproductive status on analyte variation was not specifically examined in this study as comprehensive data on reproductive status of an animal at each sampling occasion were not available for analysis. Results from other cetacean studies have not consistently demonstrated specific differences in analyte values between the sexes and their reproductive status. Though significant differences between male and pregnant bottlenose dolphins (*Tursiops truncatus*) were reported for HB, HCT, and RBC,[5] sex alone was associated with only a few differences in circulating analytes in wild Canadian belugas, mainly thyroid hormones.[27]

Circannual rhythms, observed in many of the analytes in this study, could obscure data interpretations if investigators are unaware of this cyclic variation. It is thought that the circadian rhythms in hematologic variables are endogenously (i.e., genetically) determined and are modulated by environmental factors such as climate.[1] Comparison of analyte values within a season may serve as a useful clinical reference when evaluating test results for a particular animal and for providing insight into changes potentially associated with reproductive changes such as pregnancy.

The inter- and intrawhale variation presented in Tables 2 and 3 demonstrate the influence of physiologic processes on analyte levels of many repeated measurements from 31 clinically normal managed belugas. Intrawhale variability in hematologic values such as WBC and granulocytes may be strongly influenced by stress-related events over the course of an individual animal's life, such as pregnancy, changes in environment, or surrounding social structure. The latter two events also occur in wild populations but on a more severe scale, through exposure to heavy parasitism burdens, more extreme environmental conditions, and predators. Analytes such as AP with greater interwhate, compared to intrawhale, variation represent differences in physiologic events between whales such as immaturity and transient

Table 3. Extended.

Sex Coefficient (SE)	*P*	Photoperiod Coefficient (SE)	*P*	Standard deviation	
				Interwhale	Intrawhale
3.69 (4.52)	0.42	0.07 (0.75)	0.93	10.46	13.39
0.10 (0.09)	0.26	0.05 (0.01)	**0.001**	0.21	0.24
27.56 (25.12)	0.27	13.87 (3.84)	**<0.0001**	58.00	67.81
0.32 (0.24)	0.18	0.05 (0.03)	**0.05**	0.55	0.49
0.14 (0.22)	0.53	0.03 (0.02)	**0.10**	0.51	0.34
0.19 (0.15)	0.20	0.01 (0.02)	0.64	0.34	0.43
0.44 (2.12)	0.84	0.27 (0.38)	0.47	4.86	6.72
0.13 (0.18)	0.48	0.02 (0.04)	0.68	0.39	0.74
29.88 (21.70)	0.17	16.38 (2.55)	**<0.0001**	48.20	42.02
0.52 (1.46)	0.72	0.21 (0.18)	0.24	3.41	3.14
0.33 (4.49)	0.94	1.54 (0.81)	0.06	10.26	14.33
0.08 (0.04)	**0.05**	0.02 (0.01)	**<0.0001**	0.08	0.09
0.92 (15.62)	0.95	3.43 (2.06)	0.10	36.53	36.70
3.25 (2.89)	0.26	0.08 (0.38)	0.83	6.74	6.34
9.46 (19.01)	0.62	11.87 (2.39)	**<0.0001**	44.49	42.38
3.18 (14.71)	0.83	3.74 (3.13)	0.23	32.77	55.57
0.10 (0.22)	0.65	0.01 (0.03)	0.65	0.53	0.45
0.96 (0.90)	0.29	0.03 (0.16)	0.84	1.96	2.82
0.03 (0.14)	0.82	0.02 (0.02)	0.35	0.31	0.36
0.80 (1.26)	0.53	0.01 (0.22)	**0.01**	2.89	3.86
10.88 (22.78)	0.63	3.39 (5.13)	0.51	50.82	89.66
32.25 (13.01)	**0.01**	3.71 (2.61)	0.16	27.20	39.90

enzyme elevation, as demonstrated in a number of cetacean species.[22]

Environment factors

Seasonal effects were seen in several hematologic analytes. Greater RBC and TP were seen in spring and summer, which could be due to increased erythropoietic activity during these times of the year or hemoconcentration. Elevated monocytes and segmented neutrophils were observed in late spring and summer, accompanied by decreased lymphocytes and eosinophils, suggesting possible stress from reproductive events or normal physiologic seasonal variation in these cell types.[2,17,19] Seasonal changes in blood analytes may also be connected to seasonal acclimatization to high and low ambient temperatures, or seasonal variation in body condition, which has been observed in a variety of mammalian and avian species.[5,24,28]

Mean monthly ambient temperature, as a proxy for seasonal differences, was investigated and was found to be an important independent predictor for many of the analytes. Differences between seasonal patterns across facilities led to consideration of ambient air temperature as a predictor; although significant, it did not fully account for differences between the facilities.

Photoperiod or some unknown factor may account for a portion of the seasonal partitioning in addition to temperatures. Photoperiod has been found to influence significant differences in analytes such as HCT, lymphocytes, PHOS, and CA between sexes during winter, and is presumably due to the influence of shortened daylight length.[7] Because the facilities in this study were located at very similar latitudes (between 28°N and 32°N), effects of photoperiod on analyte variation most likely reflects circannual variation within a facility rather than between them.

The whale's housing facility at the time of sampling was a significant determinant of 14 analytes after controlling for sex, age, and photoperiod. Of these 14, 8 were hematologic parameters (Table 2). More than half (n 9) of the significant analyte associations stemmed from the San Diego facility. In this study the significance of a facility may have been due in part to ambient temperature fluctuations, photoperiod length, or some other as yet unspecified characteristic associated with a facility or its location. The contribution of ambient temperature and photo-

Table 4. Blood analytes of belugas under managed care differing significantly by sex, 1988–2010.

Analyte	Males (*n* 11)	
	Mean (SD, SE)	Range (95% CI)
Hematology		
Hemoglobin (g/dl)	19.97 (1.72, 0.09)	9.3–24.7 (19.80–20.15)
Hematocrit (%)	54.90 (4.70, 0.24)	28.0–68.0 (54.42–55.37)
Red blood cells (×10⁶/l l)	3.23 (0.20, 0.01)	2.50–3.83 (3.24–3.28)
Mean corpuscular volume (fl)	168.93 (7.71, 0.40)	134–189 (168.15–169.71)
Mean corpuscular hemoglobin (pg)	61.33 (4.29, 0.22)	34.0–74.0 (60.90–61.76)
Mean corpuscular hemoglobin concentration (g/dl)	36.40 (0.99, 0.05)	33.0–42.0 (36.30–36.50)
Nucleated red blood cells (no./100 WBC)	0.23 (0.61, 0.03)	0.0–4.0 (0.17–0.30)
Reticulocytes (%)	0.43 (0.59, 0.03)	0.0–3.0 (0.36–0.49)
White blood cells (×10³/l l)	7,406.39 (1941.29, 99.46)	1,290–13,700 (7,210.84–7,601.94)
Bands (×10³/l l)	76.13 (107.87, 5.54)	0.0–590.0 (65.23–87.02)
Segmented neutrophils (×10³/l l)	4,110.47 (1297.33, 66.46)	312–10,412 (3,979.78–4,241.15)
Lymphocytes (×10³/l l)	2,206.45 (1003.95, 51.43)	297–5,461 (2,105.32–2,307.59)
Monocytes (×10³/l l)	575.49 (352.08, 18.13)	0–1,955 (539.83–611.14)
Protein		
Globulin (g/dl)	2.27 (0.50, 0.03)	0.9–3.4 (2.21–2.32)
Kidney function		
Blood urea nitrogen (mg/dl)	54.16 (9.30, 0.49)	29.0–112.0 (53.19–55.13)
Phosphorus (mg/dl)	5.79 (0.69, 0.04)	3.0–8.3 (5.72–5.86)
Liver function		
Total bilirubin (mg/dl)	0.23 (0.13, 0.007)	0.0–0.8 (0.22–0.25)
Alkaline phosphatase (IU/L)	158.23 (83.05, 4.94)	27.0–328.0 (148.51–167.95)
Alanine aminotransferase (IU/L)	7.13 (4.20, 0.22)	1.0–24.0 (6.69–7.57)
Aspartate aminotransferase (IU/L)	62.32 (17.53, 0.93)	29.0–148.0 (60.49–64.16)
Gammaglobulin transferase (IU/L)	15.56 (7.73, 0.41)	0.0–52.0 (14.75–16.37)
Other		
Glucose (mg/dl)	104.70 (14.86, 0.79)	71.0–169.0 (103.15–106.25)
Cholesterol (mg/dl)	214.34 (38.88, 2.06)	91.0–344.0 (210.29–218.39)
Triglyceride (mg/dl)	202.12 (92.82, 5.03)	32.0–597.0 (192.24–212.01)
Creatinine phosphokinase (IU/L)	143.97 (58.89, 3.14)	31.0–383.0 (137.80–150.14)
Lactate dehydrogenase (IU/L)	188.14 (65.53, 3.48)	80.0–552.0 (181.29–194.99)
Sodium (mmol/L)	155.26 (2.83, 0.16)	145.0–170.0 (154.94–155.57)
Iron (l g/dl)	264.64 (107.00, 5.69)	0.0–716.0 (253.46–275.83)
Iron binding (l g/dl)	88.46 (33.08, 2.05)	0.1–189.0 (84.43–92.49)

period to changes in hematologic analytes has been studied in other species.[23] Given that the belugas are maintained in a fairly constant water temperature, the partitioning noted in this study may occur in combination with diet, photoperiod, and other biologic and environmental factors. Though the whales are fed a relatively consistent diet throughout the year, individual variation in energy and nutrient content of the food items, due to the individual fish's age class, health, and overall body condition, may result in mild variations in nutrient content ingested by the whales over the course of the year.

Free-ranging belugas use estuaries to gather for molting and socializing, exposing them to diverse water parameters that originate from terrestrial sources. Sodium concentrations in free-ranging belugas were more wide-ranging compared to the present managed whales, which may represent changing environmental salinities.[27] Blood levels of CRE were significantly greater in summer than in winter, a pattern also noted in managed[6] and wild dolphins,[12] which may be attributed to nutritional status such as consumption of a high-protein diet, as well as dehydration, renal disease resulting in decreased glomerular filtration (renal azotemia), and potentially acute myositis or muscle trauma.[11] In the present study, greater CRE levels in the spring and summer most likely reflect changing seasonal muscle mass despite a rather constant diet and moderate changes in seasonal activity level.

Table 4. Extended.

Females (*n* 20)		
Mean (SD, SE)	Range (95% CI)	*P*
19.31 (1.43, , 0.05)	12.9–23.1 (19.21–19.39)	<0.001
52.73 (3.88, , 0.12)	36.3–63.8 (52.49–52.97)	<0.001
3.09 (0.20, 0.01)	2.29–4.07 (3.08–3.11)	<0.001
170.27 (6.28, 0.20)	141–188 (169.88–170.66)	<0.001
62.42 (2.96, 0.09)	49.2–76.0 (62.24–62.61)	<0.001
36.64 (0.98, 0.03)	33.8–43.0 (36.58–36.70)	0.004
0.33 (0.72, 0.02)	0.0–7.0 (0.29–0.38)	0.030
0.66 (0.60, 0.02)	0.0–4.0 (0.62–0.70)	<0.001
7,175.46 (1921.15, 60.63)	2,470–14,600 (7,056.48–7,294.44)	0.050
90.76 (126.83, 4.06)	0.0–588.0 (82.79–98.73)	0.050
4,490.29 (1527.32, 48.23)	275–10,414 (4,395.65–4,584.92)	<0.001
1,748.59 (984.44, 31.08)	119–9,636 (1,687.59–1,809.59)	<0.001
512.43 (318.79, 10.13)	0–1,985 (492.56–532.30)	0.002
2.43 (0.69, 0.02)	1.0–5.2 (2.39–2.47)	<0.001
52.14 (7.28, 0.23)	6.1–96.0 (51.68–52.60)	<0.001
5.68 (0.91, 0.03)	2.0–9.7 (5.62–5.73)	0.037
0.18 (0.10, 0.003)	0.0–0.9 (0.17–0.18)	<0.001
110.94 (69.50, 2.37)	9.0–310.0 (106.28–115.60)	<0.001
5.44 (4.34, 0.14)	1.0–25.0 (5.16–5.72)	<0.001
57.73 (16.40, 0.53)	27.0–150.0 (56.69–58.77)	<0.001
17.63 (9.36, 0.31)	0.0–59.0 (17.03–18.23)	<0.001
102.71 (14.46, 0.47)	40.0–201.0 (101.79–103.62)	0.027
208.66 (47.39, 1.53)	65.0–407.0 (205.67–211.66)	0.043
188.29 (89.47, 2.89)	44.0–518.0 (182.62–193.97)	0.015
131.92 (57.10, 1.85)	31.0–396.0 (128.29–735.55)	<0.001
178.11 (62.56, 2.03)	18.0–596.0 (174.14–182.09)	0.011
156.16 (3.40, 0.11)	144.0–175.0 (155.94–156.38)	<0.001
239.89 (97.58, 3.23)	0.0–703.0 (233.56–246.22)	0.001
129.74 (49.55, 1.86)	35.0–414.0 (126.10–133.39)	<0.001

Study design factors

Information (misclassification) bias was possible as a result of the methods used to extract blood values for use in the analyses. This could have resulted from incorrectly classifying the analyte as being from a healthy animal rather than a diseased one and vice versa, that is, misclassification of an animal's health status based on bloodwork alone. Additionally, because routine samples are collected at the animal's discretion, missing data were present in several cases, but were accounted for in the linear mixed effects models, which can handle unbalanced longitudinal data by ignoring missing data in the computations.

In any long-term study, there are important barriers to compiling a data set on blood analytes, especially one that covers 22 yr. Analyzers are necessarily replaced over time and methodologic consistency is difficult to maintain.[14] This study was not designed with the replicate assays needed to characterize fully the differences in mean levels or in precision associated with specific analyzers. Instead, it is acknowledged that the use of different analyzers is part of the typical variation seen in general clinical practice; this variation is thus included in the estimates of inter- and intrawhale variation. Some of the differences across facilities may be due to equipment or technologic differences, but this should not matter for purposes of tracking animal health and looking for changes in an individual animal.

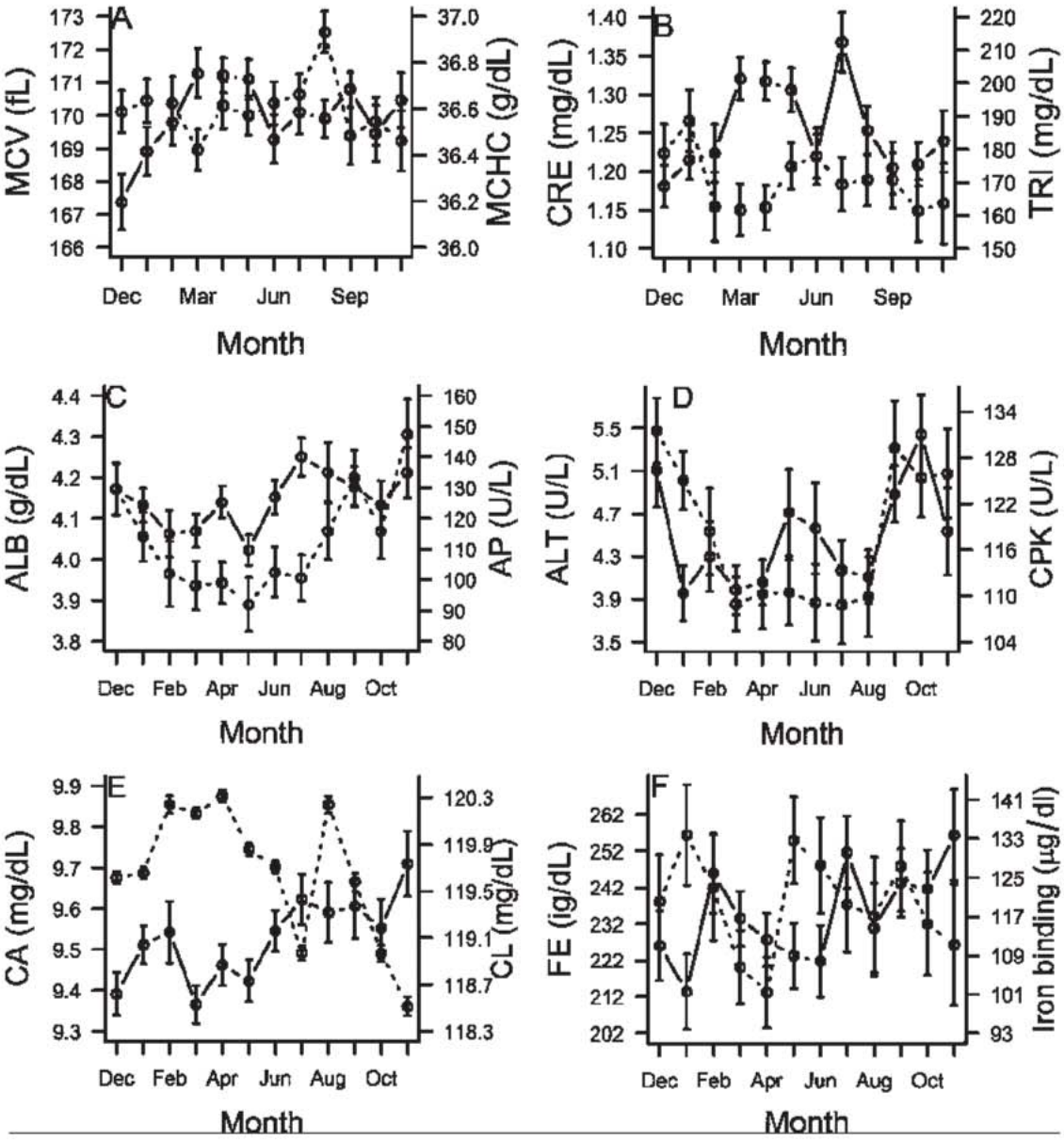

Figure 1. Mean (±SE; vertical lines) highly significant ($P < 0.001$) monthly analyte values for managed belugas (*Delphinapterus leucas*) pooled across whales, facilities, and years (1988–2010) for (**A**) mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC), (**B**) creatinine (CRE) and triglycerides (TRI), (**C**) albumin (ALB) and alkaline phosphatase (AP), (**D**) alanine transferase (ALT) and creatinine phosphokinase (CPK), (**E**) calcium (CA) and chloride (CL), and (**F**) iron (FE) and iron binding. Solid and dashed lines represent values of the analytes on the left and right y-axes, respectively.

Variation patterns observed here suggest that analyte values may be influenced by the animal's sex and age as well as environmental factors such as temperature and photoperiod. In general, analyte patterns for the managed belugas followed those of their wild counterparts suggesting there may be intrinsic physiologic factors responsible for changes in analyte levels that are independent of the individual's housing or nutritional status. The individual's reproductive status should also be considered when evaluating blood values for clinical health assessments and making treatment

decisions, but is less of a concern when assessing analyte patterns over a long time period across a population of individuals. This study sought to further explore the association of season with analyte variation by evaluating ambient temperature as a proxy for season.

Though this study may not identify all variables influencing circulating levels of the examined blood analytes in managed whales, the factors of age, seasonality (ambient temperature), and sex were found to be important determinants for a majority of the analytes measured in managed belugas, regardless of the interlaboratory variation in results, and thus should be considered when investigating health trends over time. Our findings suggest that beluga blood analytes are partitioned by these factors and by intrinsic biologic variation, particularly in analytes related to fluid balance and thermoregulation, and may also be affected by other factors such as nutritional status and photoperiod. These factors and their variability should be considered when interpreting blood analyte values and animal health in managed and wild populations.

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