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Pregnancy profiles in the common bottlenose dolphin (*Tursiops truncatus*): Clinical biochemical and hematological variations during healthy gestation and a successful outcome

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ABSTRACT

The physiological demands of pregnancy inevitably result in changes of both biochemical and hematological parameters as the fetus develops. Alterations in blood parameters have been observed to shift according to both trimester and species, to support fetal physiological needs and maternal basal requirements. Establishing normal reference ranges for each stage in gestation is important to facilitate diagnosis of underlying health concerns and prevent over-diagnosing abnormalities. Despite bottlenose dolphins (*Tursiops truncatus*) being one of the most highly studied cetaceans, the blood profile changes occurring as a result of pregnancy have not been previously described. A retrospective analysis was performed from blood samples obtained from 42 successful pregnancies from 20 bottlenose dolphins in a managed population over 30 years. Samples were compared to non-pregnant states and among trimesters of pregnancy. Blood profile fluctuations occurred throughout gestation, however significant alterations predominantly occurred between the 2nd and 3rd trimester. Hematological changes from the 2nd to the 3rd trimester included a decrease in lymphocytes, decrease in platelet count, and hemoconcentration with increased hematocrit and hemoglobin. Biochemical changes in the 3rd trimester included significant reductions in ALKP (alkaline phosphatase), ALT (alanine aminotransferase) and AST (aspartate aminotransferase) with significant increases observed in albumin, globulins, total protein, cholesterol, triglycerides and CO_2. It's important to note that despite significant shifts occurring between the 2nd and 3rd trimester, there was no significant change in platelets, hematocrit, hemoglobin, lymphocytes or CO_2 between non-pregnant and 3rd trimester blood samples. The normal reference ranges for each trimester established herein, will enable future identification of abnormalities occurring during pregnancy and help improve our understanding of factors potentially influencing a failed or successful pregnancy outcome.

1. Introduction

Natural physiological changes that occur during pregnancy are known to impact some biochemical and hematological parameters during different stages of gestation in many species [1–5]. Despite homeostatic mechanisms aiming to maintain constant levels of blood components, the additional demands of pregnancy predictably result in some variation [6]. Understanding the expected variations which occur during gestation are essential to interpret any abnormalities or health concerns which may arise during pregnancy, parturition, or lactation [7,8]. The increased metabolic

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demands of pregnancy are known to affect some parameters more than others with inter-species variations also observed [1,2].

In veterinary medicine, blood parameters have been monitored throughout pregnancy in domestic species and laboratory animals [3,9,10]. However, little is known about the effects of pregnancy on blood variables in marine mammals with the exception of managed killer whales (*Orcinus orca*), and the Yangtze and Indo-pacific finless porpoises (*Neophocaena asiaeorientalis ssp. Asiaeorientalis*, *Neophocaena phocaenoides*) [11–13]. Killer whales demonstrated a progressive inflammatory state prior to parturition, and a reduction in hematocrit, hemoglobin and red blood cell counts due to plasma volume expansion in late gestation. In equids, which share multiple reproductive similarities with dolphins, changes observed during gestation include a marked reduction in triglycerides, potassium, total bilirubin and creatinine [9]. Hematological changes observed in pregnant mares include increases in plasma fibrinogen and white blood cells at the time of parturition, most likely due to increased glucocorticoid release or due to an inflammatory response associated with normal placental detachment [14].

In pregnant mammals, the volume of blood markedly increases to facilitate fetal growth with a 1.5 L increase observed in humans, approximately 1 L of which is contained within the uterus and placenta [7]. Extensive plasma volume expansion accounts for some of the hematological changes observed during pregnancy such as decreased total protein, hematocrit, hemoglobin and red blood cell counts [15]. Establishing the expected shift in hematocrit is important in diagnosing conditions such as anemia or dehydration [16]. Increased oral fluid intake is usually observed during gestation in terrestrial mammals to maintain hydration, however homeostasis of osmolality in cetaceans is different due to the lack of oral intake of fresh water [17–20]. Adaptations of the urinary system ensure adequate hydration from oral food intake, with dolphin urine osmolality known to be capable of concentrating to at least 2658 mOsm/kg [21]. Mechanisms of ensuring hydration status and increasing plasma volume in pregnant dolphins are potentially identifiable through monitoring estimated glomerular filtration rate (GFR) and establishing if the changes occurring during gestation are different than other mammals. In humans, the kidneys increase in length and volume and physiologic hydronephrosis occurs in up to 80% of women during pregnancy due to the remarkable orchestration of physiologic changes [22]. A 50% rise in GFR is recorded during pregnancy in humans with subsequent decreases in creatinine and BUN (blood urea nitrogen) [22].

The higher oxygen demand faced by pregnant females requires additional adaptation for diving mammals to prevent hypoxia to the fetus. Continual placental perfusion is observed in diving Weddell seals (*Leptonychotes weddellii*) [23] and increased oxygenation of the blood in pregnant California sea lions (*Zalophus californianus*) [24]. Variation in placental structure among species from a diffuse epitheliochorial placenta in the horse and dolphin, to a zonary placenta in dogs and seals may account for some of the physiological adaptations observed [25,26]. Both otariids and cetaceans can be simultaneously pregnant and lactating, resulting in a high energetic demand on the female [27]. Lactation and concurrent pregnancy are unusual occurrences physiologically in mammals due to the suckling stimulus inhibiting ovulation via suppression of GnRH. A negative energy balance from mobilization of body stores during lactation can also suppress ovulation. Dolphins can ovulate within the second year of lactating demonstrating different physiological adaptations as compared to other mammals [28]. Simultaneous lactation and pregnancy are not typically observed in this managed study population, most likely due to pre-weaning sex separation. Determining the changes in hematology that enable adequate oxygenation of the fetus during pregnancy, is important to assess if any hypoxia or malnutrition is occurring.

Changes in morphology as a result of pregnancy affect the hydrodynamics of female dolphins with increased drag and gait alterations which can result in a reduction of swim speeds [29]. Free-ranging pregnant dolphins have been observed to select alternative prey items, which could be linked to obtaining increased dietary water content or easier to obtain prey items, in an attempt to optimize foraging efficiency. A dietary shift to easily obtainable squid compared to harder to catch fish can occur in free-ranging dolphins and also influence bloodwork changes [30,31], however no dietary shift was implemented in the managed care dolphins.

The aim of this study was to establish whether hematological and serum biochemical blood analytes changed during pregnancy in bottlenose dolphins compared to non-pregnancy and to establish normal reference intervals (RI) for blood analyte levels according to trimester in healthy pregnancies with a positive outcome.

2. Materials and methods

Retrospective data analyses were performed using blood data from U.S. Navy Marine Mammal Program's (MMP) bottlenose dolphins living in open-ocean netted enclosures in San Diego Bay, California. The MMP is AAALAC-accredited and adheres to the national standards of the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Animal Welfare Act. The MMP's animal care and use program is routinely reviewed by an institutional animal care and use committee, and the Department of Defense Bureau of Medicine and Surgery. Inclusion criteria for the analyses were clinically healthy adult female pregnant dolphins who successfully carried a calf to full term, parturition occurred without complication, and the calf survived >30 days. Inclusion criteria for the non-pregnant dolphin samples involved a routine blood sample obtained in the month of June prior to pregnancy (ranging 1–6 months pre-pregnancy). Exclusion criteria included any pre-existing health conditions which could affect pregnancy, or significant health conditions which occurred during pregnancy and required initiation of treatment by the attending clinician. Any administration of systemic antibiotics, antifungal, steroid or non-steroidal anti-inflammatory medications during pregnancy resulted in exclusion from the study. Medications that did not result in exclusion of the study were any medications administered as part of normal preventative medicinal care (e.g. parasiticides), vitamin supplementations routinely administered to pregnant females (e.g. Mazuri® Vita-Zu® vitamins), gastrointestinal medications used for short episodes of inappetence, or topical medications. Samples included in the study were both fasted and non-fasted blood samples.

Dolphins included in the study were fed a variety of capelin, herring, squid, eulachon and mackerel, varying from year to year based on fish species availability. Diets for individual dolphins were based upon their calorific requirement according to their body weight. Dolphins were fed a gradually increased diet according to individual kilocalorie requirements, throughout gestation to achieve, on average, a 25% increase of normal intake at the time of parturition per previously established guidelines [32]. In addition, pregnant dolphins were supplemented with oral hydration *ad lib* with an additional 1–3 L of water and electrolytes as needed. Nutritional supplementation included Mazuri® Vita-Zu® Mammal 5M26 tablets administered from 1996 onwards and Sea Tabs from 1985 to 1996. The shift in supplements resulted in the removal of iron supplementation post improved knowledge regarding iron storage disease in dolphins [33].

Blood was collected as part of the MMP's comprehensive preventative health program. All blood samples were collected via

voluntary presentation or manual restraint from the peripheral periarterial venous rete on the ventral fluke using a 22-gauge butterfly needle and vacutainer collection system or from the periarterial venous rete in the caudal peduncle using a 20/18-gauge 1.5-inch needle attached to a vacutainer collection system. Blood was collected into BD vacutainers (BD, Franklin Lakes, NJ) containing K-EDTA, sodium citrate and serum separator for analysis. Serum separator tubes were chilled for 30 min and centrifuged within 2 h at 21 °C for 10 min. Whole blood and serum samples were submitted on ice packs to Quest diagnostic laboratory or Naval Medical Center San Diego clinical pathology laboratory for analysis. Previous analyses confirmed the comparability of these two laboratories [34,35]. Blood samples were assessed for routine biochemistry and hematology including 46 total analytes. Serum biochemistry analyses included total protein (TP), albumin (Alb), total globulins (Glob), total albumin to total globulin ratio (Alb:Glob ratio), glucose (Gluc), BUN, creatinine (Crea), blood urea nitrogen to creatinine ratio (BUN:Creat ratio), GFR, uric acid (UA), total bilirubin (Bili), cholesterol (Chol), triglycerides (Trig), creatine kinase (CPK), alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), and serum electrolyte concentrations: sodium (Na), potassium (K), chloride (Cl), calcium (Ca); phosphorus (P), magnesium (Mg) carbon dioxide (C02), iron. Hematology analysis included CBC data: hemoglobin (HGB), hematocrit (HCT), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets, mean platelet volume (MPV), erythrocyte sedimentation rate (SED60), total white blood cell (WBC), absolute neutrophils (Neut), neutrophil %, absolute monocytes (Mono), monocyte %, absolute eosinophils (Eos),eosinophil %, absolute lymphocytes and lymphocytes %.

2.1. Statistical analysis

Data were analyzed using RStudio v 1.1.383 using R v 3.5.2 [36,37]. Data were divided into trimester with three 127-day intervals. Gestational age at sampling was calculated from the known parturition date based on a 380 day gestation. A single blood draw was randomly selected from each pregnancy as well as each trimester of each pregnancy using the dplyr package in R [38]. Non-parametric 95th percentile RIs and associated 90% confidence intervals were calculated with bootstrapping (n = 5000), as recommended by ASVCP guidelines [39] using the RIs package across pregnancy and for each trimester. Prior to calculation of RIs, statistical outliers were detected with Horn's algorithm and removed. Blood data RIs were likewise established for the same group of adult female dolphins when they were not pregnant. The closest sample taken prior to gestation during the month of June under a routine blood draw was selected. Samples collected due to a possible health concern were excluded from analysis.

Samples were initially tested to determine if there were general differences in hematology and/or biochemistry using two repeated measures MANOVA with Pillai's Trace statistic for both large panels (hematology and biochemistry). Analytes with >20% missing data points were excluded from the analysis. When the Pillai's Trace statistic indicated significance, more specific differences in parameters were then assessed using repeated measures ANOVA (RM-ANOVA) with Tukey's HSD post-hoc tests to assess the differences among trimesters with a significance of $P < 0.05$. Statistical comparisons were made among all three trimesters and between non-pregnant samples with each trimester. If needed, data were Box-Cox transformed prior to analyses.

3. Results

From 1985 to 2017, there were 42 successful pregnancies included in the study with a total of 305 routine blood samples obtained from 20 pregnant female dolphins throughout the gestation. A single blood sample from each trimester of each pregnancy was randomly selected to be included in the study. The non-pregnant group contained 42 blood samples selected from 218 routine blood collections during the month of June from 1978 to 2015, from the same 20 dolphins while not pregnant. These samples were selected such that the non-pregnant sample collected closest to the start of gestation was included in analyses. The n per analyte ranged from 19 to 42 with a median value of 34.5 for the non-pregnant group and across trimesters of pregnancy. There were variations in the number of samples available within each trimester for each pregnancy with 41 cases in the 1st trimester (n_{ave}/analyte = 35), 40 in the 2nd (n_{ave}/analyte = 34) and 34 in the 3rd trimester (n_{ave}/analyte = 29). The 20 dolphins included in the study ranged in age from 9 to 42 years, and ranged from 222 to 287 cm in total length and 125–258 kg in body weight pre-pregnancy. Parity (number of pregnancies per individual) ranged from the 1st pregnancy to the 9th pregnancy.

RIs were created with outliers removed, and calculated for each analyte, after subset by non-pregnant, pregnant (all trimesters combined), and by each trimester. The upper and lower values per trimester for each analyte are reported in Table 1 (Hematology) and Table 2 (Biochemistry). RIs from the non-pregnant blood draws as normal controls are provided as comparison.

An extensive interrogation of the data was performed, comparing non-pregnant and individual trimesters by repeated measures MANOVA for the biochemical and hematological panels independently. Both were significant with values of $P = 0.001$, and $P = 0.002$ respectively. Further analyses by repeated measures ANOVA, to examine the phasing of changes in blood analytes throughout pregnancy, revealed significant differences in 19 of the 46 analytes assessed in the study, ($P < 0.05$, Table 3).

Hematological changes with significant differences occurring among trimesters included neutrophil %, HCT, HGB, and RBC which all showed significant increases in the 3rd trimester. Lymphocytes decreased significantly in the third trimester compared to the 1st and 2nd trimester along with platelets, (RDW), and MCH (Figs. 1 and 2). Biochemical changes among trimesters included significant reductions in the 3rd trimester in ALKP, AST, ALT, and iron (Fig. 3). Significant increases in the 3rd trimester were observed in albumin, protein, cholesterol, triglycerides and CO_2 (Figs. 1 and 3).

4. Discussion

The RIs established herein are crucial to accurately assess health concerns and abnormalities during pregnancy. The RIs for non-pregnant dolphins in this study are similar to previously published RIs for this population but indicate a broader range of values for healthy dolphins [34]. As expected there is variation between these non-pregnant RIs and previously established RIs in free-ranging dolphins [40]; however, many analytes and the general gestational trends are comparable between managed and wild populations. Nonetheless, caution should be exercised if extrapolating the RIs calculated here to free-ranging animals. While it is likely that the general trends observed during gestation and discussed below in managed populations are also common to free-ranging animals, the natural variability is expected to be different.

Changes in blood analytes during pregnancy can be used to diagnose subclinical syndromes which could influence pregnancy outcome. For example, blood work is utilized to diagnose anemia, pre-eclampsia, and HELLP (hemolysis, enzyme elevation and low

Table 1
Mean values and 90% confidence intervals of hematology for non-pregnant females and for each trimester. The number of samples used to calculate the 90% confidence intervals is shown as well as the upper and lower values.

Variable	Not Pregnant			Pregnant			First Trimester			Second Trimester			Third Trimester		
	N	Lower Ref 90% CI	Upper Ref 90% CI	N	Lower Ref 90% CI	Upper Ref 90% CI	N	Lower Ref 90% CI	Upper Ref 90% CI	N	Lower Ref 90% CI	Upper Ref 90% CI	N	Lower Ref 90% CI	Upper Ref 90% CI
WBC	42	**4.43**	**13.53**	42	**4.14**	**10.39**	39	**5.20**	**12.50**	38	**5.60**	**12.00**	32	**3.87**	**11.40**
1000/μL		3.74–4.48	13.35–16.15		3.11–4.18	10.37–11.14		5.20–5.20	12.50–14.00		5.40–5.60	12.00–14.00		3.14–3.87	11.40–12.80
RBC	42	**2.53**	**3.93**	42	**2.63**	**3.85**	38	**2.65**	**3.59**	38	**2.63**	**3.57**	32	**2.88**	**3.88**
106/μL		2.25–2.54	3.93–4.12		2.55–2.63	3.83–4.12		2.59–2.65	3.59–3.67		2.55–2.63	3.57–3.67		2.80–2.88	3.88–4.03
HGB	42	**11.20**	**16.29**	42	**11.72**	**15.99**	39	**11.10**	**16.50**	38	**12.00**	**16.10**	32	**12.70**	**16.70**
g/dL		9.79–11.30	16.27–16.53		11.3–11.75	15.99–16.21		10.10–11.10	16.50–17.20		11.80–12.00	16.10–16.60		12.30–12.70	16.70–17.40
HCT	42	**36.81**	**50.71**	42	**34.54**	**48.03**	39	**34.60**	**49.00**	38	**34.40**	**45.80**	32	**36.30**	**49.60**
%		36.20–36.82	50.51–53.65		32.61–34.69	47.97–50.42		32.40–34.60	49.00–50.60		32.50–34.40	45.80–47.10		33.20–36.30	49.60–50.90
MCV	42	**114.61**	**148.65**	40	**117.83**	**146.59**	39	**115.00**	**153.10**	38	**119.00**	**150.60**	32	**117.80**	**145.40**
fL		113.9–114.6	148.6–150.2		114.70–117.86	146.58–147.16		110.2–115.0	153.1–160.0		117.0–119.0	150.6–156.2		114.30–117.80	145.40–148.00
MCH	37	**39.70**	**49.50**	35	**40.30**	**47.30**	32	**40.70**	**46.90**	31	**42.40**	**47.00**	25	**40.30**	**46.70**
pg		39.20–39.70	49.50–52.50		39.80–40.30	47.30–47.70		39.10–40.70	46.90–47.50		42.10–42.40	47.00–47.50		39.80–40.30	46.70–46.90
MCHC	36	**30.20**	**35.30**	38	**30.40**	**37.80**	35	**30.40**	**36.00**	34	**29.90**	**37.00**	28	**29.90**	**36.80**
g/dL		29.90–30.20	35.30–35.50		30.20–30.40	37.80–39.10		30.20–30.40	36.00–36.20		28.90–29.90	37.00–37.40		29.40–29.90	36.80–37.80
RBCDist	37	**11.80**	**19.00**	35	**11.40**	**16.90**	32	**12.10**	**17.90**	31	**11.50**	**16.90**	25	**11.40**	**16.20**
%		11.3–11.8	19.0–20.5		10.20–11.40	16.90–17.10		11.10–12.10	17.90–18.70		10.20–11.50	16.90–17.20		10.70–11.40	16.20–16.90
NRBC	36	0.00	**2.00**	34	0.00	**6.00**	30	0.00	**4.00**	30	0.00	**34.00**	25	0.00	**1.63**
%		NA	2.00–2.00		NA	6.00–8.00		NA	4.00–5.00		NA	34.00–65.00		NA	0.93–2.32
Platelets	34	**48.00**	**126.00**	36	**54.00**	**115.00**	35	**44.00**	**141.00**	34	**51.00**	**138.00**	28	**37.00**	**137.00**
1000/μL		39.00–48.00	126.0–130.0		45.00–54.00	151.0–161.0		20.00–44.00	141.0–153.0		43.0–51.0	138.0–146.0		11.0–37.0	137.0–147.0
MPV	30	**9.80**	**19.10**	27	**11.80**	**17.90**	27	**9.40**	**16.90**	27	**10.70**	**16.40**	20	**10.10**	**20.5**
fL		8.90–9.80	19.10–21.60		11.60–11.80	17.90–20.10		6.80–9.40	16.90–18.30		9.80–10.70	16.40–17.10		8.00–10.10	20.50–24.90
Neutrophils	42	**45.45**	**79.93**	40	**50.05**	**85.85**	39	**45.00**	**78.00**	38	**45.00**	**87.00**	32	**38.00**	**89.00**
%		37.68–45.90	79.85–82.00		44.03–50.10	85.70–91.83		41.00–45.00	78.00–82.00		41.00–45.00	87.00–98.00		21.00–38.00	89.00–92.00
Neutrophils	42	**2.92**	**10.02**	42	**2.91**	**7.05**	38	**2.61**	**8.19**	38	**2.70**	**7.80**	30	**2.67**	**7.64**
1000/μL		2.78–2.92	9.91–11.93		2.54–2.92	7.03–7.33		1.90–2.61	8.19–8.57		2.46–2.70	7.80–8.90		2.06–2.67	7.64–8.98
Lymphocytes	42	**5.00**	**30.93**	42	**4.08**	**34.85**	39	**4.00**	**35.00**	38	**5.00**	**38.00**	32	**1.00**	**29.00**
%		3.93–5.00	30.85–37.85		3.00–4.15	34.70–41.00		3.00–4.00	35.00–43.00		1.00–5.00	38.00–43.00		0.00–1.00	29.00–36.00
Lymphocytes	42	**0.37**	**2.27**	42	**0.21**	**2.90**	38	**0.30**	**2.80**	38	**0.40**	**3.10**	32	**0.06**	**2.32**
1000/μL		0.29–0.37	2.26–2.64		0.10–0.21	2.86–3.54		0.20–0.30	2.80–3.58		0.28–0.40	3.10–3.73		0.00–0.06	2.32–2.96
Monocytes	33	**1.00**	**8.00**	42	**1.00**	**7.93**	34	**1.00**	**7.00**	32	**1.00**	**7.00**	29	1.00	**10.00**
%		1.00–1.00	8.00–9.00		1.00–1.00	7.85–9.85		1.00–1.00	7.00–7.00		0.00–1.00	7.00–10.00		NA	10.00–13.00
Monocytes	33	**0.07**	**0.62**	42	**0.05**	**0.69**	32	**0.07**	**0.50**	32	**0.07**	**0.42**	32	**0.04**	**1.00**
1000/μL		0.04–0.07	0.62–0.71		0.04–0.05	0.68–0.94		0.06–0.07	0.50–0.51		0.02–0.07	0.42–0.50		0.02–0.04	1.00–1.38
Eosinophils	42	**6.00**	**38.40**	39	**7.00**	**25.00**	39	**4.00**	**32.00**	36	**6.00**	**31.00**	30	**3.00**	**33.00**
%		5.70–6.00	37.80–50.17		6.00–7.00	25.00–30.00		1.00–4.00	32.00–38.00		4.00–6.00	31.00–37.00		0.00–3.00	33.00–47.00
Eosinophils	40	**0.40**	**2.00**	40	**0.46**	**1.84**	38	**0.21**	**2.40**	34	**0.40**	**2.04**	32	**0.13**	**3.78**
1000/μL		0.33–0.40	2.00–2.06		0.43–0.46	1.84–1.95		0.00–0.21	2.40–2.80		0.30–0.40	2.04–2.50		0.00–0.13	3.78–5.67

Table 2
Mean values and 90% confidence intervals of biochemistry for non-pregnant females and for each trimester. The number of samples used to calculate the intervals is shown as well as the upper and lower values and a 90% confidence interval for each reference value.

	Not Pregnant			Pregnant			First Trimester			Second Trimester			Third Trimester		
Variable	N	Lower Ref 90% CI	Upper Ref 90% CI	N	Lower Ref 90% CI	Upper Ref 90% CI	N	Lower Ref 90% CI	Upper Ref 90% CI	N	Lower Ref 90% CI	Upper Ref 90% CI	N	Lower Ref 90% CI	Upper Ref 90% CI
Glucose	41	**63.30**	**132.85**	40	**67.05**	**136.88**	37	**67.00**	**147.00**	38	**71.00**	**161.00**	31	**64.00**	**136.00**
mg/dL		51.50–63.60	132.70–137.3		62.95–67.10	136.75–141.90		60.00–67.00	147.00–161.00		67.00–71.00	161.00–193.00		55.00–64.00	136.00–159.00
BUN	40	**34.05**	**88.75**	40	**34.03**	**70.88**	38	**31.00**	**71.00**	37	**33.00**	**75.00**	30	**35.00**	**61.00**
mg/dL		32.00–34.10	88.50–103.78		32.03–34.05	70.75–79.00		25.00–31.00	71.00–78.00		28.00–33.00	75.00–84.00		34.00–35.00	61.00–66.00
Creatinine	41	**0.90**	**2.09**	42	**0.90**	**2.29**	39	**0.90**	**2.00**	37	**1.00**	**2.10**	32	**1.00**	**2.00**
mg/dL		0.90–0.90	2.08–2.29		0.79–0.90	2.27–2.67		0.90–0.90	2.00–2.30		0.90–1.00	2.10–2.30		0.90–1.00	2.00–2.30
BUN:Creat	41	**17.24**	**80.81**	40	**20.01**	**145.97**	39	**18.00**	**53.08**	37	**20.63**	**63.75**	28	**21.33**	**61.00**
Ratio		14.76–17.28	80.71–94.95		19.32–20.02	45.93–47.63		12.15–18.00	53.08–58.66		16.88–20.63	63.75–72.88		16.95–21.33	61.00–74.50
Uric Acid	34	**0.10**	**1.60**	35	**0.10**	**1.10**	31	**0.10**	**1.10**	29	**0.10**	**1.00**	25	**0.10**	**1.00**
mg/dL		0.10–0.10	1.60–2.00		0.10–0.10	1.10–1.30		0.10–0.10	1.10–1.30		0.10–0.10	1.00–1.30		0.10–0.10	1.00–1.50
Sodium	37	**152.00**	**159.00**	42	**151.08**	**160.00**	38	**150.00**	**160.00**	36	**152.00**	**159.00**	32	**151.00**	**159.00**
mEq/L		151.00–152.00	159.00–159.00		150.08–151.15	160.00–161.00		147.00–150.00	160.00–161.00		152.00–152.00	159.00–160.00		151.00–151.00	159.00–160.00
Potassium	41	**3.21**	**4.60**	42	**3.10**	**4.20**	38	**3.10**	**4.2**	38	**3.00**	**4.6**	**32**	**3.20**	**4.30**
mEq/L		3.10–3.21	4.59–4.80		2.99–3.10	4.20–4.30		2.90–3.10	4.20–4.30		2.80–3.00	4.60–5.00		2.90–3.20	4.30–4.50
Chloride	41	**109.20**	**125.00**	42	**111.30**	**128.85**	39	**115.00**	**127.00**	38	**112.00**	**124.00**	31	**112.00**	**124.00**
mEq/L		105.35–109.40	125.00–126.00		107.53–111.60	128.70–130.93		115.00–115.00	127.00–129.00		107.00–112.00	124.00–124.00		109.00–112.00	124.00–125.00
CO2	38	**19.00**	**30.00**	39	**17.00**	**29.00**	36	**18.00**	**30.00**	35	**18.00**	**28.00**	29	**22.00**	**29.00**
mEq/L		17.00–19.00	30.00–31.00		15.00–17.00	29.00–31.00		17.00–18.00	30.00–32.00		17.00–18.00	28.00–30.00		22.00–22.00	29.00–30.00
Protein	40	**5.70**	**7.80**	41	**6.11**	**8.20**	37	**6.20**	**8.00**	37	**6.10**	**8.30**	31	**6.30**	**8.60**
g/dL		5.40–5.70	7.79–8.09		5.91–6.11	8.19–8.40		6.20–6.20	8.00–8.50		5.90–6.10	8.30–8.90		6.20–6.30	8.60–9.20
Albumin	40	**3.61**	**5.69**	41	**3.53**	**5.80**	37	**3.70**	**5.40**	36	**3.90**	**5.80**	31	**4.10**	**7.00**
g/dL		3.22–3.62	5.68–6.18		3.05–3.55	5.79–5.90		3.40–3.70	5.40–5.40		3.80–3.90	5.80–6.30		4.10–4.10	7.00–8.30
Globulins	25	**1.30**	**3.30**	29	**1.30**	**3.70**	26	**1.40**	**3.70**	26	**1.80**	**3.50**	19	**0.90**	**3.90**
g/dL		0.70–1.30	3.30–3.70		0.80–1.30	3.70–3.80		1.10–1.40	3.70–4.20		1.50–1.80	3.50–3.60		0.00–0.90	3.90–4.50
Alb:Glob	25	**1.40**	**4.00**	27	**1.14**	**3.22**	21	**1.30**	**1.90**	26	**1.14**	**3.22**	19	**1.05**	**7.78**
Ratio		1.40–1.40	4.00–5.20		0.98–1.14	3.22–3.34		1.20–1.30	1.90–2.00		1.08–1.14	3.22–4.04		0.80–1.05	7.78–12.77
Calcium	40	**8.30**	**9.70**	42	**6.34**	**9.89**	37	**8.50**	**9.90**	38	**8.40**	**9.70**	30	**7.90**	**9.70**
mg/dL		8.30–8.30	9.70–9.80		4.43–6.47	9.87–10.07		8.30–8.50	9.90–10.10		8.30–8.40	9.70–9.80		7.60–7.90	9.70–9.90
Phosphorus	41	**3.21**	**6.48**	41	**3.44**	**6.10**	36	**4.00**	**5.90**	38	**3.80**	**6.50**	31	**3.40**	**6.10**
mg/dL		2.89–3.21	6.45–6.97		2.76–3.47	6.10–6.22		3.50–4.00	5.90–5.90		3.50–3.80	6.50–7.20		3.20–3.40	6.10–6.30
ALKP	40	**138.00**	**523.95**	42	**70.18**	**561.95**	39	**63.00**	**566.00**	33	**155.00**	**474.00**	32	**68.00**	**425.00**
U/L		123.30–138.00	523.90–613.15		13.30–72.35	557.90–660.20		0.00–63.00	566.00–624.00		117.00–155.00	474.00–539.00		23.00–68.00	425.00–510.00
LDH	39	**300.00**	**695.00**	42	**213.98**	**1148.63**	39	**218.00**	**1151.00**	35	**226.00**	**718.00**	32	**213.00**	**911.00**
U/L		274.00–300.00	695.00–767.00		**171.23–214.95**	1145.25–1146.53		136.00–218.00	1151.00–1388.00		148.00–226.00	718.00–840.00		132.00–213.00	911.00–1102.00
AST	41	**167.25**	**485.15**	42	**138.30**	**629.83**	36	**149.00**	**438.00**	35	**159.00**	**367.00**	32	**138.00**	**302.00**
U/L		156.15–167.50	482.30–548.30		119.78–138.60	618.65–827.65		112.00–149.00	438.00–474.00		144.00–159.00	367.00–411.00		123.00–138.00	302.00–322.00
ALT	40	**12.05**	**74.63**	40	**11.03**	**122.25**	39	**11.00**	**160.00**	35	**10.00**	**97.00**	27	**13.00**	**40.00**
U/L		7.03–12.10	74.25–96.35		7.95–11.05	121.50–158.10		7.00–11.00	160.00–233.00		3.00–10.00	97.00–129.00		10.00–13.00	40.00–47.00
GGT	38	**19.00**	**51.00**	38	**14.00**	**103.00**	38	**14.00**	**931.00**	34	**21.00**	**151.00**	28	**14.00**	**55.00**
U/L		18.00–19.00	51.00–57.00		9.00–14.00	103.00–124.00		7.00–14.00	931.00–1757.00		21.00–21.00	151.00–229.00		12.00–14.00	55.00–71.00
Bilirubin	30	**0.10**	**0.40**	33	**0.10**	**0.40**	30	**0.10**	**0.40**	28	**0.10**	**0.30**	30	**0.00**	**0.40**
mg/dL		NA	0.40–0.50		NA	0.40–0.50		NA	0.40–0.50		0.10–0.10	0.30–0.30		NA	0.40–0.50
Cholesterol	40	**128.38**	**349.53**	42	**141.08**	**365.40**	36	**111.00**	**275.00**	38	**122.00**	**321.00**	32	**146.00**	**349.00**
mg/dL		111.55–128.75	349.05–404.45		131.33–141.15	361.80–447.05		76.00–111.00	275.00–307.00		114.00–122.00	321.00–356.00		131.00–146.00	349.00–382.00
Triglycerides	39	**29.00**	**247.00**	42	**33.08**	**327.28**	38	**33.00**	**538.00**	38	**20.00**	**408.00**	32	**36.00**	**377.00**
mg/dL		23.00–29.00	247.00–325.00		30.58–33.15	322.55–407.28		27.00–33.00	538.00–912.00		3.00–20.00	408.00–609.00		15.00–36.00	377.00–502.00
Iron	37	**101.00**	**444.00**	38	**104.00**	**633.00**	36	**73.00**	**665.00**	34	**103.00**	**686.00**	26	**100.00**	**479.00**
µg/dL		81.00–101.00	444.00–552.00		89.00–104.00	633.00–790.00		0.00–73.00	665.00–868.00		63.00–103.00	686.00–921.00		88.00–100.00	479.00–649.00
CPK	35	**73.00**	**251.00**	33	**67.00**	**212.00**	31	**75.00**	**230.00**	30	**62.00**	**195.00**	22	**90.00**	**212.00**
U/L		61.00–73.00	251.00–275.00		50.00–67.00	212.00–262.00		71.00–75.00	230.00–256.00		47.00–62.00	195.00–230.00		71.00–90.00	212.00–263.00
SED60	32	**2.00**	**27.00**	39	**2.00**	**46.00**	32	**2.00**	**38.00**	35	**2.00**	**60.00**	27	**4.00**	**44.00**
		1.00–2.00	27.00–31.00		0.00–2.00	46.00–55.00		1.00–2.00	38.00–48.00		0.00–2.00	60.00–87.00		2.00–4.00	44.00–53.00
GFR	41	**127.98**	**338.00**	42	**115.90**	**338.00**	39	**135.00**	**338.00**	37	**127.00**	**299.00**	32	**135.00**	**299.00**
mL/min		112.70–128.76	338.00–338.00		88.80–116.80	338.00–379.33		108.00–135.00	338.00–338.00		111.00–127.00	299.00–330.00		108.00–135.00	299.00–330.00

platelets) syndrome in humans, which can cause maternal and neonatal morbidity and mortality [41–43]. RIs, which are both species and trimester specific, aid in detection of blood analyte changes which allows for the early diagnosis and treatment of underlying conditions.

As anticipated, the results from this study confirm that pregnant bottlenose dolphins experience significant alterations in their blood profile during gestation. The majority of significant differences observed were between the 2nd and 3rd trimesters rather than between non-pregnant and pregnant samples due to the shifts occurring gradually over the course of gestation as the fetus increased in size and demand. Several of the hematological and biochemical changes observed differed from changes documented in other mammals, which could be linked to the lower than expected plasma volume expansion observed in bottlenose dolphin pregnancy in this study in relation to other species. Clinically, the most relevant finding in this study was the presence of hemoconcentration in the 3rd trimester; this is contrary to other mammals where hemodilution is the predominant finding due to plasma volume expansion. This was denoted by an increase in hematocrit, total protein and red blood cell count (Figs. 2 and 3).

HGB and HCT increased significantly in the 3rd trimester, which is a change not observed in humans, terrestrial mammals or other cetaceans. This is an important observation, because in humans, an increase in hematocrit at any stage of gestation can be a sign of reduced plasma volume expansion, which can increase risk of preterm labor [44]. Both parameters showed similar patterns (Fig. 1) with stable levels in the first two trimesters and a significant increase in the 3rd (not however significantly higher than non-pregnant levels). This increasing shift in the 3rd trimester is the opposite of reports in humans, other cetaceans and terrestrial mammals [11,15,45]. RBC initially demonstrated a slight decrease when comparing non-pregnant samples to the second trimester, which could be a relative decline linked to plasma volume expansion. A marked increase in RBC count from the 2nd to the 3rd trimester then occurred. RBC mass in humans increases by approximately 20% during the course of gestation, however this usually results in a fall in hemoglobin concentration [46]. In humans the opposing fall in hemoglobin levels during pregnancy is caused by a greater expansion of plasma volume compared with the increase in red cell volume. In dolphins increased RBC mass, with increased HGB indicates hemoconcentration during pregnancy, particularly in the 3rd trimester, rather than hemodilution due to significant plasma volume expansion as observed in other species. A significant sequential increase in total protein in dolphins from non-pregnant to the third trimester also supports this. Similar increases in total protein were observed in pregnant killer whales and the finless porpoises but the increasest in red blood cells or hemoglobin in the 3rd trimester were not observed [11,13]. It is possible the anatomic constraints within cetacean pregnancy prevent the plasma volume expanding to the same extent as observed in terrestrial mammals, which could explain why increased total protein was observed across all three cetacean species studied.

The concurrent changes occurring in MCH, RDW, HCT, HGB, iron and RBC count are demonstrated in Fig. 1. The hematological changes observed to occur in the 3rd trimester demonstrate a microcytic, hypochromic polycythemia compared to the 2nd trimester. The relative reduction in iron between the 1st and 3rd trimesters, likely due to maternal fetal transfer, could trigger the late gestation polycythemia resulting in the increased hemoglobin levels observed in the 3rd trimester [7]. HCT levels are known to vary with age in bottlenose dolphins with younger animals having higher levels [34]. All female dolphins included in this study were > 8 years old therefore age was not seen to be influencing the 3rd trimester increase in HCT levels observed in this study.

Reduction in serum iron concentrations during pregnancy are well documented in humans, with iron supplementation during pregnancy a routine practice [6,47]. Serum iron may decrease rapidly in compromised cetaceans due to cytokine production by inflammatory cells with levels observed to plummet to <20% of normal within 24 h [48]. Determining normal RI for iron during gestation is important to discern whether a shift in iron levels is a normal reduction as a result of pregnancy or a pathological drop in iron, where iron is being sequestered in the liver to prevent availability to pathogenic bacteria and iron supplementation is therefore not indicated [49]. In this study, iron levels decreased significantly between 1st and 3rd trimesters, however iron levels did not drop below normal reference ranges throughout gestation. This decrease in iron could be linked to increased demand from the fetus or due to expected inflammation occurring during pregnancy with the expansion of the uterus. The range of iron for pregnant dolphins in this study is 100–686 μg/dL, which is higher that the RI previously published for pregnant and non-pregnant dolphins in this population (i.e. 100–332 μg/dL) [34] suggesting that either iron depletion does not result from fetal growth in cetaceans to the same extreme as it does in humans [6], or dolphins are capable of preempting this decrease by increasing levels in the 1st trimester. This could be facilitated by the extensive myoglobin stores in the muscle and the iron stores in the bone marrow and liver of the dolphin resulting in a surge in circulating iron serum levels during the 1st and 2nd trimester. Iron demands likely increase throughout gestation due to increased fetal and placental requirements resulting in the decline in levels observed in the 3rd trimester. Iron is no longer routinely administered to dolphins under the Navy Marine Mammal program when they are pregnant due to the possible increased risks for hemochromatosis at this time [50].

Platelet count gradually increased in the first two trimesters resulting in a relative decrease in platelets observed, in the third trimester compared to the second. However, it is important to note this was not a significant decrease from non-pregnant levels, such as gestational thrombocytopenia observed in < 10% of human pregnancies [51,52]. Fig. 1 shows the oscillation in platelet levels between the 1st and 3rd trimesters. Platelet count is documented to decrease throughout pregnancy particularly in the third trimester in humans [53], with gestational thrombocytopenia resulting from hemodilution, increased activation and accelerated clearance [51]. Hemodilution is unlikely to be the underlying cause of a relative decrease in platelets in dolphin pregnancy due to the hemoconcentration observed. The timing of the decrease in platelets in the 3rd trimester is consistent with the decrease in iron availability and could therefore result from decreased bone marrow thrombocyte production, however this is less likely with levels of RBC and WBC being maintained. Platelet lifespan has been documented to be shorter during pregnancy with hyper destruction of platelets observed in humans [54]. Numerous changes in coagulation factors are observed during pregnancy in humans, the most prominent being elevated D-dimers [8,55]. This is a result of increased fibrin from increased thrombin generation. Additional research is required to assess the effects of pregnancy on coagulation in bottlenose dolphins.

Total WBC counts did not vary significantly between pregnant and non-pregnant dolphins, or among gestational periods. In examining the differential shifts within trimesters, the most significant finding was the reduction in absolute lymphocytes observed between the 2nd and 3rd trimester. Consequentially a relative increase in neutrophil % was also observed due to the absolute decrease in lymphocytes. Close monitoring of the changes in the differential in the 3rd trimester is important to avoid unnecessary antibiotic therapy due to a perceived neutrophilia in the third trimester (Fig. 2).

Table 3
Repeated measures ANOVA results. Least square mean is shown for each trimester. Statistically significant analytes are bolded Tukey's HSD $P < 0.05$.

	Not Pregnant	1st Trimester	2nd Trimester	3rd Trimester	p-value
WBC 1000/μL	7.58	7.47	7.3	7.29	0.935
RBC 106/μL	**3.23**	**3.22**	**3.14**	**3.34**	**0.006**
HGB g/dL	**13.97**	**14.17**	**14.04**	**14.64**	**0.024**
HCT %	**41.4**	**41.96**	**41.01**	**43.26**	**0.004**
MCV fL	128.24	130.63	130.94	129.4	0.648
MCH pg	**43.62**	**44.64**	**45.07**	**43.97**	**0.011**
MCHC g/dL	33.87	33.88	34.27	34.04	0.501
RBCDist %	**14.41**	**14.86**	**14.73**	**13.83**	**0.008**
NRBC %	0.35	0.8	1.59	0.21	0.340
Platelets 1000/μL	**90.97**	**96.95**	**101.62**	**89.13**	**0.048**
MPV fL	13.73	13.88	13.83	14.07	0.952
Neutrophils %	**65.5**	**63.56**	**62.94**	**70.35**	**0.002**
Neutrophils 1000/μL	5	4.81	4.59	5.09	0.402
Lymphocytes %	**13.7**	**17.61**	**18.62**	**12.94**	**0.001**
Lymphocytes 1000/μL	**1.03**	**1.29**	**1.35**	**0.95**	**0.002**
Monocytes %	2.73	2.93	2.46	2.71	0.746
Monocytes 1000/μL	0.21	0.22	0.18	0.21	0.632
Eosinophils %	18.8	15.83	15.81	14.14	0.120
Eosinophils 1000/μL	1.38	1.19	1.17	1.05	0.132
Glucose mg/dL	98.04	101.41	100.54	98.99	0.768
BUN mg/dL	53.81	46.74	47.37	47.74	0.295
Creatinine mg/dL	1.32	1.39	1.42	1.37	0.575
BUN:Creatinine	43.14	34.3	34.59	36.15	0.180
Uric Acid mg/dL	0.42	0.33	0.29	0.3	0.250
Sodium mEq/L	155.44	155.34	155.35	155.22	0.987
Potassium mEq/L	3.82	3.73	3.74	3.71	0.661
Chloride mEq/L	119.7	120.55	120.58	118.87	0.062
CO2 mEq/L	**24.76**	**23.28**	**23.01**	**25.1**	**0.001**
Protein g/dL	**6.73**	**6.93**	**7.07**	**7.31**	**< 0.001**
Albumin g/dL	**4.53**	**4.57**	**4.65**	**4.85**	**0.011**
Globulins g/dL	2.43	2.66	2.66	2.75	0.491
Albumin:Globulins	1.89	1.78	1.79	2.04	0.815
Calcium mg/dL	8.87	8.98	9.06	8.93	0.773
Inorganic Phosphate mg/dL	4.58	4.99	4.86	4.74	0.154
Alkaline Phosphatase U/L	**288.48**	**319.4**	**289.26**	**209.54**	**< 0.001**
LDH U/L	437.08	500.78	458.42	444.69	0.289
AST U/L	**266.17**	**265.38**	**257.71**	**217.34**	**0.001**
ALT U/L	**27.04**	**39.82**	**36.8**	**27.99**	**< 0.001**
GGT U/L	**28.89**	**71.63**	**51.53**	**53.52**	**0.012**
Bilirubin mg/dL	0.14	0.15	0.16	0.16	0.966
Cholesterol mg/dL	**222.01**	**200.71**	**199.89**	**227.5**	**0.003**
Triglycerides mg/dL	**74.3**	**98.98**	**104.18**	**142.08**	0.001
Iron μg/dL	**204.56**	**265.53**	**261.46**	**225.83**	**0.016**
CPK U/L	149.8	132.27	127.13	138.47	0.650
SED60	10.81	12.87	16.75	16.75	0.078
Magnesium mEq/L	2.1	2.15	2.11	2.06	0.428
GFR mL/min	226.57	213.08	209.83	218.05	0.702

The lower lymphocyte concentrations observed in the 3rd trimester were not significantly different from those in non-pregnant dolphins. This is in contrast to humans whose lymphocytes significantly increase during pregnancy and are highest in the 3rd trimester [2,56]. Leukocytosis in humans results from the elevated cortisol due to physiological stress induced by pregnancy, with neutrophilia being the predominant differential, resulting from impaired neutrophilic apoptosis in pregnancy [57]. Interestingly a significant reduction in lymphocytes in the 3rd trimester relative to the 2nd has not been reported in other cetaceans and only recently described in humans [11,58]. When lymphopenia has occurred in humans one of the possible pathophysiological mechanisms is that the relative lymphopenia could result from down-regulation of lymphopoiesis during pregnancy, with bone marrow alterations selective for B lineage precursors with myeloid and erythroid markers unaffected by pregnancy [59]. This could be a possible explanation for the 3rd trimester lymphopenia observed in the dolphin. There were no significant changes in eosinophils or monocytes throughout gestation.

Significant increases in steroid hormones during dolphin gestation could also be influencing the relative leukopenia observed. While steroid hormones were not evaluated in the current study, variations in cortisol levels in pregnant dolphins have been assessed alongside other glucocorticoids [60] with significantly higher levels of cortisol observed in late gestation (>241 days) and a significant peak occurring in the last month of pregnancy [48]. Glucocorticoids play an essential role in end stage of pregnancy in lung maturation and respiratory development [61,62]. Identifying a lower than expected increase in steroid hormones throughout the progression of pregnancy could impact the lung maturation at the time of parturition, thus establishment of RIs for steroid hormones in successful pregnancies may be useful to clinicians.

Multiple changes in liver enzymes were observed among trimesters with ALKP, AST and ALT all significantly lower in the third trimester when compared to the 1st and/or 2nd as shown in Fig. 3. In humans, normal changes in liver function tests during pregnancy include significantly lower albumin in all trimesters due to hemodilution, and increased ALKP due to increased bone turnover and placental production [63,64]. In dolphins, a dramatic reduction in

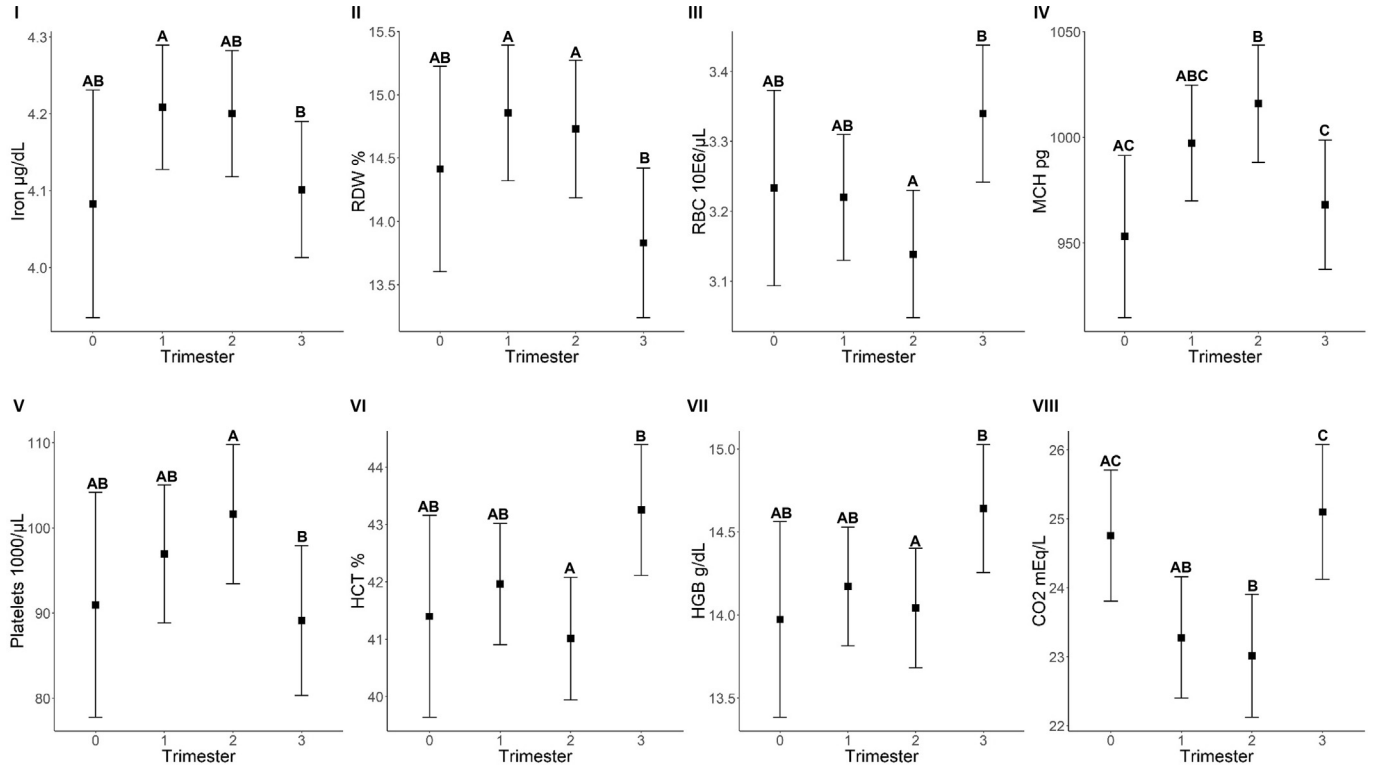

Fig. 1. Significant hematological changes occurring throughout gestation among non-pregnant (0) and trimesters 1, 2, and 3 (RM-ANOVA $P < 0.05$). Least square mean and 95% CI are plotted. Means with statistically significant different pairwise means (Tukey's HSD $P < 0.05$) are denoted by different letters. (I) Iron showed significant decrease from 1st to 3rd trimesters ($P = 0.032$). (II) RBC Dist (RDW) showed a significant decrease in the 3rd trimester from the 1st ($P = 0.010$) or 2nd trimesters ($P = 0.034$). (III) RBC showed a significant increase in the 3rd trimester compared to the 2nd ($P = 0.004$). (IV) MCH showed a significant increase between non-pregnant and the 2nd trimester ($P = 0.044$) and a significant decrease between the 2nd and 3rd trimesters ($P = 0.043$). (V) Platelets showed a significant decreased from the 2nd to the 3rd ($P = 0.041$). HCT (VI) and HGB (VII) showed a significant increase between the 2nd and 3rd trimesters ($P < 0.029$). (VIII) CO2 showed a significant decrease between non-pregnant and 2nd trimester ($P = 0.042$) and an increase in the 3rd compared to the 1st ($P = 0.029$) or the 2nd ($P = 0.010$).

ALKP is a reliable indicator of inflammation. Establishing expected ALKP RI within pregnancy is therefore imperative to discern whether additional inflammatory processes are occurring [65]. In the current study, upper reference values are similar between non-pregnant and pregnant animals (523.95 and 561.95 U/L, respectively), but the lower reference values are drastically different (138.00 and 70.18 U/L, respectively). Whilst the drop in ALKP is often dramatic in cetaceans the high sensitivity with poor specificity results in vast differential diagnoses. These results confirm the significant drop in ALKP in the 3rd trimester is an expected change during dolphin gestation, and most likely a result of normal inflammation as the uterus expands and during parturition as the placenta detaches. This is consistent with the observed decrease in iron potentially due to inflammatory processes. A significant decrease in AST in the third trimester is likely not of clinical significance and is not associated with inflammation in cetaceans [66]. Whilst ALT and GGT decreased after the 1st trimester levels did not decrease below non-pregnant RI. SED 60 which is also used as an inflammatory marker in cetaceans, showed a non-significant increase during pregnancy compared to non-pregnant which was also observed in pregnant killer whales [11].

Total protein also showed a significant increase throughout gestation, with the 3rd trimester levels markedly increased compared to non-pregnant. The sequential increase in total protein in each trimester indicates the hemoconcentration occurring rather than the plasma volume expansion documented in humans. Both albumin and globulin contributed to the total protein increase in the 3rd trimester compared to non-pregnant samples. Globulins also increased when compared to non-pregnant samples but no significant differences were observed. These results appear to be a difference in the dolphin when compared to humans but of a similar pattern to total protein levels documented in pregnant killer whales [11,63]. Interestingly hemoconcentration was not observed in the killer whale with reduced hemoglobin, hematocrit and RBC count in addition to the increased total protein.

Hyperlipemia likely occurs during pregnancy to enhance availability of triglyceride fatty acids for placental transfer to the fetus [67]. Increases throughout gestation are consistent across species and have been documented in harbor seals and dolphins [68,69]. This study confirmed dolphin triglycerides were increased compared to non-pregnant states particularly in the 3rd trimester. In managed care, the increased dietary intake offered could account for some of the triglyceride increase observed, which therefore may not be observed to the same degree in the wild counterparts where prey intake may shift to the most easily obtainable [31]. Cholesterol levels decreased during the 1st and 2nd trimesters then increased in the 3rd trimester similar to non-pregnant levels. Physiologically this likely results from mobilization of fat stores early in pregnancy as observed in other species [70]. Interestingly, equids were found to show a reduction in triglycerides during the 3rd trimester due to a peak occurring in mid gestation from increased hepatic triglyceride synthesis and VLDL secretion [9]. Insufficient sample size within each trimester prevented statistical analysis of HDL, LDL and VLDL in this study and the inclusion of both fasted vs non-fasted samples likely increased variability and could have influenced the results.

Calcium and phosphorus remained relatively stable throughout gestation. Decreases in calcium and phosphorus are frequently

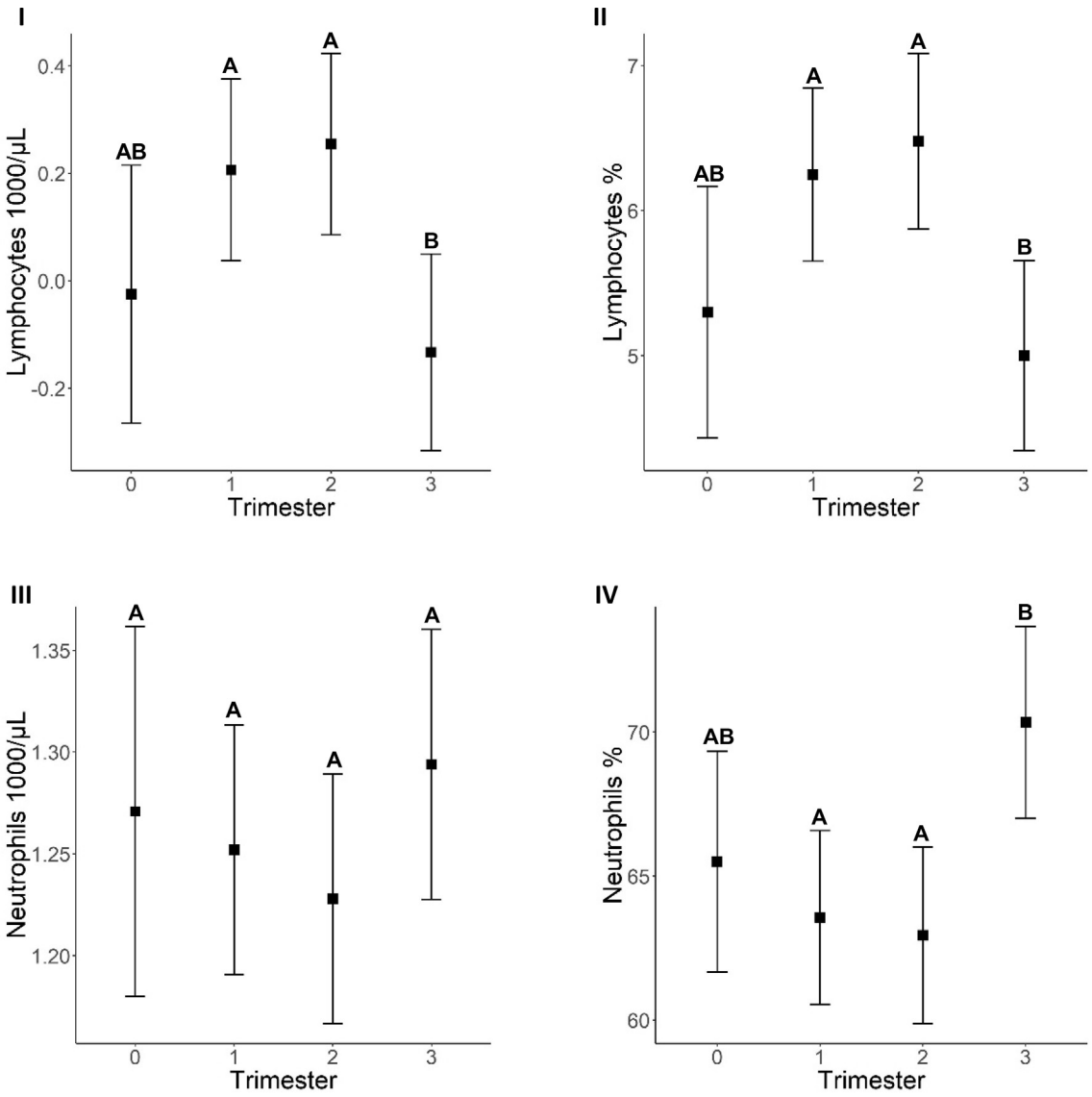

Fig. 2. Significant differences among non-pregnant (0) and trimesters 1, 2, and 3 in neutrophil and lymphocyte absolute count and differentials (RM-ANOVA $P < 0.05$). Least square mean and 95% CI are plotted. Means with statistically significant different pairwise means (Tukey's HSD $P < 0.05$) are denoted by different letters. Lymphocytes showed an absolute decrease in the 3rd trimester (I) resulting in a decrease in lymphocyte (II) and neutrophil (IV) percentages. No significant differences were observed in absolute count of neutrophils (II).

observed throughout gestation in other mammals due to mobilization of reserves for fetal bone development in other species, however the natural diet of many marine mammals is potentially higher in calcium and phosphorus than terrestrial species potentially preventing the reduction observed in mares, cows and pigs [9,71]. Magnesium decreased slightly throughout gestation, but not significantly, which could be due to a small sample size. Hypomagnesemia etiology is complex during pregnancy. The limited ability to mobilize magnesium reserves results in heavy dependence on dietary intake to maintain normal levels [72]. While hypomagnesemia during pregnancy is reported in other species, in dolphins low magnesium is uncommon and it is unlikely that this result would be significant with a larger sample size [73].

A decrease in GFR was observed when comparing non-pregnant vs pregnant samples, which was coupled with a decrease in BUN in the 1st and 2nd trimesters. The lower GFR (averaging 208 ml/min as opposed to 226 ml/min in non-pregnant [74]) observed throughout gestation is an interesting finding in the dolphin, as some species show an increase of up to 85% to excrete the additional creatinine produced by the fetus [75–77]. Further research is needed to understand why the GFR decreases during dolphin gestation. This again highlights the need for RI per trimester as in other species a reduction of GFR during pregnancy has been linked to renal failure and preterm labor [78]. Concurrent increases in creatinine resulted in a decrease in BUN:creatinine ratio. Changes of creatinine are not consistent across species with the highest levels of creatinine recorded in the 3rd trimester in mares and swine but decreasing levels recorded throughout gestation in humans and killer whales [9,11,71,79]. Measured creatinine in dolphins showed slightly elevated levels but not significantly above non-pregnant levels. Creatinine has also been shown to have seasonal variation in dolphins with higher levels recorded in the summer (in Japan) which could correlate to the insignificant slight increases in the 1st and 2nd trimesters observed [80]. Avoiding the seasonal variation in blood results was attempted by standardizing the month of sample selection of non-pregnant samples as June, as

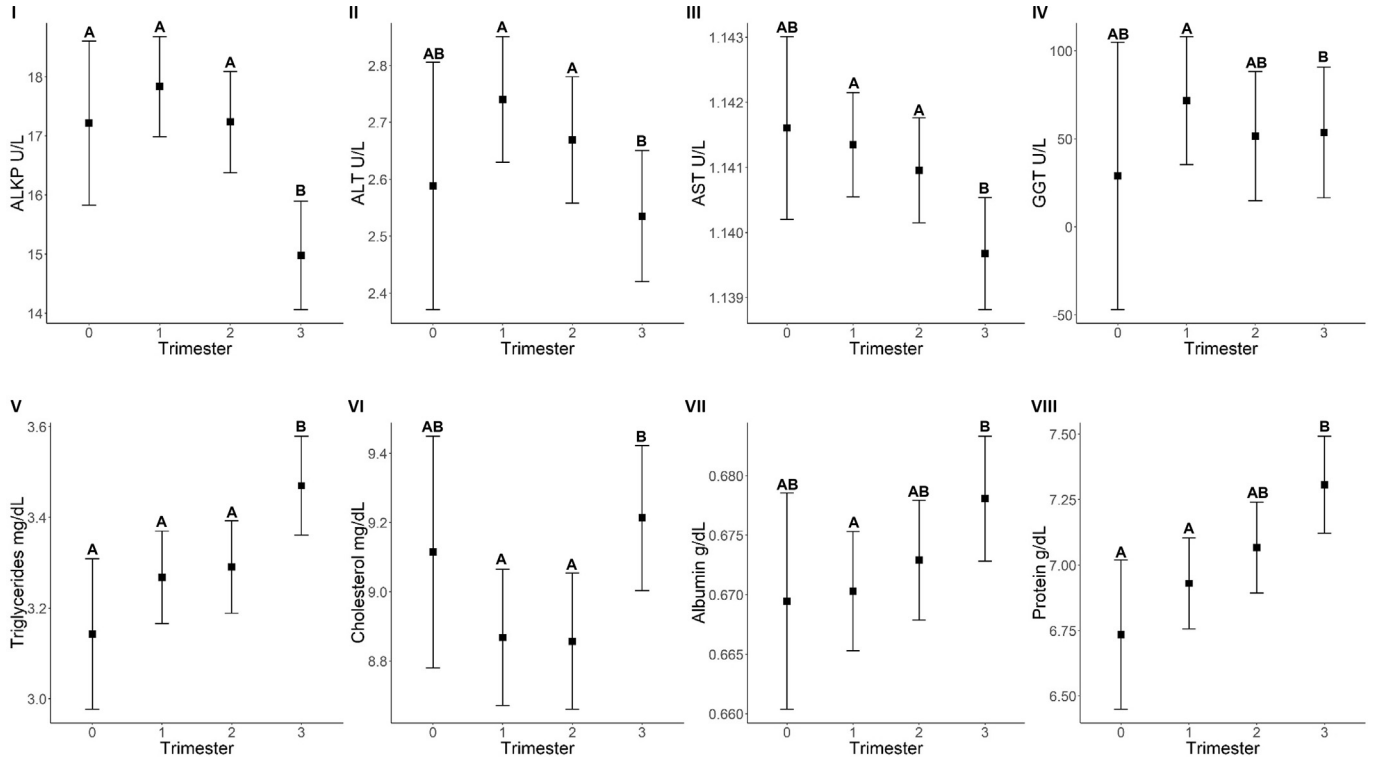

Fig. 3. Significant biochemical changes occurring throughout gestation among non-pregnant (0) and trimesters 1, 2, and 3 (RM-ANOVA $P < 0.05$). Least square mean and 95% CI are plotted. Means with statistically significant different pairwise means (Tukey's HSD $P < 0.05$) are denoted by different letters. (I) ALKP exhibited a significant decrease in the 3rd trimester compared to all other time points ($P < 0.040$). ALT (II) and AST (III) were significantly lower in the 3rd trimester than in the 1st ($P < 0.001$) or 3rd ($P < 0.023$). (IV) GGT significantly decrease between 1st and 3rd trimesters ($P = 0.025$). (V) Triglycerides were significantly increased during the 3rd trimester, relative to any other time point ($P < 0.020$). (VI) Cholesterol significantly increased between 1st and 3rd ($P = 0.0115$) or 2nd and 3rd ($P = 0.009$). (VII) Albumin showed significant increases between 1st and 3rd trimesters ($P = 0.009$). (VIII) Total protein significantly increased between non-pregnant and the 3rd trimester ($P = 0.0057$) and the 1st and 3rd ($P = 0.0016$).

minimal seasonal variation occurs in this month in San Diego.

In the 2nd trimester, CO_2 is lower than in non-pregnant dolphins and returns to non-pregnant dolphin levels in the 3rd trimester (Fig. 1 VIII). Insufficient samples were available to assess the anion gap to fully interpret this CO_2 data, however the presence of a possible metabolic alkalosis in the 3rd trimester with increased bicarbonate during a successful dolphin pregnancy could be the result of hemoconcentration, hyperalbuminemia or the lack of renal excretion of lactic acid during pregnancy due to the decrease observed in the GFR [81]. In humans, CO_2 usually remains decreased throughout gestation. Hyperventilation is observed during human pregnancy, stimulated by increased circulating progesterone and can compensate for any metabolic changes in acid base. Dolphins have also been observed to alter their respiration patterns to enable compensation for metabolic changes therefore a more likely explanation for the change in CO_2 levels is the diaphragmatic compression by the fetus, limiting the large lung tidal volume gas exchange reducing efficient gas exchange [82].

Limitations of this study included the variation of the time within each trimester that the blood sample was obtained and inclusion of both fasted vs non-fasted samples. As samples were taken as part of a comprehensive preventative medicine program for pregnant Navy dolphins and via voluntary behavior not specifically for this study, the intervals between the blood samples varied. To address this concern and to prevent non-independence of samples from the same individual and pregnancy, only a single sample from each pregnancy was included in each trimester and the samples were randomly selected in an attempt to negate any bias. Other potential variables include the use of oral water hydration and the use of nutritional supplements, whilst this should be considered in data interpretation, the rationale behind nutritional supplementation is to account for a diet of frozen vs fresh fish and would not therefore add variability to the data set due to consistent administration to all individuals but rather be an additional source of discrepancy between managed and free-ranging populations.

4.1. Key clinical points

- HCT, HGB, RBC increase significantly during 3rd trimester
- Total WBC remains consistent throughout all three trimesters
- Inflammatory hemogram of ALKP decreasing significantly in 3rd trimester
- Total Protein, Albumin and Globulins increase consistently throughout gestation.

5. Conclusion

Multiple physiological changes occur during successful pregnancy to sustain both the growing fetus and to support the maternal basal requirements. This study demonstrates the physiological demands of pregnancy do affect blood analytes in healthy bottlenose dolphins. The RIs for each trimester of pregnancy established herein will allow more accurate interpretation of blood sample results in managed pregnant animals to prevent over or underdiagnosing abnormalities and administering unnecessary treatments. While these RIs are not directly applicable to wild counterparts, the physiological changes described provide a basis for comparative use in maternal health assessment. In addition, the RIs and physiological changes assessed in these successful

pregnancies can also be used to improve the understanding of the pathophysiology of reproductive failure.

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