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Short Note

Assessment of Serum Amyloid A, Haptoglobin, and Protein Electrophoresis in Clinically Healthy and Abnormal Bottlenose Dolphins (*Tursiops truncatus*)

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Various inflammatory processes (including neoplasia, infection, stress, and trauma) activate a complex, nonspecific innate immune response referred to as the acute phase response (APR). Acute phase proteins (APPs) are a core part of the APR and have been used in a variety of species, including several marine mammals, as part of health assessments and prognostication (Murata et al., 2004; Petersen et al., 2004; Harr et al., 2006; Kakuschke et al., 2010; Sheldon et al., 2017). Assays for C-reactive protein (CRP), serum amyloid A (SAA), and haptoglobin (Hp) have also been validated in healthy bottlenose dolphins (*Tursiops truncatus*) (Cray et al., 2013). Previous studies have shown higher circulating SAA in clinically abnormal animals ($N = 3$), a correlation between SAA and Hp with commonly used hematological and biochemical inflammatory parameters, and physiologic increases of SAA during late term pregnancy and postpartum (Segawa et al., 2013; Flower et al., 2016; Miller et al., 2017). In addition, a study identified SAA as a sensitive indicator of onset and resolution of respiratory disease in a population of captive bottlenose dolphins (Trumbull et al., 2017). In total, these studies support the premise that SAA and Hp can serve as useful markers for identification and monitoring of disease in bottlenose dolphins.

APPs can increase with systemic inflammation including that observed with infection, stress, neoplasia, and trauma. APP levels can vary by etiology and time frame of expression. For example, in cattle (*Bos taurus*), SAA and Hp can be utilized to discriminate between active and chronic inflammatory diseases (Horadagoda et al., 1999; Harr et al., 2006). Another study of northern elephant seals (*Mirounga angustirostris*) identified elevations in SAA during preclinical *Otostrongylus circulitis* infection, whereas CRP only became elevated after clinical signs developed (Sheldon et al., 2017). Understanding how SAA and Hp vary over time in diseased bottlenose dolphins would improve the clinical utility of these biomarkers.

Protein electrophoresis (EPH) is another tool for assessing the APR in many animal species (Tatum et al., 2000; Cray et al., 2009). EPH has been evaluated in healthy and ill dolphins (Bossart et al., 2008, 2011, 2012; Reif et al., 2009; Schwacke et al., 2009). Elevated total globulins and alpha-2 globulins have been identified in dolphins with orogenital papillomas (Bossart et al., 2008). In addition, morbillivirus seropositive Atlantic bottlenose dolphins were found to have increased gamma globulins and decreased albumin which resulted in a low albumin:globulin (A:G) ratio (Bossart et al., 2011). Dolphins with lobomycosis were shown to have increased total protein, total globulin, alpha-1, beta, and gamma globulins as well as decreased albumin and A:G ratio (Reif et al., 2009). Overall, these studies demonstrate that EPH can be an effective marker of disease in cetaceans even in the absence of significant hematological and serum chemistry changes.

The objective of this retrospective study was to compare SAA, Hp, and EPH fractions between clinically healthy and abnormal bottlenose dolphins within a managed care population.

Furthermore, APP levels as well as EPH fractions were compared between dolphins with active and chronic disease states. The hypothesis was that unhealthy bottlenose dolphins will have elevations in SAA and Hp as well as alterations in EPH that will differ between active and chronic disease processes.

Sixty-seven banked serum or plasma samples from 27 (nonpregnant) bottlenose dolphins under managed care were collected from Georgia Aquarium, Inc. facilities in Atlanta, Georgia, and St. Augustine, Florida. This population included 11 females and 16 males varying in age from 0.1 to 50.7 years. Medical records were reviewed, and animals were divided by health status into normal ($N = 30$) and abnormal ($N = 37$) groups. Animals were considered normal if they did not have a documented disease, were behaviorally normal at the time of sample collection, and did not have significant deviations from established reference intervals on standard hematology and biochemistry analysis. Some animals had samples taken during both clinically normal and abnormal time periods. Serum and plasma was submitted at the time of collection or frozen and stored at a minimum of -20ºC for up to 10 years prior to analysis.

Clinically abnormal animals were further stratified into active and chronic disease processes. Animals with clinical, hematological, or biochemical abnormalities that required antibiotic therapy were classified as acute illness. Animals with a history of chronic illness or convalescent at the time of sampling were classified as chronic. Chronic diseases included diagnosed squamous cell carcinoma, pulmonary fungal disease, undiagnosed pulmonary disease, chronic liver abnormalities, and chronic renal abnormalities.

Banked serum and plasma samples were analyzed at the University of Miami Avian & Wildlife Laboratory in Miami, Florida. SAA was analyzed using a dolphin-specific enzyme-linked immunosorbent assay (ELISA) kit (Dolphin SAA ELISA, SAA-18; Life Diagnostics, Inc., West Chester, PA, USA) and read with an ELISA reader (Spectramax; Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's recommendations and protocol. Hp levels were analyzed using a commercial kit of reagents and calibrators (Phase Haptoglobin kit; Tridelta Tri-DD, Boonton, NJ, USA) as previously described (Cray et al., 2013). EPH was run according to the manufacturer's procedure using split beta gels (Helena SPIFE 3000; Helena Laboratories, Inc., Beaumont, TX, USA) as previously described (Bossart et al., 2012). Fraction percentages were determined by gel scanning and software analysis (Helena software; Helena Laboratories, Inc.). Absolute values were obtained by multiplying the fraction percentage by the total protein concentration. The A:G ratio was calculated by dividing the sum of pre-albumin and albumin by the sum of globulin fractions.

Descriptive statistical analysis was conducted to calculate the mean, standard deviation, and range of values for all test variables. Normality was assessed using the Shapiro-Wilks test. Outliers were evaluated using Horn's algorithm (Horn & Pesce, 2003, 2005). To compare mean values across study populations for normal and abnormal dolphins, a nested general linear model (GLM) was used. Study location and gender were entered as fixed effects, age was a covariate, and individuals were a random effect. Post-hoc comparisons of variable means between locations were conducted by least significant differences.

Total white blood cell (WBC) and erythrocyte sedimentation rate (ESR) run at the time of blood collection were abstracted from the medical record and utilized as a comparative inflammatory parameter. Pearson's correlation coefficients were generated to examine the relationships of SAA and Hp with WBC and ESR. Statistical significance was established at $p < 0.05$. All analyses were conducted using analytical software (*SPSS*, Version 22; IBM Corp., Armonk, NY, USA).

There were 30 samples from 23 clinically normal animals and 37 samples from 15 clinically abnormal animals. There was no significant difference between clinically healthy vs clinically abnormal animals for either SAA or Hp. Levels of alpha-1, alpha-2, and gamma globulins were significantly higher whereas the percent albumin and albumin:globulin ratio were significantly lower in clinically abnormal animals compared to the clinically healthy animals ($p < 0.05$; Table 1).

Results from samples obtained from animals classified as active vs chronic disease were compared (Table 2). Twenty samples were from nine animals classified as active, and 11 samples were from six animals that were classified as chronic disease. Six samples from three animals were excluded from this portion of the analysis because they did not meet the definition of acute or chronic illness, although they had minor abnormalities in serum chemistry. There was no significant difference between animals with active vs animals with chronic disease for either SAA or Hp. Animals classified as active disease had significantly lower total protein, albumin, pre-albumin, alpha-1, and gamma globulins compared to those with chronic disease ($p < 0.05$).

Significant positive correlations were noted between SAA and both WBC ($r = 0.38$, $p < 0.01$) and ESR ($r = 0.38$, $p < 0.01$). There were no significant correlations between Hp and either WBC ($r = 0.07$, $p > 0.1$) or ESR ($r = 0.24$, $p > 0.05$). There was, however, a weak but significant

Table 1. Nested univariate analysis: adjusted means (standard error) for samples, adjusted for age and sex, by health status

	Clinically healthy ($N = 30$)	Clinically abnormal ($N = 37$)	p value
Serum amyloid A (mg/L)	1.86 (1.96)	4.36 (2.29)	0.45
Haptoglobin (mg/ml)	0.12 (0.03)	0.70 (0.03)	0.29
Total protein (g/dl)	6.36 (0.12)	6.64 (0.14)	0.17
A:G ratio (g/dl)	1.89 (0.04)	1.73 (0.04)	0.01*
Pre-albumin (g/dl)	0.18 (0.01)	0.15 (0.01)	0.24
Pre-albumin (%)	2.81 (0.17)	2.26 (0.20)	0.07
Albumin (g/dl)	3.95 (0.07)	3.95 (0.08)	0.98
Albumin (%)	62.27 (0.44)	59.87 (0.52)	< 0.01*
Alpha-1 globulins (g/dl)	0.24 (0.01)	0.29 (0.01)	0.03*
Alpha-1 globulins (%)	3.82 (0.15)	4.38 (0.18)	0.04*
Alpha-2 globulins (g/dl)	0.50 (0.02)	0.60 (0.03)	0.01*
Alpha-2 globulins (%)	7.79 (0.23)	9.10 (0.27)	< 0.01*
Beta globulins (g/dl)	0.55 (0.03)	0.54 (0.03)	0.76
Beta globulins (%)	8.73 (0.36)	7.97 (0.42)	0.22
Gamma globulins (g/dl)	0.94 (0.03)	1.12 (0.04)	<0.01*
Gamma globulins (%)	14.58 (0.45)	16.46 (0.52)	0.02*

*Significantly different at $p < 0.05$

positive correlation between SAA and Hp ($r = 0.12$, $p < 0.01$). In some animals, samples were collected during early and mid-disease state as well as during convalescence. In animals with increased SAA and Hp, there was a consistent return to baseline that mirrored changes in WBC and ESR.

As has been previously shown, EPH can provide valuable information in clinically abnormal dolphins (Bossart et al., 2008, 2011, 2012; Reif et al., 2009; Schwacke et al., 2009). In the present study, EPH fractions indicated significantly lower percent albumin in abnormal animals compared to clinically healthy animals ($p < 0.01$). Within the abnormal animal group, there was significantly lower total albumin ($p < 0.01$) in the active disease group. As albumin is a negative APP, a decrease during acute inflammation to provide additional amino acids for APP production is the anticipated response.

Percent and total alpha globulins (1 & 2) were significantly higher in abnormal compared to clinically healthy animals (p range = < 0.01 to 0.04). In animals with active disease, total alpha-1 globulins ($p = 0.01$) were significantly lower than in animals with chronic disease. In mammals, alpha globulins include several APPs which typically increase during active disease; the change in the current study may indicate the presence of negative APPs in this fraction in the dolphin species. Although the adjusted mean was lower for alpha-1 globulins during active disease, more samples from dolphins with active (12/20, 60%) than chronic (4/11, 36%) illness exceeded the laboratory reference interval (0.06 to 0.26 g/dl). The gamma globulin fraction was higher in animals with chronic disease ($p = 0.01$). This EPH fraction contains immunoglobulins, which could be constituently higher in animals with chronic immune stimulation.

There were no significant differences for SAA or Hp between animals with active and chronic disease; however, there were also no differences for other standard markers of inflammation (WBC and ESR). The University of Miami Acute Phase Protein Laboratory (UMAPPL) currently utilizes a reference interval of 0.15 to 5.98 mg/L for SAA. Only one healthy animal had an SAA value slightly elevated beyond these reference intervals (6.95 mg/L) whereas dolphins with active illness had elevations in their SAA which were nearly 2-fold or greater than the reference value in all but one instance (range: 6.03 to 48.42 mg/L). No animals classified as chronic disease had elevations

Table 2. Adjusted means (standard error) for samples, adjusted for age and sex, by active and chronic stage of disease using a nested regression analysis

	Active disease ($N = 20$)	Chronic disease ($N = 11$)	p value
Serum amyloid A (mg/L)	4.36 (19.64)	8.78 (12.43)	0.68
Haptoglobin (mg/ml)	0.06 (0.23)	0.16 (0.15)	0.55
Total protein (g/dl)	3.30 (0.68)	9.17 (0.43)	< 0.01*
A:G ratio (g/dl)	1.62 (0.17)	1.77 (0.10)	0.58
Pre-albumin (g/dl)	0.01 (0.07)	0.31 (0.04)	0.02*
Pre-albumin (%)	1.80 (0.95)	3.44 (0.60)	0.30
Albumin (g/dl)	1.89 (0.39)	5.55 (0.25)	< 0.01*
Albumin (%)	59.29 (2.45)	60.37 (1.55)	0.79
Alpha-1 globulins (g/dl)	0.10 (0.05)	0.39 (0.35)	0.01*
Alpha-1 globulins (%)	3.52 (0.61)	4.30 (0.38)	0.44
Alpha-2 globulins (g/dl)	0.30 (0.14)	0.79 (0.09)	0.05
Alpha-2 globulins (%)	9.31 (1.52)	8.35 (0.96)	0.70
Beta globulins (g/dl)	0.19 (0.19)	0.79 (0.12)	0.08
Beta globulins (%)	7.04 (2.30)	8.72 (1.45)	0.66
Gamma globulins (g/dl)	0.80 (0.12)	1.35 (0.08)	0.01*
Gamma globulins (%)	19.06 (2.06)	14.83 (1.30)	0.22
White blood cell count (K/ul)	8.85 (3.94)	6.40 (1.32)	0.64
Erythrocyte sedimentation rate (mm/hr)	15.03 (36.52)	15.31 (11.23)	0.99

*Significantly different at $p < 0.05$

in SAA outside the reference intervals. This supports a level of concern for active disease when SAA values exceed the available reference intervals. Elevation in Hp was observed to be less consistent, with one normal (0.85 mg/ml) and three abnormal (0.47 to 0.61 mg/ml) animals exceeding the UMAPPL reference intervals (0 to 0.37 mg/ml).

There were no significant differences observed for either SAA or Hp levels between clinically normal and abnormal animals. Although the SAA concentration in abnormal animals was more than twice that in normal animals, the mean for the abnormal dolphins fell within the reference range. Given the large variability in SAA, further studies with a larger population would improve the power of the statistical analysis.

There was a significant correlation between SAA and other inflammatory parameters (WBC and ESR). This is consistent with a prior study which identified significant correlation of SAA with ESR, fibrinogen, WBC, and absolute neutrophil count and of Hp with ESR and fibrinogen in dolphins under managed care (Flower et al., 2016). In manatees (family Trichechidae), persistent elevation of SAA during treatment is a negative prognostic indicator (Harr et al., 2006). In the current study, all animals with elevated SAA in this study had resolution of illness associated with a decrease in their SAA. There were insufficient sample time points to determine whether elevations or declines in SAA preceded the changes in other inflammatory markers. Additional clinical studies assessing the prognostic value of APP expression in dolphins would be valuable.

There are multiple limitations to this study. Due to its retrospective nature, some inflammatory markers utilized for cetacean monitoring were not available for all samples and, therefore, not included in the analysis (i.e., fibrinogen and

iron). Further, the definitive diagnosis was not available for all abnormal animals. Additional potential limitations to this study are variations between facilities in animal housing and laboratory analysis. Further, as the analysis was adjusted for potential confounding variables (age and sex) and the dataset was small and utilized banked samples, the significance of this comparison could have been adversely affected.

The current study suggests that the response of SAA and Hp during clinical disease in dolphins is variable. Although not consistent, the data within this note supports a clinical concern when SAA shows a 2-fold elevation beyond UMAPPL reference intervals and potential value in the prognostic monitoring value of this biochemical marker.

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