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Short Note

Serum Amyloid A in Healthy Female Bottlenose Dolphins (*Tursiops truncatus)* During and After Uncomplicated Pregnancy

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The acute phase response is a nonspecific complex systemic reaction to various types of tissue injury, including inflammation, infection, trauma, surgery, or neoplasia (Gabay & Kushner, 1999). A predominant part of this response is the cytokine-induced production or decrease of certain proteins referred to collectively as acute phase proteins (APP). Major APP can increase > 100-fold in 24 to 48 h during inflammatory processes, making them sensitive markers for the diagnosis and monitoring of systemic inflammatory disease in many species (Eckersall & Bell, 2010). This diagnostic indicator may provide an additional means to evaluate inflammation in cetaceans. This is valuable because diagnosis of disease may be difficult in cetaceans due to limitations in the physical examination and species-specific behavior (McBain, 2001).

Serum Amyloid A (SAA) has been identified as a major APP in humans, horses, dogs, mice, rabbits, manatees, and swine, and a moderate to major APP in cattle (Murata et al., 2004; Petersen et al., 2004; Harr et al., 2006; Eckersall & Bell, 2010). A commercially available assay for SAA based on polyclonal anti-human antibodies was recently validated for bottlenose dolphins (*Tursiops truncatus*) (Cray et al., 2013). Since publication of that study, a dolphin-specific SAA kit has been developed. Based on diagnostic samples run using standardized protocols with this kit, SAA concentrations appear to increase in clinically ill dolphins (C. Cray, pers. obs.). This observation was supported by a recent study characterizing mRNA expression and circulating SAA isoforms in bottlenose dolphins in which SAA in plasma was found to be higher in animals with decreased appetite and inflammatory leukograms compared to healthy animals (Segawa et al., 2013).

In addition to increasing due to pathological inflammatory processes, APP will also increase during physiologic inflammatory processes such as pregnancy (Nunokawa et al., 1993; Sacks et al., 2004; Humblet et al., 2006; Coutinho da Silva et al., 2013). Dolphins are loosely defined as seasonally polyestrous spontaneous ovulators but can give birth year-round and have an approximately 12-mo gestation period. Complete blood count (CBC) and serum biochemistry analysis of pregnant killer whales (*Orcinus orca*) and bottlenose dolphins are consistent with a progressive inflammatory state characterized by increased neutrophils, fibrinogen, and other inflammatory parameters (Robeck & Nollens, 2013; Deming et al., 2015). Peripartum SAA levels have been evaluated in horses and cattle with elevations beginning after parturition and returning to baseline values by 60 h to 4 wks and 1 wk, respectively (Nunokawa et al., 1993; Humblet et al., 2006; Coutinho da Silva et al., 2013). SAA concentrations during and after pregnancy in dolphins have not been investigated to date. The knowledge of SAA concentrations in healthy pregnant dolphins is important for the accurate interpretation of hematological, biochemical, and APP results. The objective of this study was to measure SAA concentrations in healthy female bottlenose dolphins before, during, and after uncomplicated pregnancies to aid in further characterizing the pregnancy-associated inflammatory response in dolphins.

Medical records from a single facility were reviewed to identify healthy bottlenose dolphins that delivered healthy calves (nursing; no

clinical signs of illness for the first 14 d after birth) between 2005 and 2015. Banked serum samples from each animal were identified at up to five different time points: (1) within 12 mo prior to conception (nonpregnant), (2) first trimester (1 to 4 mo), (3) second trimester (5 to 8 mo), (4) third trimester (9 to 12 mo), and (5) postpartum (1 mo). Conception was defined as 12 mo prior to delivery unless a date for artificial insemination was known. All pregnant dolphins had a reference sample within 12 mo prior to conception and a third trimester sample. Additional time points were analyzed as available.

Samples were included if the review of medical records identified animals as clinically healthy surrounding the time of sample collection. A clinically normal animal was defined as alert, responsive, active, and exhibiting normal behavior. Exclusion criteria included abnormal behavior requiring medical intervention (excluding minor traumatic injuries or ocular disease), evidence of inflammation in gastric or forced exhale cytology, or radiographic evidence of respiratory disease. A CBC and chemistry profile with fibrinogen was evaluated in the nonpregnant sample, and the pregnancy samples were only included if there was no evidence of inflammation in this nonpregnant sample. A normal CBC in this study was defined as a total white blood cell count of 2,500 to 9,000 K/μL and band neutrophils less than 5%. This definition is similar to published reference intervals by Reidarson (2010); however, the definition used herein is more conservative and based on clinical observations (pers. obs. by M. Davis, J. St. Leger, and N. I. Stacy).

Serum was stored at an ultra-low freezer temperature (-80º C) for up to 10 y prior to analysis. Samples were assembled and sent to the University of Miami Avian & Wildlife Laboratory (UMAWL) for analysis. SAA was analyzed using the Dolphin SAA enzyme-linked immunosorbent assay kit (ELISA) (Life Diagnostics, Inc., West Chester, Pennsylvania, USA) according to the manufacturer's recommendations and protocol on the Spectramax ELISA reader (Molecular Devices, Sunnyvale, California, USA). Samples were initially screened in duplicate at a 1:50 dilution. Samples that exceeded the high end of the standard curve were further diluted to 1:750 to obtain a final value.

Normal values referenced in manufacturer's instructions were 0.47 ± 0.11 mg/l (n = 6), and abnormal values were 3.27 ± 2.47 mg/l (n = 7). Through further research and utilization of this kit, UMAWL established the reference interval 0.15 to 5.98 mg/l (n > 40), which is currently being used for diagnostic submissions (C. Cray, pers. obs.). For review of our data, we utilized the UMAWL reference interval because it was determined from a larger number of animals.

Descriptive statistics (i.e., mean and standard deviation, median, and minimum and maximum) were calculated using *Statistix for Windows* (Analytical Software, Tallahassee, Florida, USA) for the variables of time of sampling (i.e., days to conception, days to calving, and days postpartum) and SAA concentrations. We then tested whether preconception SAA levels were significantly higher than measures during and after pregnancy using the nonparametric Wilcoxon Sign Rank Test. Specifically, we tested the null hypotheses that there were no differences in paired SAA concentrations before conception vs first month of pregnancy (n = 8), before conception vs second month of pregnancy (n = 4), before conception vs third month of pregnancy (n = 11), and before conception vs postpartum (n = 6). Values of p < 0.05 were considered significant.

The study sample included 10 bottlenose dolphins (11 pregnancies). The median age of study dolphins was 17 y (minimum, 10; maximum, 23). Although there was a trend for median SAA concentrations to increase during pregnancy, SAA concentrations were only significantly higher (p < 0.001) during the third month of pregnancy (n = 11; median, 5.23 mg/l) compared to before conception (n = 11; median, 1.42 mg/l) (Table 1). Furthermore, SAA concentrations were significantly higher (p < 0.03) during postpartum (n = 6; median, 8.00 mg/l) than before conception (n = 6; median, 1.32 mg/l).

Five of six postpartum samples were collected at 5 to 7 d postpartum (DPP), with one sample at 16 DPP. Three of these five samples collected at 5 to 7 DPP exceeded the UMAWL reference interval. The one sample that was collected at 16 DPP (1.39 mg/l) was lower than compared to this animal's third trimester SAA (11.91 mg/l) but remained greater than twice the concentration of that individual's nonpregnant or first trimester samples (0.62 mg/l and 0.42 mg/l, respectively). This could indicate that pregnancy-associated elevations in SAA will continue longer than 1 wk postpartum but will begin to decline by 16 DPP. However, there were few postpartum samples evaluated in this study (n = 6), and additional samples are needed to make any conclusions.

This study provides new information on SAA before, during, and after pregnancy in healthy bottlenose dolphins. Median SAA concentrations showed a progressive increase from the first trimester to the postpartum time points, with some samples exceeding the UMAWL reference interval during the third trimester and postpartum periods. In addition, median SAA concentrations were significantly higher during the third trimester

Table 1. Paired comparisons of Serum Amyloid A (SAA) concentration (mg/l) in healthy bottlenose dolphins (*Tursiops truncatus*) before conception vs during pregnancy (first, second, and third month) and after pregnancy

	Before conception	Pregnant, first semester	*p (n = 8)*
Mean ± SD	1.47 ± 0.64	1.29 ± 0.84	
Median (min, max)	1.47 (0.62, 2.53)	1.00 (0.42, 2.79)	0.31
	Before conception	Pregnant, second semester	*p* (*n* = 4)
Mean ± SD	1.38 ± 0.56	3.56 ± 0.79	
Median (min, max)	1.25 (0.86, 2.16)	3.41 (2.78, 4.66)	0.12
	Before conception	Pregnant, third semester	*p (n = 11)*
Mean ± SD	1.64 ± 0.94	6.54 ± 4.55	
Median (min, max)	1.42 (0.62, 3.95)	5.23 (2.07, 16.00)	< 0.001
	Before conception	Postpartum	*p (n = 6)*
Mean ± SD	1.46 ± 0.74	15.74 ± 16.35	
Median (min, max)	1.32 (0.62, 2.53)	8.00 (1.39, 37.94)	0.03

(5.2 mg/l) and postpartum (8.0 mg/l) compared to the nonpregnant group (1.4 mg/l). These results support the idea that dolphins have a pregnancy-associated inflammatory response as described in other species.

The inflammatory response in pregnant mammals, including humans, presents an active, carefully controlled process that attracts inflammatory cells to the site of fetus implantation for the purpose of pregnancy facilitation and protection, and is consistent with a physiologic, modulated immune response (Mor et al., 2011). This pregnancy-associated inflammatory response has been well studied in humans. In one study, increases in the major APP C-reactive protein were greater than two-fold in pregnant women as compared to nonpregnant at as early as 4 wks of gestation (Sacks et al., 2004). In contrast to humans, the majority of the first trimester samples in dolphins had lower SAA than in nonpregnant dolphins, but sample sizes were low. The highest SAA concentrations in pregnant dolphins were observed during the postpartum period. In cattle and horses, it is suggested that the trauma of parturition contributes to the acute phase response (Koets et al., 1998; Jacobsen & Andersen, 2007). Similarly, tissue trauma during parturition could contribute to increased postpartum SAA concentrations in dolphins.

Our results indicate that SAA concentrations are elevated during late pregnancy and in the early postpartum period, contributing to the understanding of inflammatory changes associated with pregnancy in the bottlenose dolphin. These data provide value when determining inflammation due to infection vs the expected SAA changes associated with pregnancy. This information is important to the clinician since higher SAA concentrations in clinically normal, pregnant dolphins are consistent with the presumptive physiological pregnancy-associated inflammatory response. The study was limited by low sample size. A study using a larger sample size could statistically confirm the trend we observed that SAA concentrations are higher during pregnancy than before conception.

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