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Serum proteins in healthy and diseased Florida manatees (Trichechus manatus latirostris)

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ORIGINAL ARTICLE

Serum proteins in healthy and diseased Florida manatees (*Trichechus manatus latirostris*)

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Abstract

A major goal of this study was to determine whether serum protein fractions of healthy Florida manatees differ with age, sex, or living environments (wild versus housed). A second goal was to determine which serum protein fractions vary in diseased versus healthy manatees. Serum protein fractions were determined using agarose gel electrophoresis. Healthy adults had slightly higher total serum protein and total globulin concentrations than younger animals. This largely resulted from an increase in gamma globulins with age. Total serum protein, albumin, alpha-1 globulin, beta globulin, and total globulin concentrations were slightly higher in housed manatees compared to wild manatees, but there was no significant difference in the albumin/globulin (A/G) ratio, suggesting a difference in hydration between these groups. No significant differences were attributable to sex or pregnancy. Serum albumin concentrations and A/G ratios were significantly lower for manatees with boat trauma, entanglements, emaciation, or cold stress compared to healthy manatees. Variable increases were seen in alpha-1globulins, alpha-2 globulins, beta globulins, and gamma globulins. These globulin fractions contain positive acute-phase proteins and immunoglobulins, and their increases may reflect acute or chronic active inflammation. Changes in serum protein fractions were not consistent enough to justify the use of serum protein electrophoresis as a routine diagnostic test for manatees. However, serum (or plasma) protein electrophoresis is required when accurate values for albumin and globulins are needed in manatees and in determining which protein fractions may account for a hyperproteinemia or hypoproteinemia reported in a clinical chemistry panel.

Keywords Healthy · Diseased · Manatee · Serum proteins · Acute-phase proteins

Introduction

The Florida manatee (*Trichechus manatus latirostris*), a subspecies of the West Indian manatee, lives primarily in coastal and inland waters of Florida. The most common causes of illness and death are trauma from watercraft collisions, prolonged exposure to cold water (cold stress syndrome), inflammation/infectious disease, entanglement, perinatal complications, and exposure to biotoxins, notably red tide brevetoxins (Ackerman et al. 1995; Bossart et al. 1998; Bossart et al. 2002; Wright et al. 1995). Some disorders, for example, trauma, entanglement, and chronic hypothermia, can lead to secondary bacterial or fungal infections (Bossart et al. 2002; Bossart et al. 2004). Diseased (ill/injured), wild (free-ranging) manatees are rescued and rehabilitated for release (Adimey et al. 2016).

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It is important to establish reference intervals for each serum protein fraction to interpret serum protein values obtained from diseased manatees. The bromcresol green dye-binding method, typically used to measure albumin with clinical chemistry analyzers in other mammals, results in erroneously high albumin concentrations in manatees. Total globulin concentrations, calculated by subtracting the albumin concentration from the total protein concentration, are erroneously low in clinical chemistry profiles from manatees. Protein gel electrophoresis is the gold standard for measurement of albumin and protein fractions (Eckersall 2008) and serum or plasma protein electrophoresis is required to obtain accurate albumin and total globulin concentrations in serum or plasma of manatees (Harvey et al. 2007). In addition to measuring albumin accurately, protein electrophoresis allows for the measurement of subsets of globulins. Fibrinogen is present in plasma, but absent in serum. Fibrinogen typically migrates between beta and gamma globulins, making separation of these groups of proteins (largely immunoglobulins) difficult in plasma (Eckersall 2008). Consequently, serum protein electrophoresis has generally been used in veterinary and human medicine to determine which types of proteins may be elevated or depressed during disease (Eckersall 2008; Vavricka et al. 2009).

Previous clinical chemistry and hematology studies in manatees have revealed significant differences between healthy wild and healthy housed manatees for many analytes; consequently, differences in serum proteins between these groups also seem likely (Harvey et al. 2007; Harvey et al. 2009). Total globulins were significantly lower in young manatees than in adults (Harvey et al. 2007); consequently, one or more globulin fractions are expected vary with age in manatees. Differences related to sex are also possible, but few have been recognized in earlier clinical chemistry and hematology studies of manatees (Harvey et al. 2007; Harvey et al. 2009).

One goal of this study was to determine whether significant differences were present in serum protein fractions for manatees with regard to location (wild versus housed), age, and sex of healthy manatees. Based on these findings, reference intervals were established for healthy manatees from different age groups. A second goal was to compare serum protein fractions from diseased manatees to values from healthy manatees and determine what protein fraction might be useful in the diagnosis and/or prognosis of manatees with various disorders.

Materials and methods

Animals and sample collection

Blood was collected from the brachial vascular bundle (pectoral arteriovenous plexus) during routine diagnostic health assessments of wild manatees during captures performed by the Florida Fish and Wildlife Conservation Commission and the US Geological Survey, Sirenia Project between 2002 and 2010. Wild manatees were captured in nets deployed either alongshore or from a specialized net boat in open water (Deutsch et al. 1998; Weigle et al. 2001). Blood samples were collected throughout the year, but most samples from wild animals were collected during December and January, when manatees aggregate in springs and power plant warm water discharge areas. Most of these wild animals were captured in Tampa Bay and Crystal River, Florida, with lower numbers captured between Sarasota and Naples, Florida, and in the Florida Everglades. Manatees were assigned to four age groups using body length measurements established by the US Geological Survey's Sirenia Project (Bonde et al. 2012).

Animals were included in the healthy group if they were assessed as healthy on physical examination by an attending veterinarian in the field and had serum amyloid A (SAA) concentrations no higher than 50 mg/L (Harr et al. 2006). Increased SAA concentration has been shown to be a sensitive indicator of inflammation in manatees (Cray et al. 2013; Harr et al. 2006). Consequently, high SAA measurements were used to remove animals that might appear healthy, but have occult inflammatory disease. Even though otherwise deemed healthy, manatees that were visibly pregnant ($n = 7$) were not included in the healthy group(s) used for reference interval determinations and statistical studies; however, these pregnant females were assayed for comparison to results from adult females that did not appear to be pregnant. A total of 139 wild manatees (90 adults, 32 subadults, 15 large calves, and 2 small calves) were classified as healthy and included in the study.

Blood also was collected from 94 housed manatees (34 adults, 40 subadults, 16 large calves, and 4 small calves). To facilitate blood sample collection, housed manatees were dry-docked through pool drainage or rising false bottom floors. Housed manatees were deemed healthy using the same criteria described for wild manatees. They were housed primarily at the Lowry Park Zoo and SeaWorld Orlando, with small numbers housed at the Miami Seaquarium, Epcot's The Living Seas, and two other facilities from 2002 to 2010. Manatees were assessed to be healthy by physical examination and SAA values no higher than 50 mg/L as described for wild manatees. Some healthy housed manatees were assayed more than once. In those instances, only the results from the first assay were used for reference interval determinations and statistical studies.

Blood from wild, diseased manatees was also analyzed between 2002 and 2010. Diseased manatees were rescued by the Florida Fish and Wildlife Conservation Commission teams and transported to one of the facilities listed previously for rehabilitation. Samples from diseased manatees were collected by the staff of the facility housing the manatees. In most cases, the blood was collected the day of arrival at the rehabilitation facility. In some cases, multiple blood samples were analyzed from a manatee during rehabilitation. In those cases, the results from the first blood sample collected were used in this study. A diagnosis was rendered by the examining veterinarian at the facility based on history and clinical signs.

Diseased manatees were assigned to groups based on primary diagnosis. Sufficient numbers of animals with boat trauma ($n = 32$), entanglement ($n = 8$), emaciation ($n = 10$), cold stress ($n = 25$), and red tide ($n = 5$) were available for comparison to healthy controls. The original cause of the emaciation was not always known. Two manatees had evidence/history of past boat trauma, and gastrointestinal problems were suspected in two other manatees.

Laboratory analysis

Blood samples without anticoagulant were centrifuged within 1 h of collection, and serum was removed and stored on ice or refrigerated at 4 °C until assayed. Diagnostic Chemical Limited (DCL) reagents (Diagnostic Chemical Limited, 160 Christian Street, Oxford, CT 06478, USA) were used to measure total protein in a Hitachi 911 analyzer (Boehringer Mannheim/Roche Applied Science, 9115 Hague Road, Indianapolis, IN 46250, USA). Serum amyloid A was measured in duplicate using a manual anti-bovine SAA ELISA method (Tridelta Development Limited) validated for use in manatees (Harr et al. 2006). The lower limit of detection was 10 mg/L. Samples were diluted up to 1 to 10 when SAA results were outside the standard curve. Values above 1200 mg/L were recorded as greater than 1200 mg/L. For statistical analysis, values below lower limit of detection (10 mg/L) were recorded as 10 mg/L, and values above the standard curve (> 1200 mg/L) were recorded as 1200 mg/L. Serum protein electrophoresis was performed using an agarose gel (Paragon Electrophoresis System, Beckman Coulter Inc., 4300 North Harbor Boulevard, Fullerton, CA 92835, USA). Stained gels were scanned and percentages of each protein band were determined using a densitometer (Beckman Appraise Densitometer, Beckman Coulter, Inc.). Globulin fractions were identified as alpha-1, alpha-2, beta, and gamma. Concentrations of serum proteins were calculated by multiplying the total protein concentration by the albumin and globulin fraction percentages.

Statistical analysis

Statistical analyses were performed using SigmaStat (Systat Software, Inc., Richmond, CA, USA). A two-way ANOVA with Holm-Sidak method for comparison of pairs was used to compare test results from manatees by location (wild versus housed) and age group; however, data from small calves were not included because of inadequate animal numbers in this age group. A two-way ANOVA was also used to compare location and sex, but only adult animals were used for this comparison. A Kruskal-Wallis one-way ANOVA on rank test with Dunn's method for comparison of pairs was used when small calves were included in age comparisons and when diseased manatee groups were compared to healthy wild manatees. A Mann-Whitney rank sum test was used to compare protein fractions from each diseased group to healthy wild manatees, as well as adults versus subadult/large calf groups of healthy manatees used to establish reference intervals.Test results were considered statistically significant at $P < 0.05$. Reference intervals (95% double-sided) were determined following the American Society for Clinical Pathology guidelines (MedCalc Statistical Software, Acacialaan 22, 8400 Ostend, Belgium) (Friedrichs et al. 2012). All protein assays for these healthy groups were normally distributed as assessed by the D'Agostino-Pearson test, except for alpha-1 globulin in the adult healthy group. Log transformation resulted in a normal distribution for this group.

Results

Healthy manatees

To gain insight into the variability of serum protein assays over time in healthy manatees, the coefficient of variation (CV) for each analyte was determined for each of three healthy adult manatees that were bled multiple times. Total serum protein showed the least variability, followed by albumin (Table 1).

Slightly, but significantly, higher total serum protein ($P < 0.001$), albumin ($P < 0.001$), and total globulin ($P = 0.028$) concentrations were measured in healthy housed compared to healthy wild manatees (data not shown). However, there was no significant difference for albumin/globulin (A/G) ratio ($P = 0.223$) between wild and housed manatees. Adults had slightly higher total serum protein concentrations than large calves (Fig. 1), slightly higher total globulins than subadults and large calves (Fig. 2), and slightly lower A/G ratios than subadults (Fig. 3). There were no significant age-related differences in albumin concentration.

In addition to albumin, alpha-1 ($P < 0.001$) and beta ($P < 0.001$) globulin concentrations were slightly higher in housed compared to wild manatees (Fig. 4). There were no significant differences in alpha-2 ($P = 0.099$) or gamma globulin ($P = 0.894$) concentrations between wild and housed manatees. Adults had slightly lower alpha-1 globulin concentrations (Fig. 5) and noticeably higher gamma globulin concentrations than subadults and large calves (Fig. 6). Mean serum gamma globulin concentration in six small calves was less than half the mean value for adults (Fig. 7). There were no significant age-related differences in alpha-2 or beta globulin concentrations.

Sample sizes were not large enough for optimal reference interval calculations when manatees were separated in groups

Table 1 Coefficient of variation (CV) of serum protein assays performed on multiple samples from healthy manatees over periods of 3 to 4 years

Analyte	Manatee 1 ($n = 7$), %	Manatee 2 ($n = 8$), %	Manatee 3 ($n = 12$), %
Total serum protein	4.1	2.3	4.2
Albumin	7.2	8.0	9.9
Alpha-1 globulins	19.0	10.6	26.3
Alpha-2 globulins	16.4	9.6	10.4
Beta globulins	13.6	13.8	11.7
Gamma globulins	13.6	13.4	10.7
Total globulins	15.0	9.9	10.0
Albumin/globulin ratio	19.2	16.2	18.9

Values in parenthesis represent the number of samples assayed for that manatee

by location and age. However, serum protein differences between wild and housed manatees and between subadults and large calves were small; therefore, reference intervals for adults and reference intervals that combined subadults and large calves were calculated (Table 2). Total serum proteins ($P = 0.040$), gamma globulins ($P < 0.001$), and total globulins ($P < 0.001$) were significantly higher and alpha-1 globulins ($P < 0.001$), beta globulins ($P = 0.022$), and A/G ratio ($P = 0.006$) were significantly lower in the adult group compared to the subadult/large calf group. Albumin and alpha-2 globulin concentrations were not significantly different between these two groups. For all measures, however, there was a considerable degree of overlap in the reference intervals between these two age groups. Data from small calves are difficult to obtain, and the number of small calves in this study ($n = 6$) is not sufficient to create a reference interval. However, minimum and maximum values from healthy small calves provided in Table 3 may be useful when diseased small calves are examined.

There were no significant differences in serum protein concentrations between healthy adult male and adult female manatees. Likewise, no significant differences were identified when serum protein concentrations were compared between wild pregnant manatees ($n = 7$) and healthy wild adult females ($n = 35$) that were not visibly pregnant. In addition, serum amyloid A (SAA) concentrations were not significantly different between sexes or between pregnant and healthy wild adult females that were not visibly pregnant.

Diseased manatees

Serum protein concentrations of diseased manatees were compared to values from healthy wild manatees to determine whether

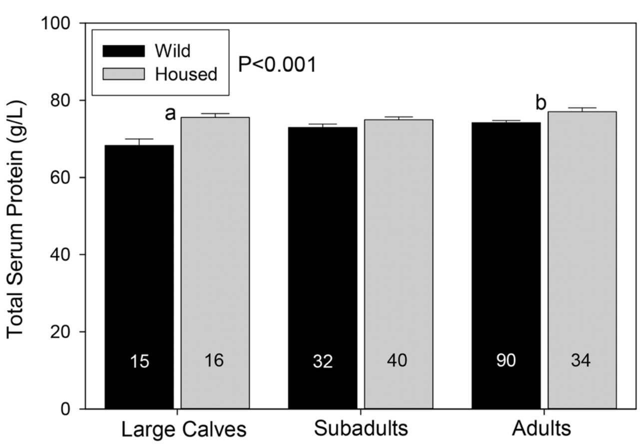

Fig. 1 Comparisons of total serum protein concentrations between age groups in wild versus housed manatees using a two-way ANOVA test with location and age groups as sources of variation. The P value displayed in the figure refers to the overall difference between wild and housed groups. Age groups identified by different letters are significantly different ($P < 0.05$). Each bar shows the numbers of manatees included and the standard error

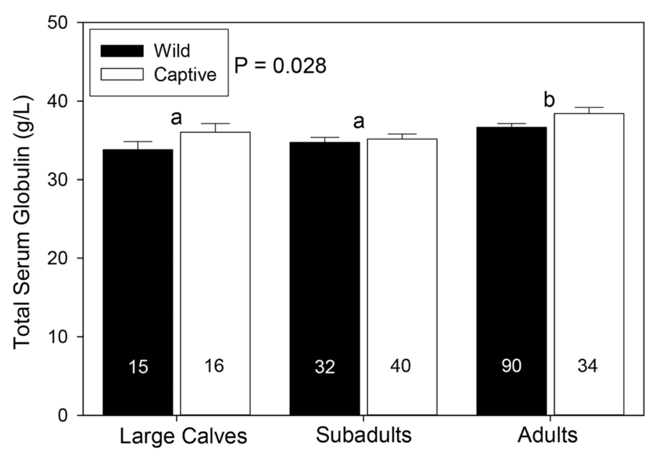

Fig. 2 Comparisons of total globulin concentrations in serum between age groups in wild versus housed manatees using a two-way ANOVA test with location and age groups as sources of variation. The P value displayed in the figure refers to the overall difference between wild and housed groups. Age groups identified by different letters are significantly different ($P < 0.05$). Each bar shows the numbers of manatees included and the standard error

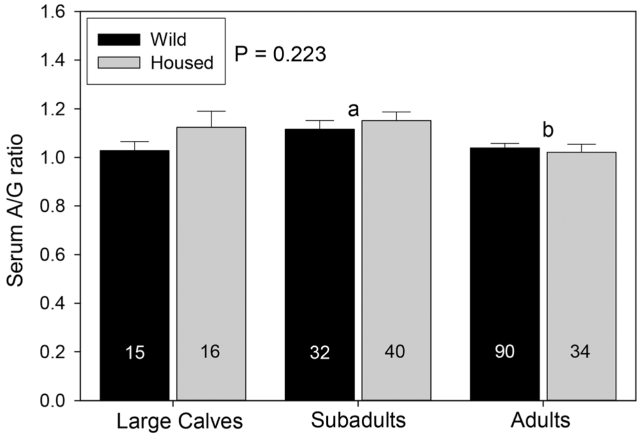

Fig. 3 Comparisons of serum albumin/globulin (A/G) ratios between age groups in wild versus housed manatees using a two-way ANOVA test with location and age groups as sources of variation. The *P* value displayed in the figure refers to the overall difference between wild and housed groups. Age groups identified by different letters are significantly different ($P < 0.05$). Each bar shows the numbers of manatees included and the standard error

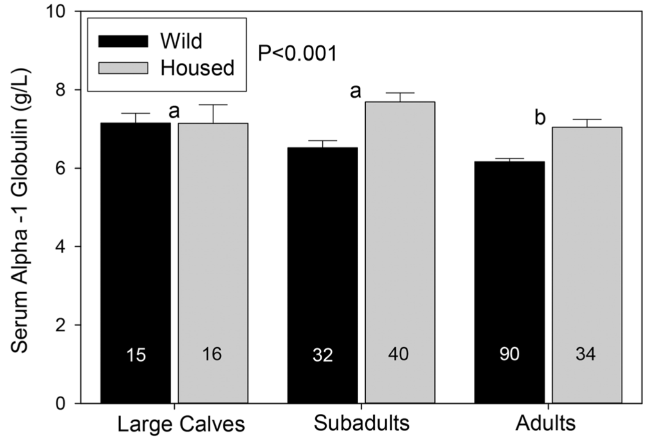

Fig. 5 Comparisons of serum alpha-1 globulin concentrations between age groups in wild versus housed manatees using a two-way ANOVA test with location and age groups as sources of variation. The *P* value displayed in the figure refers to the overall difference between wild and housed groups. Age groups identified by different letters are significantly different ($P < 0.05$). Each bar shows the numbers of manatees included and the standard error

serum protein electrophoresis might be useful in the diagnosis and/or prognosis of manatees with various disorders.

Manatees diagnosed with red tide toxicity (1 adult, 3 subadults, 1 small calf) were compared to the healthy group of wild manatees of all ages (90 adults, 32 subadults, 15 large calves, 2 small calves). There were no significant differences for any of the serum protein fractions or the A/G ratio between these groups, but the number of manatees with red tide toxicity was small.

Manatees diagnosed with boat trauma (16 adults, 9 subadults, 5 large calves, 2 small calves) were also compared to the healthy group of wild manatees ($n = 139$) described above (Fig. 8). Serum albumin was significantly lower ($P = 0.001$) in these injured manatees, but no significant differences were present in total protein or globulin fractions. The A/G ratio in injured manatees (mean = 0.95, standard error [SE] = 0.04) was slightly, but significantly ($P = 0.004$), lower than healthy manatees (mean = 1.06, SE = 0.02).

Entanglements typically involve rope or fishing line constricting flippers or the caudal peduncle. All but one of the entangled manatees were adults; consequently, manatees with entanglements ($n = 8$) were compared to a population of healthy wild adult manatees ($n = 90$). Serum albumin was significantly lower ($P = 0.003$) and alpha-1 globulins ($P < 0.001$), alpha-2 globulins, ($P = 0.001$) and

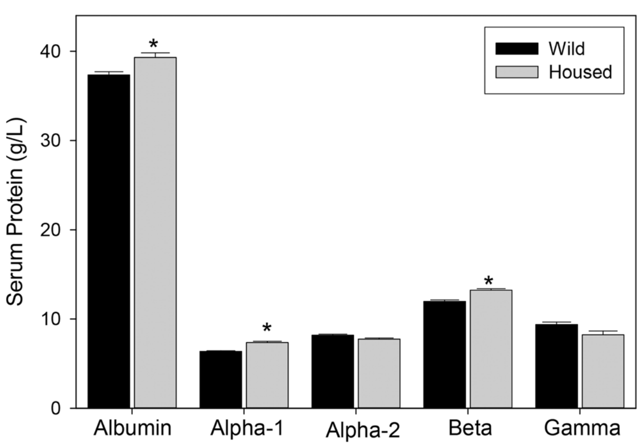

Fig. 4 Comparisons of serum albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin concentrations in wild versus housed manatees. Asterisks indicate significant differences ($P < 0.05$) between groups using a two-way ANOVA test with location (wild versus housed) and age (adult, subadult, large calf) groups as sources of variation. Small calves were not included. Error bars denote standard errors

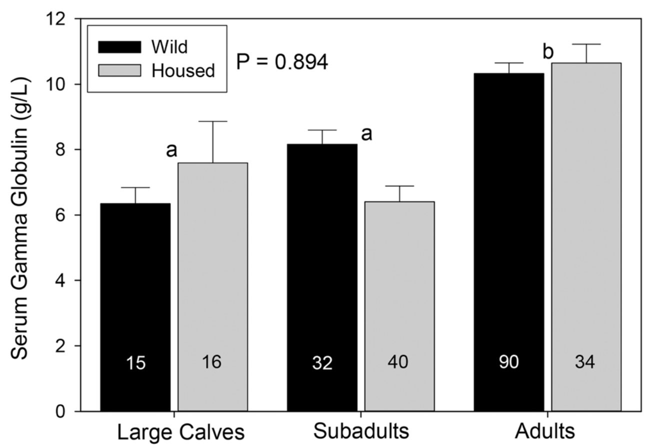

Fig. 6 Comparisons of serum gamma globulin concentrations between age groups in wild versus housed manatees using a two-way ANOVA test with location and age groups as sources of variation. The *P* value displayed in the figure refers to the overall difference between wild and housed groups. Age groups identified by different letters are significantly different ($P < 0.05$). Each bar shows the numbers of manatees included and the standard error

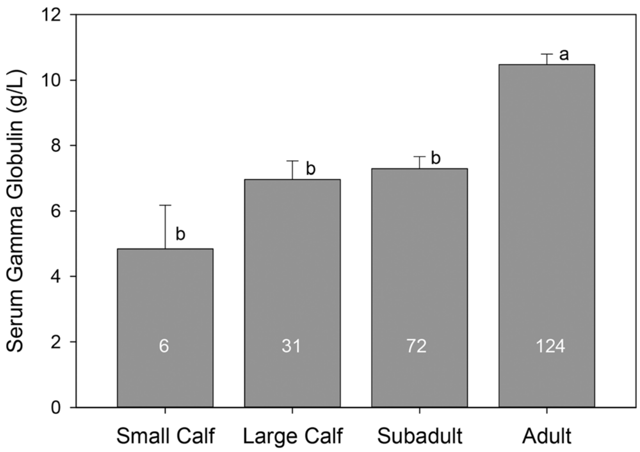

Fig. 7 Comparisons of serum gamma globulin concentrations between age groups in manatees using a Kruskal-Wallis one-way ANOVA on rank test with Dunn's method for comparison of pairs. Small calves were included. Wild and housed manatees were combined. Age groups identified by different letters are significantly different ($P < 0.05$). Each bar shows the numbers of manatees included and the standard error

Table 3 Mean, minimum, and maximum values for serum proteins from six healthy small calves

Analyte	Mean	Minimum–maximum
Total serum protein (g/L)	70.3	64.0–83.0
Albumin (g/L)	35.9	30.3–40.1
Alpha-1 globulins (g/L)	7.2	3.9–8.8
Alpha-2 globulins (g/L)	6.4	5.0–8.6
Beta globulins (g/L)	14.0	8.6–19.1
Gamma globulins (g/L)	4.8	3.2–6.7
Total globulins (g/L)	34.5	26.2–42.9
Albumin/globulin ratio	1.06	0.85–1.44

total globulins ($P = 0.026$) were significantly higher in entangled manatees (Fig. 9). The A/G ratio in entangled manatees (mean = 0.80, SE = 0.06) was significantly ($P < 0.001$) lower than the healthy manatee group (mean = 1.04, SE = 0.02).

Emaciated manatees ($n = 10$) included all age groups, except small calves. Consequently, emaciated manatees were compared to a population of healthy wild manatees without inclusion of small calves ($n = 137$). Serum albumin was significantly lower ($P < 0.001$) and beta globulins and total globulins were significantly higher ($P = 0.009$ and $P = 0.004$, respectively) in emaciated manatees (Fig. 10). The A/G ratio in emaciated manatees (mean = 0.78, SE = 0.06) was significantly ($P < 0.001$) lower than the healthy manatee group (mean = 1.06, SE = 0.02).

Cold stress was diagnosed in all age groups ($n = 24$). Consequently, manatees with cold stress ($n = 24$) were compared to all healthy wild manatees ($n = 139$), including small calves. Serum albumin was significantly lower ($P < 0.001$), and alpha-1 ($P = 0.002$), alpha-2 ($P < 0.001$), gamma globulins ($P = 0.005$), and total globulins ($P < 0.001$) were significantly higher in manatees with cold stress (Fig. 11). The A/G ratio in manatees with cold stress (mean = 0.73, SE = 0.03) was significantly ($P < 0.001$) lower than the healthy manatee group (mean = 1.06, SE = 0.02).

Table 2 Mean values and reference intervals for serum proteins in healthy adults and in combined subadults (SubA)/large calves (LC) groups*

Analyte	Adults Mean	Adults Reference interval	SubA/LC Mean	SubA/LC Reference interval
Total serum protein (g/L)	75.0**	65.1–85.9	73.8	65.6–85.0
Albumin (g/L)	37.8	28.4–45.9	38.4	28.5–48.3
Alpha-1 globulins (g/L)	6.4**	5.1–8.7	7.2	4.7–10.0
Alpha-2 globulins (g/L)	8.1	6.0–10.7	7.9	5.5–1.04
Beta globulins (g/L)	12.2**	8.4–16.1	12.8	9.3–16.2
Gamma globulins (g/L)	10.2**	5.7–15.9	6.9	2.4–13.6
Total globulins (g/L)	37.1**	20.5–45.0	35.0	27.7–43.4
Albumin/globulin ratio	1.03**	0.69–1.33	1.11	0.72–1.53

*Reference intervals represent the prediction interval between which 95% of values from the healthy group fall

**Indicates a significant difference between mean values of adults versus SubA/LC groups using a Mann-Whitney rank sum test

The adult group ($n = 124$) includes 90 wild and 34 captive animals. The SubA/LC group ($n = 103$) includes 32 wild and 40 captive subadults and 15 wild and 16 captive large calves

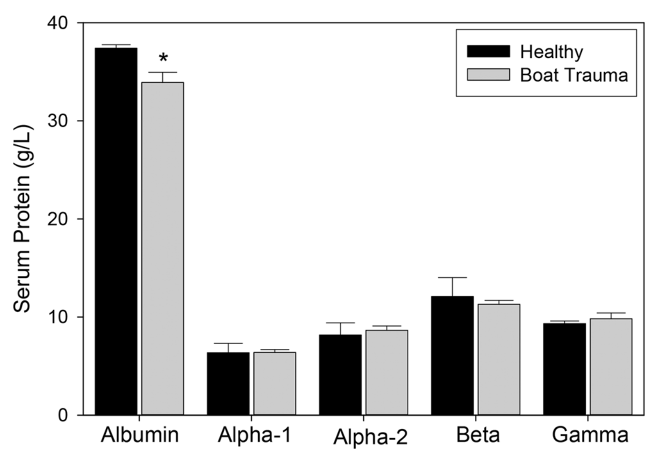

Fig. 8 Comparisons of serum albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin concentrations in healthy wild manatees ($n = 139$) versus manatees with boat trauma ($n = 32$) using a Mann-Whitney rank sum test for each protein fraction. Asterisk indicates a significant difference ($P < 0.05$) between groups. Error bars denote standard error

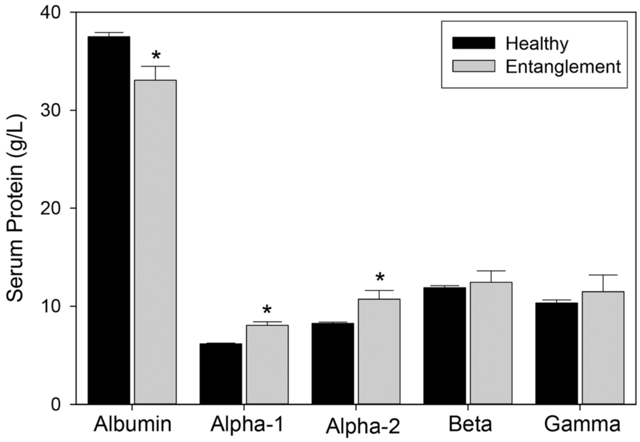

Fig. 9 Comparisons of serum albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin concentrations in healthy wild adult manatees ($n = 90$) versus entangled manatees ($n = 8$) using a Mann-Whitney rank sum test for each protein fraction. Asterisks indicate significant differences ($P < 0.05$) between groups. Standard errors are shown

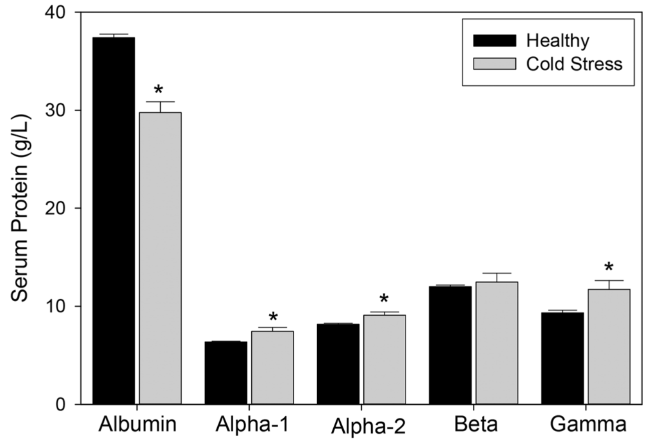

Fig. 11 Comparisons of serum albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin concentrations in healthy wild manatees ($n = 139$) versus manatees suffering from the cold stress syndrome ($n = 24$) using a Mann-Whitney rank sum test for each protein fraction. Asterisks indicate significant differences ($P < 0.05$) between groups. Error bars denote standard error

Values for SAA for each diseased group were compared to healthy wild manatees, as well as each other (Fig. 12). SAA values in all diseased groups were significantly higher than healthy manatees, but diseased groups were not significantly different from one another.

Discussion

Serum proteins in healthy manatees

Although serum proteins vary in concentration in health and disease, only changes in the most abundant serum proteins are capable of significantly influencing the concentrations of alpha, beta, and gamma globulins identified by routine electrophoresis. Major alpha-1 globulins include alpha-1 proteinase inhibitor, alpha-1 apolipoprotein, and alpha-1 acid glycoprotein. Major alpha-2 globulins include alpha-2 macroglobulin, haptoglobin, and ceruloplasmin. Major beta globulins include transferrin, beta apolipoprotein, and several complement components, most notably C3. IgG is the major gamma globulin. IgA and IgM tend to bridge the beta and gamma globulin regions (Abate et al. 2000; Eckersall 2008; Vavricka et al. 2009).

The inter-day within animal CVs depends on both biological variation within the animal and the analytical CV for the analyte being evaluated. The total serum protein CVs and, to a lesser extent, the albumin CVs indicate there is little variation

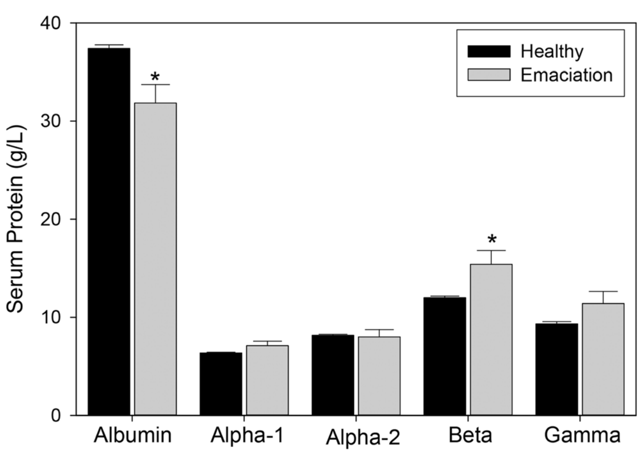

Fig. 10 Comparisons of serum albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin concentrations in healthy wild manatees ($n = 137$) versus emaciated manatees ($n = 10$) using a Mann-Whitney rank sum test for each protein fraction. Asterisks indicate significant differences ($P < 0.05$) between groups. Error bars denote standard error

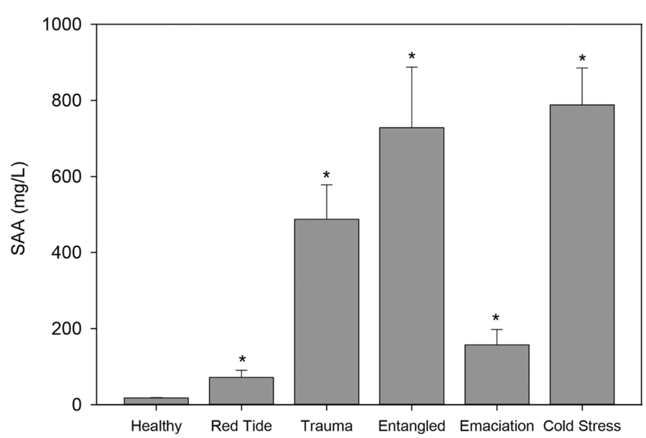

Fig. 12 Comparisons of serum amyloid A (SAA) concentrations in healthy wild manatees ($n = 139$) versus diseased manatees (red tide = 5, boat trauma = 32, entangled = 8, emaciated = 10, cold stress = 24) using a Kruskal-Wallis one-way ANOVA on rank test with Dunn's method for comparison of pairs. Asterisks indicate significant differences ($P < 0.05$) between healthy wild manatees and diseased groups. Error bars denote standard error

in these analytes over several years in healthy manatees. Analytical CVs were not determined in this study. Analytical CVs for serum protein electrophoresis, determined from eight repeated (within run) assays from a single serum sample from a healthy dolphin, were albumin 4.7%, alpha-1 globulin 15.5%, alpha-2 globulin 8.8%, beta globulin 6.3%, and gamma globulin 4.0% (Bossart et al. 2012). Consequentially, substantial portions of the observed variation among manatee samples may be attributed to the limits of analytical precision.

Heathy housed manatees had slightly higher serum protein, albumin, alpha-1 globulin, beta globulin, and total globulin concentrations than wild manatees. No significant difference was measured in the A/G ratio or in SAA. This pattern suggests a difference in the state of hydration, which might be related to dietary/environmental differences between groups or to a fluid shift during the capture process in wild manatees (Harvey et al. 2007).

The findings in the present study are in contrast to, and less dramatic than, results comparing serum protein fractions from healthy dolphins (*Tursiops truncatus*) housed in Florida and Georgia aquariums to free-ranging dolphins in the Indian River Lagoon area of Florida. Free-ranging dolphins had higher total serum protein, alpha-2 globulin, beta globulin, and gamma globulin concentrations, and lower albumin concentration and A/G ratios than housed dolphins (Bossart et al. 2012). This pattern suggests occult inflammation may be present in wild dolphins; however, SAA was not assayed in the animals.

There were several slight age-related differences in serum protein profiles. Compared to younger age groups, adults had higher concentrations of total serum protein and total globulin. The major age-related difference was the substantial increase in gamma globulins with age, which is a consistent finding in many species (Eckersall 2008). Gamma globulins are absent in newborn animals deprived of colostrum. They increase rapidly after colostrum ingestion and then decrease below adult concentrations as maternal immunoglobulins are removed from the circulation. A gradual increase in gamma globulins occurs as young animals begin to produce their own immunoglobulins in response to exposure to various foreign antigens (MacDonald et al. 2004).

Serum proteins in diseased manatees

Serum proteins may be increased or decreased in various disease states. Proinflammatory cytokines, especially interleukin 6, can upregulate or downregulate the synthesis of plasma proteins by the liver (Castell et al. 1990). Proteins that increase in concentration in response to these cytokines are called positive acute-phase proteins (APPs), and those that decrease in concentration are called negative APPs. SAA and C-reactive protein are diagnostically important positive APPs (Eckersall 2008), but they do not occur in sufficient concentrations to contribute significantly to serum protein electrophoresis profiles.

The primary biotoxin-producing organism recognized in the Gulf of Mexico is *Karenia brevis*, a dinoflagellate that produces brevetoxins. An algal bloom of this organism is commonly referred to a red tide. Manatees exposed to red tide inhale and/or ingest large amounts of brevotoxins. Although some evidence of inflammation is present at necropsy (Bossart et al. 1998), and SAA was increased in this group, no significant differences were present in measured serum proteins in manatees with red tide toxicity. However, the sample size for red tide animals was quite small and intoxication can be acute in nature, resulting in symptoms or death prior to detectable serum protein responses in the affected animals.

Albumin concentration may be low because of decreased hepatic synthesis that may occur with liver failure or with inflammation (negative APP). Albumin may also be decreased secondary to plasma protein loss from the body or overhydration; however, total globulins should also be decreased with these latter disorders, which was not the case in diseased manatee groups. For many years, hypoalbuminemia associated with starvation was largely attributed to dietary protein deficiency. However, it is now recognized that protein and energy deprivation does not result in decreased liver-synthesized plasma proteins in humans in the absence of inflammation (Fuhrman et al. 2004; Winston 2012). Consequently, the decreased albumin in diseased manatees, including emaciated manatees, is primarily attributable to inflammation.

Alpha-1 globulins may be increased in acute traumatic and inflammatory diseases because this fraction generally contains substantial amounts of the APPs alpha-1 acid glycoprotein and alpha-1 proteinase inhibitor. Alpha-2 globulins are more commonly increased in acute traumatic and inflammatory diseases than are alpha-1 globulins. This fraction contains APPs alpha-2 macroglobulin, haptoglobin, and ceruloplasmin (Eckersall 2008). Haptoglobin has previously been reported to increase with inflammatory disease in a manatee (Harr et al. 2006). Alpha-1 and alpha-2 globulin concentrations were increased in manatees with entanglement and cold stress, indicating the increase of positive APPs associated with acute inflammation (Vavricka et al. 2009). Increases in alpha globulins may be accompanied by increases in beta and/or gamma globulins when there is also an increase in immunoglobulins, as seen in chronic active inflammation (Vavricka et al. 2009). This pattern was only observed in manatees with cold stress.

Major beta globulins include transferrin, beta apolipoprotein, and several complement components, most notably C3. IgA and IgM tend to bridge the beta and gamma globulin regions (Abate et al. 2000; Eckersall 2008; Vavricka et al. 2009). Increases in beta globulins are most likely to occur if immunoglobulins (especially IgM) are increased. Increased serum beta globulins alone are seldom detected. Mean beta globulins and gamma globulins were higher in emaciated manatees, but only beta globulins were significantly increased.

Increases in IgG, IgM, and/or IgA can result in increased gamma globulin measured by serum protein electrophoresis. These are typically polyclonal (broad protein band on electrophoresis) gammopathies that result from many different immunoglobulins being produced by B lymphocytes and plasma cells. Significantly increased gamma globulin concentration was only identified in the cold stress group, which also had increased alpha-1 and alpha-2 globulins. This pattern of serum protein concentrations, along with markedly increased SAA values, is consistent with chronic active inflammation (Vavricka et al. 2009), as described for a manatee with prior boat trauma that died with a pyothorax (Harr et al. 2011).

The red tide group had no significant changes detected in any protein fraction. The remaining diseased groups displayed somewhat different patterns in serum protein changes measured by electrophoresis. The cold stress group had the highest mean SAA concentration and exhibited changes in all serum protein fractions except beta globulins. Markedly increased SAA concentrations have previously been reported in manatees with cold stress (Cray et al. 2013). Excluding the red tide group, the most consistent findings across the diseased groups were hypoalbuminemia and decreased A/G ratios. A previous study of manatees also reported decreased A/G ratios in serum and plasma of diseased animals (Harr et al. 2006). The A/G ratio determined in plasma had greater sensitivity as a test for inflammation than the A/G ratio determined in serum because the APP fibrinogen is present in plasma, but not in serum.

Conclusions

Total serum protein, albumin, alpha-1 globulin, beta globulin, and total globulin concentrations were slightly higher in healthy housed manatees compared to healthy wild manatees, but there was no significant difference in the A/G ratio. The major age-related difference in serum proteins was a substantial increase in gamma globulins with age, which resulted in slightly higher total globulin and total serum protein concentrations in adults.

Serum protein electrophoresis provides evidence of acute and chronic inflammation in diseased manatees, but changes in protein fractions are not consistent enough to justify the use of serum protein electrophoresis as a routine diagnostic test for manatees. In domestic animals and humans, this assay is usually performed to identify which globulin fractions are abnormal when total globulin concentrations are found to be substantially decreased or increased based on biochemical analysis. However, the current biochemical assay for albumin is not accurate for manatees, resulting in erroneously increased albumin values. Because total globulins are calculated by subtracting albumin concentrations from total protein concentrations, calculated total globulin concentrations are erroneously low in manatees. Unless an accurate biochemical method is identified for albumin in manatees, serum (or plasma) protein electrophoresis is required to obtain accurate albumin concentration, total globulin concentration, and A/G ratio. The benefit of using plasma for electrophoresis is that fibrinogen will be included in the total globulins, and the calculated plasma A/G ratio will more often be decreased than the serum A/G ratio in manatees with inflammation. The benefit of serum protein electrophoresis is that contributions of proteins (other than fibrinogen) in the beta and gamma regions will be more clearly identified because fibrinogen is an abundant protein that migrates between beta and gamma regions with electrophoresis. For manatees, serum protein electrophoresis will be most useful in determining which proteins contribute to a hyperproteinemia or hypoproteinemia reported in a clinical chemistry panel.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. This project was approved by the University of Florida Institutional Animal Care and Use Committee approval no. D882 and performed under the US Fish and Wildlife Service research permit nos. MA791721-3, MA067116-0, and MA773494. This article does not contain any studies with human participants performed by any of the authors.

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