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Identifying coagulopathies in the pathophysiology of cold stress syndrome in the Florida manatee *Trichechus manatus latirostris*

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ABSTRACT: Cold stress syndrome (CSS) in the Florida manatee *Trichechus manatus latirostris* has been defined as morbidity and mortality resulting from prolonged exposure to water temperatures <20°C. The pathophysiology is described as multifactorial, involving nutritional, immunological and metabolic disturbances; however, the exact mechanisms are unknown. We hypothesized that thromboembolic complications contribute to the pathophysiology of CSS in addition to the previously described factors. During the winter of 2014–2015, 10 Florida manatees with clinical signs of CSS were presented to Lowry Park Zoo, Tampa, FL, USA. Thromboelastography (TEG) and coagulation panels were performed at admission. In addition, coagulation panel data from 23 retrospective CSS cases were included in the analyses. There were numerous differences between mean values of TEG and coagulation parameters for healthy manatees and those for CSS cases. Among TEG parameters, reaction time (R), clot formation time (K) and percentage of clot lysed after 30 min (LY30) values were significantly different ($p < 0.05$) between the 2 groups. CSS cases also had significantly higher mean D-dimer concentration and coagulation factor XI activity, prolonged mean activated partial thromboplastin time (aPTT) and significantly decreased mean antithrombin activity. These combined abnormalities include clinicopathologic criteria of disseminated intravascular coagulation, indicating an increased risk of thromboembolic disease associated with manatee CSS.

KEY WORDS: Coagulopathy · Cold stress syndrome · Florida manatee · Thromboelastography · Thromboembolic disease · *Trichechus manatus latirostris*

INTRODUCTION

The Florida manatee *Trichechus manatus latirostris* is an aquatic mammal found in the subtropical waters of the southeastern USA with a minimum population estimate of 6350 individuals (95% CI: 5310–7390) (Martin et al. 2015). Their geographical range and distribution are limited due to a thermoregulatory requirement for warm water (Reep & Bonde 2006). Cold stress syndrome (CSS) is recog-

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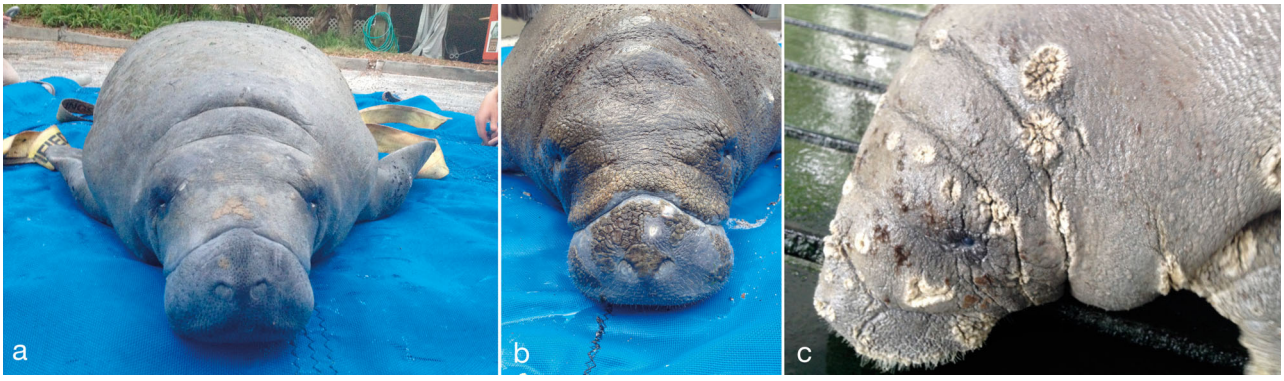

Fig. 1. Progression of epidermal lesions in cold stress syndrome (CSS) in manatees admitted for rehabilitation to Lowry Park Zoo. (a) First stage of epidermal bleaching. (b) Hyperkeratosis of verruciform grey irregular plaques. (c) More advanced epidermal lesions characterized by orthokeratotic hyperkeratosis. Photographs taken by Ashley Barratclough

nized as one of the leading natural causes of mortality in the Florida manatee (Laist et al. 2013), accounting for approximately 18% of annual manatee deaths (Bossart et al. 2004). The condition has been defined as mortality caused by prolonged exposure (>72 h) to water temperatures <20°C (68°F) (Dierauf & Gulland 2001). The pathophysiology is not fully understood and has previously been described as a series of physical and behavioral changes, such as bleaching of the epidermis and anorexia, that can progress to multisystemic organ failure and death in severe cases (Bossart 1999, Owen et al. 2013).

The risk of succumbing to CSS is linked directly to the duration and intensity of the cold weather and the quality of forage nearby the warm-water site (Campbell & Irvine 1981). Compared with CSS seen in dugongs *Dugong dugon*, lack of available forage within warm-water sites compounds the effects of CSS in Florida manatees, preventing heat generation via fermentation, whereas dugongs usually have forage available, potentially allowing improved internal thermoregulation (Owen et al. 2013). The highest recorded mortality (n = 252) attributed to cold temperatures occurred during the unusual mortality event of 2010, where 480 manatees died in a 3 mo timeframe with 252 deaths attributed to CSS (Barlas et al. 2011). During this period, water temperatures consistently fell below 20°C for prolonged periods of time with some of the coldest temperatures on record occurring in South Florida during January. Warm-water refuges, such as natural springs or artificial sources from power plants, provide manatees with the ability to thermoregulate, thereby preventing CSS. Manatee exposure to cold water is expected to increase as older power plants are closed in coming years. These artificial sources of warm water have provided refuge from cold water temperatures encountered in winter months for manatees over the last 55 yr, and some animals do not distinguish between these temporary, human-made refuges and natural refuges (i.e. artisanal springs and warm-water basins) (Bossart 1999, Stith et al. 2012).

The most noticeable clinical signs in CSS involve epidermal lesions. Grossly, these lesions can vary from mild epidermal bleaching to multifocal epidermal ulceration and thickening (Fig. 1). Verruciform grey irregular plaques are frequently observed first, which degenerate into epidermal ulceration. The more advanced epidermal lesions are frequently characterized by orthokeratotic hyperkeratosis, which can be focal or diffuse (Bossart et al. 2002). Histologic examination of blood vessels in bleached skin in early stages of CSS reveals cell debris and fibrin within vessel walls, fibrinoid necrosis and intravascular fibrin thrombi (Fig. 2).

CSS lesions are frequently noted at the extremities, including the peduncle, fluke and flippers, where vessel diameter is naturally smaller and dramatically reduced compared with the peripheral vasculature of other species (Rommel & Caplan 2003). Peripheral vasoconstriction due to cold exposure and the occurrence of endothelial dysfunction could contribute to thromboembolic disease through a combination of luminal narrowing of arterioles, perturbation of blood flow, and concomitant activation of inflammatory and coagulation pathways. We hypothesized that thromboembolic disease could be contributing to the manatee's morbidity and mortality during hypothermia. We therefore undertook studies of hemostasis in cold-stressed manatees to determine whether a systemic coagulation imbalance characterized by hypercoagulability is a pathophysiological feature of CSS. This hemostasis testing included the traditional coagulation screening tests prothrombin

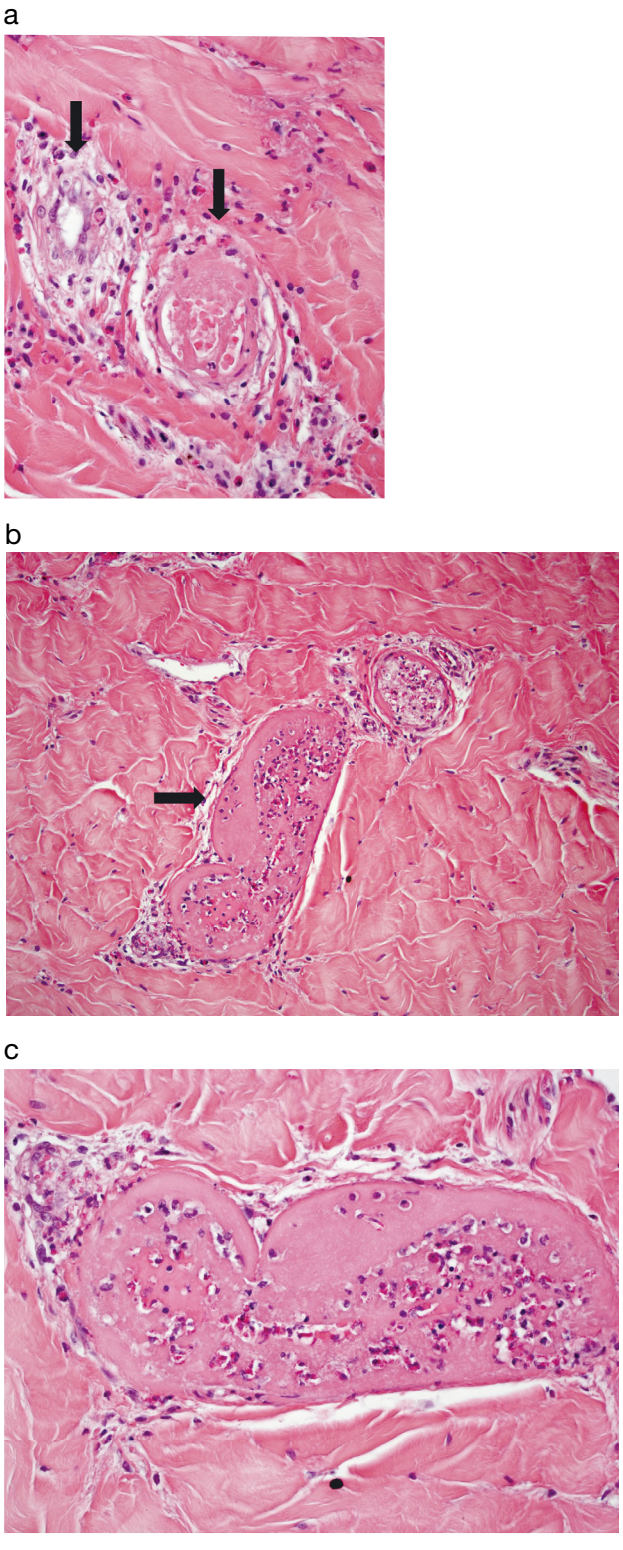

Fig. 2. (a) Difference between a vessel with heterophils in the wall demonstrating vasculitis (left arrow) and a vessel with fibrinoid necrosis of the wall and a thrombus present (right arrow). (b) A large thrombus with evidence of fibrinoid necrosis (arrow) and surrounding heterophils. (c) Higher magnification of the thrombus and occlusion of the vessel

time (PT), activated partial thromboplastin time (aPTT) and fibrinogen concentration, as well as individual coagulation factor activity assays, antithrombin activity and D-dimer concentration. We also performed 'global' hemostasis assays, including whole blood thromboelastography (TEG) (Wiinberg et al. 2005, Epstein et al. 2009, Hall et al. 2012, Sommerey et al. 2014) to assess fibrin clot formation, strength and dissolution, and a thrombin generation assay that measures coagulation potential based on the rate of thrombin formation in plasma (Hemker et al. 2003).

Our goal was to characterize hemostasis in manatees with CSS to facilitate further understanding of this complex syndrome and to improve management and treatment of these cases.

MATERIALS AND METHODS

In preparation for CSS studies, we previously performed a panel of hemostasis assays on samples from 40 healthy Florida manatees collected from the wild during health assessments in winter 2014–2015 from both the east and west coasts (Bonde et al. 2012). Confidence intervals were established for the following tests: PT, aPTT, D-dimer and fibrinogen concentrations (Barratclough et al. 2016a), coagulation factor activity assays, thrombin generation (n = 30 due to sample volume limitations) and TEG (Barratclough et al. in press).

From 5 December 2014 to 3 March 2015, 10 debilitated manatees were admitted into Lowry Park Zoo in Tampa, Florida, USA, for rehabilitation. CSS was diagnosed using previously described clinical criteria (Bossart et al. 2002), which included weight loss, hypothermia and characteristic epidermal lesions, correlated with a cold weather period. For each animal in this cohort, blood collection was performed immediately on arrival for the following tests: TEG, coagulation factor activity assays, and a coagulation panel involving PT, aPTT, D-dimer and fibrinogen concentrations, and thrombin generation. Samples were obtained on arrival prior to the manatee being placed into the rehabilitation pool. Samples were refrigerated and submitted for analysis within 24 h or centrifuged at 1000 × *g* for 15 min to separate the plasma and frozen at −80°C for future analysis as described below.

Retrospective analysis of an additional 23 CSS cases admitted to Lowry Park Zoo from March 2009 to December 2014 were added to the clinical database. These manatees had also been rescued and admitted with clinical evidence of CSS pathology. Blood samples were taken at the time of admission

using the same methodology as previously described. Inclusion criteria involved citrated blood samples for PT, aPTT, D-dimer concentration and fibrinogen levels (IDEXX Laboratories). No TEG values or coagulation factor activity results were available for this cohort due to the retrospective analysis. The IDEXX laboratory used comparable methodology to that used by Cornell University in this study using a commercially available automated clot analyzer (Coag Dx Analyzer™, IDEXX Laboratories). The 33 CSS cases included both male and female calf, juvenile and adult manatees and were from both the east and west coasts of Florida.

Blood was obtained at triage using standard manatee phlebotomy techniques from the capillary bed located between the radius and ulna (Walsh & Bossart 1999). For TEG and coagulation factor analysis, an initial non-additive tube (BD Vacutainer 3.0 ml serum blood collection tube (ref. 366668), Becton & Dickinson) was obtained to prevent tissue contamination of blood, followed by 2 anti-coagulated 0.32% sodium citrate tubes (BD Vacutainer 1.8 ml buffered sodium citrate 3.2% (ref. 366392), Becton & Dickinson) to obtain plasma. Biochemistry and hematology (including platelet count) were assessed on all manatees; however, the results are not included in this study.

The TEG assays were performed using a commercially available TEG® 5000 analyzer (Hemonetics) at 37°C using a TEG kit provided by the manufacturer (Donahue & Otto 2005). These kits were stored at 4°C in a refrigerator until 30 min prior to use. Samples were assayed 3 h post collection to facilitate comparison with the healthy dataset (Barratclough et al. in press). Machine balance and e-test was confirmed prior to running each batch of samples. Citrated whole blood (1 ml) was added to a proprietary blood tube with kaolin (Haemoscope) present. A small volume (340 µl) of the kaolin-activated blood was added to the cup along with calcium chloride (20 µl, 0.2 M, Haemoscope), and the pin was then lowered into the blood (Donahue & Otto 2005). The apparatus oscillated the cup for 10 s at an angle. Once a fibrin clot started to form, the pin moved within the cup and the resistance against movement was transferred up a wire to an electrical transducer, which quantified the critical parameters of the clot (Palmer & Martin 2014). TEG output was recorded on a laptop computer using commercial software (TEG V4 4.2.97). The program displays the thromboelastogram, which was visually inspected for asymmetry or lack of activation prior to accepting the results into analysis.

Immediately following TEG, the samples were centrifuged at 1000 × *g* for 15 min to separate the plasma. This plasma was maintained at –80°C for up to 6 wk before batch shipment on dry ice to the Comparative Coagulation Laboratory at Cornell University for coagulation screening tests (D-dimer, functional (Clauss) fibrinogen, antithrombin activity, coagulation factor activities (factors VII to XII) and thrombin generation assays). The aPTT, PT and fibrinogen assays were performed using an automated clot detection instrument (STA Compact, Diagnostica Stago) and commercial reagents (Dade Actin FS, Dade Behring; Thromboplastin LI, Helena Diagnostics; Fibrinogen, Diagnostica Stago). Antithrombin activity was measured based on inhibition of thrombin (anti-IIa assay) using a commercial chromogenic kit (Stachrom ATIII, Diagnostica Stago) and the manufacturer's automated analyzer (STA Compact, Diagnostica Stago). The standard curves for determination of fibrinogen concentration (Clauss 1957) and antithrombin activity were derived from a calibrated human plasma standard (STA Unicalibrator, Diagnostica Stago). D-dimer concentration in ng ml^{-1} was measured with a quantitative, immunoturbidometric method using a commercial kit (HemosIL, Instrumentation Laboratory) according to the manufacturer's instructions and a human D-dimer standard (HemosIL, D-dimer calibrator, Instrumentation Laboratories). The coagulant activities of factors VII and X (FVII:C and FX:C, respectively) were measured in 1-stage PT assays and activities of factors VIII, IX, XI and XII (FVIII:C, FIX:C, FXI:C and FXII:C, respectively) were measured in 1-stage aPTT assays (Triplett & Harms 1981) configured with a series of human substrate-deficient plasmas (George King Biomedical).

Thrombin generation analysis was performed on all samples with sufficient plasma remaining post initial testing; this included 30 healthy individuals and 15 CSS samples (10 current and 5 archived cases). Thrombin generation was measured by the calibrated automated thrombogram (CAT) method using a dedicated spectrofluorometer (Thrombinoscope, Diagnostica Stago) and the manufacturer's thromboplastin reagent containing 20 pM tissue factor (PPP-Reagent High, Diagnostica Stago). Replicate assays were performed according to the manufacturer's instructions in microtiter plates in reaction mixtures containing 80 µl test plasma, 20 µl activating reagent and 20 µl fluorogenic substrate/calcium trigger reagent (FluCa buffer, Diagnostica Stago). The thrombin generation measurements for each sample were calibrated against reactions run in parallel that con-

tained the test plasma and a thrombin α2 macroglobulin complex reagent (Thrombin Calibrator, Diagnostica Stago). The parameters' lag time (min), peak thrombin (nM) and overall endogenous thrombin potential summarized as area under the curve (AUC) were calculated from the thrombin generation curve with Thrombinoscope software (Thrombinoscope v. 5.0, Diagnostica Stago).

Statistical analysis was performed to compare the group mean and SD results for the CSS cases with those for the healthy manatee group using the statistical software program R (www.R-project.org.) for data analysis. Data distribution was examined for normality by the Kolmogorov-Smirnov test. A Mann-Whitney *U*-test was performed to compare the TEG results for the CSS cases with the healthy manatee normal values (Barratclough et al. in press). An unpaired *t*-test was performed to compare coagulation panel results for PT, aPTT, and D-dimer and fibrinogen concentrations with established normal values (Gerlach et al. 2015). CSS cases with prolonged clotting times beyond the aPTT upper threshold of 180 s (n = 3) or PT threshold of 90 s (n = 7) were assigned numeric values of 200 s and 100 s, respectively. Statistical significance was defined as a p-value of <0.05. The TEG data are reported as mean (SD).

RESULTS

The cumulative number of manatees included in each diagnostic test is presented in Table 1. The 10 manatees sampled during the study period all survived; the 23 retrospective cases had a 30% mortality rate. TEG results revealed several significant differences between the CSS cases and the healthy manatees. The results of the TEG comparison are presented in Table 2. The parameters reaction time (*R*), clot formation time (*K*) and percentage of clot lysed after 30 min (LY30) showed significant differences ($p < 0.05$): *R* was significantly lower in CSS cases, and *K* and LY30 were significantly higher (Fig. 3).

Comparison of the mean (SD) of the 33 CSS coagulation panel results with the 40 healthy manatees revealed significant increases in both D-dimer concentration (p = 0.0001) and aPTT (p = 0.0156) for CSS manatees (Table 3).

The results of thrombin generation for healthy (n = 30) and CSS (n = 6) manatees are displayed in Table 4 and sample thrombograms are shown in Fig. 4. Out of 15 CSS cases, only 6 generated thrombograms; 6 of the 7 CSS cases whose plasma had no clot endpoint in the PT assay also failed to generate thrombin in the CAT assay. In addition, 2 CSS cases were excluded from CAT analyses due to visible clot fragments in the plasma sample that may have resulted from *ex vivo* plasma activation. Overall, the 6 CSS cases with measurable CAT parameters demonstrated lower thrombin generation than their healthy counterparts (Table 4, Fig. 4).

The mean antithrombin and coagulation factor activities for the 10 CSS manatees compared with the healthy manatee reference range are presented in Table 5. Significant differences were found for coagulation factor XI activity (p = 0.00012) in CSS cases, with increased activity in CSS animals compared with healthy manatees, and for antithrombin activity (p = 0.00014), with decreased activity in CSS animals compared with healthy manatees.

Table 1. Number of manatees included in each diagnostic test for the 10 cold stress syndrome (CSS) cases admitted during the study, retrospective CSS cases and wild, healthy individuals. Due to variations in sample availability and volumes obtained, there were different numbers of manatees used in each test

Test	Prospective CSS	Retrospective CSS	Healthy
Thromboelastography	10	–	29
Coagulation panel	10	23	40
Coagulation factor analysis	10	–	40
Thrombin generation	10	5	30

DISCUSSION

Our previous studies to establish reference ranges of TEG parameters for healthy manatees revealed that compared with other species, results from clini-

Table 2. Thromboelastography results for cold stress syndrome (CSS) cases and wild, healthy manatee cases. The p-value indicates statistical comparison. Values are means (SD). *R*: reaction time; *K*: clot formation time; α angle: clot formation rate; MA: maximum amplitude (clot strength); LY30: percentage of clot lysed after 30 min

Sample set	*R* (min)	*K* (min)	α angle (°)	MA (mm)	LY30 (%)
Healthy (n = 29)	2.1 (0.8)	0.8 (0.0)	83.3 (2.0)	75.1 (7.8)	0.4 (0.7)
CSS (n = 10)	1.5 (0.3)	0.9 (0.2)	82.6 (3.2)	76.6 (12.7)	1.2 (1.6)
p-value	0.019	0.020	0.60	0.50	0.048

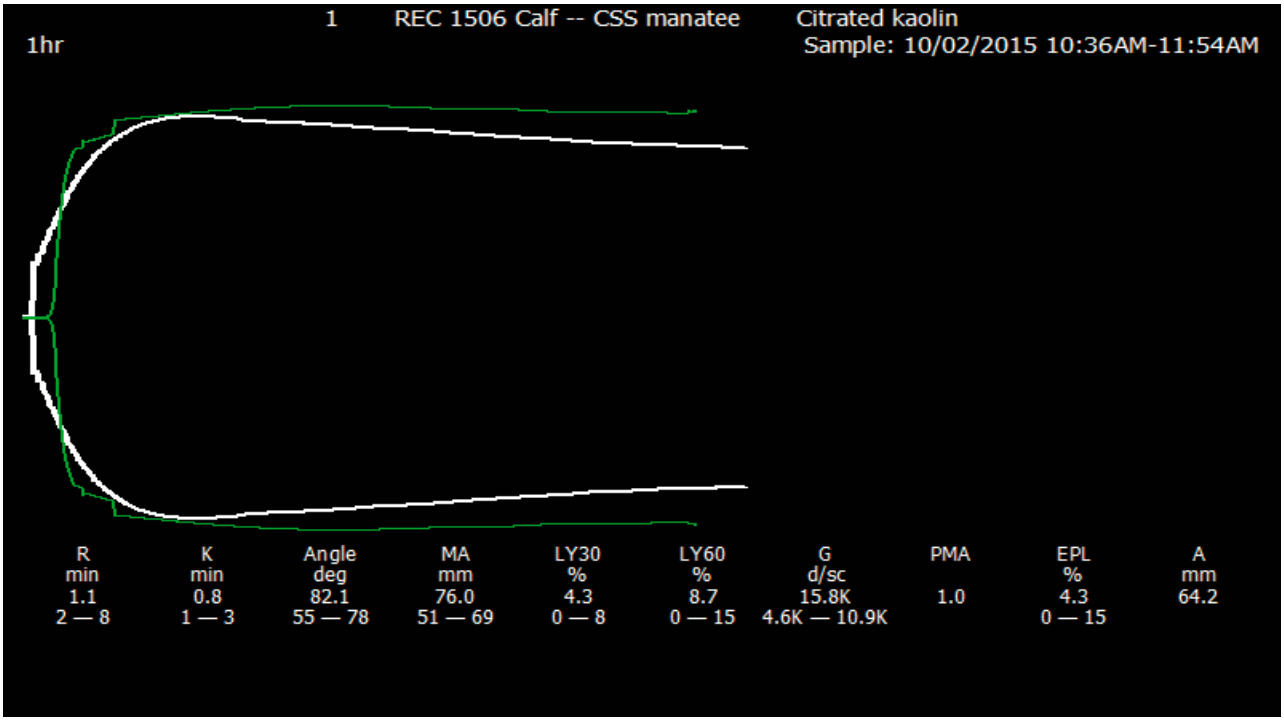

Fig. 3. Example of a hypercoagulable manatee thromboelastogram (white) superimposed on a normal thromboelastogram (green) from the same individual at the time of release for comparison. Note the reduction in reaction time (*R*) with clot initiation commencing after just 1.1 min. Also the lines begin to diverge, showing 4.3 % fibrinolysis at 30 min (LY30), increasing to 8.7 % at 60 min (LY60)

Table 3. Coagulation panel results for cold stress syndrome (CSS) cases compared with wild, healthy manatee cases. The p-value indicates statistical comparison. Values are means (SD). PT: prothrombin time; aPTT: activated partial thromboplastin time

Sample set	PT (s)	aPTT (s)	D-dimer (ng ml^{-1})	Fibrinogen (mg dl^{-1})
Healthy (n = 40)	9.5 (1.5)	10.7 (0.5)	142 (122)	369 (78)
CSS (n = 33)	12.6 (16.1)	25.9 (38.6)	924 (814)	424 (226)
p-value	0.1876	0.0156	0.0001	0.126

Table 4. Thrombin generation results for 30 wild, healthy samples and 15 cold stress syndrome (CSS) samples. Nine of the CSS samples failed to produce any response; therefore, the mean (SD) for the CSS cases is for the 6 samples that generated thrombin. Included are the peak amount of thrombin produced (nM), lag time (min) and the area under the curve (AUC), which is representative of the total amount of thrombin produced

Sample set	Peak thrombin (nM)	Lag time (min)	AUC
Healthy mean (SD)	39 (36.7)	3.7 (1.6)	233.3 (50.3)
Range (n = 30)	18.3–93.9	2.0–7.0	146.9–324.0
CSS mean (SD)	32.3 (16.2)	4.9 (1.5)	178 (64.1)
Range (n = 6)	13.4–51.4	2.2–6.2	88.2–240.3

cally healthy manatees suggest relative hypercoagulability based on short *R* and *K* times and increased clot formation rate (α angle) and maximum amplitude (clot strength) (MA) (Barratclough 2015). In addition, we found minimal variation among individuals compared with the relatively broad reference ranges described for other species.

TEG is one of the few available diagnostic testing options for the assessment of procoagulant imbalance (Fenty et al. 2011). In comparing the TEG results from healthy manatees with CSS cases, we found subtle but significant differences that support the hypothesis that cold stress may be associated with a relative procoagulant excess. The *R* values were the most significantly different variables (p = 0.019), indicating that CSS manatees formed blood clots at a faster rate than their healthy counterparts. The speed of clot formation after initiation (*K*) was also significantly higher in CSS cases (p = 0.02), which is particularly remarkable considering the standard deviation in the healthy subset was zero (i.e. no healthy animals had a result different from 0.8). CSS-affected manatees took longer to attain the predefined clot strength that defines the *K* parameter. Clot strength can be influenced by procoagulant factor activities in addition to hematocrit, platelet

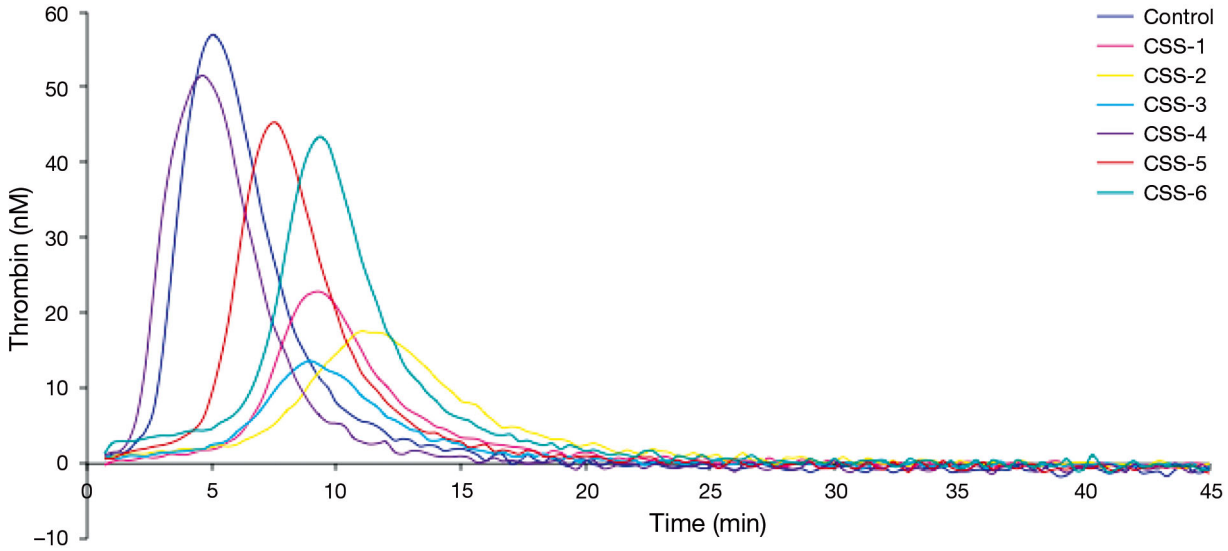

Fig. 4. Time-course curves displaying thrombin generation (thrombograms) for a healthy control manatee and 6 cold stress syndrome (CSS) individuals

count, thrombin formation and fibrin concentration (Donahue & Otto 2005). In combination, the *R* and *K* values reflect that CSS manatees start to form clots quicker but take longer to obtain predefined clot strength. An example of a hypercoagulable manatee thromboelastogram is illustrated in Fig. 3 and is superimposed on a normal TEG from the same individual at the time of release for comparison.

Neither MA or α angle were found to differ significantly in CSS cases compared with healthy manatees, indicating that the final clot strength achieved is the same. LY30 was significantly higher in CSS cases. This parameter is a measure of clot and the activity of the fibrinolytic pathway. The largest value of LY30 in a CSS case was 4.3%. This is still classified as a normal percentage of clot breakdown in other species (<7.5% at 30 min in dogs; Kelley et al. 2015); however, the difference was significantly higher in CSS cases due to the minimal occurrence of fibrinolysis in normal healthy manatees (95% CI: 0.18–0.70%). Dysregulated fibrinolysis may contribute to thrombosis and/or hemorrhage in patients with disseminated intravascular coagulation (DIC) (Fourrier et al. 1992, Toh & Downey 2005). Increased LY30 identified via TEG may reflect concomitant activation of procoagulant and fibrinolytic pathways that occurs in thrombotic DIC (Levi 2005, Barratclough et al. 2017)

In other veterinary species, TEG has been used as a prognostic survival indicator and has been found to be more accurate than routine coagulation tests such as PT and aPTT (Bentley et al. 2013). Our studies to date have defined TEG reference intervals for healthy manatees and revealed significant differences between healthy individuals and CSS cases in several parameters. Future studies are worthwhile to determine whether TEG analyses have prognostic value in manatee cases. All of the 10 CSS manatees that had TEG performed in the current study survived, which precluded our use of survival as an outcome measure. Further analysis would need to be performed on more severe CSS cases to identify TEG changes that correlated with a decreased survival.

Table 5. Cold stress syndrome (CSS) coagulation factor results compared with healthy normal values. The p-value indicates statistical comparison. Mean values are reported followed by SD. FVII:C to FXII:C are the coagulant activities of factors VII to XII, respectively

Sample set	Antithrombin (%)	FVII:C (%)	FVIII:C (%)	FIX:C (%)	FX:C (%)	FXI:C (%)	FXII:C (%)
Mean CSS (n = 10)	103.9	113.8	114.7	118.3	116.0	181.8	132.3
SD CSS	32.0	23.2	68.7	41.6	33.8	64.5	43.2
Mean healthy (n = 40)	131.5	120.1	145	126.4	113.9	124.5	113.4
SD healthy	10.7	23.1	35.0	31.9	19.0	22.6	25.6
p-value	0.0001	0.440	0.054	0.503	0.793	0.0001	0.078

In addition to TEG, we compared coagulation profiles, including PT, aPTT and D-dimer concentration, as well as fibrinogen concentrations obtained at the time of entry for the 33 CSS cases admitted since March 2009. Statistical comparison between CSS and healthy manatees demonstrated significant differences in aPTT ($p = 0.0156$) but not PT ($p = 0.1876$) (Table 3). PT values of healthy and CSS manatees were not significantly different, but this is most likely due to the large standard deviation caused by wide variation among individuals. By increasing our sample size over the 10 cases obtained during this study period, the bias of extreme outliers could be reduced. The extreme results of 180 s are relevant, however, as these values represent delayed or abnormal fibrin clot formation in the assay's reaction mixture.

Normal D-dimer concentrations in humans are <250 ng ml^{-1} (Eichinger et al. 2003). Healthy manatees ($n = 40$) were all below this threshold with a mean (SD) of 132 (126) ng ml^{-1} (Barratclough et al. 2016). The 33 CSS manatees had a wide variation in D-dimer concentrations, with a range of 250–3309 ng ml^{-1}, and mean (SD) of 924 (814) ng ml^{-1}. This is significantly different from the healthy manatees, with a p-value of 0.0001. High D-dimer concentration indicates thrombus formation and lysis, and can be considered among the most clinically useful difference identified in this study. The measurement of D-dimer concentration in CSS cases may represent a prognostic indicator. Monitoring D-dimer concentration may assist in the clinical assessment of the extent of thromboembolic disease (Nelson 2005, Gerlach et al. 2015) and may indicate disease progression or resolution (Stokol et al. 2000). From comparing D-dimer concentrations in the 33 cases with survival outcome, we propose that any value >500 ng ml^{-1} be considered abnormal, with values exceeding 1500 ng ml^{-1} resulting in a grave prognosis. Increasing values throughout rehabilitation were also indicative of a poor survival outcome.

Fibrinogen has limited utility as a prognostic indicator in patients with DIC due to the fact that it is also an acute phase protein. Fibrinogen levels may be increased due to inflammation and infection, as well as decreased due to consumption and depletion (Sato et al. 1995). Healthy manatees were found to have a mean (SD) of 369 (79) mg dl^{-1} with CSS cases having a mean of 424 (226) mg dl^{-1}. There was no significant difference between the healthy and CSS cases ($p = 0.24$). This is likely due to the very wide variation in values in the CSS cases, ranging from 15 to 860 mg dl^{-1}, resulting in a large SD. The CSS cases with low values, such as the 15 mg dl^{-1}, may represent consumptive coagulopathy, and such marked reduction may be of great clinical relevance for diagnosis and management strategies. Animals presenting with significant hemorrhage could indicate the presence of consumptive coagulopathy.

The CSS manatees had lower antithrombin concentrations (103.9 (32)%) than healthy manatees (131.5 (10.7)%) ($p = 0.00014$). Antithrombin is a plasma coagulation inhibitor that binds to and inactivates thrombin and other serine protease coagulation factors responsible for the generation of thrombin. Disseminated intravascular coagulation causes an acquired deficiency of antithrombin due to antithrombin–thrombin formation and complex clearance. Antithrombin may also be depleted secondary to protein losing nephropathy and enteropathy, and burn wounds. (Marder et al. 2012). Previous studies have found that a reduction in plasma antithrombin below 70% of normal reflects a threshold in the pathogenesis of venous thrombosis in humans (Marder et al. 2012). Reduced activity in humans has been related to poor prognosis, and as a result, treatment with antithrombin has been used to prevent DIC and death (Fourrier et al. 1992). Three of the CSS cases showed antithrombin activity <70% suggesting consumption in process. This was corroborated by the extremely prolonged aPTT at >180 s and prolonged PT at >90 s noted in these 3 individuals. This is in stark contrast to the healthy sample set where the lowest antithrombin value was 112% compared with the lowest CSS value of 41%.

While low sample numbers and missing data precluded statistical analyses of CSS thrombin generation parameters, the preliminary results in this study are suggestive of impaired thrombin formation or prothrombin depletion in this patient population. Using a trigger reagent containing high concentration of tissue factor and phospholipid, we obtained thrombograms for all 40 healthy individuals; however, the values for peak thrombin and AUC were generally lower than values reported for healthy human beings. Our data indicate that pre-analytic processing to minimize plasma activation and further optimization of the CAT assay are required to enhance its ability to reliably detect low levels of endogenous thrombin generation for comparisons between healthy and compromised manatees.

Coagulation factor XI was the only procoagulant factor measured that showed statistical difference in activity level between healthy and CSS manatees ($p = 0.00012$). In humans, increased coagulation factor XI activity has been associated with increased risk for venous thrombosis (Meijers et al. 2000, Siege-

mund et al. 2004). This further supports the hypothesis that CSS individuals are likely to have a greater occurrence of thromboembolisms; however, a larger sample size and further mechanistic studies are needed to adequately explore this hypothesis.

Manatee age categories are defined according to their length. Current guidelines for age categories are >260 cm adult, 236–260 cm juvenile and <236 cm calves (Bonde et al. 2012). All 3 categories were represented in this study. We did not include any animals less than 210 cm (neonatal) in length in this study, so all animals in our study are presumed to be over 6 mo old (Bonde et al. 2012). There were no significant differences in the coagulation parameters based on age or sex. The CSS samples used in this study for TEG and coagulation factor-level analysis were opportunistically obtained within the time frame of the project over winter 2014. This was considered a mild winter in Florida. The coastal waters of the Gulf of Mexico did however drop below 20°C, necessitating manatees to change their behavior and seek warm-water refuges during the study period. All CSS cases in this sample set survived, including the 3 individuals demonstrating the most profound coagulation abnormalities. Their clinical signs at presentation were relatively mild, and it is possible that early treatment prevented progression to overt signs of thrombosis. In a more severe winter, it is likely that the survival outcome would be less favorable, as demonstrated in the archived 23 cases where there was a 30% mortality rate. Future studies are needed to characterize the pattern of coagulation abnormalities that accompany more severe CSS cases. The naturally narrowed blood vessels in manatee extremities and the hypercoagulation observed in manatee TEGs could have wider implications in other conditions experienced by manatees in addition to CSS and should be taken into consideration when treating conditions such as entanglements and boat strike injuries.

In conclusion, we found numerous differences in coagulation parameters for CSS cases compared with healthy manatees that warrant further investigation for their prognostic value. CSS has had a profound effect on this species and a thorough understanding of the pathophysiology of this multifactorial condition is important for future management and conservation of this threatened species.

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LITERATURE CITED

Barlas ME, Deutsch CJ, de Wit M, Ward-Geiger LI (eds) (2011) Florida manatee cold-related unusual mortality event, January–April 2010. Final report to USFWS. Florida Fish and Wildlife Conservation Commission, St Petersburg, FL

Barratclough A (2015) Establishing the coagulation profile of the Florida manatee (*Trichechus manatus latirostris*) and identifying coagulopathies in the pathophysiology of cold stress syndrome in the Florida manatee. MS thesis, University of Florida, Gainesville, FL

Barratclough A, Francis Floyd R, Conner B, Reep R, Ball R, Stacy N (2016) Normal hemostatic profiles and coagulation factors in healthy free-living Florida manatees (*Trichechus manatus latirostris*). J Wildl Dis 52:907–911

Barratclough A, Ball R, Francis-Floyd R, Reep R, Conner B (2017) Identifying disseminated intravascular coagulation in the Florida manatee (*Trichechus manatus latirostris*) and understanding its clinical implications. J Zoo Wildl Med 48:152–158

Barratclough A, Francis Floyd R, Reep R, Ball R, Conner B (in press) Determining normal references for thromboelastography in wild Florida manatees (*Trichechus manatus latirostris*). Vet Clin Pathol

Bentley AM, Mayhew PD, Culp WT, Otto CM (2013) Alterations in the hemostatic profiles of dogs with naturally occurring septic peritonitis. J Vet Emerg Crit Care 23: 14–22

Bonde RK, Garrett A, Belanger M, Askin N, Tan L, Wittnich C (2012) Biomedical health assessments of the Florida manatee in Crystal River — providing opportunities for training during the capture, handling, and processing of this endangered aquatic mammal. J Mar Anim Ecol 5: 17–28

Bossart GD (1999) The Florida manatee: on the verge of extinction? J Am Vet Med Assoc 214:1178–1183

Bossart GD, Meisner RA, Rommel S, Ghim S, Jenson AB (2002) Pathological features of the Florida manatee cold stress syndrome. Aquat Mamm 29:9–17

Bossart GD, Meisner RA, Rommel SA, Lightsey JD, Varela RA, Defran R (2004) Pathologic findings in Florida manatees (*Trichechus manatus latirostris*). Aquat Mamm 30: 434–440

Campbell HW, Irvine AB (1981) Manatee mortality during the unusually cold winter of 1976–1977. In: Brownell RL Jr, Ralls K (eds) The West Indian manatee in Florida: Proc Workshop held in Orlando, Florida 27–29 March 1978. Florida Department of Natural Resources, Tallahassee, FL, p 86–91

Clauss A (1957) Rapid physiological coagulation method in determination of fibrinogen. Acta Haematol 17:237–246

Dierauf L, Gulland FM (2001) CRC handbook of marine mammal medicine: health, disease, and rehabilitation, Vol 2. CRC Press, Boca Raton, FL

Donahue SM, Otto CM (2005) Thromboelastography: a tool for measuring hypercoagulability, hypocoagulability, and fibrinolysis. J Vet Emerg Crit Care 15:9–16

Eichinger S, Minar E, Bialonczyk C, Hirschl M and others (2003) D-dimer levels and risk of recurrent venous thromboembolism. JAMA 290:1071–1074

Epstein KL, Brainard BM, Lopes MA, Barton MH, Moore JN (2009) Thrombelastography in 26 healthy horses with and without activation by recombinant human tissue factor. J Vet Emerg Crit Care 19:96–101

Fenty RK, Shaw SE, O'Toole TE (2011) Identification of hypercoagulability in dogs with primary immune-mediated hemolytic anemia by means of thromboelastography. J Am Vet Med Assoc 238:463–467

Fourrier F, Chopin C, Goudemand J, Hendrycx S and others (1992) Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies. Chest 101:816–823

Gerlach TJ, Bandt C, Conner B, Ball RL (2015) Establishment of reference values for various coagulation tests in healthy Florida manatees (*Trichechus manatus latirostris*) and evaluation of coagulation in debilitated manatees during rehabilitation. J Am Vet Med Assoc 247: 1048–1055

Hall DJ, Rush JE, deLaforcade AM, Shaw SP (2012) Kaolin-activated thromboelastography in echocardiographically normal cats. Am J Vet Res 73:775–778

Hemker HC, Giesen P, Al Dieri R, Regnault V and others (2003) Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb 33:4–15

Kelley D, Lester C, Shaw S, de Laforcade A, Webster CR (2015) Thromboelastographic evaluation of dogs with acute liver disease. J Vet Intern Med 29:1053–1062

Laist DW, Taylor C, Reynolds JE III (2013) Winter habitat preferences for Florida manatees and vulnerability to cold. PLOS ONE 8:e58978

Levi M (2005) Pathogenesis and treatment of DIC. Thromb Res 115:54–55

Marder VJ, Aird WC, Bennett JS, Schulman S, White GC (2012) Hemostasis and thrombosis: basic principles and clinical practice, Wolters Kluwer Health, Philadelphia, PA

Martin J, Edwards HH, Fonnesbeck CJ, Koslovsky SM, Harmak CW, Dane TM (2015) Combining information for monitoring at large spatial scales: first statewide abundance estimate of the Florida manatee. Biol Conserv 186: 44–51

Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR (2000) High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 342: 696–701

Nelson OL (2005) Use of the D-dimer assay for diagnosing thromboembolic disease in the dog. J Am Anim Hosp Assoc 41:145–149

Owen HC, Flint M, Limpus CJ, Palmieri C, Mills PC (2013) Evidence of sirenian cold stress syndrome in dugongs *Dugong dugon* from southeast Queensland, Australia. Dis Aquat Org 103:1–7

Palmer L, Martin L (2014) Traumatic coagulopathy – Part 1: Pathophysiology and diagnosis. J Vet Emerg Crit Care 24:63–74

Reep R, Bonde RK (2006) The Florida manatee: biology and conservation, Vol 1. University Press of Florida, Gainesville, FL

Rommel SA, Caplan H (2003) Vascular adaptations for heat conservation in the tail of Florida manatees (*Trichechus manatus latirostris*). J Anat 202:343–353

Sato N, Takahashi H, Shibata A (1995) Fibrinogen/fibrin degradation products and D-dimer in clinical practice: interpretation of discrepant results. Am J Hematol 48:168–174

Siegemund A, Petros S, Siegemund T, Scholz U, Seyfarth HJ, Engelmann L (2004) The endogenous thrombin potential and high levels of coagulation factor VIII, factor IX and factor XI. Blood Coagul Fibrinolysis 15:241–244

Sommerey CC, Williams TL, McCrone I, Ruiz-Ferreras A, Freeman D, Archer J (2014) Thromboelastography in healthy dairy cows. J Dairy Sci 97:5474–5480

Stith B, Slone D, de Wit M, Edwards H and others (2012) Passive thermal refugia provided warm water for Florida manatees during the severe winter of 2009–2010. Mar Ecol Prog Ser 462:287–301

Stokol T, Brooks MB, Erb HN, Mauldin GE (2000) D-dimer concentrations in healthy dogs and dogs with disseminated intravascular coagulation. Am J Vet Res 61: 393–398

Toh CH, Downey C (2005) Performance and prognostic importance of a new clinical and laboratory scoring system for identifying non-overt disseminated intravascular coagulation. Blood Coagul Fibrinolysis 16:69–74

Triplett D, Harms C (1981) Factor assays. Procedures for the coagulation laboratory. American Society of Clinical Pathologists Press, Chicago, IL, p 38–57

Walsh M, Bossart G (1999) Manatee medicine. In: Fowler ME, Miller RE (eds) Zoo and wild animal medicine current therapy, 4th edn. W. B. Saunders, Philadelphia, PA, p 507–516

Wiinberg B, Jensen AL, Rojkjaer R, Johansson P, Kjelgaard-Hansen M, Kristensen AT (2005) Validation of human recombinant tissue factor–activated thromboelastography on citrated whole blood from clinically healthy dogs. Vet Clin Pathol 34:389–393

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