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TRACE AND NON-TRACE ELEMENTS IN BLOOD CELLS OF BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*): VARIATIONS WITH VALUES FROM LIVER FUNCTION INDICATORS

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ABSTRACT: Alterations in trace and non-trace element homeostasis have been associated with both normal physiologic and pathologic processes of many species. Changes in copper and zinc, for instance, have been associated with liver disease in humans and dogs. While liver disease has been documented in marine mammals, associations of liver disease with trace and non-trace elements have not been determined. The goal of this study was to assess potential elemental associations with clinically relevant changes in liver enzymes of bottlenose dolphins (*Tursiops truncatus*) and to compare observed associations to what has been reported in other species. Blood cell samples were collected from 37 healthy bottlenose dolphins, maintained by the Navy Marine Mammal Program (MMP), between 1991 and 1992. Twenty-one trace and non-trace elements were assessed along with a standard liver enzyme function profile, and trace element associations to specific liver enzymes were determined. In this study, of the 21 blood cell elements assessed, 19 were measured within detectable limits in at least one of the blood samples, and 10 trace elements were found to be associated with at least one of the liver function indicators. Many of these same associations have been documented in various forms of liver disease in other species, including the associations of increases in copper and decreases in zinc with both elevated alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT). The observed analogous associations between changes in blood trace and non-trace elements and liver function indicators of bottlenose dolphins and other species may indicate similar pathologic processes and functions of some elements. Given the results of this study, additional research is warranted to further elucidate associations of trace and non-trace elements to liver disease in bottlenose dolphins.
Key words: Blood cell, elements, liver disease, liver function, metals, serum biochemistry, trace and non-trace, *Tursiops truncates*.

INTRODUCTION

Both trace and non-trace elements are involved in normal physiologic and pathologic processes. Trace elements are defined as elements that are found in the body at minuscule levels (micro, nano, or pico/gram of weight [dry or wet]) (Guidotti et al., 1997; Mullally et al., 2004). Both essential (e.g., zinc, copper, chromium, selenium) and non-essential (e.g., mercury, cadmium, lead) elements fall under this definition.

While trace and non-trace element analysis of body fluids can provide information regarding body burden or internal dose of a particular element in the body, detectable concentrations of most elements usually reflect recent exposure (Mertz, 1975; Guidotti et al., 1997). Internal concentration changes of trace and non-trace elements can cause disease through deficiency, imbalance, or toxicity. In turn, disease itself can cause fluxuations of elements (Van Gossum and Neve, 1998; Fuentealba and Aburto, 2003; Choi and Kim, 2005). Trace elements and trace element ratios are frequently reported as markers for diagnosing diseases (Daglish et al., 2004; Mullally et al., 2004; Cesur et al., 2005). In many species, increases in serum or plasma copper, for example, have been associated with liver disease, while elevated copper:zinc ratios have been associated with severity of disease or infection (Suzuki et al., 1996; Cesur et al., 2005; Seyrek et al., 2005). Current knowledge on trace and non-trace elements in dolphins, with respect to pathologic and physiologic changes, is fairly limited. Much of the existing guidelines for potential health impacts have been extrapolated from terrestrial mammals (Bowles, 1999; Das et al., 2003). Dolphins are known, however, to differ from terrestrial mammals in many aspects of their physiology (O'Hara et al., 2003; Ponganis et al., 2003). Dolphins and other cetaceans have relatively large livers compared to most terrestrial animals (Slijper, 1962). There are several features of the liver, including size, which may be related to a high level of food consumption and a high metabolic rate (Ridgway and Patton, 1971). Other features of cetacean livers that differ from terrestrial mammals include unusually thick walls of the arteries in the triads, sphincter-like thickenings of vascular walls, unusual prominence of the centrilobular sinusoids, and cytoplasmic secretion vacuoles. These differences are probably related to diving (Cowan, 2002). The large posterior vena cava has a sphincter at the level of the diaphragm that likely controls blood flow back to the heart during diving and therefore slows blood flow in the large veins and sinuses of the liver (Cowan, 2002). These physiologic differences may impact overall liver function as compared to terrestrial mammals.

Suspected elevated levels of both trace and non-trace elements have been documented in many marine mammal tissues, including the liver (Bowles, 1999; Woshner et al., 2001; Das et al., 2003; Ikemoto et al., 2004; Jaber et al., 2004). The exact ramifications of these findings, however, remain unclear.

Liver disease has been documented in dolphins (Medway et al., 1966; Ridgway and Dailey, 1972). While parasitic and infectious etiologies have been identified in some cases of liver disease, and a nutritional component has been hypothesized (Medway et al., 1966; Sweeney and Ridgway, 1975; Jaber et al., 2003, 2004), the etiology of liver disease in most marine mammals remains unknown. Similar to terrestrial mammals, alanine aminotrasferase (ALT) is considered a liver-specific indicator in bottlenose dolphins (Ridgway et al., 1970; Geraci and St. Aubin, 1979; Bossart et al., 2001). Ridgway and Dailey (1972) compared the blood of three normal dolphins captured at sea with three stranded animals with jaundice and fluke damage. Blood chemistry values for bilirubin, ALT, and aspartate aminotransferase (AST) were more than five times greater in the stranded dolphins with liver damage compared to the normal dolphins. Based on presence of adult trematodes in the liver, and on histologic confirmation of liver disease post-necropsy, these elevations were found to be directly related to liver damage and not stress-induced by the stranding event (Ridgway and Dailey, 1972). Further, chronic and phasic elevations of serum ALT were associated with high serum iron, hyperlipidemia, hyperglobulinemia, and thrombocytopenia in our managed dolphin population (Venn-Watson et al., 2008).

Excluding perhaps mercury (Rawson et al., 1993; Caurant and Navarro, 1994, 1995) and iron (Mazzaro et al., 2004), a direct causal link between elements (trace and non-trace) and liver disease, including associations with clinically relevant liver function indicators, have not been identified (Law, 1996; Bowles, 1999).

The goals of this study were twofold. First, to retrospectively assess potential associations of trace and non-trace elements with clinically relevant changes in liver function indicators of healthy bottlenose dolphins (*Tursiops truncates*); and second, to compare and contrast identified associations with current understandings of trace and non-trace elements and liver disease in other species.

MATERIALS AND METHODS

Demographics

A total of 37 whole blood and serum samples from 37 bottlenose dolphins were collected from Navy Marine Mammal Program (MMP) animals between September

1991 and August 1992. Animals were housed in open-bay netted enclosures and fed quality controlled frozen-thawed fish. In addition to their fed diet, some animals may have fed on low amounts of local fauna. The study population consisted of 20 (54%) females and 17 (46%) males. The median age was 11.4 yr (range 6.6 yr to 33.4 yr).

Sample collection methods

Whole blood samples were collected by voluntary fluke presentation from adult male and female bottlenose dolphins from the MMP animal population. Selection criteria for animals included in this study were based upon health status (no clinical signs of disease), age (>5 yr of age), sex (distribution of both male and female), and the collection of a non-hemolyzed sample. Animals were not fed overnight prior to morning blood sample collection. A total of 20 ml whole blood was collected from the ventrum of the presented fluke of each animal. The dolphins were trained to present their tails for puncture of the central plexus of the ventrum of the fluke (the peduncle periarterial vascular rete) for blood collection. Following puncture, two glass vacutainer tubes, one 10 ml serum tube (serum biochemistry), and one 10 ml sodium heparinized tube (trace element analysis) were used for sample collection. The sodium heparinized tube was a commercially available trace-mineral free vacutainer tube (BD Vacutainer Systems, Franklin Lake, New Jersey, USA) provided by the diagnostic laboratory (Doctor's Data Inc. [DDI], Chicago Illinois, USA). The Navy Marine Mammal Program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care Use and adheres to the national standards of the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Animal Welfare Act. As required by the Department of Defense, the Navy Marine Mammal Program's animal care and use program is routinely reviewed by an Institutional Animal Care and Use Committee and the Department of Defense Bureau of Medicine.

Multi-element analyses

Whole blood was collected into a 10 ml sodium heparinized tube, (BD Vacutainer Systems), and the plasma component was separated from the red blood cell (RBC) component within 1 hr after collection. The RBC component was shipped on ice, on the same day as collection, to DDI for examination. Twenty-one elements were incorporated in the analysis, including calcium, chromium, copper, iron, potassium, magnesium, manganese, sodium phosphorus, sulfur, selenium, silicon, strontium, zinc, zirconium, aluminum, cadmium, cobalt, lithium, nickel, and vanadium. Methodology for analysis (excluding selenium) was an open beaker digestion technique with measurement on ICP-AES (Atomic Emission Spectroscopy) (McLeod et al., 1984; Budde, 1991). Selenium was analyzed using a hydride method and flow injection analysis with absorption spectroscopy (Crock and Lichte, 1982). Serum controls for trace element analysis were obtained from CIBA-Corning (Ramsey, Minnesota, USA) and used as quality control. All samples were digested the same day that the specimens were received.

Biochemical analyses

The second 10 ml blood sample was collected into a glass vacutainer tube (BD Vacutainer Systems) and allowed to clot at room temperature. The serum was separated via centrifugation (15 min, 1,500 $\times$ G), removed, and then shipped on ice to a commercial laboratory for analysis (Quest Diagnostics, San Diego, California, USA). The following liver function indicators (LFIs) were measured using an Olympus 5400 spectrophotometer (Olympus, Central Valley, Pennsylvania, USA): alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and lactate dehydrogenase (LDH). Serum controls for liver enzyme analysis were from Quest Diagnostics.

Statistical analyses

Statistical analyses were conducted using SAS® software (Release 8e; SAS Institute, Inc., Cary, North Carolina, USA). Sixteen of the 21 trace and non-trace elements were analyzed including blood cell calcium, magnesium, potassium, copper, zinc, iron, manganese, chromium, phosphorus, selenium, silicon, sulfur, strontium, zirconium, copper:zinc ratio, and strontium:calcium ratio. Elements for which there were <10 measurements were excluded from the analysis. Mean, standard deviation, median, and range values were determined for each trace and non-trace element.

Age and sex as predictors of blood cell element values

An analysis of variance (ANOVA) via a general linear model was used to assess age as a significant predictor of blood cell element values (PROC GLM; MODEL [blood cell elements]=AGE). When age was identified as a significant predictor, a GLM model was applied using three age categories (<10-yr-old, 10–20-yr-old, and >20-yr-old) (PROC GLM; CLASS AGEGROUP; MODEL [blood cell elements]=AGEGROUP). A post-hoc Scheffe's test was used to assess differences among each age group comparison. A similar model was used to assess sex as a predictor of blood cell element values (PROC GLM; CLASS SEX; MODEL [blood cell elements]=SEX; MEANS SEX) (SAS/Assist, 1999).

Blood cell element values as predictors of liver function indicators

Simple linear regressions were initially conducted to assess the ability for blood cell elements to predict the values of serum ALT, AST, GGT, LDH, and iron. In the case of blood cell copper, an analysis of covariance (ANCOVA) was used to control for the effects of age on blood cell copper levels.

Two case-control studies were conducted to determine the clinical significance of relationships between blood cell elements and liver function indicators. Based upon a sample set of 1,113 blood samples collected from 52 healthy MMP dolphins during the period 1998 through 2005, a previous study determined that normal reference ranges for serum ALT, AST, and GGT levels in healthy MMP dolphins vary significantly by age, and normal reference ranges for serum iron and GGT in healthy MMP dolphins also vary significantly by both sex and age (Venn-Watson et al., 2007). The first case-control study defined cases as samples with high serum ALT levels (>53 u/l ages >5–10 yr; >54 u/l ages >10–30 yr; and >42 u/l ages >30 yr). The second case-control study defined cases as samples with high serum GGT levels (Females: >37 u/l ages >5–10 yr; >77 u/l ages >10–30 yr; and >46 u/l ages >30 yr; Males: >40 u/l ages >5–10 yr; >48 u/l ages >10–30 yr; and >44 u/l ages >30 yr). Controls for both studies were limited to samples with normal serum ALT, AST, GGT, and iron levels. The ANOVA model used for both case-control studies was PROC GLM; CLASS TYPE (case or control); MODEL [blood cell elements]=TYPE (SAS/Assist, 1999).

Among all analyses, significance was defined as a P value $\leq$0.05. If covariates were included in a model, a Type I sum of squares P value was used to control for potential confounders.

RESULTS

Of the 21 elements measured, 19 were detectable in at least one of the blood cell samples assessed. Mean, median, and ranges for all elements are provided in Table 1.

Associations of trace and non-trace blood cell elements with age and sex

Of 16 trace and non-trace blood cell elements analyzed, age was a significant predictor for copper only (P=0.008). Specifically, dolphins aged 10 yr to 20 yr were more likely to have higher copper compared to dolphins aged greater than 20 yr (mean copper value for dolphins <10 yr=0.65 ppm; 10 yr to 20 yr=0.66 ppm; greater than 20 yr=0.52 ppm). Sex was not a significant predictor of blood cell elements assessed in the study.

Blood cell element predictors of liver indicators: Of 16 blood cell elements analyzed, 10 elements (calcium, copper, iron, potassium, phosphorus, manganese, selenium, sulfur, strontium, and zinc) and one ratio (copper:zinc) were found to be significant predictors of four liver function indicators (ALT, AST, GGT, LDH) in the managed bottlenose dolphin population (Table 2). Zinc, iron, sulfur, and the copper:zinc ratio were found to be significant predictors of ALT, GGT, and LDH. In addition, calcium, potassium, phosphorus, selenium, sulfur, and strontium were found to be significant predictors of GGT and LDH. Manganese was the only significant predictor of AST. No other significant associations were identified.

Blood cell element and liver function indicator case-control studies

Four blood cell elements and one ratio were found to be significantly associated with clinically relevant levels of serum ALT. Animals with clinically high levels of ALT (n=5) were more likely to have higher blood cell calcium and a higher copper:zinc ratio and lower blood cell iron, sulfur, and zinc as compared to animals with normal ALT values (Table 3). No other statistically significant correlations between other elements with ALT were found.

In this study, chromium was the only blood cell element which demonstrated a statistically significant association with

TABLE 1. Summary values of trace and non-trace elements in blood cell[a] of a managed bottlenose dolphin (*Tursiops truncatus*) population.

Trace or non-trace element (blood cells)	*n*	Mean	SD	Median	Range
Aluminum	1	0.063	NA[b]	NA	NA
Calcium	37	21.0	16.5	15.6	10.1–75.6
Cadmium	0			Not detected	
Cobalt	5	0.03	0.008	0.02	0.02–0.04
Magnesium	37	57.8	75.8	45.2	33.7–505.0
Potassium	37	76.8	16.1	79.0	38–109
Copper	37	0.63	0.13	0.61	0.09–0.92
Zinc	37	7.2	1.6	7.5	2.8–10.4
Iron	37	1095	235	1165	437–1535
Lithium	2	0.027	0.005	0.027	0.023–0.03
Manganese	35	0.018	0.013	0.015	0.008–0.081
Chromium	34	0.10	0.10	0.08	0.03–0.64
Nickel	0			Not detected	
Phosphorus	37	488.9	78.8	508	287–653
Selenium	37	1.2	0.6	1.1	0.2–2.9
Silicon	37	1.0	0.3	1.1	0.4–1.6
Sodium	4	82.8	3.6	83.5	78–86
Sulfur	36	1428	124	1419	1183–1756
Strontium	31	0.02	0.02	0.01	0.01–0.08
Zirconium	37	0.11	0.03	0.10	0.06–0.19
Vanadium	0			Not detected	
Copper:Zinc	37	0.10	0.06	0.08	0.01–0.30
Strontium/Calcium	37	0.0008	0.0004	0.0008	0.0000–0.0020

[a] Units are reported as ppm wet weight unless otherwise indicated.

[b] NA = not applicable.

clinical changes in GGT. Animals with clinically elevated levels of GGT ($n=4$) were more likely to have higher blood cell chromium as compared to animals with normal GGT values (Table 4). No other statistically significant associations between other elements and GGT were found.

DISCUSSION

Our current understanding of changes in trace and non-trace elements in dolphins, with respect to liver pathologic and physiologic changes, has been limited. Alterations in element homeostasis associated with liver disease are known to occur in other species (Thornburg, 2000; Bersenyl et al., 2003; Fuentealba and Aburto, 2003; Cesur et al., 2005). Thus, the goal of this study was to determine if similar trends could be identified in bottlenose dolphins. Clinically significant ALT and GGT levels, as determined by established normal reference ranges in our animal population (Venn-Watson et al., 2007), were used as markers for potential liver pathology.

In this study, both trace and non-trace elements (calcium, copper, zinc, copper: zinc ratio, iron, phosphorus, strontium, potassium, manganese, sulfur, and silicon) were associated with high levels of at least one of the liver function indicators assessed. Many of the associations observed in this study have been observed in other species including humans, domestic dogs, ruminants, and rodents (Table 5). For purposes of brevity, the remainder of this discussion will focus on copper, zinc, and iron associations.

Copper associations

Copper is an essential trace element and is required for catalytic activity of a variety of enzymes, including several

TABLE 2. Significant blood cell element predictors of liver function indicators in a managed bottlenose dolphin population (*Tursiops truncatus*), n=37 blood samples, 1991–1992.

Liver function indicator[a]	Significant blood cell element predictors	P value
ALT	Zinc	0.002
	Iron	0.03
	Sulfur	0.03
	Copper:Zinc	0.0009
AST	Manganese	0.04
GGT	Copper	0.003
	Calcium	<0.0001
	Potassium	0.002
	Zinc	<0.0001
	Iron	<0.0001
	Phosphorus	<0.0001
	Selenium	0.004
	Sulfur	0.001
	Strontium	0.0005
	Copper:Zinc	<0.0001
LDH	Copper	0.004
	Calcium	<0.0001
	Potassium	0.002
	Zinc	<0.0001
	Iron	<0.0001
	Phosphorus	<0.0001
	Selenium	0.008
	Sulfur	0.0005
	Strontium	<0.0001
	Copper:Zinc	<0.0001

[a] ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = gammaglutamyltransferase; LDH = lactate dehydrogenase.

TABLE 3. Comparisons of mean blood cell element values by high or normal serum alanine aminotransferase levels in bottlenose dolphins (*Tursiops truncatus*).

Blood cell element	Mean value (SD)[a] high ALT	Mean value (SD)[a] normal ALT, AST, GGT, and iron	P value
	n=5	n=25	
Calcium	35.7 (26.1)	17.6 (12.5)	0.02
Copper:Zinc	0.16 (0.11)	0.08 (0.02)	0.0006
Iron	887 (400)	1146 (165)	0.02
Sulfur	1323 (111)	1450 (115)	0.03
Zinc	5.6 (2.5)	7.6 (1.0)	0.005

[a] Units are reported as ppm wet weight unless otherwise indicated: ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = gammaglutamyltransferase.

TABLE 4. Comparisons of mean blood cell element values by high or normal serum GGT levels in bottlenose dolphins (*Tursiops truncatus*).

Blood cell element	Mean value (SD)[a] high GGT	Mean value (SD)[a] normal ALT, AST, GGT, and iron	P value
	n=4	n=25	
Chromium	0.21 (0.28)	0.09 (0.04)	0.03

[a] Units are reported as ppm wet weight unless otherwise indicated: ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = gammaglutamyltransferase.

hepatic enzymes (Gubler, 1956; Linder, 1991; Linder and Hazegh-Azam, 1996). As a result, copper is typically found in large quantities within the liver (Beck et al., 1997; Fuentealba and Aburto, 2003). Elevations in tissue or body fluid copper have been associated with various forms of liver disease in other species, including hepatitis, hepatic necrosis, cirrhosis, biliary cirrhosis, hepatic lipidosis, porphyria cutanea tarda, Wilson's disease, and cholestatic liver disease (Fredricks et al., 1960; Pramoolsinsap et al., 1994; Van Gossum and Neve, 1998; Noaker et al., 1999; Dabrowska et al., 2000; Thornburg, 2000; Webb et al., 2002; Fuentealba and Aburto, 2003; Halifeoglu et al., 2004; Cesur et al., 2005). To our knowledge, direct associations between changes in copper and liver disease have not been documented in bottlenose dolphins. In this study, the mean blood cell copper concentration was 0.63 ppm, and elevated levels of copper were found to be a predictor of elevated GGT and LDH levels. In both humans and dolphins, elevations in GGT can be an indicator of chronic cholestatic disease associated to liver disease (Castro-E-Silva Jr. et al., 1990). In marine mammals, elevated GGT has been reported in cases of hepatic cirrhosis, cholestasis, and biliary disease of bottlenose dolphins (Bossart et al., 2001). A similar association among elevated levels of GGT, liver disease, and elevated levels of serum copper has been documented in other species including humans (Pramool-

TABLE 5. Trace and non-trace element associations with liver disease, other serum chemistry and other species.

Associated diseases	Species	Elements[a]	Other serum chemistry	References
Acute liver disease				
Hepatitis	Human	↓ S-Ca, S-Mg, S-Zn ↑ S-Cu, S-Fe, S-P, ↓ Cu/Zn	↑ GGT, ALP, & ↓ Bilirubin and Albumin	Cesur et al., 2005; Suzuki et al., 1996; Süleyman et al., 2005; Van Gossum and Neve, 1998
Chronic liver disease				
Alcohol associated	Human	↓ S-Mg, S-Zn, P-Mg, P-Zn, P-Se, P-Cr ↑ S-Cu, S-Fe	↑ AST, ALT, GGT, & Bilirubin	Avsaroglu et al., 2005; Cook et al., 1991
Hepatopathy, Hepatitis, Liver Disease, Porphyria Cutanea Tarda	Domestic Dog, Human, & Ferret	↑ S-Cu, S-Fe, S-Mg, & T-Cu	↑ ALT, & AST	Fuentealba and Aburto, 2003; Dabrowska et al., 2000*
Asymptomatic or non-specific liver disease				
	Human	↓ P-Fe, P-Cu, S-Zn, S-Cu	↑ AST & ALT	Johnston, 1999; Pramoolsinsap et al., 1994*
	Dolphin	↑ T-Hg		Law, 1996*; Rawson et al., 1993*
Known copper-associated liver disease				
Copper exposure or Copper storage, Wilson's Disease	Sheep, Domestic Dog, & Mice	↑ S-Cu, ↑ T-Cu, ↓ S-Cu	↑ Hemolysis, Hemoglobin, AST, GGT, ALP, BUN, ALT	Fuentealba and Aburto, 2003 Thornburg, 2000; Webb et al., 2002; Noaker et al., 1999*
Cirrhosis				
	Cat, Domestic Dog, & Human	↓ S-Zn, ↓ P-Zn ↑ P-Cu, Cu/Zn, ↓ S-Ca, S-Mg, S-P, ↑ S-Cu, ↓ S-Zn	↑ ALP & GGT ↓ Leukocytes	Fuentealba and Aburto, 2003 Fredricks et al., 1960; Halifeoglu et al., 2004; Walravens, 1979; Suzuki et al., 1996; Pramoolsinsap et al., 1994; Cesur et al., 2005

[a] S = serum; P = plasma; T = tissue; B = blood; ↑ = increased; ↓ = decreased; AST = aspartate aminotransferase; ALT = alanine aminotransferase; GGT = gammaglutamyltransferase; ALP = alkaline phosphorus; BUN = blood urea nitrogen.

* Other serum chemistry was not assessed in these studies.

sinsap et al., 1994; Avsaroglu et al., 2005) and sheep (Fuentealba and Aburto, 2003).

Compared to GGT, the association of LDH and blood cell copper is slightly more ambiguous. Like terrestrial mammals, marine mammal LDH is located in various body tissues. Thus, due to its widespread location, it is not a popular diagnostic tool in veterinary medicine (Bossart et al., 2001). Although LDH may be less specific for assessing liver function than other tests in humans, it is disproportionately elevated in liver injury (Johnston et al., 1999). Thus, increased activities of serum LDH paired with increased ALT, AST, and ALP are well known diagnostic indicators of hepatic injury in humans as well as in other species (Johnston, 1999; Celik et al., 2005).

To our knowledge, this is the first report of copper concentrations in red blood cells of bottlenose dolphins. Previous studies on Weddell seals (*Leptonychotes weddellii*), harp seals (*Phoca groenlandica*), and striped dolphins (*Stenella coeruleoalba*) have reported whole blood concentrations ranges 0.11–0.91 ppm, 0.28–0.92 ppm, and 0.87–1.05 ppm, respectively (Honda et al., 1982; Ronald et al., 1984; Yamamoto et al., 1987). The copper range reported in this study is similar to what has been reported in whole blood of other marine mammal species.

Zinc associations

Like copper, zinc is an essential element and vital for a variety of normal physiologic processes, including growth and cell replication (Bartholomay et al., 1956; Vallee, 1959; Walravens, 1979). It is involved in several hepatic enzyme systems and is typically found in large quantities in the liver (Vallee, 1959; Fredricks et al., 1960; Bennett et al., 2001). In several species, a decrease in serum or plasma zinc has been associated with a variety of liver diseases including cirrhosis, Wilson's disease, fulminant hepatic failure, and hepatitis (Walravens, 1979; Nandi et al., 1989; Cook et al., 1991; Halifeoglu et al., 2004; Cesur et al., 2005). In this study, blood cell zinc levels were a significant predictor of ALT, GGT, and LDH levels. Animals with clinically high serum ALT levels were more likely to have lower blood cell zinc compared to dolphins with normal ALT levels. Interestingly, five (14%) of the dolphins in our study, including those with clinically high serum ALT at the time of the study, have been recently characterized as animals with chronic, phasic increases in transaminases due to suspected hepatitis (Venn-Watson et al., 2008). One of these animals had chronic hepatitis confirmed on histopathology, and two animals had excessive iron deposition in liver tissue, indicating that liver pathology was likely the source of chronically elevated ALT.

While elevations in zinc within the liver have been associated with an increase in infection in harbour porpoises (Bennett et al., 2001), to our knowledge no direct associations between elevated zinc levels and liver function indicators have been documented in bottlenose dolphins. In bottlenose dolphins, ALT activity appears to be liver-specific (Ridgway et al., 1970; Geraci and St. Aubin, 1979; Bossart et al., 2001). Similar associations between serum or plasma zinc and serum ALT have been documented in humans (Cook et al., 1991; Pramoolsinsap et al., 1994; Cesur et al., 2005) and other species (Bersenyl et al., 2003; Fuentealba and Aburto, 2003). Associations among decreased serum or plasma zinc, elevations in serum GGT, and liver disease have also been documented in humans (Cook et al., 1991; Cesur et al., 2005).

To our knowledge, this is the first report of zinc concentrations in red blood cells of bottlenose dolphins. Previous studies on Weddell seals and striped dolphins have reported whole blood concentration ranges 4.02–5.34 ppm and 3.67–3.96 ppm, respectively (Honda et al., 1982; Yamamoto et al., 1987; Honda and Tatsukawa, 1983). The range reported herein is similar to what has been reported in whole blood of other marine mammal species.

Copper:zinc ratio associations

Due to the biologically significant homeostatic relationship between copper and zinc, the copper:zinc ratio has been used in various studies as an indicator of severity of disease (Van Gossum and Neve, 1998; Halifeoglu et al., 2004). For example, Al-Bader et al. (1998) reported a positive correlation between the copper: zinc ratio and inflammatory response in patients undergoing coronary bypass surgery. Seyrek et al. (2005) reported a positive correlation between an increased copper:zinc ratio and systemic inflammatory response in malaria patients. In this study, the copper:zinc ratio was found to be a predictor of ALT, GGT, and LDH, and elevations in the copper:zinc ratio were associated with elevations in ALT. This may be an indication of inflammatory response to liver disease; further studies are needed, however, to address associations between the copper:zinc ratio and the severity of disease.

Iron associations

Iron is involved in a variety of biologic processes, including xenobiotic metabolism, oxygen transport and storage, and cellular reproduction and is considered an essential element of the body (Berger et al., 1999; Agrawal et al., 2001; Merguro et al., 2003). Elevations in iron have been associated with signs of liver disease in both terrestrial and marine mammals (Fuentealba et al., 1997; Mazzaro et al., 2004; Venn-Watson et al., 2008).

In this study, iron was a predictor of ALT, GGT, and LDH. Decreases in blood cell iron were correlated to elevations in serum ALT. Interestingly, the opposite has been documented when measuring serum versus whole blood iron. Dabrowska et al. (2000) reported an increase in serum iron associated with an increase of ALT in porphyria cutanea tarda patients, a disease similar to hemochromatosis. Johnston (1999), however, reported a decrease in plasma iron associated with an increase of ALT in asymptomatic liver disease patients. A direct association between elevated serum iron levels and changes in liver function tests has been observed in this population of bottlenose dolphins (Venn-Watson et al., 2008), and iron overload and its potential association with hemochromatosis has been documented in northern fur seals (Mazzaro et al., 2004). Thus, the observed differences in this study, as compared to what has been reported in the literature, may also be a factor of substrate (comparing blood cell versus serum or plasma) differences. Further studies are warranted to clarify these differences.

Other contributory variables

Due to the nature of this study, several limitations exist including relatively small sample size, lack of prospective cases and controls, potential seasonal variations, and substrate limitations (e.g., comparison of blood cell versus serum or plasma, or differences in collection or analysis techniques). Some of these contributory factors will be discussed herein.

Age and sex associations: Many trace and non-trace elements have been associated with both age and sex in both terrestrial and marine mammals. Honda (1982) reported both age and sex differences for iron, manganese, and copper in striped dolphins. Storelli et al. (2000) reported a difference in liver copper concentrations between neonates, calves, and an adult female bottlenose dolphin. Honda et al. (1983) reported an age association with whole blood zinc concentrations in striped dolphins. Whole blood zinc concentrations gradually decreased until about 8 yr of age, and then stabilized out. This pattern of higher copper concentrations in newborns/young as opposed to adults has been documented in both terrestrial mammals (humans and goats) and other marine mammal species (Wagemann and Muir, 1984; Fujise et al., 1988; Law et al., 1992). This is thought to be due in part to the fact that younger mammals have higher levels of cystine-rich copper binding proteins (Luckey and Venugopal, 1977).

In this study, copper was the only element for which age was found to be a significant predictor. Those animals between 10–20 yr were more likely to have higher copper concentrations then animals either younger or older. Juvenile animals were not assessed in this study, so it is unknown if copper concentrations would be higher in those animals as compared to the 10–20 yr group. It is unclear why this particular age group had higher copper concentrations, but perhaps it is due, in part, to either pathologic or physiologic changes. Liver-associated pathologic changes and elevated copper were discussed above. Physiologic changes and diet have been attributed to elevations in serum and plasma copper levels of adult goats and cattle (Gromadzka-Ostrowska et al., 1986; Yokus and Dilek-Cakir, 2006). This is believed, in part, to be due to increased ceruplasmin production as a result of hormonal changes (Yokus and Dilek-Cakir, 2006).

Substrate differences: In this study, blood samples were routinely drawn for routine health physicals from the MMP dolphin population. Typically, the RBC component is discarded. Red blood cells were selected as a means of minimizing the amount of blood to be drawn, thereby maximizing usefulness of each sample and decreasing potential contamination.

For many elements, however, measured concentrations and their potential associations to disease have varied between matrices (serum, plasma, whole blood, and red blood cells) (Herring et al., 1960; Versieck et al., 1974; Akanli et al., 2003). Versieck et al. (1974) reported an association between elevations in serum copper and acute hepatitis but not with red blood cells. Akanli et al. (2003) reported red blood cell zinc levels typically to be 10 times higher then that of plasma zinc levels. Decreases in both, however, were found to be associated with lung disease in humans. A study by Herring et al. (1960) looked at differences in trace metals of human plasma and red blood cells between healthy individuals and patients with hematologic diseases. Plasma copper levels were associated with disease, while whole blood copper levels were not. Changes in both plasma and RBC zinc were found to be associated to disease. On the other hand, many studies have shown substantial agreement between reported RBC levels of copper and zinc and reported serum levels (Fredricks et al., 1960; Davies et al., 1968; Carson et al., 1986). Thus, often the optimal matrix of choice is greatly dependent on the element of interest, mechanism of interest (intracellular vs. extracellular), and available collection and storage techniques (Anand et al., 1975; Savory and Wills, 1992; Subramanian, 1995).

Healthy versus diseased animals: It is important to note that blood was drawn from animals with no clinical signs of disease. However, those animals found to have elevated serum ALT and GGT in this study have recently been characterized with chronic, phasic elevated transaminases (Venn-Watson et al., 2008). It is plausible that some of the animals sampled in this study could have had preclinical liver changes as early as the 1990s.

CONCLUSIONS

While strong associations between trace and non-trace elements and several liver function indicators were observed, caution should be taken in interpreting these results. Retrospective associations of trace and non-trace elements with liver disease function indicators do not establish cause-effect relationships without further data to support the results (Mertz, 1975; Guidotti et al., 1997).

In this study, associations between trace and non-trace elements and all assessed liver function indicators were detected. Many of these associations have been documented in various forms of liver disease in other species, including the elevation of copper and decrease in zinc and their concurrent association with elevations of ALT and GGT. Similar associations between changes in blood cell elements and liver function indicators in bottlenose dolphins and other species may indicate similar pathologic processes and functions of some trace and non-trace elements. In addition, the potential association of observed changes in trace and non-trace elements, as a cofactor to disease progression, should be considered when reporting element concentrations. Observed concentrations may be a result of exposure but may also be attributed to both normal pathologic and physiologic changes within the animal. Additional characterization of element analysis and a better understanding of baselines, however, are needed to further illuminate potential associations to liver disease and other potential disease processes.

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