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NMR-based metabolomic profile of cold stun syndrome in loggerhead *Caretta caretta*, green *Chelonia mydas* and Kemp's ridley *Lepidochelys kempii* sea turtles in North Carolina, USA

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In North Carolina, winter weather events causing sea surface temperatures to acutely drop below approximately 12°C may result in strandings of threatened loggerhead *Caretta caretta*, green *Chelonia mydas* and endangered Kemp's ridley *Lepidochelys kempii* sea turtles due to cold stun syndrome. Clinically, these turtles have been shown to have metabolic and respiratory acidosis. The purpose of this study was to apply NMR-based metabolomics to further our understanding of the pathogenesis of cold stun syndrome in sea turtles. Heparinized whole blood and plasma samples were collected from apparently healthy loggerhead (whole blood n = 8), green (whole blood n = 12, plasma n = 10) and Kemp's ridley (whole blood n = 14, plasma n = 10) sea turtles. Blood and plasma samples were also collected from juvenile, in-water or beach-cast cold stun affected sea turtles (loggerhead n = 9, green n = 11, Kemp's n = 6). We identified 18 metabolites in our spectra including amino acids, energy compounds, glycerol, ketone bodies, nucleosides/nucleotides, organic acids and osmolytes. There were several significant differences between metabolites in control and affected turtles, likely secondary to anorexia, such as differences in dimethyl sulfone, glycerol, leucine, isoleucine and trimethylamine N-oxide concentrations. Significant differences in multiple metabolites related to exertion and anaerobic metabolism included concentrations of acetate, creatine, lactate and succinate. Changes in glycerol and propylene glycol concentrations suggest some metabolic changes may have cold protective properties. The primary metabolomic findings were consistent with what is known about clinical biochemistry and blood gas panel perturbations in cold stun affected turtles. This study provides a baseline for the metabolic profile of control and cold stun affected sea turtles in North Carolina and we have identified several metabolites worthy of further investigation with regards to their roles in hypoxia, ischemia and reperfusion.

Keywords: anaerobic metabolism, hypothermic stunning, ischemia, metabolic acidosis, nuclear magnetic resonance spectroscopy

In North Carolina, winter weather events result in strandings of threatened loggerhead *Caretta caretta*, green *Chelonia mydas* and endangered Kemp's ridley *Lepidochelys kempii* sea turtles due to cold stun syndrome. Cold stun syndrome occurs when sea surface temperatures drop below approximately 12°C and has been documented along the western Atlantic coast, western Gulf of Mexico, Uruguay and the South Adriatic Sea (Standora et al. 1989, Morreale et al. 1992, Bentivegna et al. 2002, Still et al. 2005, Roberts et al.

2014, Innis and Staggs 2017, Shaver et al. 2017, Vélez-Rubio et al. 2017). Cold stunned turtles become lethargic, anorexic, positively buoyant due to gas accumulation from gastrointestinal stasis (Schwartz 1978), and are frequently washed ashore by currents and winds (Innis and Staggs 2017). Annually, the number of sea turtle strandings due to cold stun syndrome in North Carolina varies, but has been as high as 1700 in a single event (Christiansen et al. 2016). Living turtles recovered during events are triaged, have varying diagnostic tests run, and receive supportive care including slow warming. Mortality rates for turtles receiving care can range from approximately 8 to 30% (Foley et al. 2007, Roberts et al. 2014, Shaver et al. 2017).

At presentation, many sea turtles with cold stun syndrome have metabolic and respiratory acidosis (Kraus and Jackson

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1980, Innis et al. 2007, Keller et al. 2012). They may also be dehydrated with either hypo- or hyperglycemia, which can vary by species and stranding circumstances (Turnbull et al. 2000, Innis et al. 2009, Anderson et al. 2011b). Turtles without complications are released as soon as warmer water is available, while rehabilitators treat those with other issues until release is appropriate. Sequelae may include pneumonia, skin and bony lesions similar to those seen in human frostbite injury, and hemolytic anemia (Solano et al. 2008, Occhiello et al. 2009, Stockman et al. 2013, Innis and Staggs 2017). A variety of factors, including blood gas parameters, biochemical values, and the presence of buoyancy disorders, have been identified as mortality predictors (Keller et al. 2012, Stacy et al. 2013, Li et al. 2015).

Metabolomics is concerned with metabolites, the small molecular weight end products of metabolism, such as amino acids, fatty acids and sugars. Metabolomics studies may employ a variety of modalities, such as gas chromatography, mass spectroscopy and nuclear magnetic resonance (NMR) spectroscopy (Lindon et al. 2007). Mass spectroscopy requires separation via chromatography prior to analysis and is most practical for targeted studies, but it is more sensitive than NMR spectroscopy and can be used to separate out single metabolites (Lindon et al. 2007). Our laboratory focuses on NMR-based metabolomics because samples can be analyzed with relatively little processing, solvents are generally safer or not needed, and analysis is nondestructive of samples, meaning that they can be used in other modalities after NMR analysis (Reo 2002, Lindon et al. 2007).

The purpose of this study was to apply NMR-based metabolomics to further our understanding of the physiologic mechanisms of cold stun syndrome in sea turtles. We hypothesized that we would see changes in biochemical pathways consistent with what has been seen with clinical diagnostic tests, namely, evidence of anorexia, acidosis, anaerobic metabolism and ischemic injury. It is our goal that with more detailed knowledge of the physiology of this syndrome we may eventually be able to improve treatment and outcomes for the turtles.

Material and methods

Sample collection

Heparinized (sodium heparin) whole blood and plasma samples were collected from apparently healthy, hereafter referred to as control, loggerhead (whole blood $n=8$), green (whole blood $n=12$, plasma $n=10$) and Kemp's ridley (whole blood $n=14$, plasma $n=10$) sea turtles captured in pound nets as part of population biology research by the National Marine Fisheries Service in North Carolina in October 2012 and between October and November of 2014. Control loggerhead plasma was not obtained, as this species was sampled directly on small boats, to streamline sampling and avoid potentially poor quality plasma samples. Heparinized whole blood and plasma samples from juvenile, in-water or beach-cast cold stun affected sea turtles of the same three species (loggerhead $n=9$, green $n=11$, Kemp's ridley $n=6$) were collected for diagnosis and treatment purposes prior to rehabilitation of stranded turtles from 2012 to

2016. All samples were collected from the external jugular (dorsal cervical sinus) with a needle appropriate to the size of the individual turtle. Whole blood sample volumes were less than 6 ml or 5 ml kg⁻¹, whichever was smaller. Aliquots of approximately 100 μ l of whole blood and plasma from each individual were reserved for this study.

For cold stun affected turtles, transport time to intake facility varied, but all were sampled immediately upon arrival, with body temperatures less than 15°C (minimum = 4.8°C, median = 9.7°C, maximum 13.8°C) and prior to receiving any treatment. All loggerheads recovered during rehabilitation and were released. One green turtle died in rehabilitation approximately four months post-stranding. There were no significant findings on necropsy. One Kemp's ridley was found to be near death one day post-stranding and died on the second day post-stranding. All other green and Kemp's ridley turtles recovered during rehabilitation and were released.

We placed all whole blood samples directly into pre-drilled wells in a block of dry ice (Nagase and Niwa 1964, Krause and Grove 1967). We immediately centrifuged additional whole blood for plasma samples (1000 \times g for approximately 5 min), separated the plasma, and froze it in the same manner. No hemolysis was noted. We transferred the frozen whole blood and plasma samples to cryogenic vials, which were stored at -80°C until final sample processing.

Sample preparation

Before use, we soaked centrifugal filters (Amicon Ultra 10K 0.5-ml) overnight (approximately 17 h) in ultrapure water and then rinsed each filter four times with 500 μ l of fresh ultrapure water at 14 000 g for 20 min at 20°C. We thawed samples at room temperature (20–22°C), mixed 40 μ l of sample with 400 μ l of Drabkin's reagent (Ricca Chemical Co.) to induce complete and consistent hemolysis, and incubated the mixture at room temperature for 10 min (Niemuth and Stoskopf 2017). For consistency, plasma samples were treated in the same manner with the same solvent. We then filtered the samples at 14 000 g for 20 min and froze the filtrate at -80°C. We lyophilized the samples until dry (about 6 h), sealed each tube with laboratory wax film, and stored them at -80°C.

NMR data collection

We transported the frozen lyophilized samples to the NMR facility at -20°C. We thawed the sealed, lyophilized samples at room temperature. Once thawed, we rehydrated each sample with 70 μ l of 100% D₂O containing 0.1 mM of the reference standard trimethylsilyl propanoic acid (TSP), 1 mM formate as a secondary reference standard (Kriat et al. 1992), and 20 mM phosphate buffer. After vortexing, we filtered (Fisherbrand SureOne 101, extended, filter, low-retention, universal-fit pipet tips) the rehydrated samples as well as aliquots of the rehydration solution at 3000 g for 2 min. These filtration steps were standard for operation of the microcoil NMR probe. We used the filtered aliquots of rehydration solution as 'blanks' ($n=16$) run prior to and after each group of samples. We also removed 10 μ l from each sample and combined them to create a control pool, cold stun pool and

combined pool sample for each species and sample type for quality assurance. We capped all rehydrated samples, blanks and pooled samples and stored them at -20°C until NMR analysis.

We used a Varian Inova 600 MHz multinuclear INOVA NMR spectrometer equipped with a Protasis microcoil NMR probe to obtain one-dimensional proton-NMR spectra at 25.08°C with a 1.138 s acquisition time. The sweep width of 7193.60 Hz acquired 8189 complex points and 4096 transients.

Data analysis

We used ACD labs 12.0 1D NMR processor (Advanced Chemistry Development, Toronto, Ontario, Canada) for spectral processing using our laboratory's standard protocol, as follows. We zero-filled each spectrum to 16 000 points and applied a Fourier transformation. We applied automatic phasing and baseline adjustments, making additional adjustments by hand when the automatic process was inadequate. We then referenced all spectra to the TSP peak at 0 ppm (Hz/MHz).

We identified metabolites using Chenomx NMR Suite 8.43 and the Human Metabolome Database (Wishart et al. 2013). We performed two-sided, two-sample, random sampling permutation tests ($\alpha=0.05$, $R=1,000$) to test for significant differences in metabolite concentrations between sample types, species and cold stun status using R ver. 3.5.3 (<www.r-project.org>). One-sided, two-sample, random sampling permutation tests were used only if a significant difference was found with a two-sided test. Heat maps were constructed with the base heatmap function in R.

Results

All of our samples resulted in good quality spectra with narrow reference standard and water peak widths and acceptable signal-to-noise ratios for additional analysis, with the exception of one cold stun affected, loggerhead whole blood sample where noise precluded identifying glucose and propylene glycol. Our TSP peaks were consistent and narrow in appearance, indicating binding to albumin was not a substantial issue and formate was not needed as a secondary reference compound. We saw two presumed contaminants frequently in our blank spectra, methanol and an unknown singlet at 2.07 ppm. The unknown was a persistent issue for this system and was disregarded because it did not overlap with any endogenous metabolites in this study. The median methanol concentration observed in blank samples by species was calculated and subtracted from each sample's methanol concentration. A single blank, run prior to any samples, contained acetate and glycerol at concentrations approximately 3-fold and 44-fold lower concentration than the observed mean concentration, respectively. Rare contaminants in the blanks, ran between or after sample groups, included lactate ($n=1$), propylene glycol ($n=2$) and trimethylamine N-oxide (TMAO; $n=3$), which we considered to be sample washout and had concentrations approximately 12 to 43-fold lower than the observed mean metabolite concentrations by species. We subtracted the median formate concentration of the

blank samples by species from each sample and negative values were adjusted to 0 mM.

Example spectra for each species and group are shown in Fig. 1 for whole blood samples and Fig. 2 for plasma samples. We identified 18 metabolites (not including formate) in our spectra. Table 1 shows the list of metabolites identified along with the chemical shifts and multiplicities with presence in whole blood versus plasma indicated. Every metabolite was not identified in every spectrum, but no metabolite was species-specific. The median metabolite concentrations and interquartile ranges (quartile 1–3) are summarized in Table 2. The metabolite concentrations in each sample are available in the Supplementary material Appendix 1. Heat maps are presented by species to graphically display the metabolite concentration differences (Fig. 3–5). A summary of the statistically significant metabolite differences between cold stun and controls, as well as between whole blood and plasma, is given in Table 3.

Several differences were noted between whole blood and plasma spectra. Adenosine, 2'-inosine-5'-monophosphate (IMP), and taurine were not identified in plasma samples. Methanol, myo-inositol and TMAO concentrations were significantly greater in all whole blood samples versus plasma samples ($p<0.001$ for two-sided and one-sided tests for all three metabolites). The concentration of methanol in loggerhead whole blood samples (controls and cold stuns) was significantly greater than in all other species' samples ($p<0.001$ for two- and one-sided test). Succinate whole blood concentrations were greater than plasma concentrations in green turtles ($p<0.001$ for two-sided and one-sided test). Blood isobutyrate concentrations were significantly less than plasma in both green and Kemp's turtles (green: two-sided $p=0.027$, one-sided $p=0.018$; Kemp's: two-sided $p=0.011$, one-sided $p=0.014$). Blood glucose concentrations in green turtles were also significantly lower than in plasma ($p<0.001$ for two- and one-sided test).

Multiple metabolite concentration differences were found between control and cold stun affected individuals. Blood samples for loggerheads with cold stun syndrome had significantly greater concentrations of 3-hydroxybutyrate (two-sided $p=0.03$, one-sided $p=0.005$) and significantly lower concentrations of leucine (two-sided $p=0.008$, one-sided $p=0.007$) and propylene glycol ($p=0.004$). In both whole blood and plasma green turtle samples, 3-hydroxybutyrate, acetate and leucine were significantly lower in individuals with cold stun syndrome (whole blood 3-hydroxybutyrate: two-sided $p=0.03$, one-sided $p=0.003$; plasma 3-hydroxybutyrate: $p<0.001$ for two- and one-sided test; whole blood acetate: two-sided $p<0.001$, one-sided $p=0.001$; plasma acetate: $p<0.001$ for two- and one-sided test; whole blood leucine: two-sided $p=0.034$, one-sided $p=0.016$; plasma leucine: two-sided $p=0.006$, one-sided $p=0.002$). Creatine and glycerol concentrations were significantly greater in plasma samples from green turtles with cold stun syndrome (two-sided $p=0.032$, one-sided $p=0.006$; two sided $p=0.014$, one-sided $p=0.012$, respectively). Isoleucine and TMAO plasma concentrations were lower in green turtles with cold stun (two-sided $p=0.004$, one-sided $p=0.004$; two-sided $p=0.002$, one-sided $p<0.001$, respectively). In Kemp's ridleys, acetate and taurine concentrations in whole blood and dimethyl sulfone concentrations in plasma were significantly

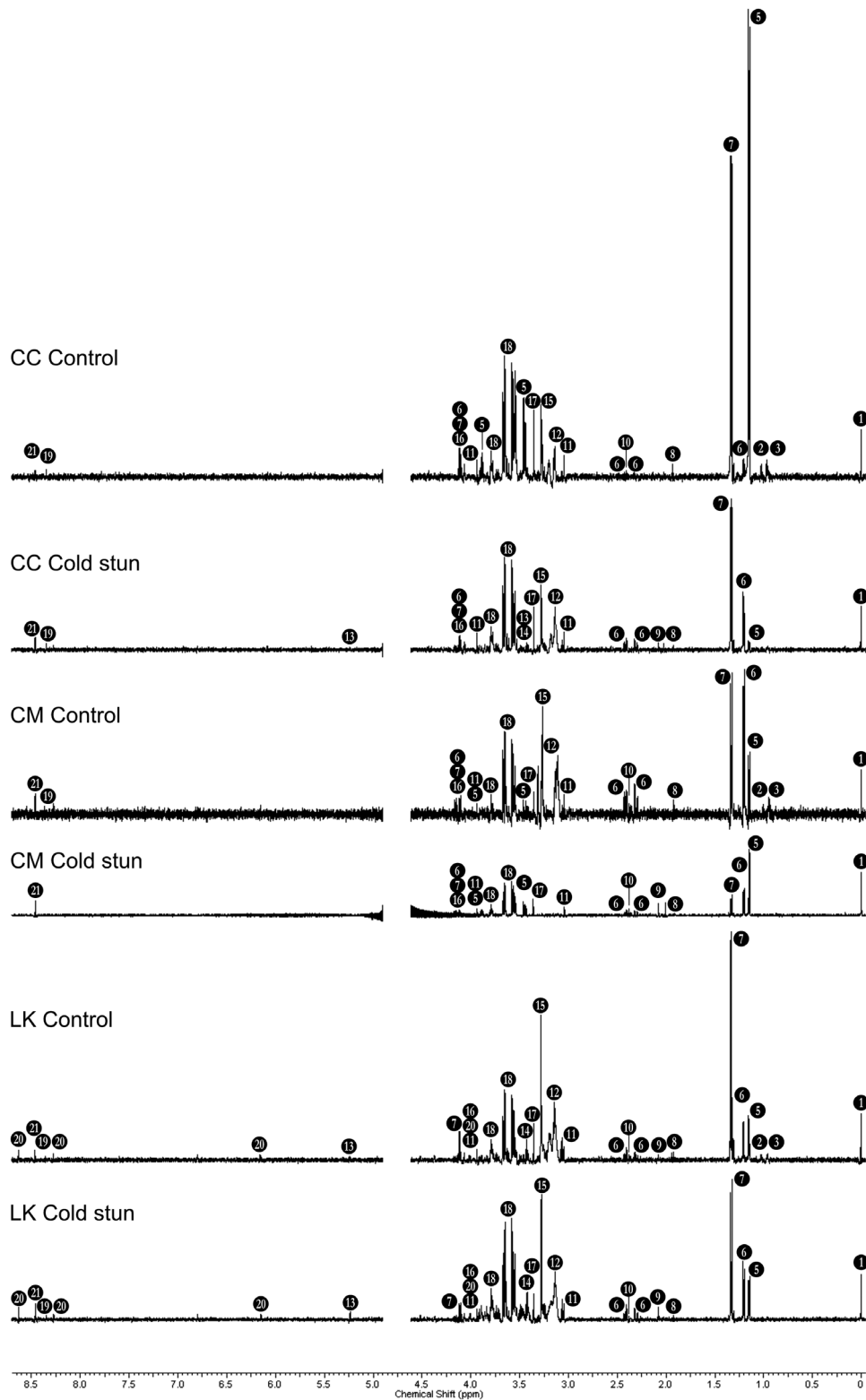


Figure 1. Example one-dimensional proton-NMR spectra of whole blood samples from loggerhead *Caretta caretta* (CC), green *Chelonia mydas* (CM) and Kemp's ridley *Lepidochelys kempii* (LK) sea turtles in North Carolina, USA. Individuals with and without (control) cold stun syndrome were sampled between 2013–2016 and 2012–2014, respectively. We saw some variance in metabolite presence among individuals in the same group. The labeled peaks are as follows: (1) trimethylsilyl propanoic acid (TSP; 0 ppm [Hz/MHz], added as a reference standard), (2) isoleucine, (3) leucine, (4) isobutyrate, (5) propylene glycol, (6) 3-hydroxybutyrate, (7) lactate, (8) acetate, (9) unidentified, presumed contaminant, (10) succinate, (11) creatine, (12) dimethyl sulfone, (13) glucose (alpha anomer most frequently labeled at 5.2 ppm due to overlapping peaks ~3.2–3.9 ppm), (14) taurine, (15) trimethylamine N-oxide, (16) myo-inositol, (17) methanol, (18) glycerol, (19) adenosine, (20) 2'-inosine-5'-monophosphate (IMP), (21) formate (added as a secondary reference standard). Not all peaks for each metabolite are labeled. The water peak has been deleted.

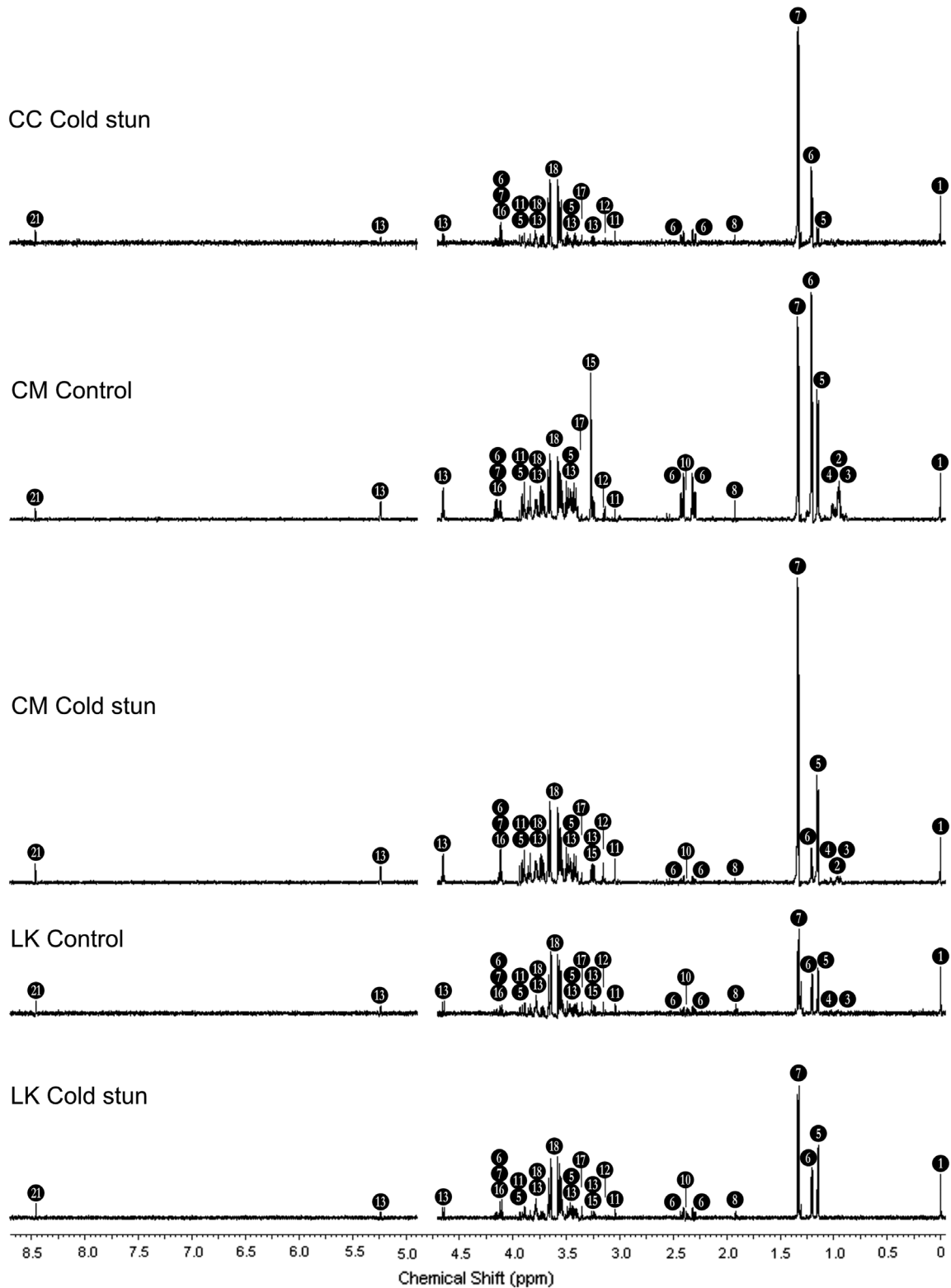


Figure 2. Example one-dimensional proton-NMR spectra of plasma samples from loggerhead *Caretta caretta* (CC), green *Chelonia mydas* (CM) and Kemp's ridley *Lepidochelys kempii* (LK) sea turtles in North Carolina, USA. Individuals with and without (control) cold stun syndrome were sampled between 2013–2016 and 2012–2014, respectively. We saw some variance in metabolite presence among individuals in the same group. The labeled peaks are as follows: (1) trimethylsilyl propanoic acid (TSP; 0 ppm [Hz/MHz], added as a reference standard), (2) isoleucine, (3) leucine, (4) isobutyrate, (5) propylene glycol, (6) 3-hydroxybutyrate, (7) lactate, (8) acetate, (10) succinate, (11) creatine, (12) dimethyl sulfone, (13) glucose (the alpha anomer at 5.2 ppm is most frequently labeled due to the overlap of its peaks at ~3.2–3.9 ppm), (14) taurine, (15) trimethylamine N-oxide, (16) myo-inositol, (17) methanol, (18) glycerol, (21) formate (added as a secondary reference standard). Not all peaks for each metabolite are labeled. The water peak has been deleted. Adenosine, 2'-inosine-5'-monophosphate (IMP), and taurine were not identified in plasma samples.

Table 1. Metabolites identified in the whole blood and plasma of loggerhead *Caretta caretta*, green *Chelonia mydas* and Kemp's ridley *Lepidochelys kempii* sea turtles in North Carolina, USA and their respective chemical shifts (ppm = Hz/MHz) and multiplicities (d: doublet, dd: doublet of doublets, m: multiplet, s: singlet, t: triplet, tt: triplet of triplets) (Wishart et al. 2013). Individuals with and without cold stun syndrome were sampled between 2012–2016 and 2012–2014, respectively. Presence in whole blood versus plasma samples is indicated. No metabolite was species-specific.

Metabolite	¹ H chemical shift (ppm) and multiplicity	Whole blood	Plasma
Amino acids			
Isoleucine	0.926(t), 0.997(d), 1.248(m), 1.457(m), 1.968(m), 3.661(d)	•	•
Leucine	0.948(t), 1.700(m), 3.722(m)	•	•
Taurine	3.25(t), 3.42(t)	•	
Energy compounds			
Creatine	3.04(s), 3.93(s)	•	•
Glucose	3.233(dd), 3.398(m), 3.458(m), 3.524(dd), 3.728(m), 3.824(m), 3.889(dd), 4.634(d), 5.223(d)	•	•
Fatty acid metabolism			
Glycerol	3.551 (m), 3.644 (m), 3.775 (tt)	•	•
Ketone bodies			
3-Hydroxybutyrate	1.2(d), 2.31(dd), 2.40(dd), 4.15(m)	•	•
Isobutyrate	1.163(d), 2.59(m)	•	•
Nucleosides/Nucleotides			
Adenosine	3.857(dd), 3.937(dd), 4.300(q), 4.438(dd), 4.808(s), 6.021(d), 8.117(s), 8.281(s)	•	
2'Inosine-5'-monophosphate (IMP)	4.02(m), 4.36(m), 4.50(m), 6.13(d), 8.21(s), 8.55(s)	•	
Organic acids/Osmolytes			
Acetate	1.92(s)	•	•
Formate ^a	8.44(s)	•	•
Lactate	1.33(d), 4.11(m)	•	•
myo-Inositol	3.27(t), 3.61(t), 4.05(t), 3.52(dd)	•	•
Succinate	2.39(s)	•	•
Trimethylamine N-oxide	3.26(s)	•	•
Other			
Dimethyl sulfone	3.138(s)	•	•
Methanol	3.36(s)	•	•
Propylene glycol	1.130(d), 3.434 (dd), 3.537(dd), 3.870(m)	•	•
Unkown ^b	2.06(s)	•	•

^a Added as a secondary reference compound.

^b Unidentified, presumed contaminant (seen on rehydration solution 'blanks').

lower in cold stun affected individuals (two-sided $p < 0.001$, one-sided $p = 0.002$; two-sided $p = 0.027$, one-sided $p = 0.015$; two-sided $p = 0.021$, one-sided $p = 0.01$, respectively). Kemp's ridley plasma lactate, propylene glycol and succinate concentrations were significantly greater in individuals with cold stun syndrome (two-sided $p = 0.03$, one-sided $p = 0.027$; two-sided $p = 0.034$, one-sided $p = 0.033$; two-sided $p = 0.003$, one-sided $p = 0.003$, respectively).

Discussion

Overview

Wildlife studies are frequently limited by sample size and availability due to species rarity, body size, legal considerations and other factors, as was the case in this study. However, we demonstrated that high quality one-dimensional proton NMR spectra could be obtained from relatively small volume samples of whole blood or plasma. We were able to identify 18 metabolites involved in a variety of metabolic pathways (Table 1). The use of Drabkin's reagent was necessary to achieve complete and consistent hemolysis, but does limit the output to polar (i.e. aqueous) metabolites. Studies combining Drabkin's reagent with methods and solvents designed for nonpolar

(i.e. lipophilic) metabolite extraction are necessary for a more complete metabolic profile, which would include metabolites such as triacylglycerides, cholesterol and fatty acids. While we were able to identify the majority of the peaks present in our spectra, there were sporadic peaks that did not fit with commonly identified metabolites. Additionally, across multiple whole blood spectra there was a very wide complex at approximately 3.1–3.2 ppm. We have not identified this metabolite, but believe it to be erythrocytic in origin, as it appears in relatively greater concentration in whole blood spectra compared to plasma spectra. In future studies, obtaining additional two-dimensional spectra, including those that incorporate other atoms such as carbon, could be useful for identification of unknown metabolites.

We elected to use a commercially available software suite for metabolite quantification and to assist in identification (Chenomx NMR Suite 8.43). This software is relatively user-friendly, has a large library of metabolites, and is widely used in the field (Viant et al. 2008, Wishart 2008, Grimes and O'Connell 2011, Mercier et al. 2011, Psychogios et al. 2011). One of the main benefits is the ability to visually examine the fit of proposed metabolites and make small adjustments if shifts have occurred, such as those due to individual variation in pH. This allowed us to fit metabolites in areas of the spectra where the peaks of multiple metabolites

Table 2. Median and interquartile range (IQR; quartile 1–3) concentrations (mM) for metabolites identified in the whole blood and plasma of loggerhead *Caretta caretta*, green *Chelonia mydas* and Kemp's ridley *Lepidochelys kempii* sea turtles in North Carolina, USA. Individuals with and without (control) cold stun syndrome were sampled between 2012–2016 and 2012–2014, respectively.

	Loggerhead						Green						Kemp's ridley					
	Whole blood			Plasma			Whole blood			Plasma			Whole blood			Plasma		
	Cold stun	Control		Cold stun	Control		Cold stun	Control		Cold stun	Control		Cold stun	Control		Cold stun	Control	
3-Hydroxybutyrate	Median	0.5805	0.1506	0.7667	0.0788	1.0187	0.2487	2.5109	0.5245	0.2300	0.4631	0.2300	0.4631	0.2300	0.4631	0.2300	0.4631	0.2300
	IQR	0.4432–0.9851	0.1031–0.2258	0.6231–1.135	0.0435–0.2421	0.5095–2.0867	0.0924–0.4639	1.1207–3.4778	0.3303–0.6153	0.1608–0.6908	0.3702–0.5654	0.1608–0.6908	0.3702–0.5654	0.1608–0.6908	0.3702–0.5654	0.1608–0.6908	0.3702–0.5654	0.1608–0.6908
Acetate	Median	0.0629	0.0512	0.0355	0.0213	0.0574	0.0240	0.0667	0.0276	0.0468	0.0447	0.0468	0.0447	0.0468	0.0447	0.0468	0.0447	0.0468
	IQR	0.0362–0.0928	0.0303–0.0991	0.0313–0.037	0.0184–0.0292	0.0463–0.0715	0.0224–0.0263	0.0543–0.0917	0.0213–0.0407	0.0449–0.054	0.0363–0.051	0.0449–0.054	0.0363–0.051	0.0449–0.054	0.0363–0.051	0.0449–0.054	0.0363–0.051	0.0449–0.054
Adenosine	Median	0.1646	0.1332	0	0.0468	0	0	0	0.0159	0	0	0	0	0	0	0	0	0
	IQR	0.1288–0.1706	0.0593–0.1596	0–0	0–0.1312	0–0.0874	0–0	0–0	0–0.0733	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0
Creatine	Median	0.1146	0.1398	0.0644	0.1130	0.0807	0.0954	0.0526	0.0598	0.0886	0.0456	0.0886	0.0456	0.0886	0.0456	0.0886	0.0456	0.0886
	IQR	0.0995–0.1251	0.0929–0.151	0.0511–0.0822	0.0948–0.1411	0.0559–0.1116	0.0744–0.1288	0.0482–0.06	0.0544–0.0721	0.0652–0.1021	0.0436–0.0489	0.0652–0.1021	0.0436–0.0489	0.0652–0.1021	0.0436–0.0489	0.0652–0.1021	0.0436–0.0489	0.0652–0.1021
Dimethyl sulfone	Median	0.0722	0.1327	0.0174	0.0288	0.0864	0.0405	0.0248	0.0767	0.1138	0.0051	0.1138	0.0051	0.1138	0.0051	0.1138	0.0051	0.1138
	IQR	0.012–0.1533	0.0844–0.1742	0.0121–0.0204	0.0167–0.1468	0.0618–0.1133	0.027–0.0805	0.0132–0.0381	0.0293–0.1358	0.0972–0.1209	0–0.0102	0.0293–0.1358	0.0972–0.1209	0–0.0102	0.0293–0.1358	0.0972–0.1209	0–0.0102	0.0293–0.1358
Formate ^{ab}	Median	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	IQR	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0	0–0
Glucose	Median	0	0.2610	0.7642	0.2128	0.4396	1.3659	1.8690	0.7775	0.4902	0.5915	1.8690	0.7775	0.4902	0.5915	1.8690	0.7775	0.4902
	IQR	0–0.4804	0–0.582	0–1.0078	0–0.5268	0–0.3731	0.9365–2.5478	1.3999–2.0607	0.6326–0.8141	0.4357–0.5464	0.4606–0.7533	1.3999–2.0607	0.6326–0.8141	0.4357–0.5464	0.4606–0.7533	1.3999–2.0607	0.6326–0.8141	0.4357–0.5464
Glycerol	Median	2.5081	2.6642	1.5101	2.0703	1.9976	1.8184	1.5601	2.5010	1.7280	1.4562	1.5601	1.7280	1.4562	1.7280	1.4562	1.7280	1.4562
	IQR	2.1952–2.8506	2.4158–2.9614	1.2104–1.7576	1.6483–2.2477	1.7515–2.3003	1.6851–1.8584	1.4461–1.5954	1.692–2.7221	1.6475–1.8602	1.1953–1.7577	1.692–2.7221	1.6475–1.8602	1.1953–1.7577	1.692–2.7221	1.6475–1.8602	1.1953–1.7577	1.692–2.7221
2-Inosine-5' monophosphate	Median	0	0	0	0.0786	0.0970	0	0	0.2203	0.2910	0	0	0.2203	0.2910	0	0	0	0
	IQR	0–0	0–0	0–0	0–0.1166	0.0792–0.1208	0–0	0–0	0.139–0.2449	0.1921–0.3268	0–0	0.139–0.2449	0.1921–0.3268	0–0	0.139–0.2449	0.1921–0.3268	0–0	0.139–0.2449
Isobutyrate	Median	0	0	0	0	0	0.0114	0.0048	0	0	0.0055	0.0048	0	0	0.0055	0.0048	0	0.0055
	IQR	0–0	0–0	0–0	0–0	0–0.0037	0–0.0134	0–0.0159	0–0.0159	0–0	0–0.0141	0–0.0159	0–0	0–0.0141	0–0.0159	0–0	0–0.0141	0–0.0159
Isoleucine	Median	0	0.0976	0	0.0335	0.0523	0.0593	0.1270	0	0.0468	0.0165	0.1270	0	0.0468	0.0165	0.1270	0	0.0468
	IQR	0–0	0.0767–0.1879	0–0	0.0054–0.0379	0.0313–0.0757	0.0479–0.0842	0.0697–0.1464	0–0	0–0.0846	0–0.0397	0.0697–0.1464	0–0	0–0.0846	0–0.0397	0.0697–0.1464	0–0	0–0.0846
Lactate	Median	1.3599	2.4851	1.6208	1.9589	1.1451	3.1030	1.4932	1.3046	0.7454	1.2455	1.4932	1.3046	0.7454	1.2455	1.4932	1.3046	0.7454
	IQR	1.1891–2.1641	1.6694–5.5352	1.6037–2.9725	0.7094–2.4337	0.854–1.8539	1.7653–3.9422	1.0378–2.9004	0.9839–1.4794	0.4105–3.7294	1.0292–1.6217	1.0378–2.9004	0.9839–1.4794	0.4105–3.7294	1.0292–1.6217	1.0378–2.9004	0.9839–1.4794	0.4105–3.7294
Leucine	Median	0	0.1523	0	0.0295	0.0953	0.0853	0.2584	0	0.0757	0.0396	0.2584	0	0.0757	0.0396	0.2584	0	0.0757
	IQR	0–0	0.059–0.2221	0–0.0378	0–0.06	0.0367–0.2049	0.0415–0.0982	0.1819–0.3613	0	0.0101–0.1899	0.0074–0.0769	0.0415–0.0982	0.1819–0.3613	0.0101–0.1899	0.0074–0.0769	0.0415–0.0982	0.1819–0.3613	0.0101–0.1899
Methanol ^a	Median	0.3316	0.4221	0.0461	0.1393	0.1222	0.0413	0.0235	0.1254	0.1914	0.0273	0.1254	0.1914	0.0273	0.1254	0.1914	0.0273	0.1254
	IQR	0.1853–0.358	0.2528–0.6173	0.0408–0.0591	0.1116–0.176	0.1067–0.1527	0.0352–0.0806	0.0078–0.0364	0.1047–0.1357	0.1698–0.2143	0.0171–0.0409	0.1047–0.1357	0.1698–0.2143	0.0171–0.0409	0.1047–0.1357	0.1698–0.2143	0.0171–0.0409	0.1047–0.1357
myo-Inositol	Median	0.3140	0.3971	0.0845	0.1865	0.2063	0.0770	0.0589	0.2176	0.2671	0.0613	0.2176	0.2671	0.0613	0.2176	0.2671	0.0613	0.2176
	IQR	0.291–0.373	0.3277–0.4181	0.0713–0.0881	0.1668–0.2051	0.195–0.2298	0.0607–0.0971	0–0.0735	0.16–0.2353	0.2418–0.3508	0.0458–0.076	0.16–0.2353	0.2418–0.3508	0.0458–0.076	0.16–0.2353	0.2418–0.3508	0.0458–0.076	0.16–0.2353
Propylene glycol	Median	0.0598	4.3919	0.0909	0.2577	0.5188	0.7705	0.8438	0.4062	0.4320	0.5456	0.8438	0.4062	0.4320	0.5456	0.8438	0.4062	0.4320
	IQR	0–0.1966	2.5358–6.0192	0.0403–0.2763	0.1175–0.6047	0.1783–1.5883	0.3944–1.4533	0.6723–1.7803	0.2114–0.5493	0.2415–0.7108	0.1638–0.2954	0.2114–0.5493	0.2415–0.7108	0.1638–0.2954	0.2114–0.5493	0.2415–0.7108	0.1638–0.2954	0.2114–0.5493
Succinate	Median	0.0084	0.0197	0.0118	0.0297	0.0321	0.0096	0.0048	0.0125	0.0153	0.0199	0.0096	0.0125	0.0153	0.0199	0.0096	0.0125	0.0153
	IQR	0–0.0124	0.0067–0.0261	0.0113–0.016	0.0255–0.0357	0.0287–0.0373	0.0069–0.0126	0–0.0073	0.0096–0.0144	0.0136–0.0194	0.0334–0.0374	0.0096–0.0144	0.0136–0.0194	0.0334–0.0374	0.0096–0.0144	0.0136–0.0194	0.0334–0.0374	0.0096–0.0144
Taurine	Median	0	0	0	0.4077	0.4364	0	0	0.2550	0.4474	0	0	0.2550	0.4474	0	0	0.2550	0.4474
	IQR	0–0.1672	0–0	0–0	0.302–0.4568	0.3273–0.5116	0–0	0–0	0.2232–0.2864	0.3937–0.5282	0–0	0.2232–0.2864	0.3937–0.5282	0–0	0.2232–0.2864	0.3937–0.5282	0–0	0.2232–0.2864
Trimethylamine	Median	0.1410	0.1529	0	0.1022	0.1510	0	0.0428	0.2169	0.3033	0	0.2169	0.3033	0	0.2169	0.3033	0	0.2169
	IQR	0.0531–0.1766	0.1007–0.1845	0–0.0076	0.0566–0.3191	0.1211–0.1843	0–0.0035	0.0247–0.2067	0.1786–0.2447	0.2225–0.3479	0–0	0.1786–0.2447	0.2225–0.3479	0–0	0.1786–0.2447	0.2225–0.3479	0–0	0.1786–0.2447

^a The median concentration seen in solvent-only 'blank' samples run with each species was subtracted prior to calculating summary statistics.

^b Formate was added to each sample as a secondary reference compound.

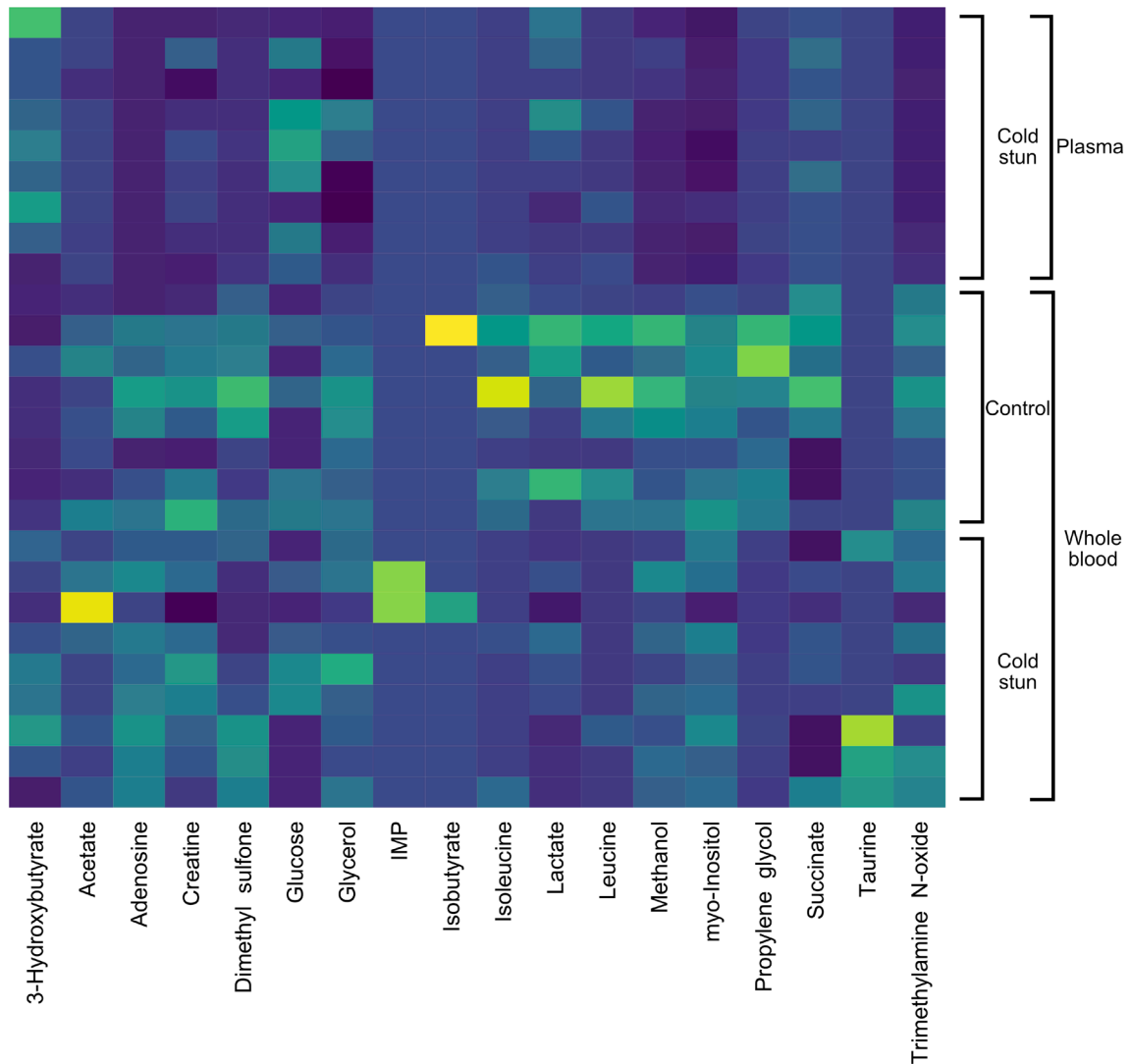


Figure 3. Heat map of metabolite concentrations from one-dimensional proton-NMR spectra of whole blood and plasma samples from loggerhead *Caretta caretta* sea turtles in North Carolina, USA (data scaled by column; low = dark purple, high = yellow). Individuals with and without (control) cold stun syndrome were sampled from 2013 to 2016 and in 2014, respectively. Methanol, myo-inositol and trimethylamine N-oxide had significantly higher concentrations in all whole blood samples across species ($p < 0.001$). Blood samples for loggerheads with cold stun syndrome had significantly greater concentrations of 3-hydroxybutyrate ($p = 0.005$) and significantly lower concentrations of leucine ($p = 0.007$) and propylene glycol ($p = 0.004$). Adenosine, 2'-inosine-5' monophosphate (IMP), and taurine were not identified in plasma samples.

were overlapping, which is in contrast to methods that divide spectra into bins and quantify concentration based on integration, limiting use to areas without metabolite overlap. However, care must be taken to maintain consistency across spectra, such as always fitting metabolites in the same order and likely using measurements conducted by a single person. Quantification is semi-automated at best, and in our experience, the absolute concentrations obtained are often underestimated. While we expect relative differences in concentrations to be valid, the concentrations presented in Table 2 should not be used as reference values.

Comparing whole blood to plasma

We expected, and found, a number of differences between whole blood and plasma metabolic profiles. Taurine was

not identified in plasma samples and TMAO was found in significantly greater concentration in whole blood samples. Both metabolites are osmolytes important in maintaining cellular volume (Yancey 2005) and would be expected to be primarily intracellular. Concentrations of myo-inositol were also significantly greater in whole blood samples, which is likely due to protein linkages containing inositol that help protect erythrocytes from lysis (Hooper 1997). Differences in metabolites involved in cellular metabolism were also distinct in whole blood samples, such as the presence of adenosine and IMP, a purine nucleoside and nucleotide, respectively (Murray 2009). Glucose concentrations in green turtle whole blood were significantly lower than in plasma. This has been seen with green turtle samples analyzed by glucometer (Perrault et al. 2017) and may be due to in vitro glycolysis during the thawing and incubation steps prior

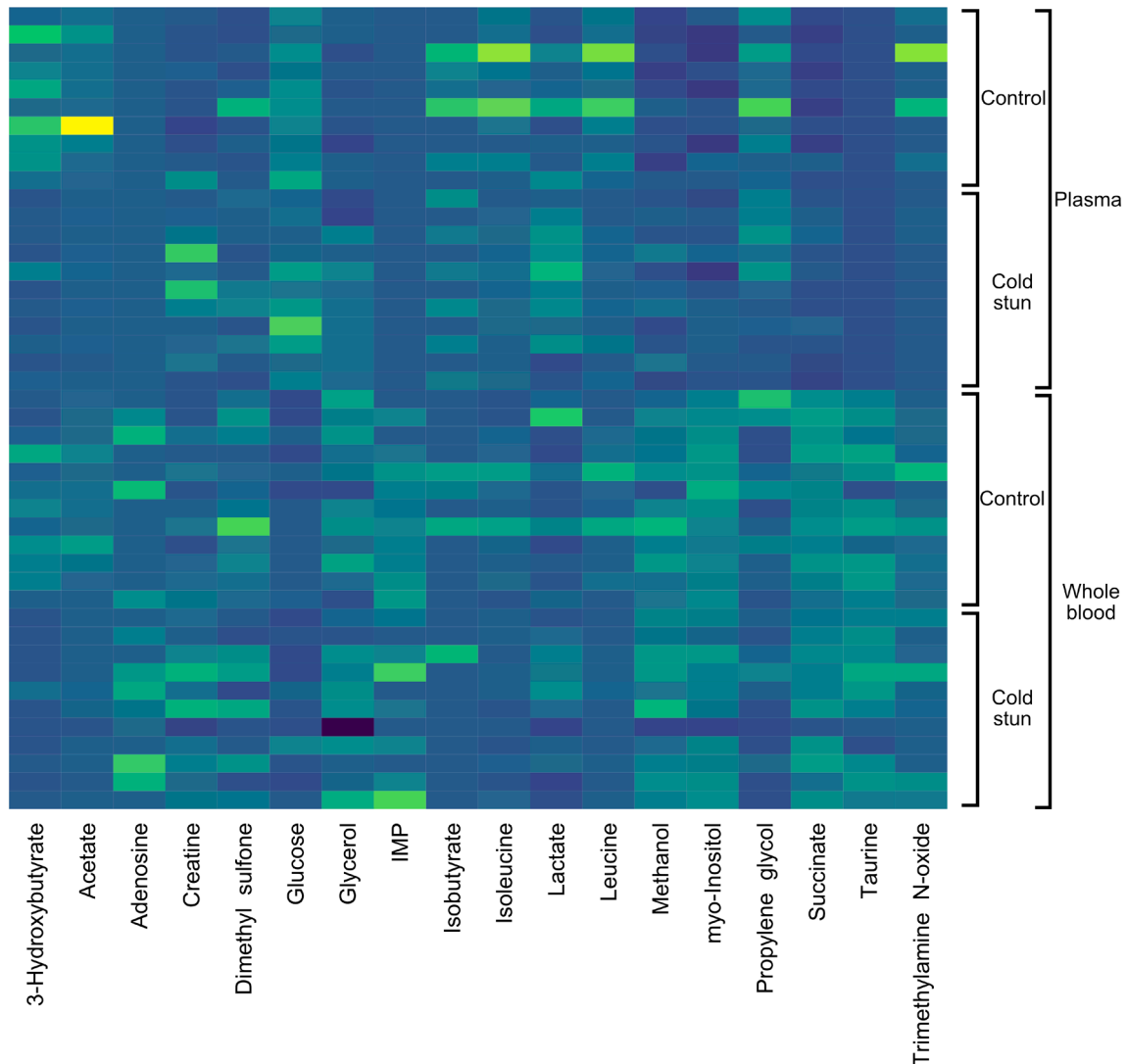


Figure 4. Heat map of metabolite concentrations from one-dimensional proton-NMR spectra of whole blood and plasma samples from green *Chelonia mydas* sea turtles in North Carolina, USA (data scaled by column; low = dark purple, high = yellow). Individuals with and without (control) cold stun syndrome were sampled between 2012–2015 and 2012–2014, respectively. Methanol, myo-inositol and trimethylamine N-oxide had significantly higher concentrations in all whole blood samples across species ($p < 0.001$). Glucose and isobutyrate concentrations were significantly less in green turtle whole blood samples versus plasma samples ($p < 0.001$ and 0.018 , respectively), whereas succinate concentrations were significantly greater in whole blood samples ($p < 0.001$). In both whole blood and plasma samples, 3-hydroxybutyrate and acetate was significantly lower in individuals with cold stun syndrome ($p = 0.005$ and 0.003 and $p = 0.001$ and < 0.001 , respectively). Creatine and glycerol concentrations were significantly greater in plasma samples from individuals with cold stun syndrome ($p = 0.006$ and 0.012 , respectively). Isoleucine, leucine and trimethylamine N-oxide plasma concentrations were lower in individuals with cold stun ($p = 0.004$, 0.002 and < 0.001 , respectively). Adenosine, 2'inosine-5' monophosphate (IMP) and taurine were not identified in plasma samples.

to hemolysis in sample preparation (Stockham and Scott 2008), as glucose concentration in loggerhead samples have been shown to decrease over time (Eisenhawer et al. 2007). Lastly, succinate, which is generated in mitochondria via the carboxylic acid cycle (Murray 2009), was significantly greater in green turtle whole blood compared to plasma.

Differences between the whole blood and plasma concentrations of isobutyrate and methanol from control and cold stun affected groups were not expected. Isobutyrate can be formed by the oxidation of valine (Smith and Macfarlane 1997) and has been identified in the plasma of women and in human fecal extracts (Le Gall et al. 2011, Bahado-Singh

et al. 2012, Shao et al. 2016). Gastrointestinal bacteria produce isobutyrate (Le Gall et al. 2011). Methanol was identified as a contaminant from our blank samples, but concentrations were greater than the background contamination. Methanol has also been identified as a human fecal metabolite (Ahmed et al. 2016, Shao et al. 2016) and gastrointestinal flora easily trap methanol (Garner et al. 2007). Methanol interacts with lipid bilayers (Sonmez et al. 2013), which may explain the significantly greater concentration in whole blood, but we do not have an explanation for why isobutyrate concentrations would be significantly less in whole blood versus plasma.

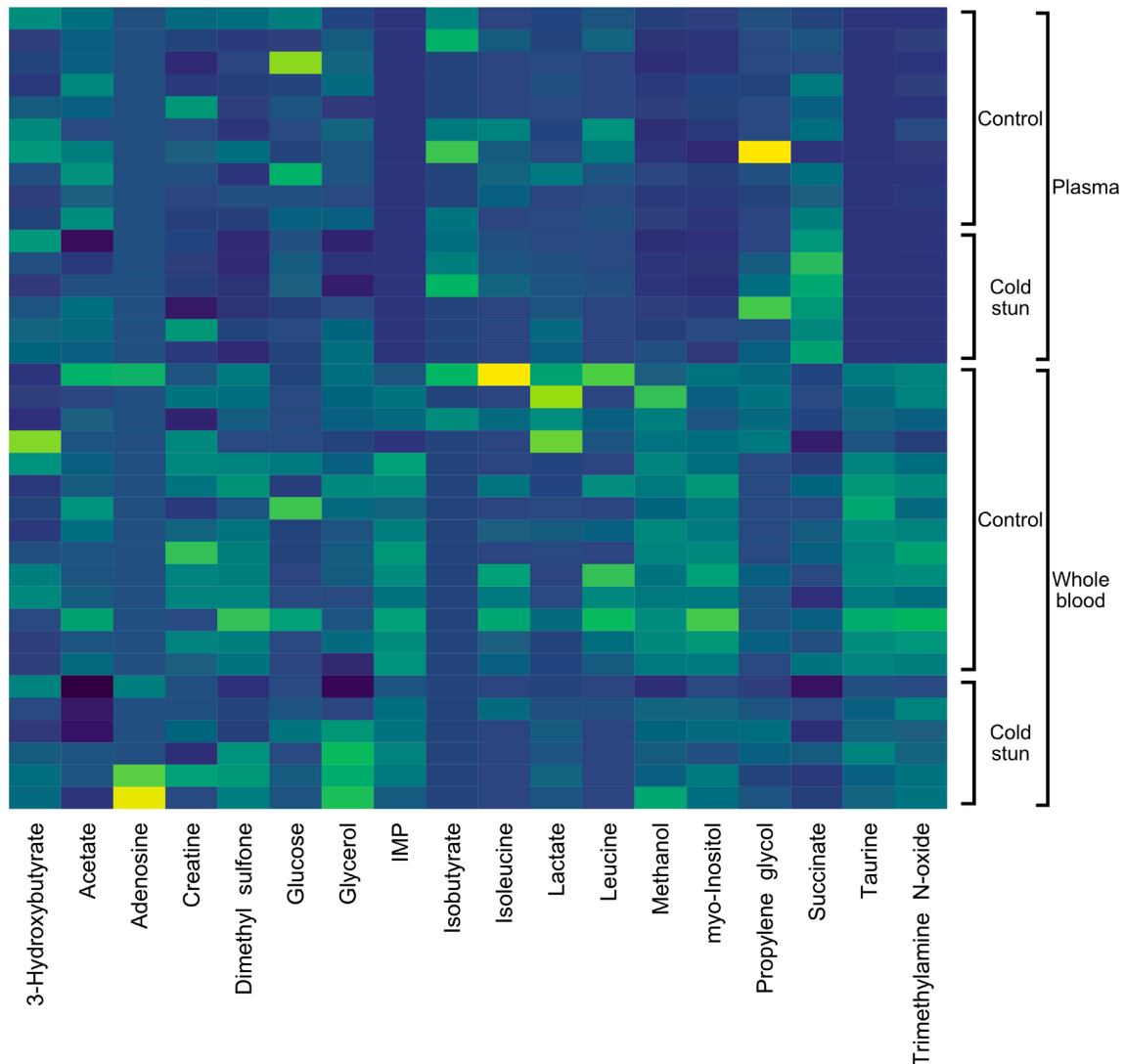


Figure 5. Heat map of metabolite concentrations from one-dimensional proton-NMR spectra of whole blood and plasma samples from Kemp's ridley *Lepidochelys kempii* sea turtles in North Carolina, USA (data scaled by column; low = dark purple, high = yellow). Individuals with and without (control) cold stun syndrome were sampled between 2013–2015 and 2012–2014, respectively. Methanol, myo-inositol and trimethylamine N-oxide had significantly higher concentrations in all whole blood samples across species ($p < 0.001$). Isobutyrate concentrations were significantly less in Kemp's ridley whole blood samples versus plasma samples ($p = 0.014$). Acetate and taurine concentrations in whole blood and dimethyl sulfone concentrations in plasma were significantly lower in cold stun affected individuals ($p = 0.002$, 0.015 and 0.01 , respectively). Plasma lactate, propylene glycol and succinate concentrations were significantly greater in individuals with cold stun syndrome ($p = 0.027$, 0.033 and 0.003 , respectively). Adenosine, 2'-inosine-5'-monophosphate (IMP), and taurine were not identified in plasma samples.

Comparing cold stuns to controls and species differences

Sea turtles affected by cold stun syndrome become lethargic and cease feeding. We identified differences in several metabolites that are likely secondary to anorexia. Isoleucine and leucine are nutritionally essential in humans (Murray 2009) and both were significantly decreased in plasma samples from cold stunned green turtles. Leucine was also significantly decreased in whole blood from cold stunned loggerhead and green turtles. Dimethyl sulfone was significantly decreased in cold stunned Kemp's ridley plasma, which is likely a result of a combination of decreased dietary intake, endogenous metabolism and bacterial metabolism (Engelke et al. 2005). A significantly lower concentration of TMAO was found

in cold stunned green turtle plasma samples, which may be related to decreased gastrointestinal microbial metabolism and decreased intake of choline and carnitine due to anorexia (Wang et al. 2011, Koeth et al. 2013). However, decreased TMAO concentrations may also reflect osmoregulatory disturbance (Yancey 2005). Significantly increased glycerol concentrations in cold stunned green turtle plasma are likely the result of triacylglycerol metabolism during anorexia (Rosenthal and Glew 2009). However, glycerol has also been noted as a cytoprotectant involved in redox balance and response to hypoxia (Yancey 2005). Additionally, increased glycerol concentrations may afford some cryoprotection, as seen in other species, including the painted turtle *Chrysemys picta* (Storey and Storey 1983, Kukal et al. 1988, Storey et al. 1988).

Table 3. Summary of statistically significant differences identified in the whole blood and plasma of loggerhead *Caretta caretta*, green *Chelonia mydas* and Kemp's ridley *Lepidochelys kempii* sea turtles in North Carolina, USA. (A) Statistically significant changes in metabolite concentrations in cold stunned versus control sea turtles. Individuals with and without cold stun syndrome were sampled between 2012–2016 and 2012–2014, respectively. (B) Statistically significant differences in metabolite concentrations in whole blood versus plasma samples of sea turtles.

Metabolite	Species	Sample type	Direction of change	Function/interpretation
(A) Statistically significant changes in metabolite concentrations in cold stunned versus control sea turtles				
3-Hydroxybutyrate	<i>Caretta caretta</i>	whole blood	↑	Anorexia, ketogenesis; may be impacted by body size and/or early/late stranding timing
	<i>Chelonia mydas</i>	whole blood	↓	
	<i>Chelonia mydas</i>	plasma		
Acetate	<i>Chelonia mydas</i>	whole blood	↓	Exertion/anaerobic metabolism
	<i>Chelonia mydas</i>	plasma		
	<i>Lepidochelys kempii</i>	whole blood		
Creatine	<i>Chelonia mydas</i>	plasma	↑	Exertion/anaerobic metabolism, ischemia, platelet aggregation
Dimethyl sulfone	<i>Lepidochelys kempii</i>	plasma	↓	Anorexia, endogenous metabolism, bacterial metabolism
Glycerol	<i>Chelonia mydas</i>	plasma	↑	Anorexia (triacylglycerol metabolism), potential cryoprotectant properties
Isoleucine	<i>Chelonia mydas</i>	plasma	↓	Anorexia
Lactate	<i>Lepidochelys kempii</i>	plasma	↑	Hypoventilation, anaerobic metabolism, abnormal perfusion
Leucine	<i>Caretta caretta</i>	whole blood	↓	Anorexia
	<i>Chelonia mydas</i>	whole blood		
	<i>Chelonia mydas</i>	plasma		
Propylene glycol	<i>Caretta caretta</i>	whole blood	↓	Unknown; potentially energy production, cryoprotection, gastrointestinal microbial metabolism or environmental contaminant; may be impacted by body size and/or early/late stranding timing
	<i>Lepidochelys kempii</i>	plasma	↑	
Succinate	<i>Lepidochelys kempii</i>	plasma	↑	Exertion/anaerobic metabolism, ischemia
Taurine	<i>Lepidochelys kempii</i>	whole blood	↓	Osmoregulatory disturbance, antioxidant consumption
TMAO	<i>Chelonia mydas</i>	plasma	↓	Anorexia, decreased gastrointestinal microbial metabolism, osmoregulatory disturbance
(B) Statistically significant differences in metabolite concentrations in whole blood versus plasma samples of sea turtles				
Glucose	<i>Chelonia mydas</i>		↓	Potentially due to in vitro glycolysis
Isobutyrate	<i>Chelonia mydas</i>		↓	Unknown; may be related to gastrointestinal bacteria
	<i>Lepidochelys kempii</i>			
Methanol	all species*		↑	Unknown; may be related to gastrointestinal bacteria, also interacts with lipid bilayers
Succinate	<i>Chelonia mydas</i>		↑	Generated in mitochondria via the carboxylic acid cycle
TMAO	all species		↑	Osmolyte
myo-Inositol	all species		↑	Protein linkages protect against erythrocyte lysis

* Additionally, the concentration of methanol in *Caretta caretta* whole blood samples (cold stuns and controls) was significantly greater than in all other species' samples.

We also identified differences in multiple metabolites related to exertion and anaerobic metabolism. Succinate was significantly increased in plasma of cold stunned Kemp's ridleys and is generated via ATP production in the carboxylic acid cycle (Murray 2009). Accumulation of succinate may also be an indicator of ischemia and has been associated with reperfusion injury (Chouchani et al. 2014, Pell et al. 2016). Similarly, increased consumption of acetate into the carboxylic acid cycle to attempt to generate energy aerobically (Rosenthal and Glew 2009) initially explains the significantly decreased acetate concentrations seen in cold stunned green turtle whole blood and plasma samples and Kemp's ridley whole blood samples. Lactate concentrations in cold stunned Kemp's ridley plasma were significantly increased, which has been found in cold stunned sea turtles and presumed secondary to hypoventilation, anaerobic metabolism and abnormal perfusion (Innis et al. 2007, Keller et al. 2012). Lactate is produced

under hypoxic conditions and used by the liver for gluconeogenesis (Murray 2009). Creatine, a product of using creatine phosphate to generate ATP, is released from ischemically damaged tissue (Wyss and Kaddurah-Daouk 2000) and concentrations were significantly greater in the plasma of cold stunned green turtles. Additionally, creatine may help inhibit platelet aggregation and in humans with acute myocardial infarction, individuals with greater creatine concentrations had a lower incidence of death and complications up to two years later (Delanghe et al. 1991, Wyss and Kaddurah-Daouk 2000). Green and Kemp's ridley turtles with cold stun syndrome have lower clot strength and take longer to form clots compared to controls (Barratclough et al. 2019). Finally, while not a significant difference ($p = 0.065$ in Kemp's whole blood samples), adenosine trended toward greater concentrations in turtles with cold stun syndrome and has also been identified as a signal of hypoxia (Bickler and Buck 2007).

Changes in taurine and propylene glycol, in addition to glycerol, may potentially be a response to cold temperatures. Raheem (1980) found a marked increase in taurine in hibernating *Varanus griseus* and suggested that it may participate in central nervous system depression. However, we found taurine at significantly lower concentration in whole blood samples from Kemp's ridleys affected by cold stun. This may be due to changes in osmoregulation or consumption as an antioxidant (Yancey 2005) as efflux of taurine has been seen in avian erythrocytes before other more osmotically important amino acids presumably as a protective measure (Shihabi et al. 1989). Propylene glycol concentrations were significantly lower in whole blood samples from cold stunned loggerheads, but significantly greater in plasma samples from cold stunned Kemp's ridley turtles. This metabolite may be of bacterial or yeast origin (Enebo 1954, Suzuki and Onishi 1968), made biogenically by the turtles (Saxena et al. 2010), or an environmental contaminant. If a true metabolite, the turtles could be using it to provide energy and protect against ketosis through gluconeogenesis, the carboxylic acid cycle and glycogenesis (Shull and Miller 1960, Ruddick 1972, Morshed et al. 1988, Nielsen and Ingvarsten 2004) or potentially as a cryoprotectant (Niemuth et al. 2018). We do not have an explanation for the observed species difference. Species and seasonal changes in diet and foraging ground could contribute to the variance. Loggerheads are larger and strand later in the season than Kemp's ridleys (Still et al. 2005). The loggerheads could have depleted their propylene glycol as a result of being more chronically cold. Alternatively, the Kemp's ridleys early in the season could be stranding because they are physiologically disadvantaged in some way.

Loggerheads with cold stun syndrome were also found to have significantly greater concentrations of 3-hydroxybutyrate in whole blood samples versus green turtle whole blood and plasma samples, which had lower concentration in cold stun affected animals. Three-hydroxybutyrate is a ketone body produced secondary to anorexia, which allows the brain to function without glucose thereby decreasing the need for gluconeogenesis (Rosenthal and Glew 2009). As previously mentioned, loggerheads have greater body mass and strand later in the season, which may mean that they have been anorexic longer and have had greater time to convert to ketosis. Green turtles strand earlier and may have consumed their normal circulating ketones prior to increasing ketogenesis.

In the single Kemp's ridley that died, concentrations of both whole blood and plasma 3-hydroxybutyrate, acetate, dimethyl sulfone, glucose, myo-inositol, creatine and lactate were greater than the calculated third quartile value (Table 2) concentrations for its cohort with the creatine and lactate concentrations being much greater (greater than two-fold and 1.5-fold the median concentrations, respectively). Blood concentrations of adenosine and plasma concentrations of glycerol and methanol were also much greater than the third quartile value. The plasma concentration of succinate was below the calculated first quartile value (Table 2). These more severe perturbations suggest that this animal had been anorexic for some time, had hypoxia, was using anaerobic metabolism and had experienced acidosis, muscle damage and ischemia.

Sources of variation

We acknowledge that, as is the case in any study sampling wildlife, there are numerous sources of variation. The control cohort for this study was sampled in October and November, earlier in the season than most cold stun strandings occur in North Carolina. It was not possible to sample non-stranded, free-ranging turtles during cold stun season, so we do not know if those turtles that do strand are representative. Feeding status is also an unknown variable for free-ranging sea turtles and can result in some changes to biochemical parameters (Anderson et al. 2011a). The individuals sampled also varied somewhat in size and, except for the control green and Kemp's ridley turtles, were of unknown sex. Both body size and sex can affect blood parameters (Bolten and Bjorndal 1992). Additionally, variation in hydration status and red blood cell count could influence metabolite concentrations. No obvious relationship was apparent in our preliminary assessments, but it may be that a complex combination of parameters might provide some additional insights. Lastly, it took several years to accumulate the samples for this study and despite storage at ultralow temperature some metabolite degradation is likely. To our knowledge, there is no comprehensive review available of metabolite stability during storage in whole blood or plasma, but changes of varying degree have been documented in samples stored for clinical biochemistry panels for a variety of species (Thoresen et al. 1995, Cray et al. 2009, Brinc et al. 2012, Eshar et al. 2018).

Conclusion

Future studies should consider other NMR techniques including two-dimensional experiments and evaluation of carbon atoms. Other metabolomic techniques, such as mass spectrometry, may also be useful. Extraction methods aimed at nonpolar metabolites could help elucidate fatty acid metabolism during cold stun syndrome. Finally, examining specific tissues and the fecal metabolome both at stranding and tracked over rehabilitation may help explain the prevalence of lung, bone, joint and skin lesions (Solano et al. 2008, Stockman et al. 2013, Innis and Staggs 2017) and provide new treatment opportunities.

This study provides a baseline for the metabolic profile of control and cold stun affected sea turtles in North Carolina. We recognized several differences between whole blood and plasma samples, which can inform future research decisions and may simplify sample collection. The primary metabolic findings were consistent with what is known about cold stun affected turtles from clinical biochemistry and blood gas panels demonstrating evidence of anorexia, acidosis, anaerobic metabolism and ischemic injury. Cold stun affected turtles can frequently spend months in rehabilitation and identification of those unlikely to survive or those likely to develop complications could increase rehabilitation efficiency. We have identified several metabolites, including adenosine, creatine, isobutyrate, methanol and taurine that are worthy of further investigation with regards to their roles in hypoxia, ischemia and reperfusion.

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Permits – Sample collection was performed under National Marine Fisheries Service Permit no. 16733-01, North Carolina State University Institutional Animal Care and Use Committee 15-001-O, and North Carolina Wildlife Resources Commission Permit no. 17ST42.

References

- Ahmed, I. et al. 2016. Investigation of faecal volatile organic metabolites as novel diagnostic biomarkers in inflammatory bowel disease. – *Aliment. Pharmacol. Ther.* 43: 596–611.
- Anderson, E. T. et al. 2011a. The effects of feeding on hematological and plasma biochemical profiles in green (*Chelonia mydas*) and Kemp's ridley (*Lepidochelys kempii*) sea turtles. – *Vet. Med. Int.* 2011: 890829.
- Anderson, E. T. et al. 2011b. Evaluation of hematology and serum biochemistry of cold-stunned green sea turtles (*Chelonia mydas*) in North Carolina, USA. – *J. Zoo Wildl. Med.* 42: 247–255.
- Bahado-Singh, R. et al. 2012. Metabolomics and first-trimester prediction of early-onset preeclampsia. – *J. Matern. Fetal Neonatal Med.* 25: 1840–1847.
- Barratclough, A. et al. 2019. Baseline plasma thromboelastography in Kemp's ridley (*Lepidochelys kempii*), green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) sea turtles and its use to diagnose coagulopathies in cold-stunned Kemp's ridley and green sea turtles. – *J. Zoo Wildl. Med.* 50: 62–68.
- Bentivegna, F. et al. 2002. Cold stunned loggerhead turtles in the South Adriatic Sea. – *Mar. Turtle Newsl.* 97: 1–3.
- Bickler, P. E. and Buck, L. T. 2007. Hypoxia tolerance in reptiles, amphibians and fishes: life with variable oxygen availability. – *Annu. Rev. Physiol.* 69: 145–170.
- Bolten, A. B. and Bjorndal, K. A. 1992. Blood profiles for a wild population of green turtles (*Chelonia mydas*) in the southern Bahamas: size-specific and sex-specific relationships. – *J. Wildl. Dis.* 28: 407–413.
- Brinc, D. et al. 2012. Long-term stability of biochemical markers in pediatric serum specimens stored at -80°C : a CALIPER substudy – *Clin. Biochem.* 45: 816–826.
- Chouchani, E. T. et al. 2014. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. – *Nature* 515: 431.
- Christiansen, E. F. et al. 2016. 2016 North Carolina sea turtle cold stunning event. – In: Proc. Int. Assoc. for Aquatic Animal Medicine. Virginia Beach, Virginia.
- Cray, C. et al. 2009. Effects of storage temperature and time on clinical biochemical parameters from rat serum. – *J. Am. Assoc. Lab. Anim. Sci.* 48: 202–204.
- Delanghe, J. et al. 1991. Can creatine predict further major cardiovascular events after acute myocardial infarction? – *Ann. Clin. Biochem.* 28: 101–102.
- Eisenhawer, E. et al. 2007. Relationship between separation time of plasma from heparinized whole blood on plasma biochemical analytes of loggerhead sea turtles (*Caretta caretta*). – *J. Zoo Wildl. Med.* 39: 208–25.
- Enebo, L. 1954. Studies in cellulose decomposition by an anaerobic thermophilic bacterium and two associated non-cellulolytic species. – Victor Pettersons Bokindustri Aktiebolag.
- Engelke, U. F. H. et al. 2005. Dimethyl sulfone in human cerebrospinal fluid and blood plasma confirmed by one-dimensional ^1H and two-dimensional ^1H - ^{13}C NMR. – *NMR Biomed.* 18: 331–336.
- Eshar, D. et al. 2018. Effects of time and storage temperature on selected biochemical analytes in plasma of red-eared sliders (*Trachemys scripta elegans*). – *Am. J. Vet. Res.* 79: 852–857.
- Foley, A. M. et al. 2007. Characteristics of a green turtle (*Chelonia mydas*) assemblage in northwestern Florida determined during a hypothermic stunning event. – *Gulf. Mex. Sci.* 25: 131–143.
- Garner, C. E. et al. 2007. Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease. – *FASEB J.* 21: 1675–1688.
- Grimes, J. H. and O'Connell, T. M. 2011. The application of micro-coil NMR probe technology to metabolomics of urine and serum. – *J. Biomol. NMR* 49: 297–305.
- Hooper, N. M. 1997. Glycosyl-phosphatidylinositol anchored membrane enzymes – *Clin. Chim. Acta* 266: 3–12.
- Innis, C. J. and Staggs, L. A. 2017. Cold-stunning. – In: Manire, C. A. (ed.), Sea turtle health and rehabilitation. J. Ross Publishing, pp. 675–688.
- Innis, C. J. et al. 2007. Metabolic and respiratory status of cold-stunned Kemp's ridley sea turtles (*Lepidochelys kempii*). – *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 177: 623–630.
- Innis, C. J. et al. 2009. Hematologic and plasma biochemical findings in cold-stunned Kemp's ridley turtles: 176 cases (2001–2005). – *J. Am. Vet. Med. Assoc.* 235: 426–432.
- Keller, K. A. et al. 2012. Metabolic and respiratory derangements associated with death in cold-stunned Kemp's ridley turtles (*Lepidochelys kempii*): 32 cases (2005–2009). – *J. Am. Vet. Med. Assoc.* 240: 317–323.
- Koeth, R. A. et al. 2013. Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. – *Nat. Med.* 19: 576.
- Kraus, D. R. and Jackson, D. C. 1980. Temperature effects on ventilation and acid–base balance of the green turtle. – *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 239: R254–R258.
- Krause, D. and Grove, D. 1967. Deep-freezing of jackass and stallion semen in concentrated pellet form. – *J. Reprod. Fertil.* 14: 139–141.
- Kriat, M. et al. 1992. Quantitation of metabolites in human blood serum by proton magnetic resonance spectroscopy. A comparative study of the use of formate and TSP as concentration standards. – *NMR Biomed.* 5: 179–184.
- Kukul, O. et al. 1988. Glycerol metabolism in a freeze-tolerant arctic insect: an in vivo ^{13}C NMR study. – *J. Comp. Physiol. B* 158: 175–183.
- Le Gall, G. et al. 2011. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. – *J. Proteome Res.* 10: 4208–4218.
- Li, T. et al. 2015. Development of a summarized health index (SHI) for use in predicting survival in Sea Turtles. – *PLoS One* 10: e0120796.
- Lindon, J. C., Nicholson, J. K. and Holmes, E. 2007. The handbook of metabolomics and metabolomics. – Elsevier.
- Mercier, P. et al. 2011. Towards automatic metabolomic profiling of high-resolution one-dimensional proton NMR spectra. – *J. Biomol. NMR* 49: 307–323.
- Morreale, S. J. et al. 1992. Annual occurrence and winter mortality of marine turtles in New-York waters. – *J. Herpetol.* 26: 301–308.
- Morshed, K. M. et al. 1988. Kinetics of propylene glycol elimination and metabolism in rat. – *Biochem. Med. Metab. Biol.* 39: 90–97.
- Murray, R. K. 2009. Harper's illustrated biochemistry. – McGraw-Hill Medical.

- Nagase, H. and Niwa, T. 1964. Deep freezing bull semen in concentrated pellet form. – In: 5th Int. Congr. on Animal Reproduction and Artificial Insemination III, pp. 410–415.
- Nielsen, N. I. and Ingvartsen, K. L. 2004. Propylene glycol for dairy cows: a review of the metabolism of propylene glycol and its effects on physiological parameters, feed intake, milk production and risk of ketosis. – *Anim. Feed Sci. Technol.* 115: 191–213.
- Niemuth, J. N. and Stoskopf, M. K. 2017. A novel extraction method for the preparation of heparinized chicken (*Gallus gallus domesticus*) and horse (*Equus caballus*) whole blood for ¹H-NMR metabolomics using Drabkin's reagent. – *JIOMICS* 7: 1–6.
- Niemuth, J. N. et al. 2018. Propylene glycol in free-ranging green sea turtle (*Chelonia mydas*) plasma. – *J. Fish. Wildl. Manage.* 9: 617–623.
- Occhiello, A. et al. 2009. Skin necrosis in sea turtle cold stunning: regeneration following rMnSOD topic treatment in a specimen of *Caretta caretta*. – *Comp. Clin. Path.* 18: 365–369.
- Pell, V. R. et al. 2016. Succinate metabolism: a new therapeutic target for myocardial reperfusion injury. – *Cardiovasc. Res.* 111: 134–141.
- Perrault, J. R. et al. 2017. Comparison of whole blood and plasma glucose concentrations in green turtles (*Chelonia mydas*) determined using a glucometer and a dry chemistry analyzer. – *J. Wildl. Dis.* 54: 196–199.
- Psychogios, N. et al. 2011. The human serum metabolome. – *PLoS One* 6: e16957.
- Raheem, K. A. 1980. Taurine – a possible inhibitory substance in the brain of a hibernating reptile. – *Comp. Biochem. Physiol.* B 65: 751–753.
- Reo, N. V. 2002. NMR-based metabolomics. – *Drug Chem. Toxicol.* 25: 375–382.
- Roberts, K. et al. 2014. Weather patterns associated with green turtle hypothermic stunning events in St. Joseph Bay and Mosquito Lagoon, Florida. – *Phys. Geogr.* 35: 134–150.
- Rosenthal, M. D. and Glew, R. H. 2009. Medical biochemistry: human metabolism in health and disease. – Wiley.
- Ruddick, J. A. 1972. Toxicology, metabolism and biochemistry of 1,2-propanediol. – *Toxicol. Appl. Pharmacol.* 21: 102–111.
- Saxena, R. K. et al. 2010. Microbial production and applications of 1,2-propanediol. – *Indian J. Microbiol.* 50: 2–11.
- Schwartz, F. J. 1978. Behavioral and tolerance responses to cold water temperatures by three species of sea turtles (Reptilia, Cheloniidae) in North Carolina. – *Fla Mar. Res. Publ.* 33: 16–18.
- Shao, T. et al. 2016. Characterization of ankylosing spondylitis and rheumatoid arthritis using ¹H NMR-based metabolomics of human fecal extracts. – *Metabolomics* 12: 70.
- Shaver, D. J. et al. 2017. Hypothermic stunning of green sea turtles in a western Gulf of Mexico foraging habitat. – *PLoS One* 12: e0173920.
- Shihabi, Z. K. et al. 1989. The taurine content of avian erythrocytes and its role in osmoregulation. – *Comp. Biochem. Physiol.* A Physiol. 92: 545–549.
- Shull, K. H. and Miller, O. N. 1960. Formation in vivo of glycogen by certain intermediates of the lactate-propanediol pathway. – *J. Biol. Chem.* 235: 551–553.
- Smith, E. A. and Macfarlane, G. T. 1997. Dissimilatory amino acid metabolism in human colonic bacteria – *Anaerobe* 3: 327–337.
- Solano, M. et al. 2008. Scintigraphic and radiographic evaluation of appendicular skeletal lesions in cold-stunned Kemp's ridley sea turtles. – *Vet. Radiol. Ultrasound* 49: 388–394.
- Sonmez, M. et al. 2013. The effect of alcohols on red blood cell mechanical properties and membrane fluidity depends on their molecular size. – *PLoS One* 8: e76579.
- Stacy, N. I. et al. 2013. Development and evaluation of three mortality prediction indices for cold-stunned Kemp's ridley sea turtles (*Lepidochelys kempii*). – *Conserv. Physiol.* 1: cot003.
- Standora, E. A. et al. 1989. Growth rates and behavior of captive and free-ranging juvenile Kemp's ridley sea turtles from Long Island, NY. – In: AAZPA Regional Conference Proceedings 1989, pp. 954–961.
- Still, B. M. et al. 2005. Climatic and oceanographic factors affecting daily patterns of juvenile sea turtle cold-stunning in Cape Cod Bay, Massachusetts. – *Chelonian Conserv. Biol.* 4: 883–890.
- Stockham, S. L. and Scott, M. A. 2008. Fundamentals of veterinary clinical pathology. – Blackwell Publishing.
- Stockman, J. et al. 2013. Prevalence, distribution and progression of radiographic abnormalities in the lungs of cold-stunned Kemp's ridley sea turtles (*Lepidochelys kempii*): 89 cases (2002–2005). – *J. Am. Vet. Med. Assoc.* 242: 675–681.
- Storey, J. M. and Storey, K. B. 1983. Regulation of cryoprotectant metabolism in the overwintering gall fly larva, *Eurosta solidaginis*: temperature control of glycerol and sorbitol levels. – *J. Comp. Physiol.* B 149: 495–502.
- Storey, K. B. et al. 1988. Hatchling turtles survive freezing during winter hibernation. – *Proc. Natl Acad. Sci. USA* 85: 8350–8354.
- Suzuki, T. and Onishi, H. 1968. Aerobic dissimilation of L-rhamnose and the production of L-rhamnonic acid and 1,2-propanediol by yeasts. – *Agric. Biol. Chem.* 32: 888–893.
- Thoresen, S. I. et al. 1995. Effects of storage time and freezing temperature on clinical chemical parameters from canine serum and heparinized plasma. – *Vet. Clin. Pathol.* 24: 129–133.
- Turnbull, B. S. et al. 2000. Medical implications of hypothermia in threatened loggerhead (*Caretta caretta*) and endangered Kemp's ridley (*Lepidochelys kempii*) and green (*Chelonia mydas*) sea turtles. – In: Proc. AAZV and IAAAM Joint Conference, New Orleans, Louisiana.
- Vélez-Rubio, G. M. et al. 2017. Hypothermic stunning in juvenile green turtles (*Chelonia mydas*) in Uruguayan coastal waters: learning for future events. – *Chelonian Conserv. Biol.* 16: 151–158.
- Viant, M. R., Ludwig, C. and Günther, U. L. 2008. 1D and 2D NMR spectroscopy: from metabolic fingerprinting to profiling. – In: Griffiths, W. J. (ed.), *Metabolomics, metabonomics and metabolite profiling*. R. Soc. Chem., pp. 44–70.
- Wang, Z. et al. 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. – *Nature* 472: 57.
- Wishart, D. S. 2008. Quantitative metabolomics using NMR. – *Trends Analyt. Chem.* 27: 228–237.
- Wishart, D. S. et al. 2013. HMDB 3.0 – the human metabolome database in 2013. – *Nucleic Acids. Res.* 41: D801–D807.
- Wyss, M. and Kaddurah-Daouk, R. 2000. Creatine and creatinine metabolism. – *Physiol. Rev.* 80: 1107–1213.
- Yancey, P. H. 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. – *J. Exp. Biol.* 208: 2819–2830.

Supplementary material (available online as Appendix wlb-00587 at <www.wildlifebiology.org/appendix/wlb-00587>). Appendix 1.