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Sea Turtle Tears: A Novel, Minimally Invasive Sampling Method for ^{1}H-NMR Metabolomics Investigations with Cold Stun Syndrome as a Case Study

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ABSTRACT: We investigated a method for collecting and processing tear samples from loggerhead (*Caretta caretta*), green (*Chelonia mydas*), and Kemp's ridley (*Lepidochelys kempii*) sea turtles and to identify tear biomarkers and potential differences between unaffected sea turtles and those affected by cold stun syndrome. Tear samples from unaffected and cold-stunned loggerhead, green, and Kemp's ridley sea turtles were collected with sterile, cellulose, latex-free ophthalmic eye spears. We pooled spears to achieve acceptable concentrations, which we extracted and analyzed with proton nuclear magnetic resonance spectroscopy. Using principal components analysis, we identified five tear biomarkers (propylene glycol, glycerol, lactate, formate, and an unidentified metabolite) that distinguished unaffected sea turtles from those with cold stun syndrome. The formate concentration was significantly lower (one-sided, exact, two-sample permutation, P=0.019) in unaffected sea turtles, which is consistent with clinical metabolic acidosis reported in cold-stunned animals. Collection of sufficient sample volume for analysis required multiple spears per sample cohort, but tear sample collection from sea turtles was easy to perform and well tolerated by the animals. Sea turtle tears can be an appropriate sample for some metabolomics research questions.

Key words: *Caretta caretta*, *Chelonia mydas*, green sea turtle, Kemp's ridley sea turtle, *Lepidochelys kempii*, loggerhead sea turtle, proton nuclear magnetic resonance spectroscopy, tear biomarkers.

Metabolomics is the study of metabolites, small molecular weight end products of metabolism such as sugars, amino acids, and fatty acids. It uses technologies including gas chromatography, mass spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy (Lindon et al. 2007). Sample types range from tissues and biofluids to excreta and whole organisms.

Several ocular disease biomarkers have been identified (Pieragostino et al. 2015) in the human tear metabolome, but veterinary studies are limited (Ahamad et al. 2017). Sea turtle lacrimal glands, which also excrete excess salt, and smaller Harderian glands, which provide lubrication, are responsible for tear formation (Wyneken 2001; Southwood Williard 2013). Increased tear production may result from elevated plasma sodium concentration, which can be affected by diet and potentially by stress (Southwood Williard 2013). We explored a method for collecting and processing tear samples from loggerhead (*Caretta caretta*), green (*Chelonia mydas*), and Kemp's ridley (*Lepidochelys kempii*) sea turtles for proton NMR to identify potentially useful biomarkers based on differences between unaffected and cold stun syndrome affected turtles.

We sampled presumed healthy, free-ranging turtles (unaffected) in North Carolina, US between 2012 and 2014, concurrent with National Marine Fisheries Service research activities (Permit 16733-01). We acquired samples from eight unaffected loggerhead, two green, and four Kemp's ridley turtles (Table 1). We collected samples from cold-stunned turtles between 2012 and 2015 as part of the activities of the North Carolina Sea Turtle Stranding and Salvage Network (NCSU IACUC 15-001-O, NC WRC Permit 17ST42). We sampled eight loggerhead, 11 green, and six Kemp's ridley cold stun-affected turtles (Table 1). Individuals with eye pathology were excluded.

TABLE 1. Pooling details of extracted supernatants from individual sea turtle tear samples. Presumed healthy, free-ranging (unaffected) sea turtles were collected for sampling between 2012 and 2014 in North Carolina, USA, and sea turtles affected by cold stun syndrome were collected and sampled between 2012 and 2015, including loggerhead (*Caretta caretta*), green (*Chelonia mydas*), and Kemp's ridley (*Lepidochelys kempii*) species. Samples were pooled (unaffected $n=4$, cold stuns $n=7$) to provide greater volume for improved detection potential via proton nuclear magnetic resonance spectroscopy.

	Unaffected		Cold stun	
Species	No. pools	No. turtles per pooled sample	No. pools	No. turtles per pooled sample
Caretta caretta	2	4, 4	2	4[a], 4
Chelonia mydas	1	2	3	4, 4, 3
Lepidochelys kempii	1	4	2	3, 3

[a] Excluded from metabolite concentration analysis due to low sample signal-to-noise ratio making it unsuitable for analysis.

We collected tear samples using ophthalmic eye spears (76211, AMD Ritmed, Montréal, Québec, Canada) without local anesthesia. Highly viscous turtle tears thwarted attempts to collect tears directly using pipettes. Using a single spear per turtle, sample collectors chose an eye, placed the tip of the spear at the medial canthus, and pulled away slightly while rolling the spear to maximize sample volume. We froze the spear inside a predrilled well in a block of dry ice and stored in a cryovial at –80 C.

All samples, including five blank spears, were processed by cutting the sponge from the handle and roughly dividing it in half. We weighed each half, cut it into smaller strips, and placed them into a 1.7-mL microtube (Xtreme series microtubes, Phenix Research Products, Candler, North Carolina, USA). We added Drabkin's reagent (12:1 volume:weight; Ricca Chemical Company, Arlington, Texas, USA) and incubated the sample at room temperature (19–20 C) for 10 min (Niemuth and Stoskopf 2017). We added a 0.9–2.0-mm stainless steel bead blend (1:1 volume:volume; Next Advance, Inc., Troy, New York, USA), homogenized the samples (4 min, speed 10; Bullet Blender, Next Advance, Inc., Troy, New York, USA), and then centrifuged them at 14,000 × G for 20 min. We pipetted the supernatants into Eppendorf tubes and pooled them (Table 1) to provide greater volume for improved detection. We froze the supernatants at –80 C, lyophilized them at –85 C (Labconco, Kansas City, Missouri, USA), sealed the tubes (Parafilm, Beemis NA, Neenah, Wisconsin, USA), and stored them at –80 C.

We thawed each sealed, pooled sample at room temperature, rehydrated each with 300 µL 10% deuterium oxide (Sigma-Aldrich, St. Louis, Missouri, USA) containing 0.1 mM trimethylsilylpropanoic acid (TSP; Sigma-Aldrich), then vortexed, centrifuged at 3,000 × G for 3 min, and transferred the samples into 3-mm NMR tubes (Wilmad-LabGlass, Vineland, New Jersey, USA).

We recorded spectra on a Bruker Avance III 500 MHz spectrometer (Bruker Corporation, Billerica, Massachusetts, USA) equipped with a room temperature BBI S2 inverse detection probe with Z-gradient operating at 500.193 MHz frequency for ^{1}H nuclei. We used a zgpr water presaturation pulse sequence. Spectra were obtained with a 2.0447-s acquisition time, 8012.33 Hz sweep width, 16,384 complex points, and 256 transients.

We processed all spectral data using ACD Labs 12.0 1D NMR Processor (Advanced Chemistry Development, Toronto, Ontario, Canada). We Fourier transformed, automatically phased, and baseline corrected (adjusted where necessary) the spectra and then referenced to TSP at 0 parts per million (ppm) (Hz/MHz). For statistical analyses, we eliminated regions up and downstream of the data (–5 ppm to –0.06 ppm or –5 ppm to 0.04 ppm [to exclude TSP] and 8.55 ppm to 15 ppm) as well

as the water peak (4.59 ppm to 5.02 ppm). We divided spectra into 0.04-ppm bins using intelligent binning (50% width looseness).

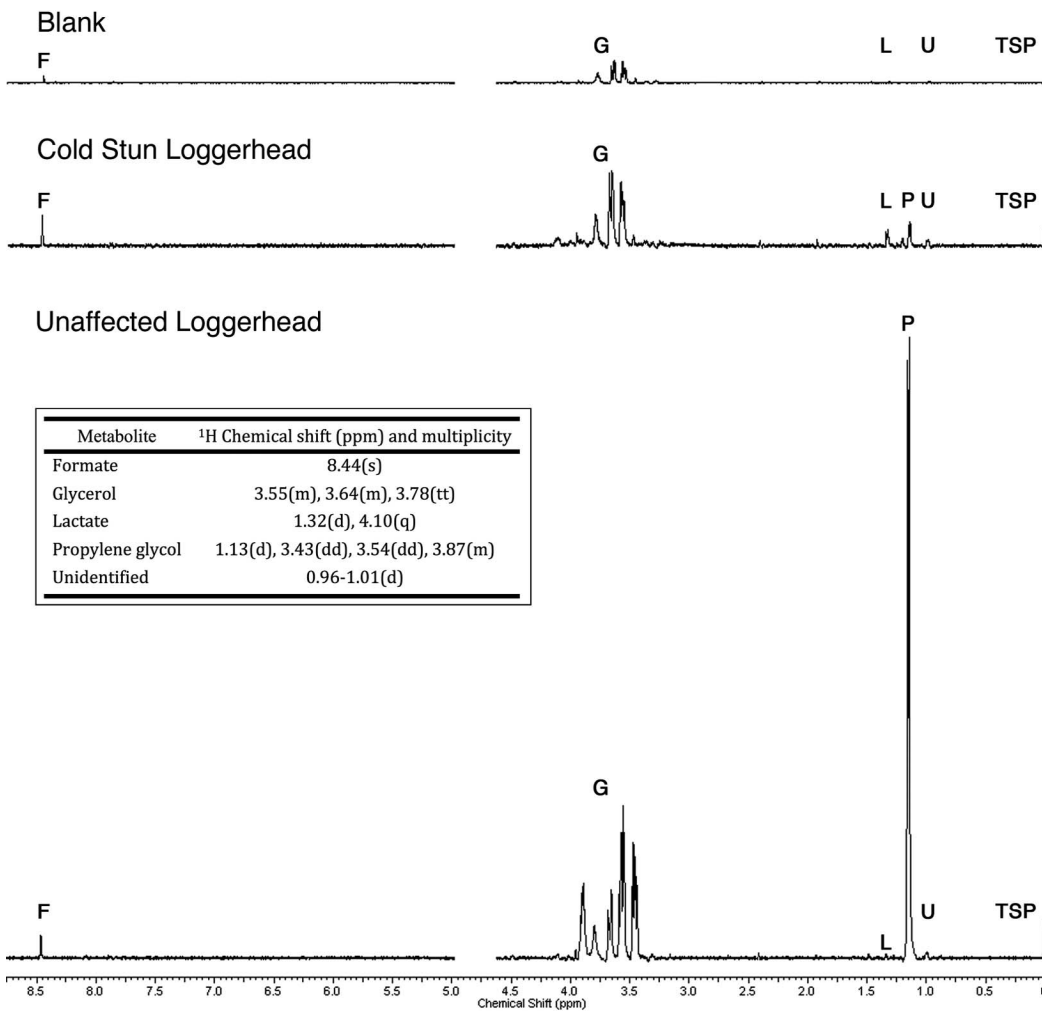
We used nontargeted statistical analysis to evaluate spectral integrals (excluding TSP) via principal components analysis (PCA; JMP Pro 13.0.0, SAS Institute, Inc., Cary, North Carolina, USA). We identified bins responsible for the separation between unaffected and cold-stunned turtles based on PCA results. We identified targeted metabolites and quantified them in turtle and blank samples using Chenomx NMR Suite 8.1 (Chenomx, Edmonton, Alberta, Canada). One metabolite was unidentified (0.96–1.01 ppm, doublet). We calculated the relative concentrations using integrals and the TSP concentration. We normalized all turtle metabolite concentrations to the number of individuals pooled per sample after subtracting the median concentration of target metabolites in the blanks (0 mM for negative values). We performed two-sided, exact, two-sample permutation tests (α=0.05, median difference) for each target metabolite using R version 3.3.3 (R Core Team 2017) with significant differences tested via one-sided, exact, two-sample permutation tests (α=0.025, median difference).

All samples, including blanks, yielded spectra (Fig. 1). As expected, the blank samples contained minor contaminants. The PCA biplots showed separation of unaffected and cold-stunned turtles and all but one of the blanks, which had a slightly lower glycerol concentration versus other blanks (Fig. 2). No separation by species was evident. Metabolites responsible for the separation of unaffected and cold-stunned turtles were propylene glycol, glycerol, lactate, formate, and an unidentified metabolite (0.96–1.01 ppm, doublet). One pooled cold-stunned loggerhead sample had a low signal-to-noise ratio and was excluded from further analysis. Boxplots of identified metabolite concentrations, and the relative concentration of the unidentified metabolite, suggested potential differences in lactate and formate concentration between groups (Fig. 3). Results of two-sided, exact, two-sample permutation tests supported a significant difference in formate (P=0.019) concentrations of unaffected and cold-stunned turtles, but no difference in concentrations of propylene glycol, glycerol, lactate, or the unidentified metabolite (P=0.276, 0.524, 0.148, and 0.914, respectively). There was significant (P=0.019, one-sided, exact, two-sample permutation test) evidence that concentration of formate was lower in unaffected turtles. Tear collection was well tolerated and samples were relatively easy to collect, but some variance in the volume collected was noted.

We acknowledge this study suffered from lack of power due to sample availability and volume. We anticipated variation in the volume of sample obtained but expected acceptable spectra based on experience with small samples. However, the results of pilot samples necessitated that we pool samples. We recommend collecting at least two spears per individual and ideally three to four. We anticipate that this would allow comparisons between species and would facilitate two-dimensional NMR experiments, which assist identification of unknown metabolites.

The tear sample processing method was similar to that for processing biofluid and tissue samples. Drabkin's reagent provides at least temporary metabolic quenching while being invisible to NMR (Niemuth and Stoskopf 2017). The method may be simplified by using a single, larger solvent volume (so as no longer to be limiting), thus eliminating the weighing step. Extraction of only polar metabolites is a drawback, but the cellulose ophthalmic spears complicate the selection of other extraction solvents.

The ophthalmic eye spears were not contaminant-free. Of the five blank spear samples analyzed, all but a single sample (with a lower glycerol concentration than the other blanks) was separated from the turtle samples on PCA. In this study, we subtracted the median blank metabolite concentrations from the turtle samples. In a human tear metabolome study, tears collected via Schirmer tear strips were similarly separated from analytic samples on PCA, but the authors elected to exclude metabolites from their pooled test samples if concentrations were less than five times the



Metabolite	^{1}H Chemical shift (ppm) and multiplicity
Formate	8.44(s)
Glycerol	3.55(m), 3.64(m), 3.78(tt)
Lactate	1.32(d), 4.10(q)
Propylene glycol	1.13(d), 3.43(dd), 3.54(dd), 3.87(m)
Unidentified	0.96-1.01(d)

FIGURE 1. Proton nuclear magnetic resonance spectral exemplars for plain ophthalmic spears (blank) and pooled loggerhead (*Caretta caretta*) tear samples from presumed healthy, free-ranging individuals (unaffected, *n*=4; collected in 2014) and individuals that stranded due to cold stun syndrome (*n*=4; collected between 2013 and 2015) in North Carolina, USA. The reference compound trimethylsilylpropanoic acid (TSP) and the biomarker metabolites formate (F), glycerol (G), lactate (L; doublet), propylene glycol (P; doublet), and an unidentified doublet (U) are labeled. Chemical shifts and multiplicities (s=singlet, d=doublet, q=quartet, dd=doublet of doublets, tt=triplet of triplets, m=multiplet) for each metabolite are included in the inset (Wishart et al. 2013). Propylene glycol was not found in the blank samples. The water region has been deleted.

blank strips (Chen et al. 2011). Additional research into the appropriate cutoff for contaminants in spears is necessary for this to be a future option. Though no cross-contamination of wells was observed during sampling, logistics required reuse of freezing wells, and microcontamination between samples within a collecting event could not be ruled out. Considering the composition of turtle tears, any cross-contamination would not have been detected within the resolution of the sensitivity of the NMR methods.

We identified five tear biomarkers (four identified and one unidentified metabolites) that differed between unaffected and cold-stunned turtles. Formate was the only metabolite with a statistically significant difference between unaffected and cold-stunned turtles.

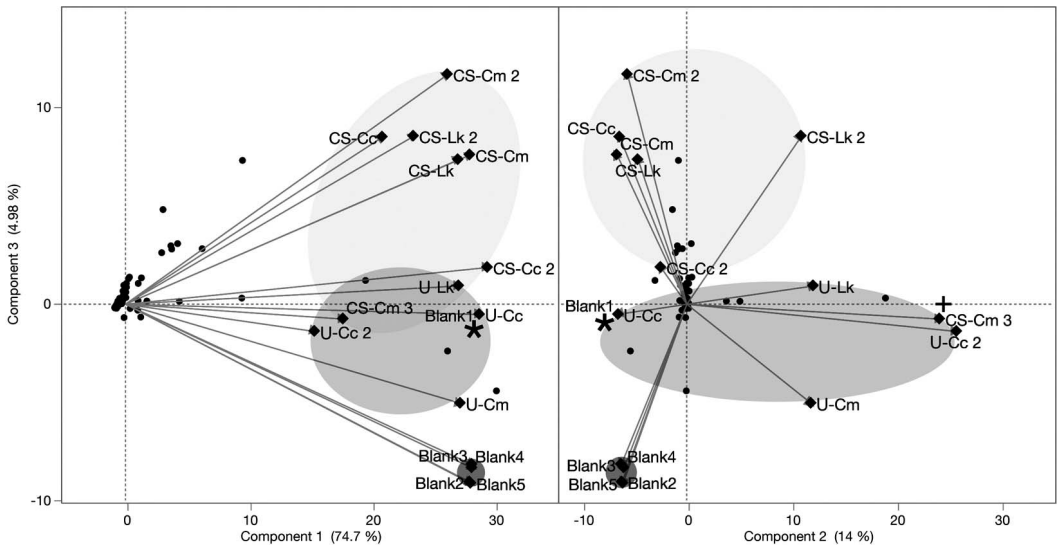


FIGURE 2. Principal components analysis biplots (percent variation for each component listed on the axes) for proton nuclear magnetic resonance spectra of plain ophthalmic spears (blanks) and tear samples from presumed healthy, free-ranging (unaffected) sea turtles (U-Cc=unaffected loggerhead [*Caretta caretta*], U-Cm=unaffected green [*Chelonia mydas*], U-Lk=unaffected Kemp's ridley [*Lepidochelys kempii*], collected between 2012 and 2014) and sea turtles affected by cold stun syndrome (CS-Cc=cold stun loggerhead, CS-Cm=cold stun green, CS-Lk=cold stun Kemp's ridley, collected between 2012 and 2015) in North Carolina, USA. In general, there was separation between the unaffected (light gray ellipses), cold stun (medium gray ellipses), and blank samples (dark gray ellipses), indicating that the spectra for the groups were different. One of the blanks (marked with an *) did not group with the others and was most likely a consequence of it having a slightly lower glycerol concentration than the other blanks.

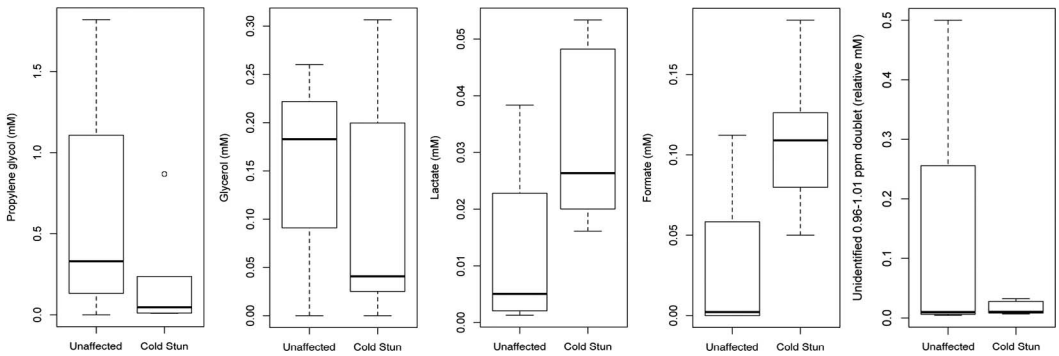


FIGURE 3. Boxplots of targeted metabolite concentrations from proton nuclear magnetic resonance spectra of tear samples of presumed healthy, free-ranging (unaffected) sea turtles (collected between 2012 and 2014) and those affected by cold stun syndrome (collected between 2012 and 2015) in North Carolina, USA, including loggerhead (*Caretta caretta*), green (*Chelonia mydas*), and Kemp's ridley (*Lepidochelys kempii*) sea turtles. The unidentified metabolite at 0.96–1.01 parts per million (doublet) is given as a relative concentration, as only the peak in the specified region was used. The median concentration of any metabolites present in the five blank samples was subtracted and all concentrations were normalized to the number of individuals that composed each pooled sample. Results of two-sided, exact, two-sample permutation tests supported a significant difference in formate (P=0.019) concentrations of unaffected and cold-stunned turtles with lower concentrations in unaffected turtles (P=0.019) but no difference in concentrations of propylene glycol, glycerol, lactate, or the unidentified metabolite (P=0.276, 0.524, 0.148, and 0.914, respectively). Variation in propylene glycol and unidentified metabolite concentrations appear lower in cold-stunned turtle samples due to most being near 0 mM.

Formate occurs in the human tear metabolome (Galbis-Estrada et al. 2014), is a carboxylic acid, a byproduct of acetate production, and is associated with metabolic acidosis (Wishart et al. 2013). Metabolic and respiratory acidoses are common disturbances in cold stun syndrome (Innis and Staggs 2017). Lactate is also associated with acidosis and tended to have higher blood concentrations in cold-stunned turtles. Propylene glycol has previously been identified in loggerhead whole blood (Bembenek Bailey et al. 2017) and green sea turtle plasma (Niemuth et al. 2018) and was not present in blank samples. Propylene glycol can be produced from glycerol (Saxena et al. 2010).

Depending on the research question, tears may be a useful sample. With an increased sample number, reliable classification of an unknown sample as unaffected or cold stunned is possible, which could be potentially useful in recently deceased individuals with an unknown cause of death. Tear samples could be useful for identification of other conditions if disease-specific biomarkers are identified, such as early identification of cold-stunned individuals likely to have sequelae during rehabilitation. This novel sample provides a minimally invasive option for specific questions about sea turtle metabolism.

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