Notes

Propylene Glycol in Free-Ranging Green Sea Turtle Plasma

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Abstract

Metabolomics is the study of metabolites, the small-molecular-weight end products of metabolism. Propylene glycol is a synthetic diol commonly used as antifreeze, as a humectant, and in the production of polyester compounds. In otherwise healthy animals, propylene glycol has generally been considered a contaminant, iatrogenic, or unexplained. We demonstrate the presence of propylene glycol in plasma of free-ranging apparently healthy green sea turtles *Chelonia mydas* and individuals impacted by cold stun syndrome, without iatrogenic administration or known sample processing contamination, using one- and two-dimensional proton nuclear magnetic resonance spectroscopy techniques. There was not a statistically significant difference in relative propylene glycol concentration between the two cohorts (two-sided random sampling two-sample permutation test, $P = 0.842$, $R = 1{,}000$). The presence of this metabolite raises important questions about sea turtle physiology and potential latent environmental contamination and serves as a starting point for future characterization of lipid metabolism and glycolysis in green sea turtles.

Keywords: antifreeze; cold adaptation; green sea turtles; metabolomics; nuclear magnetic resonance spectroscopy; propylene glycol

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Introduction

Metabolomics is the study of metabolites, the small-molecular-weight end products of metabolism. Propylene glycol (PG) is a synthetic diol commonly used as antifreeze, as a humectant, and in the production of polyester compounds (Agency for Toxic Substances and Disease Registry [ATSDR] 1997). As a metabolite, PG was

described over 60 y ago as a product of cellulose decomposition in bacteria (Enebo 1954) and aerobic dissimilation of L-rhamnose in yeasts (Suzuki and Onishi 1968). Contemporary studies of metabolism in humans most often consider PG presence as a sample contaminant (Wishart et al. 2008; Takeda et al. 2009; Psychogios et al. 2011; O'Sullivan et al. 2013; Stringer et al. 2015). Speculation about the source of this possible contamination includes sample collection-tube plastics or additives (Wishart et al. 2008; Stringer et al. 2015), drug vehicles (O'Sullivan et al. 2013), or products used by the subject, such as lotions, cosmetics, toothpaste (Psychogios et al. 2011), or cigarette smoking (Takeda et al. 2009). Additionally, in some veterinary studies, presence of PG could be iatrogenic because of administration as an antiketogenic treatment (Nielsen and Ingvartsen 2004). Most studies fail to determine a source of observed PG, but some report statistically significant differences in PG concentrations between study cohorts unrelated to any documented contamination. Examples include PG concentrations in saliva in males and females (Takeda et al. 2009) or in plasma from women who developed early-onset preeclampsia compared with those who did not (Bahado-Singh et al. 2012).

Our laboratory studies sea turtle health and adaptation using metabolomics techniques. Along the Atlantic Coast of the United States, hundreds to thousands of endangered and threatened (U.S. Endangered Species Act [1973, as amended]) green *Chelonia mydas* (U.S. Fish and Wildlife Service 1978), loggerhead *Caretta caretta* (U.S. Fish and Wildlife Service 1978), and Kemp's ridley *Lepidochelys kempii* (U.S. Fish and Wildlife Service 1970) sea turtles are reported to the Sea Turtle Stranding and Salvage Network each year (Southeast Fisheries Science Center 2018). Rescued and rehabilitated individuals may spend months under human care, but our knowledge of sea turtle physiology remains largely incomplete. Nuclear magnetic resonance (NMR) spectroscopy can be used to produce untargeted profiles of a turtle's metabolic state, which can identify metabolites and pathways overlooked by more traditional methods or targeted studies.

Our study prepared and analyzed plasma samples from free-ranging, presumed healthy, green sea turtles and those suffering from cold stun syndrome. Cold stun is a condition that occurs along the Atlantic Coast when water temperatures acutely and persistently drop below approximately 12°C and frequently results in stranding events (Burke and Standora 1991; Morreale et al. 1992; Bentivegna et al. 2002; Still et al. 2005; Roberts et al. 2014; Innis and Staggs 2017). Using proton NMR-based metabolomics, we demonstrated and confirmed the presence of PG in the plasma of both cohorts of green sea turtle, without iatrogenic administration or known sample processing contamination. The presence of this metabolite raises important questions about sea turtle physiology, including possible cold adaptation and research avenues for prevention or treatment of clinical sequelae to cold stun syndrome (e.g., bone, joint, and skin lesions), and potential latent environmental contamination.

Methods

Sample collection

We collected plasma samples (separated from heparinized whole blood) from $n = 10$ apparently healthy free-ranging green sea turtles (October 2012; National Marine Fisheries Service Permit No. 16733-01) and $n = 11$ green sea turtles affected by cold stun syndrome (December–January 2012–2015; North Carolina State University Institutional Animal Care and Use Committee 15-001-O and North Carolina Wildlife Resources Commission Permit No. 17ST42) from North Carolina. We sampled all cold stun affected turtles immediately upon reaching the staging area; they had body temperatures less than 15°C. Transport time resulted in some variation in sample timing, but sampling was always on the day of rescue and before any treatment, including rewarming. We froze all samples directly on dry ice and stored them in cryovials at −80°C until analysis. All cold stun affected turtles, except one, survived rehabilitation to release. The nonsurviving turtle died approximately 5 mo post-stranding and had no significant findings on necropsy.

Sample preparation

We soaked Amicon Ultra 10K 0.5-mL centrifugal filters (Millipore, Carrigtwohill, County Cork, Ireland) in ultrapure water overnight (approximately 17 h) before use. We then washed the filters four times with 500 μL of ultrapure water at 14,000 × g for 20 min. We thawed plasma samples at room temperature (21°C) before use. We mixed a 40-μL aliquot of each sample with 400 μL of a dilute aqueous solution of sodium bicarbonate, potassium ferricyanide, and potassium cyanide (Drabkin's reagent, Ricca Chemical Co., Arlington, TX) and incubated it at room temperature for 10 min (Niemuth and Stoskopf 2017). We selected this solvent to maintain consistent technique with planned future studies utilizing whole blood where this solvent is a necessity. We then filtered samples at 14,000 × g for 20 min. We froze the filtered samples at −80°C. We also filtered a sample containing only the dilute aqueous potassium cyanide solution in the same manner as the plasma samples and froze it. All filtration steps were necessary to ensure proper function of the microcoil and are standard for samples evaluated with this spectrometer. We lyophilized all samples (−57 to −56°C, 0.010 to 0.021 mBar) (FreeZone 2.5, Labconco, Kansas City, MO) until dry (approximately 6 h), sealed them with laboratory wax film (Parafilm, Beemis NA, Neenah, WI), and returned them to −80°C until final sample preparation.

NMR data collection

We thawed the sealed samples at room temperature and rehydrated them with 70 μL of D_2O with 20 mM phosphate buffer, 0.1 mM of the reference standard trimethylsilyl propanoic acid, and 1 mM formate as a secondary reference standard (Kriat et al. 1992). We vortexed and filtered all samples (Fisherbrand SureOne

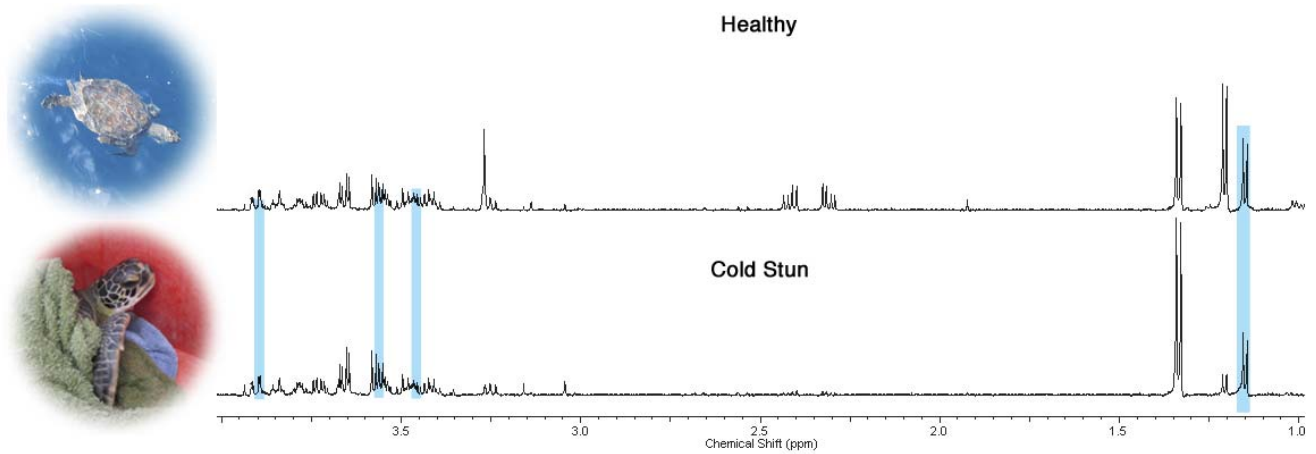

Figure 1. Section of proton nuclear magnetic resonance (NMR) spectra from pooled samples of apparently healthy and cold stun affected green sea turtles *Chelonia mydas* from North Carolina (collected from 2012-2015) highlighting the presence of propylene glycol (PG; blue bars). Concentrations of metabolites in NMR experiments are calculated using integrals of the spectral curve, but can be approximated by examining peak heights when the peaks are relatively narrow. The concentration of PG is generally similar in magnitude to the other metabolite peaks present and is neither at trace nor overwhelming concentrations.

10 μL, extended, filter, low-retention, universal-fit pipet tips, Thermo Fisher Scientific, Waltham, MA) via centrifugation (3,000 × g; 2 min; room temperature) into vials and capped them. We removed a 10-μL aliquot from each sample and combined them to form pooled samples for the apparently healthy and cold stun cohorts, as well as a combination sample for quality assurance. We also analyzed blank samples of only the D_2O solution, which had undergone the final filtration step, before and after the apparently healthy and cold stun sample groups. We stored all samples at −20°C until NMR analysis.

We performed NMR analysis with a Varian Inova 600-MHz multinuclear INOVA NMR spectrometer (Varian Medical Systems, Palo Alto, CA) equipped with a Protasis microcoil NMR probe (Protasis Corp.n, Marlboro, MA) to obtain one-dimensional ^{1}H-NMR spectra at 25.0°C with a 1.1384-s acquisition time. The sweep width of 7,193.60 Hz acquired 8,189 complex points and 4,096 transients. We performed the two-dimensional experiment, (^{1}H-^{1}H) total correlation spectroscopy with (0.1500 s, 0.0356 s) acquisition times; the sweep widths (7188.51 Hz, 7188.51 Hz) acquired (1079, 256) complex points and 256 transients.

Data analysis

We processed all spectral data with ACD labs 12.0 one-dimensional NMR processor (Advanced Chemistry Development, Toronto, Ontario, Canada). We zero-filled spectra to 16,000 points and Fourier transformed them. Spectral phasing and baselines were corrected automatically, and we adjusted them manually when automatic correction was insufficient. We referenced all spectra to the trimethylsilyl propanoic acid peak at 0 parts per million (ppm). We performed peak identification with Chenomx NMR Suite 8.1 (Chenomx, Edmonton, Alberta, Canada) and the Human Metabolome Database (Wishart et al. 2013).

We performed intelligent binning to divide the spectra for integration. We used a bin width of 0.04 ppm and 50% width looseness to prevent division of peaks across multiple bins. We chose the bin from 1.12 to 1.17 ppm for targeted statistical comparisons of PG concentrations, as the selected peak had minimal overlap with other metabolites. We statistically compared relative PG concentrations with a two-sided random-sampling two-sample permutation test ($R = 1000$ permutations) via R version 3.3.2 (R Core Team 2017) using integrals from the 1.12 to 1.17 ppm PG peak.

Propylene glycol metabolite identification confirmation experiment

We used PG (Phoenix Pharmaceutical, Inc., St. Joseph, MO) to confirm identification of PG as a metabolite. We added a 50-μL aliquot of 100% PG to 950 μL of D_2O; we then added 10 μL of this dilution to 895 μL of D_2O (final concentration of approximately 1%). We analyzed an unfiltered, solvent-free (i.e., not treated with Drabkin's reagent), lyophilized, and rehydrated green sea turtle plasma sample by NMR as described previously, with the exception of running it in a 3-mm NMR tube. We added a 5-μL aliquot of the PG dilution and reanalyzed the sample.

Results

Our analysis showed detectable concentrations of PG in both apparently healthy and cold stun affected green sea turtle plasma (Figure 1). We detected no PG in blank samples of the D_2O solution or the Drabkin's reagent. Trimethylsilyl propanoic acid peaks were consistent and narrow across spectra, and we used them as the reference standard. There was not a statistically signifi-

Table 1. Relative propylene glycol (PG) concentrations (mM) measured by proton nuclear magnetic resonance spectroscopy in apparently healthy and cold stun affected green sea turtle *Chelonia mydas* plasma samples from North Carolina (collected from 2012-2015). The bin from 1.12 to 1.17 parts per million (ppm) was chosen for targeted statistical comparisons of PG concentrations, as the selected peak had minimal overlap with other metabolites. No statistically significant difference between the two cohorts was found (two-sided random sampling two-sample permutation test, $P = 0.842$, $R = 1000$).

Relative PG concentration (mM)	
Apparently healthy	**Cold stunned**
0.18	0.57
0.43	0.83
0.16	0.27
0.13	0.13
0.28	0.13
0.58	0.06
0.23	0.28
0.46	0.67
0.13	0.13
0.59	0.43
	0.47

cant difference in relative PG concentration (Table 1) between the two cohorts (two-sided random sampling two-sample permutation test, $P = 0.842$, $R = 1000$). Propylene glycol was also present in the unfiltered, solvent-free plasma sample (Figure 2). We confirmed identification of the metabolite as PG by addition of commercial PG (Figure 2) and by two-dimensional proton NMR spectroscopy via a total correlation spectroscopy experiment (Figure 3). In addition to PG, we detected a variety of organic acids/osmolytes, amino acids, and ketone bodies, as well as energy compounds and metabolites related to fatty acid metabolism, including glycerol. Raw spectral data are available as supplemental material (Data S1, *Supplemental Material*).

Discussion

We initially hypothesized that the presence of PG was due to contamination during sample collection or processing, and we performed a thorough investigation to identify the contamination. We collected all samples before administration of any treatment or pharmaceutical, ruling out contamination via drug vehicle. We examined variations in sample processing in the field. To minimize the freezing time, we placed our samples in drilled-out wells in a block of dry ice with the sample in direct contact with the ice (Nagase and Niwa 1964; Krause and Grove 1967). Similar PG concentrations were present in test samples frozen sealed inside of a cryovial, suggesting that the dry ice procedure was not introducing PG. We ruled out the use of heparin-prepared syringes for blood collection by observations of similar PG concentrations in another study looking at sea turtle organ tissues not treated with heparin (Bembenek-Bailey, Environmental Medicine Consortium, unpublished data). We carefully examined our use of a lyophilizer. Other studies using the same equipment did not have detectable PG concentrations.

As part of quality-assurance measures, we analyzed blank samples (processed in the same manner as the plasma samples) of the rehydration D_2O solution via NMR before and after each sample cohort. These blank samples did not contain PG, eliminating the NMR spectrometer's microcoil as a source of PG. Additionally, we filtered, lyophilized, and analyzed a sample of the Drabkin's reagent used in this study via the same processing methods as the plasma samples, and it was also free of PG. The unfiltered, untreated plasma sample used for the spike experiment did contain PG, demonstrating that its

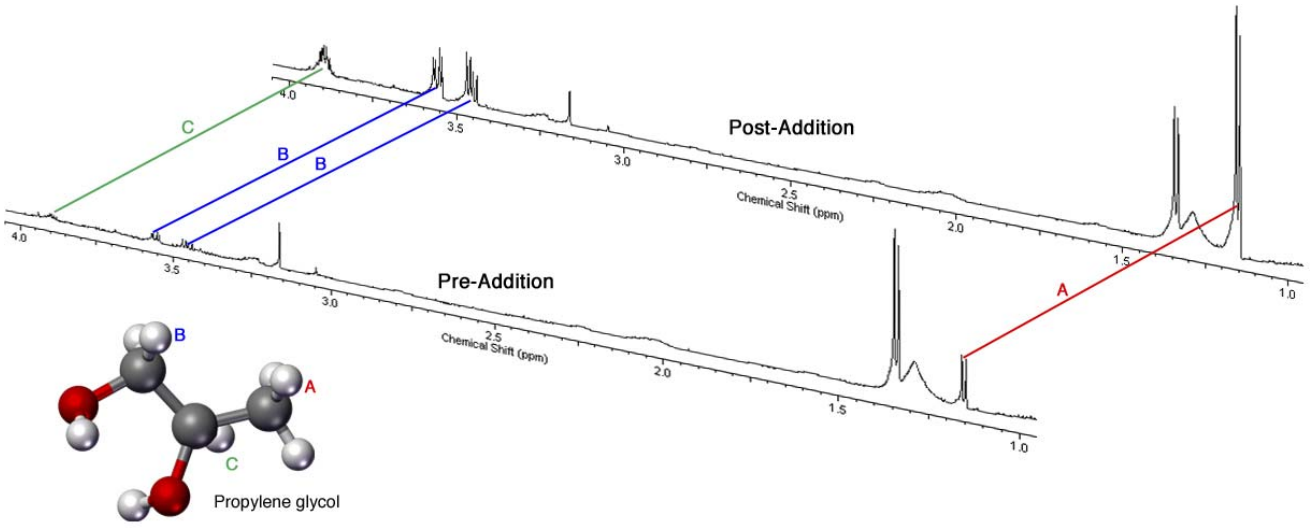

Figure 2. Propylene glycol (PG) metabolite identification confirmation experiment. We added an aliquot of commercially available PG to an unfiltered, solvent-free (i.e., not treated with Drabkin's reagent) green sea turtle *Chelonia mydas* plasma sample from North Carolina (collected in 2012). The resultant spectra demonstrated an increase in concentration at the peaks suspected to be PG, thus confirming its identification.

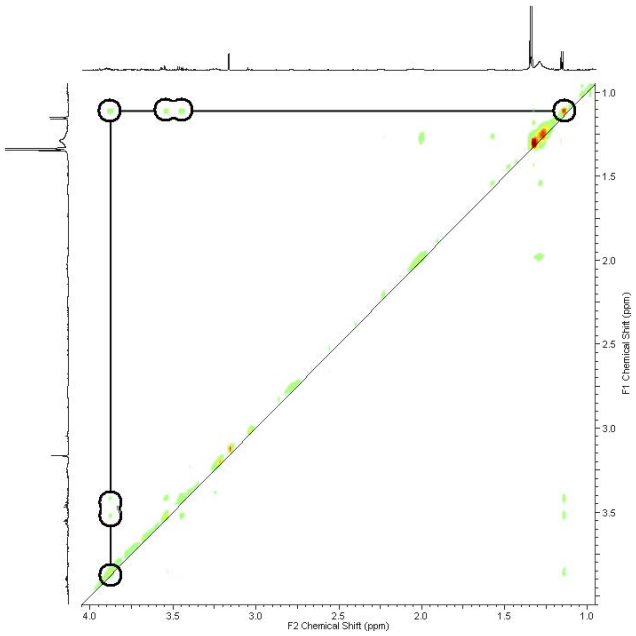

Figure 3. Two-dimensional proton nuclear magnetic resonance spectrum (total correlation spectroscopy) confirming the identification of propylene glycol (PG) in green sea turtle *Chelonia mydas* plasma from North Carolina (collected in 2012). The purpose of total correlation spectroscopy is to identify distant protons that are coupled (i.e., belonging to the same metabolite). The circled resonances are coupled and the chemical shifts match those of PG reference spectra.

presence was not due to sample interaction with Drabkin's reagent or from the microcoil. These spectra rule out contamination from the reagent, tube and filter system, lyophilizer, and microcoil. We used the same stock of syringes, tubes, vials, etc. for all of our laboratory's research activities, and PG is not present in our studies involving other (i.e., non-sea turtle) species (M.K.S., unpublished data). Last, a study of loggerhead sea turtle hatchlings performed by our group using the same processing methods and spectrometer also confirmed the presence of PG (Bembenek Bailey et al. 2017), whereas research in other species using the same processing methods and spectrometer has not (M.K.S., unpublished data).

The presence of PG in studies of metabolism is not uncommon, though apparently infrequently investigated. The supposition that PG contamination in samples from human research subjects may be due to pharmaceuticals, hygiene products, or other goods is not unreasonable (Wishart et al. 2008; Takeda et al. 2009; Psychogios et al. 2011; O'Sullivan et al. 2013; Stringer et al. 2015). However, if the PG in our study is due to contamination, the most likely source would seem to be environmental exposure. The relative concentrations of PG observed in this study are similar in magnitude to the other metabolites present (Figure 1); PG is neither present at trace nor overwhelming concentrations. The green sea turtles in this study were free ranging in North Carolina and were either netted as part of a larger ongoing study or recovered from beaches as stranded animals during cold stun events. In a metabolomics study in Hawaiian green sea turtles, which used different processing methods (Schock et al. 2013), PG appears to be present in the spectral images shown, but is not identified or mentioned in the manuscript. This suggests that either PG presence due to contamination is consistent across geographically distant regions or that it is an endogenous metabolite and not a contaminant.

As previously noted, PG production by various bacteria and yeast species is reported (Enebo 1954; Suzuki and Onishi 1968). In animals, oxidation of PG can provide energy through alterations of gluconeogenesis, the tricarboxylic acid cycle, or glycogenesis and can protect against ketosis (Shull and Miller 1960; Ruddick 1972; Morshed et al. 1988; Nielsen and Ingvartsen 2004). Propylene glycol can be biogenically produced from glycerol (Saxena et al. 2010), which we also identified as a metabolite in our samples. It is possible that sea turtles can reduce one of the alcohol functional groups in glycerol to form PG. Several studies have also pointed to glycerol as a cryoprotectant and important metabolite for cold adaptation (Storey and Storey 1983; Ahlquist et al. 1984; Kukal et al. 1988).

It is unknown if most sea turtle species have adaptations for cold, but observations of apparently torpid sea turtles have been reported (Felger et al. 1976; Carr et al. 1980; Ogren and McVea 1982). On the basis of what is known about PG, we hypothesize that higher concentrations of PG may be cold protective, but our available data trend toward lower PG concentrations in cold stun affected turtles. This raises the question of whether cold stun affected turtles that strand are potentially physiologically disadvantaged and either cannot produce enough PG or utilize PG at a higher rate, depleting their stores, compared with individuals that do not strand. Future research could compare PG concentrations in stranded individuals with those that do not strand during cold stun events or track PG concentrations of stranded individuals in rehabilitation to determine if there is correlation to commonly seen clinical sequelae (e.g., bone, joint, and skin lesions).

In conclusion, the discovery of PG, while ruling out reasonable sources of sampling and laboratory contamination, suggests either a previously unreported vertebrate biochemical pathway or a latent, widespread environmental contamination. If PG is shown to be a component of sea turtle metabolism, there are potential implications for our understanding of sea turtle cold adaptation. These findings serve as a starting point for future characterization of lipid metabolism and glycolysis to understand the source, regulation, and metabolic impact of PG in green sea turtles.

Supplemental Material

Please note: The Journal of Fish and Wildlife Management is not responsible for the content or functionality of any supplemental material. Queries should be directed to the corresponding author for the article.

Data S1. Raw spectral data from apparently healthy and cold stun affected green sea turtle *Chelonia mydas* plasma from North Carolina (collected from 2012-2015).

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