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The blood biochemistry of overwintering diamondback terrapins (*Malaclemys terrapin*)

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ABSTRACT

Estuarine ectothermic vertebrates, such as diamondback terrapins (*Malaclemys terrapin*, Schwartz 1955), inhabit a dynamic environment, and many aspects of their biology reflect their ability to withstand and respond to these environmental challenges. The physiological adjustments necessary to maintain water and salt balance and the metabolic adjustments that accompany seasonal changes in activity and behavior have not been well-characterized for overwintering terrapins under field conditions. To investigate terrapin osmotic and metabolic physiology during winter when activity levels are depressed, we obtained repeat blood samples from 10 radio-tagged female terrapins maintained in a semi-natural, open-air salt marsh enclosure in southeastern North Carolina, USA. From November 2011 to April 2012, we measured monthly plasma osmolality, plasma concentrations of inorganic osmolytes (Na^+, K^+, Cl^-), and protein catabolic indices (urea and uric acid), as well as monthly plasma concentrations of total Ca^{2+}, lactate, and glucose as metabolic indices. We used linear mixed models to determine the best predictors of blood chemistry, where time (i.e., day) and environmental variables were fixed factors and individual terrapins were random factors. Day was a poor predictor of blood chemistry concentrations, indicating that the progression of winter did not elicit corresponding changes in biochemical indices as documented in other semi-aquatic turtles exposed to more severe winter and/or laboratory conditions. Carapace temperature was the most common predictor of blood chemistry concentrations in all models, underscoring its relative influence on physiology. In contrast to previous laboratory-based studies on the overwinter physiology of terrapins, our study demonstrates that terrapins in their natural environment are able to maintain biochemical homeostasis throughout winter. The use of evasive behavioral strategies may be an important factor for terrapins to reduce the passive exchange of water and salts with the estuarine environment.

1. Introduction

Seasonal changes in environmental conditions such as temperature, photoperiod, and food and water availability can have profound effects on the physiology of ectotherms. Temperature, in particular, has direct effects on biochemical reaction rates, physiological processes, and whole animal metabolism (Dubois et al., 2009; Hochachka and Somero, 2002). For aquatic ectothermic vertebrates, water and salt concentrations can fluctuate as environmental conditions change, posing osmoregulatory challenges that require morphological, behavioral, and physiological adjustments to minimize dehydration due to excess water loss and salt accumulation (Dunson and Mazzotti, 1989; Dunson and Travis, 1994). Osmoregulation is particularly difficult for estuarine and marine ectotherms that experience periods of curtailed physiologic rates associated with seasonal cold exposure (Gilles-Baillien, 1973). For euryhaline fish, ionoregulatory processes, regulated by the gill membrane enzyme Na^+/K^+ ATPase, can be affected by cold winter temperatures, resulting in functional, ultrastructural and/or morphological changes in Na^+/K^+ ATPase to maintain osmotic balance (Anderson, 2013). Alternatively, some marine turtles undergo a behavioral shift during the cold winter months, in which they make periodic prolonged, aerobic dives to rest on the seafloor (Hochscheid et al., 2005). This intermittent dormancy reduces energetic costs while still permitting physiological functions associated with foraging (Hochscheid et al., 2007), movement, and osmoregulatory processes (i.e., lachrymal salt gland secretion).

Few estuarine species, and even fewer estuarine reptiles, are obligate to this dynamic estuarine environment (Hart and Lee, 2006). One of the true obligate estuarine reptiles, the diamondback terrapin (*Malaclemys terrapin*) is one such estuarine turtle species endemic to tidally-influenced temperate zone habitats, where salinities range from 11 to full strength seawater (≥34, Dunson, 1970). In this desiccating environment, terrapins use behavioral and physiological adjustments in order to prevent water loss and combat excessive influx of

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salts. Both compensatory and evasive strategies are employed to maintain plasma osmotic pressure 1/3 to 1/2 that of environmental water (Gilles-Baillien, 1970; Kirschner, 1970). Evasive strategies that reduce evaporative water loss and salt and water exchange with environment include impermeable integument, hypophagy, winter mud burial, aestivation, basking, and water retention via urine reduction (Bentley et al., 1967; Brennessel, 2006; Gilles-Baillien, 1973), which all ultimately reduce metabolic costs associated with maintaining osmotic balance with the environment (Bentley et al., 1967). Compensatory strategies that involve active (i.e., energy-requiring) uptake or extrusion of water or salts include the active exchange of organic osmolytes across cell membranes, the secretion of salts from body to environment via lachrymal salt gland in order to adjust osmotic pressure (Cowan, 1981; Dunson, 1970), and the detection, active uptake, and extracellular storage of fresh rain water when readily available (Bels et al., 1995; Davenport and Macedo, 1990; Robinson and Dunson, 1976).

In addition to osmoregulatory adjustments, terrapins may also experience seasonal shifts in metabolic capacity. Overwintering aquatic emydid turtles exhibit various physiological adaptations that allow them to survive the sometimes harsh conditions of winter. The effects of low ambient temperatures, hypoxia, and downregulation of molecular and cellular mechanisms result in a drastic depression of the metabolic rates of overwintering turtles (Jackson, 2000; Southwood Williard and Harden, 2011). Low metabolic rates may be supported by aerobic metabolic pathways for those turtles capable of gas exchange via extrapulmonary respiration in open, flowing water such as: *Pseudemys rubriventris*, *Apalone spinfera*, and *Graptemys geographica* (Crocker et al., 2000; Dunson, 1960; Graham and Guimond, 1995; Jackson, 1979; King and Heatwole, 1999; Ultsch, 1989).

In contrast, other freshwater species such as painted turtles (*Chrysemys picta*) and snapping turtles (*Chelydra serpentina*) often overwinter in anoxic environments (e.g., iced-over water bodies, mud) and thus rely heavily on anaerobic metabolism to meet their low metabolic needs (Jackson, 2000; Ultsch, 1989). This overwintering strategy results in the gradual accumulation of plasma lactate (Reese et al., 2001, 2004) that can exceed 200 mmol/L under stable anoxic conditions (Ultsch and Jackson, 1982; Ultsch et al., 1999). The degree to which turtles accumulate lactate and the means by which they buffer lactate play a major role in determining survival during anoxic submergence (Ultsch and Jackson, 1995). Previous studies have demonstrated that an increased reliance on anaerobic metabolic pathways during winter dormancy is reflected by elevated blood lactate concentrations and subsequent changes in blood Ca^{2+} and Mg^{2+} indicative of lactate buffering with their shell in order to avoid metabolic acidosis (Jackson, 2000, 2002; Jackson and Heisler, 1982; Jackson et al., 1996; Reese et al., 2004).

Terrapins experience shifts in environmental temperatures from summer to winter and correspondingly exhibit dramatic changes in their activity levels in winter, at which point they burrow in the interior subtidal mud and can remain inactive for extended time periods (Butler, 2002; Coker, 1906; Haramis et al., 2011; Harden and Williard, 2012; Southwood Williard and Harden, 2011; Yearicks et al., 1981). However, the physiological adjustments underlying the observed behaviors have not been thoroughly investigated. The overwintering strategy of terrapins is interesting from an osmoregulatory and metabolic perspective, given the estuarine habitat in which they live and the potentially high energetic costs of maintaining osmotic and ionic balance. Insight into seasonal adjustments in osmoregulation and metabolism in reptiles may be gained by blood biochemical variables (Costa and Sinervo, 2004) such as ion concentrations, and metabolic products (Dessauer, 1970; Jackson, 2000; Shoemaker and Nagy, 1977; Somero and Hochachka, 1971; Tracy et al., 2006).

We investigated changes in osmotic and ionic status of terrapins throughout the winter by measuring plasma osmolality, inorganic osmolytes (Na^+, K^+, Cl^-), the organic osmolyte urea, and uric acid. We also explored the metabolic status of terrapins by measuring plasma Ca^{2+}, lactate, and glucose levels. Although we did not measure bound forms of the lactate buffer, calcium (i.e., CaLactate+), we did measure free Ca^{2+} and free lactate, which are indicative of the lactate buffering mechanism described for aquatic turtles (Jackson, 2000, 2002; Jackson and Heisler, 1982; Jackson et al., 1996; Reese et al., 2004). These data were interpreted in light of terrapin habitat use and environmental data. We then predicted that throughout winter months, blood chemistry would 1) reflect physiological adjustments to minimize water loss and salt gain (e.g., increase in osmolality, increase in urea), 2) reflect increased reliance on anaerobic metabolism throughout winter burial (i.e., increase in lactate and potential buffer Ca^{2+}), and 3) vary predictably with alterations in environmental factors that affect water availability and rate processes.

2. Materials and methods

2.1. Study site and field methods

To test these predictions regarding osmotic and metabolic physiology, we maintained terrapins in an open-air enclosure on the landward side of Masonboro Island National Estuarine Research Reserve (NERR) in Byron's Creek, North Carolina, USA (34° 08′ 08″ N, 77° 50′ 57″ W, Fig. 1) that encompassed typical terrapin habitat and allowed terrapins to experience natural environmental shifts (see Harden et al., 2014 for details on materials, dimensions, and construction of enclosure).

Environmental data were obtained from a National Oceanic and Atmospheric Administration, Office of Ocean and Coastal Resource Management, National Estuarine Research Reserve System-wide Monitoring Program station located 2 km from our Byron's Creek terrapin enclosure. Salinity and tidal creek temperature data from this monitoring station were collected at 30 minute intervals by a YSI 6600EDS data sonde (YSI Inc., Yellow Springs, OH, USA) and total rainfall (mm) was also recorded at 30 minute intervals collected by a tipping bucket rain gauge (Campbell Scientific, Inc., Logan, UT, USA, Model# TE525, rainfall per tip: 0.01 in.) mounted on the monitoring station. We also measured shallow mud temperature (2 cm) salinity with HOBO data loggers (HOBO® UTBI-001 and U24-002, respectively Onset Computer Corporation, Bourne, MA) located within the terrapin enclosure, but due to equipment malfunction, salinity measurements were not recorded consistently throughout the duration of the study thus the NOAA monitoring station data were used for analyses. We were confident with this substitution in data because long-term salinity measurements from NOAA monitoring station and from the enclosure conductivity logger were significantly correlated ($r = 0.527$, $p < 0.001$) and these tidal creeks are well-mixed estuarine systems. Mud salinity was also measured by centrifuging core samples taken from the enclosure periodically throughout the winter and using a refractometer on the suspended liquid.

In nearby tidal creeks and coves, we used large >100 m gillnets to collect 10 female terrapins (300–700 g, Table 1), which were relocated to the enclosure in Byron's Creek. Terrapin collection sites were within 5 km of the enclosure site. Terrapins were sexed, aged, measured, and given a unique 3-letter code (e.g., APV) notched into the marginal scutes following processing protocols outlined by Dorcas et al. (2007). Temperature data loggers (iButtons, programmed to record carapace temperature, T_c, every 30 min) and radio transmitters (frequencies: 150.162–150.838 MHz) were attached to the anterior carapace using quick-setting marine grade epoxy putty (see Harden et al., 2014 for more details). This datalogger measured the temperature of the carapace surface, thus reflected the immediate environment of the turtle. Furthermore, previous studies have found carapace temperatures T_c to be strong indicators of body temperature T_b in small to medium-sized turtles (*C. picta*: Grayson and Dorcas, 2004; *Cuora flavomarginata*: Chen and Lue, 2008; *Platysternon megacephalum*: Shen et al., 2013), such as the terrapins used in this study.

Terrapins were released into the enclosure on 22 September 2011, and were allowed a period of 45 days to acclimate behaviorally and

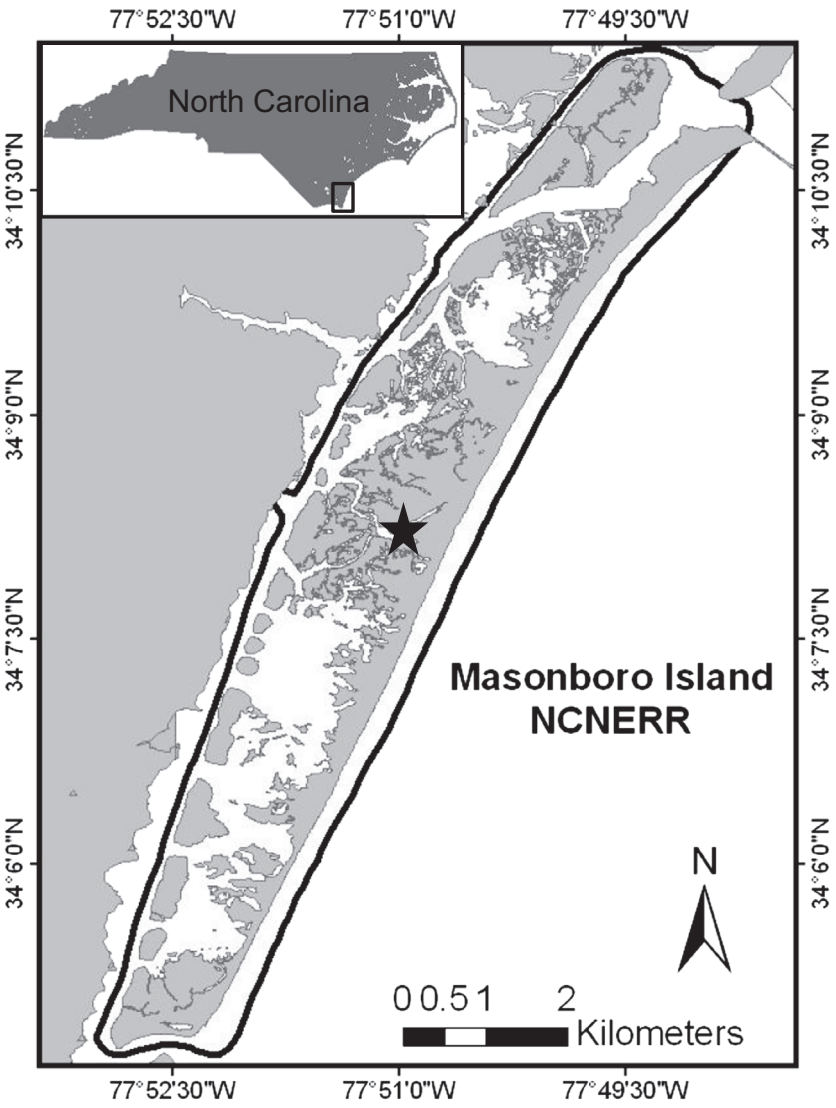

Fig. 1. Map of Byron's Creek on the landward side of Masonboro Island North Carolina Estuarine Research Reserve (NCNERR) in southeastern North Carolina. The reserve boundary is outlined with a thick, black line, and the starred area within the reserve marks the location of the enclosure.

physiologically to their new environment, before any measurements were taken. Terrapins within the enclosure were located monthly (approx. every 4 weeks) from 6 November 2011 to 5 April 2012. Immediately after locating each individual, 2 mL blood samples were obtained from the cervical sinus of each terrapin using a 25G 5/8 in. needle and 3-cm^3 syringe. Terrapins were weighed using a 1000-g spring balance (d = 10 g, Medio-Line 41000 Spring Scale, Pesola®, Baar, Switzerland) immediately after blood collection to document any changes in mass. Terrapins were released from the enclosure in late April and early May 2012 and returned to their original capture locations.

2.2. Blood chemistry analysis

Whole blood samples were immediately placed in heparinized tubes on ice, and small sub-samples were used for determination of blood concentrations of Na^+, Cl^-, K^+, and glucose with an i-STAT point-of-care (POC) handheld blood analyzer (Abaxis Veterinary Diagnostics, Union City, CA). The i-STAT blood analyzers have been used in previous studies examining the blood biochemistry and health status of various organisms such as sharks, sea turtles, and teleosts (Gallagher et al., 2010; Wolfe et al., 2008; Harrenstien et al., 2005, respectively) and have demonstrated to be an affordable and convenient method of measuring blood variables in the field.

The remaining blood was transferred immediately to a 2 mL tube and centrifuged using a portable microcentrifuge (Zipspin, LW Scientific, Lawrenceville, GA) to separate plasma from blood cells and be transported back to the laboratory at the University of North Carolina Wilmington (UNCW Lab), where samples were stored in a −80 °C freezer. Blood osmolality (mOsm) was determined using a vapor pressure osmometer (Vapro model 5600, Wescor Inc., Logan, UT). More specifically, we first calibrated the vapor pressure osmometer using 10 μL samples of 290 and 1000 mOsm standards, which were loaded into the machine to vaporize for 75 s. We then ran our 10 μL plasma samples in duplicate following the same vaporizing procedure. Concentrations of lactate, Ca^{2+}, blood urea nitrogen (urea), and uric acid were determined using commercially available reagent kits (Pointe Scientific Inc., Canton, MI, USA; lactate cat # L7596-50; Ca^{2+} cat # C7503-480; urea cat #B7550-150; uric acid cat # U7581-120) and standard spectrophotometric techniques (Lambda 25 UV/Vis, PerkinElmer, Waltham, MA) in the UNCW Lab. We used lactate standards of 2.5, 10.0, and 15.0 mmol/L, Ca^{2+} standards of 2.5, 5.0, and 10.0 mg/dL, urea standards of 20.0 mg/dL, and uric acid standards of 1.0, 2.0, and 5.0 mg/dL to generate regression equations to describe the relationship between absorbance and blood variable concentration. Based on previous studies

Table 1
Grand mean carapace temperatures (T_c) and concentrations (mmol/L; uric acid in μmol/L) of blood variables of 10 female terrapins from November 2011 to April 2012 (dark gray rows). T_c mean was calculated by averaging weighted mean T_c across all individuals for each month (T_c min and T_c max were calculated similarly, using weighted minimum or maximum T_c). Light gray columns include blood variables measured in free-ranging female terrapins in winter (January to March) 2010 from a study in southeastern North Carolina (Harden and Williard, unpublished results). White columns include blood variables measured in controlled laboratory experiments investigating "hibernation and osmoregulation in terrapins" (Gilles-Baillien, 1973). The 2011–2012 values in bold represent variable concentrations that overlap in standard error (SE) with those of the corresponding month in 2010. All values are ±SE.

	n	mOsm	Urea	Na+	K+	Cl-	Ca2+	Lactate	Uric acid	Glucose	*Tc* mean	*Tc* max	*Tc* min
Nov 2011	8	331.0 ± 8.05	34.2 ± 2.9	145.0 ± 1.4	2.7 ± 0.1	105.1 ± 2.2	2.6 ± 0.2	2.0 ± 0.6	168.1 ± 19.0	2.1 ± 0.2	15.50 ± 0.1	18.69 ± 0.3	12.92 ± 0.2
Nov	4-6		85.8 ± 24.4	179.3 ± 19.9	2.9 ± 0.6	134.0 ± 27.3							
Dec 2011	9	335.4 ± 8.5	37.5 ± 2.3	145.1 ± 1.5	2.6 ± 0.1	103.7 ± 1.8	2.5 ± 0.1	2.5 ± 0.6	136.8 ± 13.1	2.7 ± 0.4	16.18 ± 0.1	20.55 ± 0.4	13.03 ± 0.2
Dec	3-4		83.6 ± 29.7	162.7 ± 17.2	2.4 ± 0.3	149.1 ± 16.6							
Jan 2012	10	345.7 ± 9.1	44.9 ± 3.2	**152.8 ± 2.8**	**2.7 ± 0.1**	**108.8 ± 3.5**	**2.7 ± 0.1**	**1.9 ± 0.6**	**188.1 ± 14.7**	**2.2 ± 0.2**	11.35 ± 0.2	13.64 ± 0.4	9.51 ± 0.3
Jan 2010	8		37.2 ± 3.2	**155.7 ± 2.9**	**2.9 ± 0.1**	**110.1 ± 1.9**	**2.5 ± 0.1**	**1.5 ± 0.2**	**234.2 ± 34.9**	**2.6 ± 0.5**			
Feb 2012	10	332.3 ± 8.9	**40.6 ± 3.7**	146.6 ± 2.4	2.6 ± 0.1	**106.2 ± 2.7**	**2.6 ± 0.1**	**1.3 ± 0.3**	153.1 ± 14.4	**2.3 ± 0.1**	13.24 ± 0.3	15.41 ± 0.4	11.18 ± 0.5
Feb 2010	8		**41.1 ± 2.9**	155.1 ± 2.1	2.8 ± 0.1	**107.7 ± 3.8**	**2.5 ± 0.1**	**1.4 ± 0.5**	261.7 ± 12.6	**2.1 ± 0.2**			
Feb	3		80.7 ± 11.2	152.4 ± 13.5	4.2 ± 1.1	138.5 ± 3.5							
Mar 2012	10	318.1 ± 6.1	**37.1 ± 3.4**	**146.0 ± 1.3**	2.7 ± 0.1	109.1 ± 2.3	2.1 ± 0.2	**2.2 ± 0.6**	**129.8 ± 17.9**	**2.5 ± 0.1**	16.29 ± 0.0	19.51 ± 0.8	11.27 ± 0.4
Mar 2010	3		**30.6 ± 3.1**	**144.3 ± 1.7**	3.1 ± 0.1	104.3 ± 1.2	2.5 ± 0.2	**2.5 ± 0.6**	**134.8 ± 37.0**	**3.3 ± 0.4**			
Apr 2012	8	329.1 ± 8.3	39.2 ± 3.7	142.9 ± 1.2	3.5 ± 0.1	108.4 ± 1.1	2.5 ± 0.2	1.8 ± 0.4	168.3 ± 17.4	3.3 ± 0.1			
Apr	4-5		95.5 ± 23.1	199.6 ± 14.3	3.0 ± 0.6	150.5 ± 13.6							

(e.g., Gilles-Baillien, 1973; Harden and Williard, unpublished results), urea levels typically exceed the range encompassed by our standards (80 mg/dL), and thus, prior to spectrophotometric analysis, we diluted our 10 μL plasma samples with 0.9% saline 1:1 and multiplied the final results by two. Urea concentrations were calculated by measuring the change in absorbance of NADH in plasma samples (UA) between readings at time $t = 30$ s and $t = 120$ s and dividing by the change in absorbance of NADH of the standard (SA) between readings recorded at the same time intervals. NADH is directly proportional to urea concentration. The full equation is as follows:

$$\text{Urea mg/dL} = \left(\frac{\text{UA}_{t=30} - \text{UA}_{t=120}}{\text{SA}_{t=30} - \text{SA}_{t=120}}\right) \times \text{standard 20 mg/dL} \times 2.$$

All plasma samples were run in duplicate, and we used the mean of duplicate or triplicate (Ca^{2+} and uric acid) absorbance values to estimate plasma concentrations using the standard regression. We assayed buffer solutions and standard solutions simultaneously with plasma samples as a quality control measure (Tris buffer solutions served as blanks during spectrophotometric trials).

To evaluate the accuracy of the i-STAT POC blood analyzer and the spectrophotometry reagent kits for measuring terrapin blood chemistry, we compared results from these two methods with results obtained from a veterinary diagnostics laboratory (Antech Diagnostics, Inc., Southaven, MS) for the same blood samples. Specifically, we took ~2 mL blood samples from female terrapins from this study ($n = 10$) and from Kiawah Island, South Carolina ($n = 9$, see Dorcas et al., 2007 for details) and immediately measured blood variables Cl^-, K^+, Na^+, and glucose with the i-STAT POC analyzer ($n = 19$), centrifuged the remaining whole blood and separated the plasma into two cryogenic tubes to be frozen and analyzed. We followed the same spectrophotometry techniques as described above to analyze urea, Ca^{2+}, and uric acid in ten plasma samples. The remaining plasma was sent to Antech for analysis of Cl^-, K^+, Na^+, and glucose ($n = 19$) and urea, Ca^{2+}, and uric acid ($n = 10$).

A subsample of plasma (≥0.5 mL) was collected in November 2011 and February, March, and April 2012 from overwintering terrapins in the enclosure ($n = 30$) and corticosterone levels were measured to investigate any potential stress experienced by terrapins restricted to the fenced enclosure. Corticosterone from these plasma samples were compared to corticosterone levels measured from plasma samples obtained from free-ranging female terrapins captured by hand in January, February, and April of 2009 and 2010 ($n = 8$) around Masonboro Island, NC. All plasma samples were sent to the College of Charleston Grice Marine Laboratory for corticosterone analysis following radioimmunoassay (RIA) protocols described in Valverde (1996) and Snoddy et al. (2009).

2.3. Statistical analyses

To compare blood concentrations between i-STAT and Antech ($n = 19$), and UNCW Lab and Antech ($n = 10$), we used Spearman's rank–order correlation coefficient. Strong (Spearman's $\rho > 0.5$) and significant (at the $\alpha = 0.05$ level) correlations were considered adequate for us to substitute the i-STAT blood analyzer and laboratory spectrophotometric reagent kits (UNCW Lab) for veterinary diagnostic analysis (Antech).

To investigate potential changes in terrapin physiology throughout the winter months (i.e., the effect of time on blood chemistry concentration), we used linear mixed-effects models (LMM) run within in R (R Core Team, 2013). Each blood variable was considered a response variable, time (measured in days) was the fixed effect, and subject as the random effect (i.e., an unconditional LMM without other predictors). Values for day were taken directly from sample collections, which ranged from 6 November 2011 (day 6) to 15 March 2012 (day 137). We then performed a post-hoc analysis in which Markov chain Monte Carlo sampling (MCMC, $n = 10{,}000$) was used to calculate the posterior distribution (i.e., Highest Posterior Density [HPD] intervals, or 95% credible interval) of fixed effect estimates based on the unconditional LMM specified above. This approach provided credible intervals (upper and lower bounds) of the likelihood distribution and took into account the uncertainty in both fixed and random effect variables (Bolker et al., 2009), thus quantifying uncertainty around the mean of the slope of the LMM. If the range of the upper and lower HPD bounds did not include zero, the slope of the LMM was considered significantly

different than zero and thus, day had a significant effect on a particular blood variable.

To investigate the effect of temperature on blood chemistry concentrations throughout winter we used similar unconditional LMMs with each blood variable as a response variable, temperature (T_c) as the fixed effect, and subject as the random effect. T_c was measured as a weighted mean of daily mean readings recorded seven days prior to blood sample (based on correlation strength, which decreased noticeably after day seven). Days closer to blood collection day were weighted heavier than day further from blood collection day ($weights_{days1:7} = 0.3, 0.3, 0.2, 0.1, 0.05, 0.025, 0.025$). We then performed a post-hoc analysis in which MCMC sampling ($n = 10{,}000$) calculated the HPD intervals of fixed effect estimates based on the unconditional LMM specified above. If the range of the upper and lower HPD bounds did not include zero, the slope of the LMM was considered significantly different than zero and thus, T_c had a significant effect (±) on a particular blood variable.

We also created LMM with combinations of other measured fixed effects (mass, T_c, and standard deviation of carapace temperature [σ_{T_c}]) and used Akaike Information Criterion (AIC_c), an information-theoretic approach, to assess the models (Akaike, 1974; Burnham and Anderson, 1998). Subject variance was high and accounted for >50% of the overall variance in all LMM and strength and constituents of models varied among blood variables. All models included T_c, therefore, we decided to omit these models from the results and only report results from the unconditional T_c LMM (but see Supplementary data).

3. Results

Female terrapins ($n = 10$) equipped with radio transmitters and thermal iButtons were placed within the enclosure 22 September 2011 and were located monthly from 6 November 2011 to 5 April 2012. We were able to locate and draw blood from 8 terrapins in November, 9 in December, and all 10 in January, February and March.

3.1. Environmental factors

Terrapins experienced a warmer than usual winter in 2011–2012 (supporting data available at www.ncdc.noaa.gov/), where from 23 October 2011 to 15 March 2012 mean air temperature (±SD) was 12.5 ± 5.4 °C (range = −5.1 °C to 26.7 °C), mean shallow (2 cm) mud temperature was 13.6 ± 3.6 °C (range = −1.8 to 24.8 °C), and mean tidal creek water temperature was 14.0 ± 2.9 °C (range = 2.4 to 23.7 °C). Weighted mean T_c for the week prior to blood sample for all terrapins from November 2011 to March 2012 did not drop below 11.3 ± 0.2 °C while the weighted mean T_c minimum measured for the same time period did not drop below 9.5 ± 0.3 °C (Table 1). Sub-zero T_c was rare and individual T_c measurements remained > −1.0 °C, with the exception of two terrapins on 4 January 2012 and three terrapins on 12 February 2012. Variation in weighted mean T_c among individuals was low throughout the study (SD range = ±0.04 to ± 0.89 °C), with the greatest variability in February (SD = ±0.89 °C).

Tidal creek salinity from 23 October 2011 to 15 March 2012 remained >30, 94% of time with mean levels of 33.3 ± 1.8 and occasional fluctuations between 25 and 35 (see Harden et al., 2014 for more details). Additionally, rainfall throughout the same winter time period was low, with an average total rainfall of 0.014 ± 0.13 mm (range = 0 to 3.8 mm). Mud salinity remained between 33 and 36 throughout the study period. Because salinity and rainfall did not change dramatically throughout time period of winter blood sampling, and were not strongly correlated with blood variables, they were removed as fixed effects in the LMM for any of the blood variables.

During the sampling events between 8 December 2011 and 15 March 2012 all terrapins were observed buried below the mud surface, suggesting that terrapins exhibited some degree of dormancy during this time. However, terrapins were found buried in different places within the enclosure between most months, indicating periodic movement in the winter. Emergence from dormancy likely occurred between 15 March and 5 April 2012, as half of the terrapins were observed basking on the mud surface or swimming in shallow creek bed water at the final sampling event. These observed habitat and activity shifts are supported by previous radio telemetry research that documented occasional movements in the winter and timing of spring emergence for free-ranging terrapins (Harden and Williard, 2012; Harden et al., 2014).

3.2. Blood chemistry

Overall, plasma concentrations of Cl^-, K^+, Na^+, urea, Ca^{2+}, lactate, glucose, and uric acid measured in January, February, and March 2012 all fell within, or very close to, plasma concentrations measured in the same months in 2010, when opportunistic blood samples were taken of free-ranging female terrapins inhabiting a Lower Cape Fear tidal creek ~30 km south of our enclosure site with similar marsh habitat and fluctuations in salinity (Table 1; Harden and Williard, unpublished results). However, plasma concentrations of Cl^-, K^+, Na^+ and urea from this study were substantially lower than those measured in laboratory overwintering terrapins (Table 1, Gilles-Baillien, 1973, see discussion for details). Plasma corticosterone levels of terrapins were low throughout the duration of the enclosure time (November 2011 to April 2012; mean = 0.76 ng/mL ± 0.58 SD) and were within range of corticosterone levels of free-ranging terrapins measured in similar environmental conditions (mean = 0.55 ng/mL ± 0.45 SD).

Correlations between i-STAT-measured and Antech-measured blood concentrations of Cl^-, K^+, Na^+, and glucose were all statistically significant ($\rho \geq 0.91$, $p < 0.0001$). Correlations between UNCW Lab-measured and Antech-measured blood concentrations of uric acid and urea were statistically significant ($\rho = 0.88, 0.85$; both $p < 0.001$), but Ca^{2+} was not statistically significant ($\rho = 0.61$, $p = 0.054$). However, because our study focused on changes in blood variable concentrations over time rather than precise values, and we are confident in the use of both the i-STAT POC blood analyzer and UNCW Lab protocols for assessing temporal trends in all of our blood variables.

Blood biochemical variables did not change significantly over time, as indicated by the mean slope estimates of day not differing significantly from zero (i.e., all HPD intervals included zero, Table 2). However, blood biochemical variables did significantly change with temperature (T_c). More specifically, osmolality (mOsm), Na^+, Cl^-, K^+, and uric acid were all negatively correlated with T_c, while glucose was positively correlated with T_c (Table 2).

4. Discussion

4.1. Overwintering osmotic homeostasis

Diamondback terrapins, like most other species of estuarine reptiles, use a combination of compensatory and evasive strategies to

Table 2
Nine blood chemistry variables used to evaluate physiological changes in 10 female terrapins during winter (November to March). Effect of day and effect of temperature are represented as mean slope estimates [95% credible interval] that were taken from individual unconditional models with the blood variables as a response. Markov chain Monte Carlo (MCMC) bounds were calculated from the highest posterior density interval based on 10,000 draws. Significance was assessed based on the 95% credible interval not overlapping 0 and is denoted by *.

Blood variable	Effect of day	Effect of temperature (T_c)
Osmolality	−0.08 [−0.20, 0.04]	−3.59 [−6.06, −1.11]*
Urea	0.03 [−0.02, 0.08]	0.01 [−0.03, 0.05]
Sodium (Na^+)	0.01 [−0.03, 0.05]	−0.07 [−0.12, −0.02]*
Chloride (Cl^-)	0.03 [0.00, 0.07]	−1.72 [−2.70, −0.78]*
Potassium (K^+)	0.00 [0.00, 0.00]	−1.35 [−2.09, −0.58]*
Calcium (Ca^{2+})	0.00 [0.00, 0.00]	−0.48 [−1.32, 0.35]
Lactate	0.00 [−0.01, 0.01]	0.17 [−0.04, 0.40]
Uric acid	−0.19 [0.47, 0.65]	−8.91 [−14.38, −3.73]*
Glucose	0.00 [0.00, 0.00]	0.12 [0.01, 0.23]*

maintain internal osmotic pressures that are 1/3 to 1/2 that of seawater (Gilles-Baillien, 1970). Our study is the first to shed light on the physiological status of dormant terrapins in their natural estuarine environment and our findings underscore the ability of terrapins to maintain osmotic control throughout winter. Research on aquatic vertebrates has found urea accumulation to play a significant role in water balance during periods of osmotic stress (increased salinity, dehydration), such as those experienced during dormancy, and is a primary source of increased plasma/tissue osmotic pressure (Muir et al., 2007, 2008, 2010). Evidence from laboratory studies suggests that regulation of blood and tissue urea levels may play a role in water balance for terrapins, both under conditions of changing salinity and prolonged mud burial during winter dormancy (Gilles-Baillien, 1970, 1973). However, our findings do not provide evidence of significant urea accumulation in terrapin plasma that would suggest its role as an osmoeffector during the winter. Urea concentrations measured in our study were more than half those from previous laboratory-based studies (e.g., Gilles-Baillien, 1973, see Table 1). Plasma Na^+ and Cl^- concentrations were also lower in our study (Table 1).

Difference in osmolyte concentrations is likely due to environmental differences between the controlled lab study and our study, in which the terrapins experienced the natural and dynamic changes and fluctuations of the estuarine environment. Many early laboratory studies were conducted under more stable conditions with captive animals kept in tanks with constant and/or regulated salinity and temperature of freshwater, 50% seawater (~17), or full strength seawater (35) (Bentley et al., 1967: 21 °C; Dunson, 1970: 7–15 °C for freshwater exposed terrapins; Gilles-Baillien, 1970: no temp. recorded; Gilles-Baillien, 1973: no temp. recorded; Robinson and Dunson, 1976: 20–25 °C; Cowan, 1981: no temp. recorded), thus resulting in monotonic changes in osmolytes throughout the manipulated winter. Furthermore, the one study exploring winter osmotic regulation (Gilles-Baillien, 1973) only provided terrapins with aquatic overwintering habitats, whereas free-ranging terrapins are typically observed buried in intertidal or subtidal mud during dormancy (Yearicks et al., 1981; Harden and Williard, 2012; this study). Without the option of muddy substrate to overwinter in, behavioral control over osmotic balance is reduced, and there is likely more water and salt exchange between the aquatic saline environment and the terrapin's body fluids.

Terrapins in our study may be relying more on evasive adaptive mechanisms to maintain osmotic balance (e.g., behavioral/habitat changes, hypophagy), rather than compensatory mechanisms (e.g., urea accumulation and/or synthesis and salt gland excretion). Additionally, April concentrations of plasma glucose and K^+ were noticeably elevated above those of all other months (Table 1), suggesting an onset of feeding following a prolonged fast, also hypothesized by Gilles-Baillien (1973). Elevated K^+ levels following spring emergence may be explained by high K^+ levels in terrapin prey items: *Uca pugilator* (116.0 ± 2.3 m-equiv./kg body water, Holmes and McBean, 1964) and *Littorina littorea* (277–473 mg/100 g live wet weight, McCance and Shipp, 1933). Finally, body water turnover rates estimated using the stable isotope deuterium [^{2}H] show a two-fold increase between pre- and post-spring emergence (Harden et al., 2014). Taken together, these results indicate relatively low water exchange during winter dormancy (e.g., fresh rain water uptake, salt water ingestion via feeding, and excessive urine excretion). Because salt glands are energetically expensive to maintain (Bentley et al., 1967; Borut and Schmidt-Nielsen, 1963; Whittam, 1963) and may function primarily as a means to excrete excess salts ingested while foraging (i.e., in response to salt loading, Cowan, 1981), it is unlikely to be triggered during dormancy, when we have evidence to suggest they are principally hypophagic. Finally, there is little evidence to suggest that terrapins were maintaining osmotic balance during winter via extracellular storage of water (see Davenport and Macedo, 1990; Robinson and Dunson, 1976) because mean total body water (TBW%) did not change between pre- and post-spring emergence in terrapins (Harden et al., 2014). The TBW indicate that 1) terrapins do not appear to be markedly dehydrated during dormancy (based on the 60–80% total body water range for freshwater turtles, Minnich, 1982; Crawford, 1994; Roe et al., 2008), and 2) terrapins do not appear to be storing more water in their body during dormancy to combat osmotic stress of their hyperosmotic environment.

4.2. Overwintering metabolism

We found no significant change over time in metabolic indicators lactate and Ca^{2+} (Tables 1 and 2), suggesting there is little evidence of elevated reliance on anaerobic metabolism during terrapin dormancy as seen in the anoxia-tolerant painted turtle (*C. picta*). Previous studies investigating anoxia tolerance in overwintering emydids have documented an increase in plasma lactate levels from ~1 mmol/L to >200 mmol/L (*C. picta* at 3 °C for four months, Ultsch et al., 1999) accompanied by Ca^{2+} levels increasing up to 25-fold above normal Ca^{2+} levels (2.4 mmol/L). In contrast, plasma lactate and Ca^{2+} concentrations in our female terrapins overwintering in the salt marsh were much closer to normal plasma levels. Unlike most other emydid turtles, terrapins live in tidally-influenced systems and therefore have the ability to choose intertidal overwintering locations in which they are submerged at high tides and exposed to air at low tides, allowing periodic access to atmospheric oxygen.

Although aquatic respiration is an established overwintering survival mechanism for several emydids (Graham and Forsberg, 1991) including the terrapin's closest relative, *Graptemys* (Crocker et al., 2000), this is an unlikely strategy for terrapins that overwinter in anoxic mud and are surrounded by poorly oxygenated salt water. Throughout this study and a previous radio telemetry study conducted in southeastern NC, terrapins were observed overwintering in the intertidal zone of the marsh 3–10 cm from the mud surface, which may allow maintenance of aerobic metabolism (Harden and Williard, 2012; Southwood Williard and Harden, 2011) via aerial breathing. Furthermore, unlike their northern counterparts that likely experience true anoxia due to iced-over tidal creeks (Brennessel, 2006), terrapins in southeastern NC experience more moderate winter temperatures. Periodic elevated ambient temperatures, particularly in winter 2011–2012, may have allowed terrapins the occasional opportunity to emerge from mud to access aerial oxygen, which is supported by our visual observations.

Thermal influence on metabolism and subsequent behavior and activity levels is well-documented (Dubois et al., 2009; Grayson and Dorcas, 2004; Hochachka and Somero, 2002; Southwood Williard and Harden, 2011), and is supported by our results, which underscore T_c as a predictor for multiple blood biochemical variables of overwintering terrapins. This indicates that the temperature of the terrapin one week prior to blood sample acquisition is a more important indicator of physiological status than time, suggesting that their overwintering state is not continuous and progressive like other more northern latitude emydid turtle species (Crocker et al., 2000; Graham and Forsberg, 1991; Ultsch and Jackson, 1995) and thus, cannot be documented as such via consecutive blood samples throughout winter. Instead, the overwintering status of terrapins in southeastern NC is dynamic in which they often overwinter in the anoxic shallow mud of the salt marsh intertidal zone (Harden and Williard, 2012; Southwood Williard and Harden, 2011) and experience vacillating water levels and temperatures with periodic warm bouts that can trigger above ground movements. Thus, terrapins can potentially avoid the physiological stress of anaerobic metabolism (Graham and Forsberg, 1991; Storey, 1996).

4.3. Conclusion

Overall, despite changes in temperature during the winter and the associated reduction in metabolic rates, rates of physiological processes (Southwood Williard and Harden, 2011), and rates in body water flux (Harden et al., 2014), our finding show that terrapins in southeastern

NC are able to maintain osmotic and ionic balance throughout dormancy. Our results provide some evidence that use of evasive strategies, particularly behavioral adjustments, may be important in order for terrapins to reduce the passive exchange of water and salts with the environment. Further investigation of the importance of behavior and habitat use to maintain homeostasis is warranted and may be addressed by eliminating the option for such adjustments (e.g., mud burial) in a more controlled laboratory setting. Extending this study out to the spring and summer months would also be beneficial to understanding seasonal changes in terrapin salt and water balance and how their osmotic strategy may differ during the active season, when metabolism, and thus rates of physiological functions, are elevated. Finally, comparing our blood chemistry data to those of terrapins inhabiting the most northern part of their geographic range (Cape Cod, MA) would elucidate differences (or similarities) in osmotic and metabolic strategy of terrapins exposed to extremely harsh winters. This experimental field study is strengthens our knowledge of the behavioral and physiological adjustments employed by terrapins inhabiting an estuarine environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2015.01.017.

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