

RESEARCH ARTICLE

Effects of temperature and salinity on body fluid dynamics and metabolism in the estuarine diamondback terrapin (*Malaclemys terrapin*)

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ABSTRACT

The diamondback terrapin is the only temperate turtle species that exclusively inhabits estuarine environments. Morphological, behavioral and physiological features contribute to the terrapin's ability to regulate body fluid osmotic pressure in a euryhaline environment. Low integument permeability combined with aquatic–terrestrial shuttling behavior limits passive exchange of water and salts with the environment, and terrapins regulate active uptake of salts via alterations in drinking and feeding behavior. The lachrymal salt gland facilitates excretion of excess sodium (Na⁺) and chloride (Cl⁻) ions through active transport mechanisms. We investigated body fluid dynamics, oxygen consumption (\dot{V}_{O_2}) and osmotic status of terrapins exposed to an acute increase in salinity (12 to 35 psu) at 10 and 25°C to gain insight into the relative importance of behavioral versus physiological osmoregulatory adjustments over a range of seasonally relevant temperatures. Linear mixed models were used to evaluate the effects of experimental temperature, salinity and mass. Overall, temperature effects were stronger than salinity effects. Terrapins acclimated to 25°C had significantly lower blood osmolality and Na⁺, and higher water turnover rates, daily water flux (DWF) and \dot{V}_{O_2} compared with terrapins acclimated to 10°C. Salinity effects were restricted to DWF, which significantly decreased in response to acute exposure to 35 psu. Our results support the notion that behavioral adjustments predominate in the osmoregulatory strategy of terrapins.

KEY WORDS: Osmoregulation, Energetics, Oxygen consumption, Salt gland, Water balance, Reptile

INTRODUCTION

While many species of terrestrial, freshwater or marine vertebrates take advantage of temporally available resources in estuaries, the diversity of species that live entirely within the estuarine habitat is low compared with that in more stable environments (Greenberg and Maldonado, 2006). Among reptiles, a limited number of crocodylian and snake species utilize brackish waters (Dunson, 1970, 1980; Grigg, 1981; Dunson and Mazzotti, 1989; Lillywhite and Ellis, 1994; Leslie and Spotila, 2000), and several turtle species occur in tidally influenced habitats (see Agha et al., 2018, for

review). Regulation of water and salt balance in a highly variable environment is one of the primary physiological challenges facing estuarine reptiles, and the distribution of reptiles in coastal environments is constrained in large part by water salinity (Dunson and Mazzotti, 1989; Brischoux et al., 2012). Marine and estuarine reptiles generally regulate body fluid osmotic pressure within the range observed for terrestrial vertebrates, although some species are tolerant of higher body fluid ion concentrations and osmolality (Dunson, 1984; Brischoux et al., 2013; Lewbart et al., 2015). In highly variable hyperosmotic environments, these reptiles maintain osmotic homeostasis using a combination of both evasive strategies – to reduce passive exchange of water and salts between the organism and environment (Robinson and Dunson, 1976; Mazzotti and Dunson, 1989; Davenport and Magill, 1996) – and compensatory (i.e. energy-requiring) strategies – to actively take up or extrude salts (Schmidt-Nielsen and Fange, 1958; Dunson, 1970).

The diamondback terrapin (*Malaclemys terrapin*) is a semi-aquatic, emydid turtle found exclusively in marshes, tidal creeks and estuaries along the East and Gulf coasts of the USA (Hart and Lee, 2006; Ernst and Lovich, 2009). Terrapins experience a broad range of salinities (11–35 psu) in their coastal habitats (Dunson, 1970; Harden and Williard, 2012), and provide a good illustration of how morphological, behavioral and physiological features contribute to osmoregulation in estuarine environments. Body fluid osmotic pressure of terrapins under natural conditions falls within the range 300–350 mOsm, and is typically hyposmotic to the surrounding aquatic environment (Harden et al., 2015). Passive exchange of water and salts between terrapins and their environment is minimized as a result of low integument permeability (Robinson and Dunson, 1976). Furthermore, terrapins may engage in basking or terrestrial shuttling behavior to regulate salt and water exchange across the integument (Davenport and Magill, 1996). Davenport and Magill (1996) noted that terrapins held in seawater tanks spent more time on basking platforms if they were deprived of periodic access to freshwater, even when air temperature was cooler than water temperature. In natural habitats, it is common for terrapins to bask or bury in the mud of the intertidal zone during low tide, which could reflect thermoregulatory and/or osmoregulatory behavior (Spivey, 1998; Harden et al., 2007; Harden and Williard, 2012). Active uptake of water and salts may be controlled through alterations in drinking and feeding behavior. Terrapins selectively drink low salinity water (≤ 20 psu) or freshwater if given the opportunity to do so (Cowan, 1981; Davenport and Macedo, 1990). The terrapin's typical invertebrate prey (*Uca pugnator* and *Littorina littorea*) are isosmotic with the variable estuarine environment (Dunson, 1985; Tucker et al., 1995; Whitelaw and Zajac, 2002), so at higher salinities, prey consumption results in a large salt load that could disrupt osmotic homeostasis. Laboratory studies have illustrated that terrapin appetite is gradually suppressed during

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prolonged exposure to seawater with no access to supplemental freshwater (Davenport and Ward, 1993; Holliday et al., 2009).

While it is widely recognized that behavioral adjustments are an integral component of the terrapin's osmoregulatory strategy, terrapins also exhibit a variety of physiological responses to changes in environmental salinity. Reptiles are limited in their capacity to modulate osmotic pressure via waste excretion because their kidneys are not capable of generating hyperosmotic urine (Dantzler, 2016). However, production and retention of the nitrogenous waste product urea and other compatible osmolytes may contribute to osmotic balance in estuarine chelonians, as accumulation of these molecules increases body fluid osmotic pressure and facilitates water retention during exposure to high salinity or dehydration (Gilles-Baillien, 1970, 1973; Lee et al., 2006). Terrapins may also regulate body fluid osmotic pressure through the active extrusion of inorganic ions (e.g. Na^+ , Cl^-) via a lachrymal salt gland (Schmidt-Nielsen and Fange, 1958; Dunson, 1970). The terrapin salt gland is intermediate in secretory capacity between those of marine and terrestrial reptile species (Dunson, 1970; Harden and Williard, 2018). Terrapins increase rates of ion excretion through the salt gland in response to conditions associated with prolonged or extreme salt accumulation (Dunson, 1970; Robinson and Dunson, 1976; Cowan, 1981); however, the role of the salt gland in addressing routine exposure to variable salinity has not been well studied. Salt gland activation in response to a salinity challenge incurs a metabolic cost, as active transport mechanisms are employed (Bentley et al., 1967; Dunson and Dunson, 1975).

The relative importance of behavioral versus physiological strategies for osmoregulation in terrapins could vary seasonally with changes in resource availability and temperature. Terrapins exhibit distinct seasonal patterns in behavior and habitat utilization, which appear to be driven largely by variations in environmental temperature (Yearicks et al., 1981; Harden and Williard, 2012). At water temperatures (T_w) of $\leq 20^\circ\text{C}$, terrapins enter a dormant state characterized by mud burial, hypophagy and decreased metabolic capacity (Southwood Williard and Harden, 2011; Harden and Williard, 2012; Harden et al., 2015). Overwintering in the mud may reduce passive exchange of water and salts across the integument and a reduction in feeding decreases active uptake of salts from the environment (Davenport and Magill, 1996); these behavioral changes contribute to the terrapin's ability to maintain osmotic balance during a period when both metabolic capacity and resource availability are low (Harden et al., 2015). During the warm weather active season, terrapins may rely more heavily on active ion extrusion via the salt glands, given the higher rates of passive and active water and salt exchange with the environment and the need for continual energy intake to support higher metabolic demands (Baker et al., 2013; Harden et al., 2014). The decrease in metabolic rate that accompanies a decrease in temperature may limit the ability of terrapins to respond physiologically to salt ingestion or an environmental salinity challenge (Baker et al., 2013; Southwood Williard and Harden, 2011). Consequently, terrapins may rely more heavily on energetically efficient behavioral strategies to regulate water and salt exchange with the environment or simply tolerate higher blood ion concentration and osmotic pressure (Brischoux et al., 2013; Harden et al., 2015; Lewbart et al., 2015).

The primary goal of this study was to assess the role of behavioral adjustments versus energy-requiring physiological adjustments in the osmoregulatory strategy of terrapins across a range of seasonally relevant temperatures. We documented body fluid dynamics and metabolic rates of terrapins exposed to an acute increase in salinity at 25 and 10°C . At both temperatures, terrapins were acclimated

over the course of 3 weeks to an environmental salinity that approximated body fluid osmolality (12 psu), and then exposed to full-strength seawater (35 psu) for 3–5 days to assess the response to an increase in salinity at each temperature. The osmotic status of terrapins was assessed by measuring plasma osmolality, inorganic ions and organic osmolytes. The stable isotope deuterium (^2H) was used to assess total body water (%TBW), water turnover rate (WTR) and daily water flux (DWF) as indicators of active and passive water exchange with the environment. Oxygen consumption (\dot{V}_{O_2}) was monitored as a measure of metabolic rate. We predicted that in order to maintain osmotic homeostasis during acute exposure to high salinity (35 psu), terrapins would exhibit (1) a decrease in WTR and DWF reflective of behavioral adjustments (i.e. modulation of food and water ingestion) to reduce water and salt exchange with the environment and (2) an increase in \dot{V}_{O_2} reflective of activation of energy-requiring physiological compensatory mechanisms, such as ion excretion via the salt gland.

MATERIALS AND METHODS

Animal husbandry

Eleven *M. terrapin* (Schoepff 1793) (6 females, 5 males) were captured by seine in tidal creeks adjacent to the Grice Marine Laboratory in Charleston, SC, USA (32.7520°N , -79.8982°W) on 26–27 September 2013 (Table 1). T_w at the capture site was 23°C and salinity was 20 psu. Terrapins were transferred to the University of North Carolina Wilmington Center for Marine Science (UNCW CMS; 34.1419°N , -77.8678°W) on 27 September 2013, and subsequently housed in outdoor, partially shaded, flow-through tanks ($107 \times 107 \times 56$ cm, length \times width \times depth) with seawater (mean \pm s.d. 35.6 ± 1.4 psu) supplied from the Intracoastal Waterway (ICW). T_w in the outdoor holding tanks varied with natural variation in air temperature and ICW T_w (see below). Cinderblocks were placed in the tanks to provide shelter and basking platforms for terrapins. While housed in outdoor holding tanks, terrapins were exposed to natural photoperiod and precipitation. On days when it did not rain, freshwater was sprayed into the tanks for 15 min to offer terrapins the opportunity to drink freshwater. Terrapins were fed 7% of body mass every other day on a diet of natural prey items (*Uca* spp. and *L. littorea*) acclimated to holding tank salinity (Davenport and Ward, 1993; Tucker et al., 1995). All terrapins maintained or gained mass during the initial holding period of 2 weeks. This research was authorized and conducted under North Carolina Wildlife Resource Commission Permit #ES-00235, and UNCW Institutional Animal Care and Use Permit #1112-015.

Table 1. Descriptive information for the 11 diamondback terrapins used to investigate the effects of temperature and salinity on metabolism and osmotic status

Terrapin ID	Sex	Initial mass (g)	SCL (cm)
25°C acclimated			
__BHP	F	1002.8	18.0
__CHV	F	766.8	16.5
__CHX	F	1218.3	19.2
__CIL	M	291.2	12.2
__CIM	M	269.6	11.8
10°C acclimated			
__AHV	F	770.4	16.5
__CHW	F	914.8	17
__CIJ	F	1078.8	18.6
__CIN	M	232.6	11.7
__CIO	M	359.1	13.8
__CIP	M	303.2	12.3

SCL, straight carapace length.

Temperature and salinity treatments

Terrapins were transferred to an indoor, temperature-controlled, recirculating water tank system for acclimation to treatment conditions (details below). The system consisted of two oval holding tanks (132×94×53 cm, length×width×depth) so that male and female terrapins could be housed separately, as well as two sump tanks (81×38 cm, diameter×depth) to house chiller and heater units for temperature control. The tank system design resulted in uniform T_W and salinity in the two holding tanks. Water volume for the entire system was approximately 835 l, and full water change-outs occurred weekly. Water was recirculated through two mechanical filters (Aquatic Ecosystems, Inc., Apopka, FL, USA) to remove particulate matter. Water salinity was monitored with a refractometer, and modified to necessary treatment conditions by mixing freshwater with seawater supplied from the ICW. Cinderblocks placed on the tank floor provided sheltering opportunities for terrapins, but were submerged at a depth that prevented terrapins from hauling themselves out of the water during acclimation to treatment conditions. During acclimation to treatment conditions, terrapins were offered a ration (7% of body mass) of natural prey items acclimated to treatment salinity every other day, and we documented whether the entire food ration was consumed. Terrapins were not provided with supplemental freshwater.

Metabolic and osmotic status were assessed for four water temperature–salinity treatments: warm temperature and low salinity (25°C–12 psu), warm temperature and high salinity (25°C–35 psu), cold temperature and low salinity (10°C–12 psu), and cold temperature and high salinity (10°C–35 psu). Given the limitations of the indoor tank system, we tested one temperature treatment at a time, with 25°C tested first and 10°C tested second to correspond with seasonal temperatures in the Carolinas.

On 17 October 2013, 5 terrapins (3 females, 2 males) were transferred to the indoor tank system for acclimation to the 25°C–12 psu treatment. During the 2 weeks prior to this transfer, the outdoor holding tank T_W varied between 20.5 and 26.2°C (23.2±2.3°C) with a salinity of 32–37 psu (35.8±1.0 psu). Response variables indicative of osmotic status, body fluid dynamics and \dot{V}_{O_2} were assessed after a 3 week acclimation to 25°C–12 psu, and then the water salinity was increased to 35 psu. Response variables were assessed again after 3–5 days of exposure to 25°C–35 psu, and terrapins were transferred back to outdoor holding tanks upon completion of the experiments (15 November 2013). The exposure time of 3–5 days for the acute high salinity treatment was selected based on previously published work that demonstrated the time course for stabilization of blood variables (Gilles-Baillien, 1970).

On 17 November 2013, the remaining 6 terrapins (3 females, 3 males) were transferred to the indoor tank system for acclimation to the 10°C–12 psu treatment. During the 2 weeks prior to this transfer, the outdoor holding tank T_W varied between 10.9 and 21.5°C (16.4±2.9°C) with a salinity of 32–39 psu (36.8±1.5). The same response variables were assessed after a 3 week acclimation to 10°C–12 psu, and then the water salinity was increased to 35 psu. Response variables were assessed again within 3–5 days of exposure to 10°C–35 psu, and then terrapins were transferred back to the outdoor holding tanks on 6 January 2014. All terrapins were released at the site of capture in March 2014.

Osmotic status

Blood samples for determination of osmotic status and body fluid dynamics were collected after 3 weeks acclimation to the low salinity (25°C–12 psu and 10°C–12 psu) treatments, and after a subsequent acute exposure of 3 days to the high salinity

(25°C–35 psu and 10°C–35 psu) treatments. Blood samples (1–2 ml) were collected from the subcarapacial vein using a heparinized vacuum tube and 23–25 G needle (Vacutainer, BD, Franklin Lakes, NJ, USA). A sub-sample (70 µl) of whole blood was taken for immediate analysis of Na^+ (mmol l⁻¹), Cl^- (mmol l⁻¹) and glucose (mg dl⁻¹) using an i-STAT Point of Care (POC) handheld blood analyzer with 6+ cartridge (Abaxis Veterinary Diagnostics, Union City, CA, USA; Harden et al., 2015). The remainder of the blood was centrifuged at 2800 g to separate plasma from blood cells. The plasma was transferred to 0.5 ml cryogenic storage vials (Safe-Lock Tube, Eppendorf, Hamburg, Germany), wrapped in Parafilm® (Pechiney Plastic Package, Inc., Chicago, IL, USA), and stored in a –80°C freezer for subsequent analyses. A sub-sample (10 µl) of plasma was used to measure osmolality (mOsm) with a vapor pressure osmometer (Vapro model 5600, Wescor Inc., Logan, UT, USA), and a second sub-sample (30 µl) of plasma was used for determination of urea (mg dl⁻¹) with a commercially available reagent kit (Pointe Scientific Inc., Canton, MI, USA) and standard spectrophotometric techniques (Lambda 25 UV/Vis, PerkinElmer, Waltham, MA, USA). Plasma urea concentrations were measured and analyzed following the protocol and equation detailed in Harden et al. (2015).

Body fluid dynamics

Changes in terrapin body fluid dynamics in response to an increase in environmental salinity at different acclimation temperatures were evaluated using the stable isotope deuterium (²H). Percentage TBW (%TBW), WTR (ml day⁻¹) and DWF (%TBW day⁻¹) were assessed after 3 weeks acclimation to the low salinity (25°C–12 psu and 10°C–12 psu) treatments, and after a subsequent acute exposure of 3 days to the high salinity (25°C–35 psu and 10°C–35 psu) treatments. A sub-sample of plasma collected for assessment of osmotic status was reserved for determination of background enrichment of ²H ($E_{background}$) in terrapin body water. After the $E_{background}$ sample was collected, terrapins were weighed on a top-loading balance (Ohaus® Model SP40, 4000 g capacity, 0.01 g readability; Parsippany, NJ, USA). Dosage of ²H injectate solution (82.6 atom%; Isotec, Inc., Miamisburg, OH, USA; verified by Metabolic Solutions, Nashua, NH, USA) was calculated using published equations (Speakman, 1997, eqn 12.1) and data on % TBW for terrapins (Harden et al., 2014), with the goal of achieving a desired initial enrichment (DIE) of ≥600 ppm above $E_{background}$. The mass of the injectate syringe (1 ml) and needle (25 G) was measured with a digital scale (Mettler Toledo Model AB 304-S/FACT, 320 g capacity, 0.1 mg readability; Columbus, OH, USA) prior to injecting the ²H dose into the coelomic cavity of the terrapin. The empty injectate syringe and needle were weighed again after injection to determine the exact dose of ²H administered to each terrapin. Terrapins were transferred to plastic bins and maintained in room air (20–22°C) for 4–5 h to allow ²H to equilibrate with body water (Harden et al., 2014). A second blood sample was collected and processed in the manner described above in order to assess ²H concentration in body fluids after the equilibration period (E_{equil}), and terrapins were returned to indoor acclimation tanks. A third blood sample (E_{final}) was collected and processed after approximately 2 days (1.80–1.93 days) for terrapins acclimated to 25°C and 3 days (2.93–2.95 days) for terrapins acclimated to 10°C. Terrapins were offered food rations once between collection of E_{equil} and E_{final} , but were not offered supplemental drinking water.

Plasma samples were stored at –80°C for a maximum of 4 months prior to isotope analysis. An isotope ratio mass

spectrometer (IRMS, Delta V Plus, Thermo Fisher Scientific, Waltham, MA, USA) with gas bench interface (ThermoFinnigan GasBench II, Thermo Fisher Scientific) was used to determine ^2H levels in plasma samples (see Harden et al., 2014, for details). Delta values (‰) were obtained using Isodat software (Thermo Fisher Scientific, s.d. $<7\text{‰}$). A calibration equation ($y=5.0915x+4096.2$, $R^2=0.9988$) generated from two standards (USGS W-64444 and W-67400, -399.1‰ and 1.25‰ , respectively) and three dilutions of 99.9 atom% ^2H in Evian spring water ($-75.14\pm 5.2\text{‰}$, $435.51\pm 2.8\text{‰}$, $1180.58\pm 9.2\text{‰}$) was used to calculate corrected delta values for terrapin plasma. Corrected delta values were converted to ppm using the following equation:

$$\text{ppm } ^2\text{H} = \frac{1,000,000}{1 + (1/\{[(\delta^2\text{H}/1000) + 1] \times 0.00015576\})}, \quad (1)$$

where $\delta^2\text{H}$ is per million ^2H with respect to the Vienna Standard Mean Ocean Water (VSMOW), and the constant 0.00015576 is the $^2\text{H}/^1\text{H}$ ratio of VSMOW.

The two-sample technique (Speakman, 1997) was used to estimate the turnover rate of ^2H (k_d) during the time between E_{equil} and E_{final} sample collection, and the plateau method was used to estimate isotope dilution space (N_d) for calculations of %TBW and WTR. Daily water flux was estimated by multiplying %TBW by k_d .

Oxygen consumption

We used closed-circuit respirometry to measure \dot{V}_{O_2} of terrapins during exposure to each of the four temperature–salinity treatments. Respirometry trials were coordinated with feeding schedule, such that terrapins had been fasted for 40–48 h prior to the trial. For each trial, an individual terrapin was placed in a 90 l circular tank filled with water adjusted to the treatment temperature and salinity. Trials were conducted in a cold room, and T_w in the experiment tank was controlled with submersible heaters for warm trials and by adjusting room temperature for cold trials. Salinity of water in the experiment tank was adjusted using Instant Ocean aquarium salt (Spectrum Brands, Blacksburg, VA, USA). A partially submerged acrylic dome with input and output air portals was placed on top of the tank. Portals could be left open to allow the dome airspace to equilibrate with room air, or connected to a series of tubes that closed the system and recirculated air through the dome and Drierite and soda lime cartridges using a pump (Gast Manufacturing, Benton Harbor, MI, USA). Two dome sizes were used, depending on the size of the terrapin; the volume of dome airspace, cartridges, tubing and fittings was approximately 760 ml for the first dome and 2350 ml for the second dome. The water surface comprised the bottom seal for closed airspace within the dome; pressure changes within the dome airspace were mitigated by changes in water level, as the water within the submerged portion of the dome was in contact with ambient air pressure through submerged vents in the acrylic dome. Nitrogen dilution was used to calibrate the respirometry system.

Terrapins were transferred to the experiment tank and permitted an acclimation period of 20–40 min with the dome secured in place prior to the start of the trial. During the acclimation period, the input portal was open to ambient air, but the output portal was connected to the tubing system and pump. An initial air sample was drawn from the dome after completion of the acclimation period and prior to sealing the input portal of the dome system. The pump was turned off immediately prior to sample collection, and sample collection was timed to occur when the terrapin submerged after a breathing episode. Air samples (40–50 ml) were drawn from the dome through Drierite and soda lime cartridges connected to the output

portal using a syringe attached to a three-way stopcock. Samples were subsequently transferred through the stopcock to an evacuated 500 ml respiratory bag (A. M. Bickford Inc., Wales Center, NY, USA), and sealed for transfer to the oxygen analyzer (see below). Following collection of the initial air sample, the input portal was connected to the tubing system, and the pump was turned on to recirculate air through the dome and ensure adequate air mixing throughout the trial. Respirometry trial duration was selected based on dome volume and estimates of metabolic rate from previously published work (Bentley et al., 1967; Baker et al., 2013). Mean (\pm s.d.) trial duration at 25°C was 30.7 ± 5.7 min and mean trial duration at 10°C was 121.7 ± 17.3 min. Terrapins were monitored continuously during trials, and the percentage time spent submerged versus at the water surface in the dome was recorded. Percentage time submerged served as a proxy for activity level during trials, as increases in activity level are associated with decreases in dive duration in turtles (Hays et al., 2000; Okuyama et al., 2012; Baker et al., 2013). Given the limited space in the respirometry chamber, terrapin activity during trials was limited to slow paddling or walking along the tank bottom. A final air sample was collected at the conclusion of the trial in the manner described for the initial sample. The pump was turned off immediately prior to sample collection, and sample collection was timed to occur when the terrapin submerged after a breathing episode. Terrapins were returned to indoor acclimation tanks upon completion of trials.

The percentage O_2 in initial and final air samples was analyzed with an Ametek S-3A Oxygen Analyzer (AEI Technologies, Pittsburgh, PA, USA). Air samples were drawn from the respiratory bag, through the three-way stopcock and into the oxygen analyzer at a rate of 85 ml min^{-1} . Only $\frac{1}{2}$ to $\frac{3}{4}$ of the air sample volume was used for analysis to ensure that pressure effects were not generated in the analyzer. Data were acquired and recorded from the analyzer at a frequency of 1 Hz using a universal interface and ExpeData software (Sable Systems International, North Las Vegas, NV, USA). The oxygen analyzer was calibrated daily using the following gas mixtures: 100% N_2 , 5% O_2 , 18.5% O_2 and 20.94% O_2 (CalGas, Conyers, GA, USA). Calibration gases were run through the oxygen analyzer in the same manner as described for air samples from respirometry trials.

For calculation of \dot{V}_{O_2} , percentage O_2 was converted to a fractional concentration of O_2 , and Eqn 2, based on Lighton (2008), was used to determine \dot{V}_{O_2} (ml h^{-1}) during trials:

$$\dot{V}_{\text{O}_2} = \frac{V(F''_{i,\text{O}_2} - F''_{e,\text{O}_2})}{(1 - F''_{e,\text{O}_2}) \times T}, \quad (2)$$

where V is chamber volume, F''_{i,O_2} is the fractional concentration of O_2 in air scrubbed of CO_2 and water vapor upon initiation of the trial, F''_{e,O_2} is the fractional concentration of oxygen in air scrubbed of CO_2 and water vapor at the end of the trial, and T is trial duration. Values for \dot{V}_{O_2} are reported at standard temperature and pressure, dry (STPD).

Statistical analysis

We used linear mixed models to evaluate the effects of experimental temperature, salinity and individual turtle mass on response variables. Terrapins exhibit sexual dimorphism in mass; sex was not included as a factor in our analysis given the likelihood for covariance with mass. We fitted nine separate models for each of the responses (Na^+ , Cl^- , glucose, urea, osmolality, \dot{V}_{O_2} , %TBW, WTR, DWF). All nine models had the same three predictors, with the exception of \dot{V}_{O_2} , which had an additional predictor of percentage submerged (i.e. time spent submerged during the respirometry trial)

as a proxy for activity level (Hays et al., 2000; Okuyama et al., 2012; Baker et al., 2013). For all models, we included a random effect for subject (individual turtle), because of the potential for the correlated information coming from the repeated use of turtles before and after a treatment. We used the R package MCMCglmm (Hadfield, 2010), which allowed us to fit linear mixed models that account for correlated random effects while also including a Markov chain Monte Carlo sampler so that posterior means for parameter estimates and their associated credible intervals could be used for parameter interpretation and comparison. Markov chain Monte Carlo settings for all models included 70,000 iterations, 20,000 burn-in samples, and a thinning rate of 3, which resulted in a final posterior sample of $n=16,667$ for each model. Model convergence was evaluated with traceplots and density plots, in addition to running each model three independent times to assess convergence of chains among models. We examined 95% credible intervals of posterior estimates: effects were considered significant if the 95% credible intervals for the estimate excluded zero. Data used for generating models are provided in Table S1.

RESULTS

Osmotic status

Plasma osmolality was significantly lower at 25°C versus 10°C, but there was no significant effect of salinity or mass on this response variable (Table 2). Plasma Na^+ also was significantly lower at 25°C than at 10°C, but there was no significant effect of temperature on Cl^- (Table 2). Neither plasma Na^+ nor Cl^- was affected significantly by salinity or mass (Table 2). The two organic osmolytes that were included in our study, glucose and urea, were not significantly affected by temperature, salinity or mass (Table 2). Comparisons of plasma osmolality, inorganic ions and organic osmolytes for each temperature–salinity treatment are presented in Fig. 1.

Body fluid dynamics

There was no significant effect of temperature, salinity or mass on % TBW (Table 2). Temperature had a significant effect on both WTR and DWF (Table 2); these variables were positively correlated with temperature. An increase in salinity resulted in a decrease in DWF (Table 2). There was a significant, positive correlation between mass and WTR (Table 2). A comparison of %TBW, WTR and DWF for each temperature–salinity treatment is provided in Fig. 2.

Oxygen consumption

The \dot{V}_{O_2} response to an increase in salinity was not statistically significant, but temperature had a strong, significant effect on \dot{V}_{O_2} (Table 2, Fig. 3). For terrapins exposed to low salinity (12 psu),

the average \dot{V}_{O_2} at 25°C ($68.9 \pm 34.8 \text{ ml h}^{-1}$, mean \pm s.d.) was 10.9 times higher than the average \dot{V}_{O_2} at 10°C ($6.3 \pm 4.4 \text{ ml h}^{-1}$), with a calculated Q_{10} of 4.9 over this temperature range. During exposure to high salinity, the average \dot{V}_{O_2} at 25°C ($76.6 \pm 36.4 \text{ ml h}^{-1}$) was 15.3 times higher than the average \dot{V}_{O_2} at 10°C ($5.0 \pm 3.2 \text{ ml h}^{-1}$), with a calculated Q_{10} of 6.2 over this temperature range. Mass had a significant effect on \dot{V}_{O_2} ; larger terrapins had higher \dot{V}_{O_2} (Table 2). The percentage time submerged during the respirometry trial did not have a significant effect on \dot{V}_{O_2} [$7.981e^{-4}$ ($-0.199, 0.192$); mean (95% credible interval)]. Terrapins acclimated to 25°C spent an average of $56.5 \pm 24.5\%$ and $45.8 \pm 27.0\%$ of trial time submerged at 12 psu and 35 psu, respectively. Terrapins acclimated to 10°C spent an average of $77.9 \pm 15.1\%$ and $74.9 \pm 12.4\%$ of trial time submerged at 12 psu and 35 psu, respectively.

DISCUSSION

Reptiles that inhabit estuarine environments with highly variable salinity must regulate body fluid composition to maintain proper physiological functioning. They do so through a combination of energetically efficient behavioral strategies, and physiological strategies that incur an energetic cost. The results of our study indicate that terrapins do not rely heavily on energy-requiring osmoregulatory adjustments when challenged with an acute increase in environmental salinity across a broad range of seasonally relevant temperatures. Rather, a reduction in active rates of water and salt exchange with the environment, as indicated by a decrease in DWF with exposure to high salinity, is sufficient to defend osmotic homeostasis in the short term. In general, temperature had a greater effect on osmotic status, body fluid dynamics and \dot{V}_{O_2} than did salinity.

Osmotic status

The osmotic status of terrapins was not altered by an acute increase in salinity at either temperature treatment. Maintenance of stable levels of body water and blood osmolytes in the short term is facilitated by the low rates of passive water and salt exchange over the terrapin's integument (Robinson and Dunson, 1976). Additionally, terrapins may draw on their exceptionally large extracellular water stores in interstitial fluids and hyposmotic urine in the bladder to stabilize blood osmotic pressure and regulate cell volume during periods of water deprivation or dehydration (Thorson, 1968; Robinson and Dunson, 1976; Cowan, 1981). Other studies have shown a decrease in urine volume and an increase in urine osmotic pressure occurs when terrapins are exposed to seawater (Bentley et al., 1967; Gilles-Baillien, 1970). Water conservation strategies may be effective means to stabilize

Table 2. Results of linear mixed models to assess the effects of temperature, salinity and mass on blood variables indicative of osmotic status (osmolality, Na^+ , Cl^- , urea, glucose) and body fluid dynamics (%TBW, WTR, DWF), as well as oxygen consumption in diamondback terrapins

Response	Intercept	Temperature	Salinity	Mass
Osmolality	393.64 (366.84, 420.73)	-1.54 (-2.74, -0.33)*	0.10 (-0.08, 0.28)	-0.01 (-0.04, 0.01)
Sodium	171.44 (163.00, 179.66)	-0.82 (-1.18, -0.45)*	0.00 (-0.12, 0.11)	-0.01 (-0.01, 0.00)
Chloride	116.3 (103.8, 130.3)	-0.21 (-0.78, 0.34)	-0.09 (-0.32, 0.11)	-0.01 (-0.02, 0.00)
Urea	47.86 (27.21, 68.00)	0.17 (-0.60, 0.93)	0.09 (-0.37, 0.61)	-0.01 (-0.02, 0.01)
Glucose	59.05 (38.68, 77.35)	-0.49 (-1.36, 0.31)	0.12 (-0.19, 0.45)	0.00 (-0.02, 0.02)
%TBW	66.87 (60.73, 73.10)	0.09 (-0.14, 0.33)	0.06 (-0.05, 0.18)	0.00 (-0.01, 0.01)
WTR	-61.05 (-96.53, -24.21)	3.95 (2.68, 5.29)*	-0.57 (-1.39, 0.19)	0.10 (0.07, 0.12)*
DWF	0.21 (-2.82, 3.12)	0.60 (0.49, 0.70)*	-0.07 (-0.14, -0.00)*	-0.00 (-0.00, 0.00)
\dot{V}_{O_2}	-67.05 (-108.70, -26.54)*	4.16 (2.51, 5.84)*	0.15 (-0.04, 0.33)	0.05 (0.01, 0.07)*

TBW, total body water; WTR, water turnover rate; DWF, daily water flux; \dot{V}_{O_2} , oxygen consumption.

Model estimates are the posterior means and the values in parentheses are the 95% credible interval. Negative and positive values for 0.00 represent values that were beyond significant figures. *Statistically significant.

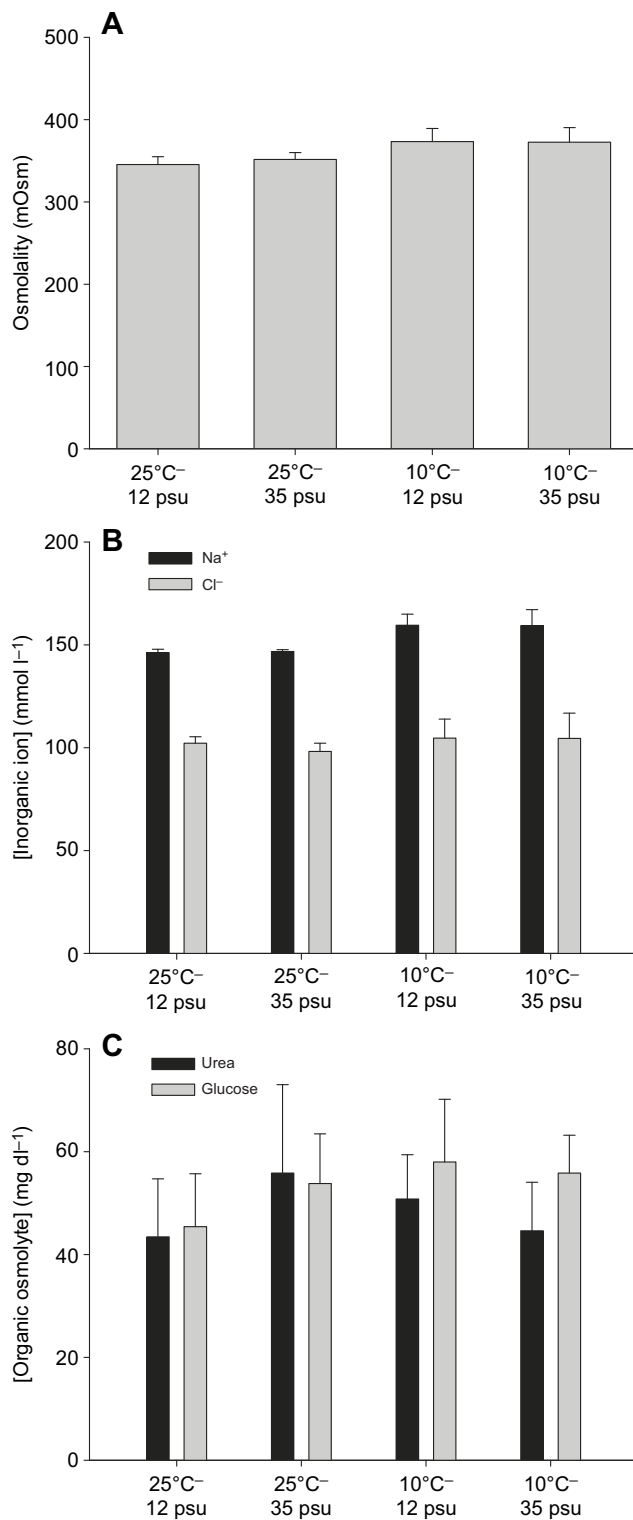


Fig. 1. Osmotic status of terrapins during exposure to four temperature-salinity treatments. (A) Results of linear mixed models indicated that plasma osmolality was significantly lower in terrapins acclimated to 25°C ($n=6$) versus 10°C ($n=5$), but salinity did not have a significant effect on plasma osmolality. (B) Temperature had a significant effect on inorganic ion concentration, with higher temperatures resulting in lower $[\text{Na}^+]$; however, there was no significant effect of temperature on $[\text{Cl}^-]$. There was no significant effect of salinity on $[\text{Na}^+]$ or $[\text{Cl}^-]$. (C) Neither temperature nor salinity had a significant effect on the concentration of the organic osmolytes urea and glucose. Data are presented as means \pm s.d. Model estimates are presented in Table 2.

blood osmotic pressure during initial exposure to high salinity, but prolonged exposure (i.e. months) results in a gradual increase in plasma osmolality and Na^+ concentration, and weight loss indicative of dehydration (Cowan, 1974; Robinson and Dunson, 1976). Several other species of estuarine and marine reptiles also exhibit weight loss in response to long-term exposure to seawater (Dunson, 1970; Lillywhite and Ellis, 1994; Lillywhite et al., 2008). These observations suggest that periodic access to freshwater or lower salinity water is a critical component of water balance for estuarine reptiles. Reptiles that inhabit fully marine environments, such as sea turtles, marine iguanas and pelagic sea snakes, have a greater capacity for ion excretion via the salt glands compared with estuarine reptiles (Holmes and McBean, 1964; Dunson, 1969, 1970; Reina and Cooper, 2000; Reina et al., 2002), and can osmoregulate effectively without access to freshwater.

Although we found no significant effect of acute salinity exposure on osmotic variables, 10°C-acclimated terrapins had significantly higher blood osmolality and Na^+ concentrations compared with 25°C-acclimated terrapins. Gilles-Baillien (1973) documented an increase in blood osmolality of terrapins overwintering in seawater in the laboratory, but this was due to an increase in urea rather than inorganic ions. Use of urea as an osmoeffector to maintain water balance during exposure to desiccating conditions has been documented for other species of turtles (*Pelodiscus sinensis*; Lee et al., 2006), and even anurans that tolerate brackish water habitats (*Rana cancrivora*, *Xenopus laevis*, *Bufo viridis*; Gordon et al., 1961; Balinsky, 1981). In contrast, we found no significant effect of either temperature or salinity on urea. Our results for temperature effects on osmotic status are more in line with results obtained from overwintering terrapins under natural conditions. Harden et al. (2015) found that overwintering terrapins in North Carolina marshes exhibited an increase in blood osmolality and Na^+ with a decrease in carapace temperature. The gradual increase in osmolality and Na^+ over the course of prolonged cold exposure may reflect progressive water loss or ion gain via passive exchange with the environment. A decrease in active ion transport mechanisms with a temperature-induced decrease in metabolic capacity may also play a role (Baker et al., 2013; Southwood Williard and Harden, 2011). Blood osmolality values for 10°C-acclimated terrapins in our study (354–400 mOsm) were somewhat higher than mean monthly values reported for terrapins overwintering in the high marsh in coastal North Carolina (318–345 mOsm). Terrapins in our study were maintained in water with no haul-out platforms, so the discrepancy between studies may be due in part to the inability of our captive terrapins to use aquatic-terrestrial shuttling behavior to modulate water and salt exchange with the environment. Under natural conditions, terrapins bury in the mud of the subtidal or intertidal zones and are rarely found in open water when temperatures drop below 20°C (Yearicks et al., 1981; Butler, 2002; Harden and Williard, 2012). Overwintering in terrestrial habitats may reduce passive exchange of water and salts across the integument (Davenport and Magill, 1996).

Body fluid dynamics

We observed a significant decrease in DWF and a trend towards decreased WTR in terrapins exposed to high salinity, indicative of a decrease in ingestion of water and food (Harden et al., 2014). Previous studies have demonstrated that terrapins readily consume freshwater or brackish water (<20 psu), but will not drink water at higher salinities (Bentley et al., 1967; Cowan, 1981; Davenport and Macedo, 1990). Preference for low salinity drinking water is a common characteristic of turtles (Dunson and Moll, 1980;

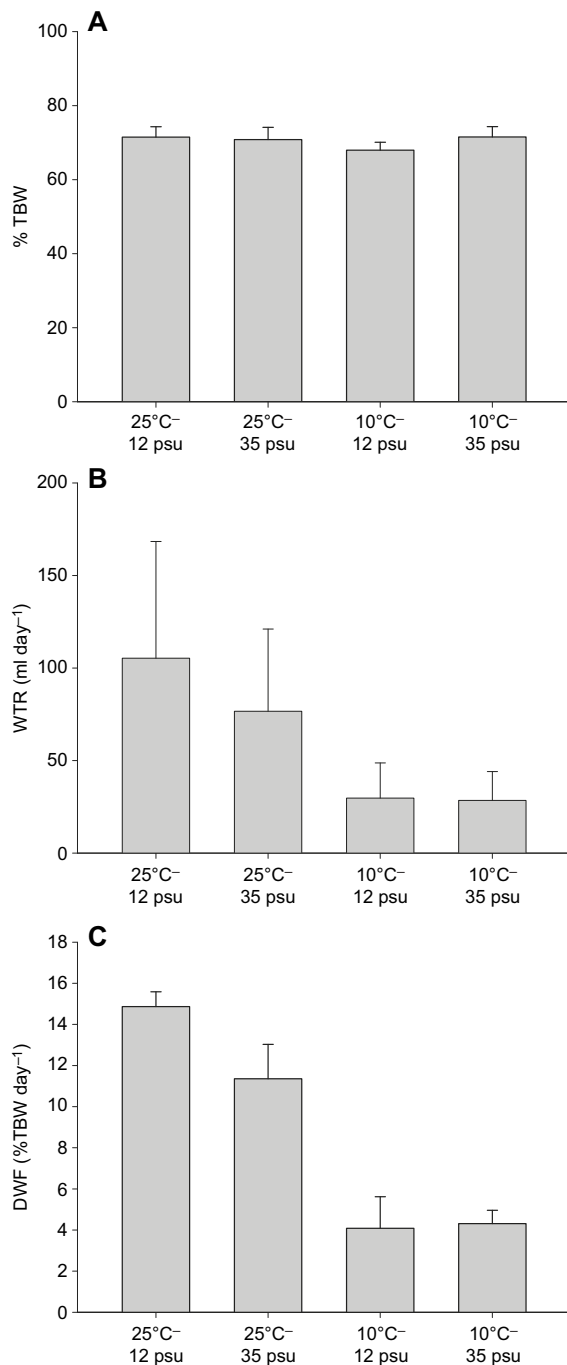


Fig. 2. Temperature and salinity have statistically significant effects on body water dynamics of terrapins. (A) Results of linear mixed models indicated that the percentage total body water (%TBW) remained stable across temperature–salinity treatments but (B) water turnover rate (WTR) significantly increased with temperature. (C) Daily water flux (DWF) significantly increased with temperature and decreased with salinity. Data are presented as means \pm s.d. Model estimates are presented in Table 2.

Davenport and Wong, 1986; Davenport and Macedo, 1990), snakes (Lillywhite and Ellis, 1994; Lillywhite et al., 2008) and crocodylians (Taplin et al., 1999) that utilize brackish water or estuarine habitats. Discrimination in drinking water is an important behavioral component of water balance and osmoregulation for terrapins and other estuarine species, as the efficacy of salt glands in estuarine reptiles is much lower than that of fully marine forms (Dunson, 1970). For example, the maximum Na^+ secretion rate of sea turtle

salt glands ($415\text{--}484 \mu\text{mol } 100 \text{ g}^{-1} \text{ h}^{-1}$) is 13–15 times higher than that of the terrapin salt gland ($31 \mu\text{mol } 100 \text{ g}^{-1} \text{ h}^{-1}$; Cowan, 1990; Reina and Cooper, 2000; Reina et al., 2002). Green turtles (*Chelonia mydas*) and leatherback turtles (*Dermochelys coriacea*) are capable of drinking seawater and excreting excess ions via the salt gland to effectively osmoregulate (Holmes and McBean, 1964; Marshall and Cooper, 1988; Reina et al., 2002). In contrast, the terrapin uses evasive behavioral strategies to reduce salt ingestion during seawater exposure, tolerates progressive dehydration, and relies on periodic access to freshwater to restore depleted body water stores (Bentley et al., 1967; Dunson, 1970; Dunson and Mazzotti, 1989).

Appetite suppression has been documented for terrapins exposed to seawater with no access to freshwater (Dunson, 1985; Davenport and Ward, 1993; Holliday et al., 2009). Terrapins in our study were offered food every other day during treatment exposure; we did not record the exact amount of food eaten but noted whether or not the entire ration was consumed. Terrapins acclimated to 25°C ate their entire ration throughout exposure to 12 psu, but left food uneaten in their tanks at 35 psu. Terrapins acclimated to 10°C did not eat their entire food ration, either at 12 psu or at 35 psu. These observations, in conjunction with the DWF results, show that reduced food intake at high salinity may contribute to maintenance of osmotic balance, particularly when supplemental sources of freshwater are not available to offset salt intake, as was the case in our study. Appetite suppression could result in a mismatch between energy supply and demand, particularly at warmer temperatures; therefore, this evasive strategy may not be sufficient to deal with osmotic stress over the long term. Several investigators have noted that terrapins cannot survive indefinitely without access to freshwater because of progressive dehydration and weight loss (Dunson, 1985; Dunson and Mazzotti, 1989; Davenport and Ward, 1993). Under natural conditions, terrapins exhibit hypophagy in combination with mud burial and reduced activity during winter dormancy. This suite of behavioral changes contributes to the terrapin's ability to maintain osmotic balance during a period when both metabolic capacity and resource availability are low (Harden et al., 2015). We found that both WTR and DWF decreased with temperature, which likely reflects a decrease in both passive water exchange across the integument and ingestion of water and food. The decrease in water turnover at lower temperature may also reflect lower metabolic and respiratory rates.

Oxygen consumption

We predicted that acute exposure to high salinity would result in an increase in energy expenditure as a result of salt gland activation or other ion transport mechanisms to maintain osmotic balance; however, when temperature, percentage time submerged and mass were controlled for, we found no significant effect of salinity on \dot{V}_{O_2} . While not statistically significant, the overall trends in the \dot{V}_{O_2} response to salinity differed between temperature treatments (Fig. 3). During acute exposure to high salinity, 25°C-acclimated terrapins increased \dot{V}_{O_2} by an average of $12.8 \pm 8.2\%$ and 10°C-acclimated terrapins decreased \dot{V}_{O_2} by an average of $10.9 \pm 28.1\%$ (Fig. 3). Variation in the metabolic response to salinity may indicate that temperature-induced differences in metabolic capacity influence strategies used to maintain osmotic balance during exposure to high salinities. The relatively low sample size of our study may have hampered our ability to detect statistically significant effects of salinity on \dot{V}_{O_2} , particularly given the level of inter-individual variation observed.

Our results for the effect of salinity on \dot{V}_{O_2} are in agreement with those of Holliday et al. (2009), who found no significant change in

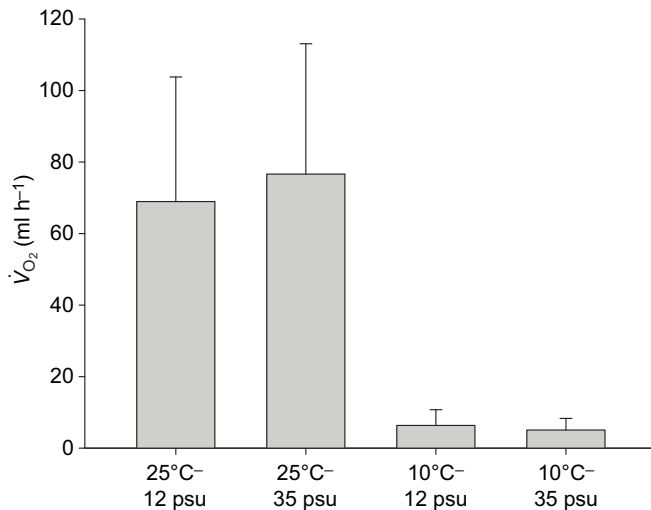


Fig. 3. Oxygen consumption measurements for terrapins in each of the four temperature–salinity treatments. Results of linear mixed models indicated that temperature had a significant effect on \dot{V}_{O_2} at the 95% credible interval level, but there was no statistically significant effect of salinity on \dot{V}_{O_2} . Data are presented as means \pm s.d. Model estimates are presented in Table 2.

metabolic rate, measured as CO_2 production, of 26°C-acclimated juvenile terrapins over a broad range of salinities (0–30 psu). These investigators did note, however, that higher salinities resulted in decreased growth rate of terrapins. It is possible that energy for growth is reallocated to meet osmoregulatory needs when terrapins are exposed to high salinities, and therefore the cost of osmoregulation is not reflected by an increase in metabolic rate (Holliday et al., 2009). Annual growth rates for adult terrapins are exceptionally low (Ernst and Lovich, 2009); nevertheless, it is feasible that energy to support osmoregulatory mechanisms is reallocated from other physiological functions. In contrast with juvenile and adult terrapins, the metabolic rate of hatchling terrapins is significantly affected by salinity. Rowe (2018) found that peak standard metabolic rate (SMR) of hatchling terrapins occurred at a salinity of 8 psu, which is similar to the salinity for peak growth rates (9 psu; Dunson, 1985); salinities greater than 8 psu resulted in lower peak SMR for hatchlings. The osmoregulatory strategy of hatchling terrapins is likely to differ substantially from that of adults, given that early life stage terrapins primarily inhabit high marsh vegetation and avoid open water (Muldoon and Burke, 2012; Selman, 2018). Promotion of growth in a low osmotic stress environment may be an important component of habitat selection in younger terrapins, particularly given the inverse relationship between rates of passive water efflux and body size in terrapins (Dunson, 1985).

Terrapins in our study did not exhibit a significant increase in \dot{V}_{O_2} with an acute increase in salinity; therefore, it is unlikely that they rely heavily on energy-requiring mechanisms to secrete accumulated salts as part of the initial response to a salinity challenge. While water conservation measures and behavioral adjustments to reduce water and salt exchange with the environment are sufficient to maintain osmotic balance in the short term, prolonged exposure to high salinity or progressive accumulation of salts may trigger physiological mechanisms to actively regulate blood ion concentrations (Bentley et al., 1967; Dunson, 1970; Gilles-Baillien, 1973; Dunson and Dunson, 1975). Terrapins acclimated to seawater for a period of weeks to months exhibit an increase in the concentration of Na^+ in salt gland secretions

(Dunson, 1970), salt gland Na^+/K^+ -ATPase activity (Dunson and Dunson, 1975) and \dot{V}_{O_2} (Bentley et al., 1967). Dunson and Dunson (1975) noted that a marked activation of Na^+/K^+ -ATPase does not occur until blood Na^+ concentration exceeds 200 mmol l⁻¹, a level not reached unless supplemental salt injections were given to seawater-acclimated terrapins. The blood Na^+ concentration of terrapins in our study was well below this level in all temperature–salinity treatments (145–169 mmol l⁻¹), and Na^+ concentrations in wild terrapins under natural conditions typically do not exceed 200 mmol l⁻¹ (Harden et al., 2015). The results from our study illustrate that terrapins initially respond to a salinity challenge by adjusting behavior to reduce water and salt exchange with the environment, thereby delaying activation of energy-requiring physiological mechanisms.

The exceedingly high levels of blood Na^+ necessary to trigger an increase in salt gland secretions in terrapins calls into question the degree to which terrapins routinely rely on the salt gland for osmotic homeostasis. Estuarine and marine snakes exhibit hypernatremia under natural and laboratory conditions, and Brischox et al. (2013) hypothesized that tolerance of high blood Na^+ concentrations in reptiles would reduce energetic costs associated with active ion transport in the salt gland. If this is the case, then the salt gland may serve to prevent dangerously high levels of blood ions but not contribute significantly to osmoregulation if energetically efficient behavioral options are available. It is interesting to note that terrapins show very little phenotypic flexibility in salt gland morphology in response to seawater exposure or increases in blood osmolality (Cowan, 1974; Dunson and Dunson, 1975). This is in contrast to marine birds, sea turtles and estuarine crocodiles, which exhibit increases in salt gland size and/or blood flow to the salt gland in response to acclimation to seawater or salt loading (Schmidt-Nielsen and Kim, 1964; Shuttleworth and Hildebrandt, 1999; Reina, 2000; Hildebrandt, 2001; Cramp et al., 2008). The lack of phenotypic flexibility in the terrapin salt gland may indicate that this structure is not as integral to osmotic balance in terrapins as it is in other species.

Temperature had a strong effect on \dot{V}_{O_2} over the range 10–25°C. Terrapins along the southeast coast of the USA typically enter winter dormancy at temperatures below 20°C (Yearicks et al., 1981; Butler, 2002; Harden and Williard, 2012), so the large Q_{10} values (4.9–6.2) we observed likely reflect the decrease in activity and feeding associated with seasonal downregulation of metabolism. Baker et al. (2013) observed a much lower Q_{10} of 1.73 for \dot{V}_{O_2} of hatchling terrapins in air over the range 10–20°C; however, these investigators did not control for the effects of activity during trials and reported high values of \dot{V}_{O_2} relative to other studies (Bentley et al., 1967). In our study, the large difference in \dot{V}_{O_2} between 10°C- and 25°C-acclimated terrapins also may reflect temperature-induced differences in feeding behavior. We fasted terrapins for 40–48 h prior to respirometry trials, based on previously published studies; Davenport and Ward (1993) reported that maximum appetite in terrapins occurs 48 h post-feeding, and \dot{V}_{O_2} of aquatic Chinese stripe-necked turtles (*Ocadia sinensis*) fed shrimp and mealworms was significantly higher than that of un-fed controls until ~45 h post-feeding (Pan et al., 2005). It is possible that the fasting period chosen for our study was not sufficient to insure a post-absorptive state in all respirometry trials, particularly given the variability in feeding behavior at the different treatment temperatures.

Conclusions

An understanding of the balance of behavioral and physiological adjustments in the osmoregulatory strategy of terrapins provides

insight into the evolutionary transition from freshwater to seawater environments. Dunson and Mazzotti (1989) proposed that the evolution of fully marine reptiles from freshwater ancestors progressed through several transitional stages related to the capacity for osmoregulation. Initial exploration of brackish environments was facilitated by behavioral adjustments to avoid high salinities and reduce passive and active exchange of water and salts with the environment (Dunson and Mazzotti, 1989; Agha et al., 2018). Extrarenal glands to facilitate salt excretion evolved in more advanced forms, and subsequent specialization of salt glands to accommodate higher rates of salt intake are evident in fully marine species (Dunson, 1970). Evidence from our research and other studies shows that terrapins represent an intermediate form in this progression. Terrapins effectively maintain osmotic balance under variable salinity conditions by using energetically efficient behavioral adjustments and water conservation strategies. Although terrapins possess lachrymal salt glands, they are still dependent on periodic access to freshwater or low salinity water in order to maintain osmotic balance and cannot survive in seawater indefinitely. This aspect of terrapin biology makes them particularly vulnerable to projected changes in coastal environments with climate change and sea level rises (Agha et al., 2018). Terrapins are a species of conservation concern (Hart and Lee, 2006), and additional studies to assess the implications of their osmoregulatory strategy for habitat utilization and resilience are warranted.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.S.W., L.A.H.; Methodology: A.S.W., L.A.H., T.T.J.; Formal analysis: A.S.W., S.R.M.; Investigation: A.S.W.; Resources: T.T.J.; Writing - original draft: A.S.W.; Writing - review & editing: L.A.H., T.T.J., S.R.M.; Visualization: S.R.M.; Project administration: A.S.W.; Funding acquisition: A.S.W.

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Supplementary information

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