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RESEARCH ARTICLE

Total body water and water turnover rates in the estuarine diamondback terrapin (*Malaclemys terrapin*) during the transition from dormancy to activity

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ABSTRACT

Water and salt concentrations in an animal's body fluids can fluctuate with changing environmental conditions, posing osmoregulatory challenges that require behavioral and physiological adjustments. The purpose of this study was to investigate body water dynamics in the estuarine diamondback terrapin (*Malaclemys terrapin*), a species that undergoes seasonal dormancy in salt marsh habitats. We conducted a field study to determine the total body water (%TBW), water turnover rate (WTR) and daily water flux (DWF) of female terrapins in south eastern North Carolina pre- and post-emergence from winter dormancy. Terrapins were injected with [^{2}H]deuterium on two occasions and washout of the isotope was monitored by taking successive blood samples during the period of transition from dormancy to activity. The WTR and DWF of dormant terrapins were significantly lower than those of active terrapins ($WTR_{dormant}$=49.70±15.94 ml day^{-1}, WTR_{active}=100.20±20.36 ml day^{-1}, $DWF_{dormant}$=10.52±2.92%TBW day^{-1}, DWF_{active}=21.84±7.30%TBW day^{-1}). There was no significant difference in %TBW between dormant and active terrapins (75.05±6.19% and 74.54±4.36%, respectively). The results from this field study provide insight into the terrapin's ability to maintain osmotic homeostasis while experiencing shifts in behavioral and environmental conditions.

KEY WORDS: Marine reptile, [^{2}H]deuterium, Dehydration, Osmoregulation, Homeostasis, Overwinter, Water flux, Salt accumulation

INTRODUCTION

Marine vertebrates are hypotonic to their environment and therefore face the continuous osmoregulatory challenges of dehydration and excess salt accumulation. Unlike marine mammals, marine reptiles cannot produce urine that is hyperosmotic to blood (Hildebrandt, 2001). Instead, they rely on extrarenal physiological mechanisms, morphological features and behavioral strategies to maintain blood osmolality lower than that of seawater (Evans, 2009). While the osmotic environment of obligate marine species remains relatively stable, estuarine species may encounter fluctuations in salinity due to tidal influences and freshwater availability. Several reptilian species take advantage of the temporal availability of resources in estuaries (Dunson, 1970; Dunson, 1980; Dunson, 1986; Ellis, 1981; Taplin et al., 1982; Mazzotti et al., 1986; Lillywhite and Ellis, 1994; Leslie and Spotila, 2000; Lee et al., 2006), but there are very few that live entirely within the estuarine habitat. Of the estuarine turtles, the diamondback terrapin, *Malaclemys terrapin* (Schwartz 1955), is the only species endemic to estuarine habitats in the temperate zone (Hart and Lee, 2006; Rasmussen et al., 2011), and can tolerate brackish to hypersaline conditions. This North American emydid turtle occurs exclusively in tidally influenced coastal salt marshes, bays, lagoons, mud and grass flats, and creeks from Cape Cod, MA, USA, to Corpus Christi, TX, USA (Ernst et al., 1994).

Terrapins regulate body fluid composition through a combination of active salt excretion via the cephalic salt glands (Schmidt-Nielsen and Fange, 1958; Bentley et al., 1967; Cowan, 1981), low integument permeability (Bentley et al., 1967), opportunistic drinking of fresh or brackish water (Davenport and Macedo, 1990), and modifications in behavior (Dunson and Seidel, 1986). Seasonal shifts between aquatic and terrestrial habitats may also play a role in osmoregulation (Spivey, 1998; Butler, 2002; Haramis et al., 2011; Southwood Williard and Harden, 2011; Harden and Williard, 2012; Akins et al., 2014). Terrapins are most active during the warm months of late spring, summer and early autumn, when they may be found swimming and foraging in flooded marshes at high tide (Tucker et al., 1995; Whitelaw and Zajac, 2002; Harden and Williard, 2012). As temperatures decrease in the autumn, terrapins enter a period of winter dormancy in which they cease eating, drastically reduce activity levels and spend the large majority of their time buried shallowly in the mud of the subtidal or intertidal zone (Harden and Williard, 2012; Akins et al., 2014). Davenport and Magill (Davenport and Magill, 1996) proposed that under certain conditions mud burial could reduce rates of water loss in terrapins. The shift to upland and intertidal mud habitats may be particularly advantageous during colder winter months when metabolic processes are slowed as a result of Q_{10} (thermal) effects and/or downregulation (Southwood Williard and Harden, 2011), making water and salt balance more difficult to actively maintain (Gilles-Baillien, 1973).

The ability of terrapins to regulate body fluid composition under various seasonal environmental regimes of the salt marsh ecosystem has not previously been investigated. Total body water and rates of water turnover for animals in a natural environment may be assessed using the stable isotope [^{2}H]deuterium (Nagy, 1989; Speakman, 1997; Jones et al., 2009). This technique involves injecting the study animal with water enriched with [^{2}H]deuterium and taking sequential blood samples to monitor the [^{2}H]deuterium level of the animal's body water over time. While the animal is at large in its natural habitat, water influx (due to ingestion or metabolic processes) will dilute the [^{2}H]deuterium in the body water. Additionally, [^{2}H]deuterium in the animal's body water will be lost

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List of symbols and abbreviations

%TBW	total body water (%)
DWF	daily water flux (%TBW day^{-1})
$E_{2,4,6h,mix}$	equilibrium [^{2}H]deuterium enrichment levels
E_{final}	final [^{2}H]deuterium enrichment levels
E_{wat}	background [^{2}H]deuterium enrichment levels
k_d	water turnover rate
N_d	isotope dilution space
T_c	carapace temperature
WTR	water turnover rate (ml day^{-1})

to the environment via evaporation, excretion and salt gland secretion (Jones et al., 2009). Deuterium has been used with success to determine water flux for several species of terrestrial chelonians (Nagy and Medica, 1986; Peterson, 1996; Henen, 1997; Penick et al., 2002; Jodice et al., 2006) and marine turtles (Oritz et al., 2000; Wallace et al., 2005; Southwood et al., 2006; Clusella Trullas et al., 2006; Jones et al., 2009); however, there have been very few studies on freshwater and/or semi-aquatic chelonians (Booth, 2002; Roe et al., 2008).

We conducted a field study to determine the total body water (TBW), water turnover rate (WTR) and daily water flux (DWF) of female diamondback terrapins in south eastern North Carolina immediately prior to and following emergence from winter dormancy. Terrapins were injected with [^{2}H]deuterium on two occasions and washout (exchange) of the isotope was monitored by taking successive blood samples during the period of transition from dormancy to activity. We hypothesized that WTR and DWF in terrapins would significantly increase upon emergence from dormancy as terrapins initiated basking and foraging behaviors, and, subsequently, TBW (%) would also increase during this seasonal shift in activity.

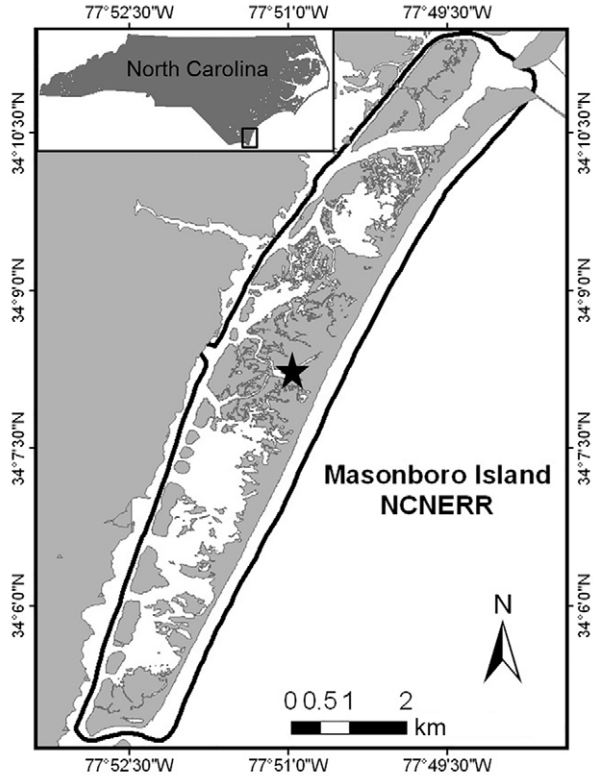

Fig. 1. Map of Byron's Creek on the landward side of Masonboro Island North Carolina Estuarine Research Reserve (NCNERR) in south eastern North Carolina. The reserve boundary is outlined with a thick, black line, and the starred area within the reserve marks the location of the enclosure.

RESULTS

We monitored terrapins within a semi-natural enclosure at Masonboro Island, NC, USA (Fig. 1) throughout the winter and observed all individuals buried below the mud surface between 8 December 2011 and 15 March 2012, suggesting that they were dormant for at least some portion of this time (Harden, 2013). Periodic movement of terrapins over the course of the winter was suggested by multiple burial sites for individual terrapins. All terrapins (N=10) were buried in the mud immediately prior to the first [^{2}H]deuterium dosing on 15 March. By 29 March (second [^{2}H]deuterium dosing event), three terrapins were observed basking on the mud surface or swimming in the shallow creek bed water, indicating an increase in activity for some animals. By 5 April, five of 10 terrapins were observed active on the surface, suggesting a progressive emergence from dormancy over the course of the sampling regime. Based on these findings and our own observations using radio telemetry within the enclosure, we designated interval 1 (15–29 March) as the time period when terrapins were less active and interval 2 (29 March to 5 April) as the time period when terrapins were more active. From this point forward, we refer to interval 1 as the 'dormant' time period and interval 2 as the 'active' time period (see Fig. 2), with the understanding that there may have been a gradual transition in activity level that spanned the two intervals. These observed habitat and activity shifts are supported by previous radio telemetry and temperature data logger research that documented infrequent movements in the winter and timing of spring emergence for free-ranging terrapins (Harden and Williard, 2012; Akins et al., 2014), and are further supported by seasonal shifts in terrapin blood biochemistry (Harden, 2013).

Over the course of the study, we measured tidal creek salinity and rainfall in close proximity to the terrapin enclosure at 30 min intervals. During the transition from dormancy to activity (15 March to 5 April), mean (±s.e.) salinity (psu) was 31.8±2.1; however, between 28 and 29 March, salinity fluctuated between 25 and 35 (Fig. 3). Additionally, there was periodic rainfall during this same time period, notably 4.1 mm on 19 March and 3.6 mm on 31 March (Fig. 3). Carapace temperatures (T_c) recorded by temperature data loggers (iButtons) were available for four of the terrapins from this study. These terrapins experienced a combined mean T_c of 21.2±0.2°C during the dormant period and a combined mean T_c of 21.3±0.2°C during the active period.

Detailed results from isotopic analyses, including [^{2}H]deuterium turnover (washout) rate (k_d), dilution space (N_d), %TBW, WTR and DWF for each terrapin, are summarized in supplementary material Table S1. WTR and DWF of dormant terrapins were significantly lower than those of active terrapins (Fig. 4A,B; F=26.33, P=0.0001; F=17.51, P=0.0009, respectively). More specifically, WTR increased from 49.70±15.94 to 100.20±20.36 ml day^{-1} during emergence from dormancy and DWF increased from 10.52±2.92 to 21.84±7.30%TBW day^{-1}. There was no significant difference in %TBW between dormant and active terrapins (Fig. 4C; t=0.13, P=0.9022). Moreover, %TBW for all terrapins fell within the normal range of 60−80% (%TBW$_{dormant}$=75.05±6.19, %TBW$_{active}$=74.54±4.36) that has been documented in other semi-aquatic turtles (Minnich, 1982; Crawford, 1994; Roe et al., 2008). Terrapin mass decreased significantly between 15 and 29 March 2012 (Z=–2.65, P=0.008).

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110411

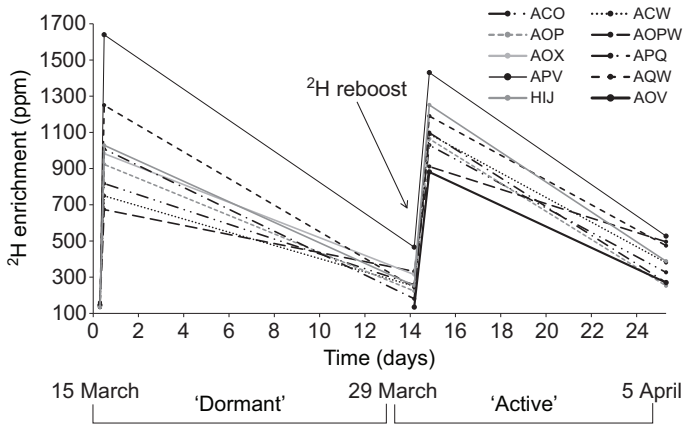

Fig. 2. Deuterium enrichment values of 10 dormant (15–29 March) and active (29 March to 5 April) female terrapins. Enrichment levels given at day 0 (15 March 2012) are background [^{2}H]deuterium (E_{wat}) and equilibration (E_{mix}). Enrichment levels given at day 14 (29 March 2012) are E_{final} and E_{mix} after [^{2}H]deuterium reboost. Enrichment levels at day 25 (5 April 2012) are E_{final}. All terrapins were buried immediately prior to initial injection of [^{2}H]deuterium on 15 March 2012 (i.e. 'dormant'), but terrapins began to emerge from dormancy during late March/early April 2012 (between 29 March and 5 April, i.e. 'active'). Turtle ID (3-letter code) is given in the key.

DISCUSSION

Terrapins in this study exhibited seasonal differences in activity patterns and habitat use (Harden and Williard, 2012; Akins et al., 2014) that are similar to patterns observed for other semi-aquatic turtle species at temperate latitudes (Grayson and Dorcas, 2004; Litzgus et al., 2004; Tuma, 2006; Harden et al., 2009; Pittman and Dorcas, 2009; Rowe and Dalgarn, 2009). The shift from quiescent burial to surface activity underlies the significant increase in terrapin body water flux observed post-dormancy. Elevated activity levels, increased aquatic habitat use for breeding and foraging (Siegel, 1980; Harden and Williard, 2012), and increased exchange of water and salts with the environment may all lead to increases in WTR and DWF. Onset of foraging upon emergence from dormancy may have a particularly large impact on body water flux, as a result of salt ingestion via invertebrate prey items and incidental drinking. This hypothesis concurs with changes in terrapin plasma K^+ and glucose concentrations documented pre- and post-emergence from dormancy (Harden, 2013). Concentrations of plasma K^+ in April are noticeably higher than concentrations measured between October and March

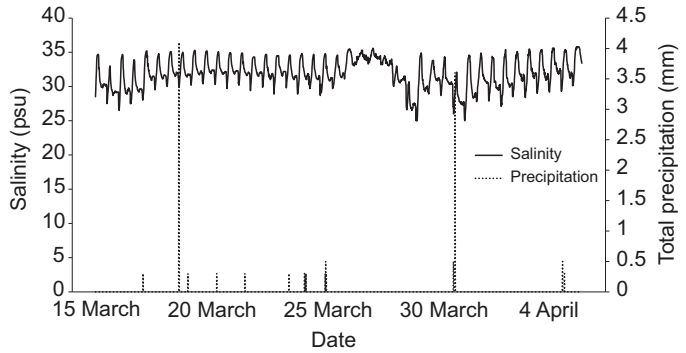

Fig. 3. Salinity (psu) and total precipitation (15 March to 5 April). Salinity was measured by a National Oceanic and Atmospheric Administration, Office of Ocean and Coastal Resource Management, National Estuarine Research Reserve System-wide Monitoring Program station located in Masonboro Island's Research Creek, 2 km from our Byron's Creek terrapin enclosure.

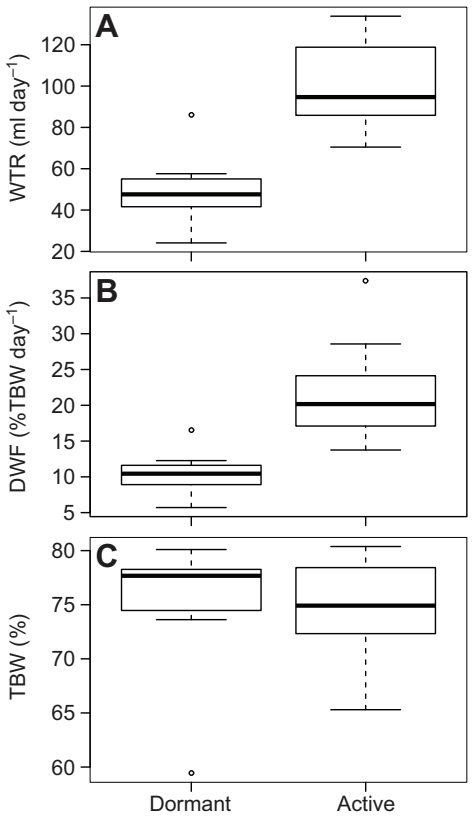

Fig. 4. Water relations of 'dormant' (15–29 March) and 'active' (29 March to 5 April) female terrapins. Data were calculated using equations from chapter 17 of Speakman (Speakman, 1997) (see Appendix, Eqns A1–A5). (A) Water turnover rate (WTR, Eqn A4) of nine terrapins, calculated using [^{2}H]deuterium turnover rates (k_d, Eqn A1) and the dilution space (N_d, Eqn A2). (B) Daily water flux (Eqn A5) of eight terrapins, calculated using Eqn A1 and total body water (%TBW) of terrapins. (C) %TBW (Eqn A3) of eight terrapins, calculated using Eqn A2 and mass of terrapins. In all three plots, the bold lines within each box represent the median of the data, the upper and lower edges of the boxes represent the 75% and 25% quartiles, respectively, the whiskers represent the minimum and maximum, and the open circles represent outliers (defined as Q3+1.5×IQR and Q1–1.5×IQR, where IQR is the interquartile range).

(Harden, 2013), suggesting an onset of feeding on isosmotic prey [e.g. *Uca pugilator* and *Littorina littorea* (Holmes and McBean, 1964; McCance and Shipp, 1933)] following a prolonged fast, as hypothesized by Gilles-Baillien (Gilles-Baillien, 1973). The increase in blood glucose upon emergence has been well documented among reptiles as an indicator of glycogenolysis and of increased intake of dietary carbohydrates, both of which are essential for fueling post-dormancy activity (Emerson, 1967; Dessauer, 1970; Crawford, 1994; Moon et al., 1999; Pereira et al., 2013). There is little evidence to suggest that the increase in terrapin WTR and DWF post-dormancy was due to a detoxifying response, as no significant increases in plasma osmolality or osmolytes have been documented in field studies of terrapins overwintering in salt marsh environments (see Harden, 2013).

The environmental or internal cues that lead to emergence from dormancy and increased body water flux have not been well investigated in terrapins. A decrease in tidal creek salinity between 28 and 29 March and corresponding notable precipitation events between 15 March and 5 April (Fig. 3) may have contributed to the increase in WTR and DWF observed in our study. Davenport and

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110411

Ward (Davenport and Ward, 1993) found that when nutrient-deprived terrapins were given access to freshwater, they consumed 7.2% of their body mass in prey in 48 h, suggesting freshwater triggers an increase in food ingestion. Terrapins have the ability to exploit rainfall by drinking thin films of freshwater from the surfaces of mud flats and water columns (Davenport and Macedo, 1990; Bels et al., 1995). They respond to surface vibrations of rainfall and have been observed drinking continuously from the surface substratum (as little as 1−6 mm thick) for up to 30 min, with the capability of fully rehydrating within 15 min (Davenport and Macedo, 1990). Terrapins exhibit acute salinity discrimination when it comes to drinking and can gain anywhere from 0% body mass (if offered 27−34 ppt salt water only) to 13.6% body mass (if offered 0−6.8 ppt salt water only) within a 30 min period. Furthermore, Davenport and Ward (Davenport and Ward, 1993) found that when nutrient-deprived terrapins at >20°C were given access to freshwater, they exhibited an increase in appetite and food ingestion that may also contribute to an increase in body water flux.

Despite increases in terrapin water flux between the dormant and active periods, there was no significant change in %TBW, suggesting terrapins maintain osmotic homeostasis during this seasonal shift in behavior. Other isotope-based water balance studies have documented osmotic homeostasis (i.e. stable %TBW) in free-ranging reptiles between wet–dry seasons, but most were tropical species experiencing stable ambient temperatures year-round [Table 1; *Chlamydosaurus kingie* (Christian and Green, 1994); *Crocodylus johnstoni* (Christian et al., 1996); *Chelonia mydas* (Southwood et al., 2006); *Chelodina longicollis* (Roe et al., 2008)]. There are just as many turtle species that experience a disruption in body water balance during periods of reduced activity due to drought or extreme temperatures. Peterson (Peterson, 1996) found that desert tortoises (*Gopherus agassizii*) tolerated temporary osmotic ‘anhomeostasis’ (e.g. reduced WTR, reduced %TBW and elevated solute concentrations; Table 1) during prolonged periods of seasonal drought until the first rainfall, when opportunistic drinking allowed them to recuperate lost body water. These physiological and behavioral drought responses in desert tortoises (Peterson, 1996) were also supported by Nagy and Medica (Nagy and Medica, 1986) and Henen (Henen, 1997). Furthermore, a study on overwintering painted turtles (*Chrysemys picta*) found that %TBW upon spring emergence was higher than that of pre-dormancy, and suggested it may have been due to osmotic and/or metabolic water (Crawford, 1994). These findings suggest many turtle species are able to tolerate seasonal osmotic anhomeostasis in order to reduce energy expenditure during periods of environmental stress.

To make comparisons with previously published laboratory studies investigating terrapin water and salt balance, we converted our WTRs to ml 100 g^{-1} h^{-1} (or hourly water flux; see Table 1 for all values). We found that hourly water flux for adult female terrapins during and post-dormancy was higher than that reported by Robinson and Dunson (Robinson and Dunson, 1976) for small adult males. The observed difference may be due to the fact that terrapins in the laboratory study were unfed and were exposed to steady changes in salinity as part of the experimental protocol. Water flux of terrapin hatchlings and yearlings (Dunson, 1985) was greater than water flux rates observed in our study, as might be expected given the differences in surface area to volume ratios between immature and adult terrapins. Mean %TBW of terrapins in our study was similar to that of terrapin hatchlings (Dunson, 1985), but higher than that of small adult male terrapins as determined by desiccation and by radiotracers (Robinson and Dunson, 1976). These previous

Table 1. A comparative table summarizing findings of previous turtle water balance studies using isotope techniques (DLW, ^2H, ^3H)

Study	Species	Mass (g)	%TBW	Method	WTR	WTR units	DWF (%TBW day^{-1})
This study	*Malaclemys terrapin*	317–658	Dormant=75.05±6.19; active=74.54±4.36	^2H	Dormant=49.70±15.94; active=100.20±20.36	ml day^{-1}	Dormant=10.52±2.92; active=21.84±7.30
Dunson, 1985	*Malaclemys terrapin*	7.0–84	77.0	^3H	1.0±0.1	ml 100 g h^{-1}	NA
Robinson and Dunson, 1976	*Malaclemys terrapin*	224–270	59.9±6.2 (desiccation); 64.5 (titrated water)	Desiccation, tritiated H_2O	0.16±0.05	ml 100 g h^{-1}	NA
Ortiz et al., 2000	*Lepidochelys kempii*	10,000±1100	78.4±1.3	^2H	123.0±6.8	ml kg day^{-1}	16
Southwood et al., 2006	*Chelonia mydas*	9800–23,800	Summer=73.7±0.9; winter=75.1±1.3	DLW	Summer=882±158; winter=747±128	ml day^{-1}	Summer=7.5±0.7; winter=6.0±0.8
Jones et al., 2009	*Chelonia mydas*	22,420±3130	Fed=66.67±3.37; fasted=58.70±7.63	DLW	Fed=1.43±0.17; fasted=0.78±0.06	ml day^{-1}	Fed=9.57±1.33; fasted=6.14±0.65
Trullas et al., 2006	*Lepidochelys olivacea*	16.91±1.9	68–85	DLW	NA	NA	NA
Wallace et al., 2005	*Dermochelys coriacea*	268±44	73.9±5.7	DLW	28,696–62,465	ml day^{-1}	23.5±5.5
Booth, 2002	*Emydura signata*, *Chelodina expansa*	1822–3090	62–64	Tritiated H_2O	2706–4090	ml day^{-1}	160–430
Jodice et al., 2006	*Gohperus polyphemus*	3400	Female=73.9±1.2; male=69.7±0.2	DLW	11–30	ml kg day^{-1}	NA
Roe et al., 2008	*Chelodina longicollis*	612	62.2–64.3 (DLW); 69.7–72.0 (3H)	DLW, ^3H	14–19	ml kg day^{-1}	NA
Crawford, 1994	*Chrysemys picta*	NA	Autumn=64.6; spring=68.3	NA	NA	NA	NA
Penick et al., 2002	*Terrapene carolina*	400	NA	DLW	Winter=8.8±5.0; spring=18.9±6.0; summer/autumn=26.4±4.5	ml kg day^{-1}	NA
Peterson, 1996	*Gopherus agassizii*	1500	52.6–72.9	DLW	<2 (during drought)	ml kg day^{-1}	NA

%TBW represents percentage total body water content, WTR represents water turnover rate, and DWF represents daily water flux. DLW, doubly labeled water.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110411

laboratory experiments address the underlying anatomical and physiological mechanisms that play important roles in terrapin osmoregulation; however, the conditions to which terrapins were exposed in these studies were not entirely representative of natural environmental conditions in the salt marsh, thus complicating comparisons with our field-based experiments.

Finally, when we consider our results in light of strategies employed by other aquatic turtles, we notice that the estuarine terrapin represents an intermediate form between freshwater and seawater. The [^{2}H]deuterium washout rates and DWF for active terrapins were high when compared with those of active marine turtles, such as the Kemp's ridley turtle (*Lepidochelys kempii*) and green turtle (*Chelonia mydas*; Table 1) (Ortiz et al., 2000; Southwood et al., 2006; Jones et al., 2009); however, WTR and DWF of active terrapins were substantially lower than those of freshwater turtles, *Emydura signata* and *Chelodina expansa* (Table 1) (Booth, 2002). %TBW of terrapins in our study fell within the range of other studies on the water balance and energetics of marine, freshwater and terrestrial chelonians (Table 1). It must be noted, however, that, unlike most of these studies that used doubly labeled water (DLW, ^{18}O and ^{2}H) to determine %TBW, we used only [^{2}H]deuterium and thus we acknowledge the tendency for N_d (dilution space established by hydrogen isotope only) to overestimate TBW by ~3−5% due to hydrogen isotopic exchange with bodily compounds other than water (Speakman, 1997; Bowen and Iversen, 1998; Król and Speakman, 1999).

Our study is the first of its kind to use the stable isotope [^{2}H]deuterium to explore seasonal changes in water balance of terrapins in their dynamic estuarine environment. The number of studies using stable isotope techniques (e.g. ^{2}H, ^{3}H, DLW) to investigate water relations and energetics of free-ranging animals has increased remarkably, providing valuable insight into body fluid dynamics and water budgets and of animals under natural conditions (Jones et al., 2009). Establishing proper protocols for isotope studies conducted with aquatic ectothermic vertebrates is difficult because of the high water turnover rates (Booth, 2002) and the pronounced seasonal changes in behavior and physiology they undergo (Jodice et al., 2006). Our study has established the first field-based labeled water protocols for diamondback terrapins and helped to provide a better understanding of the terrapin's ability to exploit dynamic and high salinity environments via behavioral and physiological adjustments. Similar to recent studies on sea snake osmoregulation (Brischoux et al., 2012; Brischoux et al., 2013), the continued study of diamondback terrapin salt and water balance may strengthen our knowledge of their eco-geography and may offer unique and valuable insights into their geographic range and the evolutionary steps that led to an invasion of salt water environments by freshwater chelonians.

MATERIALS AND METHODS

This research was approved by the University of North Carolina Wilmington IACUC committee (protocol no. A1011-006) and North Carolina Wildlife Resources Commission (Endangered Species Permit no. NC – 2011 ES 235) and the North Carolina National Estuarine Research Reserve (no. 11-2011).

Study site

We maintained terrapins in an enclosure that encompassed typical terrapin habitat and allowed terrapins to experience natural environmental shifts. The enclosure was constructed of PVC pipes (2.54 cm diameter×3 m length) and plastic fencing material [Mid-Grade Diamond Mesh Safety Fence, tensile strength: machine direction (MD): 160 lb ft^{-1}, tensile direction (TD): 100 lb ft^{-1}, mesh size: 2 mm^2, Jackson Safety Brand, Fenton, MO, USA] and encompassed an area of ~450 m^2 (30×15×2.2–4 m in height) in Byron's Creek on the landward side of Masonboro Island National Estuarine Research Reserve (NERR, 34°08′08″N, 77°50′57″W, Fig. 1). Water moved freely through the fencing material with the falling and rising of the tide. This site was chosen because it included high marsh with *Salicornia* species, *Juncus roemerianus*, and prey species *Uca pugnax* and *Littorina littorea*, low marsh with *Spartina alterniflora* and oyster beds, and creek channel where water is ~2 m at spring tides. Furthermore, regular observations of terrapins swimming in Byron's Creek by NERR scientists indicated that it was a suitable habitat for a controlled field study of terrapins.

In nearby tidal creeks and coves, we used large >100 m gillnets and seines with a mesh size of 3.2 cm to collect 10 female terrapins (300–700 g), which were relocated to the enclosure. Terrapin collection sites were within 5 km of the enclosure site. Terrapins were sexed, aged, measured and given a unique 3-letter code notched into the marginal scutes, following processing protocols outlined by Dorcas et al. (Dorcas et al., 2007). Temperature data loggers (5.9×17.4 mm, 3.12 g; iButton DS1922L-F51, Dallas Semiconductor, Dallas, TX, USA) were attached to the anterior carapace using quick-setting marine grade epoxy putty (Loctite®, Henkel Corporation, Cary, NC, USA) and coated in two layers of protective, waterproof plastic (Plasti Dip International, Blaine, MN, USA). The data loggers were programmed to record temperature every 30 min with a resolution of 0.2°C and an accuracy of 0.5°C. Previous studies have found carapace temperatures to be strong indicators of body temperature in small to medium sized turtles (Grayson and Dorcas, 2004), such as the terrapins used in this study. Radio transmitters (20×10 mm, 6–9.6 g; model PD-2, Holohil Systems Ltd, Carp, ON, Canada) were secured to the anterior carapace opposite the data logger with quick-setting epoxy putty so that terrapins could be relocated within the enclosure. The combined mass of the radio transmitters, temperature data loggers and epoxy was ≤5% of terrapin mass.

Terrapins were released into the enclosure on 22 September 2011, and were located via radio telemetry on a monthly basis from 6 November 2011 to 5 April 2012 for a series of studies on overwintering physiology (see Harden, 2013). Terrapins were released in late April and early May 2012 to return to their original capture locations.

Environmental data were obtained from a National Oceanic and Atmospheric Administration (NOAA), 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 data from this monitoring station were collected at 30 min intervals by a YSI 6600EDS data sonde (YSI Inc., Yellow Springs, OH, USA) and total rainfall (mm) was also recorded at 30 min intervals collected by a tipping bucket rain gauge [Campbell Scientific, Inc., Logan, UT, USA; Model no. TE525, rainfall per tip: 0.01 in (0.254 mm)] mounted on the monitoring station. We also measured salinity with a saltwater conductivity logger (HOBO® U24-002, Onset Computer Corporation, Bourne, MA, USA) located within the terrapin enclosure, but because of equipment malfunction, these 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.

Field isotope methods

Previous studies in south eastern North Carolina have documented terrapins overwintering buried in the intertidal mud until late March–early April, at which point they are noticeably more active at the mud and water surface (Southwood Williard and Harden, 2011; Harden and Williard, 2012). Based on these findings and our own observations using radio telemetry within the enclosure, we designated 15–29 March as the time period when terrapins were dormant and 29 March to 5 April as the time period when terrapins were active (see Results for more details). The stable isotope [^{2}H]deuterium was used to determine %TBW and WTR of terrapins during the dormant and active periods following previously outlined techniques (Speakman, 1997; Jones et al., 2009). More specifically, we used the two-sample technique that measures isotope decay over time (k_d) from the first to the last isotope determination, and the plateau method to measure isotope dilution space (N_d), from which we calculated %TBW and WTR [ml day^{-1}; see

Appendix for equations, derived from previous studies (Speakman, 1997; Jones et al., 2009)].

To determine the background enrichment of [^{2}H]deuterium (E_{wat}) in terrapin body water during dormancy, terrapins were collected for pre-[^{2}H]deuterium enrichment blood sampling (N=10). Collection occurred on 15 March 2012 during low tide, when we had access to buried dormant terrapins (Harden, 2013). We obtained a 1−3 ml blood sample from the subcarapacial vein using heparinized vacuum tubes and a 21 gauge needle (Vacutainer, BD, Franklin Lakes, NJ, USA) within 5 min of terrapin disturbance. Each blood sample was immediately placed on ice, and subsequently transferred to a 2 ml microtube and centrifuged for 5 min at 7000 rpm using a portable microcentrifuge (Zipspin, LW Scientific, Lawrenceville, GA, USA). A 0.5 ml plasma sample was then removed and placed in a 0.5 ml Safe-Lock Tube (Eppendorf, Hamburg, Germany) and wrapped in plastic paraffin film (Parafilm® M Laboratory Film, Pechiney Plastic Packaging, Inc., Chicago, IL, USA) to limit gas exchange between the sample and environment. All plasma samples were immediately stored on ice and transferred to a −80°C freezer at the University of North Carolina Wilmington within 8 h.

Within 1 h of background blood sampling, all terrapins were weighed using a battery-powered top-loading balance (Ohaus® Model SP402 Scout PRO™ Portable Balance 4000 g capacity, 0.01 g readability, Parsippany, NJ, USA). Enriched [^{2}H]deuterium (82.1 atom%; Isotec, Inc., Miamisburg, OH, US; verified by Metabolic Solutions, Nashua, NH, USA) was injected into the coelomic cavity with a pre-weighed 25 gauge needle and 1 ml syringe (BD). The amount of injectate (0.28–0.48 g, based on terrapin mass) was estimated by assuming a high washout slope of 0.5 and a TBW of 65% (Jones et al., 2009), and aiming for a final [^{2}H]deuterium enrichment of ≥100 ppm above that of E_{wat} values (see supplementary material Table S1 for injectate details). The exact dose of [^{2}H]deuterium injected into each turtle was determined by measuring the mass of the injectate syringe and needle before and after injection using a digital scale with draft shield and accuracy to four decimal places (Mettler-Toledo AB 304-S/FACT Analytical Balance Scale, 320 g capacity, 0.1 mg readability, Mettler-Toledo, LLC, Columbus, OH, USA).

Following the [^{2}H]deuterium injection, terrapins were kept in two large dry plastic containers at the field enclosure site for 6 h to permit [^{2}H]deuterium to come into equilibrium with body water. Terrapins experienced ambient temperatures of 20–24°C during the equilibration period. Equilibrium is reached when the TBW, as calculated from the [^{2}H]deuterium isotope dilution space (N_d), is equal to 60–80% of body mass (Minnich, 1982; Crawford, 1994; Roe et al., 2008), or when the [^{2}H]deuterium enrichment values reach their maximum, plateau, and start to decline over time (see supplementary material Table S1 for details). Previous studies on larger turtles have demonstrated an equilibrium time of anywhere from 2.5 to 5 h for eastern long-necked turtles (*Chelodina longicollis*, 440–634 g) at 22–26°C (Roe et al., 2008), and ~5 h at 24.1°C for green turtles [*Chelonia mydas*, 19−25 kg (Jones et al., 2009)]. Based on these studies, we chose a 2−6 h time frame for equilibration. A 0.5 ml blood sample was collected from a subset of four terrapins at 2.5, 4 and 6 h in order to confirm the [^{2}H]deuterium equilibration curve for terrapins. We collected a 0.5 ml equilibration blood sample from all remaining terrapins at 6 h (E_{mix}), as this was deemed a conservative amount of time for equilibrium to occur. All plasma samples were prepared and sealed in the same manner as background samples. Upon completion of the equilibrium period, terrapins were released into the enclosure.

After 2 weeks (13.9 days) in the salt marsh enclosure, terrapins (N=10) were relocated and 0.5 ml blood samples (E_{wat}) were taken within 5 min. Terrapins were then weighed, given a second injection of [^{2}H]deuterium (0.29–0.46 g) for determination of N_d, and placed in dry plastic containers for the ^{2}H equilibration period. Based on the 2−6 h time series of blood samples (N=4) on 15 March, [^{2}H]deuterium equilibration with terrapin body water occurred by 2.5 h post-[^{2}H]deuterium injection for three of the terrapins and by 4.5 h for the fourth terrapin. Therefore, in order to limit the amount of time turtles spent outside of their habitat, we used 4 h as our equilibration time for the 29 March ^{2}H re-boost (supplementary material Table S1). Terrapins experienced ambient temperatures of 19–31°C while in the dry container. At the end of the 4 h equilibration period, a 0.5 ml blood sample was taken for determination of E_{mix} [^{2}H]deuterium levels and terrapins were released in the enclosure. After a third week in the enclosure, terrapins were relocated and a final 1−3 ml blood sample was taken (N=9) within 5 min of capture. All blood samples were prepared and sealed for [^{2}H]deuterium enrichment determination in the same manner as previously described.

Lab isotope methods

Deuterium levels in terrapin plasma samples were determined using 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 Inc.) at the University of North Carolina Wilmington Center for Marine Science. Specifically, 100−300 µl of sample was pipetted into an exetainer (Labco International Inc., Houston, TX, USA) with a platinum catalyst (Thermo Fisher Scientific Inc.). Exetainers were capped and flushed for 10 min with compressed gas (2% hydrogen + helium) and then samples were incubated for >40 min. The deuterium concentration of body water is measured by H_2–H_2O (as water vapor, equilibrated to the headspace) exchange in the presence of a platinum catalyst, where there is isotopic exchange between the deuterated water and pure H_2 gas. Nine injections per sample were averaged to determine [^{2}H]deuterium (see 'Data analysis', below). All samples were analyzed at 25°C.

Enrichment of terrapin plasma samples exceeded the linear range of the IRMS instrument. In order to accurately determine enrichment, we diluted 99.9 atom% [^{2}H]deuterium oxide (Sigma-Aldrich Co., St Louis, MO, USA) with Evian spring water (−74‰) (Bowen et al., 2005). Dilution water was homogenized and analyzed against two USGS standards (USGS W-64444 and W-67400, −399.1‰ and 1.25‰, respectively; Reston Stable Isotope Laboratory, USGS, Reston, VA, USA) to confirm the delta value. Dilution 'references' were then created by combining Evian water and 99.9 atom% [^{2}H]deuterium oxide (435.51±2.8‰, 1180.58±9.2‰, 3919.84±17.9‰ and 12,849.65±23.9‰) to create a range of references for sample measurement. All blood samples fell within this dilution series (between −138.26±1.56‰ and 9829.61±4.35‰).

Data analysis

Delta values (‰) for all samples were determined using Isodat software (Thermo Fisher Scientific Inc.; s.d. <4‰). We used the following calibration curve: y=4.2393x+3140.7 (R^2=0.9919) generated from our two standards and four dilutions (see 'Lab isotope methods', above) to calculate our corrected delta values for our terrapin plasma samples. These delta values were then converted to ppm using the following equation: ppm ^{2}H=1,000,000/[1+(1/{[(δ^2H/1000)+1]×0.00015576})], where δ^2H is the per million [^{2}H]deuterium with respect to the International reference, Vienna Standard Mean Ocean Water (VSMOW), and the factor 0.00015576 is the accepted ^{2}H/^{1}H ratio of VSMOW.

Statistical analysis

We used Spearman's rank-order correlation coefficient to examine the relationship between terrapin mass (measured on 15 and 29 March 2012) and our isotope variable of interest (%TBW, WTR and DWF). Strong (Spearman's ρ>0.5) and significant (α=0.05 level) correlations were considered adequate to use mass as a covariate in the analysis of covariance (ANCOVA) model for a given isotope variable. As a result, we used mass as a covariate in the ANCOVA models testing for differences in WTR and DWF between dormant and active terrapins, and a Student's t-test to test for differences in %TBW. In all statistical analyses, α was set to 0.05. All statistical analyses were done in R statistical software program (R Core Team, 2013) and all values are given as means ± 1 s.d.

APPENDIX

We used the following equations from chapter 17 of Speakman (Speakman, 1997) to calculate [^{2}H]deuterium turnover (washout) rate k_d:

$$k_d = \frac{\log_e\left(E_{peak} - E_{background}\right) - \log_e\left(E_{final} - E_{background}\right)}{\text{No. of days}}, \quad \text{(A1)}$$

where E_{peak}, $E_{background}$ and E_{final} are the peak, background and final [^{2}H]deuterium enrichment level; dilution space N_d:

$$N_d = \left(\frac{\mathrm{Mol}_{inj} \times \left(E_{peak} - E_{inj} \right)}{E_{background} - E_{peak}} \right) \times \mathrm{Molmass}_{inj}, \quad \text{(A2)}$$

where N_d is in g, Mol_{inj} is the number of moles of deuterium injected, E_{inj} is the injectate enrichment in Atom% and $\mathrm{Molmass}_{inj}$ is the molecular mass of injectate; %TBW:

$$\mathrm{TBW}\% = \left(\frac{N_d}{M} \right) \times 100, \quad \text{(A3)}$$

where M is mass; WTR:

$$\mathrm{WTR} = N_d \times k_d, \quad \text{(A4)}$$

where WTR is in ml day^{-1}; and DWF:

$$\mathrm{DWF} = \%\mathrm{TBW} \times k_d, \quad \text{(A5)}$$

where DWF is given as %TBW day^{-1}.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.A.H. and A.S.W. conceived and designed the study, with extensive input from T.T.J. L.A.H. and A.S.W. collected the data, K.A.D. assisted L.A.H. in data analysis and interpretation. L.A.H. and A.S.W. wrote the manuscript with edits from T.T.J. and minor edits from K.A.D.

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Supplementary material

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Table S1. Mass, [^{2}H]deuterium injectate details, background (E_{wat}), equilibrium ($E_{2,4,6hr,mix}$) and final (E_{final}) isotope levels, water turnover rates (k_d), isotope dilution space (N_d), total body water (%TBW), water turnover rate (WTR) and daily water flux (DWF) for 10 female diamondback terrapins from 15 to 29 March (during dormancy) and from 29 March to 5 April (after dormancy).

Terrapin	ACO	ACW	AOP	AOPW	AOX	APQ	APV	AQW	HIJ	AOV	Mean ± s.d.
Plastron length (cm)	13.5	13	12	14	13.8	14	13.2	13.3	12.9	12.9	13.26 ± 0.58
Mass at capture (g)	509.20	455.10	337.10	564.80	526.10	556.00	516.30	580.80	504.80	423.30	497.35 ± 70.20
15 to 29 Mar											
Mass (g, 15 Mar)	522.10	530.10	339.10	658.80	541.50	520.10	509.10	503.10	502.70	504.80	513.14 ± 72.82
Mass (g, 29 Mar)	507.80	440.70	317.70	624.30	503.20	498.70	468.30	509.00	469.40	467.60	480.67 ± 71.84
E_{wat} (0 hrs)	134.15	160.39	134.05	137.96	137.72	135.54	145.76	136.29	134.72	134.09	139.07 ± 7.85
E_{inj} (Atom%)	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	
$MolMass_{inj}$	20.0276	20.0276	20.0276	20.0276	20.0276	20.0276	20.0276	20.0276	20.0276	20.0276	
M_{inj} (g)	0.36	0.34	0.28	0.37	0.48	0.48	0.44	0.45	0.46	0.36	0.40 ± 0.06
Mol_{inj} (moles)	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02 ± 0.00
$E_{2.5hr}$ (2.5 hrs)		758.07			963.22			229.95	1025.85		744.27 ± 313.03
$E_{4.5hr}$ (4.5 hrs)		751.17			985.51			1256.67	1027.38		1005.18 ± 179.34
E_{6hr} (6 hrs)	817.03	746.91	923.19	673.27	979.6	1007.94	1639.48	1243.08	1029.15		1006.63 ± 274.70
E_{mix} (4.5−6 hrs)	817.03	752.05	923.19	673.27	976.11	1007.94	1639.48	1256.67	1027.46		1008.13 ± 275.52
E_{final} (336 hrs, 13.9 days)	258.09	250.48	223.49	331.72	314.28	180.54	465.37	243.99	253.33	134.35	265.56 ± 85.90
k_d (15 to 29 Mar, hrs)	0.005	0.006	0.006	0.003	0.005	0.009	0.005	0.007	0.006		0.006 ± 0.00
k_d (15 to 29 Mar, days)	0.12	0.14	0.16	0.07	0.11	0.21	0.11	0.17	0.15		0.14 ± 0.04
N_d (15 Mar, Mol_{wat})	21.35	23.59	14.73	28.52	23.56	22.37	12.05	16.61	21.03		20.42 ± 4.80
Terrapin	ACO	ACW	AOP	AOPW	AOX	APQ	APV	AQW	HIJ	AOV	Mean ± s.d.
N_d (g, 15 Mar)	384.38	424.63	265.24	513.43	424.05	402.69	216.96	299	378.65		367.67 ± 86.42
%TBW	73.62	80.10	78.22	77.93	78.31	77.42		59.43	75.32		75.05 ± 6.19
WTR (ml day^{-1})	47.26	57.58	41.61	37.59	47.59	86.01	24.1	50.45	55.06		49.70 ± 15.94
DWF (%TBW day^{-1})	9.05	10.86	12.27	5.71	8.78	16.53		10.02	10.95		10.52 ± 2.92
29 Mar to 5 Apr											

Mass (g, 29 Mar)	507.80	440.70	317.70	624.30	503.20	498.70	468.30	509.00	469.40	467.60	480.67 ± 71.84
E_{wat} (0 hrs)	258.09	250.48	223.49	331.72	314.28	180.54	465.37	243.99	253.33	134.35	265.56 ± 85.90
E_{inj} (boost, Atom%)	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	820882.88	
M_{inj} (g)	0.35	0.38	0.29	0.39	0.48	0.47	0.46	0.46	0.46	0.34	0.41 ± 0.06
Mol_{inj} (moles)	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02 ± 0.00
E_{mix} (4 hrs)	1029.48	1090.2	1064.14	911.27	1188.11	1098.13	1430.19	1190.14	1251.59	881.17	1113.44 ± 153.61
E_{final} (172 hrs, 7.2 days)	326.43	380.74	252.38	495.45		270.87	526.71	473.98	388.29	270.13	376.11 ± 98.18
k_d (29 Mar to 5 Apr, hrs)	0.014	0.011	0.02	0.007		0.013	0.016	0.008	0.012	0.01	0.012 ± 0.00
k_d (29 Mar to 5 Apr, days)	0.34	0.26	0.47	0.18		0.32	0.38	0.2	0.28	0.24	0.29 ± 0.09
N_d (29 Mar, Mol_{wat})	18.42	18.57	14.12	27.2	22.47	20.8	19.45	19.95	18.95	18.79	19.87 ± 3.17
N_d (g, 29 Mar)	331.6	334.34	254.12	489.62	404.47	374.38	350.11	359.22	341.11	338.22	357.72 ± 57.04
%TBW	65.3	75.87	81.27	78.43	80.38	75.07	74.76	71.27	73.45	72.33	74.54 ± 4.36
WTR (ml day^{-1})	111.47	86.42	118.81	85.84		120.37	133.81	70.47	94.67	79.97	100.20 ± 20.36
DWF (%TBW day^{-1})	21.95	19.61	37.39	13.75		24.13	28.57	13.84	20.17	17.10	21.84 ± 7.30