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

Leigh Anne Harden[1,4*] Kimberley Anne Duernberger[2] T. Todd Jones[3] Amanda Southwood Williard[1]

[1]Department of Biology and Marine Biology, University of North Carolina Wilmington, 601 S. College Rd., Wilmington, NC 28403 USA

[2]Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K Moss Ln, Wilmington, NC 28409 USA

[3]NOAA Fisheries, Pacific Islands Fisheries Science Center, Honolulu, HI 96818 USA

[4]Department of Biology, Loyola University Chicago, Chicago, IL 60660 USA

Summary

* Corresponding author. Tel: +1 610 506 1055; *e-mail address*: lharden@luc.edu

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 southeastern 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). Results from this field study provides insight into the terrapin's ability to maintain osmotic homeostasis while experiencing shifts in behavioral and environmental conditions.

Short title: Water relations of diamondback terrapins

Key words: diamondback terrapin, [^{2}H]deuterium, estuarine, osmoregulation, homeostasis, total body water, water flux, dormancy.

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; Duson, 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,* Schoepff, 1793) 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, Massachusetts to Corpus Christi, Texas (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 fall, 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 fall, terrapins enter a period of winter dormancy in which they cease eating, drastically reduce activity levels, and spend the large majority of time buried shallowly in the mud of the subtidal or intertidal zone (Harden and Williard, 2012; Akins et al., 2014). 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 due to 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 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, 2002; Jodice et al., 2006) and marine turtles (Ortiz, 2000; Wallace et al., 2005; Southwood et al., 2006; 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, water turnover rate, and daily water flux of female diamondback terrapins in southeastern 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 water turnover rates and daily water flux in terrapins would significantly increase upon emergence from dormancy as terrapins initiated basking and foraging behaviors and subsequently, total body water (%) would also increase during this seasonal shift in activity.

Results

We monitored terrapins within a semi-natural enclosure at Masonboro Island, NC (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 (2nd [^{2}H]deuterium dosing event), 3 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, 5 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 to 29 March) as a time period when terrapins were less active and interval 2 (29 March to 5 April) as a 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 both intervals. These observed habitat and activity shifts are supported by previous radio telemetry and temperature datalogger 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 terrapin enclosure at 30 minute intervals. During the transition from dormancy to activity (15 March to 5 April), mean salinity was 31.8 ± 2.1 (± s.e.); 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 dataloggers (iButtons) were available for four of the terrapins from this study. These terrapins experienced combined mean carapace temperature (T_c) of 21.2 ± 0.2°C during the 'dormant' period and 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), total body water (TBW%), water turnover rate (WTR), and daily water flux (DWF) for each terrapin, are summarized in Table A1. Water turnover rates (WTR) and daily water flux (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% ($TBW\%_{'dormant'} = 75.05 \pm 6.19$, $TBW\%_{'active'} = 74.54 \pm 4.36$) 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 ($Z = -2.65$, $p = 0.008$).

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 and Mousseau, 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, due to salt ingestion via invertebrate prey items and incidental drinkingThis hypothesis concurs with changes in terrapin plasma K^+ and glucose concentrations documentedpre- and post-emergence from dormancy (Harden, 2013). Concentrations of plasma K^+ in April are noticeably higher than concentrations measured between October and March (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, respectively) following a prolonged fast, as hypothesized by 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; Pererira 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 WTF and DWF observed in our study. Davenport and Ward (1993) found that when nutrient-deprived terrapins were given access to freshwater, consumed 7.2% of their body mass in prey in 48 hours, 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 minutes, with the capability of fully rehydrating within 15 minutes (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 minute period. Furthermore, Davenport and Ward (1993) found that when nutrient-deprived terrapins at > 20˚C were given access to freshwater, they exhibit 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 kingii:* Christian and Green, 1994; *Crocodylus jonhsonii*: 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 (1996) found that desert tortoises (*Gopherus agassizii*) tolerate 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 supported also by Nagy and Medica (1986) and 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 • h (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 (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) were 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 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 to 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; and Jones et al., 2009), however WTR and DWF of 'active' terrapins were substantially lower than that of freshwater turtles, *Emydura signata* and *Chelodina expansa* (Table 1; Booth, 2002). Total body water (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; Krol 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 due to the high water turnover rates (Booth, 2002) and the pronounced seasonal changes in behavior and physiology they undergo (Jodice et al., 2006). Our study established the first field-based labeled water protocols for diamondback terrapins and helped to provide a better understanding of 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

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 x 3 m length) and plastic fencing material (Mid-Grade Diamond Mesh Safety Fence, tensile strength: MD: 160 lb/ft, TD: 100 lb/ft, mesh size: 2 mm^2, Jackson Safety Brand, Fenton, MO) and encompassed an area of approximately 450 m^2 (30 m x 15 m x 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 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. (2007). Temperature data loggers (5.9 mm×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 minutes 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 mm×10 mm, 6–9.6 g; model PD-2, Holohil Systems Ltd., Carp, Ontario, 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 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 for more detail). 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, 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 was collected at 30 minute intervals by a YSI 6600EDS data sonde (YSI Inc., Yellow Springs, OH, USA) and total rainfall (mm) was also recorded at 30 minute intervals collected by a tipping bucket rain gauge (Campbell Scientific, Inc., Logan, UT, USA, Model# TE525, rainfall per tip: 0.01 inch) mounted on the monitoring station. We also measured salinity with a salt water conductivity logger

(HOBO® U24-002, Onset Computer Corporation, Bourne, MA) located within the terrapin enclosure, but due to 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 southeastern 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 to 29 March as a time period when terrapins were 'dormant' and 29 March to 5 April as a 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 A2 for equations, derived from 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 (BD Vacutainer, Franklin Lakes, NJ) within five minutes of terrapin disturbance. Each blood sample was immediately placed on ice, and subsequently transferred to a 2ml microtube and centrifuged for five minutes at 7000 rpm using a portable microcentrifuge (Zipspin, LW Scientific, Lawrenceville, GA). 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) to limit gas exchange between 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 hours.

Within one hour 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, verified by Metabolic Solutions, Nashua, NH) was injected into the coelomic cavity with a pre-weighed 25-gauge needle and 1 ml syringe (BD, Franklin Lakes, NJ). The amount of injectate (0.28 to 0.48 g, based on terrapin mass) was estimated by assuming a high washout slope of 0.5 and total body water 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 Table A1 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).

Following the [^{2}H]deuterium injection, terrapins were kept in two large dry plastic containers at the field enclosure site for 6 hours 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 Figs 6,7). Previous studies on larger turtles have demonstrated an equilibrium time of anywhere from 2.5 to 5 hours for eastern long-necked turtles (*Chelodina longicollis*, 440–634 g) at 22–26°C (Roe et al., 2008), and ~5 hours at 24.1°C for green turtles (*Chelonia mydas* L., 19−25 kg, Jones et al., 2009). Based on these studies we chose a 2−6 hour timeframe for equilibration. A 0.5 ml blood sample was collected from a subset of four terrapins at hour 2.5, hour 4, and hour 6 in order to confirm the [^{2}H]deuterium equilibration curve for terrapins. We collected a 0.5ml equilibration blood sample from all remaining terrapins at hour 6 (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 two weeks (13.9 days) in the salt marsh enclosure, terrapins (n = 10) were relocated and 0.50 ml blood samples (E_{wat}) were taken within 5 minutes. Terrapins were then weighed, given a second injection of [^{2}H]deuterium (0.29 to 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 hour time series of blood samples (n = 4) on 15 March, [^{2}H]deuterium equilibration with terrapin body water occurred by 2.5 hours post-[^{2}H]deuterium injection for three of the terrapins and by 4.5 hours for the fourth terrapin. Therefore, in order to limit the amount of time turtles spent outside of their habitat, we used 4 hours as our equilibration time for the 29 March ^{2}H re-boost (Table A1). Terrapins experienced ambient temperatures of 19–31°C while in the dry container. At the end of the 4 hour 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 minutes 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) with gas bench interface (ThermoFinnigan GasBench II, Thermo Fisher Scientific Inc., Waltham, MA) 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) with a platinum catalyst (Thermo Fisher Scientific Inc., Waltham, MA). Exetainers were capped and flushed for 10 minutes with compressed gas [2% Hydrogen + Helium] and then samples incubated for > 40 minutes. 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). 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) 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) to confirm 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 12849.65 +/- 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., Waltham, MA; SD < 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 Laboratory Methods) to calculate our corrected delta values for our terrapin plasma samples. These delta values were then converted to ppm using the following equation: ppm $^2H = 1000000 / (1 + (1 / (((\delta^2H / 1000) + 1) * 0.00015576)))$, where δ^2H is the per mil [^{2}H]deuterium with respect to the International reference, VSMOW and the factor 0.00015576 is the accepted $^2H/^1H$ 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 $\rho > 0.5$) and significant ($\alpha = 0.05$ 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.

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List of symbols/abbreviations

WTR – water turnover rate (ml day^{-1})

DWF – daily water flux (%TBW day^{-1})

TBW% – total body water (%)

T_c – carapace temperature

E_{wat} – background [^{2}H]deuterium enrichment levels

$E_{2,4,6hr,mix}$ – equilibrium [^{2}H]deuterium enrichment levels

E_{final} – final [^{2}H]deuterium enrichment levels

k_d – water turnover rate

N_d – isotope dilution space

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Table 1. A comparative table summarizing findings of previous turtle water balance studies using isotope techniques (DLW, ^{2}H, ^{3}H), where TBW% represents total body water content, WTR represent water turnover rates, and DWF represents daily water flux.

Study	Species	Mass	TBW%	Method	WTR	WTR units	DWF (%TBW day^{-1})
This study	*Malaclemys terrapin*	317-658 g	'dormant' = 75.05 ± 6.19; 'active' = 74.54 ± 4.36	^{2}H	'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 g	77.0	^{3}H	1.0 ± 0.1	mL/100 g h^{-1}	NA
Robinson & Dunson, 1976	*Malaclemys terrapin*	224−270 g	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*	10.0 ± 1.1 kg	78.4 ± 1.3	^{2}H	123.0 ± 6.8	mL kg d^{-1}	16
Southwood et al., 2006	*Chelonia mydas*	9.8−23.8 kg	summer = 73.7 ± 0.9; winter = 75.1 ± 1.3	DLW	summer = 882 ± 158; winter = 747 ± 128	mL d^{-1}	summer = 7.5 ± 0.7; winter = 6.0 ± 0.8
Jones et al., 2009	*Chelonia mydas*	22.42 ± 3.13 kg	fed = 66.67 ± 3.37; fasted = 58.70 ± 7.63	DLW	fed = 1.43 ± 0.17; fasted = 0.78 ± 0.06	L d^{-1}	fed = 9.57 ± 1.33; fasted = 6.14 ± 0.65
Trullas et al., 2006	*Lepidochelys olivacea*	16.91 ± 1.9 g	68−85	DLW	NA	NA	NA
Wallace et al., 2005	*Dermochelys coriacea*	268 ± 44	73.9 ± 5.7	DLW	28696−62465	mL d^{-1}	23.5 ± 5.5
Booth, 2002	*Emydura signata*, *Chelodina expansa*	1822−3090 g	62−64	tritiated H_2O	2706−4090	ml d^{-1}	160−430
Jodice et al., 2006	*Gohperus polyphemus*	3.4 kg	female = 73.9 ± 1.2; male = 69.7 ± 0.2	DLW	11−30	mL kg d^{-1}	NA
Roe et al., 2008	*Chelodina longicollis*	612 g	62.2−64.3 (DLW); 69.7−72.0 (3H)	DLW, ^{3}H	14−19	mL kg d^{-1}	NA
Crawford, 1994	*Chrysemys picta*	NA	fall = 64.6; spring = 68.3	NA	NA	NA	NA
Pennick et al., 2002	*Terrapene carolina*	400 g	NA	DLW	winter = 8.8 ± 5.0; spring = 18.9 ± 6.0; summer/fall = 26.4 ± 4.5	mL kg d^{-1}	NA
Peterson, 1996	*Gopherus agassizii*	1500 g	52.6−72.9	DLW	< 2 (during drought)	mL kg d^{-1}	NA

Figure Captions

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

Figure 2. Deuterium enrichment values of 'dormant' (15−29 March) and 'active' (29 March−5 April) 10 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 15 March 2012 (designated as 'dormant' period), but terrapins began to emerge from dormancy during late March/early April 2012 (between 29 March and 5 April, designated as 'active' period).

Figure 3. Salinity (psu) and total precipitation measured 15 March to 5 April 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.

Figure 4. Water relations of 'dormant' (15−29 March) and 'active' (29 March−5 April) female terrapins calculated using equations from Speakman (1997, Chapter 17, See Appendix Equations A1-A5). A.) Water turnover rates (WTR, ml day^{-1}, Eqn A4) of 9 terrapins, calculated using [^{2}H]deuterium turnover rates (k_d, Eqn A1) and the dilution space (N_d, grams, Eqn A2). B.) Daily water flux (DWF %TBW day^{-1}, Eqn A5) of 8 terrapins, calculated using Eqn A1 and total body water (TBW%) of terrapins. C.) Total body water (TBW%, Eqn A3) of 8 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).

Appendix

Table A1. 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

Equations A1-A5. We used the following equations from Speakman (1997, Chapter 17) to calculate [^{2}H]deuterium turnover (washout) rate (k_d), dilution space (N_d), TBW%, water WTR, and DWF:

A1:

$$k_{\mathrm{d}}\ (rate)\ =\ \frac{log_e(E_{\mathrm{peak}}-E_{background})-log_e(E_{\mathrm{final}}-E_{background})}{days}$$

A2:

$$N_{\mathrm{d}}\ (grams)\ =\ \left(\frac{Mol_{inj}\times\left(E_{peak}-E_{inj}\right)}{E_{background}-E_{peak}}\right)\times Molmass_{inj}$$

A3:

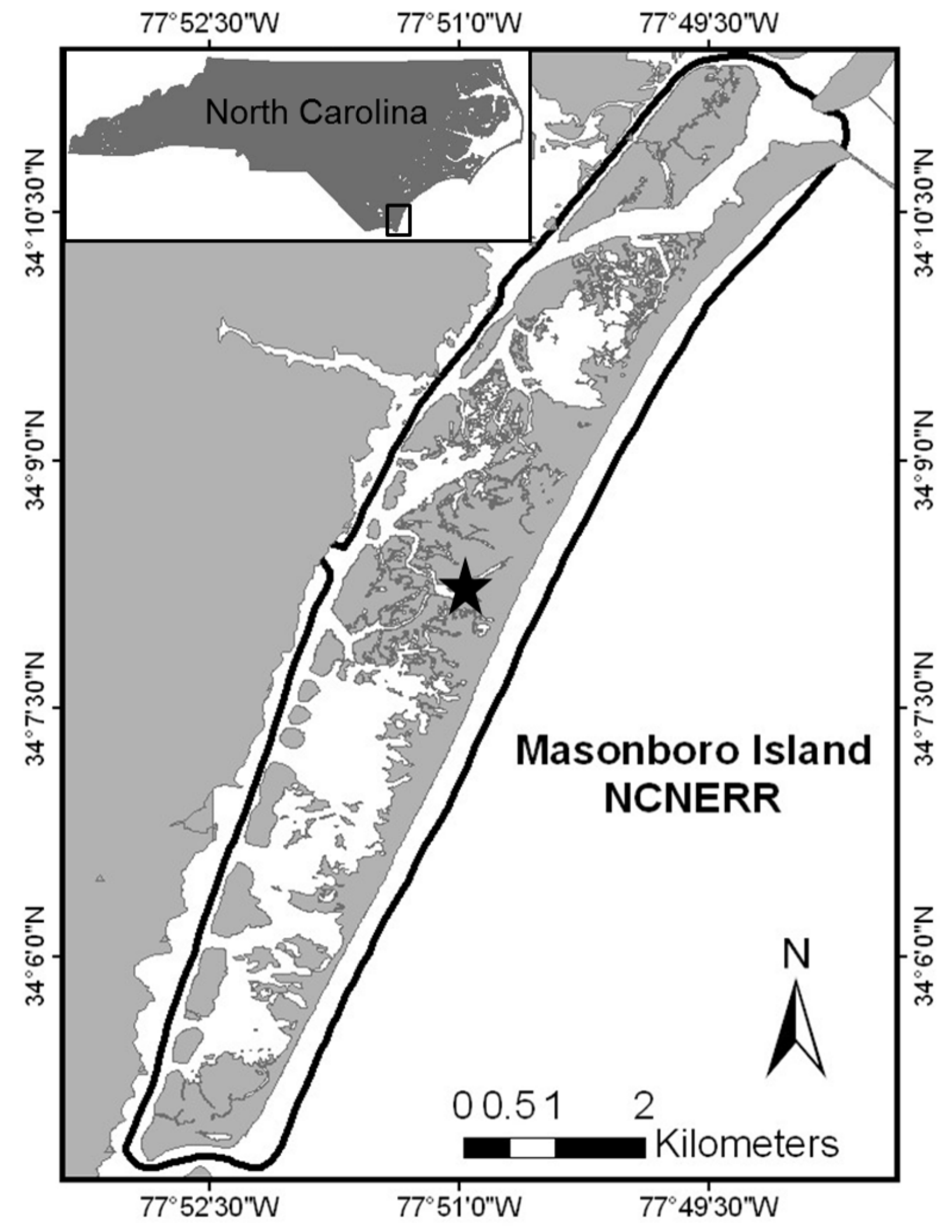
$$TBW\%\ =\ \left(\frac{N_d}{mass}\right)\times 100$$

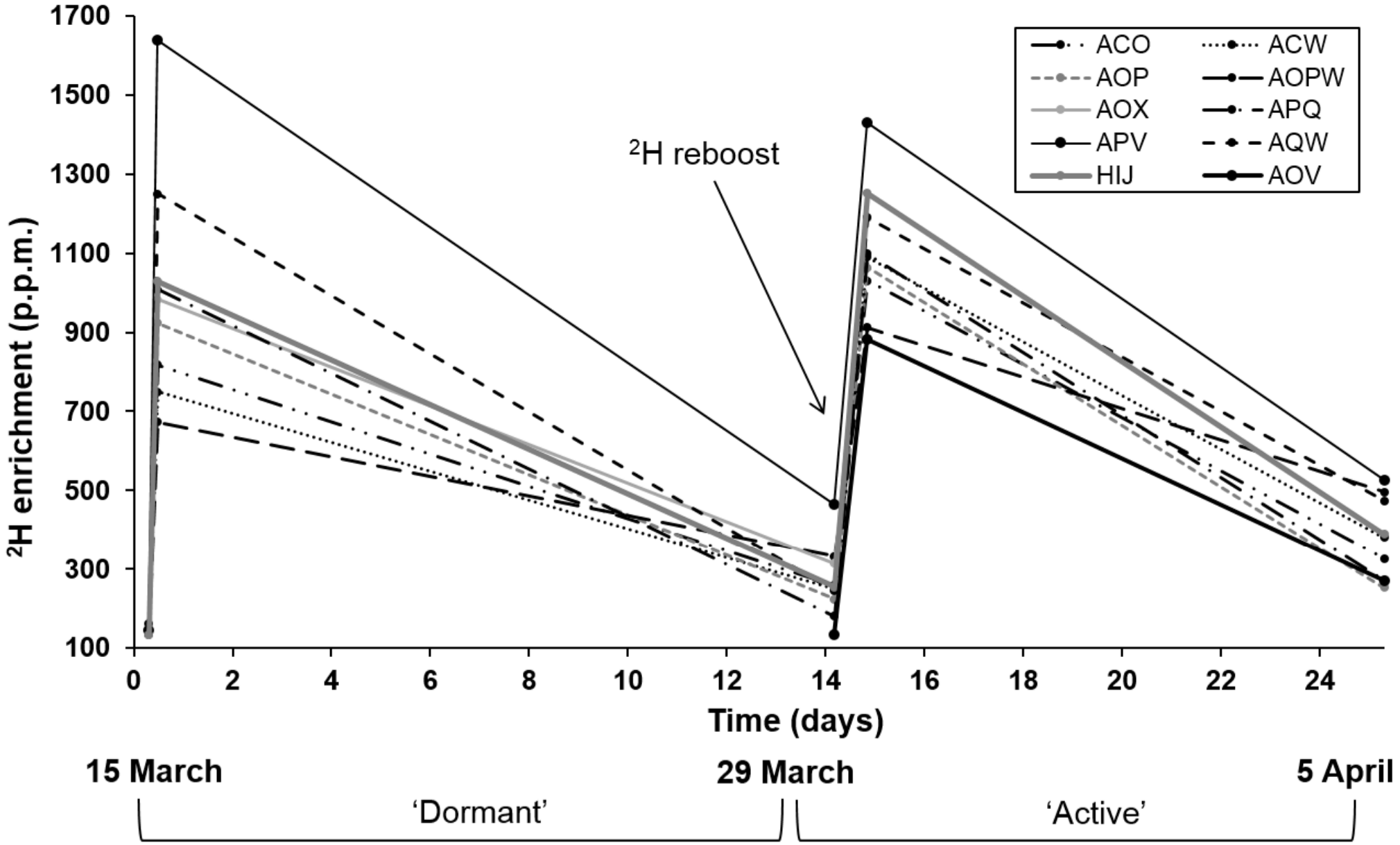
A4:

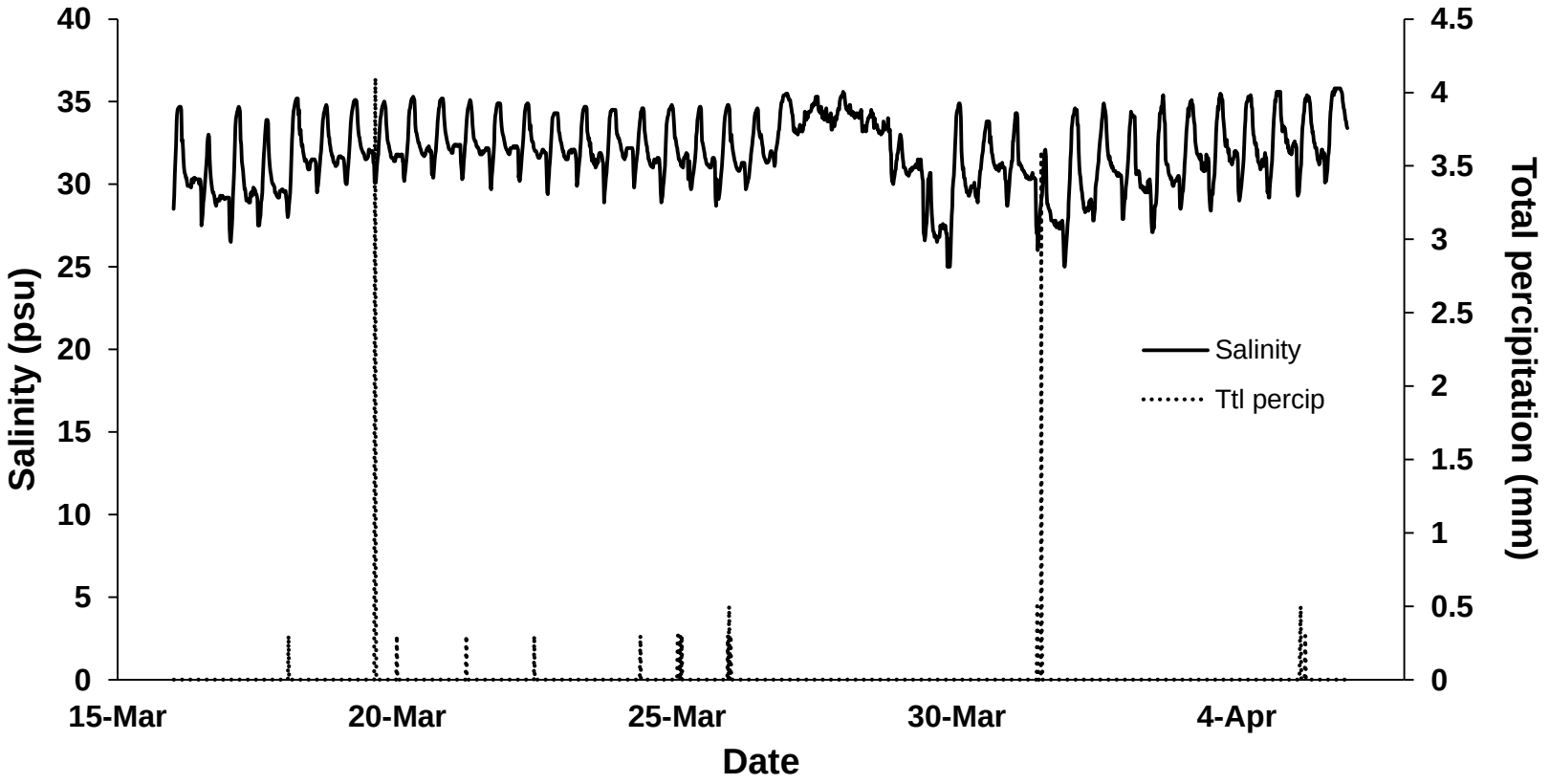
$$WTR\ (ml\ day^{-1})\ =\ N_d\ \times k_d$$

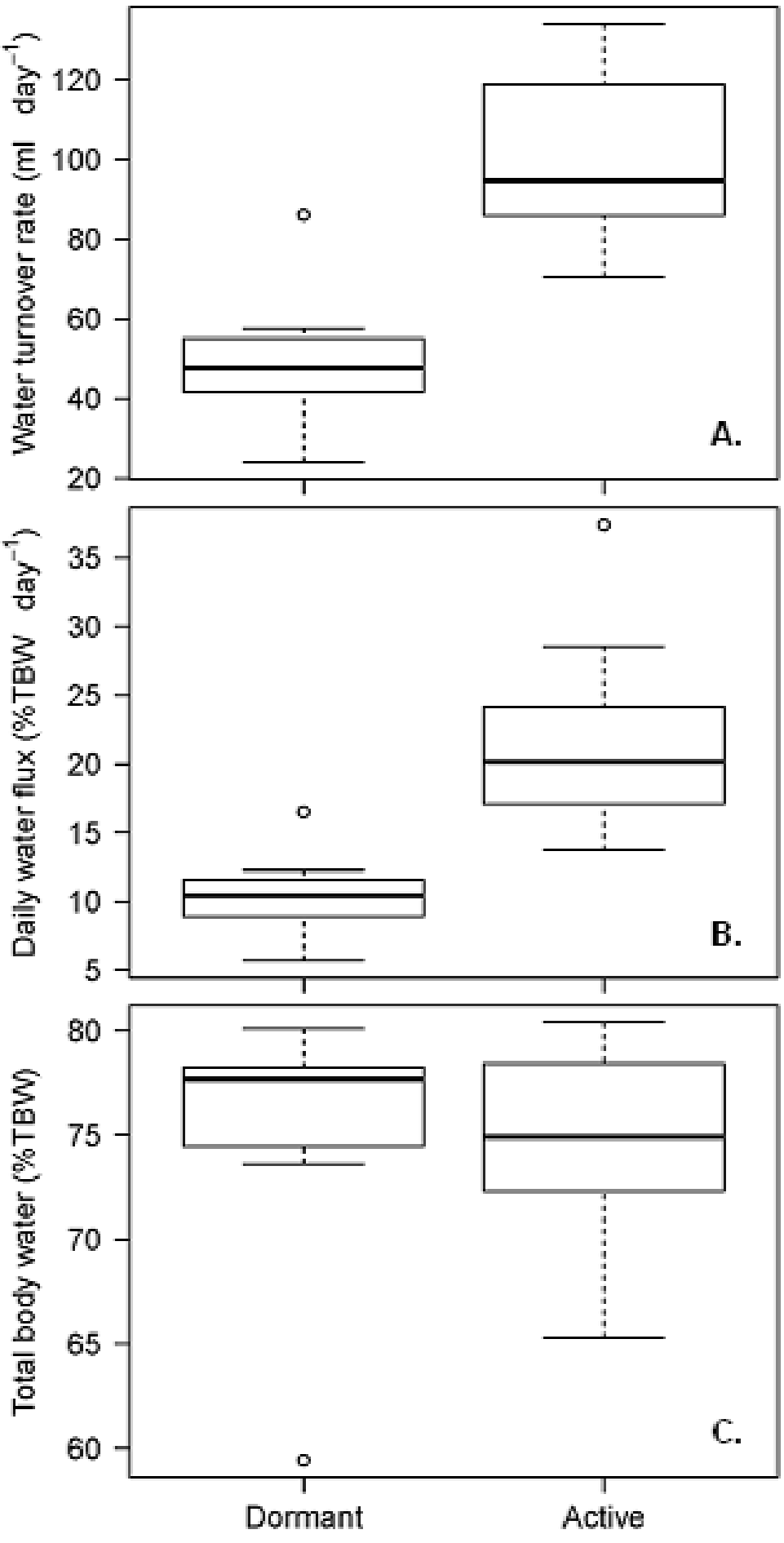
A5:

$$DWF\ (\%TBW\ day^{-1})\ =\ TBW\%\ \times k_d$$


77°52'30"W
77°51'0"W
77°49'30"W
North Carolina
34°10'30"N
34°9'0"N
34°7'30"N
34°6'0"N
Masonboro Island
NCNERR
N
0 0.5 1 2
Kilometers


1700
1500
1300
1100
900
700
500
300
100
²H enrichment (p.p.m.)
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (days)
ACO
ACW
AOP
AOPW
AOX
APQ
APV
AQW
HIJ
AOV
²H reboost
15 March
29 March
5 April
'Dormant'
'Active'


40
35
30
25
20
15
10
5
0
Salinity (psu)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Total percipitation (mm)
15-Mar
20-Mar
25-Mar
30-Mar
4-Apr
Date
Salinity
Ttl percip


Water turnover rate (ml day^{-1})
120
100
80
60
40
20
A.
Daily water flux (%TBW day^{-1})
35
30
25
20
15
10
5
B.
Total body water (%TBW)
80
75
70
65
60
C.
Dormant
Active