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RESEARCH ARTICLE

Hydrogen isotope assimilation and discrimination in green turtles

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ABSTRACT

Although hydrogen isotopes (δ^2H) are commonly used as tracers of animal movement, minimal research has investigated the use of δ^2H as a proxy to quantify resource and habitat use. While carbon and nitrogen are ultimately derived from a single source (food), the proportion of hydrogen in consumer tissues originates from two distinct sources: body water and food. Before hydrogen isotopes can be effectively used as a resource and habitat tracer, we need estimates of (net) discrimination factors (Δ^2H_{Net}) that account for the physiologically mediated differences in the δ^2H values of animal tissues relative to that of the food and water sources they use to synthesize tissues. Here, we estimated Δ^2H_{Net} in captive green turtles (*Chelonia mydas*) by measuring the δ^2H values of tissues (epidermis and blood components) and dietary macromolecules collected in two controlled feeding experiments. Tissue δ^2H and Δ^2H_{Net} values varied systematically among tissues, with epidermis having higher δ^2H and Δ^2H_{Net} values than blood components, which mirrors patterns between keratinaceous tissues (feathers, hair) and blood in birds and mammals. Serum/plasma of adult female green turtles had significantly lower δ^2H values compared with juveniles, likely due to increased lipid mobilization associated with reproduction. This is the first study to quantify Δ^2H_{Net} values in a marine ectotherm, and we anticipate that our results will further refine the use of δ^2H analysis to better understand animal resource and habitat use in marine ecosystems, especially coastal areas fueled by a combination of marine (e.g. micro/macroalgae and seagrass) and terrestrial (e.g. mangroves) primary production.

KEY WORDS: Hydrogen isotope, Trophic discrimination factor, Controlled feeding experiment, Stable isotope analysis, *Chelonia mydas*

INTRODUCTION

The use of stable isotope analysis (SIA) to characterize habitat and resource use by organisms has grown exponentially (Peterson and Fry, 1987; Post, 2002; Solomon et al., 2011) and is now routinely used to assess diet composition (Burgett et al., 2018; Díaz-Gamboa et al., 2017), the flow of energy within and among ecosystems (Doucett et al., 1996; Finlay and Kendall, 2007), trophic level and food chain length (Boecklen et al., 2011; Post, 2002), and movement/migration patterns (Hobson and Wassenaar, 2018; Zbinden et al., 2011). In particular, studies of individual- and population-level foraging strategies have greatly benefited from SIA, which provides information on resource assimilation over a variety of timescales depending on the type of tissue analyzed (Martínez del Río et al., 2009; Vander Zanden et al., 2013). This is one of the primary advantages of SIA in comparison to conventional methods that directly assess diet composition, such as observation of gut/scat content analysis, which only provides dietary 'snapshots' and may not accurately reflect variation in how different resources are assimilated by a consumer (Hobson, 1999; Newsome et al., 2010).

Carbon isotope ($\delta^{13}C$) and nitrogen isotope ($\delta^{15}N$) analyses have been most frequently used by animal ecologists to characterize resource and habitat use (DeNiro and Epstein, 1978; Hobson, 1999; Phillips, 2012), while hydrogen isotope (δ^2H) analysis has been primarily used to track the geographic origin and seasonal movement of birds (Chamberlain et al., 1996; Hobson, 2005a), insects (Flockhart et al., 2017; Hobson et al., 2012) and even humans (Ehleringer et al., 2008) across regional to continental scales. Only a few studies have used δ^2H as a tracer of food resources, specifically to quantify autochthonous (instream algae) and allochthonous (riparian plants) resources in freshwater ecosystems (Berggren et al., 2014; Doucett et al., 2007; Finlay et al., 2010), or to coarsely assess trophic level (Birchall et al., 2005; Solomon et al., 2009).

Although the use of δ^2H in ecological studies has increased rapidly over the past decade, a persistent challenge is to understand the ecological and physiological factors that contribute to variation in consumer tissue δ^2H values. Generally, as carbon and nitrogen isotopes move up a food chain, they are sorted by organisms in predictable ways, which leads to offsets in isotope values between the tissues of consumers and their diet, commonly referred to as trophic discrimination and denoted by $\Delta^{13}C$ or $\Delta^{15}N$ (Martínez del Río et al., 2009; Newsome et al., 2010). To expand the use of δ^2H analysis to study animal ecology, we must better understand how animals assimilate and sort hydrogen isotopes from the two distinct sources available for metabolism and tissue synthesis: food and body water (Ehleringer et al., 2008; Wolf et al., 2011, 2013; Fig. 1). This requires knowledge of (1) the proportion of the hydrogen in tissues from food and body water, and (2) the offset in δ^2H values of consumers and these two sources (i.e. trophic discrimination factors, Δ^2H_{Net}). In regard to the first challenge, controlled feeding experiments show that the majority (~70–80%) of hydrogen in consumer tissues is derived from diet, whereas only ~20–30% is sourced from environmental or drinking water (Rodriguez Curras et al., 2018; Newsome et al., 2017; Solomon et al., 2009; Wolf et al., 2011). Fewer studies have focused on quantifying Δ^2H_{Net}, which is mediated by physiological processes during resource assimilation, tissue synthesis and excretion. Similar to patterns observed in $\Delta^{13}C$ and $\Delta^{15}N$, variation in Δ^2H is likely

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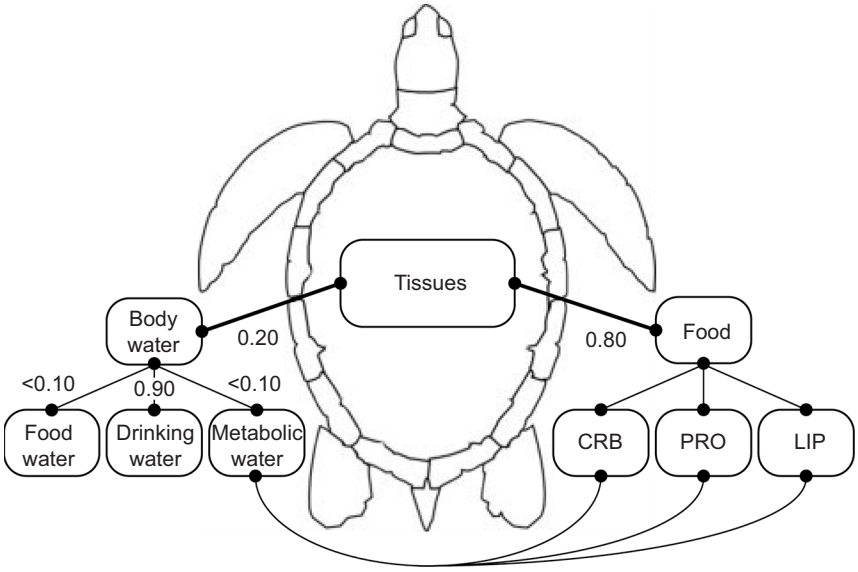

Fig. 1. Schematic of the sources of hydrogen available to green turtles (*Chelonia mydas*) in this study. The relative proportions of hydrogen from body water (p_{Water}) and food (p_{Diet}) were assumed to be 0.2 and 0.8, respectively, based on previous feeding experiments (Rodriguez Curras et al., 2018; Newsome et al., 2017; Solomon et al., 2009; Soto et al., 2013). CRB, carbohydrates; LIP, lipids; PRO, proteins.

influenced by tissue amino acid composition and the potential for disproportionate routing of dietary protein to tissue synthesis (Ambrose and Norr, 1993; Schwarcz and Schoeninger, 1991). A suite of studies have revealed that consumer tissues generally have δ^2H values greater than food sources but lower than environmental water (Peters et al., 2012; Solomon et al., 2009), a pattern that results from the mixing of these two sources and causes apparent increases in δ^2H values with increasing trophic level (Birchall et al., 2005; Hobson, 2005b; Magozzi et al., 2019).

Hydrogen isotopes have the potential to provide numerous ecological inferences. For example, significant variation in δ^2H values among marine primary producers (Estep and Dabrowski, 1980; Smith and Epstein, 1970) suggests that hydrogen isotopes may be useful for quantifying resource and habitat use in marine ecosystems (Ostrom et al., 2014), especially in nearshore ecosystems fueled by a combination of phytoplankton, macroalgae, seagrass and even terrestrial plants (e.g. mangroves). In addition, more recent work has identified predictable δ^2H discrimination among primary producer sources in marine ecosystems (Hondula and Pace, 2014), similar to that observed in freshwater aquatic and terrestrial food webs (Birchall et al., 2005; Peters et al., 2012; Soto et al., 2013). Thus, δ^2H may complement $\delta^{13}C$ and $\delta^{15}N$ data to better quantify the relative contributions of different sources of primary production and characterize food web dynamics in coastal marine ecosystems. However, before ecologists can confidently apply this tool to assess resource and habitat use by wild populations, additional controlled feeding experiments are needed to better understand the factors that influence hydrogen isotope assimilation and discrimination in a variety of marine consumers and their tissues.

Here, we investigate hydrogen isotope assimilation and discrimination in an omnivorous marine ectotherm by sampling tissues from captive green turtles, *Chelonia mydas* (Linnaeus 1758), fed controlled diets in two separate feeding experiments. Our objective was to estimate trophic discrimination factors (Δ^2H_{net}) between the sources of hydrogen available for tissue synthesis and green turtle skin (epidermis), blood serum/plasma and red blood cells. We analyzed the isotopic composition of dietary macromolecules and used a mass balance mixing model framework to examine the factors that cause variation in δ^2H values among tissue types and life stages. We anticipate that our results will help refine the use of δ^2H values as a tool to study resource and habitat use by marine consumers that inhabit nearshore ecosystems characterized by large variation in δ^2H values among primary producers.

MATERIALS AND METHODS

We analyzed green turtle tissues and diet samples collected during two previous controlled feeding experiments described in Seminoff et al. (2006) and Vander Zanden et al. (2012). For convenience, we refer to Seminoff et al. (2006) as Feeding Experiment 1 (FE1) and Vander Zanden et al. (2012) as Feeding Experiment 2 (FE2). In FE1, turtles were kept in a large oval fiberglass tank (10×3×1.5 m) filled with seawater. The water temperature was maintained at 24±1°C. Fluorescent light fixtures suspended above each tank provided full-spectrum radiation for 12 h each day; the tanks were also exposed to ambient light. Water quality was maintained between the following levels: pH=8.0–8.3, salinity=33–35 and ammonia <0.1 mg^{-1}. Monthly water changes pumped directly from the ocean prevented accumulation of high levels of ammonia, bacteria and fungi. Salinity was maintained by adding locally sourced fresh water. In FE2, turtles were kept in unshaded 40,000-gallon concrete tanks that received 400–1100 l min^{-1} of unfiltered saltwater pumped directly from the ocean. Both feeding experiments used green turtles hatched at Cayman Turtle Center (Grand Cayman, Cayman Islands). However, turtles from FE1 were transferred to the University of British Columbia (Vancouver, Canada) as yearlings. In FE1, eight juvenile green turtles with a mean (±s.d.) body mass of 11.7±0.7 kg and mean straight carapace length (SCL) of 45.2±1.2 cm were fed a pellet diet composed of 41% protein, 12% lipids and 47% carbohydrates by weight (Aquamax Grower 500; PMI Nutrition International, Brentwood, MO, USA) for 619 days (Table 1). Samples of the pelleted diet, epidermis and blood components – including whole blood, blood plasma and red blood cells – were collected at the end of FE1. In FE2, 70 green turtles (30 adults and 40 juveniles) were fed a constant pellet diet containing 35.5% protein, 4.2% lipids and 60.3% carbohydrates by weight (Southfresh Feeds, Demopolis, AL, USA) for 1460 days (Table 1); all adults in this experiment were sexually mature females. Adults had a mean (±s.d.) body mass of 125±29.2 kg and a mean curved carapace length (CCL) of 101±5.2 cm; juveniles had a mean body mass of 42±8.1 kg and a mean CCL of 73±5.5 cm. Samples of pelleted diet, epidermis, serum and red blood cells were collected at the end of FE2.

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Table 1. Diet composition information for *Chelonia mydas* in feeding experiments

	FE1	FE2
Bulk diet δ^2H (‰)	−109±3[a]	−100±3[b]
Bulk diet [H]	6.2±0.1	6.2±0.4
LE bulk diet δ^2H (‰)	−86±4[a]	−97±4[b]
LE bulk diet [H]	5.8±0.2	6.0±0.1
Protein (%)	41.0	35.5
Protein δ^2H (‰)	−149±7	−154±3
Protein [H]	4.7±0.2	4.8±0.2
Lipids (%)	12.0	4.2
Lipid δ^2H (‰)	−235±4[a]	−221±7[b]
Lipids [H]	10.6±0.4	10.7±0.7
Carbohydrates (%)	47.0	60.3
Carbohydrate δ^2H (‰)	−43	−60

Mean (±s.d.) δ^2H values, weight percent [H] of bulk diets and individual macromolecules, and relative proportions (weight percent) of each dietary macromolecule are shown for FE1 and FE2. Note that dietary macromolecular proportions sum to 100%, but they need to be multiplied by 0.8 to calculate Δ^2H_{Net} using Eqns 3–5 to account for the ∼20% contribution of hydrogen from environmental water to tissue synthesis (Fig. 1). Bulk diet was measured before and after lipid extraction (LE). Superscript letters indicate significant differences in mean δ^2H values of bulk diets or dietary macromolecules between the two diet treatments; see Results section for statistical output.

Separation of dietary macromolecules

Dietary lipid and protein δ^2H values were obtained via lipid extraction followed by hydrolysis and cation exchange separation. Lipids were extracted from homogenized diet pellets via three ∼24-h soaks in petroleum ether. After each soak, the removed petroleum ether was saved in a separate glass scintillation vial and transferred to pre-weighed silver capsules for δ^2H lipid analysis. Lipid-extracted diet samples were then rinsed with deionized (DI) water to remove any residual solvent and freeze dried. Approximately 6–7 mg of the lipid-extracted diet samples were hydrolyzed in 6 N HCl for 20 h at 110°C, breaking proteins into constituent amino acids, then dried under a flow of N_2 gas at 110°C; the HCl used for hydrolysis had a δ^2H value of −95‰. A cation exchange resin (Dowex 50WX8, 100–200 mesh) was then used to separate the amino acids from carbohydrates (Amelung and Zhang, 2001). The hydrolyzed sample was added to a column containing Dowex resin and then washed with 3–4 ml of 0.01 N HCl solution. Subsequently, 4 ml of 2 N NH_4OH was added to the column, and the effluent containing amino acids representing dietary proteins was collected in another pre-combusted glass vial. The amino acid fraction was dried down at 80°C under N_2 and temporarily re-suspended in DI water for transfer to silver capsules, which were then thoroughly dried at ∼45°C prior to δ^2H analysis (Amelung et al., 1996; Andrews, 1989). We measured δ^2H values of four to six replicates of the lipid and protein fractions isolated from each diet. δ^2H values for the carbohydrate portion of each diet were estimated with a mixing model using measured δ^2H values for dietary lipids and protein in combination with data on the relative weight percent proportion of these macromolecules in each experimental diet (Table 1). Note that dietary macromolecular proportions in Table 1 sum to 100%, but they need to be multiplied by 0.8 to calculate Δ^2H_{Net} using Eqns 3–5 (see below) to account for the ∼20% contribution of hydrogen from environmental water to tissue synthesis (Fig. 1). We acknowledge that this model does not account for potential sources of hydrogen from vitamins and minerals in each diet that could not be isolated and analyzed for their δ^2H composition, but they represent minor sources in comparison to the major macromolecular components (protein, carbohydrates, lipids) that we directly measured or modeled.

δ^2H analysis

Samples of blood serum/plasma and red blood cells were dried at 60°C for 24 h and homogenized with a mortar and pestle. Epidermis samples were rinsed with DI water, dried at 50°C for 24–48 h, and then lipid extracted in three sequential 24-h soaks with petroleum ether, followed by thorough rinsing in DI water before being dried at 50°C for ∼48 h (Seminoff et al., 2006). To evaluate the influence of lipids on δ^2H values, we compared δ^2H values of epidermis (N=20; 10 juveniles and 10 adults) from FE2 before and after lipid removal.

Approximately 0.2–0.3 mg of sea turtle tissue or pelleted diet samples were weighed into 3.5×5 mm silver capsules sealed for δ^2H analysis. Proteins can exchange ∼10–20% of their hydrogen with ambient water vapor (Bowen et al., 2005; Coplen and Qi, 2012), so we used a bench top equilibration method in which samples and associated in-house reference materials used to correct for exchangeable hydrogen sat for at least 3 weeks before analysis to ensure equilibration with local water vapor (Bowen et al., 2005; Wassenaar and Hobson, 2003).

Weight percent hydrogen concentration {[H]} and δ^2H values were measured with a high-temperature conversion elemental analyzer connected to a Delta V isotope ratio mass spectrometer (TCEA-IRMS; both Thermo Fisher Scientific, Bremmen, Germany) at the University of New Mexico Center for Stable Isotopes (UNM-CSI; Albuquerque, NM, USA). Hydrogen isotope values are expressed in delta (δ) notation using the following equation:

$$\delta^2H = [(R_{sample} - R_{standard}/R_{standard}) - 1] \times 1000, \quad (1)$$

where R represents the ratio of the heavy to the light isotope ($^2H/^1H$) in sample or standard, respectively, relative to Vienna-Standard Mean Ocean Water (V-SMOW), which is the internationally accepted standard for δ^2H analysis. Units are expressed in ppt (‰).

To correct for exchangeable hydrogen, we used a series of keratin in-house reference materials for which the hydrogen isotope composition of the non-exchangeable portion of the tissue ($\delta^2H_{non\text{-}exchangeable}$) had previously been estimated via room-temperature-exchange experiments identical to those used by Bowen et al. (2005). $\delta^2H_{non\text{-}exchangeable}$ values for the three keratin internal reference materials ranged from −55‰ to −175‰. We used a series of keratin external standards (KHS and CBS) to check the values obtained by these internal reference materials. Muscle and whole blood from cows raised in Wyoming and Florida, USA, were also used to compare δ^2H in similar tissues, which ranged from −70‰ and −150‰ for muscle and −82‰ and −160‰ for blood, respectively. We used the oil standard NBS-22 (δ^2H: −120‰) to correct lipid samples extracted from pelleted diet because lipids do not contain exchangeable hydrogen. All standards had a within-run hydrogen isotope variation (s.d.) of ≤3‰. Weight percent [H] values were measured via analysis of powdered benzoic acid analyzed in each run of unknown sea turtle tissue or diet samples.

Hydrogen isotope mixing model

The hydrogen trophic discrimination factors between tissue type and diet or water sources (food water, drinking water and metabolic

water; Fig. 1) were estimated with mass balance mixing model:

$$\delta^2H_{Tissue} = p_{Water}(\delta^2H_{Water} + \Delta^2H_{Water}) + p_{Protein}(\delta^2H_{Protein} + \Delta^2H_{Protein}) + p_{Carbohydrates}(\delta^2H_{Carbohydrates} + \Delta^2H_{Carbohydrates}) + p_{Lipids}(\delta^2H_{Lipids} + \Delta^2H_{Lipids}), \quad (2)$$

where δ^2H is the hydrogen isotope value of each source (water, protein, carbohydrates, lipids), p is the proportion of hydrogen coming from each of these sources, and Δ^2H is the discrimination factor associated with each source. This model assumed that there are four potential sources of hydrogen used by consumers to synthesize tissues, for which their relative contributions to the consumer's tissues must sum to one (Fig. 1):

$$p_{Water} + p_{Protein} + p_{Carbohydrates} + p_{Lipids} = 1. \quad (3)$$

The Δ^2H associated with each dietary macromolecule in Eqn 2 can be isolated and combined as the net trophic discrimination factor or Δ^2H_{Net}:

$$\Delta^2H_{Net} = p_{Water}(\Delta^2H_{Water}) + p_{Protein}(\Delta^2H_{Protein}) + p_{Carbohydrates}(\Delta^2H_{Carbohydrates}) + p_{Lipids}(\Delta^2H_{Lipids}). \quad (4)$$

Thus, Eqn 2 can be simplified:

$$\delta^2H_{Tissue} = p_{Water}(\delta^2H_{Water}) + p_{Protein}(\delta^2H_{Protein}) + p_{Carbohydrates}(\delta^2H_{Carbohydrates}) + p_{Lipids}(\delta^2H_{Lipids}) + \Delta^2H_{Net}. \quad (5)$$

The relative proportions of hydrogen from body water (p_{Water}) and diet (p_{Diet}) representing the combined contribution from all three dietary macromolecules were assumed to be 0.2 and 0.8, respectively, which appear to be conserved among a wide variety of taxa (Rodriguez Curras et al., 2018; Newsome et al., 2017; Solomon et al., 2009; Soto et al., 2013). We assumed that sea turtle body water had a similar δ^2H composition to ocean water because sea turtles have high water flux rates of ∼10% of their total body water per day (Jones et al., 2009) and a near-fully aquatic lifestyle. We used isoscapes of seawater $\delta^{18}O$ values (LeGrande and Schmidt, 2006) and the equation for the global meteoric water line ($\delta^2H=8\delta^{18}O+10‰$; Craig and Gordon, 1965) to estimate the δ^2H of seawater in the two locations where feeding experiments were conducted to estimate seawater δ^2H values (Kendall and Coplen, 2001). This approach resulted in a δ^2H_{Water} value for the FE1 experiment in Vancouver of −10‰, which reflects the influence of ^2H-depleted river water in coastal areas in this wet temperate region, and a δ^2H_{Water} value for the FE2 experiment in the Caribbean Cayman Islands of +10‰, which reflects the influence of evaporation of surface seawater in this relatively hot tropical region. Turtles in FE1 were also used in an experiment that utilized the doubly labeled water technique (Jones et al., 2009), which provided a mean (±s.d.) baseline ^2H concentration of 153.7 ±0.5 ppm in body water, equivalent to a mean body water δ^2H value of −13±3‰. Unfortunately, no measurements of body water δ^2H values are available for turtles from FE2. Finally, we assessed the potential impacts of our assumptions with a sensitivity analysis by varying the δ^2H value of seawater at each locality by 10‰, and the p_{water} contribution from 15% to 30%.

Data analysis

We used one- and two-way analysis of variance (ANOVA), followed by *post hoc* Tukey honest significant difference (HSD) pairwise comparisons with Bonferroni correction to evaluate differences between tissues for Δ^2H_{Net} in experiment FE1. For experiment FE2, we used a two-way ANOVA with two factors: age (juvenile or adult) and tissue (epidermis, EPI; serum/plasma, SER/PLA; red blood cells, RBC) corrected for unbalanced data (lsmeans), followed by pairwise comparison using the Bonferroni correction. When comparing between experiments, we used a two-way ANOVA with two factors: experiment (FE1 or FE2) and tissue (EPI, SER/PLA, RBC) corrected for unbalanced data (lsmeans), followed by pairwise comparison using Bonferroni correction. We used a Max-*t*-test to compare the mean δ^2H values of bulk and lipid-extracted diet and diet macromolecules (proteins and lipids) between FE1 and FE2 (Herberich et al., 2010). This procedure is useful to assess datasets that include unbalanced group sizes, non-normal distributions and non-heteroscedasticity (Herberich et al., 2010). All models were run using R software (https://www.r-project.org/).

RESULTS

Diet and water

Table 1 presents mean (±s.d.) δ^2H values, [H] and relative proportions (weight %) for bulk diet, lipid-extracted bulk diet, and dietary protein and lipids in the two feeding experiments. Mean δ^2H values of bulk diets (t=5.3, P=0.001) and dietary lipids (t=3.4, P=0.010) were higher in FE2 than in FE1. Mean δ^2H values of lipid-extracted bulk diets were lower in FE2 than in FE1 (t=−3.5, P=0.007). Mean δ^2H values of dietary protein were not different between diets (t=−1.4, P=0.19).

Results of the sensitivity analysis revealed that changing p_{Water} from 15% to 30% or varying the δ^2H value of this source of hydrogen by 10‰ resulted in an overall difference in estimated Δ^2H_{Net} within tissues or among feeding experiments of 5–6‰. These values are slightly higher than analytical precision (3–4‰) of δ^2H measurements of organic materials via TCEA-IRMS and are similar to the observed variation in δ^2H within tissues, but much smaller than differences in δ^2H among tissues (Table 2).

Tissue δ^2H patterns within and between feeding experiments

We combined isotope data for blood plasma and serum because these blood components had similar δ^2H values. Epidermis δ^2H values were higher than values for blood components in FE1 (F=47.2; d.f.=31, P<0.001; Fig. 2, Table 2). By contrast, δ^2H values of all tissues differed within and between life stages (F=560.9, d.f.=204, P<0.001; Fig. 2, Table 2) in FE2, with the exception of red blood cells and serum/plasma for the juvenile group. Adults had lower serum/plasma δ^2H values and higher weight percent [H] values than juveniles in FE2 (Fig. 3, Table 2), which resulted in a significant negative relationship between serum/plasma δ^2H values and weight percent [H] (R^2=0.88; P<0.001; Fig. 3). The only tissue that differed between feeding experiments was epidermis, which was slightly but significantly higher in juveniles in FE2 (F=313, d.f.=143, P<0.001; Fig. 2, Table 2). Lastly, there were no significant differences in mean (±s.d.) δ^2H or weight percent [H] values between bulk {δ^2H: −74±8; [H]: 5.6±0.1} and lipid-extracted epidermis {δ^2H: −73±6; [H]: 6.0±0.1} (t=−0.27, d.f.=38, P=0.79).

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Table 2. Mean (±s.d.) δ^2H values, weight percent [H] and net hydrogen discrimination factor values (Δ^2H_{Net}) of the different tissue types of *C. mydas* at different life stages in feeding experiments

		FE1			FE2		
Life stage	Tissue	δ^2H (‰)	[H]	Δ^2H_{Net} (‰)	δ^2H (‰)	[H]	Δ^2H_{Net} (‰)
Juvenile	Epidermis	−79±9^b	4.6±0.4	11±9^a	−70±5^a	6.0±0.1	8±5^a
	Serum/plasma	−108±7^c	5.8±0.4	−18±7^d	−104±5^c	5.5±0.3	−26±5^c
	Red blood cells	−109±3^c	6.0±0.1	−19±3^d	−106±3^c	5.8±0.2	−28±3^c
	Whole blood	−105±3	5.9±0.1	−15±3	–	–	–
Adult	Epidermis	–	–	–	−78±7^b	6.1±0.1	0±7^b
	Serum/plasma	–	–	–	−137±11^d	6.7±0.4	−58±11^e
	Red blood cells	–	–	–	−99±4^e	6.1±0.1	−21±4^d
	Whole blood	–	–	–	–	–	–

Δ^2H_{Net} was calculated as follows: $\Delta^2H_{Net}=p_{Water}(\Delta^2H_{Water})+p_{Protein}(\Delta^2H_{Protein})+p_{Carbohydrates}(\Delta^2H_{Carbohydrates})+p_{Lipids}(\Delta^2H_{Lipids})$. Superscript letters indicate significant differences in tissue δ^2H values between life stages and diet treatments.

Tissue Δ^2H_{Net}

Epidermis had significantly higher mean Δ^2H_{Net} values than blood components in FE1 (F=53.7, d.f.=23, P<0.001; Fig. 4, Table 2). In FE2, multiple pairwise comparisons between life stage and between tissues show that all tissues except serum/plasma and red blood cells in juveniles had significantly different Δ^2H_{Net} values (F=572.9, d.f.=205, P<0.001). Mean Δ^2H_{Net} values in epidermis and serum/plasma were higher in juveniles than in adults, whereas in red blood cells the opposite pattern was observed (Fig. 4, Table 2). Estimates of Δ^2H_{Net} for juveniles were slightly but significantly different between FE1 and FE2 experiments for serum/plasma and red blood cells (F=315.5, d.f.=143, P<0.001), but similar for epidermis (F=315.5, d.f.=143, P=0.14).

DISCUSSION

We investigated the potential for using δ^2H values as an ecological tracer in marine consumers by providing the first quantification of hydrogen isotope discrimination factors (Δ^2H_{Net}) in four tissues from juvenile and adult green turtles using samples collected during two previous controlled feeding experiments (Seminoff et al., 2006; Vander Zanden et al., 2012). In the following sections, we assess the factors that influence Δ^2H_{Net} and discuss possible physiological mechanisms responsible for the observed differences among diet treatments and age classes. Overall, our results corroborate patterns observed in previous controlled feeding experiments on other taxa (Rodriguez Curras et al., 2018; Fogel et al., 2016; Newsome et al., 2017; Wolf et al., 2013), and we anticipate that these data will enable ecologists to accurately apply δ^2H isotopes to study diet composition, habitat use and movement in marine consumers.

When comparing results from different feeding experiments, it is important to consider differences in dietary macromolecule composition and the proportional contribution of food and body water to tissue synthesis, both of which have been shown to influence tissue δ^2H and associated Δ^2H_{Net} values. Our models assumed that ∼80% and ∼20% of the hydrogen in green turtle tissues was synthesized from diet versus water (Fig. 1), respectively, which is similar to contributions found in a wide variety of vertebrate taxa (Rodriguez Curras et al., 2018; Hobson et al., 1999; Newsome et al., 2017; Soto et al., 2013). Given the relatively small contribution (20%) of hydrogen from water that is used to synthesize tissues, calculations from our sensitivity analysis showed that a small variation (5–10‰) in our estimated seawater δ^2H values did not heavily impact our estimates of Δ^2H_{Net}. With respect to diet composition, the diets used in the two feeding experiments were similar in terms of macromolecular composition and bulk diet δ^2H values (Table 1), and green turtle tissues had remarkably similar δ^2H values for nearly all tissue types (Fig. 2); the only exception to this was blood serum/plasma for adult turtles in FE2 (Fig. 3). Finally, previous δ^2H-based work on tropical seabirds (Ostrom et al., 2014) has hypothesized that variation in feather

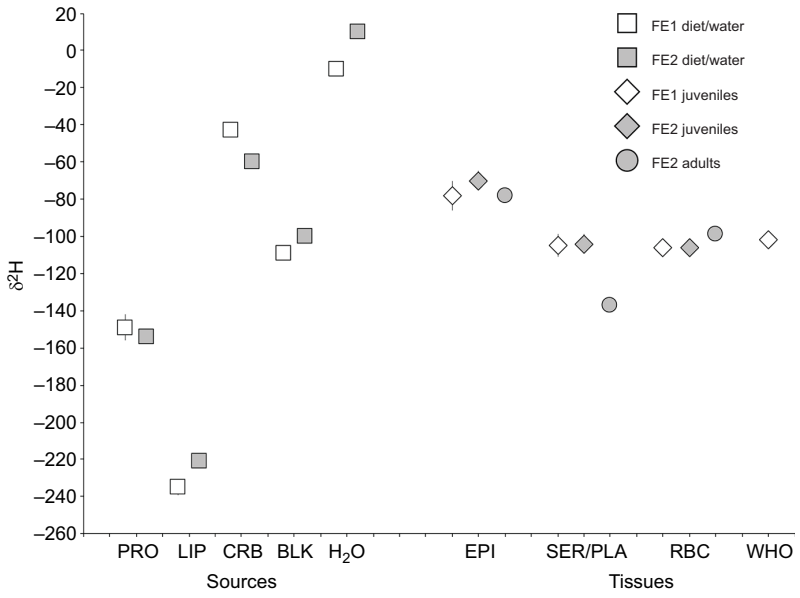

Fig. 2. Mean (±s.d.) δ^2H values of C. *mydas* tissues and potential sources of hydrogen in each feeding experiment (FE1 and FE2). Sources of hydrogen include dietary protein (PRO), lipids (LIP), carbohydrates (CARB), bulk diet (BLK) and water (H_2O). Tissues include epidermis (EPI), serum/plasma (SER/PLA), red blood cells (RBC) and whole blood (WHO). Symbols without error bars either represent a single measurement (carbohydrates and water), or the error bars are smaller than the symbol.

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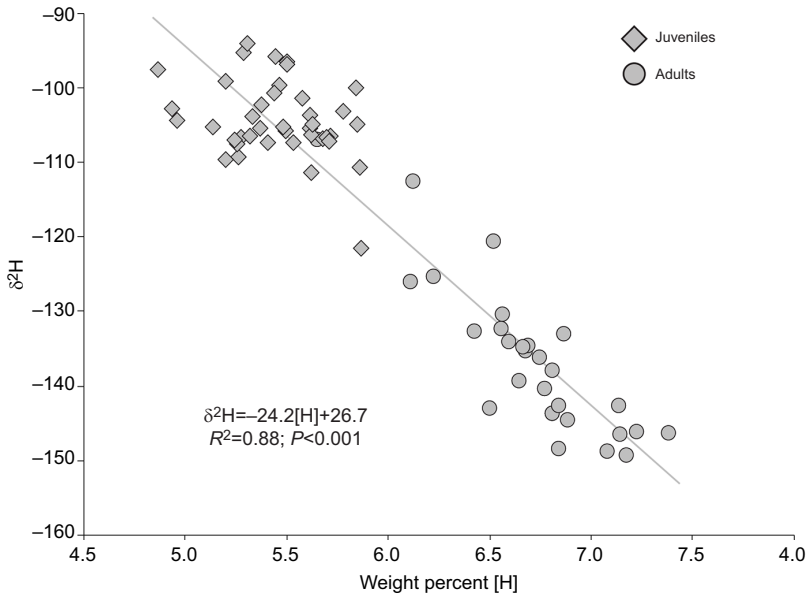

Fig. 3. δ^2H values of serum/plasma versus weight percent [H] of juvenile and adult experimental *C. mydas*. The gray line represents a significant negative linear relationship for the entire dataset (juveniles and adults).

hydrogen isotope values may be caused by isotopic fractionation associated with water loss during salt excretion via the nasal salt glands. We assume that this mechanism has a negligible isotopic effect on body water of sea turtles because they have (1) a near-fully aquatic lifestyle, (2) a large body size that dampens evaporative effects, and (3) high water flux rates relative to their body size with complete turnover of body water in ∼10 days (Jones et al., 2009). In addition, baseline ^{2}H concentrations of turtles in FE1 reported in Jones et al. (2009) show that body water δ^2H values are similar to those of local seawater. Unlike seabirds, sea turtles are considered seawater drinkers with water turnover rates as high as ∼6% of total body water per day when fasting, suggesting that water turnover is primarily driven by imbibing seawater (Jones et al., 2009).

Tissue δ^2H

Green turtle epidermis had higher mean δ^2H values relative to blood components by ∼20‰ in both feeding experiments (Fig. 2), which is similar to patterns in δ^2H values of keratinaceous tissues (feathers and claws) and blood components observed in feeding experiments on birds (Hobson et al., 1999; Wolf et al., 2011, 2012, 2013). There are two possible explanations for these patterns. First, previous feeding experiments reporting δ^{13}C and δ^2H data have suggested that such tissue-specific isotopic patterns are likely to be driven by differences in the amino acid composition among tissues (Rodriguez Curras et al., 2018; Newsome et al., 2017; Wolf et al., 2012). Hydrogen isotope values of individual amino acids within a single organism can vary by 200–300‰ (Fogel et al., 2016); thus, subtle changes in a tissue amino acid composition could easily drive variation in bulk tissue δ^2H values (Rodriguez Curras et al., 2018; Newsome et al., 2017). A second potential explanation for the observed tissue-specific δ^2H patterns between epidermis and blood components is that the hydrogen in blood is directly routed from dietary protein, which had lower δ^2H values than that of bulk diet or dietary carbohydrates in both experiments (Table 1 and Fig. 2). Here, we define protein routing as the preferred use of dietary protein to synthesize proteinaceous tissues, which decreases the costs of *de novo* synthesis of non-essential amino acids from dietary carbohydrates and/or lipids. Protein routing has been observed in

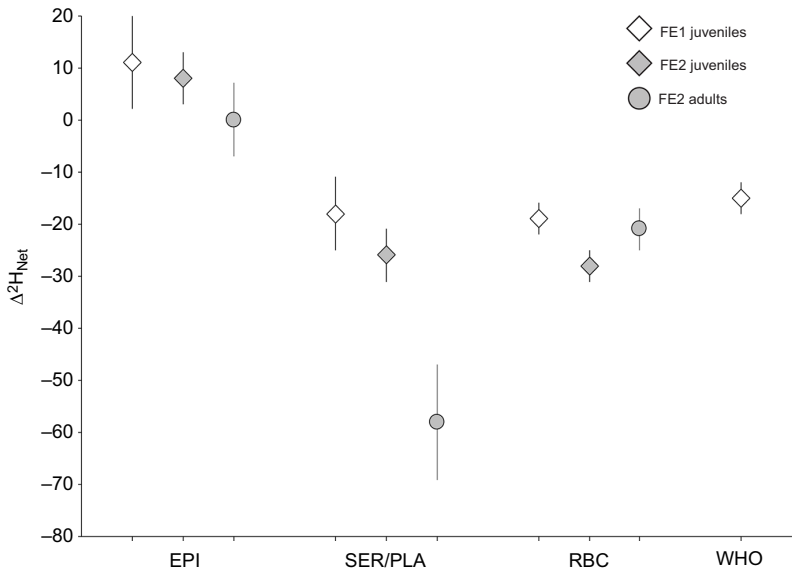

Fig. 4. Mean net δ^2H discrimination factors (Δ^2H_{Net}) for tissues collected from juvenile and adult *C. mydas* in feeding experiments. Tissue types include epidermis (EPI), serum/plasma (SER/PLA), red blood cells (RBC) and whole blood (WHO); error bars represent s.d.

other δ^2H-based controlled feeding experiments (Rodriguez Curras et al., 2018; Newsome et al., 2017); however, the animals in those experiments were fed diets that had much lower weight percent protein contents than the diets fed to green turtles in our experiments, which was exceptionally high (35.5–41.0%) for an omnivore. Because dietary protein was readily available, the potential for protein routing to particular tissues (e.g. epidermis) was likely minimal in our experiments. Furthermore, tissue-specific patterns in δ^2H values are similar among a wide range of unrelated taxa (birds, mammals and reptiles), and thus the first potential explanation above involving variation in amino acid composition among tissues is the most parsimonious reason for the observed differences in δ^2H values among green turtle tissues.

The significantly lower serum/plasma δ^2H values observed in adult female versus juvenile turtles from the FE2 experiment (Vander Zanden et al., 2012) are likely to be related to elevated lipid content in this tissue type. Lipids have relatively high weight percent [H] contents (11–12%) and lower δ^2H values in comparison to associated proteins owing to a large isotopic discrimination during the formation of acetyl CoA from pyruvate (Hayes, 2001; Schmidt et al., 2003; Sessions and Hayes, 2005). Thus, the significant negative relationship between δ^2H values and weight percent [H] observed in serum/plasma (Fig. 3) supports the idea that these samples had higher lipid content than serum/plasma collected from juveniles. Because all adults in our experiment were sexually mature females, higher concentration of free lipids in blood serum/plasma could be related to reproduction (i.e. follicle development, egg production). Females mobilize lipids prior to and throughout the nesting season to help with vitellogenesis (yolk deposition). In this process, lipids and proteins are stored in the oocytes during egg formation (Milton and Lutz, 2003) and other related reproductive processes (Hamann et al., 2002). Thus, the relationship between serum/plasma δ^2H and [H] could potentially be used as a proxy to identify sexually mature females in the field. Finally, comparison of δ^2H for lipid and non-lipid extracted epidermis suggests that this tissue has sufficiently low lipid content such that lipid extraction prior to hydrogen isotope analysis is unnecessary. These results mirror those found in previous studies that showed that δ^{13}C values of sea turtle epidermis were unaffected by lipid removal (Turner Tomaszewicz et al., 2017; Vander Zanden et al., 2012, 2014).

Tissue Δ^2H_{Net}

By definition (Eqn 5), patterns in tissue-specific trophic discrimination factors are influenced by tissue hydrogen isotope values; therefore, patterns in Δ^2H_{Net} typically mirror those in tissue δ^2H values. Thus, the observed differences in Δ^2H_{Net} values between epidermis and blood components could simply reflect differences in tissue-specific discrimination that we suggest are likely driven by variation in amino acid composition among these tissues. Δ^2H_{Net} estimates are also sensitive to the proportional contribution of dietary macromolecules (Eqn 4), especially protein because it can be directly routed from diet and thus could contribute more to proteinaceous tissue synthesis relative to its dietary content. Previous feeding experiments on tilapia (*Oreochromis niloticus*; Newsome et al., 2017) and house mice (*Mus musculus*; Rodriguez Curras et al., 2018) showed that when animals were fed a low-protein diet (5–10%), 34–48% of the hydrogen in proteinaceous tissues (muscle and liver) was derived from dietary protein. In this study, turtles in both experiments were fed diets with a high protein content (35.5–41.0%), and thus protein routing was likely minimal in comparison to the previous work mentioned above. Overall, our estimates of Δ^2H_{Net} should be considered preliminary because the protein content of the diets used in FE1 and FE2 are ∼3–4 times higher than those consumed by wild green turtles in the eastern Pacific Ocean or Caribbean (Bjorndal, 1980; López-Mendilaharsu et al., 2005; Seminoff et al., 2002). Our previous work shows that the relative importance of protein routing versus *de novo* synthesis of non-essential amino acids from dietary carbohydrates and lipids has an important influence on Δ^2H_{Net} (Newsome et al., 2017; Rodriguez Curras et al., 2018). As such, our study provides a valuable first approximation of the range in Δ^2H_{Net} values for multiple tissues of an omnivorous marine ectotherm and provides a framework for investigating this important parameter in other marine species.

Future directions

Our study is an initial step for expanding the use of δ^2H analysis to evaluate resource and habitat use of marine consumers that inhabit coastal habitats, especially those that are fueled by a combination of marine (micro/macroalgae and seagrass) and terrestrial producers (e.g. mangroves). The hydrogen isotope composition of primary producers in coastal marine ecosystems can vary by more than 100‰ (Estep and Dabrowski, 1980), providing a sufficient degree of natural variation to trace resource use by consumers with δ^2H analysis. We suggest that additional feeding experiments are needed to better understand variation in Δ^2H_{Net} among species so that mixing models can be accurately applied to quantify diet composition and energy flow in marine food webs. When possible, feeding experiments should vary diet quality (e.g. protein content) but attempt to include diets that mimic the macromolecular composition of prey consumed by wild populations. In addition to designing more realistic feeding experiments, direct δ^2H measurement of body water distilled from blood would help refine our understanding of the processes that influence discrimination of hydrogen isotopes in animals and enable us to broaden our use of this tool to study the ecology of wild populations of marine consumers.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.P.B., J.A.S., H.B.V.Z., T.T.J., K.A.B., A.B.B., W.M., S.D.N.; Methodology: L.P.B., J.A.S., G.B.-V., S.D.N.; Formal analysis: L.P.B., G.B.-V., S.D.N.; Resources: W.M.; Writing - original draft: L.P.B.; Writing - review & editing: J.A.S., H.B.V.Z., T.T.J., K.A.B., A.B.B., W.M., G.B.-V., S.D.N.; Supervision: J.A.S., S.D.N.; Funding acquisition: L.P.B., S.D.N.

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