COMPARING PREDICTED AND MEASURED TRIPLE OXYGEN ISOTOPE MEASUREMENTS ($\Delta^{17}O$) OF CAPTIVE DEER MICE (*PEROMYSCUS MANICULATUS)* BODY WATER

Zachary T. Steele

Old Dominion University – Department of Biological Sciences

Advisor: Dr. John P. Whiteman

Abstract

Animals obtain water from drinking and the food they consume (pre-formed water), and through endogenous synthesis by the metabolic pathways (metabolic water). Understanding the contributions of these water sources to an animals' total body water is critical but is challenging to measure in free-ranging wild animals. A newly developed technique, the $\Delta^{17}O_{BW}$ method, only requires one sample from an animal and measures triple oxygen stable isotopes (^{16}O, ^{17}O, and ^{18}O; $\Delta^{17}O$). This method relies upon two main assumptions: 1) the $\Delta^{17}O$ value derived for metabolic water is relatively fixed at -441 per meg; and 2) the $\Delta^{17}O$ value derived for pre-formed water is ~41 per meg. To further validate this method, I housed captive deer mice (*Peromyscus maniculatus*) in a metabolic phenotyping system within a temperature control cabinet for continuous measurements of food, water, O_2 consumption, CO_2 production, and evaporative water loss. I compared measured and predicted $\Delta^{17}O_{BW}$ values. Preliminary findings suggest that predictive models accurately estimate $\Delta^{17}O_{BW}$ values (<15 per meg difference) except when the study subjects' water economy index (WEI) is low. Slight adjustments to account for low WEI will be necessary before proceeding to the primary captive study and validation of this method in the field.

Introduction

Maintaining water balance and meeting energy requirements is critical for all animals[1]. Most animals are ~70-90% water by mass and rely on water and energy for temperature regulation, waste removal, and facilitating vital metabolic reactions[1]. The ramifications of climate change are increasing the need to understand how animals obtain water and energy because many species must acclimate to shifts in their habitat (e.g., increases or decreases in temperature and rainfall) or face extirpation[2,3]. However, few methods are available to directly measure metabolism and water intake in a natural setting[4]. Instead, most studies are conducted in captivity using methods like respirometry and many captive studies cannot easily account for the multiple environmental variables encountered by animals in the wild[5].

Alternatively, methods currently available for studies of free-ranging animals are often hampered by prohibitive costs and logistics[4]. These include methods such as the "doubly-labeled water" (DLW) technique which involves injecting a captured animal with stable isotopes of hydrogen and oxygen ($^2H_2{}^{18}O$), then releasing the animal[6]. Days to weeks later the same animal is re-captured and a blood sample is collected, and the different rates of decline in δ^2H and $\delta'^{18}O$ are used to infer water intake and metabolic rate (via CO_2 production[6]). When studying a limited or

threatened population, the necessity of multiple capture events for DLW greatly reduces the applicability of this method[4].

Recently, a potential solution to this dilemma was developed, requiring only a single blood sample to estimate metabolism and water intake[7]. This method is based upon measuring $\Delta^{17}O$ in body water ($\Delta^{17}O_{BW}$), which is a quantification of the relative amounts of the three stable oxygen isotopes (i.e., ^{17}O and ^{18}O are heavy isotopes of oxygen, compared to the common form of ^{16}O, expressed as $\delta'^{17}O$ and $\delta'^{18}O$ respectively – ' indicates linearization) that are naturally found in an animal's body water[7]. $\Delta^{17}O$ is calculated from measurements of $\delta'^{17}O$ and $\delta'^{18}O$ which have a near constant relationship when plotted against each other[7] (Figure 1A). This relationship occurs because the isotopic variation of $\delta'^{17}O$ and $\delta'^{18}O$ is typically based on mass (i.e., via mass-dependent fractionation) and means that a sample with a high $\delta'^{18}O$ value is expected to also have a high $\delta'^{17}O$ value[7]. However, there are small positive and negative deviations from this expected relationship, representing mass-independent fractionation (e.g., supersaturation, Rayleigh distillation, stratospheric intrusions, etc.[8]) which are quantified as $\Delta^{17}O$. Importantly, $\Delta^{17}O$ acts as a natural tracer of water sources[7,9].

Animals lose water through exhalation of moist breath and through evaporation from the skin, and excretion of liquid water in waste products[1]. These losses are offset by inputs from pre-formed sources (drinking and food water) and internal metabolic processes[7]. Critically, these internal processes include the de novo synthesis of water using oxygen molecules inhaled from the atmosphere[1]. This production of water, termed "metabolic

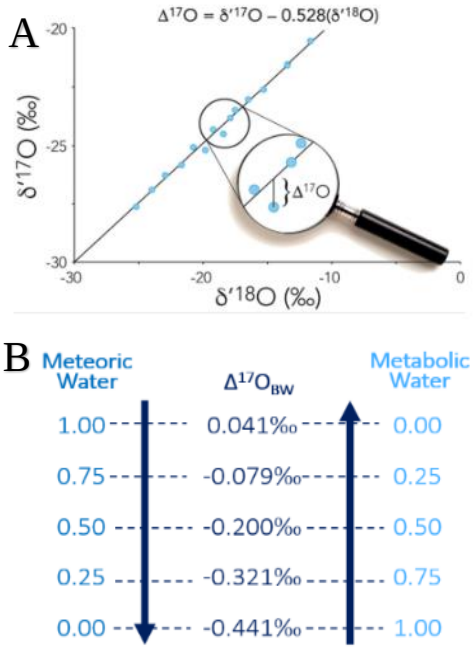

Figure 1. A) $\delta'^{17}O$ and $\delta'^{18}O$ represent the abundance of ^{17}O and ^{18}O relative to the abundance of ^{16}O. Deviations from this slope (i.e., residuals) are represented as $\Delta^{17}O$ (From reference 7; pg. 659; Figure 1). B) The variance of $\Delta^{17}O$ in an animal's body water ($\Delta^{17}O_{BW}$) depending on the contribution from meteoric and metabolic water.

water", is a byproduct of the chemical reactions of metabolism[1]. As such, metabolic water production is directly linked to the rate of energy production. Metabolic water, along with pre-formed water obtained from food or drinking water, typically accounts for 80-99% of body water[10]. Both pre-formed and metabolic water have near constant $\Delta^{17}O$ values, of 41 per meg (parts per million) and -441 per meg respectively[7,9,11]. Therefore, the $\Delta^{17}O$ value of an animal's body water should indicate their reliance on metabolic water, which varies greatly among species[7]. Some animals primarily rely on pre-formed water and have little input from metabolic water, resulting in a $\Delta^{17}O$ value near 0 per meg like African elephants (*Loxodonta africana*; Figure 1B)[7]. In contrast, desert-adapted animals like kangaroo rats (genus *Dipodomys*) may lack access to pre-formed water and instead

primarily rely on metabolic water resulting in a $\Delta^{17}O$ value closer to -200 per meg[7,12].

While early studies using the $\Delta^{17}O_{BW}$ method are promising[7,9], additional validation is required. While an initial captive study assessing $\Delta^{17}O_{BW}$ in mice found support for predicted trends of a decrease in $\Delta^{17}O_{BW}$ in response to increased metabolic rate, this study did not fully assess all relevant variables and instead only estimated real-time food and water intake[7]. This lack of precise data potentially explains a substantial, consistent underprediction of measured $\Delta^{17}O_{BW}$ values obtained in previous studies[7,9]. While previous $\Delta^{17}O_{BW}$ predictions methods were more generalized[7,9], recently described $\Delta^{17}O_{BW}$ prediction methods incorporate more variables that were previously used for detailed $\delta'^{18}O$ modeling[10,13]. More recent $\Delta^{17}O_{BW}$ predictive modeling incorporates different aspects of an animals' ecology and physiology while also integrating abiotic factors like relative humidity and ambient temperature[13]. To improve upon initial $\Delta^{17}O_{BW}$ captive studies[7,9], I housed deer mice (*Peromyscus maniculatus*) in a metabolic phenotyping system within a temperature control cabinet for continuous measurements of food, water, and O_2 consumption, CO_2 production, and evaporative water loss. I compared measured $\Delta^{17}O_{BW}$ with recently updated $\Delta^{17}O_{BW}$ predictive modeling to ascertain the accuracy of estimates[13]. In this paper, I describe the preliminary findings of this research which I aim to fully complete within the next nine months.

Methods

Captive Environment

In December 2021, eight deer mice were placed into 21x37x14cm cages as part of a metabolic phenotyping system (Promethion; Sable Systems International – NV, USA). Each cage contained three "hoppers" that were metal drop-down containers connected to mass monitors that continuously weighed each hopper (see Figure 2). The first hopper was a feeding hopper that contained 0.49% NaCl Teklad diet (TD.96208; Envigo – IN, USA) covered by a grate to provide small slits for feeding. The second hopper was a water hopper that contained a typical rodent drinker. The final hopper was a mass hopper and provided a covered walk-in platform for mice to enter. Once a mouse entered this platform for a prolonged period of time (≥5 seconds) a weight was recorded. Combined, these three hoppers allowed for continuous measurement of mass, food intake, and water intake. The metabolic phenotyping system also provided continuous measurement of O_2 consumption, CO_2 production, and evaporative water loss. The system was housed within a temperature control cabinet (Model 7000-25-1; Caron – OH, USA) which allowed for temperatures to be held constant at 25°C. This researched was approved under Old Dominion University Institutional Animal Care and Use Committee Protocol #20-001.

Figure 2. Promethion cage layout with the feeding hopper (front right), water hopper (back right), and mass hopper with red-shaded frames (center). This is a stock photo provided by Sable Systems International which includes an exercise wheel (far left) that was not used in this experiment[14].

Sample Collection & Processing

Blood samples of ≤200μL were collected via a cheek bleed on the facial vein of 6 of the 8 mice. Samples were collected in a 200μL microvette and were immediately centrifuged, and then the plasma was collected for micro-distillation following previously validated guidelines[15]. Briefly, as previously outlined[15], plasma samples were inserted into a 9" glass pipette (Kimble 883350-0009). The large opening of the pipette was then flame sealed via a torch. The narrow end of the pipette was then connected to a vacuum pump via a 3-way stopcock, the larger end of the pipette was dipped in liquid nitrogen until frozen, and then the vacuum was applied before the narrow end was quickly flame sealed. After sealing, the larger end of the pipette was placed on a slide warmer, with the narrow end extending outwards; as the sample thawed and the water slowly became vapor, the water eventually condensed at the narrow end because of the cooler temperature. A final flame seal then separated the narrow end, leaving a makeshift microcapillary tube of distilled body water.

Stable Isotope Analysis

Distilled samples were measured via a cavity ring-down spectroscopy instrument (Picarro L2140-*i*; Picarro – CA, USA). I measured samples 7–17 times (depending on final sample volume) and I removed the first 5 measurements from the data to limit the memory effect of previous measurements. Using the remaining measurements, I calculated a mean value, which in turn, provides an estimate of metabolic water production. I compiled the mean values of multiple distillation samples for each individual sample to obtain an overall average to represent that specific sample. In-house water standards (VA01, 02, and 03) verified via internationally accepted United States Geological Survey (USGS) water standards (USGS46, 47, and 48) that were measured simultaneously, were used to standardize the Picarro instrument. This allowed for corrections to be made based on instrument error. An example "run" on the Picarro is provided in Table S1 to display the setup needed to obtain accurate and precise data, with many runs lasting 72+ hours.

Data Analysis

The Picarro provides measurements of ^{17}O and ^{18}O as ratios of isotopic abundancies ($\delta'^{17}O$ and $\delta'^{18}O$, which are used to ultimately calculate $\Delta^{17}O$) in reference to the "base" form of oxygen (^{16}O). These ratios are calculated in reference to internationally accepted standards: Vienna Mean Standard Ocean Water (VSMOW) and Standard Light Antarctic Precipitation (SLAP). Normalization to what is referred to as VSMOW-SLAP scale reduces the potential for instrumental bias and allows data to be more comparable with findings within the isotope literature. Normalizing to this scale typically requires direct measurements of VSMOW and SLAP water standards, but if in possession of standards with known isotopic values already adjusted to VSMOW-SLAP scale (in this case, USGS 46,47, and 48), a correction factor may be calculated using linear regression[7]. Due to the high costs of purchasing VSMOW and SLAP, I applied the correction factor method to establish VSMOW-SLAP scale using R (version 4.0.3). Once $\delta^{17}O$ and $\delta^{18}O$ values were obtained on VSMOW-SLAP scale, I then solved for $\Delta^{17}O$.

Results

Correction Factors

Separate correction factors were applied across the two sampling runs conducted on the Picarro. Correction factors resulted in adjusted values that were on average 0.001‰ and 0.014‰ respectively from the established values for $\delta^{17}O$ and $\delta^{18}O$ of each in-house standard, and 7.46 per meg for $\Delta^{17}O$ (Table 1).

Table 1. The difference between the mean corrected and established in-house standard $\delta^{17}O$, $\delta^{18}O$, and $\Delta^{17}O$ values.

Sampling Run & Metric	Difference from Standard VA 01	Difference from Standard VA 02	Difference from Standard VA 03
Run 1 $\delta^{17}O$	0.0005‰	0.0018‰	0.0013‰
Run 1 $\delta^{18}O$	0.0047‰	0.0165‰	0.0118‰
Run 1 $\Delta^{17}O$	2.99 per meg	10.50 per meg	7.50 per meg
Run 2 $\delta^{17}O$	0.0007‰	0.0021‰	0.0014‰
Run 2 $\delta^{18}O$	0.0075‰	0.0263‰	0.0188‰
Run 2 $\Delta^{17}O$	3.37 per meg	11.89 per meg	8.52 per meg

Predicted Versus Measured $\Delta^{17}O$ Values

The predicted $\Delta^{17}O_{BW}$ was on average 26.77 per meg lower than the measured values (Table 2 & Figure 3). Measured $\Delta^{17}O_{BW}$ values ranged from -59.86 to -102.83 (mean= -81.83), whereas predicted values ranged from -68.68 to -126.84 (mean= -108.61).

Table 2. The difference between predicted and measured $\Delta^{17}O_{BW}$ of sampled captive deer mouse (*Peromyscus maniculatus*).

Mouse Number	Predicted $\Delta^{17}O_{BW}$ (per meg)	Measured $\Delta^{17}O_{BW}$ & SD (per meg)
1	-68.68	-59.86 (± 18.81)
2	-126.57	-68.82 (± 13.76)
3	-93.85	-78.48 (± 1.10)
4	-109.00	-102.83 (±2.46)
5	-126.69	-91.04 (± 15.35)
6	-126.84	-89.94 (± 14.4)

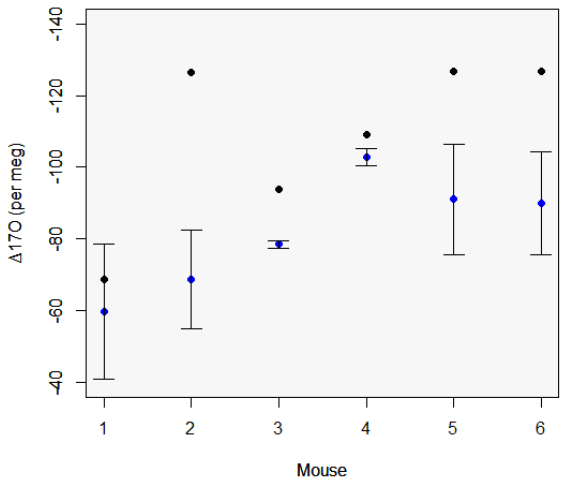

Figure 3. Measured $\Delta^{17}O_{BW}$ (blue dots) of sampled captive deer mouse (*Peromyscus maniculatus*) and standard deviation error bars along with predicted $\Delta^{17}O_{BW}$ values (black dots).

Discussion

The preliminary findings of this research suggest that adjustments need to be made to current predictive models to better

anticipate $\Delta^{17}O_{BW}$ values. Currently, predictive models are on average 26.77 per meg lower than measured $\Delta^{17}O_{BW}$ values. This likely is due to an overestimation of the contribution of metabolic water to the body water of captive deer mice.

Predictive models occasionally suggest that deer mice total body water is ~40-60% derived from metabolic water. These values would be more comparable to those of desert rodents that are adapted to be nearly water independent[12]. Both the adapted $\Delta^{17}O_{BW}$ predictive modeling and original $\delta^{18}O$ predictive modeling are heavily reliant on Water Economy Index (WEI)[10,13]. WEI is ratio of the rate of total water intake to the metabolic rate; essentially water consumed a day (mL) divided by energy exerted a day (kJ)[12]. The $\Delta^{17}O_{BW}$ predictive modeling assumes that if an animal's WEI falls below a certain threshold than this animal is essentially "water independent"[13]. Several samples of mice fell into this category resulting in $\Delta^{17}O_{BW}$ values hovering around 126 per meg; if these are removed from this preliminary work than the difference between predicted and measured improves from 26.77 to 10.12 per meg. Both the adapted $\Delta^{17}O_{BW}$ predictive modeling and original $\delta^{18}O$ predictive modeling were designed for wild animal studies and updated predictive models will likely need to be adjusted for a captive setting so a low WEI is not overstated[10,13].

Measured $\Delta^{17}O_{BW}$ values at 25°C in the preliminary findings of this study were noticeably higher than those obtained in a previous captive study assessing deer mice $\Delta^{17}O_{BW}$[7]. This would suggest that the body water of the deer mice in this preliminary work was more heavily influenced by pre-formed water than in the previous captive study despite minimal differences in housing and resources provided[7]. Although the reason for the difference is not clear, I propose the following potential explanations: 1) the mice in this study were less active than those of the previous study; or 2) the mice in this study were consuming significantly more water in this preliminary work. The supplementary materials provided in the previous captive study seem to support the first explanation but not the second[7]. In this previous captive experiment, deer mice on average consumed ~4.40 mL of water per day and expended ~ 26.77 kJ/d. In comparison, mice in this preliminary study on average consumed ~3.80 mL of water per day and expended ~ 24.37 kJ/d.

While the preliminary findings of this study are encouraging, adjustments need to be made to the predictive models to better account for variables related to metabolic water. Additionally, further assessment of the measured $\Delta^{17}O$ values needs to be conducted to confirm why these values are lower than those obtained in the previous captive deer mice study.

Future Directions

After adjustments are made, I will conduct the primary deer mice captive experiments. During the preliminary study, mice were all housed at 25°C and fed a consistent diet. During the forthcoming primary experiment, multiple experimental groups will be implemented to examine the effect of altering housing temperature and NaCl content of food (see Table 3). Lowering the temperature should result in an increase in metabolic rate, which accordingly should increase the contribution of metabolic water to total body water resulting in a lower $\Delta^{17}O_{BW}$

value. Increasing the NaCl should result in an increase of water intake, which should increase the contribution of pre-formed water to total body water resulting in a higher $\Delta^{17}O_{BW}$ value. Similar to this preliminary study, these values will be compared to values obtained via $\Delta^{17}O_{BW}$ predictive models.

Table 3. Research plan primary deer mice captive experiments. Mice will be subjected to 8 different trial periods consisting of different lengths, different temperatures, and diets of different NaCl contents. Predicted shifts in $\Delta^{17}O_{BW}$ are listed for each trial period, "---" signifies normal $\Delta^{17}O_{BW}$.

Group	Temp	Food	n	Days	Anticipated Shift in $\Delta^{17}O_{BW}$
A	25ºC	1% NaCl	4	30	---
B	25ºC	4% NaCl	4	30	Increase
C	5ºC	1% NaCl	4	30	Decrease
D	5ºC	4% NaCl	4	30	---
E	25ºC	1% NaCl	4	100	---
F	25ºC	4% NaCl	4	100	Increase
G	5ºC	1% NaCl	4	100	Decrease
H	5ºC	4% NaCl	4	100	----

After the completion of the captive experiments, I will compare the $\Delta^{17}O_{BW}$ approach with the DLW method. I will use a population of Merriam's kangaroo rat (*Dipodomys merriami*) in New Mexico as a study animal. Merriam's kangaroo rat is a desert species regarded for its' water-conservation strategies[12], which makes this an ideal species for comparing the $\Delta^{17}O_{BW}$ approach with the DLW method. At a research reserve in New Mexico, myself and collaborators at the University of New Mexico will use traps to capture and apply tracking tags to 30 kangaroo rats and seasonally sample individuals (Table 4). During each sampling period, I will collect a baseline blood sample (for later $\Delta^{17}O_{BW}$ measurement) then inject isotope tracers (for the DLW method); days later I will recapture and re-sample individuals. I will then compare DLW results, data generated from predictive models, data collected from the $\Delta^{17}O_{BW}$ novel technique, and estimated metabolic rates and water intakes from previous studies.

Table 4. Research plan for field studies using Merriam's kangaroo rat. Data collection will occur over three different time periods allowing for comparison of the seasonal shift in $\Delta^{17}O_{BW}$. Predicted shifts in $\Delta^{17}O_{BW}$ are listed for each time period, "---" signifies normal $\Delta^{17}O_{BW}$.

Time period	Avg. Rainfall (mm)	Avg. Temp.	Anticipated Shift in $\Delta^{17}O_{BW}$
May 2022	13	18ºC	---
August 2022	40	24ºC	Increase
December 2022	10	2ºC	Decrease

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Table S1. Example of a "run" completed on the Picarro. Three in-house standards (VA01, VA02, VA03) verified via internationally-accepted water standards were used throughout the run to establish a correction curve to correct the raw values obtained. A conditioning vial refers to a water sample that was used to minimize the memory effect when maneuvering between samples with significant gaps in their $\delta^{18}O$ values. A control vial refers to a water sample that is included to ensure the validity of the run after the correction curve is applied by verifying a known $\delta^{18}O$ value. Measurement order refers to the number of measurements (injections) at that point of the process, while number of measurements refers to the number of measurements for that particular item. Autosampler job number refers to the order in which each item was listed for analysis. While a sample may have 30+ measurements, most of these measurements are removed due to the memory effect and only a select number of measurements remain for analysis.

Description	**Measurement Order**	**$\delta^{18}O$**	**Number of Measurements**	**Autosampler Job Number**	**Purpose**
Conditioning Vial	1-70	~ 0	70	1	Warm-up instrument
Conditioning Vial	71-97	~ +4	27	2	Conditioning vial for positioning in-house standard
Heavy δ In-House Lab Standard (VA01)	98-157	~ +8	60	3	In-house standard for establishing correction curve
Control vial	158-184	~ +4	27	4	Control vial
Control vial	185-211	~ -2	27	5	Control vial & positioning unknown samples
Distilled Deer Mice Samples	212-361	???	150	6-17	Unknown samples
Slightly negative δ In-House Lab Standard (VA 02)	362-394	~ -4	33	18	In-house standard for establishing correction curve
Control vial	395-437	~ -10	43	19	Control vial & positioning in-house standard
Light δ In-House Lab Standard (VA03)	438-470	~ -9	33	20	In-house standard for establishing correction curve
Total Measurements	470				
Estimated run time	72.43 hours				