Journal of Visualized Experiments

Using deuterium oxide as a non-invasive, non-lethal tool for assessing body composition and water consumption in mammals

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59442R1
Full Title:	Using deuterium oxide as a non-invasive, non-lethal tool for assessing body composition and water consumption in mammals
Keywords:	animals; bat; body composition; chiroptera; cat; carnivore; deuterium oxide; endangered species; health status; insectivore; lean muscle mass; minimally invasive
Corresponding Author:	Sarah Hooper University of Missouri Columbia Columbia, MO UNITED STATES
Corresponding Author's Institution:	University of Missouri Columbia
Corresponding Author E-Mail:	hoopers@missouri.edu
Order of Authors:	Sarah Hooper
	Amanda Eshelman
	Ashley Cowan
	Alicia Roistacher
	Tyler Paneitz
	Sybill Amelon
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Columbia, MO, USA

TITLE:

Using Deuterium Oxide as a Non-Invasive, Non-Lethal Tool for Assessing Body Composition and Water Consumption in Mammals

AUTHORS AND AFFILIATIONS:

Sarah E. Hooper[1], Amanda N. Eshelman[2], Ashley N. Cowan[2], Alicia Roistacher[3], Tyler S. Paneitz[4], Sybill K. Amelon[5]

[1]Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, USA
[2]School of Natural Resources, University of Missouri, Columbia, MO, USA
[3]Department of Animal Science, University of Missouri, Columbia, MO, USA
[4]College of Veterinary Medicine, University of Missouri, Columbia, MO, USA
[5]USDA USFS Northern Research Station, Columbia, MO, USA

Corresponding author:
Sarah E. Hooper (sarahdvm.ugamizzou@gmail.com)

Email addresses of co-authors:
Amanda N. Eshelman (ane35f@mail.missouri.edu)
Ashley N. Cowan (anckv5@mail.missouri.edu)
Alicia Roistacher (aliciaroistacher@gmail.com)
Tyler S. Paneitz (tspqn5@mail.missouri.edu)
Sybill K. Amelon (samelon@fs.fed.us)

KEYWORDS:

animals, bat, body composition, chiroptera, cat, carnivore, deuterium oxide, endangered species, health status, insectivore, lean muscle mass, minimally invasive

SUMMARY:

This article describes the deuterium oxide dilution technique in two mammals, an insectivore and carnivore, to determine total body water, lean body mass, body fat mass, and water consumption.

ABSTRACT:

Body condition scoring systems and body condition indices are common techniques used for assessing the health status or fitness of a species. Body condition scoring systems are evaluator dependent and have the potential to be highly subjective. Body condition indices can be confounded by foraging, the effects of body weight, as well as statistical and inferential problems. An alternative to body condition scoring systems and body condition indices is using a stable isotope such as deuterium oxide to determine body composition. The deuterium oxide dilution method is a repeatable, quantitative technique used to estimate body composition in humans, wildlife, and domestic species. Additionally, the deuterium oxide dilution technique can be used to determine the water consumption of an individual animal. Here, we describe the adaption of

the deuterium oxide dilution technique for assessing body composition in big brown bats (*Eptesicus fuscus*) and for assessing water consumption in cats (*Felis catis*).

INTRODUCTION:

Body condition scoring systems and body condition indices are common techniques used for assessing the health status or fitness of a species[1,2]. Many domestic and zoological species have unique body condition scoring (BCS) systems that are used to assess an animal's muscle and superficial fatty tissue[3]. However, BCS assessment relies upon the evaluator—meaning that BCS is an objective or semiquantitative measurement when assessed by a trained evaluator. In wildlife species, body condition indices are commonly used rather than BCS and are based upon a ratio of body mass to body size or body mass to forearm[2]. Body condition indicis are often confounded by the effects of foraging and can be confounded by body size as well as statistical and inferential problems[4].

An alternative to body condition scoring systems and body condition indices is using a stable isotope to determine body composition. One commonly used stable isotope is deuterium oxide (D_2O), a non-radioactive form of water in which the hydrogen atoms are deuterium isotopes. The deuterium oxide dilution method described in this study can be a non-subjective, quantitative, and repeatable technique used to estimate body composition in humans[5] and a wide range of species[4,6,7]. This technique can be advantageous for studying the body composition in wildlife. For example, it can be used to assess longitudinal changes in body composition, such as before and after a management action. However, in some wildlife species deuterium oxide can overestimate the actual water content[8]. Therefore, when adapting the technique for a species, it is important to validate the method by comparing the deuterium oxide method to carcass analysis for non-endangered species. For threatened and endangered species, a non-destructive method such as dual x-ray absorptiometry (DXA) should be considered as an alternative comparison method to the gold-standard destructive method of complete carcass analysis.

In addition to body composition, the D_2O dilution technique can be used to determine the water consumption of an individual animal[9]. This unique application of D_2O can be used to answer not only research questions, but can be useful for assessing the water consumption of individual animal(s) housed in large social settings.

Here, we describe the adaption of the D_2O dilution technique for assessing body composition in an insectivore, big brown bats (*Eptesicus fuscus*), and for assessing water consumption in a carnivore, cats (*Felis catis*).

PROTOCOL:

All experiments described here were approved by the University of Missouri Animal Care and Use Committee and conducted under the Missouri Department of Conservation (MDC) Wildlife Scientific Collection permit (Permit #16409 and #17649).

1. Preparation of sterile, isotonic, salinated D_2O stock solution

1.1. Make a 50 mL stock solution of 9.0 g/L salinated D_2O.

1.1.1. Weigh 450 mg of pharmaceutical grade NaCl and transfer all NaCl into a 100 mL, sterilized beaker. Record the exact amount of NaCl to 4 decimal places in the laboratory notebook.

1.1.2. Using a sterile graduated cylinder, measure 50 g of ≥ 99.8% deuterium oxide and transfer to the sterile beaker containing the NaCl. Record the exact amount of deuterium oxide to 4 decimal places in the laboratory notebook or spreadsheet.

1.1.3. Filter 10 mL of isosmotic strength NaCl (9.0 g/L) through a non-pyrogenic sterile disk filter with submicron pores (0.2 µm).

1.1.4. Attach a 20 G needle to the non-pyrogenic sterile disk filter with submicron pores (0.2 µm) fitted with a 10 mL syringe barrel. Insert into the septum of a 100 mL sterile empty vial.

1.1.5. Attach a vacuum tube to a 22 G needle and insert the needle into the septum of the 100 mL sterile empty vial.

1.1.6. Pour 10 mL of the stock solution into the syringe barrel. Slowly turn on the vacuum until the D_2O stock solution begins to slowly filter into the sterile vial. Continue to pour the D_2O stock solution into the syringe barrel until all 50 mL is filtered.

NOTE: The stock solution may need to be diluted or concentrated depending on the dose required. The dose of D_2O will vary based upon the species and the sensitivity of the analytical method. For cats, the working solution was used to administer a dose of 0.7 g/kg D_2O. The stock solution described above minimizes the amount of NaCl solution introduced subcutaneously to the animal while still allowing accurate measurement of the dose. For small mammals such as bats, this concentration must be diluted to a working solution such as 0.1600 g/mL. This concentration allows the dose of 0.75 g/kg D_2O to be accurately measured and administered in approximately 100 µL or less NaCl solution.

2. Preparation of sterile, isotonic, salinated D_2O stock working solution for bats

2.1. Weigh a 10 mL empty sterile vial and record weight to nearest 4 decimal places. Tare scale.

2.2. Use a 1.0 mL syringe to transfer 0.65 mL of the D_2O stock solution to the tared, 10 mL empty sterile vial. Record weight of D_2O to 4 decimal places. Tare scale.

2.3. Calculate the volume of D_2O in the 10 mL empty vial. Use the following equation.

$$V = W/D$$

where *W* is recorded weight and *D* is the density of 99.8% D_2O (1.107 g/mL).

2.4. Use the calculated volume and known weight of the D_2O to determine the volume of isotonic

saline required to make ~0.1600 g/mL working solution.

2.5. Insert into the septum of the 10 mL sterile vial, the 22 G needle (attached to the vacuum tube). Insert into the septum of the 10 mL sterile vial, the 20 G needle (attached to a 0.22 µm syringe filter fitted with a 10 mL syringe barrel).

2.6. Pour the calculated mass/volume of isotonic NaCl into the syringe barrel and turn on the vacuum to allow a slow drip into the sterile 10 mL vial.

2.7. Record the weight of the vial and ensure a ~0.1600 g/mL working solution is created.

3. Determination of body composition of big brown bats (*Eptesicus fucsus*) with D_2O

NOTE: The stock solution of D_2O used in the protocol is 0.1598 g/mL. Before collecting blood, ensure that removing up to 200 µL of blood will be ≤ 10% of the total blood volume of the bat and is within the Institutional Animal Care and Use Committee's (IACUC) established guidelines for blood collection. All animals should be fasted or abdomen palpated to ensure an empty stomach. A recent meal could alter the animal's weight resulting in confounded results since calculations for determining body fat rely upon the body mass of the animal.

3.1. Anesthetize a big brown bat.

3.1.1. Use 5.0% isoflurane for induction. Maintain a stable plane of anesthesia using 0.5%−3.0% isoflurane.

3.1.2. Determine proper anesthesia depth by testing the pedal withdrawal reflex (pinching the bat's toes). The bat should not respond to the sensation and the respiratory rate should remain slow and stable. Adjust isoflurane as needed to maintain a stable plane of anesthesia.

3.1.3. Record isoflurane level, heart rate, respiratory rate, and other information as required by IACUC.

3.2. Weigh the big brown bat and record the weight to 4 decimal places.

3.3. Clean the uropatagium (tail membrane) over the interfemoral vein with an alcohol prep pad and allow to dry. Apply a thin layer of petroleum jelly over the interfemoral vein.

3.4. Use a 29 G needle to puncture the dorsal portion of the interfemoral vein and collect 100 µL of blood using plastic sodium heparin capillary tubes. Ensure adequate mixing of the whole blood with the sodium heparin by gently rolling each tube after collection and label the tube.

3.5. Using a DXA machine calibrated for small mammals, obtain three DXA scans of the bat[10].

3.6. Determine the mass (in g) of D_2O to inject by multiplying the bat weight in kg by the D_2O

dose of 0.75 g/kg. Determine the volume of the calculated D_2O dose (V) by dividing the weight of the D_2O dose by the concentration of the working solution.

$$dose = weight(kg)\ x\ \frac{0.75\ g}{kg}$$

$$V = dose\ (g) \div\ working\ solution\ concentration(\frac{g}{mL})$$

3.7. Use an insulin syringe with a 29 G needle attached to draw up the volume of D_2O calculated. Weigh the D_2O, insulin syringe, and needle. Record to 4 decimal places.

3.8. Inject the D_2O subcutaneously over the dorsal hip region of the anesthetized bat.

3.9. Allow bat to recover from anesthesia and record the time of injection.

3.10. Immediately after injection, weigh the now empty insulin syringe with the 29 G needle attached. Record the weight to 4 decimal places.

3.11. Determine the dose of D_2O injected by subtracting the post-injection weight of the insulin syringe from the pre-injection D_2O filled insulin syringe. Record to 4 decimal places.

3.12. Within 30 min post blood collection, use a hematocrit centrifuge to spin each capillary tube for 5 min. If the hematocrit centrifuge allows multiple speeds, set to 10,000 x *g*.

3.13. Use a sharp scissors to cut the plastic capillary tube between the whole blood and plasma. Use a 200 µL pipette to expel the plasma directly into a labelled, 500 µL storage tube.

3.14. After the equilibration period, collect another 100 µL of blood from the interfemoral vein.

NOTE: The equilibration period will vary by species and if the bats go into torpor. For big brown bats, typically 2 h is sufficient for the equilibration period.

3.15. Separate plasma into a second labelled, 500 µL microcentrifuge screw top tube by repeating step 3.13. Store samples at -20 °C or colder until analysis.

4. Fourier-transform infrared spectrophotometry analysis

4.1. Set the temperature of a sand bath to 60 °C to facilitate distillation (allow separation of water and D_2O from other blood components).

4.2. Pipette 50 µL of each plasma sample and standard onto the inside of a 1.5 mL conical microcentrifuge tube cap. Including standards containing known concentrations of D_2O as quality control.

NOTE: Ideally each animal will have three replicates per sample and the average of the three replicates reported. Due to the limited sample volume and the volume of sample required for the FT-IR equipment utilized by the authors, no replicates were performed for the bat samples. If any sample contains less than 50 µL of plasma, pipette the sample amount onto the conical microcentrifuge tube cap and record the volume.

4.3. Keep the microcentrifuge cap upside down and screw the 1.5 mL conical microcentrifuge tube onto the cap. Place the inverted (upside-down) tube with the cap in contact with the sand in the sand bath for a minimum of 12 h (overnight).

4.4. After 12 h, remove the cap and replace with a new, clean cap. Pulse the microcentrifuge tube for 10 s in a centrifuge.

4.5. Create the following standards: 0 ppm (0 mg D_2O in 1 L distilled water), 293 ppm (293 mg D_2O in 1 L distilled water), 585 ppm (585 mg D_2O in 1 L distilled water), 878 ppm (878 mg D_2O in 1 L distilled water), and 1170 ppm D_2O (1170 mg D_2O in 1 L distilled water).

NOTE: The values above are suggested for a standard curve. Alternative values such as 250 ppm, 500 ppm, 750 ppm, and so forth can be used.

4.6. Install a liquid transmission cell into the Fourier-transform infrared spectrophotometry (FTIR) spectrometer (**Table of Materials**). Fill the cell with methanol and connect the injection port. Slowly fill the cell with background water while carefully removing the methanol syringe to reduce the risk of air bubbles. Attach tubing to the output port to allow removal of the samples post-analysis.

4.7. Prepare the FTIR spectrometer software (**Table of Materials**) for analysis of D_2O in water. The parameter settings for the spectrometer software used in this protocol are listed in **Table 1**.

4.8. Collect a background sample using the diluent, 0.22 µm-filtered, distilled water. This should be the same water used for the standards.

4.9. Inject 40 µL of the 0 ppm D_2O and record the spectra. Save the spectra as a comma separated values (CSV) file.

4.10. Continue to inject and save the spectra of all standards to create a standard curve.

4.11. Repeat the background and standard curve every 60−90 min.

4.12. Inject 40 µL of each distilled sample into the liquid transmission cell and save the spectra.

NOTE: Alter the injection volume of the standards and distilled samples based upon the volume of liquid transmission cell. Use a smaller volume liquid transmission cell if the sample volume is

below 40 µL or dilute 1:1 with background distilled water.

4.13. Determine the concentration of D_2O of each sample from the FTIR spectra using a spreadsheet program as described by Jennings et al.[11] or the spectral software. When replicates are performed, use the average concentration to calculate the body composition.

5. Calculation of body composition

5.1. Convert the deuterium enrichment (ppm) to atom percent concentration for each sample using the following equation[12]:

$$Atom\ \% = 100 \times 0.0001557 \times (\frac{x}{1000} + 1)/(1 + 0.0001557 \times (\frac{x}{1000} + 1))$$

where x is the measured deuterium enrichment (ppm) of the sample and 0.0001557 is the mole fraction of deuterium reported in Vienna Standard Mean Ocean Water (VSMOW)[13].

5.2. Calculate total body water for each sample using the following equation[4,12,14]:

$$S(g) = 18 \times\ ((100 \times\ 0.998 \times \left(\frac{B}{20}\right))/E)\ - (0.998 \times \left(\frac{B}{20}\right)) - (0.001 \times (\frac{B}{18}))$$

where E is the measured enrichment (atom%) of deuterium in the sample after background correction, B is the injection mass in g, and 0.998 is the concentration of injected D_2O.

NOTE: Deuterium exchange with labile hydrogen causes a 2% overestimation of total body water mass. Total body water should be corrected by reducing the total body water mass estimate by 2% of the body weight.

5.3. Estimate the fat-free mass (lean body mass and all other non-fat components) of each bat using the following equation:

$$fat\text{-}free\ mass\ (g) = \frac{weight(g)}{Fractional\ moisture\ content\ of\ lean\ body\ mass}$$

NOTE: Use the conventionally accepted value of 0.732 for the fractional moisture content of lean body mass for healthy, euhydrated, non-lactating bats. The fractional moisture content of fat-free mass can change in lactating big browns based upon the post-partum week[15]. For other species, use the values published in the literature or determine the fractional moisture content of lean body mass prior to performing calculations of the lean body mass.

5.4. Estimate the body fat mass using the following equation:

$$body\ fat\ mass\ (g) = body\ weight\ (g) - fat\text{-}free\ mass(g)$$

5.5. Convert the body fat mass in g to percent body fat mass using the following equation:

$$body\ fat\ mass\ (\%) = \frac{body\ fat\ mass\ (g)}{body\ weight\ (g)} \times 100$$

6. Determination of water composition in a carnivore (*Felis catus*, domestic cat)

6.1. Prepare the stock solution as described in section 1.

6.2. Weigh each cat to the nearest 3 decimal places and record weight. Calculate the dose for each cat as described in step 3.6 using a D_2O dose of 0.70 g/kg.

6.3. Prepare each dose as described in steps 3.7–3.8. using a 3 mL or 5 mL syringe with a 22 G needle instead of an insulin syringe.

6.4. Collect 500 µL of whole blood and subsequently administer subcutaneously the 0.7 g/kg D_2O. Centrifuge whole blood at 2,000 x *g* for 15 min and store plasma in 1.5 mL microcentrifuge screw top tubes at -20°C until analysis.

6.5. Collect 500 µL of whole blood 4 h post-injection. Centrifuge whole blood at 2,000 x *g* for 15 min and store plasma in 1.5 mL microcentrifuge screw top tubes at -20 °C until analysis.

6.8. Collect 500 µL of whole blood 14 days post-injection. Centrifuge whole blood at 2,000 x *g* for 15 min and store plasma in 1.5 mL microcentrifuge screw top tubes at -20 °C until analysis.

NOTE: The number of days between blood collection can be based upon the experimental needs and the post-injection period in which D_2O can be detected above the background levels. Fourteen days was the length of the dietary treatment blocks from Hooper et al.[9].

6.9. Perform FT-IR analysis according to section 4 and calculate the body composition according to section 5 of this protocol.

6.10. Calculate the water consumption in mL/day using the following equations:

$$body\ water\ loss = TBW(kg) \times \frac{\left(initial\ D_2O\ (ppm) - final\ D_2O\ (ppm)\right)}{initial\ D_2O\ (ppm)}$$

$$Fractional\ change\ in\ H_2O = log_e \frac{final\ D_2O\ (ppm)}{initial\ D_2O\ (ppm)} \Big/ days \times (-1)$$

$$water\ consumption\ \left(\frac{ml}{day}\right) = Fractional\ change\ in\ H_2O \times initial\ TBW$$

where TBW is total body water, initial D_2O and final D_2O are the concentrations measured in ppm in the post-injection D_2O samples.

REPRESENTATIVE RESULTS:

The deuterium oxide dilution technique can be used to assess the body composition of a variety of species. To demonstrate the adaptability, we are reporting the first use of the deuterium oxide dilution technique in a North American insectivorous bat species, *Eptesicus fuscus*, the big brown bat for representative results. A timing plateau was completed by taking pre- and post-D_2O injection blood samples as should be done with any species where the equilibration period is unknown. It was determined that two hours post-injection in non-torpid bats was adequate for equilibration. With the equilibration time known, the total body water, lean body mass, and body fat mass for 13 wild-caught big brown bats and 8 captive big brown bats were determined (**Table 2**). An additional 2 wild-caught big brown bats and 5 captive big brown bats were determined to have a negative body fat mass. A negative body fat mass is calculated due to one or more of the following reasons: not receiving the entire dose of deuterium oxide, becoming torpid during the equilibration phase, having abnormally large fat masses and minimal lean mass, or bats having under 3%−5% body fat as determine by DXA (**Table 3**).

White-nose syndrome has caused many bat species to decline, so the technique was compared to the body fat measured using DXA. **Figure 1** shows the percentage of body fat determined by the D_2O dilution technique and DXA (n = 19). The two techniques were well correlated with a Pearson's r = 0.897 (**Figure 2**) and were not statistically different (one-way analysis of variance (ANOVA), F-value = 0.366, p = 0.549). The body fat showed strong correlations between body fat and body weight (**Figure 3**). The D_2O dilution technique did not consistently over or underestimate the body fat mass.

The deuterium oxide method has been previously validated in cats[16]. **Table 4** shows an example of the total body water, lean body mass, and body fat mass of a single cat[9]. Hooper et al.[9] was the first to report the use of deuterium oxide dilution to measure the water consumption of socially housed animals with the daily water consumption of the cats during each dietary block of the experiment, as shown in **Figure 4**.

FIGURE AND TABLE LEGENDS:

Figure 1: Deuterium oxide and DXA line plot. Each point represents the body fat percentage of an individual bat as determined by DXA or deuterium oxide. The mean is the light green point with error bars indicating the standard error of the mean.

Figure 2: Percentage of body fat in big brown bats. Deming regression (solid blue line, Pearson's r = 0.897) comparing the percentage of body fat determined by DXA (x-axis, the reference method) and the percentage of body fat determined by deuterium oxide (y-axis, the test method) in big brown bats with 95% confidence intervals designated by gray shading. The green dash identity line drawn represents the regression line when the methods are equal.

Figure 3: Percentage of body fat in big brown bats compared to body weight. Body weight of each bat plotted against the body fat percentage determined by D_2O or DXA. A strong correlation exists between the body weight and body fat as determined by DXA (dark blue line, Pearson's r = 0.88) and D_2O (blue line, Pearson's r = 0.86).

Figure 4: Water consumption of socially housed cats. Representative results of the daily water consumption of socially housed cats during an experiment evaluating the effects of dietary constituents on water consumption. This figure has been modified from Hooper et al.[9].

Table 1: Spectral software settings. Parameter settings used for spectral recording software.

Table 2: Body composition of big brown bats. The representative results of total body water, lean body mass, and body fat as determined by deuterium oxide dilution in big brown bats are shown in columns 5−8. Representative results of the lean body mass plus bone mineral content and body fat as determined by DXA in the same big brown bats are shown in columns 9−11.

Table 3: Body composition of big brown bats. Representative results from bats that did not receive the entire dose of deuterium oxide, became torpid during the equilibration phase, bats with abnormally large fat mass and minimal lean mass, or bats under 3%−5% body fat as determine by DXA. The representative results of total body water, lean body mass, and body fat as determined by deuterium oxide dilution are shown in columns 5−8. Representative results of the lean body mass plus bone mineral content and body fat as determined by DXA are shown in columns 9−11.

Table 4: Body composition and water consumption in a single feline. Representative results of deuterium oxide dilutional technique for assessing the lean body mass, fat mass, and water consumption of one cat at three different time points during the study conducted by Hooper et al.[9].

DISCUSSION:

The use of deuterium oxide to determine TBW has been used since the 1940s[17] and is used in humans and a variety of domestic and wildlife species[4,6,7]. Other non-destructive techniques have been developed including bioelectrical impedance analysis (BIA), DXA, and quantitative magnetic resonance (QMR). Each method has advantages and disadvantages that should be considered before selecting a particular methodology for assessing body composition. This protocol selected to use DXA as a comparison method for deuterium oxide to assess body composition, because the equipment is available as a core university resource with minimal cost, minimal time is required per scan (30 s per bat), and it is not sensitive to variables such as body temperature and skin insulation.

When adapting the deuterium oxide dilution technique to a species of interest, a pilot study should be initiated to determine the time required for equilibration[18]. This can be done by taking a background sample, and a blood sample every 15 minutes post-injection. For small species such as bats, several bats can be bled at the different time intervals instead of a single animal[18]. The

equilibration time can change when animals, such as bats, go into torpor, which explains why some of our animals had a negative percent body fat (**Table 3**). If a negative percent body fat is obtained, and the deuterium dose had sufficient time to fully equilibrate with the animal's body water, then it is likely the dose was not completely injected. Because the deuterium oxide dilution technique is highly dependent upon the full dose being administered and accurate recording of the amount of deuterium injected, this technique should only be completed by individuals skilled in performing injections. Additionally, anesthetizing or sedating animals can assist with ensuring the entire dose can be administered.

When administering the deuterium oxide, it is important to determine an appropriate concentration to administer to the animal. Using a 0.7 g/kg dose for the cats, the stock solution concentration was appropriate, whereas for the big brown bats a 0.75 g/kg dose required the stock solution of deuterium oxide to be diluted. When diluting the stock solution, an isotonic solution such as 0.9% NaCl should be used. To avoid altering the total body water of small mammals, dilute the dose of deuterium oxide as minimally as possible, just enough to ensure the dose can be measured accurately.

The doses presented here are detectible using FTIR spectrometry. FTIR spectrometry is less expensive and easier to maintain, but not as sensitive as isotope ratio mass spectrometry (IRMS)[19,20]. FTIR spectrometry can be used to measure deuterium enrichment in plasma and saliva, but it is not recommended to use an FTIR transmission cell to analyze deuterium enrichment in urine[19]. If urine is the desired sample type, then an attenuated total reflection (ATR) attachment should be used with the FTIR or IRMS should be used to assess deuterium enrichment for calculation of TBW[19].

Additionally, the doses used for the cats were adequate to allow detection of deuterium oxide 14 days post-injection. Because the concentration of the deuterium oxide 14 days post-injection was detectable, the water consumption of the cats could be calculated (**Figure 4**). This innovative use of deuterium oxide can be employed in field studies to measure body water turnover for species with high recapture rates or for animals housed in groups in ex situ or laboratory studies. However, before employing in field studies, researchers must assess if the animal can be captured and held for the duration of the equilibration period. This prolong handling period is one of the disadvantages of the deuterium oxide technique and could be problematic as many endangered species permits limit the duration that a particular animal can be held. Additionally, animals cannot have recently eaten as the washout technique relies upon the measurement of body mass; therefore, a recent meal can confound the results. An additional consideration is whether an animal must be anesthetized or sedated for subcutaneous injection and blood collections or if the animal can be restrained without sedation/anesthesia. It has been suggested that the rate of body water turnover could be a significant indicator for human health[21]. The increased water consumption in cat 5 (**Figure 4**) was documented before traditional biochemical marks of renal failure, and concentrations of creatinine and blood urea nitrogen (BUN) were elevated, suggesting that body water turnover could also be an indicator of health in animals.

ACKNOWLEDGMENTS:

This research was supported by MDC Cooperative Agreement (#416), US Forest Service Cooperative Agreement (16-JV-11242311-118), American Academy of Veterinary Nutrition and Waltham/Royal Canin, USA Grant (grant number: 00049049), NIH training grant (grant number: T32OS011126), and the University of Missouri Veterinary Research Scholars Program. The authors thank Shannon Ehlers for pre-reviewing this manuscript. We thank Dr. Robert Backus for providing the D_2O standards and allowing use of his laboratory.

DISCLOSURES:

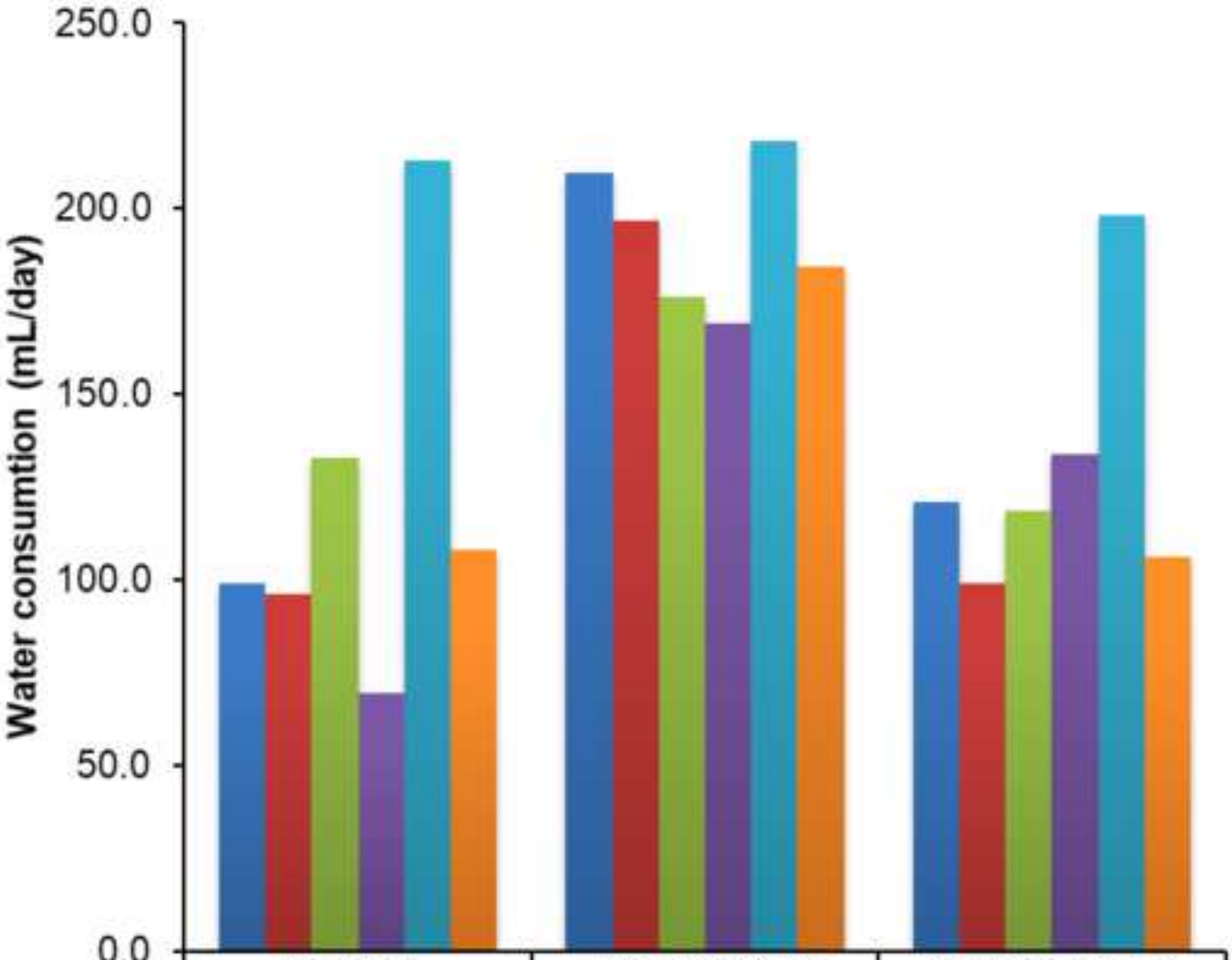
The authors have nothing to disclose.

REFERENCES:

1. Schiffmann, C., Clauss, M., Hoby, S., Hatt, J.-M. Visual body condition scoring in zoo animals – composite, algorithm and overview approaches. *Journal of Zoo Aquarium Research*. **5** (1), (2017).
2. Peig, J., Green, A. J. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos.* **118** (12), 1883−1891 (2009).
3. Bissell, H. *Body Condition Scoring Resource Center*, <https://nagonline.net/3877/body-condition-scoring/> (2017).
4. McWilliams, S. R., Whitman, M. Non-destructive techniques to assess body composition of birds: a review and validation study. *Journal of Ornithology.* **154** (3), 597−618 (2013).
5. Lukaski, H. C., Johnson, P. E. A simple, inexpensive method of determining total body water using a tracer dose of D2O and infrared absorption of biological fluids. *American Journal of Clinical Nutrition.* **41** (2), 363−370 (1985).
6. Chusyd, D. E. *et al.* Adiposity and Reproductive Cycling Status in Zoo African Elephants. *Obesity (Silver Spring).* **26** (1), 103−110 (2018).
7. Kanchuk, M. L., Backus, R. C., Calvert, C. C., Morris, J. G., Rogers, Q. R. Neutering Induces Changes in Food Intake, Body Weight, Plasma Insulin and Leptin Concentrations in Normal and Lipoprotein Lipase–Deficient Male Cats. *The Journal of Nutrition.* **132** (6), 1730S−1732S (2002).
8. Eichhorn, G., Visser, G. H. Technical Comment: Evaluation of the Deuterium Dilution Method to Estimate Body Composition in the Barnacle Goose: Accuracy and Minimum Equilibration Time. *Physiological and Biochemical Zoology.* **81** (4), 508−518 (2008).
9. Hooper, S. E., Backus, R., Amelon, S. Effects of dietary selenium and moisture on the physical activity and thyroid axis of cats. *Journal of Animal Physiolgy and Animal Nutrition (Berl).* **102** (2), 495−504 (2018).
10. Stevenson, K. T., van Tets, I. G. Dual-Energy X-Ray Absorptiometry (DXA) Can Accurately and Nondestructively Measure the Body Composition of Small, Free-Living Rodents. *Physiological and Biochemical Zoology.* **81** (3), 373−382 (2008).
11. Jennings, G., Bluck, L., Wright, A., Elia, M. The use of infrared spectrophotometry for measuring body water spaces. *Clinical Chemistry.* **45** (7), 1077−1081 (1999).
12. Beuth, J. M. Body Composition, movemement phenology and habitat use of common eider along the southern new england coast. Master of Science in Biological and Environmental Sciences (MSBES) thesis, University of Rhode Island, (2013).
13. Coplen, T. B., Hopple, J., Peiser, H., Rieder, S. Compilation of minimum and maximum isotope ratios of selected elements in naturally occurring terrestrial materials and reagents U.S. Geological Survey Water-Resources Investigations Report 01-4222. (2002).

14. Karasov, W. H., Pinshow, B. Changes in lean mass and in organs of nutrient assimilation in a long-distance passerine migrant at a springtime stopover site. *Physiological Zoology.* **71** (4), 435−448 (1998).
15. Hood, W. R., Oftedal, O. T., Kunz, T. H. Variation in body composition of female big brown bats (*Eptesicus fuscus*) during lactation. Journal of Comparative Physiology B. **176** (8), 807−819 (2006).
16. Backus, R. C., Havel, P. J., Gingerich, R. L., Rogers, Q. R. Relationship between serum leptin immunoreactivity and body fat mass as estimated by use of a novel gas-phase Fourier transform infrared spectroscopy deuterium dilution method in cats. *American Journal of Veterinary Research.* **61** (7), 796−801 (2000).
17. Moore, F. D. Determination of Total Body Water and Solids with Isotopes. *Science.* **104** (2694), 157−160 (1946).
18. Voigt, C., Cruz-Neto, A. in *Ecological and Behavioral Methods in the Study of Bats* (ed Parsons S. Kunz T.H.) Ch. 30, 621−645 (John Hopkins University Press, 2009).
19. International Atomic Energy Agency. Assessment of Body Composition and Total Energy Expenditure in Humans Using Stable Isotope Techniques. (2009).
20. International Atomic Energy Agency. Introduction to Body Composition Assessment Using the Deuterium Dilution Technique with Analysis of Saliva Samples by Fourier Transform Infrared Spectrometry. (2011).
21. Shimamoto, H., Komiya, S. The Turnover of Body Water as an Indicator of Health. *Journal of Physiological Anthropology and Applied Human Science.* **19** (5), 207−212 (2000).

Figure 4



	Control	High Moisture	High Selenium
Cat 1	98.9	209.8	120.9
Cat 2	96.1	197.0	99.3
Cat 3	132.9	176.2	118.5
Cat 4	69.6	169.0	133.9
Cat 5	213.0	218.0	198.0
Cat 6	108.4	184.6	106.1

Dietary treatments and water consumption (mL/day)

Figure 1

Click here to access/download

Video or Animated Figure

Figure 1.svg

Figure 2

Click here to access/download

Video or Animated Figure

Figure2.svg

Figure 3


Click here to access/download
Video or Animated Figure
Figure 3 jove.svg

Table1

Click here to access/download;Table;Table 1 FT-IR software settings.xlsx

Parameter	Setting
Number of scans	64
Resolution	2
Data spacing	0.946 cm^{-1}
Final format	Absorbance
Correction	None
Use fixed Y-axis limits in collection window	Min -0.01, Max 0.03
Bench range	Max 6.38, Min -5.02, Loc 1024
Total absorbing peak sensitivity	50
fringes or channeling sensitivity	80
Derivative peaks sensativity	51
Baseline error sensitivity	50
CO2 levels sensitivity	19
H2O levels sensitivity	19
Apodization mode	Happ-Genzel
Phase correction	Mertz
Filters set based upon	velocity
low pass filter	11,000
high pass filter	20

Table2

Click here to access/download;Table;Table 2 Bat Deuterium oxide.xlsx

Animal	Species	body weight (kg)	D_2O injected (g)	Total body water (g)	Lean body mass (g)	Body fat mass (g)	body fat mass (%)
1	*Eptesicus fuscus*	0.01715	0.0740	11.80	16.15	1.00	5.80
2	*Eptesicus fuscus*	0.01950	0.0920	13.80	18.83	0.69	3.50
3	*Eptesicus fuscus*	0.01677	0.08	11.33	15.47	1.30	7.74
4	*Eptesicus fuscus*	0.021292	0.097	12.51	17.09	4.20	19.7

DXA lean + bmc (g)	DXA fat (g)	DXA fat (%)
14.65	0.75	4.80
16.20	1.40	7.90
11.33	1.30	7.74
15.9	19.65	19.2

Table3

Click here to access/download;Table;Table 3 Bat Deuterium oxide 2.xlsx

Animal	Species	body weight (kg)	D_2O injected (g)	Total body water (g)	Lean body mass (g)	Body fat mass (g)	body fat mass (%)
1	*Eptesicus fuscus*	0.0277	0.1299	34.18	46.69	-19.02	-68.74
2	*Eptesicus fuscus*	0.0185	0.0810388	64.23	87.75	-69.25	-374.33
3	*Eptesicus fuscus*	0.0164	0.0719	17.38	23.74	-7.33	-44.68
4	*Eptesicus fuscus*	0.0212	0.0994	54.57	74.54	-53.37	-252.0

DXA lean + bmc (g)	DXA fat (g)	DXA fat (%)	Comment
9.90	26.55	62.80	Equilibration time insufficient
14.20	17.30	17.95	Full dose not injected
14.15	14.40	1.70	Less than 3% fat
16.41	19.01	13.65	Bat became torpid (cool to touch)

Table4

Click here to access/download;Table;Table 4 Cat dueterium oxide.xlsx

Block	Species	body weight (kg)	D_2O injected (g)	Total body water (kg)	Lean body mass (kg)	Body fat mass (kg)	body fat mass (%)	Daily water consumption (mL/day)
1	*Felis Catus*	4.830	3.36	2.69	3.68	1.149	23.8	96.8
2	*Felis Catus*	4.764	3.45	2.66	3.63	1.136	23.8	217.5
3	*Felis Catus*	4.727	3.25	2.50	3.41	1.314	27.8	125.1

Dietary Treatment
Control
High Moisture
High Selenium

Name of Material/Equipment	Company	Catalog Number
0.2 micron non-pyrogenic disk filter	Argos Technologies	FN32S
1.5 mL conical microcentrifuge tubes	USA Scientific	1415-9701
10 mL sterile glass vial for injection	Mountainside Medical Equipment	MS-SEV10
10 mL syringe	Becton Dickinson	305219
100 mL sterile glass vial for injection	Mountainside Medical Equipment	AL-SV10020
20 guage needle	Exel	26417
22 guage needle	Exel	26411
deuterium oxide	Sigma-Aldrich	151882-25G
isofluorane	Vetone	3060
OMNIC Spectra Software	ThermoFisher Scientific	833-036200
petrolium jelly	Vaseline	305212311006
plastic capillary tubes	Innovative Med Tech	100050
Sealed liquid spectrophotometer SL-3 FTIR CAF2 Cell	International Crystal Laboratory	0005D-875
sodium chloride	EMD Millipore	1.37017
Thermo Electron Nicolet 380 FT-IR Spectrometer	ThermoFisher Scientific	269-169400

Comments/Description

nylon, 30mm diameter, 0.22um, sterile
1.5 ml self-standing microcentrifuge tube, natural with blue cap

clear, sterile glass injection unit
sterile 10 mL syringe individually wrapped

clear, sterile glass injection unit
needles hypodermic 20g x 1" plastic hub (yellow) / regular bevel
needles hypodermic 22g x 1" plastic hub (black) / regular bevel
99.9 atom % D
fluriso isoflurane, USP
FT-IR standard software
Vaseline, 100% pure petroleum jelly, original, skin protectant
sodium heparin anticoagulant, 50µL capacity, 30 mm length

0.05 mm Pathlength
suitable for biopharmaceutical production

discontinued model, newer models available

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Using deuterium oxide as a non-invasive, non-lethal tool for assessing body composition and water consumption in mammals
Author(s):	Sarah E. Hooper, Amanda N. Eshelman, Ashley D. Cowan, Alicia Roistacher, Tyler S. Paneitz, Sybill K. Amelon

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☐ The Author is **NOT** a United States government employee.

☒ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Sarah Hooper
Department:	Department of Veterinary Pathobiology
Institution:	University of Missouri
Title:	Postdoctoral Researcher
Signature:	
Date:	11-26-18

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Editor and Reviewers,

We appreciate the constructive feedback on the manuscript "Using deuterium oxide as a non-invasive, non-lethal tool for assessing body composition and water composition in mammals". The comments have been addressed individually, and appropriate changes have been made to the manuscript using track changes. We look forward to continuing the review processes.

Sincerely,

The authors

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have reviewed the manuscript for spelling and grammar issues. Any errors were corrected and noted by the track changes function in Microsoft Word.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The original signed Journal of Animal Physiology and Animal Nutrition copyright transfer agreement states figures may be reused in other publications. This will be uploaded as a PDF in the Editorial Manager for review by JOVE editors and staff. The lead and senior authors do not have the file in .doc or .docx format.

3. Figure and table legends: Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

We have revised the figure and table legends to include a title and applicable description.

4. Figure 1: Please describe what the error bars represent. What do the bright green and dark green dots represent?

We had added in the legend, "The mean is the light green point with error bards indicating the standard error of the mean."

5. Figure 2: Please describe what the blue line, dashed green line and shaded area represent.

We have described the items listed above in the figure legend.

6. Table of Materials: Please revise the Table of Materials to include the name, company, and catalog number of all

relevant supplies, reagents, equipment and software in separate columns in an xls/xlsx file. Please sort the items in alphabetical order according to the name of material/equipment.

We have sorted the items in alphabetical order. Items with a numerical value (i.e. 10 mL syringe) are listed first. We have added the OMNIC Spectra Software and FTIR equipment.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Microsoft Excel, Vaseline, OMNIC Specta, etc.

We have revised the manuscript to eliminate use of commercial language except for the Table of Materials and Reagents.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the protocol text to reduce/eliminate the use of personal pronouns.

9. 3.1: Please mention how proper anesthetization is confirmed.

Step 3.1 now includes how proper anesthesia is confirmed.

10. 3.15: Please specify centrifugation parameters (force in x g and time).

Microhematocrit centrifuges typically only allow time adjustments and not speed adjustments. We listed the time, but now include additional information for centrifuges that allow multiple speeds.

11. 4.9.1-4.9.19: Please include all parameters in a table and reference the table in step 4.9.

We have created a reference table for all parameters in step 4.9.

12. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have combined the shorter protocol steps as requested.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have included single-line spaces between all paragraphs, headings, steps, etc.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Once track changes are accepted, the authors should have approximately 2.75 pages highlighted.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the

sub-steps where the details are provided must be highlighted.
17. Line 324: Please use a reference number for Hooper et al (2018) for in-text reference.

Reference number provided at the end of the sentence for line 324.

18. References: Please do not abbreviate journal titles.

The authors would like to note that the citation examples included references with abbreviated journal titles (i.e. J Med Chem), and recommend that it be changed to avoid confusion for other authors.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This study provides a good example of the use of deuterium oxide to measure body composition and water consumption in mammals. Overall the methods and conclusions are reasonable.

Major Concerns:
However, while the authors highlight many of the advantages of the use of deuterium oxide in measuring body composition they have neglected to mention many of the disadvantages in the use of deuterium oxide. Including prolonged handling times, skill in performing injections, and costs of purchasing isotopes and performing laboratory analyses. As the authors validate their body composition measures against measures determined by dual x-ray absorptiometry (DXA), some discussion on the pros and cons of deuterium oxide versus DXA (among other methods) is warranted.

We have included in the discussion that only skilled individuals should administer the deuterium oxide, considerations for prolong handling time, and considerations for sedation/anesthesia of an animal. The authors discuss that FT-IR spectrometry is less expensive than other methods. Deuterium oxide is typically considered an inexpensive method for body composition, and the readers can determine their costs by consulting the Table of Materials. We also highlight why we chose DXA. There are many excellent review articles that compare non-destructive body condition methodologies, and the authors do not believe a full discussion of all of these methods is relevant to the deuterium oxide protocol. The authors can provide suggestions of review articles to the readers if this is desired by the reviewer.

Minor Concerns:
Introduction:
Lines 55-57: But deuterium oxide relies on measures of body mass and hence it can also be confounded by recent feeding.

We have revised the discussion to expand upon the disadvantages of using deuterium oxide and addressed this comment in the discussion.

Line 66: No need to mention barnacle goose here. The inference is that the physiology of barnacle geese makes them unique when in fact it is the deuterium oxide technique itself that is imprecise and variable among species.

We revised the sentence to say, “However, in some wildlife species, deuterium oxide (D_2O) can overestimate actual water content.”

Methods:
Mention of the statistical tests performed would be helpful here.

The figure legends and results provide information on the statistical methods used. The statistical methods that readers will use depends upon how they incorporate deuterium oxide washout technique into their studies and their study design.

Results:
Lines 305-307: A figure showing the equilibration curve would be of interest.

We could provide a reference for a figure of a schematic illustration of theoretical enrichment if the reviewer and editor would find this of benefit to the readers. For our equilibration curve, we used different animals for each time point. Because the enrichment will be different in each animal due to their weight differences and different dosages, we assessed the timing based upon the timepoint with the highest level of agreement between DXA and deuterium oxide. This is difficult to visually graph for the readers, since many published examples of equilibration curves of deuterium enrichment are from repeated sampling of the same individual.

Line 320: Why use a One-way ANOVA instead of a paired t-test?

Since the t-test is a special case of one-way ANOVA, and the combined use of Deming regression and one-way ANOVA has been used widely in the literature for comparison of two or more diagnostic methodologies, we decided to use these statistical methods.

Reviewer #2:
Manuscript Summary:
First, my best wishes for this new year.
Clear and useful presentation on the technique and interesting data collection in this field.

Major Concerns:
No major concern

Minor Concerns:
- L54: BCS 9 point scale has been validated in cats (Laflamme, 1997) and shown to be accurate when used by trained observers (Bjørnvad et al , 2011; Borges et al, 2012...). As such, it would be considered as a semi quantitative/objective measure, not a subjective measure in trained observer. It is widely accepted and used in daily practice by vets as an easy tool.

We have revised this to read “ …, however BCS assessment relies upon the evaluator—meaning BCS is an objective or semiquantitative measurement when assessed by a trained evaluator.

Bjørnvad, C. R., Nielsen, D. H., Armstrong, P. J., McEvoy, F., Hoelmkjaer, K. M., Jensen, K. S., ... & Kristensen, A. T. (2011). Evaluation of a nine-point body condition scoring system in physically inactive pet cats. American Journal of Veterinary Research, 72(4), 433-437.
Borges, N. C., Vasconcellos, R. S., Carciofi, A. C., Gonçalves, K. N., Paula, F. J., Faria Filho, D. E., & Canola, J. C. (2012). DXA, bioelectrical impedance, ultrasonography and biometry for the estimation of fat and lean mass in cats

during weight loss. BMC Veterinary Research, 8(1), 111.
Laflamme, D. (1997). Development and validation of a body condition score system for cats: a clinical tool. Feline practice (Santa Barbara, Calif.: 1990)(USA).

- L316-321: it would be interesting to add information about the discrepancy between the two techniques (over/underestimation)

We have included that the deuterium technique did not consistently under or overestimate the body fat mass in the representative results (L399-400).

- L391-394: It would be interesting to remind the reader that the assumption about an lean mass hydration coefficient of 73.2% is only available in healthy subjects. As soon as a disease is involved, especially those driving a water shift in the body, the body composition assessment, whatever the technique used, need to be seen with cautious.

We have revised the methods to include the suggestions of the reviewer and highlight that in bats, the lean mass hydration coefficient can be altered post-lactation.

Reviewer #3:
Manuscript Summary:
The manuscript details a protocol for the use of deuterium dilution for body composition and water turnover analysis in examplar wildlife. The protocol is generally well written and easy to follow. I do query, however, the need or value for this protocol since there are already well-established and published protocols, for example the series of monographs published by the IAEA. In addition, the protocol is very specific, e.g., line 116 "transfer 0.65 mL" and line 110 0.1600 "g/mL". While these may be appropriate for the D2O technique in this particular species (bats), the technique is broadly applicable across many species where concentrations and dosages may differ. It would seem more appropriate to make the protocol more generic.

The lead author was asked by an editor to consider publishing the deuterium oxide washout technique that she used for measuring the water consumption in cats (Hooper et al 2018 reference). Because of the well-established and published protocols, we decided to show that the details that these general protocols lack and how using these general protocols they can be adapted for a species of interest. For those readers who are unfamiliar with the technique, the authors believe this level of detail is helpful. We also provide notes to the readers that certain concentrations like the stock solution can be used in mammals the size of domestic cats and for small mammals a working concentration would be required.

Aspects of the method are described without indication of purpose, for example what is the purpose of the sandbath heating?

We added "….to allow separation of water and deuterium oxide from other blood components (distillation)" to clarify why the sand bath is needed.

Major Concerns:
I have no major concerns regarding scientific validity of the described method but some points require attention.

Lines 109-11 and the methodology in general. The concentration of the working solution is to a large extent

immaterial. What is important is that the exact magnitude of the dose the animal receives is known and that this is administered in a volume insufficient to disturb body water volume. In practice the dose rate (expressed as D2O g/kg body-weight) is often determined by the sensitivity of the analytical method being used. This point does not come across clearly.

We revised the note after section 1 to read "The stock solution may need to be diluted or concentrated depending on the dose required. The dose of D_2O will vary based upon the species and the sensitivity of the analytical method. For cats, the working solution was used to administer a dose of 0.7 g/kg D_2O. The stock solution described above minimizes the amount of sodium chloride solution introduced subcutaneously to the animal while still allowing accurate measurement of the dose. For small mammals such as bats, this concentration must be diluted to a working solution such as 0.1600 g/mL. This concentration allows the dose of 0.75 g/kg D_2O to be accurately measured and administered in approximately 100 µL or less sodium chloride solution."

Line 197. Why are these particular concentrations chosen for the preparation of the standard curve? It is more usual to use logical intervals e.g., 0, 250 , 500, 750 mg etc.

These standards were provided by Dr. Robert Backus. We have listed this in the acknowledgements and noted on line 209 that "The values above are suggested for a standard curve. Alternative values such as 250 ppm, 500 ppm, 750 ppm, and so forth can be used."

Line 200 I question the value of the details of the FTIR spectrometry. These will be highly manufacturer specific. Problems are not addressed, for example procedures for filling the cell to ensure that no air bubles are introduced.

We have added the steps to describe using methanol to first fill the cell to ensure that there are no air bubbles introduced. Additionally, we have revised the manuscript to state "Install a liquid transmission cell into the FTIR spectrometer and prepare the FTIR spectrometer software for analysis of D2O in water. The parameter settings for the spectrometer software used in this protocol are listed in table 4. See the table of materials for specific software and FTIR spectrometer equipment."

Lines 378-83. FTIR when used with ATR attachment rather that a transmission cell can be used for urine since the optical face is resistant to chemical attack. Newer model FTIR-ATR systems , e.g. the Agilent 4500 have much improved sensitivity.

We have revised the discussion to say "FTIR spectrometry can be used to measure deuterium enrichment in plasma and saliva, but it is not recommended to use an FTIR transmission cell to analyze deuterium enrichment in urine[18]. If urine is the desired sample type, then an attenuated total reflection (ATR) attachment should be used with the FTIR or IRMS should be used to assess deuterium enrichment for calculation of TBW."

Line 370. What is appropriate, by what criteria?

In the discussion, we state "To avoid altering the total body water of small mammals, dilute the dose of deuterium oxide as minimally as possible, just enough to ensure the dose can be measured accurately."

Lines 316-21. Pearsons correlation is inappropriate to use for method comparison - use concordance correlation.

Equally for method agreement, ANOVA is inappropriate, Bland and Altman's Limits of Agreement analysis should be used.

We used Deming regression and one-way ANOVA as these are typically used for comparing two different diagnostic methodologies in veterinary medicine. Deming regression is appropriate for when both data sets can contain errors (when both methods have some degree of imprecision). Bland-Altman plots are visual representations, and we did not believe were relevant to include as a representative result.

I understand why DXA was used as the comparator method but it is incorrect to say that it is a reference method. Indeed, D2O dilution is the acknowledged reference method for body composition to which DXA is compared. DXA is the reference for bone mineral but not soft tissue. I suggest rephrasing simply in terms of comparison rather than setting one up as a gold standard.

We have altered the language in the introduction and throughout the manuscript to reflect that DXA is a comparison method and not the gold standard.

Calculation of TBW. There appears to be no correction for deuterium exchange. Why not the usual 1.04 correction? It is also not clear whether the authors are advocating back-projection to time zero or end-point calculation. If the latter are calculations based on the last sample only (it seems so) or the average of two sample taken a short period apart (IAEA protocol). There is no indication that sample analyses should be replicated.

The authors are unfamiliar with a 1.04 correction. We have added a clarity note that deuterium exchange with labile hydrogen results in a 2% overestimation of total body water mass and this should be corrected for.

Ref: Backus et. al. 2000. Relationship between serum leptin immunoreactivity and body fat mass as estimated by use of a novel gas-phase Fourier transform infrared spectroscopy deuterium dilution method in cats.

Due to the limited amount of blood that can safely be collected and the high pack cell volume (PCV) of big brown bats, we were not able to run the analysis in triplicate as was done for the cats. We revised the protocol to highlight ideally samples and average of three samples is reported.

Line 256. 0.732 is only applicable in euhydrated individuals and may not be applicable across all species. This point should be made.
We have clarified this point and specified to use the value for healthy, euhydrated, non-lactating bats. We also provide a reference showing the change in fractional moisture content based upon lactation. Furthermore, we clarify for other species that published values should be used or values determined prior to performing calculations.

Line 252. It is not lean mass that is calculated but fat-free mass, i.e., everything but fat, which is why fat can be estimated by difference with body weight. This is important since FFM includes bone mineral not just conventional lean.

We have replaced lean body mass with fat-free mass to provide clarity to the readers.

There are no details of quality assurance and control procedures that should be adopted.

We have revised where relevant to include details of quality assurance and control procedures including that a sample of known concentration should be included in the analysis, triplicate analysis should be done on each sample, etc.

Minor Concerns:
Line 97 through not thru

Changed as the review suggested.

line 137 define IACUC here on first use not later at line 145.

Changed as the review suggested.

Journal of Animal Physiology and Animal Nutrition

Published by Wiley (the "Owner")

COPYRIGHT TRANSFER AGREEMENT

Date: June 07, 2017

Contributor name: Sarah Hooper

Contributor address:

Manuscript number: JAPAN-Jan-17-047.R1

Re: Manuscript entitled Effects of dietary selenium and moisture on the physical activity and thyroid axis of cats (the "Contribution")

for publication in Journal of Animal Physiology and Animal Nutrition (the "Journal")

published by Blackwell Verlag GmbH ("Wiley")

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable the Owner to disseminate your Contribution to the fullest extent, we need to have this Copyright Transfer Agreement executed. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement shall be null and void.
Publication cannot proceed without a signed copy of this Agreement.

A. COPYRIGHT

1. The Contributor assigns to the Owner, during the full term of copyright and any extensions or renewals, all copyright in and to the Contribution, and all rights therein, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so. For the avoidance of doubt, "Contribution" is defined to only include the article submitted by the Contributor for publication in the Journal and does not extend to any supporting information submitted with or referred to in the Contribution ("Supporting Information"). To the extent that any Supporting Information is submitted to the Journal for online hosting, the Owner is granted a perpetual, non-exclusive license to host and disseminate this Supporting Information for this purpose.

2. Reproduction, posting, transmission or other distribution or use of the final Contribution in whole or in part in any medium by the Contributor as permitted by this Agreement requires a citation to the Journal suitable in form and content as follows: (Title of Article, Contributor, Journal Title and Volume/Issue, Copyright © [year], copyright owner as specified in the Journal, Publisher). Links to the final article on the publisher website are encouraged where appropriate.

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution.

C. PERMITTED USES BY CONTRIBUTOR

1. Submitted Version. The Owner licenses back the following rights to the Contributor in the version of the Contribution as originally submitted for publication (the "Submitted Version"):

a. The right to self-archive the Submitted Version on the Contributor's personal website, place in a not for profit subject-based preprint server or repository or in a Scholarly Collaboration Network (SCN) which has signed up to the STM article sharing principles [http://www.stm-assoc.org/stm-consultations/scn-consultation-2015/] ("Compliant SCNs"), or in the Contributor's company/ institutional repository or archive. This right extends to both intranets and the Internet. The Contributor may replace the Submitted Version with the Accepted Version, after any relevant embargo period as set out in paragraph C.2(a) below has elapsed. The Contributor may wish to add a note about acceptance by the Journal and upon publication it is recommended that Contributors add a Digital Object Identifier (DOI) link back to the Final Published Version.

b. The right to transmit, print and share copies of the Submitted Version with colleagues, including via Compliant SCNs, provided that there is no systematic distribution of the Submitted Version, e.g. posting on a listserve, network (including SCNs which have not signed up to the STM sharing principles) or automated delivery.

2. Accepted Version. The Owner licenses back the following rights to the Contributor in the version of the Contribution that has been peer-reviewed and accepted for publication, but not final (the "Accepted Version"):

a. The right to self-archive the Accepted Version on the Contributor's personal website, in the Contributor's company/institutional repository or archive, in Compliant SCNs, and in not for profit subject-based repositories such as PubMed Central, subject to an embargo period of 12 months for scientific, technical and medical (STM) journals and 24 months for social science and humanities (SSH) journals following publication of the Final Published Version. There are separate arrangements with certain funding agencies governing reuse of the Accepted Version as set forth at the following website: http://www.wiley.com/go/funderstatement. The Contributor may not update the Accepted Version or replace it with the Final Published Version. The Accepted Version posted must contain a legend as follows: This is the accepted version of the following article: FULL CITE, which has been published in final form at [Link to final article]. This article may be used for non-commercial purposes in accordance with the Wiley Self-Archiving Policy [http://olabout.wiley.com/WileyCDA/Section/id-828039.html].

b. The right to transmit, print and share copies of the Accepted Version with colleagues, including via Compliant SCNs (in private research groups only before the embargo and publicly after), provided that there is no systematic distribution of the Accepted Version, e.g. posting on a listserve, network (including SCNs which have not signed up to the STM sharing principles) or automated delivery.

3. Final Published Version. The Owner hereby licenses back to the Contributor the following rights with respect to the final published version of the Contribution (the "Final Published Version"):

a. Copies for colleagues. The personal right of the Contributor only to send or transmit individual copies of the Final Published Version in any format to colleagues upon their specific request, and to share copies in private sharing groups in Compliant SCNs, provided no fee is charged, and further provided that there is no systematic external or public distribution of the Final Published Version, e.g. posting on a listserve, network or automated delivery.

b. Re-use in other publications. The right to re-use the Final Published Version or parts thereof for any publication authored or edited by the Contributor (excluding journal articles) where such re-used material constitutes less than half of the total material in such publication. In such case, any modifications must be accurately noted.

c. Teaching duties. The right to include the Final Published Version in teaching or training duties at the Contributor's institution/place of employment including in course packs, e-reserves, presentation at professional conferences, in-house training, or distance learning. The Final Published Version may not be used in seminars outside of normal teaching obligations (e.g. commercial seminars). Electronic posting of the Final Published Version in connection with teaching/training at the Contributor's company/institution is permitted subject to the implementation of reasonable access control mechanisms, such as user name and password. Posting the Final Published Version on the open Internet is not permitted.

d. Oral presentations. The right to make oral presentations based on the Final Published Version.

4. Article Abstracts, Figures, Tables, Artwork and Selected Text (up to 250 words).

a. Contributors may re-use unmodified abstracts for any non-commercial purpose. For online uses of the abstracts, the Owner encourages but does not require linking back to the Final Published Version.

b. Contributors may re-use figures, tables, artwork, and selected text up to 250 words from their Contributions, provided the following conditions are met:

(i) Full and accurate credit must be given to the Final Published Version.

(ii) Modifications to the figures and tables must be noted. Otherwise, no changes may be made.

(iii) The re-use may not be made for direct commercial purposes, or for financial consideration to the Contributor.

(iv) Nothing herein will permit dual publication in violation of journal ethical practices.

D. CONTRIBUTIONS OWNED BY EMPLOYER

1. If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/institution which

must execute this Agreement (in addition to the Contributor's signature). In such case, the company/institution hereby assigns to the Owner, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

For company/institution-owned work, signatures cannot be collected electronically and so instead please print off this Agreement, ask the appropriate person in your company/institution to sign the Agreement as well as yourself in the space provided below, and upload the signed Agreement to the Wiley Author Services Dashboard. For production editor contact details, please visit the Journal's online author guidelines.

2. In addition to the rights specified as retained in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, the Owner hereby grants back, without charge, to such company/institution, its subsidiaries and divisions, the right to make copies of and distribute the Final Published Version internally in print format or electronically on the Company's internal network. Copies so used may not be resold or distributed externally. However, the company/institution may include information and text from the Final Published Version as part of an information package included with software or other products offered for sale or license or included in patent applications. Posting of the Final Published Version by the company/institution on a public access website may only be done with written permission, and payment of any applicable fee(s). Also, upon payment of the applicable reprint fee, the company/institution may distribute print copies of the Final Published Version externally.

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government, U.K. Government, and other government employees: see notes at end.)

F. COPYRIGHT NOTICE

The Contributor and the company/institution agree that any and all copies of the Final Published Version or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal.

G. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included. If the Contribution was prepared jointly, the Contributor has informed the co-Contributors of the terms of this Agreement and has obtained their written permission to execute this Agreement on their behalf. The Contribution is submitted only to this Journal and has not been published before, has not been included in another manuscript, and is not currently under consideration or accepted for publication elsewhere. If excerpts from copyrighted works owned by third parties are included, the Contributor shall obtain written permission from the copyright owners for all uses as set forth in the standard permissions form or the Journal's Author Guidelines, and show credit to the sources in the Contribution. The Contributor also warrants that the Contribution and any submitted Supporting Information contains no libelous or unlawful statements, does not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, or contain material or instructions that might cause harm or injury and only utilize data that has been obtained in accordance with applicable legal requirements and Journal policies. The Contributor further warrants that there are no conflicts of interest relating to the Contribution, except as disclosed. Accordingly, the Contributor represents that the following information shall be clearly

identified on the title page of the Contribution: (1) all financial and material support for the research and work; (2) any financial interests the Contributor or any co-Contributors may have in companies or other entities that have an interest in the information in the Contribution or any submitted Supporting Information (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership); and (3) indication of no such financial interests if appropriate.

H. USE OF INFORMATION

The Contributor acknowledges that, during the term of this Agreement and thereafter, the Owner (and Wiley where Wiley is not the Owner) may process the Contributor's personal data, including storing or transferring data outside of the country of the Contributor's residence, in order to process transactions related to this Agreement and to communicate with the Contributor. By entering into this Agreement, the Contributor agrees to the processing of the Contributor's personal data (and, where applicable, confirms that the Contributor has obtained the permission from all other contributors to process their personal data). Wiley shall comply with all applicable laws, statutes and regulations relating to data protection and privacy and shall process such personal data in accordance with Wiley's Privacy Policy located at: http://www.wiley.com/WileyCDA/Section/id-301465.html.

[X] I agree to the COPYRIGHT TRANSFER AGREEMENT as shown above, consent to execution and delivery of the Copyright Transfer Agreement electronically and agree that an electronic signature shall be given the same legal force as a handwritten signature, and have obtained written permission from all other contributors to execute this Agreement on their behalf.

Contributor's signature (type name here): Sarah Hooper

Date: 6-9-17

SELECT FROM OPTIONS BELOW:

[X] **Contributor-owned work**

[ ] **U.S. Government work**

Note to U.S. Government Employees

A contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. Government publication, is called a "U.S. Government work", and is in the public domain in the United States. In such case, Paragraph A.1 will not apply but the Contributor must type his/her name (in the Contributor's signature line) above. Contributor acknowledges that the Contribution will be published in the United States and other countries. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. Government publication, it is not a U.S. Government work.

[ ] **U.K. Government work (Crown Copyright)**

Note to U.K. Government Employees

For Crown Copyright this form cannot be completed electronically and should be printed off, signed in the Contributor's signatures section above by the appropriately authorised individual and uploaded to the Wiley Author Services Dashboard. For production editor contact details please visit the Journal's online author guidelines. *The rights in a contribution prepared by an employee of a UK government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown and must be made available under the terms of the Open Government Licence. Contributors must ensure they comply with departmental regulations and submit the appropriate authorisation to publish. If your status as a government employee legally prevents you from signing this Agreement, please contact the Journal production editor.*

[ ] **Other**

Including Other Government work or Non-Governmental Organisation work

Note to Non-U.S., Non-U.K. Government Employees or Non-Governmental Organisation Employees

For Other Government or Non-Governmental Organisation work this form cannot be completed electronically and should be printed off, signed in the Contributor's signatures section above by the appropriately authorised individual and uploaded to the Wiley Author Services Dashboard. For production editor contact details please visit the Journal's online author guidelines. *If you are employed by the Department of Veterans Affairs in Australia, the World Bank, the World Health Organization, the International Monetary Fund, the European Atomic Energy Community, the Jet Propulsion Laboratory at California Institute of Technology, the Asian Development Bank, or are a Canadian Government civil servant, please download a copy of the license agreement from http://olabout.wiley.com/WileyCDA/Section/id-828023.html and return it to the Journal Production Editor. If your status as a government or non-governmental organisation employee legally prevents you from signing this Agreement, please contact the Journal production editor.*

Name of Government/Non-Governmental Organisation:

_____

[ ] **Company/institution owned work (made for hire in the course of employment)**

For "work made for hire" this form cannot be completed electronically and should be printed off, signed and uploaded to the Wiley Author Services Dashboard. For production editor contact details please visit the Journal's online author guidelines. *If you are an employee of Amgen, please download a copy of the company addendum from http://olabout.wiley.com/WileyCDA/Section/id-828023.html and return your signed license agreement along with the addendum.*

Name of Company/Institution:

_____

Authorized Signature of Employer: _____

Date: _____

Signature of Employee: _____

Date: _____