

RESEARCH ARTICLE

Seasonal changes in background levels of deuterium and oxygen-18 prove water drinking by harp seals, which affects the use of the doubly labelled water method

Erling S. Nordøy^{1,*}, Anne R. Lager² and Pauke C. Schots³**ABSTRACT**

The aim of this study was to monitor seasonal changes in stable isotopes of pool freshwater and harp seal (*Phoca groenlandica*) body water, and to study whether these potential seasonal changes might bias results obtained using the doubly labelled water (DLW) method when measuring energy expenditure in animals with access to freshwater. Seasonal changes in the background levels of deuterium and oxygen-18 in the body water of four captive harp seals and in the freshwater pool in which they were kept were measured over a time period of 1 year. The seals were offered daily amounts of capelin and kept under a seasonal photoperiod of 69°N. Large seasonal variations of deuterium and oxygen-18 in the pool water were measured, and the isotope abundance in the body water showed similar seasonal changes to the pool water. This shows that the seals were continuously equilibrating with the surrounding water as a result of significant daily water drinking. Variations in background levels of deuterium and oxygen-18 in freshwater sources may be due to seasonal changes in physical processes such as precipitation and evaporation that cause fractionation of isotopes. Rapid and abrupt changes in the background levels of deuterium and oxygen-18 may complicate calculation of energy expenditure by use of the DLW method. It is therefore strongly recommended that analysis of seasonal changes in background levels of isotopes is performed before the DLW method is applied on (free-ranging) animals, and to use a control group in order to correct for changes in background levels.

KEY WORDS: Water drinking, Oxygen-18, Deuterium, Stable isotopes, DLW method, *Pagophilus groenlandica*

INTRODUCTION

The doubly labelled water (DLW) method is a widely used (Acquarone et al., 2006; Costa and Gales, 2003; Nagy et al., 1999) and accepted method for measuring daily energy expenditure (DEE) of both captive and free-ranging animals. In each particular study case, it is necessary to evaluate a number of assumptions (Lifson and McClintock, 1966) when applying the method. Normally, it is assumed that the natural abundance of the stable isotopes of hydrogen and oxygen in water remains constant or at least changes

very little during experimental periods. In animals that do not drink or do not have an extensive exchange of water over body surfaces, some change in background levels may be tolerated as it will not affect the background levels of the animals to any great extent.

Seals have traditionally been regarded as animals that do not need to drink freshwater/seawater (Irving et al., 1935; Ortiz et al., 1978; Tarasoff and Toews, 1972) as they have developed a thick skin that prevents cutaneous evaporative water loss (Matsuura and Whittow, 1974), an effective nasal heat exchange that reduces respiratory evaporative water loss (Folkow and Blix, 1987; Huntley et al., 1984; Skog and Folkow, 1994) and also a high urine concentrating ability (Hong et al., 1982; Nordøy et al., 1990) that reduces obligatory renal water loss. Thus, it has often been assumed in studies of seals that all water is obtained from free and metabolic water from the food they eat and that this is sufficient to maintain water balance (Depocas et al., 1971). Even so, a number of studies have shown that seals may on occasion both eat snow and drink freshwater and seawater (Gales and Renouf, 1993; How and Nordøy, 2007; Renouf et al., 1990; Schots et al., 2017; Skjalstad and Nordøy, 2000).

The purpose of the present study was to monitor seasonal changes in stable isotopes of pool freshwater and study whether these potential seasonal changes are reflected in the stable isotope abundance in the body water of the seal as a result of freshwater consumption. This study has shown that extensive seasonal variations in the background level of both deuterium and oxygen-18 in freshwater may complicate the use of the DLW method in animals that drink from this water source.

MATERIALS AND METHODS**Animals**

Four sub-adult male harp seals, *Pagophilus groenlandica* Erxleben 1777, aged 3–5 years, were used in the current study. The animals were captured as weaned pups in the pack ice of the Greenland Sea in March and kept in captivity at the Department of Arctic Biology in a freshwater pool (35,000 l) under a continuous flow with access to a dry ledge. During the 3–5 years between the time of capture and the time of use in the current study, the seals were subjected to several other experiments while kept in captivity. However, no other experiments were performed during the experimental period of this study. During the experimental period, which lasted for 1 year, the pool freshwater was continuously exchanged at a rate of three times per day. Water and ambient air temperature varied seasonally with an average of 5.2 and 10.0°C in summer (May–September), and 2.7 and 4.0°C in winter (November–February). Freshwater was delivered from a mountain freshwater reservoir at Ringvassøya, just outside Tromsø, Norway, within a day of drainage from the reservoir. Natural daylight was provided through large windows in parallel with a programmed artificial light regime using special light tubes (L 36W/72 Biolux, Osram, Germany), simulating a photoperiod at 69°N.

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Body mass was recorded just prior to and after each experiment by use of a calibrated scale (accuracy ± 0.1 kg). Recently thawed, pre-weighed (± 0.02 kg) capelin (*Mallotus villosus*) was hand-fed *ad libitum* twice daily in summer and once daily in winter. Thus, the exact amount of fish eaten by each seal was recorded by subtracting the amount of fish left over after each meal. Vitamin B complex was supplemented daily according to Blix et al. (1973).

All procedures performed in studies involving animals were approved by the National Animal Research Authority of Norway. Permission to capture the seals was granted by the Norwegian Directory of Fisheries and the Greenlandic Ministry of Fisheries, Hunting and Agriculture.

Sampling and analysis procedure

The seals were taken out of the pool for blood sampling, seven times during the experimental period of roughly 1 year with intervals of about 6–8 weeks. For technical reasons, only two blood samples were collected in April and September. A polyethylene venflon catheter (Viggo Secalon T 16G/1.7 \times 160 mm) was inserted at the level of the 4th lumbar vertebra, using local anaesthesia (1–2% xylocaine), into the intravertebral extradural vein, and a blood sample was collected for measurement of background levels of stable isotopes in body water. On one occasion, a dose of deuterium oxide (0.07 g kg⁻¹ body mass) was injected to calculate total body water and water turnover rates. A single blood sample (10 ml) was withdrawn 60 min after administration of the dose to determine isotope levels in seal body water after equilibration and another after 8 days to determine the differential turnover rate of deuterium in body water. The equilibration time of 1 h was based on several dilution studies done at our department showing that the isotope was equilibrated after 30 min. All blood samples were immediately centrifuged for 10 min at 2500 rpm. The plasma was then collected and stored at -20°C until analysis. Water samples from the pool were collected six times per year, at the same time as the seal blood samples were taken (with the exception of the first blood sample, in early April; see Fig. 1), each time in a series of three samples spaced at 2–3 days intervals to determine changes in background levels of isotopes. Concentrations of deuterium oxide and H₂¹⁸O in the water of blood plasma, which was obtained through distillation of plasma samples, and pool water samples were determined by gas-isotope-ratio mass spectrometry (Coward, 1988; Wong and Schoeller, 1990) at the Dunn Nutrition Laboratories, Cambridge, UK. Values of deuterium oxide and H₂¹⁸O are expressed in δSMOW (standard mean ocean water), which is the isotopic composition found in our water samples in relation to the international standard mean ocean water value. In order to interpret any variation in background level of stable isotopes in pool water, seasonal variation in precipitation and ambient air temperature was obtained from the Norwegian meteorological institute. These data were collected at a local weather station located about 10 km away from the freshwater source that supplied the pool.

Calculations

Total body water was calculated as the relationship between the injected isotope dose and the isotope dose found in the seal body water after equilibration time as explained by Lifson and McClintock (1966) and applied by Schots et al. (2017). The fractional turnover rate was calculated based on the difference in isotope concentration at the beginning and end of the 8 day experiment, as also explained by Lifson and McClintock (1966) and applied by Schots et al. (2017). Under the assumption that total body water and the turnover rate remain stable during the 1 week experimental period, the total water influx rate (which equals the water efflux rate) could be calculated

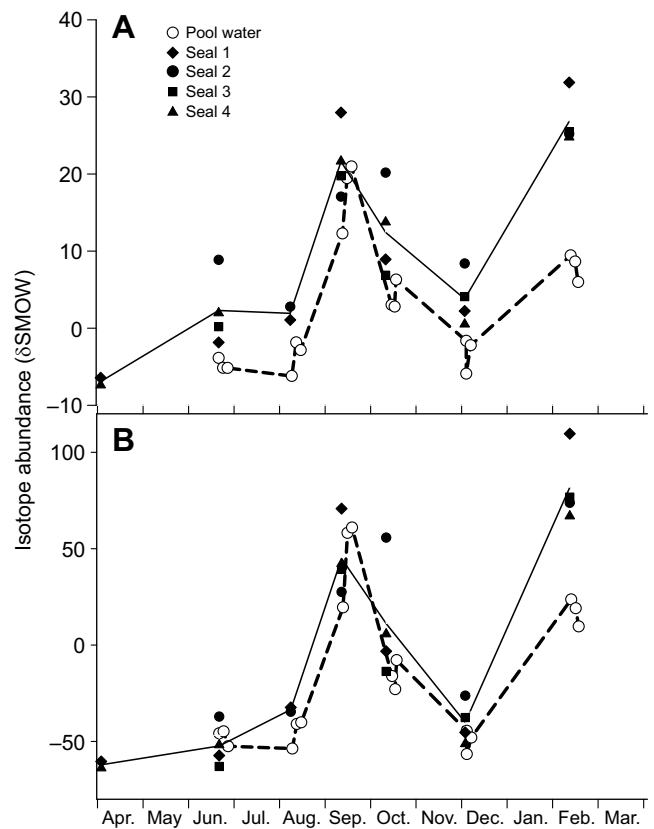


Fig. 1. Seasonal changes in isotope abundance of oxygen-18 and deuterium in pool water and individual seal body water. (A) Oxygen-18; (B) deuterium. δSMOW , standard mean ocean water. Solid line indicates the average isotope abundance in the four seals. Dashed line is pool water isotope abundance.

using eqn 8 from Lifson and McClintock (1966). The total water influx rate can be divided into the respiratory water influx, free water in the food and metabolic water influx, and water influx due to (freshwater) drinking using similar calculations as explained by Skälstad and Nordøy (2000) and Schots et al. (2017).

Statistics

The linear regression analysis in Figs 2 and 3 was done using SigmaPlot 13.0. Kruskal–Wallis tests with Dunn’s *post hoc* test of multiple comparisons were carried out in R version 3.3.2 (R Core Team 2016; www.r-project.org/), using the ‘dunn.test’ package (Dinno 2017; https://CRAN.R-project.org/package=dunn.test). We applied the Bonferroni correction for multiple comparisons.

RESULTS

Seasonal changes in oxygen-18 and deuterium abundance

Seasonal changes in the isotope abundance of deuterium and oxygen-18 in pool water and the seal’s body water is shown in Fig. 1. Both isotopes display profound seasonal variations throughout the year in the pool water and body water. A non-parametric one-way analysis of variance (Kruskal–Wallis) showed that both deuterium and oxygen-18 concentration in seal body water as well as pool water changed significantly throughout the study period (P -range: 0.01–0.002). Specifically, pool water deuterium concentration increased between June and September ($P < 0.05$) and decreased between September and December ($P < 0.05$), while pool water oxygen-18 increased between June and September ($P < 0.05$). Similarly, body water deuterium increased between June and

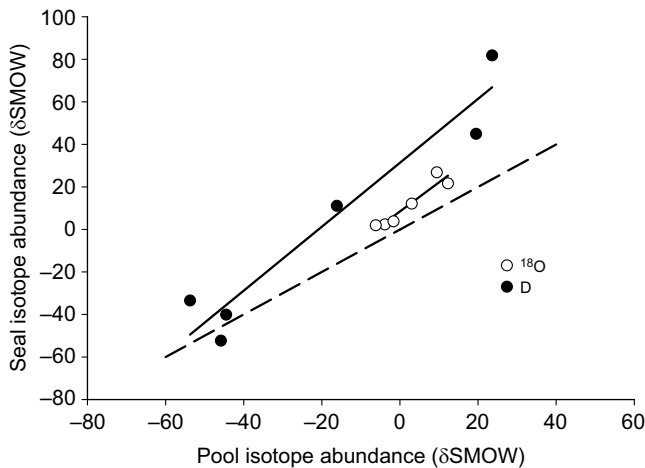


Fig. 2. Relationship between seal body water and pool water in the isotope abundance of oxygen-18 (^{18}O) and deuterium (D). The average isotope abundance of seal body water at each time point from Fig. 1 was used. Solid lines indicate the linear regression and the dashed line indicates the 1:1 relationship.

September ($P < 0.05$) and decreased from September to December ($P < 0.05$), while body water oxygen-18 values in June and August were different from values obtained in February ($P < 0.05$). Individual values of deuterium and oxygen-18 ranged from below -55 δSMOW to almost 110 δSMOW and from -5 δSMOW to more than 30 δSMOW , respectively. Changes in isotope abundance in the pool coincided with changes in isotope abundance in body water of all four harp seals.

Relationship between seal body water and pool water isotope abundance

Fig. 2 shows the relationship between pool isotope abundance and isotope abundance in the body water of the seal. There was a clear correlation between the isotopic enrichment of deuterated water between the pool water and total body water of the seals throughout the study period (Fig. 2). The relationship can be described by the following linear relationship [deuterium in seal body water] = 1.50 [deuterium in pool] + 31.34 , $R^2 = 0.93$. Similarly, there was a linear relationship between the oxygen-18 enrichment in the pool and total

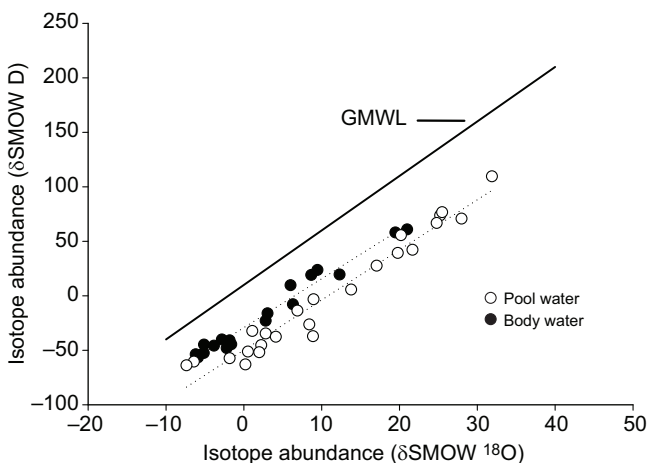


Fig. 3. Isotope abundance of δSMOW oxygen-18 versus δSMOW deuterium in the pool water and the seal's body water. $n = 4$. GMWL, global meteoric water line, as explained by Craig (1961), given as $\delta\text{D} = 5\delta\text{O}^{18} + 10$.

body water of the seals (Fig. 2), described by [oxygen-18 in seal body water] = 1.37 [oxygen-18 in pool] + 9.50 , $R^2 = 0.91$. The isotope abundance was in general higher than the $x = y$ relationship as is described by the dashed line in Fig. 2. This means that the enrichments in the seal's body water was always slightly above that of pool water.

Relationship between deuterium and oxygen-18 in seal body water and pool water

The isotope abundance of oxygen-18 (in δSMOW) versus the isotope abundance of deuterium (in δSMOW) in the seal's body water and the pool water is shown in Fig. 3. The correlation between deuterium and oxygen-18 in the pool water can be described by the following equation: deuterium = 4.49 [oxygen-18] - 29.28 , $R^2 = 0.97$, while the correlation between deuterium and oxygen-18 in the seal's body water can be described by: deuterium = 4.60 [oxygen-18] - 49.92 , $R^2 = 0.96$. These correlations are compared with the linear relationship in meteoric water presented by Craig (1961) (solid line in Fig. 3). The pool water was on the high enrichment end of the meteoric water line, fitting a line with a slope of about 5 instead of 8 as found in most of the data presented by Craig (1961). The relative enrichment of deuterium versus oxygen-18 was persistently lower over the whole range of isotope abundances experienced in the current study compared with the meteoric water line (Fig. 3).

Isotope abundance and climate

In order to investigate the large seasonal variation in the abundance of isotopes, local seasonal meteorological data from the same year were obtained from a nearby weather station and compared with the seasonal isotope data (Fig. 4). This was done to relate changes in

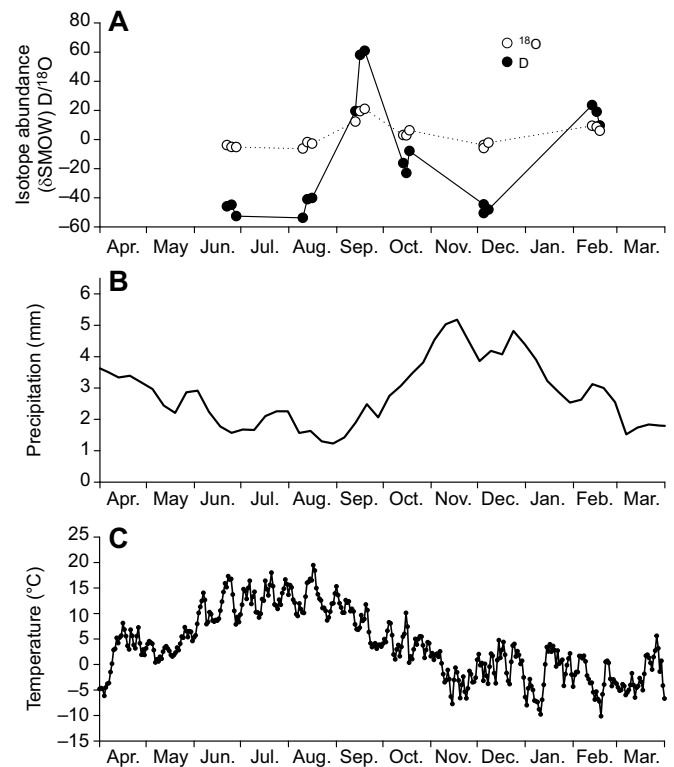


Fig. 4. Isotope abundance and climate. (A) Isotope abundance of oxygen-18 and deuterium in the pool. (B) Daily precipitation (averaged over 1 week). (C) Ambient air temperature. Data were obtained from a local weather station at the meteorological institute of Norway.

isotope abundance to seasonal weather processes, such as precipitation and condensation, resulting in gross changes in nearby freshwater supplies.

Water drinking by seals

Analysis of the seal blood samples showed that the fractional turnover rate of deuterium varied between -0.147 and -0.076 day^{-1} . Total body water of the seals varied between 24.8 and 30.8 l. The total water influx ranged between 2400 and 3800 ml day^{-1} , which is similar to the values found by Skalstad and Nordøy (2000). The respiratory water influx ranged between 240 and 380 ml day^{-1} , and the free water and metabolic water in the food ranged between 1100 and 1400 ml day^{-1} . This means that the difference between the total water influx and the respiratory water plus water in the food was due to drinking of the pool water, which ranged between 1000 and 2200 ml day^{-1} .

DISCUSSION

The harp seals studied here were kept for several years in a freshwater pool with a constant water supply originating from a freshwater lake, which is located on the coast of northern Norway. Background levels of deuterium and oxygen-18 changed markedly, even within the time frame of 1 week in the pool water, and indeed also in the seal's body water (Fig. 1). This confirms that the arctic harp seal drinks freshwater when available and, as a result, equilibrate their body water isotope levels with whatever isotope levels there are in the pool water. Such profound changes in deuterium and oxygen-18 background levels in body water may complicate measurements on energy expenditure using the DLW method, which is based on monitoring the dilution rate of deuterium and oxygen-18 after an initial injected dose, over a time period of 1 week or more.

Reasons for seasonal changes in isotope abundance

Changes in isotopic composition of the water supply to the pool may be caused by natural seasonal changes in the freshwater supply. Unlike the isotopic composition of the oceans, which is relatively stable (Dinçer, 1968), the composition of lakes may be subjected to seasonal variation. $\delta\text{Oxygen-18}$ and $\delta\text{deuterium}$ in the lake depend on the hydrological balance between water inputs (precipitation, groundwater and surface and stream inflows) and water outputs (groundwater loss, evaporation and surface and stream outflows) (Gibson et al., 1999; Leng et al., 2006). In addition, air and ground temperature might affect, for example, evaporation or sublimation rates and hence alter isotopic concentrations accordingly. Changes in the background deuterium and oxygen-18 abundance have been observed before (Craig, 1961; Dansgaard, 1964; Taylor, 1974), but records on the isotopic composition of deuterium and oxygen-18 in precipitation and lakes in northern Scandinavia have only recently become available. Changes in isotope enrichment are due to fractionation processes caused by evaporation and precipitation. The isotopic composition of deuterium and oxygen-18 in the pool water seen in the current study is similar to the seasonal cycles observed in aquifers and precipitation in northern Finland (Kortelainen and Karhu, 2004), and rivers in northern Sweden (Burgman et al., 1987). Gibson (2002) describes how $\delta\text{oxygen-18}$ and $\delta\text{deuterium}$ can change in a freshwater lake in arctic regions as a result of seasonal changes in ice cover, ice melting and snow input as well as precipitation and evaporation. In northern Scandinavia, the surface and stream inflow in spring consists mainly of meltwater from snow. Because of fractionation, precipitation is depleted in deuterium and oxygen-18. In spring time, the lake becomes

isotopically depleted as a result of the large influx of meltwater (Jonsson et al., 2009) (Figs 1 and 4). In late summer, when the air temperature increases (Fig. 4) and the precipitation and meltwater influx decrease, the lake water becomes isotopically enriched as a result of evaporation. In autumn, the lake becomes isotopically diluted again (Figs 1 and 4) because of increased precipitation. Seasonal fluctuations in the lake water isotopic composition are visible owing to the short residence time and the small lake volume.

The maximum concentrations of deuterium and oxygen-18 found in this study are significantly higher than those found in lakes in northern Sweden, and in the precipitation in East Antarctica, Bangkok and eastern Asia (Fujita and Abe, 2006; He et al., 2006; Jonsson et al., 2009; Posmentier et al., 2004). This can be explained by the fact that the lake is located at the coast; the concentration of oxygen-18 and deuterium in the precipitation is elevated along the coastline compared with precipitation that falls further inland (Andersson et al., 1990).

Seals drink water

Seasonal changes in the background level of oxygen-18 and deuterium in the body water of the seal co-varied with the seasonal changes in the background level of the pool water. This shows that the seals drink the pool water at a high rate and are continuously being equilibrated with whatever background enrichment there is of these heavy water isotopes. Based on the analysis of deuterium oxide injection, it was calculated that the seals in this study drank between 1000 and 2200 ml day^{-1} of freshwater. These values are higher than those found by Skalstad and Nordøy (2000), but considering that the seals in the current study were moulting and had a reduced food intake, i.e. reduced influx of metabolic water and free water in the food, the seals probably needed to drink more water to maintain water balance. For instance, young hooded seals do ingest snow and seawater during the respective beginning and end of their post-weaning fast to maintain water balance when water influx through food is absent (Schots et al., 2017).

Although the isotope abundance in the seal's body water co-varied with that of the pool water (Fig. 1), the abundance in the body water was in general higher than that in the pool water. This could be explained as follows. First, the free water content in the capelin can be expected to be around zero SMOW as the fish was caught in the ocean. Eating fish with water of zero SMOW will increase the isotope abundance in the body water if the abundance in the pool is SMOW negative. Second, seals lose water through urine production and cutaneous and respiratory evaporation. Both respiratory and cutaneous evaporation are subjected to fractionation (Lifson and McClintock, 1966), which would lead to an enrichment of the isotopes in the body water.

Effects on the use of the DLW method

Changes in isotopic abundance in the background level of an animal's body water do not necessarily result in errors in the DLW energy expenditure calculations (Horvitz and Schoeller, 2001; Jones, 1995). As long as the isotope dose provided to the seals and the isotopic background levels of the pool water fall along the meteoric water line, the turnover rates of deuterium and oxygen-18 will shift identically and the effect of changing background enrichment becomes self-cancelling. In this study, however, the background levels of deuterium and oxygen-18 in the pool water and the seal's body water did not fall along the meteoric water line as formulated by Craig (1961) ($\text{deuterium} = 8[\text{oxygen-18}] + 10$) (Fig. 3). The intercept of the meteoric water line is 10 whereas the intercept of the pool water and body water line was -29.28 and

–49.92, respectively. In general, the slope of the meteoric water line is 8, but for water sources at the high enrichment end of the curve, the slope is 5 (Craig, 1961). The correlation between deuterium and oxygen-18 in the pool water and body water found in this study had a slope of less than 5 in both cases (Fig. 3). In addition, as a result of the large changes in background levels of deuterium and oxygen-18, which often happened in this study, the decay curve of the stable isotopes after DLW injection may drop to background levels before the end of the experiment.

Applicability

First, this study may apply to other energy expenditure studies on captive marine mammals, provided that these species consume freshwater (drink freshwater or ingest snow or ice) that has been subjected to fractionation and thus (seasonal) variation. Second, this study may apply to studies on energy expenditure of wild marine mammals, such as the freshwater Baikal seal (*Pusa sibirica*) or ice-breeding phocids of which some have been shown to depend on freshwater in the form of snow and ice during fasting (e.g. Schots et al., 2017). Finally, the findings in this study may also apply to all DLW studies of wild populations of birds and mammals that rely on freshwater drinking for maintenance of water balance homeostasis.

Conclusions

This study has shown that captive harp seals, kept in a freshwater pool, will continuously equilibrate their body water with the isotopic abundance of deuterium and oxygen-18 in the pool as a result of drinking the pool water. These large and rapid seasonal changes in the background level of the heavy isotopes of water may complicate measurements on energy expenditure by use of the DLW method. Jones (1995) studied the effect of changing background levels of stable isotopes on the calculation of energy expenditure using this method. He suggested that as long as the background changes followed the global meteoric water line, such changes do not necessarily introduce any error. In our case, however, changes in background level were rapid and unpredictable and the values were constantly below the global meteoric water line (Fig. 3). In addition, if such rapid increases in background isotope abundance occur, this may pose a risk that after an initial DLW injection the isotope concentrations will drop below background levels before the end of the experiment, completely violating any calculation of DEE.

Such profound changes in isotope enrichment as observed in the freshwater source here, necessitate the use of a control group in order to monitor these changes accurately and to correct calculations on energy expenditure. This is of particular importance in studies done on animals that live in the proximity of freshwater sources. These animals may drink water and eat prey with a free water content that affects the isotope concentrations in the body water. For studies on migrating animals, which are surrounded by freshwater sources in different geographical locations, we also advise the use of a control group.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: E.S.N.; Investigation: E.S.N., A.R.L.; Writing - original draft: E.S.N., P.C.S.; Writing - review & editing: P.C.S., E.S.N.; Visualization: E.S.N., P.C.S.; Supervision: E.S.N.; Project administration: E.S.N.; Funding acquisition: E.S.N.

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