Applicability of a single-sample approach for the doubly labelled water method to the Streaked Shearwater *Calonectris leucomelas*

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**Abstract** The doubly labelled water method is a common means of investigating field metabolic rates (FMRs) of free-ranging animals by injecting oxygen and hydrogen isotopes. Compared with a general two-sample approach including double blood sampling, a single-sample approach, which includes an estimation of initial isotope enrichment and single blood sampling, has been developed as a less invasive technique with lower impact on the behavior of study subjects. However, little attempt has been made to improve the indirect estimation of initial isotope enrichment and to apply the two-pool model for calculating FMR from the single-sample approach. Therefore, we studied the validity of a single-sample approach in the Streaked Shearwater *Calonectris leucomelas*. We developed equations for estimating initial isotope enrichment based on the amount of injected isotopes and body mass collected from 15 shearwaters. Then, for six shearwaters subjected to a two-sample approach, we calculated the turnover rates of oxygen and hydrogen isotopes (ko and kd), and FMR using the two-pool model with measured and initial isotope enrichments. The arithmetic errors were −0.01% for the estimated initial enrichments of oxygen isotope and −0.11% for hydrogen isotope. The ko, using estimated initial isotope, is overestimated by 3.2% on average, while kd is underestimated by 0.4% in comparison with those measured by the two-sample approach. The FMR measured by the single-sample approach are overestimated by 12.0% (±12.1 SD) in comparison with those measured by the two-sample approach. We were able to estimate reliably the initial enrichments of both isotopes and apply the two-pool model in the calculation of FMR.

**Key words** Animal energetics, DLW method, Energy expenditure, Single-sample approach, Streaked Shearwater

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Patterns of energy expenditure play a significant role in the determination of life history traits (Ricklefs 1996; Costa & Shaffer 2012). Since the first application of the doubly labelled water (DLW) method to measuring human energy expenditure was described in 1982 (Schoeller & Van Santen 1982), the method has been used for energetic studies of many free-ranging animal species (Speakman 1998; Shaffer 2011).

This method relies on the measurable differences in the turnover rates of oxygen and hydrogen isotopes in animals (Lifson & MacClintok 1966; Speakman 1997). When water labelled with stable isotopes of oxygen (18O) and hydrogen (deuterium, 2H) is injected into an animal body, it takes the isotopes 1–3 hours to mix and label body water, depending on the total water content of an animal. Then, the isotopes are eliminated from the body, as is the case with chemical elements sharing the same number of protons. Because 2H leaves as H2O while 18O leaves as CO2 and H2O, there is a difference in the turnover rates of each isotope. This difference facilitates the measuring of CO2 production, which is an indi-
indicator of energy expenditure in animals. According to the standard DLW procedure to measure isotope enrichment in an animal’s body, three separate blood samples are required. The first establishes the background level making it possible to determine the natural abundances of both isotopes in animal’s body. The second provides the initial level to measure isotope enrichments at equilibration (i.e. 1–3 hours after the DLW injection) in an animal’s body fluids. The third provides the final level, measuring the extent to which the isotopes have been eliminated (compared with the initial level), during free-ranging conditions over 1–7 days. In most studies of free-ranging animals, however, the background isotope levels of animal subjects are applied to the measurements of un-injected animals because of the relatively minimal effect on estimation of metabolic rate (Speakman 1997). Therefore, it is necessary only to collect blood sample twice in order to measure isotope enrichment. This two-sample approach is the most commonly used procedure (Lifson & MacClintok 1966; Speakman 1997). However, this procedure may cause disturbance to some species, and the extent of disturbance may depend on an animal’s size and sensitivity to the duration of isotope equilibration, or handling (Webster & Weathers 1989; Schultner et al. 2010). For example, the two-sample approach may cause parental birds to alter their nest attendance or increase the possibility to them abandoning a breeding attempt (Williams & Dwinnel 1990; Schultner et al. 2010). In order to use the DLW method, when quantifying the energy expenditure of free-ranging animals, without affecting research subjects, it is important to improve the procedures used in the DLW method.

The single-sample approach, which measures only the final isotope enrichment of an animal’s body fluids, was originally proposed by Nagy et al. (1984), Ricklefs and Williams (1984), and Obst et al. (1987). This approach has been used for many avian energetic studies (Ricklefs et al. 1986; Montevecchi et al. 1992; Jodice et al. 2003; Welcker et al. 2009). Two procedures for estimating initial isotope enrichment when using the single-sample approach have been proposed (Webster & Weathers 1989; Speakman 1997; Schultner et al. 2010). One procedure assumes that the log-transformed ratio of the initial isotope enrichment [ln(18O/2H)] of each individual equals the log-transformed ratio of another individual (Webster & Weathers 1989). It has been shown that this ratio is stable across individuals (Williams & Dwinnel 1990). However, the procedure cannot derive the isotope dilution spaces for the two isotopes from the log-transformed ratio (Speakman 1997). Therefore, it is necessary to use the mean value of total body water (TBW) of subjected animal species for calculating energy expenditure. Since the calculated energy expenditure depends heavily on TBW, the lack of individual TBW values may influence the accuracy of the single-sample approach.

The other procedure estimates an initial isotope enrichment based on the relationship between initial isotope enrichment and an animal’s body mass (Speakman 1997; Schultner et al. 2010). Theoretically, if the mass of the injected isotopes is kept constant across all experimental animals (Montevecchi et al. 1992; Schultner et al. 2010), it is possible to derive a predictive relationship for the initial isotope enrichment using only body mass (Speakman 1997). However, it is difficult to insert the same mass of injectate to all experimental animals, especially under field study conditions (Speakman 1997). Therefore, it is assumed that initial isotope enrichment can be statistically estimated more accurately using both moles of injectate and body mass as independent predictors. Instead of using either of the two procedures described above, it is possible to improve the estimation of individual initial isotope enrichment and isotope dilution space, by using a combination of body mass and moles of injectate.

In this study, we validate the single-sample approach for the DLW method in the Streaked Shearwater Calonectris leucomelas, a medium-sized (450–600 g) pelagic seabird that breeds on offshore islands around the Japanese Archipelago (Oka 2004; Shirai et al. 2012). We estimated the initial isotope enrichments of 2H and 18O by using the relationship between body mass and the amount of isotopes injected and assessed the accuracy of this estimation by comparison with the two-sample approach.

**MATERIALS AND METHODS**

1) Study sites

Our field studies were conducted during August and September 2008 on Awa Island (Awa-shima 38°27′N, 139°13′E, Niigata Prefecture, Japan) and in 2013 on Funakoshi-Ohshima Island (Funakoshi-Ohshima 39°24′N, 141°59′E, Iwate Prefecture, Japan). There is no available information on the size of the breeding population on Funakoshi-Ohshima Island; however, it is estimated that approximately 84,000 pairs of Streaked Shearwaters breed on Awa
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Island (Maki Yamamoto, unpublished).

2) Field procedure

We caught 16 shearwaters (nine birds in 2008 on Awa Island and seven in 2013 on Funakoshi-Ohshima Island) at night by hand to establish the equations for estimating initial isotope enrichments (Group 1). The DLW injectate used in our study on Awa Island contained 10.0 atom percent $^{18}$O, 4.0 atom percent $^2$H, and 0.9% NaCl, while that on Funakoshi-Ohshima Island contained 21.0 atom percent $^{18}$O, 10.5 atom percent $^2$H, and 0.9% NaCl. After collecting a 1 mL blood sample as a background sample from the brachial or tarsal vein, the captured shearwaters were injected with the DLW (2 ml on Awa Island and 3 ml on Funakoshi-Ohshima Island) into their body cavities. After injection, they were kept individually in plastic boxes for 90–120 minutes. Then, we collected a 1 mL blood sample from the brachial or tarsal vein of each individual as an initial sample and measured their body mass (BM) with a Pesola spring balance accurate to the nearest 10 g.

As a second group (Group 2), we caught six shearwaters (three on each island) to investigate their field metabolic rates (FMR) by both single- and two-sample approaches. As with Group 1, background blood samples were collected from all six birds and they were injected intraperitoneally with DLW. After isotope equilibrations (90–120 mins), we collected another 1 mL blood sample from each individual as an initial sample and measured their initial body mass (BM$_i$). All individuals were ringed with individually numbered metal rings and released back into their nests. All of the injected individuals were recaptured in or near their burrows at night after they had returned from a foraging trip. Immediately after recapture, we collected a further 1 mL blood sample from the brachial or tarsal vein of each individual, as a final sample, and measured final body mass (BM$_f$).

In order to quantify the injectate, the syringe was weighed before and after each injection using an electronic balance (to the nearest 0.001 g on Funakoshi-Ohshima Island) in the field laboratory according to Speakman (1997). On average, birds on Awa Island were injected with 2.01 g DLW ($\pm$0.03 SD) and on Funakoshi-Ohshima Island with 2.916 g ($\pm$0.021 SD). All blood samples were heparinized (NT-HE 1000, Nipro, Japan in the study on Awa Island and PZ-D03, Terumo, Japan in the study on Funakoshi-Ohshima Island) and centrifuged (5 min, 6200 rpm).

After centrifugation, each serum sample was transferred to a 0.5 mL screw-topped plastic vial with an O-ring (Asahi Techno Glass Co.). Samples were stored frozen at $-25^\circ$C immediately (Awa Island), or stored at ambient temperature in the field and frozen as soon as logistically possible at $-25^\circ$C (Funakoshi-Ohshima Island) until the isotope analysis.

3) Isotope ratio analysis

The $^2$H and $^{18}$O isotope concentrations of the serum and DLW injectate samples were analyzed according to the procedure of Shirai et al. (2012), using isotope ratio mass spectrometry (IRMS; Hydra 20−20, Sercon, Crewe, UK; Yamada et al. 2009). The injectate and serum samples were diluted with distilled water measured with an electronic balance (Mettler-Toledo, Columbus, OH, USA) to the nearest 0.01 mg (Shirai et al. 2012). The enrichment of distilled water was measured using IRMS, as with the diluted serum and injectate samples. The distilled water, diluted serum and injectate samples were all put into cylindrical tubes and analyzed using the water equilibration method (Horita et al. 1989). Water standards (Iso-Analytical, Crewe, UK) were used to establish calibration curves for normalizing the values. Each sample was analyzed in duplicate. All isotope enrichments were measured in $\delta$ per mille relative to the working standards ($\delta$H$_{SMOW}$ and $\delta^{18}$O$_{SMOW}$) and converted to an absolute ratio for $^2$H by using Speakman’s (1997) equation 14.4, and for $^{18}$O by using Speakman’s (1997) equation 14.9, as follows:

$$\text{Ratio}_{2H} = \left(\frac{\delta^{2H}_{SMOW}}{1000} + 1\right) \times \text{Ratio}_{2H, SMOW}$$

$$\text{Ratio}_{18O} = \left(\frac{\delta^{18O}_{SMOW}}{1000} + 1\right) \times \text{Ratio}_{18O, SMOW}$$

where $\text{Ratio}_{2H, SMOW} = 0.00015595$, and $\text{Ratio}_{18O, SMOW} = 0.0020052$. Absolute ratios were converted to ppm for $^2$H by using Speakman’s (1997) equation 14.8, and for $^{18}$O by using Speakman’s (1997) equation 14.14, as follows:

$$pe^{2H} = \text{Ratio}_{2H} / (\text{Ratio}_{2H} + 1),$$

$$pe^{18O} = \text{Ratio}_{18O} / (1 + \text{Ratio}_{17O} + \text{Ratio}_{18O}),$$

where $\text{Ratio}_{17O} = 0.000373$. All subsequent calculations in the DLW method were performed on the mean values of each sample analyzed in duplicate.

4) Calculation procedures in the DLW method for single- and two-sample approaches

Theoretically, the increments by isotope injection from background isotope enrichments are negatively correlated with a subject’s body mass (Speakman 1997). Thus, initial isotope enrichments estimated
by body mass were also used in the calculation procedure for the single-sample approach (Welcker et al. 2009; Schultner et al. 2010). In addition, it is expected that the moles of injectate positively influence initial isotope enrichment (Speakman 1997). Thus, in our estimation of initial isotope enrichment, we established the equation for initial isotope enrichment with both body mass and the moles of the injectate. Then, the estimated initial isotope enrichments using the best fit equations were used for calculating the TBW of initial body mass and FMR of Group 2 using Speakman’s (1993) two-pool model. The initial isotope enrichments of both $^{18}$O and $^2$H for one shearwater on Funakoshi-Ohshima Island in Group 1 were much higher than for all other birds, despite this shearwater having a body mass similar to the others. This result may indicate a failure of injection volume measurement before and/or after injection. Therefore, we eliminated the data for this individual from all analyses.

We used a generalized linear model (GLM) to acquire estimation equations for the increment of each isotope ($H_{inc}$ and $O_{inc}$) between body mass (BM), injected moles (Mo$_{inj}$), and the interaction. The best-fit equation for each isotope was assessed based on the mean squared prediction error (MSPE) model selection procedure by the “leave-one-out cross-validation” process (Murtaugh 2009). The cross-validation approach was taken such that the birds used to produce the predictive equation were also used to validate it (Halsey et al. 2008). The equations for the procedure were run a further 15 times, each time with a different individual bird omitted from the analysis. After each iteration the resulting equation was used to predict the initial isotope enrichment of the omitted bird and then compared to the measured initial and samples, respectively. To convert the unit of the isotope dilution spaces, we used a conversion factor of 18.002 g mol$^{-1}$ (Speakman 1997).

To estimate total body water of Group 2, we used the plateau method to determine the isotope dilution spaces for $^2$H ($N_d$, mol) and $^{18}$O ($N_o$, mol) (Speakman 1997). $N_d$ and $N_o$ were calculated using the general equations:

$$N_d = \frac{H_{adj} \times (H_i - H_o)}{H_b - H_f}$$

$$N_o = \frac{O_{adj} \times (O_i - O_o)}{O_b - O_f}$$

where $H_{adj}$ and $O_{adj}$ represent the respective DLW injectate ($^2$H or $^{18}$O, mol), $H_d$ and $O_d$ represent the isotope concentrations ($^2$H or $^{18}$O, ppm) in the DLW injectate, and $H_b$, $H_f$, $O_b$, and $O_f$ represent the isotope concentrations ($^2$H or $^{18}$O, ppm) of the background and initial samples, respectively. To convert the unit of the isotope dilution spaces, we used a conversion factor of 18.002 g mol$^{-1}$ (Speakman 1997). For each bird, the dilution space ratio ($R_{dilspace}$, dimensionless) was calculated by dividing the total body water value obtained from $^2$H dilution by the value obtained from $^{18}$O dilution (Speakman 1997).

The turnover rates for $^2$H and $^{18}$O ($k_d$ and $k_o$, respectively, day$^{-1}$) of Group 2 were determined using the two-sample technique and calculated as follows:

$$k_d = \frac{\ln(H_f - H_b) - \ln(H_i - H_b)}{t}$$

$$k_o = \frac{\ln(O_f - O_b) - \ln(O_i - O_b)}{t}$$

where $H_f$ and $O_f$ represent the respective isotope concentrations ($^2$H or $^{18}$O, ppm) of the final samples and $t$ represents the time interval between the initial and final samples (days) (Lifson & McClintock 1966; Speakman 1997).

Although there are several different published models for calculating CO$_2$ production for the DLW method (Speakman 1997), the two-pool model of Speakman (1993) is the most suitable for Streaked Shearwater (Masaki Shirai, Yasuaki Niizuma, Maki Yamamoto, Emiko Oda, Naoyuki Ebine, Nariko Oka, Ken Yoda unpublished). Therefore, we used the model for calculating rCO$_2$ in this study as follows:

$$rCO_2 = \left(\frac{N}{2.078}\right) \times (k_o - R_{dilspace} k_d) - 0.246 N1.05 (k_o - R_{dilspace} k_d)$$

where $N = \left[\left(N_o + N_d / R_{dilspace}\right)\right] / 2$. To convert units in mLCO$_2$ day$^{-1}$ into energy equivalents, we assumed that 1 mL of CO$_2$=25.11 J (Gessaman & Nagy 1988).

All statistical analyses were performed using R (version 3.0.1).
RESULTS

1) Estimation of initial isotope enrichment for a single-sample approach in Streaked Shearwater

The increment for each isotope (H_{inc} and O_{inc}, ppm) by injection was significantly correlated with body mass (BM, g), injected moles (Mol_{inj}, mol) and their interaction (see Table 1). The equations with the lowest MSPE values are represented as follows:

\[ H_{inc} = 1.24 \times BM + 18818.56 \times Mol_{inj} - 14.36 \times BM \times Mol_{inj} - 1631.09 \]  
\[ O_{inc} = 2.79 \times BM + 40387.20 \times Mol_{inj} - 32.64 \times BM \times Mol_{inj} - 3412.20 \]  

The H_{inc} equation has an adjusted R^2 of 0.994, while the O_{inc} equation has an adjusted R^2 of 0.997. By means of the cross-validation approach, the mean arithmetic errors (i.e. accuracy) were found to be −0.01% for the estimated initial isotope enrichment by the procedure for oxygen isotope, and −0.11% for the hydrogen isotope.

2) Comparison between the single- and two-sample approaches for evaluating TBW and FMR

The total body water (TBW) of Group 2, which was measured at initial sampling by the two-sample approach, was 340.3 ml (±57.2 SD) and 341.5 ml (±42.0 SD) calculated from hydrogen and oxygen isotopes, respectively. The dilution space ratio (R_{dilspac}) was 0.99 (±0.08) (range: 0.87–1.07). The differences in TBW measured by the single- and two-sample approaches averaged −0.9% (±3.3 SD). The ko, using estimated initial isotope, was overestimated by 3.2% on average, while kd was underestimated by 0.4%, when compared with those measured by the two-sample approach (Table 2). The field metabolic rates of Group 2 measured by the two-sample approach were 903 kJ day^{-1} (±647 SD) on Awa Island and 712 kJ day^{-1} (±248 SD) on Funakoshi-Ohshima Island. The FMR measured by the single-sample approach are overestimated by 12.0% (±12.1 SD) in comparison with those measured by the two-sample approach (Table 2).

DISCUSSION

In this study, we compared the reliability of the estimation of initial isotope enrichments with different assumptions and indicate that an estimation including the moles of injectate is more reliable. Although some previous studies have assumed that the amount of injected isotope is kept constant across all experimental subjects (e.g., Schultner et al. 2010), even a slight deviation in the mass of the enriched isotopes can have a profound effect on the amount of isotope injected (Speakman 1997). In fact, our equations (including body mass, injected moles, and their interaction) for estimating initial isotope enrichments proved to have higher predictability (R^2=0.99) than the equations of Schultner et al. (2010) estimated from body mass alone (R^2=0.89–0.96). Thus, our results indicate that when using a single-sample approach it is important to note the effect of the amount of injectate isotope.

In many studies of energetics using the technique of Webster and Weathers (1989), TBW in avian subjects is estimated from the water content of other individuals, determined by desiccation of sacrificed birds or by 18O dilution (Ricklefs & Williams 1984; Ricklefs et al. 1986; Obst et al. 1987; Webster & Weathers 1989; Williams & Dwinnel 1990; Montevacchi et al. 1992). However, body components of birds, such as stored fat and body water, vary in response to changes in energy demands especially those changes that occur during the different stages of breeding (Jones 1994; Niizuma et al. 2001, 2002). Usually, parental birds store body fat as an energy supply for use when fasting during incubation and they lose that fat at the start of the chick-rearing period (Jones 1994; Hilton et al. 2000; Niizuma et al. 2001, 2002). Since it is known that the lipid mass of the whole body has a close inverse relationship

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Table 1. Model selection for estimating initial isotope enrichments of Streaked Shearwater based upon the leave-one-out cross-validation

<table>
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<th>Number</th>
<th>Model (Hydrogen)</th>
<th>Mean squared prediction error</th>
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<td>2</td>
<td>BM+Mol_{inj}</td>
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<td>3</td>
<td>Mol_{inj}</td>
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<th>Mean squared prediction error</th>
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<td>3</td>
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<td>4</td>
<td>BM</td>
<td>373075.42</td>
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</table>

Models include the variables initial body mass (BM) and the moles of injectate (Mol_{inj}).
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with water mass (Robbins 1983; Farley & Robbins 1994; Hilderbrand et al. 1998; Niizuma 2011), the percentage of TBW in birds also varies with each breeding stage. In our procedure, the TBW of each subject was estimated using the initial isotope enrichment of $^{18}O$ or $^2H$, as calculated by equations (1) and (2). We feel that the FMR estimated using our procedure might be more reliable than the previous single-sample approach. An additional advantage of our procedure is that it can apply for all rCO2 calculation models (both the one- and two-pool models; Speakman 1997). Although the two-pool model is more suitable to derive FMR and is theoretically recommended for larger birds with body masses exceeding 1,000 g (Speakman 1993, 1997), the procedure of the single-sample approach by Webster and Weathers (1989) does not allow for use of the two-pool model because the procedure cannot derive the isotope dilution space from each isotope ($^2H$ and $^{18}O$). Thus, our procedure of the single-sample approach implies to apply FMR measurements in animals over a range of different body masses.

The FMR calculated using our procedure routinely overestimated by 12% on average, compared with the two-sample approach. Theoretically, the DLW method relies on distinguishing between the elimination curves of oxygen and hydrogen isotopes. Thus, the overestimated results suggest that: 1) the turnover rate of hydrogen isotope was underestimated, 2) the turnover rate of oxygen isotope was overestimated, or 3) both processes occurred simultaneously. Compared with isotope turnover rates (i.e. $k_o$ and $k_d$) using measured initial isotope enrichments, $k_o$ using estimated initial isotope was overestimated by 3.2% by our procedure, while $k_d$ was underestimated by 0.4%. The slight differences between measured and estimated values may lead to the reduced accuracy of FMR measurements.

In conclusion, our study revealed some possibilities of improvements to the single-sample approach of the DLW method. Schlüchter et al. (2010) demonstrated a negative impact of the single-sample approach on the subsequent behavior of study subjects. Therefore, when experimental subjects are relatively small or rare, the single-sample approach of the DLW method may be preferable because it allows one to release an animal immediately after injection and to thus avoid the potential effects of initial holding. However, our results confirm that the percentage of TBW in birds also varies with each breeding stage, and our procedure might be more reliable than the previous single-sample approach. An additional advantage of our procedure is that it can apply for all rCO2 calculation models.
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those of Schultner et al. (2010) and indicate that in choosing an approach there is a trade-off between the need for accuracy and the need to reduce impacts on the behavior of the study species. Therefore, further improvements to the accuracy of the single-sample approach and relevant choices of approach are necessary for measuring the energy expenditure of free-ranging birds.

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