Non-invasive measurement of metabolic rates in wild, free-living birds using doubly labelled water

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Abstract

1. Doubly labelled water (DLW) is routinely used to measure energy expenditure and water turnover in free-ranging animals. Standard methods involve capture, blood sampling for baseline measurement, injection with isotopic tracers, captivity for an equilibration period, post-dose blood sampling, release, and subsequent re-capture for final blood sampling. Single sampling methods that minimise disturbance by reducing capture and handling time have been developed and tested. Sampling faeces rather than blood could further reduce disturbance to study animals in a range of species and study systems. However, the extent to which estimates of metabolic rate derived from blood and faecal samples diverge has not been investigated.
2. We compared isotopic enrichment in blood and faecal samples taken concurrently from captive Southern Pied Babblers *Turdoides bicolor*. Isotopic enrichment levels in faeces and in blood were used to calculate initial and final ratios of $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ for each individual. We then used these ratios to calculate daily energy expenditure (DEE) and directly compared measurements from blood samples with those from faecal samples within individuals. We found that faecal sampling resulted in estimates of DEE that agree with those based on blood sampling.
3. Additionally, we field-tested a faecal sampling protocol with a habituated population of babblers in the southern Kalahari Desert. During the field test, study animals were not captured or handled for either dosing or sampling. Field-testing confirmed the practical feasibility of non-invasive dosing and sampling techniques in free-living animals, and we obtained measurements of DEE that we used to test an *a priori* prediction that DEE is inversely related to air temperature. Our data show decreasing DEE with increasing air temperature, a pattern consistent with studies testing similar predictions in birds using traditional DLW methods.
4. We demonstrate that faecal samples can substitute for blood when measuring DEE using DLW and provide a method that will allow field-based researchers to obtain sound physiological measurements while minimising handling and removal of study animals from their natural environments.

Keywords

Avian physiology, daily energy expenditure, ecological energetics, metabolic rate, non-invasive doubly-labelled water, pied babbler

Introduction

Quantifying the energy and water requirements of free-living animals is necessary for understanding how physiological processes and behavioural patterns change in response to ecological conditions (Le Maho, 2002; McKechnie, Hockey, & Wolf, 2012; Tomlinson et al., 2014). The doubly-labelled water (DLW) method is a routine technique for measuring energy expenditure in free-living animals via the introduction of two isotopically-enriched tracers into body water and the subsequent collection of samples of body fluids for the analysis of enrichment levels (Speakman & Hambly, 2016). The DLW method is particularly important for understanding energy fluxes in animals too small for heart rate telemetry, the other widely-used technique for quantifying energy expenditure in free-ranging populations (Butler, Green, Boyd, & Speakman, 2004). The isotopes most commonly used for DLW studies, and which we have used as "labels" here, are enriched with oxygen-18 (measured as $\delta^{18}O$) and deuterium (measured as $\delta^{2}H$).

In the study of non-human vertebrates, the DLW technique traditionally involves (1) capture of a study animal, (2) injection with an isotopically-enriched solution, (3) a period of captivity for equilibration of the injectate with body water prior to release, and (4) recapture (Speakman, 1997). Typically, three blood samples are drawn within a 24 h period (or multiple thereof): a baseline sample before enrichment, followed by an initial sample after equilibration and a final enriched sample 24 h (or longer) after release (Nagy, 1983; Smit & McKechnie, 2015; Speakman & Hambly, 2016; Williams, 2001), known as the two-sample (TS) method (Butler et al., 2004; Speakman, 1997).

Handling, injecting, and blood sampling generally require temporarily removing animals from their natural environments. Most studies investigating the impact of stress associated with DLW procedures on derived measurements of energy expenditure have reported no significant differences between treated and control animals (Part, Gustafsson, & Moreno, 1992; Speakman, Racey, & Burnett, 1991; Weathers & Sullivan, 1993). However, some undesirable effects of standard DLW procedures, including reduced clutch size, nest abandonment, mass loss, behaviour change, and long absences from active nests, have been reported in birds (Culik & Wilson, 1992; Schultner, Welcker, Speakman, Nordøy, & Gabrielsen, 2010; Ward, 1996), and may alter energy expenditure. Other animal physiology studies have shown that handling time and sampling procedures affect measured cortisol levels, an indicator of stress (Pavlova et al., 2018; Romero & Reed, 2005).

Techniques that are less invasive, or entirely non-invasive, are therefore desirable to reduce any remaining risk of handling affecting physiological or concurrent behavioural measurements under otherwise natural conditions.

Techniques relying on non-invasive dosing and/or sampling may be useful for researchers working with, for example, large animals that are difficult to capture and confine (Gotaas, Milne, Haggarty, & Tyler, 1997; King, Schoenecker, Fike, & Oyler-McCance, 2018; Scantlebury et al., 2014; Williams, Anderson, & Richardson, 1997), threatened species, as suggested by Speakman and Hambly (2016), institutionalised animals in research facilities such as zoos, game reserves, rehabilitation centers or captive breeding programmes (Ballantyne, Packer, Hughes, & Dierking, 2007), very small animals from which it may be difficult or not feasible to collect blood (Chaverri, Schneider, & Kunz, 2008; Webster & Weathers, 1989), agricultural (Samuels, Cupido, Swarts, Palmer, & Paulse, 2015) or predator-naïve species (Blumstein & Daniel, 2005; Courchamp, Chapuis, & Pascal, 2003; McLean, Hölzer, & Studholme, 1999; Vitousek, Romero, Tarlow, Cyr, & Wikelski, 2010) that can be easily approached by humans, and/or habituated study populations used to such approaches (Ashton, Ridley, Edwards, & Thornton, 2018; Huchard, English, Bell, Thavarajah, & Clutton-Brock, 2016; Lazaro-Perea et al., 2000; Pozis-Francois, Zahavi, & Zahavi, 2004; Ridley, 2016; Samson & Manser, 2016; Samuni, Mundry, Terkel, Zuberbühler, & Hobaiter, 2014).

The DLW method has been extensively validated across multiple taxa (Butler et al., 2004; Congdon, King, & Nagy, 1978; Schoeller et al., 1986; Speakman & Hambly, 2016; Speakman & Racey, 1986; Tomlinson, Maloney, Withers, Voigt, & Cruz-Neto, 2013; Williams & Nagy, 1984), with typical differences of <10% in comparison with simultaneous alternative measures by other methods. Single-sample (SS) methods, in which initial isotope enrichments are determined from a separate control group of dosed animals and blood sampling of study animals is therefore limited to a single final sample (Lynn, Houtmann, Weathers, Ketterson, & Nolan, 2000; Weathers & Stiles, 1989), have also been validated and shown to produce measurements comparable to those obtained with TS procedures (Speakman, 1997; Webster & Weathers, 1989). Moreover, neither blood sampling nor injection of the dose are essential for using DLW to measure energy expenditure. Faecal samples have been used in several studies involving large mammals injected with DLW (Fairall & Klein, 1984; Gotaas et al., 1997; Klein & Fairall, 1986; Scantlebury et al., 2014; Williams et al., 1997) and one study involving birds dosed orally (Anava, Kam, Shkolnik, & Degen, 2000, 2002). Oral dosing and urine sampling are both routine practices in DLW studies involving human subjects (Burrows, Martin, & Collins, 2010; Speakman, 1997). Techniques using oral dosing and faecal sampling of animals in the field require that individuals be uniquely identifiable – for example by transponders, distinctive markings, dye-marks, or tags – and traceable.

In a recent review of the DLW method, Speakman and Hambly (2016) identified faeces as the most feasible non-invasive source of bodily fluids for sampling free-ranging animals, but noted that 1) oral dosing and faecal sampling risks being somewhat 'hit and miss' in the field, and 2) the extent of divergence between measurements derived from blood and faeces has not been investigated. We are aware of only two studies involving direct comparisons between blood and faeces collected simultaneously (Gotaas et al., 1997; Williams et al., 1997). Both studies focused on large mammals injected with DLW (n = 4 Norwegian reindeer *Rangifer tarandus tarandus*, n = 1 aardwolf *Proteles cristatus* respectively), and found that time-matched measurements from blood and faeces were correlated. Oral dosing and faecal sampling have been used concurrently in a field study on two occasions previously (Anava et al., 2000, 2002, n = 78 Arabian babblers *Turdoides squamiceps*; Scantlebury et al., 2014, n = 2 cheetah *Acinonyx jubatus*), and neither study explicitly compared blood and faecal samples within the same individuals.

Here we report the results of a study that quantified the extent to which measurements derived from blood and faeces diverged in an arid-zone bird, the Southern Pied Babbler *Turdoides bicolor*. Based on measurements in captive and free-living birds, we demonstrate the utility of a non-invasive dosing and sampling approach. To determine the efficacy of the non-invasive technique, doses of DLW were administered in food items to birds temporarily held in captivity and isotopic enrichments were measured in water derived from faecal samples and blood collected concurrently. These values were used to calculate daily energy expenditure (DEE) and compared directly. We tested two predictions: (i) the value of $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ is similar across concurrently collected samples of faeces and blood within individuals, and (ii) derived measurements of DEE for *T. bicolor* are similar when using water from concurrently collected faecal and blood samples within individuals. To ensure that a non-invasive technique is both practically feasible and sufficiently sensitive to detect relationships of biological interest, we field tested oral dosing and faecal sampling with a habituated, free-living population of *T. bicolor* in the southern Kalahari Desert. As a demonstration, we used data collected in the field to test a third prediction (iii) that measurements derived from faecal samples will detect an inverse relationship between DEE and maximum daily air temperature, expected as both endotherm resting metabolic rates (Scholander, Hock, Walters, & Johnson, 1950; Tomlinson, 2016) and activity levels in free-living birds (Smit & McKechnie, 2015) decline as temperatures rise.

Materials and Methods

Study site and system

Southern Pied Babblers (60-90 g) are cooperative breeders found in the semi-arid savannas of southern Africa's Kalahari Desert. The species lives in groups of 3-15 adults and occupy year-round territories (Ridley, 2016). We compared derived measurements from blood and faeces after oral dosing with DLW using birds captured from a wild population near the town of Askham in the southern Kalahari, South Africa (26°58'S 20°46'E). Field-testing of the non-invasive technique took place on and around the 33 km^2 Kuruman River Reserve (KRR) (26°58'S, 21°49'E), approximately 120 km east of Askham. Babblers at KRR are habituated to observation by humans at distances of 1 - 5 m (Ridley & Raihani, 2007) and regular weighing on a top-pan balance scale on which they perch in exchange for a small food reward (Ridley, 2016). All individuals are marked as nestlings with a unique combination of metal and colour rings for individual identification. Due to the importance of maintaining their habituation to human observers for the purposes of a long-term study, repeated capture and confinement of the KRR birds is undesirable. Thus, a separate population near Askham was used for the captivity study. The Kalahari region is a summer-rainfall semi-desert with mean annual precipitation of approximately 197 mm (Kong, Marsh, van Rooyen, Kellner, & Orr, 2015) and daily maximum summer temperatures ranging from 22.2 to 45.4°C (Steenkamp, Vogel, Fuls, van Rooyen, & van Rooyen, 2008). Fieldwork was undertaken between September 2016 and February 2018.

Non-invasive dosing and sampling technique in captive and free-living birds

We mixed DLW by adding one part 99.9 atom. % deuterium oxide to five parts 97 atom. % ^{18}O (Sigma-Aldrich, Kempton Park, SA, USA), a dosage based on desired initial enrichments of approximately 1000 ‰ Vienna Standard Mean Ocean Water (VSMOW) for δ^2H and approximately 400 ‰ VSMOW for $\delta^{18}O$. One batch of DLW was used for all captive individuals and for field study individuals in austral summer 2016/2017, and a second batch was used for all field study individuals in austral summer 2017/2018. We attempted to administer a standard dose of 50 µL to each bird.

We measured total body water in six pied babblers caught for a separate study at Radnor Farm (26°06'S 22°53'E) in June 2018. An initial 100 µL blood sample was obtained from each bird to establish background isotope ratios, after which 45 µL of DLW was injected into the *pectoralis* muscle. After 64.4 min (SD = 3.0), a second sample was taken to determine initial enrichment levels. These data were used to calculate total body water from the hydrogen isotope dilution space (Speakman, 1997), which average 69.3 % (SD = 4.3) of body mass. At an average body mass of 78 g, the 50 µL dose was diluted in approximately 54 ml of body water.

All DLW doses were injected into partially dehydrated darkling beetle larvae *Zophobus morio* and fed to a target bird within one minute of injection, following Anava et al. (2000). Partial dehydration of the larvae prior to injection was required to prevent loss of DLW as a result of hydrostatic pressure within the exoskeleton. Non-fasted captive birds were dosed by force-feeding while in the hand, whereas during the field test doses were presented to habituated individuals on the ground and consumed naturally within one minute of being offered. In all cases, a background faecal sample was collected prior to dosing, an initial enriched faecal sample at least 1 h after dosing, and a final enriched faecal sample as close as possible to 24 h after dosing (captive birds: mean = 23.6 h, SD = 0.5, n = 5; field test: mean = 24.5 h, SD = 2, n = 31). To avoid disrupting active breeding attempts, some captive birds could be held for only short periods and their final samples were collected after approximately 12 h (mean = 11, SD = 0.9, n=3). Between the initial and final samples, we collected many additional faecal samples (described below). All faecal samples were stored in labelled 2 ml plastic vials with silicon O-rings, double-sealed with Parafilm (Bemis NA, Neenah, USA), and refrigerated at 4 °C.

Comparing blood and faeces using captive individuals

We trapped eight birds using a combination of mist-netting off roost at dawn (n = 3) and spring-traps baited with *Z. morio* larvae (n = 5). Birds were transported in drawstring cotton pouches, no more than 20 km from the sites of capture. Although the first two individuals captured were housed in an outdoor aviary, we were able to get much more information relevant to this study by confining individuals in smaller cages. All subsequent birds were therefore housed under shade and exposed to a natural light: dark cycle in purpose-built 40x20x20 cm black shadecloth holding cages with removable Perspex floors. Cages were checked for fresh faeces every 15 minutes, faecal samples collected from the removeable floor, and the floor cleaned and dried thoroughly after the collection of each sample. All individuals were provided with perches and food (mealworms *Tenebrio molitor* larvae) *ad libitum*. Babblers are arid-zone insectivores that obtain the majority of their free water from their food (Hockey, Dean, & Ryan, 2005), and the birds were thus not provided with drinking water.

Initial faecal samples were collected from five birds after a 1 h equilibration period (Butler et al., 2004; Speakman, 1997) and from the remaining three after allowing 2 h in case equilibration was slowed by the oral administration of the dose and/or slow emptying of the stomach into the intestine (Levey & Karasov, 1994). Blood samples were obtained by brachial venipuncture using a sterile 26-gauge needle. Background, initial, and final blood samples (< 100 µL per sample) were collected concurrently with faecal samples (mean = 11 min apart, max = 21 min apart), to allow for direct comparison between the two types of sample. All individuals

were weighed after blood sampling. At the end of the study all the birds were released without incident at the sites of capture and reintegrated naturally into their groups and territories. No active nests were abandoned during the study.

Field study with free-living individuals

The field study was designed to test the practicality of the non-invasive method with completely free-living birds of the same species, and to generate data with which to address prediction (iii), that the method is sensitive enough to detect a predicted relationship between DEE and air temperature. Baseline faecal samples were collected between 5:28 and 8:48 am on the morning of dosing when excreted naturally by the focal bird. DLW doses were administered between 6:30 and 9:30 am, after collection of the baseline faecal sample. Target individuals were weighed within 20 minutes of first light on both the dosing day and final sampling day (consecutive) to ensure pre-foraging body mass measurements were obtained. Breeding stage was standardised to the incubation period to ensure that birds could be easily located around nests and avoid the dose being provisioned to young. Dosed individuals were observed from close range in their natural habitat for all daylight hours between background and final sampling and all observed faeces excreted during this time were collected. We analysed data from 31 birds that were successfully dosed and from which we collected a series of faecal samples over periods of 24 - 36 h. The birds were not handled at any time during the field study.

Air temperature (T_{air}) data were collected from an onsite weather station (Vantage Pro2, Davis Instruments, Hayward, California, USA), logged at ten-minute intervals. Daily maximum T_{air} (T_{max}) were calculated from these data for each observation day. Based on previous research on critical temperatures affecting behaviour in *T. bicolor* (du Plessis, Martin, Hockey, Cunningham, & Ridley, 2012; Wiley & Ridley, 2016), we have defined hot days as those on which $T_{max} \geq 35.5$ °C.

Laboratory extractions

To extract water from faeces we adapted a technique used for plant material (Priyadarshini et al., 2016) and sediments (S. Woodborne, unpublished data). Faecal samples ($n_{captive} = 79$, $n_{field} = 335$) were transferred to glass test tubes and connected to a vacuum extraction line via a cold finger configured to trap water vapour on the outer wall. A circular vacuum manifold with 10 cold finger ports allowed simultaneous extraction of multiple samples. The cold finger traps were cooled using liquid nitrogen and subjected to a downstream vacuum of $< 10^{-1}$ mbar. Samples were completely dehydrated after 2 h. Then, the cold finger traps were removed and rapidly

warmed. Thawed samples were transferred to sampling vials for isotopic analysis. Water was distilled from blood samples (n = 24) by cryogenic vacuum distillation as described by Speakman (1997).

Sample analysis

Serial aliquots from the water samples were analysed using a DLT-100 liquid water isotope analyser (Los Gatos Research, Mountain View, CA, USA) following the procedure described by Smit & McKechnie (2015) with an additional rinse cycle of distilled water before standards (water samples with known $\delta^{18}O$ and $\delta^{2}H$ values). All data were corrected against working standards and by removing measured baseline values for each individual from all subsequent sample values of $\delta^{18}O$ and $\delta^{2}H$.

The physiological basis of the DLW approach is the differential between elimination rates (k, in units of time d^{-1}) of both ^{18}O (k_O) and ^{2}H (k_H), and measurements typically compare the enrichments of initial and final body water samples (Lifson & McClintock, 1966; Speakman, 1997). Because the ^{18}O label is lost via both CO_2 production and water turnover, whereas ^{2}H declines due to only the latter, the rate of CO_2 production (rCO_2) is a function of the difference $k_O - k_H$ in units of time d^{-1}. The calculation of rCO_2 also requires, at a minimum, some knowledge of pool size (N, in mol) into which the isotopes distribute, most of which is body water: 18.02 g H_2O/mol (Nagy, 1980; Speakman, 1997). Oral dosing can affect the precise dose of isotope solution administered in wild animals, and therefore precludes measurement of N by isotope dilution. Thus, for the purposes of this study, we measured total body water as a percentage of mass in a separate control group of pied babblers (n = 6) and used the average N = 69.3 % as a constant throughout. This is standard practice in SS DLW methods (Lynn et al., 2000; Speakman, 1997; Webster & Weathers, 1989).

CO_2 production can be calculated from the body water pool and the rate of decline of the natural log of the ratio of $\frac{\delta^{18}O}{\delta^{2}H}$ (Nagy & Costa, 1980; Speakman, 1997). Here we use a measured initial value, $\ln(\frac{\delta^{18}O_i}{\delta^{2}H_i})$, along with each animal's measured log ratio of final enrichments, $\ln(\frac{\delta^{18}O_f}{\delta^{2}H_f})$, and a mass-specific estimate of N, to calculate rCO_2. When the same dosing solution is used on all animals, the initial log ratio, $\ln(\frac{\delta^{18}O_i}{\delta^{2}H_i})$, is independent of body mass and of the exact dose administered across study individuals (Speakman, 1997). Although we collected multiple faecal samples from each individual (Fig. 1), and the multiple-sample DLW method described in Speakman (1997) and Speakman et al. (2001) could be applied, we used the TS method to calculate turnover, as recommended by Speakman and Racey (1986). We used the single-pool model recommended for

animals smaller than 4 kg (Speakman, 1997). We used Speakman's (1997) Equation 17.7 (see eq. 1 below) for calculations of rCO_2 in mol d^{-1} because empirical testing has shown this equation to be the most accurate (Visser, Boon, & Meijer, 2000) and based on the most realistic assumptions of fractionation during evaporation (Butler et al., 2004; Speakman & Hambly, 2016):

$$rCO_2 = \left(\frac{N}{2.078}\right)(k_O - k_H) - 0.0062 * k_H * N \qquad \text{(eq. 1)}$$

where N is moles of body water and values of k represent turnover of an isotope identified by the subscript. The divisor of N (2.078) accounts for the fact that each molecule of CO_2 expired removes two molecules of oxygen from the pool and, with the inclusion of the last term ($0.0062 \bullet k_H \bullet N$), reflects a correction for fractionation. Omitting this correction causes an overestimate of rCO_2 of ~ 3 % in *T. bicolor* (see also Anava et al. 2001). We calculated k_H in the final term of eq. 1 based on change in $\ln(\delta^2H)$ between maximally-enriched faecal samples collected at early time points and final samples, where t is time (in days) elapsed between early and final samples:

$$k_H = \frac{\ln[\delta^2 H_{1-max}] - \ln[\delta^2 H_f]}{t} \qquad \text{(eq. 2)}$$

Values of $(k_O - k_H)$ can be calculated from the rate of decline of $\ln(\frac{\delta^{18}O_i}{\delta^2 H_i})$, (Nagy & Costa, 1980; Speakman, 1997):

$$(k_O - k_H) = \left(ln\left[\frac{\delta^{18}O_i}{\delta^2 H_i}\right] - ln\left[\frac{\delta^{18}O_f}{\delta^2 H_f}\right]\right) * \left(\frac{1}{t}\right) \qquad \text{(eq. 3)}$$

where $\delta^{18}O_i$ and δ^2H_i are the initial $\delta^{18}O$ and δ^2H values in faeces or blood, and $\delta^{18}O_f$ and δ^2H_f are the final $\delta^{18}O$ and δ^2H values. Using this format, ($k_O - k_H$) can be calculated even if dosing has been inconsistent among individuals, because all animals dosed with the same labelled water solution will share a similar initial $\ln(\frac{\delta^{18}O_i}{\delta^2 H_i})$. Any minor variation among individuals in $\frac{\delta^{18}O_i}{\delta^2 H_i}$ will result from interindividual variation in the distribution space for the two isotopes (Speakman, 1997). The most reliable measurements of ($k_O - k_H$) are made between one and two biological half-lives ($t_{1/2}$) of the isotopes (Nagy, 1980).

For both captive and free-living birds, rCO_2 was converted from mol d^{-1} to L d^{-1} using the conversion factor 22.4 L of ideal gas per mol at standard temperature and pressure, and L CO_2 d^{-1} was converted to kJ d^{-1} using the

relationship 27.4 kJ L^{-1} CO_2 for an insectivorous bird (Gessaman & Nagy, 1988). The metabolic rate calculated from rCO_2 is subsequently referred to as daily energy expenditure (DEE).

All measurements of DEE were normalised to 24 h and corrected for body mass changes during the measurement period (Speakman, 1997). Derived measurements were compared with estimated basal metabolic rate (BMR) for a 70 g bird using the phylogenetically-independent regression equation presented by McKechnie & Wolf (2004). We expected biologically reasonable estimates of DEE to approximate 2-3 X BMR – see Drent and Daan (1980) and Nagy (2005) – and not fall below BMR or exceed 5 X BMR (Hammond & Diamond, 1997). We compared our derived measurements with estimates of DEE for a congeneric arid-zone species, *T. squamiceps* (Anava et al., 2000), to confirm that our measurements fall within the range expected on the basis of comparable studies.

Statistical analyses

All statistical analyses were conducted in R (R Core Team, 2017). To check agreement across measurements of DEE from our two sample types, we used the Bland-Altman mean-difference method (Bland & Altman, 1987; Lehnert, 2015), taking the average of measurements per individual by each sample type against the difference between the two measurements. In exploring the relationship between DEE and T_{max}, we used both simple linear regression and one-way ANOVA in order to demonstrate that the data can be applied to questions around the effect of a predictor variable of interest.

The initial value of $\frac{\delta^{18}O_i}{\delta^2H_i}$ for one individual (out of 7), and the DEE measurement for another individual (out of 7), were excluded from statistical analyses of captive birds because of an evident analytical error (based on close inspection of multiple samples per individual) and an amplification of error associated with a short measurement duration (DEE below predicted BMR), respectively. Unlikely initial ratios were also identified for three field study birds (out of 31). In these cases, the average initial $\frac{\delta^{18}O_i}{\delta^2H_i}$ for other birds sampled close together in time was used to calculate DEE. Uncertainty around final $\frac{\delta^{18}O_f}{\delta^2H_f}$ values was detected in four individuals (out of 31) in the field study (again based on close inspection of multiple final samples per individual). In these four cases, an average of the measured final $\frac{\delta^{18}O_f}{\delta^2H_f}$ values for each individual was used.

Results

Comparing blood and faeces using captive individuals

In the captive birds, values of $\frac{\delta^{18}O}{\delta^{2}H}$ in water from faeces closely matched those in water from blood from the first to the last blood sample (n=6; Fig. 1A). The mean difference for six time-matched initial values of $\frac{\delta^{18}O}{\delta^{2}H}$ measured in blood and faeces was -0.007 ± 0.009 (paired t-test: t_5 = -1.495, P = 0.195), with a corresponding mean difference of -0.001 ± 0.005 for seven final values (paired t-test: t_6 = -0.698, P = 0.511), see Fig. 1C. Also, as expected, initial values of $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ were very similar across individuals (coefficient of variation, CV [=S.D./mean] < 0.02 in both blood and faecal samples, Fig. 1B), and the initial values of $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ in body water approximated the measured value for the injectate (mean = 0.3613, CV = 0.01, n = 4). The average measured initial $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ values for both faeces (mean = 0.3676, CV = 0.01, n = 6) and blood (mean = 0.3628, CV = 0.02, n = 6), all of which were collected between 1 and ~ 2 h of dosing, fall within the 99% confidence interval of the injectate mean (Fig. 1A and 1B). The constancy of the initial values of $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ across individuals was striking, particularly considering that three of the seven individuals received less than 75% of the intended dose, as indicated by lower initial than expected initial values of δ^{18}O and δ^{2}H (Bourne et al., 2018). This result is consistent with theory that the initial ratio $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ is robust to variation in equilibration time and total dose, and approximates the $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ value of the injectate solution (Speakman, 1997).

Most values for faecal samples collected between the initial and final samples fall along an expected general log-linear decline of the $\frac{\delta^{18}O}{\delta^{2}H}$ against time since dosing (Fig.1A). Four values (out of 62, see pink and yellow lines on Fig. 1A) fall well below the general pattern, but without replicated measures we cannot determine whether these reflect analytical errors or some other factor. These deviations occurred between initial and final sampling and did not affect our calculations using the TS method. It is theoretically consistent and methodologically confirming that fitting the log-linear decline of $\frac{\delta^{18}O}{\delta^{2}H}$ against time for each individual using all available faecal samples and estimating $\frac{\delta^{18}O}{\delta^{2}H}$ at 2 h after dosing produces an average estimated $\frac{\delta^{18}O}{\delta^{2}H}$ at 2 h of 0.3568 ± 0.0111 (n = 7; mean = 0.3591 if the four deviant points are omitted). Again, these linear regression estimates of $\frac{\delta^{18}O}{\delta^{2}H}$ at 2 h are similar to each other, to those calculated directly from initial samples, and to the measured $\frac{\delta^{18}O}{\delta^{2}H}$ in four dilutions of the injectate solution.

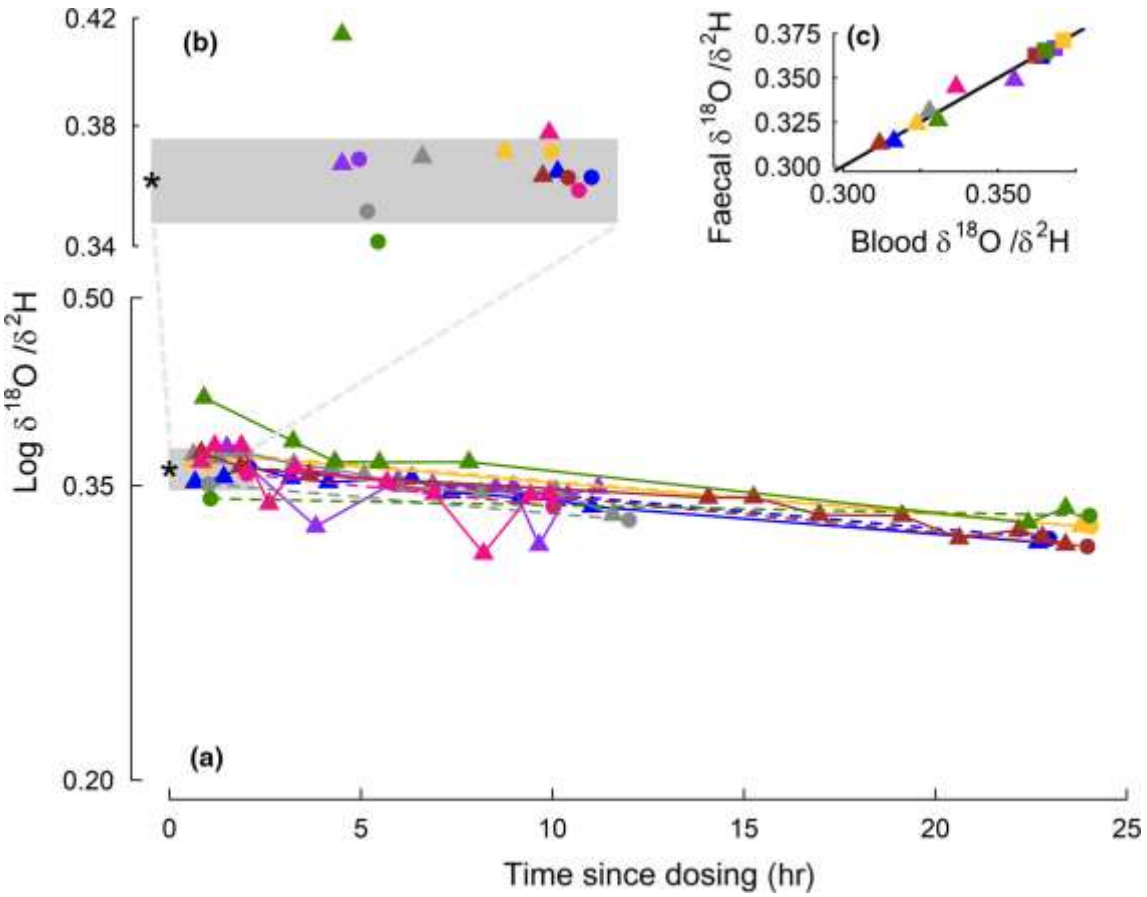

Figure 1. Values of $\delta^{18}O/\delta^{2}H$ as a function of time since dosing tracked each other very closely for each of seven captive Southern Pied Babblers Turdoides bicolor (each individual coded with a unique colour) when sampled from blood (filled circles connected by dashed lines) and faeces (filled triangles connected by solid lines). Values on the *y*-axes of panel (a) and (b) are the ratios $\ln(\delta^{18}O/\delta^{2}H$, and in panel (c), both axes are measured values of $\delta^{18}O/\delta^{2}H$, all of which are unitless. Black stars show the mean value for the labelled water injectate solution, and the lower and upper bounds of the grey rectangles correspond to the 99% confidence interval based on four measurements of the injectate. The left boundary of the grey rectangle is at time = 0 and the right boundary is at time = 2 hr, when isotopes are presumed to be fully equilibrated with body water. The upper left inset (b) shows the selection of the data from the first 2 hr which, unless otherwise indicated in the text, were used as initial values for calculations of DEE. The upper right inset (c) shows the correlation of blood values with time-matched faecal values for early time points ($n = 6$, filled squares) and final time points ($n = 7$, filled triangles). The black line represents the $y = x$ line. Both initial and final samples lie along this line of equivalence, indicating not only high correlation but good agreement between the two sample types

The use of $\frac{\delta^{18}O}{\delta^{2}H}$ values proved a powerful diagnostic tool and we were able to identify likely analytical errors on the basis of clearly unusual $\frac{\delta^{18}O}{\delta^{2}H}$ values. For example, visual inspection of Fig. 1B reveals likely analytical errors in the initial samples for the individual coded in green. Excluding this individual, 92% of initial $\frac{\delta^{18}O_i}{\delta^{2}H_i}$ values fall within the 99% confidence interval for the injectate.

Estimates of DEE averaged 1.51 ± 0.31 kJ g^{-1} d^{-1} for captive babblers, equivalent to ~2.7 x BMR and within ~2% of Anava et al.'s (2000) estimates for the congeneric *T. squamiceps* (Fig. 2A). Within-individual estimates of DEE derived from blood samples differed from those derived from faecal samples in the captive birds by ~ 4.6 ± 3.6 % (n = 6, range = 0.5% to 10%), a small and nonsignificant difference (paired t-test: t_5 = 0.425, P = 0.688), see Fig. 2A. The mean difference between measurements derived from blood and from faeces was 0.017 kJ g^{-1} d^{-1} (95 % CI = -0.086, 0.120), and the limits of agreement are small enough (-0.175 and 0.209 kJ g^{-1} d^{-1}) for us to be confident that the two sample types can be used interchangeably to calculate DEE (Fig. 2B). After 12 h, 20 ±10 % of δ^{18}O had been eliminated (n = 3, range 9 % to 27 %). After 24 h, 41±12 % of δ^{18}O had been eliminated (n = 4, range = 27 % to 57 %). We expect that precision would be further improved by extending the duration to 48 h, at which time all study animals would have eliminated more than 50% δ^{18}O.

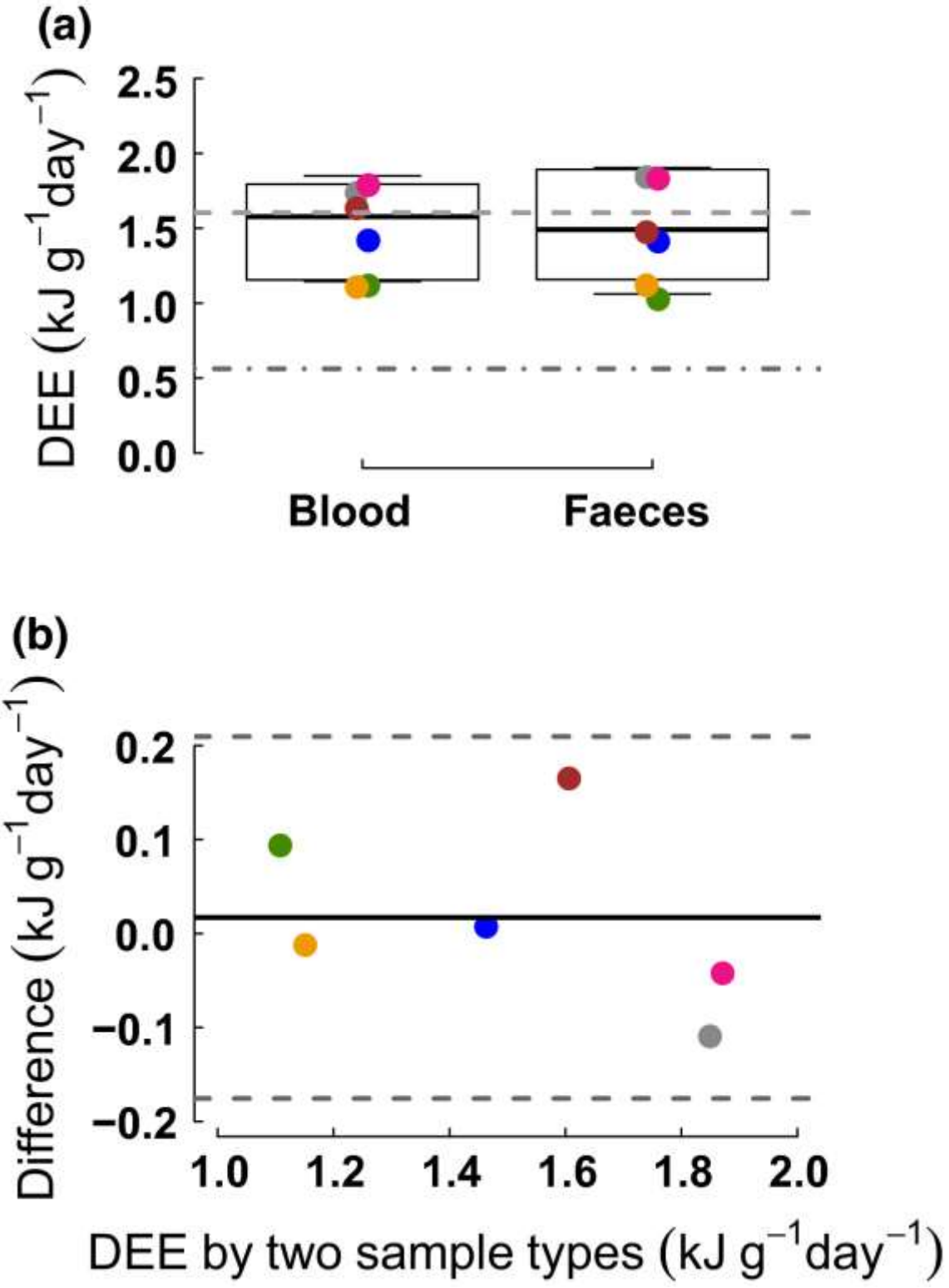

Figure 2. Daily energy expenditure (DEE) in kJ g^{-1} day^{-1} averaged 2–3 × basal metabolic rate in six captive Southern Pied Babblers Turdoides bicolor (each individual coded with a unique colour), irrespective of whether this was calculated using blood or faecal samples (Panel a). The lower dashed and dotted line represents predicted basal metabolic rate for a 70 g bird (McKechnie & Wolf, 2004), and the upper dashed line represents mean field metabolic rate in congeneric *Turdoides squamiceps* (Anava et al., 2000). Points have been jittered to improve visibility. Measurements of DEE, derived from concurrent blood and faecal samples from captive birds, were very similar within individuals (Panel b). Differences averaged 0.016 kJ g^{-1} day^{-1} (solid black line, Panel b) and fell well within two standard deviations of the mean (upper and lower grey dashed lines in Panel b). Disagreement between measurements derived from faeces and from blood did not exceed 10%

Field study with free-living individuals

In the field it was not possible to control the timing of sampling, and faecal samples were collected as they were naturally excreted by the birds. Initial samples were collected from 31 birds 2.3 h (±1.8 h) after dosing. Seven of the 31 individuals analysed here did not receive the full intended dose, indicated by lower than expected initial values of δ^{18}O and δ^{2}H (Bourne et al., 2018). As with captive individuals (demonstrated above), initial values of $\frac{\delta^{18}O}{\delta^{2}H}$ in faecal samples from the free-living birds approximated the injectate solution and were not affected by equilibration times or dose volumes. Isotopic enrichment data for each individual bird in the field study revealed very similar patterns to those in the captive birds, with $\frac{\delta^{18}O}{\delta^{2}H}$values showing the expected log-linear declines over time between initial and final samples (Table S2).

Estimates of DEE for free-living *T. bicolor* averaged 1.62 ± 0.57 kJ g^{-1} d^{-1} (n = 31), equivalent to ~2.8 x BMR and within ~3 % of Anava et al.'s (2000) estimates for free-living, congeneric *T. squamiceps* (Fig. 3). We found no evidence that estimates of DEE derived using this non-invasive technique in the field differed from those derived using faecal samples from captive individuals (difference between means = 6 %, Mann-Whitney-Wilcoxon U = 85, $n_{captive}$ = 6, n_{field} = 31, P = 0.763), or blood samples from captive individuals (difference between means = 7 %, Mann-Whitney-Wilcoxon U = 86, $n_{captive}$ = 6, n_{field} = 31, P = 0.794).

We obtained DEE measurements for days varying in T_{max} from 20.7 ° C to 38.8 ° C. We found that DEE was significantly negatively related to T_{max} (Est = - 0.07, $F_{1,29}$ = 14.72, P < 0.001; Fig. 3). With the data categorised according to the known critical temperature for *T. bicolor* (du Plessis et al., 2012; Wiley & Ridley, 2016), average DEE was 0.56 kJ g^{-1} d^{-1} lower on hot days ($T_{max} \geq$ 35.5 ° C; mean = 1.36 ± 0.36 kJ g^{-1} d^{-1}, n = 17) than on cool days (T_{max} < 35.5 ° C; mean = 1.94 ± 0.65kJ g^{-1} d^{-1}, n = 14; One-way ANOVA, $F_{1,29}$ = 9.333, P = 0.005). This represents a ~ 30 % reduction in mean DEE on hot days. After 24 h, 42 ± 7 % of δ^{18}O had been eliminated (n = 31, range 20 % to 66 %). In two individuals re-sampled at 72 h, 88 % of δ^{18}O had been eliminated. Ideally, final sampling would occur after 48 h, by which time the majority of study animals would have eliminated more than 50 % δ^{18}O.

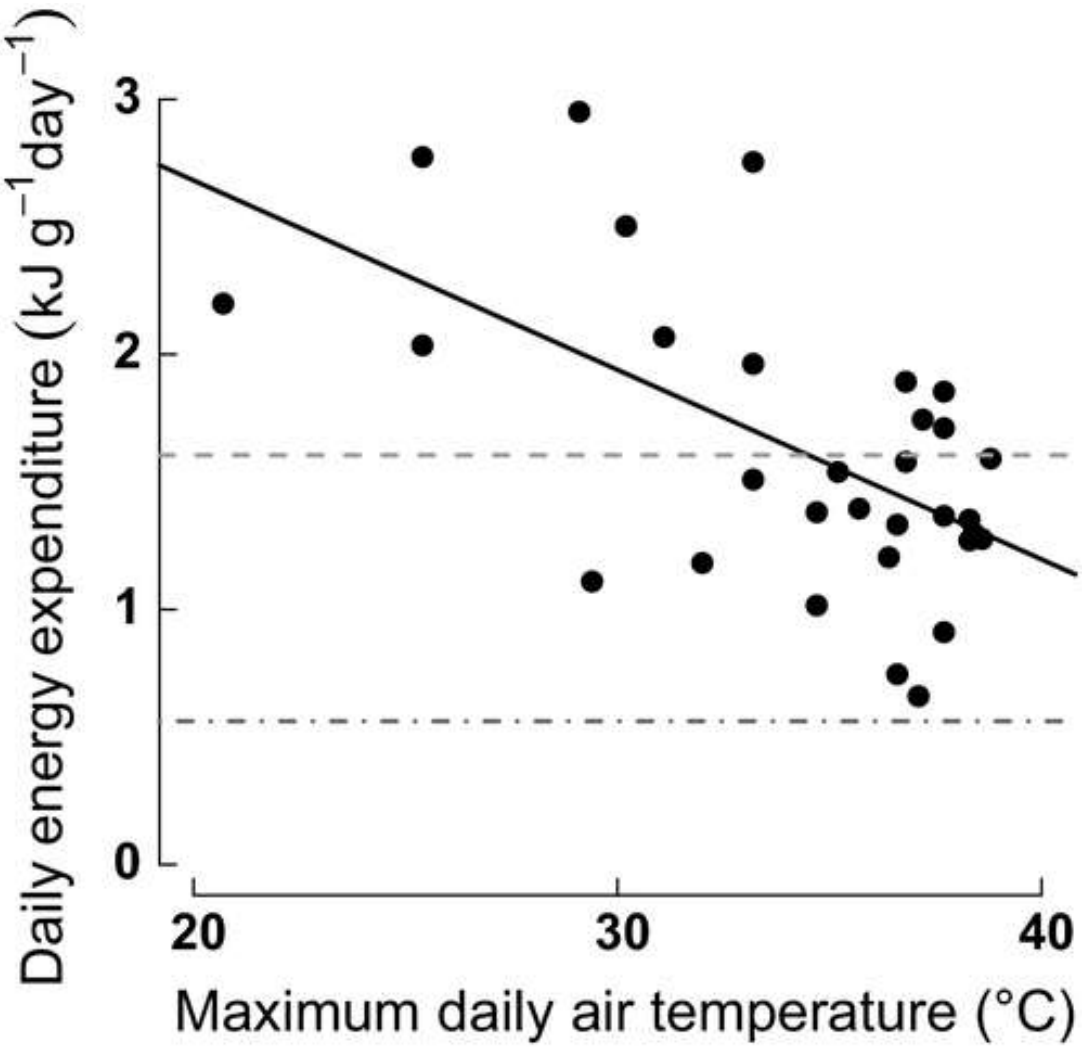

Figure 3. Daily energy expenditure (DEE) of Southern Pied Babbler *Turdoides bicolor* individuals as a function of T_{max} (maximum daily air temperature) for all data collected in the field (n = 31), showing a linear regression (solid black line). The lower, dark grey, dashed and dotted line represents predicted basal metabolic rate for a 70 g bird (McKechnie & Wolf, 2004), and the upper, light grey dashed line represents mean field metabolic rate in congeneric *Turdoides squamiceps* (Anava et al., 2000)

Discussion

Non-invasive doubly-labelled water method

By directly comparing isotopic enrichment, elimination rates, and derived measurements of DEE from concurrently-collected faecal and blood samples within individual birds, we have demonstrated that a non-invasive DLW technique using oral dosing and faecal sampling can produce measurements of $\ln(\frac{\delta^{18}O}{\delta^{2}H})$ and of DEE that, first, substantially agree between blood and faeces, and, second, are sufficiently sensitive to detect meaningful patterns in DEE. Our results support our predictions, that (i) $\frac{\delta^{18}O}{\delta^{2}H}$ is constant across samples of both faeces and blood within individuals (Fig. 1B) and (ii) measurements of DEE, derived from concurrent blood and faecal samples from captive birds, agree within individuals (Fig. 2A). These findings address the issue raised by Speakman and Hambly (2016), that the extent to which derived measurements from faecal samples diverge from more traditional applications of the method using blood samples was not known.

Our finding that $\frac{\delta^{18}O}{\delta^{2}H}$ values in blood and faecal water are so similar to each other and, in initial samples, to the injectate solution, is an empirical finding of high practical importance for effectively using oral dosing and faecal sampling in the field for non-invasive measurements of energy expenditure by isotopic turnover. This is because a core element of the calculation of CO_2 production is the difference between turnover rates of $\delta^{18}O$ and $\delta^{2}H$ (k_O-k_H), which can be calculated from the slope of the decline of $\ln(\frac{\delta^{18}O}{\delta^{2}H})$ against time since dosing (see eq. 3). Calculating CO_2 production using these ratios is useful for field applications of non-invasive DLW, which can indeed be somewhat 'hit and miss' in terms of optimal dosing and sampling (Speakman & Hambly, 2016). The ratios are robust to individual variation in equilibration times and total dose consumed, factors over which researchers have limited control in the field. Administration of a DLW dose establishes an initial value of $\frac{\delta^{18}O}{\delta^{2}H}$ that will approximate that of the injectate solution because naturally-occurring background levels of both isotopes are negligible. This value of $\frac{\delta^{18}O}{\delta^{2}H}$ in the body water pool declines over time, providing a measure of the quantity ($k_O - k_H$) irrespective of differences in individual equilibration times or dose volumes.

Comparing our estimates of DEE in *T. bicolor* with both the BMR expected for a 70-g bird and estimated DEE in a congener (Fig 2), revealed that our measurements were within the expected range. In combination with within-individual comparisons of measurements derived from blood and faeces, our field application measured the effect of daily T_{max} on DEE. A negative relationship between DEE and air temperature is expected on the basis of both the decreases in endotherm resting metabolic rates with increasing ambient temperatures

(Scholander et al., 1950; Tomlinson, 2016), and reductions in activity during hot weather (Smit & McKechnie, 2015). Both foraging efficiency (du Plessis et al., 2012) and provisioning rates to nestlings (Wiley & Ridley, 2016) decline in *T. bicolor* at daily $T_{max} \geq 35.5$ ° C, suggesting reduced activity levels at higher temperatures. We therefore predicted an inverse relationship between T_{max} and DEE in *T. bicolor* (prediction iii) and found that DEE was inversely associated with daily maximum temperature, as expected based on other studies using traditional DLW methods (Anderson & Jetz, 2005; Smit & McKechnie, 2015). Thus, field application of the non-invasive technique is certainly sensitive enough to detect relationships between DEE and predictor variables that might be expected to influence energy expenditure in animals, such as environmental variables (Klein & Fairall, 1986; Nilsson, Molokwu, & Olsson, 2016), and to detect differences in DEE between cohorts of individuals. This result strongly suggests that a non-invasive technique involving oral dosing and faecal sampling provides measurements of CO_2 production that reflect real biological variation in DEE.

Field-testing the technique with habituated study animals was useful for demonstrating that many of the practical challenges associated with applying the non-invasive method in the field referred to by Speakman and Hambly (2016) can be overcome. Because the birds in the KRR study population are individually marked and habituated (Ridley, 2016; Ridley & Raihani, 2007), a specific bird can be targeted for feeding a food item, its body mass can be obtained without handling it, and faecal samples can be collected within one minute of excretion. The arid environment meant that collecting faecal samples unaffected by environmental water was possible most of the time, as the faeces were excreted onto dry sand. The fact that *T. bicolor* is primarily a terrestrial forager (Ridley, 2016) enabled the relatively straightforward collection of faeces from the ground. Due to the nature of our study system, we were able to undertake a particularly data-rich version of the technique. We collected multiple initial and final samples and used these to identify maximally enriched samples, analytical errors, and final samples with confidence (Fig. 1A).

A duplication of our methods is probably feasible only with another habituated study population. However, a truncated version of the same approach has potential application in other types of study systems. The minimum requirements for the technique are that researchers should be able to target a particular individual for dosing, dose fairly quickly to reduce the effects of fractionation, and find the same individual again to collect a final faecal sample. Multiple samples are not necessarily required. We have demonstrated that the TS method will suffice in most cases. A SS method could even be used to calculate DEE, estimating initial $\frac{\delta^{18}O}{\delta^{2}H}$values from other individuals or even from the injectate solution if it is only possible to collect a single final sample. In

systems where the study animals are not habituated, but researchers would still like to reduce disturbance while measuring DEE, the dose could be administered to the study animal in a bait, feeder, or dart and a faecal sample collected some time later, after one or two $t_{1/2}$ of the isotope. If observing the dosing is impossible, cameras could be placed at bait sites to record which individuals ingested the dose. Ingestion of the dose from bait will work (Scantlebury et al., 2014) even if the whole dose is not consumed (provided there is a reasonable chance that the targeted study animal will consume it quickly) because (k_O - k_H) can be calculated even if dosing has been inconsistent among individuals - all individuals dosed with the same labelled water solution will share a very similar initial $\frac{\delta^{18}O}{\delta^{2}H}$value, related to the $\frac{\delta^{18}O}{\delta^{2}H}$value of the injectate, regardless of the volume of their particular dose.

Limitations of the technique

Oral dosing is not ideal for ensuring that known, or intended, quantities of isotope enter the study animal, and this was apparent in our study as substantial variation among individuals in initial, equilibrated enrichments of δ^{18}O and δ^{2}H (Table S1 and Table S2). These were lower than expected in several of our study animals in both the captive and field studies. In the SS method, initial enrichments are typically measured from a separate sample of additional individuals. As we have shown above, one could also estimate initial enrichments based on known quantities of the injectate solution if absolutely necessary. Values of $\frac{\delta^{18}O}{\delta^{2}H}$ are insensitive to volumes of isotope ingested, as they reflect the relationship between the two isotopic tracers regardless of volume. Absolute certainty about the initial dose is, therefore, not a prerequisite for calculations of metabolic rate.

However, some certainty about the initial dose is required for the measurement of the body water pool (*N)* by isotope dilution. Due to uncertainty about initial doses, we were unable to calculate *N* based on dilution spaces for each individual. We therefore had to measure body water as a percentage of body mass in a separate control group of individuals injected with DLW to acquire an estimate of *N* to apply as a constant in our calculations. This will have affected the magnitude, and therefore accuracy, of our DEE measurements. For example, scrutiny of eq. 1 indicates that if mean body water were 3% lower or higher in an individual than the average we used, then mean DEE would have been about 3% lower or higher than we calculated. Note, however, that this follows standard practice in the SS method, where percentage body water by mass is typically measured in a sample of other individuals (Niizuma & Shirai, 2015; Speakman, 1997) and applied as a constant to the study population.

In our study, equilibration time, important for determination of k_H in the fractionation correction in eq.2, was variable, taking between 0.5 h and 4 h. In the field, it was difficult to optimally time collection of initial and final samples because it was necessary to wait for the animal to excrete naturally, resulting in reduced precision. However, varying sample collection times led to small variation in $\frac{\delta^{18}O}{\delta^{2}H}$ values at early time points in our captive birds. Robust calculations of DEE were still possible as long as the interval between the early sample and final sample was long enough (i.e.at least 24 h) for sufficient elimination of the isotopes and hence a decline in $\frac{\delta^{18}O}{\delta^{2}H}$ that is large relative to routine analytical error.

Together, these limitations mean that non-invasive dosing and sampling in the field is best suited for research questions that explore relative differences in energy expenditure between cohorts, for example between sexes (Anava et al., 2000) or sites (Smit & McKechnie, 2015), or that investigate variation in relation to a predictor variable of interest, for example temperature (Cresswell et al., 2004). The method is less appropriate for studies that focus on the precise quantification of energy expenditure for a population or individual animals. The technique is also not suitable for applications in marine and aquatic environments.

Our choice of study species and of a 24 h sampling duration was based on our broader research questions seeking to correlate DEE with behaviour patterns in *T. bicolor* over 24 h periods. Whereas we clearly demonstrate that measurements from blood and faeces agree within individuals, and that the method is sufficiently sensitive to detect differences between cohorts, even at 24 h, the precision of measurements of DEE could be improved by extending the time between initial and final sampling. Where measurement durations were substantially shorter than 24 h (in three captive birds), a larger likelihood of error was introduced. The most reliable estimates would be made between one and two $t_{1/2}$ of the isotopes (Nagy, 1980), and this would occur in our study species by approximately 48 h after dosing. Due to habituation in our study population, and therefore limitations on our ability to capture and handle the birds, we were not able to compare the non-invasive with the traditional DLW technique in the same individuals. This also resulted in a relatively small sample size for the captive birds.

Suggestions for future research

Whereas we show broad agreement between measurements derived from blood and faeces within individuals, and demonstrate practical application in the field, understanding of the technique could be improved through future research in a number of ways. First, laboratory experiments comparing DEE measurements using the non-

invasive method described here with concurrent measures made by indirect respirometry (Tomlinson et al., 2013) or an energy balance feeding trial (Speakman & Racey, 1988), and/or comparing oral dosing with injecting the dose, would further validate the non-invasive technique. Second, a field trial in which DEE was measured in the same individuals using both the non-invasive technique described here and the traditional DLW technique would enhance our understanding of the extent to which measurements of DEE in the field are actually affected by handling stress, and thus inform the extent to which trickier non-invasive techniques such as those described here are really necessary. Third, an investigation into whether other measures commonly based on blood sampling (e.g. glucocorticoid levels) could be measured effectively in faeces may extend the applicability of non-invasive sampling and advance our understanding of animal stress responses under natural conditions.

Conclusion

The approach described here is likely to be suitable for research questions in which relative measures enable comparison across cohorts of animals, and for research contexts where measuring physiological costs of behavioural activities under natural conditions, avoiding disturbance, and/or maintaining habituation are important. Field applications are limited by constraints on the ability to optimally dose and time sample collection, and the technique is thus not suitable for studies aiming to precisely quantify DEE for an individual or species. Our data strongly suggest that measurements of DEE using a non-invasive technique are feasible, within the limitations that we have identified. The non-invasive technique builds on the benefits of the SS method by measuring energy expenditure in a way that reduces handling and minimises the potential contribution of handling stress to resulting estimates of DEE. It also retains the power of the TS method when both initial and final samples from which to calculate DEE can be collected, and initial values do not need to be estimated. Our finding that measurements of $\frac{\delta^{18}O}{\delta^{2}H}$ and DEE agree between blood and faecal samples collected concurrently, particularly for final values $\frac{\delta^{18}O}{\delta^{2}H}$, is potentially very useful to behavioural ecologists who wish to explore physiological correlates of behavioural strategies without disrupting the behaviour of their study organisms to collect these data. DEE can be correlated directly with observed natural behaviour and environmental variables, and we have demonstrated that a DLW technique based on oral dosing and faecal sampling, applied in the field with 31 birds, is sufficiently sensitive to detect the expected inverse relationship between DEE and temperature. Wider application of this technique could open new avenues for assessing behavioural and physiological responses concurrently in wild animals under natural conditions.

Acknowledgements

This research was undertaken at Murray Guest Farm and surrounding farms, Askham, South Africa and at the Kalahari Research Centre and surrounding farms, Van Zylsrus, South Africa. We thank the management teams of both facilities for their enthusiastic support and for making the work possible. The authors thank Ryno Kemp, Sello Matjee, and Paige Ezzey for their invaluable assistance with fieldwork, Dr Grant Hall from the University of Pretoria Stable Isotope Laboratory for his help with the extractions, Mike Butler for the extraordinary effort he put into running our samples at his lab during an emergency in ours, and Ryno Kemp for assistance in the lab. Thanks also to Dr Todd Erickson, Dr Ben Smit, and Professor Phil Withers for advice and Dr Sean Tomlinson and three anonymous reviewers for their valuable comments on previous drafts. We thank the DST-NRF Centre of Excellence at the FitzPatrick Institute for African Ornithology and the National Research Foundation of South Africa (Grant Number 110506) for funding this research. The opinions, findings and conclusions are those of the authors alone, and the National Research Foundation accepts no liability whatsoever in this regard. WHK was partially supported by a Visiting Professorship at the University of Pretoria. The Kalahari Research Centre was financed by the Universities of Cambridge and Zurich, the MAVA Foundation, ERC (Grant No. 294494 to Tim Clutton-Brock) and received logistical support from the Mammal Research Institute of the University of Pretoria.

Author contributions

AEM, ARR, and SJC initially conceived the field study, and AEM, ARB, SJC, and WHK conceived of and/or designed the captive study. AEM, ARB, and WHK undertook the fieldwork with the captive birds and ARB undertook all other fieldwork with an assistant. ARB and SMW extracted the samples in the lab, and ARB, WHK, AEM, and SMW analysed the data. ARR started habituation of the study animals in 2003 and has maintained it ever since – this was central to making the field component of the study possible. ARB completed the primary drafting of the manuscript; all authors conceived and edited the manuscript and gave final approval for publication.

Data accessibility

Data deposited in the Dryad Digital Repository, doi: 10.5061/dryad.hm04k3 (Bourne et al, 2018).

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