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Validation of saliva and urine use and sampling time on the doubly labelled water method to measure energy expenditure, body composition and water turnover in male and female cats

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Abstract

Less invasive protocols are necessary to study energy expenditure (EE) of cats living in homes for expressing their normal living conditions. The present study compared sampling times and the use of saliva, urine and blood to measure ^{2}H and ^{18}O to apply the doubly labelled water method. In the first study, four cats were used to evaluate the enrichment (2, 4, 6, 7 and 8 h) and elimination (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 d) of ^{2}H and ^{18}O (subcutaneously injected). The maximum enrichment was after 5 h (R^2 0·82) of injection, with an Ln linear elimination of both isotopes ($P < 0·001$; R^2 0·99). The results of EE were similar, regardless of the sampling time used ($P = 0·999$). In the second study, seven male cats and seven female cats were used. Before and after isotope injection (5 h, 7 d, 10 d and 14 d), blood, saliva and urine were collected. Isotope enrichment was lower in urine ($P < 0·05$) and at the similar level in blood and saliva. Isotope elimination was similar for all fluids ($P < 0·473$). The EE calculated with blood and saliva was similar but higher for urine ($P = 0·015$). According to Bland–Altman statistics, blood and saliva presented low bias and high correlation ($P < 0·001$), but this was not observed for urine ($P = 0·096$). Higher EE was observed for male cats (384 (SE 39) kJ/kg$^{0·67}$ per d) than for female cats (337 (SE 34) kJ/kg$^{0·67}$ per d; $P < 0·05$). The sampling time for the method is flexible, and saliva can be used as a substitute for blood.

Key words: **^{2}H: Energy metabolism: Felines: Fatty mass**

Two equations are proposed[1] to estimate energy requirements of cats: one for non-obese and another for obese animals. However, several other factors besides body composition may interfere with the energy expenditure (EE) of felines[2]. Male cats, for example, tend to be heavier, with more lean and less fatty mass than females, and may present higher EE[3–5]. Diet composition, neutering, genetics, physical activity, age and many other physiological and environmental factors also interfere with metabolic rate, thereby resulting in considerable differences in EE among individuals[6,7]. Most studies on energy metabolism in cats used laboratory animals[8]; although laboratory conditions are advantageous to control many variables to understand specific factors, they do not account for human–animal interactions and many other variables related to housing conditions. It is necessary to consider the effect of environment in the design of energy metabolism studies; because of this, the collection of samples in cat's natural environment as their owner's house is interesting, since they can express their normal behaviour and living conditions. However, it is required to develop study protocols that are not only precise and reliable but also easily accepted by the cats and owners.

The easiest and most traditional method to estimate the EE of an animal is measuring food intake (based on available energy) to maintain a constant body weight[9,10]. Although frequently used, this method has low precision and is laborious[11], due to the need of a strict cooperation of the feline's owner to generate reliable data. One potential alternative is the use of the doubly labelled water (DLW) method, which is considered a standard methodology that has long been validated for use with dogs and cats[12]. After the enrichment of the body fluids with ^{2}H and ^{18}O, the study of the differential elimination of both stable isotopes allows quantification of CO_2 production and EE[13,14].

Abbreviations: BC, body composition; DLW, doubly labelled water; EE, energy expenditure; FQ, food quotient; Kd, ^{2}H rate constant; LM, lean body mass; Nd, pool size of body water with ^{2}H; No, pool size of body water estimated with ^{18}O; WTR, water turnover rate.

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The method also precisely quantifies the body water pool, thereby allowing the concomitant estimation of lean and fatty mass content[15,16]. In addition, the study of ^{2}H elimination permits the determination of the body water turnover rate (WTR) for studies on water metabolism[17,18].

The DLW method has been extensively used for humans and animals, including for several physiological conditions and diseases[13,19,20]. Blood is the traditional body fluid used to measure isotope abundance; saliva and urine have also been validated and used in studies involving humans[21–24]. For cats, urine samples have been used but were collected by cystocentesis[25]. Although theoretically adequate and non-invasive, the difficulty of training animals to spontaneous eliminate urine at the necessary times is a limitation to the use of this fluid in a home environment. The authors did not localise studies about the use of saliva in cats. To determine isotope enrichment of body fluids, sampling periods of 1–8 h after administration have been adopted and periods from 2 to 15 d have been proposed to evaluate isotope elimination[14,26,27]. This serial blood sampling is a potential problem, considering that the pet cats are not acclimated to the procedure, which can generate stress for the cats and their owners. Based on studies with other species, it is possible that urine and saliva sampling are suitable alternatives to measuring isotope concentration and these body fluids can be collected with less stress on house cats. Taking this into consideration, the present study has a global objective to evaluate the use of saliva and urine compared with blood and different sampling times to establish isotope enrichment and elimination to apply the DLW in male and female cats. The time of sampling is expected to be flexible to evaluate isotope enrichment and elimination, and blood, urine and saliva present similar results of isotope abundance. It is also hypothesised that male and female cats have different body composition (BC) and EE; however, no effects of sex are expected on the type of fluid sampling. Therefore, a specific objective of the present study is to compare the use of blood, saliva and urine to measure ^{2}H and ^{18}O to determine EE, BC and WTR in male and female cats.

Materials and methods

Animals and experimental design

Two separate experiments were conducted. The first experiment was to study the kinetics of isotope enrichment and elimination in blood. The second experiment was to compare the fluids saliva and urine with blood to determine the ^{2}H and ^{18}O concentration in body fluids in the use of the DLW method. All procedures with animals followed the ethical principles adopted by the Brazilian College of Animal Experimentation and were previously approved by the Ethics Committee on the Use of Animals (protocol no. 7867/16).

In the first experiment, four mixed-breed cats were used, two males and two females; they were neutered, had 4·3 (SE 0·2) kg of body weight, were 2·25 (SE 0·91) years of age and had a body condition score of 5 for females and 6 for males on a scale from 1 to 9[28]. All animals were considered healthy after a physical examination, complete blood count, serum biochemistry (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, urea and creatinine) and urine analysis. The study lasted for 30 d: 10 d for diet adaptation and 20 d to determine the isotope kinetics. The cats were kept in a cattery with a 12 h light–12 h dark cycle and a mean temperature of 26·2 (SE 0·8)°C (Incoterms). The cats were housed for 14 h (from 18 to 8 h) in cages (all cats were previously adapted to the procedure), during which water and food were available, and released into a collective cattery for 10 h (from 8 to 18 h) for voluntary exercise and social interaction, during which they had access to water but not food. Cats had interaction with people at least three times/d: to be restricted and fed; to be released and to be brushed during the period free on the cattery. On the 10th day, the cats were sedated with acepromazine (0·05 mg/kg; Acebran, Vetnil) and zolazepam hydrochloride (2·5 mg/kg; Zoletil, Virbac do Brasil) via intramuscular injection, and a central catheter was placed in the jugular vein (Intracath, 30·5 cm, 17GA, 1·1 mm; Becton Dickinson Vascular Access). Patency was maintained by flushing the catheter three times/d with heparinised solution (5 IU/ml). After 48 h of recovery, blood samples were collected before and at 2, 4, 6, 7 and 8 h after subcutaneous injection of the isotope, when the catheter was removed. On days 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20, blood samples were also collected by direct venepuncture at a time close to the isotope injection. On each sampling, 3 ml of blood were collected and placed in vacutainers without anticoagulant (BD Vacutainer) and centrifuged (Inbras) to obtain serum. The serum was stored in a cryotube with a screw cap sealed with paraffin at −20°C until analysis.

In the second experiment, seven male cats (4·14 (SE 3·9) years; 4·57 (SE 0·45) kg; 5·85 (SE 0·83) body condition score) and seven female cats (5·57 (SE 5·12) years; 3·74 (SE 0·29) kg; 5·42 (SE 0·49) body condition score), all neutered, were used. The animals were considered healthy after the same procedures of Expt 1. The housing condition was also the same as that described for Expt 1. The second experiment lasted 24 d, 10 d for diet adaptation and 14 d to study the isotope concentration in blood, saliva and urine. Considering the results of the first experiment, samples of body fluids were collected before isotope injection, after 5 h to study the enrichment, and after 7, 10 and 14 d to evaluate isotope elimination. To collect blood, cats were physically restrained (all animals were adapted for the procedure) and a blood sample was drawn by direct puncture of the jugular vein. The blood samples were processed and stored as described for Expt 1. The saliva samples were collected with hydrophilic cotton, externally and adjacent to the mouth, next to the animal's lips. To enable this, salivation was stimulated by dropping one drop of sodium dipyrone (500 mg/ml, EMS) on the cat's mouth, equivalent to 25 mg of dipyrone per cat (all animals were adapted for the procedure). Considering that the therapeutic dose of dipyrone is 25 mg/kg per d[29], only a quarter of the recommended amount was used and the procedure can be considered safe. After dipyrone administration, a first piece of cotton was used and discarded and a second piece of cotton was used for a maximum of 5 min to collect the saliva sample. The cotton was then placed in a 20 ml syringe, squeezed, and a minimum of 1·5 ml of saliva per cat was stored in a cryotube with a screw cap sealed with paraffin at −20°C until analysis. Caution was made to cotton not absorb moisture from the air before use, keeping it in sealed

bags. The urine was collected by natural micturition. The cats were kept in their cages until spontaneous urination and sample collection. For this, the cages were continuously monitored by a trained person at 10-min intervals, and the moment of urination was recorded. Samples were collected in plastic bottles without any preservative and placed under the cage funnel. Immediately after collection, the urine was stored in a cryotube with a screw cap sealed with paraffin at −20°C until analysis. The basal urine sample (before isotope injection) was considered as the urine collected on the day before the isotope administration. The first urine produced after isotope injection was used to measure the enrichment, and this period varied from 5 up to 24 h depending on the cat. On the day of isotope elimination, the cats remained in their cages until urination, when the samples were collected, and the animals were then released to the cattery. For a more precise calculation, the exact time was recorded when the blood, saliva and urine samples were collected.

Isotope injection and analysis

The isotopes (Sercon Limited) were administrated in the approximate doses of 0·12 g of ^{2}H at 99·9 atm% and 2 g of ^{18}O at 10 atm% per kg of body water[22]. Then, a 6:100 (v/v) solution of ^{2}H at 99·9 atm% and ^{18}O at 10 atm%, respectively, was prepared and injected in a fixed dosage of 8 g of solution per cat. The solution was subcutaneously applied between the scapula after 12 h of fasting and 4 h without water. Because solution application in the first study induced discomfort, in the second study, 0·3 ml of a 20 % NaCl solution was mixed and infused together to increase the isotope solution osmolality close to the interstice. Consequently, no further discomfort was noted. To increase the accuracy of the injection, the syringe was weighed empty, with the 20 % NaCl solution, with the isotope solution and again after solution injection, according to the recommendation[22]. The body mass of the cats was calculated every 7 d and always at the same time. The scale precision was assessed each time using certified weights (INMETRO: certified weights from 1 to 10 kg OIML E1/2004).

The isotope concentration in body fluids was evaluated at the Mass Spectrometry Laboratory of the Ribeirão Preto Medical School, São Paulo, Brazil. The isotopes were analysed by isotope ratio MS (ANCA 20-20; Europe Scientific). Samples for ^{2}H were processed in triplicate (100 µl per replicate) with platinum in vacutainers, after 6 h of resting. Samples for ^{18}O were processed in triplicate (100 µl per replicate) by filling the tubes with CO_2 after 24 h of resting[22].

Diet and food management

During both studies, the cats were fed a diet formulated for maintenance[30]. The food was produced in the Extrusion Laboratory of the Faculdade de Ciências Agrárias e Veterinária da UNESP, Jaboticabal, Brazil. The food formulation and the analysed composition are presented in Table 1. Before the study, the total tract apparent digestibility of crude protein, fat and starch, and the metabolisable energy content of the food, was determined *in vivo* with six cats using the total collection of faeces and urine method. The cats were fed the diet for 10 d for adaptation, followed by 7 d of quantitative collection of faeces and urine.

Table 1. Analysed chemical composition of the food* for cats utilised in Expt 1 and Expt 2 (values on DM basis)
(Percentages)

Items	
Moisture (%)	4·7
Crude protein (%)	38·9
Acid-hydrolysed fat (%)	14·2
Starch (%)	28·7
Crude fibre (%)	3·4
Ash (%)	5·4
Ca (%)	0·81
P (%)	0·83
Metabolisable energy (kJ/g)†	15·06
Food coefficient‡	0·817

*Ingredient composition: maize grain 37·06 %; poultry by-product meal 23 %; isolate soya protein 13·9 %; poultry fat 9·4 %; swine isolate protein 5·0 %; broken rice 5·0 %; sugarcane fibre 2 % (Vit2be Fiber, SPF do Brazil); liquid palatant 1 % (SPF Brasil); common salt 0·6 %; potassium chloride 0·58 %; vitamin–mineral premix 0·5 % (Rovimix, DSM Produtos Nutricionais Brasil S.A.); choline chloride 0·4 %; calcium carbonate 0·3 %; taurine 0·12 %; mould inhibitor 0·1 % (Mold Zap Citrus: Ammonium dipropionate, acetic acid, sorbic acid and benzoic acid. Alltech do Brazil Agroindustrial Ltda); fish oil 0·1 %; antioxidant 0·04 % (Banox: Butylated hydroxy anisole, butylated hydroxytoluene, propyl gallate and calcium carbonate. Alltech do Brazil Agroindustrial Ltda).
† Evaluated in six cats by the total collection of faeces and urine method.
‡ Considering the total tract apparent digestibility of the crude protein (88·7 %), acid-hydrolysed fat (88·6 %) and starch (99·0 %) determined in six cats by the total collection of faeces method.

The crude protein, acid-hydrolysed fat and starch were analysed in food and faeces[31]. Gross energy was analysed in food, faeces and urine samples using a bomb calorimeter (IKA calorimeter, C200; IKA-Werke GmbH & Co. KG). The energy of the diet was 15·14 (SE 0·71) kJ/g (as-fed basis), and the apparent total tract digestibility of crude protein was 88·7 (SE 1·9) %, acid-hydrolysed fat was 88·6 (SE 1·6) % and starch was 99·8 (SE 0·04) %.

During Expt 1 and Expt 2, offered and refused food was weighed daily and the intake was recorded. The amounts of offered food were initially established considering the records of energy intake to constant body weight of each cat. The cats were weighed weekly, and the amount of food provided was adjusted to achieve a constant body weight. Data on food (metabolisable energy) intake to constant body weight during Expt 2 were also used to compare the results of EE with the DLW method.

Calculation procedures

The pool size for ^{2}H or ^{18}O for the total of body water was calculated as follows (considering a linear elimination response)[16]:

$$N\,(\text{mol}) = \left(\frac{WA}{18,02a}\right) \times \frac{(\delta a - \delta t)}{(\delta s - \delta p)},$$

where N is the pool size of body water; W is the amount of water used to dilute the labelled water dose; A is the weight of labelled water administration (g); a is the diluted dose for analysis; δ is the enrichment of dose (a), dilution water (t), post-dose sample (s) and pre-dose baseline (p) samples.

For ^{18}O, considering the non-aqueous exchange routes are small no correction was made. To calculate total body water with ^{2}H, a correction factor was used due to the isotope incorporation

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in non-aqueous organic molecules during biosynthesis[32] using the following formula[33]:

$$TBW = Nd/f$$

where TBW is the total body water; Nd is the pool size of body water with ^{2}H; f is the correction factor according to Nd:No (No = pool size of body water estimated with ^{18}O) ratio in each evaluated body fluid.

The lean body mass (LM) of the cats was calculated considering the hydration constant of 73·2 % for mammals by the following equation: lean body mass (kg) = body water (kg)/0·732. The fatty body mass (kg) was estimated as follows: total body mass (kg) – LM (kg) of the animal[15].

The enrichment of the body water pool with ^{2}H and ^{18}O was established using the sample obtained at the highest isotope concentration in Expt 1. In Expt 2, it was established using the sample collected 5 h after subcutaneous injection, following the results of Expt 1. The constant of isotope elimination was established with the samples obtained at different days after injection. In the second study, enrichment and elimination were calculated considering isotope concentration in blood, urine and saliva. The two-point formula was used to calculate ^{2}H and ^{18}O elimination[34]:

$$K = \frac{Ln(X(t2) - X(t1))}{t2 - t1}$$

where K is rate constants for ^{2}H (Kd) and ^{18}O (Ko); Ln is the natural logarithm; $X(t2)$ is the sampling point of isotope elimination; $X(t1)$ is the sampling point of the isotope enrichment; $t1$ is the day of isotope enrichment sampling and $t2$ is the day of isotope elimination sampling.

The amount of CO_2 produced was established using the following formula[35]:

$$rCO_2\left(\frac{mol}{d}\right) = \left(\frac{N}{2 \cdot 08}\right) \times (Ko - Kd) - 0 \cdot 015 \times Kd \times N$$

where rCO_2 is the CO_2 production; N is the dilution space for ^{18}O; Ko is the rate constant of ^{18}O in body water and Kd is the rate constant of ^{2}H in body water.

Lastly, the EE of the cats was calculated as follows[36]:

$$EE\left(\frac{kj}{d}\right) = rCO_2 \times 22 \cdot 4\left(\frac{3 \cdot 7}{FQ} + 1 \cdot 326\right) \times 4 \cdot 18$$

where EE is the energy expenditure; rCO_2 is the CO_2 production and FQ is the food quotient.

The food coefficient was calculated considering the digestible nutrient content of the food, determined *in vivo* with six cats using the total collection of faeces method[30], as previously shown. The following formula was used[37]:

$$FQ = \frac{(P \times 0 \cdot 781) + (F \times 1 \cdot 427) + (S \times 0 \cdot 746)}{(P \times 0 \cdot 996) + (F \times 2 \cdot 019) + (S \times 0 \cdot 746)}$$

where FQ is the food quotient; P is the digestible protein; G is the digestible fat and A is the digestible starch.

The body WTR was calculated as[18], assuming little to no water is lost via evaporative routes that are subject to isotope fractionation:

$$WTR\left(\frac{ml}{d}\right) = Nd \times Kd \times 18 \cdot 02$$

where WTR is the water turnover rate; Nd is the ^{2}H body water and Kd is the ^{2}H rate constant.

The EE of the cats was also computed in Expt 2 by the food intake method. For this purpose, data on food intake to constant body weight were multiplied by the metabolisable energy content of the diet, as determined *in vivo* with cats using the total collection of faeces and urine method.

Statistical analysis

The study considered each individual cat as the experimental unit. In Expt 1, only four cats were used because the protocol included central catheterisation. The sampling size of Expt 2 was established based on the results of ANOVA for EE obtained in Expt 1, considering the factorial arrangement of treatments in Expt 2. The test power was set at 0·8 (procedure Opdoe of the R software), $\alpha = 0{\cdot}05$, standard deviation = 18·5 and a standard error of approximately 25. The analysis was performed with the sample size procedure of the R software, and a sample size of seven cats per sex was obtained, which assured adequate comparison of the main outcome (EE) for each body fluid. In Expt 1, the kinetics of enrichment and elimination of ^{2}H and ^{18}O were described by common statistical analysis and polynomial regression, in a completely randomised design. The second experiment followed a 3 (body fluids) × 2 (sex) factorial arrangement (fixed effects) design, totalling six experimental treatments, for the dependent variables Nd:No ratio, CO_2 production, EE and WTR. The data were subjected to variance analysis in a completely randomised design, considering the effect of body fluid, sex and body fluid × sex interaction. This model allowed to verify the main study hypothesis, to compare the alternative fluids to blood and to verify the effects of sex on outcomes. For the variable isotope angular coefficient, LM and fatty body mass were established as a triple factorial arrangement with 3 (body fluids) × 2 (sex) × 2 (isotopes: ^{2}H and ^{18}O) totalising twelve experimental treatments. The data were submitted to variance analysis, considering the effects of body fluid, sex, isotopes and the interactions of body fluid × sex, body fluid × isotope, sex × isotope and body fluid × sex × isotope. In both situations, when differences were detected in the F test, the mean values among body fluids were compared with the Tukey test. The effect of time on isotope elimination was evaluated by polynomial contrasts, considering time on the x axis and the isotope on the y axis. As a complementary evaluation, the CI of agreement were determined as the bias (mean difference) ± 1·96 SD of the EE estimated with blood and each alternative body fluid (saliva and urine) with the Bland and Altman statistic[38]. The mean value of each body fluid was also compared using the Pearson correlation. In addition, the mean absolute error and root-mean-square errors were calculated for each body fluid using the following equations:

$$\text{Mean absolute error (MAE)} = \frac{\sum_{I=1}^{n}|P_I - O_I|}{N};$$

$$\text{Root mean square error (RMSE)} = \frac{\sqrt{\sum_{I=1}^{n}(P_I - O_I)^2}}{N}.$$

The results obtained by the DLW method using the fluid blood and the food intake method were compared by Student's *t* test, and the concordance correlation coefficient and bias correction factor (accuracy) were calculated[39]. Values of $P < 0{\cdot}05$ were considered significant. All data complied with the presuppositions of the variance analysis. The Bland–Altman and Pearson correlation was performed on Sigma Plot 14.0 (Systat Software, 2017), and the remaining analyses were performed on SAS software 9.1 using the Proc MIXED (SAS Institute, 2003).

Results

In Expt 1, cats remained health, with adequate food intake. The isotope enrichment was observed immediately after 2 h of injection (Fig. 1). Isotope concentration in blood did not differ at 2, 4, 6, 7 and 8 h after inoculation ($P = 0{\cdot}943$). However, as the basal isotope concentration was low, enrichment was described by a polynomial regression whose derivate resulted in maximum enrichment after 5 h and 20 min for both isotopes (R^2 0·82; $P < 0{\cdot}001$). The elimination of both isotopes was linear (R^2 0·99; $P < 0{\cdot}001$), which resulted in similar estimates of CO_2 production, regardless of the day of elimination used in the calculation ($P = 0{\cdot}999$) and the hour of enrichment selected ($P = 0{\cdot}999$).

In Expt 2, cats remained healthy, with proper food intake. The results of ^2H and ^{18}O levels in the fluids blood and saliva were

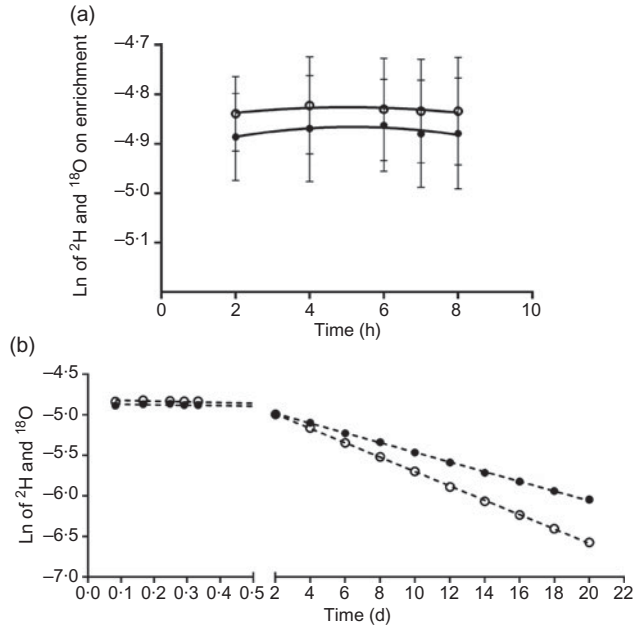

Fig. 1. (a) Polynomial regression describing the isotope enrichment in Expt 1 in blood (^2H: $y = -7{\cdot}034x^2 + 75{\cdot}15x + 174{\cdot}68$, R^2 0·82; ^{18}O: $y = -12{\cdot}19x^2 + 130{\cdot}16x + 2027{\cdot}7$, R^2 0·82; $P = 0{\cdot}943$). (b) Ln linear plateau-elimination curve describing the isotope elimination in Expt 1 in blood (^2H: $y = -0{\cdot}0598x - 4{\cdot}8645$, R^2 0·89; ^{18}O: $y = -0{\cdot}0886x - 4{\cdot}8132$, R^2 0·93; $P = 0{\cdot}999$). *n* 4 cats. (a) and (b): —●—, ^2H; -●-, ^{18}O.

obtained for all fourteen cats. However, for the fluid urine, only four of the seven female cats and six of the seven male cats were included because some cats took more than 24 h to produce the first spontaneous urine after isotope injection. It was therefore not possible to establish isotope enrichment, and the data from these animals were not used for this fluid evaluation. The body weight of the cats did not differ during Expt 2 (Table 2), and the male cats presented higher body weight and food intake than the female cats ($P < 0{\cdot}001$).

The basal concentration of ^{18}O was higher for saliva than in urine ($P < 0{\cdot}05$), but both fluids were similar to blood (Table 3). No fluid × sex interaction was found at any sampling time ($P > 0{\cdot}05$). The urine enrichment for ^{18}O was lower than those for blood and saliva ($P < 0{\cdot}05$), but during the days of elimination, the ^{18}O concentration was similar between body fluids ($P = 0{\cdot}788$). The effect of sex was observed on enrichment (5 h) and at all elimination points, with higher values for females than for males ($P < 0{\cdot}01$), which is explained by the lower body weight of the females as a fixed isotope dosage was infused. The ^{18}O pool size of body water (No) was similar between fluids ($P = 0{\cdot}178$) but different for sex ($P < 0{\cdot}01$), as male cats were bigger. The constant of isotope elimination for ^{18}O (Ko) was similar between fluids ($P = 0{\cdot}933$) and sex ($P = 0{\cdot}300$).

The basal concentration of ^2H was higher for saliva than in urine ($P < 0{\cdot}05$), but both fluids were similar to blood (Table 4). No sex × fluid interaction was observed at any time of evaluation ($P > 0{\cdot}05$). As observed for ^{18}O, in the enrichment, lower ^2H values were observed for urine than for blood and saliva ($P = 0{\cdot}002$). On the days of elimination, ^2H values were similar between body fluids ($P = 0{\cdot}562$). A sex effect was also observed for ^2H, with higher values for females ($P < 0{\cdot}01$). The ^2H pool size of body water (Nd) was lower for blood and saliva than for urine ($P < 0{\cdot}05$). The constant of isotope elimination for ^2H (Kd) was similar between fluids ($P = 0{\cdot}575$) and sex ($P = 0{\cdot}076$).

In the three body fluids, a linear ^{18}O and ^2H Ln elimination rate was observed ($R^2 > 0{\cdot}83$; $P < 0{\cdot}001$), as illustrated in Fig. 2. The angular coefficient of ^{18}O and ^2H was similar between blood and saliva (respectively, −12·7 (SE 4·6)° and −12·2 (SE 4·5)°; $P = 0{\cdot}746$), and higher than that of urine (−10 (SE 0·4)°; $P = 0{\cdot}022$), showing a slower isotope elimination in urine than in the other two body fluids. The isotope angular coefficient was also lower for males (blood; ^2H −6·9 (SE 0·7)°; ^{18}O −15·0 (SE 1·5)°) than for females (blood; ^2H −9·0 (SE 1·0)°; ^{18}O −19·5 (SE 2·3)°), showing a faster elimination from the body water pool in females ($P < 0{\cdot}01$). As expected, the angular coefficient was lower for ^{18}O than for ^2H (respectively −16·2 (SE 1·1)° and −7·3 (SE 0·6)°; $P < 0{\cdot}01$), as ^{18}O is eliminated faster from body water.

The BC calculated with ^2H (considering the mean correction factor of $f = 1{\cdot}07$) or ^{18}O was similar (data not shown), without differences for the body fluid used in calculations ($P > 0{\cdot}05$). Regarding sex, as males presented higher body mass, they showed higher LM (kg) and fatty body mass (kg) than females (Table 5; $P < 0{\cdot}05$); however, the BC as a percentage of body mass was similar between sexes ($P > 0{\cdot}05$). For all these variables, no significant statistical interactions were observed (fluid × sex, fluid × isotope, sex × isotope, fluid × sex × isotope; $P > 0{\cdot}05$).

Table 2. Body mass and food intake of male and female cats during Expt 2 (Mean values and standard deviations)

	Body mass (kg)							Food intake (g of DM/cat per d)	
	Initial	SD	Final	SD	Mean	SD	*P*	Mean	SD
Males	4·58	0·39	4·63	0·42	4·58	0·40	0·134	69·12	4·47
Females	3·74	0·24	3·84	0·24	3·76	0·22	0·138	57·05	5·86
P					<0·001			<0·001	

Table 3. Concentration of ^{18}O (parts per million) in different body fluids of male and female cats during Expt 2 (Mean values with their standard errors)

	Body fluid				SEM	*P**	
Time	Blood	Saliva	Urine	Mean	(*n* 7 cats per sex)	Fluid	Sex
Male basal	1994	1995	1986	1992	1·9		
Female basal	1990	1991	1988	1990	1·1		
Mean	1992[a,b]	1993[a]	1987[b]			0·049	0·348
Male 5 h	2278	2271	2243	2265	6·9		
Female 5 h	2356	2345	2311	2342	9·5		
Mean	2317[a]	2308[a,b]	2286[b]			0·044	<0·001
Male 7 d	2142	2140	2144	2142	2·9		
Female 7 d	2180	2178	2180	2179	4·7		
Mean	2161	2159	2162			0·788	<0·001
Male 10 d	2107	2105	2103	2105	2·7		
Female 10 d	2134	2131	2133	2133	4·5		
Mean	2120	2118	2118			0·807	<0·001
Male 14 d	2073	2071	2073	2072	3·8		
Female 14 d	2090	2088	2080	2087	3·9		
Mean	2081	2080	2076			0·819	0·018
No (mol)†							
Male	138·8	143·1	151·1	144·3	5·5		
Female	111·5	115·3	116·6	114·5	5·5		
Mean	125·2	129·2	133·9			0·179	<0·001
Ko (per d)‡							
Male	0·094	0·094	0·085	0·091	0·005		
Female	0·095	0·094	0·099	0·096	0·005		
Mean	0·095	0·094	0·092			0·933	0·300

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
*No fluid × sex interaction was observed ($P > 0·05$).
† No = pool size of body water calculated with ^{18}O.
‡ Ko = constant of ^{18}O elimination.

The Nd:No ratio, to correct the body water pool estimated with ^{2}H, did not differ between fluids or sex ($P > 0·05$; Table 6). The CO_2 production and EE differed among body fluids ($P < 0·05$), the values obtained using blood and saliva was similar, but when urine was used, the results were greater than that obtained with blood. This is explained as Ko and Kd were similar among fluids, as shown in Tables 3 and 4, but the enrichment was lower for urine. As the EE was estimated by the two-point equation on three different days of evaluation of isotope elimination (days 7, 10 and 14), the results of each day were compared and no differences among days of sampling were observed ($P > 0·05$), as illustrated in Fig. 3. Male cats presented higher CO_2 production and EE, both calculated per $kg^{0·67}$ of body weight or per $kg^{0·67}$ of LM ($P < 0·05$). No fluid × sex interaction was observed for CO_2 production and EE ($P > 0·05$).

The mean EE estimated by the three body fluids differed in variance analysis ($P = 0·015$), the EE calculated with blood and saliva was similar, but the result was higher for urine. As a complementary statistical evaluation, the EE calculated from blood was considered the reference value and was compared with the outcomes obtained from saliva, urine and by the food balance method (Fig. 4). The concordance correlation coefficient was higher between the results of blood and saliva (0·799) showing an accuracy (bias correction factor) of 0·971 and a low bias (−8·8 (SE 27·8) kJ/$kg^{0·67}$ per d; Fig. 4(a)). In addition, compared with urine, the results presented the lowest MAE (19·1) and RMSE (28·28). The values obtained with urine presented greater bias (−48·4 (SE 56·6) kJ/$kg^{0·67}$ per d; Fig. 4(b)) than those of saliva, with a lower concordance correlation coefficient of 0·360, and higher MAE (36·75) and RMSE (60·42). Considering the food intake method, the mean energy intake was 350·1 (SE 37·4) kJ/$kg^{0·67}$ per d. When the DLW using blood was compared with the food intake method, results were statistically similar ($P = 0·462$), with a low bias (−24·9 (SE 43·3) kJ/$kg^{0·67}$ per d; Fig. 4(c)) and a concordance correlation coefficient of 0·661 (MAE = 27·13; RMSE = 32·38).

Table 4. Concentration of ^{2}H (parts per million) in different body fluids of male and female cats during Expt 2 (Mean values with their standard errors)

	Body fluid				SEM	*P**	
Time	Blood	Saliva	Urine	Mean	(*n* 7 cats per sex)	Fluid	Sex
Male basal	151·0	151·4	150·6	151·0	0·2		
Female basal	150·9	151·1	150·0	150·6	0·2		
Mean	150·9^{a,b}	151·2^{a}	150·0^{b}			0·003	0·422
Male 5 h	313·5	307·5	290·6	303·9	4·1		
Female 5 h	352·9	344·4	333·9	344·9	3·7		
Mean	333·2^{a}	325·9^{a,b}	308·0^{b}			0·002	<0·001
Male 7 d	255·2	251·9	255·5	254·2	2·4		
Female 7 d	281·1	277·2	280·8	279·7	3·4		
Mean	268·2	264·5	268·2			0·567	<0·001
Male 10 d	238·2	234·9	235·4	236·2	2·2		
Female 10 d	259·5	255·7	259·5	258·2	3·4		
Mean	248·8	245·3	247·5			0·618	<0·001
Male 14 d	218·8	216·4	217·8	217·7	1·9		
Female 14 d	236·4	233·9	230·7	234·0	3·1		
Mean	227·6	225·2	222·9			0·562	<0·001
Nd (mol)†							
Male	148·5	154·1	172·7	158·4	5·4		
Female	120·0	125·7	128·7	124·8	5·4		
Mean	134·2^{a}	139·9^{a}	150·7^{b}			0·002	<0·001
Kd (per d)‡							
Male	0·063	0·064	0·053	0·060	0·004		
Female	0·067	0·066	0·068	0·067	0·004		
Mean	0·065	0·065	0·060			0·575	0·076

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
*No fluid × sex interaction was observed ($P > 0·05$).
† Nd = pool size of body water calculated with ^{2}H.
‡ Kd = constant of ^{2}H elimination.

As the isotope enrichment in urine was lower, and the isotope concentration on the days of elimination was similar between all three body fluids: a combination of body fluids was proposed considering the urine values for basal and elimination evaluations and saliva results to establish isotope enrichment (5 h after inoculation). This combined sampling procedure resulted in similar EE to blood (364·6 (SE 60·51) kJ/kg$^{0·67}$ per d; $P = 0·218$), with a high concordance correlation coefficient (0·69) and accuracy (0·839), low bias (−24·9 (SE 43·3) kJ/kg$^{0·67}$ per d), and low MAE (50·76) and RMSE (46·69). The results also showed a good Pearson correlation coefficient (R^2 0·48; $P = 0·005$), suggesting it to be an interesting alternative to blood for fluid sampling.

Discussion

As a main outcome for alternative fluid sampling, the results indicated that saliva can be used to replace blood to measure isotope abundance and to calculate EE, BC and WTR in free living owned cats. This may open opportunity to develop less invasive and more friendly study protocols, which may facilitate studies that include larger number of cats and greater diversity of breeds and living conditions, which would probably be more difficult to study in laboratory situations.

In Expt 1, about sampling time, the body water pool enrichment was confirmed on the first blood sampling, at 2 h after isotope injection, and it remained constant through the 8 h of observation. This was in accordance with previous studies on cats that adopted periods from 1 to 8 h to establish isotope enrichment in body water[25–27]. The theoretical maximum enrichment observed after 5 h and 20 min was similar to that reported in dogs, with maximum ^{18}O and ^{2}H concentration in blood between 5 and 6 h after injection[40]. The linear elimination of the isotopes also allows for adoption of a range from a few days after injection until the 20 d of sampling evaluated on the study, resulting in similar estimations of CO_2 production and EE[14,41]. This knowledge increases the flexibility in the sampling time adopted for owned animals, making it easier to adapt the sample collections to the daily routine of the owner and the cat. Thus, enrichment can be evaluated in a window of 2–8 h and the elimination from 2 to 20 d after injection with similar outcomes. Although this increases labour, it is important to consider to evaluate more than 1 d of isotope elimination, thus minimising variations or errors arising from sample collection, storage, manipulation or analysis that could reduce the precision of the procedure[22,41].

As previously reported in humans[42], blood and saliva showed similar ^{18}O and ^{2}H concentrations in all sampling times of the cats. The elimination rate of the isotopes was also similar in these two body fluids, justifying the similar outcomes of BC, water turnover, CO_2 production and EE. The cats used were not trained to allow spontaneous collection of saliva, although this is a possibility for laboratory animals which would make not necessary a chemical stimulus to salivation. This approach, however, was not feasible for owned cats, and because of this, dipyrone was used for adequate collection of saliva in cats untrained for the procedure. Dipyrone is a pain medication that is considered safe for cats when administered by the oral route[43], especially considering the low dosage necessary to

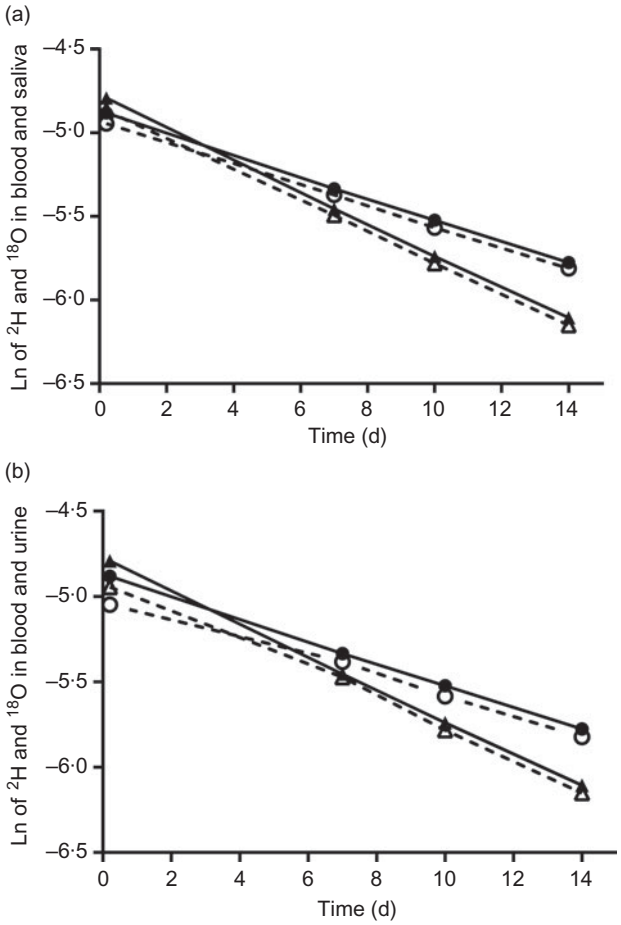

Fig. 2. (a) Ln linear regression describing the isotope elimination in Expt 2 in blood (^{2}H: y = −0·064x − 4·872, R^2 0·84; ^{18}O: y = −0·095x − 4·777, R^2 0·89; $P \leq 0·001$; *n* 14 cats) and saliva (^{2}H: y = −0·063x − 4·931, R^2 0·84; ^{18}O: y = −0·093x − 4·847, R^2 0·87; $P \leq 0·001$; *n* 14 cats). (b) Ln linear regression describing the isotope elimination in Expt 2 in blood (^{2}H: y = −0·064x − 4·872, R^2 0·84; ^{18}O: y = −0·095x − 4·777, R^2 0·89; $P \leq 0·001$; *n* 14 cats) and urine (^{2}H: y = −0·056x − 5·019, R^2 0·80; ^{18}O: y = −0·087x − 4·904, R^2 0·88; $P \leq 0·001$; *n* 10 cats). (a) —●—, ^{2}H in blood; —▲—, ^{18}O in blood; -●-, ^{2}H in saliva; -▲-, ^{18}O in saliva. (b) —●—, ^{2}H in blood; —▲—, ^{18}O in blood; -●-, ^{2}H in urine; -▲-, ^{18}O in urine.

induce salivation that is suggested in the present study. All cats behaved well after the procedure, returning to their normal activities just after the end of the collection procedure and not showing any altered attitude besides salivation for a short period (approximately 10 min).

Urine collection, on the other hand, presented some issues, especially concerning the time of sample collection and the enrichment evaluation. Different from blood and saliva, fluids for which the researcher can precisely specify the sampling time, urine is voided by cats when they want and not when the researcher needs. The moment of collection, however, is not a problem for human beings, who are able to urinate at the required times of sampling, and urine samples in humans have been used and validated in several studies[19,23,44]. The basal isotope concentration was similar between blood and urine, but the enrichment was lower in the urine. The lower estimation of body water enrichment may be justified by the dilution of the urine produced after isotope administration (when the body water was enriched with the isotopes) by the residual urine already in the urinary bladder. This problem does not occur for human beings, as the researcher may request the subject to empty the urinary bladder just before isotope administration, which is not possible in cats. Another concern is the sampling time. In the present study, four cats had to be excluded from analysis, as they delayed more than 24 h to first urination after being dosed with the isotopes. Urine is continuously produced by the kidneys, collected in urinary bladder and eliminated sporadically by the animals[45]. It was observed that until 8 h after injection, the ^{18}O and ^{2}H concentrations were stable in blood; however, it is possible that after this time the isotope concentration started to decrease as the body water pool was replaced, and the produced urine may present lower isotope concentration. Because many cats delayed more than 12 h to release the first urine, this may also account for the lower isotope concentration. To overcome this sampling problem to establish the enrichment, a previous study with cats did urine collection by cystocentesis and obtained satisfactory results[25]. This technique could be adopted in the present study, but it is too invasive and outside

Table 5. Body composition of male and female cats calculated with ^{18}O utilising different body fluids (Mean values with their standard errors; percentages)

	Body fluid				SEM	*P**		
Item	Blood	Saliva	Urine	Mean	(*n* 7 cats per sex)	Fluid	Sex	Isotope
Lean body mass (kg)								
Male	3·41	3·52	3·73	3·55	0·08			
Female	2·75	2·83	2·87	2·79	0·08			
Mean	3·08	3·18	3·19			0·108	<0·001	0·694
Lean body mass (%)								
Male	75·30	77·61	83·00	78·64	2·05			
Female	73·93	76·40	76·80	75·78	2·05			
Mean	74·76	77·23	77·23			0·070	0·326	0·691
Fat body mass (kg)								
Male	1·13	1·02	0·95	1·03	0·07			
Female	0·96	0·86	0·84	0·88	0·07			
Mean	1·04	0·94	0·98			0·166	<0·001	0·694
Fat body mass (%)								
Male	24·60	22·35	20·82	22·50	0·65			
Female	26·06	23·50	22·91	24·10	0·69			
Mean	25·23	22·76	23·70			0·070	0·325	0·691

*No interaction between fluid × sex, fluid × isotope, sex × isotope and fluid × sex × isotope was observed ($P > 0·05$).

Table 6. Nd:No ratio, carbon dioxide production, energy expenditure and body water turnover rate of male and female cats calculated utilising different body fluids
(Mean values with their standard errors)

	Body fluid				SEM	*P**	
Item	Blood	Saliva	Urine	Mean	(*n* 7 cats per sex)	Fluid	Sex
Nd:No ratio							
Male	1·06	1·07	1·14	1·09	0·01		
Female	1·07	1·09	1·09	1·08	0·01		
Mean	1·07	1·08	1·12			0·149	0·785
CO_2 (mol/d)							
Male	1·9	1·9	2·2	2·0	0·05		
Female	1·3	1·4	1·6	1·4	0·05		
Mean	1·6[a]	1·6[a]	1·9[b]			0·019	<0·001
kJ/kg $BW^{0·67}$ per d†							
Male	362·1	369·0	422·9	384·7	9·62		
Female	317·1	327·8	366·9	337·3	10·61		
Mean	339·6[a]	348·4[a]	394·9[b]			0·015	<0·026
kJ/kg $LM^{0·67}$ per d‡							
Male	458·2	457·3	506·8	474·1	12·07		
Female	391·1	395·4	445·0	410·5	13·32		
Mean	424·6	426·4	475·9			0·069	<0·001
Water turnover (ml/$kg^{0·67}$ per d)§							
Male	56·5	55·5	59·1	56·2	2·37		
Female	54·0	58·9	52·2	55·8	2·15		
Mean	55·2	57·2	55·6			0·854	0·911

Nd, pool size of body water with ^{2}H; No, pool size of body water estimated with ^{18}O; BW, body weight; LM, lean mass.
*No fluid × sex interaction was observed ($P > 0·05$).
† Daily energy expenditure per kg of metabolic BW calculated as a mean value of the 3 d of isotope elimination evaluation.
‡ LM estimated with the ^{18}O isotope.
§ Body water turnover rate calculated as a mean value of the 3 d of isotope elimination evaluation.

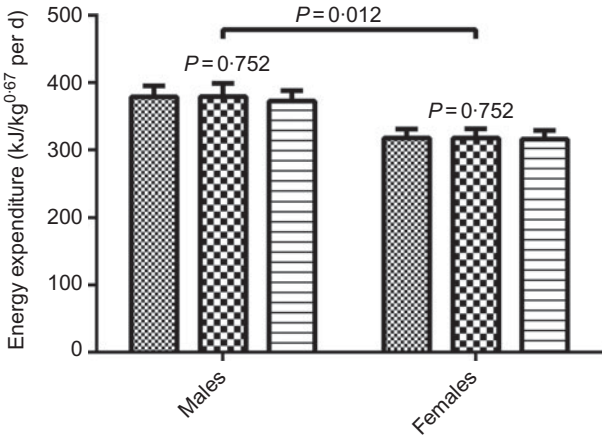

Fig. 3. Energy expenditure (kJ/$kg^{0·67}$ per d) of male (*n* 7) and female (*n* 7) cats estimated using the fluid blood and the 7th, 10th and 14th days of isotope elimination. No effect of day was verified by *F* test ($P = 0·752$). Higher values were observed for males, regardless of the day of calculation using the *F* test ($P = 0·012$). ▩, Day 7; ▩, day 10; ▭, day 14.

of the objective to develop a friendly and practical protocol. The same problem of sampling time for urine collection was also described in a study with children with severe cerebral palsy[23]. To study isotope elimination, on the other hand, urine proved to be a suitable body fluid, as the isotope concentrations on days 7, 10 and 14 did not differ among fluids. In a previous study that also used urine from cats, animals were kept for 3 h in cages, and if no urine was produced, the elimination was established by blood sampling[25]. The results of the present study suggest that this precaution may not be necessary; as long as the information about the urination time is obtained, the calculations of EE or CO_2 production can be done accurately. Another possible reason for the results on urine is that cats can concentrate urine to a higher extend than many mammals[46]. This high resorption of water or even the water exchange across the bladder mucosa[46] could also account for the differences in this fluid. The authors believe, however, that probably none of these reasons explain the observed results. Isotope abundance was lower only on enrichment time, due to a long bladder residence time for urine before initial enrichment, but the values on elimination evaluation were similar to those obtained for blood. This suggests that after the issue on the first voiding, isotope abundance remained similar on blood and urine along the observation period. Even the difference between the time of urine void by kidneys and the time of its final micturition apparently was not a problem to estimate isotope elimination, since the values at days 7, 10 and 14 were similar to blood. Summarising, the ^{18}O and ^{2}H kinetics in body fluids are apparently similar, and urine can be used to establish the elimination rate. The limitation in using urine was to obtain the samples to stablish the enrichment.

It is interesting that although the results differ among body fluids on isotope enrichment of body water, the estimations of BC were similar; in theory, any of the three fluids could be used in nutritional studies to quantify BC. However, caution is recommended in the use of urine. It is known that ^{2}H may incorporate in amino acids and proteins, resulting in increased estimation of the body water pool[40], whereas the body water content

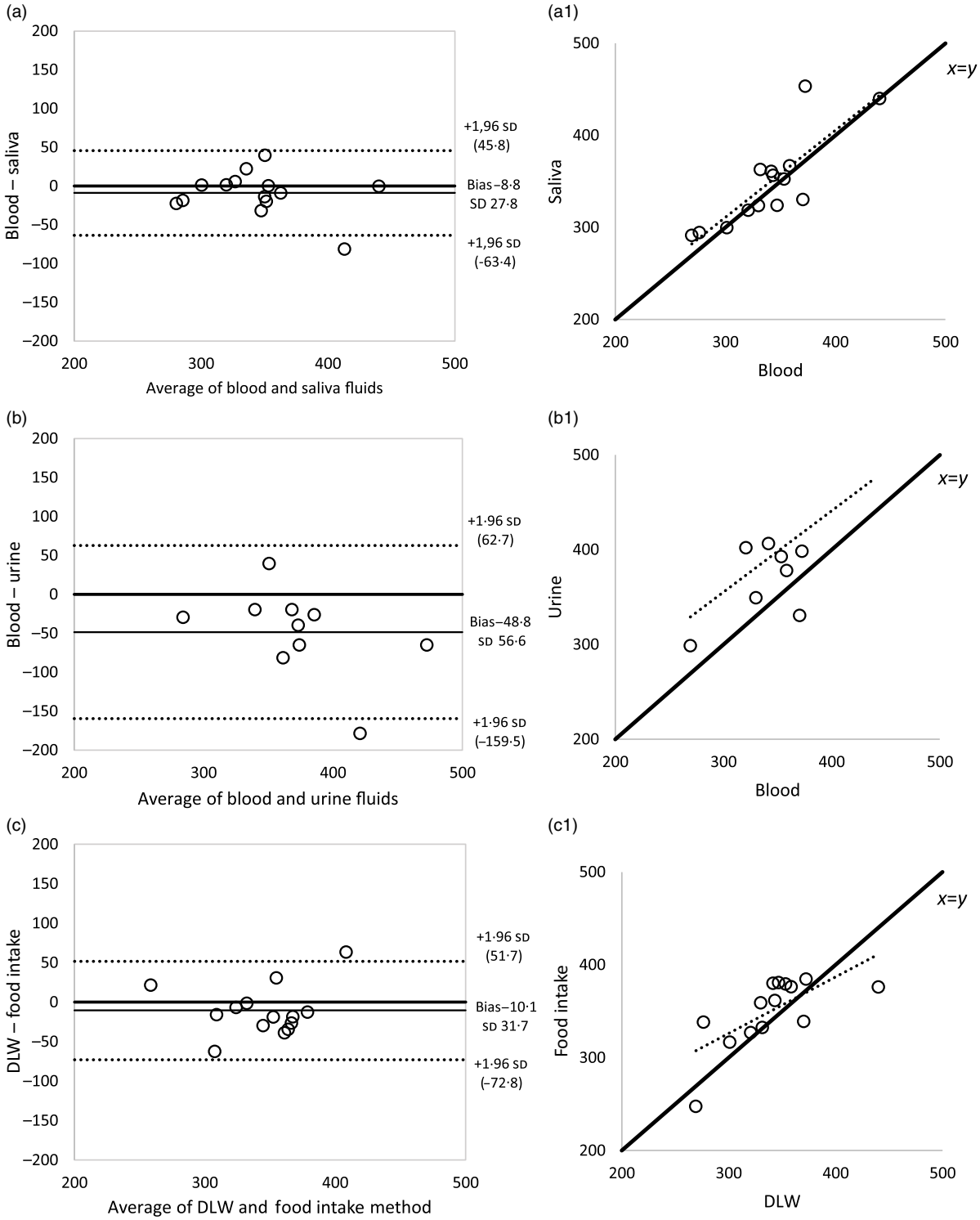

Fig. 4. Correlation between the energy expenditure (kJ/kg$^{0·67}$ per d) of cats measured by the doubly labelled water method (DLW) using the fluid blood with those estimated with (a) the fluid saliva (n 14), (b) the fluid urine (n 10) and (c) the food intake method (n 14). Bland–Altman comparisons of blood *v.* the other fluid or method. Solid horizontal lines represent mean differences between methods, and dashed lines represent the limits of agreement (mean ± 1·96 SD). Pearson correlation coefficient between DLW results using the fluid blood and the fluid saliva (a1; R^2 0·82; P = 0·003), urine (b1; R^2 0·55; P = 0·096) or the food intake method (c1; R^2 0·69; P = 0·006). Mean values of males and females estimated on days 7, 10 and 14 of isotope elimination.

estimated with ^{18}O is less variable and reflects the metabolic processes of water more accurately[47]. To correct this, the Nd:No ratio needs to be studied and a correcting factor applied when ^{2}H is used to estimate body water[41]. In the present study, although no differences between body fluids were observed, blood and saliva presented very close Nd:No ratios, and the obtained values for these two fluids were within the normal range reported for mammals of 1·01–1·10[48].

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The results of the ANOVA indicate similar estimations of CO_2 production and EE for blood and saliva, with a high concordance correlation coefficient and accuracy and low bias. For saliva, only one cat was outside of the $x = y$ axis at the Pearson correlation. Comparing urine with blood, many cats deviated from the $x = y$ axis, for which outcomes in fact did not correlate with each other and differed on variance analysis. The main problem for urine was the estimation of isotope enrichment, whose values differed from blood. When saliva was used to determine the enrichment and urine to determine the basal and elimination values, the outcomes showed a high concordance, correlation coefficient and accuracy, and a low bias in comparison with blood. Thus, urine may be a suitable fluid to measure basal and elimination isotope values, but not the enrichment, for which blood or saliva is suggested.

The results of DLW using blood and the food intake method were similar, with high correlation coefficient and accuracy. The mean value was only approximately 3 % higher for the food intake method. Discrepancies among the methods were reported in a meta-analysis on studies with cats[8]. In a study that directly compared the two methods, the metabolisable energy intake was approximately 32 % higher for males and 29 % higher for females on the food intake method than the EE estimated by the DLW[25]. In another study, the metabolisable energy intake differed from −14·7 to 10·6 % when compared with the EE obtained by the DLW, depending on the age, season or the method used to estimate food energy content (modified Atwater equation *v.* National Research Council 2006 proposed equation)[14]. In the present study, food metabolisable energy content was determined *in vivo* with urine collection, bringing more accuracy than when this value is estimated by equations[49,50]. The FQ to estimate EE was also established based on nutrient digestibility values obtained *in vivo*. One point is that the authors of the present research opted to use starch digestibility, and not N-free extract, which is commonly used. This is justified, as most of the digestible carbohydrates of dry cat foods are starch, with little sugar content, and N-free extract is an inadequate method to study digestible carbohydrates for dry cat foods[51]. The N-free extract calculation included a large part of the insoluble dietary fibre that is solubilised on the crude fibre method as well as all the soluble fibre content[51]. Because of this, it is possible to consider that the calculated FQ and EE in the present study is precise but may be different from other research studies that did not follow the same approach. These two adopted approaches, the *in vivo* digestibility and the use of starch to evaluate available carbohydrates, may justify the precision and agreement between DLW and food intake in the present study[52].

The difference in male and female cats in isotope concentration in body fluids is explained by the differences in body weight, which was higher in males, and the fact that a fixed dosage of isotopes was infused, with a higher dilution and lower proportional concentration in males. This, however, did not interfere in the accuracy or precision of the estimates. Currently, no distinction of sex is made for energy recommendations to cats[1]. A meta-analysis also did not suggest a sex effect in cat energy requirements[8]. In the present study, however, EE on a metabolic body weight basis was 14·2 % higher for males than for females. In a study that directly compared male with female cats during body weight loss and maintenance, male cats exhibited a general EE that was 12·3 % higher than that for females[5]. For several animal species, differences among sexes are recognised for energy requirements[53–56]. The observed differences cannot be attributed in the present study to BC, as the percentage of LM and fatty body mass was similar among males and females. In addition, on the basis of per kg of metabolic LM, the EE was 17 % higher for males, suggesting a true effect of sex.

The body water turnover reflects the balance between water inputs (water of the food, drinking water and metabolic water) and water excretions (insensible losses, urine and faecal water)[57,58]. It is an important parameter in physiology and nutritional studies. In the present study, all body fluids resulted in similar estimations of water turnover; thus, any of them could be selected for use. No differences regarding sex were observed, and the obtained values were in accordance with a previous study using ^2H and cats fed dry diets, which reported 57 ml/kg$^{0·67}$ per d[11]. Several factors, however, may interfere in water turnover, and the obtained results cannot be generalised[59,60]. Therefore, evaluating high-moisture foods (>80 %) by the ^3H method, a water flux of 126 ml/kg$^{0·67}$ per d was reported[18]. The nutrient composition of the diet also interferes, and high-protein or high-Na foods induce high urine output and water turnover in cats[61,62].

Conclusion

The ^{18}O and ^2H enrichment in body fluids can be evaluated from 2 to 8 h after subcutaneous injection, and their elimination rate from 2 to 20 d after administration with similar results, thus creating flexibility in the sampling protocol to conduct studies in owned cats. Saliva proved to be an alternative to blood to evaluate the isotopes at all sampling times, resulting in similar estimates of BC, energy expenditure and water flux. Urine, on the other hand, was adequate to evaluate basal concentration and isotope elimination but not ^{18}O and ^2H enrichment. Male cats presented higher energy expenditure than females, both considering their metabolic body weight and per metabolic lean mass basis.

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The authors declare that there are no conflicts of interest.

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