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Ingested water equilibrates isotopically with the body water pool of a shorebird with unrivaled water fluxes

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Visser, G. Henk, Anne Dekinga, Bart Achterkamp, and Theunis Piersma. Ingested water equilibrates isotopically with the body water pool of a shorebird with unrivaled water fluxes. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R1795–R1804, 2000.—We investigated the applicability of ²H to measure the amount of body water (TBW) and water fluxes in relation to diet type and level of food intake in a mollusk-eating shorebird, the Red Knot (*Calidris canutus*). Six birds were exposed to eight experimental indoor conditions. Average fractional ²H turnover rates ranged between 0.182 day⁻¹ (SD = 0.0219) for fasting birds and 7.759 day⁻¹ (SD = 0.4535) for birds feeding on cockles (*Cerastoderma edule*). Average TBW estimates obtained with the plateau method were within the narrow range of 75.9–85.4 g (or between 64.6 and 70.1% of the body mass). Those obtained with the extrapolation method showed strong day-to-day variations (range 55.7–83.7 g, or between 49.7 and 65.5%). Average difference between the two calculation methods ranged between 0.6% and 36.3%, and this difference was strongly negatively correlated with water flux rate. Average water influx rates ranged between 15.5 g/day (fasting) and 624.5 g/day (feeding on cockles). The latter value is at 26.6 times the allometrically predicted value and is the highest reported to date. Differences in ²H concentrations between the blood and feces (i.e., biological fractionation) were small but significant (–3.4% when fed a pellet diet, and –1.1% for all the other diets), and did not relate to the rate of water flux ($\chi^2_1 = 0.058$, $P < 0.81$). We conclude that the ingested water equilibrated rapidly with the body water pool even in an avian species that shows record water flux rates when living on ingested marine bivalves.

stable isotopes; water fluxes; evaporative water loss; isotope kinetics

STABLE AND RADIOACTIVE HYDROGEN isotopes have often been used to measure whole body rates of water turnover in captive and free-living animals (11, 12, 24). The application of heavy isotopes to measure water or energy metabolism is based on six assumptions originally outlined by Lifson and McClintock (7). Many of these assumptions have extensively been discussed in relation to stable isotope applications in humans (23) and free-living animals (11, 12, 24). Recently, Speakman

(24) has reopened the discussion of the assumption that the isotope concentrations in water leaving the body are the same as those in the body water at the same time. A difference between the isotope concentration between water leaving the body and the body water pool can potentially result from 1) physical fractionation effects due to a small mass difference between normal and heavy isotopes and 2) biological fractionation due to incomplete mixing of the label in the body water pool, especially at high turnover rates. The first issue can be accounted for mathematically, by separating the whole body water flux rates into one route of water loss in the form of liquid [e.g., urine, feces, and water from sweat glands, assumed not to be subject to fractionation effects, but see Wong et al. (33) for a discussion on fractionation in urine] and in the form of water vapor (e.g., breath water and transcutaneous evaporative water loss, subject to fractionation effects; Refs. 11, 12, 23, 24). In the past, the issue of incomplete mixing has received very little attention, and it is generally assumed that the mixing with the body water pool is complete at all water flux rates. However, in mice (*Mus musculus*) moderately suffering from diarrhea, it was found that fecal water had a 10% lower isotope enrichment than observed in the body water pool (9). Incomplete mixing therefore does occur, at least in sick animals. Subsequently, it has been argued by Nagy and Costa (12) that incomplete mixing of the labels might also occur in healthy birds eating bulky food with little energy, resulting in high passage rates through the gastrointestinal tract. These authors especially mentioned frugivorous birds exhibiting high food passage rates by consuming food with high water content but with low metabolizable energy content per gram food ingested (4, 6). However, direct measurements have been lacking.

The Red Knot (*Calidris canutus*) is a medium-sized shorebird (100–150 g) breeding on high-arctic tundra, where its main diet consists of surface-living arthropods such as Aranea (spiders) and larvae and adults of Diptera (2, 26). Insectivorous food is characterized by a high metabolizable energy content per gram food in-

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gested (4.9 kJ/g wet mass, which results in a water intake level of about 140 mg per kJ metabolized; 22). On the tundra, average water influx rates are about 80 g/day (T. Piersma et al., unpublished data), which is more than two times the prediction based on an allometric equation for free-living birds (13). In the non-breeding season, Red Knots inhabit coastal wetlands feeding on small bivalves that are swallowed whole with relatively large quantities of water adhered to the shell and external tissues (17). Although measurements of water flux rates are lacking during this stage, for several reasons it is very likely that they considerably exceed the highest values observed in tundra habitats. First, food intake rates are very high during the premigratory fattening phase when birds are accumulating fat (5). Second, during the winter in the European Wadden Sea, thermoregulatory costs are high as a result of low ambient temperatures and high wind speeds (31). Third, it is well known that during the winter, bivalves contain relatively little flesh (34). For example, during this period, cockles (*Cerastoderma edule*) consist of about 30% dry shell matter, 68% water, and often less than 2% ash-free dry matter, with a metabolizable energy content of about 0.3 kJ/g wet mass (15, 34). Ingestion of this type of food results in a water consumption of about 2,270 mg/kJ, which is about 16 times higher than when ingesting insect food. To cover their energy budgets under winter conditions, Red Knots have to ingest large quantities of bivalves. This would also result in the ingestion of large quantities of adhering water, which can potentially lead to exceptionally high water turnover rates. Therefore, the combination of a high energy demand and a diet with low energy content but with a high water content makes the Red Knot an ideal species to investigate whether under these circumstances stable isotopes can be used to measure whole body rates of water turnover.

In this study in the Red Knot, we critically evaluate the application of deuterium as a tracer to measure water turnover rates in relation to diet type, as well as during fasting. Measurements were made under controlled environmental conditions, on an indoor climatized intertidal flat.

METHODS

The artificial intertidal flat and roost. The experiments were performed in an indoor aviary system at The Netherlands Institute for Sea Research (NIOZ), consisting of a "tidal room" (1 × w × h: 7.3 × 8.0 × 3.0 m) separated from a "high tide" roost (1 × w × h: 4.7 × 1.1 × 2.5 m) with a sliding door. Both rooms were maintained at air temperatures between 16°C and 20°C, and a relative humidity between 55% and 75%. The tidal room contained 20-cm deep sandy sediment in which the cockles were buried. The sediment was daily submerged under 14 cm seawater ("high tide", between about 8 PM and 8 AM). In addition, all seawater was daily removed resulting in direct exposure of the sediment layer to the air ("low tide", between about 8 AM and 8 PM).

Experimental protocols. To evaluate the suitability of ^2H as a tracer to measure whole body water flux rates, six Red Knots (3 males and 3 females) were housed indoors. The birds had been captured in the Dutch Wadden Sea, 9 mo

before the start of the measurements, were accustomed to frequent handling and the captive conditions, and were fed ad libitum with food pellets (Trouvit; Trouw Nutrition, The Netherlands, containing 5.6% water, 48% crude protein, and 12% crude fat). From December 16 onward, the birds had only been fed with cockles (ad libitum), and the birds were weighed at least twice a week, at 9:00 AM. During this period, the six birds consumed on average 5,112 g/day (wet mass, i.e., $852 \text{ g} \cdot \text{day}^{-1} \cdot \text{bird}^{-1}$). Based on an average water content of 67.7% for the cockles during this specific adjustment period, it can be estimated that water influx rate was at least 577 g/day for an average Red Knot. During the initial period when they were fed on cockles only, the average body masses of the birds significantly decreased from 131.3 g (SD = 11.0) at the start to 117.5 g (SD = 8.1) on December 28 [repeated measures analysis (see below); $F_{4,20} = 39.5$, $P < 0.0001$]. From then onwards until January 5, body average mass did not change significantly (repeated measures analysis; $F_{2,10} = 2.25$, $P < 0.16$). Thereafter, birds were exposed to the following feeding regimes. In all cases, the birds were always fed a given diet for two subsequent days, the second day always being the measurement day. *Experiment 1* consisted of feeding on an artificial intertidal flat on cockles (performed on January 6; henceforth abbreviated as experiment IF-C₁). During this experiment, the birds had to probe into the sediment to obtain their food (whole cockles with attached water). *Experiment 2* consisted of feeding on the roost on cockles (January 22; R-C). During this experiment, the birds could see the individual cockles enabling them to immediately take the food items without probing. *Experiment 3* consisted of feeding on the artificial intertidal flat on open cockles that were dying because of anoxic conditions in the sediment (January 26; IF-C_a). During this experiment, the Red Knots could eat the cockle meat without probing into the soil, without ingesting the shell. *Experiment 4* consisted of feeding on the intertidal flat on cockles, similar to *experiment 1* (January 28; IF-C₂). *Experiment 5* studied the birds on the roost while fasting (January 30; R-F). *Experiment 6* studied birds feeding on the roost on pellets (February 2; R-P). During this experiment, birds were given the standard food pellets. *Experiment 7* consisted of feeding on the roost on boiled unshelled cockle meat (February 4; R-C_m). During this experiment, birds could eat the cockle meat without probing into the soil, without swallowing the shells of the cockle. *Experiment 8* consisted of fasting on the artificial intertidal flat after we had removed all cockles (February 13; IF-F). In the experiments where the birds could eat, food was available ad libitum. These treatments were designed to induce a wide range in water flux rates. This design enabled us to make the following comparisons. 1) What is the effect of location (intertidal flat vs. roost) and presence or absence of healthy cockles on the water fluxes of the birds (IF-C₂, R-C, IF-F, and R-F)? 2) When on the roost, what is the effect of food type on the water flux rate (R-C, R-P, R-C_m)? 3) When on the intertidal flat, what is the effect of food type on the water flux rate (IF-C₂ and IF-C_a)? Prior to each experiment, cockles were freshly collected from an intertidal flat in the western Wadden Sea. In all experiments, it was verified that the collected cockles were in normal winter condition (34) based on the relationship between shell length (L , mm) and the amount of ash-free dry mass of the soft parts of the cockle (AFDM, mg)

$$\text{AFDM} = 0.00262 \cdot L^{3.390} \quad (1)$$

The length of the cockles ranged between 6 and 15 mm. During the experiments, on average the cockles contained 68.6% water, 29.8% dry shell matter, and only 1.6% ash-free dry matter (as assessed from drying to constant mass; Dek-

inga et al., unpublished observations). The apparent metabolizable energy coefficient of cockle meat eaten by Red Knots was 71% (15).

Preparations before the actual experiments were similar throughout this study. The evening before each experiment, the birds were kept in the high tide roost and were fed cockles. At about 8 AM, when all cockles had been eaten, all six birds were captured and individually placed in small cardboard boxes. Prior to each experiment, of three randomly selected individuals small blood samples were taken to determine the natural abundance of ^2H (henceforth referred to as the background sample), following the procedures outlined by Piersma and Morrison (16). Briefly, the brachial vein was punctured with a sterile needle to obtain a blood sample for isotope analysis. Blood samples were stored as 15- μl aliquots in glass capillaries that were flame-sealed immediately with a propane torch. At least six capillaries were taken each time. Next, each bird was weighed on a Mettler balance (model BD202) to the nearest 0.1 g. Thereafter, at about 8:10 AM, a precisely known amount of $^2\text{H}_2\text{O}$ (range of quantities injected, 0.4–0.9 g; Merck) was injected subcutaneously prior to each experiment, using an insulin syringe that was weighed to the nearest 0.1 mg on a Mettler model AE160 balance before and after the administration. The doses were highest in the experiments IF-C₁, R-C, IF-C_d, and IF-C₂ to avoid potential problems of low isotope enrichments (relative to the background levels) at the end of the experiments. About 1 h thereafter (i.e., at 9:10 AM), during which the animals had been fasting in a cardboard box, the bird was reweighed, and a blood sample (henceforth referred to as initial blood sample) was collected as described above. The equilibration time of 1 h was used following the recommendation of Speakman for birds that weigh less than 1 kg (24, p. 242). Thereafter, the bird was released in the tidal room and subjected to the experimental diet until about 8 PM. The birds were recaptured and individually placed in cardboard boxes of 15 × 15 × 15 cm with plastic foil on the bottom. Next, the bird was weighed, and another blood sample (final sample, time exactly known) was taken from the brachial vein. We also aimed at the collection of fresh fecal samples from the birds when situated in the cardboard boxes. To this end, each bird was inspected every 2–3 min. Although we have given much care about proper collection of fecal samples, it cannot be ruled out that in some cases fecal water samples may have been diluted to a minor extent by water from the legs, feathers, or water vapor in the cardboard box. Thus sampling errors of fecal material are higher than that of blood (which could be flame-sealed immediately upon puncturing the vein). Fecal material was collected only if it was produced by the bird within 10 min from taking the final blood sample. Blood as well as fecal samples were stored in flame-sealed capillaries. Flame-sealed capillaries were stored at 4°C until analysis.

During the experiments, behavioral observations were made from a hide. Every 5 min a scan was made, and the behavior of each animal was categorized into foraging (i.e., walking and feeding), resting (i.e., standing, sleeping, and preening), and flying. For each bird and for each experiment, the activity scores were converted to individual time budgets, assuming that the activity of each scan lasted 5 min (i.e., the time between the scans). Thereafter, for each experiment, values for all birds were averaged. In addition, in experiment IF-C₁, individual birds were closely observed during 1-min periods to score the number of cockles swallowed. For each individual, at least four such observation periods were performed per hour. Obtained values on cockle ingestion rates were converted to daily intake rates, with the same assump-

tions as for the extrapolation of the behavioral data (see above).

Isotope analyses. Samples were analyzed in triplicate at the Centre for Isotope Research in Groningen. The remainder of the capillaries was stored as a backup. As a first step of the analytical procedure, the 15- μl sample in the capillary was microdistilled using a vacuum line. The water was trapped in a quartz vial placed in liquid air. Next, this water was passed over a uranium oven at 800°C and reduced to hydrogen gas, which was trapped in a quartz vial with activated charcoal placed in liquid air. Thereafter, the $^2\text{H}/^1\text{H}$ isotope ratios were determined with a SIRA 9 isotope ratio mass spectrometer (IRMS). During the analyses, we used internal standards in the form of enriched water (each analyzed in quadruplicate) that covered the expected enrichment range of the samples to test the effect of the uranium oven for each batch, as well as a set of two internal gas standards (at the background level and another with ^2H enrichment of about 0.15 atom percent) to correct for the cross-contamination between reference and sample channels of the IRMS (10). To increase the discriminative power of the comparison of the ^2H enrichment in the body water pool and fecal water in each animal, both types of samples were prepared and analyzed in the same batch. Typically, for the enriched samples the maximum difference between the triplicate ^2H values was nearly always less than 2%. If this difference was larger (always due to one “outlier”), then the three remaining capillaries were analyzed. We used this criterion over the entire range of isotope enrichments observed. For these samples, the average values were calculated as the average of the five determinations.

Fractional turnover rates. First, the measured $^2\text{H}/^1\text{H}$ isotope ratios of the background, initial, and final samples were averaged for each sample and converted to ^2H concentrations (henceforth abbreviated as C_b , C_i , and C_f , respectively, atom percent). Next, the fractional ^2H turnover rates (k_d , fraction/day) were calculated for each measurement with

$$k_d = 1/t \cdot \ln [(C_i - C_b)/(C_f - C_b)] \quad (2)$$

where t stands for the elapsed time interval (days) between taking the initial and final sample (7).

Total body water estimates. The size of the body water pool (TBW, g) was calculated for each bird and for each experiment on the basis of the principle of isotope dilution, i.e., on the basis of the determination of the hydrogen dilution space. The quantity (Q_d , mol) and the ^2H concentration (C_d , atom percent) of the dose are known, as well as the ^2H concentration in the bird's body water pool prior to the administration (C_b , for each experiment taken as the average value of the three birds sampled prior to the experiment) and the measured ^2H concentration after the administration (C_i)

$$\text{TBW}_{\text{plateau}} = 18.02 \cdot Q_d \cdot (C_d - C_i)/(C_i - C_b) \quad (3)$$

This method has been referred to as the plateau method (1, 24). It has to be noted that prior to all experiments, ^2H background levels were identical to those observed at the start of experiment IF-C₁. Next, for each measurement, the k_d value was used to calculate the back-extrapolated initial enrichment at the time of the injection ($C_{i,\text{extr}}$, see Ref. 24). This extrapolation procedure is meant to correct for the isotope losses during the equilibration phase. It is assumed that the rate of isotope loss during equilibration is the same as during the experimental period. This extrapolated value was also used to calculate the amount of body water (extrap-

olation method)

$$TBW_{\text{extrapolation}} = 18.02 \cdot Q_d \cdot (C_d - C_{i,\text{extr}}) / (C_{i,\text{extr}} - C_b) \quad (4)$$

Water efflux rates. Because none of the animals were in a steady state with respect to the amount of body mass (i.e., body water), first, water efflux rates (uncorrected for fractionation effects; rH_2O_{unc} in $\text{ml} \cdot \text{day}^{-1} \cdot \text{individual}^{-1}$) were calculated with Eq. 4 from Nagy and Costa (12), which takes into account changes in the size of the water pool

$$rH_2O_{\text{unc}} = (TBW_f - TBW_i) \cdot \ln \{[(C_i - C_b) \cdot TBW_i] / [(C_f - C_b) \cdot TBW_f]\} / [\ln (TBW_f / TBW_i)] \cdot t \quad (5)$$

where TBW_i and TBW_f represent the amount of body water at the start and end of the measurement, respectively (g). The TBW_i value was calculated on the basis of isotope dilution. As we found that the plateau method yielded the most reliable estimate for the amount of body water (see RESULTS), these estimates were taken for the body water pool to calculate water efflux rates. The size of the body water pool at the end of each experiment (TBW_f , g) was estimated by multiplying the final body mass by the ratio of the initial amount of body water to initial body mass. By using this method, we implicitly assumed that the percentage of body water did not change during the measurement.

Second, a correction was made for isotope fractionation effects due to evaporative water loss

$$rH_2O \text{ efflux} = rH_2O_{\text{unc}} / (x f_1 + 1 - x) \quad (6)$$

(Ref. 24, equation 7.6), where x represents the proportion of the water flux lost through evaporative pathways, and f_1 is the fractionation factor [taken as 0.94, as recommended by Speakman (Ref. 24, p. 107)]. Rather than assuming a fixed proportion of evaporative water loss relative to the total water efflux for all experiments [as applied by Lifson and McClintock (7), who took a fraction of 0.5 for all measurements], we assumed a constant rate of evaporative water loss of 10 g/day for all experiments was determined for the Red Knot in another study (27). This value is close to the predicted value of 7.9 g/day for a 125-g bird based on a general allometric relationship for birds (32). Although these assumptions have relatively little effect on the water flux estimates (see below), for the following reasons we feel that the assumption of a constant evaporation rate is much more realistic than the assumption of a fixed fractional evaporative water loss, especially at high water intake rates [see also Speakman's discussion (24)]. First, the experiments took place in climatized indoor rooms with little variations with respect to air temperature and relative humidity. Second, it is hard to imagine that a 125-g bird feeding on bivalves is able to evaporate 50% of its water efflux (i.e., about 300 g/day, on the average). Fortunately, an error analysis reveals that the effects of these assumptions are relatively small. For example, if the uncorrected water efflux rate is 600 g/day, then the assumption of an evaporative water loss of 10 g/day would result in a corrected water efflux rate of 600.6 g/day. However, the assumption of an evaporative water loss rate of 300 g/day would result in a corrected water efflux rate of 618.6 g/day, which is only 3% higher than the lowest estimate. Water influx rates (rH_2O influx, ml/day per bird) were calculated using Eq. 6 (12) and the fractionation adjusted water efflux rates

$$rH_2O \text{ influx} = rH_2O \text{ efflux} + (TBW_f - TBW_i) / t$$

Statistics. Because the Red Knot is a social species, we decided to expose all birds to the same experimental diet at

the same time (nonrandomized design). Data on body mass, fractional turnover rates, and water fluxes were analyzed using the "repeated measures" procedure in the "general linear model" option of SPSS 9.0. As a first step in each analysis, we assessed the general effect of all experiments as a within-subject factor. Next, we more specifically tested the effect of each experiment against the other experiments by using the "contrast = simple" option. Because of the fact that experiments were done with six birds (on five in experiment R-F), a maximum of five different experiments could be examined in a single test (a maximum of four experiments, if R-F was included for comparison). Therefore, tests were performed to address the three different issues outlined in the *Experimental protocols* above. Data on percentages of body water and percentages of time spent foraging were analyzed following arcsine-square root transformation (14). We used a hierarchical linear model to evaluate the difference in 2H enrichment between fecal water and body water in relation to the water efflux rate. This is a special type of regression model that is designed for a data set with a hierarchical structure (individual observations nested in experimental treatments). First, the deviance value for the null model was determined (difference in 2H enrichment between fecal water and body water is constant). Next, the significance of water flux and experimental treatments was evaluated in a stepwise procedure. To determine statistical difference, we tested the change in the deviance value with a χ^2 test (8). Analysis was performed with the computer program ML3 (21).

RESULTS

Changes in body mass. At the start of the first experiment (IF-C₁), the average initial body mass was lowest (108.2, SD = 7.38) g (Table 1; Fig. 1). A general repeated measures analysis revealed that there were significant differences in body masses between the four experiments conducted on the intertidal flat (i.e., IF-C₁, IF-C_d, IF-C₂, and IF-F; $F_{3,15} = 48.83$, $P < 0.0001$). A more specific repeated measures analysis revealed that the average initial body mass of the IF-C₁ experiment (that was performed first) was significantly lower than IF-C_d ($F_{1,5} = 28.16$, $P < 0.0001$), IF-C₂ ($F_{1,5} = 207.95$, $P < 0.0001$), and IF-F ($F_{1,5} = 57.99$, $P < 0.0001$, see also Fig. 1), which were performed later. In addition, repeated measures analysis revealed that initial body masses of the four experiments on the roost were not significantly different ($F_{3,12} = 2.58$, $P < 0.10$).

Total body water estimates. For each individual and for each experiment, the amount of body water was estimated with the plateau method as well as with the extrapolation method. In all cases, average estimates of the body water pool size were the highest using the plateau method (Table 1; Fig. 1). Average differences between both methods were smallest when the birds were fasting on the roost (R-F; 0.6% difference) and highest when feeding on cockles on the intertidal flat (IF-C₁; 36.3% difference). We compared the percentages of body water of the birds at the start of the four experiments on the intertidal flat obtained from isotope dilution experiments (plateau method). Repeated measures analysis revealed a significant effect of the experiment ($F_{3,15} = 8.73$, $P < 0.001$). A more specific

Table 1. *Experimental values*

Experiment	BM _{start} , g	BM _{avg} , g	TBW _{pl} , g	TBW _{extr} , g	k _d , day ⁻¹	Water Efflux, g/day	Water Influx, g/day
IF-C ₁	108.2(7.38)	114.6(8.59)	75.9(4.30)	55.7(3.04)	7.759(0.4535)	604.5(59.81)	624.5(64.60)
R-C	122.7(7.61)	124.8(8.11)	81.5(4.72)	69.6(3.33)	4.067(0.9487)	334.3(89.20)	340.6(92.94)
IF-C _d	120.1(7.65)	122.5(8.57)	80.1(4.95)	63.7(3.34)	5.710(0.9032)	464.8(96.62)	472.3(102.2)
IF-C ₂	127.5(7.46)	130.9(7.45)	85.4(5.36)	63.4(5.73)	6.335(0.7524)	543.2(58.2)	553.2(61.78)
R-F	127.8(7.65)	124.4(7.23)	84.1(4.71)	83.7(4.65)	0.182(0.0219)	26.0(3.28)	15.5(2.08)
R-P	127.0(7.48)	131.8(7.95)	85.2(2.87)	78.8(3.45)	1.876(0.2388)	152.2(18.33)	166.6(20.84)
R-C _m	127.2(7.26)	136.9(8.48)	83.5(3.80)	73.5(4.03)	2.906(0.3466)	229.9(21.80)	257.0(25.10)
IF-F	124.1(5.32)	123.1(5.64)	80.2(4.37)	76.5(3.43)	1.547(0.3215)	127.3(33.28)	124.6(32.01)

Values are means (SD are in parentheses) for body mass at the start of each experiment (BM_{start}), body mass during the experiment (BM_{avg}), the estimated amount of body water using the plateau method (TBW_{pl}), the estimated amount of body water using the extrapolation method (TBW_{extr}), fractional ²H turnover rate (k_d), water efflux rate (g/day), and water influx rate (g/day). In all experiments, the same 6 birds were used, except in experiment R-F (5 birds). See METHODS for complete description of experimental groups.

test revealed that the percentage of body water at the start of experiment IF-C₁ was significantly higher than the value of experiment IF-C_d (F_{1,5} = 16.33, P < 0.001), and IF-F (F_{1,5} = 15.02, P < 0.012), and almost significantly higher than the value for IF-C₁ (F_{1,5} = 5.76, P < 0.062). On the basis of a significantly lower body mass and a significantly higher percentage of body water at the start of the experiments IF-C₁, we conclude that these birds were in a relatively poor body condition. It is likely that the period during which we allowed the birds to adjust to a cockle diet may have been too short (20). Consequently, we will use only the values obtained from IF-C₂ for comparison with the other experiments. A similar comparison of the percentages of body water was made for the values at the start of the four experiments on the roost. Repeated measures analysis revealed that the values for these experiments did not differ significantly (F_{3,12} = 1.78, P < 0.21).

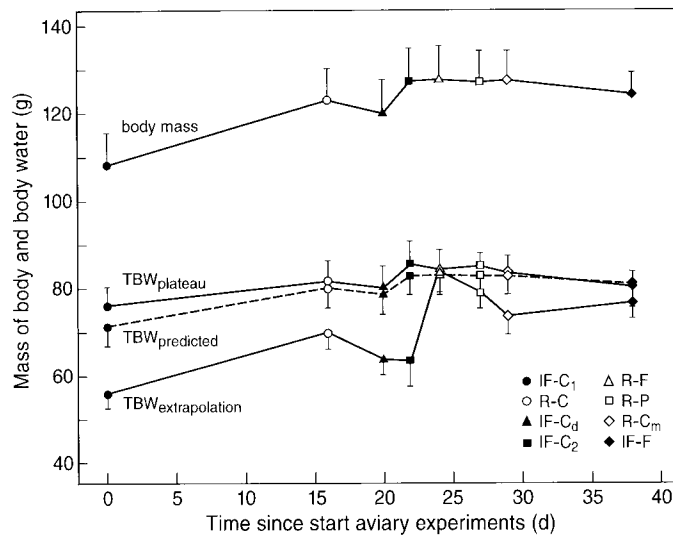


Fig. 1. Relationship between average body mass (g, and SD), the average amount of body water estimated with the plateau method (TBW_{plateau}, g, and SD), the average amount of water estimated with the extrapolation method (TBW_{extrapolation}, g, and SD), and the time since the start of the experiments. The broken line represents the predicted average amount of body water based on carcass data (TBW_{predicted}, g, and SD). See *Experimental protocols* in METHODS for complete description of groups.

When using the extrapolation method, it was found that the development of the estimated size of the total body water pool with time was less constant than when using the plateau method (Fig. 1). For example, there is a sudden change in the pool size from experiment IF-C₂ to R-F, where the size of the pool increased from 63.4 g (49.7% of the initial body mass) to 83.7 g (64.9%), in the absence of any change in body mass. In fact, values for the amount of body water below 50% are hardly ever observed in shorebirds, and only so in birds that have very large amounts of (visible) fat (3, 15).

The average total body water estimates from isotope dilution obtained with both methods (plateau and extrapolation) for the eight experiments were compared with a predictive equation based on direct measurements of actual water content of dried Red Knot carcasses [amount of body water (g) = 0.592 × body mass (g) + 7.302, r² = 0.84, n = 9, range in body mass 89.0–130.2 g; Fig. 1]. The eight average estimates obtained with the plateau method were on average 2.3% higher than derived from the predictive equation (SD = 2.05; range from 0.7% below to 6.3% above prediction; Fig. 1), and those obtained with the extrapolation method were 12.3% lower than predicted (SD = 8.70; range from 23.4% below to 0.8% above prediction). In the literature, it has been reported that estimates for the amount of body water obtained with ²H dilution are slightly higher than those obtained from dried carcasses (by about 4%; for a recent review, see Ref. 17). Apparently, ²H not only mixes with the body water pool, but it also disappears, to a minor extent, in the protein pool.

We calculated the ratio of the dilution space obtained with the extrapolation and plateau method (Fig. 2). As expected for theoretical reasons, this ratio is negatively related to the fractional turnover rate. Small deviations from the general pattern are caused by small differences in the duration of the equilibration period between the different experiments. Because all birds were subject to the same feeding schedule prior to taking the initial blood sample, in principle all birds should have the same rate of isotope loss during the equilibration period. However, to estimate the rate of isotope loss during the equilibration period, fractional turnover rates are obtained from experimental periods

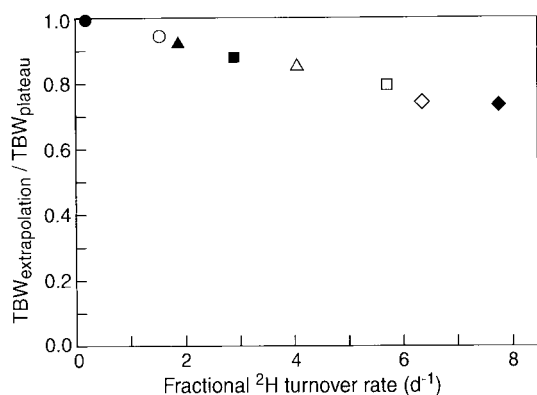


Fig. 2. Relationship between the ratio between $TBW_{\text{extrapolation}}$ (g) and TBW_{plateau} (g) and the fractional 2H turnover rate (average values are plotted for each experiment). For explanation of the symbols, see Fig. 1.

showing tremendous variation between experiments. This obviously results in biased estimates when using the extrapolation method. We conclude that the total body water estimates obtained with the plateau method are most reliable, and therefore these have been used for all subsequent calculations of the water efflux rates.

Behavioral observations. In all experiments, birds behaved normally and never showed signs of stress. In all experiments, the birds spent very little time flying (Table 2). To investigate the effect of location (intertidal flat vs. roost) and presence (normal cockles) or absence of food, percentages of foraging time were compared between R-C, IF-C₂, R-F, and IF-F (Table 2). The repeated measures analysis revealed a significant effect of location ($F_{1,4} = 65.43$, $P < 0.001$, percentage of foraging time is higher on the intertidal flat) and presence or absence of food ($F_{1,4} = 58.97$, $P < 0.002$, percentage of foraging time higher in the presence of food). The effect of the interaction term (location \times presence/absence of food) was not statistically significant ($F_{1,4} = 1.68$, $P = 0.264$). In addition, in birds feeding on the roost, we investigated the effect of food type (R-C, R-P, R-C_m) on the percentage of foraging time. A general repeated measures analysis revealed a significant effect of food type ($F_{2,10} = 7.17$, $P < 0.009$). A more specific analysis revealed significant differences between R-C and R-P ($F_{1,5} = 8.40$, $P < 0.034$) and between R-P and R-C_m ($F_{1,5} = 31.15$, $P < 0.002$), but not between R-C and R-C_m ($F_{1,5} = 0.23$, $P < 0.65$). A comparison between animals feeding on the intertidal flat (IF-C_d and IF-C₂) revealed that the foraging time was significantly higher for IF-C₂ ($F_{1,5} = 14.24$, $P < 0.013$). For experiment IF-C₁, visual observations revealed that the Red Knots consumed on average $2,253 \text{ cockles} \cdot \text{day}^{-1} \cdot \text{bird}^{-1}$ (SD = 747.6). After assuming for this experiment an average cockle mass of 0.322 g and a water content of 68.6%, this would result in an estimate for the water intake rate of $497.7 \text{ g} \cdot \text{day}^{-1} \cdot \text{bird}^{-1}$.

Fractional turnover rates of 2H . Average fractional turnover rates ranged from 0.182 day^{-1} (SD = 0.0219)

for experiment R-F to 7.759 day^{-1} (SD = 0.4535) for experiment IF-C₁. A comparison between the experiments R-C, IF-C₂, R-F, and IF-F revealed a significant effect of location (intertidal flat vs. roost, $F_{1,4} = 24.6$, $P < 0.008$) and presence or absence of cockles ($F_{1,4} = 198.83$, $P < 0.0001$). The effect of the interaction term between the two factors was not significant ($F_{1,4} = 3.19$, $P < 0.15$). When fed on the roost (i.e., experiments R-C, R-P, and R-C_m), repeated measures analysis revealed a significant effect of experiment ($F_{2,10} = 16.52$, $P < 0.01$). A more specific test revealed significant differences between R-C and R-P ($F_{1,5} = 22.16$, $P < 0.005$), R-C and R-C_m ($F_{1,5} = 6.91$, $P < 0.047$), and R-P and R-C_m ($F_{1,5} = 92.07$, $P < 0.001$). There were no significant differences in fractional turnover rates between experiment IF-C_d and IF-C₂ ($F_{1,5} = 2.08$, $P < 0.209$).

Water flux rates. In all experiments, except those during which the birds were fasting, body mass increased during the trials. Therefore, water efflux rates were lower than the influx rates (Table 1). To investigate the effect of location (intertidal flat vs. roost) and presence (normal cockles) or absence of food, water efflux rates were compared between R-C, IF-C₂, R-F, and IF-F. The repeated measures analysis revealed a significant effect of location ($F_{1,4} = 31.29$, $P < 0.005$, water efflux rates higher on the intertidal flat) and presence or absence of food ($F_{1,4} = 144.58$, $P < 0.0001$, water efflux rates higher in the absence of food). The effect of the interaction term (location \times presence/absence of food) almost reached statistical significance ($F_{1,4} = 7.35$, $P < 0.053$). The latter result may indicate that the effect of fasting is stronger on the roost than on the intertidal flat. It is likely that during experiment IF-F, water is ingested during probing in the soil, then subsequently excreted.

When fed on the roost, water efflux rates were compared between R-C, R-P, and R-C_m (see Table 1 for average values for each experiment). Repeated measures analysis revealed that there was a significant effect of the experimental treatment ($F_{2,10} = 13.62$, $P < 0.0001$). A more specific analysis revealed that water efflux rates differed significantly between R-C and R-P ($F_{1,5} = 17.75$, $P < 0.008$), between R-C_m and R-P ($F_{1,5} = 52.55$, $P < 0.001$), and almost significantly between R-C and R-C_m ($F_{1,5} = 6.44$, $P = 0.052$). Thus

Table 2. Percentage of time the birds spent foraging, resting, and flying

Experiment	Foraging, %	Resting, %	Flying, %
IF-C ₁	88.3(11.49)	11.7(11.49)	0
R-C	30.9(16.71)	69.2(16.71)	0
IF-C _d	94.4(5.49)	5.6(5.49)	0
IF-C ₂	77.7(18.05)	22.3(18.05)	0
R-F	3.8(3.25)	96.1(3.37)	0.1(0.19)
R-P	9.3(0.37)	90.7(3.71)	0
R-C _m	40.3(14.01)	59.4(13.80)	0.3(0.05)
IF-F	73.7(3.96)	26.0(3.87)	0.3(0.38)

Values are means with SD in parentheses. In all experiments, the same 6 birds were observed, except in experiment R-F (5 birds). See METHODS for complete description of experimental groups.

water efflux rates were significantly lowest during experiment R-P and were significantly higher during the experiments R-C and RC_m.

When fed on the intertidal flat, average water efflux rates were 464.8 (SD = 96.62) and 543.2 (SD = 58.2) g/day for experiments IF-C₁ and IF-C₂, respectively, which was not significantly different (repeated measures analysis: $F_{1,5} = 5.15$, $P < 0.072$).

For experiment IF-C₁, the estimate for water influx rate based on the stable isotope method (624.5 g/day) is considerably higher than that based on behavioral observations on cockle ingestion rates (497.7 g/day). However, the isotope method has revealed that during foraging on a sediment without food items (IF-F), water influx rate is 124.6 g/day. By adding this foraging mode-related water influx rate to the estimate based on cockle ingestion rates, we obtain a water influx estimate of 622.3 g/day, which is very close to the value for experiment IF-C₁ obtained with the stable isotope method. However, we have to note that the time the birds spent foraging is slightly higher for experiment IF-C₁ than for experiment IF-F. We also like to mention here that the high level of water intake during foraging in the absence of any prey items makes it very difficult to estimate rates of food intake based on estimates of water influx rates.

Is ^2H of the excreted water in equilibrium with the body water pool? At the end of experiments IF-C₁, R-C, R-F, R-P, and IF-F, samples were obtained from both blood and fecal water to evaluate whether excreted water and body water were in equilibrium at the end of the experiments. On the average, ^2H enrichment in fecal water was 1.7% (SD = 1.69) lower than that of the body water. For experiments IF-C₁, R-C, R-F, R-P, and IF-F, the average values for ^2H enrichments in fecal water were 0.74% (SD = 1.13, $n = 6$), 1.6% (SD = 1.48, $n = 6$), 2.4% ($n = 1$), 3.4% (SD = 1.96, $n = 6$), and 0.9% (SD = 1.03, $n = 6$) lower than those in the body water pool, respectively (Fig. 3). A hierarchical linear model analysis was performed to evaluate for the five exper-

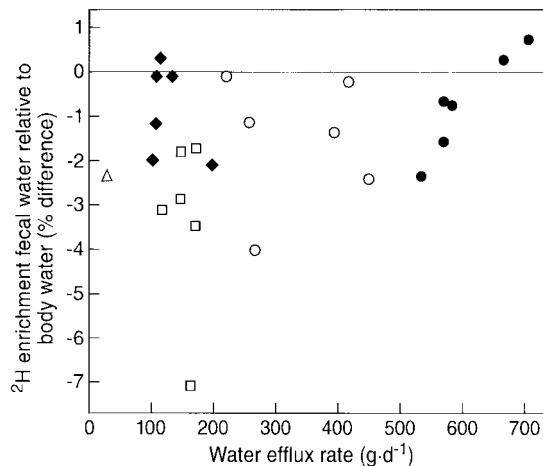


Fig. 3. The difference in ^2H enrichment of fecal water and body water (atom percent ^2H enrichment relative to the background level) in relation to the water efflux rate of each individual. For explanation of the symbols, see Fig. 1.

iments the difference in ^2H enrichment between fecal water and body water (Y , %) in relation to the water efflux rate (X , ml/day). As a first step, we determined the “deviance” value for the null model (Y is constant). The analysis revealed that the constant value (-1.6% , $SE = 0.38$) differed significantly from zero ($P < 0.01$, deviance value of the null model 96.70). Next, we included the water efflux rate in the model, which did not significantly improve the explained variance of the model (deviance value 92.96, change from null model not significant, $\chi^2_1 = 2.73$, $P < 0.098$). However, the fit of the model significantly improved after separating the effects for experiment R-P (constant value -3.4% , $SE = 0.88$) and all other experiments [constant value -1.1% , $SE = 0.40$, deviance value of entire model 82.32 (referred to as *model 1*), change in deviance between null model and *model 1* was statistically significant, $\chi^2_4 = 13.38$, $P < 0.01$]. As a last step, we evaluated whether the fit of *model 1* would improve after adding the water efflux rate to this model. The analysis revealed that this was not the case (deviance 82.26, change in deviance from *model 1* not significant, $\chi^2_1 = 0.058$, $P < 0.81$). In conclusion, over the observed range of water efflux rates (from 29.3 to 664.5 g/day), there is no effect of water flux on the difference in ^2H enrichment between fecal water and body water. However, there was a general difference of -1.1% for the experiments IF-C₁, R-C, R-F, and IF-F and a difference of -3.4% for experiment R-P.

DISCUSSION

Water flux rates. This study has revealed that average water influx rates range from a low 15.5 g/day when Red Knots are fasting on the roost (R-F) to a high of 624.5 g/day when they feed on cockles on the indoor intertidal flat (IF-C₁); corresponding average values for water efflux rates range from 26.0 to 604.5 g/day. It has also been shown that water influx rates based on stable isotope measurements are in good agreement with estimates based on behavioral observations (for experiment IF-C₁) and are in the same range as estimated for the birds fed with cockles prior to the experiments (see METHODS).

The water influx value observed in fasting birds (R-F) is 0.62 times the predicted value on the basis of the allometric relationship for captive birds having access to food ad libitum (average body mass of fasting Red Knots = 124.4 g; Ref. 13). The highest observed value for water influx rate is 26.6 times the predicted value for captive birds (average body mass 114.6 g), or 17.0 times the value predicted for free-living birds. The latter value is by far the largest relative value observed in birds and is much higher than the 95% upper confidence limit of 3.5 times the prediction (13). The highest relative values reported thus far are based on experiments on adrenalectomized domestic ducks (*Anas platyrhynchos*) yielding water flux rates of 8 times the predicted levels (25).

The average water influx rate in incubating wild Red Knots on the arctic tundra is about 80 g/day, with a

lowest value of 15 g/day during an incubation spell (T. Piersma et al., unpublished data). This minimum value is at about the same level as during our measurements on fasting animals in captivity. Maximum observed water influx rates in free-living Red Knots on the tundra (150 g/day) are much lower than maximum rates of captive Red Knots feeding on cockles in winter condition. The diet of the arctic Knots consists of arthropods that are taken from the surface of the tundra (26, and T. Piersma et al., unpublished data). The very high water efflux rates observed in captive Red Knots may have been caused by 1) a relatively high water intake because of its foraging mode, 2) the large amount of water attached to the bivalves, and 3) the fact that the cockles were in winter condition, which forced the Red Knots to ingest many prey items to make up their daily energy budget. On the basis of an average water content of the cockles of 68.6% (see METHODS), an ash-free dry matter content of 1.6% (with an average energy density of 23 kJ/g; Ref. 15), and an assimilation efficiency of 71% (15), it can be calculated that the food contains about 2,625 mg water per kJ metabolized energy. This value is very high compared with the value of 140 mg water/kJ for an average insectivorous diet (22). It is very likely that water fluxes in wild Red Knots in the Wadden Sea at least approach the maximum levels observed in captivity, especially during periods of high energy demand [e.g., when thermoregulatory costs are high (31) or when fuelling before take off on long-distance flights (18)].

Implications of high water fluxes for estimating of the amount of body water. The principle of isotope dilution has been used to calculate the amount of body water. When using the plateau method, it is assumed that all isotopes remain in the body water pool between injection and the taking of the initial blood sample (Fig. 4, the level used to calculate the amount of body water has been labeled with "A"). If some isotopes do leave from the pool, however, then this would result in an underestimation of the isotope enrichment after the equilibration period and, consequently, in a systematic overestimate of the amount of body water. The extrapolation method has been developed to correct for the isotope losses during the equilibration period, and it is generally assumed that the rates of isotope loss during the equilibration period and the measurement period are the same (24). In our study, all experimental birds were subject to the same feeding schedule prior to taking the initial blood sample. Only thereafter were the birds subjected to the different feeding experiments. During the equilibration period prior to all experiments, the fractional ^2H turnover rates may probably best be estimated from the fasting birds (i.e., from experiment R-F, Fig. 4, the level used to calculate the amount of body water has been labeled with "B"), instead of taking separate estimates obtained from each experiment. A very large error will be made if the extrapolated values are used for birds with the highest fractional turnover rates (i.e., values taken from experiment IF-C₁, the level used to calculate the amount of body water has been labeled with "C"). Therefore, the

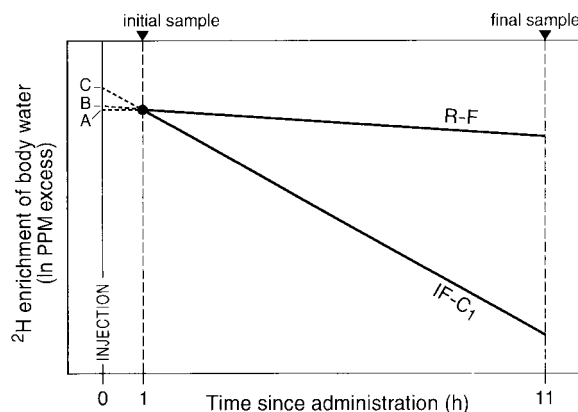


Fig. 4. The principle of isotope dilution to calculate the amount of body water in the Red Knot. The two solid lines between "initial sample" and "final sample" represent the average time courses of ^2H for the average birds during experiment IF-C₁ (obtained from birds feeding on cockles on the mudflat) and experiment R-F (obtained from birds fasting on the roost). Three different assumptions have been made to calculate the amount of body water: "A" is plateau method (assuming no loss of isotopes between injection and the taking of the initial sample), "B" is extrapolation method using average fractional turnover rates obtained from the birds when fasting (assuming that the isotope loss during equilibration is identical as the level determined during R-F), and "C" is extrapolation method using average fractional turnover rates from the birds when feeding on the intertidal flat (IF-C₁).

plateau method to estimate the amount of body water gave superior results compared with the extrapolation method (Fig. 1). Another major advantage of the plateau method is that, once equilibration has been completed, the time interval for taking the initial sample is of less importance. The reason for this is the very low rate of decrease of the ^2H concentration in a fasting bird (e.g., see the values for R-F). In contrast, when applying the extrapolation method at high turnover rates, the timing of taking the initial sample is most crucial, and it should be exactly at the time that equilibration has been completed for each individual. However, isotope equilibration curves can differ substantially between individuals, possibly due to individual differences in blood circulation (24). In addition, differences between birds with respect to the size of the fat deposits in the abdominal region may result in small differences in the administration of isotopes and, consequently, the rate of exchange between intraperitoneal water (where the isotopes are injected) and the blood. This will magnify the individual differences in equilibration curves. Therefore, given all uncertainties with respect to the individual equilibration curves, we recommend to use the plateau method for calculating the amount of body water.

Implications of high water fluxes for isotope mixing in the body water pool. To determine fractional ^2H turnover rates, it has always been assumed that the ingested water equilibrates with the body water pool during the experiments: "water lost from the body water has specific activities equal to that of the body water" (Ref. 7, assumption 4). In the past, the issue of incomplete mixing has received very little attention,

although in mice moderately suffering from diarrhea it was found that fecal water had a 10% lower isotope enrichment than observed in the body water pool (9). On the basis of this finding, it has been argued that incomplete mixing of the labels might also occur in healthy birds having high food passage rates (12). In their methodological report, Nagy and Costa (12) especially mentioned frugivorous birds exhibiting high food passage rates [like the Phainopepla (*Phainopepla nitens*)] and birds regurgitating food for their young, but they concluded that experimental data were lacking. The measured water flux rate of the Phainopepla is about twice the value allometrically predicted for free-living birds (13, 30). Similarly, water flux levels of Red Knots during their arctic breeding stage are at two times the allometrically predicted level. The highest average water efflux rates of our captive Red Knots, however, were another eight times higher. We showed that ^2H concentrations differed little but significantly between fecal water and body water (fractionation) and that this difference was insensitive to the level of water flux (even up to 665 g/day). It is well possible that Red Knots feeding on bivalves exhibit morphological adaptations of the gastrointestinal tract to facilitate a rapid food absorption at high passage rates. This may also have facilitated the rapid uptake of ingested water, resulting in a complete equilibration. Possibly, such morphological adjustments are also made by frugivorous birds (4, 6, 29). In the past, it has been assumed that urine and fecal water did not fractionate (7). However, Wong et al. (33) found a 0.7% difference between human urine and blood plasma, possibly as a result of some hydrogen exchange between urine and water in the bladder. In our birds, for most diets, we found a difference of -1.1% and a significantly lower value for birds fed a pellet diet (R-P). Unfortunately, biological fractionation issues have only been addressed in mammals (including humans), and data for birds are clearly lacking. It is well possible that the longer food passage time through the avian gut for the pellet diet resulted in an elevated level of bacterial hydrogen exchange in the hindgut.

Implications of high water fluxes for the calculation of the rate of CO_2 production in doubly labeled water experiments. The rate of CO_2 production can be calculated from differential elimination rates of ^2H and ^{18}O (7). For some processes, heavy isotopes exhibit slightly different physical properties compared with the light isotopes (fractionation effect). Therefore, the route of water loss has to be separated into a route subject to fractionation (i.e., evaporation), and a route not subject to fractionation [i.e., production of urine and feces (7)]. Lifson and McClintock (7) have assumed a fractional evaporative water loss of 0.5 for birds and mammals, a value that has been applied widely (24). However, Speakman (24) has reopened the issue of evaporative water loss in free-living animals and concluded that a value of 0.25 is more appropriate. In a recent doubly labeled water (DLW) validation study in growing shorebird chicks, Visser and Schekkerman (28) have demonstrated the importance of assumptions concern-

ing evaporative water loss. In this study, it was found that the best fit of the DLW method was obtained at a fractional evaporative water loss value of 0.13. The DLW method tended to underestimate the true rate of CO_2 production by 9% after assuming a fractional evaporative water loss value of 0.5. For the adult Red Knots of this study, after assuming a constant evaporative water loss of 10 g/day (27), it can be calculated that fractional evaporative water loss in captive Red Knots might have ranged between 1.7% in experiment IF-C₁ (when feeding on cockles on the intertidal flat) to 38.5% in experiment R-F (when fasting on the roost). In addition, in fed animals fractional evaporative water loss might have been related to the type of diet and might have ranged from 1.7% in experiment IF-C₁ to 6.6% in experiment R-P. Clearly, all these estimates are considerably lower than the fraction of 0.25 proposed by Speakman (24). At high water flux rates, the relative difference between ^{18}O and ^2H turnover is only small. Under these conditions, small alterations in the assumptions concerning evaporative water loss have a major impact on the calculated rates of carbon dioxide production. Therefore, we feel that validation studies to unravel the effects of diet type on the application of the DLW method are necessary complements to studies on the energetics of species that are likely to have high water-turnover rates.

Perspectives

Stable isotopes have frequently been used to measure some physiological processes in nonrestrained animals and humans (e.g., rates of water flux or levels of energy expenditure). The calculations for these physiological parameters have been based on mathematical models with some specific assumptions [e.g., no isotope fractionation or a level of evaporative water loss of 50% of the total efflux (7, 11, 24)]. Initially, the measurements on water fluxes have been focused on interspecific comparisons to reveal differences in relation to taxon and habitat [see for example the classic review by Nagy and Peterson (13) on water fluxes in free-living animals]. At a later stage, more emphasis was given on comparisons between and within individuals to reveal individual or seasonal adaptation processes (13, 24). It becomes clear that the results of these comparisons are very sensitive to the validity of some specific assumptions of the mathematical models used. We have demonstrated in the Red Knot that under controlled conditions, avian water fluxes can be considered to be a function of the birds' diet and that individual maximum levels can be 40 times higher than their minimum levels. These diet-induced differences in water efflux will result in differences in fractional evaporative water loss rates. We have argued that these differences have relatively little effect on the calculated water fluxes. However, differences in fractional evaporative water losses will subsequently have a major impact on the accuracy of the DLW method to measure rates of CO_2 production (see also Ref. 28). To examine this effect in more detail, validation experi-

ments are urgently required in which individual birds are repeatedly exposed to different diets that result in large differences in water flux.

Berthe Verstappen and Trea Dijkstra determined the ^2H enrichments. Dick Visser drew the figures. Dr. Maarten Loonen was of great help with the statistical analyses of the data. Dr. C. C. Peterson and two anonymous referees made many valuable suggestions to improve the manuscript.

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