# METHODS

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# **Deuterium stable isotope ratios as tracers of water resource use: an experimental test with rock doves**

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Abstract Naturally-occurring deuterium stable isotope ratios can potentially be used to trace water resource use by animals, but estimating the contribution of isotopically distinct water sources requires the accurate prediction of isotopic discrimination factors between water inputs and an animal's body water pool. We examined the feasibility of using estimates of water fluxes between a bird and its environment with a mass-balance model for the deuterium stable isotope ratio of avian body water ( $\delta D_{body}$ ) to predict isotopic discrimination factors. Apparent fractionation and thus discrimination factors were predicted to vary with the proportion of an animal's total water losses than could be attributed to evaporative processes. To test our ability to predict isotopic discrimination, we manipulated water intake and evaporative water loss in rock doves (Columba *livia*) by providing them with fresh water or 0.15 M NaCl solution in thermoneutral or hot environments. After we switched the birds from drinking water with  $\delta D = -95\%$ VSMOW (Vienna Standard Mean Ocean Water) to enriched drinking water with  $\delta D = +52\%$  VSMOW, steady-state  $\delta D_{body}$  was approached asymptotically. The equilibrium  $\delta D_{body}$  was enriched by 10-50% relative to water inputs. After isotopic equilibrium was reached, the degree of enrichment was positively related ( $r^2=0.34$ ) to the fraction of total water loss that occurred by evaporation

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*Present address:* A. E. McKechnie School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Private Bag 3, Wits, 2050, South Africa  $(r_{evap}/r_{H_2O})$  supporting the major prediction of the model. The variation we observed in discrimination factors suggests that the apparent fractionation of deuterium will be difficult to predict accurately under natural conditions. Our results show that accurate estimates of the contribution of different water sources to a bird's body water pool require large deuterium isotopic differences between the sources.

**Keywords** Evaporative water loss  $\cdot$  Isotopic discrimination  $\cdot$  Isotopic tracers  $\cdot$  Mass-balance model  $\cdot$  Water fluxes  $\cdot$  Water resources  $\cdot \delta D$ 

# Introduction

Terrestrial environments demand that animals obtain sufficient water to maintain homeostasis despite large evaporative and excretory losses. Because water is often a potentially limiting resource for animals in many environments, understanding how animals acquire and use water is a prerequisite to understanding many aspects of their ecology, as well as for predicting shifts in animal distribution associated with climate change (Wolf 2000). Water-related constraints on animal function are especially pronounced in arid ecosystems, where desiccating environmental conditions often coincide with a scarcity of water resources. Over the last few decades, plant physiological ecologists have taken significant advantage of naturally occurring differences in the oxygen and hydrogen isotope ratios ofwater resources to trace various aspects of water use in a score of plant species and communities (e.g., Lin et al. 1996; Williams and Ehleringer 2000; Zencich et al. 2002; Snyder and Williams 2003). Mixing models of the form

 $\delta D_{\text{tissues}} = p \delta D_{\text{source 1}} + (1-p) \delta D_{\text{source 2}}$ 

where  $\delta D_{\text{source 1}}$  and  $\delta D_{\text{source 2}}$  are the delta ratios of two

isotopically distinct water sources and p is the proportion of water obtained from one of the sources, have been widely used to estimate water source dependence (e.g., Snyder and Williams 2003). These mixing models for water can easily be modified to account for isotopic discrimination by including an apparent fractionation factor, such that

$$\delta D_{\text{tissues}} = p \delta D_{\text{source } 1} + (1-p) \delta D_{\text{source } 2} + \Delta$$

where  $\Delta$  is the apparent fractionation factor between the water inputs and tissues.

Although animal ecologists have had considerable success using analyses of naturally-occurring carbon and nitrogen stable isotope ratios in animal tissues to estimate the importance of isotopically distinct food resources to animal diets (Hobson 1990; Fleming et al. 1993; Szepanski et al. 1999; Hatch et al. 2002; Wolf and Martínez del Rio 2003) and to examine the relative trophic position of consumers in food webs (Hobson 1990; Post 2002; Herrera et al. 2003), they have, however, not attempted to use the natural variations in the hydrogen isotope ratios of water resources to trace the use of individual water resources by animals. Interest in hydrogen and oxygen isotopes by animal ecologists has primarily focused on the constraints that natural variation in the ratios of these isotopes impose on estimating field metabolic rates and water flux using doubly labelled water, or as tracers of animal movements. Animal ecologists, in contrast to plant ecologists, have virtually ignored the potential for tracking water resource use, even though isotopic variation in water resources in terrestrial environments can be large. Wolf and Martínez del Rio (2000, 2003, unpublished data), for example, found isotopic differences frequently greater than 100‰ between saguaro fruit and surface water resources. These differences suggest that stable hydrogen isotope ratios can potentially be used to quantify water source use by measuring the isotopic composition of body water and the source end points. Tracing water resource use is also potentially important for understanding the deuterium isotopic composition of animal tissues, since drinking water contributes significantly to the hydrogen isotopic composition of both metabolically active and inactive tissues (Hobson et al. 1999).

In this analysis we examine the potential use of stable isotope ratios as water resource tracers by experimentally testing the dependence of isotopic discrimination on water fluxes between birds and their environment. The main challenge to the use of a deuterium isotope signal as a water resource tracer is that fractionated evaporative water losses result in isotopic fractionation of the body water pool. Indeed, Wolf and Martínez del Rio (2000, 2003) found that the body water pools of a broad range of avian species were enriched 20–40‰ above the most enriched water source values, suggesting strong fractionation in this system. These results are consistent with current theory and the observation that water lost though evaporation is

depleted in deuterium relative to body water in mice and humans (Schoeller et al. 1986; Wong et al. 1988).

Understanding how the body water pool becomes enriched above water resource values requires a detailed examination of animal water budgets. Water enters an bird or mammal's body water pool by two routes: as preformed (free) water obtained from drinking and food, and as metabolic water produced during catabolic processes (Schmidt-Nielsen 1990). Water losses occur via evaporation (from the skin and respiratory surfaces) and excretion (feces and urine) (Schmidt-Nielsen 1990). At biological temperatures, evaporation across the skin and respiratory surfaces of animals tends to lead to isotopic enrichment of the body water pool, since water molecules containing the lighter hydrogen isotope (protium) evaporate more readily than the water molecules containing the heavier isotope (deuterium). This leads to the enrichment of the body water pool relative to the source water. Mass-balance models that predict the deuterium and oxygen isotopic composition of body water, and account for these avenues of water gain and loss, have been developed in the context of correcting for fractionation during measurements of field metabolic rate and water flux rate using doubly labelled water (Luz et al. 1984; Schoeller et al. 1986; Kohn 1996). A central prediction of these models, which has been confirmed in humans (Schoeller et al. 1986), is that the deuterium isotopic composition  $(\delta D)$  of body water  $(\delta D_{\text{body}})$  is a function of the ratio of evaporative (fractionated) losses to excretory (non-fractionated) losses (Luz et al. 1984; Schoeller et al. 1986; Wong et al. 1988; Kohn 1996).

These mass-balance models can be used in concert with mixing models to provide the conceptual framework for using water resource deuterium stable isotope ratios to trace water resource use by animals in the field. In theory, mass-balance models can be used to predict  $\delta D_{\text{body}}$  and hence the isotopic discrimination factors necessary to use a two end-point mixing model to quantify the contribution of isotopically distinct water sources to a bird's body water pool. Although temporal changes in  $\delta D$  and  $\delta^{18}$ O in the body water of wild birds have been investigated (Tatner 1988, 1990), the ability of mass-balance models to predict  $\delta D_{\text{body}}$  has received little attention, and it remains unclear whether these models adequately account for water fluxes and fractionation in birds under natural conditions.

Our goals in this study were to test our ability to account for variation in apparent fractionation due to the interaction between water intake and evaporative losses, and to determine whether we could predict deuterium isotopic discrimination from knowledge of water flux rates and evaporative losses in a laboratory setting. To test the ability of a mass-balance model to explain deuterium isotope ratios in the body water of rock doves, we manipulated water flux rates and the relative contribution of evaporation to total water flux, and then switched the isotopic composition of the birds' drinking water to determine turnover rates and isotopic discrimination. We used these results to explore the potential use of deuterium isotope ratios as a tracer of water resource use.

# **Materials and methods**

Mass-balance model for the deuterium isotopic composition of avian body water

Martínez del Rio and Wolf (2004) describe in detail the logic of mass-balance models and their use in animal isotopic ecology. We constructed a mass-balance model for the deuterium isotopic composition of avian body water (Fig. 1) by modifying the model of Schoeller et al. (1986), which was based on Luz et al.'s (1984) model for <sup>18</sup>O. Following Martínez del Rio and Wolf (2004) and for dimensional consistency, we use deuterium fractions ( $f_D$ ) rather than  $\delta D$  values to quantify the relative abundance of deuterium. These values can easily be transformed to the usual delta notation:

$$\mathbf{R}_{D\text{sample}} = \frac{f_D}{1 - f_D}$$

where  $R_{Dsample}$  is the ratio of deuterium to <sup>1</sup>H in the sample, and

$$\delta D = \left[ rac{R_{D ext{sample}} - R_{D ext{standard}}}{R_{D ext{standard}}} 
ight] imes 1000$$

For readers unfamiliar with these models, we provide a brief explanation of the logic followed in predicting the isotopic composition of body water using a mass-balance model (Fig. 1). Assume that an animal with body water equal to TBW (in ml) ingests preformed water at a fractional rate equal to  $r_{pre}$  and produces metabolic water at a fractional rate  $r_{met}$  ( $r_{pre}$  and  $r_{met}$  have time<sup>-1</sup> units). Also assume that the deuterium fractions contained in the hydrogen of preformed and metabolic water are  $f_{Dpre}$  and  $f_{Dmet}$ , respectively. Thus, the rate of deuterium input (I) into the animal's body water is:

$$I = TBW(f_{Dmet}r_{met} + f_{Dpre}r_{pre})$$

$$O = TBW(f_{Dbody}r_{excre} + \alpha^* f_{Dbody}r_{evap}) ,$$

where  $f_{Dbody}$  is the fraction of deuterium in the body water hydrogen pool,  $r_{excre}$  and  $r_{evap}$  are the fractional rates of water loss through excretion and evaporation, respectively, and  $\alpha^*$  is the apparent fractionation of deuterium in evaporated water relative to body water ( $\alpha^*=f_{Devap}/f_{Dbody}$ ). At steady state (dTBW/dt=0 and df<sub>Dbody</sub>/dt=0) inputs and outputs are equal ( $r_{H_2O} = r_{evap} + r_{excre} = r_{met} + r_{pre}$ ) and hence:

 $f_{Dmet}r_{met} + f_{Dpre}r_{pre} = \hat{f}_{Dbody}r_{excre} + \alpha^* \hat{f}_{Dbody}r_{evap}$ 

and

$$\hat{f}_{Dbody} = \frac{\frac{1}{r_{H_2O}} (f_{Dmet} r_{met} + f_{Dpre} r_{pre})}{\frac{1}{r_{H_2O}} (r_{excre} + \alpha^* r_{evap})} = \frac{\bar{f}_{inputs}}{1 + (\alpha^* - 1)E}$$

where  $f_{Dbody}$  is the steady state fraction of deuterium in the hydrogen of the animal's body water,  $f_{inputs}$  equals the weighted average of the isotopic composition of the inputs,  $r_{H_2O}$  is the fractional rate of water turnover, and E is the fraction of the water budget lost by evaporation (E =  $r_{evap}/r_{H_2O}$ ). The central prediction of this model is that the deuterium concentration of body water will increase with the fraction of the total water budget that the animal loses through evaporation (Fig. 1). This model also allows estimating the degree of deuterium enrichment from knowledge of the isotopic composition of the water relative to body water (Fig. 1).

In this paper, we use the terms "discrimination" as well as "fractionation". Fractionation is a change in isotope ratios arising from physical and chemical processes, whereas discrimination can include other processes, such as stoichiometric effects and isotopic routing (Martínez del Rio and Wolf 2004). In essence, we test the hypothesis that deuterium isotopic discrimination between water



**Fig. 1** A mass-balance model for the deuterium isotopic composition of body water ( $f_{Dbody}$ ), modified from Schoeller et al. (1986) and Luz et al. (1984). In this model "r" denotes water fluxes (in moles per day) and  $f_D$  refers to hydrogen isotopic composition as fraction of D in the respective total water/hydrogen pool. We use  $f_D$  rather than  $\delta D$  values for dimensional consistency. We describe in the text how to transform from  $f_D$  to  $\delta D$  values. Thus,  $r_{\rm pre}$  and  $r_{\rm met}$  represent the inputs of water through free (drinking) water and metabolic water, respectively, whereas  $r_{\rm excre}$  and  $r_{\rm evap}$  represent the outputs of water that take place through urine and feces production and evaporation (respiratory and cutaneous), respectively;  $f_{D\rm pre}$  and  $f_{D\rm met}$  are the isotopic compositions of free and metabolic water, respectively. Note that if the animal is at neutral water balance, then  $r_{\rm pre} + r_{\rm met} = r_{\rm excre} + r_{\rm evap} = r_{\rm H_2O}$ , where  $r_{\rm H_2O}$  equals the daily water

turnover. The model assumes that only evaporated water losses are fractionated with a constant apparent fractionation factor  $\alpha^* (\alpha^* = f_{Devap}/f_{Dbody})$ . We call this fractionation "apparent" because several processes can contribute to its value. The model predicts that at equilibrium the deuterium content of body water will increase with the fraction of the total daily water budget that is lost by evaporation E. For clarity and consistency with established modes of expressing isotopic composition, we transformed the results of our model into discrimination factors ( $\Delta \delta D_{body-input}$ ) in ‰. The steepness of the relationship between  $\Delta \delta D_{body-input}$  and *E* is governed by the magnitude of  $\alpha^*$ . Lower values of  $\alpha^*$  imply a larger *D* depletion in water lost through evaporation relative to body water

inputs and body water is determined entirely by the fractionation of evaporative losses.

#### Study animals and housing

We trapped 32 feral rock doves (*Columba livia*) in Albuquerque, New Mexico, using a walk-in trap. After an initial two-month period in an outdoor aviary, the birds were transferred into two rooms (1.8 m long, 1.1 m wide, 2.4 m high). Each bird occupied a separate cage (0.39 m long, 0.23 m wide, 0.28 m high) and was provided with commercial pigeon diet (Purina Nutriblend Gold) and water ad libitum. The  $\delta D$  of metabolic water was determined by measuring the  $\delta D$  of the pigeon diet [-75‰ (VSMOW)]. Samples of the diet were dried to constant mass at 60°C, ground into a fine powder, and 0.1 mg samples were loaded into pre-cleaned silver capsules. Drinking water was provided in glass drinking tubes, and drinking rates ±1.0 ml day<sup>-1</sup> were recorded daily for three weeks prior to the study, as well as for the duration of the switching experiment (see below).

The air temperature ( $T_a$ ) in each of the two rooms was measured (±0.1°C) every 5 min during the experimental period using Stowaway XTI Temperature Loggers (Onset Computer Corporation, Bourne, MA, USA). One of the rooms ("hot room") was heated to approximately 42°C for 12 h per day, using three commercially available fan heaters. Air temperature in the second room ("cool room"), and in the hot room at night, was approximately 20°C.

At the conclusion of the experiments, the body temperature  $(T_b)$  of eight doves in each of the two rooms was measured at midday by inserting a thermocouple into the cloaca, to a depth at which a slight withdrawal did not cause a reduction in the temperature reading. Each bird was handled for <30 s prior to the  $T_b$  measurement.

#### Experimental design

We manipulated water flux rates and evaporative and excretory water losses by exposing doves to different combinations of heat stress and salt loading. The 32 doves were divided into four experimental groups of eight birds each. Two groups were housed in each of the two rooms. In each room, one group was provided with fresh water, while the second group was provided with 0.15 M NaCl solution. Preliminary trials indicated that this was the maximum salt load that permitted the birds to maintain a stable body mass. The use of a heated room resulted in a fourfold increase in evaporative water loss compared to that of the doves in the cool room. This experimental approach yielded a 2×2 factorial design with four experimental groups: (1) cool, fresh, (2) cool, 0.15 M NaCl, (3) hot, fresh and (4) hot, 0.15 M NaCl. By housing groups of doves under these various combinations of thermal stress and salt loading, we generated a range of values of E (i.e.,  $r_{\rm evap}/r_{\rm H_2O}$ ). Prior to the switching experiment (see below), the birds were maintained under the conditions outlined above for seven weeks to ensure that they maintained body mass, were acclimated to the respective  $T_a$  and salt loading regimes, and that their body water  $\delta D$  was equilibrated with the drinking water.

#### Switching experiment

Two 120-l plastic drums were filled with tap water ( $\delta D$ =-95.5 ±2.5‰ VSMOW) and capped, and this water was used as drinking water for 7 weeks prior to the switching experiment (see below). Samples of the drinking water were collected from the two drums at weekly intervals, and subsequently analyzed for  $\delta D$  to ensure that these values remained stable. In order to minimize evaporation from the glass drinking tubes, we used tubes with an opening just sufficient for the birds to drink, and replaced the water in the tubes daily. Prior to the switching experiment, two more drums were filled with tap water, and the  $\delta D$  of this water was enriched to 52.0±2.4‰

VSMOW, by the addition of 99.8 Atom % D<sub>2</sub>O (Isotec, Miamisburg, OH, USA). After the initial 7-week acclimation period, a baseline blood sample (ca. 100  $\mu$ l) was taken from the brachial vein of each dove. The drinking water was then switched from the initial water supply ( $\delta D$ =-95‰ VSMOW) to the new water source with  $\delta D$ =52‰ VSMOW. Following the drinking water switch, additional blood samples were taken from each bird on days 1–4, 6, 8, 12, 16 and 20.

#### Measurement of evaporative water loss

In order to estimate the proportion of total water flux that occurred by evaporation, we measured evaporative water loss (EWL) rates at the termination of the switching experiment, in a subset of the birds using flow-through respirometry. A fed bird was placed in a 14 l glass metabolism chamber, which in turn was placed in a darkened 200 l environmental chamber. The  $T_a$  was regulated using a programmable water bath (Model 1187, VWR Scientific Products, West Chester, PA, USA), which pumped fluid through copper tubing in the chamber. Air within the insulated chamber was mixed using a small electric fan. Ambient temperature  $(T_a)$  within the metabolism chamber was measured (±0.1°C) using a 21-gauge Cu-Cn thermocouple calibrated against a NIST mercury thermometer (VWR Scientific Products, West Chester, PA, USA) and a TC-1000 thermocouple preamplifier (Sable Systems, Las Vegas, NV, USA). During the experiments, the dove rested on a wire mesh floor above a 3 cm layer of mineral oil, which covered any feces produced, preventing fecal water from influencing EWL measurements. Dry, CO<sub>2</sub>-free air was produced using a FT-IR Purge Gas Generator (Whatman, Newton, MA, USA) and flowed through the chamber at flow rates that ranged  $4-9 \pm 0.1 \text{ l min}^{-1}$ , in order to maintain dew points below 5°C in the chamber. The turnover time required to reach 99% equilibration ranged from 7 to 14 min, calculated from the equation in Lasiewski et al. (1966). Flow rates into the chamber were regulated using a FMA-series mass flow controller (Omega, Bridgeport, NJ, USA), calibrated with a 1-l soap bubble flowmeter. Excurrent air from the metabolism chamber and dry, CO2-free baseline air was alternately subsampled using a TR-RM8 Respirometer Multiplexer (Sable Systems, Las Vegas, NV, USA). The partial pressure of water vapor in subsampled air was measured using a LI-7000 CO<sub>2</sub>/H<sub>2</sub>O analyzer (Li-Cor, Lincoln, NE, USA), calibrated daily using dry, CO2-free air and span gas generated using a LI-610 portable dew point generator (Li-Cor, Lincoln, NE, USA). Dew points of the subsampled air were well below those of the surrounding environment, ensuring that no condensation occurred within the system tubing. Output from the CO2/H2O analyzer was digitized using a Universal Interface II and recorded on a PC using Datacan V data acquisition software (Sable Systems, Las Vegas, NV, USA).

Rates of EWL were measured over the range of  $T_{a}$ s experienced by the doves during the switching experiment. Thus, the EWL of doves from the hot room was measured at  $T_{a}$ =22, 32, 40 and 44°C, with measurements made on at least six birds (chosen at random) at each  $T_{a}$ . The EWL of doves from the cool room were measured at  $18^{\circ}C \leq T_{a} \leq 23^{\circ}C$ . To estimate daily EWL during the switching experiment, we fitted appropriate regression models to the massspecific EWL data for birds from each of the two rooms. We then estimated total daily EWL for each day during the switching experiment for each bird by integrating these equations using the  $T_{a}$ data recorded by the temperature loggers in each room.

Determination of the  $\delta D$  of water lost through evaporation

In order to determine the isotopic composition of water evaporated from the skin and respiratory surfaces we collected evaporated water and compared the values to  $\delta D_{\text{body.}}$ . Following the end of the switching experiment, we again provided the birds with un-enriched tap water ( $\delta D$ =ca. -95‰ VSMOW). After a minimum of 5 weeks for isotopic equilibration, we collected blood and evaporated water samples from eight birds in the hot room for  $\delta D$  analysis. After a blood sample was collected, each bird was transferred to the metabolism chamber described above at  $T_a=42^{\circ}$ C, in order to collect an evaporated water sample. Dry, CO<sub>2</sub>-free air flowed through the chamber at flow rates of 6–81 min<sup>-1</sup>. Excurrent air passed through a water trap (Ace Glass, Vineland, NJ, USA) immersed in an ethanoldry ice slurry to maintain a temperature of  $-74^{\circ}$ C. The water vapor partial pressure of the air was measured downstream of the water trap to ensure that all the water vapor had condensed in the trap. After a suitable amount of ice (1–2 g) had accumulated, the trap was removed from the slurry, sealed, and immersed in warm water until the ice melted. The water collected was then placed in a screw-cap sample vial sealed with Parafilm.

To confirm that excretory water losses were in isotopic equilibrium with body water, we collected blood, uretral urine and intestinal fluid from six birds following the switching experiment. Uretral urine was collected with a closed-end cannula, as described in Thomas et al. (1984), and intestinal fluid was collected from feces. Water was extracted from each sample as described below, and the samples were analyzed for  $\delta D$ .

#### Sample analysis

All blood samples were collected using heparinized micro-hematocrit tubes (Drummond Scientific Company, Broomall, PA, USA). The tubes were sealed using Critoseal (Oxford Labware, St. Louis, MO, USA) and stored at 2°C. Within 24 h, pure water was isolated from blood samples by cryogenic vacuum distillation (Ehleringer 1989), and flame-sealed under a vacuum. To confirm that no fractionation occurred during the extraction procedure, we repeated the procedure using tap water and confirmed that the initial and final  $\delta D$  values did not differ.

The isotopic composition of water samples was determined by isotope ratio mass spectrometry (IRMS), using a continuous flow Finnigan Delta Plus XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA), in the Department of Earth and Planetary Sciences, University of New Mexico. Laboratory standard waters, calibrated against the international standard VSMOW, were used to provide correction factors for the raw data. Stable isotope ratios were expressed as permil (‰; parts per thousand) values, using standard delta ( $\delta D$ ) notation, and referenced against VSMOW.

### Data analysis

Values are presented as mean  $\pm$  SD. The relatively high flow rates used during the EWL measurements precluded the accurate measurement of oxygen consumption ( $\dot{V}_{O_2}$ ), and we used data from Calder and Schmidt-Nielsen (1967) to estimate fasting metabolic rates and metabolic water production. We integrated the  $T_a$  data recorded by the loggers in each of the two rooms to obtain an estimate of daily  $\dot{V}_{O_2}$  and calculated daily metabolic water

**Table 1** The water flux, drinking inputs, and the ratio of evaporative water loss to total water flux  $(r_{evap}/r_{H_2O})$  in rock doves subjected to four experimental treatments differed signifi-

production from Withers (1992), based on the nutritional composition of the diet (60% carbohydrate, 14% protein, 5% fat). We assumed that  $\dot{V}_{O_2}$  during the active phase of the circadian cycle was 1.3X rest-phase  $\dot{V}_{O_2}$ . Errors in  $\dot{V}_{O_2}$  estimates are unlikely to significantly affect our conclusions. For instance, even if activephase  $\dot{V}_{O_2}$  was 2.5X rest-phase  $\dot{V}_{O_2}$ , predicted discrimination factors would change by <3%. The preformed water content of the pigeon diet was <5% by mass. We did not include preformed dietary water in our water budget calculations, since it could have represented at most 2.4% of daily water intake. Hence, total water intake was calculated as the sum of observed drinking rates and estimated metabolic water production. We assumed that the  $\delta D$  of metabolic water ( $\delta D_{met}$ ) was equal to the  $\delta D$  of the diet.

The rate of incorporation of enriched drinking water ( $\delta D$ =+52‰ VSMOW) into the doves' body water pools during the switching experiment was determined by fitting a 3-parameter exponential decay model of the form  $y=y_0-ae^{-bx}$  to the  $\delta D$  and time (days) data for each bird (see O'Brien et al. 2000 and Martínez del Rio and Wolf 2004 for a theoretical justification for the use of this equation). In this model, *b* is the daily fractional turnover rate, and  $y_0$  is the asymptotic  $\delta D$  value. We used the  $y_0$  for each bird to calculate the discrimination factor

$$\Delta \delta D_{\text{body-input}} = \delta D_{\text{body water}} - \frac{(r_{\text{pre}} \delta D_{\text{pre}} + r_{\text{met}} \delta D_{\text{met}})}{r_{\text{H}_2\text{O}}}$$

following the switching experiment. In addition, we calculated the  $\delta D$  half-life for each bird as  $t_{\frac{1}{2}} = \frac{\ln(2)}{b}$ .

# Results

Body mass, body temperature, water flux rates and evaporative water loss

Mean  $M_{\rm b}$  at the start of the switching experiment was 353.7±34.9 g and did not vary among the four treatments (ANOVA,  $F_{3,28}$ =1.496, p=0.237). There was no significant change in the  $M_{\rm b}$  of individual birds during the experimental period (Paired *t*-test, *t*=1.274, df=31, p=0.212). The mean active-phase  $T_{\rm b}$  of 16 doves was 41.4±0.5°C and did not differ between the hot and cool rooms respectively (ANOVA,  $F_{1,14}=0.721$ , p=0.721). Drinking rates varied significantly (ANOVA,  $F_{3,28}$ =45.874, p<0.001; Table 1) among treatments, and were approximately four times higher in the hot, 0.15 M NaCl treatment than in the cool, fresh treatment (Table 1).

cantly (n=8 birds per treatment, 32 birds in total). In contrast, metabolic water input and body mass did not differ significantly among treatments

|   | Cool room             |                       | Hot room               |                         |
|---|-----------------------|-----------------------|------------------------|-------------------------|
|   | Fresh water           | 0.15 M NaCL           | Fresh water            | 0.15 M NaCL             |
| Body mass (g)                           | 333.4±27.6            | 352.9±34.7            | 361.0±45.7             | 367.5±24.6              |
| Drinking input (ml day $^{-1}$ )        | $35.1{\pm}11.0^{a}$   | 41.7±9.0 <sup>a</sup> | 71.1±22.9 <sup>b</sup> | 122.4±19.8 <sup>c</sup> |
| Metabolic input (ml day <sup>-1</sup> ) | 6.6±0.4               | 7.1±0.5               | 7.5±0.9                | 7.7±0.6                 |
| Evaporative loss (ml day $^{-1}$ )      | 15.2±1.0 <sup>a</sup> | $16.3 \pm 1.2^{a}$    | $54.8 {\pm} 6.8^{b}$   | 56.3±4.0 <sup>b</sup>   |
| $r_{\rm evap}/r_{\rm H_2O}$             | $0.408{\pm}0.094^{a}$ | $0.402{\pm}0.066^{a}$ | $0.667 \pm 0.140^{b}$  | $0.462{\pm}0.077^{a}$   |

Means that differed significantly (ANOVA) are indicated by different lower-case letters

However, drinking rates did not differ significantly between the cool, fresh and cool, 0.15 M NaCl treatment (Tukey's HSD test, p>0.05).

Rates of EWL of birds in the hot room varied from 2.98  $\pm 0.37$  g H<sub>2</sub>O kg<sup>-1</sup> h<sup>-1</sup> at  $T_a=22^{\circ}$ C to 12.21  $\pm 0.92$  g H<sub>2</sub>O kg<sup>-1</sup> h<sup>-1</sup> at  $T_a=44^{\circ}$ C. At  $T_a=22^{\circ}$ C, birds in the hot room exhibited EWL rates approximately 40% higher than birds in the cool room. Estimated daily EWL was approximately 3.5 times greater in the hot room than in the cool room (Table 1).

## Incorporation rate of $\delta D_f$ into body water

The rate at which the doves incorporated the enriched drinking water into their body water pools varied among treatments (Fig. 2). Fractional turnover rates (estimated by fitted co-exponential functions to the  $\delta D_{body}$  data for each bird) were strongly correlated with daily water intake ( $r^2$ =0.82). The birds in the cool, fresh treatment exhibited the lowest fractional turnover rates and the birds in the hot, 0.15 M NaCl treatment the highest (Table 2).

Relationship between  $\delta D_{\text{body}}$  and  $r_{\text{evap}}/r_{\text{H}_2\text{O}}$ 

The doves'  $\delta D_{\text{body}}$  was enriched by 10–50‰ relative to the weighted mean of drinking and metabolic inputs (Fig. 3). Following the switching experiment, the discrimination factor ( $\Delta \ \delta D_{\text{body-input}}$ ) was positively correlated to  $r_{\text{evap}}/r_{\text{H}_2\text{O}}$  (E; Fig. 3a; r=0.62, p<0.001). In contrast,  $\Delta \ \delta D_{\text{body-input}}$  prior to the switching experiment (calculated from initial  $\delta D_{\text{body}}$  values) was not correlated to E (Fig. 3b; r=0.26, p=0.14).  $\delta D$  of evaporative and excretory water losses

Water lost through evaporation was depleted relative to body water by  $-52.7\pm4.3\%$  VSMOW ( $\delta D_{evap}$ =-95.0  $\pm4.0\%$  VSMOW,  $\delta D_{body}$ =-42.3 $\pm3.0\%$  VSMOW). Excretory water losses, in contrast, were in isotopic equilibrium with body water. This depletion allowed us to calculate the apparent fractionation ( $\alpha^*=f_{Devap}/f_{Dbody}=0.949\pm0.003$ ) of deuterium in evaporated water relative to body water. The expected relationship between  $\Delta \delta D_{body-input}$  and E, shown as a dashed line in Fig. 3a, b, shows that our model underestimated the deuterium enrichment in body water.

## Discussion

Our data show that increases in the rate of evaporative water loss relative to drinking water intake lead to the isotopic enrichment of body water relative to water sources. These results qualitatively support field observations of  $\delta D_{body}$  in white-winged doves and six other avian species (Wolf and Martínez del Rio 2000, 2003 study), and are consistent with the deuterium enrichment of body water in humans (Schoeller et al. 1986; Wong et al. 1988) and mice (Lifson et al. 1955). Below, we discuss the implications that this enrichment of avian  $\delta D_{body}$  as a function of drinking rate and environmentally generated increases in EWL has for the use of deuterium stable isotope ratios as source water tracers in ecological studies.

The degree to which dove  $\delta D_{body}$  was enriched relative to the  $\delta D$  of water influxes showed considerable variability. The significant linear relationship between isotopic discrimination ( $\Delta \delta D_{body-input}$ ) and the proportion of total water flux lost by evaporation (E) following the switching experiment (Fig. 3a) supports the mass-balance model's

Fig. 2 After the composition of drinking water was switched from  $\delta D = -95\%$  VSMOW (lower dotted line in each graph) to +52‰ VSMOW (upper dotted line in each graph), the deuterium composition of the body water of rock doves  $(\delta D_{body})$  changed in a way that can be adequately described by co-exponential functions. Both the asymptotic  $\delta D_{\text{body}}$  and the fractional turnover of the water pool were dependent on treatment. Each point is the mean value for eight doves. Bars are standard deviations





**Fig. 3a, b** The isotopic discrimination between body water and water inputs ( $\Delta \delta D_{\text{body-input}}$ ) increased significantly with the fraction of daily water flux ( $E = r_{\text{evap}}/r_{\text{H}_2\text{O}}$ ) after rock doves reached isotopic equilibrium in an experiment in which birds were shifted between water sources with different isotopic compositions (**a**). However, before the experiment when birds had been drinking water of the same composition for 7 weeks, there was no significant correlation between  $\Delta \delta D_{\text{body-input}}$  and E (**b**). The *dashed line* represents the expected value from a mass-balance model that assumes that evaporative water losses were depleted relative to body water by -52.7±4.3‰ VSMOW (i.e.,  $\alpha^*=f_{Devap}/f_{Dbody}=0.949 \pm 0.003$ ). The *solid line* is a regression line through the origin ( $r^2=0.34$ , p<0.001)

qualitative prediction that the ratio of evaporative water loss to total water flux is a major determinant of body water deuterium enrichment. These results indicate that the enrichment of  $\delta D_{\text{body}}$  relative to water influxes is directly related to patterns of water flux between a bird and it's environment, and hence to patterns of thermo- and osmoregulation. The mass-balance model (Fig. 1) makes several general predictions with regard to broad-scale patterns in the deuterium enrichment of avian body water. Firstly, the degree of  $\delta D_{\text{body}}$  enrichment should be affected by any environmental factors that influence the rate of evaporative water loss, such as  $T_a$  and relative humidity. For instance, birds in desert environments should generally exhibit more enriched  $\delta D_{\text{body}}$  relative to water inputs than birds that occupy mesic environments. Second, because EWL represents a greater proportion of overall water flux in small birds, on account of their greater relative skin and lung surface areas and higher massspecific metabolic rates, the body water of small birds should be more enriched in deuterium relative to water sources than that of larger species.

Whereas the relationship between isotopic discrimination ( $\Delta \delta D_{\text{body-input}}$ ) and the fraction of water lost through evaporation (E) following the switching experiment supported our predictions, the pattern of  $\delta D_{\text{body}}$  enrichment prior to the switching experiment did not. Following 7 weeks of acclimation to the combination of thermal conditions and salt-loading regime associated with each experimental treatment, the doves showed no relationship between  $\Delta \delta D_{\text{body-input}}$  and E (Fig. 3b). The difference in the relationship between  $\Delta \delta D_{\text{body-input}}$  and E prior to and following the switching experiment suggests that the determinants of  $\delta D_{\text{body}}$  vary over different temporal scales, and are more complex than existing models propose. Although under some conditions  $\delta D_{\text{body}}$  is related in a predictable fashion to E, our data suggest that over longer time-scales, an as yet unidentified mechanism causes the  $\delta D_{\rm body}$  of doves with different  $r_{\rm evap}/r_{\rm H_2O}$  ratios to converge.

The lack of a correlation between  $\Delta \delta D_{\text{body-input}}$  and E prior to the switching experiment could conceivably reflect drinking water intake in excess of maintenance requirements, with consequent amelioration of the effect of fractionated evaporative losses on  $\delta D_{\text{body}}$ . To investigate this possibility, we estimated maintenance water requirements, using literature  $\dot{V}_{O_2}$  data (Calder and Schmidt-Nielsen 1967), assuming a metabolizable energy coefficient (MEC) of 0.8 (Karasov 1990) and a fecal water content of 75%. Our estimates of maintenance drinking

**Table 2** In rock doves switched from tap water ( $\delta D$ =-95.5±2.5‰ VSMOW) to spiked water ( $\delta D$ =52±2.4‰ VSMOW), treatment had a significant effect on fractional deuterium turnover rate, half-life, final  $\delta D$ -value, and discrimination ( $\Delta \delta D_{body-input}$ )

|                                   | Cool room             |                        | Hot room              | Hot room                |  |
|-----------------------------------|-----------------------|------------------------|-----------------------|-------------------------|--|
|                                   | Fresh water           | 0.15 M NaCL            | Fresh water           | 0.15 M NaCL             |  |
| Fractional turnover               | $0.179{\pm}0.028^{a}$ | $0.182{\pm}0.030^{a}$  | $0.264{\pm}0.043^{b}$ | $0.359{\pm}0.054^{c}$   |  |
| Half-life (days)                  | $3.97{\pm}0.72^{a}$   | 3.91±0.71 <sup>a</sup> | $2.68 \pm 0.44^{b}$   | $1.97{\pm}0.29^{\circ}$ |  |
| Final $\delta D$ -value (% VSMOW) | $61.5 \pm 7.4^{a}$    | $59.5{\pm}6.9^{a}$     | $81.7 \pm 5.9^{b}$    | $70.4{\pm}2.7^{c}$      |  |
| $\Delta$ (‰ VSMOW)                | 9.3±7.4 <sup>a</sup>  | $7.3{\pm}6.9^{a}$      | $29.7 \pm 5.9^{b}$    | 18.2±2.7 <sup>c</sup>   |  |

Means that differed significantly (ANOVA) are indicated by different lower-case letters

rates were lower than the drinking rates we observed, suggesting that the birds were drinking more water than they required to maintain water balance. To determine whether the lack of a correlation between  $\Delta \delta D_{\text{body-input}}$ and  $r_{\rm evap}/r_{\rm H_2O}$  prior to the switching experiment was due to excessive drinking water intake, we subjected eight of the doves to a restricted water treatment, 2 months after the switching experiment. Over a 2-week period, they were provided with a daily water ration just sufficient to maintain a constant body mass  $(M_{\rm b})$ , equivalent to ca. 67% of drinking rates during the earlier ad libitum water availability. We collected a blood sample from each bird prior to the restricted water treatment, and then at 2-day intervals during the course of the treatment. In the eight birds subjected to this treatment,  $\delta D_{\text{body}}$  did not change significantly during a 2-week period of restricted water availability (paired t-test, t=0.617, df=7, p=0.557). This observation confirmed that the observed relationships between  $\Delta \delta D_{\text{body-input}}$  and E were not determined by drinking in excess of maintenance requirements.

## Deuterium depletion of water lost through evaporation

Total evaporative water losses were depleted by -52.7%relative to  $\delta D_{\text{body}}$  ( $\delta D_{\text{evap}}$ =-95.0±4.0‰ VSMOW,  $\delta D_{\text{body}}$ =-42.3±3.0‰ VSMOW). Schoeller et al. (1986) demonstrated that, in humans, the isotopic composition of water lost by evaporation was determined by respiratory evaporative water loss (REWL) fractionated under equilibrium conditions determined by body temperature, and kinetically fractionated cutaneous evaporation. Respiratory water under equilibrium conditions at a body temperature of 41.4°C (mean body temperature of 16 doves) should theoretically be depleted relative to  $\delta D_{\text{body}}$  by -57.1‰ (Clark and Fritz 1997). Assuming that respiratory water losses in doves are indeed subject to equilibrium fractionation, and that at  $T_a$ =42°C REWL comprised 46% of total evaporation (Webster and King 1987), the estimated depletion relative to  $\delta D_{\text{body}}$  of water lost by cutaneous evaporation would be -49.0%.

In practice, accounting for the partitioning of evaporative water losses into respiratory and cutaneous pathways is likely to be difficult. The partitioning of avian evaporative water losses varies with ambient temperature, but also with taxonomic affiliation (Wolf and Walsberg 1996). There is also evidence that the partitioning of evaporative water losses may vary in response to both long-term and short-term thermal acclimation (Marder and Arieli 1988; McKechnie and Wolf 2004). Moreover, the extent of kinetic fractionation may be variable. Kinetic fractionation arises as a consequence of differences in the diffusive velocities of isotopes (Clark and Fritz 1997). The extent of fractionation is dependent on the rate of net diffusion, which is primarily determined by the relative humidity of the air column (Clark and Fritz 1997). Hence, the extent of kinetic fractionation that occurs during CEWL is likely to be related to relative humidity as well as the convective environment and  $T_{a}$ .

Deuterium as a water resource tracer

Our rationale for this study was to investigate the use of deuterium as a water resource tracer in free-ranging birds under natural conditions. The feasibility of combining estimates of various water fluxes with mass-balance models to accurately predict avian  $\delta D_{\text{body}}$ , and hence the isotopic discrimination factors necessary to use deuterium as a tracer in studies of water resource utilization, appears to be limited. The fact that our model accounted for less than 40% of the variability observed in isotopic discrimination factors under laboratory conditions, suggests that accurately predicting the  $\delta D_{\text{body}}$  of free-ranging birds is likely to prove problematic. Incorporating additional factors into our experimental design, such as measuring the partitioning of total evaporative losses and ensuring that the relative humidity in the two rooms was identical to that in the metabolism chamber during EWL measurements, may have improved the model's performance under laboratory conditions. However, such refinements to the experimental design would probably not significantly improve the model's ability to predict  $\delta D_{\text{body}}$  in freeranging birds.

Our ability to predict isotopic discrimination factors for avian body water remains limited. However, these data provide new insights into the variation in discrimination factors likely to occur under natural conditions. The 95th percentile for absolute residual discrimination factors following the switching experiment (Fig. 3a) was 12.9‰. For the initial discrimination factors prior to the switching experiment (Fig. 3b), the 95th percentile differed from the mean value by 12.0%. It is encouraging that similar variability in isotopic discrimination was evident both before and after the switching experiment, and this suggests that similar variability might be expected under natural conditions. The accuracy of quantitative estimates of the contribution of isotopically distinct water sources to a bird's body water pool is dependent on the accuracy with which discrimination factors can be predicted (Fig. 4). Based on the variability we observed in rock doves, an isotopic difference between the two water sources of 260% would be a prerequisite for estimating the proportion of each source in a bird's body water pool with an accuracy of 95% (Fig. 4). In the Sonoran Desert of southern Arizona, water contained in the nectar and fruit pulp of saguaro cacti (Carnegiea gigantea) was enriched by 75-120% relative to other water sources (Wolf and Martínez del Rio 2000, 2003, unpublished data). If the variation in discrimination factors we observed in rock doves is typical of most birds, quantitative estimates of the importance of water from saguaro cacti would be subject to an error of 13-17.3% (Fig. 4). The requirement of a relatively large isotopic difference between water sources may often preclude quantitative investigations of water resource use. However, under some circumstances the necessary isotopic difference can be achieved by artificially enriching a water source of interest.

Deuterium stable isotope ratios in studies of avian migration

Hobson et al. (1999) investigated the relative contributions of diet- and water-derived hydrogen to avian tissues, and demonstrated that approximately 20% of hydrogen in metabolically active tissues such as muscle, liver and fat was derived from drinking water. A greater proportion of hydrogen in feathers and nails, 26–32%, was derived from drinking water (Hobson et al. 1999). These observations suggest that the isotopic composition of the body water pool needs to be considered when interpreting the  $\delta D$ values of various tissues. For instance, the correlation between  $\delta D$  of feathers and that of growing season precipitation is often used to track migration patterns (Hobson and Wassenaar 1997; Meehan et al. 2001; Kelly et al. 2002). Our results suggest that some of the variability in the  $\delta D$  of feathers from a single location may reflect variability in the enrichment of  $\delta D_{\text{body}}$  relative to water inputs. For instance, if we assume that in rock doves, 30% of non-exchangeable feather hydrogen is derived from body water, we would expect the  $\delta D$  of feathers to vary by 3–13‰. Feather  $\delta D$  values of adult Cooper's hawks (Accipiter cooperii) were significantly higher than expected on the basis of precipitation  $\delta D$ ,



Fig. 4 The accuracy of quantitative estimates of the proportion of an isotopically distinct water source in a bird's body water derived from a two end-point mixing model depends on the isotopic difference between the two water sources of interest. In a two endpoint mixing model for water, the deuterium isotopic composition of a bird's body water is expressed as:  $\delta D_{\text{body}}=p\delta D_{\text{source }1}+(1-p)\delta D_{\text{source }2}+\Delta \delta D_{\text{body-input}}$ , where p is the proportion of water obtained from one of the sources,  $\delta D_{\text{source }1}$  and  $\delta D_{\text{source }2}$  are the delta ratios of the two water sources, and  $\Delta \delta D_{\text{body-input}}$  is the apparent discrimination factor between the water inputs and the body water pool. For generating the relationship, we assumed a maximum absolute error in predicted values of  $\Delta \delta D_{\text{body-input}}$  of 13‰, similar to our observed 95th percentiles of 12.0‰ and 12.9‰ (see text for details)

whereas those of nestlings closely matched precipitation  $\delta D$  (Meehan et al. 2003). Age-specific differences in the relationship between precipitation and feather  $\delta D$  may reflect the relative importance of evaporative water loss. Active males, and females incubating young at high environmental temperatures, likely exhibit elevated rates of evaporation compared to nestlings and hence greater  $\delta D$  discrimination (Meehan et al. 2003).

# Conclusions

Whereas our data show that mass-balance models for the deuterium composition of body water can be used to predict isotopic discrimination factors, limits to the accuracy of these predictions will often preclude the use of hydrogen stable isotope ratios as water resource tracers. The variable enrichments we observed in  $\delta D_{\text{body}}$  relative to water influxes suggest that  $\delta D_{\text{body}}$  can be used to obtain qualitative estimates of the importance of isotopically distinct water sources only if there are large differences in the  $\delta D$  of these water sources (Wolf and Martínez del Rio 2000, 2003). Although this conclusion is sobering, it is useful. It reveals one of the limits to the potential applications of stable isotopes in animal ecology. Our data highlight some of the complexities involved in using deuterium to trace water resource use, and identify avenues for future research. Of particular interest are the mechanisms underlying the temporal variability in the relationship between  $\delta D_{\text{body}}$  and the proportion of total water flux lost by evaporation.

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