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Mechanism and rate of glucose absorption differ between an Australian honeyeater (Meliphagidae) and a lorikeet (Loriidae)

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SUMMARY

Efficient mechanisms of glucose absorption are necessary for volant animals as a means of reducing mass during flight: they speed up gut transit time and require smaller volume and mass of gut tissue. One mechanism that may be important is absorption *via* paracellular (non-mediated) pathways. This may be particularly true for nectarivorous species which encounter large quantities of sugar in their natural diet. We investigated the extent of mediated and non-mediated glucose absorption in red wattlebirds *Anthochaera carunculata* (Meliphagidae) and rainbow lorikeets *Trichoglossus haematodus* (Loriidae) to test the hypothesis that paracellular uptake accounts for a significant proportion of total glucose uptake in these species. We found that routes of glucose absorption are highly dynamic in both species. In lorikeets, absorption of L-glucose (non-mediated uptake) is slower than that of D-glucose (mediated and non-mediated uptake), with as little as 10% of total glucose absorbed by the paracellular pathway initially (contrasting previous indirect estimates of ~80%). Over time, however, more glucose may be absorbed *via* the paracellular route. Glucose absorption by both mediated and non-mediated mechanisms in wattlebirds occurred at a faster rate than in lorikeets, and wattlebirds also rely substantially on paracellular uptake. In wattlebirds, we recorded higher bioavailability of L-glucose (96±3%) compared with D-glucose (57±2%), suggesting problems with the *in vivo* use of radiolabeled D-glucose. Further trials with 3-*O*-methyl-D-glucose revealed high bioavailability in wattlebirds (90±5%). This non-metabolisable glucose analogue remains the probe of choice for measuring uptake rates *in vivo*, especially in birds in which absorption and metabolism occur extremely rapidly.

Key words: paracellular absorption, glucose absorption, red wattlebird, *Anthochaera carunculata*, rainbow lorikeet, *Trichoglossus haematodus*, 3-*O*-methyl-D-glucose, L-glucose.

INTRODUCTION

Small birds and bats maintain high digestive assimilation efficiencies, comparable to those of non-flying mammals, despite relatively shorter digestive retention times, generally smaller guts and lower absorptive surface area (Caviedes-Vidal et al., 2007; Karasov, 1990; Karasov and Hume, 1997). In non-flying mammals, total carrier-mediated absorptive capacity, usually extrapolated from *in vitro* nutrient uptake measurements by the everted sleeve technique (Karasov and Diamond, 1983), generally either meets or exceeds the daily dietary carbohydrate intake of the animal (Ferraris and Diamond, 1989). However, in many avian species, capacity for mediated glucose transport extrapolated from *in vitro* measurements is significantly lower than observed total *in vivo* glucose assimilation (Caviedes-Vidal and Karasov, 1996; Karasov and Cork, 1994; Levey and Cipollini, 1996; McWhorter et al., 2006). How, then, do small volant vertebrates maintain high digestive efficiency?

Water-soluble nutrients such as carbohydrates and amino acids are absorbed in the small intestine by both protein-carrier-mediated (transcellular) and non-mediated (paracellular) mechanisms (Hopfer, 1987). The paracellular pathway is located between adjacent epithelial cells, where the tight junctions (zonula occludens) join the cells, block the movement of integral membrane proteins between the apical and basolateral membranes, and constrain the movement of water and hydrophilic solutes across the junction (Anderson, 2001; Ballard et al., 1995; McWhorter, 2005; van Itallie and Anderson, 2006). Tight junction permeability is variable and appears to be regulated by physiological mechanisms (Mitic et al., 2000; Pappenheimer, 1987; Powell, 1981; Turner, 2000). Pappenheimer (Pappenheimer, 1993) suggested that paracellular nutrient absorption may offer a selective advantage because it provides non-saturating absorptive capacity that is matched to the rate of substrate hydrolysis in the intestine and it requires little energy. High fractional absorption, or bioavailability, of small non-transported, metabolically inert, water soluble probe molecules (e.g. stereoisomers of simple sugars) has often been taken as evidence of significant paracellular nutrient absorption in small volant vertebrates (Caviedes-Vidal et al., 2007; Chang and Karasov, 2004; McWhorter, 2005; McWhorter et al., 2006). The nutritional importance of paracellular absorption was a subject of considerable debate in the past, however, partly because differences in methodology made comparisons across studies problematic (McWhorter, 2005). More recent studies employing uniform methods have shown convincingly that small birds and bats rely more on non-mediated mechanisms of absorption than do non-flying mammals, perhaps as a compensation for smaller intestines (Caviedes-Vidal et al., 2007; Tracy et al., 2007).

A criticism that has been levelled at studies using only bioavailability data to draw conclusions about the nutritional importance of paracellular uptake is that probe compounds might be absorbed at a much slower rate than nutrients absorbed by mediated mechanisms, but over the entire length of the intestine and extended time of digesta residence in the gut (Schwartz et al., 1995). In this case, probe fractional absorption may be high, falsely indicating that paracellular absorption accounts for a high

proportion of total nutrient absorption. An elegant approach to resolving this issue, and the one which we have adopted here, is to simultaneously compare the extent and rate of absorption of passively and actively absorbed probe molecules *in vivo*. 3-*O*-methyl-D-glucose (hereafter 3-OMG) is a non-metabolisable D-glucose analogue that competes for the same co-transporters as D-glucose (Solberg and Diamond, 1987) and has a similar maximal transport rate to D-glucose in mammals (Thomson et al., 1982). Comparing the relative rates of absorption of 3-OMG (absorbed *via* both mediated and non-mediated mechanisms) and L-glucose [absorbed by non-mediated mechanisms, recently verified in birds by competitive inhibition studies (Chang et al., 2004)] in granivorous house sparrows (*Passer domesticus*), Chang and Karasov (Chang and Karasov, 2004) estimated that at least 70% of total glucose uptake was paracellular. More recently, radiolabeled D-glucose (absorbed *via* both mediated and non-mediated mechanisms and catabolised after absorption) was tested as a probe for studying the kinetics of glucose absorption *in vivo*, in the seasonally frugivorous American robin (*Turdus migratorius* Turdidae) (McWhorter et al., 2008). These authors found that the kinetics of absorption and elimination of D-glucose appear to be suitable for such *in vivo* studies, and measurements using absorption of D-glucose and 3-OMG in this species give comparable estimates of the relative contribution of paracellular absorption [60±8 *vs* 62±1% (means ± s.e.m.) of total glucose uptake averaged over the first 20 min of absorption].

To date, little data have been collected on the *in vivo* kinetics of glucose absorption by nectarivorous birds. These birds may be particularly interesting as models of digestive mechanisms, since they tend to have significantly shorter alimentary tracts compared with insectivorous species of the same body mass (Richardson and Wooller, 1986). Karasov and Cork (Karasov and Cork, 1994) studied paracellular absorption in the nectarivorous/frugivorous rainbow lorikeet (*Trichoglossus haematodus*, Loriidae) by measuring the bioavailability of L-glucose. They concluded, based only on extent of L-glucose absorption (~80%), that these lorikeets rely significantly upon paracellular absorption for glucose uptake. In the present study, we revisited glucose absorption in the rainbow lorikeet and compared this species to a large Australian honeyeater, the red wattlebird (*Anthochaera carunculata*, Meliphagidae).

Aims of the present study

Our first aim was to further assess radiolabeled D-glucose as a probe for *in vivo* pharmacokinetic studies of glucose absorption. We predicted that radiolabeled D-glucose would prove suitable for such studies. We assessed the kinetics of elimination of D-[^{14}C]glucose in red wattlebirds and rainbow lorikeets after intramuscular injection, and of absorption after oral administration. We compared our pharmacokinetic results with apparent assimilation efficiency (AE*) of non-radiolabeled D-glucose measured by a traditional 24 h mass balance protocol. Because these values were vastly different in red wattlebirds, we repeated the pharmacokinetic trials in this species using [^{14}C]3-OMG, a glucose analogue commonly used for *in vivo* glucose absorption studies (Chang and Karasov, 2004; Tracy et al., 2007). We also tracked the movement of D-[^{14}C]glucose (and L-[^{3}H]glucose, see below) into the various tissues of both study species over time to assess whether the bioavailability of D-glucose as calculated by our pharmacokinetic methods was erroneously low because of rapid post-absorption catabolism or sequestration in tissues (especially gut tissues and liver).

Our second aim was to estimate the proportional contribution of paracellular to total glucose uptake in our study species. We predicted that paracellular absorption would account for the majority of total glucose absorption in both of these nectarivorous birds. We compared the absorption kinetics of simultaneously administered D-[^{14}C]glucose and L-[^{3}H]glucose. The ratio of relative rates of absorption (or, alternately, the cumulative fraction of an oral dose absorbed at a given time point) of L-glucose to D-glucose or 3-OMG provides a robust estimate of the proportion of total glucose uptake that is non-mediated (Chang and Karasov, 2004).

MATERIALS AND METHODS

Birds and their maintenance

A total of 16 red wattlebirds (*Anthochaera carunculata* Shaw 1790, body mass M_b=108±3 g, range 92–127 g) were captured in the grounds of Murdoch University, Perth, WA, Australia, by mist netting in May 2006 (trial A, N=8) and August 2007 (trial B, N=8). Eight rainbow lorikeets (*Trichoglossus haematodus* Linnaeus 1771, M_b=134±4 g, range 122–153 g) were captured in the grounds of Perth Domestic Airport, Perth, WA, Australia, by canon-netting in July 2006, as a part of the Western Australian state Department of Environment and Conservation (DEC) project to decrease their population in the area.

The birds were housed in individual cages (46 cm×56 cm×45 cm) in a controlled environment room maintained at 21±2°C on a 12 h automatic lighting regime with the photophase from 06.00 h to 18.00 h. During the period of captivity the birds were fed a maintenance diet consisting of Wombaroo® powder (main sugar type present in the form of sucrose; Wombaroo Food Products, Adelaide, SA, Australia) supplemented with additional sucrose (~25% w/w of total dry matter). Both species received water *ad libitum*, and rainbow lorikeets also periodically received assorted fresh fruits such as apples and oranges.

For experiments, the birds were housed in individual experimental cages made from 3 mm thick opaque white polyvinyl chloride (PVC) plastic and clear acrylic sheeting (42 cm×54 cm×50 cm), with an automatic lighting regime with photophase from 07.00 h to 18.00 h. The acrylic front of the cage was coated with reflective Mylar® film to act as a one way mirror (cages lit inside, held in a darkened room) so birds would not be disturbed by outside movement. Red wattlebirds received the maintenance diet by accessing inverted stoppered syringes fixed to the outside of the back wall of the cage through a small opening cut into the cage, whereas rainbow lorikeets fed from commercially available plastic parrot feeders fixed to the inside of the back wall of the cage.

Measurement of L-glucose and D-glucose absorption *in vivo*

Food was removed 20 min prior to the experiments and measurements began approximately 2.5 h after lights on. A cocktail containing either D-[^{14}C]glucose (rainbow lorikeets and red wattlebirds, trial A) or [^{14}C]3-OMG (red wattlebirds only, trial B) and L-[^{3}H]glucose (American Radiolabeled Chemicals, Saint Louis, MO, USA) was administered orally (by gavage) and by intramuscular injection (i.m.) to each bird in separate experiments (see Table 1 for solution compositions). Hereafter these radiolabeled compounds will be referred to simply as D-glucose, 3-OMG and L-glucose. Trials were separated by at least 2 weeks to ensure complete recovery from the process of repeated blood collection and elimination of any residual radioactivity. The order of trials and the sequence of treatment type given to the birds were both randomly assigned. The volume of solution administered was measured by weighing the syringe (±0.00001 g) before and after administration. Aliquot samples of the oral and i.m. solutions were saved for radioactivity analysis.

Table 1. Composition of solutions administered by oral gavage or intramuscular injection to rainbow lorikeets and red wattlebirds

Species			Probe				NaCl (mmol l^{-1})	Unlabeled D-glucose (mmol l^{-1})
			Volume given (μl)	D-[^{14}C]glucose (Bq)	[^{14}C]3-OMG (Bq)	L-[^{3}H]glucose (Bq)		
RL	i.m.		125	4.4×10^5		1.1×10^6	175	
	Oral		300	8.9×10^5		2.1×10^6	75	200
	Oral	Probe distribution in body tissues	300	6.7×10^5		6.7×10^6	75	200
RWB	i.m.	Trial A	125	3.7×10^5	3.5×10^5	8.3×10^5	175	
	i.m.	Trial B	125			8.3×10^5	175	
	Oral	Trial A	300	7.4×10^5		1.7×10^5	75	200
	Oral	Trial B	300		7×10^5	1.7×10^5	75	200
	Oral	Probe distribution in body tissues	300	6.7×10^5		6.7×10^5	75	200

RL, rainbow lorikeet; RWB, red wattlebird; i.m. intramuscular injection. Oral solutions were made up to conditions relatively saturating for the mediated glucose transporters (Chang et al., 2004). The total osmolality of the oral and injection solutions was controlled at approximately 350 mmol kg^{-1}, so that the solutions were isosmotic with avian blood (Goldstein and Skadhauge, 2000). Differences in the amounts of radioactivity in the probe solutions reflect differences in body mass between these two species.

An ~50 μl blood sample was collected from the brachial vein prior to each trial (time zero, t_0) for background correction. Following ingestion or injection of the probe solution, eight or nine brachial vein blood samples were similarly collected at times (t_i): 2.5 (trial B only), 5, 10, 20, 30, 45, 60, 120 and 240 min. After the first hour of blood sampling, during which the birds were periodically offered maintenance diet from a feeder while held in the hand, birds were transferred to an experimental cage and received a maintenance diet solution *ad libitum*. Immediately after blood sample collection, microcapillary tubes were sealed with ChaSeal™ clay tube sealing compound (Chase Scientific Glass, Rockwood, TN, USA) and centrifuged in a microhematocrit centrifuge for 2–3 min at ~9000 ***g***. Plasma samples and probe solution aliquots were weighed (±0.00001 g), mixed with 3 ml of Ecolite+™ liquid scintillation fluid (MP Biomedicals Australasia, Seven Hills, NSW, Australia) and counted in a scintillation spectrometer (Beckman LS6500 Liquid Scintillation Counter, Beckman Coulter, Fullerton, CA, USA) as disintegrations per minute (d.p.m.). All counts were corrected for variable quenching and for spill of ^{14}C into the ^{3}H channel.

Pharmacokinetic calculations

The level of radioactivity in each plasma sample (C_i) was corrected by sample mass and plotted against actual sampling time, t_i. The extent of absorption of the radiolabeled probe was estimated using the total area under the curve (AUC_{total}), which represents the total amount of radiolabeled probe absorbed from t_0 to time infinity (t_∞). AUC_{total} is calculated using the trapezoidal rule from t_0 to the last sampling point (t_n) and estimated assuming log-linear decline for the period from t_n to t_∞ as:

$$AUC_{t_n\to\infty} = C_n / k_{el}, \quad (1)$$

where k_{el} is the elimination rate constant based on non-linear modelling of the plasma probe concentration–time curve for each bird in each experiment (Chang and Karasov, 2004; Gibaldi, 1991; Gibaldi and Perrier, 1982).

The fraction of the probe that reaches the systemic circulation is known as the fractional absorption or systemic bioavailability of the probe (Gibaldi, 1991). The fractional absorption (f) of each probe was calculated by estimating the ratio between the AUC for the plasma concentration–time curve for oral administration (AUC_{oral}) compared with the AUC of the i.m. administration ($AUC_{i.m.}$):

$$f = \frac{AUC_{oral}/dose_{oral}}{AUC_{IM}/dose_{IM}} . \quad (2)$$

Both AUC_{oral} and $AUC_{i.m.}$ were corrected to the dose given to each bird in each experiment (Gibaldi and Perrier, 1982).

Additional analyses relating to the time course and apparent rates of absorption of probes were carried out using the Wagner–Nelson or the Loo–Reigelman method as appropriate (Gibaldi and Perrier, 1982; Wagner and Nelson, 1963). Parameters were derived for each individual bird by non-linear curve fitting the plasma concentrations after i.m. administration of the probes at each time point to the mono-exponential (one compartment, $C=C_0e^{-k_{el}t}$) and bi-exponential (two compartment, $C=ae^{-\alpha t}+be^{-\beta t}$) models by use of the Marquardt–Levenberg algorithm (SYSTAT Software, SigmaPlot for Windows, San Jose, CA, USA) (Marquardt, 1963). In 30 out of 40 cases (covering L-glucose and D-glucose in both study species), a bi-exponential model did not fit the elimination data significantly better than a mono-exponential model by use of the F-test (Fig. 1A,B, insets), thus the assumption of a one-compartment model was deemed appropriate for these probes (Motulsky and Ransnas, 1987). In all eight cases for 3-OMG (this probe was used in red wattlebirds trial B only), a bi-exponential model fitted the data significantly better than a mono-exponential model by use of the F-test (Fig. 1C, inset). Analyses relating to the apparent rate of absorption were thus carried out under the assumption of an open two-compartment model and first order elimination for 3-OMG.

Measurement of D-glucose apparent assimilation efficiency by mass balance

Eight red wattlebirds and eight rainbow lorikeets were offered a range of diet solutions (250, 500, 750 and 1000 mmol l^{-1} non-radiolabeled D-glucose) *ad libitum* for 24 h (06.00 h to 06.00 h, although birds only fed during the photophase), at a constant ambient temperature of 22±1°C. Each diet concentration was fed to two birds with total N=8 for each species over the four diet D-glucose concentrations. Birds were placed in the experimental cages (as described above), with trays to collect excreta placed below; liquid paraffin was placed directly under the feeders to collect any diet

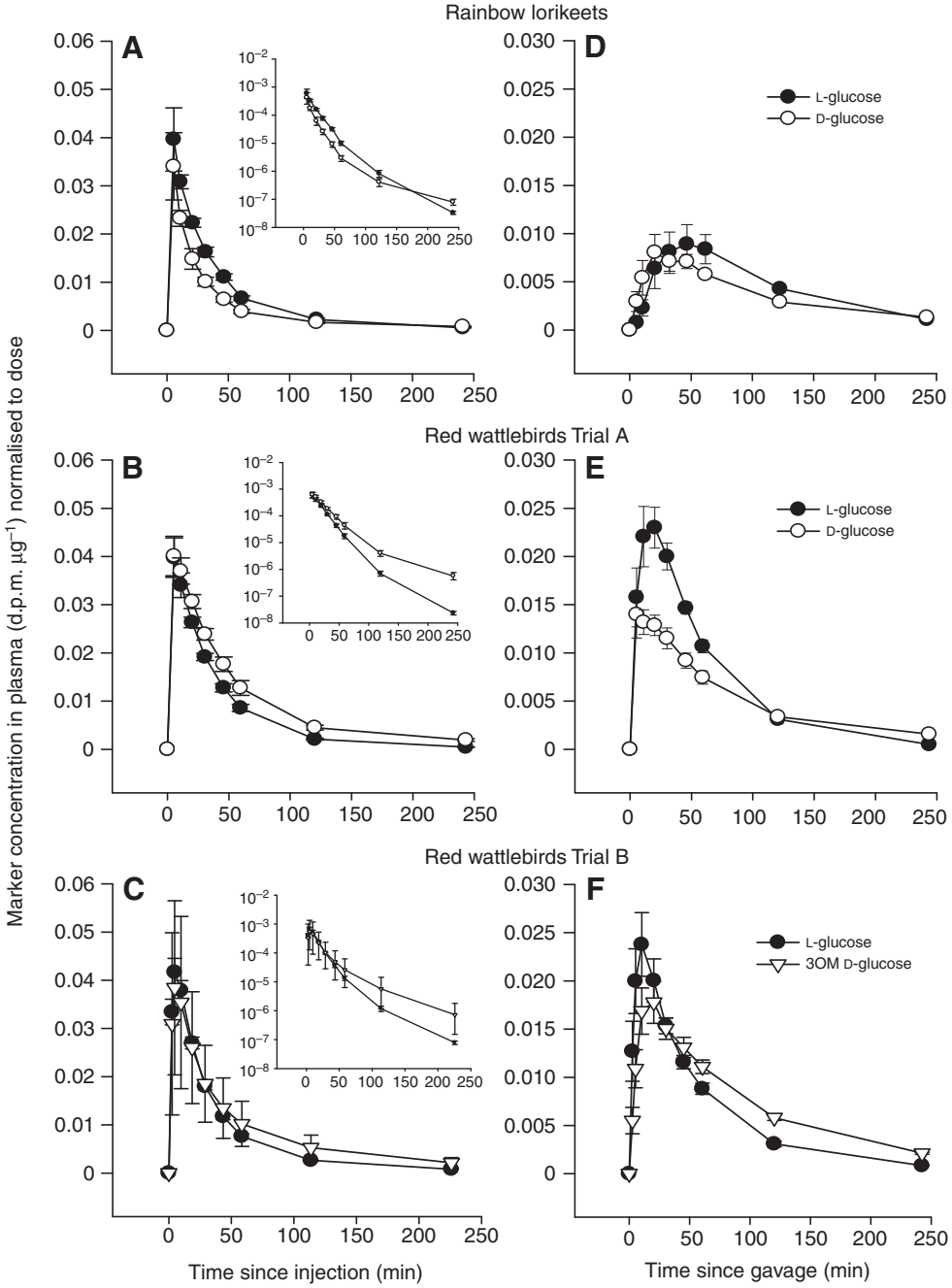

Fig. 1. Plots of the mean dose-corrected plasma concentrations of D-[^{14}C]glucose, [^{14}C]3-OMG and L-[^{3}H]glucose as a function of time since (A–C) injection (insets: semi-log$_e$ plots) and (D–F) oral gavage of the probes. In (A,D) rainbow lorikeets *N*=8 for all time points except at 20 min after injection, where *N*=6 and (B,C,E,F) red wattlebirds *N*=8 for all time points except at 30 min after injection of D-[^{14}C]glucose, *N*=7. The units for concentration are proportion per µg of plasma, as values of d.p.m. µg^{-1} plasma were normalised to injected or oral dose for each individual. Filled circles, L-[^{3}H]glucose; unfilled circles, D-[^{14}C]glucose; and unfilled inverted triangles, [^{14}C]3-OMG.

spilt. Food intake was recorded over 24 h by weighing feeders and correcting for any spillage. Assimilation efficiency (AE*) was estimated as:

$$AE^* = (\text{D-glucose}_{in} - \text{D-glucose}_{out}) / (\text{D-glucose}_{in}), \quad (3)$$

where D-glucose$_{in}$ (mg) is the concentration (mg ml^{-1}) of D-glucose in the ingested diet multiplied by the volume of food ingested (ml), and D-glucose$_{out}$ (mg) is the sugar concentration (mg ml^{-1}) in the total volume of excreta plus rinse water (ml). Two replicates of each excreta sample (100 µl) were incubated at room temperature (~22°C) for 15 min with 500 µl of the glucose hexokinase–glucose-6-phosphate dehydrogenase enzymatic assay reagent (Sigma Aldrich product code G3293, Castle Hill, NSW, Australia). Absorbance was then measured at 340 nm by spectrophotometry (UV mini 1240, Shimadzu Scientific Instruments, Balcatta, WA, Australia) relative to distilled water.

Probe distribution in body tissues following oral administration

Six red wattlebirds and five rainbow lorikeets were weighed (±0.01 g) and gavaged with a cocktail containing D-[^{14}C]glucose and L-[^{3}H]glucose (see Table 1 for cocktail composition). Individual birds were subjected to euthanasia by an overdose of the inhalation anaesthetic Isoflurane at varying time points after gavage: 2.5, 5, 10, 20, 30 and 45 (red wattlebird only) minutes. Tissues were also collected from one un-gavaged rainbow lorikeet for background radioactivity levels and quench correction of tissues. A small blood sample was collected from the heart with glass microcapillary tubes

Table 2. Values for the apparent absorption rate and fractional absorption for each of the radiolabelled probes shown in Fig. 1A-F, and apparent assimilation efficiency of unlabeled D-glucose

	Species		Probe			Comparison between probes
			D-[^{14}C]glucose	[^{14}C]3-OMG	L-[^{3}H]glucose	
Apparent absorption rate (fraction of oral dose absorbed min^{-1})	RL		0.0095±0.0018		0.0062±0.0015	***P*=0.005**
	RWB	Trial A	0.0120±0.0015		0.0273±0.0042	***P*=0.001**
		Trial B		0.0251±0.0023	0.0304±0.0032	***P*=0.003**
f (%)	RL		92.3±3.3		75.6±5.0	***P*=0.031**
	RWB	Trial A	57.4±2.4		96.1±3.5	***P*<0.001**
		Trial B		90.3±4.6	91.7±3.9	*P*=0.566
AE* (%)	RL		99.7±0.1			
	RWB		99.8±0.1			

RL; rainbow lorikeet; RWB, red wattlebird.
Apparent absorption rates are mean values over 45 min, see Results; *f,* fractional absorption; AE*, assimilation efficiency.
Values are means ± s.e.m., *N*=8 for all values. *P* values were obtained by ANOVA, repeated measures ANOVA and paired and independent *t*-tests, with a significance level of α<0.05. Significant *P* values are shown in bold text.

which were immediately sealed with clay tube sealing compound and centrifuged in a microhematocrit centrifuge for 2–3 min at ~9000 ***g***. The intestines were excised from immediately proximal to the proventriculus to the cloaca and sectioned into four (proventriculus, proximal, medial and distal sections) and flushed clean with saline solution; the intestinal contents were collected separately for each of the four sections. The whole kidneys, brain, liver and as much as possible of the pectoralis muscle were collected, weighed and frozen for later analysis. All organs and intestinal contents collected were weighed (±0.00001 g) and samples of intestinal contents, plasma and probe solution aliquots were mixed with 3 ml of Ecolite+^{TM} liquid scintillation fluid and the radioactivity counted in a scintillation spectrometer.

A small sample of each of the organs (≤200 mg) was weighed, solubilised (Solvable^{TM}, PerkinElmer, TH, Groningen, Netherlands) and decoloured with 30% H_2O_2 before being mixed with 15 ml of Ecolite+^{TM} liquid scintillation fluid and the radioactivity counted. Two samples were processed for each organ, intestinal contents and plasma, and the radioactivity in each sub-sample (d.p.m. mg^{-1}) was averaged and extrapolated to the whole organ mass to estimate the total radioactivity present in each organ (d.p.m. organ^{-1}). Plasma volume was estimated from bird body mass in mg multiplied by 0.05, the approximate plasma volume in birds (Goldstein and Skadhauge, 2000). The radioactivity of each organ was then expressed as a percentage of total radioactivity delivered by oral gavage.

Statistical analysis

Numerical data are presented as mean ± s.e.m., with *N* referring to the number of animals. Statistical analyses were performed on data for individual birds. The validity of the normal distribution of variables was tested by the one-sample Kolmogorov–Smirnov Test (SPSS, SPSS, Inc, Chicago, IL, USA). Log-transformed elimination rate constants and *f* values did not violate the assumptions of normality. Results were analysed by repeated-measures ANOVA, one-way ANOVA, paired *t*-test and independent samples *t*-test (SPSS) with degrees of freedom presented as subscripts. Linear regression was by the method of least squares. Statistical significance was accepted for α<0.05.

RESULTS

Measurement of L-glucose and D-glucose absorption *in vivo*

The elimination of D-glucose and L-glucose after i.m. administration followed single-compartment kinetics of elimination (Fig. 1A,B, insets), whereas 3-OMG followed a two-compartment model with first order elimination (Fig. 1C, inset). All three probes equilibrated rapidly when injected, with the average concentration in plasma peaking for the first sampling time of 5 min in both species, indicating little to no extended lag for distribution from the pectoralis muscle into the blood (Fig. 1A–C). In rainbow lorikeets, dose-corrected area under the curve after injection ($AUC_{i.m.}$) for D-glucose was significantly lower than for L-glucose ($F_{1,7}$=9.04, P=0.020). Based on the elimination rate constants (k_{el}) for these probes calculated from model fits for all time points (see pharmacokinetic calculations), D-glucose was eliminated significantly faster ($F_{1,7}$=74.53, P<0.001). By contrast, in red wattlebirds, both D-glucose and 3-OMG had significantly higher dose-corrected $AUC_{i.m.}$ than L-glucose ($F_{1,7}$=58.76, P<0.001 and $F_{1,7}$=70.75, P<0.001, respectively). k_{el} values were significantly lower for both D-glucose probes than for L-glucose (D-glucose: $F_{1,7}$=27.33, P=0.001; 3-OMG: $F_{1,7}$=84.74, P<0.001) in the wattlebirds.

Following simultaneous oral administration of D-glucose and L-glucose to rainbow lorikeets, average D-glucose concentration in plasma peaked by 20 min whereas L-glucose peaked later, at 45 min (Fig. 1D). D-glucose AUC_{oral} in lorikeets was not significantly different to that of L-glucose ($F_{1,7}$<0.01, P=0.988). In contrast to the injection trials in wattlebirds, D-glucose k_{el} was significantly lower than that of L-glucose ($F_{1,7}$=18.15, P=0.004) following oral administration, indicating slower elimination. The similar procedure for red wattlebirds (trial A) resulted in maximum average plasma D-glucose concentration at the first sampling time of 5 min, whereas plasma L-glucose peaked at 20 min; after these time points, both probes exhibited exponential decline (Fig. 1E). As in the lorikeets, D-glucose AUC_{oral} in wattlebirds was not significantly different from that of L-glucose ($F_{1,7}$=1.72, P=0.230). In agreement with the injection trial in red wattlebirds, D-glucose was eliminated more slowly than L-glucose, as indicated by a significant difference in k_{el} values ($F_{1,7}$=110.67, P<0.001).

Fractional absorption (*f*) of D-glucose significantly exceeded that of L-glucose in rainbow lorikeets; in red wattlebirds (trial A) there was also a significant difference between these probes, but D-glucose *f* was unexpectedly lower than that of L-glucose (Table 2). This surprising result led us to repeat the experiment using non-metabolisable 3-OMG in the red wattlebird (trial B), to remove the effects of pre-systemic catabolism of D-glucose. When red wattlebirds were gavaged with 3-OMG, average concentration in plasma peaked at 20 min after administration, and L-glucose peaked

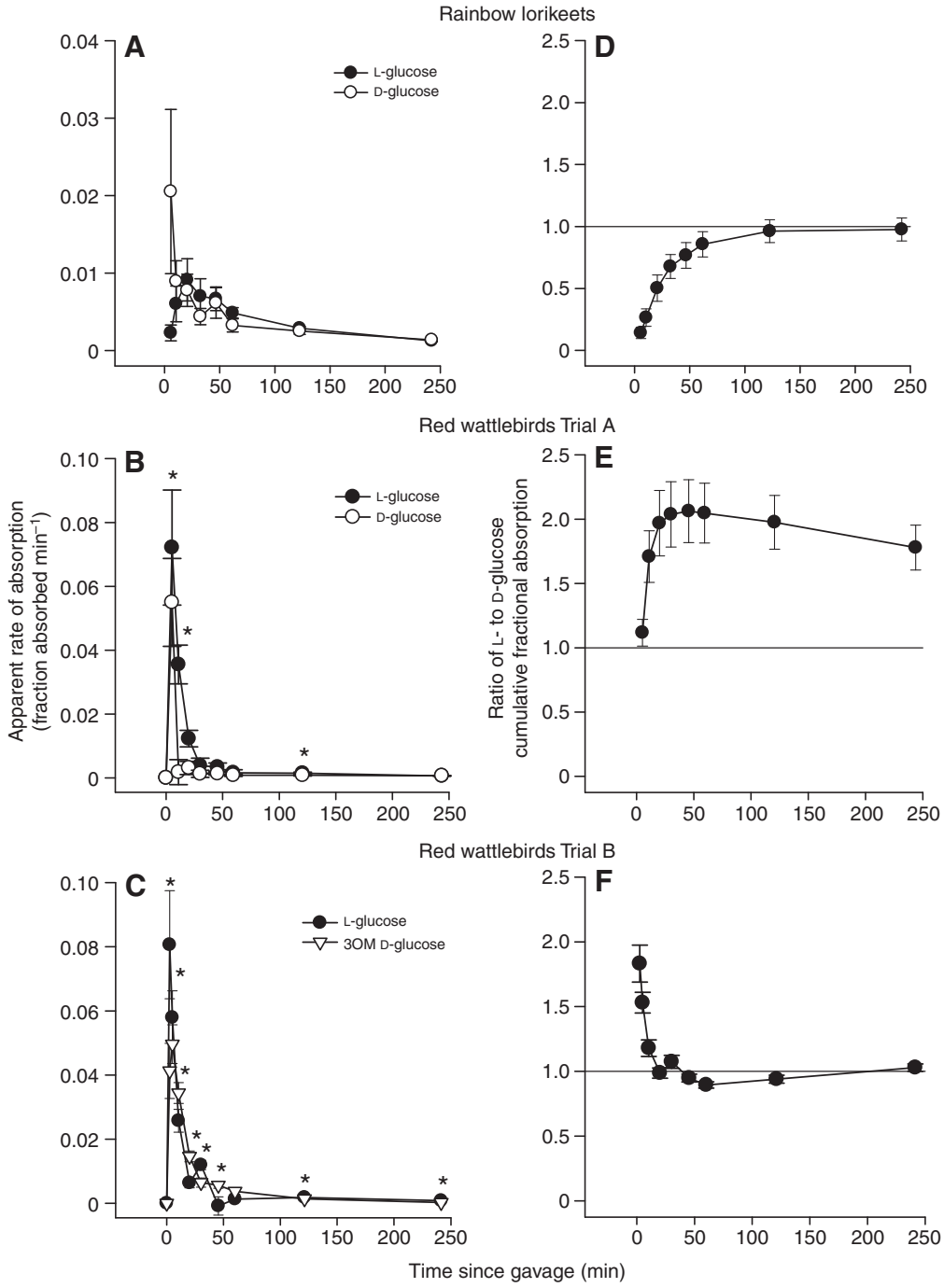

Fig. 2. Plots of (A–C) mean apparent rates of absorption (fraction of the oral dose absorbed per minute) of D-[^{3}H]glucose, [^{14}C]3-OMG and L-[^{3}H]glucose, and (D–F) the mean ratio of L-glucose to D-glucose or 3-OMG cumulative fractional absorption (fraction of the amount of the oral dose ultimately absorbed by each time point). In (A,D) rainbow lorikeets and (B,C,E,F) red wattlebirds N=8 for all time points for both species. Measurements were made under conditions saturating for mediated glucose transporters in both species. Filled circles in A–C, L-[^{3}H]glucose; unfilled circles, D-[^{14}C]glucose; unfilled inverted triangles, [^{14}C]3-OMG. Asterisks in B and C indicate a significant difference in apparent absorption rate of the two probes when data for individual birds were compared for each sampling time point separately (RM-ANOVA, P<0.05). Note different y-axis scales for A–C.

at 15 min (Fig. 1F), slightly earlier than in trial A. The observation that 3-OMG concentration in plasma peaked later that that of D-glucose (20 min *vs* 5 min; Fig. 1E,F) suggests a difference in the rate of absorption between the two D-glucose probes. As in the injection trial, 3-OMG was eliminated more slowly (k_{el} was significantly lower) than L-glucose in red wattlebirds ($F_{1,7}$=905.61, P<0.001).

For both species, mean apparent absorption rates of probes declined with time since gavage (Fig. 2A–C). There were two exceptions to this pattern: in lorikeets L-glucose absorption rate increased to a peak at 20 min before declining (Fig. 2A), and in wattlebirds 3-OMG absorption rate (trial B) peaked at 5 min before declining (Fig. 2C). Mean apparent D-glucose absorption rate in lorikeets calculated over the first 45 min after oral dosing (the approximate time required to absorb 50% of the amount ultimately absorbed) was significantly greater than that of L-glucose (Table 2). When apparent absorption rates of D-glucose and L-glucose were compared at each time point separately in rainbow lorikeets, there were no statistically significant differences (Fig. 2A). By contrast, mean L-glucose apparent absorption rate in red wattlebirds was significantly higher than that of both D-glucose and 3-OMG (Table 2). There was no significant effect of trial A *vs* B on L-glucose absorption rate ($F_{1,14}$=1.29, P=0.280). Mean apparent rate of 3-OMG absorption in wattlebirds (trial B) exceeded that of D-glucose (trial A; $F_{1,14}$=23.14, P<0.001). In wattlebirds when apparent absorption rates for the various probes were compared at each time point separately there were some significant differences (summarised in

Fig. 2B,C). Comparing between species, mean D-glucose apparent absorption rate up to 45 min in lorikeets was not significantly different to that in wattlebirds ($F_{1,14}$=1.09, P=0.320). By contrast, mean 3-OMG apparent absorption rate in red wattlebirds exceeded D-glucose absorption rate in lorikeets ($F_{1,14}$=29.08, P<0.001). Mean L-glucose apparent absorption rate up to 45 min in wattlebirds exceeded that in lorikeets ($F_{1,14}$≥22.49, P≤0.001, comparing both trial A and B).

The ratio of the cumulative fractional absorption of L-glucose to D-glucose or 3-OMG at each sampling time point was used to estimate the proportion of total glucose absorption that occurs by non-mediated mechanisms (Fig. 2D–F). The L/D ratio increased with time after gavage and ranged from 0.10 to 0.97 (Fig. 2D) in rainbow lorikeets, indicating that at least 10% of D-glucose absorption occurred *via* the paracellular pathway. During the initial absorption phase from 5 min to 45 min in rainbow lorikeets, an average of 50% of the glucose was absorbed *via* the paracellular pathway. In red wattlebirds, the L/D ratio ranged from 1.12 to 2.06 (D-glucose; Fig. 2E) and 0.89 to 1.83 (3-OMG; Fig. 2F), indicating problems either with assumptions of this method of calculation, or with the probes themselves (see Discussion).

Apparent assimilation efficiency of D-glucose

Diet concentration did not significantly influence D-glucose AE* in either rainbow lorikeets ($F_{1,6}$=1.49, P=0.270) or red wattlebirds ($F_{1,6}$=0.001, P=0.980), so the mean of all diet concentrations was calculated (Table 2). D-glucose AE* in rainbow lorikeets (99.7±0.1%) was not significantly different from D-glucose bioavailability calculated using the pharmacokinetic method (92.3±3.3%; $F_{1,7}$=5.35, P=0.054). For red wattlebirds, however, D-glucose AE* by mass balance (99.8±0.1%) was significantly higher than D-glucose bioavailability (f) estimated by the pharmacokinetic method (57.4±2.4%; $F_{1,7}$=326.71, P<0.001), although the former was not significantly different from 3-OMG bioavailability in these birds (90.3±4.6%; $F_{1,14}$=4.21, P=0.059).

Probe distribution in body tissues following oral administration

The highest proportion of both D-glucose and L-glucose recovered from the rainbow lorikeet (the sum of all organs, extrapolated to the whole organ mass) peaked at our last time point of 30 min (Fig. 3E). This contrasted with much faster recovery for red wattlebirds, with the highest proportion of gavaged D-glucose and L-glucose recovered peaking at our first sampling times (2.5 and 5 min; Fig. 3E). These data provide robust independent support for the conclusion that absorption of D-glucose and L-glucose was slower in rainbow lorikeets compared with red wattlebirds (above). However, the appearance of both probes in various tissues and organs followed similar patterns over time for the two species (Fig. 3) and from peak values, we could project a path of radiolabeled glucose through tissues and organs: gut contents→gut tissue→plasma=liver=other tissues/organs.

Significantly more ^{14}C (administered as D-[^{14}C]glucose) was recovered from the intestinal contents of red wattlebirds compared with rainbow lorikeets (Fig. 3A; independent t-tests: t_{38}=–2.23, P=0.032), intestinal tissue (Fig. 3B; t_{38}=–2.50, P=0.030) and liver (Fig. 3D; t_9=–2.59, P=0.029). Despite slower absorption by the rainbow lorikeets (Fig. 3C), there was no significant difference in the mean proportion of ^{14}C recovered from plasma between red wattlebirds (4.5±0.5%) and rainbow lorikeets (4.3±1.8%; t_9=–0.10, P=0.924). There was also no significant difference in the mean proportion of ^{14}C recovered from other organs (kidney, pectoralis and brain; Fig. 3E) between the species (red wattlebirds: 6.51±0.62%; rainbow lorikeets: 3.34±1.80%; t_{31}=–1.43, P=0.162).

Significantly more L-[^{3}H]glucose, which should not have been catabolised (Chang et al., 2004; Karasov and Cork, 1994), was recovered from the intestinal contents of red wattlebirds compared with rainbow lorikeets (Fig. 3A; t_{38}=–2.34, P=0.025). There was no significant difference in the recovery of L-[^{3}H]glucose between red wattlebirds and rainbow lorikeets from intestinal tissue (Fig. 3B; t_{37}=–1.48, P=0.147), the liver (Fig. 3D; t_9=–1.84, P=0.103) and other organs (kidney, pectoralis and brain; t_{31}=0.94, P=0.352; Fig. 3E).

DISCUSSION

Although the kinetics of D-glucose elimination after injection and absorption after oral dosing appear appropriate to warrant the use of radiolabeled D-glucose to measure glucose uptake rates *in vivo*, more detailed analyses indicated serious problems with using this probe. For example, we recorded extensive catabolism or sequestration after absorption in red wattlebirds. The second aim of our study was to determine the nutritional importance of non-mediated (paracellular) absorption in two highly nectarivorous species. Our data indicate that the contribution of paracellular uptake to overall glucose uptake is significant in both rainbow lorikeets and red wattlebirds, and that the contribution by this mechanism to total glucose absorption is highly dynamic over the absorptive phase. In the following paragraphs we discuss the inconsistencies in performance of radiolabeled D-glucose between our study species, and changes in the relative importance of paracellular uptake during nutrient absorption.

In rainbow lorikeets, the bioavailability of D-glucose calculated using our pharmacokinetic methodology was not significantly different from apparent assimilation efficiency calculated using a traditional mass-balance approach. As expected, the apparent rate of D-glucose absorption (both mediated and non-mediated uptake) over the initial absorptive period (through 45 min) significantly exceeded that of L-glucose (non-mediated uptake only) (Chang et al., 2004). Radiolabeled D-glucose thus appears to provide robust *in vivo* estimates of absorption rates in this species. The proportion of total glucose absorbed *via* the paracellular route increased over time in rainbow lorikeets, with as little as 10% of total glucose uptake *via* this non-mediated pathway at the first time point recorded (Fig. 2D). The values at the last two sampling time points (120 and 240 min, showing essentially 100% of total glucose absorption as paracellular) are probably indicative that some L-glucose absorption is occurring further along in the intestine, after D-glucose absorption was essentially complete (Fig. 2D). L-Glucose bioavailability measured in the present study (~76%) compares favourably with data collected by Karasov and Cork (Karasov and Cork, 1994) utilising a steady-state feeding methodology in these birds (~80%). L-Glucose bioavailability data, which do not provide any information on relative rates of absorption, have been previously taken as an estimate of the proportional contribution of paracellular uptake; our data show that this contribution in rainbow lorikeets is both lower on average when rates of absorption are considered, and varies through the absorptive phase.

Although our data for the rainbow lorikeet were easily interpreted, the same was not true of data for the highly nectarivorous (Pyke, 1980) red wattlebird. In these birds, estimated bioavailability of D-glucose using our pharmacokinetic methodology was low (~57%). We know this figure to be incorrect, since the apparent assimilation efficiency of D-glucose measured by mass balance was estimated at ~99%. Furthermore, the bioavailability of L-glucose (92–96%) was similarly high and

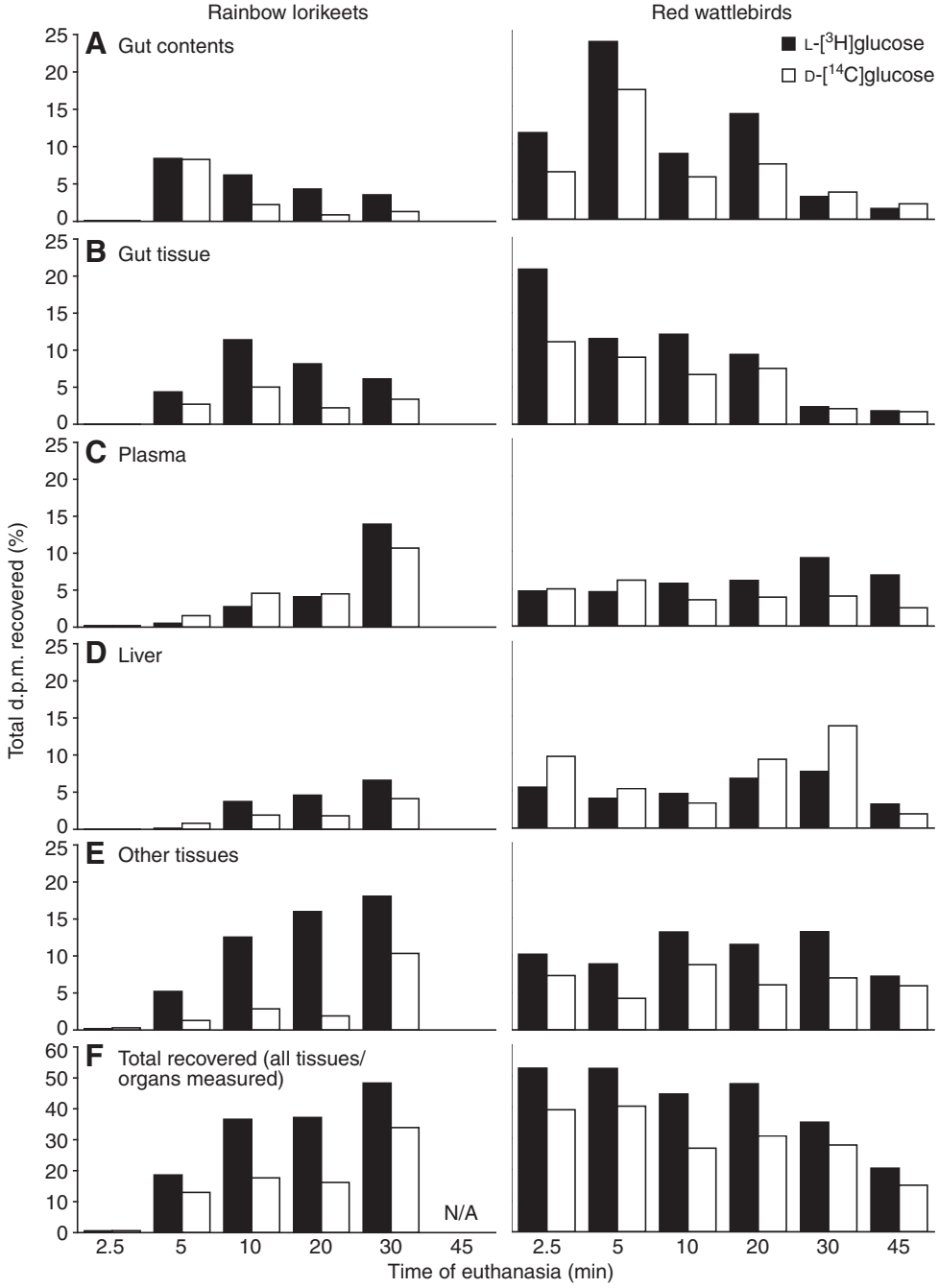

Fig. 3. Apparent total percentage of the dose of L-[^{3}H]glucose and ^{14}C (administered as D-[^{14}C]glucose) recovered in (A) intestinal contents, (B) intestinal tissues (including all section from the proventriculus distally), (C) plasma, (D) liver, (E) all other tissues including kidney, pectoralis and brain, and (F) summed for intestinal contents and all tissues measured in rainbow lorikeets (left hand panels) and red wattlebirds (right hand panels) at varying times of euthanasia (N=1 per species at each time point). Total recovery for each tissue was estimated by extrapolating the concentration of radioactivity in sub-samples to the whole tissue mass, and is expressed as a percentage of the dose delivered by oral gavage.

exceeded that of D-glucose, but based on mechanisms of absorption we would expect that L-glucose absorption should at best match that of D-glucose (Chang and Karasov, 2004). The absorption of L-glucose relative to D-glucose (L/D ratio) in red wattlebirds yielded values that exceeded 1 during the entire period of blood sampling (Fig. 2E). This erroneous result suggests that absorption rates based on the appearance of ^{14}C in plasma after oral administration of radiolabeled D-glucose do not reflect true absorption rates for this species. We suspect that pre-systemic catabolism and/or sequestering of marker in tissues (i.e. rapid removal from the bloodstream *via* hepatic and intestinal first-pass effects) (Muratoglu et al., 1986) could be responsible for these observations. We tested these hypotheses by repeating experiments in red wattlebirds with the non-metabolisable glucose analogue 3-OMG and tracing the movement of radiolabel into the various tissues and organs at specific time points after oral gavage.

We retrospectively calculated the potential impact of first-pass effects on D-glucose bioavailability (Gibaldi and Perrier, 1982) in red wattlebirds, as a frame of reference for our measurements using non-metabolisable 3-OMG. These calculations indicated that first-pass effects could have reduced calculated bioavailability by at least ~23–25%. Subsequent measurements with 3-OMG (~90% bioavailability, not significantly different from D-glucose AE*) confirmed that the erroneous result for D-glucose was indeed due to rapid absorption and subsequent catabolism and/or removal of the latter *via* hepatic and intestinal first-pass effects. However, as for the experiments with D-glucose (trial A), the apparent rate of L-glucose absorption exceeded that of 3-OMG, yielding values >1 for

the L/D ratio at the initial sampling time points. Although 3-OMG cannot be catabolised, the apparent absorption rate of 3-OMG calculated at the initial sampling time points may not directly reflect that of D-glucose. There are several concerns associated with the use of 3-OMG as an analogue for D-glucose. Firstly, the molecular mass of 3-OMG is slightly higher than that of D-glucose, lowering its diffusion coefficient in water, although this can easily be corrected (Chang and Karasov, 2004). The paracellular pathway discriminates according to molecule size (e.g. Chediack et al., 2003), so the greater molecular mass of 3-OMG may lead to apparent paracellular rates of absorption lower than for D-glucose. This effect may be especially pronounced in species in which paracellular absorption is relatively more important, and may thus lead to L/D ratios >1. Secondly, because of this structural difference, 3-OMG has a lower affinity for glucose transporters compared with D-glucose (Ikeda et al., 1989; Kimmich, 1981). In four mammal species (laboratory rat, rabbit, guinea pig and hamster) and chickens (the only avian species studied), averaging across species where data are available for both D-glucose and 3-OMG in the same study and thus avoiding a myriad of confounding methodological differences, the affinity (defined here as $1/K_m$, or reciprocal of the Michaelis constant) of the 3-OMG for carrier-mediated glucose transport systems is ~25% that of the D-glucose (Bihler, 1969; Fedorak et al., 1991; Shehata et al., 1981; Thomson et al., 1982). Lower affinity for glucose transporters may result in lower uptake rates of 3-OMG relative to D-glucose. Furthermore, because 3-OMG apparently does not stimulate the recruitment of the GLUT2 transporter to the apical membrane (Cheeseman and Harley, 1991), the mediated component may be underestimated in studies using only 3-OMG. However, we think this explanation is unlikely in the present study because birds were fed on a sucrose maintenance diet (hydrolysed to D-glucose and fructose in the intestine) immediately before and after gavage with 3-OMG. Finally, a third possible explanation for delayed 3-OMG appearance in plasma at initial sampling time points in red wattlebirds could be accumulation in intestinal tissues. Boyd and Parsons (Boyd and Parsons, 1979; Boyd and Parsons, 1978) demonstrated that as long as normal vascular perfusion is maintained, there is no significant accumulation of this probe in anuran intestinal tissues during steady-state *in vivo* perfusions (i.e. rate of appearance in blood equals apical uptake). However, this may not apply for single-dose *in vivo* uptake studies of compounds rapidly absorbed *via* mediated mechanisms where there is loading of the probe into intestinal tissue pools before washout into systemic circulation. In spite of these concerns, the apparent rate of 3-OMG absorption was significantly higher than that of D-glucose in red wattlebirds, and bioavailability calculated by our pharmacokinetic methodology was comparable to D-glucose assimilation efficiency by mass balance. Thus, 3-OMG appears to provide the best available estimate of *in vivo* glucose absorption rate of these two molecules.

A significantly higher proportion of ^{14}C (administered as radiolabeled D-glucose) was recovered in the liver and intestinal tissues of the red wattlebird than the rainbow lorikeet, lending additional support to the hypothesis that first-pass effects partly account for the low oral bioavailability and calculated apparent rate of D-glucose absorption. Although we collected gut contents and all major organs, we clearly did not succeed in locating the bulk of the radiolabel administered to rainbow lorikeets a short time after oral administration (for example, we recovered a total of only 0.6% of ^{14}C 2.5 min after oral administration of D-glucose, or 13% by 5 min; Fig. 3F). By contrast, in the red wattlebird we recovered a total of 39% of ^{14}C only 2.5 min after oral administration of D-glucose (Fig. 3F), with 17% of ^{14}C recovered from liver, kidney, muscle and brain tissues (Fig. 3D,E). Therefore, in rainbow lorikeets, absorption and utilisation of radiolabeled glucose appears to be a relatively slow process compared with red wattlebirds. Furthermore, whereas we recorded an increase in radiolabel from both L- and D-glucose in body tissues over time for the rainbow lorikeet, the proportion of radiolabel recovered from red wattlebirds declined over time (Fig. 3F). These data suggest that rapid catabolism of D-glucose and removal from the body as $^{14}CO_2$ was occurring in red wattlebirds, but over the same timeframe rainbow lorikeets were still absorbing glucose from the alimentary canal. This probably reflects differences in the physiological mechanisms of glucose uptake, but may also reflect differences in digesta passage rates between the species (not measured in red wattlebirds, mean retention time of 0.4 mol l^{-1} sugar nectar diet in rainbow lorikeets is 0.9 h, increasing to 1.6 h on 1.2 mol l^{-1} sugar) (Karasov and Cork, 1996).

Although the use of radiolabeled D-glucose appeared to be an improved method to avoid concerns with the commonly used 3-OMG, rapid absorption and catabolism of the former in the red wattlebird resulted in a significant underestimation of bioavailability. The assumption that the rate of labelled D-glucose appearance in plasma reflects apical uptake in the intestine is therefore not valid in this species. We conclude that radiolabeled D-glucose is not an appropriate probe for *in vivo* uptake measurements in animals where glucose is absorbed and/or catabolised extremely rapidly. Overall, there appears to be no real advantage in using radiolabeled metabolisable D-glucose over 3-OMG. In the American robin, the difference in the estimated contribution of paracellular absorption did not differ significantly between the two probes (McWhorter et al., 2008), however there is clearly a risk of erroneous results in birds such as the red wattlebird.

Simultaneous administration of D-glucose analogues with L-glucose provides a robust tool to evaluate the nutritional significance of paracellular absorption, avoiding concerns that non-mediated probes may be absorbed more slowly or in a different region of the intestine (Chang and Karasov, 2004; Schwartz et al., 1995). Our data revealed that glucose absorption in rainbow lorikeets and red wattlebirds is an extremely dynamic process. In spite of similar body masses and a similar nectar diet, differences in handling glucose were apparent between our study species. There appeared to be a shift in the relative importance of paracellular uptake over time in rainbow lorikeets, probably the result of decreasing substrate concentrations and thus the relative saturation mediated glucose transporters over the absorptive phase. In red wattlebirds, although the L/D-glucose cumulative fractional absorption ratio exceeded 1 in this study (and thus provided no useable data on the proportional contribution of paracellular to total glucose uptake), the fact that the apparent absorption rate of L-glucose exceeded that of D-glucose and 3-OMG suggests very significant reliance of paracellular uptake. The apparent rate of 3-OMG absorption in red wattlebirds exceeded that of D-glucose in rainbow lorikeets, and similarly the rate of L-glucose absorption in wattlebirds exceeded that in lorikeets. These observations suggest that glucose absorption overall occurs more rapidly in wattlebirds than in lorikeets. These relatively large nectarivorous birds, with their simple diets and gut structure may be ideal model species for studying the mechanisms and regulation of paracellular nutrient absorption.

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