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RESEARCH ARTICLE

Paracellular nutrient absorption is higher in bats than rodents: integrating from intact animals to the molecular level

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ABSTRACT

Flying vertebrates have been hypothesized to rely heavily on paracellular absorption of nutrients to compensate for having smaller intestines than non-flyers. We tested this hypothesis in an insectivorous bat (*Myotis lucifugus*) and two insect-eating rodents (*Onychomys leucogaster* and *Peromyscus leucopus*). In intact animals, the fractional absorption of orally dosed L-arabinose (M_r 150) was 82% in *M. lucifugus*, which was more than twice that of the rodents. Absorption of creatinine (M_r 113) was greater than 50% for all species and did not differ between *M. lucifugus* and the rodents. We also conducted intestinal luminal perfusions on anesthetized animals. Absorption of L-arabinose per nominal surface area in *M. lucifugus* was nearly double that of the rodents, while absorption of creatinine was not different among species. Using an everted sleeve preparation, we demonstrated that high concentrations of L-arabinose and creatinine did not inhibit their own uptake, validating their use as passive, paracellular probes. Histological measurements indicated that *M. lucifugus* has more cells, and presumably more tight junctions, per nominal surface area than *P. leucopus*. This seems unlikely to explain entirely the higher absorption of L-arabinose in *M. lucifugus* during perfusions, because L-arabinose absorption normalized to the number of enterocytes was still double that of *P. leucopus*. As an alternative, we investigated tight junction gene expression. *M. lucifugus* had higher expression of claudin-1 and claudin-15, and lower expression of claudin-2 relative to *P. leucopus*. Expression of claudin-7 and occludin did not differ among species. Taken together, our results support the hypothesis that bats have evolved higher paracellular nutrient absorption than non-flying animals, and that this phenomenon might be driven by both histological characteristics and differences in tight junction gene expression.

KEY WORDS: Paracellular absorption, Perfusion, Arabinose, Comparative transcriptomics, Claudin

INTRODUCTION

Vertebrates can absorb water-soluble nutrients in their intestines via two pathways: a transporter-mediated transcellular one, in which nutrients pass through enterocytes, and a non-mediated paracellular pathway, in which nutrients pass through the tight junctions between enterocytes. The degree of use of the paracellular pathway varies widely among vertebrates (Caviedes-Vidal et al., 2007; Lavin and Karasov, 2008; Fasulo et al., 2013a). For example, it is minor in terrestrial mammals, but several small birds and bats actually absorb the majority of glucose paracellularly (Karasov and Cork, 1994; Caviedes-Vidal and Karasov, 1996; Levey and Cipollini, 1996; Afik et al., 1997; Tracy et al., 2007; Karasov et al., 2012; Fasulo et al., 2013b). This phenomenon has been hypothesized to derive from an evolutionary pressure to reduce mass in flying animals (Caviedes-Vidal et al., 2007) while having as a trade-off greater exposure to water-soluble toxins (Diamond, 1991).

The physiological mechanisms driving these differences among species in paracellular nutrient absorption are poorly understood. In experiments in which isolated sections of the intestine are luminally perfused, birds (Lavin et al., 2007) and bats (Brun et al., 2014) have higher absorption of nutrient-sized probes than rodents, indicating differences in the paracellular permeability of the intestine to nutrients. These differences could arise from one or more non-mutually exclusive mechanisms. Flying vertebrates might have more tight junctions across which nutrients and paracellular probes can be absorbed. This could result from higher villous amplification or a higher density of enterocytes. Alternatively, the tight junctions of flying vertebrates might be more permeable to nutrient-sized molecules compared with non-flyers. Tight junctions are composed of a complex mixture of proteins – including occludin (OCLN) and several claudins among others – the proportions of which could affect the permeability characteristics of the tight junctions (Günzel and Yu, 2013).

We investigated the paracellular absorption of nutrients, and the mechanistic correlates thereof, in a bat and two rodents (Table 1). Because the role of diet on paracellular nutrient absorption is not yet clear, and because previous studies focused on species that consume primarily carbohydrate-rich diets (Lavin et al., 2007; Tracy et al., 2007; Caviedes-Vidal et al., 2008; Brun et al., 2014), we examined species that all rely substantially on protein-rich diets. It was previously suggested that carnivores might rely more extensively on paracellular nutrient absorption than herbivores (McWhorter et al., 2013). Our focal species thus allowed us to examine this putative dietary association as well as compare it with the influence of flight on paracellular nutrient absorption.

In intact animals, and in separate experiments in which we perfused isolated intestinal sections, we assesed the paracellular absorption of nutrients using probes that either mimic glucose or that do not interact with nutrient transporters. L-Arabinose (M_r 150) is a carbohydrate that has been used to estimate the paracellular absorption of glucose (M_r 180) (Lavin et al., 2007; Fasulo et al., 2013b; Price et al., 2013b; Brun et al., 2014) as it does not interact with glucose transporters (Lavin et al., 2007). Creatinine (M_r 113) is a nitrogenous molecule that is similar in size to proline (M_r 115), and its intestinal absorption has been generally assumed to be paracellular (Pappenheimer, 1990; Turner et al., 2000), allowing us to estimate the paracellular absorption of amino acids, which would be expected to be higher due to their smaller size. We further

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Table 1. Animal attributes

	Myotis lucifugus	*Onychomys leucogaster*	*Peromyscus leucopus*
N (males/females)[a]	12/20	4/2	11/9
Body mass (g)	7.8±0.2	38.1±3.4	22.4±0.76
Body length (cm; snout to base of tail)	4.7±0.07	10.5±0.27	8.64±0.18
Small intestine length (cm)	15.00±0.45	14.9±0.8	21.6±1.0
Small intestine circumference (mm)	4.82±0.12	7.56±0.41	6.20±0.20
Cecum mass (g wet; including contents)	Absent	0.589±0.162	0.626±0.042
Large intestine length (cm)	Absent	4.86±0.42	6.34±0.36

[a]Sample size for the morphological measurements in this table, which overlaps substantially with, but does not coincide exactly with, the total sample size. Sample sizes for each experiment are in figure legends or other tables.

demonstrate that neither of these probes is absorbed by transporter-mediated mechanisms in bat or mouse intestines. We also examined the absorption of the large carbohydrate lactulose (M_r 342), which is absorbed by the paracellular route but its absorption is expected to be low due to the size-selective nature of the tight junctions (Delahunty and Hollander, 1987; Chediack et al., 2003; Anderson and Van Itallie, 2009). To assess nutrient absorption, we measured proline and either D-glucose or its analog 3-*O*-methyl-D-glucose (3OMD-glucose), which are both absorbed by the paracellular pathway as well as transcellularly via interactions with glucose transporters. Given previous findings of high paracellular probe absorption in flying vertebrates (Caviedes-Vidal et al., 2007), we expected that absorption of L-arabinose would be higher in the bat in both intact animals and in luminal perfusions. Similarly, we expected that the percentage of glucose absorption that was paracellular (estimated using arabinose absorption as a surrogate for paracellular glucose absorption) would be higher in the bat. There are no previous comparisons among flyers and non-flyers for smaller, proline-sized molecules, although we expected creatinine absorption to be higher in bats, and similarly, that the percentage of proline absorption that is paracellular would be higher in bats.

To explain the differences among species in the absorption of these probes, we searched for differences in intestinal histology and tight junction gene expression between the bat and one rodent. We measured the villous amplification and enterocyte density of the small intestine to estimate the between-species difference in tight junctional area. We also determined expression profiles for several tight junction-associated genes that have been implicated in affecting paracellular permeability of the intestinal epithelium. Combined, our measurements provide an integrated view of among-species variation in paracellular nutrient absorption.

RESULTS

Whole animal experiments

Fractional absorption of 3OMD-glucose was uniformly high across species ($F_{2,13}$=1.34, P=0.296) and did not differ significantly from unity (P>0.14 for all species) (Fig. 1). The majority of the creatinine dose was absorbed by all species (56–83%), but varied significantly ($F_{2,14}$=4.04, P=0.041). Fractional absorption of creatinine was significantly different between *Onychomys leucogaster* and *Peromyscus leucopus* (P=0.048), but neither rodent differed significantly from *Myotis lucifugus* (P>0.2) (Fig. 1). Arabinose fractional absorption varied among species ($F_{2,15}$=5.69, P=0.014) and was notably high in the bat (82%) (Fig. 1). This was significantly higher than that of both *O. leucogaster* (40%; P=0.049) and *P. leucopus* (31%; P=0.027). Fractional absorption of lactulose varied among species ($F_{2,12}$=6.87, P=0.01) and was significantly higher in *M. lucifugus* compared with either *O. leucogaster* (P=0.026) or *P. leucopus* (P=0.032) (Fig. 1).

In the rodents, absorption of the paracellular probes generally declined with probe size. In *P. leucopus* ($F_{2,15}$=20, P<0.001), creatinine absorption was greater than that of L-arabinose (P=0.004), while L-arabinose and lactulose absorptions were statistically similar (P=0.056). In *O. leucogaster* ($F_{2,16}$=25, P=0.001), creatinine absorption exceeded that of L-arabinose (P=0.001), and L-arabinose absorption exceeded that of lactulose (P=0.033). In *M. lucifugus*, there were no significant differences detected in paracellular probe absorption ($F_{2,10}$=0.63, P=0.55).

Luminal perfusions

In the perfusion experiments, all species showed a net absorption of fluid over the duration of the perfusions (*M. lucifugus*: 1.44±0.36 ml; *P. leucopus*: 1.72±0.28 ml; *O. leucogaster*: 2.54±0.53 ml). Absorption of 3OMD-glucose in the presence of 10 mmol l^{-1} D-glucose was 33.5±14 nmol min^{-1} cm^{-2} in *M. lucifugus*, which was not significantly different from *P. leucopus* (48±5 nmol min^{-1} cm^{-2}; t_8=0.98, P=0.36). Absorption of D-glucose at 10 mmol l^{-1} was 65.3±12 nmol min^{-1} cm^{-2} in *O. leucogaster*. L-Arabinose absorption at 1 mmol l^{-1} was significantly higher ($F_{2,14}$=5.2, P=0.02) in *M. lucifugus* (2.96±0.72 nmol min^{-1} cm^{-2}) compared with *O. leucogaster* (1.33±0.15 nmol min^{-1} cm^{-2}, P=0.049) and *P. leucopus* (1.03±0.12 nmol min^{-1} cm^{-2}, P=0.028). Simlarly, L-arabinose clearance was significantly greater in *M. lucifugus* ($F_{2,24}$=5.1, P=0.022) compared with both *O. leucogaster* (P=0.049) and *P. leucopus* (P=0.03) (Fig. 2). Based on these measurements, the

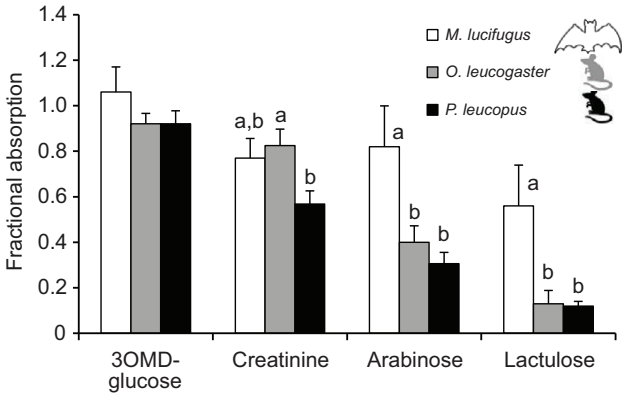

Fig. 1. Fractional absorption of orally dosed probes in the little brown bat (*Myotis lucifugus*), northern grasshopper mouse (*Onychomys leucogaster*) and white-footed mouse (*Peromyscus leucopus*). For a given probe, bars that share letters or lack letters do not differ significantly (P>0.05). For the bat, grasshopper mouse and white-footed mouse, respectively, sample sizes were as follows: 3-*O*-methyl-D-glucose (3OMD-glucose): 4, 6, 6; creatinine: 4, 6, 7; L-arabinose: 5, 7, 6; lactulose: 4, 6, 5. Data are means ± s.e.m.

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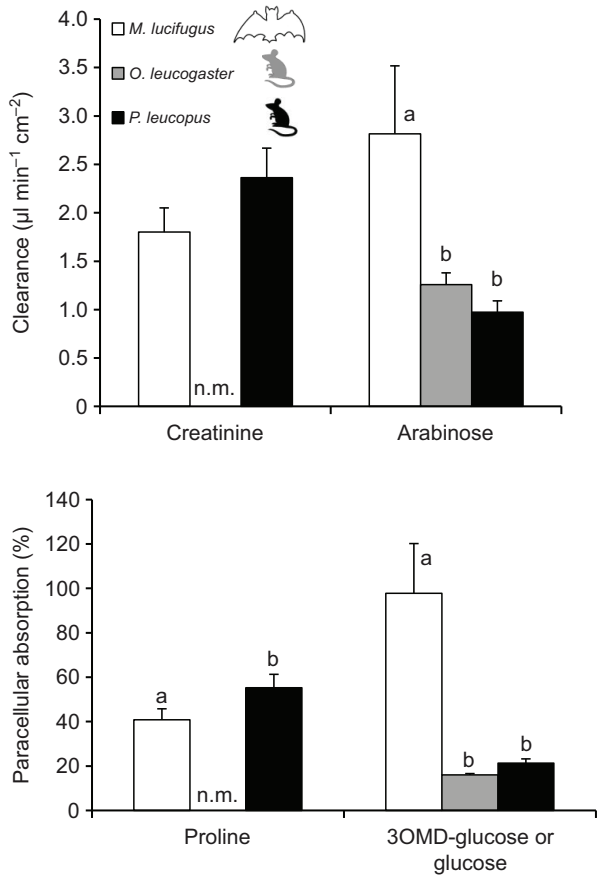

Fig. 2. Absorption of probes during intestinal perfusions. Top: clearance (µl min^{-1} cm^{-2}) of creatinine and arabinose in luminally perfused intestines of the little brown bat (*Myotis lucifugus*), northern grasshopper mouse (*Onychomys leucogaster*) and white-footed mouse (*Peromyscus leucopus*). Bottom: percentage of nutrient absorption that was paracellular, as estimated for proline using creatinine absorption, and for D-glucose or 3-*O*-methyl-D-glucose (3OMD-glucose) using arabinose absorption. For a given probe, bars that share letters or lack letters do not differ significantly (P>0.05). Sample sizes were: 6 (*M. lucifugus*), 6 (*O. leucogaster*) and 5 (*P. leucopus*). Data are means ± s.e.m.; n.m., not measured.

proportion of glucose absorption that was paracellular was significantly higher ($F_{2,14}$=723, P<0.001) in *M. lucifugus* compared with both *O. leucogaster* (P<0.001) and *P. leucopus* (P<0.001) (Fig. 2).

Proline absorption at 10 mmol l^{-1} did not vary significantly among species (*M. lucifugus*: 47±5 nmol min^{-1} cm^{-2}; *O. leucogaster*: 58±11 nmol min^{-1} cm^{-2}; *P. leucopus*: 47±6 nmol min^{-1} cm^{-2}; $F_{2,16}$=0.64, P=0.54). Creatinine absorption at 1 mmol l^{-1} did not differ between *M. lucifugus* (1.81±0.25 nmol min^{-1} cm^{-2}) and *P. leucopus* (2.38±0.30 nmol min^{-1} cm^{-2}; t=1.45, P=0.18). Creatinine clearance similarly did not differ between *M. lucifugus* and *P. leucopus* (t_8=1.4, P=0.192), although the proportion of proline absorption that was paracellular was significantly higher in *P. leucopus* than *M. lucifugus* ($F_{1,9}$=8.86, P=0.018) (Fig. 2).

Absorption of creatinine was significantly greater than L-arabinose in *P. leucopus* (t_5=4.1, P=0.009), whereas there was no significant difference between absorption of creatinine and L-arabinose in *M. lucifugus* (t_6=1.5, P=0.18).

Validation of paracellular probes with everted sleeves

As expected, D-glucose probe uptake declined significantly when measured at 100 mmol l^{-1} D-glucose compared with tracer concentration (*M. lucifugus*: % inhibition=−97.3%; *P. leucopus*: −94.1%) (P<0.001 for both species). In contrast, neither L-arabinose nor creatinine probe uptakes differed significantly when measured at tracer concentration compared with 100 mmol l^{-1} concentration (P>0.185 for all comparisons) in either species (Table 2). The lack of self-inhibition of probe uptakes by very high unlabeled substrate concentrations is evidence that the intestinal apical membranes of both *M. lucifugus* and *P. leucopus* lack mechanisms (i.e. transporters) for mediated uptake of either L-arabinose or creatinine. The contrasting results for D-glucose, which was greatly self-inhibited, is evidence that our procedures and test concentrations were sufficient for this demonstration. The total D-glucose uptake rates calculated at 100 mmol l^{-1} were 298 and 294 nmol min^{-1} cm^{-2}, respectively, for *M. lucifugus* and *P. leucopus*. These values compare favorably with the mean for eight other small mammal species [300±79 nmol min^{-1} cm^{-2}; fig. 7.15 in Karasov and Hume (Karasov and Hume, 1997)], although the latter values were for mediated uptake alone.

Histological measurements

Generally, villus length and villus width in *P. leucopus* exceeded those values in *M. lucifugus* (Table 3). However, crypt width was also greater in *P. leucopus*, implying that its villi are spaced further apart. The net result was that the amplification ratio was quite similar for the two species, particularly in the proximal and medial sections (Table 3).

Myotis lucifugus enterocytes were smaller than those of *P. leucopus* in the proximal and medial sections. *M. lucifugus* had a higher number of enterocytes per nominal surface area than *P. leucopus* (significant in the medial and distal sections) (Table 3). This higher amount of enterocytes presumably entails a similarly higher quantity of tight junctions across which our probes can absorb. To estimate the effect this would have on our calculations of arabinose clearance from our perfusion experiment (see above), we recalculated arabinose clearance normalized to the estimated number of enterocytes that were in contact with the perfusate (using

Table 2. Probe uptakes (J_i) by everted intestinal sleeves measured *in vitro* at tracer concentration (C_i) in the absence or presence of 100 mmol l^{-1} of unlabeled compound

	Myotis lucifugus			*Peromyscus leucopus*		
Incubation solution	C_i (d.p.m. µl^{-1})	J_i (d.p.m. min^{-1} mg^{-1})	*N*	C_i (d.p.m. µl^{-1})	J_i (d.p.m. min^{-1} mg^{-1})	*N*
D-Glucose tracer only	C_1=156±2.9	J_1=222±81^a	7	C_1=153±3.3	J_1=133±26^a	5
+100 mmol l^{-1} D-glucose	C_2=158±6.1	J_2=5.9±0.94^b	7	C_2=155±5.8	J_2=7.3±1.2^b	5
Arabinose tracer only	C_1=119±3.9	J_1=2.7±0.23	7	C_1=126±0.81	J_1=2.9±0.41	4
+100 mmol l^{-1} L-arabinose	C_2=118±2.9	J_2=2.9±0.21	7	C_2=135±0.87	J_2=3.4±0.82	4
Creatinine tracer only	C_1=173±19	J_1=1.5±0.91	4	C_1=207±6.2	J_1=6.5±0.15	5
+100 mmol $^{-1}$ creatinine	C_2=181±18	J_2=2.3±0.83	4	C_2=215±12	J_2=6.9±1.3	5

Values of *J* with different lettered superscripts were significantly different by a paired *t*-test.

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Table 3. Histological measurements from small intestine of little brown bats (*Myotis lucifugus*) and white-footed mice (*Peromyscus leucopus*)

	M. lucifugus (*N*=3)	*P. leucopus* (*N*=3)	*P*
Villus length (μm)			
Proximal	**397±11**	**571±59**	**0.044**
Medial	**389±4**	**518±44**	**0.045**
Distal	322±23	296±22	0.461
Villus width (μm)			
Proximal	**70±7**	**117±7**	**0.008**
Medial	**70±6**	**105±8**	**0.021**
Distal	67±5	88±10	0.139
Crypt width (μm)			
Proximal	38±3.2	47±1.3	0.062
Medial	**38±2.6**	**51±3.4**	**0.043**
Distal	38±1.3	48±4.0	0.070
Amplification ratio			
Proximal	10.3±1.0	10.5±1.2	0.919
Medial	10.0±0.8	9.5±1.0	0.768
Distal	8.5±0.9	6.5±0.9	0.166
Enterocyte width (μm)			
Proximal	**7.4±0.18**	**8.6±0.12**	**0.006**
Medial	**7.0±0.10**	**8.7±0.43**	**0.018**
Distal	7.3±0.10	8.3±0.49	0.130
Cells per nominal surface area (10^6 cells per cm^2)			
Proximal	18.8±1.7	14.2±2.0	0.153
Medial	**20.7±2.4**	**12.6±0.98**	**0.035**
Distal	**16.0±1.6**	**9.79±1.5**	**0.041**
Summed cells of the small intestine (10^6 cells)	**124±5.9**	**186±4.5**	**0.001**

Bold indicates significant differences among species (P<0.05).

histological measurements from the proximal section). After re-normalizing this way, *M. lucifugus* still had arabinose clearance values (0.153±0.042 μl min^{-1} $cells^{-6}$) that were approximately twice as large as those of *P. leucopus* (0.073±0.011 μl min^{-1} $cells^{-6}$), although this was not statistically significant (P=0.129).

Expression profiling

Myotis lucifugus and *P. leucopus* exhibited different tight junction gene expression profiles (Fig. 3). Claudin-2 (CLDN2) expression was 10-fold higher in *P. leucopus* compared with *M. lucifugus* (t_7=3.72, P=0.007), whereas CLDN15 expression was fourfold higher in *M. lucifugus* versus *P. leucopus* (t_9=3.95, P=0.003). Expression of CLDN1 was 20 times higher in the bats than the mice (t_8=2.76, P=0.02). In contrast, expression of OCLN (t_9=1.83, P=0.10) and CLDN7 (t_{10}=2.0, P=0.074) did not differ significantly between species. We tried several primer sets for amplifying CLDN4. They generally resulted in non-specific amplification in *P. leucopus* (data not shown).

DISCUSSION

We have demonstrated differences between an insectivorous bat and two protein-specialist rodents in the paracellular absorption of glucose-sized probes in both whole-animal and tissue-level experiments. In contrast, there was no difference between bats and rodents in creatinine absorption (an amino acid-sized probe) at either the whole animal or tissue level. The bat *M. lucifugus* has a higher density of enterocytes than the mouse *P. leucopus*, but it is not likely enough to explain between-species differences in absorption. These two species also differ in expression profile for several proteins that make up tight junctions, a finding that may underlie differences in the paracellular permeability to nutrients.

In the following paragraphs we first discuss the comparison of absorption at the whole animal level. We then discuss what we consider the major advances of this study; first, the demonstration that the differences at the whole animal level are also observed per square centimetre of small intestine and, second, our tests for mechanistic explanations such as differences in intestinal morphometry and tight junction molecular biology. Because our main focus is this correspondence between differences in absorption per square centimetre nominal area and putative differences in morphometry and molecular biology, from thereon we use absorption per square centimetre nominal area as our primary basis of comparison and not per whole intestine or per unit body size.

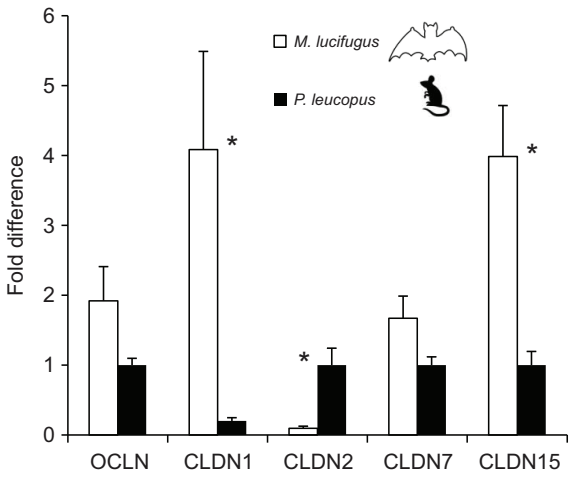

Fig. 3. Expression of some tight junction-associated genes in the little brown bat (*Myotis lucifugus*) and the white-footed mouse (*Peromyscus leucopus*). Target gene expression was normalized to the expression of ZO-1 and scaled such that mean normalized *P. leucopus* expression was unity for all genes except *CLDN1*, which was scaled to 0.2 for ease of viewing. OCLN, occludin; CLDN, claudin. Data are means ± s.e.m. *Expression of CLDN1, CLDN2 and CLDN15 differ significantly between species. Sample size was 9 for *M. lucifugus* and 8 for *P. leucopus*.

Whole animal paracellular nutrient absorption

Similar to previous findings (Brun et al., 2014), the fractional absorption of arabinose was higher in the bat (*M. lucifugus*) than both rodents. The fractional absorption of arabinose in our rodents (30–40%) falls in the range of previous measurements from several herbivorous/omnivorous rodent species (21–42%) (Lavin et al., 2007; Karasov et al., 2012; Fasulo et al., 2013a; Brun et al., 2014). In our bat, fractional absorption of arabinose (82%) was somewhat lower than that of the insectivorous bat *Tadarida brasiliensis* and the frugivore *Sturnira lilium*, which exhibited complete arabinose absorption (Fasulo et al., 2013b; Brun et al., 2014), although in all three cases the bats absorbed a substantial majority of the dose that did not differ significantly from unity. We also found a difference between *M. lucifugus* and the rodents with regard to the absorption of lactulose. The large molecular size of lactulose is thought to prevent its rapid absorption due to the size-selectivity of the tight junctions. Although lactulose absorption was not significantly lower than arabinose absorption in *M. lucifugus*, there was substantial variation in the measurement that may have reduced the power to detect a difference.

Absorption of orally dosed creatinine was not higher in our bat compared with the rodents. The absorption of creatinine by all our species was similar to the amounts previously reported in studies in laboratory mice, rats and rabbits in experiments in which glucose was provided (~70–85%) (Pappenheimer, 1990). Turner et al. (Turner et al., 2000) also found 55% creatinine absorption in humans when glucose was provided, although these trials did not include injection controls and true creatinine absorption in humans is probably even higher (see also Dominguez and Pomerene, 1945). Although we did find a statistically significant difference in creatinine absorption between *O. leucogaster* and *P. leucopus*, both values were fairly high and similar to the values for *M. lucifugus*.

The decrease in absorption of paracellular probes with increasing probe size that we observed in rodents is consistent with a size-sieving effect of the tight junction channels, a phenomenon that has been documented in a variety of species (Delahunty and Hollander, 1987; Bijlsma et al., 1995; Chediack et al., 2003; Lavin et al., 2007; Anderson and Van Itallie, 2009; Fasulo et al., 2013a; Fasulo et al., 2013b; Price et al., 2013a). This size-sieving effect seems to diminish at smaller molecule sizes, such that absorption of arabinose and creatinine was similar in *M. lucifugus*. This decreasing importance of the size-sieving effect at smaller probe sizes was also observed previously in sparrows (Chediack et al., 2003). This may be because fractional absorption increases with decreasing molecule size but cannot exceed unity. Also, a sigmoidal curve (for probe absorption versus probe size) is predicted from theoretical modeling of paracellular absorption as passage of molecules through a pore (Chediack et al., 2003).

We avoided the use of other carbohydrate probes that are closer in size to D-glucose (e.g. L-glucose or rhamnose) because of previous reports that they may have some affinity for the D-glucose transporter (Schwartz et al., 1995; Lavin et al., 2007), whereas arabinose does not (Lavin et al., 2007) (present study). The molecule size effect necessarily complicates interpretation of arabinose absorption as an indicator for paracellular glucose absorption; because arabinose is smaller than glucose (M_r 150 versus 180), it probably overestimates paracellular glucose absorption. Nonetheless, our data indicate that paracellular absorption of glucose is likely to be higher in the bat *M. lucifugus* compared with the rodents, particularly given the high fractional absorption of lactulose in *M. lucifugus*.

Creatinine (M_r 113) was chosen as a paracellular probe due to its size similarity to proline (M_r 115), and it is also similar to proline in that it contains nitrogen and a ring structure. Although we do not know exactly how various factors (e.g. size, charge distribution, shape) may affect the rate of paracellular absorption of a given probe, creatinine absorption seems a reasonable surrogate for paracellular proline absorption. As such, our creatinine absorption data, along with previous reports (Dominguez and Pomerene, 1945; Lundholm and Svedmyr, 1963; Pappenheimer, 1990; Turner et al., 2000), demonstrate that the paracellular route of absorption is important for proline across many mammals including humans. Moreover, several amino acids are of similar size or smaller than proline (and have more linear structures), implying that much amino acid absorption may occur via the tight junctions. However, a large proportion of amino acid absorption occurs in the form of dipeptide and tripeptide uptake into enterocytes (Adibi, 2003). Because of the larger size of these molecules and our results with the disaccharide lactulose, bats would presumably have higher paracellular absorption of oligopeptides than rodents.

We used different techniques for the assessment of probe absorption in the bat versus rodents so as to sacrifice fewer rodents. We have no reason to suspect that this qualitatively altered our results. Both techniques are standard pharmacokinetic procedures and we are unaware of any demonstration that the procedures provide different results. In laboratory rats the fractional absorption measured by serially sampling blood did not differ significantly from that measured by urine recovery (Caviedes-Vidal et al., 2007). In the current study, the two techniques demonstrated uniformly high absorption of 3OMD-glucose as expected, and the arabinose results of this study are similar to previous measurements using alternative techniques. For example, 34% of an arabinose dose was absorbed in rats as assessed using the serial blood sampling technique (Lavin et al., 2007). Furthermore, the differences among species were reflected in our experiments in isolated intestines (see 'Paracellular nutrient absorption in isolated intestines', below), providing further confidence that this result is not an artifact of the measurement techniques. Additionally, our findings do not result from bats having transmembrane transporters that interact with the paracellular probes. Like a previous study in rats and pigeons (Lavin et al., 2007), in our saturation kinetics experiment we saw no inhibition of either arabinose or creatinine absorption by high concentrations of the same probes, indicating that there is no transporter that can be saturated.

Overall, our measurements from intact animals provide further support for the hypothesis that paracellular absorption augments mediated absorption especially in fliers, probably to compensate for less nominal absorptive surface area in animals where flight imposes size constraints on the intestine. Among-species differences in paracellular permeability diminish with decreasing size of the permeant molecule, such that the paracellular absorption of small amino acids could be substantial, even in non-flying mammals.

Paracellular nutrient absorption in isolated intestines

The among-species variation we observed in the absorption of arabinose by intact animals might be due to differences in the paracellular permeability of the intestine, but considered alone, could also be attributed to other differences among species. For example, differences in gastric evacuation rates, contact time on the epithelium, and the presence of sufficient dietary water can affect absorption rates of paracellular probes (Pappenheimer and Reiss, 1987; Lennernäs, 1995; Turner et al., 2000; Lavin et al., 2007). Our intestinal perfusions control for these potential differences, and thus serve to confirm that the patterns observed in intact animals are in fact driven by permeability characteristics of the intestine. The

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difference between bats and rodents in arabinose absorption at the whole animal level was reflected in arabinose clearance in the perfusion experiments; additionally, the lack of a difference between *M. lucifugus* and *P. leucopus* for creatinine absorption was mirrored by their similar creatinine clearances in the perfusions.

Similar to our discussion of molecule size in the previous section, measuring arabinose absorption probably overestimates the proportion of glucose absorption that is paracellular. Nonetheless, our estimates should be comparable among species, and they indicate that the bat absorbs a much higher proportion of glucose via the paracellular pathway than the rodents. This further supports the hypothesis that flying vertebrates evolved greater dependence on the paracellular pathway as a means to evolving a smaller intestinal size (Caviedes-Vidal et al., 2007). Interestingly, this difference only occurs for larger nutrients, such as glucose and perhaps large amino acids and dipeptides, but not smaller amino acids such as proline. Our estimates for the proportion of proline absorption that was paracellular were similar for *M. lucifugus* and *P. leucopus*.

Our particular study species were not representative of the typical difference in intestinal area between bats and nonflying mammals. In terrestrial vertebrates generally, small intestinal nominal surface area scales with body mass$^{0.76}$ (Karasov, 2012), and data for multiple mammalian species with masses <40 g (the range studied here) indicate much lower intestinal nominal surface area in bats (1.02±0.09 cm^2 g$^{-0.76}$, N=16 species) compared with non-flying mammals (2.63±0.29, N=9) [t_{23}=6.99, P<0.001; data from Caviedes-Vidal et al. (Caviedes-Vidal et al., 2007)]. However, in our data set the nominal surface area for *M. lucifugus* (1.56±0.06 cm^2 g$^{-0.76}$), while similar to that of *P. leucopus* (1.35±0.09 cm^2 g$^{-0.76}$), was actually greater than that of *O. leucogaster* (0.74±0.05 cm^2 g$^{-0.76}$) (Table 1, $F_{2,36}$=20, P<0.001). Thus differences in total amount of absorption at the whole animal level (Fig. 1), while certainly based on differences in absorption per square centimetre (Fig. 2), are also possibly derived from differences in amounts of nominal intestinal surface area, especially after correcting for body size differences.

As in previous studies employing luminal perfusions, we have modeled the flow through the intestine to be similar to a continuously stirred fluid. A proportionally higher flow rate across the surface of the intestine of the bat – due to their narrower intestines – could potentially affect our findings if it causes significant deviations from this model. Suppose, instead, that absorption is dominated more by plug-flow reactor kinetics during transit through the intestinal tube. In that case, the proportionally higher flow through the bat would lead to lower fractional absorption during transit through the intestine because of less contact time, but that is opposite to what we observed. For further empirical support of our findings, we can also see from similar perfusions we conducted in three bats and three rodents, that when looking within a taxon, higher arabinose absorption was not associated with smaller intestines (Brun et al., 2014). Thus we do not believe that our results are an artifact of the small intestine size of our bat relative to the rodents.

Mechanistic correlates of paracellular nutrient absorption

The among-species differences in arabinose clearance during our perfusion experiments probably arise from two non-mutually exclusive mechanisms. Our clearance measurements were made on the basis of nominal surface area, and thus might derive from greater tight junctional area across which arabinose can absorb. Alternatively, the permeability characteristics of the tight junctions might vary across species.

We found that villous amplification was similar for *M. lucifugus* and *P. leucopus*. However, *M. lucifugus* enterocytes were smaller. Combined, the number of enterocytes per nominal surface area was slightly greater in *M. lucifugus*, which presumably corresponds to a similarly higher number of tight junctions. However, this difference does not explain the difference between species in paracellular permeability to arabinose. Although not statistically significant, the clearance of arabinose, normalized to the number of enterocytes, was nearly double in *M. lucifugus* compared with *P. leucopus*. The lack of significance probably arises from high variability in the clearance measurements in *M. lucifugus*; additionally, although three animals has been recommended for morphometric studies of the intestine (Snipes, 1994), the small sample size may not be representative of the species as a whole. The number of enterocytes summed over the entire small intestine also cannot explain the difference in whole animal arabinose absorption because *P. leucopus* greatly exceeds *M. lucifugus* in this respect (Table 3). Another interesting observation is that our bat did not differ from the rodents in creatinine absorption, even while having higher arabinose absorption. This could imply that bats have tight junctions that allow greater passage of larger molecules, while smaller molecules are equally permeant in both species.

If histological parameters cannot explain among-species differences in paracellular nutrient absorption, tight junction composition might provide an alternative. OCLN and at least 27 claudins are present in the tight junctions of mammals, and they are thought to mediate the permeability characteristics of the tight junctions, particularly to ions (Günzel and Yu, 2013). Nearly all claudins have been detected in rodent intestine, of which claudins 2, 3, 7 and 15 are the most highly expressed (Rahner et al., 2001; Holmes et al., 2006; Günzel and Yu, 2013). In studies of cultured monolayers, over-expression of OCLN, CLDN2, CLDN4 and CLDN15 cause changes in cation or anion permeability, but to date, only OCLN and CLDN1 over-expression were associated with increased permeability to mannitol, a glucose-sized molecule (M_r 182) (McCarthy et al., 2000; Van Itallie et al., 2001; Amasheh et al., 2002; Tamura et al., 2011). However, not all such studies have been consistent; for example, deletion of OCLN did not affect paracellular mannitol flux (Schulzke et al., 2005). The effect of changes in any given tight junction protein may thus be context specific and it is still unclear whether any claudins play specific roles in affecting the paracellular permeability to macronutrients (Günzel and Yu, 2013). Absorption of large molecules can also occur via a low capacity ‘leak pathway’ that results from temporary disruptions of the epithelial barrier (Shen et al., 2011). This is a potential for future research in bats, although macronutrients fall within the size range of the ‘pore pathway’, and are absorbed at higher rates than typically ascribed to the ‘leak pathway’. Additionally, non-transcriptional regulation of claudins may occur, including post-translational modification, interaction with scaffolding proteins, and exchange with intracellular pools (Shen et al., 2011; Günzel and Yu, 2013). Thus our expression data represent merely a first exploratory foray into understanding the mechanisms that drive inter-specific differences in tight junction permeability.

We found no difference between species for the expression of OCLN and CLDN7, whereas CLDN2 was less expressed in *M. lucifugus* and CLDN15 and CLDN1 were more expressed in *M. lucifugus*, relative to *P. leucopus*. CLDN2 and CLDN15 are thought to form cation channels (Amasheh et al., 2002; Van Itallie et al., 2008; Tamura et al., 2011), and between-species differences in their expression may simply represent phylogenetically distinct manners of sodium regulation. Nonetheless, our results suggest that further

experimentation is warranted to understand the effects of CLDN2 and CLDN15 expression on paracellular nutrient absorption. Interestingly, in laboratory mice, both CLDN2 and CLDN15 mRNA and protein expression change during development, with CLDN2 decreasing and becoming restricted to the crypts, and CLDN15 increasing and moving from the crypts towards the villi (Holmes et al., 2006; Tamura et al., 2011). Bats might thus have claudin profiles that represent an extension of this process. CLDN15 knockouts are also associated with glucose malabsorption, although this was attributed to the role of CLDN15 in sodium transport (Tamura et al., 2011).

The large difference between species in CLDN1 expression is also notable. Over-expression of epiptope-tagged CLDN1 in canine kidney cells caused an increase in transepithelial resistance, while simultaneously increasing mannitol flux (McCarthy et al., 2000). Over-expression of untagged CLDN1 also increased mannitol flux, but not significantly (McCarthy et al., 2000). In contrast, over-expression of CLDN1 in a lung endothelial cell line caused decreased mannitol flux (Fujibe et al., 2004). The effect of CLDN1 on paracellular permeability to nutrient-sized molecules may thus be context specific. Our results suggest the exciting possibility that CLDN1 might mediate the higher paracellular permeability to nutrients in bats, although this needs more experimentation. Further study on the molecular basis of paracellular nutrient absorption is clearly warranted.

MATERIALS AND METHODS

Animals

The little brown bat (*Myotis lucifugus* LeConte) is insectivorous (Fenton and Barclay, 1980). Both rodents of this study (white-footed mouse, *Peromyscus leucopus* Rafinesque; and northern grasshopper mouse, *Onychomys leucogaster* Wied-Neuwied) are somewhat omnivorous, but specialize on protein-rich diets more than many rodents; grasshopper mice in particular are unique amongst North American rodents in having a diet composed primarily of arthropods and vertebrates (Martin and Nelson, 1951; McCarty, 1978; Lackey et al., 1985). *Myotis lucifugus* were captured in Dane County, Wisconsin, near night roosts using mistnets and were used in experiments within 12 h of capture (Table 1). *Onychomys leucogaster* were trapped in Finney County, Kansas. *Peromyscus leucopus* were caught in Dane County, Wisconsin, and species identity was verified by molecular techniques (see 'Gene expression', below). Rodents were maintained in captivity at the University of Wisconsin-Madison, where they were housed singly and were supplied *ad libitum* with water and food (Purina 5010 Rodent Diet; 23.9% protein, 5% fat, by mass) until experiments (no longer than 2 months). To add more protein to the diet, *O. leucogaster* was supplemented with 2 g Purina Cat Chow Complete (34% protein, 13% fat) daily, which they were observed to consume entirely. All experiments were conducted at night to coincide with the active periods of the animals, as all three species are nocturnal. Experimental protocols were approved by the University of Wisconsin-Madison Animal Care and Use Committee (protocol no. A1441). Permits for trapping were obtained from the appropriate state authorities (Wisconsin Department of Natural Resources permits E/T 704 and SCP-SOD-03-2011; Kansas Department of Wildlife, Parks and Tourism permit SC-126-2012).

Assessment of paracellular absorption in intact animals

In rodents, we assessed paracellular absorption of probes by urine recovery (Fasulo et al., 2013a). We prefer urine collection over blood collection because it eliminates the need for repeated blood sampling in small animals and allowed us to reduce the number of animals used. In separate trials, individuals were dosed via intraperitoneal injection (in saline) or oral gavage (in saline with 50 mmol l^{-1} D-glucose). After dosing, rodents were put in wire-bottomed metabolic cages with access to water (with 50 mmol l^{-1} glucose) but no food. Addition of glucose to the water adds some nutrition and encourages the animals to drink and urinate more frequently. Cages were checked approximately every hour and urine was collected and weighed. Subsamples were counted in 5 ml Ecolume scintillation cocktail in 8 ml glass vials using a Wallac 1414 liquid scintillation counter (PerkinElmer, Waltham, MA, USA), and we determined total recovery of the probe after 24 h. Fractional absorption of the probes was determined by dividing the recovery (percentage of the dose recovered) after gavage by the recovery after injection.

Animals were dosed with the following probes: [^{3}H]-3-*O*-methyl-D-glucose (3OMD-glucose), [^{14}C]-L-arabinose, [^{3}H]-lactulose and [^{14}C]-creatinine. 3OMD-glucose (M_r 194) is a glucose analog that is not metabolized but is absorbed both via glucose transporters and via tight junctions. To estimate the paracellular absorption of an 'amino acid-like' molecule, we chose creatinine (M_r 113), which is similar in size to proline (M_r 115) and contains nitrogen. There are reports of some proteins that are capable of transporting creatinine in the kidney, notably OAT2 (Lepist et al., 2014). However, expression of OAT2 is either low or absent in the intestine (Maubon et al., 2007; Meier et al., 2007; Estudante et al., 2013), and creatinine absorption in the intestine has generally been ascribed to the paracellular pathway (Dominguez and Pomerene, 1945; Pappenheimer, 1990; Turner et al., 2000). In addition, we tested for saturation kinetics of intestinal creatinine absorption (see 'Validation of paracellular probes', below). Arabinose (M_r 150) and lactulose (M_r 342) are carbohydrates that have no affinity for intestinal transporters and were chosen for their similarity and dissimilarity, respectively, to the size of glucose (M_r 180). Rodents were tested in multiple trials that were separated by at least 3 days.

Bats urinate infrequently and it can be difficult to separate their urine from their feces. For these reasons, we determined fractional recovery by serial blood sampling (for details, see Caviedes-Vidal et al., 2008; Karasov et al., 2012). Individual bats were dosed with an oral gavage (in saline and 50 mmol l^{-1} glucose) or intraperitoneal injection (in saline), and a blood sample was taken from the antebrachial or uropatagial vein after ~5, 15, 30, 45, 60 and 90 min. From this, we constructed a curve of dose-corrected plasma concentration versus time. We determined the area under the curve via the trapezoidal rule. To determine the area under the curve after the final point, we plotted ln(concentration) versus time and determined the elimination constant (K_{el}) as the negative slope of the line joining the final two points in the curve. We averaged the K_{el} for all injection trials and then applied this slope to the gavage trials. The area under the curve after the final point was calculated as the final concentration divided by K_{el}. For each probe, fractional absorption was determined by dividing the average area under the curve in gavage trials by the average area under the curve in injection trials. Individual bats could only be used in single trials and were then euthanized.

In situ luminal perfusions

We performed perfusions of the intestinal lumen as described previously (Price et al., 2013a). *M. lucifugus* individuals were naïve, whereas the rodents had been previously used (at least 3 days before) in the intact animal absorption study. Animals were maintained at 37°C with a Deltaphase isothermal heating pad (Braintree Scientific, Braintree, MA, USA) while under isoflurane anesthesia. The abdomen was opened and we cannulated the small intestine close to the stomach. An exit cannula was placed 9.1±0.45 cm distally, and pre-warmed saline was pumped through the intestine to flush out any gut contents. We then began the experimental perfusion using a peristaltic pump to circulate a buffer (10 mmol l^{-1} D-glucose, 10 mmol l^{-1} L-proline, 1 mmol l^{-1} L-arabinose, 1 mmol l^{-1} creatinine, 1.2 mmol l^{-1} $NaHPO_4$, 110 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1 mmol l^{-1} $MgSO_4$, 2 mmol l^{-1} $CaCl_2$, 20 mmol l^{-1} $NaHCO_3$, pH 7.4) at a flow of 1 ml min^{-1}. In *M. lucifugus* and *P. leucopus*, we measured absorption of the probes by adding tracer amounts of [^{3}H]-proline, [^{3}H]-3OMD-glucose, [^{14}C]-creatinine and [^{14}C]-arabinose. Due to a paucity of animals, we only made measurements of *O. leucogaster* using labeled [^{3}H]-proline and [^{14}C]-L-arabinose with liquid scintillation counting, and measured glucose concentration using a kit (Sigma-Aldrich, St Louis, MO, USA). After exiting the intestine, the perfusate returned to a reservoir (which was kept at 37°C in a water bath) and recirculated for 104±4 min and was then collected. Animals remained alive throughout the perfusion.

We weighed the perfusate prior to and following the perfusion. Subsamples (50 µl) collected before and after the perfusion were counted.

After each experiment, the perfused length of the intestine was measured using calipers. The intestine was then cut longitudinally and laid flat to measure the circumference using the average of three measurements along its length. We calculated the nominal surface area (which is the surface area of a smooth bore tube and does not account for villous magnification) as the product of length×circumference. Absorption of each probe was calculated from the loss of total radioactivity during the perfusion experiment and was normalized among experiments by dividing by contact time on the intestine (min) and nominal surface area (cm^2). Readers can re-express absorption per milligram of wet intestine using the following conversion factors for *M. lucifugus* and *P. leucopus*, respectively: 78.3 mg cm^{-2} and 63.1 mg cm^{-2} (not measured for *O. leucogaster*). For arabinose and creatinine, we also calculated clearance ($\mu l\ min^{-1}\ cm^{-2}$) by dividing absorption by $(C_{initial}–C_{final})/(C_{initial}/C_{final})$, where C is concentration (Sadowski and Meddings, 1993). Because they are not transporter mediated (and therefore do not exhibit saturation kinetics), absorption of arabinose and creatinine vary linearly with concentration. Dividing absorption by the concentration to calculate clearance thus provides a value that can be compared with other studies that employ other concentrations.

To estimate the proportion of glucose absorption that was paracellular, we used arabinose, and to estimate paracellular proline absorption we used creatinine. L-Arabinose and creatinine absorptions were measured at 1 mmol l^{-1}, whereas glucose and proline absorptions were measured at 10 mmol l^{-1}. Because the absorption of arabinose and creatinine is not carrier mediated (see 'Validation of paracellular probes', below), their absorption rates are directly proportional to their luminal concentrations. Therefore, we multiplied arabinose or creatinine absorption by 10, divided by total glucose or proline absorption at 10 mmol l^{-1}, and multiplied this quotient by 100%. For this calculation, we assumed that absorption of 3OMD-glucose was representative of glucose absorption, although the larger size of 3OMD-glucose and lower affinity for the glucose transporter may make this an underestimation. The effects of probe choice for estimating paracellular nutrient absorption are considered further in the Discussion.

Validation of paracellular probes

To verify that our L-arabinose and creatinine probes have no affinity for intestinal transporters, we used an everted sleeve technique to test for saturation kinetics (Karasov and Diamond, 1983; Lavin et al., 2007). Seven naïve *M. lucifugus* and *P. leucopus* were euthanized with CO_2 and the entire small intestine was immediately removed and perfused with ice cold Ringer solution (125 mmol l^{-1} NaCl, 4.7 mmol l^{-1} KCl, 2.5 mmol l^{-1} $CaCl_2$, 1.2 mmol l^{-1} KH_2PO_4, 1.2 mmol l^{-1} $MgSO_4$ and 20 mmol l^{-1} $NaHCO_3$, pH 7.3–7.4). We everted the intestine and cut it into sections that were then secured to the tip of a metal rod (2 or 3 mm diameter). Six 1-cm sections were mounted using surgical thread with the aid of grooves at 1 and 11 mm from the rods' ends; excess tissue was cut away. Throughout preparation, tissues were kept cold in Ringer solution gassed with 95% O_2 and 5% CO_2.

We prepared experimental incubation solutions modified from the Ringer solution using isosmotic replacement of NaCl. We tested for saturation of intestinal transporters using pairs of adjacent sections of intestine; one section was incubated with 100 mmol l^{-1} mannitol (with only tracer amounts of the probe of interest, ~1 µmol l^{-1}) while the other section was incubated with a probe at high concentration (100 mmol l^{-1} D-glucose, 100 mmol l^{-1} L-arabinose or 100 mmol l^{-1} creatinine). Each pair of solutions was labeled with an impermeant marker to account for adherent fluid ([^{3}H]polyethylene glycol, M_r 4000) as well as the appropriate probe ([^{14}C]D-glucose, [^{14}C]L-arabinose or [^{14}C]creatinine).

For 5 min prior to testing, mounted intestinal tissue was pre-incubated in the Ringer solution gassed with 95% O_2 and 5% CO_2 at 37°C. Tissues were then transferred to the experimental solution where they were incubated for 2 min (glucose) or 4 min (arabinose and creatinine), during which the solution was gassed at 37°C and mixed at high speed with a spin bar. The tissue was then removed, blotted to remove excess solution, cut from the mounting rod with a scalpel, and placed in a tared scintillation vial and weighed. We added 1 ml Soluene-350 tissue solubilizer (PerkinElmer) and incubated the tissue overnight at 55°C, before adding 5 ml scintillation cocktail for counting. Samples of the incubation solutions were taken prior to incubation and prepared similarly for counting.

Probe uptake rates (J, d.p.m. $min^{-1}\ mg^{-1}$ tissue) were calculated using the equation $J=(P-RM)/tm$, where P is the disintegrations per minute (d.p.m.) of the probe in or on the tissue sample, R is the d.p.m. of the probe divided by the d.p.m. of the impermeant marker in the incubation solution, M is the d.p.m. of the marker in or on the tissue, t is the incubation time, and m is the mass of intestinal sleeve. The measurement at tracer concentration (C_1, d.p.m. μl^{-1}) yielded uptake J_1, and the measurement in the presence of 100 mmol l^{-1} unlabeled compound (C_2) yielded uptake J_2. To compare probe uptake rates with or without unlabeled compound, while also standardizing for small differences between C_1 and C_2, we calculated:

$$J_1' = J_1\{C_1/[(C_1+C_2)/2]\} \quad (1)$$

and

$$J_2' = J_2\{C_2/[(C_1+C_2)/2]\}\ . \quad (2)$$

If probes have affinity for intestinal transporters, J_2' should be substantially less than J_1', because the transporters become saturated at high concentrations of probe. This can also be expressed as percentage inhibition of the unlabeled compound, calculated as $100(J_1'–J_2')/J_1'$. Some intestines were too short for preparation of all six sections, so the samples sizes vary somewhat among probes. For comparative purposes, we also calculated D-glucose uptake (nmol $min^{-1}\ mg^{-1}$) at 100 mmol l^{-1} by dividing the probe uptake, calculated as above, by the ratio of d.p.m. of the probe per nanomole of D-glucose in the incubation solution (Karasov and Diamond, 1983). Although useful for demonstrating the presence/lack of a nutrient carrier, we do not consider the everted sleeve preparation appropriate for quantifying paracellular absorption due to the lack of blood flow.

Morphological and histological measurements

Gross morphological measurements were made in *O. leucogaster* using animals immediately following the perfusion experiments, and from *M. lucifugus* following intact absorption measurements. Additionally, gross morphology was measured from *M. lucifugus* and *P. leucopus* when taking tissue samples for RNA expression. For studies of histology and RNA expression, naïve *M. lucifugus* and *P. leucopus* were euthanized during their normal activity period (nocturnally). After euthanasia, we removed the gastrointestinal tract and measured the length of the small intestine. The small intestine was divided into thirds, cut longitudinally, and laid flat to measure circumference with calipers. A 2-cm section from the distal end of the proximal third of the intestine was placed in RNAlater for later analysis (see below). For three bats (*M. lucifugus*) and three white-footed mice (*P. leucopus*), we also fixed intestinal segments in 10% formalin and 5 µm sections were cut and stained with haematoxylin and eosin on glass slides. We made measurements of villus length, villus width and crypt width of 25 villi per section using 200× magnification with NIS-Elements D software (Nikon Instruments, Melville, NY, USA), and used this information to calculate the amplification ratio (Kisielinski et al., 2002). We also measured enterocyte size by measuring the number of enterocytes per unit length (counting along the length of 10 villi per section) at 400× magnification. The inverse of this measurement is the dimension of the width of an average enterocyte. We assumed that enterocytes were roughly square on their apical surface (assuming a different shape such as hexagonal did not change our conclusions). Therefore enterocyte width was squared (to determine the lumenal surface area of an average enterocyte) and multiplied by villous amplification ratio to determine the number of enterocytes per nominal surface area. This was multiplied by the nominal surface area to determine the total number of enterocytes in each section, and these products were summed to determine the number of enterocytes per intestine.

Gene expression

We generated mRNA expression profiles in *M. lucifugus* and *P. leucopus* for several genes that are highly expressed in the intestine and are associated with paracellular permeability of the tight junctions to ions or mannitol, including CLDN2, CLDN15, CLDN4, CLDN7 and OCLN (McCarthy et al., 2000; Van Itallie et al., 2001; Amasheh et al., 2002; Alexandre et al., 2005; Hou et al., 2006; Van Itallie and Anderson, 2006; Krause et al., 2008; Markov et al., 2010; Günzel and Yu, 2013). We were unable to detect claudin-3 or claudin-5 in *M. lucifugus* intestine. We also measured the expression of zonula occludens 1

Table 4. Primers used for quantitative PCR and species identification, with the GenBank accession numbers upon which they were based

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	GenBank accession nos
Zonula occludens 1 (ZO-1)	GCTGAGCAGTTAGCCAGTGT	CTCTTGGAGCTGCGAAG	KF906440−KF906442
Occludin (OCLN)	ACTGCCCAGGCTTCTGGA	GGGATCCACCACACAGTAGTGATA	KJ010082−KJ010085
Claudin-1 (CLDN1)	AACCCGTGCCTTGATGGT	CAAATTCATACCTGGCATTGAC	KJ420413−KJ420415
Claudin-2 (CLDN2)	TGGCCTGCATTATCTCTGTG	TCAAATTTCATGCTGTCAGG	KF906445−KF906449
Claudin-4 (CLDN4)	GCAGTGCAAGGTGTACGACT	*M. lucifugus*: GTCCAGGACACGGGCACC *P. leucopus*: GTCCAGGAGATGGGCACC	KJ406712−KJ406715
Claudin-7 (CLDN7)	TACGACTCGGTGCTCGC	AGACCTGCCACGATGAAAA	KF983355−KF983357
Claudin-15 (CLDN15)	*M. lucifugus*: GGAGTTCCCGTCCATGCT *P. leucopus*: GGAGTTCCCTTCCATGCT	AGGAAGAGGCCCAGGAAGC	KF935258−KF935259
EEF1A1	CAGCACCTACATTAAGAAAATTGG	CCCTTGAACCAAGGCATATT	KF906438−KF906439
RPLP0	GCGACCTGGAAGTCCAACTA	ATCTGCTGCATCTGCTTGG	KF935253−KF935257
COXIII (*P. leucopus*)	ACACCAATTGTACAAAAGGGACTC	GATTCCAAGTAGGATTGTAATAAGAAG	AY009173, AY513981[a]
COXIII (*P. maniculatus*)	ATCTCCCCTTTAAACCCATT	TCCAAGTAGGATTGTAATAAGAAGAGC	AY524697, AY524701

[a]The sequences we obtained for COXIII can be found in accession nos KF723289−KF723291.

(ZO-1), a scaffold protein that interacts with other tight junction proteins and that is present fairly uniformly in association with tight junctions (Holmes et al., 2006). Expression of a ribosomal protein (RPLP0) and eukaryotic elongation factor (EEF1A1) was quantified to provide reference genes.

RNA that had previously been stored in RNAlater (see above) was extracted using TRIzol (Life Technologies, Carlsbad, CA, USA), genomic DNA was digested using DNase I (New England Biolabs, Ipswich, MA, USA), and RNA was reverse transcribed to cDNA (iScript cDNA synthesis kit, Bio-Rad, Hercules, CA, USA). At the DNA digestion step, 10 µg RNA was used, determined from measurement of the absorbance at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The ratio of A_{260}/A_{280} was ≈2 for all samples, indicating high purity.

To create degenerate primers, we used known or predicted sequences for various mammals from GenBank (Benson et al., 2012). Once we obtained partial coding sequences, we designed specific primers with the aid of several programs (Table 4) (Rozen and Skaletsky, 2000; Larkin et al., 2007) (Finch TV 1.4.0, Geospiza, Inc., Seattle, WA, USA). With our specific primers, we verified that they amplified a single target by examination of melt curves, electrophoresing the product to verify the presence of a single band, and subsequent sequencing of that band. To compare expression results across species, we designed specific primers such that amplicons were of the same length in both species, and whenever possible, identical primers were used. This ensured that the fluorescence per amplification cycle (Colborn et al., 2008) and reaction efficiency was the same for both species.

Quantitative PCR reactions were run in duplicate with Platinum *Taq* polymerase (Life Technologies; reaction conditions: 1×buffer, 2.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, 0.25 µmol l^{-1} each primer, 1 unit *Taq*, 0.7× SYBR Green 1, 1 µl template in 20 µl reaction volume) on an Eppendorf Realplex2 thermocycler (Eppendorf, Hamburg, Germany). The cycling program was: 94°C for 10 min, then 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with fluorescence acquisition at the end of the 72°C step. A melt curve followed cycling. Expression for each gene was calculated as $(E+1)^{\Delta C_q}$, where E is efficiency and ΔC_q is the difference in quantification cycle between the sample and an arbitrarily chosen standard sample. We normalized target gene expression by dividing by the geometric average of the expression of two standard reference genes (*EEF1A1* and *RPLP0*) to create an expression ratio. However, this procedure may result in ratios that are not relevant to describing interspecies differences in tight junction gene expression. This is because *EEF1A1* and *RPLP0* are expressed by all cells, and the ratio of enterocytes (and their associated tight junctions) to other cells in the intestine may vary among species. Therefore, we present target gene expression normalized to the expression of ZO-1 (Holmes et al., 2006), although normalizing instead to our standard reference genes would not have qualitatively affected our conclusions.

White-footed mice (*P. leucopus*) and deer mice (*P. maniculatus*) are not readily distinguished by morphological characters (Rich et al., 1996; Tessier et al., 2004). To verify species identity, we developed a technique for use with our cDNA that was based on that developed by Tessier et al. (Tessier et al., 2004). Our technique used two species-specific primer sets (Table 4) designed to amplify part of the cytochrome c oxidase subunit III (COXIII) coding sequence. All *Peromyscus* samples were identified as *P. leucopus* by presence/absence of amplification with each primer set, amplicon length and amplicon sequencing with subsequent comparison to sequences in GenBank.

Statistical analyses

We used standard formulas for calculating means and variances of mathematical combinations of data (e.g. the ratio of AUC_{gav}/AUC_{inj} in bats) (Mood et al., 1974; National Research Council, 1980). Differences among species or probes were detected using *t*-tests, ANOVA and Tukey's *post hoc* tests (Zar, 1999). Differences from unity were detected with one sample *t*-tests. Analyses were performed with the aid of R statistical software (Fox, 2005; R Development Core Team, 2010).

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors were involved in planning the research. W.H.K. and E.C.-V. obtained project funding. E.R.P. and K.H.R. conducted experiments and wrote the manuscript. All authors helped revise the manuscript.

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