# A Comparison of Mucosal Surface Area and Villous Histology in Small Intestines of the Brazilian Free-Tailed Bat (*Tadarida brasiliensis*) and the Mouse (*Mus musculus*)

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*ABSTRACT* Studies on birds have led to the hypothesis that increased intestinal absorption between enterocytes (paracellular) evolved as a compensation for smaller intestinal size in fliers, which was perhaps selected to minimize the mass of digesta carried. This hypothesis predicts that bats will also exhibit relatively reduced intestinal size and high paracellular absorption, compared with nonflying mammals. Published studies on three bat species indicate relatively high paracellular absorption. One mechanism for increasing paracellular absorption per $cm^2$ small intestine (SI) is increased number of tight junctions (TJs) across which paracellular absorption occurs. To our knowledge, we provide the first comparative analysis of enterocyte size and number in flying and nonflying mammals. Intestines of insectivorous bats *Tadarida brasiliensis* were compared with *Mus musculus* using hematoxylin and eosin staining method. Bats had shorter and narrower SIs than mice, and after correction for body size difference by normalizing to $mass^{3/4}$, the bats had 40% less nominal surface area than the mouse, as predicted. Villous enhancement of surface area was 90% greater in the bat than in the mouse, mainly because of longer villi and a greater density of villi in bat intestines. Bat and mouse were similar in enterocyte diameter. Bats exceeded mice by 54.4% in villous area per cm length SI and by 95% in number of enterocytes per $cm^2$ of the nominal surface area of the SI. Therefore, an increased density of TJs per $cm^2$ SI may be a mechanistic explanation that helps to understand the high paracellular absorption observed in bats compared to nonflying mammals. J. Morphol. 000:000–000, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: Bats; rodents; small intestine; intestinal surface area enlargement factor; enterocytes; paracellular absorption

## INTRODUCTION

In vertebrates, the small intestine (SI) is the major site of hydrolysis of nutrient macromolecules and absorption of their breakdown products (Karasov and Hume, 1997). The intestine's surface area is a key determinant of the overall hydrolytic capacity of membrane bound digestive enzymes and absorptive capacity by transcellular and paracellular pathways. Transcellular pathways for water soluble nutrients involve mediated absorption by membrane bound transporters, whereas lipophilic nutrients (e.g., fatty acids) can passively diffuse across the lipid bilayer (Karasov and Hume, 1997). Paracellular absorption of small water soluble nutrients occurs by diffusion or solvent drag through the tight junctions (TJs) between adjacent enterocytes (Pappenheimer and Reiss, 1987). Hence, information about intestinal surface area can help explain differences among species in digestive and absorptive capacity and even reveal possible underlying mechanisms.

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There are marked differences among some species in the capacity for paracellular absorption. For example, Caviedes-Vidal et al. (2007) found that compared with nonflying mammals, small birds generally, and at least two species of bats, absorb significantly more metabolically inert nonactively transported monosaccharides, indicating an enhanced paracellular pathway for intestinal absorption of water soluble nutrients such as glucose and amino acids (Caviedes-Vidal et al., 2007; Fasulo et al., 2013a). One mechanism for increasing paracellular absorption per $cm^2$ SI is having an increased density of enterocytes per nominal area, hence an increased number of TJs across which paracellular absorption occurs. We tested this possible mechanism by comparing two species recently shown to differ in paracellular absorption. In absorption studies with intact animals, the small bat *Tadarida brasiliensis* absorbed five times more than laboratory mice (*Mus musculus*) of an oral dose of the inert carbohydrate probe L-arabinose (MM = 150.13), which is absorbed exclusively by paracellular route (Table 2; Fasulo et al., 2013a, b). Moreover, intestinal perfusion experiments with L-arabinose confirmed the higher paracellular permeability per $cm^2$ of the intestines of these bats compared to those of mice (Table 2; Price et al., 2013). Therefore, we predicted that the density of enterocytes and thus TJs would be higher in the bat than in the mouse. To our knowledge, we performed the first comparative analysis of enterocyte size and number in flying and nonflying mammals.

## MATERIALS AND METHODS

Laboratory mice, provided by the animal facility of the Universidad Nacional de San Luis (San Luis, Argentina), were provided mouse pellets and tap water ad libitum. Bats were live-trapped from a roosting colony on the campus of Universidad Nacional de San Luis and used immediately. All animal procedures adhered to institutional animal use regulations and approved animal use protocols (Institutional Animal Care and Use Committee, CICUA number protocol: B-39/07- Universidad Nacional de San Luis). Individuals of *Tadarira brasiliensis* (I. Geoffroy 1824) and *Mus musculus* (Linnaeus, 1758) were anesthetized with ketamine (40 mg/kg body weight) and acepromazine (0.5 mg/kg body weight). After removing the gastrointestinal tract and, still under deep anesthesia, the animals were killed by decapitation. The stomach and the SI in mice or the entire intestine in bats were dissected out, the lumen washed with ice cold 1% NaCl solution to remove digesta. Afterward, intestines were blotted dry, weighed, and measured for length by holding one end of the intestine against a vertical ruler while the other end was gently pulled until the intestine was taut. After release, the length was measured. Then, the intestine was divided in three equal portions, the proximal, medial, and distal regions. The pieces were carefully cut longitudinally with small surgical blunt scissor, opened, cleaned with ice cold 1% NaCl solution, and placed with the mucosa side up on a cold stainless steel plate. Using a digital caliper, two measures of length and four of width were taken to estimate nominal surface area of each regional portion.

For histological examination, tissues from both species where equally treated. Six 1-cm sections, two from each region were cut and immersed in 10% formalin solution for 48 h. Before embedding, tissue samples were washed for 3 h with distilled water every 0.5 h, and dehydrated through a graded series of ethanol solutions, and then embedded in paraffin (thawing point, 56–58°C). Transverse and vertical 5-μm serial sections were obtained (rotary microtome) from each section. Sections were mounted on slides, stained with hematoxylin and eosin and covered with cover glasses. Microphotographs were taken using an Olympus BX50 microscope connected to a video-camera (CaptureX98 software) and a PC-based image analysis system using Image J software (Schneider et al., 2012).

From each section, we measured the circumference of the serosal surface, length, and width of villi and the width of the crypts. We took 30 such measurements per section, resulting in 90 measurements per individual. We measured only those villi that were cut in their midline, from tip to base, as verified by observations of similarly sized and shaped enterocytes. These data were used to estimate the mucosal to serosal surface area enlargement factor (SEF) using the following equation according to Kisielinski et al., (2002):

$$\mathrm{SEF} = \frac{(\text{villus width} \times \text{villus length}) + \left(\frac{\text{villus width}}{2} + \frac{\text{crypt width}}{2}\right)^2 - \left(\frac{\text{villus width}}{2}\right)^2}{\left(\frac{\text{villus width}}{2} + \frac{\text{crypt width}}{2}\right)^2}$$

To avoid inflation of degrees of freedom by repeated measurements within individuals, means and standard deviation were calculated for individual animals. These means were used in statistical analyses.

Enterocyte diameter expressed in μm was calculated as the inverse of the number of enterocytes per unit length counted along the length of 10 villi segments per intestinal section at 400× magnification using ImageJ software (Schneider et al., 2012).

### Statistical Analysis

Numerical data are presented as means ±1 standard error of the mean (SEM) ($n$ = number of animals). Comparisons of single measures (e.g., body mass, intestine length, and so forth; Table 1) were made by $t$-test. Variation in morphometric measures along the intestine and between species was evaluated by repeated measures ANOVA. Statistical significance was accepted for $P < 0.05$.

## RESULTS

### Intestinal Morphometrics

*Tadarida brasiliensis* bats were smaller than mice (body mass; Table 1) and had significantly smaller stomachs, intestine masses and lengths, intestine widths, and intestinal nominal surface areas (Table 1). At a gross scale, there was no obvious division into small and large intestine in the bat due to the lack of presence of a cecum, as also reported in seven other morphometrics

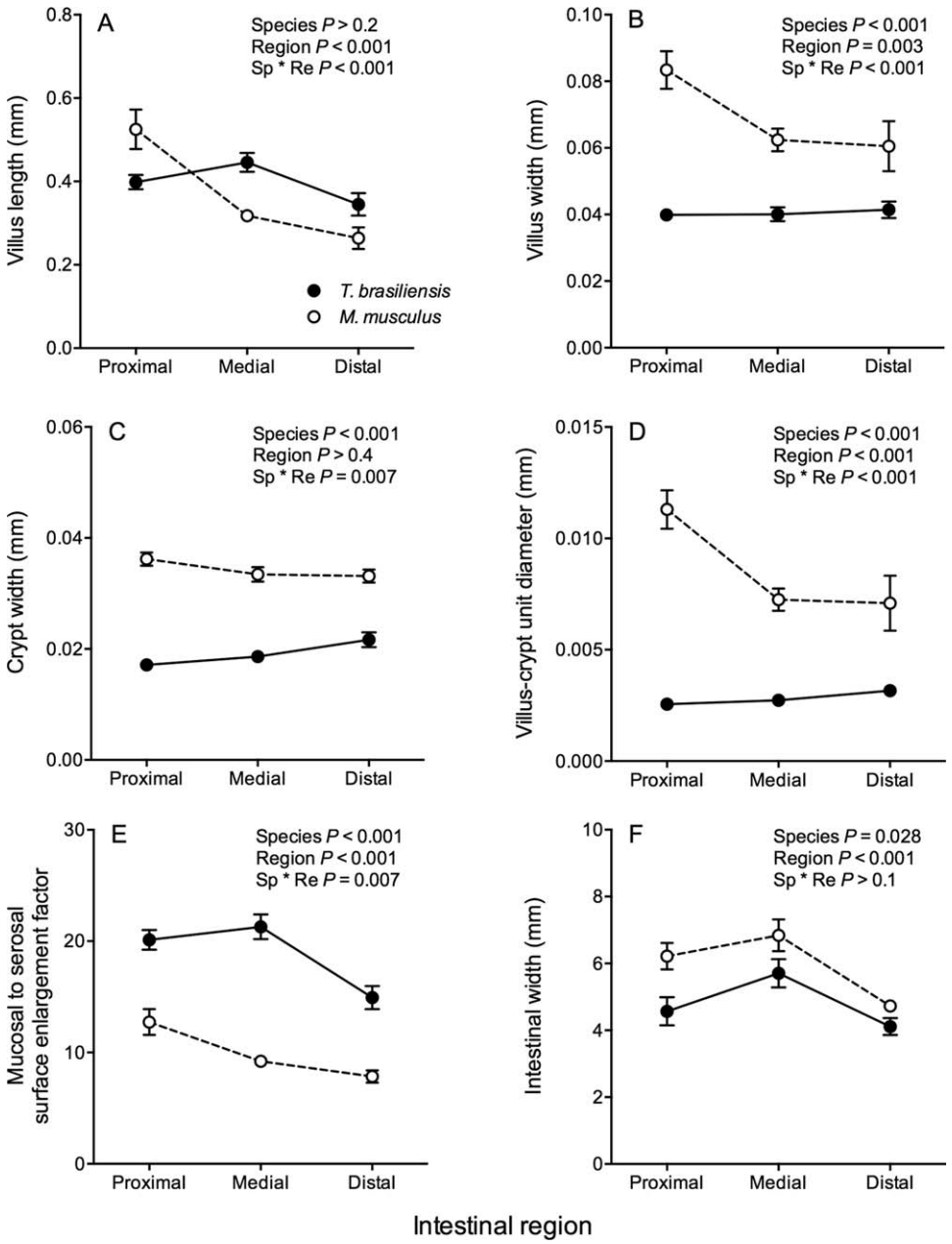


Fig. 1. Intestinal morphometrics as a function of intestinal region in the bat, *Tadarida brasiliensis* ($n = 8$) and the mouse (*Mus musculus*; $n = 6$). The mucosa (i.e., villous) to serosa (i.e., nominal) surface area enlargement factor (**E**) was calculated from measures of villus length (**A**), villus width (**B**), crypt width (**C**), and villus-crypt unit diameter (**D**). The ~100% larger mucosa surface enlargement factor in bats, compared with mice more than compensated for the 20% greater intestinal nominal widths in mice (**F**). Values are means ± SEM.

*TABLE 1. Gross morphometrics of Tadarida brasiliensis and Mus musculus*

| | (*n*) | Body mass (g) | Stomach Mass (g) | Intestine Mass[a] (g) | Intestine Length[a] (cm) | Intestine Width[a,b] (cm) | Nominal Surface Area[a] ($cm^2$) |
|---|---|---|---|---|---|---|---|
| Bat | 8 | 14.0 ± 0.2 | 0.081 ± 0.002 | 0.389 ± 0.01 | 16.71 ± 0.15 | 0.48 ± 0.0.01 | 7.972 ± 0.20 |
| Mouse | 6 | 37.3 ± 0.8 | 0.255 ± 0.008 | 1.118 ± 0.069 | 47.50 ± 0.029 | 0.59 ± 0.220.01 | 28.31 ± 0.8 |
| *P*-value[c] | | <0.001 | <0.001 | <0.001 | <0.001 | 0.022 | <0.001 |

[a]corresponds to total intestinal tube for bat and for small intestinal tube for mouse.
[b]corresponds to width averaged over proximal, medial and distal regions; see Fig. 1.
[c]*t*-test.

*TABLE 2. Paired comparisons of intestinal mucosal to serosal surface area enlargement factor (SEF), enterocyte density per $cm^2$ intestine in bats versus nonflying eutherians, and values of the absorption of the probes L-arabinose (MM = 150.1, [A]) or L-rhamnose (MM = 164.2, [R]) absorbed exclusively by the paracellular pathway*

| | | | | | | | Paracellular absorption (Type of study) | |
|---|---|---|---|---|---|---|---|---|
| Pair No. | Sources for species pair | Type of ocomotion | Species | Body mass (g) | SEF[a] | Enterocytes/$cm^2$ NSA[b] | Absorption (%)—(intestinal perfusion trials) | Fractional absorption—(*in vivo* intact animal trials) |
| 1 | This study | Flier | *Tadarida brasiliensis* | 14 | 18.8 | $1.05{\cdot}10^8 \pm 8.1{\cdot}10^6$ | $109 \pm 6.2$[c] [A] | $1.03 \pm 0.14$[d] [A] |
| | | Nonflier | *Mus musculus* | 37.3 | 9.9 | $5.47{\cdot}10^7 \pm 4.7{\cdot}10^6$ | $24.67 \pm 4.67$[e] [A] | $0.21 \pm 0.02$[f] [A] |
| 2 | Caviedes-Vidal et al. (2008) | Flier | *Artibeus lituratus* | 69.6 | 16.8 | $3.42{\cdot}10^7 \pm 3.2{\cdot}10^6$ | | $0.9 \pm 0.11$[g] [R] |
| | Lavin et al. (2008); Lavin et al. (2007) | Nonflier | *Rattus norvegicus* | 350 | 7.7 | $1.91{\cdot}10^7 \pm 2.1{\cdot}10^6$ | $0.34 \pm 0.04$[h] [A]<br>$0.23 \pm 0.02$[h] [R] | |
| 3 | Barry (1976) | Flier | *Eptesicus fuscus* | 18 | 7.0 | | | |
| | | Nonflier | *M. musculus* | 36.5 | 3.4 | | | |
| 4 | Makanya et al. (1997) | Fliers | *Miniopterus inflatus, Epomophorus wahlbergi, Lisonycteris angolensis* | 8.9–76.9 | $6.5 \pm 0.7$ | | | |
| | Mayhew and Middleton (1985) | Nonflier | *R. norvegicus* | 296 | 5.3 | | | |

In each pairing, the measurements were made by some of the same investigators using uniform methodology.
References:
[a]SEF: mucosal to serosal surface area enlargement factor = villous surface area/nominal surface area
[b]NSA: nominal surface area of the small intestine
[c]Price et al. (2013)
[d]Fasulo et al. (2013a)
[e]E. Price, A. Brun, W. Karasov, and E. Caviedes-Vidal unpublished data
[f]Fasulo et al. (2013b)
[g]Caviedes-Vidal et al. (2008)
[h]Lavin et al. (2007).

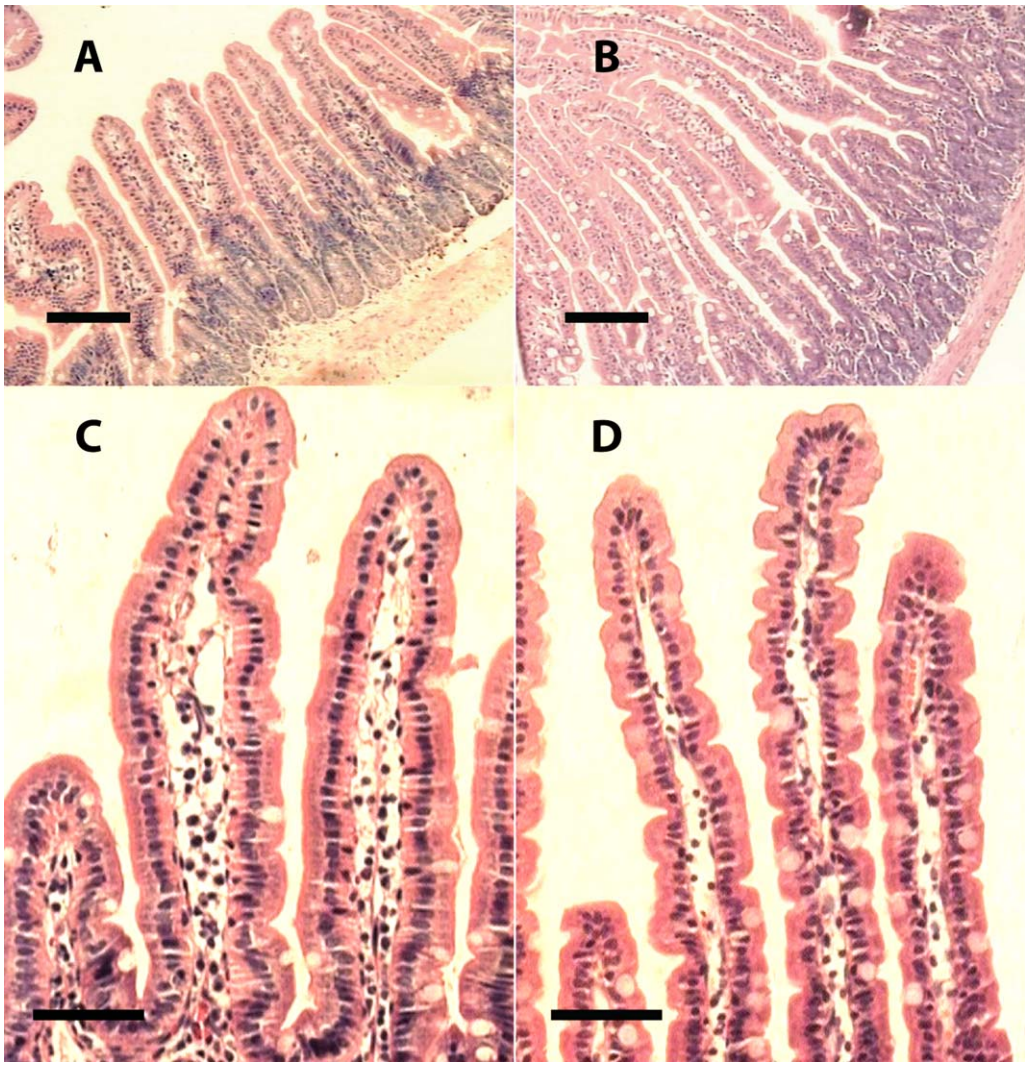


Fig. 2. Histological microphotographs of hematoxylin and eosin-stained sections of the proximal (bars: 100 μm) and medial (bars: 50 μm) regions of the small intestine of the mouse (*Mus musculus*), **A** and **C**, respectively, and the bat (*Tadarida brasiliensis*), **B** and **D**, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

studies in bats (reviewed by Caviedes-Vidal et al., 2008; Makanya et al., 2001), whereas in the mouse the SI was easily discriminated from cecum and large intestine.

Dimensions of various features of the villi and crypts were used to calculate the mucosal to serosal surface area enlargement factor, which varied significantly with intestinal position and was significantly greater in the bats than mice in all regions (Fig. 1E). Overall, the villi increased intestinal surface area by $18.8 \pm 0.3$ times in bats, which was about double that in mice ($9.9 \pm 0.2$; $P < 0.001$, *t*-test; Fig. 2). The underlying explanation is that in bats, compared with mice, villus lengths were similar or longer at every intestinal position (Fig. 1A), and had higher density per $cm^2$ nominal surface area because villus widths (Fig. 1B), crypt diameters (Fig. 1C), and villus–crypt unit diameters (Fig.1D) were narrower in bats than in mice. The ~100% larger mucosa SEF in bats, compared with mice more than compensated for the 20% greater intestinal nominal widths in mice (Fig. 1E). This yields in the bat a mucosal area per unit intestinal length of 9.0 $cm^2$/cm, which is 50% greater than that in the mouse (5.9 $cm^2$/cm; $P = 0.003$).

Enterocyte diameters did not differ significantly among intestinal regions or between species (Fig. 3A). We estimated that bats exceeded mice in enterocyte density per $cm^2$ nominal surface area of the intestine; bats had ~2 times more enterocytes per $cm^2$ than mice (respectively, $1.05 \times 10^8 \pm 8.1 \times 10^6$ cells vs. $5.47 \times 10^7 \pm 4.7 \times 10^6$ cells; $P < 0.0019$ by *t*-test; $P = 0.0002$ by *t*-test; Fig. 3B). The number of enterocytes per $cm^2$ changed along the intestine ($F_{2,24} = 11.9$, $P < 0.0001$) and the pattern of variation differed between the two species (interaction term $F_{2,24} = 11.7$, $P < 0.0001$; Fig. 3B).

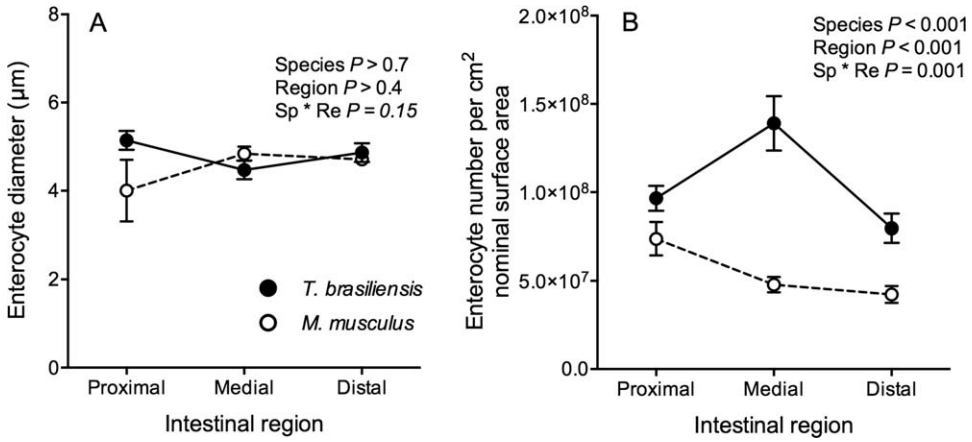


Fig. 3. Enterocyte diameter (**A**) and number of enterocytes per cm intestine (**B**) as a function of intestinal region in the bat, *Tadarida brasiliensis* ($n = 8$) and the mouse (*Mus musculus*; $n = 6$). Values are means ± SEM.

## DISCUSSION

The mouse and bat differ in body mass (Table 1), therefore, the significance at the organismal level of the greater SEF in the bat is better appreciated after correction for the size differences between bats and mice. In vertebrates, intestinal nominal surface area generally increases with mass$^{3/4}$(Karasov, 2012). Consequently, a crude, body size–corrected comparison of total intestinal areas would yield a 58% lower size-corrected nominal surface area in *T. brasiliensis* compared with the mouse (1.1 vs. 1.89 nominal cm$^2$/mass$^{3/4}$, respectively). Lower nominal surface area corresponds to a lower luminal volume and probably less digesta mass carried, which may have been selected for in flying vertebrates to reduce energy costs of flight (Caviedes-Vidal et al., 2007). However, taking into account the SEF, the size-corrected mucosal surface areas are similar in the two species (18.7 vs. 20.7 cm$^2$ mucosa/mass$^{3/4}$ in mouse and bat, respectively). Hence, the comparison shows how an increase in SEF can compensate for a shorter intestine with less nominal surface area (Table 1) to help maintain the intestinal absorptive capacity.

In the sections that follow, we consider to what extent some of our findings on *T. brasiliensis* versus mice can be generalized to flying versus nonflying eutherians. In addition, we will also discuss likely physiological significance of the differences in intestinal morphometrics.

### Greater SEF in Bats than in Nonflying Mammals

The functional surface area of the intestine is increased by villous folds and microvilli on cells. There are no comparative data of microvilli, but data are accumulating that permit comparisons with regard to villous surface area enlargement and a test of whether this could help compensate for lower nominal surface area in bats.

The ratio of villous area relative to nominal surface area, sometimes called the SEF, has been previously measured in some bat species (see list of references in Caviedes-Vidal et al., 2008) and in nonflying mammal species (Table 2) by a number of investigators using a variety of methods. Based on data available so far, SEF does not change significantly with increasing body mass (Lavin et al., 2008), which makes it easier to compare fliers and nonfliers of different sizes within eutherians. However, this measurement is sensitive to the particular method used by an investigator (Snipes et al., 1994), as is apparent in the comparison of SEF by two groups for both laboratory rats and mice (Table 2). But in comparisons of bats and nonflying mammals by the same investigators using similar methodology, bats exceed the nonfliers in SEF by 86% ± 26% ($n = 4$ paired comparisons, $P < 0.025$ by paired $t$-test). Thus, our preliminary conclusion is that bats, relative to nonflying mammals, exhibit an increased villi density relative to nominal surface area. A caveat to this conclusion must be placed because dietary habits of the two studied species are different, mice are omnivorous and *T. brasiliensis* are insectivorous. However, although the influence of diet on the intestinal architecture was not specifically addressed in this study, both bat species studied to date with very different dietary habits, one insectivorous (*T. brasiliensis*) and the other frugivorous (*Artibeus lituratus*), showed higher SEFs than the laboratory rodents. Further analyses including dietary habits as factor, different body masses and different orders of rodents and chiropterans will ultimately allow a more robust, phylogenetically informed test of the hypothesis that increased intestinal villus morphology has evolved as compensation for smaller intestinal size in flying vertebrates.

### Physiological Significance of Greater SEF

Greater SEF increases functional area of the intestine and thus has the potential to increase the reaction rates per unit nominal surface area for membrane-bound processes such as hydrolysis and transcellular absorption by diffusion (for lipophilic compounds such as fatty acids) or mediated and active transport (for water soluble compounds such as sugars and amino acids). It also has the potential to increase the capacity for paracellular absorption by increasing the number of intestinal cells and hence cell–cell TJs. Because bats have greater capacity for paracellular absorption than nonflying mammals (Caviedes-Vidal et al., 2007, 2008; Fasulo et al., 2013a, b; Price et al., 2013), we have focused on this issue. Using uniform methodology, we measured enterocyte size and calculated the density of enterocytes per $cm^2$ nominal surface area of the intestine in two bat species and two nonflying eutherian mammals (see Results, Table 2). These calculations indicate that both bat species have a greater density ($\sim$1.8–2 times) of enterocytes per $cm^2$ nominal surface area than nonflying mammals, and therefore, could be inferred a greater density of TJs per $cm^2$ as predicted based on absorption studies (see Introduction, Table 2). Future research must include stereological and electron microscopy studies to test our inference. We conclude that the observed increase in the amount of enterocytes due to an increased SEF of the nominal surface area of the intestine is given principally by a greater density of villi per nominal. This finding may be an important mechanistic explanation for the high paracellular absorption observed in bats compared to nonflying mammals. Other mechanisms that may contribute to the enhanced paracellular absorption observed in bats (e.g., differential molecular architecture of TJs) need further research.

## AUTHOR CONTRIBUTIONS

Conceived and designed experiments: Z-Q.Z., W.H.K., E. C-V. Performed experiments and gathered data: Z-Q.Z., A.B., E.P. Analyzed data: all. Contributed reagents/materials/analysis tools: W.H.K., E.C-V, A.P.C-N., Wrote the paper: Z-Q.Z., A.B., W.H.K, E.C-V.

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