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STEREOLOGICAL ESTIMATION OF SIZE-WEIGHTED ENTEROCYTE MEAN SIZE IN BAT SMALL INTESTINE

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ABSTRACT

The total absorptive surface area is often the target parameter in stereological studies of the intestine. Here we alternatively considered two descriptors of mean size of the absorptive enterocytes: their surface-weighted mean volume $\mathbb{E}_S(V)$, and volume-weighted mean surface area $\mathbb{E}_V(S)$. The practical applicability of the corresponding estimators was demonstrated using an anisotropic biological tissue, namely the bat small intestine. The volume-weighted enterocyte mean volume $\mathbb{E}_V(V)$, was also considered because it was obtained as a 'by-product' from the estimation of $\mathbb{E}_V(S)$. This preliminary study indicates that the considered descriptors of enterocyte mean size may be lower in the middle part than in the proximal and distal parts of the bat intestine.

Key words: Bat, enterocyte, intestine, isotropic sections, size-weighted size, stereology.

1. INTRODUCTION

The bat has evolved various adaptations to cope with its unique energy-expensive lifestyle. High nutrient transport rates have been reported (Keegan, 1977), and these have been attributed to a large absorbtive surface area within the intestine (Keegan & Mödinger, 1979). The latter observation was confirmed by Makanya *et al.* (1997) using stereological methods. A further parameter of potential biological importance, due to its possible correlation with the absorptive properties of the intestine, is the mean size of its absorptive enterocytes (i.e. the epithelial cells). Zoubi *et al.* (1995) have shown that in the rat small intestine, the mean enterocyte size decreases from the proximal to the distal parts. For the bat intestine, however, there appears to be no data on the latter parameter. The main purpose of this paper is therefore to demonstrate the practical estimation of the mean enterocyte size of the bat small intestine. The primary target parameters were the surface-weighted mean volume $\mathbb{E}_S(V)$, and the volume-weighted mean surface area $\mathbb{E}_V(S)$. The corresponding estimators have previously been implemented only in a materials science application (Karlsson & Cruz-Orive, 1997). The surface-weighted star

volume, which is a concept similar to $\mathbb{E}_S(V)$, was recently proposed and applied to pig lung by Reed & Howard (1998). We also considered the volume-weighted enterocyte mean volume $\mathbb{E}_V(V)$, because this parameter may be obtained from the same set of measurements collected for the estimation of $\mathbb{E}_V(S)$. The estimates of $\mathbb{E}_S(V)$, $\mathbb{E}_V(S)$ and $\mathbb{E}_V(V)$ were lower in the middle part than in the proximal and distal parts of the intestine. The functional meaning of this finding is difficult to deduce from our results since only one individual was used.

2. MATERIAL, SAMPLING AND TISSUE PREPARATION

One frugivorous bat (*Epomophorus wahlbergi*) was used in this study. The bat was captured and its intestine removed and fixed as described in Makanya *et al.* (1997). The small intestine was cut into three segments (denoted proximal, middle and distal) of about equal length, and two subsegments (about 2 cm long) were sampled in a systematic random way from each segment. After embedding in methacrylate resin, each subsegment was cut into three mutually orthogonal sections, an *or*thogonal *tri*plet *p*robe or 'ortrip' (Mattfeldt *et al.*, 1985). The first section of each ortrip was cut at an isotropic random orientation using the 'orientator' method (Mattfeldt *et al.*, 1989, 1990). All sections had a nominal thickness of 3 μm and were stained with hematoxylin and eosin. The isotropic uniform random (IUR) tissue sections were observed using a light microscope fitted with a 100× oil immersion objective and a motorized specimen stage. Colour images of the enterocytes were observed on a monitor connected to the microscope via a video camera. The final magnification on the monitor was 2800×. Stereological test systems were overlaid on the images of the enterocytes using the Visilog® software. The video camera was rotated to obtain various orientations of the test systems relative to the structure. Fields of view were systematically sampled from each section by moving the specimen stage in pre-determined steps.

The non-cryptal intestinal epithelium is columnar comprising absorptive cells (enterocytes) and goblet cells. The goblet cells are interspersed between the enterocytes and are moderate in number compared with the enterocytes. A characteristic feature of the goblet cells is the presence of numerous secretory granules, which occupy a major part of the cytoplasm. This feature makes it possible to distinguish the latter from absorptive epithelial cells. Any one section of the intestinal wall may generate oblique and/or transverse profiles of the cells. Such profiles will not cause any serious identification problems since a goblet cell profile without the characteristic granules is a rare event and would occur only if such a cell is transected through the subnuclear level. Profiles of migratory lymphocytes, which are occasionally encountered in the intercellular spaces, were easy to identify due to their irregular shape and because they don't adhere to the adjacent cells. Similarly, cell profiles from the core of the villus and other intestinal layers have characteristic morphologies and were thus identified without problems.

3. SIZE-WEIGHTED MEAN SIZE

Consider a population of N objects (e.g. cells or particles) of arbitrary shape and size. Their individual volumes are denoted $V_1, V_2, \ldots, V_N$; individual surface areas $S_1, S_2, \ldots, S_N$; total volume V_{Tot}; and total surface area S_{Tot}. The descriptors of mean

size considered here are defined as population means

$$\mathbb{E}_S(V) = \sum_{i=1}^{N} V_i \cdot \frac{S_i}{S_{Tot}}, \quad \mathbb{E}_V(S) = \sum_{i=1}^{N} S_i \cdot \frac{V_i}{V_{Tot}}, \quad \mathbb{E}_V(V) = \sum_{i=1}^{N} V_i \cdot \frac{V_i}{V_{Tot}}.$$

For instance, $\mathbb{E}_S(V)$ denotes the mean object volume V evaluated with respect to the distribution in surface area S. The weights in the three preceding means are S_i/S_{Tot} and V_i/V_{Tot} for the distribution in surface area and volume, respectively. Each object thus contributes to the mean in proportion to its size (S_i or V_i), hence the notion size-weighted mean. For an overview of this concept, see Karlsson & Cruz-Orive (1997).

There is a two-step procedure for estimating size-weighted mean size (Karlsson & Cruz-Orive, 1997). First, a collection of objects (here, enterocytes) is sampled from the proper distribution using a probe of the appropriate dimension. Second, the size of each sampled object is estimated in an unbiased way. Thus, estimators have been developed for the surface-weighted mean volume $\mathbb{E}_S(V)$ (Gittes, 1990), volume-weighted mean surface area $\mathbb{E}_V(S)$ (Jensen & Gundersen, 1987, 1989), and volume-weighted mean volume $\mathbb{E}_V(V)$ (Gundersen & Jensen, 1985; Jensen & Gundersen, 1985). The implementation of these estimators is described in the following section.

The estimators used here (Eqs. 1–4) hold for convex objects only, but may be extended to those of arbitrary shape (Gundersen & Jensen, 1985; Jensen & Gundersen, 1985, 1987, 1989); see also Cruz-Orive (1987).

4. WORKED EXAMPLES

4.1. Estimation of $\mathbb{E}_S(V)$

The surface-weighted enterocyte mean volume $\mathbb{E}_S(V)$, was estimated using the method proposed by Gittes (1990). Boundary sampled intercepts (BSI)—intercepts that emanate from a point on a profile boundary and project into the profile—are generated on a section plane using a cycloidal test system. The lengths of the BSI are then measured along the minor axis of the cycloids. For an anisotropic structure, unbiased estimation requires IUR cycloids and IUR section planes.

A test system of cycloids was overlaid with uniform random position on each of the available IUR sections (Fig. 1a). The cycloids were parallel to facilitate measurement of BSI lengths. The minor axis of the cycloids had an isotropic random orientation within the section, and a fresh orientation was chosen in a systematic way for each analysed field of view. Enterocyte profiles whose boundaries were intersected by the cycloids (or any IUR test line of arbitrary shape) correspond to enterocytes sampled from the surface-weighted distribution. Whenever a cycloid intersected a profile boundary, the corresponding BSI emanating from that intersection point was measured along the minor axis of the cycloid (Fig. 1b). This procedure ensured that the BSI had an isotropic random orientation in three dimensions, a requirement for an unbiased volume estimation of a sampled enterocyte. If n BSI were sampled, the unbiased estimator $\overline{v}_S$ of $\mathbb{E}_S(V)$ was

$$\overline{v}_S = \frac{2\pi}{3} \cdot \frac{1}{n} \sum_{i=1}^{n} l_{1i}^3, \tag{1}$$

where l_{1i} is the length of the ith ($i = 1, 2, \ldots, n$) intercept (Gittes, 1990). The subscript 1 of l_1 indicates the intercepts have been sampled with a one-dimensional probe (i.e. a line).

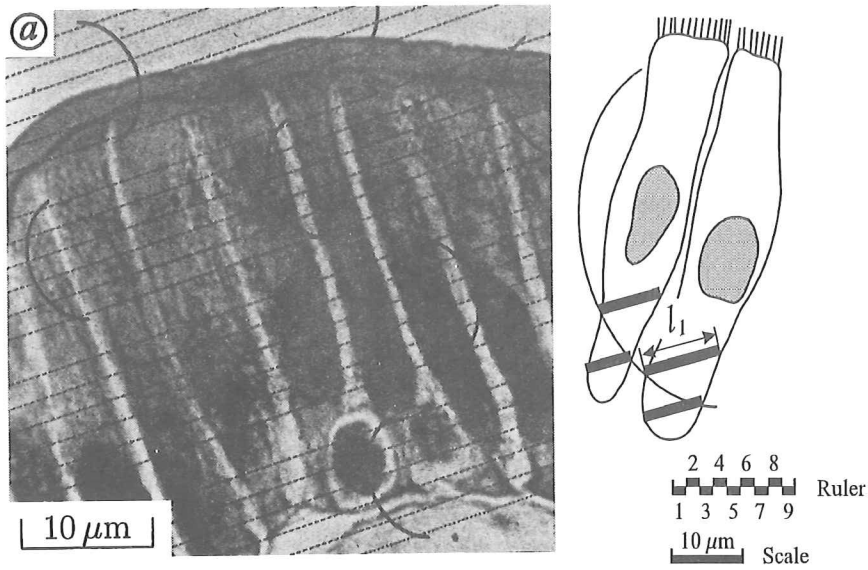

Fig. 1. The boundary sampled intercepts (BSI) method was used to estimate the surface-weighted enterocyte mean volume $\mathbb{E}_S(V)$. (a) An isotropic uniform random (IUR) test system of cycloids overlaid on an IUR section through the epithelium of the bat intestine. The dashed straight lines facilitate measuring BSI lengths along the minor axis of the cycloids. (b) Each intersection between a cycloid and a profile boundary generates a BSI of length l_1, measured along the minor axis of the cycloid. Here four BSI were generated (thick segments). The lengths of these were classified using a ruler into classes 4, 5, 4, and 6. The class width of the ruler used was 1.9 μm. Then from Eq. (1), $\overline{v}_S = (2\pi/3) \cdot (1/4) \cdot (3.5^3 + 4.5^3 + 3.5^3 + 5.5^3) \cdot 1.9^3 \approx 1230\,\mu\text{m}^3$.

Intercept lengths were classified using the transparent ruler shown in Fig. 1b . The class middle was used as class mark, that is, as an estimate of the mean of the intercept lengths within that class. To avoid edge effects, only BSI that emanated from points within a predetermined rectangular frame were considered; thus all intercepts terminated within the field of view. Estimates of $\mathbb{E}_S(V)$ were computed for each ortrip using Eq. (1), as illustrated in Fig. 1b. There is also an estimator of $\mathbb{E}_S(V)$ relevant for intercepts recorded into classes (Karlsson & Cruz-Orive, 1997, Eq. 4.3). The number of intercepts generated per ortrip varied from 107 to 274, with a mean of 166. Within the proximal, middle and distal segments, the total number of intercepts was 279, 484 and 232, respectively.

4.2. Estimation of $\mathbb{E}_V(S)$

The surfactor method proposed by Jensen & Gundersen (1987, 1989) was used to estimate the volume-weighted enterocyte mean surface area $\mathbb{E}_V(S)$. Point sampled intercepts (PSI) are generated on a section plane using a test system of points and lines. The lengths of the PSI and the angles between the PSI and tangents to the profile boundaries are then measured. For an anisotropic structure, unbiased estimation requires an IUR test system and IUR section planes.

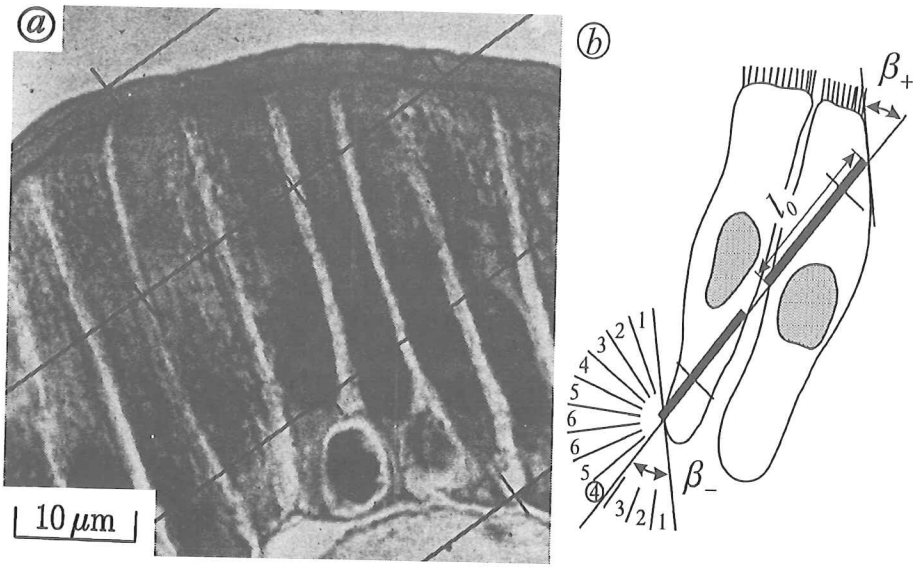

Fig. 2. The surfactor method was used to estimate the volume-weighted enterocyte mean surface area $\mathbb{E}_V(S)$. (a) An isotropic uniform random (IUR) test system of points on parallel lines overlaid on an IUR section through the epithelium of the bat intestine. (b) A test point that hits a profile generates a point sampled intercept (PSI) of length l_0 along the test line. Associated with each PSI are two acute angles β_- and β_+ between the test line and the tangents to the profile boundary at the intersections between the test line and the profile boundary. Here two PSI were generated (thick segments), and the lengths of these were classified into classes 9 and 11. The ruler shown in Fig.1b was used for this purpose. The shown protractor was used to classify the four acute angles (only two are indicated) into angle classes 4, 2, 2 and 3. The corresponding values for $c(\beta)$ were computed using Eq. (3): 1.502, 3.844, 3.844 and 2.194. The class width of the ruler used was 1.9 μm. Then from Eq. (2), $\overline{s}_V = (2\pi/3) \cdot (1/2) \cdot [8.5^2 \cdot (1.502 + 3.844) + 10.5^2 \cdot (3.844 + 2.194)] \cdot 1.9^2 \approx 3980\,\mu\text{m}^2$.

A test system of points on parallel lines was overlaid with random position on the IUR section planes (Fig. 2a). The test lines had an isotropic random orientation within the section, and a fresh orientation was chosen in a systematic way for each analysed field of view. Enterocyte profiles that were hit by test points correspond to enterocytes sampled from the volume-weighted distribution. For each test point that hit a profile, the lengths of the corresponding PSI was measured along the test line. In addition, the two acute angles between the support line of the PSI and the tangents to the profile boundary at the two intersection points were also measured (Fig. 2b). The surface area of a sampled enterocyte may be estimated from these length and angle measurements (Jensen & Gundersen, 1987, 1989). If n PSI (and hence $2 \cdot n$ acute angles) were sampled, the estimator used was

$$\overline{s}_V = \frac{2\pi}{3} \cdot \frac{1}{n} \sum_{i=1}^{n} l_{0i}^2 \cdot [c(\beta_{-i}) + c(\beta_{+i})], \qquad (2)$$

where l_{0i} is the length of the ith ($i = 1, 2, \ldots, n$) intercept, β_{i-} and β_{i+} are the acute angles between this intercept and the tangents to the enterocyte profile boundary at the two intersection points, and $c(\cdot)$ is defined as

$$c(\beta) = 1 + (\pi/2 - \beta) \cdot \cot\beta, \qquad 0 < \beta \leq \pi/2, \tag{3}$$

(Jensen & Gundersen, 1987, 1989). The subscript 0 of l_0 indicates the intercepts have been sampled with a zero-dimensional probe (i.e. a point).

Intercept lengths were classified and edge effects eliminated in the same way as previously described. The acute angles were classified using the transparent protractor shown in Fig. 2b, namely a half circle divided into twelve angle classes of equal width $\pi/12$ radians. The class middle was used as class mark. Estimates of $\mathbb{E}_V(S)$ were computed for each ortrip according to Eqs. (2)–(3), as illustrated in Fig. 2b. There is a convenient estimator of $\mathbb{E}_V(S)$ for intercepts and angles recorded into classes (Karlsson & Cruz-Orive, 1997, Eq. 5.3). The number of intercepts generated per ortrip varied from 26 to 60, with a mean of 45. Within the proximal, middle and distal segments, the total number of intercepts was 119, 69 and 80, respectively.

4.3. Estimation of $\mathbb{E}_V(V)$

The volume-weighted enterocyte mean volume $\mathbb{E}_V(V)$ may be estimated using the PSI method (Gundersen & Jensen, 1985; Jensen & Gundersen, 1985). The lengths of PSI are measured along isotropic random orientations in three dimensions. For this purpose, either IUR or vertical uniform random (VUR) sections may be used. On IUR sections, a test line with an isotropic random orientation in the section is effectively isotropic random in three dimensions. On VUR sections, the test lines must be sine-weighted (Baddeley *et al.*, 1986). The PSI method is hence in contrast to the BSI and surfactor methods where IUR sections *must* be used (unless the structure itself is isotropic). Since the surfactor method involves measuring PSI (and angles in addition), $\mathbb{E}_V(V)$ may be obtained as a 'by-product' from the same measurements collected for estimating $\mathbb{E}_V(S)$.

Intercepts were sampled as described in the previous subsection. If n PSI were measured (the acute angles were ignored), the estimator used was

$$\overline{v}_V = \frac{\pi}{3} \cdot \frac{1}{n} \sum_{i=1}^{n} l_{0i}^3, \tag{4}$$

where l_{0i} is the length of the ith ($i = 1, 2, \ldots, n$) intercept (Gundersen & Jensen, 1985; Jensen & Gundersen, 1985).

Estimates of $\mathbb{E}_V(V)$ were computed for each ortrip using Eq. (4). For an example, see Fig. 2b and consider only the PSI (not the acute angles), whereby $\overline{v}_V = (\pi/3) \cdot 1.9^3 \cdot (8.5^3 + 10.5^3)/2 \approx 6360\,\mu\text{m}^3$. There is also an estimator of $\mathbb{E}_V(V)$ relevant for intercepts recorded into classes (Karlsson & Cruz-Orive, 1997, Eq. 6.2).

5. RESULTS

Estimates of $\mathbb{E}_S(V)$, $\mathbb{E}_V(S)$ and $\mathbb{E}_V(V)$ from the proximal, middle and distal segments of the bat small intestine are shown in Fig. 3. Pooled estimates (horizontal lines in Fig. 3) among two ortrips were computed as a ratio-of-sums estimator (Cochran, 1977, Section 6); see also (Baddeley, 1993). These estimates varied along the intestine in a

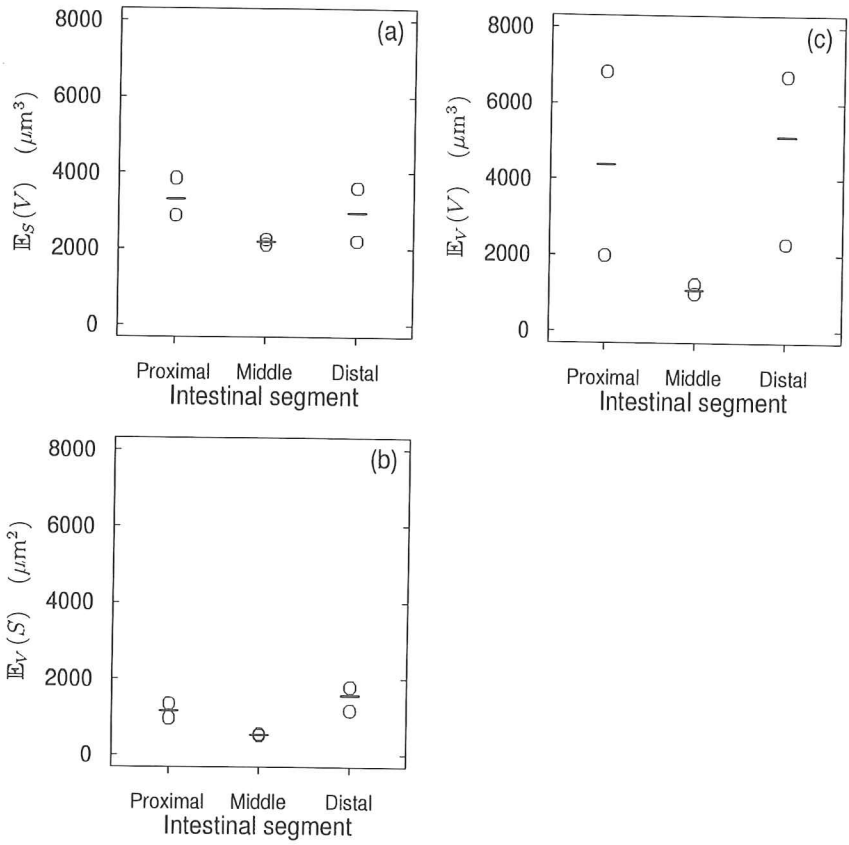

Fig. 3. Estimates of enterocyte mean size from the proximal, middle and distal segments of the bat intestine. Two ortrips were used in each segment and the pool among these is shown as a horizontal line. The following size descriptors were used: (a) the surface-weighted mean volume $\mathbb{E}_S(V)$, (b) the volume-weighted mean surface area $\mathbb{E}_V(S)$, and (c) the volume-weighted mean volume $\mathbb{E}_V(V)$.

'u-shaped' pattern, with the lowest value in the middle segment and higher values in the proximal and distal segments (Fig. 3). The pooled estimates, computed as ratios-of-sums, for the whole intestine were $\overline{v}_S = 2680\,\mu\text{m}^3$, $\overline{s}_V = 1110\,\mu\text{m}^2$ and $\overline{v}_V = 3750\,\mu\text{m}^3$.

6. DISCUSSION

The 'u-shaped' variation of enterocyte mean size along the intestine found here was unexpected, and is in contrast to what is reported in the literature for the rat (Zoubi *et al.*, 1995). Our findings must, however, be confirmed by including more individuals, which is a target of our future studies. Recall that the main purpose of this preliminary study was to demonstrate the applicability of the methods and not to establish definite biological parameters.

The parameters $\mathbb{E}_S(V)$, $\mathbb{E}_V(S)$ and $\mathbb{E}_V(V)$, together with the surface-weighted star

volume (Reed & Howard, 1998), are available from single or independent sections, that is, without using a disector (Sterio, 1984). They have a well-defined three dimensional interpretation and, possibly, a sound functional meaning in biology, as opposed to size descriptors involving 'height' (Howard, 1990). Furthermore, the descriptors of mean size considered here may prove to be important tools in applied biology. The parameter $\mathbb{E}_V(V)$ has already proved to be a powerful diagnostic and prognostic tool in cancer pathology (e.g. Sörensen, 1992, and references therein). The true usefulness of $\mathbb{E}_S(V)$ and $\mathbb{E}_V(S)$ must, however, await further investigation.

Estimation of $\mathbb{E}_S(V)$ and $\mathbb{E}_V(S)$ requires IUR sections. For this purpose, the orientator (Mattfeldt *et al.*, 1989, 1990) or isector (Nyengaard & Gundersen, 1992) methods may be used. The former method is more convenient for large specimens, whereas the latter one is suitable for small specimens. Estimation of $\mathbb{E}_V(S)$ is more time consuming than estimation of $\mathbb{E}_V(V)$, because angles are measured in addition to intercept lengths. This drawback is, however, compensated since two parameters, $\mathbb{E}_V(S)$ and $\mathbb{E}_V(V)$, are obtained from one set of measurements.

Note, that the estimation procedures described here can be carried out by manually overlaying a transparent test system on micrographs of the structure; thus no sophisticated software or equipment is a prerequisite.

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