Blackwell Publishing

Asian J Androl 2005; 7 (3): 267–275
DOI: 10.1111/j.1745-7262.2005.00042.x

AJA

·*Original Article*·

Regionalization of epididymal duct and epithelium in rats and mice by automatic computer-aided morphometric analysis

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Abstract

Aim: To establish a rat and mouse epididymal map based on the use of the Epiquatre automatic software for histologic image analysis. **Methods:** Epididymides from five adult rats and five adult mice were fixed in alcoholic Bouin's fixative and embedded in paraffin. Serial longitudinal sections through the medial aspect of the organ were cut at 10 μm and stained with hematoxylin and eosin. As determined from major connective tissue septa, nine subdivisions of the rat epididymis and seven for the mouse were determined, consisting of five sub-regions in the caput (rat and mouse), one (mouse) or three (rat) in the corpus and one in the cauda (rat and mouse). Using the Epiquatre software, several tubular, luminal and epithelial morphometric parameters were evaluated. **Results:** Statistical comparison of the quantitative parameters revealed regional differences (2–5 in the rat, 3–6 in the mouse, dependent on parameters) with caput regions 1 and 2 being largely distinguishable from the similar remaining caput and corpus, which were in turn recognizable from the cauda regions in both species. **Conclusion:** The use of the Epiquatre software allowed us to establish regression curves for different morphometric parameters that can permit the detection of changes in their values under different pathological or experimental conditions. *(Asian J Androl 2005 Sep; 7: 267–275)*

Keywords: rat; mouse; epididymis; morphometry; zonation; computer-aided image analysis; Epiquatre Software

1 Introduction

Epididymal anatomy of the rat has been studied since 1957 [1] with segments defined according to gross morphology and different divisions of the initial segment into zones [2, 3]. The increasing awareness of the regional disposition of genes and gene products in the epididymal epithelium and their influence on the migration, maturation and storage of spermatozoa in transit [4] has brought the anatomy of this organ to the forefront of studies of gene regulations [5]. The current interest in transgenic mice, many of which show infertility related to epididymal malformation [6, 7] and gene expression [8–10], has renewed interest in epididymal regional division in this species, and many differing classifications have been reported based on gross morphology, histochemical staining or ultrastructure. Computer-aided methods of im-

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Received 2004-10-09 Accepted 2005-02-12

age analysis should be able to provide objective assessment of the relative epididymal duct and its epithelial morphometry. The objective of the present study was to establish a quantitative model of the anatomy of the epididymis of rats and mice using the Epiquatre (Proiser, Buñol, Spain), a new histology analysis software system.

2 Materials and methods

2.1 Animals and tissue preparation

Five male rats (*Rattus norwegicus*, Wistar strain, 126 g–139 g, 120 days old) and five male mice (*Mus musculus*, Balb strain, 26 g–27 g, 105 days old) were killed by an overdose of diethylether and the right epididymides were removed, weighed and the volume and density were obtained by water displacement. Organs were fixed by immersion in alcoholic Bouin's fixative for 24 h and dehydrated through graded alcohols before the entire organ was embedded in paraffin. Serial longitudinal sections through the medial aspect of the organ, incorporating the caput, corpus and cauda regions (Figure 1), were cut at 10 µm and stained with Harris's hematoxylin (Merck, Darmstadt, Germany) and Eosin yellow (Panreac, Montcada i Reixac, Spain) and mounted in Entellan (Merck, Darmstadt, Germany).

2.2 Image analysis

Slides corresponding to the central area of the longi-

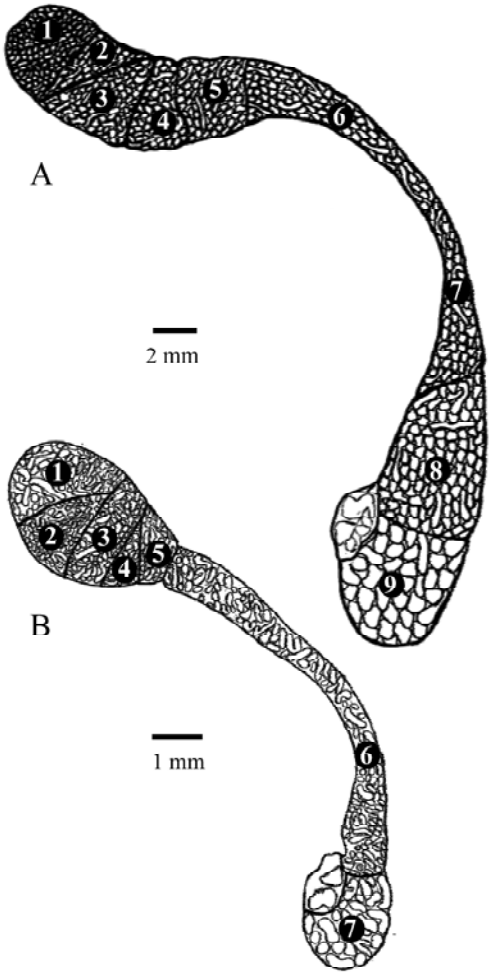

Figure 1. Diagrams representing the regionalization observed towards the medial aspect of the right epididymis of the rat (A) and mouse (B), based on longitudinal sections of the organs. Regions 1–5 exist in the rat caput, 6–8 in the corpus and 9 in the cauda (A). Regions 1–5 are present in the mouse caput, and regions 6 and 7 are in the corpus and cauda (B).

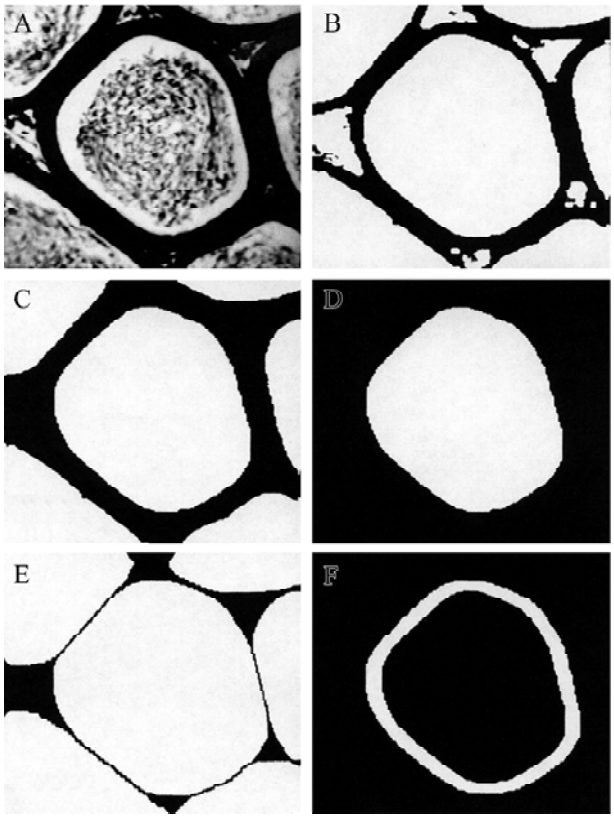

Figure 2. Sequential steps of the Epiquatre. The original micrograph (A) is rendered first with high contrast (B) and then further definition of the epithelium is provided (C) to yield an image of the entire lumen (D). Detection of the intertubular boundaries (E) and subtraction of the lumen permits the epithelium (F) to be measured.

tudinal sections were examined using an Olympus BH-2 microscope (Olympus, Tokyo, Japan) equipped with 40 and 20× bright field objectives and a 3.3× photo-ocular. As many tubule sections as possible were analyzed per zone. In the caput and corpus epididymidis, nearly circular (Tubule minimum diameter/Tubule maximum diameter [TmaxD/TminD] > 0.8) transverse tubule sections were measured; in the cauda all the transverse sections were assessed. If fewer than four tubule sections were analyzable, another complete section 20 μm deeper was examined. Thus, in both species, at least 20 tubule sections for each anatomical region (four per animal) were evaluated.

The image was acquired by a Sony CCD AVC-D7CE B/W video camera (Sony, Tokyo, Japan) interfaced with a PIP 1024 frame grabber (Microptic S.L., Barcelona, Spain). The array size of the video grabber was 256 × 256 × 8 bits providing digitized images of 65 536 pixels and 256 grey levels (0 black–255 white). The resolution of images was 0.033 (40×) μm and 0.17 (20×) μm per pixel in the horizontal and vertical axes.

The Epiquatre software performs as follows: 1) the capture and digitization of the microscopic images (Figure 2A); 2) the binarization of the micrographs, achieved by selecting high grey levels (near to 255) followed by the hole fill procedure (Figure 2B); 3) the erosion and posterior reconstruction of the images to retain only the lumen of the tubules (Figure 2C); 4) the use of the border kill function to render the lumen of the central tubule from which luminal parameters were calculated (Figure 2D); 5) the expansion of the highlighted area (Figure 2C) to embrace the inter-tubular planes, based on the premise that the epithelial height must be constant in the same epididymal zone, followed by a border kill to produce an image of the entire tubule (Figure 2E) from which tubule parameters can be analyzed; and 6) subtraction of the areas corresponding to the entire tubule and the lumen to render epithelium morphometry (Figure 2F). Each tubule section was examined for the following parameters: luminal area (La, μm^2), luminal perimeter (Lp, μm) and

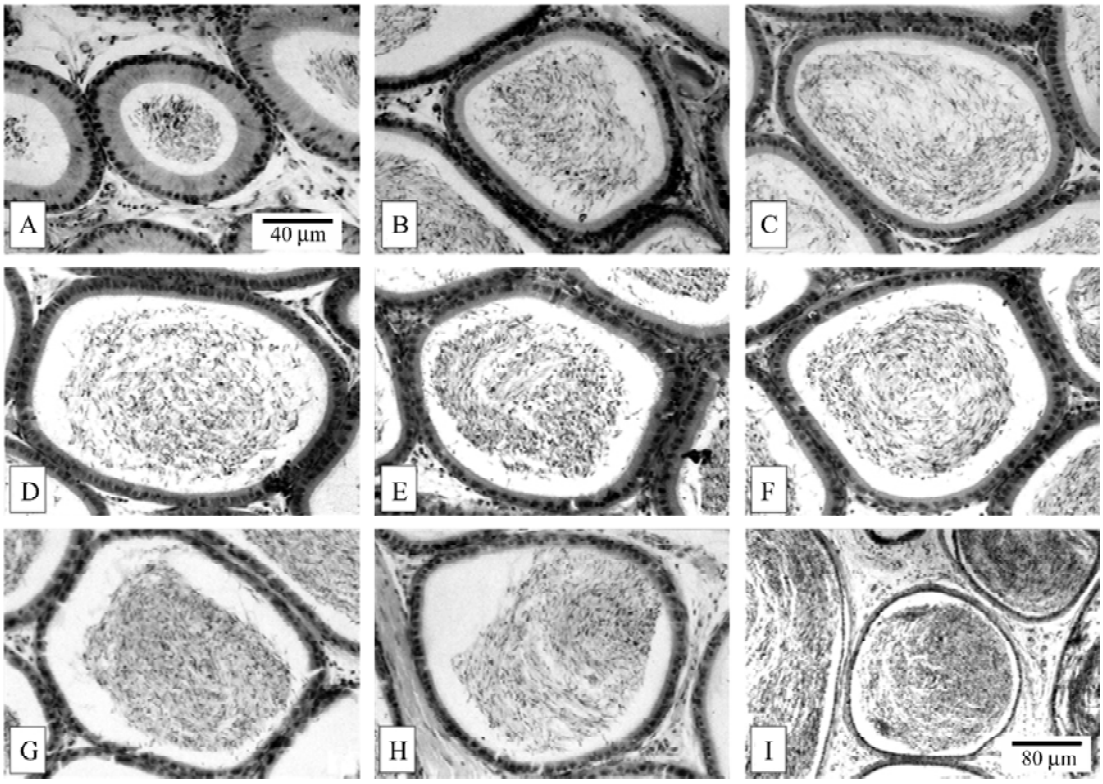

Figure 3. Representative micrographs of individual tubules in rat epididymal regions 1–9 (A-I), as indicated in Figure 1. Scale showed in region 1 (A) applies to regions 2-8 (B-H).

luminal maximum and minimum luminal diameter, (LmaxD, LminD, calculated as the maximum and minimum feret diameters, respectively, μm), tubule area (Ta, μm^2), tubule perimeter (Tp, μm) and tuble maximum and minimum diameter (TmaxD, TminD, calculated as the maximum and minimum feret diameters, respectively, μm), epithelial area (Ea, μm^2) and epithelial height (Eh, calculated as [[TmaxD-LmaxD]+[TminD-LminD]]/2, μm).

The advantage of this morphometric approach is that an average height for the epithelium surrounding the entire circumference of the lumen in all sections in all regions is provided rather than the traditional, far fewer, measurements on transverse sections. At this resolution, no data on the microvilli and/or stereocilia borders of the epithelium could be provided. It is possible that small regions may not be captured in all the longitudinal sections, but the major divisions will be objectively measured.

2.3 Epididymal zonation

The rat epididymis was seen to be divided into nine regions as judged from major connective tissue septa (Figure 1A). These correspond to generally accepted regions of the caput (regions 1–5), corpus (regions 6–8) and cauda (region 9), and representative tubule cross sections of these regions are shown in Figure 3. For the mouse epididymis only seven regions could be discerned on the basis of connective tissue septa (Figure 1B). These are equivalent to the caput (regions 1–5), corpus (region 6) and cauda (region 7), and representative tubule profiles of these regions are presented in Figure 4.

2.4 Statistical analysis

Polynomial regression curves were calculated for each parameter, with the epididymal region as the independent variable, in order to characterize the changes throughout the duct [11]. Median values for all parameters were

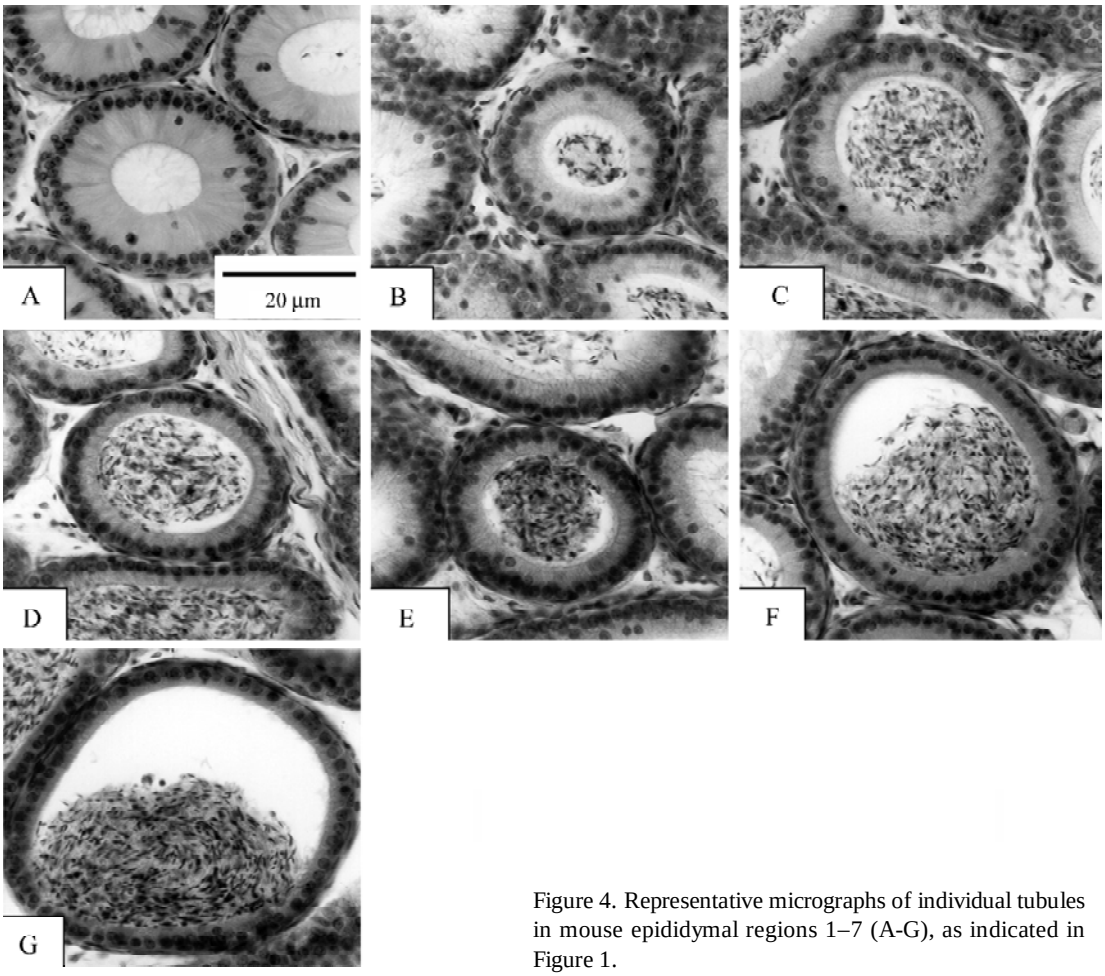

Figure 4. Representative micrographs of individual tubules in mouse epididymal regions 1–7 (A-G), as indicated in Figure 1.

Table 1. Physical characteristics of experimental rat and mouse epididymides. BW: body weight; EW: epididymal weight. Data in mean ± SEM.

Species	Rat	Mouse
BW (g)	133.00 ± 5.85	26.65 ± 0.52
EW (g)	0.49 ± 0.02	0.035± 0.007
EW/BW (%)	0.38 ± 0.01	0.13 ± 0.02
Volume (mL)	0.47 ± 0.02	0.03 ± 0.01
Density (g/mL)	1.05 ± 0.01	1.05 ± 0.04

obtained from the five animals in order to construct the regression lines. Repeated measure ANOVA was performed using the animal and epididymal regions as factors for assessing the changes in parameters within each epididymal region. When ANOVA showed significant differences ($P < 0.05$), the *a posteriori* Fisher test was used to distinguish different regions of the epididymal duct in both species on the basis of the eight parameters examined.

3 Results

3.1 Epididymis of rat

Epididymal weight as a percentage of body weight was (0.38 ± 0.01) % with a density of (1.05 ± 0.01) g/mL (Table 1).

Figure 5 displays the average values of the morphometric parameters in each region of the rat epididymis. It reveals that the areas (Figure 5A), perimeters (Figure 5B) and diameters (Figure 5C) of the tubule and lumen display a triphasic trend in magnitude, increasing 3–5-fold in magnitude from regions 1 to 4, decreasing slightly in regions 5 and 6, and increasing between regions 7 and 9.

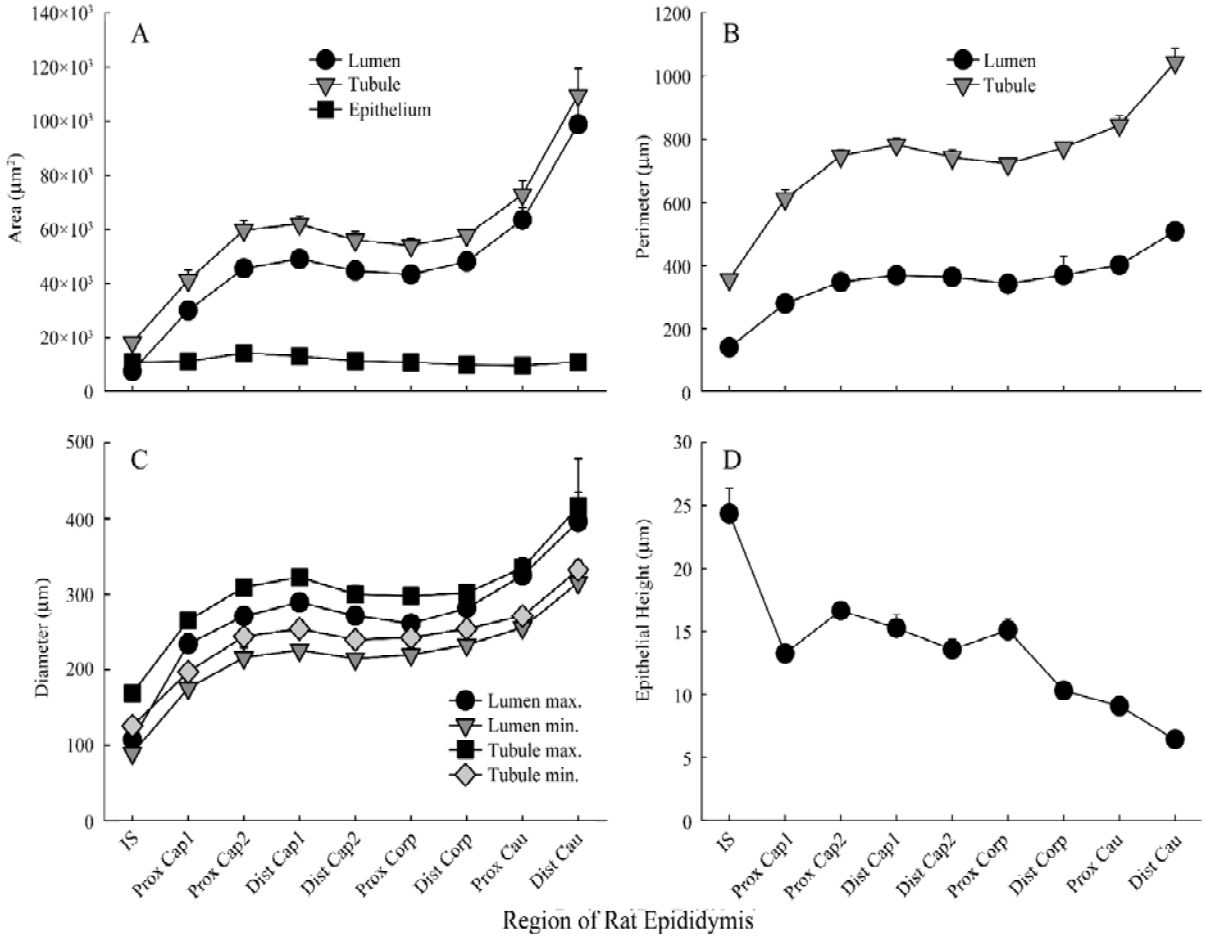

Figure 5. Rat epididymal morphometric parameters. Values are mean ± SD.

Table 2. Polynomial regression equations for morphometric parameters of the rat epididymis. [a]x refers to region of epididymis. Tubule maximum diameter (TmaxD); Tubule minimum diameter (TminD); Tubule area (Ta); Luminal maximum diameter (LmaxD) and Luminal minimum diameter (LminD); La: luminal area; Eh: epithelial height; Ea: epithelial area.

Parameter	Polynomial regression equation	R^2
TmaxD	$y= -5.68+203.34x-42.36x^2+2.76x^3$	0.997
TminD	$y= -0.22+154.33x-30.81x^2+1.97x^3$	0.993
Ta	$y= -35500+65000x-14200x^2+968.55x^3$	0.993
LmaxD	$y= -66.46+223.5x-45.65x^2+2.96x^3$	0.988
LminD	$y= -38.43+160.91x-31.94x^2+2.05x^3$	0.994
La	$y= -41600+59700x-1.3000x^2+893.61x^3$	0.997
Eh	$y= 42.85-26.97x+8.86x^2-1.18x^3+5.32x^4$	0.908
Ea	$y= 7660+3200x-381.51x^2-48.66x^3+6.23x^4$	0.812

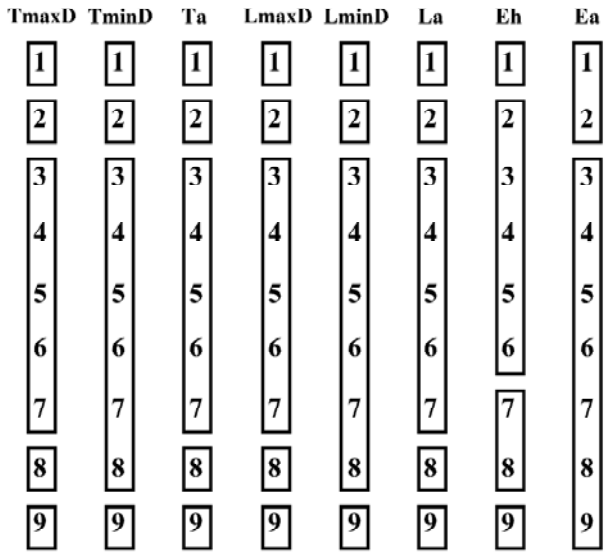

Figure 6. Zones grouping of the morphometric parameters for the rat epididymis shown in Figure 5 after repeated ANOVA and *a posteriori* Fisher test. Distinct regions are bounded by a common box ($P < 0.05$).

The epithelial area was maintained approximately constant along the zones (Figure 5A), while the epithelial height showed the tallest cells in region 1, the initial segment and shortest in region 9 (Figure 5D).

Polynomial regression analysis was applied to the parameters found in each region of the rat epididymis and revealed a triphasic trend of increase in tubule and luminal diameters and areas up to the corpus, a plateauing, and then an increase distally (Table 2). Statistical analysis of the data revealed that, depending on the parameter examined, 2 to 5 regions were identifiable by the Fisher test (Figure 6). For most parameters, regions 1 and 2 were distinct and separable from the more distal epididymal regions. The distal caput and corpus epididymidis were generally not significantly different, but the cauda (with or without the distal corpus) was also a distinct morphometric entity. No differences were observed between values corresponding to different animals for any of the parameters.

3.2 Epididymis of mouse

Epididymal weight as a percentage of body weight was 0.13 ± 0.02, lower than that in the rat, while the density maintained similar values (Table 1).

Analysis of the morphometric data obtained from these profiles revealed a tetraphasic pattern of decreasing areas, perimeters and diameters between regions 1 and 2, increasing in regions 3 and 4, declining to region 5 and increasing again towards regions 6 and 7 (Figure 7A–C). Epithelial height was highest in the initial segment and declined between regions 1 and 2, more-or-less constant in regions 2 to 5 and declined in regions 6 and 7 (Figure 7D).

Polynomial regression analysis, applied to the parameters found in each region of the mouse epididymis, revealed a tetraphasic change in tubule and lumen diameter and area with a noticeable decrease in values from region 1 followed by an increase, plateau and final distal increase (Table 3). Statistical analysis of the data revealed that essentially 3 to 6 regions were identifiable by the Fisher test (Figure 8). Regions 1 and 2 were largely distinguishable from the intervening regions, as were regions 6 and 7. As in the rat, no differences were observed between values corresponding to different animals for any of the parameters.

4 Discussion

In the present study, objective morphometric parameters of the epididymal tubule were measured in a total of nine regions in the rat epididymis (five in the caput, three in the corpus and one in the cauda) and seven in the mouse (five in the caput, one in the corpus and one in the cauda). Reid and Cleland [1] divided the rat epididymis into nine regions (six in the caput, one in the corpus and two in the cauda), including three sub-divisions of the initial segment. Fawcett and Hoffer [2] confirmed

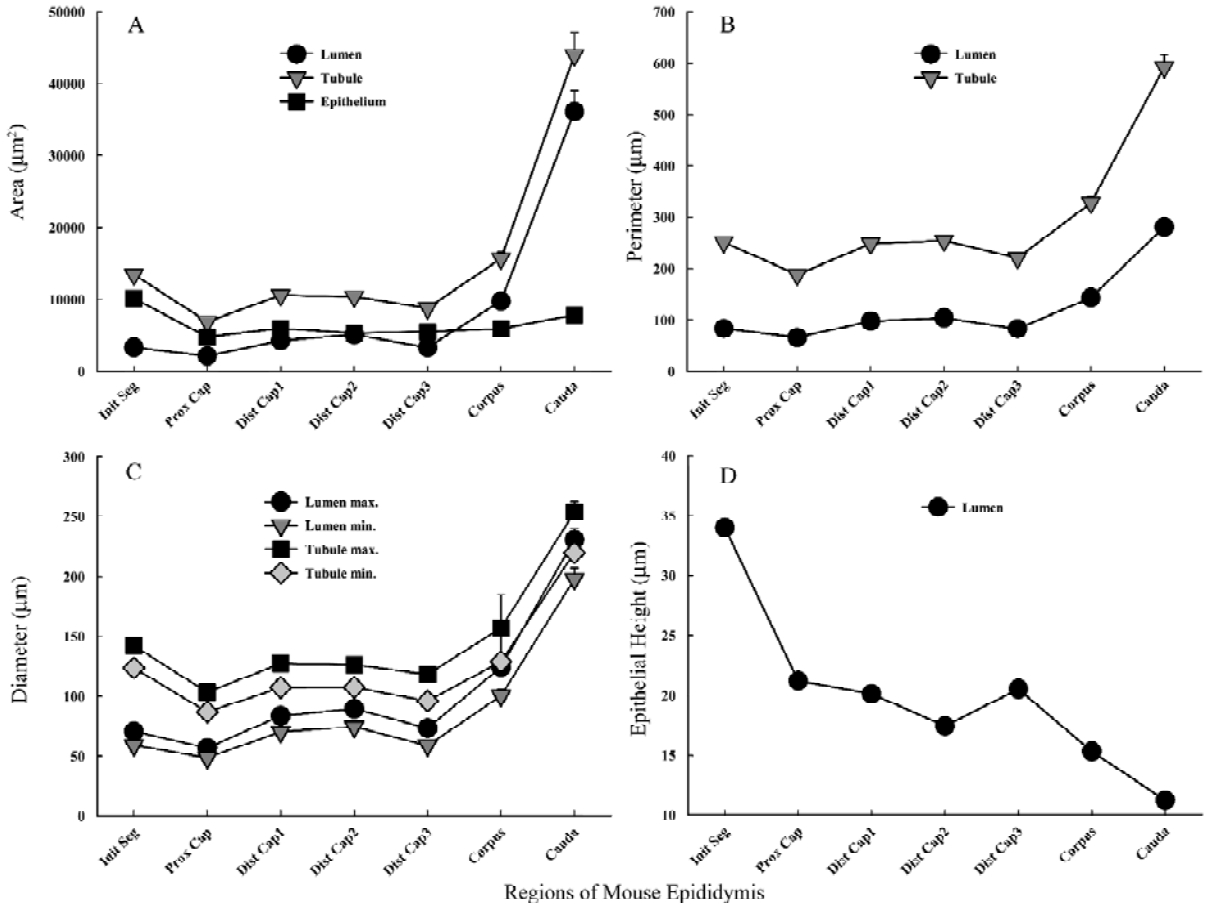

Figure 7. Mouse epididymal morphometric parameters. Values are mean ± SD.

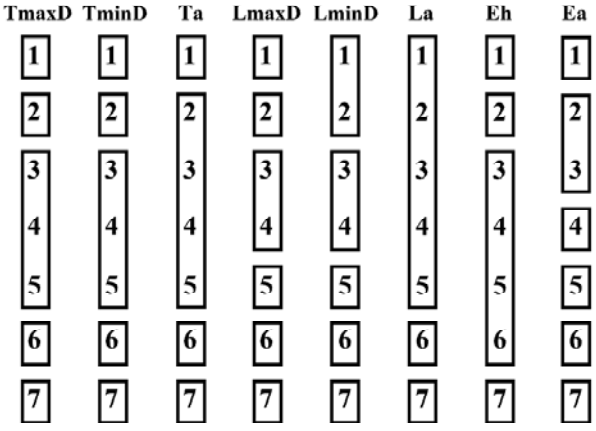

Figure 8. Zones grouping of the morphometric parameters for the rat epididymis shown in Figure 7 after repeated ANOVA and *a posteriori* Fisher test. Distinct regions are bounded by a common box ($P < 0.05$).

Table 3. Polynomial regression equations for morphometric parameters of the mouse epididymis. [a]x refers to region of epididymis. Tubule maximum diameter (TmaxD); Tubule minimum diameter (TminD); Tubule area (Ta); Luminal maximum diameter (LmaxD) and Luminal minimum diameter (LminD); La: luminal area; Eh: epithelial height; Ea: epithelial area.

Parameter	Polynomial regression equation[a]	R^2
TmaxD	$y= 280.68–228.16x+106.75x^2–20.41x^3+1.39x^4$	0.983
TminD	$y= 261.75-227.26x+106.77x^2–20.50x^3+1.40x^4$	0.984
Ta	$y= 44700–52500x+25800x^2–516.90x^3+363.75x^4$	0.998
LmaxD	$y= 152.76–149.60x+81.66x^2–17.12x^3+1.25x^4$	0.983
LminD	$y= 132.09–133.86x+74.07x^2–15.73x^3+1.15x^4$	0.986
La	$y= 20100–30500x+17200x^2–3740x^3+279.76x^4$	0.999
Eh	$y= 64.39–43.00x+14.46x^2–2.02x^3+9.60x^4$	0.973
Ea	$y= 24600–21900x+8660x^2–1430x^3+83.99x^4$	0.931

the presence of three initial segment regions in addition to the initial segment. Using major connective tissue septa as guides to define segments, as in the present study, Turner *et al*. [3] found thirteen natural cleavage planes in the rat (seven in the caput, two in the corpus and four in the cauda) and seven in the mouse (four in the caput, one in the corpus and two in the cauda). They also found that the initial segment of the rat extended to four segments but that of the mouse consisted only one. Other workers have defined other divisions of the mouse epididymis into 13 segments [12], the proximal murine epididymis into six to eight lobules [13], five lobules [14], four lobules [15] or just three lobules [16]. These differences represent the unavoidable difficulty in deciding from external morphology which tubule coils exist in which gross morphologically defined area, as well as the difficulty at the histological level of distinguishing complete connective tissue septa and semi-septa [3], particularly when serial sectioning is not performed.

From the morphometric data presented in this study, the values for epithelial height and duct diameter provided here are generally consistent with those provided from measurement of transverse sections in the rat [1] and mouse [16]. However, in the present study, epithelial height was always lower than that described in published reports. This may reflect the algorithm of obtaining an average height for the epithelium from many sections, but on the other hand it may reflect the fact that to obtain a complete longitudinal section, certain small regions (e.g., the initial segment) may not be represented in all sections. The tall epithelium of the typical initial segment (corresponding to region 1A of Reid and Cleland [1]) may well have been missed by the procedure in which sections of all the corpus and cauda are included, and this could explain why there was no decrease in parameters from region 1 to the adjacent zone (triphasic polynomial) as found in the mouse, where a more extensive initial segment is present (tetraphasic polynomial). This would explain the lower epithelial height found in region 1, here compared to published reports of both species [1, 16].

Statistical analysis of differences in tubule morphology indicated fewer subdivisions of the epididymal tubule in both species. Depending on the parameter examined, statistically significant differences between adjacent segments limited the number of distinct segments to two (epithelial area) to five (several parameters) in the rat, or six (epithelial area) to three (luminal area) in the mouse. Overall, the caput and cauda regions differed in luminal topology from the corpus epididymidis. Interestingly, this is the most usual division of the epididymis based on gross morphology, providing further evidence of an association between the epididymal capsule that divides the tubule into distinct lobules, the nature of the epithelium within each lobule [3] and the functions related to sperm maturation proximally and sperm storage distally.

A large epithelial area can be considered to represent a greater amount of active epithelium and thus to reflect greater epithelial activity (absorption or secretion) in regions displaying it. In the rat, the greatest epithelial area was in regions 3 and 4, whereas in the mouse this was in the initial segment, although in both species cell height was tallest in the initial segment. This is the region with the highest blood flow in the rat [17] and for which endocrinological activity has been proposed, based on differential testicular tubule alterations observed after efferent duct ligation and partial epididymectomy [18]. In the mouse, the initial segment displays the greatest vascularity [9,19] and specifically accumulates rhodamine [20].

On the other hand, a larger perimeter provides more areas for transport processes, be they secretion or resorption. Again, species differences were found with the luminal perimeter increasing in the first four sections of the rat epididymis in contrast to a generally constant perimeter in the mouse, in which species a drastic increase in the perimeter occurred in the cauda. In both species the luminal perimeter mirrored that of the tubule perimeter. Although it is suggested that the proximal epididymis may be more active in secretion/absorption than more distal regions, physiologically the actual area available for transport depends on the area investing microvilli and stereocilia on principal cells and surface infolds and flaps of clear and apical cells, which were not measurable with this light-microscopical technique. The luminal area naturally reflects the volume available for sperm within the tubule in the region and in both species increased in the cauda distal tubule where sperm storage occurs.

Finally, we can conclude that the use of Epiquatre software allowed us to establish regression curves for different morphometric parameters that can permit the detection of changes in their values under different pathologic or experimental conditions. This could be particularly useful in studies of hormonal regulation in different strains of normal or transgenic rodents.

Acknowledgment

We thank Miss Ana M Marqués for her technical assistance. This work was partially funded by Dirección General de Investigación Ciencia y Técnica (DGICYT) and the Conselleria de Cultura Educació i Ciència de la Generalitat Valenciana (PS88-0116 and 683.3-130, respectively). We also thank I.V. Costello for linguistic assistances.

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