

Functional Morphology of the Glomerular Filtration Barrier of *Gallus gallus*

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ABSTRACT The anionic charge barrier and the endothelial and epithelial pore sizes on the glomerular filtration barrier (GFB) were examined in white leghorn chickens (*Gallus gallus*). Ruthenium red was used to stain anionic charge sites on the GFB. The tissue was treated by normal dehydration and freeze substitution dehydration for transmission electron microscopy (TEM). In addition, the basal lamina was isolated for study. The results of our study indicate that *G. gallus* possess a thick, negatively charged glycocalyx surrounding the podocytes and slit diaphragm and on the endothelium. However, in all cases, little anionic charge is present in the basal lamina. The pores on the endothelium are elliptical and have mean dimensions of 148×110 nm. This is in contrast to mammals, which have smaller, round pores. The epithelial pores in *G. gallus* measure approximately 35 nm in length, approximately 4 times larger than those found in mammals. These results indicate that the avian glomerulus may allow the filtration of larger molecules from the plasma than occurs in mammals and that the charge on the molecule may not be as restrictive a filtration characteristic as in mammals. © 1996 Wiley-Liss, Inc.

The structure of the glomerular filtration barrier (GFB) in birds and mammals consists of the capillary endothelium, basal lamina, and visceral epithelium of Bowman's capsule containing the podocytes and slit diaphragms (Tisher and Madsen, '86). In mammals, macromolecules from the plasma are filtered through the GFB on the basis of both their size and charge (Brenner et al., '77; Rennke et al., '78). The avian GFB may operate in the same manner; however, this has not been demonstrated. A recent renal clearance study using dextrans in birds (Boykin et al., '94) has shown that the upper limit of filtration of macromolecules is at a diameter of 13 nm. A similar study in rats has shown that filtration ceases with macromolecules having a diameter of 8.8 nm and larger (Chang et al., '75). Hence, the avian kidney appears to filter macromolecules that are 30% larger than is the case for mammals. These data suggest two possibilities. First, the pore sizes on the GFB may be larger in birds, and second, the nature and arrangement of the anionic charge barrier may be different in birds.

The differences in functional morphology of the GFB between birds and mammals may be attributed to different strategies for excret-

ing metabolically produced nitrogen. Mammals excrete nitrogen largely in the form of urea and to a lesser extent ammonia. Birds are uricotelic, excreting most of their nitrogen in the form of uric acid, which occurs as small spheres within the nephron (Dantzer, '70). Chemical analysis of the spheres has shown that they contain protein (Braun et al., '87). The amount of protein in avian ureteral urine may be as much as 5 mg/ml (Boykin et al., '94), compared to 1 mg/ml in cases of severe proteinuria in humans (Behnke and Zelander, '70; Brenner et al., '77). It is not known how the protein enters the nephron in birds. One possibility is that the protein is filtered at the glomerulus.

Electron microscopic studies on rats indicate that a negative charge is present on the epithelial and endothelial layers of the GFB (Latta et al., '75; Caulfield and Farquhar, '76; Latta and Johnston, '76) and within the basal lamina (Caulfield and Farquhar, '78; Schurer et al., '78). However, no similar studies have examined the GFB in birds. Consid-

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ering that the negative charge is responsible for preventing anionic proteins from being filtered, it is important to know both the degree and arrangement of charge sites along the GFB. This is especially so for birds, considering the large quantity of protein that is present in ureteral urine (Boykin and Braun, '93).

The high protein content may be necessary to maintain uric acid in spherical form. The protein may either be filtered by the glomerulus or secreted by the cells of the renal tubules. To test whether there is a potential for proteins to be filtered by the avian glomerulus, we used transmission (TEM) and scanning electron microscopy (SEM) to examine both the charge barrier and pore sizes of the GFB in white leghorn domestic fowl (*Gallus gallus*).

MATERIALS AND METHODS

Anionic charge barrier

This study used a total of 17 female white leghorn domestic fowl (*Gallus gallus*) to examine both the anionic charge barrier and pore size of the GFB. The presence and distribution of an anionic charge on the avian filtration membrane were examined on membrane preparations from 11 chickens. Of these, six were used for whole membrane preparations (i.e., intact GFBs) and in the remaining five, the basal lamina was isolated from the GFB and examined for the presence of an anionic charge.

Whole membrane preparations

Whole membrane preparations from six birds were examined for the presence of an anionic charge. Birds were killed by decapitation. The dorsal aorta just cranial to the kidneys was cannulated and the kidneys were flushed, first with phosphate buffer to clear the blood, then with half-strength Karnovsky's fixative containing 0.2% ruthenium red (to stain the anionic sites). To ensure optimum fixation, we immersed the kidneys in the perfusion fixative for 24 h.

To demonstrate that the degree of staining of the anionic charge barrier was not a function of the time it took to perfuse the kidneys (i.e., 5–10 min), a sixth bird was infused first with ruthenium red for 1 h, then the kidneys were perfused with fixative containing ruthenium red for 5–10 min as above. In this way, we were able to determine whether infusion time influenced the staining of the GFB. The bird was restrained in a harness, and the

brachial vein was cannulated under local anesthesia (Anthocaine, 20 mg/ml). An infusion of 0.9% saline was given at a rate of 0.25 ml/kg/min to ensure a steady-state diuresis. The infusion contained 0.2% ruthenium red to stain anionic sites on the GFB. After 1 h the bird was killed by decapitation, and the kidneys were perfused with half-strength Karnovsky's fixative containing 0.2% ruthenium red and processed as above. Tissue samples from the entire kidney were cut in 1 mm³ blocks and processed for TEM.

Dehydration of tissue for electron microscopy

Previous studies on rats have found that an anionic charge occurs on the basal lamina (Kanwar and Farquhar, '79a,b). The basal lamina in both birds and mammals has been previously thought to be composed of three layers (Kristic, '84; Tisher and Madsen, '86). However, recent studies have shown that the three-layered structure is an artifact due to rapid dehydration during tissue processing for electron microscopy (Goldberg and Escaig-Haye, '86; Reale and Luciano, '90; Chan et al., '93; Chan and Inoue, '94). Therefore, to demonstrate that the presence of the anionic charge barrier in birds was not due to an artifact of processing, the tissue was processed in two ways: by standard dehydration and by freeze substitution dehydration. The latter process removes the artifact caused by the fixation process (Chan et al., '93).

The tissue prepared by standard dehydration was postfixed in 2% osmium tetroxide, dehydrated in a series of alcohols, and embedded in epon resin. Tissue blocks were selected at random, and thick sections were cut and stained with toluidine blue to locate areas containing the GFB. Thin sections (90 nm) were then cut, stained with uranyl acetate and lead citrate, and viewed on an electron microscope.

Tissue prepared by freeze substitution was immersed in 30% glycerol in phosphate buffer and placed on a stirring plate for 3 h. The tissue was then frozen in liquid Freon 22 cooled by liquid nitrogen and transferred into absolute acetone containing 1% osmium tetroxide and 0.5% uranyl acetate precooled to -70°C. The tissue was kept at -70°C for 24 h, at -40°C for 24 h, and then at -20°C for 24 h. The tissue was brought to room temperature, then washed in several changes of fresh absolute acetone and embedded in epon resin. Tissue blocks were selected at random

and thin sections were cut, stained, and viewed as described above.

Isolated basal lamina

To demonstrate that the anionic staining of the basal lamina was not due to staining of the cell coats of the adjoining endothelium or epithelium, basal laminae were isolated from the kidneys of five birds. The birds were killed by decapitation. The kidneys were removed, and areas of cortex were dissected from the kidneys and placed in a Potter-Elvehjem tissue grinder with cold, oxygen-

ated HEPES-sucrose buffer (pH 7.4). The tissue was homogenized to an even consistency to free glomeruli from proximal tubules. The glomeruli were harvested by aspirating with a small pipette and were then placed in the HEPES-sucrose buffer on ice. The glomeruli were centrifuged at 10,000 rpm for 10 min to obtain a pellet. The supernatant was decanted and the pellet was resuspended in 4% sodium deoxycholate containing 0.1% sodium azide. The suspension was stirred for 1 h, then centrifuged. The supernatant was again decanted and the pellet resuspended in

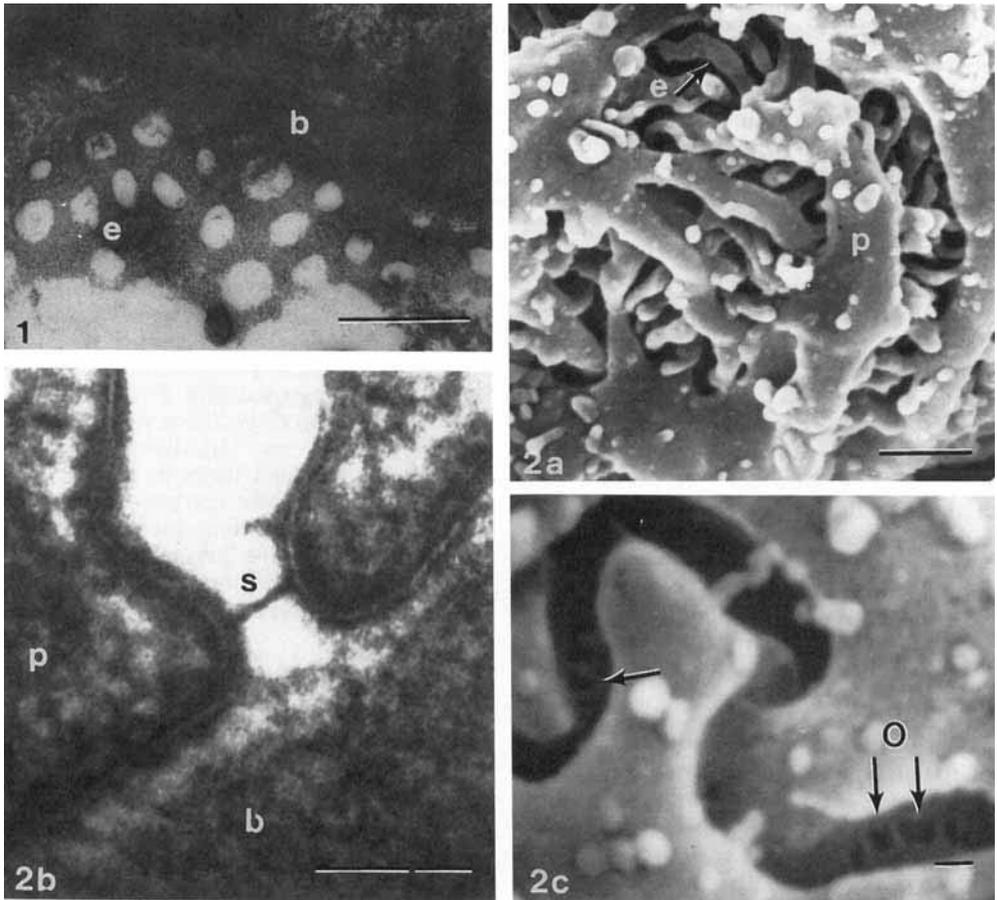


Fig. 1. *Gallus gallus*. Transmission electron micrograph of the endothelial pores of the GFB. e, endothelial pores; b, basal lamina. Bar = 500 nm. $\times 33,600$.

Fig. 2. *Gallus gallus*. a: Transmission electron micrograph showing the organization of the podocytes and pedicels in the glomerulus. b: Transmission electron micrograph of the glomerular slit diaphragm between adjacent podocytes, where the length of the slit diaphragm was measured. Note the absence of a central filament. c: Scanning electron micrograph of the slit pores. s, slit diaphragm; p, podocyte; e, pedicels; b, basal lamina; o, slit pores (arrows). Bars = 100 nm (a,b); 1 μm (c). $\times 240,000$ (a); $\times 70,000$ (b); $\times 12,000$ (c).

several changes of distilled water. Finally, the pellet was resuspended overnight in half-strength Karnovsky's fixative containing 0.2% ruthenium red. The pellet was processed routinely for TEM and embedded in Spur resin. Sections were cut, stained, and viewed as described earlier.

GFB pore sizes

The pore sizes of the endothelial and epithelial (slit diaphragm) layers of the GFB were measured in six birds. Birds were killed by decapitation and their kidneys were prepared as described earlier. The kidneys were perfused with half-strength Karnovsky's fixative containing 1% tannic acid to stain the glycoproteins, making the pores more visible (Rennke et al., '78). Cortical areas of the kidneys were dissected in 1 mm³ blocks and processed routinely for SEM and TEM, cut, stained, and viewed as described earlier.

The pores on the endothelial side of the GFB are elliptical (Fig. 1). To determine the extent to which the pores were elliptical, we measured both the long and short axes. The lengths of the slit pores were estimated by measuring the distance between the base of adjacent podocytes (not including the glycocalyx of the podocytes), along the edge of the slit diaphragm (Fig. 2a-c). Pores were selected at random and measurements were made with the electron microscope, calibrated with a diffraction replica (Ernest Fullam, Inc., Latham, NY). This measuring technique has been used previously for mammalian tissue (Farquhar et al., '61; Latta et al., '75; Larsson and Maunsbach, '80; Tisher and Madsen, '86).

Statistics

The endothelial pore size data were log transformed and analyzed using an analysis of covariance (ANCOVA) (Sokal and Rohlf, '81). In this manner, any differences in both the shape and size of the pores between birds could be determined. To determine if the shape of the endothelial pores was significantly different from circular, the 95% confidence interval for the regression coefficient was determined for pore length vs. width in each bird and compared to a slope of 1.0 (i.e., round). A one-way analysis of variance (ANOVA) was used to test whether the sizes of the epithelial pores varied between birds.

RESULTS

Anionic charge barrier

The basal lamina of the GFB in the avian kidney contains little anionic charge compared to that seen in previous studies on rats (Figs. 3, 4). However, a negative charge is present on both the epithelial and endothelial sides of the GFB (Fig. 3a,b). Neither the perfusion time nor the rapid dehydration had any effect on the amount of anionic staining (Fig. 3a,b). The isolated basal lamina preparations also show little evidence of an anionic charge compared to that of previous studies on rats (Fig. 4a,b).

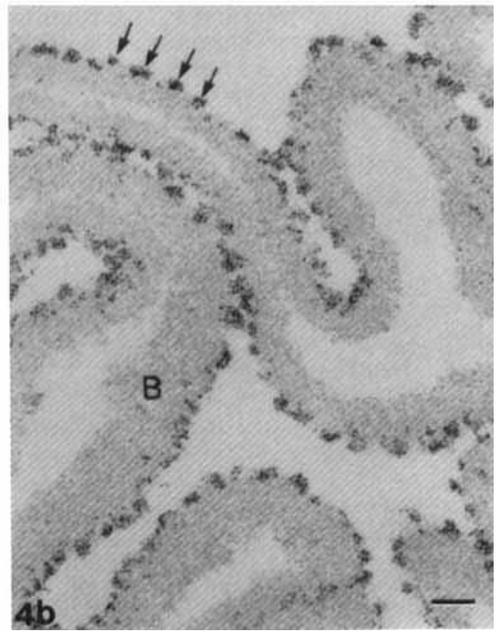
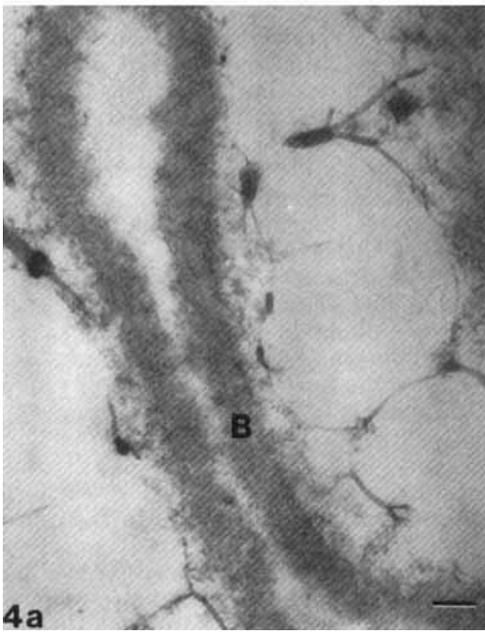
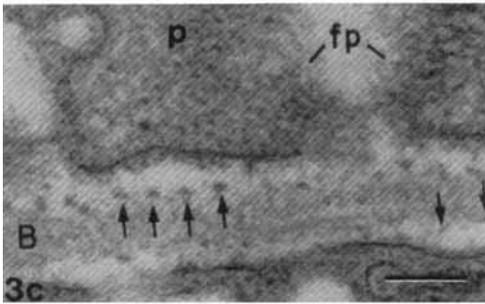
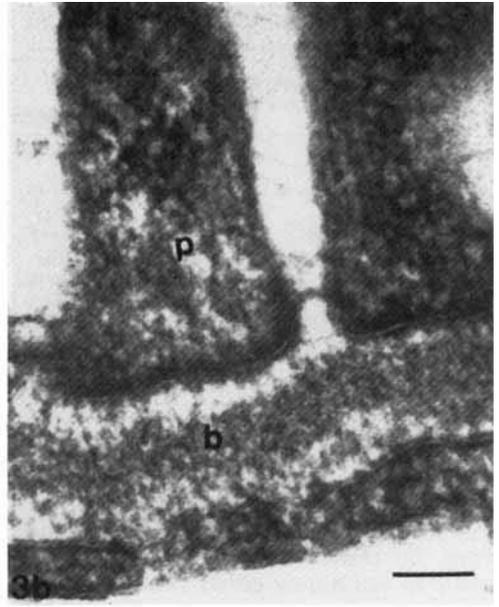
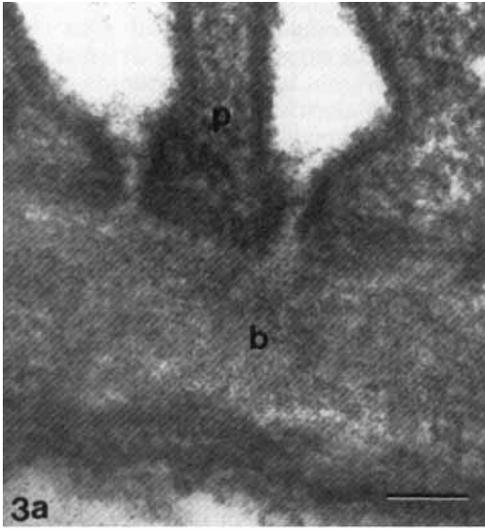
Pore sizes

The pore size of the endothelium in the GFB of *G. gallus* ranges from 102 ± 31 to 171 ± 59 nm (Table 1). The mean values for all birds are larger than found in previous studies on mammals (Table 2). In *G. gallus* the endothelial pores are elliptical. ANCOVA showed no significant differences ($P > 0.05$) in the relationship between the long and short axes of pores within individual birds. However, the overall sizes of the pores varied significantly ($P < 0.05$) between birds. When the slope coefficients for each of the individual birds were calculated, they were all significantly lower ($P < 0.05$) than a slope of 1.0 (i.e., round pores) (Fig. 5). This indicated that the pores in *G. gallus* were elliptical.

There was no significant difference ($P > 0.05$) between birds in the lengths of the long axes of the epithelial pores in *G. gallus* (Table 1). The long axes of the pores in the epithelium were larger in *G. gallus* (35 nm) than found in previous studies in rats (14 nm) and in humans (12 nm) (Tables 1, 2).

Fig. 3. *Gallus gallus*. Transmission electron micrograph of the GFB showing no anionic staining of the basal lamina after infusion of ruthenium red. **a**: Freeze substitution. **b**: Rapid dehydration. **c**: Anionic staining of the glomerular basal lamina in the rat. Arrows show the presence of the anionic charge. p, podocyte; fp, foot process; b,B, basal lamina. Bars = 100 nm. ×120,000. (Figure 3c reproduced from *The Journal of Cell Biology*, 1979, vol. 81, pp. 137-153, by copyright permission of The Rockefeller University Press.)

Fig. 4. Transmission electron micrograph of the isolated glomerular basal lamina from (a) *Gallus gallus* showing no anionic staining and (b) the rat showing anionic staining (arrows) of the glomerular basal lamina. B, basal lamina. Bars = 500 nm. ×65,000. (Figure 4b reproduced from *The Journal of Cell Biology*, 1979, vol. 81, pp. 137-153, by copyright permission of The Rockefeller University Press.)



Figures 3 and 4

TABLE 1. Mean \pm standard deviation pore size (nm) in the endothelial and epithelial layers of the GFB in the domestic fowl (*Gallus gallus*)¹

Bird No.	Endothelium		Epithelium
	Major axis	Minor axis	
1	171 \pm 59 (36)	135 \pm 44 (36)	35.8 \pm 9.6 (52)
2	130 \pm 25 (83)	102 \pm 26 (83)	35.2 \pm 9.1 (129)
3	142 \pm 39 (60)	105 \pm 25 (60)	35.3 \pm 7.5 (115)
4	—	—	36.1 \pm 6.2 (55)
5	146 \pm 39 (80)	102 \pm 31 (80)	36.0 \pm 6.4 (74)
6	152 \pm 38 (107)	107 \pm 32 (107)	34.2 \pm 6.2 (105)

¹Sample sizes are in parentheses.

DISCUSSION

Few studies have examined the GFB of the kidney. Of these studies all have been on mammals, examining either rats or humans (see Table 2). This is the first study to examine in detail the functional anatomy of the avian GFB. Our data show that the GFB in *G. gallus* possesses a negative charge on the surfaces of both the endothelial and epithelial sides of the membrane. On the epithelium, the charge occurs on the surface of the podocytes and on the slit diaphragm. However, there is little charge within the basal lamina, as opposed to the situation in mammals (Caulfield and Farquhar, '78; Schurer et al., '78; Kanwar and Farquhar, '79a,b). As the methods used in our study for *G. gallus* were the same as those used in previous studies on mammals, the absence of a charge

barrier in *G. gallus* presumably was not due to the methodology employed. Our results for *G. gallus* suggest that the avian glomerulus may present less of a barrier to negatively charged molecules, such as some proteins, than in mammals.

In birds, the only functional anionic barrier to negatively charged macromolecules may lie at the surface of the endothelium and at the slit diaphragm. However, current theory suggests that it is the basal lamina that functions as the charge barrier for the filtration of negatively charged macromolecules (Farquhar et al., '61; Caulfield and Farquhar, '74; Rennke et al., '78; Farquhar, '81). In birds, the charge barrier on the endothelium and slit diaphragm may carry out this function. However, if the GFB in birds operates in the same manner as it does in mammals (there is no evidence to the contrary), then the results of our study on *G. gallus* suggest that there is a greater probability that negatively charged molecules may be filtered through the GFB.

In the current study, *G. gallus* had larger sized pores in both the endothelium and epithelium of the GFB than those reported previously for mammals. The larger sized pores in *G. gallus* suggest that larger macromolecules (regardless of charge) may be filtered through the GFB. Our study also found that the pores in the endothelium are elliptical in *G. gallus*. Previous studies in mammals (Latta, '70; Schneeberger et al., '75; Farquhar, '81; Tisher and Madsen, '86; Kanwar and Venkatachalam, '92) have given only one measurement for pores, leading to the conclusion that they may be round. We measured pore diameters from sections cut at a tangent to the capillary. Hence, the elliptical shape did not result from cutting oblique sections through the endothelium.

The functional implication of an elliptical endothelial pore in *G. gallus* is not clear. The molecules filtered from the plasma are comprised of many shapes, ranging from globular to elongate; however, the shape of the molecules can change depending on pH and pressure (Bull, '64). One possibility is that elliptical pores may expand and contract depending on the filtration pressure, hence facilitating the movement of molecules into the nephron. The slit diaphragm pores in *G. gallus* were 40–80% larger than values reported for mammals (see Table 2). As the slit diaphragm acts as a fine filter, a larger pore size may enable larger molecules to be filtered in birds.

TABLE 2. Pore size data on the endothelial and epithelial layers of the GFB in mammals and birds

Species	Pore sizes (nm)		Reference
	Endothelium	Epithelium	
Chicken	107–147	35	Current study
Rat	30–90	8	Latta et al. (1975)
	50–100	7–10	Farquhar et al. (1961)
	50–100	4 \times 14	Rodewald and Karnovsky (1974)
	100	7	Latta (1970)
	100	4 \times 14	Kanwar and Venkatachalam (1992)
	105–108	8	Larsson and Maunsbach (1980)
Human	—	5 \times 12	Schneeberger et al. (1975)
	70	4 \times 14	Tisher and Madsen (1986)

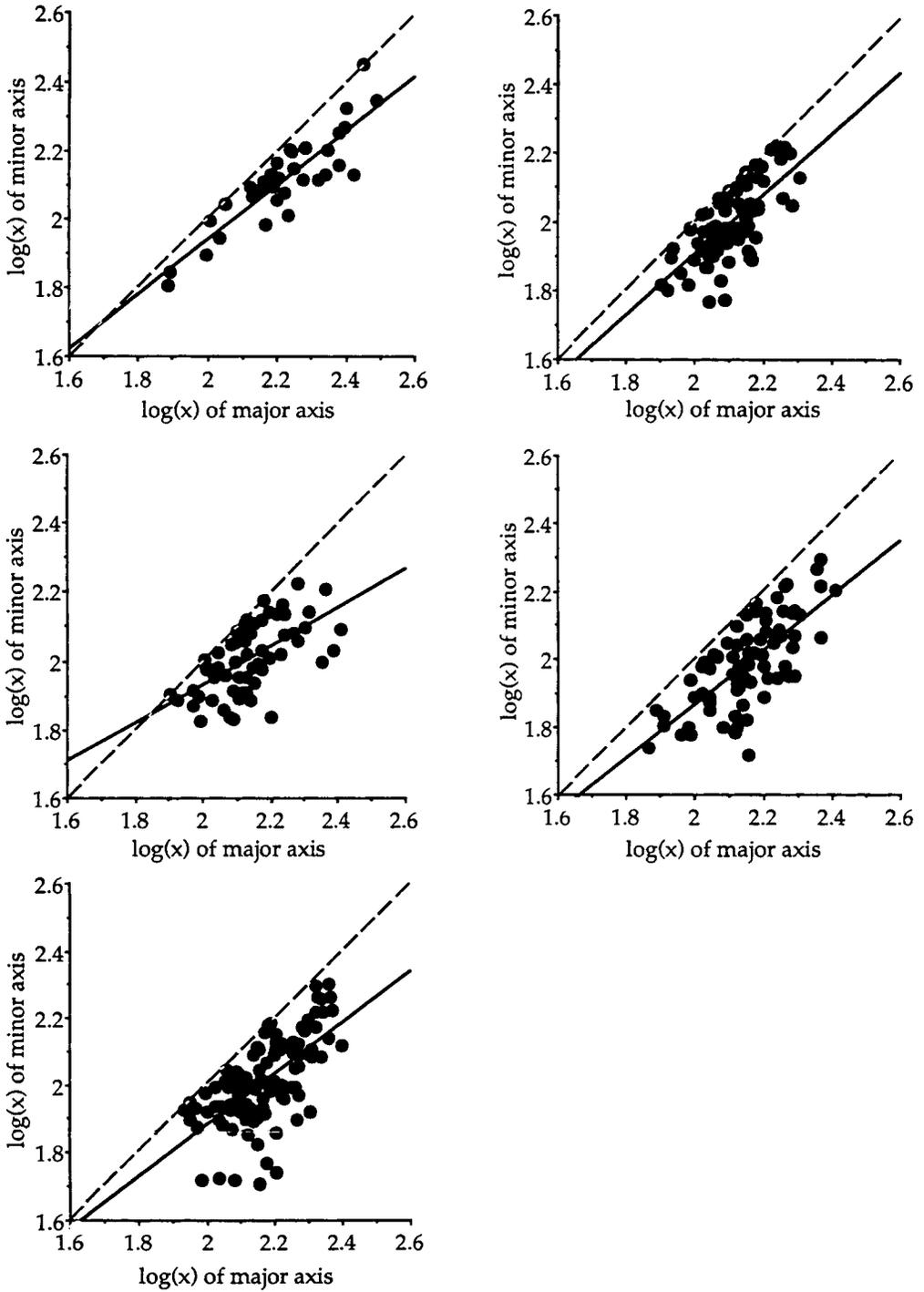


Fig. 5. *Gallus gallus*. Endothelial pore size (major and minor axes) of the GFB. Solid lines represent the regression through the data. Dotted lines represent where the regression line would be if the pores were round.

A large amount of protein is found in the ureteral urine of birds (Boykin et al., '94). However, as yet neither its source of entry into the nephron nor its function is known. The protein may be required to maintain the uric acid in sphere formation, hence preventing blockage of the nephron. Thus, the lack of a significant charge barrier on the basal lamina and the large endothelial and epithelial pores may be an adaptation to uricotelism. Current physiological studies in our laboratory are aimed at identifying and characterizing the protein component of avian urine. The information from the current study together with data from these physiological studies may enable a better understanding of the relationship between the structure of the GFB and uricotelism in birds.

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