Comparative Study of Fibrillar Collagen Arrangement in the Corneas of Primates and Other Mammals

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ABSTRACT

This study is a comparative study of the relationship between corneal structure, morphology, and function in a range of mammalian species. X-ray scattering patterns were gathered at regular spatial intervals over the excised cornea (and in most cases also the scleral rim) of humans, marmosets, horses, cows, pigs, rabbits, and mice. All patterns were analyzed to produce quantitative information regarding the predominant orientation of fibrillar collagen throughout the tissue. The predominant direction of corneal collagen varies between mammals. This variation is not related to the size, shape, or thickness of the cornea or the frequency with which the animal blinks. A relationship does, however, appear to exist between corneal collagen arrangement and visual acuity. An excess of collagen directed toward one or both sets of opposing rectus muscles is a feature of animals that have an intermediate to high level of visual acuity. There is a significant variation in the arrangement of corneal collagen between different mammalian species. This finding may be related to differences in the frequency of action and the forces generated by the various extraocular muscles during eye movement and image fixation. Anat Rec, 290:1542–1550, 2007.

Key words: cornea; species; collagen; x-ray scattering

The cornea, often referred to as "the transparent window at the front of the eye," provides a tough external barrier to protect the contents of the eye from injury while also maintaining a precise curvature to enable light to be focussed onto the retina. The mammalian cornea is composed mainly of water and type I collagen fibrils, and it is the unique arrangement of this collagen that governs the strength, shape, and transparency of the tissue. The long, cylindrical collagen fibrils have a uniform diameter, a regular separation distance, and lie parallel to each other within layers (lamellae), which are themselves stacked parallel to the surface of the cornea. The diameter and separation distance of the fibrils differs between species but the proportion of the corneal stroma occupied by hydrated collagen fibrils remains constant (Meek and Leonard, 1993).

As initially proposed by Kokott (1938) and more recently confirmed using x-ray scattering (Meek et al., 1987; Aghamohammadzadeh et al., 2004; Boote et al., 2006), most lamellae in the central region of the human

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TABLE 1. Sample details

Species	Sample details	Source	Storage
Human *Homo sapiens sapiens*	Right cornea with 3-mm scleral rim. Tagged at superior. (n = 2)	Great Wall Hospital, Bejing, China	Fixed 10% formalin
Human *Homo sapiens sapiens*	Paired left and right cornea with 3mm scleral rim. (n = 2)	Manchester Eye Hospital, UK	Frozen −80°C
Human *Homo sapiens sapiens*	Left corneas with 3-mm scleral rim. Tagged at superior. (n = 2)	Bristol Eyebank, UK	Frozen −80°C
Common marmoset *Callithrix jaccus*	Paired left and right cornea with 0.5-mm scleral rim. Tagged at superior. (n = 20)	Oxford University, UK	Frozen −80°C
Horse *Equus caballus*	Cornea with a 2-mm scleral rim. Vertical meridian marked. (n = 3)	Abattoir, Bristol, UK	Frozen −80°C
Cow *Bos indicus*	Cornea with a 2-mm scleral rim. Vertical meridian marked. (n = 5)	Abattoir, Bristol, UK	Frozen −80°C
Pig *Sus scrofa domesticus*	Cornea with a 2-mm scleral rim. Tagged at superior. (n = 3)	Abattoir, Bristol, UK	Frozen −80°C
New Zealand White Rabbit *Oractolagus cuniculus*	Cornea with a 2-mm scleral rim. Tagged at superior. (n = 3)	Heath Hospital, Cardiff, UK	Frozen −80°C
Laboratory mouse *Mus musculus*	Cornea with a 0.25-mm scleral rim. Tagged at superior. (n = 13)	Cardiff University, UK	Frozen −80°C

cornea, particularly in the deeper layers of the stroma, lie in the superior–inferior and nasal–temporal directions. As the tensile strength of connective tissue is determined in part by the orientation of collagen fibrils in relation to the direction of stress (Jeronimidis and Vincent, 1984; Hukins and Aspden, 1985), this has prompted suggestions that the preferred orthogonal orientation of fibrils in the central human cornea may be necessary to resist the mechanical forces of the four rectus muscles that insert behind the limbus at opposing positions along each meridian (Kokott, 1938; Daxer and Fratzl, 1997; Newton and Meek, 1998). A limbal annulus of collagen surrounds the human cornea and is believed to provide additional reinforcement at the point where it meets the lesser curved sclera (Maurice, 1984; Newton and Meek, 1998; Aghamohammadzadeh et al., 2004).

Based on the birefringence properties of the bovine cornea, it was suggested that a nonrandom distribution of lamellae was also present in the bovine cornea (Kaplan and Bettleheim, 1972). Small-angle light scattering of bovine and rabbit cornea confirmed this to be the case in both species (McCally and Farrell, 1982), but the authors were unable to determine the precise orientation of the stromal lamellae. Of interest, analysis of single x-ray scatter patterns obtained from the central cornea of over 50 vertebrate species (which included primates), revealed the human cornea to be unique in possessing an arrangement of stromal collagen that is preferentially orientated along both the superior–inferior and nasal–temporal meridians (Meek et al., 1987).

X-ray scattering is a powerful technique for studying the gross orientation of collagen fibrils in the cornea. As corneal collagen molecules lie near axially within the fibrils, the high-angle equatorial x-ray scattering pattern arising from the lateral packing of the molecules in the stroma can be used to determine the preferred direction of the fibrils at that position in the cornea. In recent years, this method has been used successfully to map the predominant orientation of collagen at fine spatial intervals throughout the entire human and marmoset cornea and limbus (Aghamohammadzadeh et al., 2004; Boote et al., 2004, 2006).

Given the frequent use of nonhuman species in studies of corneal biomechanics and that the size and orientation of collagen fibrils largely contributes to biomechanical calculations (Jeronimidis and Vincent, 1984; Hukins and Aspden, 1985), it is clear that a greater knowledge of the precise arrangement of corneal collagen in species other than human is needed. Here, we have addressed this issue by using x-ray scattering to map the predominant orientation of corneal and limbal collagen in the human, marmoset, horse, cow, pig, rabbit, and mouse.

MATERIALS AND METHODS

Tissue Samples

All tissue samples used in this study (Table 1) were obtained in accordance with the tenets of the Declaration of Helsinki for the use of human tissue and the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic research.

In the majority of cases, the in vivo orientation was marked on the scleral rim at the 12 o'clock position by means of a nylon suture or a surgical skin marker pen. In the case of the horse, cow, and pig cornea, where the in vivo orientation was not known, the vertical meridian was identified by the elliptical shape of the cornea. To minimize tissue dehydration, frozen samples were wrapped in Clingfilm™ (Superdrug Stores Plc., Croydon, UK) before freezing in liquid nitrogen-cooled isopentane. Each sample was defrosted at room temperature immediately before data collection.

X-ray Scattering Data Collection

High-angle x-ray scatter patterns were collected from most samples on Station 14.1 at Daresbury Synchrotron Radiation Source (Warrington, UK), using a 200 × 200

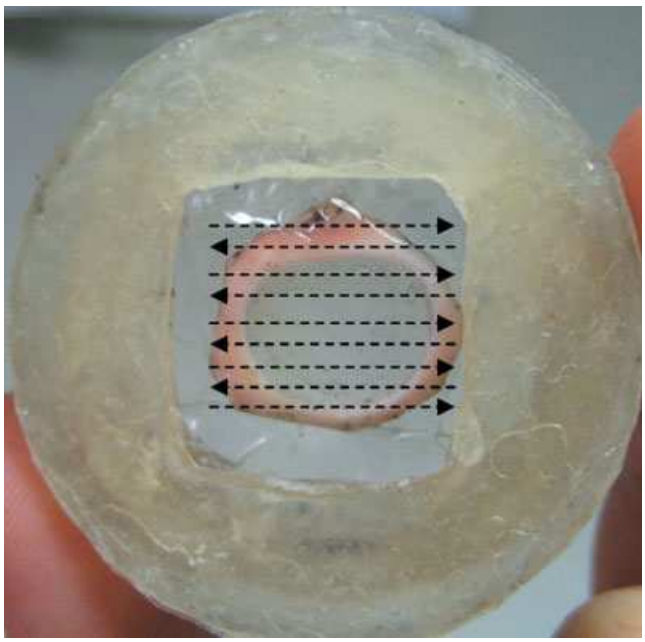

Fig. 1. A pig cornea (with scleral rim) enclosed within a sealed sample holder. X-ray scattering patterns were recorded at regular intervals across the cornea in the directions indicated by the arrows.

micron beam with a wavelength of 0.1488 nm. The images were recorded on a Quantum 4R CCD detector (ADSC, Poway, CA) placed 150 mm behind the sample. The rare opportunity to use the micro-focus x-ray beam (measuring 5 microns in diameter and with a wavelength of 0.984 nm) on Station ID-13 at the European Synchrotron Radiation Facility (Grenoble, France) was utilized to obtain comparable x-ray scattering data for an additional pig cornea.

Each cornea was placed in its correct orientation into a sealed sample holder enclosed between two sheets of Mylar (Dupont-Teijin, UK; Fig. 1). The sample holder was then carefully positioned onto a computer-operated translation stage (Newport Spectra-Physics Ltd., Newbury, UK) so that the most anterior side of the cornea faced the x-ray beam. X-ray scatter patterns resulting from an exposure time of 75 sec (human), 150 sec (marmoset), 30 sec (pig), and 45 sec (rabbit and mouse) were collected over the entire cornea and scleral rim of each specimen at regular intervals of 0.8 mm (human), 0.5 mm (marmoset), 1 mm (pig and rabbit), and 0.2 mm (mouse). Due to the large size of the horse and cow cornea and the limited capacity of the specimen holder, x-ray scatter patterns resulting from a 20-sec x-ray exposure were collected at 1-mm intervals over a 14-mm trephined corneal disc taken from the center of each cornea.

X-ray Diffraction Data Analysis

A circular x-ray scatter reflection, formed as a result of the interference of x-rays scattered by the collagen molecules within the fibrils, was present on each x-ray scatter pattern. In cases where the collagen was orientated equally in all directions within the plane of the tissue, a uniform ring of x-ray scatter was formed; however, when there was an excess of fibrils lying in a particular direction (Fig. 2A) or directions (Fig. 2B), lobes of increased scatter intensity were seen at right angles to the dominant orientation of the fibrils.

Using image analysis software (Optimas 6.5, Media Cybernetics, UK) and Microsoft Excel (UK), a 0–360 degree intensity distribution profile was produced for each x-ray pattern by measuring the intensity of x-ray scatter around the intermolecular reflection (Fig. 2C,D). Each intensity distribution profile was normalized against the exposure time and the average x-ray intensity during exposure (recorded by an ion chamber placed between the x-ray beam and the sample). At this point, the signal to noise ratio was improved by folding the intensity profile; this was possible without the loss of any data, due to the centrosymmetric nature of x-ray scatter patterns. The area under the intensity profile, which is proportional to the total amount of collagen through which the x-ray beam has passed, can be separated into two components—the scatter from collagen lying equally in all directions within the plane of the cornea (isotropic scatter) and the scatter from collagen fibrils that adopt a preferred orientation (aligned scatter) (Fig. 2C,D).

Removal of the isotropic scatter from the intensity profile leaves only the scatter from aligned fibrillar collagen. To ascertain the preferred orientation of these aligned fibrils, it was necessary to first shift the profile by 90 degrees to account for the fact that the high-angle equatorial reflection occurs at right angles to the fibril axis. Using statistical analysis software (Statistica 7, StatSoft Ltd., UK), the intensity profile was then converted into a vector plot (Fig. 2E,F), in which the distance from the center to the edge of the plot at any given angle is proportional to the intensity of x-ray scatter from aligned collagen molecules oriented in that direction. Individual vector plots were then compiled onto a grid relating to corneal position to show the preferred orientation of aligned collagen in each cornea.

The specific arrangement of corneal collagen in each species was examined alongside published values (where available) of corneal size, radius of curvature, and thickness as well as the frequency with which the animal blinks (shown as the interval between subsequent blinks) and its visual acuity (Table 2). Visual acuity, which is measured in cycles per degree of visual field, may be defined as the spatial resolving capacity of the visual system. It provides a measure of the eye's ability to distinguish one object from another in terms of degree angles of visual field.

As no values could be found in the literature for the blink interval of the pig, this value was measured by assessing videographic footage acquired from nine healthy Yorkshire/Landrace mix breed sows housed in single stalls for birthing. The time between subsequent blinks was recorded in several minute episodes using the video time counter, and the average blink interval was calculated.

RESULTS

Figure 3A shows the predominant direction of collagen in the human cornea. Within the central 5.6 mm, most fibrils lie in the superior–inferior and nasal–tem-

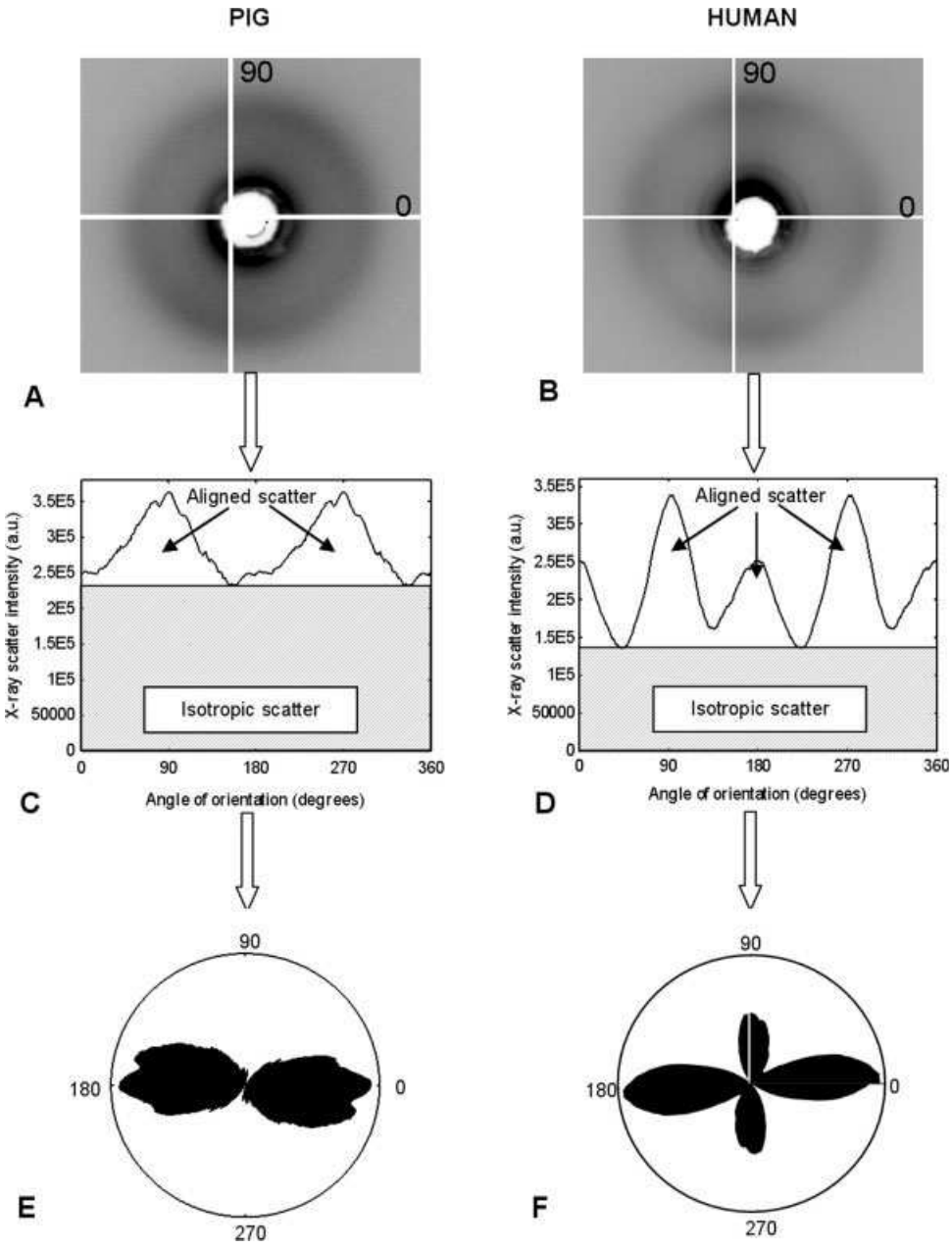

Fig. 2. **A,B:** High angle x-ray scattering patterns from the center of a pig (A) and a human cornea (B). **C,D:** The intensity of x-ray scatter around the intermolecular reflection was measured to produce a profile of total collagen scatter intensity as a function of angular position. **E,F:** The profile of x-ray scatter from aligned collagen only (isotropic scatter removed) is displayed as a vector plot, taking into account that x-rays are scattered at right angles to the fibril axis. The distance from the center of a vector plot in any given direction is representative of the amount of collagen fibrils preferentially orientated in that particular direction.

poral directions, giving rise to a characteristic cross-shaped vector plot (Newton and Meek, 2001; Aghamohammadzadeh et al., 2004; Boote et al., 2006). With increasing distance from the center of the cornea, the size of the vector plots increases, indicating that more collagen is highly aligned and the principal direction of collagen becomes increasingly tangential with respect to the edge of the cornea. A distinct annulus of aligned collagen measuring 1.6–2.0 mm spans the corneolimbal region.

A predominantly orthogonal orientation of collagen is rarely seen in the marmoset cornea (4 of 20 examined). In most cases (16 of 20 corneas examined) an excess of fibrils are oriented along the superior–inferior meridian (Fig. 3B). As with the human cornea, a well-defined limbal annulus measuring 0.5–1.2 mm is a common feature of the marmoset.

X-ray scatter patterns recorded from three equine corneas and three bovine corneas revealed that, in the central 14 mm, an excess of collagen is orientated along the

TABLE 2. Corneal collagen arrangement and published values of corneal dimensions, visual acuity and blink interval[a]

Species	Collagen direction[1]	Cornea size (horizontal × vertical) (mm)	Radius of curvature (mm)	Corneal thickness (mm)	Visual acuity (cycles/deg)	Blink interval (sec)
Human	+	11.7 × 10.6[2]	7.8[2]	0.55[9]	47.0–86.0[13]	2.8[18]
Marmoset	| (or +)	6.1 × 5.4[3]	3.5[3]	0.30[3]	30.0[3]	1.4[19,20]
Horse	|	35.0 × 28.5[4]	17.2[7]	0.81[10]	16.4–20.4[14]	2.3[19]
Cow	| (or O)	29.0 × 24.0[4]	15.8[7]	0.80[11]	12.4[15]	2.7[19]
Pig	O	15.5 × 12.5[4]	9.0[7]	0.85[9]	8.0[15]	3.8[21]
Rabbit	O	13.4 × 13.0[5]	7.3[5]	0.38[12]	4.3[16]	>30[19]
Mouse	O	3.5 horiz.[6]	1.4[8]	0.17[6]	0.5[17]	>30[19]

[a]The superscript numbers indicate the following:
[1]Corneal collagen arrangement is classified as orthogonal (+), vertical (|) or circumferential (O); [2]Tripathi and Tripathi, 1984; [3]Troilo et al., 1993; [4]Smythe, 1956; [5]Bozkir et al., 1997; [6]Zhang et al., 1996; [7]Coile and O'Keefe, 1988; [8]Schmucker and Schaeffel, 2004; [9]Wollensak et al., 2003; [10]Svaldeniene et al., 2004; [11]Scott and Bosworth, 1990; [12]Li et al., 1997; [13]Hirsch and Curcio, 1989; [14]Timney and Keil, 1992; [15]Heffner and Heffner, 1992; [16]Vaney, 1980; [17]Prusky et al., 2000; [18]Ponder and Kennedy, 1927; [19]Blount, 1927; [20]The blink interval of the marmoset is not known but is assumed to be similar to that of published values for the Sudanese monkey; [21]Personal observation.

vertical meridian (Fig. 4A,B). Collagen orientation appears to be tangential at the edge of the 14 mm trephined disks, but this effect may be an artefact caused by trephination. Of interest, examination of a further two bovine corneas that were prepared in the same manner revealed the presence of collagen lying parallel to the edge of the cornea throughout the central 14-mm region and this arrangement of collagen will be referred to as "circumferential." A predominantly circumferential arrangement of fibrillar collagen was observed in all pig (Fig. 4C), rabbit (Fig. 4D), and mouse (Fig. 4E) corneas, and as seen in the human and marmoset, the amount of aligned collagen increases at the limbus to form an annulus of collagen surrounding the cornea.

As shown in Table 2 and Figure 5, the variation in corneal collagen arrangement between species does not appear to be correlated with the size (Fig. 5A), radius of curvature (Fig. 5B), or thickness (Fig. 5C) of the cornea. The average blink interval for the pig was calculated to be 3.76 (± 2.0) sec, and when these new data were included with those taken from the literature, no relationship seemed to exist between collagen arrangement and blink interval (Fig. 5E). However, a relationship does appear to exist between the arrangement of corneal collagen and the visual acuity of the animal (Fig. 5D).

DISCUSSION

In this study, we have shown that major differences in corneal collagen arrangement exist between mammals and, as highlighted in the common marmoset and the cow, further structural differences may also exist between individuals of the same species. The observed variation in collagen arrangement does not, however, appear to be related to the size, radius of curvature or thickness of the cornea.

It has been suggested elsewhere that the presence of an annulus of aligned collagen at the limbus may play a role in maintaining the curvature of the cornea at the point where it meets the lesser curved sclera (Maurice, 1984; Aghamohammadzadeh et al., 2004; Boote et al., 2004). Of interest, a limbal annulus of circumferentially aligned collagen appears to be a common feature of all animals examined within this study group, despite the existence of large species differences in the amount of curvature change between the cornea and sclera. For example in the human, a considerable change in radius of curvature occurs between the cornea (7.8 mm) and the sclera (11.5 mm; Tripathi and Tripathi, 1984), whereas in the rabbit a far less notable change in curvature occurs between the two regions (7.5 and 9.8 mm; Hughes, 1972).

Because it is a typical property of most connective tissues that an increased level of stress produces an increased level of stress resistance (Reichel et al., 1989), it has been suggested in the human cornea that the predominantly orthogonal alignment of collagen, directed toward the insertion points of opposing rectus muscles, may provide the tissue with additional strength to resist distortion during eye movement (Daxer and Fratzl, 1997). Electrical activity studies of the human extraocular muscles have shown that, even when the eye is in the primary position, there is considerable activity in all of the rectus muscles (Bjork and Kugelberg, 1953). Furthermore, the retraction of the eye during blinking has been attributed to the co-contraction of the extraocular rectus muscles in the case of the human and the combined action of the rectus and retractor bulbi muscles in the rabbit (Evinger et al., 1984). The retractor bulbi muscle, which forms an inner muscle cone around the optic nerve and assists in retracting the globe, is present in the horse, cow, pig, rabbit, and mouse but exists only as a rudimentary structure in the monkey and is entirely absent in the human (Prangen, 1928). The presence of four extraocular rectus muscles are, however, common to all mammalian species, although the length, width, and position of insertion varies between animals (Prangen, 1928; Prince et al., 1960). For example, the rectus muscles of the monkey and human insert at locations much closer to the limbus than those of the rabbit and pig (Prangen, 1928). Despite all mammalian species possessing the same four extraocular rectus muscles, a predominantly orthogonal arrangement of corneal collagen is not a common feature of vertebrates and has only been observed in the human, a minority of marmosets (Boote et al., 2004), and the chicken (personal observation). It must be remembered at this point that x-ray scattering patterns provide an average measurement of

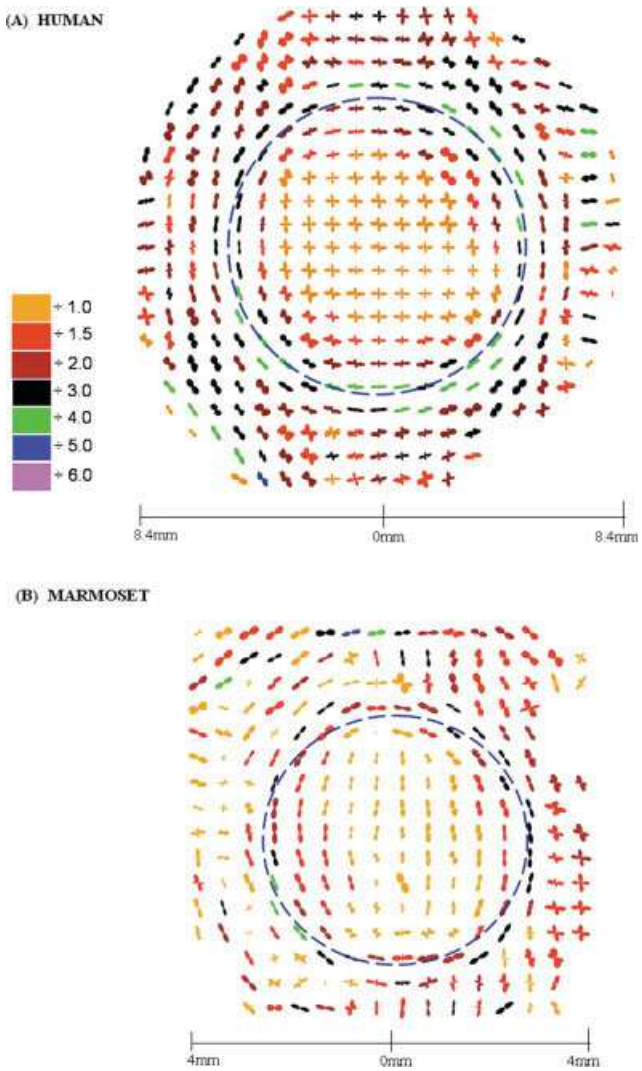

Fig. 3. **A,B:** Vector plot maps showing the predominant direction of stromal collagen in the human (A) and marmoset (B) cornea and scleral rim, sampled at 0.8-mm and 0.5-mm intervals, respectively. Vector plots are scaled down by the factors shown in the key. The position of the limbus is highlighted by a broken blue line.

collagen fibril orientation throughout the entire thickness of the cornea. It is possible therefore that other nonhuman species also possess an orthogonal arrangement of lamellae but as suggested by Gyi and colleagues (1987), they may be fewer in number or of a reduced thickness to those present in the human cornea and therefore contribute less to the overall x-ray scatter pattern. Indeed electron microscopy, a technique that can be used to provide detailed structural information at specific sites in a tissue, has provided evidence of some fibrils crossing at right angles to each other in the posterior stroma of the developing mouse cornea (Haustein, 1983), but x-ray scattering has revealed that, on average, most stromal collagen in the adult mouse cornea lie in a circumferential arrangement with respect to the edge of the cornea.

Here, we have shown that a relationship may exist between corneal collagen orientation and visual acuity (Fig. 5; Table 2). This apparent relationship may be due to animals with a higher level of visual acuity having an increased frequency of rectus muscle contraction as a result of finer eye movements (Prince et al., 1960). Primates are exclusive among mammals in their possession of a fovea—a small circular region of the retina that has a high cone photoreceptor density and affords the animal with a high level of visual acuity. The alignment of collagen in the human cornea toward the insertion points of the rectus muscles may play a role in maintaining a stable eye position during image fixation on the fovea and/or resisting corneal distortion caused by the co-contraction of antagonistic pairs of rectus muscles during blinking. It is worth noting here that, although marmosets possess a fovea, have a relatively high level of visual acuity (30 cycles/degree; Troilo et al., 1993) and likely (based on published values of the blink interval in the Sudanese monkey; Blount, 1927) blink at a similar frequency to humans, they rarely exhibit an orthogonal arrangement of corneal collagen. However, in contrast to the human retina, which shows a sharp decrease in cone density with distance from the fovea, the marmoset retina exhibits a much more gradual decline of cone density along the horizontal meridian (Troilo et al., 1993). The need for such precise horizontal eye movements during image fixation may therefore be reduced and the presence of a predominantly vertical arrangement of collagen may help to preferentially counteract the forces generated by the superior and inferior rectus muscles. It must also be considered that, if corneal collagen arrangement is indeed influenced by the stresses exerted on the cornea by the action of the extraocular muscles, then one might expect the corneas of animals born and raised in captivity to differ from their wild counterparts as a result of differences in their visual environment and, hence, the frequency and direction of eye movements. As suggested by Barmack (1976), deprivation of binocular vision in an animal that normally has excellent binocular vision may reduce the stiffness of the extraocular muscles and cause misalignments.

The need for accurate control of eye position is greatly reduced in animals that possess a retina with a visual streak (a broad strip of increased cone density), because the lateral position of their eyes affords a large field of view without the need for extensive eye movement (Prince et al., 1960; Barmack, 1976). The horse and cow possess an intermediate level of visual acuity (relative to the other species studied here), and in the majority of cases examined in this study have an excess of vertically orientated collagen within the central region of the cornea. A predominantly vertical arrangement of collagen in these species may help to counteract the forces of the superior and inferior rectus muscles, which act antagonistically to help maintain a stable retinal image on a horizontal visual streak. In lower visual acuity animals such as the pig, rabbit, and mouse, the predominantly circumferential direction of corneal collagen does not correspond to the insertion points of any of the rectus muscles. In these species, the frequency of rectus muscle contraction may be lower as a result of limited eye movement and the possession of a retractor bulbi muscle, which helps to retract the eye during blinking. Such a relationship between collagen

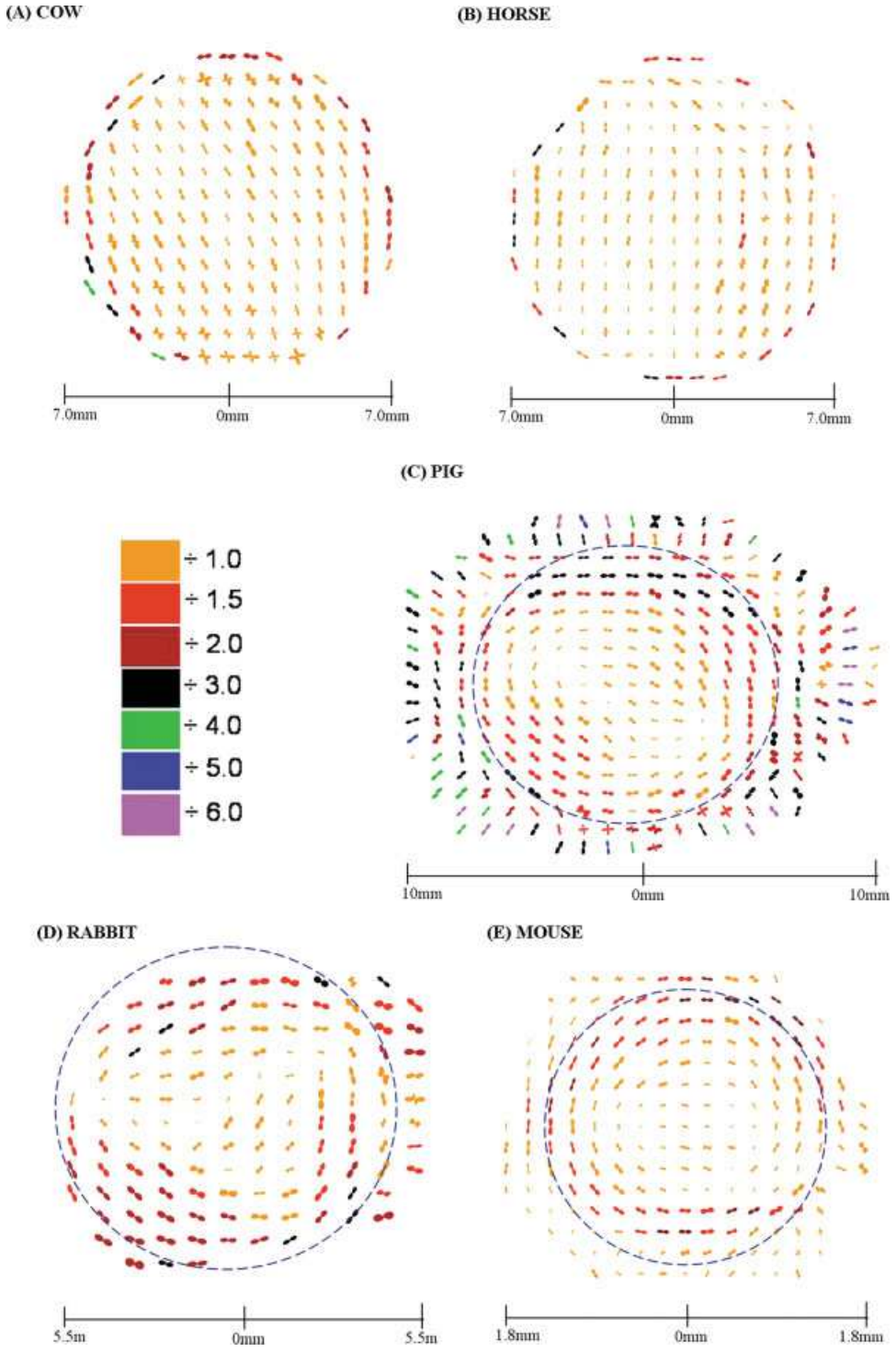

Fig. 4. **A–E:** Vector plot maps showing the predominant direction of stromal collagen in the central 14 mm of the cow (rotated anticlockwise 30 degrees off axis; A) and horse cornea (B) and in the cornea and scleral rim of a pig (C), rabbit (D), and mouse (E). Vector plots are scaled down by the factors shown in the key.

arrangement and eye movement is supported by the fact that the stiffness of both the cornea (Jue and Maurice, 1986) and the extraocular rectus muscles (Barmack, 1976) of the rabbit are far lower than that of the human.

This study has highlighted that major differences in corneal structure exist between mammalian species. These differences may be related to variations in the direction, frequency, and precision of eye movements, however, a functional study of corneal collagen arrange-

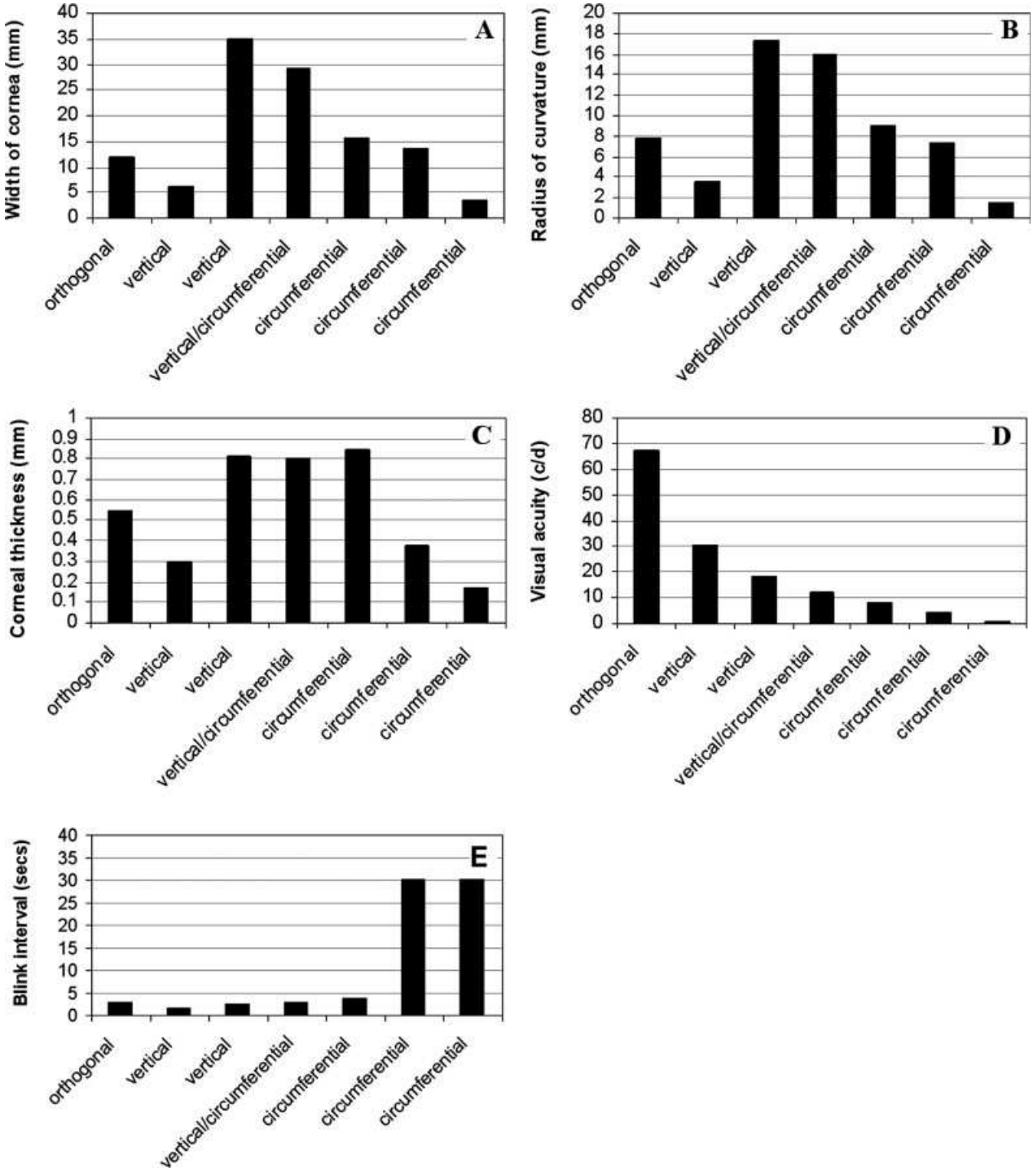

Fig. 5. **A–E:** The arrangement of corneal collagen (orthogonal, vertical, or circumferential) in the human, marmoset, horse, cow, pig, rabbit, and mouse (shown in order from left to right across each graph) is shown relative to published values of the size (A), radius of curvature (B), and thickness (C) of the cornea and the visual acuity (D) and blink interval (E) of each animal (as listed in Table 2).

ment and ocular muscle activity is needed to confirm this. As a consequence of such interspecies structural variation, one should be cautious in interpreting the clinical relevance of nonhuman corneal biomechanical studies.

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