Introduction

Articular cartilage is primarily characterized for its mechanical properties in compression due to its ability to resist loading while providing low friction in articulating joints. The mechanical properties of this anisotropic and heterogeneous material arise from its three principal components, collagen, proteoglycan and water and their physical interactions. The negatively charged proteoglycans form a gel-like material that has a tendency to swell and imbibe water. This gel is entrapped in a cross-linked collagenous fibril network that integrates the entire structure and attaches it to underlying bone via the calcified cartilage layer. While the hydrated proteoglycan phase provides much of the compressive stiffness to cartilage, the ability of the collagenous network to resist tension is just as critical to cartilage compressive strength as indicated in recent experimental and theoretical studies. For example, in the case of unconfined compression, a high tensile stiffness in the direction orthogonal to compression dramatically increases fluid pressurization that is isotropic and therefore active in the direction of load and dynamic stiffness [1,2,3]. In this case, specifying the equilibrium tensile modulus in the transverse direction to be 10 MPa and the compressive modulus in the axial direction to be 1 MPa in a biphasic model with a composite solid allows an accurate description of the high transient stiffness (~6 MPa) and strong relaxation behavior (to ~ 1 MPa at equilibrium) of cartilage in unconfined compression. Furthermore, stress relaxation data from a sequence of superimposed ramp displacements revealed an increasing transient stiffness with subsequent ramps, called compression-offset dependent stiffening, that could only be described with this composite model when the equilibrium tensile modulus was increased for subsequent ramps [4]. The latter suggests that axial compression was stretching and stiffening the fibrillar network in the transverse direction. This was reaffirmed in nonlinear finite-element analysis where other sources of nonlinearity such as strain-dependent permeability were found to be incapable of producing compression-offset dependent stiffening of this magnitude [5]. Finally, an additional nonlinear behavior, where further compression from a static offset results in a larger transient than release of the same amplitude from the same offset, was also only predicted when tensile stiffness was permitted to increase with tensile strain [6]. The net result of these latter studies was that nonlinear behavior in compression could be theoretically described if a nonlinear increase of equilibrium tensile modulus on the order of 10-20 MPa per % tensile strain was included, a property that has never been specifically assessed.

The mechanical properties of articular cartilage in tension have been characterized previously using stress relaxation, creep and constant speed protocols applied to dumbbell-shaped and/or rectangular samples derived from animals of different ages and from different depths from the articular surface. Early studies applied mostly constant speed stretching of dumbbell-shaped samples to failure, revealing a fracture stress that decreased with age (22-
81-year-old human), in the range of 10-25 MPa [7,8]. A later study indicated that this decrease in fracture stress with maturation and age (7-90-year-old human) was site specific, being much more present in the femoral head than in the talus [9]. Constant-speed stretching of bovine cartilage strips also suggested a decrease in tensile stiffness with age between only in the deeper layers of articular cartilage since no age-dependence was detected in cartilage strips containing the articular surface [10]. In contrast to a previous study [8], a more recent one [11] did not find any decrease in tensile stiffness with age (24-70-year-old human) and suggested that the previously found decrease in tensile stiffness with age was due to pathological degeneration and fibrillation in some of the tested samples. Equilibrium tensile stiffness of human and bovine cartilage derived from different depths from the articular surface revealed much lower stiffness values in general and a very significant decrease in equilibrium tensile stiffness with depth where ~10 MPa was found at the articular surface reducing to ~1 MPa in deeper zones [11] similar to previously identified depth-dependence in human [12] and bovine cartilage [21]. This decrease in tensile stiffness with increasing distance from the articular surface is most likely attributable to depth-dependent orientation of collagen where fibrils are parallel to the articular surface near to the surface and more randomly or vertically oriented in deeper zones [13]. Depth-dependent stratification of articular cartilage structure is also generally accentuated with age [14]. A video dimensional analyzer has been used to examine deformation in directions parallel and orthogonal to the constant speed tensile loading direction [15]. A second study with this system suggested stiffening at equilibrium due to large deformation stretch (5-10% per step) [16] while a different study found an essentially linear response in the range of 0 to 15% tensile strain [11]. More recently, digital image analysis of tensile deformation suggested tensile stiffening due to tensile deformation in addition to providing values of Poisson's ratio for human patellar cartilage [17]. Some of the different findings amongst these previous studies could be due to differences in species and joints sites as well as in specimen preparation, mounting, preconditioning, and the criteria used to establish equilibrium. An example of the latter is the very large range of times that are used to estimate equilibrium, ranging from 15-30 minutes in the above studies [11,16,17] to several hours in other tensile tests of articular cartilage [18,19].

Given the importance of tensile properties of articular cartilage in determining its response to compression, the ability of nonlinear stiffening of tensile modulus to describe dynamic nonlinear responses of cartilage to compression, and the established relationship between weakened tensile stiffness and incidence of osteoarthritis, we designed a study to investigate nonlinear properties of articular cartilage in tension, taking into account age and depth from the articular surface. The hypotheses were that 1) tensile equilibrium modulus of articular cartilage increases with uniaxial tensile strain and 2) tensile stiffness increases with age. To aid interpretation of our results we additionally measured longitudinal and transverse deformation using cells as fiducial markers [20] and examined the influence of some technical parameters that affect measurements of tensile stiffness including gripping conditions, tare load, and time to equilibrium. In addressing these hypotheses our results are clinically relevant to articular cartilage function in situ, to how maturation and age affects cartilage function, and they are valuable in the diagnosis and treatment of osteoarthritis and other pathologies of articular cartilage.

Materials and Methods

Tissue Isolation. Articular cartilage was isolated from the load-bearing region of bovine humeral heads of animals with ages under 12 months (young, n = 9), between 12 and 24 months (adolescent, n = 11), and over 24 months (adult, n = 6) about 16 hours after sacrifice. The shoulders were retained with joints and synovial membranes closed and intact. Once the shoulder joint was opened, blocks of bone-cartilage having dimensions of 6 to 8 mm in width by 2 cm in length and 5 to 7 mm in thickness were obtained using a cast-cutter (bone was retained to minimize distortion). Ten blocks were taken from each shoulder (5 rows × 2 columns). Exposed cartilage was kept moist during the entire procedure using Hanks Buffered Saline Solution (HBSS, 14060-57 from Gibco) with 1% Penstrep (P-0781 from Sigma). Blocks were then rinsed and placed at 4°C in sealed tubes containing sterile PBS (P-3813 from Sigma) soaked tissue paper to humidiy the inside of the tube. Cell viability was assessed using ethidium homodimer-1 (E-1169) and calcein AM (C-1430 both from Cedarlane, Missasauga, Ontario) demonstrating acceptable viability (>70% live cells, while 100% live cells in the middle of the sample) under these storage conditions for up to two weeks.

Before each tensile test, samples were obtained using a Vibratome 1000 (Scott Scientific, Montreal) with custom holders and a weight (Fig. 1a) enabling cartilage slices to be prepared by cutting parallel to the articular surface, without the need for prior freezing. The bone-cartilage block was initially immobilized in a bench vice, and then two blades with a 2 mm separation were used to cut perpendicularly into the cartilage. The cartilage was then removed from the bone using another razor blade providing a two-millimeter wide, full-thickness articular cartilage sample with no underlying bone. The cartilage-only sample was then quickly placed onto the holder in the vibratome with the articular surface facing down and a weight placed on top to force the articular surface into contact with the lower surface. This lower surface of the chamber was made of styrofoam that had just been cut with the vibratome blade so that it was parallel to this blade. The vi-

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**Fig. 1** a) Schematic of the apparatus used to cut parallel to the articular surface. The cartilage was placed articular surface down onto the styrofoam stage of the holder, which was fixed to the Vibratome. The weight ensured flat contact and cuts parallel to the articular surface. b) Schematic drawing of the mechanical tester. The upper clamp is fixed to the U support, which is fixed to the crossbeam, and the lower clamp is fixed to the pieces that move with the actuator (ring, load cell, piston of the actuator). The actuator is fixed to the crossbeam. c) Schematic representation of the cartilage strip installed in the clamps, showing the captured images and locations of cell fiducial markers (Pkij) used to evaluate deformation.
bratome blade was then raised to the desired thickness of 250 microns and a thin cartilage slice of uniform thickness retaining the articular surface was cut and removed. The slices were immediately placed in PBS/Penstrep to prevent drying of the sample during thickness measurements. Thickness was then measured at four sites using a micrometer attached to a multimeter that detects resistance resulting in an average of $267 \pm 53 \mu m$ (mean $\pm$ sd, n = 19) for the mechanical test samples and an average of $273 \pm 68 \mu m$ (mean $\pm$ sd, n = 7) for samples stretched and viewed under light microscopy. The average variation of thickness within a sample was $31 \pm 13 \mu m$ (mean $\pm$ sd, n = 19) for the mechanical test and $31 \pm 12 \mu m$ (mean $\pm$ sd, n = 7) for the samples stretched and viewed under light microscopy.

**Tension Tests.** Tension tests were performed on young (n = 7), adolescent (n = 6), and adult (n = 6) articular cartilage using a Mach-1 A400.25 mechanical tester (from BioSyntech, La-Val, Qc www.biosensing.com) with customized grips made for tensile testing (Figs. 1b and 2a). The actuator of this instrument has an accuracy of 0.1 $\mu m$ and the 150 g capacity load cell has a display resolution of 0.0075 g and a nonlinearity of $\pm 0.2\%$. Before testing, a calibration of the load cell with a $\pm 100$ g weight was performed. Tests regarding load cell drift showed a deviation maximum of $\sim 0.1$ g/h in the absence of a sample. In this setup, the load cell is fixed to the moving part of the actuator, and a metal ring, on which the lower clamp is attached, is fixed to the load cell. The actuator and the U support, on which the other (upper) clamp is attached, are both fixed to the crossbeam. Waterproof sandpaper was fixed onto the inside of each clamp using cyanoacrylate glue to prevent slipping of the sample. Initially, sandpaper with grain size 150 was used, but histology showed that this grain size was too large since cutting and damage to the sample within the clamps was observed (Fig. 3), and also appeared to induce irregular sample shape near the edge of the grips. Following these observations, we decided to use the smallest grain size available, type 1500. A gauge length of 4 mm was exposed between the grips while about 2-3 mm of the sample was present inside the grips at each end (for $\sim 10$ mm total sample length). The sample and grips were then immersed in a bath containing approximately 400 ml of PBS with 1% Pen/Strep, which was also covered with plastic film, to reduce evaporation (Fig. 2a). Because of the long duration of the test (48 hours), pH and volume were measured before and after, to ensure their stability. The average change in volume was $-3.1 \pm 2.2\%$ (mean $\pm$ sd, n = 10), and the value of initial pH and final pH were 7.34 $\pm$ 0.06 and 7.31 $\pm$ 0.17 (mean $\pm$ sd, n = 10), respectively. A sequence of 5 ramp extensions was then applied, each of 2% strain (80 $\mu m$ at 6 $\mu m/second$) after an initial offset of 2% was imposed. The relaxation time for
each step was chosen to increase for subsequent steps such that the load decay at the end of each step was similar and therefore the degree of attainment of equilibrium would be similar for different steps. This criterion was met with a relaxation time of 5 1/2 hours for the initial offset, 6 1/2 hours for the first step, 7 1/2 hours for the second, 8 1/2 hours for the third, 9 1/2 hours for the fourth, and 10 1/2 hours for the fifth and last step resulting in a total time of each test of 48 hours. Some 96-hour tests were also made using two 48-hour protocols of constant relaxation time of 8 hours, the second one after the sample was returned to its original length. This second series of 5 ramps showed weakened responses for the 1st and 2nd steps but similar responses for the 4th and 5th steps, compared to the first series, suggesting some structural alterations were induced by the stretching but not by the relatively long duration of the 48-hour test.

Equilibrium and peak force data were treated with the nominal dimensions ignoring the effect of the thickness/width variation. Equilibrium moduli were calculated using the incremental infinitesimal deformation for each step as a reasonable approximation, given that maximum strain was 10% with individual steps of 2%, according to

\[
E = \frac{\Delta F/A}{e_{zz}} \quad \text{and} \quad e_{zz} = \frac{\Delta L}{L}
\]

(1)

where \(E\) (MPa) is the equilibrium modulus, \(\Delta F\) (N) is the detected increment in axial force at equilibrium relative to the beginning of the ramp, \(A\) is the initial cross-sectional area (mm²), \(\Delta L\) (µm) is the incremental extension of the sample relative to the beginning of the ramp and \(L\) (µm) is the length of the sample at the beginning of the ramp. The initial cross-sectional area was calculated using the 2 mm width and the average measured thickness of the slice. As a simple means of representing transient and dynamic behavior, we calculated a peak modulus using exactly the same procedure as the equilibrium modulus except \(\Delta F\) (N) was the detected increment in axial force at the peak, just when the ramp was completed. A characteristic relaxation time for each ramp was also calculated as the time necessary to reach half of the load decay during relaxation. To assess the extent of attainment of equilibrium, the stress decay (g/hour) at the end of each relaxation curve was calculated with the load decay of the last two hours. The nonlinearity (MPa%) of equilibrium modulus and peak modulus was evaluated by fitting a straight line to the values of equilibrium modulus and peak modulus as a function of % strain. The ratio equilibrium-modulus/peak-modulus (%) was calculated for each curve to provide an index of the magnitude of relaxation.

Statistical analysis of equilibrium and peak moduli was performed using repeated measures ANOVA with Statistica (StatSoft, Tulsa, OK). Age was defined as a 3-level (Young, Adolescent, Adult) categorical predictor while equilibrium or peak moduli were defined as repeated measures with 5 levels each corresponding to values at 2, 4, 6, 8, 10% strain.

**Stretching as Viewed Under Light Microscopy.** Stretching as viewed under light microscopy was performed on young (n=5) and adolescent (n=5) articular cartilage using the same clamps and sandpaper as those used for the mechanical testing. Additional adapter pieces were designed to perform the stretching manually on the stage of a Zeiss inverted fluorescence microscope using a Mitotoyo non-rotating micrometer head (Fig. 2b). After the sample was cut, it was placed in 4 ml PBS with 4 µl Hoechst 33258 for at least three hours to label cells. The sample was then installed in the clamps and stretched using a protocol similar to the above mechanical test, the only differences being that the relaxation time was 30 minutes and the ramp velocity was not controlled since it was manually stretched. Although the relaxation time may influence the measured deformation, we did not detect any clear difference (within ±0.25% strain) in preliminary experiments comparing deformation at 30 minutes vs deformation at 8 hours (in contrast to the force measurements described above) so that a 30-minute relaxation was chosen. In order to visualize the entire sample, two images were captured using a 4X objective (N.A. = 0.1, resolution = 2.2 µm) 30 minutes after stretching by 2% (Fig. 1c). Care was taken to make sure that each image was taken at the same location and focus, to enable valid comparison between images at different deformation, and to maintain the same orientation of the sample image. On each of the two images taken before the first stretch, 9 cells were chosen, each of them at different periodic positions in the image (Fig. 1c). The same cells (18 overall) were then identified and marked with an imaging software (Paint Shop Pro 7) for images taken after each of the subsequent 5 stretches. Images were then analyzed using Northern Eclipse software (Empix Imaging, Missassauga Ontario) to identify the position of the cells. Data was exported to Microsoft Excel, where the distances between chosen cells were computed, in pixels, and separately for two directions (longitudinal and transverse). With reference to Fig. 1c, each chosen cell is indicated by \(P_{ij}\) where i and j designate the row and the column of the cell position and \(k\) designates the deformation step (1 to 5). The longitudinal lengths, designated \(L_{kim}\), were then evaluated as distances between chosen cells in the longitudinal direction (component of the distance parallel to the long axis of the sample) where \(i\) still indicates the transverse row while \(m\) indicates the longitudinal position-\(m = 1\) between \(j = 1\) and \(j = 2\); \(m = 2\) between \(j = 2\) and \(j = 3\); \(m = 3\) between \(j = 4\) and \(j = 5\); \(m = 4\) between \(j = 5\) and \(j = 6\). The transverse lengths were similarly designated by \(L_{kim}\), where \(j\) indicates the longitudinal column while the label \(n\) indicates the transverse position: \(n = 1\) between \(i = 1\) and \(i = 2\); \(n = 2\) between \(i = 2\) and \(i = 3\). Finally, the deformation was computed between each image, the longitudinal deformation according to

\[
D_{li}^{l} = \frac{\sum_{i=1}^{3} \left( \frac{L_{kim} - L_{kim-1}}{L_{kim-1}} \times 100 \right)}{3}
\]

(2)

and the transverse deformation as

\[
D_{ij}^{T} = \frac{\sum_{n=1}^{2} \left( \frac{L_{kim} - L_{kim-1}}{L_{kim-1}} \times 100 \right)}{2}
\]

(3)

The accuracy of these deformation estimates was found by taking images at 11 different focal planes within the sample and observing intercellular distances to vary by at most 1 pixel (1.68 µm). The resolution for the strain technique is therefore approximately one pixel width relative to the smallest intercellular distance used in this calculation (400 pixels) or 0.25%.

**Results**

**Age- and Maturation-Dependent Behavior.** The sequence of stress relaxation tests revealed significant viscoelastic behavior (Fig. 4) with a peak force being attained at the end of the ramp followed by rapid dissipation of some of the peak load, followed by a very slow relaxation that never quite reached equilibrium. Nonetheless, our relaxation times were chosen such that the rate of stress decay at the end of the relaxation period was similar for each ramp. There was an evident stiffening of peak and equilibrium stiffness with age, the latter being so weak for the young tissue that all of the developed force appeared to be dissipated by the end of the relaxation phase producing a near null equilibrium modulus. Peak moduli showed an increase with strain and with maturation and age (Fig. 5b) where modulus values for the last step (8-10%) were (mean ± sd), 10.1 ± 3.3 MPa for young (n=7), 18.7 ± 5.9 MPa for adolescent (n=6), and 28.3 ± 16.6 MPa for...
adult (n = 6). A linear fit of peak modulus versus strain for each test was made to quantify the nonlinearity of the peak modulus resulting in 0.8 ± 0.4 MPa/% for young, 1.8 ± 0.4 MPa/% for adolescent, and 3.1 ± 1.7 MPa/% for adult. For Peak moduli, the effect of strain and the strain-age interaction were statistically significant (p < 10^{-5}) while the main effect of age was not statistically significant (p = 0.163).

Equilibrium moduli showed a significant increase with strain as well as with maturation and age (Fig. 5b) with modulus values for the last ramp (8-10%) of (mean ± sd), −0.1 ± 0.5 MPa for young (n = 7), 4.4 ± 2.6 MPa for adolescent (n = 6), and 15.4 ± 9.6 MPa for adult (n = 6). There was very little increase of equilibrium modulus with strain for the young cartilage, while the adolescent and adult cartilage showed a greater increase with slopes of the linear fit resulting in (mean ± sd) −0.1 ± 0.2 MPa/% for young (n = 7), 0.3 ± 0.2 MPa/% for adolescent (n = 6), and 1.7 ± 1.0 MPa/% for adult (n = 6). The effect of strain and the strain-age interaction were statistically significant (p < 10^{-5}) as was the main effect of age (p = 0.004). We also calculated moduli corresponding to a 15 minute relaxation period using our data and found for the fifth and last step (mean ± sd) 3.6 ± 1.5 MPa instead of −0.1 ± 0.5 MPa for young (n = 7), 10.8 ± 4.3 instead of 4.4 ± 2.6 MPa for adolescent (n = 6), and 21.7 ± 13.5 instead of 15.4 ± 9.6 MPa for adult (n = 6), thus confirming the importance of relaxation time when estimating equilibrium moduli for cartilage tested in tension.

A linear regression of each of the equilibrium moduli with its corresponding peak modulus was done for all deformations showing that the percentage of relaxation (1-ratio of equilibrium modulus to peak modulus) decreases with age (Fig. 6). To quantify the ratio of the equilibrium modulus to peak modulus, a linear fit (passing through zero) for each group was calculated. For young, adolescent and adult cartilage, the slopes were 3.5%, 25.6%, and 58.3%, respectively.

The half-relaxation time was computed from each stress relaxation curve for the last three steps. There was no statistical dependence of the half-relaxation time with the age with (mean ± sd) of 308 ± 162 sec (n = 21), 562 ± 179 sec (n = 18), and 468 ± 644 (n = 18) secs for young (n = 7), adolescent (n = 6) and adult (n = 6), respectively. The load decays at the end of the relaxation period did not vary significantly with step number or age, showing (mean ± sd) 0.13 ± 0.09 (g/hour), 0.16 ± 0.08 (g/hour), and 0.14 ± 0.12 (g/hour) for young (n = 35), adolescent (n = 30) and adult (n = 30) cartilage, respectively. For a sample having 250 microns thickness, the error induced by a slope of ±0.1 g/h during a relaxation time of 2 hours, for example, is ±0.2 MPa and is less than the standard deviation of moduli measured for each age group.

Stretching as Viewed Under Light Microscopy for Tissue Strain Distribution. The deformation of both young (n = 2) and adolescent (n = 5 for the 4 first deformations and n = 4 for the last deformation) cartilage were similar and are grouped together (n = 7) below. The longitudinal deformation induced by each step showed a constant increase with each step (Fig. 7). Longitudinal deformation of 1.52 ± 0.18% (mean ± sd, n = 34) was computed.
between successive jaw-to-jaw 2% strains and the overall computed deformation (10% jaw-to-jaw) was of 7.7±0.6% (mean ± sd, n = 6). There was also no apparent spatial dependence of longitudinal deformation although we were unable to measure it immediately adjacent to the clamps. Our cell fiducial markers were generally located in the central portion of the cartilage thickness, as assessed by visual inspection while changing the focal plane.

The transverse deformation induced by each step showed an increase after the first and second step, mainly in the center of the sample (position j = 3 and 4) where the incremental deformation was −1.96±0.95% and −1.87±0.94 (n = 7) for k = 1 and −3.82±0.84% and −4.00±1.09% (n = 6) for k = 5 (Fig. 8 and Table 1). The deformation pattern found showed a smaller deformation near the clamps, approximately −1% per step, and a maximum deformation of about −5% per step in the center of the sample, for each step. The total lateral computed deformation (at 10% jaw-to-jaw longitudinally) revealed a large variation along the length of the sample. Near the clamps, the lateral deformation at 10% extension was −4.7±3.0% and −4.6±1.6% (n = 6), respectively, for both sides of the sample, while the largest deformation was in the center of the sample, with an average of −16.5±4.2% (mean ± sd, n = 6). Taking the measured average longitudinal deformation as 7.7%, an empirical Poisson’s ratio at the center can be estimated as ν = 2.1, within the range of values (1-4) found in previous studies [15,17].

We also measured the deformation after a 24-hour relaxation period following the last step to see if there was any deformation during the long relaxation time. We found changes in measured longitudinal and transverse deformation of (mean ± sd) 0.10 ± 0.53% and −0.93±1.00%, respectively (n = 46 adolescent and n = 12 young), relative to that after 30 minutes. Using a two-tailed t-test, these results did not differ significantly from zero.

**Mechanical Testing of Cartilage at Different Depths From the Articular Surface.** Tensile tests were also performed on slices from deeper regions of articular cartilage that did not include the articular surface. However, we found a non-negligible curvature was induced in the sample when it was installed in the clamps (Fig. 9). We found that increasing the distance between the clamps by approximately 800 μm could straighten out this curvature, but this offset value varied between samples. Thus, it was difficult to decide whether the force generated was attributable to extension or straightening out of sample curvature in these samples from deeper regions. We also noticed considerable plastic deformation due to gripping in samples from deeper regions suggesting irreversible sample alteration and further incertitude regarding initial boundary conditions (Fig. 9). For these reasons we did not perform extensive tests comparing the tensile properties from the different layers. However, at the last step (8% to 10%), the average for the peak modulus and the equilibrium modulus, for samples not including the articular surface was 3.96 ± 3.12 MPa (mean ± sd, n = 7) and 0.75 ± 1.05 MPa (mean ± sd, n = 7) for adolescent articular cartilage and were much lower than those found from cartilage containing the articular surface. Further research is needed to determine how these mechanical properties may differ from those in the articular surface.
thermore, these averages did not include some tests that generated little force above noise without a peak or apparent relaxation.

**Discussion**

Tensile stress relaxation tests (5 ramps of 2% strain each) of bovine articular cartilage strips that included the articular surface revealed a strong increase in stiffness with age (young = 6 months, adolescent = 18 months, adult > 24 months) with mean values ranging from 0 to 15 MPa, for young to adult cartilage at equilibrium, and 10 to 28 MPa, for young to adult cartilage at the transient peak. As can be appreciated from these relative values of equilibrium and transient stiffness, the amount of the peak force that relaxed was greatly reduced with age, where young cartilage could relax entirely (100% of its developed force whereas adult cartilage typically still retained at equilibrium about 60% of the maximum transient force. We also detected a significant nonlinear increase in tensile stiffness with tensile strain at all ages, and the magnitude of this nonlinearity also increased with age. For example, the slope of equilibrium modulus versus strain was \(-0.1\) MPa/% for young and 1.7 MPa/% for adult cartilage. In order to appreciate the state of deformation of the sample and aid interpretation of mechanical tests, longitudinal and transverse deformation fields were calculated by observing labeled cells with a microscope before and after stretching. The results indicated that longitudinal deformation was uniform across the sample but was only 77% of the jaw-to-jaw deformation calculated based on imposed displacements. This difference was possibly due to hypothetically large strain values occurring in regions where we could not measure near the grips. Equilibrium moduli reported here were calculated using jaw-to-jaw displacement rather than sample center deformation in order to directly compare with the literature and since the correction is also small (30%) relative to observed age-related differences. Transverse deformation was, however, quite non-uniform. For example, at 10% applied longitudinal strain, transverse strain was \(-5\)% near the grips and \(-15\)% in the sample center. In addition to these tests on strips that were prepared to include the articular surface, tests were performed on samples from deeper regions of the articular cartilage, however technical difficulties related to instability in sample geometry and grip-induced sample curvature precluded us from quantitatively analyzing these measurements. Nonetheless, it was evident that deeper regions were much less stiff than the articular surface for all ages confirming numerous previous studies.

Comparison of our results with those of previous studies requires examination of technical differences in terms of sample preparation (e.g., shape, fresh vs frozen) and loading protocols (e.g., gripping technique, presence of tare load, speed of stretch, determination of equilibrium, measurement of strain). Sample shape is usually in the form of a dumbbell in tests when the sample is loaded to failure [9, 21] in order to ensure failure in the mid region where stress and strain are assumed to be uniform, far away from the grips. Rectangular shapes are smaller, and more easily obtained and are used in small deformation tests (up to 15% in [11]), usually assuming that jaw-to-jaw strain is equivalent to sample strain in the mid-region. We have found in our study that this latter assumption may be subject to an error of about 25% in overestimating the sample stretch. Nonetheless, longitudinal strain was found to be uniform throughout most of the sample. Gripping technique may be more critical than previously appreciated in light of our micrographs of samples gripped with different grain sizes of sandpaper (Fig. 9). Larger grain size can clearly cut the cartilage within the grips in an unpredictable manner, possibly weakening the observed response. Freezing of cartilage could also weaken mechanical properties as has been observed recently in compression tests [22]. Yet another technical factor to account for in comparisons is speed of stretch and time to equilibrium. Our studies found that estimation of equilibrium properties required long relaxation times of 5-10 hours, in stark contrast to that of compression tests where 5-15 minutes usually suffices. Some previous studies allowed only 15 minutes to equilibrium for uniaxial tension [11, 17] while others [18, 19] have recognized the need for a much longer time to establish equilibrium. Finally, the influence of imposing a tare load is difficult to assess. For example, some samples could be highly curved due to swelling before gripping or plastic deformation induced by the grips (Fig. 9) such that a small tare load could stretch significantly such weak samples until they stiffen enough to produce a detectable load. In a similar way, weakening by freezing or by sandpaper grains cutting the sample in the grip could be uncontrollably compensated by imposing a

### Table 1 Incremental transverse deformation (%).

<table>
<thead>
<tr>
<th>j</th>
<th>k = 1</th>
<th>k = 2</th>
<th>k = 3</th>
<th>k = 4</th>
<th>k = 5</th>
<th>k = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>j = 1</td>
<td>-0.45±0.52</td>
<td>-1.30±0.81</td>
<td>-1.96±0.95</td>
<td>-1.87±0.94</td>
<td>-1.55±0.66</td>
<td>-0.56±0.46</td>
</tr>
<tr>
<td>j = 2</td>
<td>-0.83±0.60</td>
<td>-2.35±0.76</td>
<td>-3.28±1.06</td>
<td>-3.29±1.12</td>
<td>-2.65±0.83</td>
<td>-0.82±0.44</td>
</tr>
<tr>
<td>j = 3</td>
<td>-1.00±0.63</td>
<td>-2.74±0.72</td>
<td>-3.76±1.08</td>
<td>-4.10±1.30</td>
<td>-3.00±0.99</td>
<td>-0.88±0.41</td>
</tr>
<tr>
<td>j = 4</td>
<td>-1.17±0.70</td>
<td>-2.95±0.75</td>
<td>-3.88±0.88</td>
<td>-4.02±1.14</td>
<td>-3.20±0.80</td>
<td>-1.04±0.51</td>
</tr>
<tr>
<td>j = 5</td>
<td>-1.24±0.78</td>
<td>-2.87±0.71</td>
<td>-3.82±0.84</td>
<td>-4.00±1.09</td>
<td>-3.01±0.90</td>
<td>-1.21±0.35</td>
</tr>
<tr>
<td>j = 6</td>
<td>-1.24±0.78</td>
<td>-2.87±0.71</td>
<td>-3.82±0.84</td>
<td>-4.00±1.09</td>
<td>-3.01±0.90</td>
<td>-1.21±0.35</td>
</tr>
</tbody>
</table>

Fig. 9 Pictures of slices from the different layers taken from the same adult sample. AS: articular surface of \(-300\) microns thickness. MZ: median zone of \(-330\) microns thickness. DZ: deep zone of \(-320\) microns thickness. The top left panel shows the samples after they had just been cut, while images on the right show the curvature of the samples once installed in the clamps. The bottom left panel shows images of the same samples once removed from the clamps, clearly indicating differences between zones.
tare load. These technical points are important when interpreting and comparing maturation- and age-related findings and nonlinear properties.

We have found that tensile stiffness of articular cartilage increases with maturation and age in contrast to previous studies observing a decrease with age [9] or no age-dependence [11]. Possible reasons for this difference compared with previous studies is the species difference as well as the exact age range used, in addition to freezing, gripping technique, tare load, high strain rate and possible presence of fibrillation or spontaneous degeneration [9] and insufficient time to equilibrium of 15 minutes [11]. Our data is consistent with tensile tests of other non-mineralized collagenous connective tissues including tendon, lens capsules and vascular tissues showing stiffening with age in terms of tensile stiffness and fracture stress, although strain at fracture is reduced [23]. This age-dependent stiffening has been related to changes in composition, especially in the quantity and type of cross links found between fibrillar collagens. The concentration of enzymatically produced trivalent cross links (hydroxylysyl-pyrindinline) increases until maturity and is stable thereafter [24] while concentrations of non-enzymatic glucose-derived cross-links (i.e. pentosidine) increase continually from maturity until death [25]. Additionally, increased concentrations of glucose-derived cross links were found to correlate with stiffening of cartilage as indicated by microscopic observation of reduced lateral expansion under compression [26]. Thus, according to our studies, it appears that articular cartilage stiffens in tension with maturation and age, as do most other non-mineralized connective tissues.

We found that tensile stiffness of cartilage at equilibrium and during the transient, increases with tensile strain in contrast to previous studies of non-enzymatic glucose-derived cross-links [11] but in agreement with measurements made on self-assembled collagen fibres [27]. As with age-related discrepancies, contradictory results obtained in the previous study may be due to freezing, gripping technique, tare load and insufficient time to equilibrium of 15 minutes [11]. Our estimation of the nonlinear increase in equilibrium tensile stiffness of adult articular cartilage, 1.7±1.0 MPa%/9, is within the range of that of self-assembled collagen fibers, 0.5–5.0 MPa%/27, but much smaller than values needed to predict nonlinear properties of cartilage in compression, 10–30 MPa%/28. Although we have no firm explanation for this discrepancy, we speculate that our measured nonlinearity in uniaxial tension is lower than that required to explain nonlinear compressive properties since axial compression places the transverse plane in biaxial tension. The latter would be expected to generate a much higher force in both transverse directions than uniaxially. The only biaxial tension tests of young cartilage may also reveal a stress relaxation curve that does not entirely relax, compared to the uniaxial tests performed here. Taken together, our study indicates for the first time that tensile stiffness of articular cartilage increases with maturation and age and that any measured decrease could signal degeneration. Uniaxial tension tests of cartilage also revealed that stretching increases cartilage tensile stiffness at small strains (2–10%), qualitatively in agreement with measurements of compressive nonlinearities in cartilage. These results are essential when interpreting functional measurements for clinical use or when designing therapies to heal articular cartilage that is damaged due to trauma and arthritis.

Acknowledgments
This research was supported by the Natural Sciences and Engineering Research Council of Canada, the Canadian Arthritis Network, and Arthritis Society of Canada. The authors would like to thank Caroline Tanguay for preparing histological sections.

References

