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1 **Horses and cows might teach us about human knees.**

2

3 Holland, C.^{1,2}, Vollrath, F.^{1*} and Gill, H.S.^{3,4}

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5 ¹ Department of Zoology, University of Oxford, South Parks Road, Oxford, UK

6 ² Department of Materials Science and Engineering, University of Sheffield, Mappin
7 Street, Sheffield, UK.

8 ³ Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences,
9 University of Oxford, Nuffield Orthopaedic Centre, Oxford, UK.

10 ⁴ Department of Mechanical Engineering, University of Bath, Claverton Down, Bath, UK
11 Bath, UK

12

13 * corresponding author : Prof. Fritz Vollrath,

14

15 Department of Zoology, South Parks Road, Oxford, Oxfordshire, OX1 3PS, UK

16

17 Tel: +44 (0) 1865 271 216, Email: fritz.vollrath@zoo.ox.ac.uk

18

19 **Abstract**

20 Our comparative study of the knees of horses and cows (paraphrased as highly
21 evolved joggers and as domesticated couch-potatoes, respectively) demonstrates
22 significant differences in the posterior sections of bovine and equine tibial cartilage,
23 which are consistent with specialization for gait. These insights were possible using a
24 novel analytical measuring technique based on the shearing of small biopsy samples,
25 called Dynamic Shear Analysis. We assert that this technique could provide a powerful
26 new tool to precisely quantify the pathology of osteoarthritis for the medical field.

27

28 **Keywords**

29 Dynamic-Shear-Analysis, osteoarthritis, cartilage

30

31 **Introduction**

32 Millennia of natural selection have shaped the form and function of animal joints. Of
33 special importance is a joint's response to loading and the distribution of stresses and
34 strains across its surfaces. Hence it is of significant interest both academically and
35 practically to understand, firstly, how animals have co-evolved and integrated
36 morphology, materials and mobility of specific joints and, secondly, how these structural
37 and functional features are further integrated into behaviour i.e. gaits. When such highly
38 evolved systems fail there is subsequent cost; in Nature lameness reduces physical fitness
39 and thus increases the chance of predation and death, for man joint diseases represent
40 individual pain and disability(Desmeules et al. 2010; Muraki et al. 2010) as well as
41 significant socioeconomic drain(Gupta et al. 2005). There are feedback links between
42 morphology and gait; with, for example, decades of un-natural impact (for example
43 jogging with wrong shoes and foot-fall) beginning to leave their legacy in the traumatic
44 wear and tear of human knees(Felson et al. 1997). Here we propose that a comparative
45 study of the knees of horses and cows (paraphrased as highly evolved joggers and as
46 domesticated couch-potatoes, respectively) might allow us to test specific as well as
47 generic hypotheses about the form and function of integrated knee morphology and
48 cartilage.

49
50 For human knees an important failure mode is osteoarthritis (OA), which is the
51 result of degradation of the articular cartilage within the joint and often begins in specific
52 locations(Gulati et al. 2009). The role of the cartilage is to provide a low-friction, self
53 lubricating bearing surface transmitting loads and permitting movement. It is primarily
54 composed of a tightly ordered network of type II collagen fibres embedded in a highly
55 hydrated matrix of proteoglycans. Whilst much is known about the biochemistry and
56 genetics of this biological composite, relatively little is known of how these components
57 contribute to the bulk material properties and if/how they are optimised within a joint.
58 However, characterising the mechanical properties of cartilage across the surface of the
59 tibia is not trivial. Typically this has required either (i) the excision of significant
60 amounts of tissue to allow for the curvature of the underlying bony surface or (ii) the use
61 of cumbersome equipment (Appleyard et al. 2003; Young et al. 2007).

62

63 Hence, there is a clear need to develop a simple technique that will allow the
64 testing of small tissue samples at physiological conditions. Here we present one such
65 technique, Dynamic Shear Analysis (DSA) and its suitability for the study of small
66 biopsy samples of knee cartilage. DSA employs rheometry, which gives us detailed
67 measurements of the behaviour of a material in response to highly controlled shear forces
68 (Chaudhury et al. 2011). Specifically, DSA examines material properties by compressing
69 a sample with a known force between two metal plates. The sample is then oscillated
70 parallel to the plates at an extremely small fixed strain over a range of frequencies whilst
71 measuring the resistance to this deformation. The dynamic information provided (the
72 shear modulus, G^*) from such a test is indicative of the overall integrity of the collagen
73 network. Thus DSA allows us to easily and quickly examine in great detail the force
74 response curves of small *ex vivo* samples in body fluids and at body temperatures.

75

76 We chose the ‘knees’ of horses and cows for comparison in order to test the
77 hypothesis that there are correlations between the lifestyle of an animal (mobility, gait)
78 and the form and function of its knees, with our uncertainty whether the differences are
79 more in the details of knee morphology or more in the details of cartilage material
80 properties.

81

82 **Methods**

83 Sample preparation

84 Bovine and equine specimens were obtained through dissection of full intact knees from
85 cadaverous material obtained from a slaughterhouse. A pilot study was performed on 3
86 knees (2 left and 1 right) from 3 bovines and the trends in properties across the knee
87 surface observed are in agreement with those reported this study. The study presented
88 here represents data from a further 6 knees from three individuals (both left and right) of
89 bovines (all < 18 months old) and the same again of equines (18 months, mid teens and
90 late teens). Sample preparation occurred no longer than 12 hours after slaughter. Tibial
91 plateaus were inspected at time of dissection and no signs of osteoarthritis were found.
92 Subsequently 8 mm punch biopsies were taken from 6 respective locations on the upper

93 surface of the tibia (medial, lateral and anterior, middle, posterior, Fig. 1). Punches were
94 stored overnight in DMEM at 4°C in order to ensure the tissue was still viable when
95 tested. The following day the 8 mm punch biopsies were sub-sampled into two 3 mm
96 punch biopsies, one was used for this study and then other reserved for future work.
97

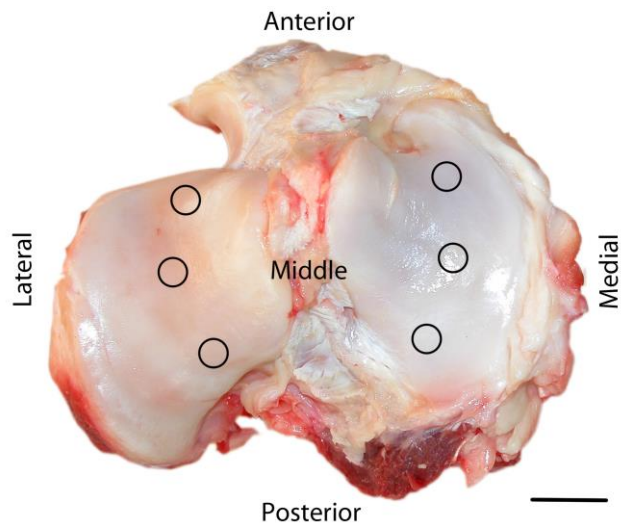


Fig. 1 Diagram depicting the location of the punch biopsies on the tibial articular cartilage used in this study. Bovine sample. Scale bar is 20mm.

98

99 Rheological testing

100 A Bohlin Gemini 200 HR Nano rheometer (torque range 3 nNm to 200 mNm, controlled
101 stress/strain oscillation, Malvern Instruments, UK) was used to collect all data. Samples
102 were loaded between two parallel plates with cartilage upper surface facing the upper
103 plate of the rheometer and surrounded by fresh DMEM kept at 37°C by an environmental
104 chamber. In order to maintain a constant grip on the samples a 0.2 N compressive load
105 was applied during testing. Prior to characterisation, samples had their linear viscoelastic
106 region characterised and subsequent tests were made within this region of strain. An
107 oscillatory test (12.5-0.1 Hz, 0.001 strain) was undertaken and valid shear modulus (G^*)
108 readings taken (as defined by a raw phase angle being within machine limits) and
109 averaged due to the frequency independent nature of the sample moduli over the tested
110 frequency range. The test-retest variation of DSA has been previously reported
111 (Chaudhury et al. 2011) and validated in this study to be >1% (n=1, data not shown).

112

113 Statistical Analysis

114 Because the distribution of the data was not normal, non-parametric statistics were
115 performed where $p < 0.05$ was regarded as significant. Mann-Whitney U tests were
116 performed on data to determine differences between species, sides and site-to-site
117 comparisons. Kruskal-Wallis tests were performed within a side. The statistical package
118 used for analysis was PASW (v.18, PASW Inc., Chicago, IL).

119

120 Results

121 Overall the material properties tested in this study showed highly significant differences
122 between bovine and equine samples (Mann-Whitney U test $p < 0.0001$ for all) and when
123 comparing the medial and the lateral sides between each species (Mann-Whitney U test p
124 < 0.0001 for all except shear modulus on the lateral side where $p = 0.009$). Compared to
125 bovine material, the equine tibial cartilage was approximately 50% thicker, 5 times less
126 compressible and considerably more resistant to shearing forces (Fig. 2); equine cartilage
127 thus would require significantly more energy to deform.

128

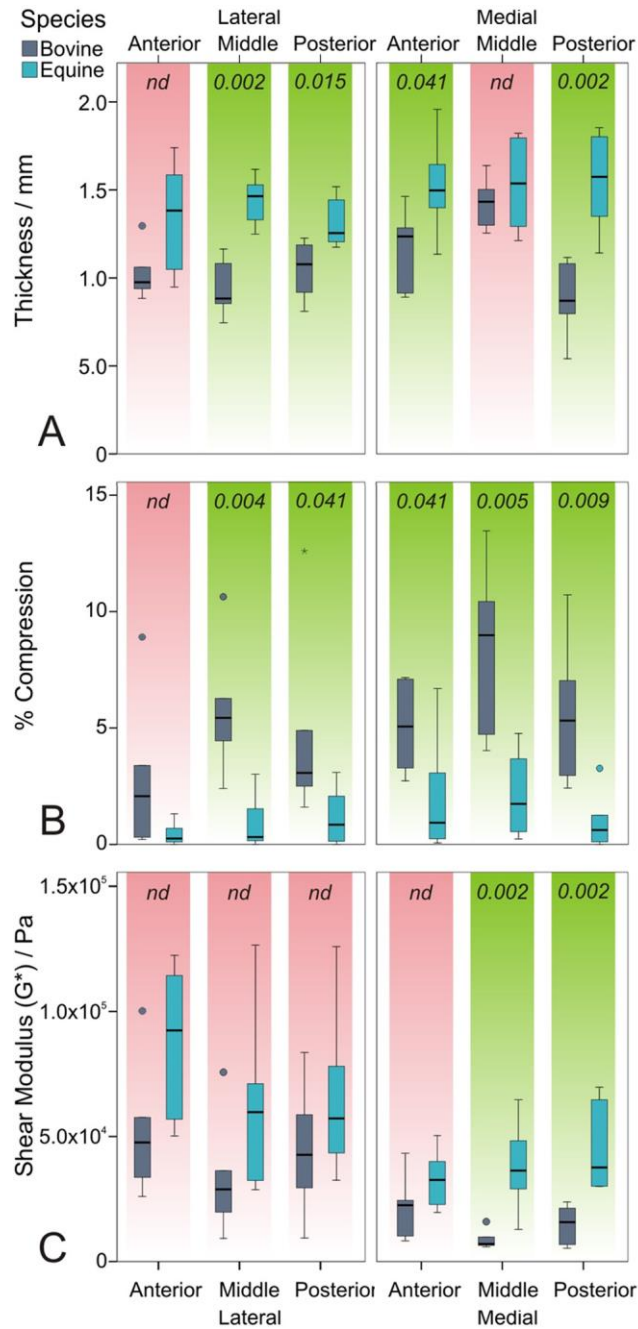


Fig. 2 The mechanical properties of bovine and equine knee cartilage as determined by dynamic shear analysis. A) Thickness, B) Compression during testing and C) Shear modulus for bovine (n=6 knees, dark-blue) and equine (n=6 knees, light-blue) tibial articular cartilage samples. Data presented as box charts indicating median, 25 and 75 percentile range and whiskers containing 95 percent range. Circles are outliers whereas stars are extreme outliers. Also shown are the p values (Mann-Whitney U) comparing

bovine to equine at each site, nd=no significant difference

129 In addition we detected some with-in species differences in material properties
130 according to location in the joint. The medial and lateral sides in the bovines differed
131 significantly in thickness, **unconfined compression** and shear modulus (**Mann-Whitney U**
132 $p = 0.042, 0.036$ and <0.001 respectively) whereas they differed only in the shear
133 modulus in the equines (**Mann-Whitney U** $p=0.001$). However, only the bovine medial
134 side displayed significant differences between the anterior, middle and posterior positions
135 (thickness, Kruskal-Wallis $p= 0.06$ and shear modulus, Kruskal-Wallis $p = 0.042$). Taken
136 together and seen in the light of the overall shape and biomechanical constraints of the
137 knees, these data strongly suggest that there may be specialisation of the joint in these
138 areas. There were measurable differences in the shear moduli at all locations in the
139 bovine samples (**Mann-Whitney U** Test anterior $p=0.015$, middle $p=0.008$, posterior
140 $p=0.026$) as well as across the equine anterior section of the tibia (**Mann-Whitney U** Test
141 $p=0.004$). Hence, it appears that cartilage material properties differ most in the medial
142 side and the posterior section of the tibial surface suggesting these to be sites of knee
143 specialisation (Fig. 2).

144

145 Furthermore the loading history of the joint will most likely have contributed to
146 the variation seen in the samples. The horses used in this study were from a range of
147 different backgrounds and ages. The youngest equine sample (18 months) displayed a 5
148 fold increase in compression of the cartilage and two thirds of the shear modulus when
149 compared to the older equine samples, despite having the same thickness of cartilage.
150 This is consistent with an age related increase of non-enzymatic crosslinking of equine
151 collagen which has been predicted to give rise to stiffer, less compressible material
152 (Brommer et al. 2003; Brama et al. 1999). However bovines are low impact grazers
153 which are all slaughtered at approximately the same age. This would explain why we saw
154 a smaller degree of inter-sample variation when compared to equines. Hence our
155 rheometric approach also appears to be well suited for investigating developmental
156 changes, or the effect of different lifestyles on the material properties of cartilage.

157

158 **Discussion**

159 Our study using DSA showed significant differences in the posterior sections of bovine
160 and equine tibial cartilage with the equine cartilage being thicker, compressing less and
161 having a greater resistance to deformation. These features support the thesis of
162 specialisation associated with gait such as e.g. faster top and trot speeds in horses
163 compared to cows (10.4 vs. 6.4 and 5 vs. 3.8 ms⁻¹ resp. at comparable body weights)
164 (Taylor 1985) with the argument that faster gaits result in larger forces, which must be
165 accommodated by the storage and recovery of the associated elastic strain energy
166 (Warner et al. 2013). Moreover, when analysing our samples for age differences we
167 observed further details, suggesting further powers of analysis.

168
169 Breeding, as well as natural selection, has contributed to both conformation and
170 gait of the horses and cows studied. Moreover, we must assume that all joints, as well as
171 the bones and indeed the hooves, of these two taxa have evolved since the mid-Miocene
172 period as units; and with ~20M years of independent evolution for each system and
173 thousands of years of selective breeding it is not surprising that most morphological and
174 anatomical features differ in detail (and to various degrees) between the taxa, species and
175 races despite many the overall similarities (Southwood 2003; Pough et al. 1989). Hence it
176 would be impertinent of us to speculate here any further on our findings other than to
177 reiterate the apparent power of the technique used, which provides a novel tool to
178 researchers whether there are interested in the evolution and behaviour of quadrupeds or
179 investigating leg/foot diseases and related joint modifications.

180
181 Our observations have implications for the study of human knee cartilage and its
182 diseases. In particular, osteoarthritis (OA) of the knee displays highly repeatable patterns.
183 Anteromedial OA was first described by (White et al. 1991) and the patterns of cartilage
184 damage in both isolated medial and lateral compartment OA of the knee was later
185 described by (Gulati et al. 2009). These damage patterns are intriguing; in isolated medial
186 OA the cartilage damage is observed on the medial side at the front of the joint, whilst
187 those for isolated lateral OA are observed on the lateral side toward the back of the joint.
188 Humans have evolved from a quadrupedal to a bipedal gait and hence the common

189 observation of repeatable but different cartilage lesion patterns in isolated medial and
190 lateral osteoarthritis in human knees might then be a result of evolutionary drag in the
191 material properties of our knee cartilage. The findings of the current study demonstrate
192 that cartilage material properties differ with location going from the front of the tibial
193 plateau to the back, and the patterns of material property variation are different between
194 bovine and equine samples. The requirements of bi-pedal gait necessitate a dramatic
195 increase in the range of motion of the knee compared to quadrupedal locomotion, thus
196 exposing the posterior part of the joint to functional loading. Evolutionary pressures
197 driving increased knee range of motion may not have driven a significant change in
198 cartilage material properties.

199

200 In conclusion our findings in this study, combined with a recent quantitative
201 validation of DSA in rotator cuff tendon pathology (Chaudhury et al. 2011), suggest that
202 the technique of DSA could offer a new, powerful means to precisely quantify the
203 pathology of osteoarthritis for medical research (although not as a diagnostic in this
204 current guise). This potentially offers a new approach to classification where previous
205 methods have relied mostly upon visual inspection (Gulati et al. 2009).

206

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212

213 **Author Contributions**

214 CH and HSG performed the experiments. All authors contributed to manuscript
215 preparation and writing.

216

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