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The biomechanical properties of the collateral ligaments in the equine distal forelimb

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Abstract

At the gallop, high loading forces are experienced in the equine distal limb, resulting in stresses to the soft tissue structures of the distal forelimb. Ligaments and tendons attenuate and reduce the concussive effects of the forces acting on the limb and their injury is the most frequent cause of musculoskeletal injury (Clayton, 2016; Clegg, 2012; Woo *et al.*, 2000).

Computer models of equine motion estimate the forces in the equine distal limb during motion, providing insight into the biomechanical factors that cause musculoskeletal injury. However, currently models do not account for all structures in the distal limb (Bullimore *et al.*, 2006; Farley *et al.*, 1993; Harrison *et al.*, 2010; McGuigan *et al.*, 2003), particularly the collateral ligaments (CL). This study aimed to determine the biomechanical properties of the collateral and distal sesamoid ligaments of the equine distal forelimb.

CL and the straight and oblique distal sesamoid ligaments were harvested from the proximal interphalangeal joint (PIP), metacarpophalangeal joint (MCP), carpus and elbow joints of the forelimbs of 10 Thoroughbred and 9 other equine breeds (total: 19 horses). The elastic moduli (EM) were determined by tensile testing the ligaments with a strain rate of 1 mms^{-1} after 10 cycles of preconditioning load.

The EM of the ligaments differed significantly between the joints, according to position and function. The highest EM was for Thoroughbred MCP joint CL (63 ± 45 MPa, $p < 0.05$) and the lowest EM for all breeds was the lateral collateral elbow ligament (3 ± 2 MPa, $p = 0.14$). Thoroughbred horses had a significantly higher EM in the CL of the PIP (27 ± 14 MPa vs. 12 ± 7 MPa) and MCP (63 ± 45 MPa vs. 35 ± 15 MPa) joints than the other breeds in the study ($p < 0.05$). There was a large variation in EM, negatively affected by age and, in the distal ligaments, wither height ($p < 0.05$). The mechanical properties described here will be of use in creating the 'Anybody' model of the equine distal forelimb being developed at Massey University to determine the effect of ground surface perturbations on the distal limb.

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List of Abbreviations

CL	Collateral Ligaments
CSA	Cross Sectional Area
CT	Computed Tomography
CV	Coefficient of Variation
EM	Elastic Modulus
GDP	Gross Domestic Product
MCP	Metacarpophalangeal joint – Fetlock
MRI	Magnetic Resonance Imaging
PIP	Proximal interphalangeal joint – Pastern
SD	Standard Deviation

Introduction

<u>Thoroughbred industry</u>

The Thoroughbred racehorse industry is of major economic importance worldwide. In 2009, there were 162,891 Thoroughbred races held in 47 countries with over a quarter of a million different horses starting a race (McManus *et al.*, 2013). The economic impact of the racing industry includes employment, export income and gambling and in the US is estimated to be US$26.1 billion (Peterson *et al.*, 2008). It is one of the largest industries in Australia, contributing 0.5% to gross domestic product (GDP) (Bailey *et al.*, 1997). In NZ, there are 71 Thoroughbred racing clubs, and approximately 500 race meetings held annually, with over 800 trainers, 140 jockeys and 5,800 horses participating in flat and jumps racing (Bolwell *et al.*, 2016, 2017; Perkins *et al.*, 2005a; Rosanowski *et al.*, 2015). The racing sector is estimated to generate over NZ$1.4 billion (approximately 1%) in GDP (Bolwell *et al.*, 2017). Numerous injuries result from racing and training which can significantly alter the ability of a horse to continue to race or result in loss of the animal (Jeffcott *et al.*, 1982; Peloso *et al.*, 1994; Williams *et al.*, 2001). Therefore, wastage is of high economic importance as well as a growing health and welfare concern within the Thoroughbred racing industry.

Wastage can consist of losses by retirement, loss of training days or even death (Bailey *et al.*, 1997; Perkins *et al.*, 2005a). The major reason for wastage is voluntary retirement due to lack of talent or poor performance, accounting for approximately 33% of all loss (Bolwell *et al.*, 2017). Involuntary retirement encompasses injury, illness or accident, and of these, musculoskeletal injury is the next most common reason for loss (Back *et al.*, 2012; Bolwell *et al.*, 2017; Clegg, 2012; Moffat *et al.*, 2008; Perkins *et al.*, 2005c). Musculoskeletal injury accounts for 80% of involuntary interruptions to training and 25% of horses exiting the industry through death or retirement from racing (Perkins *et al.*, 2005b). Musculoskeletal injury results in economic loss due to lost training time, incurring the costs of veterinary visits, diagnosis, treatment, rehabilitation and spelling, and of loss of horses from racing through either retirement or euthanasia due to severe injury (Firth *et al.*, 2004; Harrison *et al.*, 2010).

Over 90% of musculoskeletal injuries involve the distal limb (Perkins *et al.*, 2005c; Williams *et al.*, 2001). A number of risk factors for musculoskeletal injury to the distal limb have been identified in racehorses including: horse age, race type and distance, horse shoe characteristics, training surface and trainer/training schedule (Clegg, 2012; Perkins *et al.*, 2005c; Peterson *et al.*, 2008; Reardon *et al.*, 2013; Williams *et al.*, 2001). These factors indicate that chronic fatigue and ground reaction forces are significant contributors to distal limb injury. Training surface is an important factor that is able to be modified. Softer surfaces are associated with a reduced incidence of distal limb (especially tendon and ligament) injury (Williams *et al.*, 2001). Similarly, there is a reduction in the odds of injury in winter (wet) relative to summer (dry) (OR = 0.53, $p < 0.001$) (Perkins *et al.*, 2005c). In addition, surface perturbations are thought to cause acute, or indirect, loading to the structures in the distal limb, leading to musculoskeletal injury (Riemersma *et al.*, 1996).

The high-speed gallop places large stresses on the musculoskeletal system of the Thoroughbred racehorse (Peterson *et al.*, 2008). The normalised ground reaction forces on the forelimb equate to approximately 100 - 120% of the equine body weight at trot (3.7 ms^{-1}), 120 - 150% at canter (5 - 9 ms^{-1}) and 170% at gallop (14 ms^{-1}) (Hjertén, 1994; Kai *et al.*, 2000; Swanstrom *et al.*, 2005). At racing speeds of 14 - 16 ms^{-1}, these loads are even greater and likely contribute to the high incidence of musculoskeletal injuries observed in racehorses. Musculoskeletal injuries accounted for 82% of injuries in UK racehorses (Williams *et al.*, 2001), 80 - 83% of fatalities in California racehorses (Johnson *et al.*, 1994), and accounts for three times more wastage than all other medical problems in the Thoroughbred industry (Rossdale *et al.*, 1985). The forelimb is the most common site for musculoskeletal injury and fracture, with approximately 80 - 90% of musculoskeletal injuries sustained in racehorses involving the forelimbs (Cogger *et al.*, 2008; Johnson *et al.*, 1994; Perkins *et al.*, 2005c; Williams *et al.*, 2001).

Injuries to the tendons and ligaments are one of the most frequent causes of musculoskeletal injury and early retirement in the Thoroughbred racehorse (Clegg, 2012). They accounted for 46% of all limb injuries in a 3 year surveillance study of UK racehorses between 1996-1998 and were identified as the most frequent cause of injury during racing (6.9/1000 starts) in a cohort study undertaken at 6 racecourses in 2000 and 2001 (Clegg, 2012; Perkins *et al.*, 2005c; Williams *et al.*, 2001). They were the second most common limb injury in NZ after shin soreness - accounting for 17% of lower limb injuries in a selection of 1,571 NZ racehorses from 20 trainers between the years of 1997-2000 (Perkins *et al.*, 2005c).

Tendon and ligaments are mainly injured by overstrain or percutaneous trauma (Avella *et al.*, 2012). Due to low blood supply, ligaments and tendons do not recover as quickly from micro trauma (from training effects) as muscle and bone (Hodgson *et al.*, 2014). Once injured, ligaments and tendons are permanently compromised to a greater or lesser extent, with numerous different treatments and a high reliance on rehabilitation (Avella & Smith, 2012; Back & Clayton, 2012). Indeed, the risk of musculoskeletal injury is higher in horses that have sustained a previous musculoskeletal injury than those without (relative risk 1.4, 95% CI = 1.2 – 1.7; $p < 0.001$) (Perkins *et al.*, 2005b). One of the major efforts in equine research is investigation of factors which can reduce injury during training and racing (Firth *et al.*, 2004). Reducing the incidence of tendon and ligament injury would decrease the monetary costs of wastage and rehabilitation from the racing industry in addition to improving equine welfare.

<u>Anatomy of the equine distal forelimb</u>

In order to investigate the causes of musculoskeletal injury, it is important to understand the anatomy of the equine distal forelimb. Horses are cursorial animals and have unique physiological adaptations to exercise (Hodgson *et al.*, 2014). The four limbs have evolved with heavy musculature confined to the proximal limbs, while the distal limbs (below the carpus and tarsus) have long, lightweight ligaments and tendons to move and support the single functional digit. The limb acts like a spring, storing energy in the palmar soft tissue structures from early stance to end of stance phase, optimized by having a hyperextended metacarpophalangeal (MCP) joint (Avella & Smith, 2012). These energy storing ligaments and tendons also reduce the concussive effects of the forces acting on the horse during movement (Woo *et al.*, 2000). The equine forelimb supports approximately 60% of the body weight of the horse, whilst the more angulated hind legs provide propulsion (Clayton, 2016). This arrangement of proximal muscle mass and light limbs reduces the moment of inertia of the limb and the amount of energy expended in locomotion, allowing the horse to reach high speeds (Clayton, 2016); but means the ligaments and tendons are the sole soft tissue support mechanisms for the distal limb.

The forelimb has a pillar-like structure specialised for weight bearing with the antebrachial and metacarpal segments aligned at the carpus (Clayton, 2016). The suspensory apparatus is an important component of the forelimb, supporting the MCP joint which is constantly under pressure from the body weight of the horse. The suspensory ligament, check ligaments on the flexor tendons and fibrous bands of annular ligaments create a suspensory sling to support the hyperextended MCP joint. The proximal interphalangeal (PIP) joint lacks a suspensory apparatus, so overextension is limited by the distal sesamoid ligaments of the MCP joint attached to the middle phalanx (PII) (Goody *et al.*, 2000). During the stance phase, body weight exerts a force on these tendons and ligaments which hold the MCP and PIP joints steady allowing the horse to stand and graze with no effort (Hermanson *et al.*, 1992). Therefore, the ligaments and tendons of the equine distal forelimb are constantly under stress and are an integral part of both rest and locomotion.

The functions of the musculotendinous system of the equine forelimb include connecting the forelimb to the trunk, supporting the body mass, stabilizing the joints in opposition to the force of gravity during stance phase, generating forces used in locomotion (propulsion, braking, turning) and flexing the joints during the swing phase of movement (Clayton, 2016). At the beginning of stance phase during movement, the limb is passively adducted, resulting in efficient locomotion as the limbs support the body closer to its centre of mass (Back & Clayton, 2012). Power generated at the elbow in early swing phase has been shown to drive the movements of the more distal segments with the amount of motion being controlled passively by the surrounding soft tissues (Lanovaz *et al.*, 1999). The joints of the equine forelimb distal to the elbow are constrained by supporting structures such as collateral ligaments to move in the sagittal plane with relatively small amounts of abduction/adduction and internal/external rotation when under load (Back & Clayton, 2012; Degueurce *et al.*, 1996).

Collateral ligaments (CL) have the important role of supporting each joint medially and laterally, preventing lateral movement to maintain maximum energy transmission and correct limb function (Davankar *et al.*, 1996). Any movement outside the sagittal plane places additional stress on these ligaments. Their possible role in the attenuation of load in the distal limb is largely unknown. CL injuries, though uncommon, are difficult to treat and have limited recovery success (Lamb *et al.*, 2012).

Mechanical properties of ligaments

The structures of the ligaments and tendons in the distal limb are optimised to perform their mechanical roles of force transmission and skeletal support (Avella & Smith, 2012). Ligaments act passively to link bones and resist their disturbance, whereas tendons connect muscle to bone and transform mechanical force from the muscle into movement (Hodgson *et al.*, 2014; Woo *et al.*, 2000). Ligaments and tendons are both composed of linearly arranged collagen fibrils and elastin in a matrix of ground substance composed of water, proteoglycans and other non-collagenous proteins (Hodgson *et al.*, 2014; Rapoff *et al.*, 1999). However, they differ significantly in morphological appearance, biochemical contents and tensile properties (Woo *et al.*, 2000).

Collagen fibres in ligaments are more elastic than tendons and are architecturally oriented to effectively control and constrain joint motion (Martin *et al.*, 2015; Woo *et al.*, 2000). They have a lower strength and stiffness than tendons (lower elastic modulus), but higher extensibility (Ozkaya *et al.*, 1991). Within an animal, ligaments have different mechanical properties and healing responses which relate to structural demands, relative magnitudes of load experienced and their biochemical composition (Hart *et al.*, 1999; Martin *et al.*, 2015; Shetye *et al.*, 2009; Woo *et al.*, 2000).

Both ligaments and tendons are viscoelastic, which means their mechanical properties vary as they are stretched (Avella & Smith, 2012). However, when loaded they produce characteristic load-deformation patterns with a linear region which represents the stiffness of the material (Martin *et al.*, 2015). The elastic modulus (EM) of the ligament substance is dependent on the organisation, orientation and type of collagen fibres and the interaction among tissue constituents such as ground substance (Woo *et al.*, 1990a). It characterises the stiffness of a material irrespective of its geometry, and is the ratio of stress to strain in the elastic region of an uniaxial loading curve. In a uniform uniaxially loaded sample, it can be related to the stiffness of the ligament as follows (Ozkaya & Nordin, 1991):

$$EM = \frac{kl}{A}$$

Where *EM* is the elastic modulus, *k* is the stiffness of the ligament substance, *A* is the cross sectional area (CSA) and *l* is the length of the sample between the opposing forces. The EM can be determined from the slope of the linear region of an *in vitro* stress-strain curve (Bowser *et al.*, 2011; Ozkaya & Nordin, 1991; Woo *et al.*, 2000).

Injury to ligaments and tendons may occur when the functional load equals the elastic limit or ultimate stress, the load at which the tissue ruptures or breaks. This margin is called the biological safety limit. Rupture load is subject to inherent variability between structures and is influenced by factors such as strain rate, so is difficult to estimate. However, tensile strains of the equine flexor tendons of a horse with a rider have been recorded as approximately 3% at walk, 6 - 8% at the trot and 12 - 16% at gallop (Stephens *et al.*, 1989). This is close to measured *in vitro* tendon rupture strains of 12 - 16% (Martin *et al.*, 2015), indicating that tendons and ligaments experience functional strains that are very close to failure loads during peak performance (Back & Clayton, 2012). Therefore, knowledge of the mechanical properties of the ligaments and tendons in the equine distal limb and how they are affected by disparate forces during locomotion is of paramount importance in understanding the effects of ground reaction force on distal limb injury.

Variation in ligaments

A complication in the study of the behaviour of ligaments and tendons is that the variability in mechanical properties between individuals is high (Germscheid *et al.*, 2011; Gijssen *et al.*, 2004; Rapoff *et al.*, 1999; Woo *et al.*, 1983). In a population of normal horses, there is more than a twofold variation in the ultimate strength of the superficial digital flexor tendon (Avella & Smith, 2012; Thorpe *et al.*, 2010). The coefficients of variation of the elastic moduli of medial CL in one species of rabbit ranged from 13% to 52% (Woo *et al.*, 1990b). This variance is most likely caused by genetic components such as horse height/mass or ligament CSA, and environmental factors such as ageing and exercise but it could also arise from inaccuracies in measurement of mechanical properties *in vitro* (Avella & Smith, 2012).

It has long been acknowledged that accurate and reproducible measurement of ligament tensile properties is notoriously difficult. *In vitro* measurements depend on many factors, including fibril orientation, temperature and hydration of the ligament or tendon specimen, strain rate and previous storage (Martin *et al.*, 2015; Woo *et al.*, 2000). In addition, measurements *in vitro* do not completely predict *in vivo* action (Hodgson *et al.*, 2014). This was confirmed by findings that the tendon strain measurements *in vivo* were different to *in vitro* findings for the same pony used in a study measuring tendon strain in the forelimbs of ponies (Riemersma *et al.*, 1996). However, it was hypothesized that the differences were due to the restrictions necessary to complete *in vitro* tests - where normal muscular tension was absent during movement simulation and the humerus length was compromised to fit in the testing apparatus. Investigation of the disparate structures in the equine distal limb and how they interact could be integral to the understanding of ligament and tendon injury.

Genetic components and environmental factors affecting ligament and tendon properties include age, exercise, height or mass and breed. The mechanical properties of ligaments change with age - increasing to maturation and declining in old age (Martin *et al.*, 2015; Woo *et al.*, 1990a). Exercise has been shown to increase tensile strength in extensor tendons in pigs (Woo *et al.*, 1980), but in horses differences are small and more evident in young animals and when compared with immobilisation (Martin *et al.*, 2015; Moffat *et al.*, 2008). There are a number of studies that have highlighted that increased size (wither height) may predispose the horse to musculoskeletal injury (Ducro *et al.*, 2009; Dyson, 2017; Murray *et al.*, 2010; Parkes *et al.*, 2013). Larger animals have an associated faster growth rate which may be an intrinsic factor shaping structural limb quality, perhaps weakening the mechanical durability of the structures in the limb and affecting ligament and tendon quality (Barneveld *et al.*, 1999; Ducro *et al.*, 2009; Hodgson *et al.*, 2014). Indeed, the development of the CSA of equine flexor tendons has been reported to vary significantly in four different breeds of 2 year old horses during a year of observation, perhaps reflecting their different growth rates (Koster *et al.*, 2014). However, these effects are difficult to distinguish due to the high variability between individuals.

Both ligaments and tendons have been demonstrated to have large variations in mechanical properties that reflect the functional demands put upon them (Avella & Smith, 2012; Martin *et al.*, 2015; Shetye *et al.*, 2009; Woo *et al.*, 2000). The mechanical environment during maturation is thought to drive the growth of ligaments and tendons, so their structural properties at maturity relate to their function (Avella & Smith, 2012; Rogers *et al.*, 2008a). Significant differences in collagen fibril diameter distributions were observed between nine different ligaments and tendons in the equine carpus, indicating that their composition and thus properties are dependent on their function and position in the carpus (Davankar *et al.*, 1996). In five species (sheep, monkey, goat, horse and dog), the EM of the anterior cruciate ligament was greater than the posterior (Vysotskii, 1975). These differences were comparable between individuals and across species despite the high individual variation in mechanical properties.

Thoroughbred horses have been bred specifically for racing and typically have a deep chest, lean body, long flat muscles, slender legs and a light build (Willoughby, 1974). The high forces experienced by the distal limb at speed coupled with these attributes may compromise the structural stability of the Thoroughbred musculoskeletal tissues, causing Thoroughbreds to be more at risk of musculoskeletal failure (Hodgson *et al.*, 2014). Therefore, determination of the load distribution in the structures of the equine distal forelimb during high-speed locomotion could provide insight into the causative factors contributing to musculoskeletal injury in the Thoroughbred racehorse.

<u>Gait models</u>

Dynamic gait models have been designed in order to understand the biomechanics of locomotion and the forces in the distal limb of mammals. These models use the mechanical properties of ligaments and tendons to determine leg stiffness in order to model the distal limb as a passive spring in mammals of differing species and size (Bullimore & Burn, 2006; Farley *et al.*, 1993; McGuigan & Wilson, 2003; Meershoek *et al.*, 2001). Ligament and tendon properties are assumed to act similarly in all species (Bullimore & Burn, 2006), with the effect of breed and size on distal limbs largely unknown. The EM of the ligaments and tendons is required to accurately model the forces in the distal limb.

Simple models represent the distal limb as a passive elastic strut or simple spring-mass system, similar for all sizes and species of quadrupeds, with larger animals having stiffer leg springs (Bullimore & Burn, 2006; Farley *et al.*, 1993; Herr *et al.*, 2002). However, relative stride length (a dimensionless parameter used to normalise the movements of differently sized animals) measurements of experimentally observed and modelled horses found that the assumed effect of size on leg stiffness to be greater than that expected from the model (Bullimore & Burn, 2006). Due to the complexity of the many integrated structures in the equine distal limb and observed discrepancies of movement between real and modelled horses, it is evident that there are unknown factors in the leg affecting the assumed stiffness for biomechanical use.

The complementary actions of the passive suspensory apparatus and active muscular activation during movement provide challenges to computer modelling in quantifying the passive and active forces of each (Harrison *et al.*, 2012). Few studies have considered the mechanical interactions that affect the function of the entire structure of the equine forelimb. McGuigan and Wilson (2003) modelled the forelimb as 2 springs, determining that most of the length change during the stance phase of locomotion occurred in the distal limb (from elbow to hoof), mainly at the MCP joint. A pulley model was used to determine the forces in the flexor tendons during movement in the sagittal plane (Meershoek *et al.*, 2001) and was expanded to a 3D model by Rollot *et al.* (2004) which indicated that PIP joint flexion plays an important role in decreasing the strain in the distal sesamoid ligaments. More complex models represent the forelimb in 8 parts with 9 muscle-tendon structures and 6-16 ligaments (Brown *et al.*, 2003; Harrison *et al.*, 2012; Swanstrom *et al.*, 2005). These models are restricted to motion in the sagittal plane, but indicate that the passive ligament and tendons in the distal limb may have a larger contribution to torque generation and strain dispersal during movement than previously thought. In particular, the distal sesamoid ligaments limit PIP joint overextension and their role in strain dispersal is little studied.

A new computer model of the equine forelimb, using the 'Anybody' platform, is currently under development at Massey University. The biomechanical properties of bone, muscle, tendon and ligament structures will be used to accurately model the forces in the forelimb during equine motion. This will be used to investigate the effects of surface perturbations on the gait and compliance of the equine distal limb – one of the risk factors for tendon and ligament injury in Thoroughbred racehorses.

In order to understand the contribution of individual ligaments to the overall stiffness characteristics of the distal limb, data on the tensile properties of each ligament are essential. The specific properties of the CL in the equine distal limb have not been studied. This contrasts with our knowledge of the larger ligaments and tendons (deep digital flexor tendon, superficial digital flexor tendon, accessory ligament) (Becker *et al.*, 1994; Harrison *et al.*, 2010; Pourcelot *et al.*, 2005; Riemersma *et al.*, 1996; Thorpe *et al.*, 2010). CL play a critical role in limb stabilisation, bracing each joint to restrict medial/lateral movement (Abramowitch *et al.*, 2003b). In addition, little is known about the properties of the distal sesamoid ligaments, despite their important interaction with PIP and MCP joint flexion during locomotion (Rollot *et al.*, 2004). The addition of collateral and distal sesamoid ligaments to the 'Anybody' dynamic gait model will provide a more accurate representation of the forces in the distal forelimb than is currently known.

Aims

The aim of this study was to determine the elastic modulus of the collateral ligaments and distal sesamoid ligaments of the equine distal forelimb. A secondary aim was to determine if and how the elastic modulus of the collateral ligaments of the equine distal forelimb are influenced by horse breed and size.

Methods

Ligament selection

Collateral ligaments were collected opportunistically from the forelimbs of horses that were humanely euthanized for post mortem with no orthopaedic issues in their harvested limbs at Massey University's School of Veterinary Science. Horses with any obvious pathology of the ligaments were excluded from the study as not representative of the population of 'normal' ligaments. The height, weight, breed, reason for euthanasia and background of each horse was measured and recorded.

Sample size

The sample size estimates were calculated using data of the EM from the equine superficial digital flexor tendon (1217.0 ± 199.4 MPa, range 819.4-1635.8 MPa) (Thorpe *et al.*, 2010) as there were no published values for the EM of equine CL within the literature. Using this reference data, it was estimated that ligaments from 15 horses were required to give 85% power to fit a regression line with an effect size of 0.35 (measured by Cohen's $f^2 = r^2 / (1 - r^2)$) (Snedecor *et al.*, 1980).

Ligament harvest

The medial and lateral CL from the PIP, MCP, carpal and elbow joints in addition to the straight and oblique distal sesamoid ligaments of the two forelimbs were harvested post mortem within hours of euthanasia where possible, otherwise as soon as possible after refrigeration at 3°C. The ligaments were cut from their attachment sites on either side of the joint, ensuring that the band of parallel ligament fibres was left intact. The ligaments were kept hydrated in water and frozen in labelled airtight plastic bags at -20°C until subsequent testing in the materials lab (Woo *et al.*, 1986).

Measurements

The CSA of the frozen and thawed ligament samples were measured using dial callipers with a precision of ± 0.02 mm (*Mitutoyo series 505-633-50*). The ligaments were assumed to have a rectangular cross section following the methods of Germscheid *et al.* (2011); Shetye *et al.* (2009); Woo *et al.* (1983). Measurements were taken in the middle of the ligament. On 6 ligaments, the CSA was measured in 5 different places to investigate the consistency of the measurement.

Samples were thawed at room temperature for a minimum of 6 hours before testing. Ligaments were blotted on paper towels to remove excess fluid before being placed in serrated jaw clamps in a materials testing machine (*Stable Microsystems TA.XT plus texture analyser*) to elongate the ligament to failure in a controlled environment with room temperature of 20°C. The cross sectional dimensions of the ligaments were measured again, under a physiologically low tension (10 N) – a negligible load of < 1% of biological failure load (Thorpe *et al.*, 2010) and in all cases between 1 - 20% of the maximum load reached. Width and depth measurements were taken from the mid-substance of the ligament and subsequently compared with the frozen measurements. The resting length of the ligament was taken as the gauge length - the distance between the crossheads of the clamps when the sample was under 10 N of tension.

Each sample was exposed to 10 cycles of preconditioning load from 0-50 N at a crosshead speed of 1 mms^{-1} to ensure a consistent strain history was applied to each specimen (Martin *et al.*, 2015; Thorpe *et al.*, 2010; Woo *et al.*, 2000). Immediately following these cycles, the sample was tested to failure at a stretch rate of 1 mms^{-1}. The crosshead displacement and applied force were recorded simultaneously at a rate of 200 pps (data points per second) with *Exponent* software (TEE32, *version 6*). Sample rupture or slip from the clamp was recorded for each test. Each test was conducted within 3 minutes of removal of the thawed hydrated ligament from the plastic bag kept at room temperature.

Mechanical properties of ligaments

The mechanical properties of the CL were determined using engineering definitions. Stress-strain relationships were calculated for each ligament (Figure 1.) showing a toe region where the collagen crimps were removed by elongation and linear region where the collagen fibres were stretched (Martin *et al.*, 2015). The EM was measured from the linear region between a minimum of 2% and maximum of 35% of strain before the linear region was exceeded.

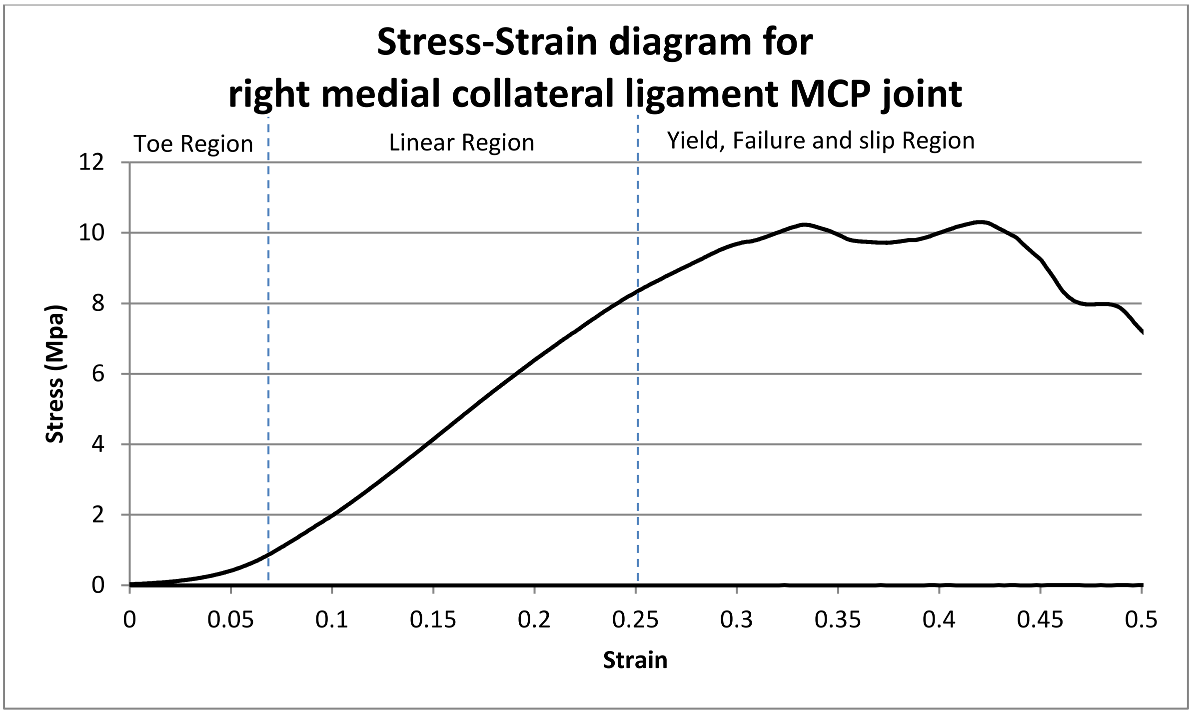

Figure 1. Typical curve showing the stress-strain relationship of the right medial collateral ligament in the MCP joint of horse 7. There is a clear linear region between the toe region and failure region, from which the elastic modulus was calculated.

The tensile stress was found from the applied force using the following equation:

$$\sigma = \frac{F}{A}$$

Where *F* is the applied force (N) and *A* is the cross sectional area (mm^2) of the tendon under 10 N of tensile force. Stress (σ) is expressed in megapascals (1 Mpa = 10^6 Nm^{-2}).

Strain was calculated as:

$$\varepsilon = \frac{\Delta l}{l}$$

Where *Δl* is the change in length (taken as the crosshead displacement in mm) and *l* is the resting length of the ligament (mm), taken as crosshead distance at 10 N. Strain (ε) is a dimensionless parameter.

The stress-strain curves were examined to determine the EM (Bowser *et al.*, 2011; Woo *et al.*, 2000). The linear region of slope of the stress – strain curve (between the toe region and failure/slippage) was used to determine the EM by finding the slope of the line of best fit. The EM has units of pressure and is expressed here in megapascals (MPa).

Repeatability of tests

The repeatability of the EM measurement was determined in 6 different ligaments (left lateral collateral PIP joint, left medial oblique sesamoid, left straight sesamoid, right medial collateral MCP joint, right lateral collateral carpus, right lateral collateral elbow). For each, the stress-strain measurements were taken 5 times consecutively by immediately re-clamping and re-testing the slipped end of the ligament. The EM was calculated separately from the CSA measurement taken before each of the runs, and compared for that sample.

Data analysis

Data were initially examined using descriptive statistics and boxplots to determine if there were any significant differences between the EM of the left and right leg, anatomical site, lateral/medial differences or breed. Data were tested for normality using the *Shapiro-Wilks* test. All data were not normally distributed and differences between groups were tested using the non-parametric *Kruskul-Wallis* test. Selective comparisons were made using pairwise *Wilcox* non-parametric tests.

The EM and CSA of the ligaments from each joint were regressed against measures of horse size, age, sex and breed. A stepwise multiple linear regression model and two-way analysis of variance (*ANOVA*) were used to determine relationships between these variables ($p < 0.05$).

Simple linear correlations were used to compare both horse height and weight and CSA measurements of the frozen and thawed (under 10 N of tension) ligaments.

All statistical tests were performed using 'R Studio' (Version 1.0.143) (RStudio, 2015).

Results

Horses

Ligaments from 39 forelimbs of 20 horses were sampled. Horse data are summarised in Table 1. The horses ranged in withers height from 102 cm – 175 cm. Height data from 2 horses were not recorded, so were estimated by extrapolating the linear height-weight correlation graph. Live weight ranged from 180 kg – 637 kg. Breeds included Thoroughbred (n=10), Standardbred (n=2), Miniature horse (n=1), Shetland (n=1) and Crossbred/Warmblood (n=6). The miniature horse was excluded from the statistical analysis due to the clinical diagnosis of laxity of the ligaments in the hind limbs, leaving 37 forelimbs from 19 horses. The horses were aged 1 to 19 years (median 12 years). Reasons for euthanasia included colic (n = 3), laminitis (n = 3), neurological disorders (n = 3), lameness (n = 2), distal limb fracture (n = 2), failed surgical recovery (n = 2) and untreatable medical conditions (n = 4). A range of horses with both active (such as race training or eventing) and inactive backgrounds (paddock mates, teaching horses) were included in the study.

Table 1. Summary of horses included in the study

Horse	Height cm	Weight kg	Age yrs	Gender	Breed	Reason for Euthanasia	Background
1	161	535	7	Gelding	Thoroughbred	Laminitis	Pleasure riding
2	160	480	7	Mare	Thoroughbred	Unwanted paddock mate	Hacking
3	151	400	2	Mare	Thoroughbred	Hind Limb fracture	Race training
4	140	400	18	Mare	Thoroughbred	Neurological Disorder	Eventer
5	149	450	19	Mare	Quarterhorse Cross	Recurring Uveitis	Pleasure horse/hack
6	152	488	16	Gelding	Stationbred	Heart problem	LATU teaching horse
7	169	564	8	Gelding	Thoroughbred	Chronic Laminitis	Race training
8		637	12	Gelding	Stationbred	Lesion on deep digital flexor tendon	-
9	175	631	8	Gelding	Crossbred	Colic	SJ/Hunting
10	156	410	14	Gelding	Stationbred	Choker	Eventer
11	147	330	1	Gelding	Thoroughbred	Wobbler - Spinal Ataxia	Yearling
12	162	485	19	Gelding	Thoroughbred	Broken Pelvis	Pleasure riding
13	102	180	10	Mare	Shetland	Laminitis	-
14	157.5	505	13	Mare	Standardbred	Chronic Hoof Abscess	LATU teaching horse
15	163.5	552	17	Gelding	Thoroughbred	Laminitis	Ex Eventer
16	153	400	1	Gelding	Thoroughbred	Wobbler - Spinal Ataxia C5/6	Yearling
17		495	15	Mare	Warmblood	Colic	Broodmare
18	158	450	16	Mare	Warmblood	Ruptured abdominal tendon	10 mths pregnant
19	170	600	11	Gelding	Thoroughbred	Colic	-

Consistency of measurement

Ligaments were harvested from within 6 hours of euthanasia in 13/19 cases, after 1-3 days of refrigeration in 5/19 cases and after more than 1 week of refrigeration in 1/19 cases. The ligament slipped out of the clamps in the majority (372/388) of cases before failure. However, a clear linear region was observed in the stress-strain curve prior to slippage or failure, allowing the EM to be calculated.

The coefficient of variation (CV) of the width measurements for the frozen ligament was 9% and that of thickness was 12%. The CV of the width measurements of the thawed ligament under 10 N of tension was 9% and that of thickness was 10%. There was a strong positive correlation between the two measurements (CSA at 10 N = 0.83* CSA frozen – 9.45, $r = 0.73$, $p < 0.05$). The CSA of the thawed ligaments varied by an average of 14% along their length. The pooled CV of the EM measurements was 32%.

Elastic modulus

There were no significant differences in EM between left or right leg. The EM of the CL differed significantly between the different joints studied ($p < 0.05$), with the MCP joint CL having the highest EM of 49.4 ± 36.5 MPa (mean ± SD) for all breeds (Figure 2) and highest EM of 63 ± 45 MPa (mean ± SD) for Thoroughbreds only.

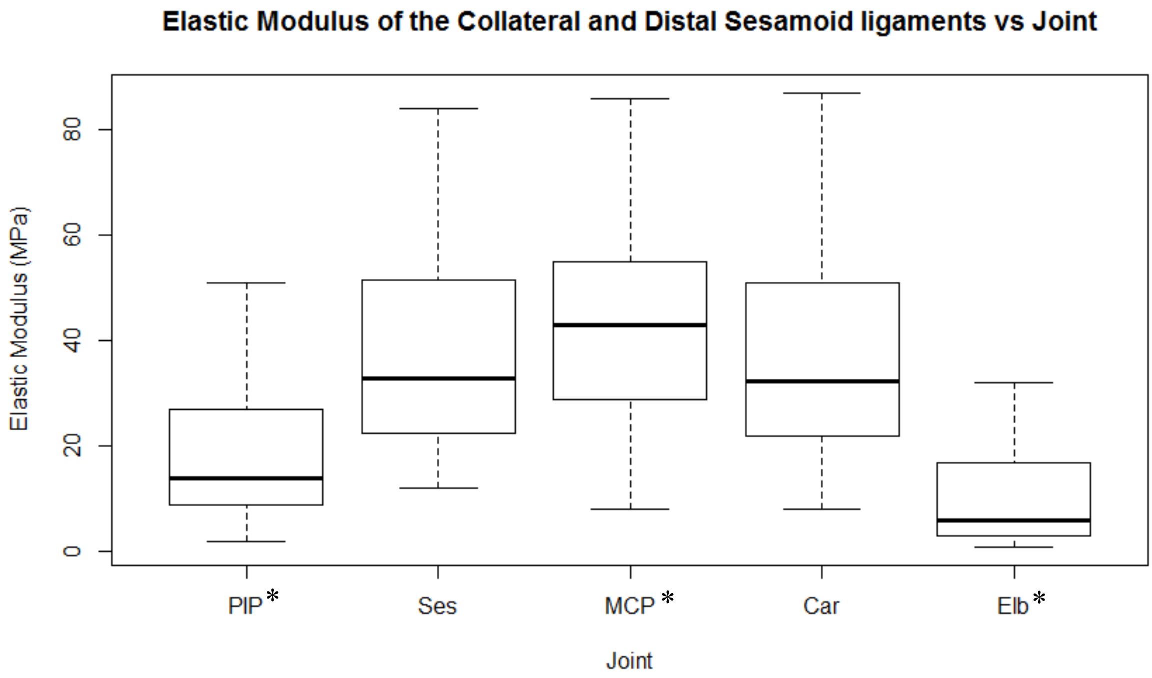

Figure 2. Elastic modulus of the CL for the PIP joint (PIP), distal sesamoid ligaments (Ses), CL for the MCP (MCP), carpal (Car) and elbow (Elb) joints of the equine distal limb. * denotes significant differences between joints (pairwise *Wilcox* tests, $p < 0.05$).

The mean and standard deviation (SD) of the EM and CSA of the CL for each joint is presented in Table 2 for Thoroughbreds separate from the other breeds included in the study. There were significant differences between the EM of the lateral and medial CL for the carpus and elbow. Lateral and medial differences between CL are presented separately where significant differences exist. The lateral elbow CL has the lowest EM (3 ± 2 MPa) for all breeds of horse. The EM of the collateral and sesamoid ligaments showed a wide variation between individual horses (Table 2.).

Thoroughbreds (n = 10) had a significantly higher EM and smaller CSA in their CL for the PIP and MCP joints than the other breeds (6 Crossbreeds, 2 Standardbreds and 1 Shetland) used in the study (Table 2).

Table 2. Mean ± standard deviation values for the elastic modulus and cross sectional area of the collateral ligaments from the distal forelimb of 19 horses. Thoroughbred (n = 10), Other – includes Crossbreeds (n = 6), Standardbreds (n = 2) and Shetland (n = 1). *Kruskal-Wallis* test for difference between breeds with significance level of 0.05. p_{breed} values < 0.05 show statistical significance between the breeds.

Collateral Ligament	Elastic Modulus (Mpa)		p_{breed}	Cross Sectional Area (mm²)		p_{breed}
PIP Joint			8.4E-06			2.0E-05
Thoroughbred	27	± 14		65	± 35	
Other	12	± 7		123	± 60	
All breeds	19	± 13		96	± 57	
Oblique Sesamoid			0.31			0.94
Thoroughbred	37	± 18		38	± 11	
Other	34	± 22		39	± 15	
All breeds	35	± 20		38	± 13	
Straight Sesamoid			0.22			0.02
Thoroughbred	50	± 25		59	± 12	
Other	39	± 18		49	± 12	
All breeds	44	± 21		53	± 13	
MCP Joint			1.4E-04			1.80E-03
Thoroughbred	63	± 45		35	± 13	
Other	35	± 15		48	± 20	
All breeds	49	± 36		41	± 18	
Lateral Carpus			0.43			0.60
Thoroughbred	47	± 21		64	± 46	
Other	40	± 14		58	± 18	
All breeds	44	± 18		61	± 35	
Medial Carpus			0.57			0.95
Thoroughbred	42	± 35		80	± 32	
Other	26	± 12		80	± 14	
All breeds	34	± 27		80	± 24	
Lateral Elbow			0.14			0.01
Thoroughbred	4	± 3		304	± 87	
Other	3	± 1		376	± 84	
All breeds	3	± 2		338	± 92	
Medial Elbow			0.06			0.30
Thoroughbred	64	± 99		69	± 41	
Other	33	± 55		94	± 64	
All breeds	51	± 84		79	± 52	

Horse height had a weak negative relationship with the EM of the PIP joint CL only (r = -0.29, p = 0.02) as shown in Figure 3.

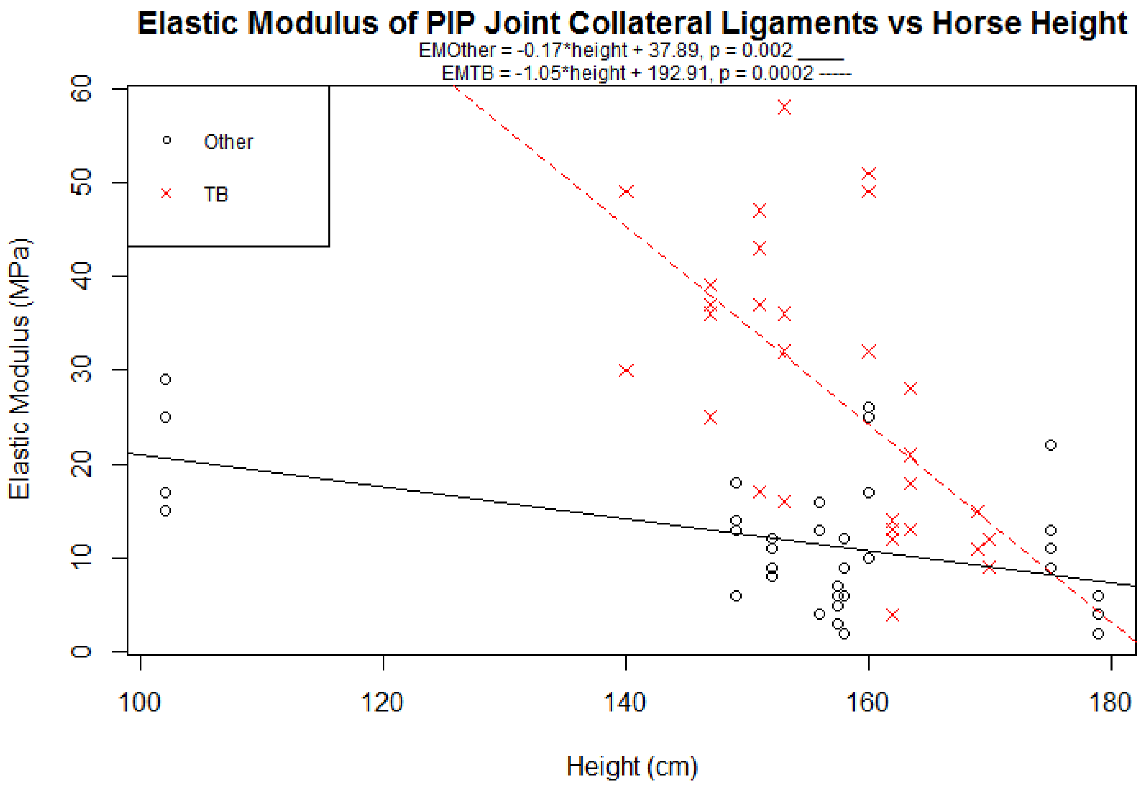

Figure 3. Elastic modulus of PIP joint CL vs horse height for Thoroughbreds and other equine breeds, TB – Thoroughbred (n = 10), Other – includes Crossbreeds (n = 6), Standardbreds (n = 2) and Shetland (n = 1). P value from linear regression model.

The EM for the MCP joint, carpus and elbow CL and the oblique and straight distal sesamoids of the equine distal forelimb were independent of horse height. There was a weak, though significant (p = 0.04) negative relationship between MCP joint EM and height for Thoroughbreds only (Figure 4). This relationship was driven by 3 outlying values, each of which came from 1 year old Thoroughbred geldings. With these values omitted, no relationship existed between MCP joint EM and horse height for Thoroughbreds.

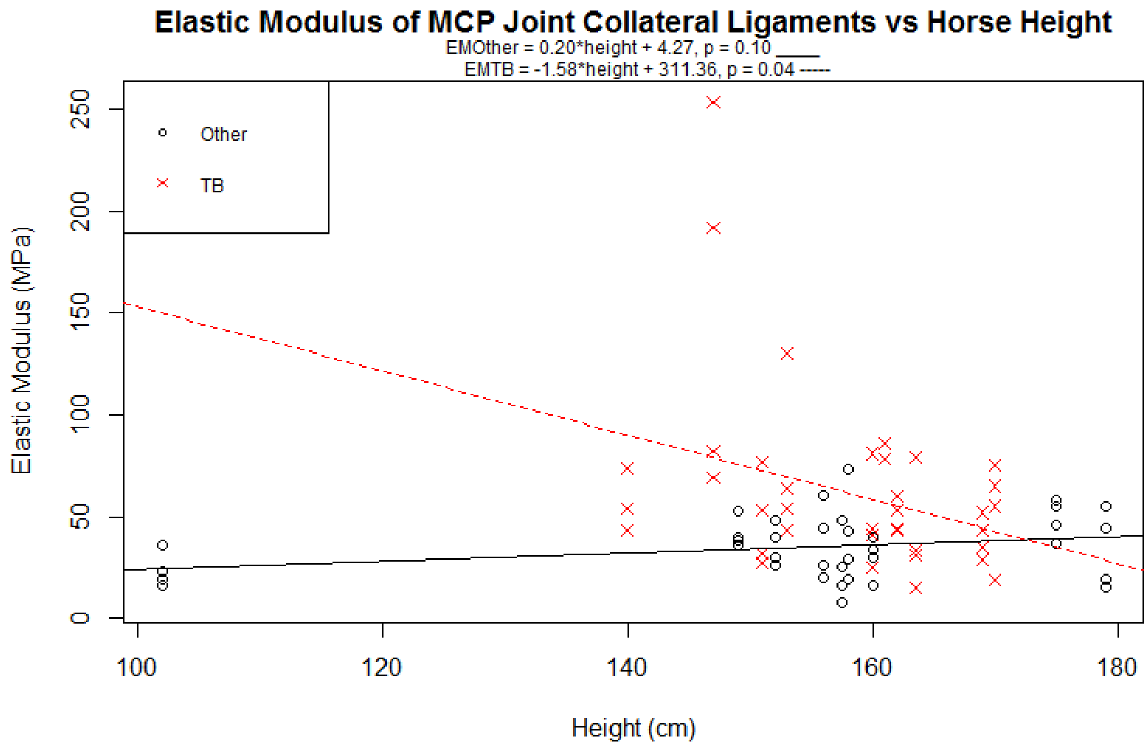

Figure 4. Elastic modulus of MCP joint CL vs horse height for Thoroughbred and other breeds of horses, TB – Thoroughbred (n = 10), Other – includes Crossbreeds (n = 6), Standardbreds (n = 2) and Shetland (n = 1). P value from linear model.

There was a significant positive correlation between the EM of the CL of the PIP, MCP and carpal joints. Figure 5 shows the inter-horse correlation between the PIP and MCP joint CL with the carpal CL.

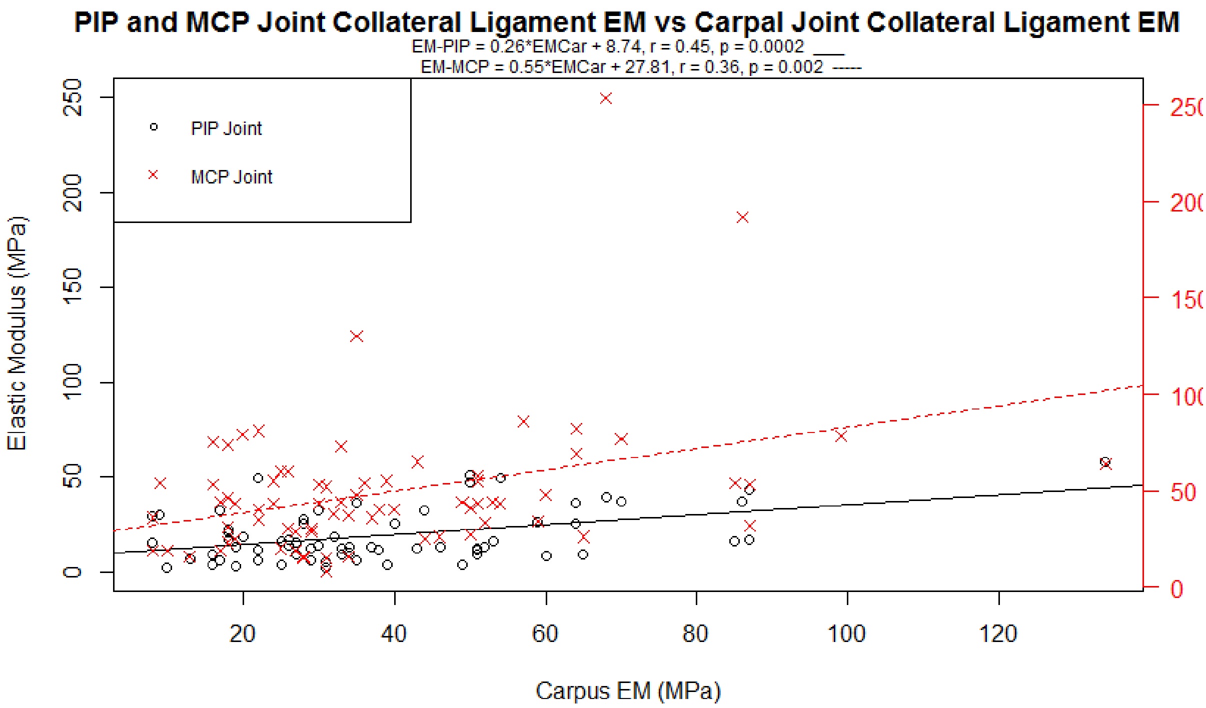

Figure 5. PIP and MCP joint elastic modulus plotted against carpus elastic modulus for the collateral ligaments.

Age had a weak negative correlation with the EM of the CL of the PIP, MCP joint, carpus, and medial elbow CL. Mares had a significantly lower EM (39 ± 19 MPa) than geldings (57 ± 44 MPa) in the MCP joint only for all horses (mare n = 8, gelding n = 11, p = 0.02), however when breed was taken into account there were no significant differences.

<u>Cross sectional area of the collateral ligaments</u>

The CSA of the CL differed significantly with each joint, and medially - laterally for the carpus and elbow as shown in Figure 6 and Table 2. The CSA of the PIP joint, oblique sesamoid ligaments and lateral elbow CL all had a weak, though significant positive relationship with horse height ($p < 0.05$). The CSA of the straight sesamoid ligaments, MCP joint, carpus, and medial elbow CL were independent of horse height.

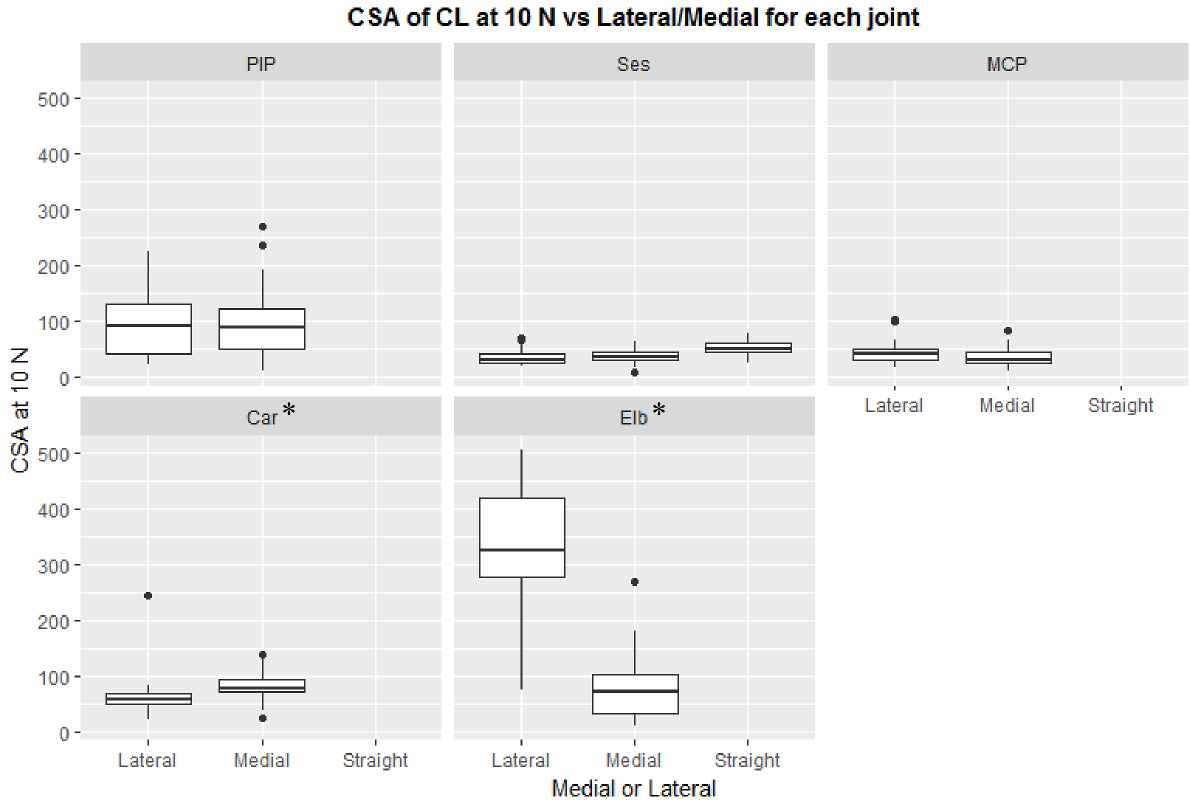

Figure 6. Cross sectional area (CSA) under 10 N of tensile force for the lateral and medial CL of each joint. *Kruskal-Wallis* and *Wilcox* pairwise tests with significance level of 0.05 show that there were significant differences (as denoted by *) between the lateral and medial CSA of the CL in the carpus and elbow only.

Discussion

This study provides an assessment of the biomechanical properties of the collateral ligaments from the PIP, MCP, carpal, and elbow joints as well as the distal sesamoid ligaments of the equine forelimb.

<u>Mechanical properties of ligaments and their function</u>

There were significant differences between the elastic moduli of the ligaments. The differences were consistent with the joint position and function of each ligament and reflected the disparate forces experienced by the ligaments in each of the joints of the equine distal forelimb. These findings complement previous research that has shown that the mechanical properties of ligaments vary according to their position and functional demands (Hart *et al.*, 1999; Martin *et al.*, 2015; Shetye *et al.*, 2009; Woo *et al.*, 2000). For example, Davankar *et al.* (1996) described that the differences between structures (and thus imputed biomechanical properties) of nine ligaments in and around the equine carpus were more significant than the differences in the same structure between horses.

This is the first study of equine limbs to show that ligament EM varied distally to proximally along both forelimbs. Previous studies have shown that the MCP and carpal joints experience the highest peak joint forces in the distal limb during locomotion (Harrison *et al.*, 2010) and that the distal sesamoid ligaments are the main limiter of PIP overextension, experiencing a large amount of force (Goody & Goody, 2000). Therefore, the higher EM observed in the CL of the MCP joint, carpus and distal sesamoids are likely a direct result of their biological function and the higher stresses to which they are exposed.

The present study also showed that the EM of the lateral and medial CL were similar in the PIP and MCP joints. Lateral-medial similarity in the PIP and MCP joints could reflect the function these ligaments play in supporting the suspensory apparatus of the equine forelimb. They form part of the network of soft tissues that tightly wraps the distal extremities and is constantly loaded with the force of body weight. These joints are less able to move due to the structure of the bones, and thus the CL experience equal stresses. Indeed, minimal extra sagittal motion has been measured in the PIP and MCP joints during trot in a straight line, as opposed to both the carpus and elbow which experience a greater degree of lateral-medial motion (Back & Clayton, 2012; Clayton *et al.*, 2004).

The EM of the medial elbow and lateral carpal CL were higher (and less elastic) than their counterparts. This was in agreement with canine carpal CL studies in which the EM of the lateral CL was greater than the medial CL, though the differences were not significant ($p = 0.196$) (Shetye *et al.*, 2009). The size disparity between horses and canines could result in an exaggeration of these differences which are consistent with their function to constrain extra sagittal movement and attenuate disparate concussive forces. During locomotion, passive adduction of the distal limb is constrained by the lateral elbow CL, which requires elasticity to allow the radius and ulna to rotate slightly outwards in flexion, supported by the stiffer medial elbow CL. The hoof then impacts the ground on the lateral side, followed by a medial rocking motion of the hoof during landing (Chateau *et al.*, 2006). This initial impact places the majority of strain on the lateral aspect of the carpus as the joint furthest from the hoof unsupported by the trunk of the body, and the high EM of the lateral carpus CL is in accordance with this function. This effect was also found in the collagen fibril distribution of Thoroughbred carpal ligaments which indicated that the lateral carpal CL was subject to significantly higher early long term stress than the medial, although this was only evident before the age of 2.5 years (Davankar *et al.*, 1996).

<u>Genetic and environmental variation in ligament elastic modulus</u>

In addition to EM differences between anatomical sites, wide variation in mechanical properties between individuals was found in the present study, as has been observed in previous studies of mammalian ligaments (Becker *et al.*, 1994; Brett *et al.*, 2014; Germscheid *et al.*, 2011; Qian *et al.*, 2014; Siegler *et al.*, 2016; Thorpe *et al.*, 2010; Woo *et al.*, 1990a). Breed, height, and age had some influence on the EM, notably the CL from the more distal PIP and MCP joints. Thoroughbreds had a significantly higher EM than other equine breeds for the CL of these joints, and a weak negative relationship with horse height and age. Thoroughbred horses have a light build with proportionally longer, more slender distal limbs than other equine breeds (Willoughby, 1974). Differences in EM with breed has also been observed in two breeds of pigs, where the EM of the carpal CL of the lighter weight breed were higher than the heavier breed (400.0 ± 47.5 MPa vs 327 ± 54.4 MPa, $p =$

0.03) (Germscheid *et al.*, 2011). In addition, a higher EM in the CL could produce a tighter, better performing joint with less deflection, resulting in selective breeding for these higher performing racehorses.

Another explanation for the higher EM values in Thoroughbreds could be the adaptation of ligaments to the higher forces that early race training experienced by most racehorses impose (Rogers *et al.*, 2008b). Adaptive response of ligaments and tendons to exercise is not well documented, but evidence suggests that musculoskeletal tissues can be conditioned during the early growth phase of the animal (Avella & Smith, 2012; Helminen *et al.*, 2000; Moffat *et al.*, 2008; Rogers *et al.*, 2008a). The high outlying EM values for the MCP joint CL were from two 1 year old Thoroughbred geldings. Horses reach appendicular skeletal maturity at approximately 3 years, with the closure of the epiphyses in the MCP joint occurring between 8 - 14 months (Strand *et al.*, 2007; Yoshida *et al.*, 1982). Skeletal maturity has been shown to influence the mechanical properties of rabbit CL, with the EM increasing by 30 - 50% until maturity and then gradually declining with age (Woo *et al.*, 1990a). Training data were unavailable, so it was unknown whether the yearlings experienced early training prior to euthanasia. However, if they did, the high EM values could be an early adaptive response of the ligaments to this training. This effect could interact with the effects of breed and age of the horse. Unfortunately there were no data available for skeletally immature horses that were not Thoroughbreds, so this was unable to be investigated.

The decline in the mechanical properties of ligaments with age is a well-documented phenomenon (Back & Clayton, 2012; Martin *et al.*, 2015; Woo *et al.*, 1990a) and was evident in the EM of the PIP, MCP, carpus and medial elbow joint CLs of this study. This is due to accumulative micro damage and degeneration of the ligament substance (Avella & Smith, 2012). The absence of a relationship in the distal sesamoid ligaments and lateral elbow CL could be due to their inherent properties, or may be affected by introduced errors from the limitations of the study and difficulties of *in vitro* ligament property measurement. This could also be the reason for the lower EM observed in the fetlock CL of mares, although this finding is consistent with generally more compliant ligaments in female humans and pigs (Kiapour *et al.*, 2015; Martin *et al.*, 2015).

The negative relationship with horse height and EM in the PIP and MCP joints contrasts with previous findings of elastic property independence between tendons and body mass (Pollock *et al.*, 1994). However, in the larger CL studied, this independence was supported. The smaller, more distal ligaments could be more affected by the faster growth rate associated with greater wither height in horses than the larger proximal CL, which would lead to the observed compromise in ligament properties (Hodgson *et al.*, 2014). Larger horses are likely to have greater bending moments in the distal aspect of their limbs (Murray *et al.*, 2010). As the larger moments lead to higher stresses in the ligament, the lack of increase in ligament EM may account for the higher rates of injury observed in taller horses as they operate closer to the safety margins of their ligaments (Dyson, 2017; Murray *et al.*, 2010; Parkes *et al.*, 2013).

<u>Methodological limitations</u>

Data were limited by the opportunistic nature of collection to 37 forelimbs from 19 horses of varying breeds. However, despite high individual variation, this was sufficient to demonstrate the aforementioned significant differences in elastic moduli of ligaments between the joints of the distal forelimb, the lateral or medial aspect, breed, height and age of the horse. The dataset provided a good cross-section of horses, being representative of horses in both active work (such as race training or eventing) and those not (paddock mates, teaching horses). The ligaments were harvested from their attachment sites in a band of parallel fibrils and tensile tested *in vitro*.

The *in vitro* measurement of EM is influenced by a number of variables, including the geometry of the tissue (CSA, length and shape), strain rate/elongation, temperature and hydration. Equine ligaments were assumed to have a rectangular CSA, following the methods of Germscheid *et al.* (2011), Shetye *et al.* (2009) and Woo *et al.* (1983) in studies of CL in pigs, dogs and rabbits. Due to the proportionally long limbs of the horse, deviations from a rectangular CSA may be greater than in other species, and the CSA was found to vary by 14% along the length of the sample. This variation likely accounted for some of the large uncertainty in the EM. However, mid-substance width and thickness measurements were used to calculate CSA of the ligaments and the CSA of the PIP and MCP joint CL and distal sesamoid ligaments were in agreement with MRI/CT data from a single Thoroughbred mare as shown in Table 3 (Swanstrom *et al.*, 2005). The difference in oblique sesamoids is likely attributable to their measurement of the broad origin of the ligament as opposed to the mid substance measurement (mean ± SD) used in this study.

Table 3. CSA measurements from the present study (mean ± standard deviation) in comparison with those measured by Swanstrom *et al.* (2005).

Collateral Ligament	**CSA (mm^2)**	
	This study	Swanstrom *et al.*, 2005
PIP Joint	65 ± 35	51
Oblique Sesamoid	38 ± 11	81.8
Straight Sesamoid	59 ± 12	60.1-73
MCP Joint	35 ± 13	24.8-32

The resting length of the ligament was defined as the length of the ligament under 10 N of tension. This represented a physiologically low load (between 1 - 20% of the max load reached), and follows tension loads and protocol used within the literature (Thorpe *et al.*, 2010; Woo *et al.*, 1983). Sample elongation was determined by using the displacement value of the crosshead of the testing machine. Measurements of tensile strain using crosshead displacement have been known to yield erroneously high strain values due to the ligament slipping at the grips and machine deformation (Martin *et al.*, 2015). However, negligible differences were observed between grip-to-grip extension and that observed using markers, or a strain gauge, in previous studies of the mechanical behaviour of ligaments (Hoffman *et al.*, 2005; Siegler *et al.*, 2016). The pooled CV of repeated EM measurements was 32%, indicating that slip may have contributed to a portion of the final error in EM. However, visual inspection of the data indicated that the variability in EM were random, and there was a positive correlation between the EM of the PIP and MCP joint with the carpal joint ligaments within a horse, indicating that the results were consistent with the *in vivo* situation. It would be recommended in future experiments to employ digital imaging technology or an external strain gauge to measure mid-substance strain as this would eliminate potential error from ligament slip and any deformation inherent in the testing machine.

The ligaments were tested at a very low strain rate which may not be representative of the high strain rates to which they are exposed during locomotion. However, strain rate has been shown to have less influence on the biomechanical properties of ligaments than age, which had a weak effect in the present study (Woo *et al.*, 1990b). Nevertheless, EM did increase with increasing strain rates, so the EM presented in this study may be lower than expected *in vivo*.

The collagen fibrils of the lateral elbow CL were oriented in a diagonal cross. This made it impossible to tensile test the ligament as an entire parallel band of fibrils, as was done with the other ligaments. The tensile force was applied vertically according to the position of the ligament in the leg – i.e. parallel to the middle of the diagonal cross. In addition, the lateral elbow CL was very short and thick, and had a large CSA compared to the other ligaments in the study. This made it difficult to grip in the clamps as it slipped out more easily, and so it experienced a lower tensile strain and a smaller linear region of the stress-strain curve was obtained. Testing was not conducted at the different angles of the parallel collagen fibrils due to the difficulties in gripping the ligament in the testing device. These factors may have resulted in an abnormally low reported EM value, as collagen fibrils are strongest along their axes (Weintraub, 2003). In addition, the reported EM was calculated using the CSA for the entire specimen, whereas in reality the collagen fibrils under direct load are only a proportion of the total CSA, thus contributing to the abnormally low EM value (due to the inverse relationship of EM and CSA). Elongation in non-uniform ligaments could be measured more accurately in two or three dimensions using speckle techniques to appreciate local variations in strain (Martin *et al.*, 2015). However, the direction of applied force approximated the ground reaction force during the stance phase of locomotion.

Both temperature and hydration affect the mechanical behaviour of ligamentous tissue (Martin *et al.*, 2015). All the ligaments were harvested by the same person using a consistent technique and kept hydrated during storage by freezing with water. Freeze thawing has been shown not to affect the mechanical properties of rabbit medial CL or equine tendons (Martin *et al.*, 2015; Thorpe *et al.*, 2010; Woo *et al.*, 1986). However, the testing environment of 20°C did not approximate the body temperature (38°C) of an *in-vivo* situation. Since there is an inversely proportional relationship of tensile load and temperature for a given strain level (Woo *et al.*, 1987), this may have resulted in higher than normal values for the EM. Ligaments were tested in the controlled air environment and there was a visible loss of tissue water content during loading and gripping in the clamps. This dehydration would also result in an over estimation of EM (Hoffman *et al.*, 2005; Lavagnino *et al.*, 2005). In the present study, the ligaments were kept hydrated in water until testing. This contrasts with the saline buffer solution used in other ligament studies (Germscheid *et al.*, 2011; Woo *et al.*, 1990a) and could have resulted in gradual enzymatic degradation of the collagen molecules, reducing material strength (Martin *et al.*, 2015). The effect of freezing and the short length of the test minimised these effects, however immersion in a physiologic saline solution before and during testing at body temperature would better approximate the *in vivo* environment of the ligaments as well as mitigate dehydration and degradation effects.

The ligaments were not tested until failure. Prior to slippage from the serrated jaw clamps, a linear region was observed in the stress-strain curve, allowing the EM to be calculated. Failure or slip occurred between strains of 7 - 35%. This was above the accepted values for the toe region of the curve of 1.5 - 3% (Martin *et al.*, 2015), indicating that the linear region of the stress strain curve was reached for each ligament. Previous findings record that ligaments with high elastin content can be strained to 30% and up to 60% without damage (Martin *et al.*, 2015; Woo *et al.*, 2000). Therefore, the results from this study fall within the predicted range of elastic behaviour for ligaments. The EM of equine carpal CL were comparable to that of adult humans of 50 - 60 MPa (Mo *et al.*, 2012) and canines of approximately 50 - 80 ± 40 MPa (Shetye *et al.*, 2009), but significantly less than the carpal MCL in pigs of 327.6 ± 54.4 – 477.8 ± 47.5 MPa (Germscheid *et al.*, 2011) and goats 516 ± 158 MPa (Abramowitch *et al.*, 2003a). These differences may reflect the more locomotory nature of the former species having more flexible and elastic CL to allow for greater movement in their distal limbs and highlight the importance of accurate breed specific EM values for use in musculoskeletal modelling.

The collateral ligaments of the distal interphalangeal joint and shoulder were not included in this study due to harvesting difficulties. Therefore, the data in this study does not represent the entire equine distal limb, and further studies are required to determine the properties of these ligaments.

Any inaccuracies in methodology due to methodological limitations were consistent throughout the study (pooled CV of 32% for each ligament) and similar to the observed high individual variability between mechanical properties of ligaments (CV of 13 - 52%) (Avella & Smith, 2012; Rapoff *et al.*, 1999; Shetye *et al.*, 2009; Thorpe *et al.*, 2010; Woo *et al.*, 1990b). In addition, the ligament property data agrees with similar data from the literature (Mo *et al.*, 2012; Shetye *et al.*, 2009; Swanstrom *et al.*, 2005). Thus, despite all the above methodological issues, the data in the present study is considered valid and of use for gait modelling.

<u>Gait models</u>

Gait models often use a single stiffness value (Bullimore & Burn, 2006; Farley *et al.*, 1993; Herr *et al.*, 2002; Herr *et al.*, 2000) or biomechanical data for the major tendons and ligaments only (Brown *et al.*, 2003; Harrison *et al.*, 2012; Meershoek *et al.*, 2001; Rollot *et al.*, 2004; Wilson *et al.*, 2001). The findings of the present study suggest that this may be inappropriate as it has found that the EM differs according to position and function in the equine distal limb, even between ligaments performing a similar function. However, due to high individual variation in ligament properties, average data is unlikely to be adequately representative of every horse. Despite this, common trends in the EM of the CL at specific joints exist in the data between horses, enabling the distribution of forces in the distal limb to be accurately modelled.

The property data for CL obtained in the present study will be within the 'Anybody' computer model of equine motion being developed at Massey University to investigate how ground surface changes interact with dynamic forces in the equine distal limb. Ground reaction forces in the equine distal forelimb will be distributed differently according to the differences in elasticity of each tendon and ligament in the limb. The addition of CL to this model will allow a more accurate representation of the path of force in the distal limb to be determined.

An accurate representation of limb loading pattern changes will help to determine factors which cause injury to the distal limb. This could lead to development and maintenance of racetrack surfaces or horseshoes which could reduce injury incidence in Thoroughbred racehorses. In addition, rehabilitation techniques for distal limb injuries could be improved as understanding of the force transmission in the limb increases. For the Thoroughbred racing industry, this information could significantly reduce wastage and rehabilitation costs from ligament and tendon injuries.

The present study has shown that a better understanding of the structures and their interactions in the equine distal forelimb is needed. Further knowledge of the properties of every ligament and its' function in the equine distal forelimb will increase the understanding of factors leading to equine musculoskeletal injury. Ultimately, a complete model of an entire horse may be able to be modelled, allowing accurate simulations to pinpoint structures that are at risk of injury and determine factors which could minimise musculoskeletal injury or lead to appropriate methods of rehabilitation.

Conclusions

Significant differences were found between the elastic moduli of the collateral and distal sesamoid ligaments from the different joints of the equine distal forelimb. These were in accordance with the disparate forces to which the joints are subjected. The EM varied distally to proximally along the limb, lower at the extremities (EM of the lateral elbow 3 ± 2 MPa) with the highest EM of the CL in the MCP joint (49 ± 36 MPa).

Individual variation in the elastic moduli was high. Thoroughbred horses had a significantly higher EM in the PIP and MCP joint CL only, perhaps due to their lightweight build or early race training. The EM of the CL in the equine distal limb were mainly independent of horse height, except for those from the PIP joint which had a weak linear negative relationship with height. This negative relationship and biomechanical property size independence of the collateral and distal sesamoid ligaments may contribute to the higher incidence of injury seen in larger horses.

The differences and variation of EM found in the present study further our understanding of the equine distal forelimb. Their addition to the 'Anybody' dynamic gait model will help to explain the dynamics of ground reaction forces in the equine distal forelimb at speed. This will afford a better understanding of soft tissue and skeletal mechanics in the equine distal limb and their reaction to surface perturbations.

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