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Thoroughbred horses in race training have lower levels of subchondral bone remodelling in highly loaded regions of the distal metacarpus compared to horses resting from training

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ABSTRACT

Bone is repaired by remodelling, a process influenced by its loading environment. The aim of this study was to investigate the effect of a change in loading environment on bone remodelling by quantifying bone resorption and formation activity in the metacarpal subchondral bone in Thoroughbred racehorses. Sections of the palmar metacarpal condyles of horses in race training ($n = 24$) or resting from training ($n = 24$) were examined with light microscopy and back scattered scanning electron microscopy (BSEM). Bone area fraction, osteoid perimeter and eroded bone surface were measured within two regions of interest: (1) the lateral parasagittal groove (PS); (2) the lateral condylar subchondral bone (LC). BSEM variables were analysed for the effect of group, region and interaction with time since change in work status. The means ± SE are reported.

For both regions of interest in the training compared to the resting group, eroded bone surface was lower (PS: 0.39 ± 0.06 vs. 0.65 ± 0.07 per mm, $P = 0.010$; LC: 0.24 ± 0.04 vs. 0.85 ± 0.10 per mm, $P < 0.001$) and in the parasagittal groove osteoid perimeter was higher (0.23 ± 0.04% vs. 0.12 ± 0.02%). Lower porosity was observed in the subchondral bone, reflected by a higher bone area fraction in the LC of the training group (90.8 ± 0.6%) compared to the resting group (85.3 ± 1.4%, $P = 0.0010$).

Race training was associated with less bone resorption and more bone formation in the subchondral bone of highly loaded areas of the distal metacarpus limiting the replacement of fatigued bone. Periods of reduced intensity loading are important for facilitating subchondral bone repair in Thoroughbred racehorses.

Introduction

Repeated cyclical loading of bone during intense exercise results in bone fatigue. Fatigued bone is bone that has undergone cyclic loading and consumed a proportion of its fatigue life (the number of cycles to failure). Bone fatigue results in microdamage, initially at the molecular level, before the development of microcracks, which can be detected microscopically (Burr et al., 1997; Muir et al., 2008). Fatigued bone is replaced by remodelling, which involves the coordinated actions of the bone-resorbing osteoclasts, and the bone-forming osteoblasts. The action of osteoclasts produces scalloped ('eroded') surfaces, which can be observed histologically, and increased bone porosity, which can be quantitated as measurements of void area in bone sections. Increased porosity is due to the normal delay in the onset of the bone formation phase following resorption, where osteoblasts deposit bone matrix (osteoid) on bone surfaces which then slowly mineralise (Boyde and Firth, 2005).

The remodelling process has been shown to vary within bones both temporally and regionally (Murray et al., 2001; Boyde and Firth, 2005). Fatigue fractures and subchondral bone injuries occur when microdamage accumulates faster than can be repaired by remodelling. Therefore, factors that alter remodelling rates affect the risk of injury (Riggs, 2002). Under conditions of high cyclic loading, remodelling activity decreases, an effect that is thought to be due to inhibition of osteoclast recruitment (Jee et al., 1991; Rubin et al., 1999). This reduction of remodelling activity may be inconsequential if the loading is of short duration. However, in circumstances of prolonged loading or in inadequately adapted bone, microdamage will accumulate (Whitton et al., 2010). Microdamage can also directly stimulate focal remodelling, even under high loading conditions (Burr et al., 1985; Whitton et al., 2013). Paradoxically, while this targeted remodelling serves to assist healing under moderate loading conditions, it may accelerate injury development in circumstances of intense loading (van Oers et al., 2011).

The Thoroughbred racehorse subjects the subchondral bone of the metacarpophalangeal joint to extreme habitual loading when in race training (Harrison et al., 2010, 2013). Training periods vary in duration but can exceed 20 weeks without interruption (Whitton

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et al., 2010). There is limited quantified information on subchondral bone turnover during this period. Previously observed reduced porosity and scant resorption surfaces within distal metacarpal subchondral bone of horses in training are suggestive of suppressed remodelling rates (Boyde and Firth, 2005; Whitton et al., 2010, 2013). The combination of low bone turnover and high repeated loading exposes this area to a high risk of fatigue damage, consistent with the large proportion of race horses that exhibit distal metacarpal subchondral bone injury (Barr et al., 2009; Pinchbeck et al., 2013). A better understanding of the influence of race training on subchondral bone remodelling is essential to understanding the pathophysiology of subchondral bone injury.

In this study, we measured remodelling activity in the subchondral bone of the distal metacarpus of Thoroughbred horses either in race training or resting from training. Two sites on the distopalmar aspect of the third metacarpal bone that are considered to undergo high loading were selected: (1) the lateral parasagittal groove where microdamage is commonly observed; (2) the lateral condyle where injury is least common (Muir et al., 2008; Pinchbeck et al., 2013). We hypothesised that bone remodelling activity would be lower in intensely training horses. Additionally, we aimed to explore the relationship between remodelling activity and time since a change in training status.

Materials and methods

Third metacarpal (MC3) bones were collected from 48 Thoroughbred horses that died or were euthanased in the period April 2007–August 2012 that underwent comprehensive post-mortem examination at the University of Melbourne. The use of animal tissues met the requirements of the University of Melbourne Animal Ethics Committee. Twenty-seven of these horses had been included in a previous study (Whitton et al., 2010). Horses were assigned to two groups: (1) training: in race training; or (2) resting controls: horses that had previously undergone race training but were not currently training. Training horses had been exercising regularly for 4 weeks or longer and had progressed to intense exercise (fast canter or gallop) in the current training period. Resting horses had been restricted to a stable or paddock and were not undergoing forced exercise.

The sex distribution was the same for both groups, with 6 females and 18 males, 14 of which were castrated. The mean age (years) ± SE was higher in the training group (4.9 ± 0.4) than the resting group (3.7 ± 0.3). Of the training group, the cause of death was fracture ($n = 15$ including two biaxial sesamoid fractures and three proximal phalanx fractures), other musculoskeletal condition ($n = 3$ including two suspensory apparatus ruptures), acute abdomen ($n = 2$) and other medical conditions ($n = 4$). Of the resting group, the cause of death was fracture ($n = 2$), other musculoskeletal condition ($n = 9$), acute abdomen ($n = 7$) and other medical conditions ($n = 6$). None of the fractures within either group were of the metacarpus.

The palmar–distal aspects of both MC3s were removed by cutting the bone at 55° to the frontal plane through the centre of rotation of the condyles prior to storing in 70% ethanol as previously described (Whitton et al., 2010). Racing and training history were obtained from race records and telephone questioning of trainers, cross-referenced with race records from an official database (Sirius, Racing Victoria). Length of current training period was defined as the length of time (weeks) that a horse had been continuously training with no rest period of more than 4 weeks. Information regarding any lameness history and current or previous treatment, housing status for horses resting from training (i.e. stable or paddock) was obtained from the trainer and the horse's regular veterinarian.

Following random selection (using a random number generator) of the left or right MC3 the specimen was serially dehydrated and embedded in methyl methacrylate. The condyles from the selected limb were then sectioned with a low speed saw (Isomet, Buehler). The cut surface was ground, polished, carbon coated and examined with a scanning electron microscope (FEI Quanta field emission gun 200) fitted with an annular solid-state backscattered electron detector. Consecutive overlapping micrographs of both the entire section and of specific regions of interest (ROI) were acquired at magnifications of ×70 and ×200. All micrographs of each ROI were transferred to a desktop computer and the whole ROI was reconstructed using photo stitching software (Adobe Photoshop Elements 3). For light microscopy thin (8 μm) sections were cut using a microtome (Polycut E sledge microtome, Leica Microsystems). Methyl methacrylate was removed with methoxyethylacetate and the tissue was dehydrated with ethanol and stained with a commercial Masson's Trichrome kit (Australian Biostain).

Assessment of bone activity was based on standard two-dimensional bone histomorphometry measurements (Parfitt et al., 1987). Bone surface (perimeter; Pm) measurements were performed as previously described (Whitton et al., 2013) in the lateral condylar subchondral bone (ROI a, Fig. 1) and the lateral parasagittal groove (ROI b, Fig. 1). Eroded bone surface (E.Pm) was measured on BSEM images whereas osteoid surface (O.Pm) was measured on Masson's trichrome images using open source imaging software (Image J, NIH). For measurement of bone area (B.Ar) and total tissue area (T.Ar), BSEM images were automatically thresholded using the 'minimum' algorithm available in the software and performed in the lateral and medial condylar subchondral and trabecular bone combined (ROI c plus d, Fig. 1), the lateral condylar subchondral and trabecular bone (ROI c, Fig. 1) and the two subchondral bone areas; the lateral parasagittal groove (ROI b, Fig. 1) and the lateral condylar subchondral bone (ROI a, Fig. 1). Values for eroded surface are presented both as a percentage of total bone surface (E.Pm/B.Pm) and relative to tissue area (E.Pm/T.Ar), and osteoid surface values are presented as a percentage of bone surface (O.Pm/B.Pm). Bone area values are presented as a percentage of total tissue area (bone area fraction; B.Ar/T.Ar).

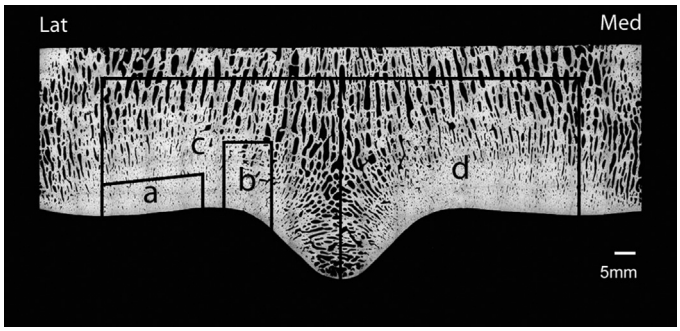

Fig. 1. Backscattered electron microscopy image of the distal palmar aspect of the third metacarpal bone of a Thoroughbred race horse in race training showing regions of interest (ROI) utilised in the study. (a) Lateral condylar subchondral bone, (b) lateral parasagittal groove, (c) lateral condyle, (d) medial condyle.

As bone resorption is the initial step in the remodelling cycle and formation can be due to both modelling and remodelling, we used eroded bone surface as our primary measure of remodelling. The mean calcified cartilage thickness for each ROI was determined by dividing the area of the calcified cartilage on the BSEM section by the length of the calcified cartilage for each ROI.

The number of microfractures per millimetre of articular surface was counted for each ROI on BSEM sections. To differentiate from artifactual fractures created by processing, microfractures were defined as discontinuity of the articular surface that also met one of the following criteria: (1) hypermineralised bone present immediately adjacent to the fracture line (Boyde, 2003); (2) direct association of the defect with bone fragmentation.

Statistical analysis

A power study determined that 26 bones were required for each group in order to detect a 20% difference in parameters of remodelling with a power of 80% and a probability of 0.05 (IBM SPSS SamplePower 3.0).

Statistical analysis was performed using IBM SPSS for Windows version 21. Equal variance between groups was confirmed by plotting the residuals. The effect of group on BSEM measures of remodelling was analysed with an independent samples *t* test. Comparisons of variables between ROI were assessed using a paired samples *t* test. A general linear model was used to test for an effect of 'time since change in exercise status' on BSEM variables within each group and the interaction between effect of 'time in training/rest' and variables in which an effect of training was found. For all analyses, age, sex, presence of a fracture, number of career starts and weeks in current training period were tested for significance or confounding. Despite the higher mean age of the training horses, age was not a confounder. Two-tailed significance was set at $P < 0.05$. All values are reported as mean (±SE).

Results

Lateral condylar subchondral bone sections (ROI a, Fig. 1) from both training and resting horses consisted predominantly of dense bone except for two of the unraced 2-year-olds, in which the bone was mostly trabecular. In contrast, sections from the parasagittal groove (ROI b, Fig. 1) contained areas of both dense and trabecular bone which was demonstrated by a difference in the percentage bone area fraction (B.Ar/T.Ar) between the two sites ($P < 0.001$, Table 1).

Measures of eroded surface were lower in training than resting horses in both subchondral bone ROI with the greatest differences observed in the lateral condylar subchondral bone (Table 1). This was accompanied by lower porosity in the subchondral bone of the

Table 1
Bone variables for training and resting Thoroughbred race horses. Values for eroded surface are presented both as a percentage of total bone surface (E.Pm/B.Pm) and relative to tissue area (E.Pm/T.Ar), and osteoid surface values are presented as a percentage of bone surface (O.Pm/B.Pm). Bone area values are presented as a percentage of total tissue area (bone area fraction; B.Ar/T.Ar).

		Training mean ± SE ($n = 24$)	Resting mean ± SE ($n = 24$)	P value
Lateral and medial condyles combined	B.Ar/T.Ar %	76.1 ± 1.5	74.3 ± 1.1	0.36
Lateral condyle only	B.Ar/T.Ar %	75.1 ± 1.4	74.2 ± 1.2	0.63
Parasagittal groove	B.Ar/T.Ar %	82.4 ± 1.7	79.2 ± 1.6	0.18
	E.Pm/B.Pm %	11.6 ± 1.6	16.1 ± 1.6	0.051
	E.Pm/T.Ar mm^{-1}	0.39 ± 0.06	0.65 ± 0.07	0.010
	O.Pm/B.Pm %	0.23 ± 0.04	0.12 ± 0.02	0.021
	Microfractures mm^{-1}	1.93 ± 0.30	0.95 ± 0.17	0.007
Lateral condylar subchondral bone	B.Ar/T.Ar %	90.8 ± 0.6	85.3 ± 1.4	0.001
	E.Pm/B.Pm %	11.1 ± 2.0	20.8 ± 1.2	<0.001
	E.Pm/T.Ar mm^{-1}	0.24 ± 0.04	0.85 ± 0.10	<0.001
	O.Pm/B.Pm %	0.25 ± 0.04	0.19 ± 0.03	0.22
	Microfractures mm^{-1}	0.12 ± 0.03	0.08 ± 0.03	0.40

B.Ar, bone area; T.Ar, total tissue area; B.Pm, bone surface; E.Pm, eroded bone surface; O.Pm, osteoid surface.

training horses than the resting horses, reflected by an increase in B.Ar/T.Ar within the lateral condylar subchondral bone, which was not detected in the parasagittal groove (Fig. 2, Table 1). The measure of bone formation (O.Pm/B.Pm) was higher in horses in training than resting horses in the parasagittal groove (Table 1).

In horses sampled early in their training period compared to those with a longer training history, eroded surfaces were lower in both the parasagittal groove and the lateral condylar subchondral bone (parasagittal groove E.Pm/B.Pm: $R^2 = 0.20$, $P = 0.027$; lateral condyle E.Pm/T.Ar: $R^2 = 0.26$, $P = 0.011$, Fig. 3). For resting horses, there was a large individual horse variation in eroded surface measures at both subchondral bone sites, which was not explained by the nature of their rest (box vs. paddock) (Fig. 3).

One training horse had pronounced porosity in the lateral condylar subchondral bone with abundant active eroded and mineralising surfaces. This horse had suffered bilateral humeral fractures during a training gallop. Despite being in training for 22 weeks, it was reported to have been unable to race or trial for the previous 18 weeks due to musculoskeletal problems and a recent change in trainer may have resulted in variation in exercise intensity within the preceding weeks.

There were more microfractures in the parasagittal groove of the training horses than the resting horses (Table 1). Within both groups microfractures were more prevalent in the parasagittal groove than the lateral condylar subchondral bone (training: 1.9 ± 0.03 vs. 0.12 ± 0.03 per mm, $P < 0.001$; resting: 1.0 ± 0.17 vs. 0.08 ± 0.03 per mm, $P < 0.001$). There was no difference detected in the calcified cartilage thickness between groups (parasagittal groove: $P = 0.97$; lateral condyle: $P = 0.56$).

Discussion

We have demonstrated that race horses in training have less eroded bone surface in both the lateral parasagittal groove and the lateral condylar subchondral bone and more osteoid surface in the lateral parasagittal groove of the third metacarpal bone than those resting from training. The difference in bone resorption is particularly prominent in the lateral condylar subchondral bone where eroded surface of horses in training is less than one-third that of the resting horses and appears to contribute to lower condylar porosity in training horses. Although overall, bone resorption is lower in association with training, bone resorption is more prominent in horses that have been training for a longer period.

Increased bone formation and reduced bone resorption in response to increased habitual loading is a widely accepted concept (Jee and Li, 1990; Roshan-Ghias et al., 2011). When compared with horses that have never trained, horses in training have lower levels

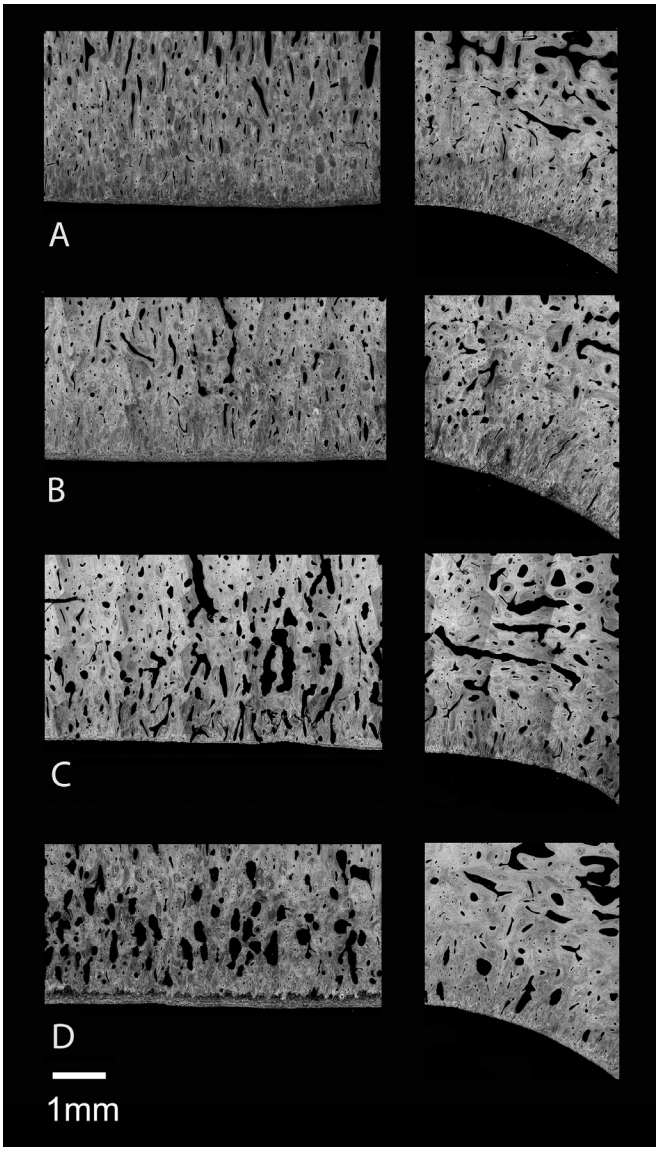

Fig. 2. Backscattered scanning electron microscopy of the lateral condylar subchondral bone (left) and lateral parasagittal groove (right) from Thoroughbred race horses that had been (a) in race training for 12 weeks, (b) resting for 1 week, (c) resting for 4 weeks, and (d) resting for 9 weeks. There is higher porosity of the subchondral bone with greater duration of rest period most prominent in the lateral condylar subchondral bone.

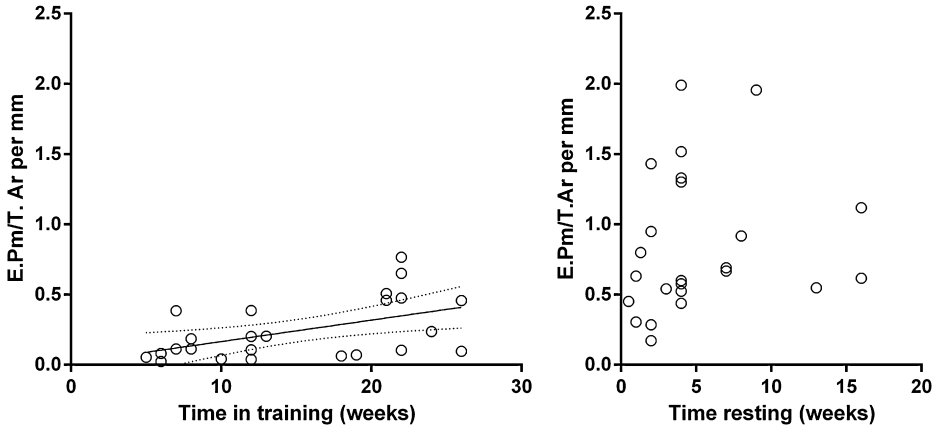

Fig. 3. Eroded bone surface (E.Pm/T.Ar) in the lateral condylar distal third metacarpal subchondral bone plotted against duration of training and of a rest period. There was a positive correlation between eroded surface and time in training ($R^2 = 0.26$, $P = 0.011$) and no significant association between duration of rest period and eroded surface ($R^2 = 0.02$, $P = 0.48$).

of circulating markers of bone resorption and lower porosity of the dorsal cortex of the third metacarpal bone (McCarthy and Jeffcott, 1992; Jackson et al., 2003). The lower bone porosity measured in response to training is consistent with lower levels of bone remodelling, as transient porosity is produced by the normal delay in onset of the bone formation phase following initial resorption.

There are limited studies comparing subchondral bone between horses in training and horses resting from training. Firth et al. (2007) were unable to detect a difference in volumetric bone mineral density (vBMD) of the distal metacarpal epiphysis when Thoroughbred horses had been in race training for 8 months compared with the same horses after 5 months paddock rest. This was despite a decrease in vBMD in the metacarpal diaphysis of these same horses (Firth et al., 2007). The differences in subchondral bone volume we observed between training and resting horses were confined to only a small proportion of the epiphysis (the lateral condylar subchondral bone, ROI a), therefore, the lack of detected difference when measuring the entire epiphysis is not surprising.

Histomorphometric observation of carpal subchondral bone in previously untrained 2-year-old horses following a 19 week treadmill training regimen also demonstrated differences in eroded bone surfaces confined to specific areas (Murray et al., 2001). A 30% reduction in eroded bone surface compared with horses that underwent a walking only regimen was observed in the dorsal aspect of the radial facet of the third carpal bone, whereas reduced remodelling was less evident at other subchondral bone sites within the carpus (Murray et al., 2001). Therefore, the current study reinforces the site specificity for the effect of training on subchondral bone remodelling activity which appears to be confined to the areas subjected to the highest loads during galloping exercise.

In morphological studies of subchondral bone of training horses, bone formation predominates and is associated with filling of trabecular spaces and higher bone volume fraction compared with resting horses (Murray et al., 2001; Boyde and Firth, 2005). In fact in 2-year-old horses 13–15 weeks following commencement of training for the first time, BSEM examination of the subchondral bone of the distal third metacarpus demonstrated extensive bone formation with very little evidence of bone resorption (Boyde and Firth, 2005). We expanded these previous findings, showing that subchondral bone resorption is lowest early in a training period (<20 weeks) but can be higher later in a training period (>20 weeks) (Fig. 3). These findings are suggestive of an initial adaptive phase to the introduction of habitual loading followed by re-instigation of remodelling once subchondral bone is better adapted. It is also possible that the accumulation of fatigue damage throughout a training period may contribute to subsequent remodelling activity.

It is acknowledged that there is considerable overlap in the adaptive and reparative response of subchondral bone following a change in loading circumstances. Although resorption is an important component of bone remodelling, a proportion of the bone resorption observed in detrained subchondral bone in our study will be due to bone modelling, or adaptation to the lower level of loading. However, subsequent re-instigation of training and adaptive filling of the previously created voids will ultimately result in net replacement of bone, and therefore repair of bone fatigue. Fewer microfractures within the parasagittal groove in the resting horses as compared to training horses are consistent with the reparative effect of resting from race training. Maximising subchondral bone repair in Thoroughbred race horses at this site is important because up to 80% of individuals have gross evidence of fatigue injury of the subchondral bone of the distal metacarpus (Barr et al., 2009; Pinchbeck et al., 2013).

Based on our findings, reducing the duration of training periods to limit the accumulation of fatigue damage and allowing regular rest periods to facilitate repair of bone fatigue would decrease the risk of subchondral bone fatigue. Alternatively, stimulating remodelling and therefore bone repair activity in horses while in training could also decrease the accumulation of fatigued bone but it is unknown whether this is possible, and the negative biomechanical effects of the increased bone porosity have the potential to outweigh the benefits as has been shown in computational models of bone under repeated loading (van Oers et al., 2011).

Determining optimal duration of periods of rest from training for Thoroughbred race horses to prevent the accumulation of subchondral bone fatigue will require further investigation. It is likely dependent on the duration and intensity of the previous training period. The largest measures of eroded surface were observed in horses that had been rested for 3–10 weeks suggesting that the benefits of rest are maximized during this time, although they are likely to continue indefinitely, albeit at a lower rate, due to ongoing remodelling.

The large variations in erosion surface and the degree of subchondral bone porosity in resting horses were difficult to explain. However, they may have been influenced by the heterogeneity of loading histories within the resting category. Differences in exercise levels between stable confinement and other types of rest did not appear to be the cause, although many horses had mixed rest types. It is likely that horses with highly porous subchondral bone

were at risk of subchondral bone injury if rapidly returned to high intensity training without sufficient time for pores to fill with bone. There is no published data on radial closure rates for resorption channels in equine subchondral bone, but for metacarpal cortical bone 1.23 μm/day has been reported (Boyde and Firth, 2005). The pore size in the most porous subchondral bone in resting horses from the current study was between 60 and 120 μm which at the published bone formation rate would take from 60 to 120 days to fill. Under current training practices this would result in some horses beginning to race before all pores were filled. Of particular concern was the one horse that had highly porous subchondral bone, despite being in race training. Although a complete training history was not available the trainer considered the horse was ready to race which demonstrates the potential for horses with poorly adapted bone to go unidentified and potentially suffer catastrophic consequences.

This study was limited by the need to section equine bone to acquire high resolution images for histomorphometry and, therefore, its inherent cross-sectional nature. Case selection was influenced by the availability of dead horses for whole bone collection, creating a bias of samples relating to the post-mortem program or clinical cases. Where possible, horses with fatigue injury were excluded but available samples and resources did not allow complete exclusion. However, it was shown that the presence of fatigue injury was not a confounder. There was also variability between individual training programs and the nature of rest each horse experienced, which may have limited the ability to assess the effect of the length of time following a change in work status on measures of bone remodelling. Moreover, although attending veterinarians and trainers were questioned about the use of bisphosphonates and none was reported we cannot be sure that they had not been previously administered in all cases resulting in exogenous inhibition of bone resorption (Flanagan and Chambers, 1991).

Conclusions

Our data are consistent with the concept that high intensity training inhibits bone remodelling in the subchondral bone of highly loaded areas of the distal metacarpus. While remodelling may restart once the bone has adapted to training, the high prevalence of subchondral bone fatigue damage in Thoroughbred race horses indicates that in many cases the level of remodelling is inadequate. This emphasises the importance of periods of less intense loading to allow subchondral bone repair. The duration of rest required for adequate bone replacement due to remodelling varies between individuals but maximal bone remodelling activity appears to occur within the first 10 weeks following discontinuing training.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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