In Vitro Fatigue Behavior of the Equine Third Metacarpus: Remodeling and Microcrack Damage

R. B. Martin, S. M. Stover, V. A. Gibson, J. C. Gibeling, and L. V. Griffin

Summary: We studied remodeling and microcrack damage in specimens of Thoroughbred racehorse third metacarpal bone that had been subjected to monotonic or fatigue failure. We asked three questions. What effects does mechanical loading have on histologically observable microcrack damage? Are there regional variations in remodeling of the equine cannon bone, and do these variations correlate with mechanical properties? To what extent are remodeling and microcrack damage age-dependent? Machined beams from the medial, lateral, and dorsal cortices were loaded to fracture in four-point bending monotonically, or cyclically at a load initially producing 10,000 microstrain. Specimens were then bulk-stained in basic fuchsin, and cross sections were prepared from loaded and load-free regions of each beam. Current and past remodeling, porosity, and microcrack density and length were determined histomorphometrically. Stained and unstained microcracks were observed. Unstained cracks were associated with regions of woven bone and appeared to be damaged Sharpey's fibers. Their density (approximately 30/mm²) did not increase after failure, but their length (approximately 25 um) did, especially near the surfaces of the beam. Stained cracks were wider and longer than unstained cracks and were located primarily near the fracture surface and on the compressed side of the beam. Stained cracks after failure were more numerous in those beams having a higher elastic modulus, a shorter fatigue life, or greater deformation at failure. The extent of past remodeling increased with age, especially in the medial region; the rate of
current remodeling generally declined with age, but not in the dorsal region, which has the best fatigue resistance. In summary, while remodeling varied with age and region, its effects on bone structure did not appear to influence microdamage. Basic fuchsin staining of damage in fractured equine bone was independent of age and region and confined to near the fracture surfaces. Distributed microdamage consisted only of what appeared to be subtle disruptions of Sharpey's fibers.

In a preceding paper (14), we described the mechanical behavior of the equine third metacarpal (or cannon) bone diaphysis in four-point bending to failure, including both monotonic and low cycle fatigue tests. The results of these tests were strongly correlated with anatomic position in the cortex. The lateral region was stronger and stiffer than the medial and dorsal regions when monotonically loaded but had the shortest fatigue life. These results raise a number of important questions concerning how the equine cannon bone is regionally adapted to resist fatigue and monotonic failure.

Regional differences in mechanical properties could depend on variations in primary bone structure, the degree of remodeling that has occurred, or both. The primary cortical structure of the equine cannon bone cortex, which is formed by saltatory bone formation (25), is different from the plexiform structure of bovine cortical bone, and both are different from canine and human bone. Remodeling of primary bovine bone by secondary osteons has been shown to reduce both the monotonic strength (20,21) and fatigue life (6,8). On the other hand, the presence of secondary osteons has been hypothesized to increase bone toughness and fatigue resistance by creating a composite structure capable of diverting cracks and absorbing energy (15,18).
Therefore, we were interested in the effects of remodeling on the fatigue properties of our specimens.

These specimens also provided an opportunity to study histologically observable damage in relationship to monotonic and fatigue failure. Burr et al. (2,16) showed that in vivo loading of canine forelimbs significantly increases the number of microcracks in the radius. It is postulated that such cracks accumulate and coalesce if their repair by remodeling cannot keep pace with their generation by loading, and that such accretion, if unchecked, ultimately produces a stress fracture. Many other in vitro studies of machined specimens have explored the mechanical behavior of bone under fatigue loading, including such mechanical measures of damage as reductions in elastic modulus or strength (4-11,17). However, we have found only a few studies that tried to correlate these data with microcrack damage, and none involving equine bone (8,12,22,23).

Consequently, the purpose of the present study was to answer the following questions concerning the material in the equine cannon bones of racehorses. What effects does mechanical loading have on histologically observable microcrack damage? Are there regional variations in remodeling of the equine cannon bone, and do these variations correlate with mechanical properties? To what extent are remodeling and microcrack damage age-dependent?

METHODS

The experimental design and mechanical loading were previously described in detail (14). Briefly, 36 beams measuring 100 x 10 x 4 mm were machined from the diaphyses of six cannon bone pairs obtained at necropsy from Thoroughbred and non-Thoroughbred racehorses. Five of these animals were female and one was male: the age range was 2-5 years (mean = 3.7 years). One beam was machined from each of the three thickest cortices
of each bone: lateral, medial, and dorsal. The 36 beams were uniformly distributed among three experimental groups so that each group was similarly represented with respect to the six horses, left and right bones, and the three cortical regions. All beams were loaded to fracture in four-point bending while immersed in a saline bath at 37°C. One group was monotonically loaded at a deflection rate of 1 mm/sec with the periosteal side in tension. The other two groups were fatigued under load control using a load that initially produced a peak strain of 1000 microstrain (approximately 900 N). The minimum load was 10 N. The periosteal group had the periosteal side of the beam in tension and the endosteal group had the endosteal side in tension.

Following failure, both fragments of the beam were bulk-stained in 1% basic fuchsin using a modification of the method of Burr and Stafford (3). The beam was sequentially soaked for 24 hours in each of four solutions: 1% basic fuchsin in 70, 80, 90, and 100% alcohol. At least the first 2 hours in each solution were spent under vacuum to enhance penetration of the stain. After an additional 24 hours in the 100% alcohol and 1% basic fuchsin solution, the beam was removed and allowed to air dry for 2 days, then rehydrated in deionized water for 4 days.

With use of a Gillings-Hamco diamond saw (Hamco, Rochester, NY, U.S.A.), complete beam cross sections were cut from control and experimental regions (Fig. 1, top). These sections were ground to a thickness of 100 ± 5µm and mounted on glass slides with Eukitt (Calibrated Instruments, Hawthorne, NY, U.S.A.) for histomorphometric analysis. When the centrally located experimental section was more than 5 mm from the fracture surface an additional experimental section, cut as close to the fracture site as possible (consistent with obtaining an intact, complete section), was analyzed.
Crack damage was quantified in the histologic sections using an Olympus BH-2 microscope (Olympus, Lake Success, NY, U.S.A.) equipped with a Merz grid. At \( \times250 \) magnification, 30 fields were examined in each section (Fig. 1, bottom). In each 0.129 \( \text{mm}^2 \) field, the number of cracks within the grid boundary was counted and divided by grid area to give crack density (\( \text{mm}^{-2} \)). Total crack length per unit area of section was defined as crack damage (\( \text{mm}^{-1} \)). The Merz grid contains rows of semi-circular test lines that are used for measuring the lengths of lineal features in a section. The number of intercepts of cracks with these lines, \( p \), was counted, and damage was calculated using the formula:

\[
\text{crack damage} = \frac{p}{L},
\]

where \( L = 3.38 \text{ mm} \) is the total length of the test lines in each field. These measurements were averaged for each three-field scan so that damage could be studied as a function of distance from the more periosteal side of the beam. In addition, mean crack length (\( \text{mm} \)) for each scan was calculated by dividing crack damage by crack density. The data for all 10 scans were averaged to obtain section values. Porosity was also determined for each field, scan, and section by counting the fraction of the 36 test points in each field (1,080 points per section) that fell in haversian or Volkmann’s canals or in remodeling spaces. Care was taken not to count crack spaces or osteocyte lacunae as porosity. [Figure 1]

All histomorphometry was done by a single observer (R.B.M.) on blinded sections. In counting cracks, we focused up and down through the sections in order to distinguish between cracks and other features, such as canaliculi and edges of canals. Thus, to be sure of what was being counted, we violated the stereological principle that features should be counted in a single focal plane. This probably caused us to overestimate crack density and damage, but equally in all sections. [Figure 2]
Microcracks could be categorized into two groups according to their appearance. The most common cracks were so narrow that their opposing surfaces usually could not be distinguished. These cracks appeared black rather than red after basic fuchsin staining and were also apparent in specimens that were not bulk-stained. We refer to them as unstained cracks (Fig. 2). Other, less numerous cracks stained red and their opposing surfaces could usually be distinguished at x250 or x500. We call these stained cracks (Fig. 3). We counted and separately tabulated both stained and unstained cracks.

Active and past remodeling were quantified in the sections from the experimental (loaded) portions of each beam. At x40 magnification, 10 Mere grid fields were examined for each section, covering about 90% of the beam cross section. The density (no./mm²) of active basic multicellular units was estimated by counting the sum of resorption spaces and refilling basic multi-cellular units with osteoid seams in each field. The extent of previous remodeling in the field was also estimated using a scale of 1 to 3, in which 1 referred to a field whose area was composed of less than 20% secondary osteons, 3 referred to a field whose area contained greater than 80% secondary osteons, and 2 referred to anything in between. Mean values of both active basic multi-cellular units/mm² and remodeling index were calculated for each beam.

For reasons related to the mechanical aspects of the study, left and right pairs of beams were placed in the same experimental groups. Problems associated with the lack of left-right independence were avoided by averaging together left-right pair data or treating "side" as an analysis of variance (ANOVA) factor.

Some variables were not normally distributed. Parametric ANOVA was used to compare groups or regions for normally distributed variables, and Wilcoxin signed rank
tests or Kruskal-Wallis ANOVA on ranks were used for variables that were not normally distributed. The Student-Newman-Keuls post hoc test was used following a significant ANOVA. Interactions not reported were not significant. Correlations were tested using the Pearson product method for normally distributed variables or the Spearman rank method when at least one variable was not normally distributed. The criterion for statistical significance was p < 0.05. [Figure 3] [Table 1]

RESULTS

Unstained cracks were ubiquitous in both control and experimental sections. There were 20-40 cracks mm⁻² (Table 1); they were about 25 µm long and oriented perpendicular to the periosteal surface (Fig. 2). They were predominantly found on the periosteal side of the layers of woven bone created during radial expansion of the metacarpus or radiating into the interstitial bone from the periosteal-facing side of the cement line of secondary osteons that had remodeled the woven bone regions. When viewed in circularly polarized light, they were usually seen to be within birefringent fibers lying across (rather than parallel to) lamellae.

Unstained crack density and damage values exhibited a Poisson or binomial-like distribution, but their lengths were normally distributed. Unstained crack density, damage, and length were independent of cortical region, but density and damage diminished with distance from the periosteal surface (Fig. 4). Three-way ANOVA (factors: horse, loading group, and experimental compared with control specimen) on unstained crack length showed that horse-to-horse variability was not significant, but lengths were 17.5% greater in the experimental specimens than in the controls when all the loading groups were pooled (0.0288 compared with 0.0245 mm, p < 0.009). Loading mode was also a significant factor, with greater crack lengths in the monotonic group than in the endosteal (p = 0.005) or periosteal (p = 0.04) groups, which were
not different from each other. Load-induced changes in length were more pronounced further from the neutral axis of the beams (Fig. 4).

Stained crack density and damage also lacked a normal distribution. Non-parametric analysis showed that loading to failure increased stained crack density by an order of magnitude when all experimental groups and cortical regions were pooled (p = 0.032) (Table 1). Stained crack damage was greater in loaded beams from the lateral region (0.191 ± 0.313 mm$^2$) than in those from medial (0.015 ± 0.030 mm$^2$) and dorsal (0.015 ± 0.041 mm$^2$) regions (p < 0.01). However, five of 18 loaded sections contained no stained cracks, compared with 12 of 18 control sections (p < 0.005, chi-square test). After monotonic or fatigue failure, the presence of cracks seemed to depend on the section's proximity to the fracture surface. Also, stained crack density was not correlated with distance from the neutral axis. Instead, stained cracks were primarily located on the compressed side of the beam. Often, a large crack extended along the beam, parallel to and about one-fourth of the way in from the compressed surface. This large crack would be the source of diagonally oriented stained cracks turning primarily toward the compressed surface. These smaller cracks would in turn be associated with numerous fine, stained cracks. Often, these secondary cracks would be associated with enhanced staining of canaliculi and calcified matrix to one side of the crack or the other, but not both (Fig. 3). We could not discern any relationship between such staining and local bone structure or the stress field. [Table 2]

Three-factor ANOVA (experimental compared with control specimen, anatomic region, and horse) showed that porosity was similar in the experimental and control specimens but was affected by horse and anatomic region. When the effects of horse-to-horse variability were blocked, porosity was significantly greater in the dorsal region than the medial (p = 0.0025)
and lateral (p = 0.0003) regions, which were not different from each other (Table 2). On the other hand, the remodeling index was greater in the medial region than either the dorsal (p = 0.022) or lateral (p = 0.0066) regions, which were not different from each other. There was significant horse-to-horse variation in this variable as well, which was blocked to obtain the above results. The numbers of active basic multicellular units also demonstrated significant horse-to-horse variability, but when this was blocked the regional variability in this measure of current remodeling was not significant (p = 0.22).

No significant correlations were found between stained and unstained crack variables. When all the beams were pooled, porosity had a positive but low correlation with unstained crack density ($r^2 = 0.12; p < 0.05$) and damage ($r^2 = 0.13; p < 0.04$) in the loaded sections but not in the control sections. Stained crack density, on the other hand, had a low and negative correlation with porosity in the loaded sections ($r^2 = 0.14; p < 0.04$). In other words, the more porous beams were inclined to gain more unstained crack damage as a result of loading than the less porous beams but were less likely to produce the larger, stained cracks at failure.

Elastic modulus had a low negative correlation with porosity ($r^2 = 0.16; p < 0.020$) but a moderate positive correlation with stained crack density ($r^2 = 0.30; p < 0.005$) and damage ($r^2 = 0.26; p < 0.002$). That is, the specimens that were stiffest initially tended to exhibit the most stained cracks after monotonic or fatigue failure. Monotonic failure loads were not significantly correlated with crack variables, but those specimens that deformed more before failure had higher stained crack densities after failure ($r^2 = 0.43; p < 0.03$). However, shorter fatigue lives were moderately correlated with greater stained crack density ($r^2 = 0.40; p < 0.001$) and damage ($r^2 = 0.42; p < 0.005$) after failure.
The dependence of remodeling on individual variability of the horses appears to be at least partially related to age. The remodeling index increased with age in the dorsal ($r^2 = 0.60; p < 0.01$) and medial ($r^2 = 0.49; p < 0.02$) regions but not in the lateral region ($r^2 = 0.26$). Conversely, active remodeling declined with age in the medial ($r^2 = 0.66; p < 0.01$) and lateral ($r^2 = 0.62; p < 0.01$) regions but not in the dorsal region ($r^2 = 0.03$; Fig. 5). The crack variables displayed no significant age-dependence, with a single exception: unstained crack length decreased with age in the dorsal region ($r^2 = 0.54; p < 0.01$).

**DISCUSSION**

We found the following answers to our three research questions. First, both monotonic and fatigue loading increased stained crack density and damage, but in a localized and inconsistent manner. Unstained crack density and damage were not significantly affected by loading, but unstained crack length was increased. Second, there were regional variations in the extent of past remodeling in the equine cannon bone, with the medial region having experienced the most remodeling. Regional differences in current remodeling activity were not statistically significant, however. Finally, the amount of past remodeling increased with age, especially in those regions with the best fatigue resistance (dorsal and medial). The rate of remodeling generally declined with age, except in the dorsal region, which had the best fatigue resistance. Micro-crack variables were age-independent, except that unstained cracks in the dorsal region were shorter in older horses.

Several limitations inherent in this study were addressed in a previous paper (14). A very important factor in this work is the variable training and racing histories of the horses. Statistical blocking on the horse variable helps suppress these variations to reveal
the effects of other variables. However, we do not know enough about the exercise and medical histories of the horses to explore the connections that almost certainly exist between these variables and, for example, remodeling activity.

Another limitation was the difficulty associated with identifying microcracks in bone sections and distinguishing those associated with fatigue from sectioning artifact. Bulk staining with basic fuchsin is the accepted means of making this distinction (3), but this method does not, in all likelihood, stain all the existing fatigue cracks or other forms of microdamage. In particular, it is unlikely to stain fine cracks or those located at some distance from a haversian canal or external surface. Thus, the method is liable to false negative results. Conversely, bone features like canaliculi may stain whether or not they are associated with a crack, producing false positive counts. We attempted to avoid the latter by using criteria in addition to staining, as suggested by previous investigators (2,3,13,16), to identify cracks. In addition, we identified and separately counted a particular class of unstained cracks that we thought were unlikely to be sectioning artifact. Until a better means of eliminating artifactual microcracks is developed, the best method of deciding whether unstained cracks are artifacts or not seems to be the comparison of measurements in loaded and control sections (22,23).

Another limitation was the use of control sections located 4-5 cm distal or proximal to the experimental sections. Although lengthwise tissue variations could confound such controls, in a separate (unpublished) study of equine cannon bones we found no significant variability in unstained cracks or static osteonal remodeling variables over the diaphyseal length used in the present study.
Frost (13) originally developed the basic fuchsin technique for isolation of fatigue cracks, reporting about 0.3 stained cracks/mm² in human rib. In verifying this technique, Burr and Stafford (3) observed 0.142 stained cracks/mm² in human rib. Recently, Schaffler et al. (24) observed zero to five stained cracks/mm² in human femurs. These numbers are consistent with our measurements (0.53-3.03 mm⁻²) for stained cracks in control sections.

Two other studies have examined the effects of experimental loading on microcracks. Mori and Burr (16) used en bloc staining after application of $10^4$ cycles of in vivo three-point bending to canine radii. Periosteal strains were 2,500 microstrain. They observed that stained crack density increased with loading, but lengths did not. Schaffler et al. (22) loaded bovine femoral and tibial bone at 0-1,200 microstrain in tension for $10^6$ cycles. Induced microcracks were identified without bulk staining by comparison with control (i.e., not loaded) specimens. In contrast to our unstained crack results, but consistent with our stained crack results and those of Mori and Burr, they found that loading significantly increased crack density but not length.

We believe that what we have termed unstained cracks are associated with Sharpey's fibers. As the cannon bone expands radially in the young racehorse, layers of periosteal woven bone form and become connected to the cortex by radial bony struts. The spaces thereby enclosed fill in with lamellar bone to form rows of primary osteons separated by the woven bone layers (25). Examination of the forming primary osteons in polarized light reveals Sharpey-like fibers passing from the filling space into the adjacent woven bone layer (see Fig. 5 in ref. 25). Examination of our beam sections in polarized light reveals that such transversely oriented fibers are distributed throughout the cortex in the same manner as the unstained cracks, but more abundantly. Most significantly, unstained
cracks lie within these birefringent fibers. We postulate that these cracks represent debonding or splitting of these fibers. The fact that the mean length of unstained cracks was greater in the loaded sections, but their number was not, suggests that the damage to individual fibers is extended with loading, but the number of damaged fibers does not change.

While our search has not been exhaustive, we have not found similar unstained cracks in canine, bovine, or human bone. However, we have observed them in the equine humerus, which has a histologic structure similar to that of the metacarpus. We postulate that fatigue damage in bone takes many forms, depending on the amount of loading and the specific structure of the tissue, and that other types of bone may contain analogous "minor" cracks. For example, Zioupos and Currey (26) discussed the relationship of microcracks in bovine bone to its histologic structure. The plexiform structure of this bone is somewhat like that of our equine specimens in that it consists of layers of lamellar and woven bone parallel to the periosteal surface, with the latter more highly mineralized (1). Stress concentrations would be expected at this interface and, indeed, that is where Zioupos and Currey saw (in tension specimens) the formation of minute, diffuse cracks that were much smaller than the basic fuchsin-stained cracks described by other authors. We hypothesize that those cracks are analogous to our unstained cracks, which also emanated from woven bone layers.

Furthermore, we postulate that these kinds of diffuse, small cracks constitute damage that is analogous to "subcritical damage" in composite materials: damage associated with energy dissipation but that is self-limiting (19). When composite materials are loaded, such damage typically plateaus at a level called the characteristic damage, which is not
increased by additional loading. Such damage is thought to diminish the energy available for propagation of more dangerous cracks through the structure. Since this form of damage is self-limiting, its capacity to divert energy is eventually exceeded, allowing truly dangerous cracks to grow and coalesce and lead to failure. We hypothesize that the Sharpey's fiber-related, unstained cracks that we have described serve such a role in the equine cannon bone. We have recently observed that unstained, Sharpey's fiber-associated cracks may also be common near the periosteal surfaces of canine radii, whether or not they have been experimentally loaded, especially near the insertions of muscles or tendons. These cracks are often longer than those in the equine specimens and occasionally stain red with basic fuchsin.

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REFERENCES


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FIG. 1. The upper diagram shows a side view of the experimental beam during four-point bending. C represents the proximal and distal locations of control sections, beyond the outer supports. X is the experimental, loaded region. The heavy, jagged line through X represents a typical fracture surface. The broken line through the middle of X shows the location of the three centrally located experimental cross sections. When these sections were greater than 5 mm from the fracture surface, additional experimental cross sections were taken closer to the fracture surface, as represented by the dotted line. The bottom diagram shows one of these 4 x 10 mm cross sections as it appeared on the microscope stage; its short edge corresponds to the dashed line in the upper diagram. Across the middle of the section arc depicted 30 microscopic fields examined for microcracks. Starting from the surface of the beam closest to the periosteal surface, these fields were examined in 10 scans.
FIG. 2. Photomicrograph of a control cross section showing numerous horizontal, unstained cracks adjacent to a woven bone region between the osteons at left and right. (One crack is marked with arrows.) Basic fuchsin stain; bar at lower left is 50 µm long.

FIG. 3. Photomicrograph of a stained crack (smaller arrows) passing through two secondary osteons. Note the enhanced staining of the bone matrix and the osteocyte-canalicular network to one side of this crack. "Ibis unilateral pattern of staining is common in the failed equine cannon bone. There is a much larger crack across the upper right corner (arrowheads) and a resorption cavity at the bottom of the field (opposed larger arrows). Bar at lower left is 50 pm long.
FIG. 4. Unstained crack density (top) and length (bottom) as functions of distance from the periosteal side of the beam for control (open circles) and loaded (solid circles) sections of all three loading groups. Each point is the value for one section. Crack density was greater near the periosteal side of the beam, but increases in length tended to occur away from the neutral axis, which was at about the 2 mm position.
FIG. 5. Active basic multicellular units (ACT BMUs)/mm$^2$ versus age of the horse for all the experimental sections. Each point is the value for one section. The density of active basic multicellular units in the lateral (triangles) and medial (squares) regions significantly decreased with age as indicated by the regression lines labeled L and M, respectively. The rate of remodeling in the dorsal region (circles) was more variable and did not change with age.
### TABLE 1. Summary of microcrack damage data in the experimental groups

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*Nearly significantly different from control, p = 0.06ₚ.
*Significantly different from control, p < 0.05.

### TABLE 2. Remodeling data

<table>
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<th>Region</th>
<th>Porosity (%)</th>
<th>Remodeling index</th>
<th>Active basic multicellular units/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal</td>
<td>4.ₚₚ (1.₀ₚ)</td>
<td>2.ₚₚ (0.ₚₚ)</td>
<td>0.ₚₚ (0.ₚₚ)</td>
</tr>
<tr>
<td>Medial</td>
<td>3.ₚₚ (0.ₚₚ)</td>
<td>2.ₚₚ (0.ₚₚ)</td>
<td>0.ₚₚ (0.ₚₚ)</td>
</tr>
<tr>
<td>Lateral</td>
<td>3.ₚₚ (0.ₚₚ)</td>
<td>2.ₚₚ (0.ₚₚ)</td>
<td>0.ₚₚ (0.ₚₚ)</td>
</tr>
<tr>
<td>All regions</td>
<td>3.ₚₚ (0.ₚₚ)</td>
<td>2.ₚₚ (0.ₚₚ)</td>
<td>0.ₚₚ (0.ₚₚ)</td>
</tr>
</tbody>
</table>

Numbers shown in parentheses are SDs. In this table only, different superscripts indicate significant differences between regions.