

High Fat Diet-Induced Obesity Negatively Affects Whole Bone Bending Strength but not Cortical Structure in the Femur

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1 **Abstract**

2 Although body mass index is positively associated with bone mineral density, suggesting obesity
3 is protective against fracture, elderly obese individuals experience greater fracture risk at certain
4 sites than non-obese peers, suggesting bone structural or material changes contribute to fragility.
5 Diet-induced obesity rodent studies have reported detrimental changes to bone microstructure and
6 some apparent-level material properties, but tissue-level material changes are not well understood.
7 Because adipose tissue is highly vascularized, and bone remodeling depends critically on
8 functional vascular supply, concurrent effects on osteovascular perfusion and structure may
9 provide insight about obesity-related bone fragility. This study aimed to determine the effects of
10 obesity on both tissue-level bone properties and osteovascular properties that could negatively
11 impact bone strength. Five-week-old male C57Bl/6J mice were fed either high fat diet (HFD) or
12 control fat diet (CFD) for 17 weeks and received daily treadmill exercise or remained sedentary
13 for eight weeks at ages 14-22 weeks. HFD negatively affected femur bending strength, with 18%
14 lower yield load than CFD. Although HFD negatively altered cancellous microstructure in the
15 distal femur, with 32% lower bone volume fraction than CFD, it did not affect cortical bone
16 geometry in the femoral metaphysis or diaphysis. HFD caused increased carbonate substitution
17 but had no effect on other composition metrics or apparent- or tissue-level material properties in
18 the femoral diaphysis. Exercise did not affect bone strength or microstructure but increased
19 endosteal mineralizing surface in the tibial diaphysis, mineral crystallinity and mineral-to-matrix
20 ratio in the femur, and blood supply to the proximal tibial metaphysis. HFD did not affect blood
21 supply in the tibia or 2D osteovascular structure in the distal femoral metaphysis, indicating that
22 HFD negatively affects cancellous bone without affecting osteovasculature. This study reveals that

23 HFD negatively affected cancellous microstructure without affecting osteovascular structure, and
24 whole-bone strength without altering cortical geometry or material properties.

25

26 **Keywords:**

27 obesity, high fat diet, bone strength, material properties, exercise

28 **1 Introduction**

29 Over half of adults worldwide are overweight or obese.⁽¹⁾ Higher bone mineral density (BMD), a
30 primary determinant of bone strength⁽²⁾ that is associated with decreased fracture incidence in
31 elderly men and women,^(3,4) is associated with increasing body mass index (BMI) in both obese
32 and non-obese individuals.⁽⁴⁻⁷⁾ However, increasing BMI in obese women is not as strongly
33 correlated with increasing BMD and estimated material strength compared to non-obese
34 women.^(7,8) A meta-analysis reported that, despite having higher BMD, elderly obese individuals
35 experience higher fracture incidence at particular sites compared to non-obese individuals – obese
36 postmenopausal women have a higher risk of fracture in the spine, humerus, and leg bones but a
37 lower risk of fracture in the hip and wrist, while older obese men have a higher risk of non-spinal
38 fractures but a lower risk of fracture in the spine.⁽⁵⁾ Since bone strength depends not only on BMD
39 but also structural and material properties,^(9,10) the differential fracture risk with obesity likely
40 results from adverse changes to bone structure and/or material properties, although these effects
41 are understudied in humans. In non-obese elderly women, mid-tibial cortical thickness and cortical
42 area, measured with high-resolution peripheral quantitative computed tomography (HR-pQCT),
43 were positively correlated with BMI.⁽¹¹⁾ Despite these beneficial changes to geometry, cortical
44 bone material strength index (BMSi) in the tibia, measured with reference point indentation, had
45 a weak negative correlation with both BMI and subcutaneous fat in the tibia.⁽¹¹⁾ Examining bone
46 structure and material properties beyond HR-pQCT and BMSi is difficult in humans, but they have
47 been examined in animal models of obesity. Previous diet-induced obesity studies in young, mostly
48 male, mice reported detrimental changes to trabecular microstructure in the femur,⁽¹²⁻¹⁷⁾ cancellous
49 bone formation rate,⁽¹⁷⁾ serum concentrations of osteocalcin, tartrate resistant acid phosphatase,⁽¹⁴⁾
50 and carboxyl-terminal collagen crosslinks,⁽¹²⁾ and cortical apparent material properties (bending

51 apparent modulus and ultimate stress) and fracture toughness,^(18,19) but no change to femoral areal
52 BMD,^(18,19) cortical volumetric BMD (vBMD),⁽¹⁶⁾ or cancellous tissue mineral density (TMD)<sup>(14-
53 16)</sup> for high fat diet (HFD) compared to control fat diet (CFD). Therefore, HFD-induced obesity
54 induces some structural and apparent-level material changes without changes to bone density,
55 further supporting the notion of tissue-level effects that need to be further examined.

56

57 Vascular properties may contribute to the detrimental changes in cancellous bone structure with
58 obesity. Adipose and bone tissues are highly vascularized and require adequate blood flow for
59 formation and homeostasis.⁽²⁰⁻²³⁾ In rodent studies, HFD increases the amount of adipose in the
60 medullary cavity of long bones,^(13,24-27) and adipose produces angiogenic cytokines that induce
61 rapid vascularization.^(20,28) Although increased bone vascularization is associated with increased
62 bone formation rate in cancellous bone in normal-weight rats,⁽²⁹⁾ HFD is associated with
63 detrimental changes to cancellous bone structure.⁽¹²⁻¹⁷⁾ In addition to the amount of blood vessels,
64 the structure of vasculature within bone is also important for remodeling; compared to non-
65 remodeling bone surfaces, active sites of bone remodeling have increased number of capillaries
66 within 50 μm of the bone surfaces.^(30,31) Exercise reduces the accumulation of adipose within the
67 long bones of mice fed HFD^(24,25) and stimulates osteovascular crosstalk pathways, such as VEGF
68 and bone morphogenetic protein 2 (BMP2), that promote bone formation.^(32,33) However, the
69 effects of HFD and exercise on the osteovasculature is understudied. In this study, we examined
70 changes in both bone and osteovascular tissues using a mouse model of diet-induced obesity, both
71 with and without moderate treadmill activity. We hypothesized that obesity decreases the integrity
72 of bone microstructure and material properties, while exercise induces new vascular and bone
73 growth.

74 **2 Materials and Methods**

75 *2.1 Study Design*

76 The protocol for this work was approved by the Institutional Animal Care and Use Committee at
77 North Carolina State University. Sixteen 5-week-old male C57Bl/6J mice (The Jackson
78 Laboratory, Bar Harbor, ME) were fed a high fat diet (D12492 60% kcal fat, Research Diets, Inc.,
79 New Brunswick, NJ) (n=8, “HFD” group) or a matched control fat diet (D12450B 10% kcal fat,
80 Research Diets, Inc) (n=8, “CFD” group) for 17 weeks (Figure 1). Mice were housed with their
81 groups (4-5 per cage) under controlled 12-hour diurnal photoperiod and fed their respective diets
82 *ad libitum*. After 9 weeks of diet (Week 9, 14 weeks of age), after the obesity phenotype was
83 established, mice were further divided into two activity groups, either daily treadmill exercise (n=4
84 from each diet group, “CFD-Exercise” and “HFD-Exercise”) or stationary treadmill groups (n=4
85 from each diet group, “CFD-Sedentary” and “HFD-Sedentary”).

86

87 Exercise mice were acclimated to a mouse treadmill (Exer 3/6, Columbus Instruments, Columbus,
88 OH) over three days of increasing speeds (day 1: 6 m/min for 10 min, day 2: 9 m/min for 10 min,
89 day 3: 12 m/min for 10 min). After acclimation, exercise groups ran on the treadmill 5 days per
90 week for 8 weeks (8 m/min for 37 min at a 5-degree incline). Mice in the HFD group were unable
91 to run for 30 min at 10 m/min, so the protocol was adjusted to 8 m/min for a longer time to provide
92 the same running distance (300 m). Sedentary groups were placed on an immobile replica treadmill
93 for the same duration as the exercise groups. Exercise and diet were continued for 8 weeks until
94 the end of the study (Week 17, 22 weeks of age).

95

96 For dynamic histomorphometry, alizarin complexone (C0875, Sigma-Aldrich, St. Louis, MO) and
97 calcein (A3882, Sigma-Aldrich) were injected intraperitoneally (30 mg/kg) at 10 and 3 days prior
98 to sacrifice, respectively. At the conclusion of the study, and immediately before sacrifice, *in vivo*
99 measurements of tibial perfusion were made under anesthesia (described below). Mice were
100 euthanized by CO₂ asphyxiation followed by cervical dislocation. For serum assays, blood was
101 immediately collected through cardiac puncture and left at room temperature for 30 min to clot,
102 after which the serum was separated by centrifugation (2,000 x g for 10 min) and stored at -80°C.
103 The left and right femora and tibiae were dissected. The left femur and both tibiae were fixed in
104 10% neutral buffered formalin at 4°C for 36 hours, then stored in 70% ethanol at 4°C. The unfixed
105 right femur was wrapped in 1X phosphate buffered saline (PBS)-soaked gauze and fresh frozen at
106 -20°C.

107

108 *2.2 Obesity Phenotype*

109 Body mass and serum glucose were measured weekly in all groups following the initiation of
110 treadmill exercise in Week 9. Serum glucose concentration was measured from the tail vein after
111 6 hours of fasting (AlphaTrak 2 Blood Glucose Monitoring System, Abbott Laboratories, Abbott
112 Park, IL). Glucose tolerance tests (GTT) were performed following 6 hours of fasting at Week 13
113 and Week 17 to assess ability to clear a bolus injection of glucose from the blood, which is a test
114 for the development of diabetes. For the test, a 0.3 g/mL (30%) glucose solution was injected
115 intraperitoneally at 1 g of glucose per kg of body mass. Serum glucose concentration was measured
116 immediately prior to the injection of glucose and 15, 30, 60, 90, and 120 min after the injection.
117 Glucose concentrations over the maximum threshold of the glucometer were recorded as 750

118 mg/dL (upper range concentration of the AlphaTrak 2). The areas under the curve for the GTT
119 results were calculated using the trapezoid rule.

120

121 *2.3 Bone Perfusion (Tibia)*

122 *In vivo* tibial perfusion was measured at the endpoint of the study in the right proximal tibial
123 metaphysis with laser Doppler flowmetry (LDF). LDF can quantify vascular perfusion – a
124 functional measure of bone blood flow comprised of amount of vasculature, velocity and direction
125 of blood flow, and vascular permeability – in murine tibiae.^(34,35) Perfusion readings were taken
126 just prior to sacrifice using an LDF monitor with 785-nm light source and selectable 3 kHz lowpass
127 filter (moorVMS-LDF, Moor Instruments Ltd., Axminster, UK) paired with a needle probe (VP4,
128 0.8 mm outer diameter, 0.25 mm fiber separation), as follows. After 6 hours of fasting, mice were
129 anesthetized with 2% isoflurane in pure oxygen. Mice were placed supine, and the shaved right
130 hindlimb was taped to a heated surgical platform. A 2-5 mm long was made over the anteromedial
131 side of the proximal tibial metaphysis, the periosteum was gently scraped away from the
132 metaphysis, and the LDF probe was held flush to the bone with a micromanipulator (MM3-ALL,
133 World Precision Instruments, Sarasota, FL) for a 30-second recording. The probe was removed
134 and replaced two more times, and the weighted mean of the three recordings was used for analysis.
135 Measurements are expressed in perfusion units (PU), arbitrary units that are standard for LDF.

136

137 *2.4 Cancellous and Cortical Bone Structure (Femur)*

138 Cancellous bone microstructure and cortical bone geometry were assessed in the left femur by
139 scanning in 70% ethanol with micro-computed tomography (micro-CT, μ CT80, SCANCO
140 Medical AG, Brüttisellen, Switzerland) using a 10- μ m voxel size, 45 kV peak X-ray tube potential,

141 177 μ A X-ray intensity, and 800-ms integration time. Volumes of interest (VOI) were analyzed
142 using the scanner's software (SCANCO v.6.6) for standard cortical and cancellous bone
143 metrics.⁽³⁶⁾ The distal metaphyseal VOI was defined as 10% of the total femur length positioned
144 proximal to the distal growth plate. The cancellous and cortical bone were contoured and analyzed
145 separately in the metaphysis. The diaphyseal VOI was defined as 15% of the total femur length,
146 centered between the distal growth plate and the middle of the third trochanter. The mid-diaphyseal
147 VOI (used for estimating apparent-level material properties with three-point bending data) was
148 defined as a 2.5-mm section with the same center as the diaphyseal VOI.

149

150 *2.5 Cortical Bone Remodeling (Tibia)*

151 Dynamic indices of cortical bone remodeling were examined in the right tibial diaphysis using
152 dynamic histomorphometry. The right tibia was embedded in methylmethacrylate, then sectioned
153 transversely in 200- μ m thick sections just distal to the tibiofibular junction under constant water
154 irrigation using a low-speed precision saw (IsoMet Low Speed Precision Cutter, Buehler, Lake
155 Bluff, IL). Sections were glued to glass slides with cyanoacrylate glue and sanded to 10-30 μ m
156 thickness with increasing grit sandpaper.⁽³⁷⁾ Two sections from each bone were imaged at 40X on
157 a Zeiss LSM 880 laser scanning microscope with Airyscan (Carl Zeiss Microscopy, Thornwood,
158 NY). Standard dynamic histomorphometry parameters – mineralizing surface per bone surface
159 (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) – were measured on
160 two sections per mouse using ImageJ (version 1.51v) and Photoshop (version CC 2018m, Adobe
161 Systems Inc., San Jose, CA),^(38,39) and the mean values were used for analysis.

162

163 *2.6 Whole Bone and Apparent-Level Mechanical Properties (Femur)*

164 The right femur underwent three-point bending to failure to measure whole bone mechanical
165 properties and estimated apparent-level material properties. Immediately prior to testing, the femur
166 was brought to room temperature and placed in a 37°C bath of 1X PBS for 60 sec. The bone was
167 centered over a 6.5-mm lower span (40% average femur length) with the anterior side facing up
168 so that the anterior diaphysis was loaded in compression. Three-point bending was performed to
169 failure using an actuator speed of 0.025 mm/sec (EnduraTec ELF 3220, Bose Corp., Minnetonka,
170 MN). Force (500-g capacity load cell, Sensotec Model 31/6775-06, Honeywell Sensotec,
171 Columbus, OH) and displacement were recorded at 100 Hz. After failure, the femur was
172 immediately wrapped in PBS-soaked gauze and returned to -20°C. Yield load (F_{yield}), maximum
173 (ultimate) load (F_{ultimate}), stiffness, post-yield deformation (PYD), and work-to-fracture were
174 calculated from load-displacement curves with MATLAB[®] (R2017, The MathWorks, Inc., Natick,
175 MA).⁽⁴⁰⁾ Yield was calculated as the point where a line with a 5% decrease in stiffness intersected
176 the force-displacement curve.⁽⁴⁰⁾ PYD was calculated as the difference between the deformation at
177 yield and the deformation at failure. The stress-strain curve was estimated using the cross-sectional
178 moment of inertia about the bending axis calculated from the mid-diaphyseal VOI in the micro-
179 CT scans of the left femur (described above).⁽⁴¹⁾ Yield stress (σ_{yield}), maximum (ultimate) stress
180 (σ_{ultimate}), and Young's modulus (E) were calculated from these estimated stress-strain curves with
181 MATLAB[®].

182

183 *2.7 Tissue-Level Mechanical Properties (Femur)*

184 Cortical bone material properties were examined with nanoindentation in the right femoral
185 diaphysis. Following three-point bending, the right femur was already divided in half at the failure
186 point (position of the top loading point); a 1-2 mm transverse section was cut just distal to the

187 distal half and affixed to a glass slide with the fractured end facing up. The remainder of the distal
188 half of the right femur was reserved for immunofluorescence (described below). The section was
189 smoothed with increasing grit sandpaper (120 followed by 600 grit) and then polished with 3 μm
190 diamond slurry (90-3DL3, Allied High Tech Products Inc, Rancho Dominguez, CA) until
191 smooth.⁽⁴²⁾ Before nanoindentation, Raman spectroscopy was performed on the proximal surface
192 of the polished cortical section (described below). Then nanoindentation was performed on the
193 same samples using a Hysitron TriboIndenter TI 980 with a diamond Berkovich tip (Bruker,
194 Billerica, MA). The instrument was calibrated by performing indentations in air and a fused quartz
195 standard. Each bone was indented in the anterior and posterior regions of the mid-cortex in a 4x4
196 grid of points equally spaced 15 μm apart. A trapezoidal loading function with 60 sec loading to
197 3000 μN , 30 sec holding at peak load, and 6 sec unloading was performed at each point.^(42,43) The
198 fused quartz standard was tested before and after each mid-cortex grid to validate calibration and
199 remove organic material from the tip. Force-displacement curves exhibiting nonlinearity during
200 loading were removed from analysis. Hardness (H) and reduced modulus (E_r) were calculated from
201 the force-displacement curve during unloading and were averaged across each region grid, giving
202 a mean for each anterior and posterior region.⁽⁴⁴⁾

203

204 *2.8 Cortical Bone Composition (Femur)*

205 Cortical bone tissue composition was measured with Raman spectroscopy (XploRA PLUS
206 confocal Raman microscope, HORIBA Scientific, Piscataway, NJ). Raman spectra were collected
207 with a 785-nm laser at 50X magnification at the endosteal edge, mid-cortex, and periosteal edge
208 in the posterior, lateral, anterior, and medial quadrants of the section (Figure 2A). Mid-cortex
209 quadrant scans were comprised of a 2 x 5 grid of point collections spaced 5 μm apart, while

210 endosteal and periosteal quadrant scans were comprised of a line of 6 points spaced 2 μm apart,
211 aligned parallel to and positioned 5-10 μm in from the bone surface. Each point was a 30-second
212 accumulation in the 800-1800 cm^{-1} range. The spectrometer software (LabSpec 6, v.6.5.1.24)
213 automatically performed baseline correction, while the remaining analysis was performed in
214 MATLAB[®].

215
216 Spectra were normalized relative to the phosphate ν_1 maximum intensity (930-980 cm^{-1}), and the
217 maximum normalized intensities were determined in the regions corresponding to the summation
218 of proline (830-863 cm^{-1}) and hydroxyproline (864-899 cm^{-1}), phosphate ν_1 (930-980 cm^{-1}),
219 carbonate ν_1 (1055-1090 cm^{-1}), amide III (1220-1300 cm^{-1}), and amide I (1616-1720 cm^{-1}) (Figure
220 2B).^(45,46) Several standard metrics were calculated, as follows.⁽⁴⁵⁾ Mineral-to-matrix ratios were
221 calculated as the ratio of the phosphate ν_1 normalized intensity relative to amide I, amide III, or
222 summed proline and hydroxyproline normalized intensity. The carbonate-to-matrix ratio was
223 calculated as the ratio of the carbonate ν_1 to amide I normalized intensities. Carbonate substitution
224 was calculated as the normalized intensity of carbonate ν_1 . Mineral maturity (crystallinity) was
225 calculated as the inverse of the full-width at half maximum (FWHM) of a single-order Gaussian
226 curve fit to the phosphate ν_1 band. Each of these Raman metrics were averaged across each
227 measurement grid within quadrants, giving a mean for each region (endosteal edge, mid-cortex, or
228 periosteal edge) at each quadrant (posterior, lateral, anterior, and medial).

229

230 *2.9 Osteovascular Structure (Femur)*

231 Vascular structure and proximity to bone surfaces were examined in the distal femoral metaphysis
232 using thick-section immunofluorescence to quantify the amount of blood vessels, labeled by
233 endomucin (EMCN), and bone surfaces, labeled by collagen type I (COL-1).⁽⁴⁷⁾ The remaining

234 distal portion of the right femur samples were fixed overnight in 10% neutral buffered formalin at
235 4°C, decalcified in 0.5M ethylenediaminetetraacetic acid at 4°C for 24 hours, and then embedded
236 in cryoprotectant embedding media comprised of 8% gelatin (G1890, Sigma-Aldrich), 2%
237 polyvinylpyrrolidone (P5288, Sigma-Aldrich), and 20% sucrose (S7903, Sigma-Aldrich) in 1X
238 PBS. Samples were sectioned longitudinally in 100- μ m thick sections on a cryotome at -23°C (HN
239 525NX, Thermo Fisher Scientific, Waltham, MA). Sections were stained overnight at 4°C using
240 unconjugated primary antibodies at 1:100 dilution for endomucin (rat anti-mouse sc-65495, Santa
241 Cruz Biotechnology, Santa Cruz, CA) and at 1:200 dilution for collagen type I (rabbit anti-mouse,
242 AB765P, MilliporeSigma, Burlington, MA). Secondary antibodies at 1:200 dilution were added
243 for 90 min at room temperature (goat anti-rat with AlexaFluor 647 ab150159, Abcam, Cambridge,
244 UK; goat anti-rabbit with AlexaFluor 488 A11006, Invitrogen, Carlsbad, CA). DAPI at 2 μ g/mL
245 was added for 10 min at room temperature to counterstain nuclei. All sections were imaged at 20X
246 on a Zeiss LSM 880 laser scanning microscope with Airyscan. Regions with positive COL-1 and
247 EMCN labeling were traced by hand in ImageJ (version 1.51v) in a region of interest (ROI) that
248 was 10% of the femur length and positioned just proximal to the distal growth plate (same as the
249 micro-CT metaphyseal VOI). Vascular structure was analyzed by calculating EMCN⁺ area per
250 total area, COL-1⁺ area per total area, and the distance between EMCN⁺ and COL-1⁺ surfaces in
251 MATLAB[®]. Several samples were destroyed or lost during sectioning, so only a subset of samples
252 were analyzed (n = 1 CFD-Sedentary, n = 3 CFD-Exercise, n = 2 HFD-Sedentary, n = 1 HFD-
253 Exercise).

254

255 *2.10 Osteovascular Crosstalk (Serum)*

256 To examine osteovascular crosstalk between endothelial cells and osteoblasts, serum
257 concentrations of bone morphogenetic protein 2 (BMP2) and vascular endothelial growth factor A
258 (VEGF-A) were measured using serum collected and stored at the endpoint of the study. Serum
259 concentrations were measured with enzyme-linked immunosorbent assays (ELISA) per the
260 manufacturers' instructions, using mouse-specific kits for BMP2 (ab119582, Abcam) and VEGF-
261 A (KMG0111, Thermo Fisher Scientific). All samples were analyzed in duplicate using a plate
262 reader (Synergy H1M, BioTek Instruments, Inc., Winooski, VT).

263

264 *2.11 Statistical Analyses*

265 All statistical models were analyzed in SAS (SAS University Edition v. 9.4, SAS Institute Inc.,
266 Cary, NC) or R (R v. 3.5.1, R Foundation for Statistical Computing, Vienna, Austria) to determine
267 the following: 1) effects of HFD and exercise on body mass and fasting serum glucose
268 concentration at each week after treadmill exercise was started; 2) effects of HFD and exercise on
269 metrics of glucose tolerance, bone perfusion, cancellous and cortical bone microstructure, cortical
270 bone remodeling, whole bone mechanical properties, apparent-level material properties,
271 osteovascular structure, and osteovascular crosstalk; 3) effects of HFD and exercise on cortical
272 bone material properties measured with nanoindentation and composition measured with Raman
273 spectroscopy. All data are presented as the group mean \pm standard deviation unless otherwise
274 stated. Results from nanoindentation and Raman spectroscopy are presented as mean across the
275 scanned regions.

276

277 For analysis #1, mouse mass and serum glucose were compared between diet and activity groups
278 across weekly timepoints using a repeated measures factorial model with interaction between all

279 terms (SAS ‘MIXED’ procedure). Diet (CFD or HFD) and activity (sedentary or exercise) were
280 modeled as fixed factors, while week was modeled as a repeated measure within each mouse. The
281 residual variance was modeled assuming compound symmetry covariance, chosen as the
282 covariance structure that provided the best fit to the data. Predicted least-squares means with
283 Tukey-Kramer adjustment for multiple comparisons were used to analyze effect differences
284 between diet and activity groups, with interaction, at each timepoint (i.e., CFD-Sedentary vs. HFD-
285 Sedentary at Week 9).

286

287 For analysis #2, outcome parameters were compared between diet and activity, with interaction,
288 using two-way analysis of variance (R ‘aov’ function). Tukey’s post-hoc tests were used to
289 compare group means. Vascular structure parameters were analyzed with a similar model, but the
290 interaction between diet and activity was not modeled due to missing data and thus insufficient
291 power to analyze the full model. Three-point bending parameters were further analyzed with two
292 analysis of covariance (ANCOVA) models, one with mass as the continuous covariate and one
293 with femur length as the continuous covariate.^(40,48)

294

295 For analysis #3, the same repeated measures factorial model used in analysis #1 was used (SAS
296 ‘MIXED’ procedure), but parameters were compared between diet and activity groups across scan
297 region (anterior and posterior for nanoindentation; posterior, lateral, anterior, and medial for
298 Raman spectroscopy). The residual variance was modeled assuming compound symmetry
299 covariance. Predicted least-squares means with Tukey-Kramer adjustment for multiple
300 comparisons were used to analyze pairwise differences between diet and activity groups, with
301 interaction (i.e., HFD-Sedentary vs. HFD-Exercise).

302 **3 Results**

303 *3.1 Obesity Phenotype*

304 Weekly measures of mouse mass, serum glucose, and monthly glucose tolerance tests confirmed
305 that the high fat diet produced an obese phenotype in this study. The HFD group had consistently
306 greater body mass at all timepoints compared to the CFD group ($p = 0.0016$, Figure 3A). At the
307 end of the study, after 17 weeks of diet, the HFD group (43.0 ± 5.2 g) weighed 33% more than the
308 CFD group (32.4 ± 1.7 g, $p < 0.0001$). Overall, the HFD group had increased fasting glucose
309 concentrations relative to the CFD group ($p = 0.0054$), but not at every timepoint (Figure 3B). At
310 the end of the study, fasting glucose concentration was 27% higher in the HFD group (246 ± 28
311 mg/dL) than in the CFD group (193 ± 36 mg/dL, $p = 0.014$). Exercise did not affect body mass (p
312 $= 0.76$) or fasting serum glucose concentration ($p = 0.57$).

313
314 The HFD group had a lower glucose tolerance, metabolizing a bolus of glucose more slowly
315 (represented by larger area under the curve) than did the CFD group at Week 13 (HFD: 33.2 ± 9.0
316 $p = 0.017$ vs. CFD: 26.2 ± 2.2) and Week 17 (HFD: 39.0 ± 9.0 , $p = 0.0004$ vs. CFD: 26.5 ± 7.4)
317 (Figure 3C). At Week 13, exercise nearly improved glucose tolerance in the HFD-Exercise group
318 (28.2 ± 2.8) relative to the HFD-Sedentary group (38.2 ± 8.1 , $p = 0.066$), bringing the glucose
319 tolerance of HFD-Exercise similar to that of CFD-Sedentary (26.0 ± 5.1 , $p = 0.92$) and CFD-
320 Exercise (26.0 ± 1.7 , $p = 0.96$) groups. The benefit of exercise in the HFD group did not persist,
321 however, and at Week 17, the HFD-Exercise (39.1 ± 4.5) and HFD-Sedentary (38.9 ± 6.7) groups
322 had similar glucose tolerance ($p = 1.00$) elevated above that of the CFD groups (CFD-Exercise:
323 28.0 ± 5.2 ; CFD-Sedentary: 25.0 ± 3.9). Several mice had glucose concentrations that were above
324 the detectible range of the glucometer, which artificially decreased the area under the curves.

325 During the Week 13 GTT, one HFD-Sedentary mouse had over-range readings at three timepoints,
326 and one CFD-Sedentary mouse had over-range readings at one timepoint. During the Week 17
327 GTT, one HFD-Sedentary and one HFD-Exercise mouse had over-range readings at two
328 timepoints each, and one CFD-Exercise had an over-range reading at one timepoint.

329

330 *3.2 Bone Perfusion (Tibia)*

331 At the end of the study, *in vivo* perfusion in the proximal tibial metaphysis was 29% greater in
332 exercise groups (12.2 ± 3.0 PU) compared to sedentary groups (9.5 ± 1.6 PU, $p = 0.044$, Figure 4).
333 Tibial perfusion was similar between HFD (11.7 ± 2.6 PU) and CFD groups (10.1 ± 2.7 PU, $p =$
334 0.23).

335

336 *3.3 Cancellous and Cortical Bone Structure (Femur)*

337 High fat diet had detrimental effects on trabecular microstructure in the distal femoral metaphysis.
338 Compared to CFD, the HFD group had 32% lower bone volume fraction (BV/TV, HFD: $11.2 \pm$
339 3.5% vs. CFD: $16.4 \pm 3.2\%$, $p = 0.0089$, Figure 5A); 20% lower trabecular number (Tb.N, HFD:
340 3.73 ± 0.23 mm⁻¹ vs. CFD: 4.64 ± 0.54 mm⁻¹, $p = 0.0010$, Figure 5B); and 26% greater trabecular
341 separation (Tb.Sp, HFD: 262.4 ± 18.4 μm vs. CFD: 208.5 ± 21.0 μm, $p=0.0001$, Figure 5C); but
342 similar trabecular thickness (Tb.Th, HFD: 51.3 ± 6.6 μm vs. CFD: 50.1 ± 3.6 μm, $p = 0.68$, Figure
343 5D). In addition, the connectivity density (Conn.D) of the trabecular network was 50% lower in
344 the HFD group compared to the CFD group ($p = 0.00053$, Table 1), but the degree of anisotropy
345 (DA) was not significantly different between HFD and CFD groups ($p = 0.11$, Table 1). HFD
346 group had a 30% lower trabecular vBMD than CFD group ($p = 0.0082$) but similar TMD ($p =$
347 0.98). Exercise did not significantly affect any of the metrics for trabecular bone microstructure.

348

349 While cancellous bone microstructure was substantially altered by HFD in the distal femoral
350 metaphysis, cortical bone geometry remained similar between HFD and CFD in the distal
351 metaphysis and diaphysis. In the femur, neither HFD nor exercise had a significant effect on
352 cortical vBMD, cortical area (Ct.Ar), total area (Tt.Ar), cortical area fraction (Ct.Ar/Tt.Ar), or
353 cortical thickness (Ct.Th) in either the cortical bone around the metaphyseal VOI or in the
354 diaphyseal VOI (Table 1). Similarly, in the mid-diaphyseal VOI, neither HFD nor exercise affected
355 medial-lateral moment of inertia (I_{ML} , $p = 0.25$ and $p = 0.38$, respectively) or anterior-posterior
356 moment of inertia (I_{AP} , $p = 0.11$ and $p = 0.28$, respectively). Overall femur length was also similar
357 across both diet ($p = 0.17$) and exercise ($p = 0.52$) groups (Table 1).

358

359 *3.4 Cortical Bone Remodeling (Tibia)*

360 Dynamic indices of cortical bone remodeling from dynamic histomorphometry were similar in the
361 HFD and CFD groups at both the endosteal and periosteal surfaces, with no significant differences
362 in MS/BS, MAR, or BFR/BS (Table 2). Exercise, however, did significantly affect the extent of
363 active remodeling bone surface, with 62% greater endosteal MS/BS compared to sedentary groups
364 ($p = 0.016$), but exercise did not affect endosteal MAR ($p = 0.74$) or BFR/BS ($p = 0.57$). The
365 periosteal surface had little labeling, and neither HFD nor exercise had a significant effect on
366 periosteal remodeling (Table 2).

367

368 *3.5 Whole Bone, Apparent, and Tissue Mechanical Properties (Femur)*

369 HFD negatively affected whole bone mechanical properties in the femur measured by three-point
370 bending (Figure 6, Table 3). Compared to the CFD group, the HFD group had 18% lower yield

371 load ($p = 0.039$) and nearly lower ultimate load (14% lower, $p = 0.058$) and stiffness (18% lower,
372 $p = 0.055$). After accounting for body mass (ANCOVA), none of the mechanical properties
373 differed between HFD and CFD groups, except whole bone stiffness tended to be lower in HFD
374 compared to CFD even after body mass adjustments ($p = 0.085$, Table 3). Femoral length was
375 similar across diet and exercise groups (Table 1), but when whole bone mechanical properties were
376 adjusted for femur length (ANCOVA), none of the mechanical properties differed between HFD
377 and CFD groups, except yield load was nearly lower in HFD compared to CFD ($p = 0.082$, Table
378 3). Similarly, none of the estimated apparent-level material properties – yield stress, ultimate
379 stress, and Young’s modulus – were significantly affected by HFD or exercise (Table 3). Cortical
380 tissue material properties assessed with nanoindentation were also not significantly affected by
381 HFD or exercise (Table 3). Both hardness and reduced modulus values were consistent across
382 regions ($p = 0.66$ and $p = 0.42$, respectively).

383

384 *3.6 Cortical Bone Composition (Femur)*

385 HFD had only a small effect on tissue composition as assessed by Raman spectroscopy (Figure 7),
386 nearly reducing carbonate substitution by 2% in the mid-cortex ($p = 0.080$) and by 3% along the
387 periosteal edge ($p = 0.083$, Figure 7F). Exercise had more pronounced effects on cortical bone
388 composition. Mineral maturity was nearly higher (2% greater phosphate crystallinity) for exercise
389 groups compared to sedentary groups near the periosteal edge ($p = 0.068$, Figure 7A). Exercise did
390 not affect mineral crystallinity in the mid-cortex ($p = 0.81$) or near the endosteal edge ($p = 0.20$).
391 Mineral-to-matrix band intensity ratios near the endosteal edge were higher for exercise groups
392 relative to sedentary groups for the phosphate/(proline+hydroxyproline) ratio (27% higher, $p =$
393 0.013, Figure 7B), phosphate/amide I ratio (18% higher, $p = 0.030$, Figure 7C), and

394 phosphate/amide III ratio (25% higher, $p = 0.023$, Figure 7D). Similarly, the carbonate-to-matrix
395 ratio (carbonate/amide I) near the endosteal edge was also increased for exercise compared to
396 sedentary (13% higher, $p = 0.023$, Figure 7E). Carbonate substitution was not affected by exercise
397 in any region (Figure 7F).

398

399 *3.7 Osteovascular Structure (Femur)*

400 Osteovascular structure in the distal femoral metaphysis, as assessed by immunofluorescence, was
401 not significantly affected by HFD or exercise (Table 4). Vessel area fraction (endomucin-positive
402 blood vessels per total area) within the bone was similar HFD and CFD ($p = 0.78$) and between
403 exercise and sedentary ($p = 0.51$) groups. Similarly, the average vessel-to-bone distance between
404 endomucin-positive blood vessels and collagen type I-positive bone surfaces did not differ
405 between HFD and CFD ($p = 0.44$) or between exercise and sedentary ($p = 0.15$). Bone surface area
406 fraction (col-1-positive bone area per total area) was 32% lower in the HFD group compared to
407 the CFD group ($p = 0.034$, Table 4), consistent with the reduced BV/TV noted above. Exercise did
408 not affect col-1-positive bone surface area fraction ($p = 0.51$), also consistent with BV/TV results.

409

410 *3.8 Osteovascular Crosstalk (Serum)*

411 Crosstalk between endothelial cells and osteoblasts, as assessed by serum ELISA, was not affected
412 by HFD or exercise (Table 5). At the end of the study, serum concentrations were similar between
413 HFD and CFD groups ($p = 0.27$ for BMP2 and $p = 0.89$ for VEGF-A) and also between exercise
414 and sedentary groups ($p = 0.36$ for BMP2 and $p = 0.43$ for VEGF-A).

415

416

417 **4 Discussion**

418 High fat diet-induced obesity reduced whole bone bending strength in the femur, without altering
419 cortical bone mineral density, geometry, or apparent- or tissue-level material properties relative to
420 control fat diet. Because bone strength depends on these parameters,^(9,10) we expected one of them
421 to be altered by HFD to explain the underlying cause for the relative strength deficits in that group.
422 The reductions in bending properties with HFD were no longer significant after adjusting for body
423 size, by including either body mass (yield and ultimate load) or femur length (ultimate load and
424 stiffness) as a covariate. Although femur length was not significantly different between HFD and
425 CFD groups, variations in body size seems to account for diet-related strength differences, as was
426 also reported in a recent study where the magnitude of the effects of HFD on cortical area and
427 bending strength were reduced after accounting for body mass.⁽⁴⁸⁾ HFD had deleterious effects on
428 cancellous bone microstructure in the distal femur, with reduced bone volume due to loss of
429 trabeculae, which reduces bone strength to a much greater extent compared to trabecular
430 thinning.⁽⁴⁹⁾ Therefore, bone strength at primarily cancellous bone sites, like vertebrae, may also
431 be reduced with HFD, as was demonstrated in the mouse L3 vertebra after HFD⁽¹⁵⁾ and the rat L6
432 vertebra after high sucrose diet induced-obesity.⁽⁵⁰⁾ HFD did not alter osteovasculature in
433 cancellous sites, with no differences in bone perfusion (proximal tibia) or vascular area and
434 proximity to bone surfaces (distal femur) relative to CFD. This work reveals that HFD negatively
435 affects cancellous bone microstructure without affecting vessel area, and cortical bone strength
436 without affecting cortical geometry or material properties, and only slight changes to tissue
437 composition.

438

439 HFD created an obese, hyperglycemic phenotype that persisted with daily treadmill exercise. After
440 9 weeks of diet, HFD groups were heavier than CFD groups, and after 13 weeks of diet, HFD
441 groups had significantly lower glucose tolerance and weekly fasting serum glucose concentrations
442 that were over 200 mg/dL, indicative of pre-diabetes.⁽⁵¹⁾ Although exercise had transient benefits
443 to glucose tolerance in the HFD group, these benefits did not persist to the end of the study at
444 Week 17, and daily treadmill exercise did not mitigate the negative effects of HFD on cancellous
445 bone microstructure. Exercise had no effect on femoral cortical mechanical properties at the whole
446 bone, apparent, or tissue levels, despite slightly increasing mineral-to-matrix ratios in the
447 diaphysis. Exercise, but not HFD, increased the extent of active remodeling bone surface in the
448 tibial diaphysis and bone perfusion in the proximal tibia but had no effect on the relative amount
449 of blood vessels or the distance between blood vessels and bone surfaces in the distal femur.

450
451 High fat diet negatively affected cancellous, but not cortical, bone structure in the femur. Our
452 reductions in cancellous microstructure and bone surface area in the distal femur with 60% fat diet
453 from age 5-23 weeks are consistent with results from several other studies, which also reported
454 cancellous bone degradation following HFD in young male C57Bl/6J mice. Compared to mice fed
455 a control fat diet, mice fed a high fat diet (either 45% or 60% fat) from 3-6 weeks of age to 15-28
456 weeks of age experienced 18-49% reductions in cancellous bone volume fraction^(12,14,15,17,52) and
457 10-18% reductions in trabecular number^(13,15) in the distal femoral metaphysis. Conversely, a 60%
458 HFD from 7-28 weeks of age induced a 14% increase in trabecular cross-sectional area in the distal
459 femur relative to CFD, but the measurements were obtained using peripheral quantitative
460 computed tomography with a large voxel size (70 x 70 x 500 μm).⁽⁵³⁾ Most studies have been
461 performed in young, male mice, though a couple of studies found similar reductions in BV/TV in

462 diets started after skeletal maturity was reached. A study comparing extended HFD from 7-28
463 weeks of age to short-term HFD from 25-28 weeks of age found a 19% decrease in cancellous
464 BV/TV in the distal femoral metaphysis with extended HFD and a 12% decrease with short-term
465 HFD.⁽¹²⁾ Similarly, a study comparing 60% HFD from 5-17 weeks of age (young) to HFD from
466 20-32 weeks of age (mature) found, compared to CFD mice of the same age, a 45% decrease in
467 BV/TV in the distal femoral metaphysis in young mice and a 29% decrease in mature mice.⁽¹⁵⁾
468 These studies demonstrate that diet-induced obesity in male mice commonly leads to detrimental
469 changes in cancellous bone microstructure, as we report here, and suggest that altered modeling
470 during skeletal growth is not solely responsible for the negative HFD effects on microstructure.

471
472 The effects of HFD on cortical bone geometry in male C57Bl/6J mice are less consistent. Similar
473 to our results, several groups report no effect on cortical bone parameters,^(13,14,16,54) but the study
474 that reported increased trabecular cross-sectional area also found a 7% increase in cortical area in
475 the diaphysis and a 21% increase in polar moment of inertia (pMOI) relative to CFD.⁽⁵³⁾ Similarly,
476 a 60% fat diet from 4-23 weeks of age resulted in an 11% increase in both diaphyseal Ct.Th and
477 Ct.Ar relative to CFD,⁽¹⁸⁾ while a 60% fat diet from 6-18 weeks resulted in slightly expanded
478 diaphyseal marrow area, lower Ct.Th, and similar pMOI relative to CFD.⁽¹⁷⁾ More research is
479 required to determine specific underlying factors that may be contributing to this variability in
480 HFD-induced effects on cortical bone structure, and whether these factors may help explain our
481 reduced femoral strength. In particular, cortical porosity, which we could not examine at the
482 resolution of our micro-CT scans, can impact bone strength,^(55,56) and changes in cortical porosity
483 with HFD are understudied. Two HFD studies have reported porosity measured with micro-CT
484 using voxel sizes between 10-12 μm ,^(13,57) but accurately measuring cortical porosity requires a

485 higher resolution with a voxel size of 1-2 μm , particularly for small animals.⁽⁵⁸⁾ To our knowledge
486 only one study has examined porosity at this appropriate resolution, and they found that porosity
487 measured with a 2- μm voxel size was up to 37% lower than porosity measured with a 1- μm voxel
488 size, and that HFD reduced vascular canal porosity by 33% relative to CFD.⁽⁵⁹⁾

489
490 HFD decreased whole bone mechanical properties in the femur, with 18% lower yield load, 14%
491 lower ultimate load, and 18% lower stiffness in three-point bending compared to CFD. Other
492 groups have also reported reduced femur bending properties for young male C57B/6J mice.
493 Studies with HFD beginning at 3-6 weeks of age and ending at 19-28 weeks of age reported a 12%
494 reduction in maximum load,⁽¹⁹⁾ 29% reduction in ultimate load, and 20% reduction in stiffness.⁽⁵²⁾
495 Similar results have also been reported in the L3 vertebra, with mice fed a 60% HFD from 5-17
496 weeks of age (young) or from 20-32 weeks of age (mature) having 17-24% lower yield load, 16-
497 26% lower maximum load, and 21-27% lower stiffness during compressive loading in both age
498 groups compared to age-matched mice fed a CFD.⁽¹⁵⁾ Conversely, in a study of cantilever bending
499 in the femoral neck, the HFD group (60% fat diet from 7-28 weeks of age) had 18% higher
500 maximum load and 29% higher bending modulus compared to the CFD group.⁽⁵³⁾

501
502 Despite reductions in whole bone mechanical properties, we found no changes in estimated
503 apparent-level material properties with HFD. The study with reduced maximum force and stiffness
504 in the L3 vertebra also found no significant changes in apparent-level material properties in either
505 young or old HFD mice compared to age-matched CFD.⁽¹⁵⁾ Other groups have reported either
506 reduced or increased apparent-level material properties for HFD vs. CFD in male C57B/6J mice.
507 For whole femurs in three-point bending, two studies found that HFD (60% fat diet starting from

508 3-6 weeks-of-age to 19-28 weeks of age) caused 19-32% lower apparent elastic modulus, 15-26%
509 lower maximum stress, and 24% lower yield stress,^(18,19) while another study found 44% higher
510 apparent elastic modulus.⁽⁵²⁾ Tissue-level material properties were also unaltered by HFD in our
511 study. To our knowledge, no previous study has examined the effects of HFD on tissue-level
512 material properties. Since we did not find HFD-induced changes in bone density, structure, or
513 tissue-level properties, the reduced whole bone strength may result from a combination of small
514 changes in several parameters that were not statistically significant in this study.

515
516 Cortical tissue composition in the femur was altered by exercise, with increased mineral-to-matrix
517 and carbonate-to-matrix ratios near the endosteal edge and increased mineral maturity near the
518 periosteal edge. Mineralization of new bone tissue occurs slowly, so higher mineral-to-matrix and
519 carbonate-to-matrix ratios are associated with older bone that is generally harder and stiffer.^(45,60)
520 However, a study using the same treadmill regimen initiated at 16 weeks of age found that
521 treadmill exercise increased ultimate strain and the mineral-to-matrix ratio of phosphate ν_1 to
522 summed proline and hydroxyproline without affecting tibial morphology, suggesting increased
523 mineral-to-matrix ratios could be a mechanism by which bone adapts to exercise to maintain local
524 functional strain.⁽⁶¹⁾ Other studies have used Raman spectroscopy to analyze the increased
525 accumulation of advanced glycation end-products (AGEs), known to cause material differences
526 that increase fracture risk,^(52,62-64) in rodent diabetic bone. Elevated glucose may lead to AGE
527 accumulation in collagen,^(62,65) which has been shown to increase resistance to plastic deformation
528 and stiffness at the material level in bone.^(64,66) A recent study in HFD mice (60% fat from 8-30
529 weeks of age) found no difference in mineral-to-matrix ratio, crystallinity, or carbonate
530 substitution compared to CFD, but an increased amount of the AGE pentosidine (PEN), which was

531 positively correlated with higher bending modulus despite lower stiffness and ultimate load.⁽⁵²⁾
532 However, the Raman spectra in our study did not contain any of these AGE bands, indicating
533 AGEs were not significantly present.

534

535 This study found no effect of HFD or exercise on 2D osteovascular structure (vessel area and
536 proximity to bone surfaces) in the distal femur, but stereological methods are not ideal for
537 measuring complex three-dimensional structures like the branching network of blood vessels,⁽⁶⁷⁾
538 so HFD may have affected osteovascular parameters that are not quantifiable with stereology.
539 Similar to our results, a recent study reported no HFD-related changes in the 3D vessel network in
540 the proximal tibia using a new contrast agent with micro-CT.⁽⁶⁸⁾ Specifically, HFD from 8-30
541 weeks of age did not affect the vessel volume per medullary volume or the distance between blood
542 vessels and bone surfaces compared to CFD. However, this study also reported that HFD reduced
543 the number of blood vessels by 3.9-fold and increased average vessel diameter by 2.7-fold, metrics
544 that cannot be accurately quantified with stereological techniques.

545

546 Perfusion is a functional measure of blood supply to tissue that incorporates not only the amount
547 of blood vessels but also the velocity and direction of the blood flow in the vessels, as well as
548 vessel permeability and diameter.⁽⁶⁹⁾ For example, if HFD increased vessel diameter but reduced
549 vessel number compared to CFD, these changes could offset each other and result in the same
550 perfusion measurement. Similarly, the increased perfusion observed with exercise may result from
551 other structural changes to the vascular network besides vessel area and proximity to bone surfaces,
552 which were similar between sedentary and exercise groups. Furthermore, bone perfusion likely
553 experiences temporal changes in response to interventions like HFD and exercise; however, it was

554 only measured at the end of the study to avoid causing inflammation in the hindlimb, as
555 recommended by the group that developed the method for assessing perfusion in mouse tibiae.⁽³⁴⁾
556
557 Diet-induced obesity has far-reaching physiological effects that can impact bone health and may
558 be responsible for the observed envelope-specific changes to cancellous but not cortical bone
559 structure. In this study, HFD led to the development of obesity and pre-diabetic levels of elevated
560 serum glucose, both of which impact metabolic pathways that influence bone metabolism.
561 Elevated glucose concentrations are associated with reduced BMD in rats and humans,^(70,71) as
562 well as *in vitro* proliferation and mineralization of osteoblasts.^(65,72,73) Obesity in humans and HFD-
563 induced obesity mouse models are associated with increases in both leptin and glucocorticoids,
564 which differentially affect cortical and cancellous bone envelopes.⁽⁷⁴⁻⁷⁷⁾ Leptin, which signals
565 satiety, also promotes the maintenance of bone mass; when the leptin receptor is knocked out
566 globally in mice they become obese, even without HFD, and gain cortical bone but lose cancellous
567 bone.⁽⁷⁷⁾ Mice with conditional knockout of the leptin receptor in bone marrow stromal cells,
568 however, do not become obese without HFD. With 12 weeks of HFD, the conditional knockout
569 prevented detrimental cancellous microstructure changes and decreased the number of
570 mesenchymal stem cells (MSC) that differentiated into adipocytes compared to wild-type mice,
571 suggesting obesity affects bone maintenance directly through leptin.⁽⁷⁸⁾ Corticosterone, a
572 glucocorticoid in rodents, is associated with increased bone resorption, but in growing mice the
573 effect is bone- and site-specific, tending to increase endosteal resorption while preventing
574 periosteal remodeling and leading to an expanded marrow cavity.⁽⁷⁶⁾ Unlike leptin, the effect of
575 obesity on increased serum glucocorticoids in either rodents or humans is unclear.^(75,79) Lastly,
576 increased amounts of marrow adipose tissue (MAT) may negatively affect cancellous bone

577 structure. We did not quantify MAT in this study, but other groups report dramatic increases in the
578 amount of metaphyseal MAT with HFD,^(13,24-27) and decreased MAT with intense exercise.^(24,25)
579 Moderate treadmill exercise did not affect bone microstructure in this study, but other studies that
580 utilize more intense exercise regimen, such as free access to running wheel^(24,25,53,80) or high
581 intensity treadmill training,⁽⁸¹⁾ found effects of exercise in HFD mice.

582

583 In conclusion, our study demonstrated that high fat diet-induced obesity caused detriments to
584 cancellous bone microstructure and whole bone bending strength in the femur that were not
585 concomitant with changes to metaphyseal perfusion or vascularity, or to cortical geometry or tissue
586 properties. We also showed that moderate treadmill activity did not reverse the deleterious effects
587 of HFD, increase intraosseous vascularity, or increase mechanical properties in this model.
588 Exercise did, however, increase intraosseous perfusion in the tibia, and stimulate changes to tissue
589 composition in the femur, without affecting geometry. These findings should be examined further
590 by characterizing changes to intraosseous perfusion at different timepoints during the development
591 of HFD, and by incorporating more intense exercise routines.

592

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610

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616

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882

Figure Legends

Figure 1: Experimental design: Mice were fed either control fat diet (CFD) or high fat diet (HFD) starting at 5 weeks of age. After 9 weeks of diet, groups either were exercised (moving treadmill) or remained sedentary (stationary treadmill) for 8 weeks. After 17 total weeks of diet and 8 weeks of exercise, various endpoint vascular and bone metrics were analyzed.

Figure 2: A) Bone composition was assessed by Raman spectroscopy at three positions in the posterior, lateral, anterior, and medial quadrants within the cortical diaphysis of right femora: mid-cortex (2x5 point array) and endosteal and periosteal edges (1x6 linear array). B) Raman spectra were normalized to the phosphate ν_1 band intensity (b), and crystallinity was calculated as the inverse of the full-width at half maximum of the phosphate ν_1 band. Mineral-to-matrix band intensity ratios were calculated for phosphate ν_1 relative to the summation of proline and hydroxyproline (a), amide I (e), and amide III (d). Carbonate substitution (carbonate ν_1 (c)/ phosphate ν_1) and carbonate-to-matrix ratio (carbonate ν_1 / amide I) were also calculated.

Figure 3: A) Body mass was consistently higher with HFD than CFD at every timepoint. B) Weekly fasting glucose concentration were higher in the HFD group at Week 11, 13, 15, 16, and 17. C) HFD had lower glucose tolerance (higher area under curve) than CFD at Weeks 13 and 17 of diet. Data in A-B presented as estimated least-squares mean \pm 95% confidence interval. a: $p < 0.05$ HFD vs. CFD (main effect), b: $p < 0.10$ HFD-Exercise (Ex) vs. HFD-Sedentary (Sed).

Figure 4: Bone perfusion in the proximal tibial metaphysis was significantly higher in exercise (Ex) than in sedentary (Sed) groups but not with HFD compared to CFD. c: $p < 0.05$ Ex vs. Sed (main effect). PU = perfusion unit (arbitrary).

Figure 5: Relative to CFD, HFD groups exhibited significantly less robust trabecular architecture in the distal femur, with A) decreased bone volume fraction and B) trabecular number and C) increased trabecular separation, but D) no differences in trabecular thickness. a: $p < 0.05$ HFD vs. CFD (main effect).

Figure 6: Representative force-displacement curves from femur three-point bending to failure. Relative to CFD, HFD significantly reduced yield load and nearly reduced stiffness and ultimate load. a: $p < 0.05$ HFD vs. CFD (main effect), d: $p < 0.10$ HFD vs. CFD (main effect).

Figure 7: Relative to sedentary, exercise groups had increased A) mineral crystallinity on the periosteal edge. Along the endosteal edge, exercise increased B) phosphate ν_1 to combined proline and hydroxyproline ratio, C) phosphate ν_1 to amide I ratio, D) phosphate ν_1 to amide III ratio, and E) carbonate ν_1 to amide I ratio but not F) carbonate substitution. Points represent mean of all quadrants per femur, lines and bars represent estimated least-squares mean \pm 95% confidence interval c: $p < 0.05$ Ex vs. Sed (main effect), d: $p < 0.10$ HFD vs CFD (main effect), g: $p < 0.10$ Ex vs. Sed (main effect).

Tables

Table 1: Cancellous and Cortical Bone Structure in the Femur (mean \pm SD)

Trait	Control Fat Diet		High Fat Diet	
	Sedentary	Exercise	Sedentary	Exercise
Distal metaphysis (cancellous and cortical)				
Conn.D (mm ⁻³)	152.7 \pm 52.9	144.1 \pm 21.8	82.4 \pm 13.5 ^a	67.0 \pm 22.8 ^a
DA	1.39 \pm 0.14	1.48 \pm 0.21	1.40 \pm 0.17	1.32 \pm 0.09
Trabecular vBMD (mg/cm ³)	190.8 \pm 59.5	165.6 \pm 18.5	141.0 \pm 33.9 ^a	106.5 \pm 21.1 ^a
Trabecular TMD (mg/cm ³)	811.3 \pm 12.8	802.7 \pm 18.0	814.0 \pm 23.9	803.2 \pm 4.9
Cortical vBMD (mg/cm ³)	682.5 \pm 31.4	670.1 \pm 21.1	678.6 \pm 17.3	670.5 \pm 9.0
Ct.Ar (mm ²)	0.99 \pm 0.11	0.94 \pm 0.06	0.99 \pm 0.17	0.90 \pm 0.07
Tt.Ar (mm ²)	3.75 \pm 0.20	3.67 \pm 0.36	3.62 \pm 0.43	3.57 \pm 0.16
Ct.Ar/Tt.Ar (%)	26.5 \pm 2.1	25.7 \pm 1.6	27.1 \pm 1.5	25.1 \pm 1.4
Ct.Th (μ m)	126.0 \pm 9.3	122.0 \pm 4.3	125.2 \pm 11.4	118.6 \pm 6.2
Diaphysis (cortical)				
vBMD (mg/cm ³)	841.5 \pm 15.0	834.6 \pm 8.3	841.0 \pm 16.7	824.6 \pm 14.7
Ct.Ar (mm ²)	1.12 \pm 0.10	1.04 \pm 0.08	1.00 \pm 0.18	0.93 \pm 0.05
Tt.Ar (mm ²)	2.62 \pm 0.22	2.42 \pm 0.35	2.33 \pm 0.37	2.27 \pm 0.11
Ct.Ar/Tt.Ar (%)	39.7 \pm 2.3	41.5 \pm 3.5	41.8 \pm 0.6	40.5 \pm 1.6
Ct.Th (μ m)	191.0 \pm 10.5	193.0 \pm 9.2	194.1 \pm 20.4	186.7 \pm 9.7
Mid-diaphysis (cortical)				
I _{ML} (mm ⁴)	0.22 \pm 0.04	0.19 \pm 0.05	0.18 \pm 0.06	0.17 \pm 0.02
I _{AP} (mm ⁴)	0.55 \pm 0.09	0.48 \pm 0.10	0.45 \pm 0.15	0.39 \pm 0.03
Femur Length (mm)	16.4 \pm 0.3	16.1 \pm 0.8	15.9 \pm 0.3	15.9 \pm 0.2

a: p < 0.05 HFD vs. CFD (main effect)

Table 2: Dynamic Indices of Cortical Bone Remodeling in the Tibial Diaphysis (mean \pm SD)

Trait	Control Fat Diet		High Fat Diet	
	Sedentary	Exercise	Sedentary	Exercise
Endosteal				
MS/BS (%)	27.8 \pm 3.6	43.1 \pm 13.4 ^c	27.0 \pm 12.7	39.8 \pm 6.8 ^c
MAR (μ m/day)	0.59 \pm 0.25	0.34 \pm 0.24	0.33 \pm 0.41	0.49 \pm 0.36
BFR/BS (μ m ³ / μ m ² /day)	0.30 \pm 0.12	0.18 \pm 0.13	0.13 \pm 0.16	0.23 \pm 0.16
Periosteal				
MS/BS (%)	4.3 \pm 5.1	13.4 \pm 15.1	4.4 \pm 6.7	8.7 \pm 15.2
MAR (μ m/day)	0.12 \pm 0.16	0.22 \pm 0.26	0.10 \pm 0.13	0.20 \pm 0.26
BFR/BS (μ m ³ / μ m ² /day)	0.02 \pm 0.02	0.09 \pm 0.12	0.01 \pm 0.01	0.05 \pm 0.10

c: p < 0.05 Exercise vs. Sedentary (main effect)

Table 3: Whole Bone, Apparent, and Tissue Mechanical Properties in the Femoral Diaphysis

Trait	Control Fat Diet		High Fat Diet	
	Sedentary	Exercise	Sedentary	Exercise
Whole bone mechanical properties (three-point bending) (mean ± standard deviation)				
F _{yield} (N)	16.0 ± 2.1	16.1 ± 3.7	13.8 ± 2.3 ^{a,f}	12.6 ± 1.3 ^{a,f}
F _{ult} (N)	22.3 ± 4.1	20.9 ± 2.1	19.1 ± 3.1 ^d	18.0 ± 1.6 ^d
Stiffness (N/mm)	137.5 ± 21.9	132.9 ± 17.6	119.3 ± 25.9 ^{d,e}	101.7 ± 26.2 ^{d,e}
PYD (mm)	0.13 ± 0.04	0.11 ± 0.05	0.11 ± 0.02	0.14 ± 0.02
Work-to-fracture (mJ)	6.09 ± 0.98	4.43 ± 0.97	3.79 ± 1.60	5.02 ± 0.75
Apparent material properties (three-point bending) (mean ± standard deviation)				
σ _{yield} (MPa)	93.4 ± 8.9	100.5 ± 22.4	96.5 ± 15.8	88.15 ± 5.0
σ _{ult} (MPa)	117.6 ± 6.1	121.9 ± 11.2	117.2 ± 12.7	117.3 ± 6.9
E (GPa)	3.65 ± 0.15	4.15 ± 0.74	3.89 ± 0.73	3.58 ± 1.19
Tissue material properties (nanoindentation) (least squares mean ± 95% confidence interval)				
H (GPa)	0.96 ± 0.21	0.85 ± 0.22	0.90 ± 0.20	0.87 ± 0.21
E _r (GPa)	25.6 ± 5.6	22.7 ± 5.9	25.2 ± 5.6	22.6 ± 5.5

(main effects) a: p < 0.05 HFD vs. CFD raw data; d: p < 0.10 HFD vs. CFD raw data; e: p < 0.10 HFD vs. CFD body mass-adjusted; f: p < 0.10 HFD vs. CFD femur length-adjusted

Table 4. Osteovascular Structure in the Distal Femoral Metaphysis (mean ± standard deviation)

Metric	CFD	HFD	Sedentary	Exercise
Vessel area fraction (% EMCN ⁺)	7.9 ± 3.6	7.2 ± 1.9	5.8 ± 3.5	8.9 ± 1.6
Bone surface area fraction (% COL-1 ⁺)	19.2 ± 2.4	13.1 ± 2.5 ^a	15.8 ± 4.3	17.1 ± 4.2
Vessel-to-bone distance (μm)	78.6 ± 41.8	60.5 ± 8.0	85.9 ± 41.7	59.5 ± 20.5

a: p < 0.05 HFD vs. CFD (main effect). Area fractions expressed at % of total area.

Table 5. Serum Concentrations of Bone-Vascular Crosstalk Markers (mean ± standard deviation)

Marker	Control Fat Diet		High Fat Diet	
	Sedentary	Exercise	Sedentary	Exercise
BMP2 (pg/mL)	80.7 ± 12.1	72.0 ± 7.5	73.8 ± 3.4	98.9 ± 31.0
VEGF-A (pg/mL)	42.8 ± 13.4	52.0 ± 8.8 ^a	55.4 ± 8.0	38.0 ± 8.5

Figures

Figure 1

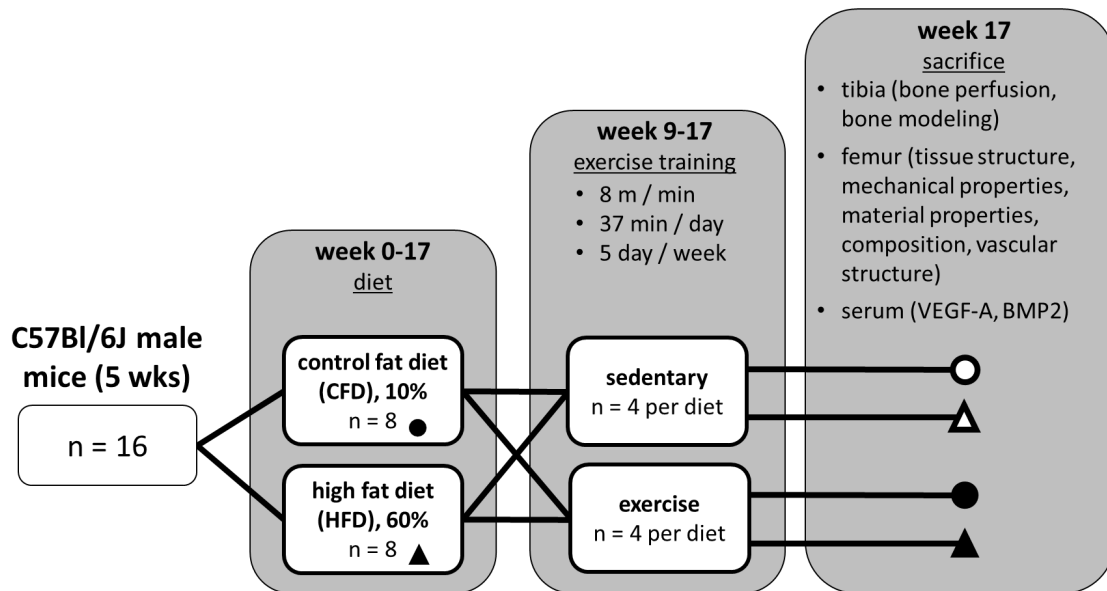


Figure 2

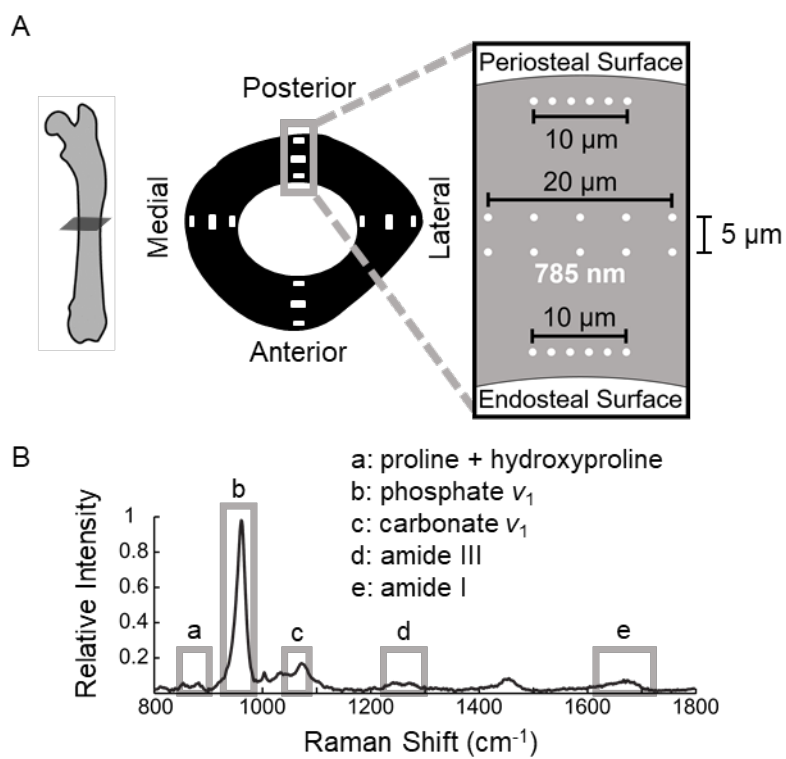


Figure 3

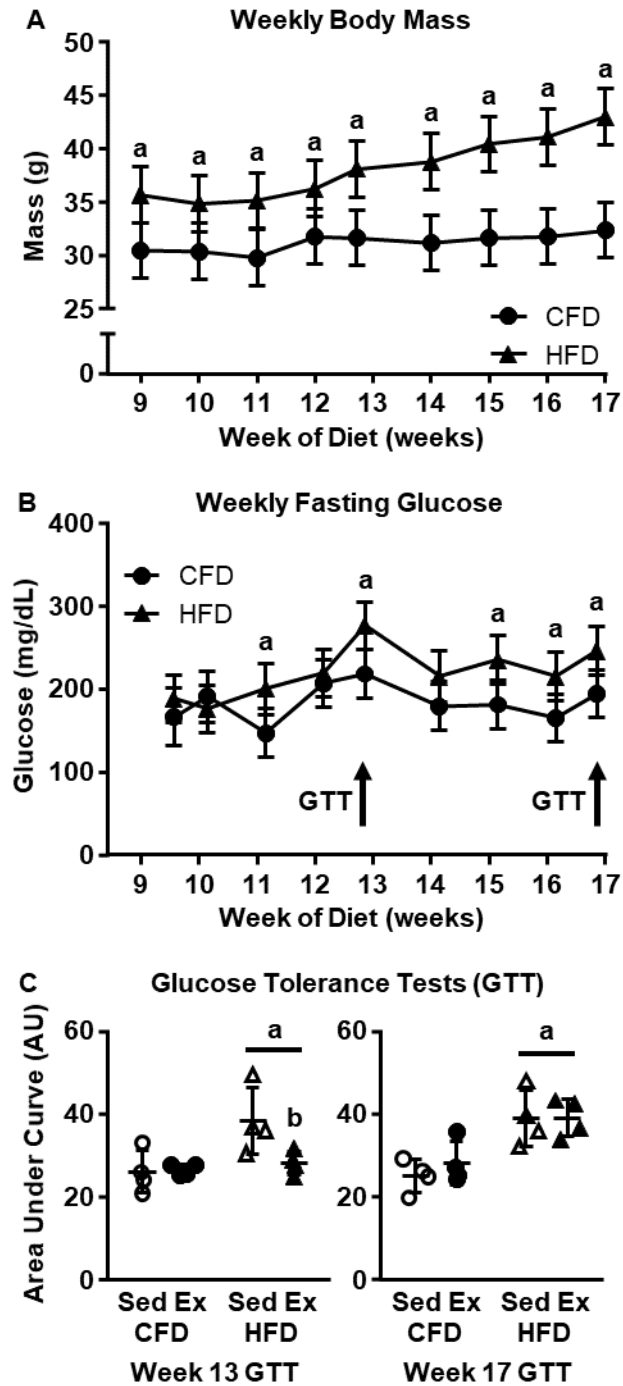


Figure 4

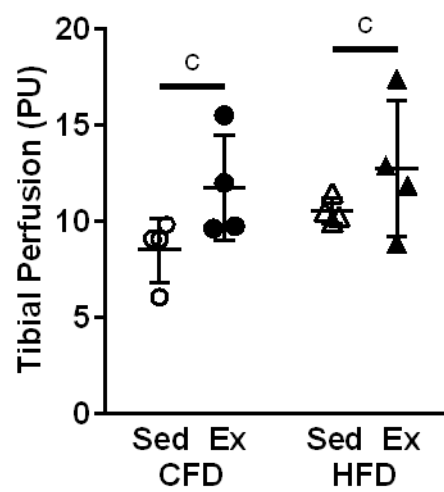


Figure 5

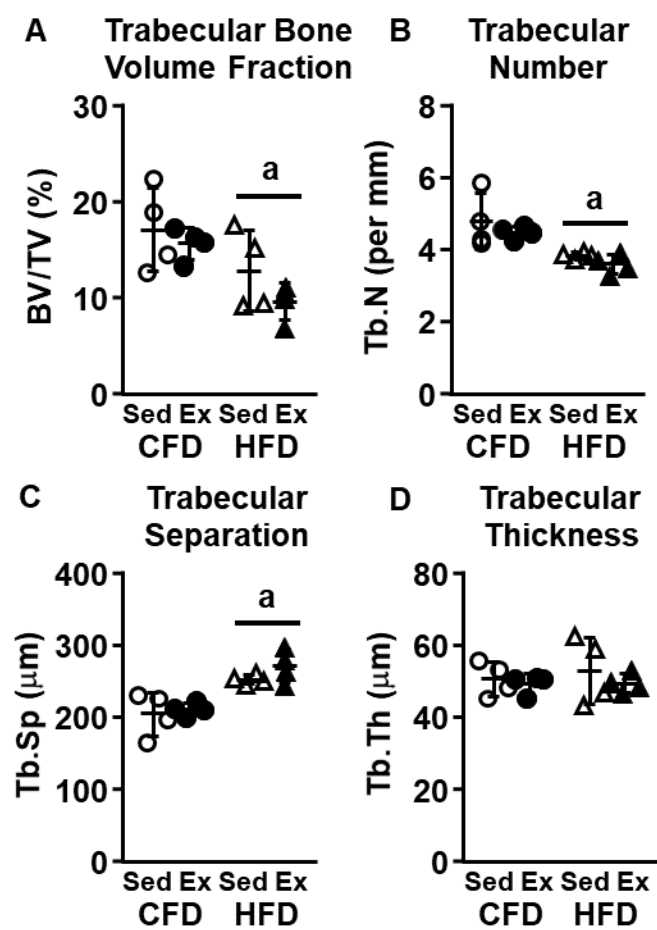


Figure 6

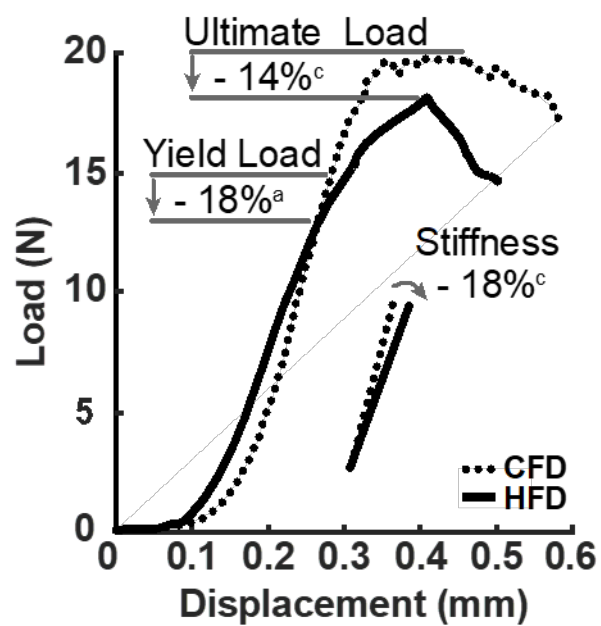


Figure 7

