nature portfolio

Peer Review File

Short-term moderate high fat diet impacts bone tissue material properties and metabolism for young adult and aged C57BL/6JN mice

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study used short-term, moderate-fat diet to investigate skeletal health without severe metabolic disruption compounded with aging. The investigation is rigorous and thorough, covering aspects of inflammation response, bone turnover, cellular activities, matrix quality, tissue strength and fracture toughness, etc.
Below are my specific comments:
1. The study title may not be accurately reflected by the results. Both aging and HFD led to decrease of Kcinit. But is there any interaction between age and diet? Is the loss of Kc with aging more pronounced in the HFD group?
2. Since the journal places method section at the end, please briefly provide information on age of the mice and study period at the end of the introduction or the beginning of the results.
3. The term “baseline glucose” creates ambiguity. Consider changing the term to “fasting glucose”. Maximum and delta glucose levels are less relevant in presenting glucose metabolism and can be removed from the results.
4. How do the authors explain the HFD increased body mass and adipose depot mass in males but not in females?
5. The 20-fold increase in adipokine leptin level in young HFD mice compared to all other groups seems unusual. What do the authors think led to this dramatic change?
6. Did the authors assess cortical TMD and was it different across groups?
7. The authors should define the outlier criterion in the methods and determine whether outlier should be included in statistical analyses. Presenting statistical outcomes both with and without outlier may complicate the interpretation.
8. As an important outcome, the authors could present the bone strength boxplots.
9. Aging didn’t seem to change global bone turnover measures (although some interactions are significant). Why do the authors think this happened?
10. Raman-measured carbonate:phosphate ratio does not directly indicate mineral maturity (line 338). Studies have used crystallinity to indicate mineral maturity, which is not different between age groups in this study. Therefore, the statement of “aging increased bone mineral maturity” based on carbonate:phosphate ratio is incorrect.
11. Why does only one carbonate:phosphate ratio measure include a covariate of glucose AUC (Figure 7 row 2)? What’s the relevance here?
12. Could the authors also compare the I1670/I1690?
13. Figure 8 is confusing. It is very difficult to understand how the correlational results are reflected in the heatmaps. Please either choose an alternative way to present Figure 8, or explain the clustering analysis in more detail in the results section.
14. Consider also conducting correlational analysis with Kcmax.

Reviewer #2

(Remarks to the Author)
The manuscript titled "High-fat diet exacerbates the loss of bone fracture toughness in aging for C57BL/6JN mice" by Brown et al. is well written and possesses considerable clinical relevance. The authors investigated how age, gender, and diet differentially affect energy metabolism, trabecular and cortical bone properties, bone mineralization and matrix composition, collagen structure, as well as cellular functions and metabolic pathway signatures in male and female young and aged C57BL/6JN mice under low-fat and high-fat diets. Given the increasing aging population and prevalent unhealthy eating

habits, this study is of significant clinical importance.

The research is comprehensive, employing a variety of methodologies to address the research question effectively. The authors utilized appropriate techniques, and the results are presented with clarity and precision. Additionally, the manuscript is well structured, featuring a thorough discussion. However, some major points should be addressed.

The abstract is written in a simplified manner and does not adequately reflect the comprehensiveness and complexity of the entire study. It should be revised to better capture the scope and depth of the research.

The authors discuss local inflammatory responses (line 582 ff); however, it is unclear what specific aspects were investigated. It would be beneficial to stain for particular immune cells or inflammatory markers locally to gain deeper insight into the effects of age, gender, and diet on local inflammation.

Body weight significantly affects movement and load-bearing, which in turn impacts load-induced bone formation and bone strength. Therefore, it would be valuable to know if there were differences in load-bearing and locomotion between the groups, as these factors may have influenced the results.

Overall, the results exhibit considerable heterogeneity based on age, sex, and diet. The effects of sex, particularly the lack of weight gain in female mice on a high-fat diet (HFD) and the observation that HFD females have stronger bones than males, warrant a more detailed discussion.

The heterogeneity of the results may also stem from the moderate HFD used in the study, which should be discussed in more detail.

Given the complexity and heterogeneity of the findings, it would be helpful for readers if the main results were illustrated in a summarizing figure at the end of the manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed most of the comments adequately, and the manuscript has shown improvement. However, I have a few remaining concerns outlined below.

Concerning comment 10, it is indeed true that aging bone tissue generally has a higher carbonate-to-phosphate ratio. Nevertheless, this ratio cannot be reliably used as an indicator of bone mineral maturity, as the quality of bone mineral also deteriorates with age. According to Farlay et al. (J Bone Miner Metab., 2010), mineral maturity is defined as “the progressive transformation of non-apatitic domains into poorly then well-crystallized apatite.” The substitution of carbonate for phosphate leads to distortions in the bone mineral lattice, making the mineral more similar to “non-apatite.” Additionally, the study by Taylor et al. (Calcif Tissue Int., 2021) demonstrated that the analytical carbonate content inversely correlated with both Raman and FTIR measurements of mineral maturity in bone. Similarly, Yerramshetty et al. (Bone, 2006) found a significant negative relationship between the carbonate-to-phosphate ratio and mineral crystallinity. Thus, while higher crystallinity is a validated measure of increased mineral maturity, it is incorrect to assume that a higher carbonate-to-phosphate ratio indicates greater mineral maturity. Please make changes in the text to reflect this body of literature and accordingly adjust the statements on carbonate-to-phosphate ratio.

Regarding comment 4, could you include a few sentences in the discussion summarizing the hypothesis for why sexual dimorphism occurs in response to diet?

Reviewer #2

(Remarks to the Author)
The study from Brown et al. is a very comprehensive and rigorous study that helps to understand, for example, sex differences in the observed effects of HFD on fat and glucose metabolism as well as bone turnover. The authors addressed all comments made by the reviewers and the paper has significantly improved.

We sincerely thank the Reviewers and Editor for their thorough review of this paper. We believe that our manuscript is much improved as a result. Detailed responses to Reviewer questions are provided below with reviewer and editor comments in **bold**. In addition, we have edited the paper for clarity and brevity in several locations. All changes to the manuscript have been tracked, and new text is highlighted in yellow in this response. Line numbers are in reference to the marked-up version of the manuscript.

Reviewer #1 (Remarks to the Author):

This study used short-term, moderate-fat diet to investigate skeletal health without severe metabolic disruption compounded with aging. The investigation is rigorous and thorough, covering aspects of inflammation response, bone turnover, cellular activities, matrix quality, tissue strength and fracture toughness, etc.

Thank you. We appreciate your thoughtful comments and have worked to further improve the manuscript.

Below are my specific comments:
1a. The study title may not be accurately reflected by the results.

Thank you. In light of other comments from both Reviewers, we updated the title to better encompass the complexity of the study and highlight key findings.

Original title [lines 1 -2]

> High-fat diet exacerbates the loss of bone fracture toughness in aging for C57BL/6JN mice

Modified title [lines 1 -2]

> Short-term moderate high fat diet impacts bone matrix properties, bone tissue metabolism, and fracture resistance for young adult and aged C57BL/6JN mice

1b. Both aging and HFD led to decrease of Kc_{init}. But is there any interaction between age and diet?

All interaction terms were included in the 3-way ANOVA models for Kc_{init} and all other dependent measures in this study. In the case of Kc_{init}, there was no statistically significant interaction found between age and diet, only significant main effects of both aging and HFD. The results of tests for interactions on fracture toughness are reported in Figure 4 and Supplementary Table S4.

We have clarified in the results and discussion that there were no significant interactions between age, diet, or sex effects on Kc_{init}.

RESULTS

Original text [lines 238 - 243]

> HFD mice had lower bone tissue intrinsic fracture toughness compared to LFD mice (Kc_{init}, -15.1%, p = 0.023, Figure 4a). There was also a trend (p = 0.056) for lower fracture toughness at maximum loading (Kc_{max}) for HFD compared to LFD groups.

Modified text [lines 238 - 243]

> HFD mice had lower bone tissue intrinsic fracture toughness compared to LFD mice (Kc_{init}, -15.1%, p = 0.023, Figure 4a). Kc_{init} also had a similar decrease for aged mice compared to young mice (-15.9%, p = 0.017) but showed no sex effect. There were no interactions between age and HFD on Kc_{init}. There was also a trend (p = 0.056) for lower fracture toughness at maximum loading (Kc_{max}) for HFD compared to LFD groups.

DISCUSSION

Original text [line 578 - 583]

> Both aging and HFD reduced the stress intensity factor at crack initiation (Kc_{init}), which measures the intrinsic toughness of bone matrix (i.e., mechanisms that act in front of the crack tip)[1], but HFD did not lower the stress intensity factor at the maximum load (Kc_{max}), which includes both intrinsic and extrinsic toughening mechanisms (i.e., mechanisms that act in front of the crack tip and behind the crack's wake)[1].

Modified text [line 578 - 583]

> Both aging and HFD reduced the stress intensity factor at crack initiation (Kc_{init}), which measures the intrinsic toughness of bone matrix (i.e., mechanisms that act in front of the crack tip)[1], in an additive rather than an interactive manner. However, HFD did not lower the stress intensity factor at the maximum load (Kc_{max}), which includes both intrinsic and extrinsic toughening mechanisms (i.e., mechanisms that act in front of the crack tip and behind the crack's wake)[1].

1c. Is the loss of Kc with aging more pronounced in the HFD group?

Aging and HFD main effects were found to be associated with additive losses in Kc_{init} not interactive effects. This means that the loss of Kc_{init} with age was similar for both LFD and HFD of each sex. The loss of Kc_{init} with diet was similar for both young and aged mice of each sex (Aged v Young: -15.9%; HFD v LFD: -15.1%).

RESULTS

Original text [lines 238 - 247]

> HFD mice had lower bone tissue intrinsic fracture toughness compared to LFD mice (Kc_{init}, -15.1%, p = 0.023, Figure 4a). There was also a trend (p = 0.056) for lower fracture toughness at maximum loading (Kc_{max}) for HFD compared to LFD groups. HFD did not interact with age or sex effects on fracture toughness. However, Kc_{max} demonstrated an interaction between sex and age (p = 0.019), where aged females had

lower fracture toughness than young females (-23.2%, adjusted p = 0.002, Figure 4b). Males did not have significant differences between age groups for Kc_{max}. Kc_{init} was higher for young mice (+15.9%, p = 0.017) but showed no sex effect.

Modified text [lines 238 - 247]

HFD mice had lower bone tissue intrinsic fracture toughness compared to LFD mice (Kc_{init}, -15.1%, p = 0.023, Figure 4a). Kc_{init} also had a similar decrease for aged mice compared to young mice (-15.9%, p = 0.017) but showed no sex effect. There were no interactions between age and HFD on intrinsic fracture toughness. There was also a trend (p = 0.056) for lower fracture toughness at maximum loading (Kc_{max}) for HFD compared to LFD groups. HFD did not interact with age or sex effects on fracture toughness. However, Kc_{max} demonstrated an interaction between sex and age (p = 0.019), where aged females had lower fracture toughness than young females (-23.2%, adjusted p = 0.002, Figure 4b). Males did not have significant differences between age groups for Kc_{max}.

2. Since the journal places method section at the end, please briefly provide information on age of the mice and study period at the end of the introduction or the beginning of the results.

Thank you. We made this information more readily accessible in the beginning of the results.

RESULTS

Original text [lines 102 – 111]

Body mass change in response to a moderate HFD varied by sex, age, and diet (sex*age*diet interaction, p < 0.001). Young (5-months-old) males gained the most body mass (Figure 1a, Supplementary Figure S1). Epididymal adipose depot mass also showed interactive effects between sex and diet (p < 0.001) and age and diet (p = 0.037), with the largest increase in HFD males compared to low-fat diet (LFD) males (+58.2%, adjusted p < 0.001, Figure 1b). Only age affected adiposity in females with aged females having higher adipose depot mass compared to young females (+56.3%, adjusted p < 0.001). Young HFD mice had heavier adipose depots than young LFD mice (+137%, adjusted p = 0.001), while aged (22-month-old) showed no significant difference in adipose depot mass between HFD and LFD.

Modified text [lines 102 – 111]

Body mass change in response to short-term (8-weeks) moderate HFD (45% fat) varied by sex, age, and diet (sex*age*diet interaction, p < 0.001) in C57BL/6JN mice. As expected, young (5-month-old) males gained the most body mass (Figure 1a, Supplementary Figure S1) and females did not grain significant mass after 8-weeks of a moderate HFD[2,3]. Epididymal adipose depot mass also showed interactive effects between sex and diet (p < 0.001) and age and diet (p = 0.037), with the largest increase in HFD males compared to low-fat diet (LFD, 10% fat) males (+58.2%, adjusted p <

0.001, Figure 1b). Only age affected adiposity in females with aged females (22-month-old) having higher adipose depot mass compared to young adult females (+56.3%, adjusted $p < 0.001$). Young HFD mice had heavier adipose depots than young LFD mice (+137%, adjusted $p = 0.001$), while aged showed no significant difference in adipose depot mass between HFD and LFD.

3a. The term "baseline glucose" creates ambiguity. Consider changing the term to "fasting glucose".

We have replaced all the instances of baseline glucose with fasting glucose in the text and Figure 1.

3b. Maximum and delta glucose levels are less relevant in presenting glucose metabolism and can be removed from the results.

Thank you. In response to your comment, we have removed the maximum and delta glucose results from Figure 1. Instead, we added the full ipGTT response curves to Figure 1. We have kept the text regarding maximum and delta glucose levels in the results because they describe different aspects of glucose metabolism, which may be informative for some readers. All body and adipose depot masses and ipGTT measures and their statistical results can be found in Supplementary Table S1.

Modified figure [lines 128 - 143]

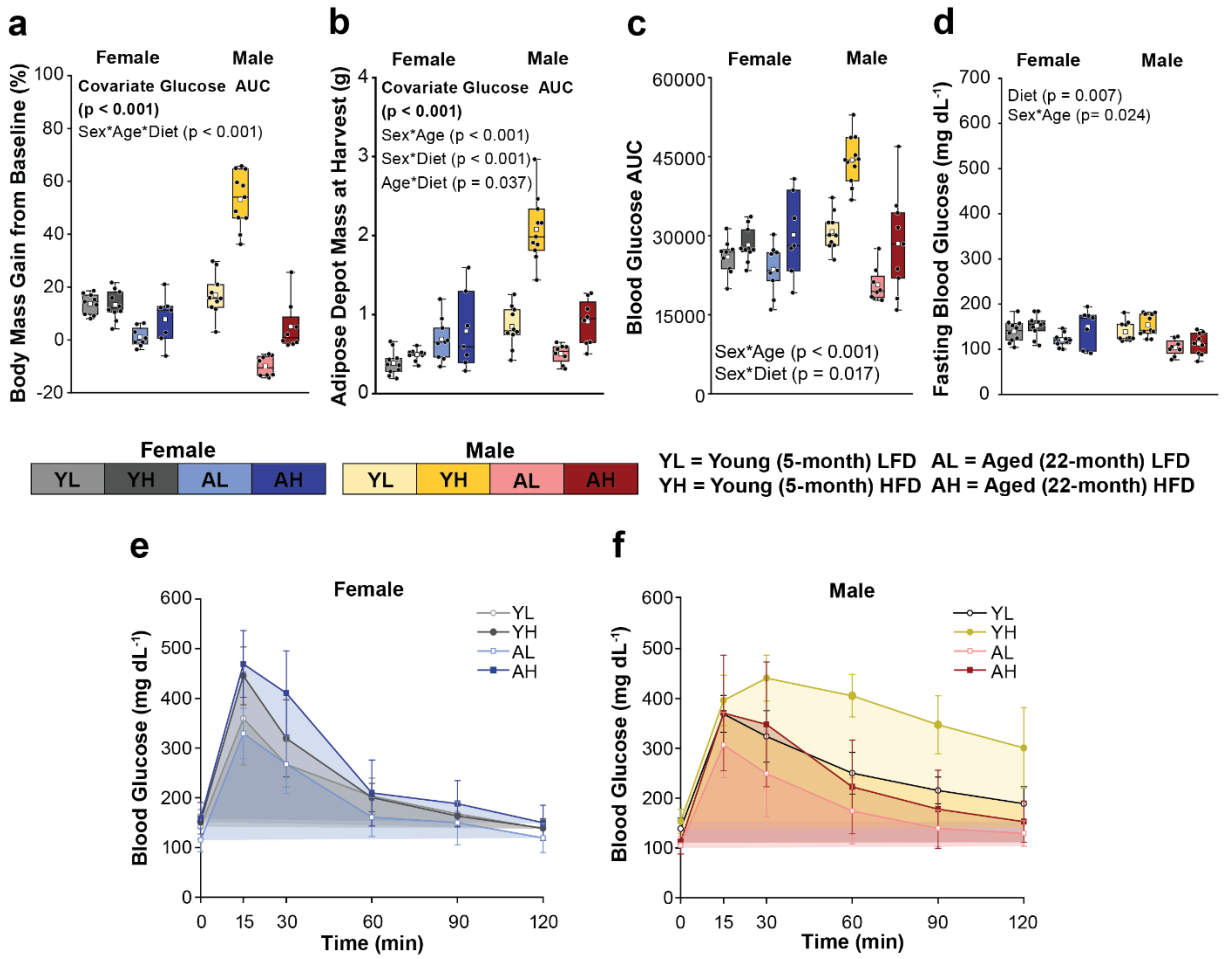

Figure 1. Eight weeks of high-fat diet disrupted glucose metabolism for young and aged mice of both sexes but only increased mass for males. (a) Percent mass change from the start to end of the diet intervention and (b) the reproductive adipose depot mass at harvest. (c) The area under the intraperitoneal glucose tolerance testing (ipGTT) curves was termed as the blood glucose area under the curve (AUC), (d) the first time point (0 min.) following six hours of fasting was the fasting blood glucose level. (e-f) The average ipGTT curves ± 1 SD for each female group and male group. Boxplots illustrate the interquartile range (box), the minimum and maximum values (whiskers), the median value (bar across the box), the mean value (white square), and individual data points (black circles). Significant p-values from 3-way ANOVA and pairwise post-hoc tests are noted on the plots. For all models unrelated to ipGTT, the glucose AUC was considered as a covariate in a 3-way ANCOVA model. If the covariate was not significant, the model was rerun without it.

4. How do the authors explain that HFD increased body mass and adipose depot mass in males but not in females?

The sex differences in body and adipose depot mass found in our study are aligned with prior work by other labs for the same strain of mice (i.e., C57BL/6 mice) fed 8 weeks of moderate high fat diet (45% fat)[2,4,5]. Casimiro and coauthors reported that after 8-weeks on a moderate

HFD (4 weeks old at start of diet), male mice gained body and adipose mass compared with a LFD group, but females did not[2]. While female mice were initially more resistant to adipose accumulation, they are expected to eventually gain a similar percentage of body mass as males if the diet duration is extended to 22-weeks[2]. Huang et al. demonstrated that males (6 weeks old at start of diet) gained in body and adipose mass in response to 5 weeks of moderate HFD but females did not[4]. Nie et al. demonstrated the same patterns for 5-week old females and males fed a moderate HFD for 14-20 weeks[5].

DISCUSSION

Original text [lines 563 – 570]

> However, glucose metabolism was disrupted for all HFD groups. We found that aging and HFD each reduced bone toughness in an additive fashion, but likely for different reasons.

Modified text [lines 563 - 570]

> Notable sex differences in the response to diet were observed, consistent with findings from other studies which have demonstrated that young male C57BL/6 mice gained significantly higher body and adipose mass in response to a similar duration of moderate HFD, while females did not[2,4,5]. We further observed that the sex difference in adipose gain is attenuated in aging, although all groups fed HFD had dysregulated glucose metabolism. Regardless of the degree of mass or adipose gain, we found that aging and HFD each reduced bone toughness in an additive fashion, but likely for different reasons.

5. The 20-fold increase in adipokine leptin level in young HFD mice compared to all other groups seems unusual. What do the authors think led to this dramatic change?

Thank you for pointing out the leptin level. An error was found in the reporting units. Our assay measured pg/mL but we accidentally reported ng/mL. Nonetheless, leptin levels were still increased by a large amount for young HFD males compared with the same age on a LFD (increased by 16-fold), and leptin levels were even greater in contrast with other groups.

Leptin is an adipokine released from adipose tissue[6] and the body's responsiveness to it is sensitive to estrogen levels. The systemic leptin response is influenced by factors such as sex, age, diet duration, and the age at which the diet intervention begins[7]. Young (2-month) female C57BL/6J mice have been shown to remain responsive to leptin with no significant alterations to serum leptin after 8-weeks of a moderate HFD (45% fat)[8]. In contrast, young C57BL/6 males (diet started at 6-weeks old) have been shown to develop resistance to leptin, and increased serum levels, within 16 days of starting a 45% fat HFD compared to young males on LFD[9]. In this prior study, leptin had a strong positive correlation with male body mass gain and duration of the diet[9].

RESULTS

Original text [line 149 - 151]

> The adipokine leptin exhibited a three-way interaction ($p = 0.018$), where young HFD male mice had over 20-fold higher levels compared to all other groups (Figure 2a).

Modified text [line 149 - 153]

> The adipokine leptin exhibited a three-way interaction (p = 0.018), where young HFD male mice had over 16-fold higher levels compared to all other groups (Figure 2a). The systemic leptin response is influenced by factors such as sex, age, diet duration, and the age at which the diet intervention begins[7], with young males being particularly prone to diet-induced hyperleptinemia[9].

DISCUSSION

Original text [lines 687 - 693]

> Leptin also indirectly regulates bone matrix turnover, through one or more mechanisms[10]. Further mechanistic studies will improve knowledge about how dietary fat can impact bone cells and their regulation of bone matrix.

Modified text [lines 687 - 693]

> The adipokine leptin also indirectly regulates bone matrix turnover, through one or more mechanisms[10]. The systemic leptin response is influenced by factors such as sex, age, diet duration, and the timing of diet intervention[7], with young males being particularly susceptible to diet-induced hyperleptinemia [9]—potentially accounting for some of the observed matrix differences between groups. Further mechanistic studies will improve knowledge about how dietary fat can impact bone cells and their regulation of bone matrix.

6. Did the authors assess cortical TMD and was it different across groups?

Yes, Ct. TMD results were reported in the supplementary data table with all micro-computed tomography measures (Table S3). There was no statistically significant effect of age or diet on Ct. TMD. But females had slightly higher Ct. TMD than males (+1.79%, p < 0.001).

RESULTS

Original text [lines 221 – 222]

> Complete data for femur cortical geometry and trabecular microarchitecture are reported in Supplementary Table S3.

Modified text [lines 221 – 222]

> Complete data for femur cortical geometry, cortical TMD, and trabecular microarchitecture are reported in Supplementary Table S3.

7. The authors should define the outlier criterion in the methods and determine whether outlier should be included in statistical analyses. Presenting statistical outcomes both with and without outlier may complicate the interpretation.

Thank you. We have added details to our description of the outlier criterion in the methods. We performed all statistical tests with and without outliers. A small number of the tests had different results with and without outliers. The results of these particular tests are reported in Supplementary Table S13. We have clarified instances where the inclusion of outliers altered conclusions with respect to diet.

RESULTS

Added text

[lines 111 - 113]

However, if outliers were included there was no interaction between diet and age or diet main effect. All information regarding outliers can be found in Supplementary Table S13.

[lines 182 - 183]

However, if outliers were included in the analysis for IL-6, the diet effect was no longer significant (Supplementary Table S13).

[lines 332 - 333]

When two outlier LFD females are included, there were no longer any significant effects of diet on *Atp6v1g1* (Supplementary Table S13).

METHODS

Original text [lines 938 - 944]

Analyses was performed with and without outliers identified by Grubb's Test. Results are presented without outliers, but the majority of conclusions made from statistical analyses did not differ whether outliers were included or not. The 11 measures that did not have the exact same conclusions with and without outliers are presented in Supplementary Table S13.

Modified text [lines 938 - 946]

Analyses were performed with and without outliers identified a single Grubb's Test. Grubb's outlier test identifies up to one sample per group if a point has a low probability ($p < 0.05$) of occurring given the group's data distribution. If no samples in a group fit this criterion, then no samples were removed from a group. Results are presented without outliers, but the majority of conclusions made from statistical analyses did not differ whether outliers were included or not. The 11 measures that did not have the exact same conclusions with and without outliers are presented in Supplementary Table S13. We have noted in the results where the inclusion of outliers led to significantly different conclusions regarding diet effects.

8. As an important outcome, the authors could present the bone strength boxplots.

Figure 4 has been updated to include the ultimate strength results and the relevant text has been updated.

RESULTS

Original text [lines 248 - 249]

> Bone strength showed a significant interaction between sex and diet (p = 0.021), with HFD females having stronger bones than HFD males (+16.9%, adjusted p < 0.001).

Modified text [lines 248 - 249]

> Bone strength showed a significant interaction between sex and diet (p = 0.021, Figure 4c), with HFD females having stronger bones than HFD males (+16.9%, adjusted p < 0.001).

Modified figure [lines 260 - 268]

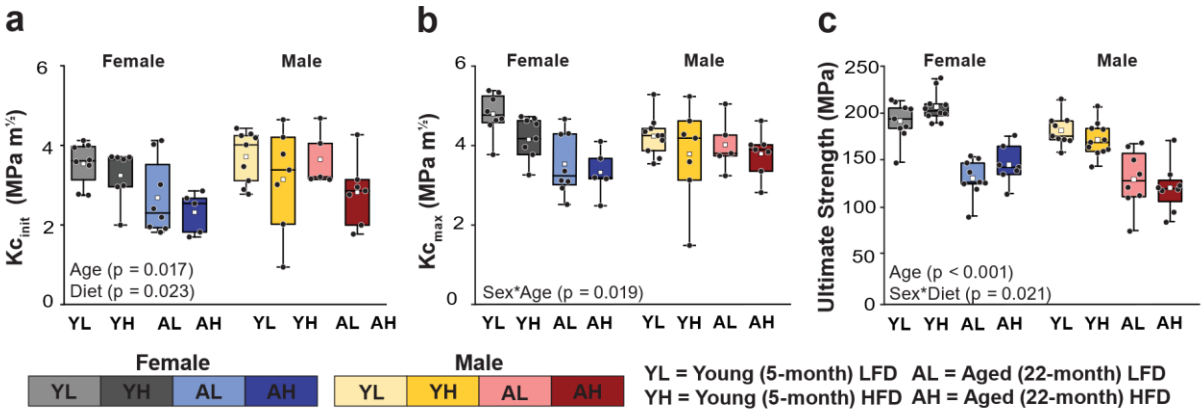

Figure 4. Aging and high-fat diet reduce bone intrinsic toughness to fracture. (a) Intrinsic fracture toughness (Kc_{init}), (b) fracture toughness at maximum load (Kc_{max}), and (c) ultimate strength. Boxplots illustrate the interquartile range (box), the minimum and maximum values (whiskers), the median value (bar across the box), the mean value (white square), and individual data points (black circles). Significant p-values from 3-way ANOVA and pairwise post-hoc tests are noted on the plots. For all models, the glucose area under the curve (AUC) was considered as a covariate. If the covariate was not significant, the model was rerun without it.

9. Aging didn't seem to change global bone turnover measures (although some interactions are significant). Why do the authors think this happened?

In the current study, we find that the P1NP, CTX1, and the ratio of P1NP:CTX1 were unchanged with aging. Our findings are in partial agreement with prior work from our group that showed that both P1NP and CTX1 increased with aging for female C57BL/6 mice on a standard chow diet (22 vs 5 month-old), but the ratio of these measures did not change[11]. We note that changes to

P1NP and CTX1 in aging are variable, as evidenced by the literature. Shahnazari et al. found that P1NP and CTX1 decreased with aging (24 vs 6 month-old male C57BL/6 mice), but did not report how the ratio of these measures changes in aging[12]. Similarly, Xu et al. found decreased P1NP and CTX1 with aging (20 vs 6 month-old male C57BL/6 mice) but also did not report the change in ratio[13]. On the other hand, some groups find no change in P1NP or CTX1 with aging in either sex[14] or mixed effects of age on P1NP and CTX1 serum levels[15], demonstrating that there is considerable biological variability in global measures of bone turnover for C57BL/6 mice. There may be differences in experimental conditions, diets, mouse housing, or assay choice, that could contribute to variance in these results.

Gene expression from PCR is another method to estimate changes to global bone turnover. We found that there was an age-diet interaction on the *Opg:Rankl* ratio, such that this measure was decreased with aging but only for HFD mice. Other measures related to global bone turnover include *Acp5*, which increased in aging. In aging, bone turnover is dysregulated, and it is common for bone resorption to have increased[12,16–18].

Because of the variability intrinsic to global measures of bone turnover, we also measured bone turnover at specific locations. Quantitative histomorphometry showed that age decreased mineralization along the periosteal surface while HFD decreased mineralization along the endocortical surface. Osteoclast counts at the tibia metaphysis demonstrated age-diet and sex-diet interactions, such that aged HFD mice had lower osteoclast number density than young HFD mice and that LFD females had higher osteoclast number density than HFD females but there were no diet differences in males.

DISCUSSION

Added text [589 - 596]

> We found that aging and HFD both decreased bone toughness but differently impacted bone turnover and matrix properties. As measured through quantitative histomorphometry, aging decreased bone turnover on the periosteal surface, while HFD decreased bone turnover on the endocortical surface. Notably, while there were differences in bone turnover measured from quantitative histomorphometry at the femur with aging and HFD, differences were not detected with either factor in serum measures of global bone turnover, demonstrating that these effects are site specific. The local changes in bone turnover to the femur were mirrored by changes in bone matrix detected by Raman spectroscopy.

10. Raman-measured carbonate:phosphate ratio does not directly indicate mineral maturity (line 338). Studies have used crystallinity to indicate mineral maturity, which is not different between age groups in this study. Therefore, the statement of "aging increased bone mineral maturity" based on carbonate:phosphate ratio is incorrect.

Thank you for your comment. Carbonate:phosphate and crystallinity are both measures of bone maturity used by bone researchers. Carbonate:phosphate is a measure of the B-type substitution of carbonate into the mineral crystal lattice, which can correlate with mechanical properties, mineral kinetics, and bone turnover[19,20], and has a minor effect on crystal size[20]. Crystallinity is related to the crystal size (length and thickness) as well as stoichiometric perfection (impacted by carbonate and acid-phosphate content), which also correlates to mechanical properties and tissue turnover[19]. As bone matures, both carbonate:phosphate and crystallinity are expected to increase[21–23].

In some studies, variation in carbonate:phosphate and crystallinity measured by Raman Spectroscopy show the same pattern[24,25], while in others only one of the measures changes in response to aging, disease, or an intervention[26,27] . In a recent study, it was demonstrated that there is a discernable relationship between Raman measures of carbonate:phosphate and biological variation in carbonate:phosphate for murine, ovine, and human bone measured through gold-standard analytical techniques[19]. However, Raman measures of crystallinity had very low biological variation and did not appreciably correlate with gold-standard measures of crystal length and thickness[19]. For these reasons, we decided to interpret carbonate:phosphate and crystallinity together in identifying changes to bone mineral maturity[19], as opposed to just one of the measures. However, in response to this comment we clarified the text as follows:

DISCUSSION

Original text [601 - 608]

> These measurements recapitulated several changes in aging rodent[25] and human bone[28] that have been seen in prior work, such as increased mineral maturity carbonate content and crystallinity. At the endocortical surface, the effect of HFD on bone matrix became apparent, but in a manner that interacted with age and sex.

Modified text [601 - 608]

> These measurements recapitulated several changes in aging rodent[25] and human bone[28] that have been seen in prior work, such as increased mineral maturity (i.e., carbonate content and crystallinity). While carbonate:phosphate was increased as a main effect of aging, crystallinity was only increased for aging males and not females. Both of these measures are indicators of mineral maturity, yet carbonate:phosphate as measured by Raman Spectroscopy can show a stronger relationship with gold-standard analytic measures of mineral maturity than crystallinity[19]. At the endocortical surface, the effect of HFD on bone matrix became apparent, but in a manner that interacted with age and sex.

11. Why does only one carbonate:phosphate ratio measure include a covariate of glucose AUC (Figure 7 row 2)? What's the relevance here?

Glucose AUC was chosen as a covariate since glucose dysregulation would be expected to produce variation in bone outcomes and does not simply vary with one measure. Indeed, there are significant interactions between sex and diet, and sex and age on this measure and a few mice across groups with high glucose AUC values (Figure 1c).

All statistical models were first run with glucose AUC as a covariate, since this allows testing the effect of the independent fixed effects (e.g., diet, aging, sex) on the dependent measure of interest after accounting for the linear relationship between the dependent measure and glucose AUC. In the case that the covariate was not significant, it was removed, and the model was run again without it. All results of covariate significance are available in Supplementary Tables S1-11.

In the case of Raman Spectroscopy measures, most did not have significant covariates of glucose AUC. However, the carbonate:phosphate ratio measured at the endocortical surface did have a significant covariate. Even after accounting for the linear relationship between glucose

AUC and this measure, there was still an interaction between sex and diet such that HFD males had higher carbonate:phosphate than HFD females (+24.4%, p = 0.02).

RESULTS

Original text [line 390]

> Males showed no significant diet differences in either measure.

Modified text [lines 390 - 394]

> Males showed no significant diet differences in either measure. However, HFD males had higher endocortical median carbonate:phosphate ratio and I1670/I1690 than HFD females (carbonate:phosphate: +24.4%; I1670/I1690: +14.1%, adjusted p < 0.001 for both) after accounting for the linear relationship with glucose AUC. Age and diet did not impact endocortical measures for I1670/I1690 for females.

12. Could the authors also compare the I1670/I1690?

Thank you for the suggestion. We have added I1670/I1690 to the Raman Spectroscopy results.

RESULTS

Original text

> [lines 366 - 377]
>
> Males had lower median mineral:matrix than females (-12.1%, p < 0.001), and aged female mice had higher mineral results of maturity, as estimated by the median carbonate:phosphate ratio, than young mice (+11.6%, p < 0.001). Bone median mineral crystallinity had an interaction between sex and age p = 0.016), where aged males, but not females, had modestly higher median mineral crystallinity compared to young counterparts (+2.5%, adjusted p = 0.018). There was no impact of diet on the median of either periosteal matrix measures (Figure 7 Row 1).
>
> [lines 390]
>
> Males showed no significant diet differences in either measure.

Modified text

> [lines 366 - 377]
>
> Males had lower median mineral:matrix (-12.1%, p < 0.001) and a moderate increase in collagen secondary structure (median I1670/I1690: +4.9%, p = 0.010) compared to females. Other measures of collagen secondary structure (I1670/I1640 and I1670/I1610) were not significant. Aged mice had higher mineral maturity, as estimated by the median carbonate:phosphate ratio, than young mice (+11.6%, p < 0.001) and decreased collagen secondary structure (median I1670/I1690: -4.1%, p = 0.023). Other measures of collagen secondary structure (I1670/I1640 and I1670/I1610) were not significant. Bone median mineral crystallinity had an interaction between sex and age p = 0.016),

where aged males, but not females, had modestly higher median mineral crystallinity compared to young counterparts (+2.5%, adjusted p = 0.018). There was no impact of diet on the median of either periosteal matrix measures (Figure 7 Column 1).

[lines 390 - 394]

Males showed no significant diet differences in either measure. However, HFD males had higher endocortical median carbonate:phosphate ratio and I1670/I1690 than HFD females (carbonate:phosphate: +24.4%; I1670/I1690: +14.1%, adjusted p < 0.001 for both) after accounting for the linear relationship with glucose AUC. Age and died did not impact endocortical measures for I1670/I1690 for females.

Modified Figure [399 - 412]

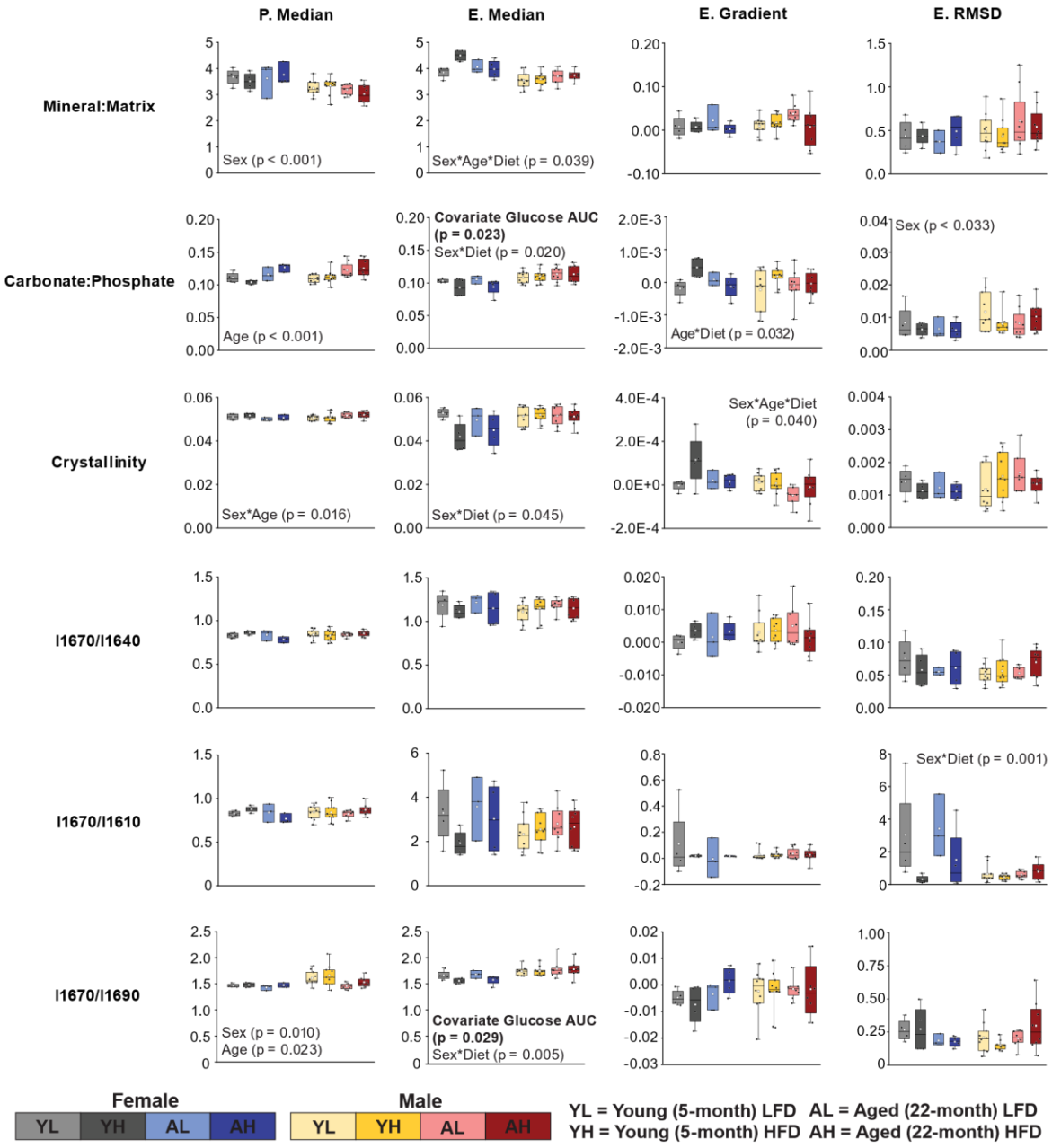

Figure 7. Bone matrix composition depends on high-fat diet in an age- and sex-dependent manner. The measurements of matrix maturity from hydrated Raman spectroscopy for the periosteal surface and the endocortical region (anterior-lateral location). Column 1 – the median measures for the periosteal surface (P. Median). Column 2 – the median measures for the endocortical region (E. Median). Column 3 – the gradient (slope of linear fit) of the measures across the endocortical region (E. Gradient). Column 4 – Estimate of the root mean square deviation (RMSD) from linear fit residuals of each measure across the endocortical region (E. RMSD). Boxplots illustrate the interquartile range (box), the minimum and maximum values (whiskers), the median value (bar across the box), the mean value (white square), and individual data points (black circles). Significant p-values from 3-way ANOVA and pairwise post-

hoc tests are noted on the plots. For all models, the glucose AUC was considered as a covariate. If not significant, the model was rerun without the covariate.

13. Figure 8 is confusing. It is very difficult to understand how the correlational results are reflected in the heatmaps. Please either choose an alternative way to present Figure 8, or explain the clustering analysis in more detail in the results section.

Thank you. We agree that the correlation results panel in Figure 8 could be improved. We removed the panel from the figure and added more information on the correlation cluster analysis using ECCO in the results text. We have also altered the text on the color bar to specify that it represents the scaled median data values for each measurement (minimum-maximum scaling), which was previously shown as a standard deviation scale on transformed data. The conclusions remain the same.

RESULTS

Original text [lines 414 - 417]

> Since the intrinsic fracture toughness of bone tissue was lower with HFD and aging, and multiple, often intercorrelated, features of matrix properties contribute to intrinsic toughening, we utilized clustering techniques based on the strength of absolute correlations to assess the association of matrix properties with fracture toughness (Kc_{init}).

Modified text [lines 414 - 423]

> Since the intrinsic fracture toughness of bone tissue was lower with HFD and aging, and multiple, often intercorrelated, features of matrix properties contribute to intrinsic toughening, we utilized clustering techniques based on the strength of absolute correlations to assess the association of matrix properties with fracture toughness at crack initiation (Kc_{init}) and fracture toughness at maximum load (Kc_{max}). Ensemble clustering with cluster optimization (ECCO) was utilized for clustering data to ensure a robust solution that minimized sensitivity to the choice of distance and linkage functions. Correlational ECCO used combinations of two distance (correlation or square root correlation) and three linkage (Ward, average, and complete) methods to generate clusters. Bone matrix measures co-occurring in 100% of combination solutions were used as the final clusters.

Modified Figure [lines 443 – 458]

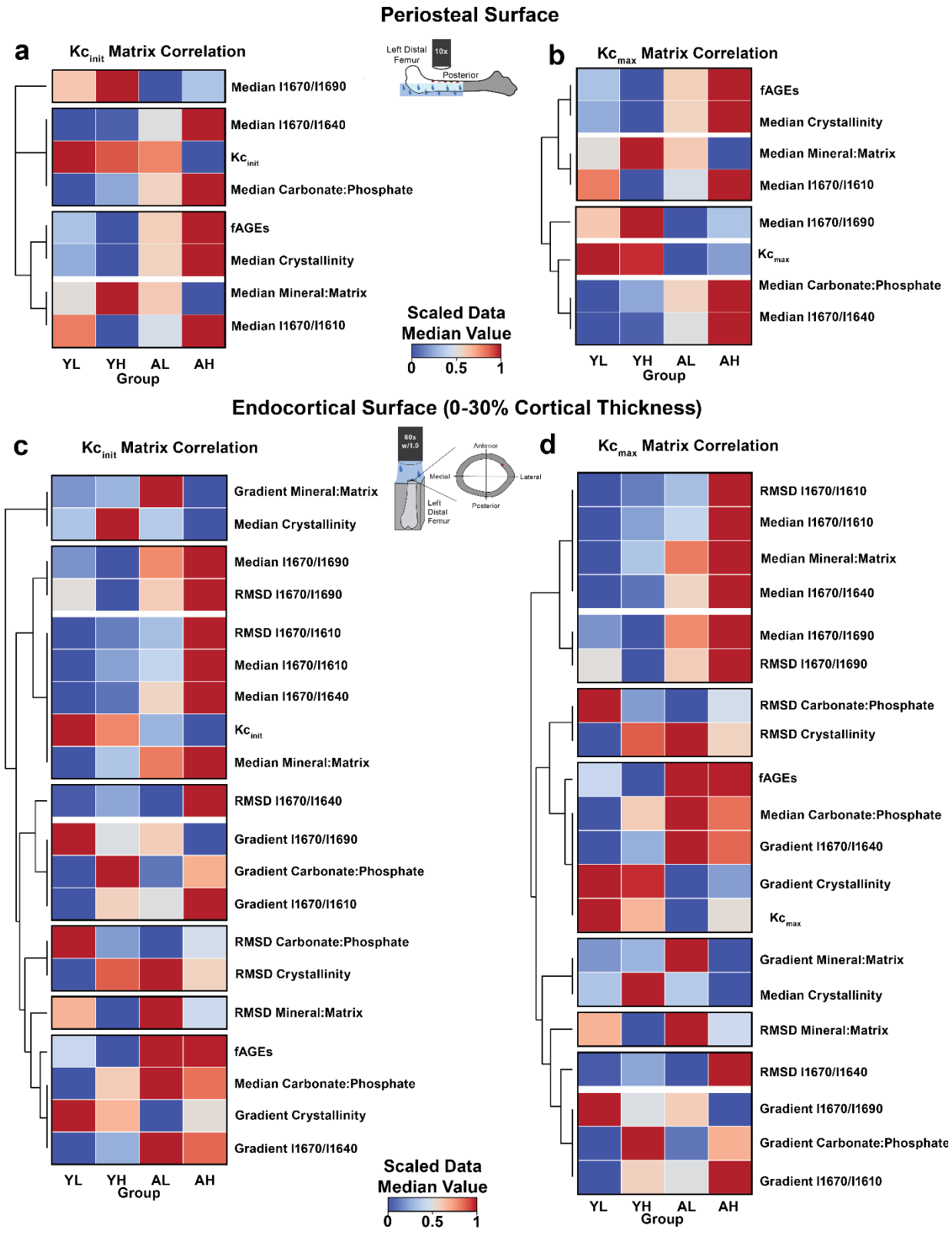

Figure 8. Bone matrix alterations at the endocortical surface are associated with decreased fracture toughness. (a) Heatmap of periosteal hydrated Raman spectroscopy

measures correlated with Kc_{init} and (b) Kc_{max}. Periosteal measures were collected with the set up illustrated on the left distal femur. Measures for male samples were clustered by magnitude of absolute correlation and the fill color corresponds to the scaled group median value for each measure on the heatmaps. (c) Heatmap of endocortical hydrated Raman spectroscopy measures with Kc_{init} and (d) Kc_{max} collected from the anterior-lateral area of the left distal femur cross-section. YL = Young (5-month) low-fat diet, YH = Young (5-month) high-fat diet, AL = Aged (22-month) low-fat diet, AH = Aged (22-month) high-fat diet, fAGEs = fluorescent advanced glycation end products, Kc_{init} = Intrinsic fracture toughness, Kc_{max} = fracture toughness at maximum stress, Gradient = Slope of the linear fit of the data, RMSD = Root mean square deviation of the data about the linear fit.

14. Consider also conducting correlational analysis with Kc_{max}.
Thank you for your suggestion. We have added the heatmaps for Kc_{max} at the periosteal and endocortical surfaces to Figure 8 as shown in response to the previous comment (comment 13).

RESULTS

Original text [lines 424 - 442]

> At the periosteal surface, the carbonate:phosphate ratio and collagen secondary structure (I1670/I1640) measures had the strongest correlations (inverse) with fracture toughness as evident through representation in the same cluster (Figure 8a). Kc_{max} had the same periosteal measures correlate closely with it, in addition to another measure of collagen secondary structure (I1670/I1690, Figure 8b). At the endocortical surface, the median collagen secondary structure (I1670/I1610), RMSD of collagen secondary structure (RMSD I1670/I1610), and the median mineral:matrix ratio had the strongest (inverse) correlations to fracture toughness. Decreased fracture toughness was associated with higher values for these measures. fAGEs were not within the closest clusters with Kc_{init} for either the periosteal or endocortical region of interest (Figure 8 b and c).
>
> Together, these data show that HFD and aging differently impact bone matrix. HFD alters endocortical bone mineral, and for females, the RMSD of collagen secondary structure. By contrast, aging increases periosteal bone mineral maturity, but not mineral content. The variation of fracture toughness seen across males in this study was more related to variation in collagen structure and mineral:matrix ratio than other measures.

Modified text [lines 424 - 442]

> At the periosteal surface, the carbonate:phosphate ratio and collagen secondary structure (I1670/I1640) measures had the strongest correlations (inverse) with Kc_{init} as evident through representation in the same cluster (Figure 8a). Kc_{max} demonstrated equally robust correlations with the same periosteal measures as Kc_{init}, while also showing a strong association with an additional measure of collagen secondary structure (I1670/I1690, Figure 8b). At the endocortical surface, the median collagen secondary structure (I1670/I1610), RMSD of collagen secondary structure (RMSD I1670/I1610),

and the median mineral:matrix ratio had the strongest (inverse) correlations to fracture toughness. Decreased fracture toughness was associated with higher values for these measures. fAGEs were not within the closest clusters with Kc_{init} for either the periosteal or endocortical region of interest (Figure 8 c). However, fAGEs, the endocortical gradient of mineral crystallinity and collagen secondary structure (I1670/I1640), and mineral carbonate:phosphate content had the strongest correlations with Kc_{max} (Figure 8d).

Together, these data show that HFD and aging differently impact bone matrix. HFD alters endocortical bone mineral and collagen secondary structure for females more than males. By contrast, aging increases periosteal bone mineral maturity, but not mineral content and decreases collagen secondary structure order. The variation of fracture toughness seen across males in this study was more related to variation in collagen structure and mineral:matrix ratio than other measures.

Reviewer #2 (Remarks to the Author):

The manuscript titled "High-fat diet exacerbates the loss of bone fracture toughness in aging for C57BL/6JN mice" by Brown et al. is well written and possesses considerable clinical relevance. The authors investigated how age, gender, and diet differentially affect energy metabolism, trabecular and cortical bone properties, bone mineralization and matrix composition, collagen structure, as well as cellular functions and metabolic pathway signatures in male and female young and aged C57BL/6JN mice under low-fat and high-fat diets. Given the increasing aging population and prevalent unhealthy eating habits, this study is of significant clinical importance.

The research is comprehensive, employing a variety of methodologies to address the research question effectively. The authors utilized appropriate techniques, and the results are presented with clarity and precision. Additionally, the manuscript is well structured, featuring a thorough discussion. However, some major points should be addressed.

Thank you for your kind words. We have addressed your comments, as detailed in responses below.

1. The abstract is written in a simplified manner and does not adequately reflect the comprehensiveness and complexity of the entire study. It should be revised to better capture the scope and depth of the research.

Thank you. Given the 150-word limit on abstract length, we are limited in the depth that we can add to the abstract (original was 147 words). However, we have made several changes to better represent the scope and depth of this research project that only modestly increases the abstract length (170 words).

Original abstract text [lines 20 -32]

> The elderly are at increased risk of bone fracture and more often consume poor quality diets, such as high-fat diet (HFD). We hypothesized that HFD would exacerbate the loss of bone fracture resistance in aging. To test this hypothesis, we fed 5-month and 22-month C57BL/6JN mice of both sexes HFD or low-fat diet for 8 weeks. Aging and HFD lowered bone fracture toughness, while only aging reduced bone strength. At the tissue-scale, aging increased bone mineral maturity, while HFD altered bone mineralization and maturity as well as collagen structure. Cortical bone metabolism was also dysregulated with aging and HFD. Dysregulated pathways in aging and HFD were related to cellular function and viability, or glucose regulation, respectively. Aging with HFD also impacted osteoclast and adipocyte abundance and osteocyte viability. Together, these data demonstrate that HFD may exacerbate the loss of bone matrix quality and fracture resistance in aging.

Modified abstract text [lines 20 -32]

The elderly are at increased risk of bone fracture and more often consume poor quality diets, such as high-fat diet (HFD). We hypothesized that HFD exacerbates the loss of bone fracture resistance in aging. Female and male 5-month and 22-month C57BL/6JN mice were fed moderate HFD (45%) or low-fat diet (10%) for 8 weeks. All HFD groups showed disrupted glucose metabolism. Aging and HFD lowered bone fracture toughness, while aging alone reduced bone strength. Raman Spectroscopy demonstrated that aging and HFD differently impact bone matrix. Aging increased mineral maturity while HFD increased mineral maturity in males but decreased mineral maturity and altered collagen structure in females. Untargeted metabolomics revealed that cortical tissue metabolism was dysregulated with aging and HFD. Aging and HFD affected pathways related to cellular function and viability, or glucose regulation, respectively. In aging mice, HFD also impacted osteoclast and adipocyte abundance and osteocyte viability in both sexes. Together, these data demonstrate that HFD exacerbates the loss of bone matrix quality and fracture resistance in aging C57BL/6JN mice.

2. The authors discuss local inflammatory responses (line 582 ff); however, it is unclear what specific aspects were investigated. It would be beneficial to stain for particular immune cells or inflammatory markers locally to gain deeper insight into the effects of age, gender, and diet on local inflammation.

We agree that the term local inflammatory responses used in this context (metabolomic data) was unclear. We have modified the language to specify that we mean the tissue scale inflammatory response from the flushed cortical bone metabolomic data rather than systemic inflammatory markers from blood serum.

We agree it would be beneficial for future studies to explore the sources of these inflammatory metabolites in greater detail and their consequences in bone. However, we do not have sufficient tissues remaining for *in situ* analyses of inflammatory markers.

RESULTS

Original text [lines 511 - 520]

Inflammatory metabolic pathways, such as butanoate, histidine, and nicotinamide pathways, were upregulated in young HFD females compared to young LFD females. Aged HFD females exhibited minor downregulation of these inflammatory pathways compared to young HFD females. Aged HFD males demonstrated upregulation of the same inflammatory pathways compared to their young counterparts and aged females. Young HFD males downregulated these pathways compared to young LFD males and females of both diets, while upregulating other inflammatory pathways, such as the folate-mediated one carbon pool. Differences in the HFD response between young females and males may reflect sex differences or variation in adipose gains.

Modified text [lines 511 - 520]

Cortical bone metabolic pathways associated with inflammatory responses, such as butanoate, histidine, and nicotinamide pathways, were upregulated in young HFD females compared to young LFD females. Aged HFD females exhibited minor downregulation of these inflammatory pathways compared to young HFD females. Aged HFD males demonstrated upregulation of the same inflammatory pathways compared to their young counterparts and aged females. Young HFD males downregulated these pathways compared to young LFD males and females of both diets, while upregulating other inflammatory pathways, such as the folate-mediated one carbon pool. Differences in the HFD inflammatory response between young female and male bone may reflect sex differences or variation in adipose gains.

DISCUSSION

Original text

[lines 666 - 671]

Although neither HFD nor aging promoted a clearly elevated systemic inflammatory response, metabolites from cortical bone tissue showed that all HFD and aging groups had local inflammatory responses. Young females on HFD demonstrated resistance to the inflammatory environment through upregulation of histidine metabolism and branched chain amino acid degradation, which are necessary to attenuate oxidative stress and inflammation.

[lines 680 - 682]

These data suggest that HFD induces sex- and age-specific responses to local inflammation, potentially contributing to variations in bone matrix regulation.

Modified text

[lines 666 - 671]

Although neither HFD nor aging promoted a clearly elevated systemic inflammatory response, metabolites from cortical bone tissue showed that all HFD and aging groups had inflammatory responses at the tissue scale. Cortical bone in young females on HFD demonstrated resistance to the inflammatory environment through upregulation of histidine metabolism and branched chain amino acid degradation, which are necessary to attenuate oxidative stress and inflammation.

[lines 680 - 682]

These data suggest that HFD induces sex- and age-specific responses in cortical bone to inflammation, potentially contributing to variations in bone matrix regulation.

3. Body weight significantly affects movement and load-bearing, which in turn impacts load-induced bone formation and bone strength. Therefore, it would be valuable to know

if there were differences in load-bearing and locomotion between the groups, as these factors may have influenced the results.

We agree understanding the effects of body weight and movement would be valuable information. Casimiro et al. showed that young HFD C57BL/6J males on a moderate HFD (45% fat) had decreased activity and increased adipose gain while young HFD females neither gained significant adipose tissue nor was their activity changed after 12-weeks of intervention. However, given the co-housing design of our experiment, it was not possible to track activity. Further, individually housing these mice would have introduced other issues, especially for older mice[29–31].We included glucose AUC as a covariate in our statistical models to partially mitigate the question of how adiposity influence bone outcomes.

DISCUSSION

Original text [lines 710 – 716]

> The sex differences identified in young female versus male mice may reflect differences in adiposity rather than true sex differences. To address this challenge, we used the AUC from glucose tolerance tests as a covariate in statistical models.

Modified text [lines 710 – 717]

> The sex differences identified in young female versus male mice may reflect differences in adiposity rather than true sex differences. In addition, increased adiposity could affect mobility[2] and load bearing on bone[32]. However, our study did not track movement due to the cohabitation design, so the effects of moderate HFD-induced adiposity and movement on bone between sexes requires further studies. To partially address this challenge, we used the AUC from glucose tolerance tests as a covariate in statistical models as it was highly correlated with adiposity and is a measure of metabolic disruption.

4. Overall, the results exhibit considerable heterogeneity based on age, sex, and diet. The effects of sex, particularly the lack of weight gain in female mice on a high-fat diet (HFD) and the observation that HFD females have stronger bones than males, warrant a more detailed discussion.

Thank you for this comment. Indeed, there is considerable variance in bone outcomes across the age, sex, and diet groups. Some of this is to be expected, since aging increases variability in bone biomechanics and tissue properties[17,25,33] . Females were found to gain less body and adipose mass in response to moderate high fat diet than males. This finding is not surprising and has been seen in additional studies, such as Casimiro et al.[2], Huang et al.[4], and Nie et al.[5]. Even within males, there are mice that gained much more weight than others. These findings are also seen by others in the literature[34–36]. As discussed in depth in the response to Reviewer 1 – comment 5, our choice of short-term moderate (45%, 8 weeks) HFD as opposed to a 60% HFD was in part to reduce the variability in response to the diet. We believe that variation with sex in bone properties and responses to interventions such as diet is a critically understudied aspect of bone biology and mechanics and hope that our findings help to address this gap in knowledge. Therefore, we have updated the language in our discussion to better highlight sex differences in the response to our dietary intervention.

The Reviewer points out an interesting finding that HFD females had stronger bones (~17%) than HFD males. Since differences in strength can relate to differences in shape (i.e., rounder as opposed to more slender bones experience more shear during three point bending and their strength is consequently underestimated), we ran an additional analysis in response to this comment to ask whether HFD affected the shape of the femora. We ran three-factor ANOVA for I_{min}/c, since this measure (section modulus) approximates roundness, where the higher I_{min}/c means more round. We found that there were no diet differences between groups in the shapes of the bone. We then ran a three-factor ANCOVA for ultimate strength, with I_{min}/c as a covariate. We found that the section modulus was not a statistically significant covariate and thus the changes to the strength of the bones was not explained by the shape changes. Indeed, the LFD males have somewhat more round bones and thus, if anything, their strength could be slightly underestimated. We added text to the discussion where we further consider the sex*diet interaction on ultimate strength.

DISCUSSION

Added text

[lines 559 - 566]

Moderate HFD can induce variable body and adipose mass gain in male and female C57BL/6J mice, with this variability expected to increase as the duration of the diet intervention is extended [37]. To address this, the short-term intervention used in this study was designed to minimize the variability in adipose gain within groups while still inducing metabolic disruption. Notable sex differences in the response to diet were observed, consistent with findings from other studies which have demonstrated that young male C57BL/6 mice gained significantly higher body and adipose mass in response to a similar duration of moderate HFD, while females did not[2,4,5].

[lines 589 - 620]
Updated language in discussion on matrix.

[lines 617 – 620]

We note that there was an interaction between sex and diet on ultimate strength, such that HFD females had higher strength than HFD males. These findings are likely not because of shape differences between these groups (Supplementary Figure S4) and may instead reflect sex differences in material-level properties in response to diet.

SUPPLEMENTAL FIGURES FILE

We have added the results of the ANOVA on Imin/c, the ANCOVA on ultimate strength with Imin/c as a covariate, and a figure to the supplemental figures (Supplementary Figure S4).

ANOVA on I_{min}/c

There was a significant interaction between sex and age ($p = 0.014$) but no significant effects of diet. Both aged females and males had higher I_{min}/c than their young counterparts (females: +25.0%, males: +36.2%, adjusted p for both < 0.001). There was no difference between sexes when they were young but aged males had higher I_{min}/c than aged females (+14.6%, $p < 0.001$).

ANCOVA on ultimate strength with a covariate of I_{min}/c
I_{min}/c was not a significant covariate for ultimate stress.

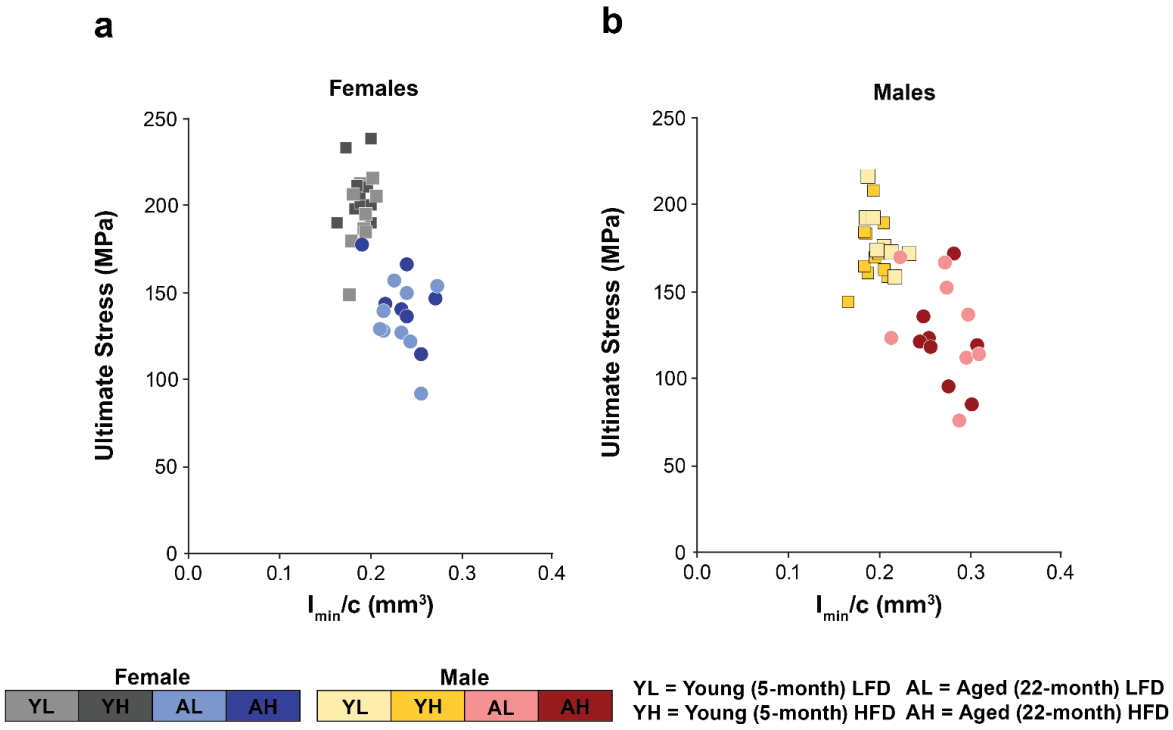

Figure S4. I_{min}/c (section modulus) did not change with HFD. Estimate of bone shape versus ultimate stress for (a) females and (b) males.

5. The heterogeneity of the results may also stem from the moderate HFD used in the study, which should be discussed in more detail.

Thank you. As the Reviewer notes, there is considerable variability in some bone metrics. Moderate HFD does produce variability in adipose mass gain. Yang and co-authors demonstrated that for C57BL/6 mice (8 weeks old at start of diet) fed 45% fat diet, the variability in adipose mass gain increased with the length of the diet (which was studied over 35 weeks)[37]. We chose a shorter-term diet (8 weeks) in part to mitigate this variability.

Additionally, moderate-fat HFD (45%) can produce less variability in bone outcomes than higher fat diets (60%). Devlin et al. found that female C57BL/6 mice (3 weeks old at start of diet) fed a 45% HFD for 9 weeks had a standard deviation of the femoral maximum force from three point bending of 7% of the mean, while mice fed LFD had a variability of 8% of the mean[3]. By contrast, Gautam et al. found that C57BL/6 mice (4 weeks old at start of diet) fed 60% HFD for 10 weeks had standard deviations of the femoral maximum force from three point bending that were 40% of the mean for females and 28% for males. The standard chow controls had a variability of 13% for males and 31% for females[38].In our study, the variability for the maximum force measured from three-point bending was 11% and 10% of the mean for young adult females and males fed HFD, compared with 14% and 9% of the mean for young adult females and males fed LFD. In aged mice, the variability for the maximum force measured from three-point bending was 12% for both aged HFD females and males. LFD variability for aged females

was 20% and for aged males it was 23%. Therefore, HFD does not always increase variability in bone measures, and short-term moderate HFD may actually reduce variability compared with other higher-fat diets for at least some measures.

We now include a brief discussion of the variability in bone outcomes with HFD in text, as outlined below.

DISCUSSION

Original text [lines 558 - 559]

> Mice were fed 45% HFD for 8 weeks, which resulted in varying degrees of adiposity within and between experimental groups.

Modified text

> [lines 558 - 563]
>
> Mice were fed 45% fat HFD for 8 weeks, which resulted in varying degrees of adiposity within and between experimental groups. Moderate HFD can induce variable body and adipose mass gain in male and female C57BL/6J mice, with this variability expected to increase as the duration of the diet intervention is extended[37]. To address this, the short-term intervention used in this study was designed to minimize the variability in adipose gain within groups while still inducing metabolic disruption.

> [lines 709 - 710]
>
> However, the short-term moderate HFD can decrease some bone measures' variability compared to higher fat diets (e.g. 60% fat).

6. Given the complexity and heterogeneity of the findings, it would be helpful for readers if the main results were illustrated in a summarizing figure at the end of the manuscript.

Thank you for your suggestion. We have added a summarizing figure at the end of the manuscript (Figure 10).

DISCUSSION

Added figure [lines 734 – 736]

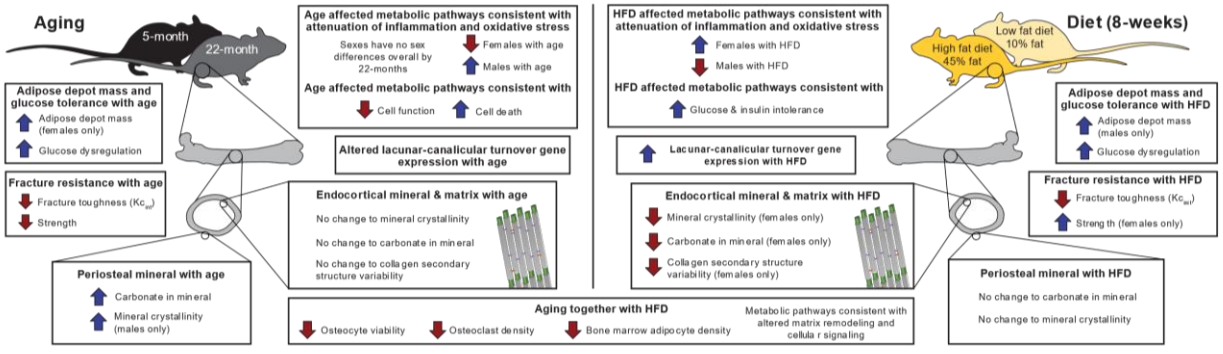

Figure 10. A summary of the effects of aging and high-fat diet on C57BL/6JN mice.

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Thank you for the additional comments to further improve this paper. Detailed responses to Reviewer questions are provided below with reviewer and editor comments in **bold**. All changes to the manuscript have been tracked, and new text is highlighted in yellow in this response. Line numbers are in reference to the marked-up version of the manuscript.

Reviewer #1 (Remarks to the Author):

The authors have addressed most of the comments adequately, and the manuscript has shown improvement. However, I have a few remaining concerns outlined below.

Concerning comment 10, it is indeed true that aging bone tissue generally has a higher carbonate-to-phosphate ratio. Nevertheless, this ratio cannot be reliably used as an indicator of bone mineral maturity, as the quality of bone mineral also deteriorates with age. According to Farlay et al. (J Bone Miner Metab., 2010), mineral maturity is defined as "the progressive transformation of non-apatitic domains into poorly then well-crystallized apatite." The substitution of carbonate for phosphate leads to distortions in the bone mineral lattice, making the mineral more similar to "non-apatite." Additionally, the study by Taylor et al. (Calcif Tissue Int., 2021) demonstrated that the analytical carbonate content inversely correlated with both Raman and FTIR measurements of mineral maturity in bone. Similarly, Yerramshetty et al. (Bone, 2006) found a significant negative relationship between the carbonate-to-phosphate ratio and mineral crystallinity act. Thus, while higher crystallinity is a validated measure of increased mineral maturity, it is incorrect to assume that a higher carbonate-to-phosphate ratio indicates greater mineral maturity. Please make changes in the text to reflect this body of literature and accordingly adjust the statements on carbonate-to-phosphate ratio.

Thank you for your comment. We have altered the language related to mineral maturity to reflect this discussion.

Abstract

Original text [lines 24 - 29]

Aging increased mineral maturity while HFD increased mineral maturity in males but decreased mineral maturity and altered collagen structure in females.

Modified text [lines 24 - 29]

Aging altered matrix properties but the effect depended on sex. Both sexes had higher carbonate content and altered collagen structure (I1670/I1690) with age but males also had increased crystallinity. HFD decreased mineral maturity (i.e., crystallinity) as well as altered collagen structure in females but not males.

Results

Original text [lines 354 - 356]

Aged mice had higher mineral maturity, as estimated by the median carbonate:phosphate ratio, than young mice (+11.6%, $p < 0.001$) and decreased collagen secondary structure (median I1670/I1690: -4.1%, $p = 0.023$).

Modified text [lines 354 - 356]

Aged mice had altered mineral composition, as estimated by the median carbonate:phosphate ratio, than young mice (+11.6%, $p < 0.001$) and decreased collagen secondary structure (median I1670/I1690: -4.1%, $p = 0.023$).

Original text [lines 420 - 422]

By contrast, aging increases periosteal bone mineral maturity, but not mineral content and decreases collagen secondary structure order.

Modified text [lines 420 - 422]

By contrast, aging alters periosteal bone mineral composition, but not mineral content and decreases collagen secondary structure order.

Discussion

Original text [lines 546 - 547]

Aging primarily impacted bone tissue characteristics related to mineral maturity, while HFD mineral content and maturity as well as collagen structure.

Modified text [lines 546 - 547]

Aging primarily impacted bone tissue characteristics related to mineral composition, while HFD mineral content and maturity as well as collagen structure.

Original text [lines 574 - 586]

These measurements recapitulated several changes in aging rodent[1] and human bone[2] that have been seen in prior work, such as increased mineral maturity (i.e., carbonate content and crystallinity). While carbonate:phosphate was increased as a main effect of aging, crystallinity was only increased for aging males and not females. Both of these measures are indicators of mineral maturity, yet carbonate:phosphate as measured by Raman Spectroscopy can show a

stronger relationship with gold-standard analytic measures of mineral maturity than crystallinity[3]. At the endocortical surface, the effect of HFD on bone matrix became apparent, but in a manner that interacted with age and sex. For young females, HFD increased bone mineral:matrix ratio and also increased the spatial gradient of mineral maturity, as seen in the measures of carbonate:phosphate and crystallinity. However, HFD females of both ages had reduced median mineral maturity near the endocortical surface and decreased spatial variation in collagen secondary structure.

Modified text [lines 574 - 586]

These measurements recapitulated several changes in aging rodent[1] and human bone[2] that have been seen in prior work, such as altered mineral properties (i.e., carbonate content and crystallinity). While carbonate:phosphate was increased as a main effect of aging, crystallinity was only increased for aging males and not females. Both of these measures are indicators of mineral quality and potentially maturity[3–5]. At the endocortical surface, the effect of HFD on bone matrix became apparent, but in a manner that interacted with age and sex. For young females, HFD increased bone mineral:matrix ratio and also increased the spatial gradient of mineral maturity, as seen in the measure of crystallinity. However, HFD females of both ages had reduced median mineral maturity near the endocortical surface and decreased spatial variation in collagen secondary structure.

Original text [lines 602 - 604]

Indicators of mineral maturity, such as carbonate:phosphate and crystallinity, and fAGEs were less associated with intrinsic fracture toughness.

Modified text [lines 602 - 604]

Indicators of mineral maturity, such as crystallinity, and fAGEs were less associated with intrinsic fracture toughness.

Regarding comment 4, could you include a few sentences in the discussion summarizing the hypothesis for why sexual dimorphism occurs in response to diet?

Certainly. We have added the following paragraph to the discussion:

Discussion

Added text [lines 665 - 684]

Mice exhibit well-documented sexually dimorphic responses to HFD[14,76–78], but this is the first study to characterize sex differences in the impact of dietary fat to multi-scale material properties and the metabolomics of cortical bone. Several factors may contribute to these between-sex differences including the influence of sex hormones[76], differences in the

inflammatory response[14,78], and differences in metabolic flexibility[14,77]. Previous work in adipose tissues has demonstrated that female C57BL/6J mice aged between 6- and 40-weeks at the initiation of a 60% fat HFD, exhibited greater anti-inflammatory responses (e.g. accumulation of anti-inflammatory macrophages and regulatory T-cells in adipose tissue)[14,78] and greater oxidative capacity than males[14]. In contrast, males also developed more severe hyperinsulinemia, glucose intolerance, systemic inflammation, and less metabolic adaptivity compared to females[78]. When both sexes undergo gonadectomies, their response to HFD converge, indicating that sex hormones likely play a central role in mediating the metabolic effects of HFD on tissues and systemic metabolism[76]. Additionally, sex differences have been observed in the cortical bone metabolome, with notable differences in lipid metabolism[54,55] in C57BL/6J mice on control diets. As our study challenged lipid metabolism, it is reasonable to expect that the downstream effects of HFD would differ by sex, likely reflecting distinct inflammatory responses and metabolic adaptation within the cortical bone. However, further studies are necessary to establish mechanisms driving/regulating the observed sex-specific responses to HFD in cortical bone, as well as whether crosstalk with adipose tissues influences these responses[26].

Reviewer #2 (Remarks to the Author):

The study from Brown et al. is a very comprehensive and rigorous study that helps to understand, for example, sex differences in the observed effects of HFD on fat and glucose metabolism as well as bone turnover. The authors addressed all comments made by the reviewers and the paper has significantly improved.

Thank you for your constructive feedback for improving this manuscript.

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Response to Reviewers

We have supplied all documents requested by the editors.