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Femur EPA and DHA are correlated with femur biomechanical strength in young *fat-1* mice

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Abstract

Evidence suggests that n-3 polyunsaturated fatty acids (PUFA) are beneficial for maintenance of bone health and possibly bone development. This study used the *fat-1* mouse, a transgenic model that synthesizes n-3 PUFA from n-6 PUFA, to determine if outcomes of bone health were correlated with n-3 PUFA in femurs. Control and *fat-1* mice were fed an AIN-93G diet containing 10% safflower oil from weaning through 12 weeks of age. Femur bone mineral content (BMC) and density were determined by dual-energy X-ray absorptiometry, and biomechanical strength properties, surrogate measures of fracture risk, were measured by a materials testing system. Femur fatty acid composition was determined by gas chromatography. At 12 weeks of age, femur n-3 PUFA were higher among *fat-1* mice compared to control mice. The n-6/n-3 PUFA ratio in the femur was negatively correlated with BMC (r=−.57, P=.01) and peak load at femur midpoint (r=−.53, P=.02) and femur neck (r=−.52, P=.02). Moreover, long-chain n-3 PUFA, eicosapentaenoic acid, and docosahexaenoic acid were significantly and positively correlated or displayed a trend suggesting positive correlations, with BMC and peak load. In conclusion, the results of the present study suggest that n-3 PUFA have a favorable effect on mineral accumulation and functional measures of bone in *fat-1* mice at young adulthood.

Keywords: Bone mineral density; n-3 fatty acids; Biomechanical bone strength; Mice

1. Introduction

The National Institutes of Health defines osteoporosis as a skeletal disorder characterized by compromised bone strength predisposing an individual to an increased risk of fragility fracture [1]. Preventive strategies against osteoporosis may target one or more specific stages of the life cycle such as childhood, adolescence and/or late adulthood. With respect to bone development, attainment of peak bone mass, the maximum amount of bone an individual achieves during the life cycle, is important to protect against osteoporosis during aging.

Although genetics is a significant contributor to peak bone mass, an individual's full genetic potential can be achieved through proper nutrition and exercise [2]. Emerging evidence suggests that dietary fat, particularly n-3 polyunsaturated fatty acids (PUFA), can favorably modulate bone development [3–7]. Plant-derived α-linolenic acid (ALA; 18:3n-3) and eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) from marine sources have been suggested to be beneficial in the prevention and/or treatment of cardiovascular diseases, dyslipidemia, diabetes, inflammatory diseases and cancer [8].

To date, many studies have used ovariectomized or aged rodent models to study the effect of n-3 PUFA on bone metabolism. Dietary n-3 PUFA reduced bone loss in aging female mice [9] and middle-aged male mice [10]. In

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ovariectomized mice, a fish oil diet protected against the loss of bone mineral and strength in the femur and vertebrae [11]. Inflammation-induced bone resorption in mice with periodontal diseases was also attenuated with n-3 PUFA [12]. In contrast, conflicting findings have arisen from studies investigating the effect of n-3 PUFA on developing animals [3,5,13,14]. In growing rats, n-3 PUFA were positively correlated with bone formation markers [3] and reversed compromised structural integrity in previously n-3 PUFA-deficient animals [5]. However, a study in mice fed a flaxseed oil diet (high in ALA) reported no effect on bone mineral density (BMD) and strength in femurs and vertebrae [13]. In another study, young female rats fed a fish oil diet had reduced bone growth and vertebral peak load [14]. It is possible that the conflicting findings are a result of differences in the amount and the type of experimental diets; thus, it is necessary to develop a strategy to eliminate diet as a confounder.

Therefore, the goal of this study was to investigate the effect of n-3 PUFA on bone strength in the unique *fat-1* mouse model. Due to the presence of the *fat-1* transgene from *Caenorhabditis elegans*, *fat-1* mice are able to endogenously synthesize n-3 PUFA from n-6 PUFA [15]. Mammals lack the n-3 desaturase enzyme that is coded by the *fat-1* gene and must obtain ALA from the diet, from which EPA and DHA can be endogenously synthesized. For example, *fat-1* mice fed an n-6 PUFA-enriched diet have elevated levels of n-3 PUFA in many tissues and organs compared to their wild-type littermates on the same diet [15]. Only one diet for all groups is required, reducing the possibility of introducing variability through diet preparation.

The objectives of this study were to determine the differences in bone mineral and biomechanical strength of femurs among young wild-type and *fat-1* mice in both genders and to correlate femur fatty acid concentrations with measures of femur mineral and biomechanical strength.

2. Methods and materials

2.1. Animals and diet

All procedures were approved by the Animal Ethics Committee at the University of Toronto, Toronto, Canada. C57BL/6 × C3H *fat-1* breeders were obtained from Dr. Jing X. Kang (Harvard Medical School). Mice were housed in temperature- and humidity-controlled standard clean environmental conditions with a 12-h light/dark cycle during the study period. F1 progeny (n=12 male wild-type mice, n=14 male *fat-1* mice, n=11 female wild-type mice, n=13 female *fat-1* mice) were obtained by mating male C57BL/6 × C3H *fat-1* breeders with female C57BL/6 mice from Charles River Laboratories (Saint-Constant, Quebec, Canada). At 3 weeks of age, mice were weaned and housed two to four per cage with their littermates of the same gender.

Fat-1 mice convert n-6 PUFA to n-3 PUFA; thus, these mice may potentially become n-6 PUFA deficient. All mice were fed a modified AIN-93G diet containing 10% safflower oil, high in linoleic acid (LA; 18:2n-6) (Product #D04092701; Research Diets, New Brunswick, NJ), to prevent deficiency. The fatty acid composition of the diet is provided in Table 1. All mice had ad libitum access to food and water.

At 12 weeks of age, mice were euthanized with CO_2. Body weights were measured using a digital scale. Femurs were excised, cleaned of soft tissue and stored at −80°C until analyses were performed.

2.2. Femur bone mineral content (BMC) and BMD

Whole right femurs were placed on a plastic tray and scanned in air at room temperature for determination of bone mineral using dual-energy X-ray absorptiometry (pDEXA Sabre, Model 932937; Stratec Medizintechnik GmbH, Germany) and a specialized software program (Host software version 3.9.4, Scanner software version 1.2.0). A quality-control scan was performed on each day of scanning to ensure accuracy. An 8 cm×8 cm scout scan was performed initially at a resolution of 1 mm×1 mm and a speed of 40 mm/s, followed by the measurement scan (a 1 cm×2 cm scan at a resolution of 0.1 mm×0.1 mm and a speed of 2 mm/s). The percentage of coefficient of variance for femur area, BMC and BMD was 0.6%, 1.4% and 1.8%, respectively.

2.3. Biomechanical strength testing of femurs

Biomechanical strength testing of right femurs was performed at the femur midpoint and femur neck as previously described [11]. Femurs were soaked in physio-

Table 1
Fatty acid composition of the diet

	Percentage of total fatty acids
Fatty acid	
14:0 (myristic)	0.1±0.1
16:0 (palmitic)	7.4±0.2
18:0 (stearic)	3.0±0.1
18:1 n-9 (oleic)	14.1±0.2
18:1 n-7 (*cis*-vaccenic)	0.5±0.2
18:2 n-6 (linoleic)	74.5±0.5
18:3 n-6 (γ-linolenic)	ND
18:3 n-3 (α-linolenic)	ND
20:0 (arachidic)	0.3±0.1
20:4 n-6 (arachidonic)	ND
20:5 n-3 (eicosapentaenoic)	ND
22:0 (behenic)	0.2±0.1
22:6 n-3 (docosahexaenoic)	ND
Total saturates	11.0±0.1
Total unsaturates	89.0±0.1
Total monounsaturates	14.5±0.4
Total polyunsaturates	74.5±0.5
Total n-6 PUFA	74.5±0.5
Total n-3 PUFA	ND

All data are expressed as mean±S.E.M. ND, not detected.

logical saline (9 g NaCl/L) for 4 h at room temperature prior to testing. Three-point bending at the femur midpoint and femur neck fracture was performed using a materials testing system (Model 4442 Universal Testing System; Instron Corp., Canton, MA) and a specialized software program (Instron Series IX Automated Materials Tester, Version 8.15.00; Instron Corp.).

2.3.1. Femur dimensions

Immediately prior to testing, femurs were weighed using an electronic scale in grams to four decimal places. Femur length and widths were measured using electronic precision calipers in millimeters to two decimal places. Width measurements were taken at the femur midpoint in both the anteroposterior and mediolateral directions as femurs are not true cylinders.

2.3.2. Three-point bending at femur midpoint

Three-point bending was performed at the femur midpoint to determine the elastic and plastic properties contributing to bone strength (yield load, resilience, stiffness, peak load and toughness) as previously described [11].

2.3.3. Femur neck fracture

Femur neck fracture test was performed on the right femurs after the three-point bending test. Individual femurs were placed in a customized holder, and the crosshead was lowered at a constant speed of 2 mm/min. Force was applied directly onto the proximal femur head until the femur neck fractured.

2.4. Femur fatty acid analysis

Fatty acid analysis was performed on left femurs to determine the femur lipid profile. Five wild-type mice and five *fat-1* mice were chosen from each gender for analysis. Bone marrow was isolated by transecting the proximal and distal ends of the femur and gently flushing with physiological saline (9 g NaCl/L). Bone tissue was placed in liquid nitrogen and pulverized using a pestle and mortar. Lipids were extracted from the bone tissue with chloroform/methanol (2:1, v/v) as described by Folch et al. [16]. The upper aqueous layer was removed and the lower chloroform fraction was evaporated under a gentle stream of nitrogen. Lipids (0.2 mg) were transferred to a clean screw cap tube with Teflon cap and saponified using 2 ml of 0.5 M KOH in methanol for 1 h at 100°C. After cooling, 2 ml of hexane and 2 ml of 14% boron trifluoride in methanol were added to transesterify the fatty acids for 1 h at 100°C. Two milliliters of water was then added to stop the methylation process, and the mixture was centrifuged at ~100×*g* for 10 min to separate phases. The upper hexane layer containing the fatty acid methyl esters (FAMEs) was extracted and then quantified on an Agilent 6890 gas chromatograph equipped with a flame ionization detector and separated on an Agilent J&W fused silica capillary column (DB-23; 30 m, 0.25 μm film thickness, 0.25 mm i.d.; Agilent, Palo Alto, CA). Samples were injected in splitless mode. The injector and detector ports were set at 250°C. FAMEs were eluted using a temperature program set initially at 50°C and held for 2 min, increased at 20°C/min and held at 170°C for 1 min, increased at 3°C/min and held at 212°C for 10 min to complete the run.

Table 2
Femur dimensions, bone mineral and biomechanical strength properties at femur midpoint and femur neck

	Males		Females		Two-way ANOVA (*P* value)		
	Wild-type	*fat-1*	Wild-type	*fat-1*	Gender	Genotype	Interaction
Whole femur							
Weight (mg)	90.0±3.3	88.8±3.3	71.9±4.7	67.7±3.4	<.001	NS	NS
Length (mm)	15.0±0.1	15.1±0.1	14.6±0.1	14.4±0.1	<.001	NS	NS
Depth (mm)	1.31±0.02	1.32±0.02	1.24±0.01	1.21±0.02	<.001	NS	NS
Width (mm)	1.89±0.02^{b}	2.00±0.03^{a}	1.66±0.02^{c}	1.65±0.03^{c}	<.001	NS	.013
BMC (mg)	23.2±1.1	25.0±0.7	18.6±0.6	18.6±0.6	<.001	NS	NS
BMD (mg/cm^2)	58.7±2.0	61.7±1.3	52.1±1.0	51.9±1.1	<.001	NS	NS
Femur midpoint							
Yield load (N)	11.8±0.5	11.2±0.4	10.6±0.7	9.6±0.7	.027	NS	NS
Resilience (J) (×10^{-3})	0.6±0.1	0.50±0.04	0.6±0.1	0.5±0.1	NS	NS	NS
Stiffness (N/mm)	147.5±7.5	143.6±3.8	132.9±7.1	134.0±5.7	.045	NS	NS
Peak load (N)	22.0±1.1	22.2±0.7	19.4±0.8	18.0±0.8	<.001	NS	NS
Toughness (J) (×10^{-3})	12.9±1.5	11.9±0.5	8.8±1.1	10.0±1.2	.007	NS	NS
Femur neck							
Peak load (N)	18.3±1.1	20.3±1.2	16.3±0.8	16.6±0.9	.012	NS	NS

All data are expressed as mean±S.E.M.; for femur midpoint measurements, *n*=12 male wild-type, *n*=14 male *fat-1*, *n*=11 female wild-type and *n*=12 female *fat-1*; for all other measurements, *n*=12 male wild-type, *n*=14 male *fat-1*, *n*=11 female wild-type and *n*=13 female *fat-1*. Within a row, statistically different values are marked with different superscripts when a significant interaction was observed. NS, not significant ($P \geq .05$).
Depth refers to the AP width at the midpoint of the femur, and width refers to the ML width at the midpoint of the femur.

The carrier gas was helium set to a 0.7 ml/min constant flow rate. Fatty acids were identified by comparing the relative retention times with those of a known standard mixture (GLC 463; Nu-Chek-Prep, Elysian, MN). The area under each peak was determined using ChemStation (version B.01.01; Agilent). Values are expressed as the percentage of total fatty acids. The same procedure was used to determine the fatty acid composition of the diet.

2.5. Statistical analyses

Statistical analyses were performed using SigmaStat version 2.0 (Jandel Corp., San Rafael, CA). All data are expressed as mean±standard error of the mean (S.E.M.). Data were analyzed by two-way analysis of variance (ANOVA) with gender and genotype as main effects and the interaction between gender and genotype. The Tukey post hoc test was used to determine the differences among the four groups when a statistical Gender×Genotype interaction was observed. Pearson's correlation was performed to correlate femur fatty acid concentrations with femur BMC and the peak load at femur midpoint and femur neck. Differences were considered significant at $P<.05$.

3. Results

3.1. Final body weight

Final body weight of male mice (25.68±0.43 g) was significantly higher ($P<.001$) than that of the female mice (19.46±0.45 g). The final body weight of wild-type mice (22.51±0.45 g) was not significantly different from that of the transgenic mice (22.63±0.42 g). There was no significant Gender×Genotype interaction.

3.2. Femur weight and dimensions

Femur weight, length, anteroposterior (AP) width and mediolateral (ML) width were significantly greater ($P<.001$) among males compared to females (Table 2). Genotype had no effect on all four parameters (Table 2). There was a significant Gender×Genotype interaction for ML width ($P=.013$) (Table 2).

Table 3
Femur fatty acid composition

	Percentage of total fatty acids				Two-way ANOVA (*P* value)		
	Males		Females		Gender	Genotype	Interaction
	Wild-type	*fat-1*	Wild-type	*fat-1*			
Fatty acid							
14:0 (myristic)	1.3±0.1	1.4±0.1	1.44±0.04	1.57±0.02	.048	NS	NS
16:0 (palmitic)	19.3±0.8	19.5±0.6	19.7±0.5	20.4±0.5	NS	NS	NS
16:1 n-7 (palmitoleic)	4.4±0.5	5.9±0.8	5.6±0.5	5.9±0.4	NS	NS	NS
18:0 (stearic)	8.4±0.4	8.5±0.5	7.6±0.4	6.8±0.5	.014	NS	NS
18:1 n-9 (oleic)	16.4±0.8	17.6±0.4	19.9±0.6	21.6±0.5	<.001	.027	NS
18:1 n-7 (*cis*-vaccenic)	1.5±0.1	1.6±0.1	1.7±0.1	1.6±0.1	NS	NS	NS
18:2 n-6 (linoleic)	28.7±0.6	30.3±1.1	29.2±0.6	30.9±0.7	NS	NS	NS
18:3 n-6 (γ-linolenic)	ND	0.1±0.1	ND	ND	NS	NS	NS
18:3 n-3 (α-linolenic)	ND	0.1±0.1	ND	0.1±0.1	NS	.004	NS
18:4 n-3 (stearidonic)	0.28±0.04	0.2±0.1	0.12±0.05	0.15±0.04	NS	NS	NS
20:0 (arachidic)	0.8±0.4	0.3±0.1	0.10±0.04	0.3±0.1	NS	NS	NS
20:2 n-6 (eicosadienoic)	1.1±0.3	0.9±0.1	0.9±0.1	0.8±0.1	NS	NS	NS
20:3 n-6 (eicosatrienoic)	1.1±0.2	1.2±0.2	0.8±0.1	0.8±0.2	.074	NS	NS
20:3 n-3 (eicosatrienoic)	0.1±0.1	0.3±0.2	ND	ND	NS	NS	NS
20:4 n-6 (arachidonic)	7.5±0.6	4.3±0.1	6.5±0.4	3.3±0.3	.015	<.001	NS
20:5 n-3 (eicosapentaenoic)	ND	1.0±0.1[a]	ND	0.6±0.1[b]	.004	<.001	.004
22:0 (behenic)	1.6±0.5	0.7±0.1	1.13±0.03	0.5±0.1	NS	.012	NS
22:1 (erucic)	0.6±0.3	0.1±0.1	0.2±0.2	0.1±0.1	NS	NS	NS
22:2 n-6 (docosadienoic)	0.2±0.2	ND	ND	0.1±0.1	NS	NS	NS
22:4 n-6 (adrenic)	2.4±0.5	1.2±0.2	1.9±0.2	1.1±0.2	NS	.003	NS
22:5 n-6 (docosapentaenoic)	3.9±0.5	0.6±0.2	2.8±0.2	0.7±0.1	NS	<.001	NS
22:5 n-3 (docosapentaenoic)	ND	1.1±0.1[a]	ND	0.7±0.1[b]	.001	<.001	.001
22:6 n-3 (docosahexaenoic)	0.3±0.1[c]	3.0±0.2[a]	0.4±0.1[c]	2.1±0.1[b]	.009	<.001	.002
Total saturates	31.5±0.4	30.4±1.0	30.0±0.8	29.5±1.1	NS	NS	NS
Total unsaturates	68.5±0.4	69.6±1.0	70.0±0.8	70.5±1.1	NS	NS	NS
Total monounsaturates	22.9±1.5	25.3±1.2	27.4±0.9	29.3±1.0	.003	NS	NS
Total polyunsaturates	45.6±1.3	44.3±0.9	42.6±0.7	41.2±0.8	.006	NS	NS
Total n-6 PUFA	45.0±1.2	38.7±0.6	42.2±0.7	37.5±0.8	.036	<.001	NS
Total n-3 PUFA	0.7±0.2[c]	5.7±0.4[a]	0.47±0.05[c]	3.7±0.2[b]	<.001	<.001	.002
n-6/n-3	96.8±29.3	6.9±0.4	94.0±12.5	10.3±0.7	NS	<.001	NS

All data are expressed as mean±S.E.M.; $n=5$ per gender per genotype. Within a row, statistically different values are marked with different superscripts when a significant interaction was observed. NS, not significant ($P\geq.05$). ND, not detected.

3.3. Femur BMC and BMD

Male mice had significantly greater BMC and BMD than female mice ($P<.001$) (Table 2). BMC and BMD were unaffected by genotype (Table 2). There was no significant gender × genotype interaction in either BMC or BMD (Table 2).

3.4. Biomechanical strength properties at femur midpoint and femur neck

Male mice had significantly greater yield load ($P=.027$), peak load ($P<.001$), toughness ($P=.007$) and stiffness ($P=.045$) at femur midpoint compared to female mice (Table 2). There was no significant effect of gender on resilience (Table 2). Genotype and its interaction with gender did not have a significant effect on all five strength properties (Table 2). Gender did have a significant effect on peak load at femur neck ($P=.012$) with male mice demonstrating a greater peak load than females (Table 2). The peak load at femur neck was not affected by genotype (Table 2).

3.5. Femur fatty acid composition

Fat-1 mice had significantly greater percentage composition of total n-3 PUFA ($P<.001$), specifically ALA ($P=.004$), EPA ($P<.001$) and DHA ($P<.001$), than wild-type mice (Table 3). Male mice also had significantly greater percentage composition of total n-3 PUFA ($P<.001$), specifically EPA ($P=.004$) and DHA ($P=.009$), than female mice (Table 3). Significant Gender×Genotype interaction was observed in total n-3 PUFA ($P=.002$), EPA ($P<.004$) and DHA ($P=.002$) (Table 3).

Fat-1 mice had significantly less percentage composition of total n-6 PUFA ($P<.001$), specifically arachidonic acid (AA; 20:4n-6) ($P<.001$), than wild-type mice (Table 3). The percentage composition of LA in *fat-1* mice appeared to be lower than that of wild-type mice, but significance was not achieved ($P=.055$) (Table 3). Male mice also had a significantly greater percentage composition of total n-6 PUFA ($P=.036$), specifically AA ($P=.015$), than female mice (Table 3). No significant Gender×Genotype interaction was observed in total n-6 PUFA, LA and AA (Table 3).

Fat-1 mice had a significantly lower n-6/n-3 PUFA ratio ($P<.001$) than wild-type mice (Table 3). Gender and its interaction with genotype resulted in no significant difference in n-6/n-3 PUFA ratio (Table 3).

3.6. Correlations

Correlation analyses were performed between PUFA (LA, AA, ALA, EPA and DHA) and BMC and peak load at femur midpoint and femur neck (Table 4 and Fig. 1). No significant correlations were present between LA, AA and ALA and bone outcomes (BMC and peak load at femur midpoint and femur neck) (Table 4). EPA and DHA were significantly and positively correlated with BMC (Fig. 1). EPA was also significantly and positively correlated with the peak load at femur neck (Fig. 1). Although the correlations between EPA and DHA and other peak load measures did not achieve significance (EPA and peak load at femur midpoint, $P=.08$; DHA and peak load at femur midpoint, $P=.07$; DHA and peak load at femur neck, $P=.06$), a trend suggesting positive correlations was observed (Fig. 1). The n-6/n-3 PUFA ratio was significantly and negatively correlated with all three bone outcomes (Fig. 2).

4. Discussion

In this study, we investigated the potential role of n-3 PUFA in bone development in male and female mice using the *fat-1* mouse model that is capable of de novo synthesis of n-3 PUFA. Our findings linked bone n-3 PUFA status to measures of bone health, for example, bone mineral and biomechanical strength properties. We demonstrated that both male and female *fat-1* mice have higher levels of n-3 PUFA in femur tissue relative to wild-type mice. Although femur BMC, BMD and peak load were not significantly different between *fat-1* and wild-type mice of either gender, these measures correlated significantly and negatively with the n-6/n-3 PUFA ratio. Moreover, EPA and DHA, long-chain n-3 PUFA of interest, showed either a significant positive correlation or a trend in the positive direction with femur BMC and peak load. These findings suggest that a reduction of the n-6/n-3 PUFA ratio and an increase in n-3 PUFA from EPA and DHA may be linked to greater bone strength in healthy young male and female mice. As expected, male mice had greater bone mass and stronger femurs than female mice of either genotype. The gender difference is attributed to the fact that the body size of males is typically larger than that of females and ultimately results in larger bones that can withstand greater force before being fractured.

Table 4
Correlations between fatty acid concentrations and femur outcomes

Fatty acid	BMC		Peak load at femur midpoint		Peak load at femur neck	
	r	*P*	*r*	*P*	*r*	*P*
18:2 n-6 (linoleic)	.09	NS (.714)	.05	NS (.829)	−.03	NS (.907)
20:4 n-6 (arachidonic)	−.05	NS (.836)	.06	NS (.794)	.23	NS (.336)
18:3 n-3 (α-linolenic)	.41	NS (.07)	.18	NS (.458)	.00	NS (.999)

$n=5$ per gender per genotype. NS, not significant ($P\geq.05$).

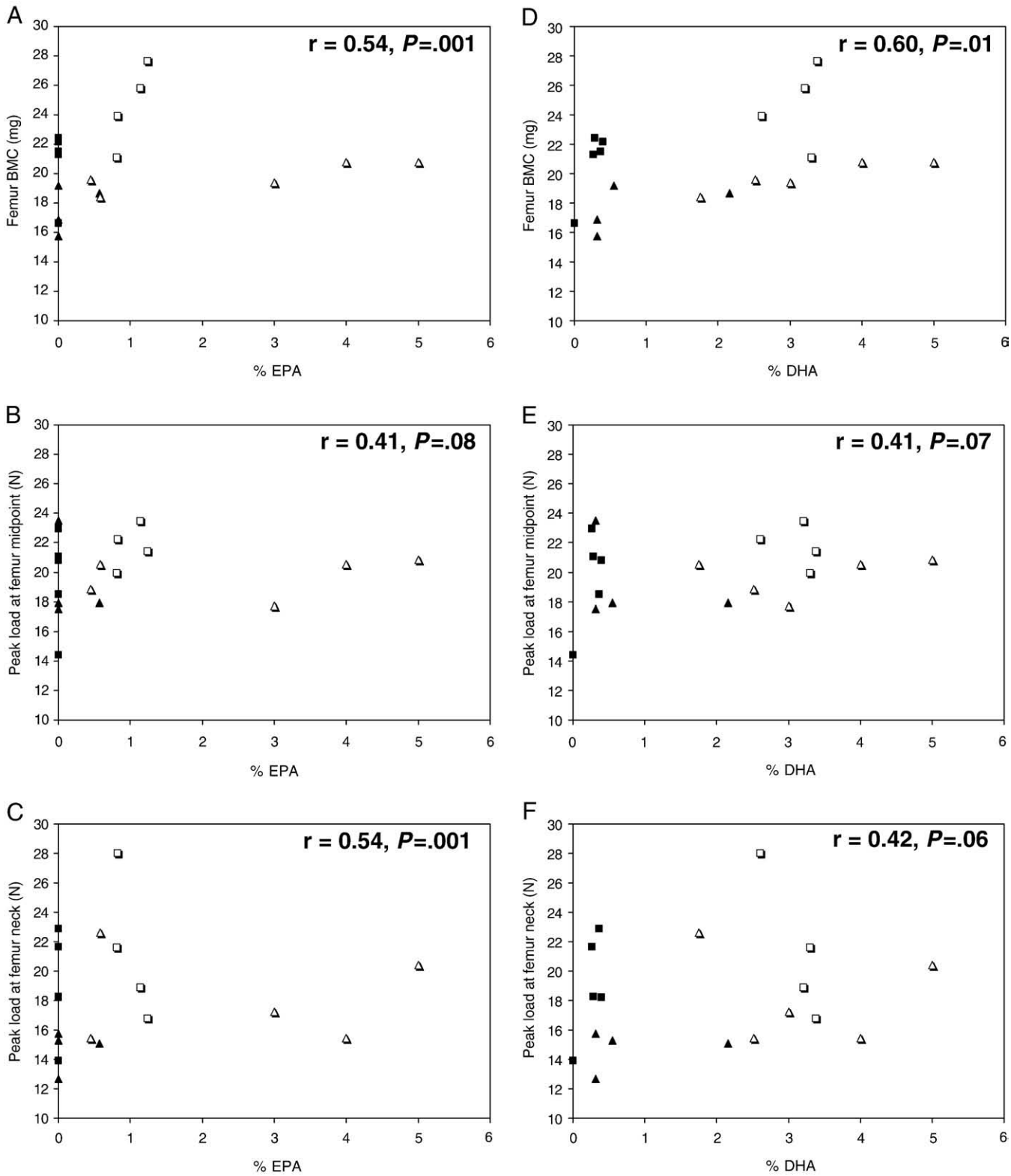

Fig. 1. Relationships between femur EPA content and (A) BMC, (B) peak load at femur midpoint and (C) peak load at femur neck and between femur DHA content and (D) BMC, (E) peak load at femur midpoint and (F) peak load at femur neck. *n*=5 per gender per genotype. Symbols used: ■, male wild-type; □, male *fat-1*; ▲, female wild-type; △, female *fat-1*.

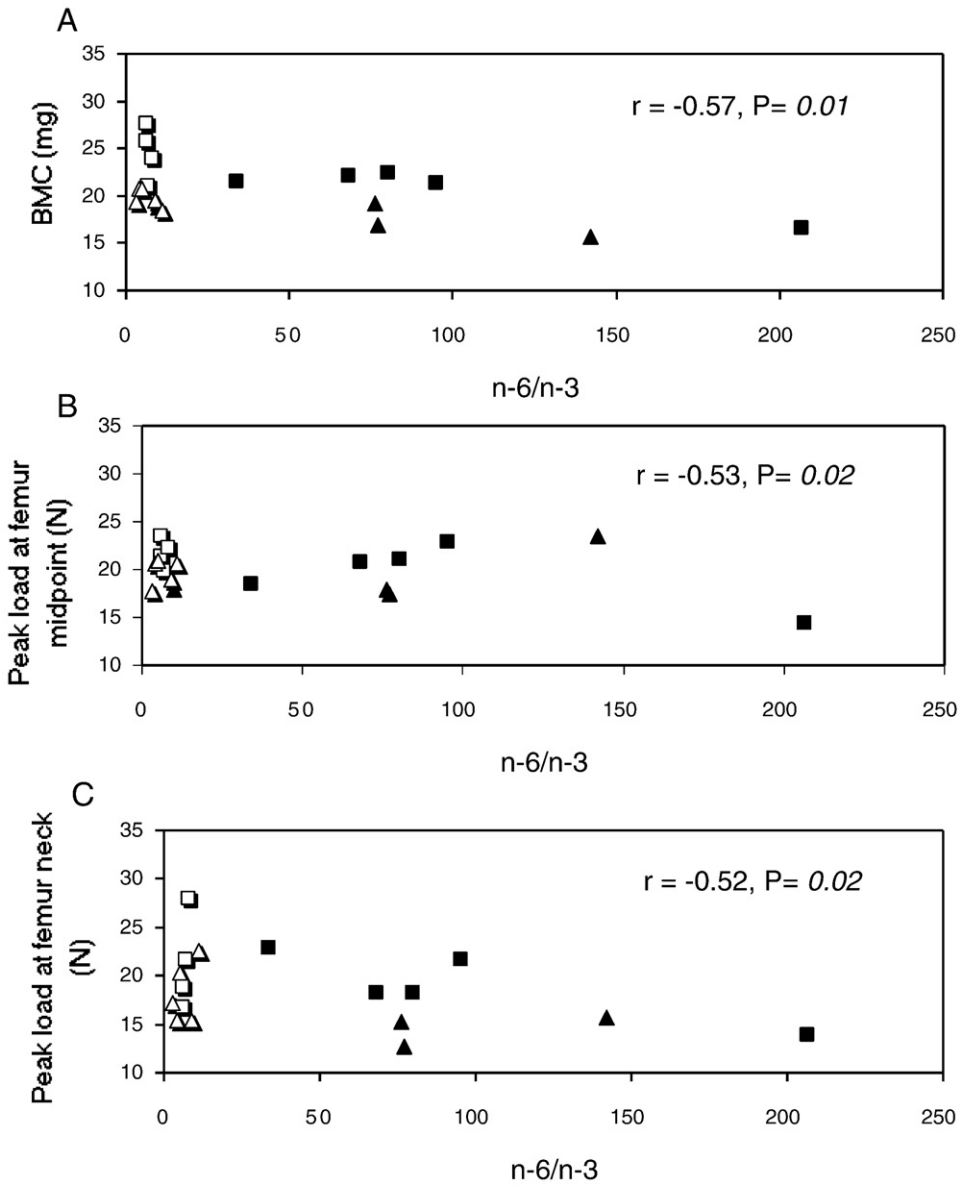

Fig. 2. Relationships between n-6/n-3 PUFA ratio in femur and (A) BMC, (B) peak load at femur midpoint and (C) peak load at femur neck. *n*=5 per gender per genotype. Symbols used: ■, male wild-type; □, male *fat-1*; ▲, female wild-type; △, female *fat-1*.

Fat-1 mice of either gender had higher levels of n-3 PUFA and a lower n-6/n-3 PUFA ratio compared to wild-type mice. The n-6 PUFA, n-3 PUFA and the n-6/n-3 PUFA ratio of the femur in the wild-type mice were 45.0%, 0.7% and 96.8, respectively, which are comparable to the values (48.9%, 0.8% and 77.8) achieved in a dietary study where the control group was fed a safflower oil diet [17]. The fatty acid levels in *fat-1* mice were also comparable to those fed a high n-3 PUFA diet in the same study [17]. The similar results indicate that the *fat-1* mouse model mimics the changes in fatty acid levels observed in dietary studies. Thus, the *fat-1* mouse is a physiological and viable model for studying the effects of n-3 PUFA on bone metabolism. The relevance of the model is further strengthened by observations made by our group about the phenotype of the mouse. One concern of all transgenic mice is the unknown compensatory effects due to the insertion of a transgene, which may lead to the misinterpretation of study results. In our experience, outwardly, the *fat-1* mice do not have physical defects, produce viable young of expected litter size, have a life expectancy of about 2 years and have successfully transmitted the gene through more than eight generations.

The levels of EPA and DHA were significantly different between male and female mice. Male mice had a 1.6-fold and a 1.3-fold higher increase in EPA and DHA, respectively, as compared to females. A similar 1.5-fold increase in total n-3 PUFA was also observed. This finding suggests that the two genders incorporate n-3 PUFA into femur tissue at a different rate and/or through a different mechanism. In fact, sex steroid hormones may regulate this

incorporation process. Studies have suggested that females tend to have higher plasma levels and synthesis of n-3 PUFA than males [18]. In contrast, our study has shown that males had higher levels of n-3 PUFA than female mice in the femur. The higher levels of n-3 PUFA among males may be due to gender differences in rate of uptake and/or turnover of n-3 PUFA in bone tissue.

A possible reason for not observing differences in BMC, BMD and peak load among *fat-1* and wild-type mice is that these mice were killed at early adulthood when endogenous sex steroid hormone production is high, and there were no concurrent insults to bone health at this relatively early and healthy stage of the life cycle. In contrast, previous studies have suggested that n-3 PUFA may have greater effects on bone health during aging as sex steroid production declines or in a diseased state. In studies using older rodents, n-3 PUFA intake attenuated deterioration of bone tissue by suppressing bone turnover [19], decreasing pro-inflammatory cytokine production [9] and altering systemic hormone levels such as insulin-like growth factor-1, parathyroid hormone and vitamin D [10], all of which have an impact on bone formation and resorption. Dietary n-3 PUFA have also been negatively correlated with serum N-telopeptides, a biochemical marker of bone resorption in humans [20]. In rats with periodontal disease, an oral inflammatory state, dietary treatment of fish oil, rich in EPA and DHA, reduced bone resorption [12]. Although femur BMC, BMD and peak load in our study were not significantly different among groups, future studies should determine if lifelong exposure to n-3 PUFA programs bone metabolism, resulting in greater protection against deterioration of bone tissue during aging.

Our finding that the n-6/n-3 PUFA ratio was negatively associated with BMC and the peak load at two different sites of the femur (midpoint and femur neck) suggests that the reduction of n-6/n-3 PUFA ratio in bone may be linked to better bone status. Indeed, other studies have linked a low n-6/n-3 PUFA ratio to a variety of measures of bone health [3,17,21–23]. A low dietary ratio of n-6/n-3 PUFA in rats resulted in a higher femur BMC [17], higher bone formation markers and lower prostaglandin production [3]. In humans, a high dietary n-6/n-3 PUFA ratio was associated with lower hip BMD [21] and a low n-6/n-3 PUFA ratio was positively correlated with bone formation markers [22] and BMD [23].

Findings from the present study show that ALA is not associated with improved bone outcome measures given that only trace levels were found in *fat-1* mice. In contrast, the results suggest that the efficaciousness of n-3 PUFA on bone mineral and strength is associated with EPA and DHA. Hence, the effects of ALA may be indirectly due to its metabolic conversion to EPA and DHA. Therefore, the type of n-3 PUFA may explain some discrepancies in previous studies [3,5,13,14].

In conclusion, the *fat-1* mouse is a valuable tool to study the effects of n-3 PUFA on bone health. Future studies should investigate potential mechanisms of n-3 PUFA using this transgenic model as well as potential effects at other skeletal sites that differ in the composition of trabecular and cortical bone. The vertebrae contain a higher proportion of trabecular bone compared to cortical bone, and thus may be more responsive to n-3 PUFA. Furthermore, the potential benefit of n-3 PUFA at other stages of the life cycle such as during aging, when bone metabolism is compromised due to declining production in sex steroids, should be investigated.

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