

The Effect of Pre- and Postmolt Diets High in n-3 Fatty Acids and Molt Programs on Skeletal Integrity and Insulin-Like Growth Factor-I of White Leghorns

H. Mazzuco,^{*,†1} J. P. McMurtry,[‡] A.Y. Kuo,[‡] and P. Y. Hester^{*,2}

**Department of Animal Sciences, Purdue University, West Lafayette, Indiana 47907; †Embrapa Swine & Poultry Research Center, Concordia, Brazil 89700-000; and ‡USDA, Growth Biology Laboratory, Beltsville, Maryland 20705*

ABSTRACT This study investigated changes in bone integrity and circulating concentrations of insulin-like growth factor-I (IGF-I) of hens subjected to 2 distinct molting regimens and fed pre- and postmolt diets high in n-3 or n-6 fatty acids. A dual-energy x-ray absorptiometer determined bone mineral density (BMD) of the tibia and humerus of 45 live hens from 62 to 76 wk of age. Densitometric scans were also conducted in excised tibia and humerus at 66, 71, and 76 wk of age. Concentrations of IGF-I were monitored using an homologous RIA at the same ages. The molting treatments consisted of 10 d of fasting + cracked corn for 7 d + pullet developer diet for 10 d or a nonfasting molt (wheat-middlings-based diet for 27 d). Five weeks prior to and after either molt treatment, birds were fed 1 of 2 diets containing dietary n-6/n-3 fatty acids ratios of 0.6 or 8.0. At the end of the molt (71 wk of age), tibial BMD decreased 30% in fasted and 11% in nonfasted molt regimens, and the fatty acid con-

tent of the premolt diet had no effect on the decline in BMD. The BMD of the humerus also decreased during molt with the exception of hens subjected to a nonfasted molt and fed n-3 fatty acid diets in which their BMD values were similar to or greater (at 73 wk of age) than those of controls during the entire experimental period (treatment by bone by age, $P \leq 0.0001$). Induced molt affected circulating IGF-I concentrations (treatment by age interaction, $P \leq 0.0001$), and the response was the same regardless of molt regimen (fasting vs. nonfasting) or diet (n-3 vs. n-6 fatty acids). A decrease in IGF-I 54 h postmolt was noted; however, from 13 to 43 d postmolt, all molted birds had elevated IGF-I as compared with controls. In conclusion, a nonfasted molt as compared with fasted molt was less detrimental to bone mineralization; dietary n-6/n-3 fatty acid ratios in the pre- and postmolt diets had little effect on the decline of skeletal integrity during molt, and circulating IGF-I concentrations were affected by molt.

(Key words: bone mineral density, n-3 fatty acids, insulin-like growth factor-I, nonfeed removal molt, hen)

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INTRODUCTION

The 18-carbon fatty acids, linoleic and linolenic acids, are recognized as metabolically essential fatty acids, but linoleic acid is the only essential fatty acid for which a dietary requirement has been demonstrated in poultry (National Research Council, 1994). The n-3 and n-6 fatty acids are largely derived from the dietary intake of linolenic and linoleic acids, respectively. Emerging evidence from animal studies indicates that a low n-6:n-3 ratio exerts beneficial effects on bone (Xu et al., 1994; Watkins et al., 1996; Li et al., 1999; Weiler and Fitzpatrick-Wong, 2002). Possible mechanisms that might be influenced by

the low dietary ratios of n-6:n-3 fatty acids on bones include increased intestinal calcium absorption, lowered bone turnover (Claassen et al., 1995), improved calcium deposition (Coetzer et al., 1994), enhanced synthesis of bone collagen (Kruger and Horrobin, 1997), increased circulating insulin-like growth factor-I (IGF-I), and decreased locally produced prostaglandins (Watkins et al., 1996; Li et al., 1999). Further evidence of the protective action of n-3 fatty acids on bone coincides with elevated levels of bone formation markers that are indicative of enhanced bone formation activity (Watkins et al., 2001; 2003).

A polypeptide hormone, IGF-I is structurally related to insulin (McMurtry et al., 1997). The synthesis and secretion of IGF-I is under both growth hormone-dependent and independent control (McMurtry et al., 1997). It is

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¹Student sponsored by National Council for Scientific and Technological Development (CNPq) - Brasilia-DF, Brazil.

²To whom correspondence should be addressed: phester@purdue.edu.

Abbreviation Key: BMC = bone mineral content, BMD = bone mineral density; CT = cycle threshold; IGF-I = insulin-like growth factor-I; PGE₂ = prostaglandin E₂; PUFA = polyunsaturated fatty acids.

synthesized by various tissues, where it acts via autocrine and paracrine mechanisms to effect an array of anabolic pathways (McMurtry et al., 1997). Accumulating evidence strongly suggests that IGF-I plays an important role in bone metabolism (Bonjour et al., 2005). The skeleton is a major source of IGF-I through de novo synthesis by osteoblasts and by virtue of their release during active skeletal resorption (Conover and Rosen, 2002). Blood-borne IGF-I, derived mainly from the liver, exerts its actions via endocrine mechanisms (McMurtry et al., 1997). The IGF-I in serum and most body fluids are complexed with high affinity IGF-binding proteins (Thissen et al., 1994).

Dietary components affect circulating concentrations of IGF-I (McMurtry, 1998). The production and action of this growth factor are selectively influenced by the dietary supply of proteins whose deficiency was associated with low plasma IGF-I, decreased bone mineral mass, and increased risk of osteoporotic fracture (Bonjour et al., 2005). Circulating IGF-I was 2-fold lower in chicks fed a 12% CP diet than in chicks fed a 21% CP diet (McMurtry et al., 1998). Previous findings revealed that plasma IGF-I concentrations and hepatic IGF-I mRNA gene expression in young chickens are responsive to alterations in nutritional conditions and are reduced by feed restriction (Morishita et al., 1993; Kita et al., 1996; Roberson et al., 2002) and increased after refeeding (Kita et al., 1998; Kita et al., 2002). The plasma concentration of IGF-I was highest in chicks fed diets rich in n-3 polyunsaturated fatty acids (PUFA) as compared with chicks fed low dietary n-3 PUFA (Watkins et al., 1996; 1997).

Avian osteoporosis has been recognized as a problem in the laying hen industry for at least 45 yr (Korver et al., 2004). Commercial laying hens are susceptible to structural bone osteoporosis due to their high rates of sustained egg production (Webster, 2004). Modern strains of laying hens produce more eggs than in the past, resulting in a greater reliance on bone mineral stores of Ca for eggshell production (Korver et al., 2004). Cage layer osteoporosis in hens severely reduces bone strength because of high bone turnover related to eggshell formation and inadequate physical activity (Rath et al., 2000). Husbandry systems such as battery cages maintain the birds during their entire productive life in a condition of reduced activity, which most likely contributes to the severity of the problem. Osteoporosis is not so severe as to result in caged layer fatigue, but widespread structural bone loss can lead to high incidences of fractures at various sites throughout the skeleton (Whitehead and Fleming, 2000).

Induced molting is currently an integral part of the replacement programs used on table egg production farms in the United States (Bell et al., 2004). However, existing molting induction procedures (by light manipulation and feed removal) have become increasingly criticized on the grounds of animal well-being (Braw-Tal et al., 2004). Therefore, new research has been directed toward finding alternative methods for molt induction that

do not require complete removal of feed (Donalson et al., 2005).

It has previously been reported that a fasted molt is deleterious to the skeletal integrity of White Leghorns (Mazzuco and Hester, 2005a). Therefore, the objectives of the current study were 2-fold. The first objective was to monitor the carryover effect of pre- and postmolt diets containing high n-3 PUFA on the skeletal integrity of White Leghorns before, during, and after a fasted or non-fasted molt. The second objective was to determine the influence of diets containing high n-3 PUFA and molting programs on plasma IGF-I concentrations and hepatic IGF-I mRNA expression.

MATERIALS AND METHODS

A 14-wk trial was conducted from 62 to 76 wk of age using 310 White Leghorns nearing the end of their first cycle of lay. The study was conducted under guidelines approved by the Purdue University Animal Care and Use Committee. At 59 wk of age, hens were housed at random in 10 rows of cages (31 birds per row) with 1 bird placed in a cage, resulting in 1,084 cm² of floor space per hen. Birds were weighed individually at 61 wk of age for purposes of eliminating potential culls prior to molt and to ensure similar BW means among treatments prior to initiation of the experiment. There were no culls; therefore, at 62 wk of age, all hens were assigned to 1 of the following 5 treatments with 2 rows of cages per treatment (see Table 1 for diet composition and Table 2 for fatty acid composition of diets).

Treatment 1: n-6—Fasted Molt

Birds were fed n-6 diets 5 wk before and after molt (starting at 67 wk of age) containing n-6/n-3 PUFA ratios of 7.66 and 8.18, respectively, and were subjected to a 10-d fast followed by the consumption of cracked corn (n-6/n-3 PUFA ratio of 28.3) for 7 d and a pullet developer diet (n-6/n-3 PUFA calculated ratio of 15.2 based on analyzed fatty acid profile of the dietary ingredients) for 10 d (see Schreiweis et al., 2004 for diet composition).

Treatment 2: n-3—Fasted Molt

This treatment was the same as treatment 1 except that pre- and postmolt n-3 diets were fed that contained n-6/n-3 PUFA ratios of 0.69 and 0.35, respectively

Treatment 3: n-6—Nonfasted Molt

Five weeks before and after molt, birds were fed n-6 diets containing n-6/n-3 PUFA ratios of 7.66 and 8.18, respectively, and a wheat-middlings-based molt diet (n-6/n-3 PUFA calculated ratio of 11.85) for 27 d as part of the nonfasted molt

Treatment 4: n-3—Nonfasted Molt

This treatment was the same as treatment 3 with the exception that pre- and postmolt n-3 diets were fed and

Table 1. Ingredients and nutrient composition of experimental diets

Ingredients (%)	n-3 diet	n-6 diet	Nonfasted molt diet ¹
Ground yellow corn (6.12% CP)	54.70	54.70	23.00
Dehulled soybean meal (47.5% CP)	29.17	29.17	—
Wheat middlings	—	—	71.39
Dicalcium phosphate	1.84	1.84	0.10
Limestone, ground	8.07	8.07	4.96
Linseed oil ²	5.00	—	—
Soybean oil	—	5.00	—
Salt	0.50	0.50	0.30
Vitamin-mineral premix ³	0.25	0.25	0.25
DL-Met	0.15	0.15	—
Mold inhibitor ⁴	0.05	0.05	—
Antioxidant ⁵	0.02	0.02	—
Microtracer ⁶	0.25	0.25	—
Total	100.0	100.0	100.0
Calculated nutrient composition ⁷			
ME, kcal/kg	2,952	2,963	2,198
CP, %	17.20	17.20	12.83
Lys, %	1.01	1.01	0.55
Met, %	0.44	0.44	0.19
Met + Cys, %	0.75	0.75	0.46
Ca, %	3.53	3.53	2.00
Nonphytate P, %	0.44	0.44	0.25

¹Diet composition from Biggs et al. (2004).

²Barleans Organic Oils LLC, Ferndale, WA.

³Provided per kilogram of diet: vitamin A, 6,600 IU; vitamin D₃, 2,695 IU; vitamin E, 33 IU; vitamin K, 1.2 mg; riboflavin, 4.4 mg; pantothenic acid, 6.6 mg; niacin 21 mg; choline, 358 mg; vitamin B₁₂, 0.006 mg; Mn, 83 mg; Zn, 61 mg; Fe, 32 mg; Cu, 3.9 mg; I, 1.1 mg; and Se, 0.256 mg.

⁴Myco Curb Dry, Kemin Industries Inc., Des Moines, IA.

⁵Ethoxyquin (66%), Prime Quality Feeds, North Little Rock, AR.

⁶Microtracers, San Francisco, CA.

⁷Based on NRC (1994) feed composition tables.

contained n-6/n-3 PUFA ratios of 0.69 and 0.35, respectively.

Treatment 5: Controls

Hens were not subjected to a molt and were fed n-6 diets containing n-6/n-3 PUFA ratios of 7.66 to 8.18 throughout the experimental period. All diets and water were provided ad libitum to hens. Water restriction was not used during molt. Control hens were maintained on a constant 16 h photoperiod. Prior to molt, molted hens were also maintained on a 16 h photoperiod. Hens of both molting regimens were restricted to 8 h of light beginning on the first day of molt. At 28 d postmolt, molted hens were returned to a photoperiod of 16 h. Mortalities and eggs laid by each hen were recorded daily throughout the entire experimental period. Egg production for the flock was calculated as hen-day egg production (Bell, 2002).

Dietary lipid was extracted from feed samples through acid hydrolysis according to the methods of the Association of Official Analytic Chemists (2000), section 4.5.02, method 954.02. Fatty acid composition of the diets (Table 2) was determined using gas liquid chromatography as described by the American Oil Chemists' Society (1990; method Ce 2 to 66) and were analyzed using AOCS methods Ce 1b-89, Ce 1f-96, and Ce 1d-91. The amounts of

saturated fatty acids, monounsaturated fatty acids, PUFA, total n-3 and n-6 PUFA, and the n-6/n-3 ratios were expressed as a percentage of total fat.

Forty-five hens, or 9 hens per dietary treatment, were monitored at weekly intervals beginning at 62 wk of age and ending at 76 wk of age for in vivo skeletal integrity using dual energy x-ray absorptiometry (Norland Medical Systems, Ft. Atkinson, WI). The bone mineral density (BMD; g/cm²) and the bone mineral content (BMC; g) of the left leg (tibia and fibula) and wing (humerus) were measured in live, nonanaesthetized hens. Birds were restrained on their backs in a foam holding device and secured with straps (Schreiweis et al., 2003). Scanning began at the proximal end of the bone and took approximately 10 min for each bone. Individual BW was recorded after each live scan.

The 45 hens subjected to weekly dual-energy x-ray absorptiometry scans were bled (3 mL/hen per age) from the brachial vein at each of the following ages: 62, 66, 67, 69, 70, 72, 73, and 76 wk of age. A 2% solution of disodium EDTA (12 mg/blood sample; Sigma Diagnostics, St. Louis, MO) was used as the anticoagulant. Blood was centrifuged at 350 × g for 15 min, and the plasma was collected. Plasma samples were stored at -80°C for later analysis of IGF-I via an homologous RIA (McMurtry et al., 1994).

Table 2. Fatty acid composition¹ of the pre- and postmolt diets (analyzed values)

Fatty acids	n-6 diet ²		n-3 diet ³	
	Premolt	Postmolt	Premolt	Postmolt
14:0	0.07	ND ⁴	0.05	ND
14:1	ND ⁴	ND	ND	ND
15:0	ND	ND	ND	ND
16:0	11.52	11.61	7.66	8.22
16:1(n9)	0.10	ND	0.08	ND
17:0	0.10	ND	0.14	ND
17:1(n10)	ND	ND	0.07	ND
18:0	4.06	3.68	3.67	3.60
<i>trans</i> 18:1	0.04	ND	ND	ND
18:1(n9)	21.40	21.18	19.70	20.34
18:2(n-6)	54.06	54.99	27.66	30.65
18:3(n-3)	7.03	6.72	40.07	52.48
18:4(n-3)	0.03	ND	0.05	36.36
20:0	0.40	0.34	0.23	0.24
20:1(n9)	ND	ND	ND	ND
20:3(n-3)	ND	ND	ND	ND
20:4(n-6)	ND	ND	ND	ND
20:4(n-3)	ND	ND	ND	ND
20:5(n-3)	ND	ND	ND	ND
22:0	0.48	0.33	0.25	ND
22:1(n9)	ND	ND	ND	ND
22:5(n-3)	ND	ND	ND	ND
22:6(n-3)	ND	ND	0.04	ND
23:0	0.06	ND	0.05	ND
24:0	0.22	ND	0.21	ND
24:1(n9)	ND	ND	ND	ND
SAT ⁵	16.91	15.96	12.26	12.06
MUFA ⁶	21.54	21.18	19.85	20.34
PUFA ⁷	61.12	61.71	67.73	119.49
Σ (n-6)PUFA ⁸	54.06	54.99	27.66	30.65
Σ (n-3)PUFA ⁹	7.06	6.72	40.12	88.84
(n-6)/(n-3) ¹⁰	7.66	8.18	0.69	0.35

¹ Expressed as percentage total fat.

² Diet high in n-6 fatty acids fed from 62 to 66 wk of age (premolt) and from 71 to 76 wk of age (postmolt).

³ Diet high in n-3 fatty acids fed from 62 to 66 wk of age (premolt) and from 71 to 76 wk of age (postmolt).

⁴ ND = not detected.

⁵ SAT = total saturated fatty acids.

⁶ MUFA = total monounsaturated fatty acids.

⁷ PUFA = total polyunsaturated fatty acids.

⁸ Σ (n-6)PUFA = Sum of 18:2(n-6) + 20:4(n-6).

⁹ Σ (n-3)PUFA = Sum of 18:3 (n-3) + 18:4(n-3) + 22:6 (n-3).

¹⁰ (n-6)/(n-3) = Σ (n-6)PUFA / Σ (n-3)PUFA.

Egg traits were measured and averaged from 2 eggs collected from each scanned hen at weekly intervals from 62 to 76 wk. Individual egg weight was determined. The yolk and albumen were siphoned from the egg with a syringe, the shell with its membrane intact was rinsed and dried at 60°C, and the dry shell weight was recorded. Shell thickness and percentage of shell were determined as described by Klingensmith and Hester (1985).

Additional hens, apart from those subjected to weekly scans, were euthanized during the premolt, molt, and postmolt periods. Specifically, at 66 and 71 wk of age (5 hens per treatment), and at 76 wk of age (9 hens per treatment), birds were bled as described previously and euthanized using carbon dioxide. Liver samples (caudal half of the right lobe) were removed from each hen, wrapped in aluminum foil, snap frozen in liquid nitrogen, and stored at -80°C for later determination of IGF-I mRNA expression. Total RNA was isolated from liver

tissue using the TriReagent protocol (Molecular Research Center Inc., Cincinnati, OH). The integrity of RNA was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring optical density at 260 and 280 nm. Three micrograms of total RNA was used to synthesize single-strand cDNA using reverse transcription reactions (SuperScript III First Strand Synthesis System, Invitrogen Life Technologies, Carlsbad, CA). We used PCR (DNA Engine Opticon, MJ Research Inc., Waltham, MA) to analyze chicken IGF-I gene expression with the iTaqSYBR Green Supermix with Rox PCR kit (Bio-Rad, Hercules, CA). Amplification was carried out in a total volume of 25 μ L containing 1% SYBR Green master mix, 0.5 μ M of each primer, RNase-free water, and 1 μ L of cDNA. Each sample was amplified with chicken IGF-I primers (Guernec et al., 2003) and chicken β actin primers (external control) that formed a product of 300 bp. Cycle threshold (CT), defined as the

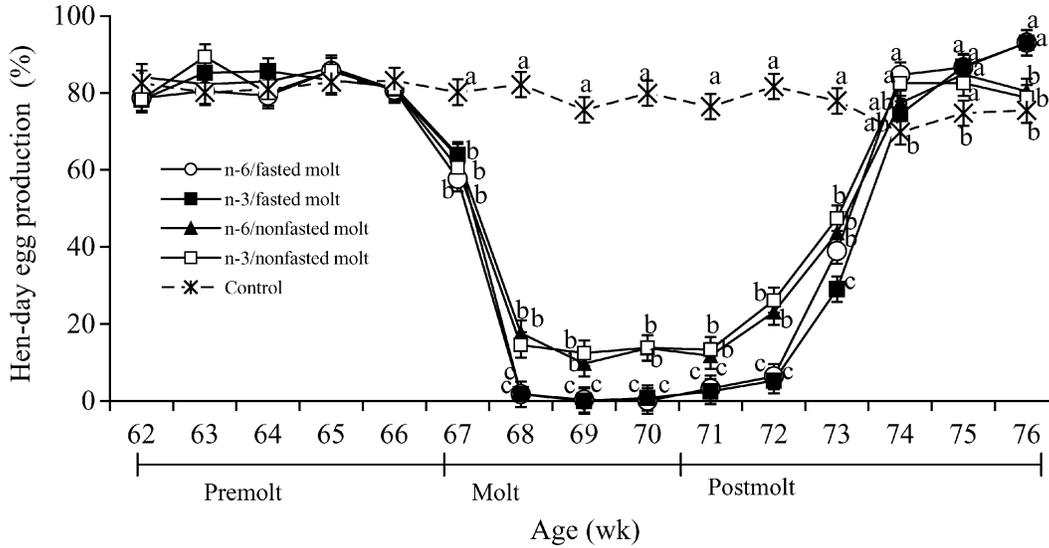


Figure 1. Weekly hen-day egg production of 310 hens fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. ^{a-c}Within age and among dietary treatments, least square means \pm SEM with no common letter are significantly different (treatment by age interaction, $P \leq 0.0001$). Each value represents the average of 2 rows of cages with 31 hens per row.

cycle number at which fluorescence was distinguishable from noise, appeared during the exponential phase of the PCR reaction and was inversely proportional to the initial number of template molecules in the sample. Results were expressed as a ratio of IGF-I CT to the chicken β actin CT (Meijerink et al., 2001).

After the collection of liver samples, the left humerus and tibia with its fibula were excised. The bones were cleaned of all tissue, wrapped in 0.85% saline-soaked gauze, placed in a plastic bag, and frozen at -7 to -10°C for later analysis of bone ash (Schreiweis et al., 2003).

Statistical Analyses

The BMD and BMC of live birds were analyzed by an analysis of covariance with BMD or BMC at 62 wk as the covariate and repeated measurements (from 62 to 76 wk of age) using treatment (type of molt and pre- and postmolt diets high or low in n-3 fatty acids) as the whole plot with the type of bone (tibia and humerus) within a bird as a subplot. An ANOVA was used to test for the fixed effects of age (66, 71, and 76 wk of age) and type of bone (tibia and humerus) on excised bone traits. Pro-

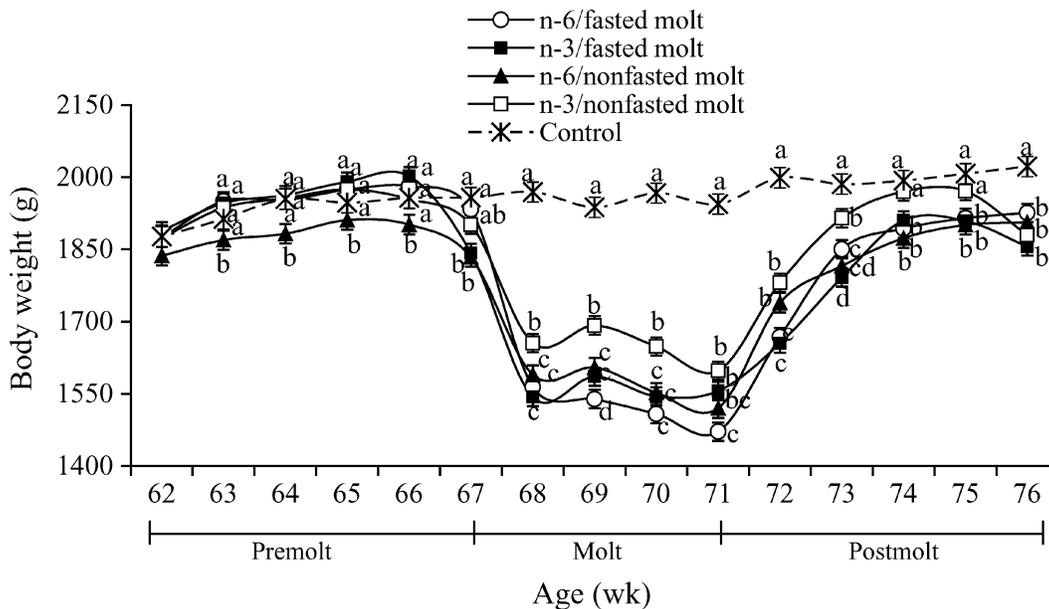


Figure 2. Body weight of White Leghorns fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. ^{a-d}Within age and among dietary treatments, least square means \pm SEM with no common letter are significantly different (treatment by age interaction, $P \leq 0.0001$). Each value represents the average of 8 to 9 hens per treatment.

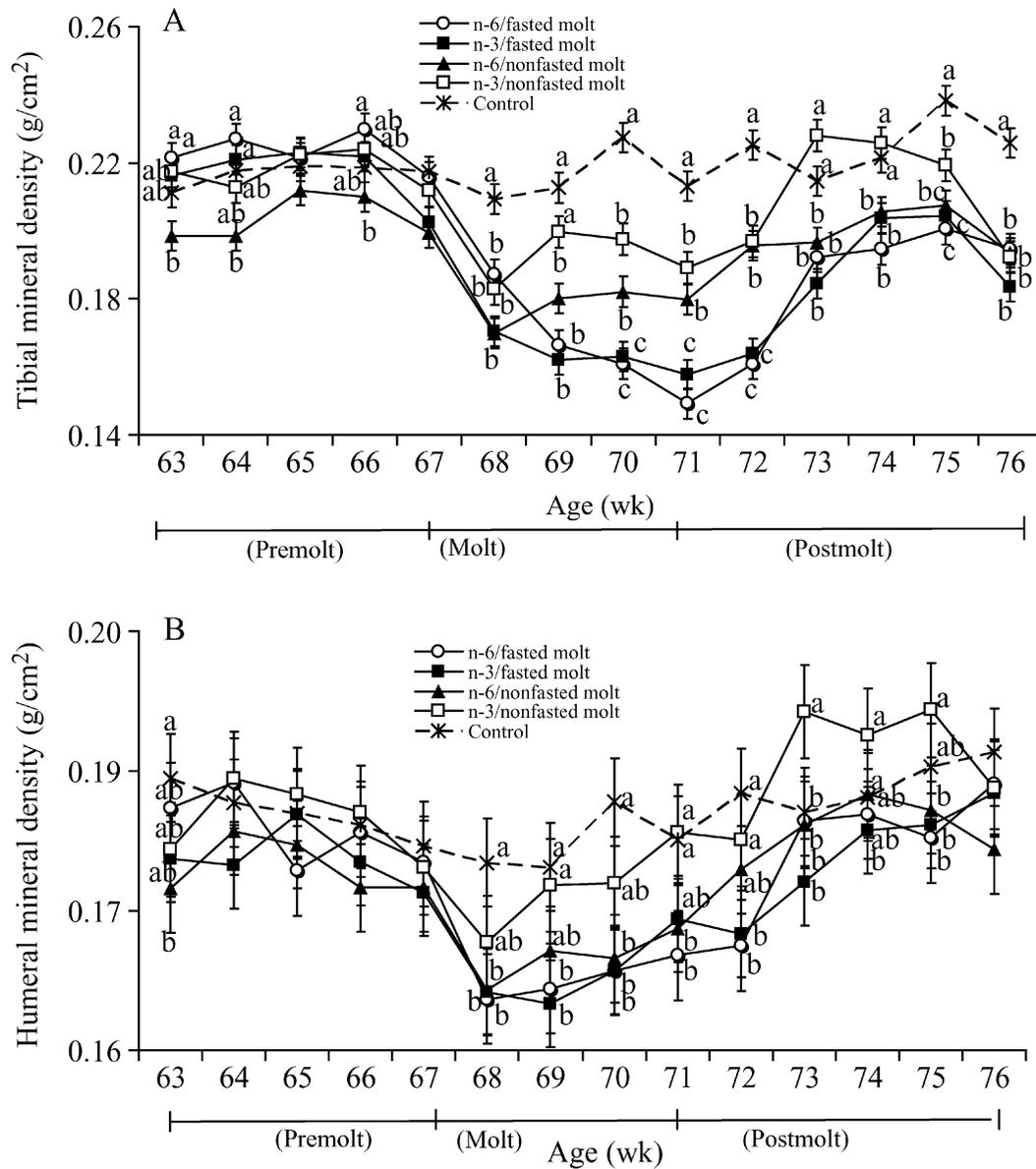


Figure 3. Tibial (A) and humeral (B) mineral density of White Leghorns fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. ^{a-c}Within age and among dietary treatments, least square means \pm SEM with no common letter are significantly different (treatment by bone by age interaction, $P \leq 0.0001$). Each value represents 8 to 9 hens per treatment.

duction traits (BW, hen-day production, livability, and shell quality) were analyzed by an ANOVA with repeated measurements with treatment and age as fixed effects. Plasma concentrations of IGF-I for birds repeatedly sampled at 66, 67, 69, 70, 72, 73, and 76 wk of age were analyzed by an analysis of covariance using IGF-I concentrations at 62 wk as the covariate, with treatment and sample times as fixed effects. For samples obtained from hens euthanized at 66, 71, and 76 wk of age, an ANOVA was used for the IGF-I plasma concentrations and hepatic IGF-I expression with treatment and age as main fixed effects. The individual hen was the experimental unit, except for egg production in which a row of birds was the experimental unit. Differences of least squares means were used to partition means for significant interactions (Oehlert, 2000). The mixed model procedure of the SAS

system was used to conduct the statistical analysis (SAS Institute, 2001). Pearson correlation coefficients were performed among variables of interest (Steel et al., 1997).

RESULTS

Molt induced by fasting caused cessation of lay by 68 wk of age with a return to normal egg production by 74 wk of age (Figure 1). Postmolt, the fasted groups showed higher hen-day egg production than controls at 75 and 76 wk of age and higher hen-day egg production than both nonfasted groups at 76 wk of age. Egg production during the induced molt for hens of the nonfasted molt regimens decreased to approximately 12% but never achieved 0%. Birds subjected to a nonfasted molt returned to control or above control egg laying production levels

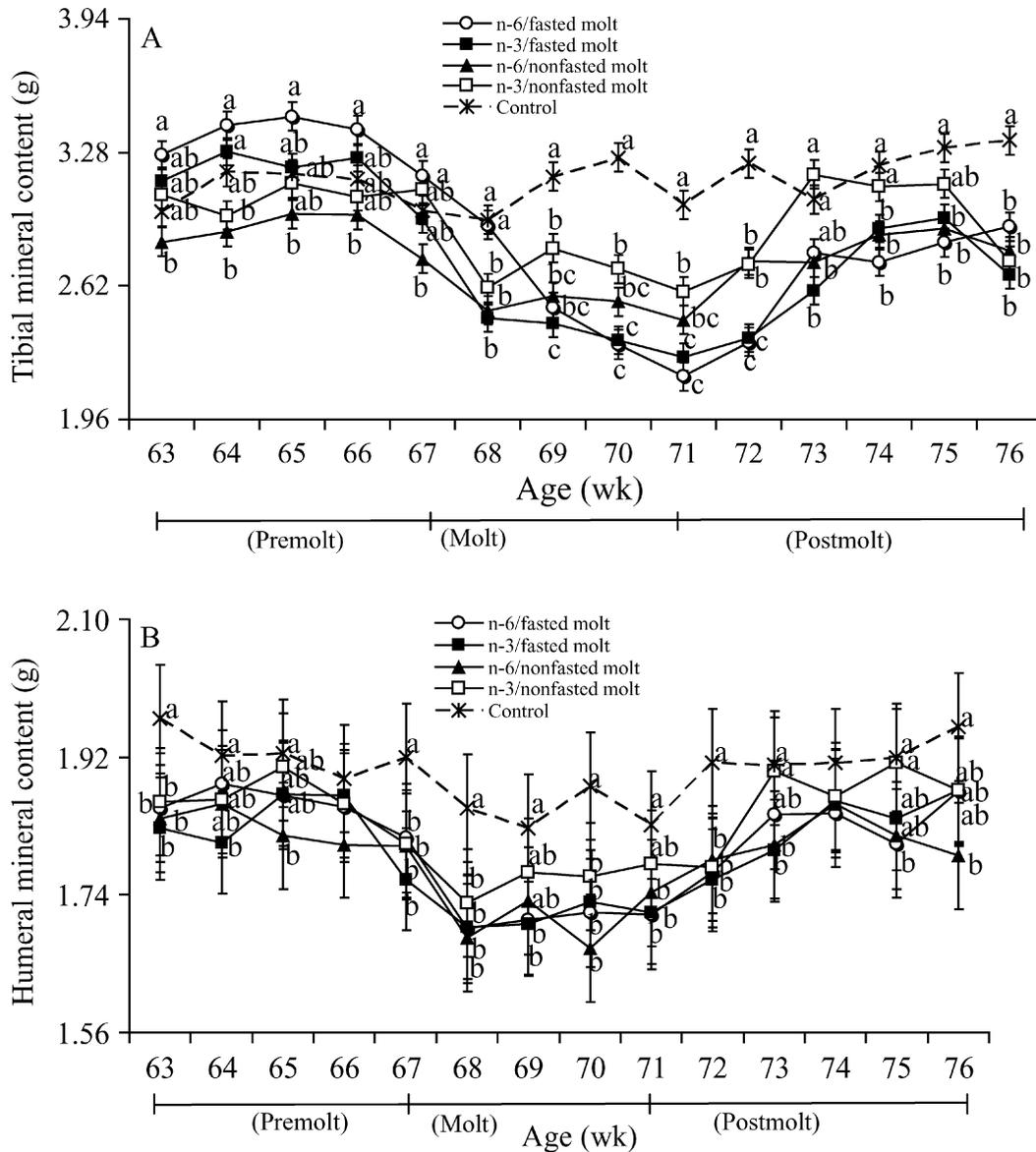


Figure 4. Tibial (A) and humeral (B) mineral content of White Leghorns fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. ^{a-c}Within age and among dietary treatments, least square means \pm SEM with no common letter are significantly different (treatment by bone by age interaction, $P \leq 0.0001$). Each value represents 8 to 9 hens per treatment.

by 74 wk of age (treatment by age interaction, $P \leq 0.0001$). The feeding of n-3 enriched diets before or after the molt did not affect egg production during the experimental period of 62 to 76 wk of age.

Premolt, hens assigned to the nonfasted molt and fed the n-6 diet had lower BW than hens of all other groups from 63 to 66 wk of age (Figure 2). During molt, all hens except controls lost weight. Hens subjected to feed withdrawal for 10 d resulted in a 24% reduction in BW with nonfasted molting hens losing approximately 20% of their BW. Hens consuming the n-3 diets and subjected to a nonfasted molting regimen did not lose as much weight during molt as compared with the other molted hens leading to a faster recovery postmolt. All hens showed increases in BW after molt (treatment by age interaction, $P \leq 0.0001$).

Livability was not affected by the type of molt (fasted vs. nonfasted) or the diet (high vs. low n-3 fatty acids). Mortality rates for the molt and diet treatments were similar to those of controls. Livability decreased as hens aged ($P \leq 0.0001$) with 94% during the premolt period and 91 to 90% during the induced molt and postmolt periods.

During the premolt period of 63 to 67 wk of age, there were small differences among dietary treatments in tibial BMD, but none were significantly different from controls (Figure 3A). Tibial mineral density loss was more pronounced in birds subjected to a fasted molt as compared with birds subjected to a nonfasted molt (70 to 72 wk of age). For example, a 30 and 11% decrease in tibial BMD occurred in fasted and nonfasted hens, respectively, by 71 wk of age as compared with premolt values at 63 wk of age. Feeding diets high in n-3 fatty acids prior to or

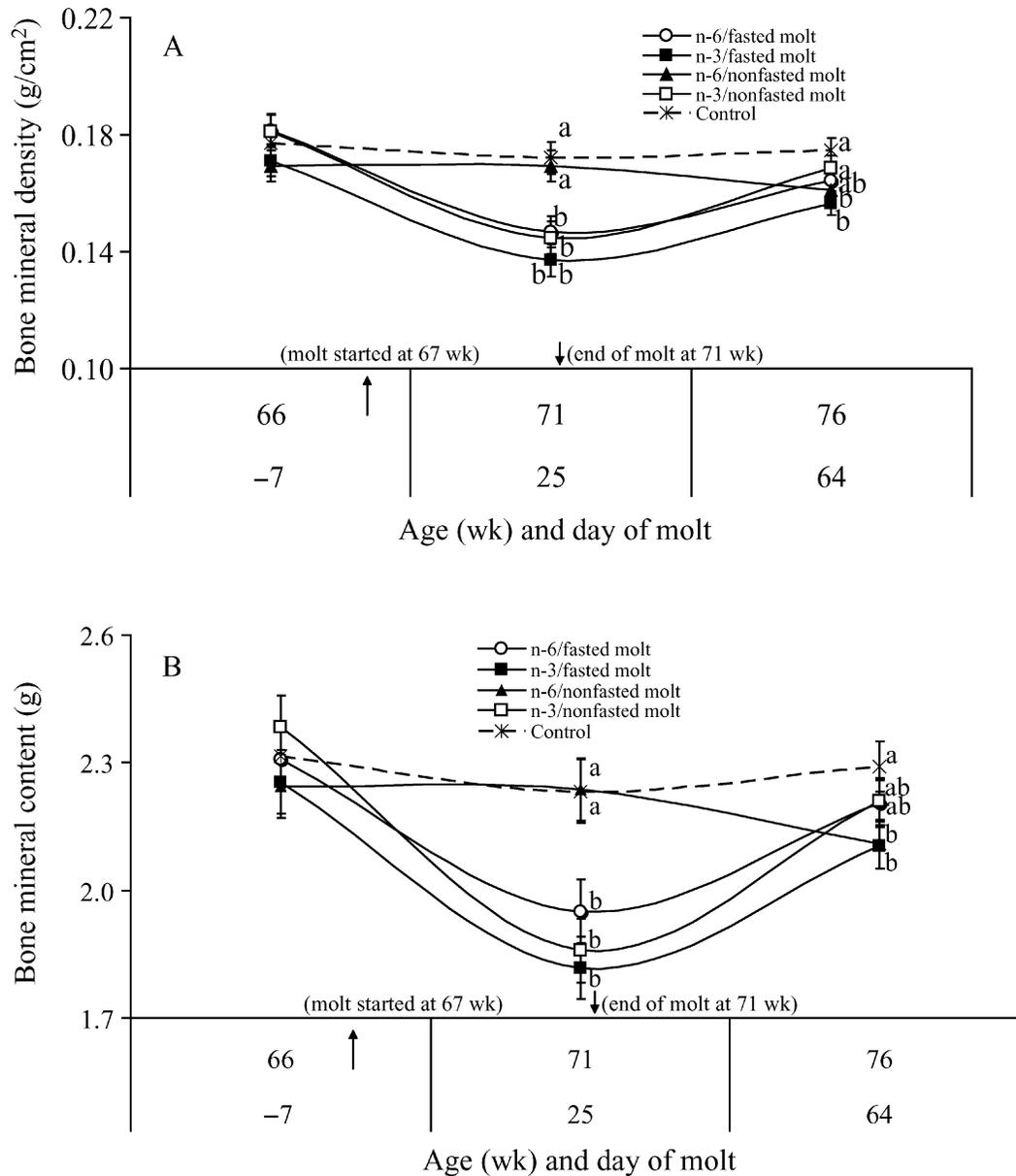


Figure 5. Bone mineral density (A) and bone mineral content (B) of excised bones (humerus and tibia) from White Leghorns fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. ^{a,b}Within age and among dietary treatments, least square means \pm SEM with no common letter are significantly different (treatment by age interaction, $P \leq 0.01$). Each value represents 5 hens per treatment at 66 and 71 wk of age and 8 to 9 hens per treatment at 76 wk of age.

after a molt had no effect in altering the decline in the BMD of the tibia during and after a fasted or nonfasted molt. A brief period of recovery was noted in tibial BMD between 73 and 74 wk of age in nonfasted hens consuming diets high in n-3 fatty acids when compared with controls, but the effect was not sustained. By the end of the study at 76 wk of age, the tibial BMD of hens subjected to either molt (fasted and nonfasted) or feeding (high and low n-3 fatty acids) regimen was significantly lower than those of the controls.

During the premolt period of 63 to 67 wk of age, humeral BMD did not differ among dietary treatments except for hens of the nonfasted group fed n-6 diets whose BMD was lower than controls at 63 wk of age (Figure 3B). With the exception of hens subjected to a nonfasted

molt and fed n-3 diets, molting also caused a decline in BMD of the humerus, although the 6% decrease from premolt values was less pronounced than what occurred with tibial BMD. The humerus of hens fed diets high in n-3 fatty acids and subjected to a nonfasted molt had BMD similar to those of controls throughout the entire length of the study and actually surpassed controls at 73 wk of age. Feeding n-3 fatty acids to hens prior to a fasted molt did not prevent the decline in humeral BMD nor did its feeding postmolt affect its recovery. There were no differences among dietary treatments in the BMD of the humerus by 76 wk of age.

The BMC of the tibia and humerus responded to treatments in a similar manner as BMD (Figure 4, treatment by bone by age interaction, $P \leq 0.0001$). The main differ-

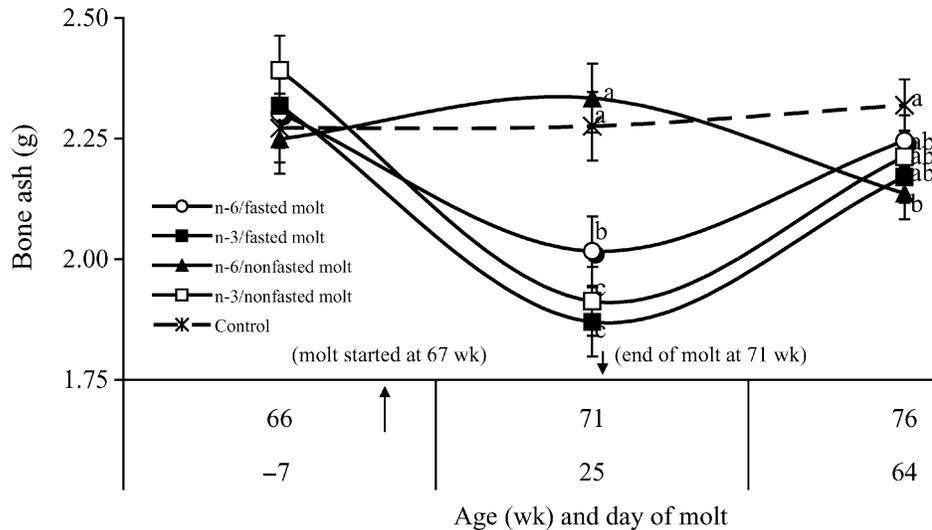


Figure 6. Bone ash weight of excised humerus and tibia of White Leghorns fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. ^{a-c}Within age and among dietary treatments, least square means \pm SEM with no common letter are significantly different (treatment by age interaction, $P \leq 0.0001$). Each value represents the ash weight of the humerus and tibia collected from 5 hens per treatment at 66 and 71 wk of age and 8 to 9 hens per treatment at 76 wk of age.

ence in response between BMD and BMC is that the feeding of n-3 fatty acids did not appear to alleviate the decline in humeral BMC of hens subjected to a nonfasting molting regimen.

Excised bones from hens subjected to molt and n-3 diets (Figure 5) responded similarly to bones that were scanned from live hens. By the end of molt at 71 wk of age, BMD and BMC (Figure 5) and bone ash (Figure 6) were lower in all treatment groups when compared with controls, except for bones of hens subjected to a nonfasted molt and fed n-6 diets. For this latter group, a decrease in BMD and BMC and bone ash due to molt was not observed until 76 wk of age as compared with controls. In addition, the BMD and BMC of excised bones from fasted hens consuming n-3 diets had not recovered by 76 wk of age as compared with controls (treatment by age interaction, $P \leq 0.01$).

An induced molt affected plasma concentrations of IGF-I (treatment by age interaction, $P \leq 0.0001$, Figure 7A). Decreased concentrations of plasma IGF-I were observed in hens subjected to an induced molt by 2 d postmolt when compared with controls. However, from 13 to 43 d postmolt, all molted birds had higher plasma IGF-I than controls. By 64 d postmolt, concentrations of plasma IGF-I in molted hens had returned to control concentrations. By this time, the molted hens had recovered most of their loss in BW due to the molting process. Feeding diets high in n-3 fatty acids 5 wk prior to or after molt or use of nonfasted vs. fasted molt programs did not affect the IGF-I response of molted hens.

Premolt values of circulating concentrations of IGF-I from birds euthanized at 66 wk of age were similar among treatments, but by 25 d into the molt, plasma IGF-I concentrations from birds euthanized at 71 wk of age differed among treatments (treatment by age interaction, $P \leq 0.01$,

Figure 7B). The fasted group consuming premolt diets high in n-6 fatty acids showed significantly higher IGF-I concentrations as compared with the other dietary treatments and premolt values. All other groups subjected to a molt had IGF-I values intermediate between the fasted group consuming premolt n-6 diets and controls at 25 d postmolt. There were no differences in plasma IGF-I concentrations among treatments by 64 d postmolt (or 76 wk of age).

Hepatic IGF-I gene expression patterns were similar among treatments prior to molt (-7 d or 66 wk of age, Figure 8). Near the end of molt (25 d postmolt), hepatic IGF-I gene expression of molted hens was higher (as indicated by the low ratio of IGF-I CT/chicken β actin CT) than controls with the exception of the group of hens subjected to a nonfasted molt consuming premolt diets high in n-6 fatty acids. This latter group at 25 d postmolt had gene expression levels for IGF-I similar to controls. By 64 d postmolt, after the hens had been consuming the breeder diet for 36 d, only the hens of the fasted molt consuming diets high in n-6 fatty acids continued to have higher hepatic IGF-I gene expression than controls (treatment by age interaction, $P \leq 0.01$). The correlation between IGF-I plasma concentrations and hepatic IGF-I mRNA expression was 0.52 ($P \leq 0.0001$).

Egg traits measured during premolt, molt, and postmolt are presented in Figure 9. Prior to the molt from 62 to 66 wk of age, only small subtle differences in shell traits were noted among dietary treatments with values seldom or consistently different from controls. After molt, shell traits improved for all molted hens as compared with nonmolted controls, especially from 74 to 75 wk of age (Figure 9, C and D, for percentage of shell and shell thickness, respectively). Feeding diets high in n-3 fatty acids before or after molt had no effect on egg traits.

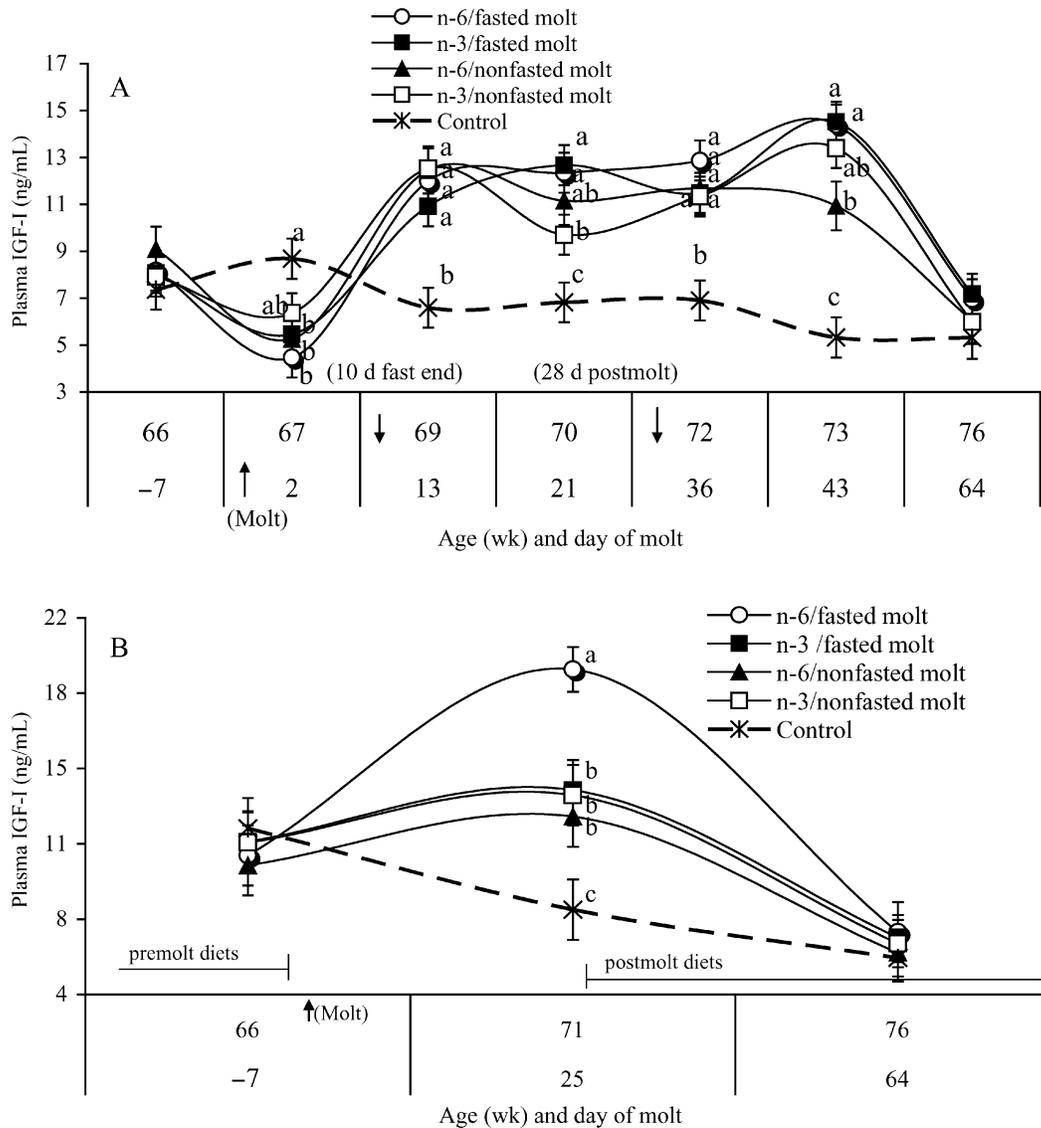


Figure 7. Plasma insulin-like growth factor (IGF-I) levels of live (A) and euthanized (B) White Leghorns fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. ^{a-c}Within age and among treatments, least square means \pm SEM with no common letter are significantly different (treatment by age interaction, $P \leq 0.0001$ and $P \leq 0.01$ for plasma IGF-I from live and euthanized hens, respectively). Each value represents 8 to 9 hens per treatment for panel A and 5 hens per treatment at 66 and 71 wk of age and 8 to 9 hens per treatment at 76 wk of age for panel B.

DISCUSSION

The results of this study indicated that the use of pre- and postmolt diets high in n-3 fatty acids did not alleviate the molt-induced decline and subsequent recovery of hen BMD and BMC. The only exception was the humerus of hens subjected to a nonfasted molt consuming n-3 fatty acids in which the BMD response was similar to controls. However, a similar effect did not occur in the fasted hens consuming diets high in n-3 fatty acids. The lack of response to n-3 fatty acids on bone mineralization in the current study may be related to the length of time that the diets were fed. Feeding fish-oil-based diets rich in n-3 fatty acids for 28 wk increased tibial ash, Ca, and P in 34 wk-old quail as compared with the bones of quail fed diets low in n-3 fatty acids (Liu et al., 2003). Our study was unable to show a similar effect on bone mineralization

perhaps due to the shorter amount of time (two 5-wk periods for a total of 10 wk) that the hens were allowed to consume the n-3 fatty acid diets as compared with the long-term feeding of n-3 fatty acids to quail (Liu et al., 2003). Bone mineralization and metabolism is a cumulative process, and perhaps long-term dietary treatment is needed to amplify the effects of lipids on bone.

An additional reason for the lack of improvement in bone mineralization due to dietary n-3 fatty acids may be related to the older age of the hens when they consumed the diets. Previous research conducted on the skeleton of young birds showed a positive response to the consumption of n-3 fatty acids diets. Bone histomorphometric measurements indicated beneficial effects of dietary n-3 fatty acids enrichment on the skeleton of young birds (e.g., bone formation rate, medullary cavity area, bone volume, and trabecular volume were all higher in

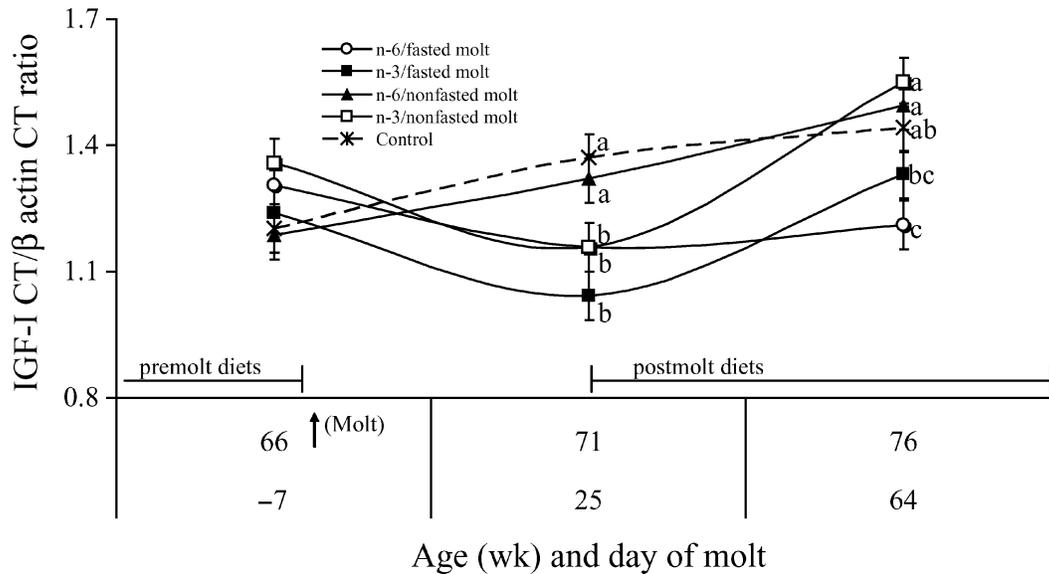


Figure 8. Insulin-like growth factor (IGF-I) mRNA expression from the liver of White Leghorns fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. ^{a-c}Within age and among dietary treatments, least square means \pm SEM with no common letter are significantly different (treatment by age interaction, $P \leq 0.01$). Each value represents 5 hens per treatment at 66 and 71 wk of age and 8 to 9 hens per treatment at 76 wk of age. The expression of IGF-I mRNA is presented as a ratio of IGF-I cycle threshold (CT): β -actin CT. A low ratio for IGF-I CT: β -actin CT indicates high hepatic IGF-I gene expression.

bones from birds fed n-3 fatty acids as compared with bones from birds fed n-6 fatty acids; Watkins et al., 1996, 1997). Diets high in saturated fatty acids caused decreased bone mineralization when consumed by juvenile chickens and turkeys (Atteh et al., 1983; Stevens et al., 1983; Atteh and Leeson, 1984). Juvenile birds consuming n-3 fatty acid diets had lower production of prostaglandin E_2 (PGE_2) in bone, concomitant with a decrease in the formation of bone arachidonic acid, the precursor of PGE_2 (Watkins et al., 1996, 1997; Liu and Denbow, 2001; Liu et al., 2003). Higher concentrations of PGE_2 at specific bone sites act as a potent stimulator of bone resorption (Watkins et al., 2001). Different responses to diet may be associated with age-related metabolic changes taking place in the bone, with bone modeling and growth occurring in the skeleton of young animals as opposed to remodeling or mature bone turnover in adult bone (Wohl et al., 1998).

This study is the first description regarding circulating IGF-I and its mRNA IGF-I gene expression in the liver of hens during molt and leading into the second cycle of egg laying. Findings of the current study indicated that circulating IGF-I in laying hens was markedly sensitive to nutritional status. Molt had a profound effect on circulating concentrations of IGF-I and hepatic IGF-I mRNA expression. Regardless of molting regimen, whether it was induced by fasting or nutrient restriction (wheat-middling-based diet), the IGF-I response was the same with reduced plasma concentrations on d 2 and an overshooting phenomenon by d 13 of molt that was sustained through 43 d postmolt as compared with controls. The decrease in circulating IGF-I during d 2 of molt as compared with the concentration in control hens is similar to the response of juvenile birds subjected to fasting or feed restriction (Kita et al., 1996; Schew et al., 1996; McMurtry

et al., 1998). By 13 d postmolt of the current study, plasma IGF-I concentrations had rebounded in molted hens, exceeding control and premolt concentrations by about 70 and 52%, respectively. At this point in the study, the fasted hens had consumed cracked corn for 3 d, and the nonfasted hens were on d 13 of consuming the nutrient-restricted diet of wheat middlings. This sustained elevation in circulating IGF-I persisted in all molting hens for another 20 d. By 43 d postmolt, molted hens still had elevated concentrations of IGF-I after they had been consuming the nutritionally balanced control laying hen diet for 16 d. The circulating concentrations of IGF-I in molted hens did not return to control concentrations until they had been consuming the laying diet for 37 d (64 d postmolt). It has been previously reported that refeeding juvenile birds after a period of feed restriction elevated IGF-I to levels that exceeded controls (Schew et al., 1996; Kita et al., 2002). Similarly, IGF-I concentration in birds euthanized at 71 wk of age increased during the refeeding phase at the end of molt (Figure 7B). Why hens fed the n-6 diet prior to the fasted molt showed higher concentrations of plasma IGF-I is unknown (Figure 7B) because this did not occur in hens that were repeatedly sampled for IGF-I (Figure 7A) as compared with the other dietary treatments.

In general, molt-induced changes in the expression of hepatic IGF-I (Figure 8) paralleled the changes in circulating concentrations of IGF-I (Figure 7). At 25 d postmolt, when IGF-I concentrations were elevated (Figure 7B), gene expression was also high with the exception of the nonfasted hens consuming the n-6 fatty acid diet (Figure 8). These were the same hens that did not experience a decrease in bone mineralization until 64 d postmolt, which is unlike the other molted hens who showed deteri-

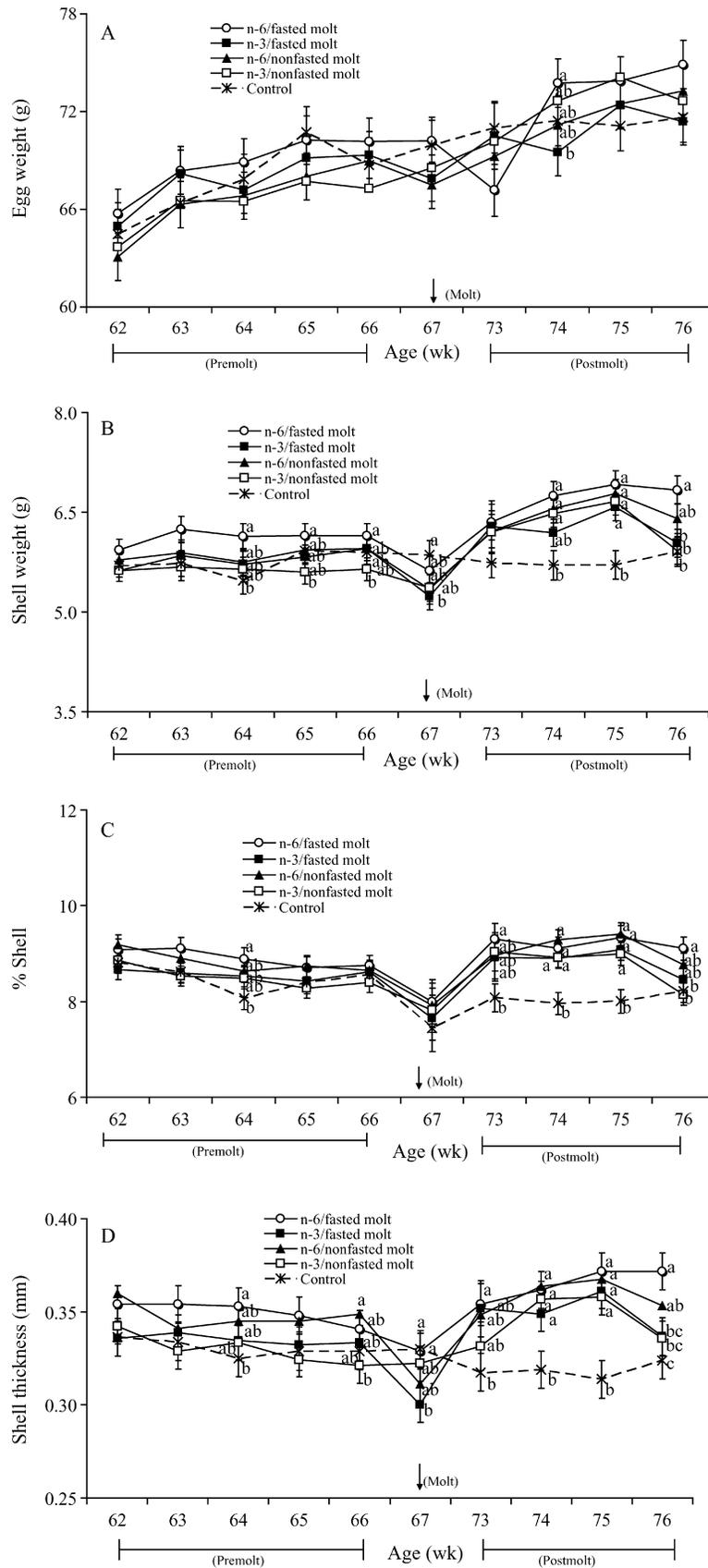


Figure 9. Egg traits measured from White Leghorns fed pre- and postmolt diets high in n-6 or n-3 fatty acids and subjected to a fasted or nonfasted molt as compared with controls. Induced molt was initiated at 67 wk of age. Levels of significance for treatment by age interaction are $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.05$, and $P \leq 0.0001$ for egg weight, shell weight, percentage shell, and shell thickness, respectively. ^{a-c}Within age and among dietary treatments, least square means \pm SEM with no common letter are significantly different. Values represent 2 to 4 eggs collected from each of 8 to 9 hens per treatment.

oration in skeletal integrity as early as 25 d postmolt (Figures 5 and 6). These results on gene expression of hepatic IGF-I are perplexing because one would have expected an increase in nonfasted hens consuming n-6 fatty acids, especially because these hens showed elevated concentrations of plasma IGF-I at 25 d postmolt. The positive correlation between IGF-I mRNA and circulating IGF-I in the current study ($r = 0.52$, $P \leq 0.0001$) was in agreement with Kita et al. (1998) who showed a similar correlation.

Linseed oil clearly increases the n-3 fatty acid content of diets in the form of linolenic acid (Table 2), the precursor of the n-3 fatty acid family (Lopez-Ferrer et al., 2001). In the present study, PUFA manipulation through dietary inclusion of linseed oil or soybean oil did not affect IGF-I concentrations. Li et al. (1999) also reported that type of PUFA did not significantly affect circulating IGF-I concentrations in rats fed n-6 or n-3 fatty acids. On the other hand, Watkins et al. (1997) observed that plasma concentration of IGF-I was highest in chickens (56 d of age) consuming a diet rich in n-3 fatty acids (n-6/n-3 ratio of 1.38) as compared with chickens fed a diet with low n-3 fatty acid content (n-6/n-3 ratio of 7.63).

The alternative molting procedure using a nonfasted molt indicated a less deleterious effect on hen's bone integrity as compared with the conventional molting regimen when a 10-d fast was adopted to induce molt. The amount of decrease in tibial BMD and BMC as a result of a fasted or nonfasted molt was similar to that observed by Mazzuco and Hester (2005a,b). Past research has also pointed out the effect of deprivation of food on hen's bone integrity. Garlich et al. (1984) reported that the femur density of White Leghorns molted at 71 wk of age decreased due to feed removal. Yosefi et al. (2003) also observed that tibial ash decreased during an induced molt (feed withdrawal for 8 or 11 d at 67 wk of age). Likewise, Park et al. (2004) showed that, although there were no differences in tibia breaking strength between molted (66 wk old) and control hens, tibia ash of fasted hens was less than those of nonmolted controls. Recently, Kim et al. (2005) indicated that alfalfa based molt diets (90, 80, or 70% alfalfa in the diet composition) led to reduced bone loss of White Leghorns (60 wk old) as compared with a feed withdrawal regimen during the same 9-d molting period. Similar to our study in which the medullary tibia was more adversely affected by molt than the pneumatic humerus, the medullary bone was the most compromised (Kim et al., 2005), confirming its role in the provision of Ca during long-term Ca deficiency.

Unlike live scanned hens (Figures 3 and 4), the BMD, BMC, and ash of 71-wk-old euthanized hens subjected to a nonfasted molt and fed the n-6 premolt diet did not show a decline in skeletal integrity at 25 d postmolt (Figures 5 and 6). The reason for this lack of a response could be attributed to sample size. Live and euthanized hens were administered treatments simultaneously, and sampling was done at the same age. The skeletal system of the 5 hens that were randomly selected for euthanasia at 71 wk of age may have been able to cope with the nonfast-

ing molting regimen for a longer period of time than the 9 live hens. By 76 wk of age or 64 d postmolt, the bone mineralization response to the nonfasted molt between live and euthanized hens was similar, with these hens showing poorer bone mineralization than the controls.

The cage density used in the present study (1,084 cm² of floor space/hen) is not typical of hen space allocation used by the egg industry (432 cm² of floor space/hen; United Egg Producers, 2000). Under more crowded conditions, a hen's skeletal integrity may be more compromised during molt due to increased competition for feeder space and less exercise. The experimental conditions of the current study showed that feeding hens diets high in n-3 fatty acids prior to and after an induced molt did little to prevent the decline in BMD and BMC during molt and had few benefits during recovery. Supplementation of diets with n-3 fatty acids during pre- and postmolt periods may be more beneficial in improving bone mineralization if fed to hens subjected to more competitive conditions typically used in industry. Future research on molting should consider using industry hen space allocation and should also elucidate the long-term impact of dietary manipulation of n-6/n-3 ratios on skeletal integrity by feeding these diets to birds throughout the life cycle, especially during the pullet phase.

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