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Bone resorption varies as a function of time of day and quantity of dietary long chain polyunsaturated fatty acids☆

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Abstract

It is unclear whether dietary arachidonic acid (AA) and docosahexaenoic acid (DHA) alter the circadian rhythms of bone turnover markers, plasma osteocalcin (OC) and urinary *N*-telopeptide (NTx). We hypothesize that dietary AA and DHA will influence the circadian rhythm of NTx and OC. Piglets were randomized to receive one of four formulas for 15 days: control or control with AA:DHA (0.5:0.1, 1.0:0.2 or 2.0:0.4 g/100 g of fat). Measurements included polyunsaturated fatty acids (PUFA) and plasma OC (sampled at 0900, 1500 and 2100 h on day 15) and urinary NTx:creatinine (collected from 2100 h on day 14–0900 h, 0900–1500 h and 1500–2100 h on day 15). Main effects (litter, diet, time) were identified by mixed model repeated measures ANOVA. In those fed AA and DHA, regression identified relationships among plasma PUFA and NTx. There was a diet (P=.0467) and time (P<.0001) effect on urinary NTx:creatinine, whereby those receiving 1.0:0.2 g/100 g of fat as AA:DHA had the lowest values and values were lowest at 2100 h. Likewise, diet (P=.0001) and time (P<.0001) affected plasma AA and DHA; higher dietary AA and DHA elevated values and time reduced values. There was a diet by time interaction on eicosapentaenoic acid and DHA proportions, suggesting dietary AA and DHA altered their circadian rhythm. In regression, plasma AA and DHA were not associated with urinary NTx:creatinine. Dietary AA and DHA at amounts similar to that found in breast milk reduce bone resorption, but do not alter its circadian rhythm.

Keywords: Plasma polyunsaturated fatty acids; Piglets; Urinary *N*-telopeptide; Osteocalcin; Circadian rhythm

1. Introduction

Arachidonic acid (AA) and docosahexaenoic acid (DHA) are not considered essential fatty acids but may be beneficial for a variety of health outcomes when included in the diet. Over the past decade, our research group has investigated the effects of these long-chain polyunsaturated fatty acids (LCPUFA) supplemented in formula on bone mass in piglets and observed higher values of bone mineral content or density with dietary supplementation of AA:DHA in amounts ranging from 0.5:0.1 to 0.75:0.1 g/100 g of fat [1–4]. It has been proposed that these changes in bone mass result from alterations in bone metabolism as indicated by bone turnover markers [1–4].

There are several biomarkers available and validated to measure bone turnover in the pig, including osteocalcin (OC) and *N*-telopeptide of type 1 collagen cross-links (NTx) [5,6]. OC is a noncollagenous protein secreted by osteoblasts and is accepted as a bone turnover markers [7]. OC is also released into circulation when bone is broken down so the amount found in the plasma can represent both bone formation and resorption [7]. Thus, OC is an indicator of bone remodelling during growth or turnover during maintenance of bone mass [7]. NTx refers to the amino-terminal telopeptide that is found in Type I collagen and is released during bone resorption [7]. Measurement of urinary NTx is a sensitive and specific marker of bone resorption [8].

A circadian rhythm is a pattern based on a 24-h cycle. OC concentrations follow a circadian rhythm characterized by a decline during the morning (0300 h) to a noontime low

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followed by a gradual rise that peaks after midnight in humans [9] and in piglets [10]. NTx is highest in the early morning (peak excretion between 0300 and 0700 h) with a decline over the day to the lowest point in the afternoon (between 1500 and 1900 h) [11]. Other researchers have only found a circadian rhythm for NTx when corrected to creatinine [12–14]. Consequently, time of sampling is a major consideration when measuring these bone turnover markers.

In many studies conducted within our research group, elevations in bone mass in response to dietary AA:DHA (0.5:0.1–0.75:0.1) are not clearly explained by bone turnover markers [1,3,4]. In addition, 0.8:0.1 [15] and 1.0:0.2 g/100 g of fat [4] as AA:DHA suppresses bone resorption [4,15]. In each of these piglet studies, the bone turnover markers were measured at one time of day — typically between 0800 and 1000 h and in the nonfed state. In one research study using piglets, DHA status was inversely related to bone resorption [16], and in another study, liver AA was positively associated with bone resorption [3].

To our knowledge, there is limited research examining how plasma polyunsaturated fatty acids (PUFA) changes over the day in response to feeding (vs. the nonfed state). In addition, it is unknown whether the circadian rhythms of OC and NTx are altered by various amounts of dietary LCPUFA. The circadian rhythm (also referred to as circadian variation) of bone resorption over the day is associated with food intake and is diminished during fasting [17]. The response of bone turnover markers to fat, glucose and protein are similar [17]. In the fed state, urinary NTx and plasma OC are lower than during the nonfed state [18].

In order to establish if consuming dietary LCPUFA alters bone metabolism throughout the day, samples collected at multiple time points over the day and from both nonfed and fed states need be examined. Hence, the objectives of this study were to determine: (1) the effects of varying amounts of dietary AA:DHA at a constant ratio (5:1) on the circadian rhythms of biomarkers of bone metabolism; (2) whether time of sampling affects the identification of differences between dietary groups and (3) the associations between plasma PUFA proportions and biomarkers of bone metabolism. These objectives were a part of and accomplished within a large piglet study that was also designed to study the effects of dietary AA and DHA on bone mass [4].

2. Methods and materials

2.1. Animals and diet

Male piglets (n=32), born at The Glenlea Swine Research Unit, University of Manitoba, were transported to the housing facility at the main campus of the University. Piglets were selected with a birth weight of ≥1.4 kg, from eight litters consisting of 10–12 piglets each with at least four males. Piglets were adapted over 2 days to housing and diet as per Mollard et al. [4]. Throughout the study, and based on 0900-h weight, the piglets were offered 350 ml/kg of liquid formula per day. This amount was divided into three equal portions provided at 0900, 1500 and 2100 h for 15 days (from 5 to 21 days of life) as per Weiler and Fitzpatrick-Wong [19]. Piglets consumed formula within 2 h following feeding. Animal care and procedures were examined by the University of Manitoba Committee on Animal Use and were within the guidelines of the Canadian Council on Animal Care [20].

Piglets were randomized to receive one of four dietary treatments as per Mollard et al [4]. Treatments were control formula or control supplemented with 0.5:0.1 g AA:DHA, 1:0.2 g AA:DHA or 2:0.4 g AA:DHA per 100 g of fat. Fatty acid composition of the diets are presented in Table 1. Supplementation was held constant at an AA:DHA ratio of 5:1 and a total n-6:n-3 ratio of 9:1. The AA was provided in the form of RBD-ARASCO (40.6 g/100 g of fatty acids as AA), and DHA, in the form of RDB-DHASCO (40.0 g/100 g of fatty acids as DHA). AA was derived from a common soil fungi, and DHA was derived from a marine microalgae (Martek Biosciences). These sources were chosen because they were previously used in our laboratory and because they are used in the manufacturing of many infant formula products. Formulas were isocaloric with equal amounts of fat. The formula was based on nutritional requirements for healthy growing piglets between 3 and 10 kg as set by The National Research Council [21] and currently proven to support growth [19]. The dietary composition of the control formula and dietary composition of PUFA in each

Table 1
Dietary PUFA (g/100 g of fat) measured in formula fed to piglets for 15 days[a]

	Control diet (g/100 g fatty acids)	AA:DHA diets (g/100 g of fatty acids)		
		0.5:0.1	1.0:0.2	2.0:0.4
C12:0	10.78	10.64	10.50	10.24
C14:0	4.34	4.33	4.32	4.30
C16:0	8.04	8.12	8.19	8.34
C18:0	3.11	3.20	3.27	3.43
C18:1 (n-9)	38.33	37.97	37.63	36.96
C18:1 (n-7)	1.79	1.77	1.75	1.71
C18:2 (n-6), LA	27.12	26.82	26.53	25.97
C18:3 (n-3), ALA	3.11	3.07	3.03	2.95
C20:0	0.31	0.32	0.33	0.34
C20:1	0.15	0.15	0.15	0.15
C20:4 (n-6), AA	0	0.49	0.96	1.88
C20:5 (n-3), EPA	0	0	0	0
C22:0	0.25	0.26	0.28	0.31
C22:5 (n-3)	0	0	0	0
C22:6 (n-3), DHA	0	0.10	0.19	0.38
20:4 (n-6)/22:5 (n-3)	0	5.05	5.05	5.05
AA:DHA				

[a] Data expressed as g/100 g of fatty acids. Fatty acids with zero for specific fatty acids indicate not detected.

Table 2
Composition Control of diet fed to piglets for 15 days

Diet Composition of Control Formula	
	U/L
Oil blend	
Soybean [a] (g)	23
High oleic safflower [b] (g)	23
Coconut [c] (g)	14
Dry mix	
Skim milk powder [d] (g)	110
Whey powder [e] (g)	35
Vitamin [f] and mineral [g] mix	
DL-α-tocophyeryl acetate (mg)	5
Cholecalciferol (mg)	0.11
All transretinol acetate (mg)	1
Thiamine (mg)	30
Riboflavin (mg)	60
Niacin (mg)	440
Pantothenic acid (mg)	284
Pyridoxine (mg)	36
Folacin (mg)	20
Vitamin B_{12} (mg)	0.4
D-Biotin (mg)	2
$CaCO_3$ (g)	1
Choline chloride, g	42.4
$MnSO_4$ (mg)	40
Ferrous sulfate (mg)	167

[a] Vita Health, Winnipeg, Manitoba.
[b] Bestfoods Food Service, Division of Bestfoods, Toronto, Canada.
[c] Harlan Teklad, Madison, WI, USA.
[d] Parmalat Canada Production and Distribution, Winnipeg Canada.
[e] Lactose reduced whey powder (as Avaonlac 134, Glanbia Ingredients, Monroe, WI, USA).
[f] Harlan Teklad for all listed except all transretinol acetate from Sigma–Aldrich.
[g] Sigma–Aldrich Canada.

treatment formula are presented in Table 2. Formula contained 1050 kcal/L, 60 g/L fat, 50 g/L protein, 2.1 g/L calcium and 1.4 g/L phosphorous. Piglets were allowed approximately 1 h of exercise before each feed.

2.2. Sample collection

Urine was collected at intervals between feedings over a 24 h period using metabolic cages starting on day 14 and ending on day 15 of study. Urine was collected from 2100–0900, 0900 h–1500 h and 1500–2100 h. Samples were stored at −20°C until analysis of NTx and creatinine. On Day 15, before piglets were fed (0900, 1500 and 2100 h), blood (5 ml) was sampled using the internal jugular blind stab technique. Anticoagulated blood (heparin) was separated into plasma and erythrocyte fractions. Plasma and erythrocytes were obtained by centrifugation at 2000*g* for 10 min at 4°C, and plasma was stored at −80°C until analysis of OC and polyunsaturated fatty acids. The next morning, piglets were anaesthetized by ip injection of sodium pentobarbital (30 mg/kg, 65 mg/ml concentration) or isofluorane gas followed by sodium pentobarbital overdose (180 mg/kg).

2.3. Fatty acid analysis

Total lipids were extracted from plasma according to an adapted method of Folch et al [22] as previously described [3]. Plasma was extracted in chloroform:methanol 2:1 containing 0.01% butylhydroxytoluene (BHT). An internal standard, heptadecanoic acid (C17:0) was added to each sample. Crude lipid extracts were transmethylated in 1.2 ml of methanolic HCl (3N, Supleco, Bellefonte, PA, USA) at 80°C for one h. Fatty acid methyl esters were separated by gas-liquid chromatograph (Varian Star 3400, Varian, Mississauga, ON, Canada), equipped with a 30 m capillary column (J&W Scientific, Folsom, CA, USA), a flame ionization detector and using hydrogen as the carrier gas. The column is made of fused silica coated with DB225 (25% cyanopylphenyl) and run at 180–220°C with a between-sample temperature of 240°C to clean the column. The detector oven is set at 300°C and produces a sensitivity of 1–5 ng/ml. Fatty acid methyl esters (C12–24) were identified by comparison with retention times of Supelco 37 component FAME mix (Supelco) and expressed as proportion of total fat (g/100 g of fat).

2.4. Bone turnover markers

Plasma OC was measured in duplicate using an I^{125} radioimmunoassay (DiaSorin, Stillwater, MN, USA). This assay is based on rabbit antiserum to bovine OC that has been proven to be a valid approach (porcine OC standard) for measuring porcine OC [5]. NTx in urine was measured in duplicate by a competitive inhibition enzyme-linked immunosorbent assay (Osteomark, Ostex, Seattle, WA, USA). Although a human assay, it has been validated for the use in samples from growing piglets by cross-calibrating to a human standard [6]. Urinary NTx sample values were corrected to creatinine as determined by the Jaffe method (procedure no. 555; Sigma–Aldrich Canada, Oakville, Canada) to account for urinary dilution. For NTx kit, the CV % was <20%, and for OC kit, it was <15%. Creatinine in urine was measured colorimetrically (Sigma, St. Louis, MO, USA). The CV % for triplicate analysis of creatinine in all samples was <10%.

2.5. Statistical analysis

Main effects were detected using mixed-model, repeated-measures analysis of variance with diet and time as fixed effects; the random effect was litter. Residual plots were done to determine outliers. Differences among times and dietary groups were identified with estimate statements (*t* tests with Bonferroni correction). Relationships between plasma PUFA and bone resorption were detected using linear regression analysis while also accounting for the effect of time of day. Relationships between PUFA in plasma, selected to reflect whole body PUFA status, and bone resorption were conducted with the control group excluded since it was not a component of the dose–response relationship. A *P* value of less than .05 was accepted as

significant. Data is expressed as mean±S.E.M. All data were analyzed using SAS statistical software (SAS software release 8.2; SAS Institute, Cary, NC, USA).

3. Results

To examine the influence of dietary LCPUFA on the circadian rhythm of bone turnover markers, urinary NTx:creatinine (a marker of osteoclast activity) and plasma OC (a marker of osteoblast activity) were measured. There was a significant effect of diet on NTx (P=.0467) (Fig. 1A). The 1.0:0.2 g AA:DHA group had lower NTx:creatinine compared to all other groups. There was also a significant effect of time on NTx values (P<.0001), with lower NTx values at 2100 h compared to 0900 and 1500 h (Fig. 1A). There was no time by diet interaction on NTx:creatinine (P=.4093). There were no significant main effects of diet (P=.4445), time (P=.0808) or time by diet interaction (P=.1605) on OC values (Fig. 1B).

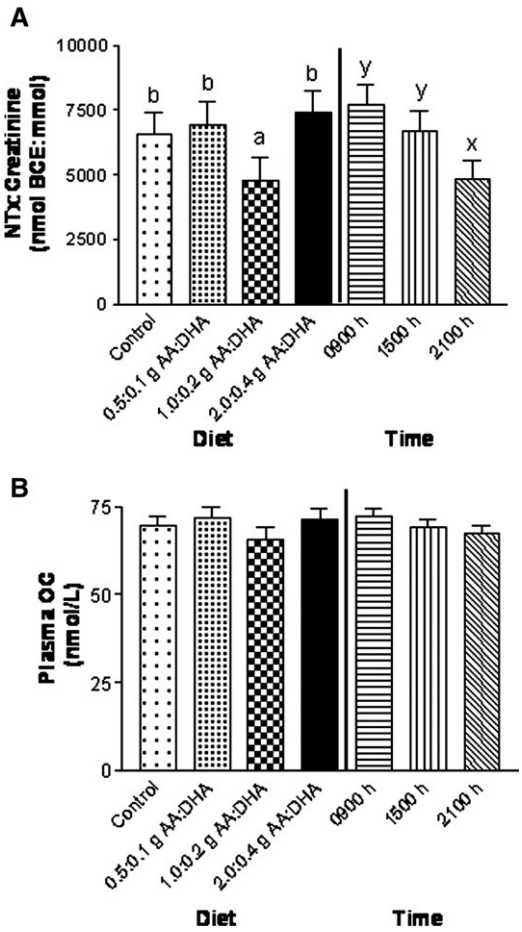

Fig. 1. The effect of dietary AA:DHA and time on urinary NTx concentration (A) and plasma OC concentration (B) in piglets fed formula for 15 days. Data are mean±S.E.M. (n=32). Bars with different subscripts indicate significant differences where $a<b$ for diet and $x<y$ (P<.05). BCE, bone collagen equivalents.

The analysis of diet on plasma PUFA and LCPUFA proportions (Table 3) revealed a significant main effect of diet on the proportion of AA in plasma. The control group had the lowest proportion of AA in the plasma and as the dietary AA increased the proportion of AA significantly increased. There was a significant effect of diet on linoleic acid (LA), with proportions decreasing as AA and DHA increased in the diet. There was no dietary effect on the proportion of alpha-linolenic acid (ALA). There was a significant main effect of diet on the proportion of total n-6 PUFA; however, post hoc analysis did not identify differences among groups. There was also a significant main effect of diet on total n-3 PUFA; proportions decreased with supplementation of AA and DHA.

The effect of time on plasma PUFA and LCPUFA proportions are presented in Table 1. There was a significant effect of time on the proportion of plasma AA; it was significantly higher at 0900 h (12 h postprandial) compared to 1500 (6 h postprandial) and 2100 h (6 h postprandial). There was a significant effect of time on plasma PUFA proportions; LA and ALA increased with time, with proportions lower at 0900 h compared to 1500 and 2100 h. Total n-6 PUFA and total n-3 PUFA significantly decreased with time. Total n-6 was significantly higher at 0900 h compared to 1500 and 2100 h, while total n-3 was significantly higher at 0900 compared to 1500 and 2100 h, but also significantly higher at 1500 h compared to 2100 h.

There was a main effect of diet on plasma DHA, but not eicosapentaenoic acid (EPA) proportions. However, both EPA and DHA decreased over time. A diet-by-time interaction was found for EPA (P=.0243) (Fig. 2A) and DHA (P=.0187) (Fig. 2B). Post hoc analysis identified that plasma EPA and DHA responded differently to dietary AA and DHA than control formula between 0900 h and 1500 h but were not different between 1500 and 2100 h. Plasma EPA (P=.0042) and DHA (P=.0055) decreased from 0900 to 1500 h in the 0.5:0.1 g AA:DHA, 1.0:0.2 g AA:DHA and 2.0:0.4 g AA:DHA groups, but did not change in the control group. The 1.0:0.2 g AA:DHA diet had a different effect on DHA proportions compared to the 0.5:0.1 g AA:DHA diet from 0900 to 2100 h (P=.0370). These interactions indicate that the response of plasma EPA and DHA proportions to dietary AA and DHA is different in a nonfed vs. fed state. It also indicates that the presence of AA and DHA within the diet alters the how EPA and DHA concentrations change in the plasma over the day when compared to a diet with no AA or DHA.

Regression analysis was conducted to determine whether changes in urinary NTx:creatinine related to changes in specific plasma n-6 and n-3 PUFA and LCPUFA when adjusted for time of day (Table 4). Only plasma LA was negatively associated with urinary NTx:creatinine: y=17,302 (intercept coefficient)−177.88 (time coefficient)−71.14 (LA coefficient). Urinary NTx:creatinine was not related to ALA, AA, EPA, DHA, total n-6 PUFA, total n-3 PUFA or total n-6:

Table 3
The effect of dietary AA:DHA and time on plasma PUFA proportions (g/100 g) in piglets fed formula for 15 days vs. control [a]

	Diet				Time (h)			*P* value		
	Control	AA:DHA (g/100 g of fat)			0900	1500	2100	Diet	Time	Time x Diet
		0.5:0.1	1.0:0.2	2.0:0.4						
LA	33.37^{d}±0.37	31.73^{c}±0.38	30.73^{b}±30.73	28.10^{a}±0.37	30.15^{x}±0.40	30.98^{y}±0.30	31.42^{y}±0.26	<.0001	.0011	.2652
ALA	1.47±0.05	1.44±0.05	1.38±0.05	1.40±0.05	1.16^{x}±0.03	1.53^{y}±0.04	1.51^{y}±0.04	.3671	<.0001	.7513
AA	6.05^{a}±0.47	7.65^{b}±0.49	9.12^{c}±0.49	10.99^{d}±0.48	9.80^{y}±0.42	7.86^{x}±0.42	7.70^{x}±0.43	<.0001	<.0001	.2049
EPA	0.15±0.02	0.17±0.02	0.14±0.02	0.15±0.02	0.20^{y}±0.01	0.14^{x}±0.01	0.12^{x}±0.01	<.0001	<.0001	.0243
DHA	1.29^{a}±0.14	1.61^{b}±0.14	1.73^{b}±0.14	1.89^{b}±0.14	2.03^{y}±0.13	1.46^{x}±0.13	1.40^{x}±0.13	.1520	<.0001	.0187
Total n-6 PUFA	39.58±0.40	39.77±0.42	40.49±0.42	40.43±0.41	41.28^{y}±0.56	40.08^{x}±0.54	39.45^{x}±0.30	.0492	<.0001	.8182
Total n-3 PUFA	3.37^{a}±0.13	3.67^{b}±0.13	3.65^{b}±0.13	3.81^{c}±0.13	3.91^{y}±0.13	3.55^{x}±0.13	3.40^{x}±0.13	<.0001	<.0001	.2718

[a] Data expressed as means±S.E.M. (n=32). Differences among groups are identified by different subscripts: for dietary differences, a<b<c<d; for time differences: x<y<z.

n-3 ratio. Although, the n-6:n-3 ratio approached significance (P=.0579) and may be a reflection of the LA and urinary NTx:creatinine relationship.

4. Discussion

Previously reported in these piglets, NTx in overnight (12 h) samples was significantly lower in the group fed 1.0:0.2 g/100 g of fat as AA:DHA compared to the group fed 2.0:0.4 g/100 g of fat as AA:DHA, but neither had values different from control or 0.5:0.1 g/100 g as AA:DHA (P=.039) [4]. A similar observation was made herein when urine was sampled over the day, except that 1.0:0.2 g/100 g of fat as AA:DHA resulted in significantly lower

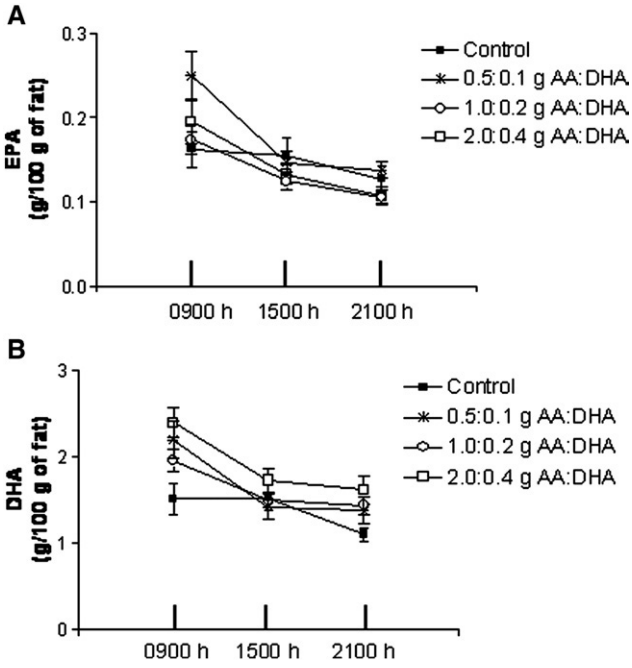

Fig. 2. The interaction effect of dietary AA:DHA and time on EPA (A) and DHA (B) proportions in piglets fed formula for 15 days. Plasma EPA (P=.0042) and DHA (P=.0055) decreased from 0900 to 1500 h in the 0.5:0.1 g AA:DHA, 1.0:0.2 g AA:DHA and 2.0:0.4 g AA:DHA groups, but did not change in the control group. The 1.0:0.2 g AA:DHA diet had a different effect on DHA proportions compared to the 0.5:0.1 g AA:DHA diet from 0900 to 2100 h (P=.0370). Data are mean±S.E.M. (n=32).

Table 4
Relationships among urinary *N*-telopeptide:creatinine with time of days (h) and selected plasma n-6 and n-3 polyunsaturated fatty acids (g/100 g of fat) in piglets fed formula with AA and DHA [a, b]

Predictor	R^2	Coefficient	*P* value
Time alone	0.1273		.0033
Intercept		10,027	<.0001
Time		−253.53	.0033
Time and LA	0.1857		.0015
Intercept		20,814	.0002
Time		−232.41	.0058
LA		−368.87	.0375
Time and ALA	0.1374		.0095
Intercept		11,498	<.0001
Time		−203.18	.0503
ALA		−1586.39	.3946
Time and AA	0.1479		.0065
Intercept		7067.05	.0120
Time		−204.42	.0294
AA		240.57	.2219
Time and EPA	0.1346		.0105
Intercept		8429.02	.0016
Time		−208.41	.0490
EPA		6056.44	.4692
Time and DHA	0.1704		.0028
Intercept		5493.77	.0557
Time		−151.88	.1301
DHA		1723.58	.0753
Time and total n-6 PUFA	0.1393		.0089
Intercept		17,140	.0298
Time		−282.55	.0022
Total n-6 PUFA		−165.83	.3527
Time and total n-3 PUFA	0.1441		.0074
Intercept		5284.10	.2411
Time		−199.89	.0411
Total n-3		1053.93	.2710
Time and total n-6:n-3 PUFA	0.1761		.0022
Intercept		17,302	<.0001
Time		−177.88	.0530
Total n-6:n-3 PUFA		−771.14	.0579

[a] n=24.
[b] Equation: y=intercept coefficient−time coefficient−PUFA coefficient.

values compared to all groups. Other dietary PUFA such as gamma-linolenic acid (GLA) plus EPA and DHA in a 3:1 ratio [23] or fish oil (EPA and DHA) [24] have been shown to reduce bone resorption as indicated by urinary pyridinolines measured over a 4–5-day period. Overall, it seems that a sample collected over a 24-h period is better than an overnight (12 h) sample collection to accurately assess bone resorption in response to PUFA.

In contrast, OC values at 0900 h were not affected by dietary AA and DHA over the range of intakes studied [4], and this finding is the same when plasma is sampled at different time points across the day. Previously, plasma OC values were lower in response to AA:DHA supplementation at 0.5:0.1 g/100 g of fat compared to a similar control formula [2]. Another study did not find differences in plasma OC values in response to dietary AA:DHA at 0.5:0.1 g/100 g of fat [1]. However, changes in bone formation have been seen in growing rats fed EPA and DHA at high amounts using different biomarkers [25]. Watkins et al [25] found that a diet high in n-3 LCPUFA (EPA and DHA) increased serum bone-specific alkaline phosphatase compared to those fed a diet high in n-6 PUFA (LA) but only observed a downward trend when OC was used to assess bone formation. It is possible that OC is not sensitive enough to detect changes in bone formation due to PUFA. Thus, other markers such as bone-specific alkaline phosphatase should be used in combination with OC to assess the response of bone to dietary PUFA.

Bone turnover markers follow a circadian rhythm. The majority of variation seen in biomarkers of resorption over the day and night are not purely circadian but rather induced by food intake [15]. The circadian variation in bone resorption is diminished during fasting, and food intake reduces bone resorption [15,16] and formation [16] acutely. This is the first study to investigate the effects of dietary LCPUFA supplementation on the circadian variation of bone turnover markers. While the pattern of circadian variation was unaltered for both NTx and OC, the amplitude of NTx was reduced in the piglets fed 1.0:0.2 g/100 g of fat as AA:DHA.

In piglets, the circadian rhythm of NTx adjusted to creatinine (highest in the morning with a significant drop over the day) is similar to that in humans [10–12]. Plasma OC did not significantly drop over the day. This could be due to the fact that blood was sampled over a 12-h period rather than 24 h. If blood had been sampled at 0300 h, this may have revealed that the OC follows a similar rhythm, as reported in humans. Previously, in piglets, researchers found that plasma OC was significantly lower at 0900 and 1700 h, compared to 0100 h [8].

Tissue proportions of PUFA are influenced by and reflect the amount of PUFA in the diet, including plasma [3]. Over the day, LA and ALA proportions increased, and the LCPUFA decreased, suggesting that plasma PUFA and LCPUFA have their own circadian rhythms. These changes could be in response to feeding since they were measured in the nonfed (0900 h) followed by the fed (1500 h) state. Our fed sample was taken approximately 5 h after feeding; it would have been additive to measure plasma PUFA and LCPUFA levels within 1 h postprandial. In addition, it is possible that PUFA and LCPUFA plasma levels follow similar circadian rhythms when fasted over the day. Repeated sampling over the day while in the fasted and fed state, in addition to 1 h postprandial (after each feed) would help clarify PUFA and LCPUFA response to feeding.

In breast milk, EPA and DHA peak at 24 h after a meal rich in EPA and DHA, whereas other fatty acids (linoleic acid and linolenic acid) peaked between 10 and 14 h in meals rich in the respective PUFA [26]. It is possible that changes in EPA, DHA and AA in plasma in response to LCPUFA feeding are delayed in comparison to the shorter chain PUFA, and that is why we see different plasma diurnal variations over the day. In addition, highly unsaturated fatty acids are transferred from the intestine to plasma and from plasma to liver and skeletal muscle at a higher rate than those that are more saturated [27]. While muscle AA and DHA were not measured in the present study, previously, we reported that liver and adipose AA and DHA increased with increasing dietary amounts [4] that may account for the reduced postprandial DHA in the supplemented groups.

Plasma LA was negatively associated with urinary NTx, even after accounting for time. This is surprising, since a reduction of dietary LA by inclusion of GLA with EPA and DHA or EPA and DHA alone decreases bone resorption [23,24]. Plasma AA proportions were not related to urinary NTx after adjusting for time. A positive relationship was found in piglets between liver AA and urinary NTx when AA:DHA was supplemented as 0.30, 0.45, 0.60 or 0.75:0.1 g/100 g of fat [3]. In rats, a significant negative correlation was found between bone formation rate and the ratio of AA:EPA in bone [25]. In the present study, regression analysis accounting for time found no relationship between plasma EPA, AA:EPA or DHA proportions and urinary NTx. In piglets fed a PUFA diet with no DHA or AA, higher plasma DHA concentrations were associated with reduced bone resorption [14].

In conclusion, dietary AA:DHA at 1.0:0.2 g/100 g of fat resulted in lower bone resorption compared to all other groups; however, there was no effect of dietary LCPUFA on the circadian rhythms of bone turnover markers. Also, whether different types of LCPUFA or changes in the total n-6:n-3 ratio lead to changes in the circadian rhythm of bone turnover markers requires further investigation. Here, plasma was sampled before feeding and urine was collected for a period of 6 h following feeding. *It would be interesting to determine how bone biomarkers and plasma LCPUFA respond to dietary LCPUFA sooner after feeding (within 1 h) and at multiple time points (every hour). This would help to clarify whether there is a more immediate response and if the effects of LCPUFA were missed because of measuring after over a longer period of time.* Understanding this physiology further is important in the design of human studies. Bone loss as a result of aging and estrogen

deficiency (menopause) occurs due to an increase in resorption over formation [28]. Animal studies demonstrate the potential for LCPUFA to result in acute changes in bone turnover, and a human trial demonstrates the potential for enhancing bone mass later in life [29].

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