Dose response of bone mass to dietary arachidonic acid in piglets fed cow milk–based formula

Janice L Blanaru, June R Kohut, Shirley C Fitzpatrick-Wong, and Hope A Weiler

ABSTRACT
Background: The addition of arachidonic acid (AA) and docosahexaenoic acid (DHA) to infant formula was recently approved in North America. In piglets, dietary AA is linked to elevations in bone mass.

Objective: The objective was to investigate the effects of varied amounts of dietary AA on bone modeling and bone mass with the use of the piglet model for infant nutrition.

Design: Male piglets (n = 32) were randomly assigned to receive 1 of 4 formulas supplemented with AA (0.30%, 0.45%, 0.60%, or 0.75% of fat) plus DHA (0.1% of fat) from days 5 to 20 of life. Measurements included biomarkers of bone modeling, fatty acid status, and whole-body and femur bone mineral content; bone area was measured by dual-energy X-ray absorptiometry. Differences among groups were detected with two-factor analysis of variance. Regression analyses were used to determine factors responsible for bone mineral content after dietary AA was accounted for.

Results: Proportions of AA in plasma, liver, and adipose were modified by the dietary treatments, but bone modeling was not affected. Liver AA was positively related to plasma insulin-like growth factor 1 and calcitriol and urinary resorption. Body bone mineral content was elevated in the piglets fed 0.60% AA and 0.75% AA and was best predicted by dietary AA and bone resorption.

Conclusions: This study confirms that dietary AA alters bone mass and clarifies the best amount of AA to add to the diet of pigs born at term. Because the amount of dietary DHA was held constant, whether other amounts of DHA are related to bone mass requires investigation. Am J Clin Nutr 2004;79:139–47.

KEY WORDS Arachidonic acid, bone, growth, piglet

INTRODUCTION
Infant formula products are designed to mimic the qualities of human milk and are formulated based on the most current state of science. The quality of fat in infant formula was recently revised in North America. For decades, infant formulas have contained the essential n-6 and n-3 polyunsaturated fatty acids (PUFAs) linoleic acid (18:2n-6) and linolenic acid (18:3n-3) in a ratio of 9:1. Some commercially available formulas now contain the long-chain PUFAs arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3). Similac Advance (AA:DHA = 0.4%:0.15%, a 2:7:1 ratio; Ross Products Division of Abbott Laboratories, Columbus, OH) and Enfamil Lipil (AA:DHA = 0.64%:0.32%, a 2:1 ratio; Mead Johnson Nutritional, Evansville, IN) are sold in the United States, and Enfalac A+ (AA:DHA = 0.64%:0.32%, a 2:1 ratio; Mead Johnson Nutritional) is sold in Canada. The evidence supporting these ratios is based on decades of research regarding safety and on the relation between infant fatty acid status and visual and cognitive development (1–4).

The ratio of dietary n-6 to n-3 PUFAs is becoming recognized as also playing a role in bone biology (5–7). Candidate PUFAs with the potential to enhance bone mass include γ-linolenic acid (GLA, 18:3n-6), AA, eicosapentaenoic acid (EPA, 20:5n-3), and DHA. In young rats, a dietary total n-6:n-3 ratio of 3:1 combined with GLA and EPA in 4:1:1 ratios enhance bone calcium and calcium balance after 42 d (8). A lower ratio of GLA to EPA of 1:8:1 and a total n-6:n-3 ratio of 1:1 only moderately elevates femur bone calcium (8). In rats of similar ages, a reduction in the total n-6:n-3 dietary ratio of from 23.8:1 to 2.6:1 or to 1:2:1 by the addition of fish oil elevates total alkaline phosphatase over 42 d, but does not affect the bone formation rate (9). In our laboratory, the addition of AA and DHA to formula in ratios of ~5:1 (0.5:0.1% of dietary fat) elevates bone mass over 14–15 d in piglets when the total n-6:n-3 ratio is 5:1 (10) or 9:1 (11).

On the basis of the studies highlighted above, the optimal amount and type of PUFA required to enhance bone mass is not clear. Claassen et al (8) and Watkins et al (9) not only modified diets by adding GLA, EPA, or both but also modified the total 18:2n-6 and 18:3n-3 contents of the diet. This resulted in confusion as to whether it is the total n-6:n-3 ratio or the specific PUFA that affects bone. Previously, we added AA and DHA while keeping the total n-6:n-3 ratio relatively stable (10, 11), which suggests that AA, DHA, or both were responsible for the elevated bone mass and not the total n-6:n-3 ratio.
ratio. It is notable that the studies that report higher bone mineral mass used both n–6 and n–3 PUFA supplementation; Claassen et al (8) used GLA:EPA in a 4:1 ratio and Weiler (10) used AA:DHA in a ratio of ≈5:1. By design, the status of EPA and DHA was unchanged in our previous study, but the status of AA was elevated (10). However, it is not clear how much AA is required to optimize the development of bone mass. Because AA and DHA are now added to some infant formulas, it is important to investigate their potential to affect bone during early life. Therefore, this study was designed to investigate the effects of varied amounts of dietary AA, while keeping the DHA status and the total n–6:n–3 PUFA constant, on bone modeling and bone mass. Whether varying the amount of DHA affects bone will be investigated separately. The piglet model was selected for continued study to enable comparison with our previous studies (10, 11).

MATERIALS AND METHODS

The experimental design used was a 15-d researcher-blind, randomized block design. Male piglets (3 d old) were obtained from Glenlea Research Station, University of Manitoba. All piglets were randomly assigned within litters to 1 of 5 groups on day 3 of life so that only 1 piglet per litter represented each group. Eight of the piglets remained with the sows and were sow-reared for the remainder of the study. Thirty-two piglets were removed from the sows and transported to the University of Manitoba Animal Care Facility. Piglets in the formula groups were to receive 1 of 4 cow milk-based formulas for 15 d. The formulas provided optimal nutrition for healthy growing piglets as set by the National Research Council (12). Group 1 received AA:DHA at a 3.0:1.0 ratio (0.3:0.1% wt/wt of total dietary fat), group 2 received AA:DHA at a 4.5:1.0 ratio (0.45:0.1% wt/wt of total dietary fat), group 3 received AA:DHA at a 6.0:1.0 ratio (0.6:0.1% wt/wt of total dietary fat), and group 4 received AA:DHA at a 7.5:1.0 ratio (0.75:0.1% wt/wt of total dietary fat). Ratios of AA to DHA referred to in dietary treatment groups are the target ratios. The formulas used in the study were provided by Ross Products Division of Abbott Laboratories (product no. P96-AV2). The composition of the base formula is presented in Table 1. AA was provided in the form of RBD-ARASCO (40.6% AA) and DHA as RBD-DHASCO (40.0% DHA). DHA is derived from a marine microalgae (DHASCO), and AA is derived from a common soil fungi (ARASCO); both were provided by Martek Biosciences Corp (Columbia, MD) and selected as the source because these products are also used in the manufacture of human infant formula. Group 5 was a sow-reared reference group. For comparison, the milk from the sows in this study contained 0.54% of the fat as AA and 0.07% as DHA and had a total n–6:n–3 fatty acid ratio of 6.9:1.0.

Formula-fed piglets were housed individually in stainless steel cages under heat lamps to maintain an ambient temperature of 28–30°C. On day 3 of life, the piglets were taught to lap liquid formula and were fed standard formula at half strength. By the end of day 4 of life, the formula strength was increased to three-fourths the full strength. Before each morning feeding, the piglets in each of the 4 formula treatment groups were weighed by digital scale (Mettler-Toledo Inc, Hightstown, NJ) with an animal-weighing program (average of 3 weights). Those in the sow-reared group were weighed at 0900 daily, which represents a fed state.

On the basis of morning weights, the formula-fed groups received formula at 350 mL · kg⁻¹ · d⁻¹. This amount was divided into 3 equal amounts and fed to piglets at 0900, 1500, and 2100. Piglets were allowed ≈1 h of exercise in a pen outside their cages before each feeding. Piglets in group 5 (sow-reared) were maintained according to standard housing conditions at the research station. The pigs were exposed to 16 h of light and 8 h of darkness and were penned with the sow, allowing some room for physical activity. Piglets within a litter and not part of the study were allowed to be cross-fostered to the sow in this study, but a maximum of 10 piglets per sow was maintained throughout the study.

The experimental procedures were approved by the Fort Garry Campus Protocol Management and Review Committee, University of Manitoba, and were in agreement with the Guide to the Care and Use of Experimental Animals (13).

### TABLE 1

Composition of piglet formula before supplementation with arachidonic acid and docosahexaenoic acid

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Analytic value (per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g)</td>
<td>59</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>56.5</td>
</tr>
<tr>
<td>Linoleic acid (% of dietary fat)</td>
<td>22.14</td>
</tr>
<tr>
<td>Linolenic acid (% of dietary fat)</td>
<td>2.45</td>
</tr>
<tr>
<td>n–6:n–3</td>
<td>9.1</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>64</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>1005</td>
</tr>
<tr>
<td>Ash (g)</td>
<td>13.3</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>2261</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>1553</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>183</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>820</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>2719</td>
</tr>
<tr>
<td>Chloride (mg)</td>
<td>1226</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>28.5</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>25</td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>1.5</td>
</tr>
<tr>
<td>Manganese (µg)</td>
<td>912</td>
</tr>
<tr>
<td>Iodine (mg)</td>
<td>0.4</td>
</tr>
<tr>
<td>Selenium (µg)</td>
<td>34</td>
</tr>
<tr>
<td>Vitamin A (IU)</td>
<td>872</td>
</tr>
<tr>
<td>Vitamin D (IU)</td>
<td>175</td>
</tr>
<tr>
<td>Vitamin E (IU)</td>
<td>32</td>
</tr>
<tr>
<td>Vitamin K (µg)</td>
<td>60</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>168</td>
</tr>
<tr>
<td>Thiamine (mg)</td>
<td>1.4</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>2</td>
</tr>
<tr>
<td>Vitamin B-6 (mg)</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin B-12 (µg)</td>
<td>8</td>
</tr>
<tr>
<td>Pantothenic acid (mg)</td>
<td>7</td>
</tr>
<tr>
<td>Folic acid (µg)</td>
<td>237</td>
</tr>
<tr>
<td>Nicacin (mg)</td>
<td>13</td>
</tr>
<tr>
<td>Biotin (µg)</td>
<td>50</td>
</tr>
<tr>
<td>Choline (mg)</td>
<td>300</td>
</tr>
<tr>
<td>α-Inositol (mg)</td>
<td>57</td>
</tr>
<tr>
<td>Taurine (mg)</td>
<td>85</td>
</tr>
</tbody>
</table>

¹ Values are per the manufacturer, except for linoleic and linolenic acids.
The growth rate of all piglets was determined by calculating an average daily weight gain as follows:

\[
\text{Weight gain} = \frac{\text{change of weight in g}(\text{weight in kg on day 1}) + \text{weight in kg on day 15}}{15} \text{ d} = \frac{g \cdot \text{kg}^{-1} \cdot \text{d}^{-1}}{1}
\]

Sow-fed piglets were transferred from the Glenlea Research Station to the University of Manitoba on the evening of the 15th day of treatment. All piglets were fed only water ad libitum after 1800, which resulted in 14 h in the nonfed state. On the morning of the 16th day, piglets were anesthetized with an intraperitoneal injection of 30 mg sodium pentobarbital/kg (65 mg/mL). The animal’s length was then measured with a nonstretchable measuring tape from the tip of the snout to the base of the tail (to the nearest 0.1 cm). Once deep anesthesia was achieved, a cardiac puncture was performed and ~50 mL blood was drawn into heparin-containing syringes. Samples were centrifuged at 2000 × g and 4 °C for 15 min (model TJ-6R tabletop centrifuge; Beckman, Palo-Alto, CA) to separate red blood cells from plasma and were then flushed with liquid nitrogen and stored at ~80 °C until analyzed for fatty acid composition and biomarkers in plasma. After the removal of blood, the animals were terminated with a sodium pentobarbital overdose (180 mg/kg). Liver and tibia (~1.0 g) samples were excised, rinsed with 0.9% NaCl, and weighed to the nearest 0.1 g. Liver tissue was flash frozen in liquid nitrogen and stored at −80 °C. Urine was collected in a syringe via bladder puncture and stored at −20 °C.

Total lipids in plasma and tissues were extracted with the use of methanol:chloroform (1:2, by vol) according to the method of Folch et al (14) as previously described (10, 11). An internal standard, heptadecanoic acid (17:0), was added to each sample. Crude lipid extracts were transmethylated in 1 mL methanolic HCl (3N; Supelco Inc, Bellefonte, PA) at 100 °C for 15 min. Brain was extracted and methylated by the same method but was methylelated over 90 min. Fatty acid methyl esters were separated by gas-liquid chromatography (Varian Star 3400; Varian, Mississauga, Canada) with the use of hydrogen as the carrier gas. The gas chromatograph was equipped with a 30-m long capillary column made of fused silica and coated with DB225 (25% cyanopropylphenyl; J&W Scientific, Folsom, CA), an 8100 autosampler, an integrator, and a flame ionization detector. Samples were injected (0.5 μL) at an initial temperature of 180 °C, and then the oven temperature was increased to a final temperature of 220 °C at a rate of 3 °C/min. Fatty acid methyl esters (carbon chain lengths of 10-24) were identified by comparison with retention times of Supelco 37 component FAME mix (Supelco Inc) and expressed as a percentage of total fatty acids.

Plasma osteocalcin was analyzed by using a radioimmunoassay (Diasorin, Stillwater, MN). Urinary N-telopeptide (NTX) was measured in urine by using an enzyme-linked immunosorbent assay (Osteomark; Ostex, Seattle). NTX was corrected to creatinine as measured by using the Jaffe method (procedure no. 555; Sigma-Aldrich Ltd, Oakville, Canada). The concentration of intact parathyroid hormone (PTH) was determined in plasma by using an immunoradiometric assay (Diasorin), and calcitriol concentrations were measured in plasma by using a radioimmunoassay (Diasorin). Total transforming growth factor β-1 (TGFβ-1) was assayed in plasma by using an enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN) as used in recent cell culture experiments (15, 16). Plasma concentrations of insulin-like growth factor I (IGF-I) were measured with an enzyme-linked immunosorbent assay (R & D Systems).

The ex vivo release of prostaglandin E2 (PGE2) and TGFβ-1 was measured in an approximately 1-g segment of tibia incubated in Hank’s Balanced Salt Solution for 2 h at 37 °C, according to the method of Dekel et al (17). This method was selected because it has been used in chicks (18), rats (9), and piglets (19) to assess prostanoid metabolism after dietary treatment. Briefly, bone was sampled from the mid-diaphysis to represent cortical bone after removal of periosteum and marrow. The released PGE2 was analyzed in diluted samples by using an enzyme-linked immunosorbent assay (R&D Systems) and corrected to the weight of the tibia segment studied. Total TGFβ-1 was measured by using the same enzyme-linked immunosorbent assay kit that was used for plasma but was expressed per unit of tibia segment studied. This method provides for total TGFβ-1 and not TGFβ-1 activity, which requires bioassays (20). Release of TGFβ-1 by using a similar approach was conducted in tissue culture, in which osteoid matrix breakdown was measured 2 h after the addition of osteoclasts (20). Whether 2 h is the optimal duration of incubation for both PGE2 and TGFβ-1 requires clarification in subsequent experiments.

After the tissues were removed, the abdominal cavity was closed with sutures to maintain tissue depth. Piglet carcasses were then transported to a dual-energy X-ray absorptiometer (QDR4500W; Hologic Inc, Waltham, MA). Single scans were completed to determine the bone mineral content (BMC) and areas of the whole body (software version V8.16a:5) and left femur with the use of the subregion array hip program. All scans were performed with the piglets in the anterior-posterior position with limbs extended.

**Statistical analysis**

Values are expressed as means ± 1 SD unless stated otherwise. Statistical significance was set at a P value ≤ 0.05. The sample size, n = 8 per group, was calculated on the basis of a change of 25 ± 13 g in whole-body BMC as reported in the same species of animal (10) fed a formula with an AA:DHA ratio (5.0:1.0) similar to that in the present study. The power was set at 0.80 and α = 0.05. Differences in outcome measurements were detected by using a two-factor (litter and dietary treatment) analysis of variance for a randomized block design and post hoc analyses with the Bonferroni post hoc test to indicate differences among the formula-fed groups. Interactions among the litters and diets were not tested, because only one piglet per litter represented each diet. Pearson’s correlation analyses were used to identify factors measured in tissues or the circulation (AA in liver and plasma IGF-I, TBFβ-1, PTH, calcitriol, NTX, and osteocalcin) or localized to bone (PGE2 and TGFβ-1) that were related to the dietary treatment and bone mass. To determine which variables, after diet was accounted for, contributed to the prediction of bone mass, the same factors used in the correlation analyses with significant
linkages to diet or bone were entered into backward stepwise regression. The sow-reared group was not compared with the use of statistical tests but was used as a target reference group for outcome measurements.

**RESULTS**

The growth of piglets was measured daily to determine whether differences existed between treatment groups. The average weights of the piglets were not significantly different whether differences existed between treatment groups. The sow-reared group paralleled that of the formula-fed groups throughout the study. Length was also not significantly different between the formula groups at the end of study. The size and growth rate of the piglets in the sow-reared reference group paralleled that of the formula-fed groups (Table 2). No significant differences in formula intakes were observed between the treatment groups (sow-reared group intake was not measurable).

After 15 d of dietary treatment, plasma, liver, adipose, and brain fatty acids were analyzed. No significant differences in the saturated and monounsaturated fatty acid content of all tissues were found between formula groups (data not shown).

### TABLE 2

<table>
<thead>
<tr>
<th>Initial and final weights, average weight gains, final lengths, and average formula intakes for piglets that were fed formula supplemented with long-chain polyunsaturated fatty acids over 15 d&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acid</strong></td>
</tr>
<tr>
<td>Initial weight (kg)</td>
</tr>
<tr>
<td>(reference group, 2.4 ± 0.5)</td>
</tr>
<tr>
<td>Final weight (kg)</td>
</tr>
<tr>
<td>Average weight gain (g · kg&lt;sup&gt;−1&lt;/sup&gt; · d&lt;sup&gt;−1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Final length (cm)</td>
</tr>
</tbody>
</table>

<sup>1</sup>n = 8 piglets per group with 1 piglet per litter per group. The sow-reared reference group represented the same 8 litters; n = 1 per litter, n = 8 total. AA, arachidonic acid; DHA, docosahexaenoic acid; NA, not analyzed because of randomization within litters.

<sup>2</sup>Ratios represent the percentage of fatty acids in the formula.

<sup>3</sup>Post hoc testing was conducted only to determine differences between formula groups.

<sup>4</sup>± SD.

### TABLE 3

<table>
<thead>
<tr>
<th>Polynsaturated fatty acids in the plasma of piglets that were fed formula supplemented with long-chain polyunsaturated fatty acids for 15 d&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acid</strong></td>
</tr>
<tr>
<td>% by wt</td>
</tr>
<tr>
<td>18:2n−6 (reference group, 21.39 ± 1.76)</td>
</tr>
<tr>
<td>18:3n−6 (reference group, 0.88 ± 0.21)</td>
</tr>
<tr>
<td>20:4n−6 (reference group, 9.87 ± 1.13)</td>
</tr>
<tr>
<td>18:3n−3 (reference group, 0.75 ± 0.05)</td>
</tr>
<tr>
<td>20:5n−3 (reference group, 0.16 ± 0.09)</td>
</tr>
<tr>
<td>22:6n−3 (reference group, 2.48 ± 0.44)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are for n−6 and n−3 fatty acids >0.1% of total fatty acids; n = 8 piglets per group, with 1 piglet per litter per group. The sow-reared reference group represented the same 8 litters; n = 1 per litter, n = 8 total. AA, arachidonic acid; DHA, docosahexaenoic acid. Values in a row with different superscript letters are significantly different. P ≤ 0.05.

<sup>2</sup>Ratios represent the percentage of fatty acids in the formula.

<sup>3</sup>Post hoc testing was conducted only to determine differences between formula groups.

<sup>4</sup>± SD.
TABLE 4
Polyunsaturated fatty acids in the liver of piglets fed formula supplemented with long-chain polyunsaturated fatty acids for 15 d

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>AA:DHA in supplemented formula</th>
<th>Litter</th>
<th>Formula group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1, 0.3:0.1</td>
<td>Group 2, 0.45:0.1</td>
<td>Group 3, 0.6:0.1</td>
</tr>
<tr>
<td>18:2n−6 (reference group, 13.48 ± 0.72)</td>
<td>17.15 ± 0.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.16 ± 1.27</td>
<td>16.37 ± 1.48</td>
</tr>
<tr>
<td>18:3n−6 (reference group, 0.64 ± 0.20)</td>
<td>0.27 ± 0.03</td>
<td>0.31 ± 0.03</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>20:4n−6 (reference group, 12.89 ± 2.97)</td>
<td>17.24 ± 1.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.41 ± 1.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.75 ± 1.05&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:3n−3 (reference group, 0.58 ± 0.30)</td>
<td>0.42 ± 0.09</td>
<td>0.46 ± 0.12</td>
<td>0.46 ± 0.18</td>
</tr>
<tr>
<td>20:5n−3 (reference group, 0.39 ± 0.06)</td>
<td>0.27 ± 0.02</td>
<td>0.29 ± 0.02</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>22:6n−3 (reference group, 4.51 ± 0.91)</td>
<td>5.51 ± 0.85</td>
<td>5.74 ± 0.41</td>
<td>5.51 ± 0.99</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are for n−6 and n−3 fatty acids >0.1% of total fatty acids; n = 8 piglets per group, with 1 piglet per litter per group. The sow-reared reference group represented the same 8 litters; n = 1 per litter, n = 8 total. AA, arachidonic acid; DHA, docosahexaenoic acid. Values in a row with different superscript letters are significantly different, P ≤ 0.05.

<sup>b</sup> Ratios represent the percentage of fatty acids in the formula.

<sup>c</sup> Post hoc testing was conducted only to determine differences between formula groups.

After correction for the effects of litter, supplemental AA in the formula was reflected in plasma, liver, and adipose tissues but not in brain (Tables 3-5). Elevated AA did not alter the proportion of other n−6 and n−3 fatty acids, except for brain DHA, which was lower in the 4.5:1 formula group than in the other 3 groups (Table 5). A main effect of litter was observed for 18:2n−6 and DHA in plasma, 18:2n−6 and AA in liver, and AA and DHA in brain. No effects of litter were observed for adipose.

TABLE 5
Polyunsaturated fatty acids in the adipose and brain of piglets fed formula supplemented with long-chain polyunsaturated fatty acids for 15 d

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>AA:DHA in supplemented formula</th>
<th>Litter</th>
<th>Formula group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1, 0.3:0.1</td>
<td>Group 2, 0.45:0.1</td>
<td>Group 3, 0.6:0.1</td>
</tr>
<tr>
<td>Adipose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n−6 (reference group, 12.34 ± 3.51)</td>
<td>20.72 ± 1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.71 ± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.60 ± 0.72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:3n−3 (reference group, 0.37 ± 0.09)</td>
<td>0.29 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35 ± 0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:6n−3 (reference group, 0.06 ± 0.05)</td>
<td>0.07 ± 0.03</td>
<td>0.08 ± 0.05</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n−6 (reference group, 1.08 ± 0.02)</td>
<td>1.36 ± 0.10</td>
<td>1.34 ± 0.10</td>
<td>1.35 ± 0.10</td>
</tr>
<tr>
<td>20:4n−6 (reference group, 9.21 ± 0.40)</td>
<td>9.28 ± 0.19</td>
<td>9.28 ± 0.32</td>
<td>9.33 ± 0.31</td>
</tr>
<tr>
<td>18:3n−3 (reference group, 0.04 ± 0.01)</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>22:6n−3 (reference group, 7.73 ± 0.41)</td>
<td>7.94 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.65 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.74 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> n = 8 piglets per group, with 1 piglet per litter per group. Note that 18:3n−6 and 20:5n−3 were not detected in adipose or brain in amounts >0.05% of total fatty acids. The sow-reared reference group represented the same 8 litters; n = 1 per litter, n = 8 total. AA, arachidonic acid; DHA, docosahexaenoic acid. Values in a row with different superscript letters are significantly different, P < 0.05.

<sup>b</sup> Ratios represent the percentage of fatty acids in the formula.

<sup>c</sup> Post hoc testing was conducted only to determine differences between formula groups.

<sup>d</sup> ± SD.

<sup>e</sup> Post hoc testing indicated that formula groups were not significantly different.
Results of the two-factor analysis of variance indicated that after 15 d of treatment, the AA:DHA ratio of the formula had a significant main effect on whole-body BMC ($P = 0.038$). A significant effect of litter was not observed. Post hoc analysis indicated that groups 3 and 4 had higher values than did groups 1 and 2 ($P < 0.05$) (Figure 1). Whole-body bone area followed the same pattern as did BMC, but the main effects of litter ($P = 0.318$) and diet ($P = 0.055$) were not significant (Figure 1).

Femur BMC was not significantly different between groups: $2.0 \pm 0.4$ g for group 1, $2.2 \pm 0.7$ g for group 2, $2.2 \pm 0.4$ g for group 3, $2.4 \pm 0.2$ g for group 4, and $3.7 \pm 0.8$ g for the sow-reared group. Femur bone area was affected significantly by litter ($P = 0.018$) but not by dietary treatment ($P = 0.351$); mean values were $8.4 \pm 1.5$ cm$^2$ for group 1, $8.2 \pm 1.6$ cm$^2$ for group 2, $8.6 \pm 1.2$ cm$^2$ for group 3, and $9.1 \pm 0.6$ cm$^2$ for group 4. The value for bone area in the sow-reared group was $11.0 \pm 1.6$ cm$^2$. Although statistical comparisons were not made to determine significance, for all measurements of BMC the sow-reared group had higher values on average than did the formula-fed groups, regardless of formula group.

Modeling and resorption of collagen matrix was assessed by analyzing NTX in urine samples. The results were then normalized for urine dilution by dividing values by urinary creatinine; no significant differences were found between groups (Table 6). Plasma osteocalcin was analyzed to assess osteoblast activity. No significant differences were observed between groups (Table 6). Differences in plasma PTH, IGF-I, calcitriol, and TGFβ-1 and in urinary cortisol were not observed between the formula-fed groups. Release of TGFβ-1 and PGE$_2$ from bone was not significantly different among formula groups and litters (Table 6). Effects of litter were observed for plasma PTH and urinary NTX.

Dietary AA correlated with AA in plasma ($r = 0.44, P = 0.01$) but not with liver AA ($r = 0.35, P = 0.055$) or the variables related to bone metabolism (data not shown). The proportion of AA in plasma and liver did, however, correlate with plasma IGF-I (plasma: $r = 0.50, P = 0.004$; liver: $r = 0.56, P < 0.001$), calcitriol (plasma: $r = 0.48, P = 0.006$; liver: $r = 0.37, P = 0.035$), and urinary NTX (liver: $r = 0.533, P = 0.002$).

Pearson’s correlation analysis was conducted to determine any relations between the biochemical variables and bone mass. Whole-body BMC was related to bone TGFβ-1, NTX, and PGE$_2$: PTH ($r = -0.12, P = 0.55$), bone TGFβ-1 ($r = 0.40, P = 0.04$), IGF-I ($r = 0.28, P = 0.12$), plasma TGFβ-1 ($r = 0.08, P = 0.07$), calcitriol ($r = 0.04, P = 0.81$), NTX ($r = 0.44, P = 0.01$), osteocalcin ($r = -0.09, P = 0.06$), and PGE$_2$ ($r = 0.38, P = 0.03$). Backward stepwise regression was conducted to examine which variables in combination with dietary treatment were most likely responsible for the higher BMC with increasing AA. The only variables that contributed to BMC were dietary AA ($P = 0.018$) and urinary NTX ($P = 0.033$), in the regression equation $y = 38.01 + 1.82$ (dietary AA as % of fat) + 0.90 (NTX as nmol:mmol creatinine), $R^2 = 0.607, P = 0.008$.

Whole-body bone area was correlated with bone TGFβ-1, NTX, and PGE$_2$ but not with plasma PTH, IGF-I, TGFβ-1, calcitriol, or osteocalcin: bone TGFβ-1 ($r = 0.40, P = 0.04$), NTX ($r = 0.32, P = 0.07$), and PGE$_2$ ($r = 0.48, P = 0.006$); plasma PTH ($r = -0.05, P = 0.81$), IGF-I ($r = -0.05, P = 0.81$), TGFβ-1 ($r = 0.04, P = 0.85$), calcitriol ($r = 0.05, P = 0.78$), and osteocalcin ($r = -0.03, P = 0.89$). The only variable that contributed significantly to bone area after dietary AA was accounted for ($P = 0.004$) was urinary NTX ($P = 0.034$), in the regression line $y = 79.04 + 4.49$ (dietary AA as % of fat) + 1.74 (NTX as nmol:mmol creatinine), $R^2 = 0.66, P = 0.002$.

**DISCUSSION**

The main objective of this research was to examine the effect of varied amounts of AA added to cow milk–based formula on biomarkers of bone modeling and bone mass in the piglet model for infant nutrition. After as little as 15 d, proportions of AA in tissues (plasma, liver, and adipose) were elevated in proportion to diet and, by design, DHA was unaltered. These observations were accompanied by elevations in BMC when AA was >0.5% of dietary fat, which confirmed the results of previous studies (10, 11). Whether elevations in bone mass

**FIGURE 1.** Whole-body bone mineral content and bone area in 4 groups of piglets ($n = 8$ piglets per group with 1 piglet per litter per group) fed formula with different amounts of arachidonic acid relative to docosahexaenoic acid for 15 d and in a sow-reared suckling reference group from the same litters ($n = 1$ per litter). Formula-fed groups with different letters are significantly different, $P \leq 0.05$ (two-factor ANOVA and Bonferroni tests). Upper panel: main effects of litter, $P = 0.280$; main effects of formula, $P = 0.038$. Lower panel: main effects of litter, $P = 0.318$; main effects of formula, $P = 0.055$. Ratios of arachidonic acid to docosahexaenoic acid (as percentages of dietary fatty acids) in the formula were as follows: group 1, 0.3:0.1; group 2, 0.45:0.1; group 3, 0.60:0.1; and group 4, 0.75:0.1.
continue after 15 d remains to be determined. This is an important consideration because fatty acid status does not plateau in piglets fed vegetable oil–based formula for ≥25 d (21). However, our values for AA and DHA were almost identical to those in liver after 29 d (22) and in plasma after 16 d (23) in piglets fed the same dietary sources and similar amounts of AA and DHA. Additionally, after piglets are fed fish oil for 12 d, incorporation of EPA into platelets reaches a steady state plateau (24). Thus, it is possible that the PUFA status plateaus quicker when dietary AA and DHA are provided in comparison with diets devoid of these fatty acids (21). This theory is supported by an insignificant change in serum AA in human infants after 2–6 wk of consuming formula made with AA and DHA in contrast with significant reductions after 2 wk in infants fed unsupplemented formula (25, 26).

The mechanisms behind the elevations in bone mass with >0.5% AA are probably linked to the resorption and modeling of bone. Whole-body BMC was correlated with NTX, bone TGFβ-1, and bone PGE2, but not with the circulating growth factors or hormones (PTH, calcitriol, IGF-I, and TGFβ-1). In rats, TGFβ-1 is linked to the stimulation of bone formation (27) and healing (28). However, AA status was not related to bone TGFβ-1 or PGE2, but was related to IGF-I, calcitriol, and NTX in the piglets. Regression analyses indicated that after the dietary groups were accounted for, whole-body BMC was best predicted by NTX. This was the case for bone area as well, which suggests that a higher turnover was associated with modeling of bone and not with loss of mineral. The observation that both BMC and bone area were elevated supports this interpretation. This observation agrees with that by Claassen et al (8), in which a ratio of GLA to EPA of 1.8:1 resulted in lower values for bone resorption and lower bone calcium than did a ratio of 4:1, although the total n-6:n-3 ratios varied as well.

It is important to note that NTX values in the piglets were within the expected range (10, 11) and were not elevated by the dietary treatments directly. AA status was modified by diet and was related to NTX as well as to IGF-I and calcitriol but not to TGFβ-1 or PGE2. In chicks, EPA and DHA (fish oil) elevate TGFβ-1 (18), but this was not the case in piglets fed AA and DHA. Thus, whereas calcitriol, IGF-I, TGFβ-1, and PGE2 are all linked to bone modeling (7, 27), the role of each in modulating bone modeling and mass as a result of feeding dietary AA is unclear.

There are no reports of bone mass in infants fed formula with AA and DHA and, thus, the results of this study should not be extrapolated to human infants. In the piglets, liver AA was positively related to urinary NTX, which suggests that lower amounts of AA are linked to lower bone modeling and resorption. Infants who are born prematurely have elevated rates of bone resorption and delayed bone growth (29–31). Whether dietary AA would benefit bone growth in preterm infants is unknown. Interestingly, high rates of bone turnover continue up to 8–12 y of age in children who were born preterm (32) and also in males aged 19–21 y who were born of low birth weight (33). In addition, growth in the first year of life is known to relate to adult bone mass (34–36). These studies suggest that bone mass and metabolism are programmed by events in early life. Whether dietary AA can program bone modeling or pre-

### Table 6

<table>
<thead>
<tr>
<th></th>
<th>AA:DHA in supplemented formula</th>
<th>P (two-factor ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1, 0.3:0.1</td>
<td>Group 2, 0.45:0.1</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
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<tr>
<td>PTH (pmol/L) (reference group, 0.514 ± 0.168)</td>
<td>0.500 ± 0.16²a</td>
<td>0.514 ± 0.116</td>
</tr>
<tr>
<td>Calcitriol (pmol/L) (reference group, 534.0 ± 255.5)</td>
<td>474.3 ± 284.5</td>
<td>562.8 ± 213.7</td>
</tr>
<tr>
<td>IGF-I (pmol/L) (reference group, 16.2 ± 3.9)</td>
<td>15.6 ± 1.9</td>
<td>15.4 ± 7.2</td>
</tr>
<tr>
<td>Osteocalcin (nmol/L) (reference group, 15.1 ± 3.1)</td>
<td>15.8 ± 5.8</td>
<td>15.1 ± 5.3</td>
</tr>
<tr>
<td>TGFβ-1 (μmol/L) (reference group, 126.0 ± 27.2)</td>
<td>111.9 ± 45.5</td>
<td>82.4 ± 42.4</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
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<tr>
<td>NTX (nmol:mmol creatinine) (reference group, 9.5 ± 6.2)</td>
<td>9.1 ± 2.6</td>
<td>9.8 ± 3.7</td>
</tr>
<tr>
<td>Cortisol (nmol:mmol creatinine) (reference group, 136.6 ± 38.1)</td>
<td>57.4 ± 18.2</td>
<td>53.8 ± 19.6</td>
</tr>
<tr>
<td><strong>Bone organ culture</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE2 (ng/g bone) (reference group, 5.4 ± 2.8)</td>
<td>3.6 ± 2.0</td>
<td>5.5 ± 2.8</td>
</tr>
<tr>
<td>TGFβ-1 (ng/g bone) (reference group, 37.5 ± 15.0)</td>
<td>52.7 ± 14.4a</td>
<td>30.0 ± 10.9b</td>
</tr>
</tbody>
</table>

* n = 8 piglets per group, with 1 piglet per litter per group. The sow-reared reference group represented the same 8 litters; n = 1 per litter, n = 8 total. PTH, parathyroid hormone; IGF-I, insulin like growth factor I; TGFβ-1, transforming growth factor β-1; NTX, N-telopeptide of type I collagen; PGE2, prostaglandin E2; AA, arachidonic acid; DHA, docosahexaenoic acid. Values in a row with different superscript letters are significantly different, P < 0.05.

² Ratios represent the percentage of fatty acids in the formula.

²² Post hoc testing was conducted only to determine differences between formula groups.

²³ SD.
vent the long-term sequelae of low bone mass and high turn-
over osteopenia due to prematurity or size at birth is not
known. Longer-term supplementation studies are needed in
which AA is supplemented early in life and in which bone mass
is assessed at maturity in humans and other mammals.

A suckled piglet group was included as a target reference
group because the objective of designing infant formula is to
mimic the qualities of mothers’ milk. Although the BMC of the
reference group was higher, the piglets fed the higher amounts
of AA (0.6–0.75%) had BMC values closest to that of the
reference group. The weight and length of the piglets were not
affected by feeding mode (suckling compared with formula),
which indicated that the growth rate was not a confounding
variable. The higher BMC in the suckled piglets may have been
linked to environment and other nutritional qualities of mater-
nal milk other than those from PUFAs. The suckled piglets
would be exposed to shorter more frequent bouts of weight-
bearing activity than would the formula-fed groups, for which
exercise was permitted 3 times daily. In rats, shorter more
frequent loading sessions lead to higher bone masses than does
the same amount of loading delivered over one session daily
(37). In human infants the environment is more similar be-
tween infants who are breastfed or fed infant formula. Yet a
positive effect of duration of feeding mothers’ milk on bone
mass has been observed in prepubertal children who were born
preterm (38) or term (39). It is postulated that greater nutrient
bioavailability of minerals in maternal milk (40) and physio-
logic responses such as elevated circulating IGF-I (41) and
osteocalcin (42) may be linked to enhanced bone mass in
human neonates fed mothers’ milk. Both IGF-I and osteocalcin
in human neonates are measured in the fed state. The lack
of effect of AA and DHA on IGF-I and osteocalcin in the piglets
might have been due to measurement in the nonfed state.

As part of a global objective of research designed to estab-
lish whether dietary AA enhances bone mass during growth,
our research group is interested in other effects in vulnerable or
rapidly growing tissues such as the brain. Brain fatty acids
were determined to ensure that the amounts of AA and DHA
were supportive of expected tissue amounts of AA and DHA in
human neonates fed mothers’ milk. Both IGF-I and osteocalcin
in neonates are measured in the fed state. The lack of
effect of AA and DHA on IGF-I and osteocalcin in the piglets
might have been due to measurement in the nonfed state.

In summary, although it is unclear whether continued dietary
supplementation with AA will result in sustained elevations in
bone mass, it is clear that short-term supplementation with AA
elevates BMC in piglets. It was shown previously that 0.5% of
AA as dietary fat has positive effects on BMC when the content
of DHA is kept constant at 0.1% of total fat and the total
n−6:n−3 ratio is <9:1 (10, 11). Given the narrow range of AA
used in the current study (0.30–0.75% of dietary fat), the
findings are significant and important for clarifying the optimal
amount of AA to be added to infant formula products. Whether
the amount of DHA interacts with AA to affect bone remains
unclear because our studies to date have only evaluated sup-
plementation with DHA at 0.1% of dietary fatty acids. Further
studies in which AA is held constant at 0.5–0.6% of dietary fat
but in which DHA is added in amounts ranging from 0.1% to
0.3% of dietary fatty acids are needed to ensure that the role of
both n−6 and n−3 fatty acids early in development are eluci-
dated. Interestingly, some commercial infant formula products
(Mead Johnson Nutritional) contain 0.64% of dietary fat as
AA and 0.32% as DHA. Whether there is benefit to human
infant bone mass of such a formula has not been reported.
Nonetheless, although the design of milk formula substitutes
to mimic the qualities of human milk is important and because
mothers’ milk is associated with enhanced bone mass in hu-
mans and pigs, strategies to prolong the duration of breastfeed-
ing continue to be in the best interest of whole-body infant
development.

HAW wrote the manuscript. JLB (fatty acid analysis in plasma, liver,
and adipose) and JRK (growth factors and calciotropic hormones)
conducted part of this research during graduate training. SCF-W measured
the calcitriol, prothrombin, NTX, and osteocalcin concentrations. None of
the authors had any conflicts of interest.

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