Influence of dietary calcium and phosphorus content in a fixed ratio on growth and development in Great Danes

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Objective—To study the musculoskeletal development of Great Dane puppies fed various dietary concentrations of calcium (Ca) and phosphorus (P) in fixed ratio by use of dual energy x-ray absorptiometry (DEXA), determination of serum insulin-like growth factor I and parathyroid hormone concentrations, radiography, and blood chemistry analysis results.

Animals—32 purebred Great Dane puppies from 4 litters.

Procedure—At weaning, puppies were assigned randomly to 1 of 3 diets. Blood was collected for biochemical analyses and hormone assays, and radiography and DEXA were performed through 18 months of age. Changes in body weight, bone mineral content, fat tissue weight, lean mass, result of serum biochemical analyses, hormonal concentrations, and radius lengths were analyzed through 18 months of age.

Results—Bone mineral content of puppies correlated positively with Ca and P content of the diets fed. Significant differences between groups in bone mineral content, lean mass, and body fat were apparent early. The disparity among groups increased until 6 months of age and then declined until body composition was no longer different at 12 months of age. Accretion rates for skeletal mineral content, fat, and lean tissue differed from each other and by diet group.

Conclusions and Clinical Relevance—Ca and P concentrations in the diet of young Great Dane puppies are rapidly reflected in the bone mineral content of the puppies until 5 to 6 months of age, after which hormonal regulation adjusts absorption and excretion of these minerals. Appropriate Ca and P concentrations in diets are important in young puppies < 6 months of age. (*Am J Vet Res* 2002;63:1036–1047)

Large- and giant-breed puppies such as Great Danes continue to grow for a longer period than smaller breeds, and within that growth period they experience extremely rapid growth primarily between 3 and 5 months of age.[1] For many years, maximal growth was thought to be synonymous with optimal growth, and consequently, highly palatable and digestible diets with an increase in nutrient density were developed to maximize genetic growth potential. For large- and giant-breed puppies, this resulted in rapid growth to reach mature size at an earlier age. In American Kennel Club breeds for which no maximum height limitations are stated, a consistent movement toward favoring larger dogs has been seen. These nutritional and conformational traits have converged in the development of foods for rapid, maximal growth and selective breeding for larger dogs. For example, a Great Dane growth chart published in 1971[2] indicated a mature weight of 60 kg by 25 months of age. The American Kennel Club standard for the Great Dane states that a height of > 32 in (81 cm) is preferred. Today, it is not uncommon to see mature male Great Danes weighing well over 70 kg and standing > 35 in (89 cm) tall. However, recognition of an increase in developmental disorders, particularly of the musculoskeletal system, in these puppies has resulted in reevaluation of the definition of optimal growth and the impact of the nutritional components on this growth, particularly during the early period of life.

For this reason, the development of diets designed specifically for balanced nutrition during the early phases of rapid growth and development of large- and giant-breed puppies is critical.[3-6] Differences in the energy requirement among giant breeds such as Great Danes and Newfoundlands[7] have also been observed.[1,8-11] Several dietary components have the potential to profoundly influence the period of rapid growth. Energy is essential for growth and maintenance, and excess dietary energy positively affects the rate of weight gain and can contribute to the expression of developmental musculoskeletal disorders in many species, including puppies.[10,12-18] Anecdotal reports have suggested a similar effect on growth and the development of musculoskeletal disorders with high protein diets, but Nap et al[19] failed to confirm such effects in growing Great Danes puppies fed varying amounts of protein.

Although vitamins C and D can affect growth, the need for exogenous sources for these vitamins in dogs is controversial. Both vitamins are believed to be synthesized by dogs in quantities sufficient for proper bone and cartilage development.[20] However, the **Association of American Feed Control Officials (AAFCO)**[21] requires that a minimum of 500 IU of vitamin D/kg be included in commercial diets formulated for growth and maintenance, whereas no exogenous

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source of vitamin C is required in the content of commercial diets.

Currently, the AAFCO recommends a range of 1.0 to 2.5% **calcium (Ca)** in canine foods formulated for growth whereas the recommended range of **phosphorus (P)** in diets formulated for growth is 0.8 to 1.6%. The ratio of Ca to P in the diet is of considerable importance as was discussed by Richardson and Zentek.[1] The accepted ideal ratio of Ca:P, 1.2:1 to 1.4:1,[21] is a narrow range, but the AAFCO allows a range of 1.0:1.0 to 2.0:1.0 in canine diets formulated for growth.

The purpose of the study presented here was to evaluate the effects of diets containing various concentrations of Ca and P in a fixed ratio on various variables of growth and development of rapidly growing Great Dane puppies. Three concentrations of Ca and P in the ratio 1.2:1.0 were chosen for study. All diets contained moderate amounts of protein and energy (14% fat, 26% crude protein). Concentrations of vitamin D were in accordance with AAFCO requirements.[21] No exogenous source of vitamin C was added, and all other nutrients were present in accordance with the requirements of the AAFCO.

Previous work has shown that **dual energy x-ray absorptiometry (DEXA)** is an accurate, precise, and noninvasive technique for the quantitative measurement of body composition in dogs.[22] Dual energy x-ray absorptiometry was used in our study to serially measure variables of growth in giant-breed puppies.

Materials and Methods

This project was approved by and conducted within the guidelines of the Auburn University Institutional Animal Care and Use Committee. Thirty-two Great Dane puppies from 4 dams bred and housed at Auburn University College of Veterinary Medicine were whelped, weaned, and assigned randomly to 3 diets. Data from noninvasive measurements were collected periodically.

Animals—Four clinically normal vaccinated[a] purebred adult female and 3 purebred adult male Great Danes, all unrelated and between 1 and 4 years of age, were used as breeding stock. Four litters (1 from each of the 4 breeding bitches) were born during a 5-month period. After whelping, each puppy was randomly assigned a letter designating the litter into which it was whelped and a consecutive number. Puppies were identified by a combination of letter and number designation. Coat pattern, color, sex, and birth weight were recorded for each animal. Dietary group assignments were made after birth and according to the order of numbering (ie, A1, A2, and A3 puppies from the first litter were assigned to the **low concentration Ca and P diet [LCP]**; A4, A5, and A6 puppies were assigned to the **medium concentration Ca and P diet [MCP]**; and A7 and A8 puppies were assigned to the **high concentration Ca and P diet [HCP]**). Some attempt was made to resolve male to female distribution inequities in groups, but males were necessarily overrepresented in all groups as a result of birth distribution. The final sex distribution was 21 males and 11 females, divided into groups containing 8 males and 3 females, 6 males and 5 females, and 7 males and 3 females in the LCP, MCP, and HCP groups, respectively.

At 4, 6, and 12 months of age, 2 puppies from each group were selected randomly for euthanasia to obtain tissues for histologic evaluation of skeleton and joints at these time points (to be reported elsewhere). The remaining dogs were euthanatized at 18 months of age to provide tissues for similar histologic examination at 18 months of age. All dogs were euthanatized with an IV injection of pentobarbital sodium and phenytoin sodium.

Housing and environment—Puppies were whelped in an environmentally-controlled building where they remained with their dams until 8 weeks of age, at which time they were transferred to a kennel building containing indoor-outdoor runs. In addition to exercise in the runs, puppies were allowed to exercise in large grassed pens (4 × 28 m) twice weekly for periods of about 2 hours each. Puppies were exercised with their kennel mates early in our study and later were exercised individually to minimize potential for injury.

Diets—Breeding dogs were fed a uniformly formulated maintenance dry food[b] (**Appendix 1**), which was available ad libitum via large feeders hung inside the runs. Fresh water was available at all times.

Following ultrasonographic confirmation of pregnancy at 29 days, maintenance diet was replaced with pregnancy and lactation diet (**Appendix 2**).[b] The pregnancy and lactation diet and water were available ad libitum from mid-pregnancy through lactation.

Weaning of puppies began naturally at 4 to 5 weeks of age. At this time, the puppies were placed into their assigned groups and fed diets twice daily.

The 3 diets were isocaloric and isonitrogenous, varying only in the amount of Ca and P with the ratio of Ca to P remaining the same (Ca:P, 1.2:1.0; **Appendix 3**).[b] Minor differences were found in the chemical analysis in trace elements and fiber, which are considered unimportant. Manipulated dietary components are not in quantities that are anticipated to adversely interact with the absorption and metabolism of other nutrients. Diet designations were as follows: LCP, 0.47% Ca and 0.38% P; MCP, 0.78% Ca, and 0.67% P; and HCP, 2.67% Ca, and 2.27% P.

Initially, dry diets were blended with water to facilitate ingestion by young puppies. The food was made available for 30 minutes, twice daily, after which puppies were returned to their littermates and dam in the whelping pens. At 8 weeks of age, fully weaned puppies were separated from their dams and housed in the indoor-outdoor kennel in groups containing 2 or 3 puppies, according to litter and group assignment. The same feeding protocol of two 30-minute ad libitum feeding periods daily was continued throughout our study. Fresh water was available to puppies at all times. Behavior problems that might affect food intake (ie, dominance at feeding times) were corrected by housing puppies separately.

Health maintenance—Puppies were immunized at 8, 12, and 16 weeks of age with canine distemper virus, adenovirus type 2, parainfluenza virus, parvovirus vaccines, and leptospira bacterin. Puppies also received monthly ivermectin heartworm preventative and were dewormed quarterly with pyrantel tartrate.

Anesthesia—For any procedures requiring anesthesia, the following protocol was followed. Food was withheld for 12 hours prior to administration of anesthetics. Puppies were premedicated with a SC injection of atropine sulfate (0.4 mg/ml) at a dose of 0.04 mg/kg, and anesthesia was induced with an IV injection of xylazine hydrochloride and butorphanol tartrate (0.4 mg/kg). Anesthesia was maintained by inhalation of approximately 1 L of oxygen/min with either 1.5% 2-bromo-2-chloro-1,1,1-trifluoroethane during the early parts of our study or 1.5% isoflurane during the later parts of our study. Procedures requiring anesthesia were scheduled together to reduce the number of anesthetic events.

Monitoring of food intake—Weight of food consumed was determined by recording weight of feed bins before and after feeding (which included any remaining uneaten food), with estimates made for spillage.

Dual energy x-ray absorptiometry scans—Puppies were scanned while under anesthesia as described previously.[22] Positioning of the puppies was selected to mimic that recommended for the DEXA software, which was developed for human absorptiometry scans.

All scans were performed with a dual energy x-ray absorptiometer[c] that had been used to validate DEXA in dogs.[22] Tests of precision and accuracy for this equipment in dogs and cats were described previously.[22,23] Because of the wide range of body weights (4 kg to > 60 kg) and age (8 weeks to 18 months) of the puppies during the course of our study, 2 software programs were required to measure body composition. Pediatric software[d] was used for total body scans of puppies weighing between 4 and 35 kg. Within the pediatric program were selections for small, medium, and large patients, with body weight ranges of 5 to 15 kg, 15 to 25 kg, and 25 to 35 kg, respectively. Once a puppy exceeded the pediatric software maximum weight limit (> 35 kg), adult software[e] was used to perform total body scans. Positioning techniques remained the same regardless of the software program used for total body scans.

In addition to total body scans, ventrodorsal scans of the vertebral column were performed on the lumbar region of the dogs at each time point. The pediatric software[d] was used for dogs < 35 kg, and the adult software in medium and fast modes[e] was used on heavier and older puppies. The head of the last rib was used as the cranial landmark, with the crest of the ileum as the caudal landmark to identify the lumbar vertebrae. Lumbar vertebrae 1 (L1) through 4 (L4) were identified and analyzed to measure **bone mineral content (BMC)**, area, height, and width of the selected vertebrae. Total body, ventrodorsal vertebral column, and forelimb DEXA scans were performed at monthly intervals from 2 to 8 months of age and then bimonthly until puppies were 18 months of age or euthanatized.

Radiography—To assess longitudinal bone growth, measurements were made from mediolateral radiographic views of the antebrachium. All radiographs were obtained with puppies anesthetized as already described beginning at 8 weeks of age and repeated every 8 weeks until euthanasia. Radiographs were made by use of standard radiographic techniques and evaluated by board-certified veterinary radiologists. Mediolateral radiographic views of the radius were digitized by use of a scanner.[f] Measurements were made of the length of the radius and ulna from proximal physis to distal physis in triplicate with imaging software.[g] The physis-to-physis length of the radius was measured from the central point of the proximal physis to the central point of the distal physis. The physis-to-physis length of the ulna was measured from the most proximal point of the proximal physis at the olecranon to the most distal point of the distal physis.

Clinicopathologic testing—Blood samples were collected from all puppies via jugular venipuncture monthly from 8 weeks to 8 months of age and then bimonthly until completion of our study or euthanasia. Plasma and serum samples were obtained, and portions were used immediately for full biochemical analysis or frozen at –80 C until used for endocrine assays. Complete blood counts including WBC, RBC, and platelet counts and full serum biochemical analysis to assess organ function, including ionized Ca concentration determination, were conducted; results were compared with reference range values established by Auburn University College of Veterinary Medicine Clinical Pathology Laboratory for dogs > 12 months of age and with published values for puppies.[24] Urine samples were collected by cystocentesis monthly from 8 weeks to 8 months of age and then bimonthly until completion of our study or euthanasia. Urine samples were analyzed and results compared with reference data for the laboratory. Fractional excretion of Ca and P was calculated as described previously.[25]

Serum insulin-like growth factor-I assay—Serum samples from puppies 12 months of age or younger underwent extraction to remove binding proteins by a modification of the method described by Cox et al.[26] Samples were assayed in duplicate by use of a commercially available kit on the basis of a heterologous double antibody radioimmune assay.[h] Serial dilutions of canine serum following binding protein extraction were paralleled to the recombinant **insulin-like growth factor-I (IGF-I)** standard curve, as determined by linear regression analysis. Addition of recombinant IGF-1 standard to the canine sera resulted in the recovery of 100.5% of the standard. Intra- and interassay variation was 4.96 and 10.6%, respectively. The sensitivity of the assay was 0.25 ng/ml.

Serum parathyroid hormone assay—Serum samples from puppies 12 months of age and younger were assayed in duplicate by use of a commercially available kit,[i] which had been validated for canine serum.[27] This assay is a 2-site immunoradiometric assay for the measurement of biologically intact **parathyroid hormone (PTH)**.

Table 1—Mean (± SD) serial dual energy x-ray absorptiometry (DEXA) measurements of body weight (kg) of growing Great Danes by sex and diet

	Sex		Diet		
Age (m)	Male	Female	LCP	MCP	HCP
2	6.42 ± 1.30	6.03 ± 1.79	5.10^{a} ± 1.34	6.85^{a,b} ± 0.92	6.97^{b} ± 1.35
3	12.41 ± 3.17	11.88 ± 3.34	8.99^{a} ± 2.45	13.97^{b} ± 1.41	13.87^{b} ± 2.56
4	18.53 ± 4.69	18.09 ± 4.47	13.36^{a} ± 4.44	20.54^{b} ± 1.87	20.49^{b} ± 3.00
5	24.04 ± 5.87	23.65 ± 6.22	19.13^{a} ± 6.22	27.29^{b} ± 3.40	25.46^{b} ± 4.37
6	30.76 ± 6.27	30.13 ± 3.85	26.96^{a} ± 3.97	34.24^{b} ± 3.46	30.40^{a,b} ± 6.40
7	38.85 ± 6.20	34.26 ± 4.11	33.15 ± 4.66	40.67 ± 4.57	39.54 ± 6.35
8	42.93^{c} ± 5.70	36.72^{d} ± 3.78	36.96^{a} ± 4.06	43.82^{b} ± 5.81	42.35^{a,b} ± 5.79
10	48.49^{c} ± 5.50	40.65^{d} ± 3.41	42.41 ± 4.10	49.81 ± 6.11	46.96 ± 6.32
12	50.26^{c} ± 5.75	42.56^{d} ± 2.71	44.14 ± 3.77	50.99 ± 6.66	49.49 ± 5.96
14	50.88^{c} ± 5.63	42.64^{d} ± 4.09	43.46 ± 4.94	51.86 ± 5.20	50.49 ± 6.47
16	52.24^{c} ± 5.54	42.04^{d} ± 3.98	44.16 ± 5.67	50.32 ± 6.63	54.02 ± 5.75
18	52.04^{c} ± 5.70	42.05^{d} ± 5.07	43.88 ± 6.29	52.85 ± 7.00	50.79 ± 5.50

LCP = Low concentration calcium and phosphorus diet. MCP= Medium concentration calcium and phosphorus diet. HCP = High concentration calcium and phosphorus diet.

^{a,b,c,d}Values with different superscripts within a row differ significantly ($P < 0.05$) from each other.

Statistical analysis—A commercially available software package[j] was used to analyze data from our study by use of either a 1- or 2-way ANOVA. Other tests included Student *t* test and regression analysis. For parametric data where significant differences were revealed by use of an ANOVA, Tukey and Kruskal-Wallis tests were performed to identify differences. Significance was determined by a value of $P < 0.05$.

Results

Food intake—No significant differences were found in food intake among groups at any time point in our study, either by absolute volume or when data were normalized for body weights.

Dual energy x-ray absorptiometry scans—Calculations of total body weight (kg) were made from total body scan output data (**Table 1**). Significant differences were found at 2, 3, 4, and 5 months of age, with puppies fed LCP weighing less than the puppies fed MCP or HCP. At 6 and 8 months of age, puppies fed LCP weighed significantly less than those fed the MCP. Differences between males and females became apparent at 8 months of age at which time male puppies were significantly heavier than females. Males remained heavier than females throughout the duration of our study (ie, 18 months).

At 2, 3, 4, 5, and 8 months of age, LCP-group dogs had significantly less lean mass than the MCP- and HCP-group dogs (**Table 2**). At 6 months of age, LCP-group dogs had less lean mass than MCP-group dogs. Significant differences by group were no longer evident after 8 months of age. Differences between males and females became apparent in the measurements of lean tissue at 10, 12, 16, and 18 months of age, with males having more lean tissue than females.

Puppies in the LCP group had less fat tissue than puppies in the MCP or HCP group at 2, 3, and 4 months of age (**Table 3**). Differences between males and females were also significant for this component of body composition at 8 and 10 months of age, with male puppies having more body fat that female puppies.

Differences among groups were apparent by 2 months of age, with the puppies in the LCP group having significantly less BMC than puppies fed MCP or HCP (**Table 4**). These differences persisted through 10 months of age. At 12 months of age, puppies in the HCP group had significantly more BMC than puppies in the LCP group. Differences between males and females were detected in BMC at 10 and 12 months of age, with males having more BMC than females.

Ventrodorsal scans of lumbar vertebrae (L1-L4) were analyzed for height, width, and area of each vertebra, with no differences in vertebral size detected among groups. However, the BMC of vertebrae was significantly different among groups at 2, 3, 5, 7, 8, and 10 months of age, with HCP group puppies having higher BMC in lumbar vertebrae (**Table 5**).

Comparisons of serial increases in the BMC, body weight, fat, and lean tissues of dogs were made during our study. Serial rates of increases of bone mineral content between 2 and 3 months of age were 130 to 230%, whereas the increases in body weights were only 75 to 100%. Percentage increases in bone mineral content

Table 2—Mean (± SD) serial DEXA measurements of lean tissue weight (kg) of growing Great Danes by sex and diet

	Sex		Diet		
Age (mo)	Male	Female	LCP	MCP	HCP
2	6.06 ± 1.20	5.71 ± 1.68	4.84 ± [a]1.26	6.47[b] ± 0.86	6.56[b] ± 1.27
3	11.36 ± 2.71	11.09 ± 3.04	8.44[a] ± 2.25	12.73[b] ± 1.00	12.77[b] ± 2.24
4	16.54 ± 3.84	16.48 ± 3.83	12.38[a] ± 3.93	18.25[b] ± 1.47	18.33[b] ± 2.26
5	20.99 ± 4.46	21.17 ± 5.28	17.14[a] ± 5.17	23.55[b] ± 2.34	22.62[b] ± 3.20
6	26.35 ± 4.15	26.80 ± 3.05	24.00[a] ± 3.03	28.88[b] ± 2.13	26.65[a,b] ± 4.36
7	32.24 ± 3.88	29.92 ± 3.29	28.75 ± 3.22	33.09 ± 3.21	33.68 ± 3.12
8	35.61 ± 3.16	32.07 ± 2.88	31.93[a] ± 2.73	35.76[b] ± 3.28	36.30[b] ± 2.67
10	39.59[c] ± 2.96	34.65[d] ± 2.78	36.06 ± 3.27	39.46 ± 3.63	39.49 ± 3.36
12	41.40[c] ± 3.42	36.14[d] ± 3.17	37.30 ± 3.57	41.02 ± 3.79	42.48 ± 3.56
14	42.65 ± 3.84	33.61 ± 8.73	34.56 ± 8.27	42.69 ± 3.96	43.44 ± 3.94
16	43.75[c] ± 3.50	33.86[d] ± 8.57	35.81 ± 8.64	42.33 ± 4.35	45.18 ± 4.07
18	44.49[c] ± 4.03	34.00[d] ± 8.51	36.18 ± 9.04	44.40 ± 5.68	43.94 ± 3.33

See Table 1 for key.

Table 3—Mean (± SD) serial DEXA measurements of fat tissue weight (kg) of growing Great Danes by sex and diet

	Sex		Diet		
Age (mo)	Male	Female	LCP	MCP	HCP
2	0.27 ± 0.07	0.24 ± 0.07	00.21[a] ± 0.06	0.28[b] ± 0.05	0.30[b] ± 0.07
3	0.78 ± 0.54	0.53 ± 0.19	0.42[a] ± 0.19	0.92[b] ± 0.59	0.73[a,b] ± 0.39
4	1.47 ± 0.93	1.14 ± 0.49	0.72[a] ± 0.47	1.75[b] ± 0.76	1.47[a,b] ± 0.80
5	2.31 ± 1.50	1.71 ± 0.82	1.50 ± 0.97	2.88 ± 1.35	1.91 ± 1.32
6	3.34 ± 2.12	2.31 ± 0.82	2.17 ± 0.96	4.18 ± 1.80	2.59 ± 2.24
7	5.17 ± 2.90	3.12 ± 1.05	3.32 ± 1.31	6.07 ± 2.98	4.22 ± 3.00
8	5.68[c] ± 2.79	3.33[d] ± 1.31	3.75 ± 1.27	6.44 ± 2.88	4.26 ± 3.02
10	6.96[c] ± 2.78	4.49[d] ± 2.28	4.80 ± 1.67	6.63 ± 2.76	5.44 ± 2.92

See Table 1 for key.

Table 4—Mean (± SD) serial DEXA measurements of bone mineral content (kg) of growing Great Danes by sex and diet

	Sex		Diet		
Age (mo)	Male	Female	LCP	MCP	HCP
2	0.09 ± 0.04	0.09 ± 0.04	0.054[a] ± 0.02	0.09[b] ± 0.02	0.12[b] ± 0.04
3	0.27 ± 0.13	0.27 ± 0.12	0.130[a] ± 0.05	0.32[b] ± 0.06	0.37[b] ± 0.09
4	0.53 ± 0.22	0.47 ± 0.24	0.263[a] ± 0.12	0.54[b] ± 0.19	0.68[b] ± 0.14
5	0.74 ± 0.28	0.77 ± 0.30	0.481[a] ± 0.22	0.87[b] ± 0.18	0.92[b] ± 0.20
6	1.06 ± 0.32	1.02 ± 0.21	0.790[a] ± 0.17	1.19[b] ± 0.19	1.17[b] ± 0.27
7	1.44 ± 0.36	1.22 ± 0.32	1.07[a] ± 0.22	1.52[b] ± 0.21	1.64[b] ± 0.30
8	1.64 ± 0.30	1.32 ± 0.32	1.29[a] ± 0.28	1.63[b] ± 0.26	1.78[b] ± 0.30
10	1.95[c] ± 0.29	1.50[d] ± 0.27	1.56[a] ± 0.29	1.95[b] ± 0.26	2.03[b] ± 0.31
12	2.08[c] ± 0.27	1.60[d] ± 0.25	1.70[a] ± 0.30	2.05[a,b] ± 0.29	2.18[b] ± 0.24

See Table 1 for key.

Table 5—Mean (± SD) serial DEXA measurements of bone mineral content (g) in L1 to L4 of growing Great Danes by diet

	Diet		
Age (mo)	LCP	MCP	HCP
2	1.60[a] ± 0.77	2.37[a] ± 0.57	3.01[b] ± 0.90
3	3.79[a] ± 1.53	7.18[b] ± 1.37	7.00[b] ± 3.73
4	7.18 ± 3.22	14.05 ± 3.02	15.16 ± 4.04
5	11.59[a] ± 5.36	20.41[a,b] ± 4.78	20.66[b] ± 6.00
6	17.41 ± 4.69	26.12 ± 7.57	25.85 ± 9.05
7	24.73[a] ± 6.58	34.37[a] ± 4.17	40.56[b] ± 10.63
8	31.69[a] ± 8.76	37.48[b] ± 10.82	44.93[b] ± 13.72
10	38.98[a] ± 10.96	49.39[a,b] ± 8.34	53.41[b] ± 16.05

See Table 1 for key.

Table 6—Mean (± SD) radial diaphyseal lengths (mm) in growing Great Danes by diet

		Diet		
Sex	Age (m)	LCP	MCP	HCP
Combined	2	78.6 ± 14.7 (11)	79.6 ± 5.5 (11)	80.0 ± 5.0 (10)
Male	2	82.1 ± 15.5 (8)	82.4 ± 4.7 (6)	79.9 ± 4.5 (7)
Female	2	68.0 ± 6.2 (3)	76.0 ± 4.7 (5)	79.8 ± 7.5 (3)
Combined	4	127.1 ± 13.8[a] (11)	140.8 ± 6.2[b] (11)	139.8 ± 7.7[b] (10)
Male	4	130.0 ± 15.2 (8)	143.4 ± 6.0 (6)	140.9 ± 8.4 (7)
Female	4	118.4 ± 9.1 (3)	136.5 ± 3.4 (5)	135.6 ± 8.3 (3)
Combined	6	169.1 ± 10.4[a] (11)	181.0 ± 11.0[b] (11)	175.4 ± 9.9 (10)
Male	6	170.6 ± 12.3 (8)	187.8 ± 3.9 (6)	179.6 ± 6.5 (6)
Female	6	163.1 ± 5.6 (3)	173.5 ± 12.9 (5)	171.9 ± 4.5 (3)

Numbers in parentheses indicate number of dogs.
See Table 1 for remainder of key.

achieved were greater than the increases in body weight until after 7 months of age. Serial increases in lean mass paralleled those of body weight.

Longitudinal bone growth—Diet effects on longitudinal bone growth were significant at 4 and 6 months of age when bones from puppies fed LCP were significantly shorter than those of puppies fed MCP and HCP (**Table 6**). Differences between males and females in bone length were found as early as 4 months of age. Radii of males were significantly longer than in females (diets combined) throughout the course of our study, with differences increasing from 4 months to 10 to 18 months of age. By 6 months of age, radii of males were longer than in females only in the MCP group. This group continued to have differences between males and females as they aged (males > females at 8, 10, and 12 months of age). Significant differences between males and females fed LCP were observed by 8 months of age and continued throughout the remainder of our study. Radii of males were longer than in females (8, 10, 12, 14, 16, and 18 months). Puppies fed HCP and MCP could not be evaluated on the basis of sex after 6 and 12 months of age, respectively, because too few female puppies were present within the groups. Measurements of ulna length yielded similar results, with similar differences at common time points (data not shown).

Clinicopathologic testing—The LCP-group dogs had total RBC counts and Hct that were greater than reference range values at 2 months of age and were significantly greater than those of the other diet-group dogs at 2 and 3 months of age (**Table 7**). Hemoglobin concentrations in LCP-group dogs were significantly higher than those of the other group dogs at 2 and 3 months of age. Mean corpuscular volume and mean corpuscular hemoglobin concentration were not significantly different among groups at any age.

Mean total WBC counts, mean absolute lymphocyte numbers, and mean absolute basophil numbers were within reference limits for all groups at each age. Absolute numbers of eosinophils were increased 2-fold above reference range in the HCP-group dogs at 2 and 3 months of age. The HCP-group dogs had significantly greater absolute monocyte counts when compared with LCP-group dogs at 3 months of age, but all values were within reference ranges. The HCP-group dogs had significantly greater absolute numbers of neutrophils than LCP-group dogs at 5 months of age and significantly greater numbers than the MCP-group dogs at 6 months of age; however, all mean values were within reference ranges at all time points. Mean platelet counts were within reference limits, and no significant differences were found among the 3 groups of dogs as they aged.

Serum biochemical analysis—Mean serum alkaline phosphatase activities were within reference ranges at all time points for HCP group dogs but were greater than reference range values in LCP- and MCP-group dogs at 3, 4, and 5 months of age (**Table 8**). Serum alanine transferase activities were within reference ranges for all groups at all time points evaluated. However, significant differences were found within the groups at 2, 3, 4, and 6 months of age. Puppies in the LCP group had significantly lower alanine transferase

activities than puppies in MCP and HCP groups at 2 and 3 months of age and than puppies in the MCP group at 4 months of age. Mean creatine phosphokinase activity, regardless of group, was high at 2 through 5 months of age, compared with values from clinically normal puppies of similar ages.[24] After 6 months of age, creatine phosphokinase activity was within reference range for similarly aged puppies.

Total serum bilirubin concentrations were within reference limits at all ages evaluated, and no significant differences were found among the groups (data not shown). Serum creatinine concentrations were within reference limits for all groups at all time points but were significantly higher in puppies in the LCP group, compared with those in the HCP group at 2 months of age and those in the MCP group at 3 months of age.

Mean blood urea nitrogen concentrations from the MCP- and HCP-group dogs were less than those of clinically normal puppies at 2 months of age. These values were lower than mean blood urea nitrogen concentration of LCP-group dogs (which had concentrations within reference range) at 2 and 3 months of age.

Total protein concentrations for puppies in the LCP group were greater than reference range values at 2, 3, 4, and 5 months of age. Mean protein concentration in LCP-group dogs was significantly greater than that of MCP- and HCP-group dogs at 2, 3, and 4 months of age.

In the LCP-group dogs, serum albumin concentrations were greater than reference range values at 2

Table 7—Mean (± SD) serial measurements of hematologic variables in growing Great Danes by diet

Variable	Age (mo)	Diet			RR values*
		LCP (n = 11)	MCP (n = 11)	HCP (n = 10)	
RBC (cells X 10^6/mm^3)	2	5.17[a] ± 0.31	4.11[b] ± 0.24	4.08[b] ± 0.22	3.3–4.5
	3	5.51[a] ± 0.58	4.70[b] ± 0.34	4.53[b] ± 0.35	4.7–6.3
Hematocrit (%)	2	33.2[a] ± 2.06	26.8[b] ± 1.24	26.13[b] ± 1.67	22–33.9
	3	34.58[a] ± 3.28	30.65[b] ± 2.56	29.13[b] ± 2.06	30.9–42.7
Hemoglobin (g/dl)	2	11.68[a] ± 0.62	9.55[b] ± 0.43	9.32[b] ± 0.53	7.4–10.3
	3	12.23[a] ± 1.23	10.83[b] ± 1.0	10.43[b] ± 0.76	11–15
Neutrophils (cells/mm^3)	2	8517 ± 2182	8782 ± 3886	9283 ± 3770	4769–13505
	3	7672[a] ± 2945	6698 ± 928	7488[b] ± 3295	4370–12500
	4	7631 ± 2972	7898 ± 2809	9490 ± 2859	4370–12500
	5	7078[a] ± 1541	7979 ± 3050	10962[b] ± 3780	4370–12500
	6	6839 ± 3264	5204[a]±1924	8976[b] ± 3320	4370–12500
Eosinophils (cells/mm^3)	2	243 ± 152	367 ± 316	2093 ± 1024	0–640
	3	275 ± 296	1210 ± 440	2157 ± 1291	0–978
	4	381 ± 159	1051 ± 657	1098 ± 736	0–978
Monocytes (cells/mm^3)	2	580 ± 312	1045 ± 1044	837 ± 630	0–366
	3	349[a] ± 342	655 ± 411	928[b] ± 470	0–445
	4	552 ± 282	465 ± 322	530 ± 400	0–445

*Values from reference 24, which are based blood samples from 200 puppies representing 11 breeds at each age group.
RR = Reference range.
See Table 1 for remainder of key.

Table 8—Mean (± SD) serial measurements of serum biochemical variables in growing Great Danes by diet

Variable	Age (mo)	Diet			RR values*
		LCP	MCP	HCP	
Alkaline phosphatase (U/L)	3	230.4 ± 83.5	204.9 ± 51	174.1 ± 33.6	77–199
	4	230 ± 62.8	207 ± 39.5	183 ± 51.8	77–199
	5	218.7 ± 44.6	201.7 ± 45.2	172.6 ± 47.3	77–199
Alanine transferase (U/L)	2	13.3[a]± 2.2	21.8[b] ± 3.7	21[b] ± 3.9	9–30
	3	17.5[a] ± 6.7	27.3[b] ± 7.9	29.4[b] ± 6.6	9–30
	4	26.5[a] ± 4.3	35.4[b] ± 7.1	37.1 ± 9.2	9–30
Creatine phosphokinase (U/L)	2	540.6 ± 133.2	462.5 ± 162.9	477.7 ± 86.3	103–391
	3	451.6 ± 51.6	457.8 ± 68.3	528.9 ± 114.5	34–272
	4	418.9 ± 82.4	418.2 ± 65.7	405.5 ± 159.9	34–272
	5	442.3 ± 143.7	349.3 ± 89.2	352.7 ± 151.6	34–272
Creatinine (mg/dl)	2	0.47[a] ± 0.06	0.40 ± 0	0.43[b] ± 0.05	0.3–0.8
	3	0.57[a] ± 0.05	0.51[b] ± 0.08	0.48 ± 0.08	0.3–0.9
Blood urea nitrogen (mg/dl)	2	8.36[a] ± 2.66	4.18[b] ± 1.99	4.5[b] ± 1.43	6.2–15.2
	3	14.73[a] ± 7.19	8.0[b] ± 3.55	9.7[b] ± 5.33	7–19
Total protein (g/dl)	2	5.52[a] ± 0.54	4.47[b] ± 0.2	4.74[b] ± 0.25	4.0–5.2
	3	5.90[a] ± 0.49	5.31[b] ± 0.64	5.23[b] ± 0.54	4.6–5.8
	4	5.90[a] ± 0.41	5.54[b] ± 0.29	5.44[b] ± 0.25	4.6–5.8
Albumin (g/dl)	2	2.97[a] ± 0.36	2.26[b] ± 0.24	2.25[b] ± 0.17	2.2–2.9
	3	2.98[a] ± 0.24	2.51[b] ± 0.31	2.37[b] ± 0.2	2.4–3.1
	4	2.02[a] ± 0.26	2.72[b] ± 0.25	2.55[b] ± 0.26	2.4–3.1
	5	3.06[a] ± 0.26	2.74 ± 0.23	2.39[b] ± 0.62	2.4–3.1

At 2, 3, and 4 months of age, data are based on 11 puppies in each LCP and MCP group and 10 puppies in the HCP group. At 5 and 6 months of age, data are based on 10 puppies in each LCP and MCP group and 9 puppies in the HCP group.
See Tables 1 and 7 for remainder of key.

months of age. At all other time points, mean serum albumin concentrations were within reference ranges for age. Serum albumin concentrations for the LCP-group dogs were greater than those of the MCP- and HCP-group dogs at 2, 3, and 4 months of age and those of the HCP-group dogs only at 5 months. Serum globulin concentrations were within reference range at all ages; no significant differences were found among groups (data not shown).

Serum calcium concentration—Mean total serum Ca concentrations decreased after 12 months of age, as has been shown previously (**Table 9**).[24,28] Mean total serum Ca concentrations were within reference ranges for age in the LCP- and MCP-groups. In the HCP group, total serum Ca concentrations were less than the reference range for puppies at 4, 5, 6, and 7 months of age. In HCP-group dogs, total serum Ca concentrations were significantly lower than those in MCP-group dogs at 4 months of age. At 5 months of age, HCP-group dogs had significantly lower total serum Ca concentrations than MCP- and LCP-group dogs.

Mean serum ionized Ca concentrations were lower in the LCP-group dogs than in MCP- and HCP-group dogs at 2 months of age. At 3 months of age, MCP-group dogs had mean serum ionized Ca concentrations that were higher than LCP- and HCP-group dogs. The MCP-group dogs had higher serum ionized Ca concentrations than did HCP-group dogs at 5 and 6 months of age. As shown previously,[28] albumin concentration affects the concentrations of ionized Ca and total Ca in blood.

Mean total serum inorganic P concentrations for HCP-group dogs were greater than the reference range values at 4 and 7 months of age. Mean total serum inorganic P concentrations for HCP-group dogs were significantly greater than those for the LCP-group dogs at 4 months of age.

Fractional excretion values were calculated for Ca and P (**Table 10**). Mean Ca fractional excretion was not significantly different among the 3 groups, except at 12 months of age when the HCP-group dogs excreted more Ca than the LCP- and MCP-group dogs (data not shown). Mean P fractional excretion in the LCP-group dogs was significantly less than that in the MCP- and HCP-group dogs at 2 and 3 months of age as well as lower than MCP-group dogs at 4 and 5 months of age. Mean fractional excretion of P in HCP-group dogs was significantly greater than that of MCP-group dogs at 3 months of age.

Table 9—Mean (± SD) serial measurements of serum calcium and phosphorus concentrations in growing Great Danes by diet

Variable	Age (m)	Diet			RR values*
		LCP	MCP	HCP	
Total calcium (mg/dl)	4	10.34 ± 0.54	10.82[a] ± 1.06	9.32[b] ± 1.29	9.8–12.4
	5	10.74 ± 0.49[a]	10.65[a] ± 0.57	8.96[b] ± 1.3	9.8–12.4
Ionized calcium (mmol/L)	2	1.48[a] ± 0.04	1.58[b] ± 0.02	1.60[b] ± 0.06	NA
	3	1.48[a] ± 0.05	1.60[b] ± 0.03	1.49[a] ± 0.16	NA
	4	1.50 ± 0.04	1.54 ± 0.03	1.33 ± 0.21	1.32–1.52
	5	1.44 ± 0.06	1.54[a] ± 0.06	1.36[b] ± 0.19	1.32–1.52
	6	1.47 ± 0.04	1.49[a] ± 0.06	1.38[b] ± 0.12	1.32–1.52
Total inorganic phosphorus (mg/dl)	4	8.89[a] ± 0.93	9.0 ± 0.66	10.37[b] ± 1.97	7.2–10.1
	6	9.46 ± 0.79	9.30 ± 1.41	8.86 ± 1.83	4.4–9.2
	7	9.56 ± 1.08	9.66 ± 0.86	9.96 ± 0.72	4.4–9.2

NA = Data not available. At 2, 3, and 4 months of age, data are based on 11 puppies in each LCP and MCP group and 10 puppies in the HCP group. At 5 and 6 months of age, data are based on 10 puppies in each LCP and MCP group and 9 puppies in the HCP group. At 7 months of age, data are based on 8 puppies in each LCP and MCP group and 10 puppies in the HCP group

See Tables 1 and 7 for remainder of key.

Table 10—Mean (± SD) serial measurements of urinary fractional excretion of calcium and phosphorus in growing Great Danes by diet

Variable	Age (m)	Diet			RR values*
		LCP	MCP	HCP	
Fractional excretion of calcium (%)	2	0.092 ± 0.059	0.064 ± 0.033	0.065 ± 0.058	0.42 ± 0.06 (9 wk)
	3	0.094 ± 0.085	0.082 ± 0.067	0.058 ± 0.051	0.53 ± 0.09 (13 wk)
	4	0.15 ± 0.14	0.195 ± 0.21	0.222 ± 0.32	0.44 ± 0.26 (17 wk)
	5	0.116 ± 0.06	0.082 ± 0.036	0.594 ± 0.82	0.58 ± 0.17 (21 wk)
	6	0.113 ± 0.08	0.167 ± 0.184	0.419 ± 0.934	0.48 ± 0.17 (25 wk)
Fractional excretion of phosphorus (%)	2	3.25[a] ± 4.3	14.9[b] ± 5.93	15.7[b] ± 3.3	22.92 ± 5.85 (9 wk)
	3	7.11[a] ± 6.45	12.9[b] ± 4.66	25.0[c] ± 9.3	23.19 ± 7.53 (13 wk)
	4	4.97[a] ± 5.61	11.6[b] ± 3.93	10.5 ± 3.87	27.44 ± 3.27 (17 wk)
	5	6.35[a] ± 6.59	12.6[b] ± 3.75	10.8 ± 3.99	37.69 ± 12.08 (21 wk)

At 2, 3, and 4 months of age, data are based on 11 puppies in each LCP and MCP group and 10 puppies in the HCP group. At 5 and 6 months of age, data are based on 10 puppies in each LCP and MCP group and 9 puppies in the HCP group.

*Values from reference 35, which are based on data from 6 healthy Beagle puppies fed a commercial puppy growth diet containing Ca and P in a ratio of 1.44:1.16.

RR = Reference range.

See Table 1 for remainder of key.

Serum insulin-like growth factor I concentrations—Mean serum IGF-1 concentrations were within reference range for all puppies. The only significant difference in serum IGF-1 concentrations among groups was at 3 months of age (684.0 ± 167.9 ng/ml for HCP-group dogs vs 538.9 ± 79.4 ng/ml for MCP-group dogs).

Serum parathyroid hormone concentrations—Serum PTH concentrations for individual puppies in our study varied greatly from month to month and were often higher than reported reference range values for clinically normal adult dogs.[27] Highest values (> 500 pg/ml) were found in puppies at 4, 5, and 6 months of age. However, by 12 months of age, only 4 of the 19 puppies evaluated had PTH values > 60 pg/ml. All 4 were male puppies, and all diet groups were represented. The highest concentration at 12 months of age (142 pg/ml) was seen in a puppy fed LCP. A significant difference in serum PTH concentration was found between the LCP- and MCP-group dogs at 3 months of age (175.8 ± 130.4 pg/ml for LCP-group dogs vs 47.3 ± 27.9 pg/ml for MCP-group dogs).

Discussion

Research into the proper amounts of dietary Ca has been on going since the 1930s,[29] yet absolute requirements for puppies of specific breeds and ages remain controversial, particularly because most testing has been done with small-breed dogs. Currently, AAFCO recommends a range of 1.0 to 2.5% Ca in canine foods formulated for growth. In general, less attention is paid to P concentration, and indeed P is not as closely regulated in the body. Phosphorus is plentiful in plants and animal tissues and is rarely deficient in the diet. The recommended range of P in diets formulated for growth is 0.8 to 1.6%. The accepted ideal ratio of Ca:P, 1.2:1 to 1.4:1,[21] is a narrow range, but the AAFCO allows a range of 1.0:1.0 to 2.0:1.0 in canine diets formulated for growth. In our study, a ratio of Ca to P at 1.2:1.0 was maintained in all of the diets. The maintenance of this ratio probably played an important role in the overall health and growth of the puppies by ameliorating the effects of excess or deficient Ca or P.

Comparison of previous studies to our study is difficult because of the interactions of other nutrients (ie, energy) in the diets. Effects of various concentrations of dietary Ca and P in a fixed ratio, in a diet containing moderate amounts of protein and energy and fed during the early rapid growth period in giant-breed puppies, had not previously been evaluated sufficiently. Complex interactions among nutrients influence growth. For instance, dogs fed ad libitum may have subclinical effects of excess energy,[10] whereas feeding fixed amounts to rapidly growing dogs may actually result in deficiencies of nutrients if careful adjustments for rates of growth and individual differences are not made.[6] To control for the effects of other components on growth and development, diets for our study were formulated to provide complete dietary needs according to the AAFCO, with moderate energy and protein content. The Ca and P content was altered while maintaining Ca and P in a fixed ratio.

In our study, food consumption of various diets was equal in absolute values and as a percentage of body weight. When compared with the Hedhammar study,[10] caloric intake in our study was determined to be slightly less than for puppies fed ad libitum in that study. However, the Ca and P contents as well as the ratio of Ca to P in the diets were greater in the Hedhammar study.

Evaluating growth and development in large puppies required serial assessment of growth variables. Traditionally, growth has been represented primarily by charts that indicate changes in total body weight with time. This method of evaluation, however, does not permit independent assessment of the rate of growth of various components of body composition such as fat accretion, muscle development, and skeletal mineralization. The recent adaptation of DEXA for use in companion animals has provided a precise and accurate method to evaluate alterations in body composition of dogs and cats.[22,23] The use of DEXA in the evaluation of growing cats indicates an unequal growth rate for muscle, bone, and fat.[23]

In our study, BMC increased at a greater rate than body weight, and body fat increased at a greater rate than lean mass until approximately 8 months of age when these rates become similar (percentage change data not shown). The unequal growth rate of these components is consistent with previous observations in kittens that revealed similar inequities in rates of growth of tissue components.[23] Results of our study indicate that in giant-breed dogs, valuable information can be obtained from examining the rates of growth of the various components of body composition when considering the effects of nutrition, treatments, and disease processes.

The serial measurements of BMC by use of total body DEXA provided strong evidence that Ca and P concentration in the diet had a rapid, direct effect on the amount of mineral in the maturing skeleton of Great Dane puppies. This was apparent in the initial DEXA scan results (Table 8). Puppies in our study began the weaning process naturally at 5 to 6 weeks of age and were fed appropriate diets, although testing was not scheduled to begin until puppies were 8 weeks of age. As a result, the total body DEXA scans obtained at 8 weeks of age reflected approximately 2.5 weeks of supplementation of bitch milk with respective diets. Our study followed the growth and development of puppies from 2 through 18 months of age, providing more information about the maturation of this giant breed and elucidating the maturation of tissues long after the 6-month period previously reported.[6] In our study, the magnitude of differences in the BMC among puppies on the various diets began to decline at 6 months of age and had disappeared completely by 12 months of age. These data suggest that the puppies began to regulate absorption and excretion of dietary Ca at 5 to 6 months of age and eventually produced BMC that were equal regardless of the diet fed.

Differences in weight gain, lean mass, and fat mass were also observed among puppies on diets varying in Ca and P concentration. Puppies fed LCP weighed significantly less than puppies fed MCP or HCP from 2 to

5 months of age. Body weight of puppies fed MCP or HCP were not significantly different throughout our study. At the conclusion of our study, puppies fed LCP still weighed approximately 6 kg less than those fed MCP or HCP. This was attributable in part to sex distribution, because there were 3 females in the LCP group and 1 female in each of the MCP and HCP groups. Evaluation of only male puppies at 18 months of age indicated that puppies in the LCP group weighed less (mean of 8 kg; approx 5 kg of lean tissue and 3 kg of fat) than dogs in the MCP and HCP groups. The reason for this is unclear, but the accretion of fat tissue was significantly greater in puppies fed MCP than in puppies fed LCP at the first DEXA scan, and the difference remained significant through 4 months of age. Differences in activity levels of the various groups of puppies that might have affected this measurement were not apparent, and serum biochemical analysis indicated that the dogs were not deficient in nutrients. Additionally, results of monthly physical examinations of dogs (data not shown) did not indicate clinical nutritional deficiencies.

Differences between males and females were observed after 8 to 10 months of age for all components quantified by use of DEXA. Male puppies had significantly greater body weight than females from 8 through 18 months of age. Similar differences were found for measurements of soft and lean tissue mass. The only time that whole body BMC was significantly different on the basis of sex was at 10 and 12 months of age, with males having a higher BMC than females. Fat tissue was significantly greater in male puppies than females at 8 and 10 months of age only; however, not enough female puppies were available after 12 months for complete analysis.

When lumbar vertebrae were examined for mineral accretion by use of DEXA, the results were similar to the whole body results as already described, suggesting that such a partial scan might be indicative of whole body trabecular bone mineral accretion. During the early part of our study, LCP-group puppies had significantly less BMC in the lumbar vertebral column than MCP- and HCP-group puppies. However, no differences were found among groups at 12 months of age or older.

Results of our study support the use of DEXA for dietary research in dogs. The sensitivity of DEXA in measuring BMC, its efficacy in analyzing soft-tissue components, the minimal radiation exposure when compared with standard radiography, and the opportunity for serial measurements in the same dog as it ages, or for a prescribed period, provide opportunities for longitudinal studies in nutrition, growth and development, disease progression, and response to treatment.

Radiographs of the antebrachium of all puppies were obtained on the same day as DEXA scans and were used to measure the lengths of the radii. Comparing physis-to-physis length of the radii, significant differences among groups were found only at 4 and 6 months of age as follows: MCP > HCP > LCP group dogs.

Clinical laboratory data were within reference range values reported for puppies for most variables and time points. Interestingly, significant differences in variables were found among groups when puppies were 2 to 6 months of age, the period of most rapid growth, suggesting a metabolic effect of mineral intake in the diet along with differences in bone mineralization. These differences occurred even though each group was comprised of puppies from 4 litters born 2 to 6 months apart and, therefore, placed on diets at staggered starting dates as they were weaned. This factor reduced the likelihood that alterations were the result of environmental factors such as housing or exposure to pathogens.

Insulin-like growth factor-1 is known to affect growth directly and has been considered a reliable, fairly stable indicator of growth hormone concentration in blood.[30] Hazewinkel et al[30] reported to the contrary that plasma IGF-1 concentration does not correlate well with growth hormone concentration but was influenced by dietary protein and Ca content in growing Great Danes from 7 to 27 weeks of age. They showed that a Ca:P ratio of 1.1:1.0 maintained IGF-I concentration, whereas concentrations of IGF-I decreased in puppies fed high concentrations of Ca with regular concentrations of P (Ca:P ratio, 3.3:0.9).[30] In our study, IGF-1 concentrations for all group dogs were similar at all ages tested, indicating that IGF-1 was not affected by alteration of dietary Ca and P concentrations when the ratio of these 2 minerals was held constant and when moderate amounts of protein and fat were fed. The IGF-1 concentrations rapidly increased from means of approximately 400 ng/ml at 2 months of age to > 650 ng/ml at 6 and 7 months, concentrations considerably higher than those reported by Hazewinkel et al.[30] The reason for this difference is unclear but cannot be attributed to insufficient dietary protein in our study. Concentrations decreased after 8 months of age so that by 18 months of age, mean concentrations were 300 to 400 ng/ml.

The LCP-group puppies had high Hct, total RBC counts, and total hemoglobin concentrations at 2 and 3 months of age. Hemoconcentration is most commonly attributed to dehydration, but no other biochemical variables supported dehydration as a cause. Differences in renal maturity could be a factor; however, other blood chemistry results appear to indicate that the kidneys of these dogs were mature at early ages. Adrenal function of our dogs was not examined. All puppies had ad libidum access to fresh water at all times. Because puppies were not maintained in metabolic cages, actual fluid intake and excretion was not determined, but no differences were found in specific gravities of urine samples collected.

Puppies fed HCP had significantly more eosinophils than puppies fed other diets and had more monocytes. Eosinophilia is most commonly seen in puppies with parasitic infections or with allergic conditions. Periodic deworming treatments make parasitic infection unlikely. Allergic inflammatory processes were not observed in these puppies during our study. Additionally, this pattern was seen in puppies fed HCP regardless of litter of origin.

An explanation for changes seen in RBC count in puppies fed LCP and in WBC count in puppies fed

HCP could be that dietary Ca and P concentrations affect bone marrow directly or indirectly. The balance of proliferation and maturation of various cell types in the bone marrow is influenced by the concentration of many factors within the microenvironment of the marrow cavity, including the active form of vitamin D, Ca, calcitonin, and PTH. Signaling mechanisms mediating the effects of erythropoietin on erythrocyte precursors are poorly understood, but appear to involve Ca and 1,25-dihydroxycholecalciferol as well as other cytokine factors.[31] The active form of vitamin D influences osteoclastogenesis and myelopoiesis by mechanisms that are not well defined.[32] Evaluation of bone marrow function was beyond the scope of our study.

The high serum alkaline phosphatase activity in LCP-group dogs up to 5 months of age and in MCP-group dogs at 3 and 4 months of age was not surprising. Others have shown that alkaline phosphatase activity increases in puppies as a reflection of the bone isozyme activity during rapid bone growth and remodeling.[33] Puppies fed HCP would not be as likely to remodel bone to maintain serum Ca concentration, thus accounting for the lower alkaline phosphatase activity in the serum of these puppies. The cause of significantly lower alkaline phosphatase activities found in the puppies fed LCP at 6 months of age is unclear but could reflect reduced bone production in these puppies.

Serum activities of creatine phosphokinase were greater than reference range values in puppies of all dietary groups from 2 to 5 months of age. Creatine phosphokinase activities increase rapidly after periods of high muscle activity, manipulation, or damage, as well as after halothane anesthesia.[34] These puppies were active and were excitable when handled. Radiographic and DEXA procedures required anesthesia for up to 2 hours, with manipulation of limbs to achieve appropriate positioning. Therefore, changes in creatine phosphokinase activity are likely to be induced by factors other than diet.

Blood urea nitrogen concentrations were less than reference range values at 2 months of age for MCP- and HCP-group puppies. Blood urea nitrogen concentrations for these groups were significantly lower than those for LCP-group puppies at 2 and 3 months of age. Total protein concentrations were significantly higher in the LCP-group puppies at 2, 3, and 4 months of age, compared with other group puppies, and this was attributable in large part to high serum albumin concentrations in LCP- group puppies at those times. These data suggest differences in protein metabolism as a result of differences in dietary Ca and P concentrations during times of rapid growth. Data derived from DEXA indicated that MCP- and HCP-group puppies have more of a rapid increase in lean tissue than did LCP-group puppies. These findings could also reflect diet-associated differences in functional maturation of liver and kidney, organs involved directly in protein metabolism and excretion. Regardless of the causes, these differences in protein and blood urea nitrogen concentrations were not observed after 5 months of age.

Serum Ca and P concentrations in these puppies were enigmatic. Although total serum Ca concentrations were within reference range for LCP- and MCP-group puppies, HCP-fed puppies had total serum Ca concentrations that were less than reference range values from 4 through 7 months of age. In part, this may reflect the lower concentrations of serum albumin in puppies fed HCP, but ionized Ca concentrations were also low in these puppies. The DEXA data indicate that HCP-group puppies had substantially more BMC at these ages, suggesting that they moved substantially more Ca out of the serum and into the bone. It is interesting to note that 88% of blood samples from LCP-group puppies, 49.4% from MCP-group puppies, and only 35.3% from HCP-group puppies had ionized Ca concentrations within reference limits as reported for puppies 4 to 12 months of age.[28] Almost all of the outlying values for MCP-group puppies were greater than reference range values. The outlying values for HCP-group puppies were more evenly divided between less than and greater than reference range values. Fractional excretion rates of Ca at 2 and 3 months of age indicated that none of the puppies in our study were eliminating this mineral in urine in amounts considered typical for Beagle puppies of the same age.[35] Among HCP-group puppies, Ca excretion gradually increased at 5 and 6 months of age to values similar to values reported for Beagles. Reference range values for fractional excretion of Ca for Great Dane puppies have not been reported; but the values reported here in our study for LCP- and MCP-fed puppies are similar to those reported for adult dogs ($0.15 \pm 0.13\%$).[36]

In addition to influences on skeletal development, an increase in Ca in the diet may produce long-term effects in the kidneys of growing puppies. High dietary concentrations of Ca combined with an imbalance in the Ca to P ratio produced by a static dietary concentration of P caused irreversible nephrocalcinosis in weanling rats.[37] In that study, rats fed high concentrations of Ca from 4 to 12 weeks of age retained 2 to 3 times more Ca in the kidneys than rats fed the same diet from 12 to 37 weeks of age. Similarly, Schoenmakers et al[38] reported Ca deposits in the kidneys of young Great Dane puppies fed diets high in Ca content.

Parathyroid hormone is produced in response to a need for an increase in Ca in the blood. One would expect that puppies with inadequate dietary Ca should have an increase in PTH hormone concentration. Our puppies had wide individual variations in serum PTH concentrations throughout the study, resulting in no significant differences among groups.

Serum P concentrations were greater than reference range values in HCP-group puppies from 4 to 7 months of age but were significantly different from that in the other 2 groups only at 4 months of age. Fractional excretion for P was significantly lower in LCP-group puppies at 4 to 6 months of age, suggesting impressive conservation of P by these puppies. DiBartola et al[36] reported mean fractional P excretion in adult dogs of various breeds to be $21 \pm 9\%$. In our study, only the HCP-group puppies had mean P excretion that equaled or exceeded this value, but data were quite variable from month to month. The greatest

excretion of P of 38.0 ± 22.7% was recorded in puppies at 14 months of age. The kidneys are the primary regulator of serum P homeostasis but are only indirectly involved in the mechanisms of Ca homeostasis. Although it is possible to explain diet-related effects on serum biochemical values in clinically normal adult dogs, it is difficult to predict the effect of dietary mineral content on the potentially immature organ systems of Great Dane puppies at 8, 12, and 16 weeks of age.

Schoenmakers et al[6,38] attempted to normalize diet after a prescribed feeding period of either excess or deficient Ca concentrations and concluded that Ca and P metabolism becomes balanced after diet normalization. The results of our studies indicate that as regulatory systems mature within Great Dane puppies, the normalization process occurs naturally. No change of diet was necessary to accomplish the balance of BMC in the maturing skeletons of our puppies. This is evidenced by the diminishing BMC differences seen among all puppies during the period of 6 to 12 months, while adjustments were being made within the skeletal system. After 12 months of age, no significant differences were found in BMC among all groups. Following the growth patterns of giant-breed dogs for periods of longer than 8 months, it is necessary to understand how giant breeds grow and mature. Although studies of shorter duration may be appropriate for smaller-breed dogs, our study clearly documents continually changing growth patterns during the first 12 months of life of Great Danes. It is important to recognize that mature body development is not achieved in Great Danes until 2 to 3 years of age. Maximum body weight for our puppies was not attained during the 18-month course of our study.

Although our study was expected to induce changes in growth patterns in the puppies fed contrived high concentrations of Ca and P, it should be noted that it is possible to purchase commercial pet foods that contain high concentrations of Ca and P, similar to those found in our study. In addition, these experimental conditions were designed with an optimal ratio of Ca to P, which likely ameliorated many of the possible adverse effects associated with high mineral content diets. It is common practice for many dog owners to add Ca supplements, eggs, cottage cheese, meats, or other table scraps to the commercially prepared diets of growing puppies. Such supplementation not only changes the absolute amounts of Ca and P in the diet but also alters the accepted ratio of Ca:P, which can further exacerbate the negative effects of improper amounts of these minerals.

[a]Merial Ltd, Iselin, NJ.
[b]Diet and feed analysis, The Iams Company, Dayton, Ohio.
[c]Lunar model DPX-L densitometer, GE Lunar Corp, Madison, Wis.
[d]Pediatric software version 1.5g, GE Lunar Corp, Madison, Wis.
[e]Adult Fast Mode, version 1.31, GE Lunar Corp, Madison, Wis.
[f]UMAX Mirage II scanner and UMAX MagicScan 4.3 software, UMAX Technologies Inc, Fremont, Calif.
[g]Scion Image, Scion Corp, Frederick, Md.
[h]Nichols Institute Diagnostics, #40–2100, San Juan Capistrano, Calif.
[i]Nichols Institute Diagnostics, #40–2170, San Juan Capistrano, Calif.
[j]Sigma Stat version 2.0 and Sigma Plot version 5.0, SPSS, Inc, Chicago, Ill.

Appendix 1

Maintenance diet* fed to breeding dogs

Variable	Amount
Crude protein (%)	25.5
Crude fat (%)	16.1
Crude fiber (%)	2.1
Moisture (%)	7.9
Energy (kcal/kg)	4380

*Primary ingredients: chicken, chicken by-product meal, rice flour, ground corn, ground grain sorghum, fish meal, chicken fat, dried beet pulp, chicken digest, dried egg product, and brewers dried yeast.

Appendix 2

Pregnancy and lactation diets* fed to dams

Variable	Amount
Crude protein (%)	31.48
Crude fat (%)	20.60
Crude fiber (%)	2.32
Calcium (%)	1.13
Phosphorus (%)	0.88
Moisture (%)	6.62
Energy (kcal/kg)	5145

*Primary ingredients: chicken, chicken by-product meal, ground corn, rice flour, fish meal, chicken fat, ground grain sorghum, dried beet pulp, chicken digest, dried egg product, brewers dried yeast, and flax.

Appendix 3

Diets* fed to puppies

	Amount		
Variable	LCP	MCP	HCP
Crude protein (%)	26.0	26.2	26.3
Crude fat (%)	14.8	14.8	14.7
Crude fiber (%)	2.1	1.5	1.4
Calcium (%)	0.47	0.78	2.67
Phosphorus (%)	0.38	0.67	2.27
Magnesium (%)	0.06	0.05	0.05
Zinc (mg/kg)	217.0	233.0	242.0
Copper (mg/kg)	23.6	26.5	25.8
Manganese (mg/kg)	47.6	47.6	51.5
Moisture (%)	8.9	7.9	7.8
Energy (kcal/kg)	4,897	4,911	4,907

*Primary ingredients: chicken, chicken by-product meal, ground corn, rice flour, ground grain sorghum, fish meal, dried beet pulp, chicken fat, chicken digest, dried egg product, and brewers yeast.
LCP = Low concentration Ca and P diet. MCP = Medium concentration Ca and P diet. HCP = High concentration Ca and P diet.

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