

# Evaluation of cognitive learning, memory, psychomotor, immunologic, and retinal functions in healthy puppies fed foods fortified with docosahexaenoic acid-rich fish oil from 8 to 52 weeks of age

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**Objective**—To assess effects of foods fortified with docosahexaenoic acid (DHA)-rich fish oil on cognitive, memory, psychomotor, immunologic, and retinal function and other measures of development in healthy puppies.

**Design**—Evaluation study.

**Animals**—48 Beagle puppies.

**Procedures**—Puppies were assigned to 3 groups after weaning (n = 16/group) and received 1 of 3 foods (low-DHA, moderate-DHA, or high-DHA food) as their sole source of nutrition until 1 year of age. Visual discrimination learning and memory tasks, psychomotor performance tasks, and physiologic tests including blood and serum analysis, electroretinography, and dual-energy x-ray absorptiometry were performed at various time points. Anti-rabies virus antibody titers were evaluated 1, 2, 4, and 8 weeks after vaccination at 16 weeks of age.

**Results**—Foods had similar proximate analysis results but varied in concentration of DHA from fish oil; the high-DHA food also contained higher concentrations of vitamin E, taurine, choline, and L-carnitine than did other foods. The high-DHA group had significantly better results for reversal task learning, visual contrast discrimination, and early psychomotor performance in side-to-side navigation through an obstacle-containing maze than did the moderate-DHA and low-DHA groups. The high-DHA group had significantly higher anti-rabies antibody titers 1 and 2 weeks after vaccination than did other groups. Peak b-wave amplitudes during scotopic electroretinography were positively correlated with serum DHA concentrations at all evaluated time points.

**Conclusions and Clinical Relevance**—Dietary fortification with fish oils rich in DHA and possibly other nutrients implicated in neurocognitive development following weaning improved cognitive, memory, psychomotor, immunologic, and retinal functions in growing dogs. (*J Am Vet Med Assoc* 2012;241:583–594)

Neurologic and physical development in growing dogs is influenced by genetic predisposition, environment, disease, and nutrition.<sup>1</sup> Nutritional interventions for neurocognitive development and maintenance, specifically learning, memory, and motor function tasks, in dogs have been undervalued, compared with nutritional interventions for humans.<sup>2</sup> In aged Beagles (mean age at the start of the study,  $\geq 8$  years old), complex mixtures of antioxidants added to maintenance-type foods have been shown to slow the onset of cognitive decline and associated neuropathologic changes.<sup>3,4</sup> Feeding growth foods supplemented with fish oils rich in DHA to bitches during gestation and lactation has been reported to improve trainability and ERG-recorded retinal activity in their puppies.<sup>5,6a</sup> Cognitive evaluations of puppies in

## ABBREVIATIONS

ALA	$\alpha$ -Linolenic acid
BALP	Bone alkaline phosphatase
BCS	Body condition score
BMD	Bone mineral density
DEXA	Dual-energy x-ray absorptiometry
DHA	Docosahexaenoic acid
DNMP	Delayed nonmatching to position
ERG	Electroretinography
LCPUFA	Long-chain polyunsaturated fatty acids
TGTA	Toronto general test apparatus

those studies usually included only 1 measure of cognitive function, and testing was performed at only 1 developmental time point.

The objective of the study reported here was to further increase our knowledge of the effectiveness of feeding foods fortified with specific nutrients, including DHA from fish oil, early in development by comparing the effects of 3 growth foods on learning, memory, psychomotor, immunologic (response to anti-rabies

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virus vaccination), and retinal functions in healthy Beagle puppies from weaning to 1 year of age. Other measures of health and development, including body weight, BCS, CBC, serum biochemical values, serum biomarkers of bone and cartilage growth, and DEXA-measured variables (body fat, lean mass, bone content, and BMD), were also evaluated. The rationale for selection of the foods used in the study was our intent to compare variables among puppies fed different foods (2 commercially available foods and 1 growth food that was not commercially available at the time of the study) with similar proximate analysis results but various concentrations of other assayable nutrients implicated in neurocognitive health (DHA from fish oil, choline, vitamin E, taurine, and L-carnitine).

Cognitive learning and memory test protocols were used to characterize the learning, memory, and psychomotor developmental processes in puppies and to determine associations between the foods fed to puppies and test outcomes. These included a series of tests previously developed for use in studying the cognitive decline associated with aging in dogs. Tests performed to assess cognitive learning and memory included visual learning tasks of discrimination and reversal learning,<sup>7</sup> an oddity discrimination task,<sup>8</sup> and a landmark discrimination learning test.<sup>9</sup> A short-term memory test, DNMP, was also performed.<sup>10</sup>

## Materials and Methods

**Animals**—All dogs (dams and puppies) were evaluated for signs of systemic disease by means of physical examination, CBC, serum biochemical analysis, urinalysis, and fecal examination for parasites. Dams and puppies were included in the study only if they were determined to be healthy on the basis of results of this evaluation. Puppies that had clinical signs of disease were excluded or removed from the study and received treatment appropriate for their disease condition. All dogs (including puppies  $\geq$  8 weeks old) were vaccinated against canine distemper virus, canine adenovirus-1 and canine adenovirus-2, canine parvovirus, and *Bordetella bronchiseptica* according to standard protocols, and all puppies were vaccinated against rabies virus beginning at 16 weeks of age.

Fourteen mature female Beagles were used for the breeding portion of the study. Dams were maintained in group housing at a licensed breeding facility until confirmed pregnant and then moved to maternity housing facilities for whelping. Following whelping, puppies and dams were maintained in the same housing until the puppies were 8 weeks old, at which time they were weaned. Weaned puppies were moved and housed in groups (4 puppies/room) in indoor runs (1.5  $\times$  5.2 m) with natural light that varied with seasonal changes and were fed once daily. Dogs were provided behavioral enrichment through interactions with each other, daily interaction and play with caretakers, daily opportunities to run and exercise outside, and access to toys. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Hill's Pet Nutrition Incorporated.

**Foods and group assignment**—For all foods, nutritional analysis was conducted in accordance with

standard food analytic procedures at a commercial laboratory.<sup>b</sup> Mature female Beagles were fed a commercially available low-DHA food adequate for gestation and lactation<sup>c</sup> for  $\geq$  2 weeks prior to conception and throughout gestation and lactation (Appendix). Puppies had access to the same food as the dam until weaning at 8 weeks of age.

Following weaning, 48 puppies (22 males and 26 females) were assigned to 3 groups. Assignment into these groups was controlled for maternal influences by assigning equal numbers of offspring from a given litter to each group. For example, if a dam had 8 puppies, only 6 were included in the study, with 2 puppies assigned to each group.

Each group was fed 1 of 3 foods (low-DHA,<sup>d</sup> moderate-DHA,<sup>e</sup> or high-DHA<sup>f</sup> food; Appendix). Sixteen puppies (7 males and 9 females) were assigned to the low-DHA food group, 16 (7 males and 9 females) were assigned to the moderate-DHA food group, and 16 (8 males and 8 females) were assigned to high-DHA food group.

**Feeding protocol**—The 2 commercial foods were repackaged in white paper bags, as was the test food, and all were coded with color-coded labels. Food was offered in portions in accordance with feeding guidelines copied from the original packaging. Puppies were fed individually within their group housing until they were 6 months old, at which time a group feeding protocol was instituted. Throughout the study, body weights were recorded weekly and food intake of the group-housed puppies was recorded daily. Body condition scoring<sup>11</sup> was performed on a weekly basis. Portions were adjusted to maintain BCS close to 3 on the 5-point scale, but portions were not allowed to be above or below the feeding guideline ranges recommended by the manufacturer on the bag.

**Blood sample collection and analysis**—During the 10-month test period, blood samples were collected from a jugular vein at predetermined time points from 7 weeks (1 week prior to weaning; baseline values) to 52 weeks of age. A CBC, serum biochemical analysis, and measurement of whole-blood taurine concentration were performed at 7, 12, 24, 36, and 52 weeks of age. Serum fatty acids, including DHA, and vitamin E concentrations were measured at 7, 12, 16, 24, 36, and 52 weeks of age. Anti-rabies virus antibody titers were measured at 16 weeks (immediately prior to vaccination against rabies virus) and at 17, 18, 20, and 24 weeks of age.

Bone and cartilage growth biomarkers were assessed at 7, 16, 24, 36, and 52 weeks of age. Serum samples were stored at  $-20^{\circ}\text{C}$  ( $4^{\circ}\text{F}$ ) in 1-mL aliquots until testing. Samples were analyzed for BALP activity<sup>g</sup> and serum concentrations of amino-terminal cross-linked telopeptide,<sup>h</sup> cartilage synthesis protein II,<sup>i</sup> total D-pyridinoline,<sup>j</sup> pyridinoline,<sup>k</sup> osteocalcin,<sup>l</sup> and carboxy-terminal cross-linked telopeptide<sup>m</sup> by use of commercially available kits in accordance with the manufacturers' instructions.

**Ophthalmic examinations and ERG**—At weaning, a complete ophthalmic examination was performed by a veterinary ophthalmologist to assess any ocular abnormalities, and all puppies were considered to have

normal results. At 2, 4, 6, 9, and 12 months of age, puppies underwent ERG of 1 eye (the same eye for each exam) for assessment of retinal development.

The ERG was performed with a handheld, portable machine that included a miniature Ganzfeld-type of stimulator.<sup>12,n</sup> Dogs were allowed to adapt to darkness for  $\geq 1$  hour and were then anesthetized with medetomidine<sup>o</sup> (0.1 mg/kg [0.045 mg/lb], IM) and ketamine<sup>p</sup> (5.0 mg/kg [2.27 mg/lb], IM) under red light. Pupils were maximally dilated with a topically applied 1% tropicamide solution,<sup>q</sup> and corneal anesthesia was induced with a topically applied 0.5% proparacaine hydrochloride solution.<sup>r</sup> A contact lens ERG electrode<sup>s</sup> was positioned on the eye and cushioned with a methylcellulose solution.<sup>t</sup> Subdermal platinum needle electrodes<sup>u</sup> were placed at the occiput and approximately 0.5 cm from the lateral canthus of the eye and served as ground and reference electrodes, respectively. The standard flash for the instrument (0 log) was 1.7 candelas/s/m<sup>2</sup> (produced by white LEDs). The flash intensity was adjustable in 0.3 log increments, from -3.0 to 1.2 log candelas/s/m<sup>2</sup>. For scotopic stimulation, -3.0 log, 0 log, and 1.2 log candelas/s/m<sup>2</sup> intensity settings of the standard flash were used. After 10 minutes of adaptation to light (produced by ordinary fluorescent lights in the room), photopic recordings were performed at 0 and 1.2 log candelas/s/m<sup>2</sup> and then responses to a 30-Hz flicker stimulation were obtained. All recordings were saved on the ERG unit and later transferred to a computer for further evaluation.

**DEXA**—Dual-energy x-ray absorptiometry<sup>v</sup> was performed in puppies at 2, 4, 6, 9, and 12 months of age. General anesthesia was induced as described for the ERG, and DEXA was performed according to standard protocols for body composition on the basis of recommendations by the manufacturer. Data collected via whole-body DEXA included measurements of lean mass, body fat, bone content, and BMD.

**Cognitive function tests**—Learning was assessed via a series of visual discrimination tasks for tests performed with a T-maze<sup>w</sup> or TGTA<sup>7</sup> throughout the study period. Correct choices were associated with access to a food reward; to obscure olfactory cues associated with correct choices, equal amounts of the food were allocated to incorrect choices but were inaccessible to puppies and thus could not serve as a reward. Short-term memory was tested with a food reward-associated DNMP task that also required a response to visual stimuli as previously described.<sup>7</sup> Puppies underwent 10 trials/d for 2-choice tasks and 12 trials/d for 3-choice tasks. To acquire a reward, puppies were required to physically move an object or door hiding the reward. To pass a task and continue to the next phase of study, puppies were required to have 90% correct responses in 1 day or 80% correct responses during 2 consecutive days. The total number of errors accumulated before passing the task was used for statistical analysis. All personnel responsible for cognitive testing of the dogs were blinded to food group assignments.

The food reward in all instances was a complete and balanced wet food appropriate for the puppy's life stage; the reward was standardized to approximately

1 g of as-fed weight, equivalent to approximately 250 mg of dry matter/reward. Most tasks had a maximum of 10 correct answers/d; thus, the maximum amount of daily food reward was 2.5 g of dry matter/dog. At weaning, the puppies' weight ranged from 2.5 to 4 kg (5.5 to 8.8 lb); on this basis, the daily ration intake for each puppy was approximately 100 to 150 g of dry matter/d of the food evaluated in the study. In general, the maximum amount of reward was calculated to total  $\leq 2.5\%$  of the daily food intake for each puppy.

#### T-MAZE TESTING

Cognitive learning function was first assessed with a positional T-maze paradigm at approximately 8 to 13 weeks of age. Before testing, puppies were taught how to access a food reward from a goal box in either arm of the maze. Puppies were then tested with a positional task in which they responded to 1 side of the maze to obtain a food reward in a goal box on that side. Once they learned this positional discrimination, a reversal task phase was initiated in which puppies were only rewarded for choosing the side opposite from the previously rewarded side (ie, nonpreferred side). The reversal procedure was then repeated, and the rewarded side was switched each time the puppy learned the new correct choice. The reversal and multiple reversal phases were used to assess learning associated with frontal lobe function representative of a higher level of learning than the original positional discrimination.

#### TGTA TESTING

The remaining cognitive learning assessment tests were performed with a TGTA.<sup>7</sup> The protocol consisted of an initial pretest training phase, in which puppies approximately 14 to 16 weeks of age were trained to approach and displace objects positioned before them to obtain the food reward in preparation for subsequent evaluative tests. At 16 to 20 weeks of age, puppies were tested with a simple object discrimination and reversal task.<sup>7-9</sup> A third cognitive testing protocol was used to evaluate discrimination learning at approximately 20 to 25 weeks of age; puppies were tested on 2 tasks, both of which required them to displace the odd object out of a set of 3.<sup>9</sup>

At approximately 27 to 33 weeks of age, contrast discrimination learning<sup>13</sup> was tested and compared among groups. Puppies were first trained and tested (with images at maximum [100%] contrast) to selectively respond to a card that showed either a black triangle or black circle, each on a white background. They were then tested with cards showing the same images in which contrast was reduced (25% contrast), with the triangle and circle as well as the background shown in shades of gray. A third component of the contrast discrimination test protocol examined each puppy's performance with images of varying contrast; in this test, 6 sets of object pairs (circle and triangle) were used that varied in contrast on a scale from 1% (minimum contrast) to 25% by different shades of gray for the object and background.

A DNMP test was used to evaluate short-term memory function in puppies approximately 33 to 44 weeks of age.<sup>10</sup> The protocol included both acquisi-

tion and memory assessment phases; in the acquisition phase, an object (red block) was placed before the puppy so that it was positioned to the left or right of midline and was associated with a reward when selected; the object was then removed from view for 5 seconds, and 2 identical blocks were presented with the novel position representing the correct choice associated with the reward. Dogs that passed the acquisition phase of the test moved to the memory assessment phase. In the memory assessment phase, the same protocol was used with delays of either 25 or 50 seconds.

The final cognitive learning test protocol examined the puppies' ability to perform a landmark discrimination task and evaluate performance of the 3 groups at approximately 44 to 51 weeks of age.<sup>9</sup> A landmark (yellow wooden peg) was placed on a food reward tray and 2 flat, round discs (coasters) were placed over food wells equidistant from the midline. Puppies were rewarded with food for responding to the coaster in closest proximity to the landmark. Landmark task 0 was defined as having the landmark indicate the center of the correct coaster; in successive tasks, the landmark was placed progressively farther away from the correct coaster and closer to the midline by 1 cm (task 1) or 2 cm (task 2) to increase the difficulty of the task.

**Psychomotor performance evaluation**—The puppies' motor skills were assessed on the basis of their ability to rapidly run through a T-maze containing obstacles. The time (in seconds) required to run through the maze and retrieve a food reward (ie, latency score) was used as a quantitative measure of psychomotor ability (sensory and motor coordination). Psychomotor testing was conducted at 3 time points (approx 3.3, 6.1, and 12.3 months of age). There were 2 sessions (in which cutouts and half panels were used) with 10 trials/session performed at each time point. Prior to the initial test session, puppies were given 2 practice sessions to ensure they were performing at a high level (mean time under 60 seconds) in the maze with no obstacles present.

In the first obstacle test, full-width panels with circular cutouts were placed in the runway (long arm) and branch (short arms perpendicular to the runway) sections of the T-shaped maze. Dogs had to navigate through the cutouts in the panels to enter one of the goal-box containing branches of the maze. The size and height of the panel holes were adjusted to adapt to the growth of puppies at each time point. The second obstacle test used half panels placed on opposite sides of the T-maze runway, requiring puppies to run in an S-shaped pattern (side to side) around the obstacles to reach the goal box. For both tasks, food rewards were placed inside the food wells of goal boxes in both branches of the maze. Latency (time in seconds) to retrieve 1 food reward from either goal box was used as the primary measure of psychomotor performance.

**Statistical analysis**—Data are reported as mean  $\pm$  SEM (cognitive learning) or mean  $\pm$  SD (all other tests). Data were analyzed by use of statistical software with various modules<sup>8</sup> as deemed appropriate for the data set and design of experiments. To minimize bias for cognitive tasks, data were analyzed by a statistician prior to re-

vealing the color-coded assignment groups. All *P* values were determined with 2-tailed tests. Values of  $P \leq 0.05$  were considered significant.

Data for weight, results of DEXA, and serum concentrations of bone and cartilage growth biomarkers (amino-terminal cross-linked telopeptide, cartilage synthesis protein II, BALP, total D-pyridinoline, pyridinoline, osteocalcin, and carboxy-terminal cross-linked telopeptide) were evaluated via ANOVA.<sup>x</sup> Data were compared among groups at the beginning and end of the study, with dietary treatment (food group assignment) used as the dependent variable.

Anti-rabies virus antibody titers, ERG data, and serum concentrations of DHA, vitamin E, and taurine were evaluated via a mixed procedure,<sup>x</sup> with time and treatment as variables in the model. Means separation was performed with Tukey-Kramer analysis for ERG data and with least squares means for the other data.

Cognitive and psychomotor test data were investigated with either single or repeated-measures ANOVA as appropriate, with food group used as a between-subject variable. In all situations, conformity with assumptions of normality and homogeneity of variance were assessed with a Shapiro-Wilk test (significance level set at  $\alpha = 0.05$ ) or Levene and (where appropriate) Box tests (both with  $\alpha = 0.001$ ), respectively. Failure to fully satisfy either assumption was addressed by applying the transformation (square root or logarithmic) that most reduced the number of violations. Significant results were explored further with pairwise comparisons performed via a Tukey least significant difference test, Dunnett test, or Tamhane T2 test, dependent on the heterogeneity of variances and adjusted for the number of comparisons (Bonferroni correction).

Associations between serum concentrations of DHA and contrast discrimination scores were evaluated via multiple regressions comparing the variation in the contrast response with the variation in circulating concentration of DHA.

## Results

Proximate analysis results were similar for the composition of crude protein, fat, calcium, and phosphorus on the basis of acceptable analytic variance as described by the Association of American Feed Control Officials.<sup>14</sup> Concentrations of other assayable nutrients, in addition to DHA from fish oil, varied among the 3 foods. The high-DHA food had higher concentrations of other nutrients implicated in neurocognitive development (vitamin E [DL- $\alpha$ -tocopherol], taurine, choline, and L-carnitine) than did the other foods (Appendix). Concentrations of vitamin E and taurine were higher in the moderate-DHA food than in the low-DHA food, and choline concentration was higher in the low-DHA food than in the moderate-DHA food.

All puppies assigned to the 3 food treatment groups on the basis of dietary DHA concentration (low-DHA, moderate-DHA, and high-DHA food) completed the study. There was no significant difference in weight or BCS among the 3 groups at the beginning or end of the study. Results of routine serum biochemical analysis and CBCs were within the laboratory reference ranges for all groups at all described time points during the

study; no statistical comparisons were made for these variables among groups.

**Circulating concentrations of DHA, vitamin E, and taurine**—Concentration of DHA in serum was not significantly different among groups prior to dietary intervention (Table 1). Concentration of DHA in serum from puppies of the high-DHA group was significantly greater than that from puppies of the low-DHA or moderate-DHA groups at all subsequent time points. In addition, concentration of DHA in serum was significantly greater in the moderate-DHA group than in the low-DHA group at all time points after baseline (7 weeks of age).

There was no significant difference in concentrations of vitamin E in serum or taurine in whole blood among groups prior to dietary intervention (Table 1). At all subsequent time points, puppies in the high-DHA group had significantly greater circulating concentrations of vitamin E and taurine, compared with those of the low-DHA and moderate-DHA groups, except for taurine concentrations at 12 weeks in the moderate-DHA group. The moderate-DHA group also had significantly higher concentrations of vitamin E in serum than did the low-DHA group at all time points after baseline. Finally, the moderate-DHA group also had significantly higher concentrations of taurine in whole blood at 24 and 36 weeks of age, compared with the low-DHA group.

**Immunologic response to vaccination against rabies virus**—Puppies in the high-DHA group had significantly higher serum anti-rabies virus antibody titer responses (12.4 and 13.7 U/mL) than did those in the moderate-DHA (1.5 and 3.7 U/mL) and low-DHA (2.4 and 3.5 U/mL) groups 1 and 2 weeks after vaccination, respectively (Figure 1). However, values were not significantly different at  $\geq 4$  weeks after vaccination.

**Analysis of biomarkers of bone and cartilage growth**—Serum BALP activity was not significantly dif-

ferent among groups at baseline. However, at all subsequent time points, puppies of the high-DHA and moderate-DHA groups had significantly lower activities of BALP than did those of the low-DHA group (Table 2). No differences between the moderate-DHA and high-DHA groups were detected for this variable.

Concentration of cartilage synthesis protein II in serum was not significantly different among puppies of the 3 groups at baseline or any other time point until 36 weeks of age (Table 2). However, the concentration of this protein in the high-DHA group was significantly less than that in the low-DHA group at 36 and 52 weeks of age, and concentration in the moderate-DHA group was significantly less than that in the low-DHA group at 52 weeks. There were no differences among groups in the concen-

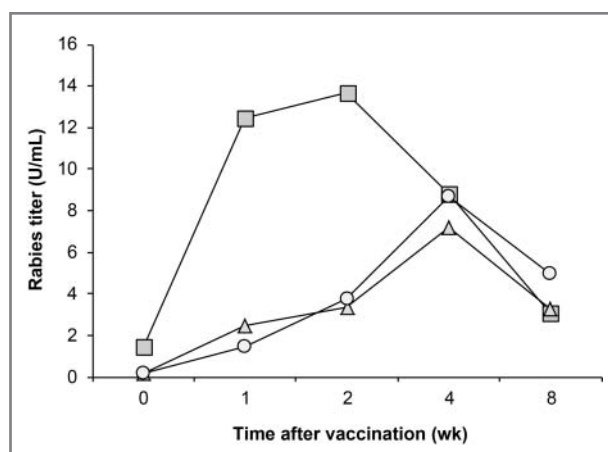


Figure 1—Mean serum anti-rabies antibody titers (U/mL) following rabies vaccination at 16 weeks of age in 48 Beagle puppies assigned to 1 of 3 dietary treatment groups (low DHA [triangles], moderate DHA [circles], or high DHA [squares];  $n = 16/\text{group}$ ). The foods had similar proximate analysis results but concentrations of other assayable nutrients varied among the 3 diets. Puppies in the high-DHA group had a significantly ( $P < 0.05$ ) greater overall mean antibody response than did puppies in the low-DHA or moderate-DHA groups as determined via mixed-procedure statistical analysis.

Table 1—Mean  $\pm$  SD serum concentrations of DHA and vitamin E and whole-blood concentrations of taurine in 48 Beagle puppies fed 1 of 3 foods from weaning to 52 weeks of age.

Variable	Group	Baseline	Age (wk)				
			12	16	24	36	52
DHA ( $\mu\text{g/L}$ )	Low-DHA	2.3 $\pm$ 0.6*	3.4 $\pm$ 0.9*	4.7 $\pm$ 1.5*	4.3 $\pm$ 2.4*	3.8 $\pm$ 1.7*	4.3 $\pm$ 4.1*
	Moderate-DHA	2.4 $\pm$ 0.7*	13.6 $\pm$ 2.5†	15.4 $\pm$ 2.9†	15.0 $\pm$ 4.6†	12.8 $\pm$ 2.4†	12.9 $\pm$ 2.7†
	High-DHA	2.3 $\pm$ 0.6*	19.0 $\pm$ 5.3‡	24.2 $\pm$ 7.4‡	24.4 $\pm$ 5.1‡	21.9 $\pm$ 4.6‡	20.3 $\pm$ 6‡
Vitamin E ( $\mu\text{g/mL}$ )	Low-DHA	14.5 $\pm$ 2.8*	10.8 $\pm$ 1.9*	13.2 $\pm$ 4.1*	14.5 $\pm$ 2.2*	15.7 $\pm$ 2.7*	16.8 $\pm$ 2.9*
	Moderate-DHA	14.4 $\pm$ 3.3*	19.7 $\pm$ 4.9†	21.0 $\pm$ 4.2†	22.3 $\pm$ 3.4†	20.6 $\pm$ 2.8†	21.9 $\pm$ 2.3†
	High-DHA	14.4 $\pm$ 2.7*	26.1 $\pm$ 7.5‡	32.1 $\pm$ 21.1‡	36.9 $\pm$ 28.5‡	33.3 $\pm$ 6.8‡	33.4 $\pm$ 6.8‡
Taurine ( $\mu\text{mol/L}$ )	Low-DHA	354 $\pm$ 112*	328 $\pm$ 91*	—	302 $\pm$ 38*	298 $\pm$ 24*	289 $\pm$ 26*
	Moderate-DHA	337 $\pm$ 45*	369 $\pm$ 55*†	—	331 $\pm$ 30†	322 $\pm$ 29†	298 $\pm$ 26*
	High-DHA	342 $\pm$ 65*	409 $\pm$ 66†	—	371 $\pm$ 43‡	360 $\pm$ 27‡	334 $\pm$ 39†

At weaning, puppies were assigned to dietary treatment groups (low-DHA, moderate-DHA, or high-DHA group;  $n = 16/\text{group}$ ). Baseline values were obtained at 7 weeks of age (1 week prior to weaning). Proximate analysis results were similar, but concentrations of other assayable nutrients varied among the 3 foods. The high-DHA food had higher concentrations of other nutrients implicated in neurocognitive development (vitamin E, taurine, choline, and L-carnitine) than did the other foods; concentrations of vitamin E and taurine were higher in the moderate-DHA food than in the low-DHA food, and choline concentration was higher in the low-DHA food than in the moderate-DHA food.

\*†‡Within a variable for a given time point, values with different superscripts are significantly ( $P < 0.05$ ) different among groups as determined via least squares means.

— = Not applicable.

trations of amino-terminal cross-linked telopeptide, total D-pyridinoline, pyridinoline, osteocalcin, or carboxy-terminal cross-linked telopeptide in serum at any time point.

**DEXA**—There were no significant differences among groups for any DEXA-measured variables (body fat, lean mass, bone content, and BMD) at any time points, except for body fat differences between moderate-DHA and high-DHA groups at 4 months of age. In addition, no significant differences for any of these variables were detected when values were compared at baseline and 12 months of age. Absolute lean mass and body fat content were summarized to show relative growth rates in puppies of the 3 groups (Table 3).

**ERG**—A significant effect of time for all measured variables of the ERG analysis was observed. Significant group effects were revealed for peak b-wave amplitude during scotopic ERG at 1.2 log candelas/s/m<sup>2</sup>

stimulation at 4, 6, and 12 months of age and at 0 log candelas/s/m<sup>2</sup> stimulation at 6 months of age (Table 4). In addition, peak b-wave amplitudes measured at both intensities had a significant group-by-time interaction; the b-wave amplitudes of puppies in the high-DHA and moderate-DHA groups were significantly higher than those in the low-DHA group at both intensities, while no difference was detected between the high-DHA and moderate-DHA groups.

Concentration of DHA in serum and peak b-wave amplitude during scotopic ERG at 1.2 log candelas/s/m<sup>2</sup> stimulation were significantly correlated at all time points ( $P < 0.001$ ;  $r^2 = 0.21$ ). No significant differences were detected for any of the other ERG variables measured (photopic responses, a-wave amplitudes, implicit times, or flicker fusion) among groups.

**Cognitive function tests**—Relevant results of cognitive learning assessment and short-term memory

Table 2—Mean  $\pm$  SD serum activities of BALP and concentrations of cartilage synthesis protein II in the same 48 puppies in Table 1.

Biomarker	Group	Baseline	Age (wk)				
			16	24	36	52	
BALP (U/L)	Low-DHA	142 $\pm$ 38*	98 $\pm$ 30†	66 $\pm$ 15†	44 $\pm$ 10†	30 $\pm$ 10†	
	Moderate-DHA	132 $\pm$ 30*	76 $\pm$ 15*	53 $\pm$ 12*	37 $\pm$ 7*	23 $\pm$ 3*	
	High-DHA	127 $\pm$ 22*	72 $\pm$ 15*	50 $\pm$ 10*	36 $\pm$ 8*	23 $\pm$ 5*	
Cartilage synthesis protein II (ng/mL)	Low-DHA	667 $\pm$ 116*	871 $\pm$ 135*	1,044 $\pm$ 159*	820 $\pm$ 127†	991 $\pm$ 110†	
	Moderate-DHA	673 $\pm$ 115*	811 $\pm$ 111*	1,002 $\pm$ 247*	786 $\pm$ 59*†	863 $\pm$ 109*	
	High-DHA	650 $\pm$ 54*	825 $\pm$ 130*	1,100 $\pm$ 287*	742 $\pm$ 104*	908 $\pm$ 122*	

\*†For a given biomarker, values with different superscripts within a column are significantly different among groups as determined via least squares means.  
See Table 1 for remainder of key.

Table 3—Mean  $\pm$  SD results of DEXA analysis of lean mass and body fat content in the same 48 puppies in Table 1.

Variable	Group	Age (mo)				
		2	4	6	9	12
Lean mass (kg)	Low-DHA	2.65 $\pm$ 0.53*	5.25 $\pm$ 0.84*	7.20 $\pm$ 1.09*	8.13 $\pm$ 1.31*	8.49 $\pm$ 1.40*
	Moderate-DHA	2.49 $\pm$ 0.61*	5.14 $\pm$ 0.94*	6.93 $\pm$ 1.11*	7.80 $\pm$ 1.16*	7.99 $\pm$ 1.12*
	High-DHA	2.54 $\pm$ 0.43*	5.10 $\pm$ 0.81*	7.03 $\pm$ 0.80*	8.14 $\pm$ 0.96*	8.39 $\pm$ 0.90*
Body fat (kg)	Low-DHA	0.29 $\pm$ 0.16*	0.81 $\pm$ 0.32*†	1.76 $\pm$ 0.75*	2.88 $\pm$ 1.30*	2.96 $\pm$ 1.51*
	Moderate-DHA	0.25 $\pm$ 0.14*	0.94 $\pm$ 0.34†	1.77 $\pm$ 0.60*	2.74 $\pm$ 0.98*	2.42 $\pm$ 1.17*
	High-DHA	0.26 $\pm$ 0.13*	0.67 $\pm$ 0.32*	1.59 $\pm$ 0.82*	2.63 $\pm$ 1.27*	2.47 $\pm$ 1.15*

See Table 1 for key.

Table 4—Mean  $\pm$  SD peak b-wave amplitudes in the same 48 puppies in Table 1 during scotopic ERG.

Flash intensity (log candelas/m <sup>2</sup> )	Group	Age (mo)				
		2	4	6	9	12
1.2	Low-DHA	43 $\pm$ 1.2*	232 $\pm$ 14*	217 $\pm$ 13*	197 $\pm$ 14*	195 $\pm$ 6*
	Moderate-DHA	41 $\pm$ 1.2*	271 $\pm$ 14†	259 $\pm$ 13†	232 $\pm$ 14*	256 $\pm$ 16†
	High-DHA	40 $\pm$ 1.2*	276 $\pm$ 14†	253 $\pm$ 13†	230 $\pm$ 14*	214 $\pm$ 16*
0	Low-DHA	44 $\pm$ 7*	316 $\pm$ 64*	309 $\pm$ 59*	290 $\pm$ 59*	313 $\pm$ 65*
	Moderate-DHA	46 $\pm$ 7*	336 $\pm$ 57*	348 $\pm$ 78*	286 $\pm$ 65*	317 $\pm$ 63*
	High-DHA	43 $\pm$ 6*	349 $\pm$ 80*	368 $\pm$ 83†	318 $\pm$ 52*	328 $\pm$ 94*

The standard flash intensity for the instrument (0 log) was 1.7 candelas/s/m<sup>2</sup>.  
\*†For each intensity for a given time point, means with different superscripts are significantly different among groups as determined via a Tukey-Kramer test.

function tests performed via T-maze and TGTA testing were summarized (Tables 5 and 6).

#### T-MAZE TESTING

Group effects were nonsignificant for the positional learning task; however, a significant effect for group was found for the reversal task (Table 5). The mean number of errors for the reversal task was significantly lower for the high-DHA and moderate-DHA groups, compared with the low-DHA group. There was a significant effect of task (positional vs reversal) that was a result of increased errors to acquire the reversal for all groups, compared with initial learning of the positional task. Additionally, a significant task-by-group interaction was found. Analysis of the number of reversals successfully acquired during multiple reversal testing revealed that the high-DHA group acquired significantly more reversals than did the moderate-DHA group, but values were not significantly different between the high-DHA and low-DHA groups ( $P = 0.10$ ).

#### TGTA TESTING

There were no significant differences among groups for results of simple object discrimination tasks. In addition, no significant differences were detected via regression analysis of associations between serum DHA concentration and simple object discrimination results. There were no significant group effects for either of the 2 oddity discrimination tasks as determined by analysis of correct choices according to criterion.

For contrast discrimination testing performed at 27 to 33 weeks of age, means separation analysis revealed that the high-DHA group had significantly better results (mean  $\pm$  SEM number of errors,  $42.8 \pm 8.6$ ) than did the moderate-DHA ( $72.1 \pm 11.7$ ) or low-DHA ( $72.2 \pm 11.2$ ) groups when evaluated by use of the maximal contrast image. Multiple regression analysis revealed a significant ( $P < 0.01$ ;  $r^2 = 0.21$ ) relationship between serum DHA concentration and error scores for maximal contrast discrimination.

In DNMP tests of short-term memory function, all puppies successfully completed the acquisition phase and no significant group effects were detected. There were also no significant differences detected among groups for the memory phase of the evaluation.

Evaluation of combined errors for landmark discrimination tasks 0, 1, and 2 revealed significant differences among groups. Analysis of task errors revealed that puppies of all groups performed landmark discrimination task 2 with significantly fewer errors than for task 0 or 1, yielding a significant task effect. Means separation analysis revealed that puppies in the high-DHA group had significantly fewer errors than did puppies in the moderate-DHA group for tasks 0 and 2, but these values were not significantly different from those of the low-DHA group.

**Psychomotor tests**—Effects of group over time were examined by comparing results for the first (circular cutout) and second (side-to-side) T-maze obstacle navigation tests among the 3 groups at 3, 6, and 12 months of age (Figure 2). For cutout ob-

Table 5—Mean  $\pm$  SEM number of errors in performance of positional and reversal tasks and total number of reversals acquired for the same 48 puppies in Table 1 during T-maze testing at approximately 8 to 13 weeks of age.

Group	Positional task errors	Reversal task errors	No. of reversals acquired
Low-DHA	2.5 $\pm$ 1.2*	16.8 $\pm$ 1.6*	6.8 $\pm$ 0.4*†
Moderate-DHA	2.9 $\pm$ 1.0*	11.3 $\pm$ 1.4†	6.6 $\pm$ 0.2*
High-DHA	3.4 $\pm$ 1.3*	9.5 $\pm$ 1.4†	7.7 $\pm$ 0.4†

After a preliminary training period, positional task assessment was performed in which puppies responded to one side of a T-shaped maze to obtain a food reward concealed in a goal box. For the reversal task, puppies were only rewarded for choosing the side opposite from the previously rewarded side. The number of errors in selection of the correct side was counted for a total of 10 attempts/d for each task until a passing score was attained. For multiple reversal testing, the rewarded side was switched each time the puppy learned the new correct choice; the maximum number of successful reversals possible was 10.

\*†Within a column, values with different superscripts are significantly different among groups as determined via least squares means.

Table 6—Mean  $\pm$  SEM number of errors in performance of landmark discrimination tasks for the same 48 puppies in Table 1 at approximately 44 to 51 weeks of age.

Group	Landmark task			
	0	1	2	All (combined)
Low-DHA	28.5 $\pm$ 3.6*†	36.8 $\pm$ 3.9*	12.4 $\pm$ 1.7*†	25.9 $\pm$ 2.5*
Moderate-DHA	32.2 $\pm$ 2.4†	38 $\pm$ 3.9*	14.3 $\pm$ 1.4†	28.2 $\pm$ 2.5*
High-DHA	21.9 $\pm$ 3*	27.7 $\pm$ 3.8*	9.2 $\pm$ 1.3*	19.6 $\pm$ 2.5†

After a preliminary training period, a landmark (rectangular peg) was placed in variable locations on a food tray along with 2 flat, round discs (coasters) placed over 2 food wells. The landmark was placed on top of (task 0) or 1 or 2 cm away (tasks 1 and 2, respectively) from the landmark toward the midline between the coasters. Puppies received a food reward for selecting the coaster that was closest to the landmark.

See Table 5 for remainder of key.

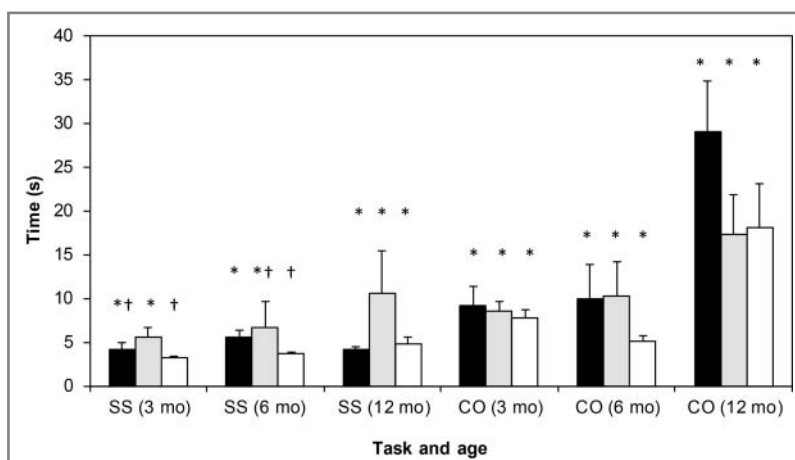


Figure 2—Mean  $\pm$  SD time for psychomotor task completion of the same 48 puppies in Figure 1 at 3, 6, and 12 months of age. Two timed tasks required puppies in the 3 groups (low DHA [black bars], moderate DHA [gray bars], or high DHA [white bars]) to traverse a T-maze containing panels; in the first task, solid panels containing circular cutouts were placed in the maze in a manner that required puppies to pass through the cutouts to complete the maze for a food reward. In the second task, solid panels were placed in a manner that required side-to-side maneuvering to complete the maze. \*†Within a time period, values with different symbols are significantly different between groups. CO = Cutout. SS = Side to side.

stacle navigation tests, analysis revealed significant effects of age and trials and a significant age-by-trials interaction; however, there was no significant effect of group on these results at any time point. Significant effects of trials revealed within-session differences, generally indicating that the time required for puppies to complete the test decreased between trial 1 and trial 10.

Analysis of side-to-side obstacle navigation test results at 3, 6, and 12 months of age revealed a significant age-by-group interaction. Means separation comparisons at each time point indicated that at 3 months of age, puppies of the high-DHA group required significantly less time to complete the course (mean  $\pm$  SD, 3.2  $\pm$  0.1 seconds) than did those of the moderate-DHA group (5.6  $\pm$  1.1 seconds), but time for the high-DHA group was not significantly different from that of the low-DHA group (4.2  $\pm$  0.8 seconds). At 6 months of age, puppies of the low-DHA group required significantly more time to complete the course (mean  $\pm$  SD, 5.5  $\pm$  0.8 seconds) than did those of the high-DHA group (3.6  $\pm$  0.3 seconds), but time for the high-DHA group was not significantly shorter than that for the moderate-DHA group (6.6  $\pm$  3.1 seconds) even though the numeric value was lower. No differences were detected among groups for this task at 12 months of age.

## Discussion

Nutritional adequacy has been historically defined as the amount of a nutrient required to support a desired outcome for a selected physiologic variable when other nutrients are supplied at required concentrations in the diet. Previous nutritional studies have typically used maximized aspects of growth in young growing animals or reproductive performance in adult animals as the defining factor for determining nutritional adequacy. Recently, the use of nutrients at concentrations in excess of historically defined minimum requirements or in combination with other nutrients has been advocated to maximize physiologic outcomes other than growth, such as physical development, immunologic responses, and cognitive function.

For example, antioxidants are proposed to mitigate the effects of free radicals via complex networks with multiple steps, which may require multiple fortifications at each point of the network to achieve a maximal response of the system.<sup>15</sup> In addition, vitamin E in supraphysiological doses has been shown to delay the onset of institutionalization in humans with Alzheimer's disease.<sup>16</sup> In aging dogs, a complex mixture of antioxidants has been reported to slow the progression of cognitive decline.<sup>4,17</sup>

Results of the study reported here support the premise that fortification of a complex food with concentrations of specific nutrients in excess of the minimum daily recommended amounts determined by the Association of American Feed Control Officials may enhance specific physiologic outcomes in healthy, growing puppies. In the present study, 3 foods (2 that were commercially available at the time of the study [low DHA<sup>d</sup> and moderate DHA<sup>e</sup>] and 1 that was not [high DHA<sup>f</sup>]) were fed to Beagle puppies after weaning (from 8 to 52 weeks of age). These foods had similar

proximate analysis results but contained various concentrations of nutrients considered to support learning, memory, and eye development (choline, DHA from fish oil, vitamin E [DL- $\alpha$ -tocopherol], taurine, and L-carnitine) and immunologic function (vitamin E) and also to modulate oxidative stress (vitamin E).<sup>1,4,5,12,13,18-20</sup> The high-DHA food contained higher concentrations of L-carnitine, vitamin E, choline, and taurine than did the other 2 foods; concentrations of vitamin E and taurine were higher in the moderate-DHA food than in the low-DHA food, and choline concentration was higher in the low-DHA food than in the moderate-DHA food.

Our results suggested various benefits of the high-DHA food, compared with the other foods evaluated. Detailed evaluation of specific nutrients via multiple regression analysis indicated that serum concentration of DHA was positively correlated with results of contrast discrimination tests and retinal function (peak b-wave amplitude during scotopic ERG) outcomes, even in the presence of a complex background matrix.

Gross development of the canine brain is extremely rapid during the first 4 weeks after birth and then slows considerably until reaching adult size.<sup>21</sup> To our knowledge, the effects of diet on such morphological changes have not been evaluated in dogs. However, inclusion of fish oil rich in n-3 fatty acids in maternal foods has been shown to increase learning ability and ERG-assessed retinal function in growing puppies.<sup>5,a</sup> In the present study, we assessed cognitive (visual discrimination learning), memory, and psychomotor function in puppies throughout the first year of life using tests that have been extensively used in adult dogs and evaluated the influence of postweaning dietary treatments on results of those tests as well as ERG outcomes.

Supplementation of foods fed during gestation and lactation and before weaning with fish oil rich in DHA has been reported to improve the response to training in young puppies, suggesting improved cognitive development.<sup>4,v</sup> Results of the present study suggest that dietary supplementation with fish oil rich in DHA after weaning may also have a positive impact on cognitive development as measured by a series of cognitive function tests. It should be noted that the effects attributed to DHA may in fact be attributable to other nutrients (eg, eicosapentaenoic acid) that are also found in fish oils. As such, the DHA content of the 3 foods evaluated should be considered to reflect the fish oil content of those foods.

Results of T-maze testing at approximately 8 to 13 weeks of age indicated that the reversal task, a test of learning associated with frontal lobe function, was performed with significantly fewer errors by puppies of the high-DHA and moderate-DHA groups, compared with results for puppies in the low-DHA group. Further evaluation by means of multiple reversal task testing showed that puppies in the high-DHA group performed this task significantly better than did puppies in the moderate-DHA group but not significantly ( $P = 0.10$ ) better than the low-DHA group. This was probably attributable to the variation being higher than anticipated and therefore a lack of power.

Contrast discrimination testing at 27 to 33 weeks of age and landmark discrimination testing at 44 to 51



weeks of age both had the same general results as T-maze testing, suggesting that the learning differences identified early after weaning were still detectable up to 1 year of age. Multiple regression analysis of maximal contrast discrimination data also revealed a significant positive association between serum concentrations of DHA and improved task performance (ie, fewer errors) at all time points. Inconsistency in outcomes of cognitive tasks was observed with respect to group effects. This inconsistency may be attributable to the task or ability of dogs to learn the task at each time period. In addition, multiple factors may impact cognition, and it is possible that other factors contributed to the observed inconsistency. Nonetheless, the group fed higher amounts of fish oil rich in DHA performed better overall, although not consistently, on multiple cognitive tasks performed at various ages than did those fed foods that contained smaller amounts of these nutrients.

When results of the various cognitive function tests performed in the present study are considered together, a reasonable conclusion is that increased fish oil concentration in foods provided to puppies after weaning was associated with improved neurocognitive development and that this was related to increased serum concentrations of DHA. However, comparisons among complex foods should be made cautiously, and these results cannot exclude the possibility that factors other than DHA, such as taurine, vitamin E, L-carnitine, other fatty acids, or other nutrients, may have contributed to the observed differences.<sup>22,23</sup>

Acetyl-L-carnitine has been shown to have positive effects in slowing cognitive decline in people and mice.<sup>22,24</sup> Because the high-DHA food also had the highest concentration of L-carnitine, this may have contributed to the improved cognitive scores described for puppies of this group. In addition, a metabolomic study<sup>25</sup> of transgenic mice that develop an Alzheimer's-like disease revealed decreased concentrations of several metabolites in brain tissue, including taurine and combined choline plus phosphocholine. It could be hypothesized that replacement of these factors may enhance cognitive abilities, and indeed a dietary study<sup>18</sup> involving the use of a complex matrix of nutrients (acetyl-L-carnitine, glycerophosphocholine, DHA, and phosphatidyl serine) resulted in decreased oxidative stress and improved cognitive performance in mice.

In a previous study,<sup>5</sup> ALA:LCPUFA ratios were modulated to achieve variable enrichments of ALA:LCPUFA in foods from low-to-low (0.14%:0.06%) to low-to-high (0.2%:11.6%) ratios as well as high-to-low (6.8%:0.14%) ratios. The modified foods were fed to bitches during gestation and lactation and to puppies for 6 weeks after weaning. The low-ALA, low-LCPUFA food in that study was roughly equivalent to the low-DHA food of the present study. Results of the previous study<sup>5</sup> indicated that dietary supplementation with ALA and LCPUFA might have positive effects on ERG-measured responses of a-wave amplitudes and implicit times in 12-week-old puppies, and it was noted that dietary supplementation with ALA alone was not as effective as the addition of DHA-rich fish oil. This is slightly different from results of the present study, in which significant positive group effects (ie, increased values for

puppies in the high-DHA and moderate-DHA dietary treatment groups, compared with those for the low-DHA group) were detected only for b-wave amplitudes at a specific intensity stimulation (1.2 log candelas/m<sup>2</sup>) during scotopic ERG at 4 and 6 months of age. The concentration of DHA in the moderate-DHA food of the present study was similar to the low to moderate concentrations previously reported to have no effect on ERG-measured responses in the study<sup>5</sup> in which investigators examined ALA:LCPUFA variables. The present study also had the strength of repeated measures, compared with a single evaluation at 12 weeks of age in the previous study,<sup>5</sup> to evaluate sustainability of the observed effects of different foods at multiple ages, highlighting the positive results in puppies fed high-DHA and moderate-DHA foods. It should also be noted that the high-DHA food of the present study contained 4 times the ALA concentration and approximately one-third the DHA concentration, compared with the low-to-high ALA:LCPUFA food described to have positive benefits in the previous study.<sup>5</sup>

Increased b-wave amplitudes during scotopic ERG indicated improved activity of the inner cell layer of the retina and increased ability to see in low-light or dark conditions. In the present study, when data were assessed across all time points, a significant correlation was detected between serum DHA concentration and peak b-wave amplitude during scotopic ERG. This differs from what has been previously reported for growing dogs, in which a-wave amplitudes, indicative of outer retinal cell layer activity, was improved following supplementation of the dam's diet during gestation with long-chain n-3 fatty acids, which include DHA.<sup>5</sup> The differences between these 2 studies could be attributable to the amount or timing of the DHA administration, but in both instances, dietary DHA concentration appeared to have a detectable effect on ERG results in growing dogs.

In the study reported here, puppies in different dietary treatment groups differed in their immunologic response to vaccination against rabies virus for at least 2 weeks but not longer than 4 weeks after vaccine administration. The high-DHA group, which received food that also provided the highest concentrations of vitamin E and taurine, had a significantly improved response, compared with the other 2 groups. Increased concentrations of vitamin E in serum have been associated with improved humoral immune responses to vaccination in geriatric humans.<sup>19</sup> Foods that include complex mixtures of antioxidants have also been shown to increase antibody production more quickly than do unfortified foods in adult dogs.<sup>26</sup> The observed results in the present study may have been related to the complexity of the foods rather than the total amount of any single nutrient.

No significant effects of dietary treatment group on static measures of bone development, as assessed via DEXA, were detected in the present study. Puppies in the low-DHA group appeared to have the greatest weight and BMD values, but when BMD was regressed against weight, a significant correlation was observed between the variables that could serve as a confounding factor. When the BMD for each puppy was divided

by its weight, the resulting ratios were not significantly different among groups.

In contrast, serum BALP activity, which is an indicator of bone matrix synthesis,<sup>27</sup> was significantly higher in the puppies of the low-DHA group than in those of the moderate-DHA and high-DHA groups at all time points after weaning. Serum concentration of cartilage synthesis protein II, an indicator of cartilage synthesis, was also significantly higher in puppies of the low-DHA group than in other groups at 52 weeks of age. Dietary micronutrient variation has been shown to have variable effects on other markers of bone synthesis and degradation in other species.<sup>28</sup> Specifically, inclusion of n-3 fatty acids has been shown to increase BMD in young men, whereas decreasing the ratio of n-6 to n-3 fatty acids is associated with increased BMD in both sexes.<sup>29,30</sup> Conversely, increased amounts of oxidized dietary lipids had negative effects on measures of growth and bone formation in young growing dogs.<sup>31</sup> In addition, increased dietary vitamin E content has been associated with increased trabecular bone formation and growth plate cartilage in poultry.<sup>32</sup> Results of the present study suggest that various nutrients administered following weaning may have effects on indirect markers of growth rate in young dogs, similar to results reported in other species.<sup>33,34</sup> These effects may be very complex, and further investigation is needed to determine the influences of specific nutrients during growth and whether the effects are extended or altered in mature dogs. In addition, the effects of body mass may need to be accounted for, as in the DEXA analysis, to ensure differences are not attributable to mechanical forces rather than to nutritional influences.

In the present study, when side-to-side obstacle navigation was added to T-maze tasks performed by puppies at 3 and 6 months of age, puppies in the high-DHA group had significantly better task completion times than did those of the low-DHA group at both time points and also had better results than did the moderate-DHA group at 3 months of age. This improved navigation could be attributed to a variety of cognitive or extracognitive processes related to the nutrients provided.<sup>20,35,36</sup> It is possible that neurologic function and vision were enhanced, improving navigation of these obstacles or anticipation of the next change in directional movement. It may be that extraneous physiologic factors of muscle strength, cartilage strength, cardiac output, or flexibility also impacted results for this task. However, it is of interest that although no significant differences in physical composition among dogs of the 3 dietary treatment groups were detectable via DEXA, psychomotor performance measured according to the side-to-side obstacle navigation task was significantly different among groups at 2 ages during development, with better performance observed in puppies of the high-DHA group.

Finally, no difference was detected among groups for DNMP tasks (targeted to assess short-term memory) in the present study. This is somewhat surprising, given that concentrations of nutrients such as choline and vitamin E, which are reported to increase memory retention in other species,<sup>23,37,38</sup> were considerably higher in the high-DHA food than in the other 2 foods.

It may be that visual discrimination memory function is maximal in young animals and that improvement is only amenable to dietary alteration in animals with impaired function. Alternatively, the nutrients provided in all foods may have been in sufficient quantities to support the function of memory in growing puppies.

Results of the study reported here support the hypothesis that feeding foods rich in nutrients that enhance neurologic development (DHA, vitamin E, and taurine) and immune function (vitamin E) and combat oxidative stress (vitamin E and taurine) results in improved outcomes of various tests for discrimination learning, psychomotor ability, retinal function, and immunologic response to anti-rabies virus vaccination. Elucidation of which nutrient, or combination of nutrients, was responsible for the differences detected was not possible in the present study because of the complexity of the foods tested. Nonetheless, serum concentrations of DHA were positively correlated with contrast discrimination learning and ERG-measured retinal function, suggesting that this nutrient may be an important component in neurocognitive development in puppies.

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- f. Puppy Growth Formula, Hill's Pet Nutrition Inc, Topeka, Kan.
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- h. Amino-terminal cross-linked telopeptide ELISA, Wampole Laboratories LLC, Princeton, NJ.
- i. Type II collagen synthesis ELISA, IBEX Technologies Inc, Montreal, QC, Canada.
- j. Total D-pyridinoline ELISA, Quidel Corp, San Diego, Calif.
- k. Pyridinoline ELISA, Quidel Corp, San Diego, Calif.
- l. Osteocalcin ELISA, 3OSC4000, Nordic Bioscience Diagnostics Inc, Herlev, Hovedgade.
- m. Carboxyterminal cross-linked telopeptide ELISA, OD06099, Orion Diagnostica Corp, Denmark.
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- o. Domitor, Pfizer Inc, Exton, Pa.
- p. Vetaket, Lloyd Laboratories Inc, Shenandoah, Iowa.
- q. Tropicamide Ophthalmic Solution USP, Alcon Laboratories Inc, Fort Worth, Tex.
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## Appendix

Results of nutritional profile analysis for foods used in a study to evaluate learning, psychomotor, immunologic, and retinal functions in 48 Beagle puppies from weaning until approximately 52 weeks of age (all values are stated on an as-fed basis).

Nutrient	Diet			
	Maternal <sup>c</sup>	Low-DHA <sup>d</sup>	Moderate-DHA <sup>e</sup>	High-DHA <sup>f</sup>
Moisture (%)	7.44	6.6	7.18	6.09
Crude protein (%)	27.9	30.2	29.43	29.4
Fat (%)	14.2	15.1	16.42	17.6
Calcium (%)	1.46	1.37	1.35	1.43
Phosphorus (%)	0.96	1.02	1.24	1.20
Eicosapentaenoic acid (%)	< 0.01	< 0.01	0.13	0.31
DHA (%)	0.01	< 0.01	0.095	0.19
Linoleic acid (%)	2.14	2.6	2.8	3.6
Linolenic acid (%)	0.17	0.12	0.12	0.8
Arachidonic acid (%)	0.06	0.03	0.03	0.11
Total n-3 fatty acids (%)	0.19	0.12	0.35	1.40
Total n-6 fatty acids (%)	2.6	2.6	2.8	3.7
L-carnitine (ppm)	< 30	< 30	< 30	312.1
Vitamin E (DL- $\alpha$ -tocopherol; IU/kg)	43.8	60.5	311.6	816
Choline (ppm)	1,703	1,822	888	4,876
Taurine (%)	0.08	0.08	0.15	0.14

The maternal diet was fed to adult female Beagles used in the breeding portion of the study and was also available to all puppies until weaning. At weaning, puppies were assigned to low-DHA, moderate-DHA, or high-DHA dietary treatment groups.



From this month's *AJVR*

### Pharmacokinetics of methylprednisolone acetate after intra-articular administration and subsequent suppression of endogenous hydrocortisone secretion in exercising horses

Maria I. Menéndez et al

**Objective**—To determine the pharmacokinetics of methylprednisolone (MP) and the relationship between MP and hydrocortisone (HYD) concentrations in plasma and urine after intra-articular (IA) administration of 100 or 200 mg of MP acetate (MPA) to horses.

**Animals**—Five 3-year-old Thoroughbred mares.

**Procedures**—Horses exercised on a treadmill 3 times/wk during the study. Horses received 100 mg of MPA IA, then 8 weeks later received 200 mg of MPA IA. Plasma and urine samples were obtained at various times for 8 weeks after horses received each dose of MPA; concentrations of MP and HYD were determined. Pharmacokinetic-pharmacodynamic estimates for noncompartmental and compartmental parameters were determined.

**Results**—Maximum concentration of MP in plasma was similar for each MPA dose; concentrations remained greater than the lower limit of quantitation for 18 and 7 days after IA administration of 200 and 100 mg of MPA, respectively. Maximum concentration and area under the observed concentration-time curve for MP in urine were significantly higher (approximately 10- and 17-fold, respectively) after administration of 200 versus 100 mg of MPA. Hydrocortisone concentration was below quantifiable limits for  $\geq 48$  hours in plasma and urine of all horses after administration of each MPA dose.

**Conclusions and Clinical Relevance**—Pharmacokinetics of MP may differ among IA MPA dosing protocols, and MP may be detected in plasma and urine for a longer time than previously reported. This information may aid veterinarians treating sport horses. Further research is warranted to determine whether plasma HYD concentration can aid identification of horses that received exogenous glucocorticoids. (*Am J Vet Res* 2012;73:1453–1461)



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