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The oil-rich alga *Schizochytrium sp*. as a dietary source of docosahexaenoic acid improves shape discrimination learning associated with visual processing in a canine model of senescence

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ABSTRACT

Whole cell *Schizochytrium sp*. is a rich source of omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) including docosahexaenoic acid (DHA), an important nutrient for brain health. Aged beagle dogs experienced on a visuospatial task of working memory, variable-delay delayed-non-matching-to-position were used to assess efficacy of DHA-rich microalgae based upon DHA wt% of total phospholipids and 8-iso-$PGF_{2\alpha}$ concentrations in plasma, and performance on cognitive assessments of visual object discrimination, learning, and memory consolidation after 25 weeks on fortified diet.

Improved DHA status ($p < 0.001$) and initial learning of the protocols for visual and variable contrast discrimination ($p < 0.05$), but not long-term recall of the concurrent discrimination task were observed in animals fed the algal-fortified diet. Overall, results were consistent with dried *Schizochytrium sp*. as a source of n-3 LCPUFA nutrition to support DHA status in large mammals, and healthy brain function in a canine model of senescence.

1. Introduction

Industrial monoculture of microbes, including marine algae, has been recognized for more than four decades as an important means of large-scale production of biomolecules such as, antibiotics, vitamins and other nutrients. Marine algae, is a rich source of omega-3 (n−3) long-chain polyunsaturated fatty acids (LCPUFAs), DHA (22:6n−3) and eicosapentaenoic acid (EPA, 20:5n−3). The composition of dried whole cell *Schizochytrium sp*. in particular includes approximately 20% DHA, on a per weight basis, making it a rich source of this nutritionally important n-3 LCPUFA. Extraction and refinement from whole cell algae result in oils in which DHA comprises greater than 50% of the total fatty acid for use as a concentrated source of dietary n-3.

DHA is the major n-3 LCPUFA in brain and comprises 30–40% of the aminophospholipid fatty acids in neuronal cell membranes [1,2]. As an integral component of neural cell membranes, DHA is involved in numerous processes including membrane order, receptor activation, and signal transduction [3]. There has also been considerable speculation that suboptimal DHA concentrations may contribute to cognitive dysfunction and dementia over the course of aging, in some individuals [4]. Epidemiological investigations associate low DHA status in plasma with age-related cognitive decline in aged healthy and in individuals with dementia [5–8]. Some investigations have indicated that long-term supplementation with algal DHA supports learning and memory in healthy elderly individuals with age-related mild cognitive decline [9]. Other investigations provide evidence of a positive relationship between supplementation with adequate amounts of n-3, increased status of DHA in plasma, and cognitive performance in aged subjects with subjective memory complaints [10].

The canine model of brain aging has emerged as an important tool for the study of age-related changes in cognitive functions in humans. Visual processing is among the earliest functions for which decrements in cognitive performance attain measureable limits during aging in both humans and dogs [11–14]. Gradual impairments in visuospatial learning and memory involving object recognition and discrimination are also common features of aging, in both humans and dogs [13,15]. To date, there are no published investigations of the relationship between the status of DHA during senescence and cognitive function in large non-human mammals. Dried whole cell biomass may provide an important alternate source of dietary n−3s for use in applications which do not require a highly refined source of DHA or EPA.

The objective of this study was therefore to assess whole cell algal biomass as a source of DHA based upon changes in the concentrations

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in plasma of n-3 LCPUFAs and 8-isoPGF$_{2\alpha}$. Evidence was also investigated for possible interactions between fortification with whole cell algae and performance on various cognitive tasks, using a well characterized canine model of aging [11,15–18].

2. Materials and methods

2.1. Animals and diets

Twenty-six colony beagles (CanCog Technologies, Toronto, Canada) ranging in age from 8.6 to 11.1 years (mean=9.8 years, SD=0.8, 10 females) were utilized in the study. The research protocol was approved by the CanCog Technologies Animal Care Committee, and in accordance with the guidelines of the Ontario Ministry of Agriculture. The animals were group housed at the CanCog site throughout the trial. Each animal used for the investigation was visually examined daily by a trained veterinary and animal research staff. All dogs had similar exposure to cognitive testing, as previously described [19]. Each animal underwent examination by a veterinarian to ensure that auditory, motor, and visual functioning was normal. Housing temperature was maintained at 21±6 °C and relative humidity levels ranged between 15% and 75%. Animals had free access to fresh water and approximately 1 h, to consume their food. Animals were acclimated for a period of 42 days to a commercially available, standard extruded dry diet which lacked the n-3 LCPUFAs (PUFAs) docosahexaenoic acid (DHA, 22:6n–3) or eicosapentaenoic acid (EPA, 20:5n–3). Metabolizable energy and nutrient composition, on an as fed basis, of the control diet were ~1.47×10^4 kJ/kg and 26%, 12%, 4% and 10% of protein, fat, fiber, and moisture, respectively. Total fat and protein content of dried *Schizochytrium sp*. (DSM Nutritional Lipids, Columbia, MD) were 45.3 and 12 wt%, respectively (Tables 1 and 2). The dehydrated single-cell algal (DHA) biomass comprised 0.4% of the experimental diet. Minor adjustments to the amounts of tallow and corn were made to the control diet, in order to maintain the same energy contents between the control and the test diets. The final content of DHA in the test diet, as fed, was 1 mg/g. Body condition scores were monitored on a weekly basis throughout the investigation. The amount of diet fed daily to each animal was 26 g/kg of body weight. Dietary amounts were adjusted weekly in increments of 50 g, as needed in order to maintain body condition score approximating 3 on the 5-point scale.

2.2. Group assignments

Twenty six dogs, which had been previously trained on the variable-delay delayed-non-matching-to-position (DNMP) task [15], were selected at baseline in order to identify 24 for use in experimental assessments. DNMP score was used to rank visuospatial performance for each of the animals at baseline (Fig. 1). To do so, the dog making the fewest incorrect responses was ranked as 1 and the dog making the most incorrect responses was ranked as 26. Two subjects were dropped as they showed the most frequency response failures. The remaining animals were assigned based upon descending rank to one of two groups (e.g. 1,2,2,1, etc.). The two cognitively equivalent groups (n=12/group) were then randomly assigned by lot drawing to the base diet (control) or to the DHA-fortified diet for the duration of the investigation.

The study was blinded to all personnel in the investigation with the exceptions of the person(s) involved with administering the investigational and control diets and the person responsible for performing allocation. Collection of data from the study by these individuals was limited to food consumption. The code assignments and other records and document that would reveal treatments to people collecting data remained archived in a secured room at the test facility until all data were collected and analyzed.

The treatment phase started on the day following group placement. The first 51 days provided the initial wash-in and no other procedures were introduced during this time. Over the next 124 days, the groups were tested on a battery of cognitive tests, including concurrent discrimination learning (days 51–90), contrast sensitivity learning (days 100–140), retest on DNMP (days 145-154), performance on variable contrast protocol (days 157–164) and retention of concurrent discrimination learning task (days 167–174).

Table 1
Nutritional profile analysis for diets used to evaluate cognitive behavior in 24 aged beagle dogs (all values are stated on an on as-fed basis).

Nutrient	Diet	
	Control	Fortified
Moisture (%)	5.9	7.4
Crude protein (%)	28.8	29.1
Fat (%)	11.8	11.8
Fiber (%)	1.9	2.2
Ash (%)	9.5	9.5
Dried whole cell algae (%)	0.0	0.4

Table 2
Nutritional profile, fatty acid, and vitamin composition of dried whole cell algae.

Basic components	Per 100 g
Moisture	2.5
Protein	12.1
Carbohydrates	32.0
Ash	8.2
Fiber	0.6
Crude fat	45.3
Fatty acid	
12:0 (Lauric)	0.2
14:0 (Myristic)	5.1
16:0 (Palmitic)	12.1
18:0 (Stearic)	0.02
18:3n6 (γ-linolenic)	0.1
20:3n6 (DHGLA)	0.2
20:4n6(Arachidonic)	0.2
20:5n3 (Eicosapentaenoic)	0.5
22:5n6 (DPAn6)	**6.3**
22:6n3 (DHA)	**18.0**
24:0 (Lignoceric)	**0.1**
Vitamins	
Biotin	0.3 mg
Choline	1440 mg
Folic Acid	0.1 mg
Niacin	14 mg
Vitamin A	33.6 μg
Beta Carotene	2.3 μg
Vitamin B_1	4.4 mg
Vitamin B_2	2.9 mg
Vitamin B_6	1.4 mg
Vitamin B_{12}	54.9 μg
Vitamin C	0
Vitamin E	0.45 μg
Pantothenic Acid	3.5 mg

2.3. Blood collection

Whole blood was collected on days −42, 1 and 175 and placed into EDTA tubes for separation into plasma and red blood cell. Plasma was prepared for the purpose of measuring 8-iso-PGF$_{2\alpha}$ by centrifugation at 1300*g* for 25 min to remove platelets. Samples were stored at −80 °C until analysis.

Fig. 1. Timeline of scheduled events. A commercial diet was provided as the sole source of nutrition for all of the animals in the investigation during the acclimation period of 42 days (dotted line). Animals underwent cognitive testing on the Delayed-non-matching-to-position task in order to determine their individual score and rank. On day-0 each animal was assigned to either of the two cognitively equivalent dietary groups for treatment beginning from day-1 with the standard commercial (control) diet or fed diet fortified with dehydrated algal (DHA) comprised of *Schizochytrium sp* . biomass until completion of the trial on day-175. Testing to assess cognitive abilities began 50 days after the dietary treatment with the algal fortified diet. The days on which training and assessment on various cognitive activities were scheduled to begin are indicated by the numbers which appear below the time-line. Specific cognitive activities are listed above the expanded sections, which correspond to the duration of each activity, along the time-line.

2.4. Fatty acid analysis

Whole blood was collected from each animal following a fasting period of 8 h. Total plasma lipids were extracted from heparinized plasma using the method by Folch [20]. The major neutral plasma phospholipids (PL) were separated by thin layer chromatography using 60/40/3 hexane/ether/acetic acid on 20×20 silica gel 60 plates, and tricosanoic free fatty acid (23:0, Sigma-Aldrich, St. Louis, MO) added as a reference internal lipid standard. Fatty acid methyl esters (FAMEs) were analyzed by gas liquid chromatography (GLC) using a Hewlett Packard 6890 equipped with a flame ionization detector. FAME separation was achieved using 30 m FAMEWAX capillary column (Restek, Bellefonte, PA; 0.25 mm diameter, 0.25 μm coating thickness) using hydrogen at a flow rate of 40 mL/min with a split ratio of 48:1. The chromatographic run parameters included an oven starting temperature of 130 °C and was increased at 6 °C/min to 225 °C, and held for 20 min and then increased to 250 °C at 15 °C/min, for a final hold of 5 min. The injector and detector temperatures remained constant at 220 °C and 230 °C, respectively. The resulting peaks were identified by comparison of retention times with external FAME standard mixtures from NuCheck Prep (Elysian, MN). The fatty acid profiles were expressed as weight percent values for total plasma PL.

2.5. Measurement of isoprostanes

To measure 8-iso-$PGF_{2\alpha}$, a deuterated internal standard 8-iso-PGF_2-D4, was purchased from Cayman Chemical (Ann Arbor, MI). Butylated hydroxytoluene (BHT), and hydrochloric acid were purchased from Sigma-Aldrich (St Louis, MO). All other reagents and solvents were high performance liquid chromatography (HPLC) grade and were purchased from VWR (Radnor, PA). HPCL -mass spectrometry analysis of 8-iso-PGF_2, was performed as previously described [21] with minor modifications. Briefly: freshly thawed dog plasma from the study animals, and from samples intended for use as reference controls between sessions, were added with BHT solution (1% in ethanol) to, 8-iso-PGF_2-D4 (50 ng/mL in methanol) in 130 μl of water, as an internal reference, then hydrolyzed with 1:9KOH 50% wt: wt in water: methanol. The resulting mixture was incubated at 40 °C for 60 min. The reaction was stopped on ice by adding formic acid 0.1% (v/v) and 5 N hydrochloric acid. Extraction of the isoprostanes was performed as described by Janicka et al. [22] using SPE Cartridges Oasis HLB 60 mg (Waters, Milford, MA).

The SPE cartridges were conditioned with methanol followed by 5 mM HCl. The hydrolyzed dog plasma was loaded with 5 mM HCl, onto the SPE cartridge, then washed with 5 mM HCl followed by hexane. Isoprostanes were eluted with ethyl acetate containing 1% CH_3OH directly in conical glass tubes with conical bottom. Solvent was removed in a stream of nitrogen at 34 °C using a turbovap evaporator (Biotage, Charlotte, NC). The dried sample were then re-dissolved in HPLC mobile phase at time 0 (50:15:35 – V:V:V of mobile phase A, B, C, (see below) respectively, and transferred to HPLC vials with glass inserts. A correction factor based upon the control samples of 1.3433 was used to adjust measured values obtained between assay sessions.

The chromatography was carried out using an Agilent 1200 quaternary pump system (Agilent, Santa Clara, CA) using a Kinetex XB-C18 100 Å column (100×4.6 mm, 2.6 μm) Phenomenex (Torrance, CA) kept at 4 °C. The mobile phase consisted of a three solvent system: Solvent A was aqueous 0.01% acetic acid, solvent B acetonitrile 0.01% acetic acid, and solvent C methanol 0.01% acetic acid. The flow rate was 600 μl/min and the injection volume was 40 μl. Post column flow was split down to 1:5 ratio and only the HPLC output from 5 to 15 min was delivered to the electrospray ionization source.

The HPLC was coupled to a 4000 QTRAP LC/MS/MS system from AB Sciex (Concord, ON, Canada) through a Turbo V ion source using the electrospray ionization probe and operating in negative ion mode. Curtain gas, ion source gas 1, and ion source gas 2 were set at 55, 25, 25 °C respectively. The ions spray voltage was set at −4500 V and the source temperature was set at 600 °C for 8-iso-$PGF_{2\alpha}$ 500 V and set at 600 °C for 8-iso-PGF_2ex (Concord, ON, Canada) through a 193.2 *m/z* and 357.3 to 197.2 *m/z,* respectively. Quantification was performed using Analyst® 1.6 software (AB Sciex).

2.6. Cognitive test apparatus

All cognitive testing utilized a Toronto General Test Apparatus designed for cognitive assessment of dogs. The apparatus consisted of a plastic box (approximately 3′×5′), and has been described elsewhere [19]. The front of the apparatus contained three height-adjustable stainless steel gates through which the dogs responded. An experimenter was separated from the dog by a partition to which was affixed a one-way mirror and a hinged door that, when opened allowed a Plexiglas tray and stimuli to be presented to the dog. The tray contained one medial and two lateral food wells, and was accessible through the adjustable gates by the dog.

The computer program, Varicog© (CanCog Technologies Inc.), was used for all cognitive testing. The software controls all test sequences and was under administrator control, assuring that the parameters, once set, could not be modified by technicians doing the testing. In addition, the same sequence of correct locations was used on each test session for all subjects.

2.7. Cognitive test protocols

2.7.1. Delayed non-matching-to-position-task

Variable delay, DNMP has been previously described [23]. Testing on the variable delay task occurred at baseline over 10 consecutive days (d −11 to −2). There were three delay intervals, set at 5, 55 and 105 s, and there were 18 trials daily, 6 at each delay interval. The inter-trial interval was set at 30 s. Subjects were allowed to correct their first incorrect response on each session. In scoring, response failures were counted as 0.5 correct responses. Animals were again tested on this task using identical parameters from days 145–154 to examine long-term memory.

2.7.2. Concurrent discrimination learning

Each dog was tested on the Concurrent Discrimination task over a maximum of 40 consecutive days, from days 51–90. The task assessed the animals' ability to retain information about complex object to

Table 3
Fatty acid composition of experimental diets.[a]

Fatty acid	Diet	
	Control	Fortified
∑ of saturated FA (%)	3.76	4.04
∑ of monosaturated FA (%)	3.86	3.93
18:2n–6 (%)	1.64	1.54
[b]20:4n–6 (%)	0.03	0.02
18:3n–3 (%)	0.1	0.24
DHA (%)	**[b] < 0.01**	**0.1**
∑ of n–3 fatty acids (%)	0.11	0.35
∑ of n–6 fatty acids (%)	1.7	1.62

[a] Values represent the mean of the weight % of diet.
[b] Below the limit of detection.

object associations in the presence of novel objects with similar but different features (e.g., shape, size) and thus learn to discriminate four different pairs of objects (A vs. B, C vs. D, E vs. F, and G vs. H) simultaneously. The object pairs used were constructed from Lego blocks in such a way that each pair differed on three dimensions, color, size and shape. On day 51, subjects received a preference test, which consisted of 20 presentations of the object pairs, with each pair presented on 5 occasions. Selecting either object led to food reward; for each pair, the object selected most frequently was designated as the preferred object. On the subsequent training days, the dogs were given 24 presentations per session with an inter-trial interval of 15 s and with each object pair presented 6 times. On each trial, the dogs were rewarded only if they choose their initially non-preferred object. For the first incorrect response for each object pair, a correction procedure was used, in which the subject was allowed to reverse its choice. The dogs were tested for either a maximum of 40 consecutive daily sessions following the preference test or until they achieve a score of 85% or higher on two consecutive days.

2.7.3. Contrast sensitivity

Measurement of contrast sensitivity has been described previously [11]. On days 100–140, all subjects were trained on a contrast sensitivity protocol, which was designed to assess visual processing abilities. On day 100 the dogs were given a preference test, in which they were given 10 presentations of two objects, a black circle on a white background vs a black triangle on a white background. The object selected most frequently was deemed to be the preferred object. In the case of ties, the preferred object was designed by a coin toss. Starting on day 101, subjects were trained to approach their initially non-preferred object to obtain reward. On the initial training, the contrast was at 100%. Subjects were given 10 trials per day until they either completed 40 test sessions, or successfully completed the following two stage criterion: Stage 1 criterion: 90% correct on a given day or 80% or better on 2 consecutive days with no response failures. Stage 2 criterion: on 3 consecutive days following completing the stage 1 criterion, subjects must have achieved at least 70% or higher and to respond correctly on at least 30 trials.

After acquiring the Stage 2 criterion, subjects were tested at progressive lower contrasts, in which the contrasts were reduced to 40%, 25%, and 5% by decreasing the luminance of the shapes. At each new level of contrast, the dogs had to complete the two stage criteria defined above. If a dog completed the Stage 2 criterion at 5% luminance, testing was then discontinued. During both training and testing on Contrast Sensitivity, animals were allowed to correct their first incorrect response of each session.

On days 157–164 subjects were retested for contrast sensitivity using the variable contrast task. This entailed testing every animal for 8 successive sessions with 2 trials per session at contrasts luminance of 1%, 3%, 5%, 15%, 20% and 40%.

2.8. Statistical analysis

Repeated measures of analysis of variance (ANOVA), and when appropriate, non-parametric statistics were used to assess the effects of diet on performance on cognitive testing. A 2-tailed analysis, unless otherwise indicated, was used to compare between independent samples *post hoc* as necessary using Student *t*-test. Statistical analyses were completed using both Statistica and Excel, with an alpha of 0.05 considered significant.

The statistical analysis also took into consideration consistency of responding. The general procedure for dealing with response failures was to give the subject a score of 0.5, which would have been obtained by chance. However, response failures also prolonged training because of the *a prior* criterion that dogs respond on at least 30 trials before passing the learning criterion. Because response failures depress performance scores, but are not necessarily indicative of cognitive impairment, we used a criterion of 1.5 failures per session on the average, to justify excluding any given animal from the statistical analysis of the data. Generally, all data sets were analyzed twice: the first included the data from all of the subjects and the second analysis was after excluding data from animals that showed frequent response failures (non-responders).

3. Results

3.1. Diets

The experimental diet was made by fortification with 0.4% dried whole-cell algal biomass (as fed) in place of minor amounts of dietary fat in the base diet (Table 1). Protein, fiber, and ash content in the algae fortified diet were comparable to concentrations in the base diet. Results from analysis of the fatty acid (FA) concentration for each diet confirmed the presence of DHA in the diet fortified with whole cell marine algae, compared to concentrations below the limit of detection in the control diet (Table 3). Food intake was monitored daily for each animal. Mean dry matter intake, following adjustments in the amounts fed in order to maintain a constant body score, was 18±4 g/kg/d and was not different ($p = 0.406$) between animals fed the control or experimental diets. Mean intake of the control and algal biomass supplemented diets were 17.4±4.4 and 18.4±3 g/kg/d, respectively. There was no evidence of any adverse effects on palatability or consumption as a result of fortification of the base diet with the DHA-rich dried algae (data not shown).

3.2. Fatty acid content in plasma total phospholipids

Weight percent (wt%) of polyunsaturated fatty acid (PUFA) in total plasma PL were measured at baseline prior to treatment, but following randomization of animals to the control or experimental diet group, and then again after 175 days of treatment on the respective diets. At baseline the mean±s.d. wt% of 22:6n–3 was 0.31±0.05 in animals (n = 12) assigned to the control diet (data not shown) and was not different after 175 days of treatment or from values at baseline in the experimental diet group (n = 12). ANOVA indicated that consumption of the DHA-enriched diet for 25 weeks was associated with a 10-fold increase [$F(3,44) = 416.37$; $p < 0.001$] 22:6n–3 wt% PL of 22:6n–3, (Fig. 2). In contrast, wt% of 20:4n–6 in control animals was 18.5±1.9 and was not different [$F(3,47) = 1.75$;$p = 0.171$] between the two groups before or after treatment.

3.3. Group placement and DNMP

Twenty four aged beagle dogs, were fed a commercially available dog food, which lacked n-3 LCPUFAs for 42 days. The DNMP task is used to measure performance of episodic memory retention related to visuospatial function. DNMP score was used to rank visuospatial

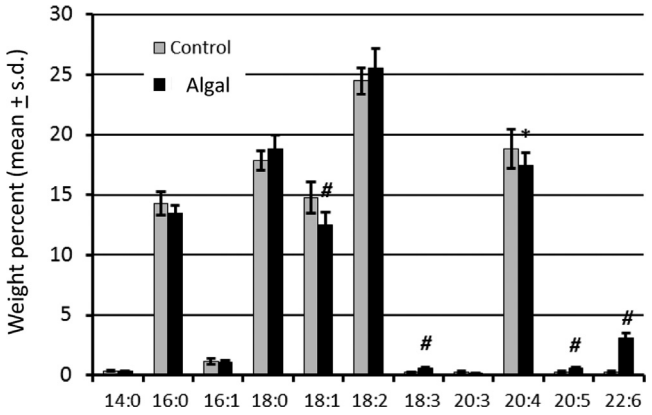

Fig. 2. Total phospholipid composition in plasma after 25-week dried algal fortified diet. Control animals were fed with a standard commercial diet. Animals in the algal group were fed the same diet fortified with 0.4% dried whole-cell algae. $P < 0.05$ (compared to control). The data are expressed as means±standard deviation (n=12).

performance for each of the animals at baseline. The dog making the fewest incorrect responses was ranked as 1 and the dog making the most incorrect responses was ranked as 26. Two subjects were dropped as they showed the most frequency response failures. The score from the 10 trials at baseline on the DNMP task represents a mean ratio of correct to total responses for each subject. Each of the cognitively experienced animals in this study was assigned to the control or experimental group (mean age, 9.82±0.2 and 9.71±0.23 yrs., respectively) for dietary treatment based solely upon their individual performance on the DNMP task. Repeated ANOVA with performance on DNMP from training at day −11 to day −2, for each animal as the between subjects effects revealed a significant effect of delay [$F(1,2) = 30.8$; $p < 0.001$], as the within subjects factor, with equivalent performance [$F(1,2) = 0.09$; $p = 0.916$] between assigned groups on the accuracy of performance on the DNMP task at baseline. Final ranks for the animals designated to receive the control diet (n = 12) and the experimental dietary treatment (n = 12) were (13.46±2.08 and 13.54±2.08 (mean± SEM), respectively.

Testing on the DNMP task was repeated for each animal between days 145 and 154, but showed no effect related to either of the diets [$F(1.2) = 0.182$; $p = 0.834$]. Performance of the groups was equivalent at both baseline and during dietary treatment and the delay effect was due to more accurate performance at 5 s than at 55 or 105 s, both of which differed significantly from performance at the 5 s delays ($p < 0.001$; Fisher LSD test, Fig. 3).

3.4. Isoprostanes

The concentration in plasma of 8-iso-$PGF_{2\alpha}$ was measured at baseline and after 25 weeks in each animal from control (n = 12) or algal-fortified (n = 12) dietary treatment groups. Analysis of variance of the mean concentrations of 8-iso-$PGF_{2\alpha}$ indicated no difference ($p = 0.508$) between values observed at baseline (91.57±24.98 pg/mL) and after 25 weeks of dietary intake of the control or fortified diet.

3.5. Performance on cognitive test protocols

3.5.1. Concurrent discrimination (CCD) learning

Initial training and testing on CCD began 51 days after the initiation of both dietary treatments and was continued for 39 days. One animal from the treatment group was dropped from this phase of the study after the 17th session because of unreliable responding. (In the 17 sessions, this animal did not respond on 270 of 408 trials). The initial analyses included the data from all of the subjects, independently of the subjects' consistency in responding. The subjects on the DHA supplemented food required a mean±s.d. of 27.5±9.8 testing

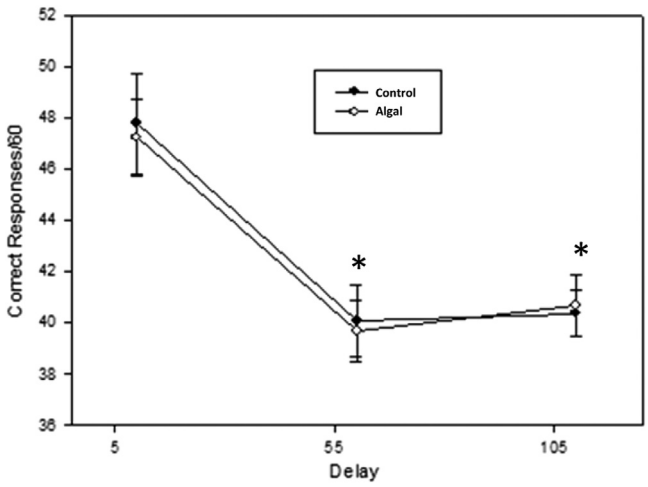

Fig. 3. Performance on DNMP during baseline (from training day −11 to day −2) as a function of group placement and delay. Mean (±s.d.) number of correct responses after delays of 5, 55, or 105 s by groups fed control (open) or algal-fortified (filled) diets. Asterisks (*) indicate $p < 0.0001$ compared to performance after 5 s.

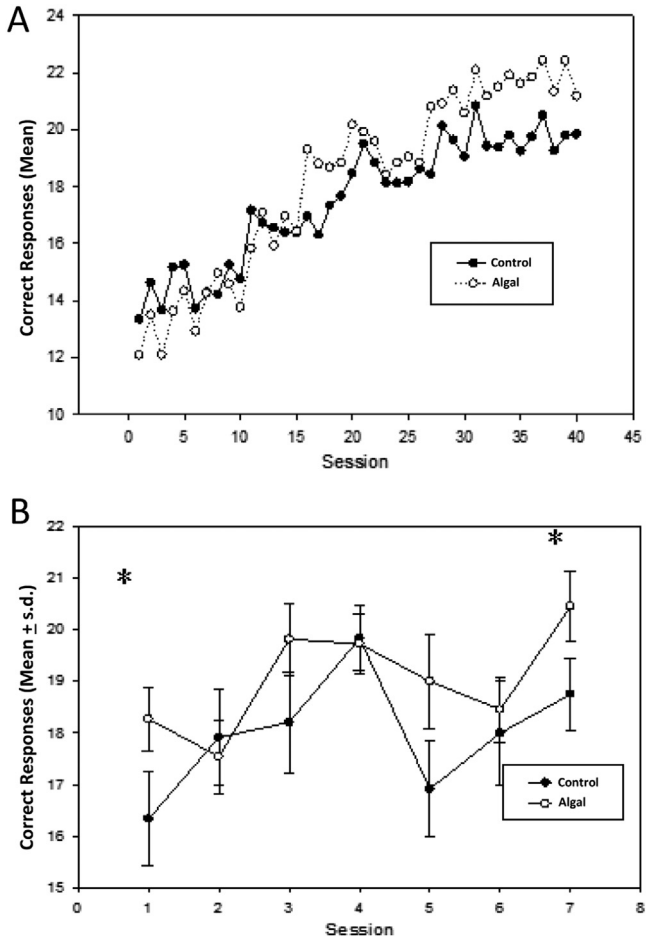

Fig. 4. Performance on the concurrent discrimination learning task as a function of test session and group. The Y-axis depicts mean correct out of a maximum of 24 sessions. (A) Results obtained following initial training on CCD task during days 51–90 of treatment on the control (filled) or algal-fortified (open) diet, and (B) effects of dietary treatment on concurrent discrimination learning task (days 167–174). Asterisks (*) indicate $p < 0.05$.

sessions and 232±124.4 incorrect responses to complete testing. By contrast, the control subjects required a mean of 32.9±10.5 and 259.5±113.8 errors to complete testing (Fig. 4). The next analysis was done after dropping the data from 4 subjects (2 from each group)

who averaged more than 1.5 incorrect responses per trial. The groups were then compared on both errors to criterion and sessions to criterion. The results of the analysis revealed better apparent performance by the animals receiving the algal supplemented diet (means +s.d. for errors were, 25.1±8.2 vs. 30.4±10.9 for trials, and 199.5±103.7 vs. 235.65±109.6 for errors), but the differences did not achieve statistical significance (p=0.4 and 0.871).

A third analysis focussed on the probability of a given subject successfully achieving the learning criterion. In the control group, 5 of 12 animals were able to successfully complete training. By contrast, 9 of 12 animals in the DHA group passed the learning criterion within 40 test sessions. A chi squared test was used to compare the probability of success between the groups. The difference between the groups did not reach significance overall χ^2 ((1) 2.74, N = 24, p = 0.098).

The final analysis compared groups on every test session using a repeated measures ANOVA. After 25 weeks, the mean number of correct responses as a function of test session and group revealed a highly significant effect of session [F(1,39) = 26.8; $p < 0.02$] on the mean correct responses, shown as the change from baseline for each group. A significant interaction was also observed between group and session [F(1,39) = 1.55; $p < 0.02$] which reflected the algal-fortified group showing higher levels of performance over the last part, but not during the initial part of training on CCD (Fig. 5).

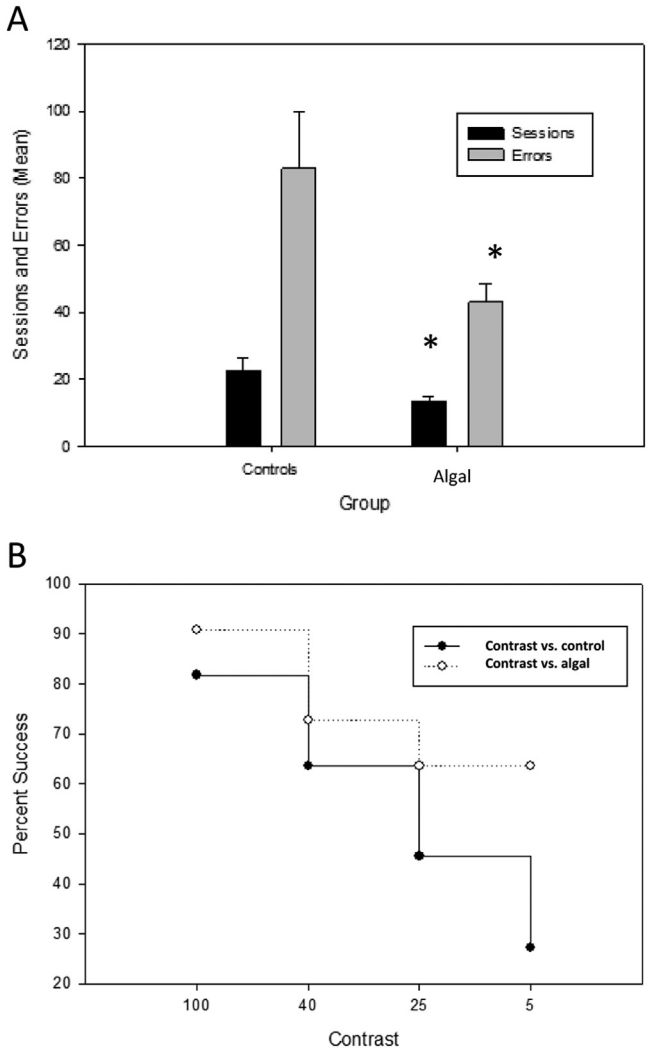

Fig. 5. Effects of diet contrast sensitivity (A) both trials and errors to criterion initial learning of the contrast sensitivity test and, (B) percent of subjects passing contrast sensitivity protocol at each successive contrast plotted as a function of group. Asterisks (*) indicate significant differences of $p < 0.05$ between groups.

Retention of CCD task was evaluated between days 167 and 174. One animal was not included in the results because it had been dropped from the original learning phase. Repeated measures ANOVA with test session as a within subject variable and group as a between subject variable indicated a statistically significant effect of session [F(6,1) = 6.04; $p < 0.001$) and no significant interaction between session and group (p = 0.119).

The results from each of the test sessions suggested that both groups showed improved performance over repeated testing and that the DHA group tended to perform better overall than the controls. A closer look at the results from the two groups at each test session using a Student *t*-test revealed statistically significant differences on the first and last retention test day indicating that a statistically significant effect of session ($p < 0.05$) remained, but there were no other statistically significant main effects or interactions.

3.5.2. Contrast sensitivity learning

Animals in both treatment groups were trained on the contrast sensitivity learning task between days 100 and 140. Note that one animal from the control group only completed 29 training trials. This was because at the initial start of testing, the animal was removed from testing because of too frequent response failures and was only started after undergoing a remedial training protocol. Because the animal did not complete the training, it was considered to be non-responsive to training and therefore not included in any of the statistical analysis. Three other animals, all from the group fed the experimental diet, were dropped from the statistical analysis because they averaged more than 1.5 failures per session. *T*-test results (n = 12/group) comparing the control and algal supplemented groups on errors and trials to criterion are illustrated in Fig. 6. Mean±s.d. for sessions in order to complete initial training on contrast sensitivity and incorrect responses from control and algal-supplemented animals were 23.3±11.8 and 19.3±11.3, and 88.7±57.8, and 81.4±74.1 and were not significantly different (p = 0.208 and 0.395), respectively. Similar analysis including only responders, defined as animals which averaged < 1.5 failure per session, from both groups indicated that subjects fed the control diet (n = 11) required a mean±s.d. of 22.7±12.2 sessions in order to complete initial training on contrast sensitivity learning. The mean number of incorrect responses by control animals during training was 82.9±56.7. Dogs fed the diet algal-fortified diet (n = 9) required fewer sessions 13.6±4.2 p = 0.023) and made fewer incorrect responses (43±18.7, p = 0.029) compared to control diet fed animals.

A chi-square test was used to examine the relationship between

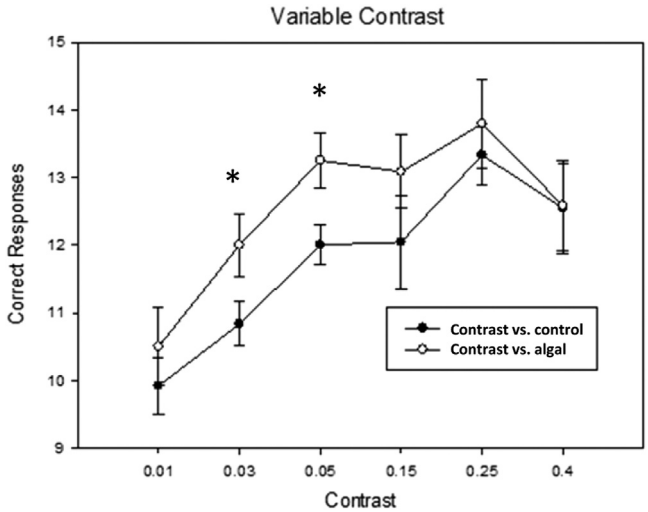

Fig. 6. Performance as a function of contrast and group on the variable contrast discrimination task. Asterisks (*) indicate differences in performance between control (filled) and algal (open) dietary treatment groups that were significant at the 0.05 level.

dietary treatment and performance on the contrast sensitivity protocol. The relationship between these variables was not significant (p=0.098). Three of 12 animals in the control group successfully completed all of the contrast levels. Successful completion of all test was demonstrated by 7 of 12 animals fed the algal fortified diet.

3.5.3. Performance on the variable contrast object discrimination task

Performance on the variable contrast object discrimination task was assessed on days 157–164 for each dog in the investigation. This entailed testing every animal for 8 successive sessions with 2 trials per session, at each contrast, for a total of 16 sessions. The grouped data were first analyzed with a repeated Measures ANOVA with contrast level as a within subject variable and group as a between subject variable. The initial analysis was based on all 12 animals in each group and revealed a highly significant effect of contrast [$F(1,5)=13.9$; $p < 0.001$] but not group ($p = 0.13$) (Fig. 6). Results indicate that the contrast effect reflected progressively poorer performance with decreasing contrasts beyond 25% for both treatment groups. In addition, the group differences were greater at intermediate contrasts. Further analysis by Student *t*-test was used to compare the results at each contrast level. P-values at contrasts 3% and 5% were 0.052 and 0.023, respectively, and were not statistically significant at either lower or higher contrast. Results of the analysis indicated statistically significant differences at contrast of 0.03 and 0.05 (p=0.026 and 0.011, respectively).

A second analysis was performed in which two animals, one from each group, were dropped because of inconsistent responding. The results of a 2-tailed analysis again revealed a statistically significant main effect of contrast ($p < 0.001$) but not group ($p = 0.092$). One-tailed analysis indicated $p = 0.046$ for group. Both 2-tailed and 1-tailed analysis were used to compare the groups at each level of contrast and again revealed statistically significant effects of $p = 0.012$ and 0.006 at contrasts of 0.03 and similarly $p = 0.012$ and 0.006 at contrast 0.05.

4. Discussion

Age-related impairment of cognitive performance has been well characterized in canines and shown to involve a number of cognitive domains including visual contrast sensitivity, spatial memory, learning, and working memory [15,16,24–26]. Neuropathological correlates include reduced mitochondrial respiratory activity [27], brain-derived neurotrophic factor [28], and hippocampal neuron populations, due at least in part to decreased neurogenesis within the hilus region of the dentate gyrus [29,30], in addition to increased oxidative damage [31,32], and deposition in brain of Aβ-amyloid subunits [33,34].

Previous investigations have provided evidence of the potential efficacy of long-term dietary fortification with brain-specific nutrients, as part of a multi-interventional strategy, in order to support neuronal homeostatic regulation of metabolic [35], bioenergetic [27], secretory [36], and neurogenic [28] pathways in the aging canine brain. Furthermore, efficacy as a result of the long-term use of dietary enrichment has been extended to include behavioral outcomes. Learning of the reversal discrimination task was improved relative to controls in animals fed a diet enriched with supplemental antioxidants and mitochondrial enzymatic cofactors in a longitudinal trial [17,37]. Improved utilization of allocentric spatial information has also been demonstrated as a result of dietary fortification with the same antioxidant cocktail [17].

Determining the effects of daily consumption of DHA-rich algae on the activation of pathways involved in either short- or long-term memory following visual stimuli in senescent dogs was also a main goal of this investigation. Age-related changes in performance in visuospatial function become discernable by 6 years in dogs [33,34]. Other signs of brain aging in dogs includes the deposition and accumulation of β-amyloid protein affecting the prefrontal cortex and then later, the medial temporal regions [33,34]. Therefore the age range used in this investigation was selected in order to include animals likely to demonstrate mild to moderate age-related decrements on cognitive tasks dependent upon visuospatial functions [16,25]. Additional steps taken in an effort to isolate possible effects by the condition of interest included selection to animals which a record of previous training on the variable-DNMP task, and masking of olfactory signals in all cognitive assessment tasks by placement of inaccessible food equal to the amount in the correct choice, within immediate proximity for all incorrect choices [23].

4.1. Studies of DHA in canines

This study is the first to investigate possible benefits from dietary fortification with DHA-enriched algae on cognitive functions in a model of canine senescence. Previous studies of the effects of DHA-rich fish oil supplemented during perinatal growth on neurodevelopment in puppies indicated improved visual acuity related to greater retinal sensitivity, and increased learning on CCD attributed to frontal lobe function [38,39]. A standard commercially available dog food was fortified with dehydrated whole cell alga, in order to evaluate *Schizochytrium sp*. as a nutritional source of marine n-3 LCPUFAs. Dried alga was added to the base diet in order to meet intake amounts of n-3 LCPUFA considered to be adequate for canines [40]. The PUFA composition of the base diet also included α-linolenic acid at concentrations which met or exceeded those considered as adequate for the maintenance of healthy adult animals. There were no measurable effects as a result of fortification on the concentrations other dietary FAs, however, the negative change in the n−6:n−3 LCPUFA ratio (6 vs. 17) observed in animals fed algal biomass was consistent with the influence of 22:6n−3 and 18:3n−3 intake from the diet.

DHA from *Schizochytrium sp*. was sufficient to induce an increase in the DHA wt% of total PL in plasma which corresponded with consumption of algal-fortified diet. In addition, a small but significant increase in 20:5n−3 was observed in the algal-fortified group. These results are consistent with results from other studies in which healthy dogs were fed diets supplemented with omega-3 LCPUFAs [38,39,41].

Increased concentrations of $PGF_{2\alpha}$ isomers of non-cyclooxygenase generated products of peroxidized arachidonic acid, and in particular 8-iso-$PGF_{2\alpha}$, have been shown in clinical investigations of canine cardiovascular diseases, including congestive heart failure [42] and experimentally induced subarachnoid hemorrhage [43]. In addition to progressive deterioration of tissue function, oxidative stress is believed to contribute to observed negative changes in cognitive performance during aging in both humans [44] and dogs [45]. DHA and EPA administered at therapeutic doses have been shown to decrease the concentrations of isoprostanes in plasma from dysmetabolic subjects [46] and in tissues from a rodent model of lung inflammation [47]. In contrast, concentrations of F_2-isoprostanes were increased in urine from normal dogs after 21 days of supplementation with 200 mg/kg of DHA and EPA [48]. In addition to marine n-3 LCPUFAs, epidemiological studies have provided evidence that regular dietary intake of carotenoids and tocopherol may reduce 8-iso-$PGF_{2\alpha}$ [49,50]. Results from the present investigation showed no evidence of interactions between algal biomass and generation of 8-iso-$PGF_{2\alpha}$. This outcome was not totally unexpected, considering the use of normal dietary concentrations, the small change in concentrations in plasma of the 8-iso-$PGF_{2\alpha}$ precursor, ARA, and the absence of any overt signs of disease or inflammation following a health exam, as a criteria for selection for treatment in this investigation. It is also reasonable to consider that the concentrations of 8-iso-$PGF_{2\alpha}$ in plasma were already within a normal physiological range for concentrations in the apparently healthy older animals used in the present investigation.

4.2. Contrast sensitivity

Senescence is accompanied by progressive deficits in contrast sensitivity, visual processing, working memory, and learning in human, non-human primates, and other animals including dogs [12,13,15,16,51,52]. Contrast sensitivity results here provided the most compelling evidence of age-countering effects on visual processing as a result of dietary fortification with dried whole cell *Schizochytrium sp*. [53]. During the initial acquisition phase, the algal group learned the initial discrimination more rapidly and with fewer errors compared to control animals. In addition, results from the variable contrast phase showed highly significant differences between the two groups at contrasts of 3% and 5%. Although on average, the algal fed dogs performed more accurately on all but the highest contrast, variability between subjects within each group may explain the lack of significant differences as a result of dietary treatment. Performance at contrasts $\geq$25% was equivalent between dietary treatments. These results were consistent with previous observations in canines which indicate a contrast threshold beyond which age-effects on the ability to distinguish differences between objects becomes greatly diminished [11].

Performance on the contrast sensitivity protocol depends on the ability to detect and then discriminate between objects or shapes presented at low contrasts to background ratio, and is a function of size and spatial frequency [53]. Contrast sensitivity declines with age in humans [12,13] and dogs [11,38,39]. DHA accretion in the retinal membrane supports photoreceptor spectral sensitivity under conditions of low contrast by lowering the threshold of photon-induced activation of the phosphodiesterase cascade by the rod outer segments, during signal transduction to the visual cortex [54,55]. Electroretinography has been used to demonstrate sensitivity by photoreceptors to diet-induced changes to the status of DHA, during the early stages of canine development [38,39]. Therefore, a similar effect on vision by DHA-rich dried whole cell algae in this trial may at least partially account for the differences in performance on contrast sensitivity tasks by each group. Here, selection of the correct response required retention of information related to the shape and spatial relationship between objects based upon the results from a previous experience. Information about spatial relationships is received from the retina and maintained according to various attributes in the visual cortex as stimulus representations for short periods during working memory [56,57]. In order to differentiate between the contributions by the retina and visual processing centers, non-periodic patterns, or images on a contrasting background, were used, as opposed to periodic patterns such as sine waves, in order to evaluate contrast sensitivity in this study. The differences between groups on the results for contrast sensitivity and for performance at variable contrast may therefore be interpreted to indicate that algal-DHA supports retention of information during visual processing related to spatial memory during testing in a canine model of brain aging. Notably, significant differences in group accuracy during performance on variable contrast were only detected at intermediate contrasts, which afford increased difficulty during object recognition by older animals [11]. These results could represent group differences at the level of the retina, cerebral cortex and possibly both, and are consistent with roles hypothesized for the visual cortex. Additional studies in this area may further the current understanding about interactions, or lack thereof, between DHA and other possible underlying factors which may contribute to persistent signs in some individuals of cognitive deficits during senescence.

4.3. Concurrent discrimination learning

Concurrent discrimination (CCD) task assesses the ability to retain information about complex object to object associations in the presence of novel objects with similar but different features (e.g., shape, size). Results in this investigation did not support rejection of the null hypothesis. Future investigations should consider the use of middle-age adult dogs and or increasing sample size.

5. Conclusions

Results from this investigation provides support for the use of dried *Schizochytrium sp*. biomass as a suitable source of the n-3 LCPUFA DHA for canine diets. Microalgae has been widely recognized as a source of nutrients and compounds associated with health and well-being in humans and animals. Carotenoids, including β-carotene, polyphenols, and tocopherols are valued components of algae due to the potential application as natural antioxidants in food products [58]. Dietary supplementation using antioxidants, including beta-carotene and tocopherol have been associated with improved cognitive function related to contrast sensitivity in the context of age-related cognitive aged dogs [11]. Antioxidant activities vary among microalgae [59,60]. Total antioxidant activity was not measured in the dried algal biomass used in this study and remains to be determined by future investigations. Low fat *Schizochytrium sp*. may be considered for possible use in a control diet. Contributions by other nutrients in the algal biomass to the observed results in this investigation cannot be ruled out. However, the dietary concentrations of these other nutrients were arguably insufficient to attribute positive outcomes associated with intake of the algal-fortified diet. DHA is therefore proposed as the primary mechanism associated with improved performance on cognitive tasks. Additional investigations are needed in order to confirm the observations related to performance on cognitive assessments of spatial learning and memory, and visual processing in pre-senescent and aged animals. Overall, these observations are consistent with an association between consumption of a diet fortified with DHA-rich algae and support of healthy brain function during senescence in a canine model of brain aging.

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Conflict of interest notification

Dr. Kevin B. Hadley is employed by DSM Nutritional Products. The study was funded by DSM Nutritional Products.

Contributors

Milgram, Bauer, and Hadley contributed to the study concept and design. Milgram and Hadley participated in the study conduct. Milgram was responsible for the statistical analysis. Milgram and Hadley contributed to the interpretation of data. Hadley was responsible for drafting the manuscript. Bauer, Milgram, and Hadley contributed participated in the revision of the manuscript.

References

[1] L. Lauritzen, H.S. Hansen, M.H. Jorgensen, K.F. Michaelsen, The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina, Prog. Lipid Res. 40 (2001) 1–94.
[2] H.Y. Kim, N. Salem Jr., Phospholipid molecular species analysis by thermospray liquid chromatography/mass spectrometry, Anal. Chem. 58 (1986) 9–14.
[3] N. Salem Jr., B. Litman, H.Y. Kim, K. Gawrisch, Mechanisms of action of docosahexaenoic acid in the nervous system, Lipids 36 (2001) 945–959.
[4] W.J. Lukiw, N.G. Bazan, Docosahexaenoic acid and the aging brain, J. Nutr. 138 (2008) 2510–2514.
[5] M.A. Beydoun, J.S. Kaufman, J.A. Satia, W. Rosamond, A.R. Folsom, Plasma n-3 fatty acids and the risk of cognitive decline in older adults: the atherosclerosis risk in communities study, Am. J. Clin. Nutr. 85 (2007) 1103–1111.
[6] B. Heude, P. Ducimetiere, C. Berr, E.V.A. Study, Cognitive decline and fatty acid composition of erythrocyte membranes – the EVA study, Am. J. Clin. Nutr. 77 (2003) 803–808.
[7] J.A. Conquer, M.C. Tierney, J. Zecevic, W.J. Bettger, R.H. Fisher, Fatty acid

analysis of blood plasma of patients with Alzheimer's disease, other types of dementia, and cognitive impairment, Lipids 35 (2000) 1305–1312.
[8] E.J. Schaefer, V. Bongard, A.S. Beiser, S. Lamon-Fava, S.J. Robins, R. Au, K.L. Tucker, D.J. Kyle, P.W. Wilson, P.A. Wolf, Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham heart study, Arch. Neurol. 63 (2006) 1545–1550.
[9] K. Yurko-Mauro, D. McCarthy, D. Rom, E.B. Nelson, A.S. Ryan, A. Blackwell, N. Salem Jr., M. Stedman, M. Investigators, Beneficial effects of docosahexaenoic acid on cognition in age-related cognitive decline, Alzheimer'S. Dement.: J. Alzheimer'S. Assoc. 6 (2010) 456–464.
[10] K. Yurko-Mauro, D.D. Alexander, M.E. Van Elswyk, Docosahexaenoic acid and adult memory: a systematic review and meta-analysis, PLoS One 10 (2015) e0120391.
[11] C. de Rivera, I. Boutet, S.C. Zicker, N.W. Milgram, A novel method for assessing contrast sensitivity in the beagle dog is sensitive to age and an antioxidant enriched food, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 29 (2005) 379–387.
[12] J.E. Ross, D.D. Clarke, A.J. Bron, Effect of age on contrast sensitivity function: uniocular and binocular findings, Br. J. Ophthalmol. 69 (1985) 51–56.
[13] G. Schlotterer, M. Moscovitch, D. Crapper-McLachlan, Visual processing deficits as assessed by spatial frequency contrast sensitivity and backward masking in normal ageing and Alzheimer's disease, Brain: a J. Neurol. 107 (Pt 1) (1984) 309–325.
[14] Y. Xing, T. Ledgeway, P.V. McGraw, D. Schluppeck, Decoding working memory of stimulus contrast in early visual cortex, J. Neurosci.: Off. J. Soc. Neurosci. 33 (2013) 10301–10311.
[15] A.D. Chan, P.M. Nippak, H. Murphey, C.J. Ikeda-Douglas, B. Muggenburg, E. Head, C.W. Cotman, N.W. Milgram, Visuospatial impairments in aged canines (Canis familiaris): the role of cognitive-behavioral flexibility, Behav. Neurosci. 116 (2002) 443–454.
[16] E. Head, R. Mehta, J. Hartley, M. Kameka, B.J. Cummings, C.W. Cotman, W.W. Ruehl, N.W. Milgram, Spatial learning and memory as a function of age in the dog, Behav. Neurosci. 109 (1995) 851–858.
[17] N.W. Milgram, E. Head, B. Muggenburg, D. Holowachuk, H. Murphey, J. Estrada, C.J. Ikeda-Douglas, S.C. Zicker, C.W. Cotman, Landmark discrimination learning in the dog: effects of age, an antioxidant fortified food, and cognitive strategy, Neurosci. Biobehav. Rev. 26 (2002) 679–695.
[18] N.W. Milgram, S.C. Zicker, E. Head, B.A. Muggenburg, H. Murphey, C.J. Ikeda-Douglas, C.W. Cotman, Dietary enrichment counteracts age-associated cognitive dysfunction in canines, Neurobiol. Aging 23 (2002) 737–745.
[19] N.W. Milgram, E. Head, E. Weiner, E. Thomas, Cognitive functions and aging in the dog: acquisition of nonspatial visual tasks, Behav. Neurosci. 108 (1994) 57–68.
[20] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[21] J. Larose, P. Julien, J.F. Bilodeau, Analysis of F2-isoprostanes in plasma of pregnant women by HPLC-MS/MS using a column packed with core-shell particles, J. Lipid Res. 54 (2013) 1505–1511.
[22] M. Janicka, A. Kot-Wasik, J. Paradziej-Lukowicz, G. Sularz-Peszynska, A. Bartoszek, J. Namiesnik, LC-MS/MS determination of isoprostanes in plasma samples collected from mice exposed to doxorubicin or tert-butyl hydroperoxide, Int. J. Mol. Sci. 14 (2013) 6157–6169.
[23] N.W. Milgram, J.A. Araujo, T.M. Hagen, B.V. Treadwell, B.N. Ames, Acetyl-L-carnitine and alpha-lipoic acid supplementation of aged beagle dogs improves learning in two landmark discrimination tests, FASEB J.: Off. Publ. Fed. Am. Soc. Exp. Biol. 21 (2007) 3756–3762.
[24] A. Gonzalez-Martinez, B. Rosado, P. Pesini, S. Garcia-Belenguer, J. Palacio, A. Villegas, M.L. Suarez, G. Santamarina, M. Sarasa, Effect of age and severity of cognitive dysfunction on two simple tasks in pet dogs, Vet. J. 198 (2013) 176–181.
[25] C.M. Studzinski, L.A. Christie, J.A. Araujo, W.M. Burnham, E. Head, C.W. Cotman, N.W. Milgram, Visuospatial function in the beagle dog: an early marker of cognitive decline in a model of human aging and dementia, Neurobiol. Learn. Mem. 86 (2006) 197–204.
[26] P.D. Tapp, C.T. Siwak, J. Estrada, D. Holowachuk, N.W. Milgram, Effects of age on measures of complex working memory span in the beagle dog (Canis familiaris) using two versions of a spatial list learning paradigm, Learn Mem. 10 (2003) 148–160.
[27] E. Head, V.N. Nukala, K.A. Fenoglio, B.A. Muggenburg, C.W. Cotman, P.G. Sullivan, Effects of age, dietary, and behavioral enrichment on brain mitochondria in a canine model of human aging, Exp. Neurol. 220 (2009) 171–176.
[28] M. Fahnestock, M. Marchese, E. Head, V. Pop, B. Michalski, W.N. Milgram, C.W. Cotman, BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog, Neurobiol. Aging 33 (2012) 546–554.
[29] C.T. Siwak-Tapp, E. Head, B.A. Muggenburg, N.W. Milgram, C.W. Cotman, Neurogenesis decreases with age in the canine hippocampus and correlates with cognitive function, Neurobiol. Learn. Mem. 88 (2007) 249–259.
[30] C.T. Siwak-Tapp, E. Head, B.A. Muggenburg, N.W. Milgram, C.W. Cotman, Region specific neuron loss in the aged canine hippocampus is reduced by enrichment, Neurobiol. Aging 29 (2008) 39–50.
[31] E. Head, J. Liu, T.M. Hagen, B.A. Muggenburg, N.W. Milgram, B.N. Ames, C.W. Cotman, Oxidative damage increases with age in a canine model of human brain aging, J. Neurochem. 82 (2002) 375–381.
[32] A. Skoumalova, J. Rofina, Z. Schwippelova, E. Gruys, J. Wilhelm, The role of free radicals in canine counterpart of senile dementia of the Alzheimer type, Exp. Gerontol. 38 (2003) 711–719.
[33] E. Head, R. McCleary, F.F. Hahn, N.W. Milgram, C.W. Cotman, Region-specific age at onset of beta-amyloid in dogs, Neurobiol. Aging 21 (2000) 89–96.
[34] M.J. Russell, M. Bobik, R.G. White, Y. Hou, S.A. Benjamin, J.W. Geddes, Age-specific onset of beta-amyloid in beagle brains, Neurobiol. Aging 17 (1996) 269–273.
[35] W.O. Opii, G. Joshi, E. Head, N.W. Milgram, B.A. Muggenburg, J.B. Klein, W.M. Pierce, C.W. Cotman, D.A. Butterfield, Proteomic identification of brain proteins in the canine model of human aging following a long-term treatment with antioxidants and a program of behavioral enrichment: relevance to Alzheimer's disease, Neurobiol. Aging 29 (2008) 51–70.
[36] V. Pop, E. Head, M.A. Hill, D. Gillen, N.C. Berchtold, B.A. Muggenburg, N.W. Milgram, M.P. Murphy, C.W. Cotman, Synergistic effects of long-term antioxidant diet and behavioral enrichment on beta-amyloid load and non-amyloidogenic processing in aged canines, J. Neurosci. : Off. J. Soc. Neurosci. 30 (2010) 9831–9839.
[37] N.W. Milgram, E. Head, S.C. Zicker, C.J. Ikeda-Douglas, H. Murphey, B. Muggenburg, C. Siwak, D. Tapp, C.W. Cotman, Learning ability in aged beagle dogs is preserved by behavioral enrichment and dietary fortification: a two-year longitudinal study, Neurobiol. Aging 26 (2005) 77–90.
[38] K.M. Heinemann, M.K. Waldron, K.E. Bigley, G.E. Lees, J.E. Bauer, Long-chain (n-3) polyunsaturated fatty acids are more efficient than alpha-linolenic acid in improving electroretinogram responses of puppies exposed during gestation, lactation, and weaning, J. Nutr. 135 (2005) 1960–1966.
[39] S.C. Zicker, D.E. Jewell, R.M. Yamka, N.W. Milgram, 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, J. Am. Vet. Med. Assoc. 241 (2012) 583–594.
[40] N.R.C.A.H.C.o. Dog, C. Nutrition, Nutrient requirements of dogs and cats, National Academies Press, 2006.
[41] C.J. LeBlanc, J.E. Bauer, G. Hosgood, G.E. Mauldin, Effect of dietary fish oil and vitamin E supplementation on hematologic and serum biochemical analytes and oxidative status in young dogs, Vet. Ther.: Res. Appl. Vet. Med. 6 (2005) 325–340.
[42] L.M. Freeman, J.E. Rush, P.E. Milbury, J.B. Blumberg, Antioxidant status and biomarkers of oxidative stress in dogs with congestive heart failure, J. Vet. Intern. Med. 19 (2005) 537–541.
[43] M. Sakamoto, E. Takaki, K. Yamashita, K. Watanabe, S. Tabuchi, T. Watanabe, K. Satoh, Nonenzymatic derived lipid peroxide, 8-iso-PGF2 alpha, participates in the pathogenesis of delayed cerebral vasospasm in a canine SAH model, Neurol. Res. 24 (2002) 301–306.
[44] D. Pratico, C.M. Clark, F. Liun, J. Rokach, V.Y. Lee, J.Q. Trojanowski, Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease, Arch. Neurol. 59 (2002) 972–976.
[45] E. Head, J. Rofina, S. Zicker, Oxidative stress, aging, and central nervous system disease in the canine model of human brain aging, Vet. Clin. North Am. Small Anim. Pract. 38 (2008) 167–178.
[46] E. Mas, R.J. Woodman, V. Burke, I.B. Puddey, L.J. Beilin, T. Durand, T.A. Mori, The omega-3 fatty acids EPA and DHA decrease plasma F(2)-isoprostanes: results from two placebo-controlled interventions, Free Radic. Res. 44 (2010) 983–990.
[47] H. Yin, W. Liu, K. Goleniewska, N.A. Porter, J.D. Morrow, R.S. Peebles Jr., Dietary supplementation of omega-3 fatty acid-containing fish oil suppresses F2-isoprostanes but enhances inflammatory cytokine response in a mouse model of ovalbumin-induced allergic lung inflammation, Free Radic. Biol. Med. 47 (2009) 622–628.
[48] J.M. Walters, T.B. Hackett, G.K. Ogilvie, M.J. Fettman, Polyunsaturated fatty acid dietary supplementation induces lipid peroxidation in normal dogs, Vet. Med. Int. 2010 (2010) 619083.
[49] J. Helmersson, J. Arnlov, A. Larsson, S. Basu, Low dietary intake of beta-carotene, alpha-tocopherol and ascorbic acid is associated with increased inflammatory and oxidative stress status in a Swedish cohort, Br. J. Nutr. 101 (2009) 1775–1782.
[50] O. Ishihara, M. Hayashi, H. Osawa, K. Kobayashi, S. Takeda, B. Vessby, S. Basu, Isoprostanes, prostaglandins and tocopherols in pre-eclampsia, normal pregnancy and non-pregnancy, Free Radic. Res. 38 (2004) 913–918.
[51] R.J. Robitsek, N.J. Fortin, M.T. Koh, M. Gallagher, H. Eichenbaum, Cognitive aging: a common decline of episodic recollection and spatial memory in rats, J. Neurosci.: Off. J. Soc. Neurosci. 28 (2008) 8945–8954.
[52] M.T. Schmolesky, Y. Wang, M. Pu, A.G. Leventhal, Degradation of stimulus selectivity of visual cortical cells in senescent rhesus monkeys, Nat. Neurosci. 3 (2000) 384–390.
[53] C. Owsley, Contrast sensitivity, Ophthalmol. Clin. North Am. 16 (2003) 171–177.
[54] S.L. Niu, D.C. Mitchell, S.Y. Lim, Z.M. Wen, H.Y. Kim, N. Salem Jr., B.J. Litman, Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency, J. Biol. Chem. 279 (2004) 31098–31104.
[55] D.G. Birch, E.E. Birch, D.R. Hoffman, R.D. Uauy, Retinal development in very-low-birth-weight infants fed diets differing in omega-3 fatty acids, Invest. Ophthalmol. Vis. Sci. 33 (1992) 2365–2376.
[56] S.J. Luck, E.K. Vogel, The capacity of visual working memory for features and conjunctions, Nature 390 (1997) 279–281.
[57] T. Pasternak, M.W. Greenlee, Working memory in primate sensory systems, Nat. Rev. Neurosci. 6 (2005) 97–107.
[58] K. Goiris, K. Muylaert, I. Fraeye, I. Foubert, J.D. Brabanter, L.D. Cooman, Antioxidant potential of microalgae in relation to their phenolic and carotenoid content, J. Appl. Phycol. 24 (2012) 1477–1486.
[59] H.B. Li, K.W. Cheng, C.C. Wong, K.W. Fan, F. Chen, Y. Jiang, Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae, Food Chem. 102 (2007) 771–776.
[60] M.S. Miranda, R.G. Cintra, S.B.M. Barros, J. Mancini-Filho, Antioxidant activity of the microagla Spirulina maxima, Braz. J. Med. Biol. Res. 31 (1998) 1075–1079.