Learning ability in aged beagle dogs is preserved by behavioral enrichment and dietary fortification: a two-year longitudinal study

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Abstract
The effectiveness of two interventions, dietary fortification with antioxidants and a program of behavioral enrichment, was assessed in a longitudinal study of cognitive aging in beagle dogs. A baseline protocol of cognitive testing was used to select four cognitively equivalent groups: control food-control experience (C-C), control food-enriched experience (C-E), antioxidant fortified food-control experience (A-C), and antioxidant fortified food-enriched experience (A-E). We also included two groups of young behaviorally enriched dogs, one receiving the control food and the other the fortified food. Discrimination learning and reversal was assessed after one year of treatment with a size discrimination task, and again after two years with a black/white discrimination task. The four aged groups were comparable at baseline. At one and two years, the aged combined treatment group showed more accurate learning than the other aged groups. Discrimination learning was significantly improved by behavioral enrichment. Reversal learning was improved by both behavioral enrichment and dietary fortification. By contrast, the fortified food had no effect on the young dogs. These results suggest that behavioral enrichment or dietary fortification with antioxidants over a long-duration can slow age-dependent cognitive decline, and that the two treatments together are more effective than either alone in older dogs.

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1. Introduction
Over the past several years, our laboratories have been studying a novel model of cognitive aging, that of the aged beagle dog. We have previously established that dogs show marked age-dependent decline in learning and memory, which varies as a function of task. The pattern of cognitive decline mirrors that seen in humans in several respects. Aged dogs also develop neuropathology that is similar to that seen in both successfully aging humans and patients with Alzheimer’s disease. Like humans, beta amyloid protein is deposited in the aging dog brain, and shows a selective brain distribution that changes as a function of age. To date, our analysis of canine cognitive aging has been restricted to a cross-sectional study, in which selected groups of aged animals are compared with selected groups of younger animals. This strategy has limitations, which include cohort effects and the possibility of selective bias in mortality. 

The present study sought to further extend the canine model of cognitive aging with a two-year longitudinal investigation of discrimination and reversal learning ability in groups of young and aged beagle dogs. We had two major goals. The first was to obtain longitudinal data of age-dependent decline in learning ability. The second was to assess the effectiveness of two interventions in counter-
acting age-dependent decline, behavioral enrichment and a maintenance food fortified with a broad spectrum of antioxidants and mitochondrial cofactors.

1.1. Behavioral enrichment and age-dependent cognitive decline

The behavioral enrichment intervention included three components: increased exercise, environmental enrichment, and possibly most important, a program of cognitive enrichment. Exercise was suggested primarily by studies indicating that physical activity is associated with improved cognitive function and lower risks of cognitive impairment and dementia [12,13]. The second component, environmental enrichment, was suggested by evidence that rearing in enriched environments improves learning ability, produces beneficial changes in cellular structure and increases the resistance of neurons to injury [28]. The effect can be sufficiently robust as to reduce or eliminate age-dependent cognitive decline [15].

The rationale for including a cognitive enrichment intervention was based on retrospective studies of human subjects suggesting a link between cognitive experience and the development of age-dependent cognitive dysfunction. People characterized as having a low level of cognitive function are more likely to develop severe cognitive dysfunction than people characterized as having a high level of cognitive function [20]. Similarly, several studies have reported an inverse relationship between amount of education and rate of cognitive decline later in life. More direct evidence has been obtained in studies demonstrating that special training protocols improve cognitive performance in the elderly [5], and in patients with dementia [36].

1.2. Antioxidant supplementation and age-dependent cognitive decline

The dietary intervention consisted of providing a dry maintenance dog food fortified with a broad spectrum of antioxidants and mitochondrial cofactors. The food was intended to reduce damage to tissue by reactive oxygen species as well as support mitochondrial function. Reactive oxygen species are formed as by-products of cellular metabolism and, when produced in amounts in excess of detoxification, are purported to cause oxidative stress [7]. Oxidative damage to proteins and lipids has been linked to the development and accumulation of neuropathology associated with degenerative disease as well as normal aging [3,18,27,35], and is therefore a likely causal factor in age-dependent cognitive decline.

There is also more direct evidence of beneficial effects of dietary supplementation with antioxidants on cognition. In aged rats, antioxidants improved spatial learning [25,43], motor learning and cerebellar function [8,10]. The effectiveness of antioxidants in counteracting age-related cognitive decline has also recently been demonstrated in mice deficient in apolipoprotein [48], Vitamin E deficient aged rats [17], and in the SAMP8 mouse [16]. In clinical trials, antioxidant supplementation of Alzheimer’s patients with Vitamin E was found to delay the onset of institutionalization [41]. We have also found that short-term maintenance on an antioxidant fortified food improved discrimination learning in beagle dogs [31,32]. Furthermore, we have previously shown evidence of increased oxidative stress in the aged canine brain [23].

The present investigation started with a period of baseline testing, which was used to separate beagle dogs, approximately 8–11 years of age, into four cognitively equivalent groups, which differed in food provided and behavioral enrichment. We hypothesized that both the behavioral enrichment and dietary supplementation treatments would have beneficial effects on cognitive function, and that the two treatments combined would be more effective than either by itself. To partially evaluate this hypothesis, one year after the treatment phase was initiated the animals were tested successively on a size discrimination learning task and a size discrimination reversal learning task. These tasks were selected because they were conceptually similar to the object discrimination learning and object discrimination reversal tasks used in assessing baseline cognitive function. Furthermore, we have previously found the size discrimination and size discrimination reversal tasks to show age-sensitivity [21,45]. The one-year results have been previously reported [34]. Both the fortified food and behavioral enrichment improved learning, most notably in the reversal learning task. However, these findings were mainly attributable to the superior performance of the combined treatment group (fortified food and enriched experience), when compared to the other three groups. Approximately two years following the start of the treatment phase, the animals were tested on a black/white discrimination and a black/white discrimination reversal learning task, to provide a protocol for assessing longitudinal changes in discrimination and reversal learning and the effectiveness of the fortified food and behavioral enrichment interventions over two years.

2. Materials and methods

2.1. General design

Forty-eight old and 17 young beagle dogs were trained on a battery of cognitive tests over approximately 9 months. Performance on the baseline testing was then used to divide the aged dogs into four cognitively equivalent test groups of 12 animals each, with two treatment conditions—dietary fortification and behavioral enrichment. Group, C-C, was both fed the control food and provided with control experience; group, C-E, received the control food and a program of enriched experience. Group, A-C, was fed food fortified with antioxidants and mitochondrial cofactors and also given control experience: while group A-E received both the fortified food and the enriched experience.
The young beagle dogs were divided into two cognitively equivalent groups, one of which was given the antioxidant enriched food (N = 9) and the other the control food (N = 8). One control dog was subsequently dropped from the study during the first treatment year because of motivational problems, reducing the size of the control group to seven. Both young groups received the behavioral enrichment protocol.

One year after the start of the dietary manipulation, all dogs were tested on both a size discrimination and a reversal learning task. Prior to the one-year test, dogs in the enriched environment groups had participated in a landmark discrimination task [31] and an oddity discrimination task [32]. The size discrimination task evaluated ability to learn to distinguish two objects that differed only in size in order to locate a food reward. In the size discrimination reversal task, the association between the objects and reward was switched. Thus, if an animal was rewarded for approaching the smaller of two objects during the initial discrimination learning task, it was rewarded for approaching the larger of the two objects during the reversal task. The size discrimination results have been previously reported [34].

The enrichment condition between years 1 and 2 of treatment consisted of training on a size concept learning task [46] and on a repeated reversal learning task. At the completion of the two-year enrichment phase, all animals were trained on a black/white discrimination learning task, in which the subjects were presented with two blocks that were identical in size and shape but differed in color, with one object painted black and the other white. The animals were first trained to approach one of the two to obtain a food reward. After achieving a criterion level of performance, the rewarded objects were then switched for the black/white discrimination reversal phase.

2.2. Subjects

Two groups of beagle dogs (Canis familiaris) served as subjects. The first consisted of 48 aged dogs (24 males and 24 females) ranging from 7.2 to 11.6 years at the start of baseline testing, and from 8.05 to 12.04 years of age at the start of the treatment phase. The second group consisted of 17 young dogs (6 males and 11 females), 1.3–3.9 years of age at the start of baseline testing and 1.95–4.6 at the start of the treatment phase. Half the dogs (young and old) came from a closed colony of beagle dogs (Cohort 1). The other half were obtained from a second, independent, closed colony (Cohort 2). Both groups were naïve with respect to cognitive test experience before starting the study.

The aged dogs were housed, either singly or in pairs, in pens with continual access to fresh water. Because of space considerations, the young dogs were housed in a separate animal facility, from two to four per room. In all other respects, the old and young animals were treated identically. All dogs were fed approximately 300 g of the control food once daily. In each case, however, the quantity of food was adjusted so that the animals maintained a relatively constant body weight throughout the duration of the study.

The aged subjects were all administered complete physical and neurological examinations prior to the dietary intervention and again every six months after the start of intervention. Dogs were also initially examined by slit-lamp for ocular abnormalities that might have impaired the animals’ visual capabilities. The initial physical examinations did not reveal neurological, musculoskeletal, ocular or physical abnormalities that justified exclusion from the study.

2.3. Apparatus

The test apparatus was a 0.609 m × 1.15 m × 1.08 m wooden chamber that was based on a canine adaptation of the Wisconsin General Test Apparatus [29]. The testing chamber was equipped with a sliding Plexiglas food tray with three food wells. Vertical stainless steel bars covered the front of the box. The height of the bars was adjustable to allow the size of the opening to each food well to be uniquely adjusted for each dog. The experimenter was separated visually from the dog, by a wooden screen containing a one-way mirror, and a hinged wooden door at the bottom. Testing was conducted in darkness, except for a light with a 60 W bulb attached to the front of the box. Each test trial commenced with the hinged door being opened for the presentation of the tray. A 1 cm³ amount of Hill’s® Prescription Diet® Canine p/d™ canned food was used as the reward.

2.4. Dietary intervention

The control and antioxidant foods were formulated to meet the nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs (AAFCO 1999) [4]. The two foods were identical, except for the inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the test food. The control and enriched foods had the following differences in formulation on an as fed basis, respectively: d,l-alpha-tocopherol acetate (120 ppm versus approximately 000 ppm), L-carnitine ([20 ppm versus approximately 275 ppm], d,l-alpha-lipoic acid (≥20 ppm versus approximately 125 ppm), ascorbic acid as Stay-C (≤30 ppm versus approximately 80 ppm), and 1% inclusions of each of the following (1:1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp. The rationale for these inclusions is as follows: Vitamin E is lipid soluble and acts to protect cell membranes from oxidative damage; Vitamin C is essential in maintaining oxidative protection for the soluble phase of cells as well as preventing Vitamin E from propagating free radical production; alpha-lipoic acid is a cofactor for the mitochondrial respiratory chain enzymes, pyruvate and alpha-ketoglutarate dehydrogenases, as well as an antioxidant capable of redox recycling other antioxidants and raising intracellular glutathione levels; L-carnitine is a precursor to acetyl-L-carnitine and is involved in mitochon-
trait lipid metabolism and maintaining efficient function; fruits and vegetables are rich in flavonoids and carotenoids and other antioxidants. The diet was produced by an extrusion process and was fed for no more than six months before a new lot was manufactured.

2.5. Behavioral enrichment intervention

The behavioral enrichment condition commenced after completion of the baseline cognitive testing. The animals in the enriched group were housed with kennel mates, exercised twice a week for 15 min intervals, and given sets of toys that were alternated weekly. None of these were provided to the control animals. The enrichment condition also included a cognitive enrichment protocol. The first year of cognitive enrichment started immediately after baseline with testing on a series of discrimination learning tasks, as previously described [31]. After completing the landmark task, the subjects were tested on a series of oddity discrimination learning problems [32]. After completing the oddity problems, the dogs were then tested for retention of the landmark task. The cognitive enrichment provided during the second year consisted of a series of nine discrimination learning tasks that were intended to study size concept learning [46] and a series of repeated reversal learning tasks that were intended to study learning set formation.

2.6. Cognitive test protocol

2.6.1. Pretraining

All subjects underwent a standard pretraining cognitive testing protocol. It consisted of reward approach and object approach learning [29], which were procedural learning tasks designed to train animals to displace an object on a tray to obtain a food reward consisting of approximately 1 g of Hill’s® Prescription Diet® Canine p/d® canned food. The dogs responded to the objects by pushing them away from the food well with their noses, and then eating the food. After completing the procedural learning tasks, all subjects were trained on an object discrimination learning task, which was followed by an object reversal learning task [29]. The animals were then tested on an object recognition memory task [9] and delayed non-matching-to-position task (DNMP) [11]. The initial group assignment took into consideration age, sex, cohort and the subjects combined performance on the reversal learning task, the object recognition task, and the DNMP task. The baseline cognitive data has previously been reported [34]. The four test groups of aged dogs did not differ on any of the baseline tests used in classification. Similarly, the two test groups of young animals did differ from each other on the baseline evaluations. There were, however, significant differences between the old and young animals in performance on the reversal and visuospatial tasks. All animals were maintained on the control food during the pretraining period, which continued for approximately nine months.

2.6.2. Year 1 assessment

Testing on the size discrimination learning task commenced approximately 20 months following the start of baseline testing, and approximately one year after the start of the treatment phase. For the animals in the behaviorally enriched group (group C-E and group A-E), a one-week non-test interval preceded the start of size discrimination learning. The dogs in the control experience (A-C, C-C) condition did not undergo any cognitive testing for approximately nine months after completing the baseline testing. The procedure followed in the size and size reversal learning has been described previously [34,45].

2.6.3. Year 2 assessment

Training on the black/white discrimination task started approximately two years after starting the treatment conditions. We used two wooden blocks that were identical in all respects except color: one was covered with white enamel paint and the other black enamel paint. The subjects were first administered a preference test, which consisted of a single test session of 10 trials used to establish object preferences; the total choices of one the blocks out of 10 provided the absolute preference score. On this and all subsequent test sessions, the objects were placed over the two lateral food wells, and the location of the objects varied randomly, with the constraint that each object was placed on each lateral food well on exactly 50% of the trials. A customized computer program controlled all timing and randomization procedures. The program also assured that on each trial, the locations of the objects were the same for each animal. Before the beginning of each trial, the computer emitted a tone that served as a cue for the dog and instructed the experimenter to present the food tray. Each trial was started when the experimenter pressed a key and simultaneously presented the tray to the subject. The dogs’ responses to the two objects were recorded by a key press, which also indicated the end of the trial and signalled the beginning of the inter-trial interval.

Training on the black/white discrimination problem started on the day following the preference test. The animals were given 10 trials per day, constituting one session, with an intertrial interval of 30 s. Testing was six days per week. The animals received a maximum of 40 training sessions to achieve a two-stage criterion. The first stage was successfully met once the animal either averaged 80% over two sessions, or at least 90% on a single session. To complete the second stage, the dog was required to respond correctly on at least 70% of the trials over three successive sessions. Thus, passing both stages took a minimum of four test sessions. One dog failed to learn the black/white discrimination task within the 40 trials, and was consequently administered a program of remedial training, so that the dog could be tested on the reversal task. During the remedial training phase, an additional 13 training sessions were allotted for each animal to reach the criterion performance level.
The black/white reversal task started on the day following completion of the black/white discrimination learning task. The testing procedures were identical, except that the reward contingencies of the positive and negative block were reversed. Thus, if an animal was rewarded for approaching the white block during the initial testing, it was now rewarded for approaching the black block.

2.7. Statistics

For individual subjects, rate of learning was characterized by error scores, which were calculated by adding the total number of errors to either pass the two-stage learning criterion, or the total number of errors made over 40 training trials. The data were analyzed with factorial and repeated measures analysis of variance (ANOVA) [42]. When required, post hoc analysis was performed by Tukey’s studentized range test (HSD), using the 0.05 level of significance. In addition, chi-square analysis was performed on the frequency of failure for the three assessment periods.

The initial analyses took into consideration food, behavioral enrichment condition, cohort and sex. There were no significant effects of sex on any of the dependent variables and sex was therefore dropped from all subsequent analyses.

3. Results

3.1. Survival

Table 1 shows the sample size and mean ages of each group at the start of discrimination testing during baseline, after one year of treatment and after two years. As indicated in Table 1, completed sets of discrimination and reversal data were not obtained from four animals assigned to group C-C, and 1 animal assigned to group C-E: Four of these died or had to be euthanized for medical reasons; the fifth was dropped from the study because of motivational problems.

3.2. Effect of food and experience on learning a black/white discrimination and reversal task

For the aged animals, the results of the black/white task were first analyzed with a repeated measures analysis of variance, with discrimination and reversal learning as within subject measures, and cohort, food, and behavioral enrichment as between subject measures. The results revealed significant main effects of food \((F(1,34) = 4.678; P < 0.05)\), behavioral enrichment \((F(1,34) = 31.89; P < 0.01)\) and task \((F(1,34) = 78.93; P < 0.01)\). As expected, the task effect was due to the reversal task being more difficult than the original discrimination learning task.

To further breakdown the food and behavioral enrichment effects, we performed separate factorial analyses for the black/white discrimination task and for the black/white discrimination reversal task. On the black/white discrimination task, the ANOVA revealed a significant main effect of behavioral enrichment \((F(1,38) = 22.35; P < 0.01)\), and no other significant effects or interactions. Fig. 1A illustrates that each of the groups that received behavioral enrichment (group A-E and group C-E) made fewer errors than either of the two non-enriched groups (group A-C and group C-C). Tukey’s LSDs indicated that each behavioral enrichment groups performed significantly better than its respective control enrichment group (group C-C versus group C-E and group A-C versus group A-E). By contrast, the control groups did not differ from each other.

Analysis of black/white discrimination reversal learning, on the other hand, revealed significant effects of both behavioral enrichment \((F(1,34) = 27.2094; P < 0.01)\) and food \((F(1,34) = 5.11; P < 0.05)\). These results are largely due to the high level of performance of group A-E (see Fig. 1B), which received the combined treatment of antioxidant enriched food and behavioral enrichment. Further, post hoc analysis revealed that group A-E did significantly better than group C-C and group A-C. The only other significant group differences were between group C-E and group C-C.

We also analyzed the data from the young animals using a repeated measures ANOVA, and found a significant effect of

### Table 1

<table>
<thead>
<tr>
<th>Age and treatment group</th>
<th>Object discrimination (baseline)</th>
<th>Size discrimination (year 1)</th>
<th>Black/white discrimination (year 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Age (years)</td>
<td>N</td>
</tr>
<tr>
<td>Old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C</td>
<td>12</td>
<td>9.95 ± 1.01</td>
<td>12</td>
</tr>
<tr>
<td>C-E</td>
<td>12</td>
<td>9.96 ± 0.93</td>
<td>11</td>
</tr>
<tr>
<td>A-C</td>
<td>12</td>
<td>9.76 ± 1.24</td>
<td>12</td>
</tr>
<tr>
<td>A-E</td>
<td>12</td>
<td>9.45 ± 1.15</td>
<td>12</td>
</tr>
<tr>
<td>Young</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9</td>
<td>2.50 ± 1.08</td>
<td>9</td>
</tr>
<tr>
<td>C</td>
<td>8</td>
<td>2.30 ± 1.08</td>
<td>7</td>
</tr>
</tbody>
</table>

Mean ages and standard deviations of the four groups of old dogs and two groups of young dogs at the start of baseline discrimination learning testing, after one year of treatment, and after two years of treatment.
Fig. 1. Effect of antioxidant fortified food and behavioral enrichment on acquisition of a black/white discrimination learning task (A) and reversal learning task (B) in aged beagle dogs. Error bars represent standard errors. Group C-C: control food-control experience, Group C-E: control food-enriched experience, Group A-C: antioxidant fortified food-control experience, Group A-E: antioxidant fortified food-enriched experience. Bars with different superscripts are significantly different by Tukey’s studentized range test (HSD).

There were no significant differences in the black/white discrimination learning task. On the black/white discrimination reversal task, by contrast, there was a highly significant effect of age ($F(1, 12) = 17.12; P < 0.01$). As indicated in Fig. 3, this was largely due to poor performance of the old animals on the control food. Post hoc analysis indicated this group performed significantly more poorly than both groups.

3.3. Age differences

Because the young animals all received behavioral enrichment, age differences on both the black/white discrimination and reversal tasks were assessed with the use of a repeated measures ANOVA that compared the two young groups with the old groups provided with behavioral enrichment. In this analysis, task (discrimination versus reversal learning) served as the within subject variable and food and age were between subject variables. The results revealed a highly significant effect of task ($F(1, 34) = 48.93; P < 0.01$), age ($F(1, 34) = 15.26; P < 0.01$), and a significant age by task interaction. ($F(1, 34) = 5.799; P = 0.03$). To further clarify these results, we carried out separate factorial analysis for the black/white discrimination and black/white discrimination reversal learning.

Fig. 2. Effect of antioxidant fortified food on acquisition of a black/white discrimination and black/white discrimination reversal learning task in young beagle dogs. Error bars represent standard errors.

Fig. 3. Age differences in acquisition of black/white discrimination and black/white discrimination reversal learning task. The old animal data was taken only from subjects in the behaviorally enriched groups. Error bars represent standard errors. Groups with different superscripts are significantly different by Tukey’s studentized range test (HSD).
of young animals and than the old animals on the fortified food.

3.4. Longitudinal changes in discrimination and reversal learning between baseline and year 2 assessment

3.4.1. Overall results with all animals, young and old included

To evaluate longitudinal changes in discrimination and reversal learning, the data over the three years were first analyzed with an omnibus repeated measures ANOVA with age (young versus old) and food (enriched versus control) as between subject variables and test year (object, size and black/white) and task type (discrimination versus reversal) as within subject factors. There were highly significant main effects of age ($F(1, 54) = 44.277; P < 0.0001$), test year ($F(2, 108) = 21.149$) and task type ($F(1, 54) = 273.67; P < 0.0001$). There were also significant interactions between age and test year ($F(2, 108) = 10.446; P < 0.001$) and age and task type ($F(1, 54) = 24.76; P = 0.00001$).

3.4.2. Effects of age within behaviorally enriched groups

To examine the effects of age, we next performed the same analysis on the behaviorally enriched dogs only, which included all of the young dogs and half of the old dogs. The results revealed a highly significant effects of food ($F(1, 34) = 7.22; P = 0.02$), test year ($F(2, 68) = 15.34; P < 0.0001$) and task type ($F(1, 34) = 142.04; P < 0.0001$). There were also significant interactions between age and test year ($F(2, 68) = 7.75; P = 0.001$) and between age and task type ($F(1, 34) = 18.9153; P < 0.001$). As illustrated in Fig. 4, the task and age effects are attributable to animals generally showing faster learning of the black/white discrimination task than of the black/white discrimination reversal task, and second, consistently more accurate learning by the young animals.

3.4.3. Effects of food and experience on aged animals

We then looked at treatment effects in the aged animals alone, looking at discrimination and discrimination reversal learning separately. For the discrimination learning task, there were highly significant main effects of task ($F(2, 68) = 34.11; P < 0.0001$) and behavioral enrichment ($F(1, 34) = 17.95; P < 0.001$) and significant two way interactions between task and behavioral enrichment ($F(2, 66) = 7.475; P = 0.001$) and between task and food ($F(2, 66) = 34.11; P < 0.01$). Fig. 5 shows that the task effect reflects the animals showing a progressive slowing in learning over the three years. The interaction with experience reflects the behaviorally enriched group performing better than the non-enriched group over the third year only. Finally, the task by food effect reflects consistently better performance of the enriched animals over the controls on the last two years, after the start of the antioxidant treatment.

The reversal learning was first analyzed with a repeated measure ANOVA over the three years and revealed significant main effects of behavioral enrichment ($F(1, 34) = 9.78 P < 0.001$), food ($F(1, 34) = 4.198, P < 0.05$), and task ($F(2, 68) = 52.11, P = 0.001$). There were also significant interactions between task and enrichment ($F(2, 68) = 15.60, P = 0.0001$) and between task and cohort ($F(2, 68) = 3.37, P < 0.05$). The behavioral enrichment effect is shown in Fig. 6A, which illustrates that the animals provided with the behavioral enrichment treatment learned more accurately than the animals provided with control experience, and the differences increased over repeated testing. Fig. 6B illustrates that the food effect is due to improved performance of the group given both the antioxidant fortified food in the second treatment year on the black/white reversal task.

The subjects tested in these experiments were allowed a maximum of 40 sessions to solve the reversal learning task, and some of the animals were unsuccessful. The error score assigned to the subjects that failed was based on the 40 test sessions administered, which underestimated the true error rate because of a ceiling effect. The number of failures for each of the test groups is shown in Table 2. The contrast between the animals given the combined treatment and the animals in the control-control group was notable. All 12 of the animals in the combined treatment condition were able to solve all of the reversal learning problems, while only two of eight control-control animals showed learning. The chi-squared value obtained by comparing frequency of failure for the discrimination reversal learning over all time periods with expected frequencies was highly significant ($P = 0.02$). Subsequent chi-square analysis of the failure at each measured point showed no significant difference at baseline or the first year of assessment. However, the second year of assessment revealed a highly significant chi-square of 0.0033. This could be attributed to the high failure rate in the C-C group compared to the other groups.

Table 2

<table>
<thead>
<tr>
<th>Treatment group (food-experience)</th>
<th>Year 1 (object reversal)</th>
<th>Year 2 (size reversal)</th>
<th>Year 3 (black/white reversal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-control</td>
<td>0/12</td>
<td>3/12</td>
<td>6/8</td>
</tr>
<tr>
<td>Control-enriched</td>
<td>0/12</td>
<td>3/12</td>
<td>2/10</td>
</tr>
<tr>
<td>Antioxidant-control</td>
<td>0/12</td>
<td>3/12</td>
<td>4/12</td>
</tr>
<tr>
<td>Antioxidant-enriched</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>Total</td>
<td>0/48</td>
<td>9/48</td>
<td>12/42</td>
</tr>
</tbody>
</table>
4. Discussion

This project had three goals: to evaluate the cognitive effectiveness of long-term maintenance on an antioxidant-fortified food; to evaluate the effectiveness of a long-term program of behavioral enrichment, and; to assess cognitive decline in the beagle dog in a longitudinal study. The data presented in this report was obtained from aged dogs tested on a black/white discrimination learning and reversal task after two years receiving one of four treatment conditions: control food-control experience; fortified food-control experience; control food-enriched experience; and fortified food-enriched experience. We also tested two groups of dogs that were young at the start of the study. One group received the control food and the other the fortified food. Both groups of young dogs received behavioral enrichment.

Both treatment conditions improved performance of the aged group. However, the effectiveness of the various treat-
ment combinations varied as a function of both experience and food. Performance of the black/white discrimination learning task was significantly improved in the aged dogs provided with behavioral enrichment, relative to the control enrichment condition. The reversal task, by contrast, was significantly affected by both experience and food. Furthermore, because the experimental design limited the number of the training trials, the magnitude of the treatment effects was likely underestimated. Eight of 18 animals in the control food condition failed the reversal test in the allotted 40 ses-
sions and 10 of 20 animals in the control experience failed.

By contrast, all 12 animals in the combined treatment condi-
tion successfully achieved the a-priori learning criterion. Finally, on both tasks, the two treatments combined were more effective than either treatment alone.

The performance of the subjects in the young group by contrast, was unaffected by the dietary manipulation, which was not unexpected. The effectiveness of the food is theoretically linked to its' ability to arrest or reverse cellular dys-
function produced by excessive free radicals and improve-
ment of aged mitochondrial function. However, free-radical based brain damage is minimal in younger animals [23].
Fig. 6. Performance on discrimination reversal learning tasks as a function of antioxidant fortification and behavioral enrichment over three years in aged subjects. (A) The scores over three years for the animals in the enriched experience and control experience groups. Group differences are apparent in the first test year, after one year of treatment. (B) Comparison of the aged animals on the antioxidant fortified and control foods at baseline and over the next two years.

The results of this study extend our previous report on the effects of the antioxidant fortification and behavioral enrichment on size discrimination and reversal learning, which was carried out after one year of treatment. Whereas the present study revealed a significant main effect of food by itself on the reversal learning, the one-year results indicated that antioxidant supplementation was only effective when it was combined with behavioral enrichment [33]. The present results indicate that the effect of fortified food on cognition is more robust after two years on the food than after only one year. We have also found, however, that dietary fortification has significant beneficial effects after a short time frame among animals provided with behavioral enrichment [31,32].

4.1. Effects of behavioral enrichment

The behavioral enrichment condition included a program of cognitive enrichment, increased physical activity and environmental enrichment. The present results do not allow
us to distinguish the relative importance of each of these interventions. We suspect, however, that the cognitive enrichment was particularly important. First, previous animal studies in which aged subjects are provided with environmental enrichment have had small inconsistent effects on cognition [47]. By contrast, training animals on particular tasks early in life can produce long-lasting changes in the animals’ abilities to learn those tasks later in life [49].

Cognitive enrichment protocols have also been found to produce beneficial effects in elderly human subjects. Ball et al. [5] examined the effect of three distinct cognitive interventions (memory training, reasoning training, and speed of processing training). They were all effective, but the effectiveness was selective, and improved only the targeted cognitive ability. These results from human subjects suggest that cognitive enrichment protocols produce task-specific improvement. The present results are consistent with this suggestion. Although the cognitive enrichment protocol consisted of a broad range of tasks, they could all be solved with a discrimination learning strategy. This was also true of the black/white discrimination task, and the discrimination reversal task, which requires two cognitive skills: learning to inhibit the response to a previously rewarded stimulus and learning to respond to a previously non-rewarded stimulus.

4.2. Effects of dietary intervention

The finding of improved performance in the groups on the antioxidant fortified food is consistent with our previous reports, which were obtained in animals provided with the fortified food for under a year [31,32]. The present results extend these findings to indicate that the fortified food remains an effective therapeutic after two years of treatment. The dietary intervention used in this study has been previously described and discussed [31,32]. Briefly, the food was enriched with a cocktail containing both antioxidants and mitochondrial cofactors. Because of the numerous ingredients, we do not know which if any specific component is particularly important, or, alternatively, whether beneficial effects depend on the use of a broad spectrum of ingredients. The latter interpretation is consistent with the moderately large literature on the effects of antioxidant supplementation on cognition. In several studies, in which only a limited group of antioxidants were used, the effects on cognition were small and restricted. Thus, Sano et al. [41] found that supplementation with α-tocopherol over two years did not improve scores on cognitive measures, such as on the mini-mental state examination, in moderately demented Alzheimer’s disease patients. Socci et al. [43] found no effect on passive avoidance memory of long-term antioxidant supplementation with Vitamin E, phenyl-α-tert-butylnitrone and ascorbic acid, although the antioxidant treatment did improve rate of water maze learning. Finally, Joseph et al. [25] did not find differences between control treatment and a variety of antioxidants on water maze learning, although they reported evidence suggesting that supplements resulted in greater improvement, and also were able to erase other age-dependent deficits.

4.3. Cross sectional age differences

The analysis of age-dependent cognitive decline was partially confounded by the absence of a non-enriched young-animal group. The young animals, consequently, could only be compared with the behaviorally enriched aged animals. The results revealed significant differences between the age groups on the reversal learning, but not on the discrimination learning. The absence of an age-dependent difference in discrimination learning contrasts with data obtained from these animals after only one year of treatment [33], and with other studies showing age-dependent deficits in complex discrimination learning tasks [45]. We attribute these results to two factors: the first is the extensive test experience given to our behaviorally enriched aged animals. The second relates to the age range of the young group. Although we have used the term young, the mean age of the young group at the time of final testing was greater than five and some of the animals were over seven, which we have previously considered to be middle aged [29].

We did get significant age differences in the reversal learning task, as expected based on our previous work [29,45] and studies with non-human primates [6,26,39,50]. However, the differences were largely a result of poorer performance of the aged group that received the control food, suggesting that the antioxidant food was able to reduce age-dependent deficits in reversal learning.

4.4. Longitudinal changes in cognitive ability in the young and old groups

The other aim of this study was to obtain longitudinal evidence of changes in discrimination and reversal learning ability in the beagle dog. To characterize cognitive decline, we studied two groups of beagle dogs, an aged group and a young group. The aged group consisted of 48 dogs that ranged from approximately 8–10 years of age. The young group consisted of 17 dogs. The results demonstrated progressive deterioration in performance over three years in both discrimination and reversal learning in the aged dogs.

At the start of the study, the old group performed significantly worse than the young on object discrimination reversal learning, but not on object discrimination learning, suggesting overall cognitive impairment in 8- to 10-year-old beagle dogs. As a group, the aged animals showed progressively poorer performance throughout the course of the study on both discrimination and reversal tasks. The performance differences between the size and black/white tasks are likely due to marked age-dependent decline over the course of the study, rather than to differences in task difficulty. In a previous experiment, we used a crossover design to compare acquisition of a size discrimination and black/white discrim-
ation tasks and found that the size task was significantly more difficult than the black/white task for aged beagle dogs [30]. In the present study, by contrast, the aged dogs did more poorly on the black/white discrimination, which was tested in the second year of the study, than they did on the size discrimination task, which was tested in the first year of the study. Since this is the opposite of what we have seen previously, it’s likely that the poorer performance reflects age differences (the animals were one year older) rather than differences in task difficulty. This conclusion is further reinforced by the data from the young dogs, which performed comparably on the black/white, size and size discrimination tasks, as well as on the discrimination reversal tasks.

Another notable observation was the high incidence of failures with increased age—particularly by the control group on the reversal learning task. Because these tasks are not particularly difficult for young or middle aged beagle dogs, learning failures provide strong evidence of age-associated cognitive decline or dementia. The present results, therefore, suggest that the likelihood of cognitive decline increases precipitously after the age of 10. This conclusion is also consistent with the results of the Patronek et al. [38], in which a 10-year-old beagle was found to be equivalent in physiological age to a 66.6-year-old human. We have also found evidence of cognitive deterioration in the performance of dogs used in this study on a delayed non-matching to position task, a measure of visuospatial function, which will be reported separately. Several aged dogs failed the task in the third year of the study, despite the fact that they had successfully learned the task when tested one or two years earlier.

We have focused primarily on longitudinal changes in the old dogs. We also found evidence of cognitive deterioration in the young dogs. The performance of the young group showed an overall, but not significant, decline in the second year of the study (on black/white discrimination and reversal), when compared to the first (size discrimination and reversal). However, we expected the dogs to do better on the black/white test than on the size discrimination based on previous findings [30]. In fact, by the second treatment year of the study, the designation of the group as young dogs was no longer appropriate, as the mean age of the dogs was now over five years. One possibility is that the performance on the black/white discrimination task actually represents impairment, relative to younger dogs. This suggested time frame for cognitive aging in the beagle dog is consistent with imaging studies of aged dogs and time course studies of beta amyloid deposition. Su et al. [44] found that after the age of 10, ventricular volume increases exponentially. Head et al. [22] found that neuropathy, manifested by the occurrence of beta amyloid deposition, first appears when the beagle dog at about eight years, when it is most prominent in the prefrontal cortex. At this time, many cognitive functions are unimpaired, although deficits are manifested in functions, such as reversal learning, that are likely frontal lobe dependent. After 10 years of age, beta amyloid accumulation is also notable in entorhinal, parietal, and occipital cortices, which is also the time frame when more severe and widespread cognitive dysfunction occurs.

The suggestion of an increasing proportion of subjects severe cognitive decline after about 12 is also consistent with observational data obtained from studies of pet dogs, in which cognitive impairment characterized by disorienta- tion, disturbances in social interactions, impairment in house training and disruption of sleep–wake cycles shows an increasing prevalence with advanced age. Thirty percent of 11- to 12-year-old animals show impairment in one or more category and 10% show impairments in two or more of these categories. In animals between 15 and 16, the percentages increase to 68 and 35 [37].

5. Conclusions

To conclude, the present results demonstrates that both discrimination and reversal learning ability decline progressively with advanced age in beagle dogs, but that the rate of decline can be delayed by both behavioral enrichment and antioxidant supplementation. Possibly the most important outcome of this study was the demonstration that the behavioral enrichment and the antioxidant supplementation condition combined were more effective than either alone. This study is the first that we know of to look at both interventions in combination. The dietary intervention was based on a cocktail of compounds, and it will be important in future studies to determine which of the ingredients are most effective, and whether the cocktail can be improved. The behavioral intervention also involved a cocktail of treatments (activity, environmental enrichment and cognitive enrichment), and the contribution of each to the present results remains to be determined.

6. Conflict of interest statement

The following conflict of interest was declared by the authors with respect to publication of this paper: Steven Zicker is an employee of Hill’s Pet Nutrition Inc., which has commercialized the antioxidant fortified food used in the study. The following conflict of interest was declared by the authors with respect to publication of this paper: Steven Zicker is an employee of Hill’s Pet Nutrition Inc., which has commercialized the antioxidant fortified food used in the study.
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