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Award Number: DAMD17-98-1-8622

TITLE: The Effects of Antioxidants and Experience on the Development of Age Dependent Cognitive Dysfunction and Neuropathology in Canines

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REPORT DATE: October 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Final (15 Sep 98 - 14 Sep 02)

4. TITLE AND SUBTITLE
The Effects of Antioxidants and Experience on the Development of Age Dependent Cognitive Dysfunction and Neuropathology in Canines

5. FUNDING NUMBERS
DAMD17-98-1-8622

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Progression of individual rates of age-dependent cognitive dysfunction and the potential for antioxidants and environmental enrichment to slow the rate of decline are being evaluated over a 3-year span in beagle dogs. The study started with dogs between the ages of 9 and 13 years. Dogs underwent baseline screening of cognitive function and general health evaluation including clinical pathology and physical examinations. Magnetic resonance image scans (MRIs) are being used to obtain in vivo measures of brain and cerebrovascular function. Each dog has been placed into one of four groups counterbalanced with respect to cognitive ability, sex, and age: 1) control, 2) enriched environment, 3) dietary enrichment, and 4) combined dietary and environmental enrichment. A broad spectrum of antioxidants is added for dietary enrichment. The environmental enrichment condition consists of additional cognitive experience and enriched sensory environment. Cognitive function, physical health, and brain MRIs are being monitored yearly to establish ongoing effects of the treatment. We now demonstrate significant improvements in learning and memory, particularly in animals receiving the combined treatment. In addition, we provide the first evidence that cognitive function can be maintained in treated animals, and hippocampal atrophy, measured by MRI volumetric analysis, has been slowed in animals receiving either or both of the interventions. We have now begun the neurobiological studies proposed for the study and are currently measuring the extent of β-amyloid deposition and several endpoint markers of oxidative damage. Neuroanatomical endpoint measures, once collected, will be correlated with cognitive function and MRI measures of brain atrophy and cerebrovascular function to establish the effectiveness of the treatments on delaying or preventing the development of age-dependent neuropathologies.

14. SUBJECT TERMS	15. NUMBER OF PAGES
aging, cognitive dysfunction, antioxidants	235
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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I. INTRODUCTION

The purpose of the current research project is to determine the effects of both antioxidants and environmental enrichment on age-dependent cognitive decline in a 3-year longitudinal design using beagle dogs. Forty-eight dogs have undergone baseline screening of cognitive function and a general health evaluation including clinical pathology and physical examinations. Magnetic resonance image scans (MRIs) are being used to obtain in vivo measures of brain and cerebrovascular function. Each dog is in one of four treatment groups, which are counterbalanced with respect to baseline cognitive ability, sex, and age: (1) enriched environment/antioxidant diet, (2) enriched environment/control diet, (3) control environment/antioxidant diet, and (4) control environment/control diet. A broad spectrum of antioxidants is being added for dietary enrichment using a specially formulated geriatric canine diet. The environmental-enrichment condition consists of additional cognitive experience, enriched sensory environment, and physical exercise. Cognitive function, physical health, and brain MRIs are being monitored annually to establish ongoing effects of the treatment. At the end of 3 years on study, half of the study subjects have been euthanized, and anatomical and biochemical studies are in progress to determine correlates with cognitive function and MRI measures of brain atrophy and cerebrovascular function. Further, these anatomical studies will be used to establish the effectiveness of the treatments on delaying or preventing the development of age-dependent neuropathologies.

II. BODY OF THE REPORT

In year 4, we proposed to have completed the third year of dietary and environmental enrichment in the study and the third treatment year's annual re-evaluation of cognitive ability in all the dogs. This was also the final year of study for one-half of the animals and as per our protocol, animals were euthanized in May 2002.

A. Study Status

A total of 24 dogs from Lovelace Biomedical and Environmental Research Institute (LBERI) ranging in age from 9.3–13.8 years were placed into the study in October 1998 and were supported by the current grant. These animals were euthanized for anatomical and biochemical studies in May 2002. A second group of 24 beagles ranging in age from 9.5–12.9 years from Hill's Pet Nutrition were added to the study in April 1999 and are supported by Hill's Pet Nutrition. Dogs provided by Hill's are part of a survival study and will continue being fed

the antioxidant diet until age-related health issues require euthanasia. All dogs are beagles. The current age of surviving individual Hill's animals at the time of submitting this progress report is listed in Appendix A, along with the age at which animals from LBERI were euthanized.

Hill's dogs are still receiving either or both an antioxidant-enriched diet and environmental enrichment. Dogs receiving the environmental enrichment have been given additional learning experience on an oddity and landmark discrimination task. As per the study plan, animals in the enriched environment groups (enriched environment/control diet and enriched environment/antioxidant diet) are walked outdoors twice per week for 20 minutes each time. Last, environmentally enriched dogs are housed in pairs and are provided with play toys that are rotated through the kennels at weekly intervals.

Table 1 provides a summary of all completed or in-progress cognitive tasks for each treatment group. Dogs in the enriched environment groups have provided the most cognitive data since they are tested continuously, and new data are presented in the current annual report. Dogs in the control environment groups do not receive additional learning experience and thus, the annual evaluations are the major source of cognitive data.

Table 1. Cognitive Tasks Completed or Ongoing in Each Treatment Group

		Environment	
		Control	Enriched
Diet	Control	1. size discrimination 2. size reversal 3. spatial memory 4. object recognition memory 5. intensity discrimination	1. landmark discrimination 2. oddity learning 3. landmark retention 4. size discrimination 5. size reversal 6. spatial memory 7. object recognition memory 8. intensity discrimination
Diet	Antioxidant	1. size discrimination 2. size reversal 3. spatial memory 4. object recognition memory 5. intensity discrimination	1. landmark discrimination 2. oddity learning 3. landmark retention 4. size discrimination 5. size reversal 6. spatial memory 7. object recognition memory 8. intensity discrimination

In this report, we provide the evidence based upon an evaluation of all the animals in the study and provide comparisons among each of the four treatment groups.

B. Health Status

Medical evaluations of the dogs have been completed through year 3 of the study for the LBERI dogs and through 2.5 years for the Hill's dogs. These evaluations have included physical examinations, blood samples for clinical chemistry, and blood cell counts at baseline and every 6 months on study. Urinalysis has been done at baseline and annually during the study. Eight dogs have died during the study, six during the past year (October 2001–September 2002). To date, four dogs have died in the control environment/control diet group, three dogs in the enriched environment/control diet group, one dog in the control environment/antioxidant diet group, and none in the enriched environment/antioxidant diet group (Tables 2A–2D).

The LBERI dogs alive at the end of the third year of testing were sacrificed as planned between of May 14–17, 2002. A gross necropsy was performed, and any significant gross lesions were sampled and placed in formalin for possible future examination. The lesions observed at gross necropsy are listed by dog below:

1494D, testes, interstitial cell tumor

1502S, lung, metastatic mammary tumors

1506B, lung, pleura, fibrosis and hyperplasia

1510A, meninges, fibrosis over frontal cortex

1518D, meninges, fibrosis over frontal cortex

1523B, testes, atrophy, unilateral

1529S, lung, left caudal lobe, nodular hyperplasia

1541B, skin, tumor, keratoacanthoma; eye, right, limbus, lymphoma

1542S, meninges, fibrosis, periventricular

1543S, liver, nodular hyperplasia and fatty degeneration; Thyroid, right, adenoma; skin, multiple flat papillomas

1581S, oviducts, enlarged fimbria, bilateral

1585A, spleen, hyperplastic nodule

B2150, liver, hyperplastic nodule

With the exception of the metastatic mammary tumor in the lung of dog 1502S, all of the lesions are commonly found in older dogs.

Illnesses found in the dogs over the past year were unrelated to treatment groups and were typical of an older dog population. Several dogs were treated for gastroenteritis (1508A and D053), spondylosis (D054, D056, and D066), urinary tract infection (D052 and D071), and hypothyroidism (D081). Mammary tumors were removed from 1509U and 1529S. A mass was located in the limbus of the right eye of 1541B. Standard veterinary procedures were used to treat these conditions.

Table 2A. Treatment Group: Control Environment/Control Diet

Dog Number	Colony of Origin	Alive	Sacrificed	Died	Age (yr)	Cause of Death
1494D	LBERI	no	5/16/02		14.9	
1508U	LBERI	no	–	7/26/01	12.4	Chronic heart failure
1510A	LBERI	no	5/17/02		13.2	
1521S	LBERI	no	5/17/02		12.6	
1543S	LBERI	no	5/17/02		11.9	
B2150	LBERI	no	5/17/02		13.5	
D051	Hill's	10/1/02			12.2	
D059	Hill's	no		4/16/02	10.5	Hyperadrenocorticism
D062	Hill's	no		10/20/01	10.1	Chronic heart failure
D063	Hill's	10/1/02			11.5	
D066	Hill's	no		9/12/02	11.3	Discospondylosis
D071	Hill's	10/1/02			12.1	
	Totals	3	5	4		

Table 2B. Treatment Group: Enriched Environment/Control Diet

Dog Number	Colony of Origin	Alive	Sacrificed	Died	Age (yr)	Cause of Death
1492B	LBERI	no		11/24/99	12.5	Liver degeneration, pancreatitis and atrophy
1506B	LBERI	no	5/16/02		14.3	
1518D	LBERI	no	5/17/02		13.7	
1523U	LBERI	no		2/2/02	12.3	Chronic enteritis, nephritis
1529S	LBERI	no	5/16/02		13.3	
1542S	LBERI	no	5/16/02		12.9	
D052	Hill's	10/1/02			14.3	
D053	Hill's	10/1/02			12.3	
D072	Hill's	10/1/02			12.8	
D073	Hill's			4/17/02	12.6	Hemangiosarcoma, spleen
D074	Hill's	10/1/02			13.0	
D080	Hill's	10/1/02			13.1	
	Totals	5	4	3		

Table 2C. Treatment Group: Control Environment/Antioxidant Diet

Dog Number	Colony of Origin	Alive	Sacrificed	Died	Age (yr)	Cause of Death
1491B	LBERI	no	5/14/02		14.0	
1508A	LBERI	no	5/15/02		13.3	
1509U	LBERI	no		1/21/02	12.9	Abscess, left axilla
1523B	LBERI	no	5/15/02		11.5	
1532S	LBERI	no	5/15/02		12.3	
1581S	LBERI	no	5/16/02		10.0	
D048	Hill's	10/1/02			13.2	
D056	Hill's	10/1/02			12.8	
D064	Hill's	10/1/02			12.2	
D067	Hill's	10/1/02			11.0	
D081	Hill's	10/1/02			11.8	
D082	Hill's	10/1/02			10.0	
	Totals	6	5	1		

Table 2D. Treatment Group: Enriched Environment/Antioxidant Diet

Dog Number	Colony of Origin	Alive	Sacrificed	Died	Age (yr)	Cause of Death
1502S	LBERI	no	5/14/02		14.8	
1521B	LBERI	no	5/15/02		13.6	
1541B	LBERI	no	5/16/02		13.0	
1542T	LBERI	no	5/14/02		12.9	
1581T	LBERI	no	5/14/02		11.0	
1585A	LBERI	no	5/15/02		10.8	
D054	Hill's	10/1/02			12.4	
D055	Hill's	10/1/02			14.0	
D060	Hill's	10/1/02			13.1	
D065	Hill's	10/1/02			13.3	
D070	Hill's	10/1/02			11.9	
D075	Hill's	10/1/02			12.7	
	Totals	6	6	0		

C. Maintenance on Dietary Enrichment, Environmental Enrichment, or Combination Improves Visuospatial Learning

The dogs have all been tested on the same visuospatial task over 3 consecutive years. Spatial memory was tested by showing animals a single red lego block covering one of the three recessed food wells in the Toronto General Canine Text Box. After displacing the single object and obtaining the food reward, the presentation tray was withdrawn from the animal's sight for either a 5- or 10-second delay. After this delay, dogs were shown two identical objects. One

object covered the same well as seen previously, and the second covered one of the two remaining wells. Dogs were rewarded for selecting the object covering the novel location.

The initial testing was carried out during baseline screening. In this instance, each dog was given up to 50 sessions with 12 trials per session to achieve a criterion performance level on the task, with the delay fixed at 10 seconds. Unexpectedly, we found that only eight out of 48 old dogs could achieve the criterion performance level. Accordingly, when the dogs were retested at year 2, which was 1 year after the start of treatment, we lowered the delay to 5 seconds for all dogs that failed to reach criterion at 10 seconds during the baseline test. To equate difficulty, in analysis of these data, the animals were given error scores based on either the total number of errors required to pass the task at 10 seconds or the total number of errors after 50 training sessions. Thus, animals that were started at 5 seconds and failed to learn after 50 sessions were given an error score based on the total errors made during the 50 trials. If an animal did learn at 5 seconds, its total error score was based on errors made at both 5 and 10 seconds. For example, if an animal made 50 errors over 10 sessions at a 5-second delay, and 200 errors over the first 40 sessions at a 10-second delay, its error score was 50 + 200 = 250.

The results over 3 years are summarized in Figure 1. Performance improved, overall, over the 3 years, which was expected because of practice effects. There was no significant treatment effect after 1 year on the study. As shown in Table 3, by the second year, the antioxidant diet groups (includes enriched environment/antioxidant diet and control environment/antioxidant diet groups) had shown improvement and were performing significantly better than the control diet groups.

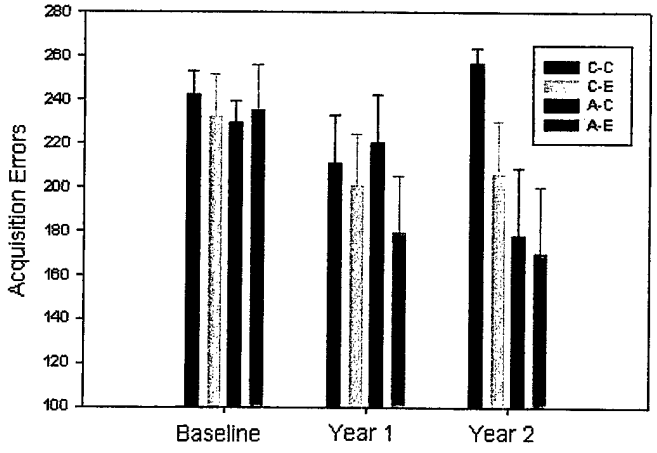

Figure 1. Visuospatial learning is plotted as a function of time on the study for each treatment condition. All dogs showed some improvement in the second test session suggesting a practice effect. However, each treatment group showed further improvement in the third test session with the combined treatment group showing the greatest improvement. C-C (control/control), C-E (enriched environment/control diet), A-C (antioxidant diet/control environment), and A-E (enriched environment/antioxidant diet). Error bars indicate standard error of the mean.

Table 3. Visuospatial Learning Errors as a Function of Diet and Time

Diet	Time	Mean	SE
Control	Baseline Errors	234.4875	12.48116
Control	Year 1 Errors	198.5625	19.42141
Control	Year 2 Errors	225.3125	20.96893
Antioxidant	Baseline Errors	226.7500	10.96589
Antioxidant	Year 1 Errors	193.4500	17.06356
Antioxidant	Year 2 Errors	175.1917	18.42321

To further clarify the treatment effects in the year-2 visuospatial acquisition, a factorial ANOVA was used with diet, enrichment, and cohort (source of animal) as dependent variables and total errors as the independent variable. We found a highly significant effect of diet [$(F(1,34) = 4.52, p < 0.04]$ and of cohort [$F(1,34) = 5.95, p < 0.02$]. Two cohorts of dogs were included in the study, and because the source of the animal may be a variable that interacts with the treatments used, a separate analysis compared the two groups (LBERI vs. Hill's). As shown in Table 4, at year 2 the significant cohort effect was due to the fact that the Hill's-reared animals performed more poorly than the LBERI-reared animals, suggesting more rapid cognitive aging.

Table 4. Effect of Cohort on Visuospatial Learning

Cohort	Time	Mean Errors	SE
LBERI	Baseline Errors	226.0208	11.57219
LBERI	Year 1 Errors	207.6375	18.00700
LBERI	Year 2 Errors	171.0458	19.44182
Hill's	Baseline Errors	235.2167	11.92117
Hill's	Year 1 Errors	184.3750	18.55005
Hill's	Year 2 Errors	229.4583	20.0000

The original rationale for using the visuospatial task was to provide a means of assessing visuospatial memory. To do this, we used a program of increasing delays over 40 sessions after successfully completing the task at a 10-second delay. Table 5 summarizes the maximal memory data in seconds of delay for those animals that could pass the Delayed Non-Match to Position (DNMP) task on at least one occasion.

Table 5. Changes in Maximum Memory Over Course of Study in Animals that Learned the Task

The values are the time of delay in seconds.

Subject	Baseline	Year 1	Change (Year 1 – Base)	Year 2	Change (Year 2 – Year 1)
		Control Environment/Control Diet			
1508U	0	30	+30	deceased	
1543S	0	0	0	5	+5
DO59	10	20	+10	deceased	–5
DO66	0	10	+10	5	–5
DO71	0	10	+10	5	–5
		Enriched Environment/Control Diet			
1518D	0	30	+30	30	0
1542S	0	0	0	30	+30
DO53	30	50	+20	10	–40
DO72	0	5	+5	5	0
DO74	20	20	0	5	–15
DO80	0	0	0	5	+5
		Control Environment/Antioxidant Diet			
1508A	0	30	+30	30	0
1523B	30	5	–25	10	+5
1532S	0	0	0	10	+10
1581S	30	50	+20	110	+60
DO81	0	20	+20	10	–10
		Enriched Environment/Antioxidant Diet			
1541B	0	0	0	50	+50
1585A	0	20	+20	20	0
1521B	0	0	0	20	+20
1581T	50	110	+60	110	0
1542T	10	5	–5	20	+15
DO55	0	10	+10	5	–5
DO70	30	50	+20	30	–20

Table 5 shows the maximal memory scores (length of delay/seconds) for every animal that reached the acquisition criterion at any time during baseline and 2 years on the treatments, which included a total of 23 animals. Note that only eight animals learned during the baseline testing period. During year 1, 14 dogs improved or remained the same as baseline. Two dogs (1542T and 1523B) failed to improve on the retest and were assigned maximal memory

scores of 5, since they were retested at 10 seconds. One other dog, D72, learned at 5 seconds, but could not pass at 10. Retest in year 2 on study resulted in 13 dogs remaining the same or improving and eight dogs with lower memory scored.

D. Maintenance of Cognitive Function

To measure maintenance of cognitive function, three tests of learning that were given at baseline, year 1, and year 2 were compared. All tests involved discrimination learning and subsequent reversal learning. The tests included simple visual discrimination/reversal, size discrimination/reversal, and intensity discrimination/reversal.

We had hypothesized that young dogs would maintain relatively stable cognitive function over the study period and that old dogs would show progressive deterioration. Young dogs have shown consistent error scores over the 3 years of the study on measures of discrimination learning (Figure 2) and on reversal learning (Figure 3). In contrast, aged dogs show significant impairments over time with year 2 showing the largest age effects.

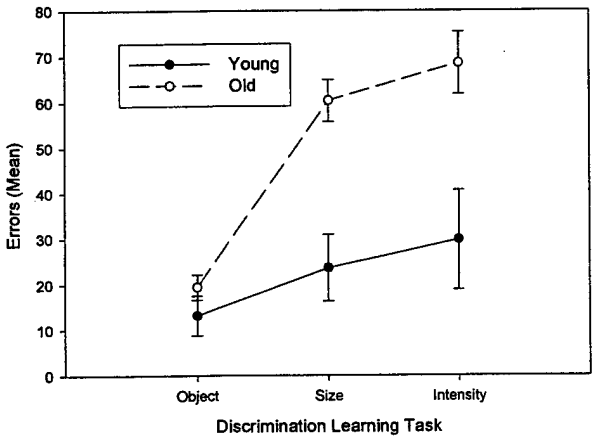

Figure 2. Average error scores for discrimination learning are plotted as a function of time to illustrate age effects. Note that old dogs show a progressive deterioration in discrimination learning over the longitudinal study. Error bars represent standard error of the mean.

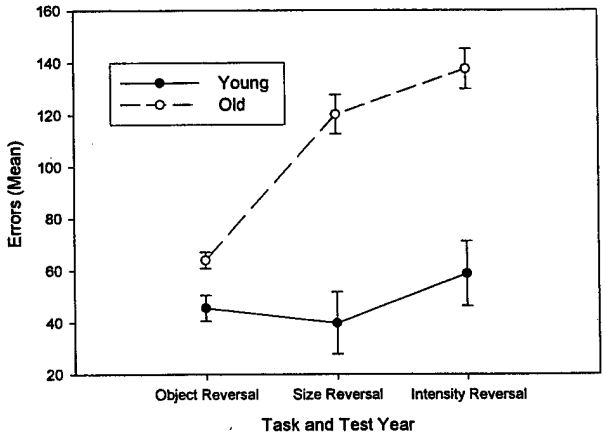

Figure 3. Average error scores for discrimination reversal learning are plotted as a function of time to illustrate age effects. Note that old dogs showed a progressive deterioration in discrimination learning over the longitudinal study. Error bars represent standard error of the mean.

To analyze treatment effects, a repeated measures analysis was used and indicated a significant effect of task overall [$F(2,68) = 33.41$, $p < 0.0001$] and of environmental enrichment [$F(1,33) = 19.76$, $p < 0.0001$]. A significant interaction of task by environmental enrichment

[F(2,66) = 8.24, $p < 0.0006$] reflects improved performance in the size and intensity discrimination learning in the animals provided with environmental enrichment (Figure 4).

A significant task by diet interaction [F(2,68) = 3.76, $p < 0.03$] indicates improved performance on the size and intensity discrimination learning tasks in animals provided with the diet rich in antioxidants (Figure 5).

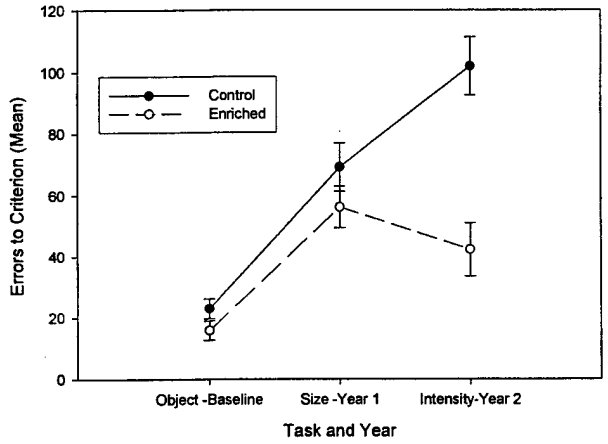

Figure 4. Average error scores on a discrimination learning task are plotted as a function of time for dogs receiving the enriched environment vs. controls. After 2 years of treatment, the dogs receiving the enriched environment show significantly lower error scores (improved cognition) relative to controls. Bars represent standard error of the mean.

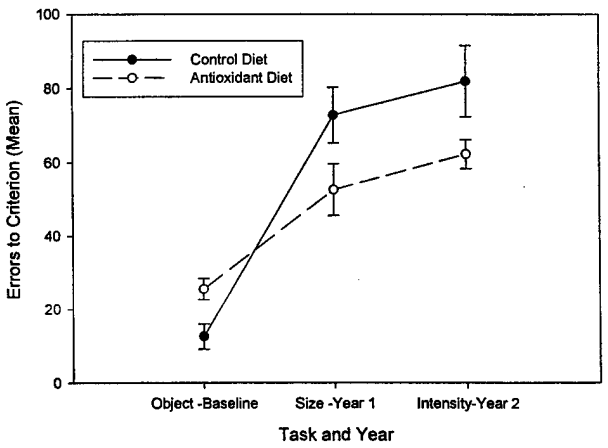

Figure 5. Average errors to criterion on a discrimination learning task plotted as a function of time for dogs receiving the antioxidant diet vs. control diet. Overall, dogs receiving the antioxidant-enriched diet perform consistently better than controls in years 1 and 2 of the study but not at baseline. Bars indicate standard error of the mean.

Reversal learning was also compared across baseline to 2 years of treatment as shown in Figure 6.

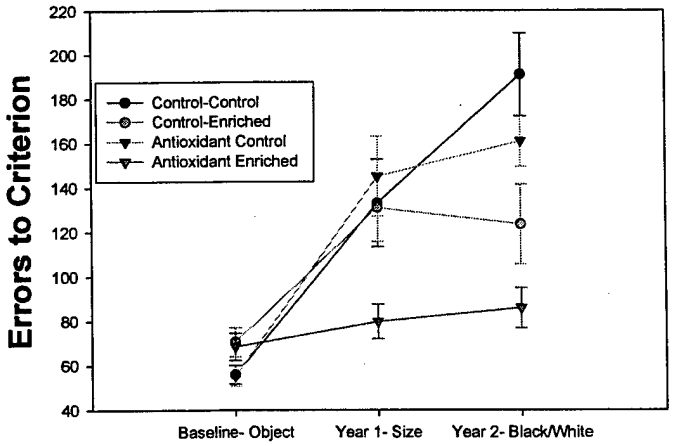

Figure 6. Average error scores for reversal learning in each treatment group are plotted as a function of time. Note the progressive increase in error scores in untreated aged animals indicating cognitive deterioration. The combined treatment group shows maintenance in cognitive function with the single treatment groups exhibiting intermediate error scores. Bars indicate standard error of the mean.

Reversal learning is mediated by the prefrontal cortex and measures the animal's ability to inhibit a previously learned behavior. Striking treatment effects were observed over time. Overall, a significant effect of the antioxidant diet [$F(1,34) = 4.42$, $p < 0.043$] and environmental enrichment [$F(1,34) = 10.19$, $p < 0.0003$] indicated that animals receiving either intervention performed significantly better than the control group. Further, a significant 3-way interaction between task by diet and by enriched environment [$F(2,68) = 3.19$, $p < 0.047$] indicated that the best performers were animals receiving the combined treatment, particularly in intensity discrimination learning. This is the first evidence to suggest that the interventions used in the current study can lead to the maintenance of cognitive function in aging animals [1].

E. Open Field Activity Remains Relatively Unaffected by Treatment Condition

The results from last year's progress report have now been published [2]. Year 1.5 evaluations are being analyzed for the open field test in the LBERI dogs. Data from the human-dog interaction test and curiosity test are complete up to the 1.5-year point. The only significant finding to date is a decrease in the time spent playing with dog toys in the curiosity test from baseline to the years 1 and 1.5 evaluations [$F(2, 38) = 6.39$, $p = 0.004$]. The largest decrease was in the enriched environment/antioxidant diet group. The enriched environment/control diet and control environment/control diet groups showed smaller declines, while the levels of the control environment/antioxidant diet group remained the same at the evaluation points. The curiosity test is used as a measure of exploratory behavior, and evidence indicates that treatment with antioxidants reduces exploratory behavior in female rats. Although in this case the enriched environment is also necessary. No other measures of spontaneous behavior were significantly affected, and all results are summarized in Table 6.

Year 1 evaluations for the open field test are being analyzed for the Hill's dogs. Data from the human-dog interaction and curiosity test are complete up to the 1-year point. The only significant finding among this group of dogs is a decrease in locomotion in the open field test from baseline to the 6-month evaluation point [$F(1, 20) = 28.02$, $p = 0.000035$]. Locomotor activity decreased in all four treatment groups indicating the effect is not a result of the treatments.

Table 6. Summary of Open Field Tests[a]

	Open Field Locomotion	Human Interaction		Curiosity Test	
		Contact	Near	Contact	Sniffing
LBERI Dogs					
C/C[b]	no difference	no difference	no difference	small decrease	no difference
C/A	no difference	no difference	no difference	no difference	no difference
E/C	no difference	no difference	no difference	small decrease	no difference
E/A	no difference	no difference	no difference	decrease	no difference
Hill's Dogs					
C/C	decrease	no difference	no difference	no difference	no difference
C/A	decrease	no difference	no difference	no difference	no difference
E/C	decrease	no difference	no difference	no difference	no difference
E/A	decrease	no difference	no difference	no difference	no difference

[a]LBERI dogs have data to 1.5 years on treatment and Hill's dogs to 1 year.
[b]C/C = control/control
C/A = control environment/antioxidant diet
E/C = enriched environment/control diet
E/A = enriched environment/antioxidant diet

The baseline data for the mirror test have been analyzed. The LBERI dog groups [$F(3, 20) = 0.33$, $p = 0.80$] or the Hill's dog groups [$F(3, 21) = 0.89$, $p = 0.46$] did not differ significantly in the baseline period.

Activity data were also obtained using the Actiwatch® activity monitoring system [3]. Consistent with the open field data, the treatment groups for the LBERI dogs [$F(3, 19) = 0.08$, $p = 0.96$] at the 1.5-year point or the Hill's dogs [$F(3, 20) = 2.05$, $p = 0.14$] at the 1-year point did not differ significantly for overall distance traveled.

F. Blood Biochemistry and Blood Coagulation Studies Suggest no Adverse Consequences of Long-Term Dietary Intervention

In general, the blood biochemistry values for all animals were within normal limits. The values were not significantly different from baseline values. Raw data from samples to date are provided in Appendix B.

As shown in Figure 7, coagulation profiles were obtained after 1 and 2 years on intervention to assess the effects of supplemented antioxidants and mitochondrial cofactors on

coagulation. This was done for two reasons. Intakes of vitamin E in extreme excess have been reported to decrease coagulation time and predispose animals to bleeding disorders [4]. Second, this appears to be only a problem when an antagonistic factor to vitamin K is present such as warfarin [5]. As such, we examined the coagulation profiles of older dogs in the study after 1 and 2 years of intervention. All levels were well within normal ranges.

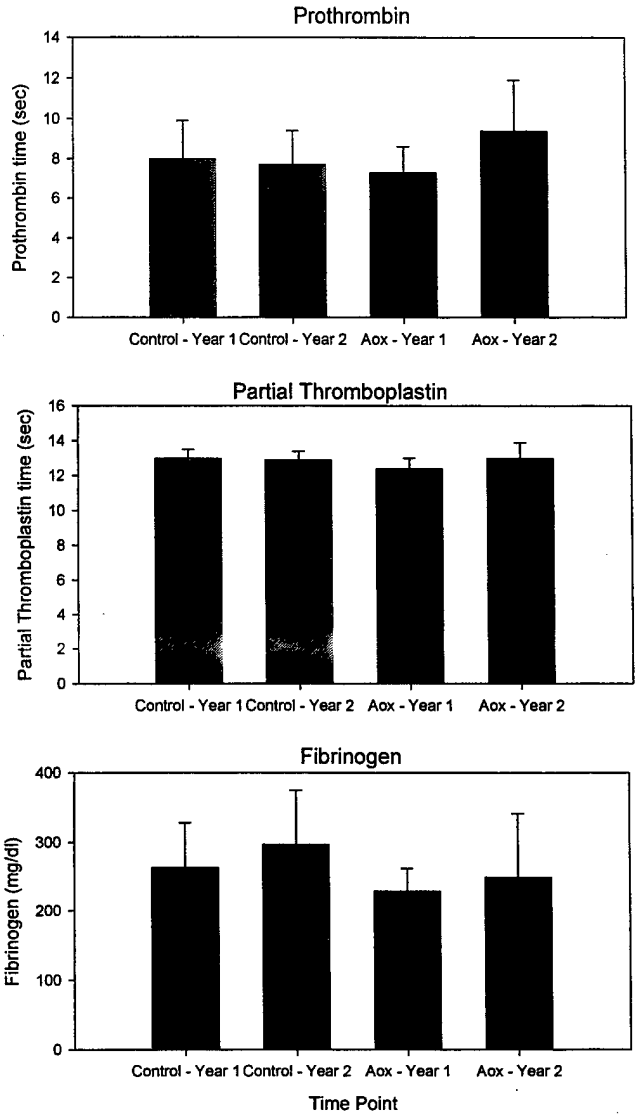

Figure 7. Blood coagulation factors are plotted as a function of time point (years 1 and 2) and treatment group. Each panel shows no significant effects of the antioxidant diet on prothrombin times, partial thromboplastin times, or fibrinogen, respectively. Gray bars indicate animals receiving the control diet and the black bars the animals being fed the antioxidant diet.

G. Vitamin E Levels Remain High in Dogs Provided with the Antioxidant Diet

As in previous progress reports, the animals receiving the diet rich in antioxidants continue to maintain significantly high vitamin E levels relative to control diet animals [$F(3,41) = 12.91$, $p < 0.0001$] (Figure 8).

H. Lipid Peroxidation in Plasma Samples

Our previous report provided preliminary data describing the results from one measure of lipid peroxidation conducted in plasma samples from dogs in the study [6] and the results of a study of age effects on several other measures of oxidative damage [7]. We showed at that time that malondialdehyde (MDA) in plasma was increased in the dogs provided with environmental enrichment and significantly reduced in the group of animals (Hill's beagles) provided with the antioxidant diet [8]. MDA may reflect lipid peroxidation from either or both peripheral or central nervous system tissue.

To confirm and extend these findings, we are collaborating with Dr. Jason Morrow at Vanderbilt University (Nashville, TN). Dr. Morrow is an expert on measuring F_2-isoprostane in several tissue types as an index of oxidative damage in vivo [9]. The rationale for measuring the isoprostanes includes the following: (1) isoprostanes are specific products of lipid peroxidation; (2) they are stable compounds; (3) levels of isoprostanes are detectable in many tissue types; (4) the formation of isoprostanes is modulated by antioxidant status; and (5) isoprostane level is unaffected by lipid content of the diet. The amount of F_2-isoprostane (ng/mg) was determined by gas chromatography/negative chemical ionization mass spectrometry as described [10].

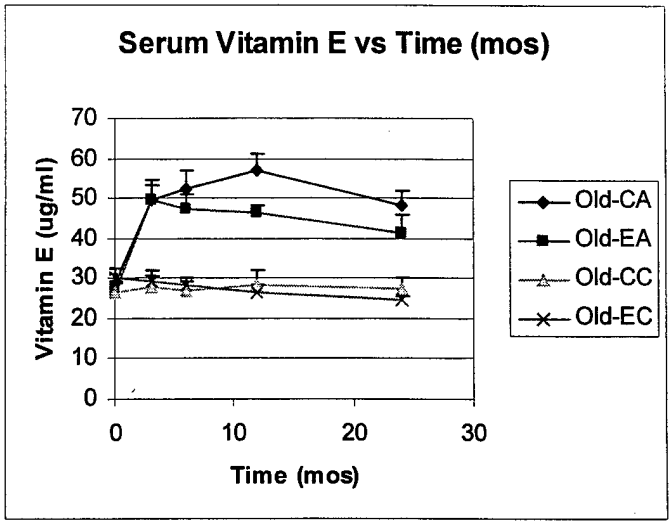

Figure 8. Vitamin E level in the serum of aged dogs is plotted as a function of treatment group. Five time points have now been assayed and illustrate that animals receiving the antioxidant-enriched diet show significantly higher levels of vitamin E than untreated dogs. CA (control/antioxidant diet), EA (enriched environment/antioxidant diet), CC (control/control), and EC (enriched environment/control diet). Bars indicate standard error of the mean.

In previous reports, the amount of isoprostane formation in transgenic mice (a model for Alzheimer's disease) ranged from 0.2–0.6 ng/ml, while human plasma levels ranged from 0–0.25 ng/ml in nondemented controls and 0.25–1.2 ng/ml in Alzheimer's disease. In dogs, plasma isoprostane formation ranged from 0.019–1.07 ng/ml.

Experiments to measure isoprostane levels in dogs on the study were conducted in 2002 using archived plasma samples. Young and old dogs exhibited similar isoprostane levels in year 1 of the study (Figure 9). In contrast, year 2 plasma isoprostane levels were significantly higher in the old dogs relative to the young dogs [$t(59) = 2.02$, $p < 0.048$].

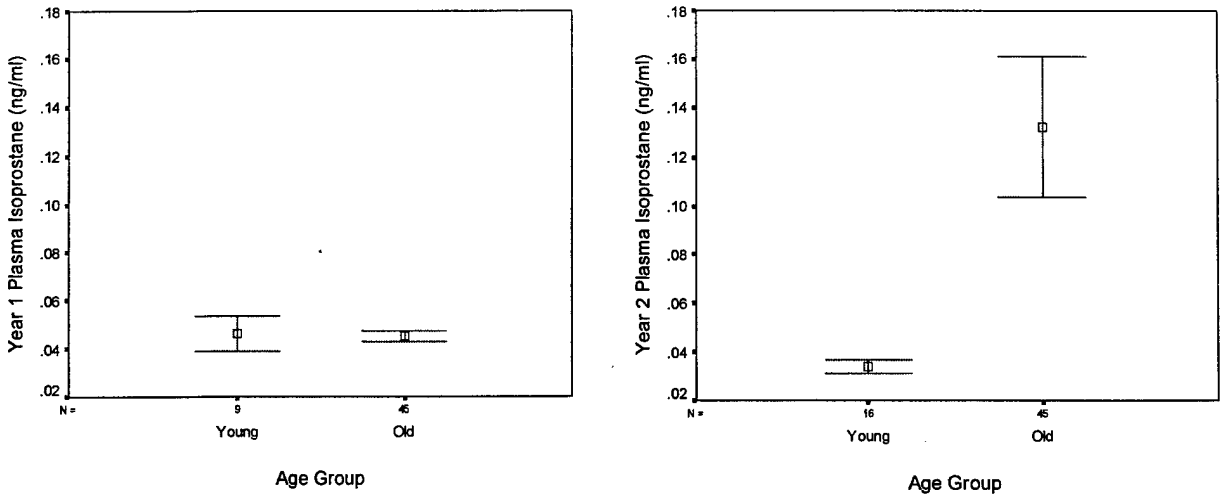

Figure 9. The left panel illustrates a lack of age effects on overall level of plasma lipid peroxidation (isoprostane) in the first year of the study. The right panel shows that age effects were observed in the second year of the study with the aged dogs showing significantly higher isoprostane in the plasma than young dogs. Bars indicate standard error of the mean.

There were no treatment effects on isoprostane levels when the four groups were compared. Combining dogs into two groups of antioxidant diet vs. no antioxidant diet also did not reveal any differences in plasma isoprostane levels. The two measures of lipid peroxidation, MDA and isoprostane, at year 1 were significantly correlated ($r = 0.316$, $p < 0.035$) with each other, but most of this effect was due to one animal (1529S) that had both high levels of MDA and isoprostane. A significant correlation was found between landmark discrimination learning (L0: $r = 0.556$, $p < 0.007$) and the year-1 plasma level of isoprostane (Figure 10). Plasma isoprostane at year 2 did not correlate with any cognitive test scores.

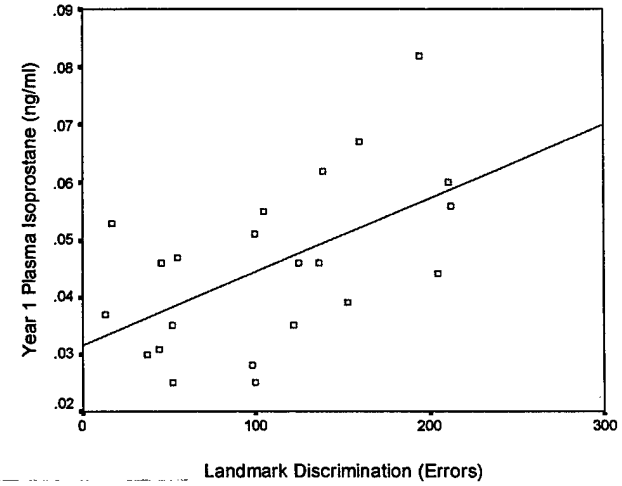

Figure 10. Poor landmark discrimination learning (higher error scores) is associated with higher levels of plasma lipid peroxidation (isoprostane) during year 1 on treatment. Each point represents an individual animal, and the line represents the regression analysis.

I. Anatomical Changes Measured by MRI and Treatment Effects

In the last MRI performed in May 2002, many of the LBERI dogs ($n = 22$) had developed brain lesions. The examinations of MRI scans from Hill's dogs are in progress. The lesions were most often seen in the caudate nucleus, then in the olfactory bulb. Some small

lesions were also seen in the cortical regions. In the exam performed 1 year ago, some lesions were present in 10 of these 22 dogs. The locations of lesions (indicated by image number) found during 2002 and 2001 examinations are summarized in Table 7. A Kruskal-Wallis nonparametric test comparing the presence or absence of a lesion in each treatment group indicated a lack of treatment effect in both 2001 [$\chi^2(3) = 2.22$, $p > 1$] and 2002 [$\chi^2(3) = 0.23$, $p > 1$]. Figure 11 shows selected images from one 13-year-old dog. The largest lesion appeared to be an infarction near the hippocampus. Two lesions were located in the olfactory bulb. Four small lesions were found in the caudate nucleus and the nearby periventricular space. Several small lesions were found in the cortical regions. In the exam performed 1 year earlier, only the large lesion in the olfactory bulb was present. This dog also showed a rapid decline in her cognitive ability during the last year. The brain has been fixed and is currently undergoing histological examination. We will correlate the pathology of these lesions with the imaging appearance when the data become available.

Table 7. The Locations of Lesions (Indicated by Image Number) During 2002 and 2001 MRI Exams from the LBERI Animals

	Lesion Location	
Tattoo	2002	2001
1491B	L43 L49-50	
1502S	R26	
1506B	L35	
1508A	R47-50, L56-57	R48-51
1510A	L44-45	
1521S	L35	L32
1523B	R54	
1529S	L36-37, L38-40	
1541B	L11-13, L13-31	L9-10, L11-29
1542S	many	
1542T	many	R48-49, R54-55
B2150	several	R39-40
D048	L28-29, L34-35	
D051	L43-44	L43-44
D052	L40	L41-42
D054	L38-39	
D055	R31, L37	
D064	L24	L22
D071	many	R35
D075	R17, R23-24, R34-35, L38-39	
D081	L18, L37-38, R36-37	L37-38
D063	L37-38	

L: the left-hand side of the image, dog's left brain
R: the right-hand side of the image, dog's right brain

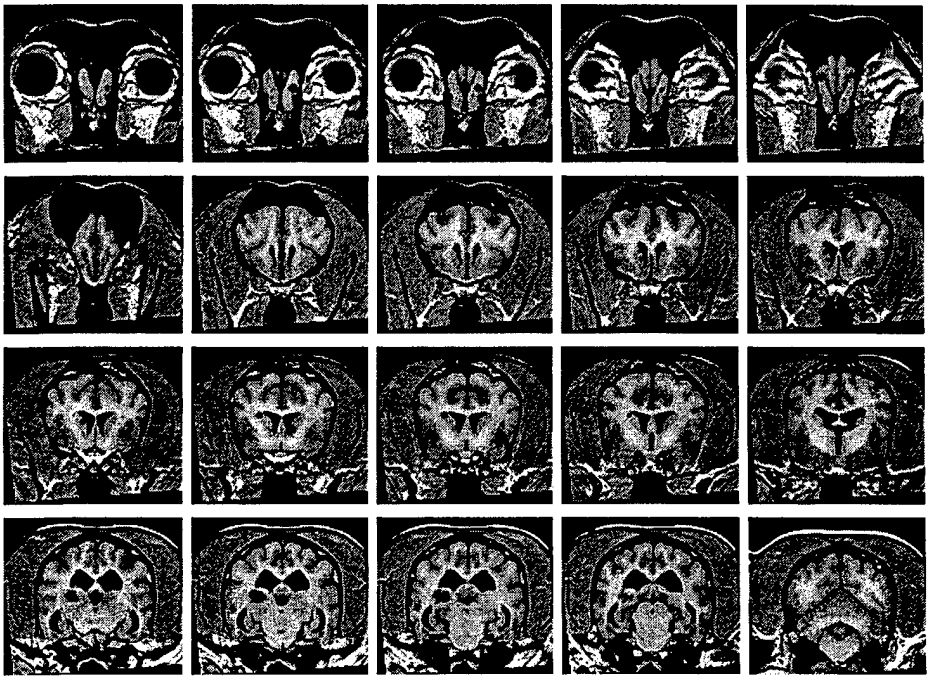

Figure 11. Selected images from one 13-year-old dog. Many lesions were present in the brain. The largest lesion appeared to be an infarction near the hippocampus. Two lesions were located in the olfactory bulb. Four small lesions were found in the caudate nucleus and the nearby periventricular space. Several small lesions were also found in cortical regions.

The volume of the total cerebrum, lateral ventricles, caudate nucleus, cerebellum, olfactory bulbs and the hippocampus was determined for images taken in 2001 by outlining regions of interest in serial sections. Each brain region volume was subsequently calculated as a proportion of the total cerebrum volume. As shown in Figure 12, an analysis of variance comparing the four treatment groups indicates a significant difference among the hippocampal volumes [$F(3,43) = 4.047$, $p < 0.013$]. The significant difference reflects a smaller hippocampal volume in animals in the control environment/control diet group relative to the animals receiving the combined treatment

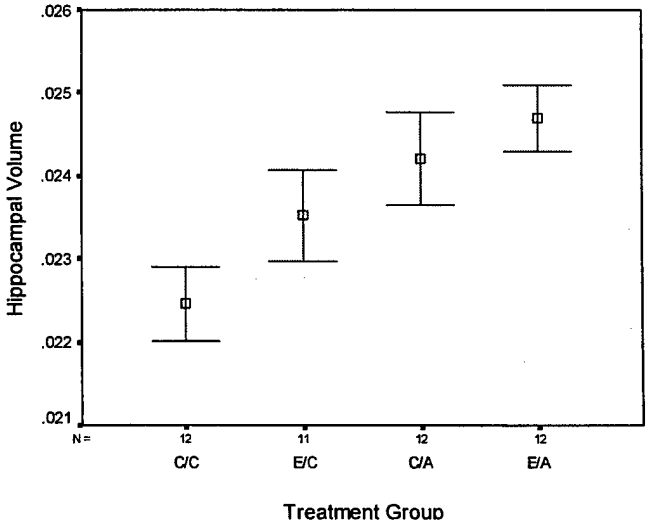

Figure 12. The hippocampal volume was lowest in the control/control group as compared to the three other intervention groups. The group with the largest average hippocampal volume was the combined treatment. Hippocampal volume was adjusted for the total cerebral volume and is expressed as a proportion. Bars indicate standard error of the mean.

of environmental enrichment and an antioxidant diet. As a separate analysis, these data comparing the two sources of dogs (LBERI vs. Hill's) showed a significant treatment effect in the Hill's dogs but not the LBERI dogs.

J. Preliminary Anatomical Data Studies

We euthanized the 20 remaining LBERI beagle dogs in May 2002 as per our study design. Two weeks prior to euthanasia, dogs were administered bromo-deoxyuridine (BrdU) that was incorporated into the DNA of new brain cells, as per our study protocol. The left half of all the canine brains was fixed in 4% paraformaldehyde, and the right half was frozen at –80°C. The left half has been serially and exhaustively sectioned on all 24 animals (including the four animals that were euthanized or had died earlier in the study) for anatomical studies. We have completed immunostaining for four measures of β-amyloid (Aβ) including Aβ1-16, Aβ1-40, Aβ1-42, and isomerized Aβ1-16. One of these markers is an additional endpoint that we have included in the study. The marker was developed during the last 3 years. Four brain regions are being quantified: the prefrontal, occipital cortex, parietal cortex, and entorhinal cortex. We anticipate that this quantification will be completed by the end of 2002.

Frozen samples from the same four brain regions listed above, in addition to cerebrospinal fluid and plasma were sent to Dr. Paul Murphy at the Mayo Clinic in Jacksonville, Florida, for Aβ quantification studies using sensitive sandwich ELISA techniques. Similar samples were also sent to Dr. Jiankang Liu at the University of California, Berkeley, to measure levels of malondialehdye, protein carbonyl formation, and glutamine synthetase activity. We have published our early data on these endpoint markers [5].

We have also obtained additional funds from the National Institute on Aging to conduct stereology-based cell counts for total neuron number and for total new neurons. These studies are labor intensive and will be completed over the next year of the study.

Western blots will be used to quantify the extent of synapse loss or maintenance as a function of treatment group and also the extent of survival factors including brain-derived neurotrophic factor. The procedures are currently being optimized, and we anticipate that these studies will be completed over the next year in four different brain regions.

III. KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 4

- We have completed the cognitive testing portion of the study and accomplished each goal set out in the original statement of work.
- Visuospatial learning performance improved overall during the 3 years, which was expected because of practice effects. Although there was no significant treatment effect after 1 year of treatment, at year 2, there was a significant treatment effect – with the animals on the antioxidant diet making fewer errors than the animals on the control diet.
- Visuospatial memory showed no significant treatment effect after 1 year. However, by the last year of the study, animals in the antioxidant groups showed improvement on the average. There was a cohort effect for the antioxidant diet groups, with the LBERI animals performing better than the Hill's animals. Further, performance deteriorated from year 2 to year 3 in the animals on the control diet, indicating progressive impairment in visuospatial function.
- We provide the first evidence to suggest that the interventions used in the current study can lead to the maintenance of cognitive function in aging animals based upon tests of discrimination and reversal learning.
- Open field activity showed no consistent treatment effect.
- Vitamin E levels in serum continue to remain higher in the antioxidant groups than in controls over the treatment period.
- Peripheral measures of lipid peroxidation were expanded over the last year of the study to include a measure of isoprostane in plasma. Plasma isoprostane levels were sensitive to longitudinal age effects but not treatment effects. The extent of lipid peroxidation was correlated with a measure of visuospatial attention (landmark discrimination) but not with all tests of cognition.
- Several dogs developed lesions over the last year of the study that were not associated with any specific treatment. The volume of the hippocampus measured by MRI in 2001 was smallest in control environment/control diet animals relative to animals receiving the combined treatment of enriched environment/antioxidant diet.

IV. REPORTABLE OUTCOMES

We have eight papers published or "in press" that describe the results of our study in the last year [2, 7, 11-16]. Two more manuscripts have been submitted [3, 17]. (See Appendix C for reprints and preprints.) Two manuscripts actively being prepared for submission are listed below.

1. Milgram, N.W., Head, E., Zicker, S., Ikeda-Douglas, C., Murphey, H. Muggenberg, B., Siwk, C., Tapp, D., Cotman, CW. Effects of age, behavioral enrichment and dietary fortification with antioxidants on discrimination and reversal learning in aged beagle dogs: A two year longitudinal investigation (in preparation).

2. Milgram, N.W., Head, E., Zicker, S., Ikeda-Douglas, C., Murphey, H. Muggenberg, B., Siwak, C., Tapp, D. Cotman, CW. Dietary antioxidant fortification and behavioral enrichment combined improve cognitive performance of aged beagles on visual discrimination learning and reversal (in preparation).

Six abstracts have been or will be presented in 2002 and are included in Appendix D.

1. Head, E., Liu, J., Ames, B., Su, L., Muggenburg, B.A., Ikeda-Douglas, C., Murphey, H., Zicker, S., Milgram, N.W., and Cotman, C.W. An antioxidant enriched food improves learning and reduces lipid peroxidation in aged canines: A longitudinal study. Presented at the 8th International Conference on Alzheimer's Disease and Related Disorders. July 20-25, 2002, Stockholm, Sweden. Abstract published in Neurobiology of Aging, 23 (1S), p. S115.

2. Head, E., Liu, J., Muggenburg, B.A., Murphey, H., Ikeda-Douglas, C., Zicker, S., Ames, B.N., Milgram, N.W., and Cotman, C.W. A longitudinal dietary antioxidant intervention in aged canines improves learning reduces peripheral measures of oxidative damage. To be presented at Neuroscience 2002, Society of Neuroscience, November 2-7, Orlando, FL.

3. Ikeda-Douglas, C.J., Murphey, H., Muggenburg, B., Head, E., Cotman, C.W., Zicker, S.C., and Milgram, N.W. Long term maintenance of an antioxidant enriched food plus behavioral enrichment markedly delays age related cognitive decline in beagle dogs. To be presented at Neuroscience 2002, Society of Neuroscience, November 2-7, Orlando, FL.

4. Siwak, C.T., Tapp, P.D., and Milgram, N.W. Measuring complex working memory processes using a spatial list learning paradigm in a canine model of aging. To be presented at Neuroscience 2002, Society of Neuroscience, November 2-7, Orlando, FL.

5. Studzinksi, C., Araujo, J.A., and Milgram, N.W. Inter-session retention: Why is it hard for an old dog to learn new tricks. Poster Presentation, 2002 Summer Undergraduate Research Program, Institute of Medical Science, Toronto 2002 (First Prize Winner).

6. Tapp, P.D., Siwak, C.T., Su, M.-Y., Chiou, G., Black, S.E., McCune, S., Cotman, C.W. Head, E. and Milgram N.W. Effects of age on frontal and hemispheric brain symmetry in the canine. To be presented at Neuroscience 2002, Society of Neuroscience, November 2-7, Orlando, FL.

V. CONCLUSIONS

The goals for year 4 were to complete the cognitive portion of the intervention study, to acquire a final set of MRIs, and to begin the anatomical studies. The results of the study indicate that both treatments, the enriched environment and the antioxidant diet, significantly affect cognitive aging. The results from the combination treatment group suggest that the two interventions are additive. The study of nontreated aged animals has led to the first longitudinal study of aging reported in a higher mammalian species and shows progressive declines in several cognitive domains, particularly those that rely upon the prefrontal cortex. Age-dependent decline can be prevented through the interventions used in the current study with a combination of treatments resulting in a preservation of cognitive function.

We have introduced additional endpoint markers and have now initiated a collaboration with a group that can measure lipid peroxidation by the formation of F_2-isoprostane. This new indicator of oxidative damage appears sensitive to the aging process but peripheral assays do not vary as a function of treatment condition. We anticipate that direct measures of brain oxidative damage will provide a stronger correlate of cognitive dysfunction and brain atrophy measured by MRI.

In vivo imaging data in the longitudinal study are now yielding evidence that the hippocampus may indeed progressively decrease in size in untreated animals and that the interventions may slow the rate of atrophy. In addition, gross measures of total cerebral volume and ventricle size are less sensitive to progressive deterioration with age. We also provide the

first evidence of in vivo responses of the brain to an intervention. Over the next year of the study, we will finalize the analysis and begin a correlation analysis of the association between cognition, neurobiological measures, and cognition. To accomplish this aim, a large dataset is currently being maintained where all quantitative data (cognitive test scores, biological measures, MRI measures) are being captured.

In anticipation of the neurobiological component of the longitudinal study, we obtained additional funding to conduct neuron counts using stereology-based techniques. This is currently the "gold standard" for determining the extent of cell loss in defined brain regions. To prepare for these studies, one-half of each canine brain in the longitudinal study (LBERI animals) has been serially and exhaustively sectioned from the anterior to posterior of the left hemisphere. The sections include an entire half-hemisphere and will allow us to count neurons in defined brain regions (e.g., hippocampus, entorhinal cortex, prefrontal cortex, etc.) and to measure the extent of Aβ deposition in many more brain regions than originally proposed. The number of sections far exceeds the studies described for the current study and thus a bank of archived sections will now be available for other studies or for collaborative work.

A great deal of interest has been generated in the human aging and Alzheimer's disease fields upon presentation of this study at several meetings. We were invited to submit a review of the study to Neurobiology of Aging; the review is now in press (Appendix C) [12].

The next year of the study will be devoted to writing manuscripts summarizing the cognitive testing portion of the study and the in vivo imaging results. In parallel, neurobiological studies will be completed that will complement the cognitive and MR portions of the study.

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Appendix A. Status of Individual Animals in the Longitudinal Study

Dog	Date of End of Study	Intervention Start Date	Birthdate	Age at Start of Study (yrs)	Current Age (yrs)	Time on Intervention (yrs)	Group		Source	Comments
							Diet	Environment		
1532S	05/15/02	08/06/99	02/09/89	10.38	13.27	2.89	Aox	Control	LRRI	Completed Study
1581S	05/16/02	09/13/99	05/15/91	8.12	11.01	2.89	Aox	Control	LRRI	Completed Study
1523B	05/15/02	09/13/99	11/26/89	9.58	12.47	2.89	Aox	Control	LRRI	Completed Study
1508A	05/15/02	08/06/99	02/12/88	11.37	14.26	2.90	Aox	Control	LRRI	Completed Study
1509U	01/21/02	08/06/99	03/03/88	11.31	13.90	2.58	Aox	Control	LRRI	Dead
1491B	05/14/02	09/13/99	05/13/87	12.12	15.01	2.89	Aox	Control	LRRI	Completed Study
1541B	05/16/02	09/03/99	05/25/89	10.09	12.98	2.89	Aox	Enriched	LRRI	Completed Study
1542T	05/14/02	08/06/99	06/03/89	10.07	12.95	2.89	Aox	Enriched	LRRI	Completed Study
1585A	05/15/02	09/13/99	08/29/91	7.83	10.72	2.89	Aox	Enriched	LRRI	Completed Study
1581T	05/14/02	09/13/99	05/15/91	8.12	11.01	2.89	Aox	Enriched	LRRI	Completed Study
1502S	05/14/02	08/06/99	08/16/87	11.86	14.75	2.89	Aox	Enriched	LRRI	Completed Study
1521B	05/15/02	09/13/99	10/06/88	10.72	13.61	2.90	Aox	Enriched	LRRI	Completed Study
1543S	05/17/02	07/18/99	06/04/89	10.06	12.96	2.90	Control	Control	LRRI	Completed Study
B2150	05/17/02	07/18/99	11/12/87	11.62	14.52	2.90	Control	Control	LRRI	Completed Study
1521S	05/17/02	08/15/99	10/06/88	10.72	13.62	2.90	Control	Control	LRRI	Off Study
1494D	05/16/02	08/15/99	05/27/87	12.08	14.98	2.90	Control	Control	LRRI	Completed Study
1510A	05/17/02	09/13/99	03/22/88	11.26	14.16	2.90	Control	Control	LRRI	Completed Study
1508U	07/26/01	08/15/99	02/12/88	11.37	13.46	2.09	Control	Control	LRRI	Dead
1529S	05/16/02	07/18/99	01/23/89	10.42	13.32	2.89	Control	Enriched	LRRI	Completed Study
1523U	02/02/02	08/15/99	11/26/89	9.58	12.19	2.61	Control	Enriched	LRRI	Dead
1542S	05/16/02	07/18/99	06/03/89	10.07	12.96	2.89	Control	Enriched	LRRI	Completed Study
1506B	05/16/02	08/15/99	01/04/88	11.47	14.37	2.90	Control	Enriched	LRRI	Completed Study
1492B	11/24/99	08/15/99	05/23/87	12.09	12.52	0.42	Control	Enriched	LRRI	Dead
1518D	05/17/02	07/18/99	09/18/88	10.77	13.67	2.90	Control	Enriched	LRRI	Completed Study
D056	10/15/02	01/31/00	12/05/88	10.66	13.87	3.211	Aox	Control	Hills	On Study
D048	10/15/02	01/31/00	09/15/88	10.88	14.09	3.211	Aox	Control	Hills	On Study
D064	10/15/02	01/31/00	08/15/89	9.96	13.18	3.214	Aox	Control	Hills	On Study
D067	10/15/02	01/27/00	10/01/90	8.83	12.05	3.214	Aox	Control	Hills	On Study
D081	10/15/02	02/07/00	02/23/90	9.44	12.65	3.211	Aox	Control	Hills	On Study
D082	10/15/02	01/31/00	09/18/91	7.87	11.08	3.211	Aox	Control	Hills	On Study
D058	10/03/00	02/07/00	09/20/88	10.87	12.04	1.178	Aox	Enriched	Hills	Dead
D060	10/15/02	01/31/00	09/20/89	9.87	13.08	3.211	Aox	Enriched	Hills	On Study

Appendix A. Status of Individual Animals in the Longitudinal Study (Concluded)

D054	10/15/02	01/27/00	05/15/90	9.22	12.43	3.211	Aox	Enriched	Hills	On Study
D055	10/15/02	01/27/00	10/16/88	10.79	14.01	3.211	Aox	Enriched	Hills	On Study
D065	10/15/02	01/27/00	06/10/89	10.14	13.36	3.214	Aox	Enriched	Hills	On Study
D075	10/15/02	01/27/00	02/08/90	9.48	12.69	3.211	Aox	Enriched	Hills	On Study
D051	10/15/02	01/15/00	08/15/89	9.96	13.18	3.211	Control	Control	Hills	On Study
D059	10/15/02	01/15/00	10/06/90	8.82	12.03	3.211	Control	Control	Hills	Dead
D062	10/20/01	01/15/00	10/01/90	8.83	11.06	2.227	Control	Control	Hills	Dead
D063	10/15/02	01/15/00	04/08/90	9.32	12.53	3.214	Control	Control	Hills	On Study
D066	10/15/02	11/20/99	05/28/90	9.18	12.39	3.214	Control	Control	Hills	Dead
D071	10/15/02	01/15/00	09/24/89	9.85	13.07	3.214	Control	Control	Hills	On Study
D052	10/15/02	02/07/00	07/08/88	11.07	14.28	3.211	Control	Enriched	Hills	On Study
D053	10/15/02	02/07/00	07/19/91	8.04	11.25	3.211	Control	Enriched	Hills	On Study
D080	10/15/02	02/07/00	08/04/89	9.99	13.21	3.211	Control	Enriched	Hills	On Study
D074	10/15/02	02/07/00	09/26/89	9.85	13.06	3.214	Control	Enriched	Hills	On Study
D073	10/15/02	02/07/00	09/21/89	9.86	13.07	3.214	Control	Enriched	Hills	Dead
D072	10/15/02	02/07/00	12/26/89	9.60	12.81	3.214	Control	Enriched	Hills	On Study

Appendix B. Blood Biochemistry Parameters for Individual Animals

Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D056	07/31/99	Pre	12/05/88	10.66	A	C	H	28.00
D048	07/31/99	Pre	09/15/88	10.88	A	C	H	51.00
D064	07/30/99	Pre	08/15/89	9.96	A	C	H	22.00
D067	07/30/99	Pre	10/01/90	8.83	A	C	H	21.00
D081	07/31/99	Pre	02/23/90	9.44	A	C	H	29.00
D082	07/31/99	Pre	09/18/91	7.87	A	C	H	32.00
1532S	06/25/99	Pre	02/09/89	10.38	A	C	L	32.00
1581S	06/25/99	Pre	05/15/91	8.12	A	C	L	21.00
1523B	06/25/99	Pre	11/26/89	9.58	A	C	L	18.00
1508A	06/23/99	Pre	02/12/88	11.37	A	C	L	32.00
1509U	06/23/99	Pre	03/03/88	11.31	A	C	L	19.00
1491B	06/23/99	Pre	05/13/87	12.12	A	C	L	27.00
D058	07/31/99	Pre	09/20/88	10.87	A	E	H	35.00
D060	07/31/99	Pre	09/20/89	9.87	A	E	H	29.00
D054	07/31/99	Pre	05/15/90	9.22	A	E	H	19.00
D055	07/31/99	Pre	10/16/88	10.79	A	E	H	25.00
D065	07/30/99	Pre	06/10/89	10.14	A	E	H	20.00
D075	07/31/99	Pre	02/08/90	9.48	A	E	H	24.00
1541B	06/25/99	Pre	05/25/89	10.09	A	E	L	22.00
1542T	06/25/99	Pre	06/03/89	10.07	A	E	L	20.00
1585A	06/25/99	Pre	08/29/91	7.83	A	E	L	27.00
1581T	06/25/99	Pre	05/15/91	8.12	A	E	L	24.00
1502S	06/23/99	Pre	08/16/87	11.86	A	E	L	34.00
1521B	6/23/99	Pre	10/06/88	10.72	A	E	L	29.00
	AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START							26.67
								7.30

Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D051	07/31/99	Pre	08/15/89	9.96	C	C	H	24.00
D059	07/31/99	Pre	10/06/90	8.82	C	C	H	23.00
D062	07/30/99	Pre	10/01/90	8.83	C	C	H	34.00
D063	07/30/99	Pre	04/08/90	9.32	C	C	H	35.00
D066	07/30/99	Pre	05/28/90	9.18	C	C	H	22.00
D071	07/30/99	Pre	09/24/89	9.85	C	C	H	28.00
1543S	06/25/99	Pre	06/04/89	10.06	C	C	L	28.00
B2150	06/25/99	Pre	11/12/87	11.62	C	C	L	27.00
1521S	06/23/99	Pre	10/06/88	10.72	C	C	L	24.00
1494D	06/23/99	Pre	05/27/87	12.08	C	C	L	26.00
1510A	06/23/99	Pre	03/22/88	11.26	C	C	L	34.00
1508U	06/23/99	Pre	02/12/88	11.37	C	C	L	32.00
D052	07/31/99	Pre	07/08/88	11.07	C	E	H	30.00
D053	07/31/99	Pre	07/19/91	8.04	C	E	H	29.00
D080	07/31/99	Pre	08/04/89	9.99	C	E	H	26.00
D074	07/30/99	Pre	09/26/89	9.85	C	E	H	22.00
D073	07/30/99	Pre	09/21/89	9.86	C	E	H	36.00
D072	07/30/99	Pre	12/26/89	9.60	C	E	H	14.00
1529S	06/25/99	Pre	01/23/89	10.42	C	E	L	29.00
1523U	06/25/99	Pre	11/26/89	9.58	C	E	L	32.00
1542S	06/25/99	Pre	06/03/89	10.07	C	E	L	22.00
1506B	06/23/99	Pre	01/04/88	11.47	C	E	L	25.00
1492B	06/23/99	Pre	05/23/87	12.09	C	E	L	27.00
1518D	06/23/99	Pre	09/18/88	10.77	C	E	L	63.00
	AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START							28.83
								8.87

Appendix B. Blood Biochemistry Parameters for Individual Animals

Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D056	08/23/00	0.50	12/05/88	11.72	A	C	H	30.00
D048	08/23/00	0.50	09/15/88	11.95	A	C	H	47.00
D064	08/23/00	0.50	08/15/89	11.03	A	C	H	22.00
D067	08/23/00	0.50	10/01/90	9.90	A	C	H	19.00
D081	09/06/00	0.50	02/23/90	10.54	A	C	H	22.00
D082	08/23/00	0.50	09/18/91	8.94	A	C	H	22.00
1532S	02/09/00	0.50	02/09/89	11.01	A	C	L	25.00
1581S	03/21/00	0.50	05/15/91	8.86	A	C	L	28.00
1523B	03/21/00	0.50	11/26/89	10.32	A	C	L	24.00
1508A	02/09/00	0.50	02/12/88	12.00	A	C	L	35.00
1509U	02/09/00	0.50	03/03/88	11.95	A	C	L	19.00
1491B	03/21/00	0.50	05/13/87	12.87	A	C	L	24.00
D058	09/06/00	0.50	09/20/88	11.97	A	E	H	30.00
D060	08/23/00	0.50	09/20/89	10.93	A	E	H	34.00
D054	08/23/00	0.50	05/15/90	10.28	A	E	H	29.00
D055	08/29/00	0.50	10/16/88	11.88	A	E	H	24.00
D065	08/29/00	0.50	06/10/89	11.23	A	E	H	17.00
D075	08/23/00	0.50	02/08/90	10.55	A	E	H	27.00
1541B	03/21/00	0.50	05/25/89	10.83	A	E	L	27.00
1542T	02/09/00	0.50	06/03/89	10.69	A	E	L	22.00
1585A	03/21/00	0.50	08/29/91	8.57	A	E	L	40.00
1581T	03/21/00	0.50	05/15/91	8.86	A	E	L	25.00
1502S	02/09/00	0.50	08/16/87	12.49	A	E	L	36.00
1521B	03/21/00	0.50	10/06/88	11.46	A	E	L	35.00
	AVERAGE FOR ANTIOXIDANT GROUP AFTER 6 MONTHS ON DIET							27.63
								7.23
	AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START							26.67
								7.30
Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D051	08/29/00	0.50	08/15/89	11.05	C	C	H	26.00
D059	09/06/00	0.50	10/06/90	9.93	C	C	H	19.00
D062	09/06/00	0.50	10/01/90	9.94	C	C	H	28.00
D063	09/06/00	0.50	04/08/90	10.42	C	C	H	26.00
D052	08/29/00	0.50	07/08/88	12.15	C	E	H	32.00
D053	09/06/00	0.50	07/19/91	9.14	C	E	H	22.00
D066	08/29/00	0.50	05/28/90	10.26	C	C	H	22.00
D080	09/06/00	0.50	08/04/89	11.10	C	E	H	36.00
D074	08/23/00	0.50	09/26/89	10.92	C	E	H	22.00
D073	08/29/00	0.50	09/21/89	10.95	C	E	H	27.00
D072	08/29/00	0.50	12/26/89	10.68	C	E	H	23.00
D071	09/06/00	0.50	09/24/89	10.96	C	C	H	22.00
1529S	01/25/00	0.50	01/23/89	11.01	C	E	L	28.00
1543S	01/25/00	0.50	06/04/89	10.65	C	C	L	19.00
1523U	02/16/00	0.50	11/26/89	10.23	C	E	L	22.00
B2150	01/25/00	0.50	11/12/87	12.21	C	C	L	36.00
1542S	01/25/00	0.50	06/03/89	10.65	C	E	L	27.00
1521S	02/16/00	0.50	10/06/88	11.37	C	C	L	28.00
1506B	02/16/00	0.50	01/04/88	12.13	C	E	L	24.00
1494D	02/16/00	0.50	05/27/87	12.73	C	C	L	29.00
1510A	03/21/00	0.50	03/22/88	12.01	C	C	L	31.00
1508U	02/16/00	0.50	02/12/88	12.02	C	C	L	32.00
1492B	02/16/00	0.50	05/23/87	12.75	C	E	L	.
1518D	01/25/00	0.50	09/18/88	11.36	C	E	L	38.00
	AVERAGE FOR CONTROL GROUP AFTER 6 MONTHS ON DIET							26.91
								5.38
	AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START							28.83
								8.87

Appendix B. Blood Biochemistry Parameters for Individual Animals

Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
1541B	09/20/00	1.00	05/25/89	11.33	A	E	L	24.00
1542T	08/22/00	1.00	06/03/89	11.23	A	E	L	21.00
1585A	09/19/00	1.00	08/29/91	9.07	A	E	L	37.00
1532S	08/22/00	1.00	02/09/89	11.54	A	C	L	14.00
1581S	09/20/00	1.00	05/15/91	9.36	A	C	L	23.00
1581T	09/19/00	1.00	05/15/91	9.36	A	E	L	25.00
1523B	09/20/00	1.00	11/26/89	10.82	A	C	L	22.00
1502S	08/22/00	1.00	08/16/87	13.03	A	E	L	28.00
1508A	08/22/00	1.00	02/12/88	12.53	A	C	L	30.00
1521B	09/19/00	1.00	10/06/88	11.96	A	E	L	36.00
1509U	08/22/00	1.00	03/03/88	12.48	A	C	L	18.00
1491B	09/20/00	1.00	05/13/87	13.37	A	C	L	21.00
D056	01/30/01	1.00	12/05/88	12.16	A	C	H	26.00
D048	01/30/01	1.00	08/15/88	12.47	A	C	H	47.00
D060	01/30/01	1.00	09/20/89	11.37	A	E	H	37.00
D054	01/30/01	1.00	05/15/90	10.72	A	E	H	29.00
D055	01/30/01	1.00	10/15/90	10.30	A	E	H	22.00
D064	01/30/01	1.00	10/15/88	12.30	A	C	H	20.00
D065	01/30/01	1.00	06/10/89	11.65	A	E	H	19.00
D067	01/30/01	1.00	10/01/90	10.34	A	C	H	17.00
D081	02/06/01	1.00	02/23/90	10.96	A	C	H	24.00
D075	01/30/01	1.00	02/08/90	10.98	A	E	H	17.00
D082	01/30/01	1.00	09/18/91	9.38	A	C	H	31.00
D070	02/06/01	1.00	10/25/90	10.29	A	E	H	33.00
D056	01/30/01	1.00	12/05/88	12.16	A	C	H	26.00
	AVERAGE FOR ANTIOXIDANT GROUP AFTER 12 MONTHS ON DIET							25.88
								7.75
	AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START							26.67
								7.30

Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
1529S	07/24/00	1.00	01/23/89	11.51	C	E	L	29.00
1543S	07/24/00	1.00	06/04/89	11.15	C	C	L	22.00
1523U	08/22/00	1.00	11/26/89	10.75	C	E	L	20.00
B2150	07/24/00	1.00	11/12/87	12.71	C	C	L	45.00
1542S	07/24/00	1.00	06/03/89	11.15	C	E	L	29.00
1521S	08/22/00	1.00	10/06/88	11.88	C	C	L	22.00
1506B	08/22/00	1.00	01/04/88	12.64	C	E	L	24.00
1494D	08/22/00	1.00	05/27/87	13.25	C	C	L	24.00
1510A	09/19/00	1.00	03/22/88	12.50	C	C	L	27.00
1508U	08/22/00	1.00	02/12/88	12.53	C	C	L	32.00
1492B	08/22/00	1.00	05/23/87	13.26	C	E	L	.
1518D	07/24/00	1.00	09/18/88	11.85	C	E	L	32.00
D051	01/30/01	1.00	08/15/88	12.47	C	C	H	24.00
D059	01/31/01	1.00	10/06/90	10.33	C	C	H	18.00
D062	02/06/01	1.00	10/01/90	10.36	C	C	H	26.00
D063	02/06/01	1.00	04/08/90	10.84	C	C	H	29.00
D052	01/31/01	1.00	07/15/88	12.56	C	E	H	26.00
D053	02/06/01	1.00	07/15/91	9.57	C	E	H	17.00
D066	01/31/01	1.00	05/28/90	10.69	C	C	H	25.00
D080	02/06/01	1.00	08/04/89	11.52	C	E	H	29.00
D074	01/31/01	1.00	09/26/89	11.36	C	E	H	28.00
D073	01/31/01	1.00	09/21/89	11.37	C	E	H	27.00
D072	01/31/01	1.00	12/26/89	11.11	C	E	H	24.00
	AVERAGE FOR CONTROL GROUP AFTER 12 MONTHS ON DIET							26.32
								5.78
	AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START							28.83
								8.87

Appendix B. Blood Biochemistry Parameters for Individual Animals

Animal ID	Date	Period	Birthdate	Age	Diet	Enrichment	Source	AST (SGOT)
D056	08/10/01	1.50	12/05/88	12.69	A	C	H	39.00
D048	08/10/01	1.50	08/15/88	12.99	A	C	H	65.00
D060	08/10/01	1.50	09/20/89	11.90	A	E	H	40.00
D054	08/10/01	1.50	05/15/90	11.25	A	E	H	30.00
D055	08/10/01	1.50	10/15/90	10.83	A	E	H	29.00
D064	08/10/01	1.50	10/15/88	12.83	A	C	H	28.00
D065	08/10/01	1.50	06/10/89	12.18	A	E	H	24.00
D067	08/10/01	1.50	10/01/90	10.87	A	C	H	23.00
D081	10/07/01	1.50	02/23/90	11.63	A	C	H	41.00
D075	08/10/01	1.50	02/08/90	11.51	A	E	H	24.00
D082	08/10/01	1.50	09/18/91	9.90	A	C	H	26.00
D070	10/07/01	1.50	10/25/90	10.96	A	E	H	31.00
1491B	03/10/01	1.50	05/13/87	13.84	A	C	L	24.00
1502S	02/20/01	1.50	08/16/87	13.53	A	E	L	48.00
1508A	02/17/01	1.50	02/12/88	13.02	A	C	L	36.00
1509U	02/17/01	1.50	03/03/88	12.97	A	C	L	23.00
1521B	03/10/01	1.50	10/06/88	12.43	A	E	L	31.00
1523B	03/10/01	1.50	11/26/89	11.29	A	C	L	24.00
1532S	02/17/01	1.50	02/09/89	12.03	A	C	L	17.00
1541B	03/10/01	1.50	05/25/89	11.80	A	E	L	28.00
1542T	02/17/01	1.50	06/03/89	11.72	A	E	L	28.00
1581S	03/10/01	1.50	05/15/91	9.83	A	C	L	24.00
1581T	03/10/01	1.50	05/15/91	9.83	A	E	L	29.00
1585A	03/10/01	1.50	08/29/91	9.54	A	E	L	42.00
	AVERAGE FOR ANTIOXIDANT GROUP AFTER 18 MONTHS ON DIET							31.42
								10.35
	AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START							26.67
								7.30

Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D051	08/12/01	1.50	08/15/88	13.00	C	C	H	24.00
D059	08/12/01	1.50	10/06/90	10.86	C	C	H	29.00
D062	10/07/01	1.50	10/01/90	11.02	C	C	H	24.00
D063	10/07/01	1.50	04/08/90	11.51	C	C	H	24.00
D052	08/12/01	1.50	07/15/88	13.08	C	E	H	34.00
D053	10/07/01	1.50	07/15/91	10.24	C	E	H	25.00
D066	08/12/01	1.50	05/28/90	11.22	C	C	H	24.00
D080	10/07/01	1.50	08/04/89	12.18	C	E	H	37.00
D074	08/12/01	1.50	09/26/89	11.88	C	E	H	30.00
D073	08/12/01	1.50	09/21/89	11.90	C	E	H	37.00
D072	08/12/01	1.50	12/26/89	11.64	C	E	H	25.00
D071	08/12/01	1.50	09/24/89	11.89	C	C	H	30.00
1492B	.	1.50	05/23/87	.	C	E	L	.
1494D	03/10/01	1.50	05/27/87	13.80	C	C	L	35.00
1506B	03/10/01	1.50	01/04/88	13.19	C	E	L	28.00
1508U	03/10/01	1.50	02/12/88	13.08	C	C	L	31.00
1510A	03/10/01	1.50	03/22/88	12.98	C	C	L	27.00
1518D	02/17/01	1.50	09/18/88	12.42	C	E	L	29.00
1521S	03/10/01	1.50	10/06/88	12.43	C	C	L	30.00
1523U	03/10/01	1.50	11/26/89	11.29	C	E	L	27.00
1529S	02/18/01	1.50	01/23/89	12.08	C	E	L	28.00
1542S	02/18/01	1.50	06/03/89	11.72	C	E	L	28.00
1543S	02/19/01	1.50	06/04/89	11.72	C	C	L	21.00
B2150	02/18/01	1.50	11/12/87	13.28	C	C	L	41.00
	AVERAGE FOR CONTROL GROUP AFTER 18 MONTHS ON DIET							29.04
								4.99
	AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START							28.83
								8.87

Appendix B. Blood Biochemistry Parameters for Individual Animals

Animal ID	Date	Period	Birthdate	Age	Diet	Enrichment	Source	AST (SGOT)
1491B	11/15/01	2.00	05/13/87	14.52	A	C	L	25.00
1502S	10/06/01	2.00	08/16/87	14.15	A	E	L	47.00
1508A	10/06/01	2.00	02/12/88	13.66	A	C	L	25.00
1509U	10/06/01	2.00	03/03/88	13.60	A	C	L	39.00
1521B	11/15/01	2.00	10/06/88	13.12	A	E	L	34.00
1523B	11/15/01	2.00	11/26/89	11.98	A	C	L	29.00
1532S	10/06/01	2.00	02/09/89	12.66	A	C	L	25.00
1541B	11/15/01	2.00	05/25/89	12.48	A	E	L	29.00
1542T	10/06/01	2.00	06/03/89	12.35	A	E	L	31.00
1581S	11/15/01	2.00	05/15/91	10.51	A	C	L	24.00
1581T	11/15/01	2.00	05/15/91	10.51	A	E	L	28.00
1585A	10/06/01	2.00	08/29/91	10.11	A	E	L	48.00
D056	05/17/02	2.00	12/05/88	13.45	A	C	H	31.00
D048	05/17/02	2.00	08/15/88	13.76	A	C	H	31.00
D060	05/17/02	2.00	09/20/89	12.66	A	E	H	33.00
D054	05/17/02	2.00	05/15/90	12.01	A	E	H	24.00
D055	05/17/02	2.00	10/15/90	11.59	A	E	H	26.00
D064	05/17/02	2.00	10/15/88	13.59	A	C	H	20.00
D065	05/17/02	2.00	06/10/89	12.94	A	E	H	20.00
D067	05/17/02	2.00	10/01/90	11.63	A	C	H	20.00
D081	05/31/02	2.00	02/23/90	12.27	A	C	H	35.00
D075	05/17/02	2.00	02/08/90	12.28	A	E	H	31.00
D082	05/17/02	2.00	09/18/91	10.67	A	C	H	26.00
D070	05/31/02	2.00	10/25/90	11.61	A	E	H	35.00
	AVERAGE FOR ANTIOXIDANT GROUP AFTER 24 MONTHS ON DIET							29.83
								7.39
	AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START							26.67
								7.30

Animal ID	Date	Period	Birthdate	Age	Diet	Enrichment	Source	AST (SGOT)
1492B	.	2.00	05/23/87	.	C	E	L	.
1494D	08/12/01	2.00	05/27/87	14.22	C	C	L	35.00
1506B	10/06/01	2.00	01/04/88	13.76	C	E	L	31.00
1508U	03/10/01	2.00	02/12/88	13.08	C	C	L	.
1510A	10/06/01	2.00	03/22/88	13.55	C	C	L	32.00
1518D	08/10/01	2.00	09/18/88	12.90	C	E	L	29.00
1521S	10/06/01	2.00	10/06/88	13.01	C	C	L	27.00
1523U	10/06/01	2.00	11/26/89	11.87	C	E	L	28.00
1529S	08/10/01	2.00	01/23/89	12.55	C	E	L	31.00
1542S	08/10/01	2.00	06/03/89	12.19	C	E	L	29.00
1543S	08/10/01	2.00	06/04/89	12.19	C	C	L	87.00
B2150	08/10/01	2.00	11/12/87	13.75	C	C	L	46.00
D051	05/21/02	2.00	08/15/88	13.77	C	C	H	26.00
D059	08/12/01	2.00	10/06/90	10.86	C	C	H	
D062	10/07/01	2.00	10/01/90	11.02	C	C	H	
D063	05/31/02	2.00	04/08/90	12.15	C	C	H	51.00
D052	05/21/02	2.00	07/15/88	13.86	C	E	H	26.00
D053	05/31/02	2.00	07/15/91	10.88	C	E	H	23.00
D066	05/21/02	2.00	05/28/90	11.99	C	C	H	22.00
D080	05/31/02	2.00	08/04/89	12.83	C	E	H	34.00
D074	05/21/02	2.00	09/26/89	12.66	C	E	H	22.00
D073	08/12/01	2.00	09/21/89	11.90	C	E	H	
D072	05/21/02	2.00	12/26/89	12.41	C	E	H	17.00
D071	05/21/02	2.00	09/24/89	12.66	C	C	H	28.00
	AVERAGE FOR CONTROL GROUP AFTER 24 MONTHS ON DIET							32.84
								15.34
	AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START							28.83
								8.87

Appendix B. Blood Biochemistry Parameters for Individual Animals

Animal ID	Date	Period	Birthdate	Age	Diet	Enrichment	Source	AST (SGOT)
1491B	04/05/02	2.5	05/13/87	14.91	A	C	L	22
1502S	04/05/02	2.5	08/16/87	14.65	A	E	L	39
1508A	04/05/02	2.5	02/12/88	14.15	A	C	L	35
1509U	.	2.5	03/03/88	.	A	C	L	
1521B	04/05/02	2.5	10/06/88	13.50	A	E	L	36
1523B	04/05/02	2.5	11/26/89	12.36	A	C	L	28
1532S	04/05/02	2.5	02/09/89	13.16	A	C	L	21
1541B	04/05/02	2.5	05/25/89	12.87	A	E	L	35
1542T	04/05/02	2.5	06/03/89	12.85	A	E	L	37
1581S	04/05/02	2.5	05/15/91	10.90	A	C	L	27
1581T	04/05/02	2.5	05/15/91	10.90	A	E	L	33
1585A	04/05/02	2.5	08/29/91	10.61	A	E	L	37
	AVERAGE FOR ANTIOXIDANT GROUP AFTER 30 MONTHS ON DIET							31.82
								6.29
	AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START							26.67
								7.30

Animal ID	Date	Period	Birthdate	Age	Diet	Enrichment	Source	AST (SGOT)
1492B	.	2.5	05/23/87	.	C	E	L	
1494D	04/05/02	2.5	05/27/87	14.87	C	C	L	31
1506B	04/05/02	2.5	01/04/88	14.26	C	E	L	28
1508U	.	2.5	02/12/88	.	C	C	L	
1510A	04/05/02	2.5	03/22/88	14.05	C	C	L	28
1518D	04/05/02	2.5	09/18/88	13.55	C	E	L	21
1521S	04/05/02	2.5	10/06/88	13.50	C	C	L	28
1523U	.	2.5	11/26/89	.	C	E	L	
1529S	04/05/02	2.5	01/23/89	13.21	C	E	L	35
1542S	04/05/02	2.5	06/03/89	12.85	C	E	L	32
1543S	04/05/02	2.5	06/04/89	12.84	C	C	L	25
B2150	04/05/02	2.5	11/12/87	14.41	C	C	L	38
	AVERAGE FOR CONTROL GROUP AFTER 30 MONTHS ON DIET							29.56
								5.13
	AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START							28.83
								8.87

Appendix B. Blood Biochemistry Parameters for Individual Animals

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
40.00	0.10	226.00	5.00	5.20	2.70	2.50	1.10
146.00	0.10	397.00	19.00	6.20	3.30	2.90	1.10
42.00	0.20	217.00	2.00	6.40	3.30	3.10	1.10
48.00	0.10	93.00	12.00	6.10	3.50	2.60	1.30
22.00	0.10	76.00	4.00	6.10	3.60	2.50	1.40
42.00	0.10	354.00	7.00	5.90	3.30	2.60	1.30
127.00	0.10	57.00	6.00	6.40	2.40	4.00	0.60
29.00	0.30	43.00	6.00	5.40	3.20	2.20	1.50
36.00	0.30	94.00	2.00	6.00	2.90	3.10	0.90
61.00	.1 (lipemic)	166.00	7.00	5.90	3.10	2.80	1.10
17.00	0.10	128.00	5.00	5.70	2.90	2.80	1.00
51.00	0.10	139.00	6.00	5.70	3.10	2.60	1.20
50.00	0.10	63.00	2.00	6.70	3.00	3.70	0.80
38.00	0.10	136.00	2.00	5.70	2.90	2.80	1.00
23.00	0.10	81.00	4.00	5.70	3.30	2.40	1.40
26.00	0.10	58.00	7.00	6.20	3.20	3.00	1.10
56.00	0.10	171.00	5.00	7.10	3.40	3.70	0.90
36.00	0.10	220.00	4.00	5.90	3.60	2.30	1.60
38.00	0.10	78.00	1.00	6.20	3.60	2.60	1.40
20.00	0.10	239.00	1.00	6.10	3.00	3.10	1.00
31.00	0.10	68.00	1.00	5.40	2.80	2.60	1.10
49.00	too lipemic	163.00	1.00	5.90	3.50	2.40	1.50
26.00	.1 (lipemic)	98.00	6.00	6.30	3.20	3.10	1.00
36.00	0.10	43.00	10.00	5.70	3.30	2.40	1.40
45.42	0.12	142.00	5.21	6.00	3.17	2.83	1.16
30.48	0.06	94.33	4.11	0.42	0.31	0.46	0.25
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
38.00	0.10	265.00	4.00	5.70	3.20	2.50	1.30
34.00	0.10	222.00	13.00	6.30	3.60	2.70	1.30
29.00	0.10	99.00	1.00	6.00	3.30	2.70	1.20
36.00	0.10	270.00	3.00	6.70	2.60	4.10	0.60
31.00	0.10	125.00	6.00	6.10	2.90	3.20	0.90
105.00	0.10	142.00	5.00	6.00	3.50	2.50	1.40
52.00	0.10	105.00	1.00	5.80	2.80	3.00	0.90
46.00	0.20	67.00	7.00	5.70	3.10	2.60	1.20
32.00	0.10	48.00	4.00	5.70	2.70	3.00	0.90
45.00	0.10	85.00	1.00	6.20	2.70	3.50	0.80
61.00	0.10	96.00	3.00	6.40	3.20	3.20	1.00
29.00	0.20	90.00	7.00	6.30	3.20	3.10	1.00
38.00	0.10	358.00	4.00	5.40	3.40	2.00	1.70
46.00	0.20	140.00	2.00	6.70	3.50	3.20	1.10
25.00	0.10	150.00	2.00	6.10	3.20	2.90	1.10
28.00	0.20	269.00	1.00	5.70	3.00	2.70	1.10
444.00	0.10	472.00	24.00	6.40	3.10	3.30	0.90
17.00	0.10	100.00	5.00	6.10	3.10	3.00	1.00
49.00	0.20	91.00	1.00	5.70	3.10	2.60	1.20
29.00	0.10	79.00	3.00	6.10	3.10	3.00	1.00
24.00	0.30	261.00	6.00	5.60	2.50	3.10	0.80
35.00	0.10	402.00	3.00	6.40	3.20	3.20	1.00
22.00	0.10	74.00	11.00	4.90	2.30	2.60	0.90
74.00	0.10	218.00	5.00	7.00	3.10	3.90	0.80
57.04	0.13	176.17	5.08	6.04	3.06	2.98	1.05
84.56	0.06	115.75	5.06	0.46	0.32	0.46	0.23

Appendix B. Blood Biochemistry Parameters for Individual Animals

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
30.00	0.10	275.00	7.00	5.60	2.90	2.70	1.10
195.00	0.20	574.00	24.00	6.20	3.50	2.70	1.30
27.00	0.20	239.00	3.00	5.90	3.50	2.40	1.50
33.00	0.20	97.00	4.00	6.20	3.80	2.40	1.60
22.00	0.10	79.00	4.00	6.50	3.90	2.60	1.50
24.00	0.20	255.00	8.00	5.90	3.40	2.50	1.40
38.00	0.30	389.00	1.00	6.20	3.10	3.10	1.00
61.00	0.10	72.00	6.00	5.40	3.40	2.00	1.70
32.00	0.10	91.00	3.00	6.00	3.40	2.60	1.30
61.00	0.30	246.00	1.00	6.30	3.30	3.00	1.10
17.00	0.20	134.00	2.00	6.30	3.40	2.90	1.20
41.00	0.20	187.00	5.00	6.00	3.40	2.60	1.30
37.00	0.10	66.00	2.00	6.80	3.40	3.40	1.00
36.00	0.20	190.00	7.00	6.00	3.10	2.90	1.10
24.00	0.10	89.00	6.00	5.90	3.30	2.60	1.30
23.00	0.10	58.00	3.00	6.30	3.40	2.90	1.20
36.00	0.10	161.00	1.00	6.40	3.50	2.90	1.20
31.00	0.20	125.00	6.00	5.70	3.30	2.40	1.40
29.00	0.20	91.00	3.00	5.70	3.60	2.10	1.70
19.00	0.30	186.00	4.00	6.60	3.50	3.10	1.10
39.00	0.10	100.00	3.00	5.80	3.20	2.60	1.20
38.00	0.10	139.00	3.00	6.20	3.70	2.50	1.50
57.00	0.20	122.00	6.00	6.70	3.20	3.50	0.90
36.00	0.10	60.00	4.00	6.00	3.50	2.50	1.40
41.08	0.17	167.71	4.83	6.11	3.40	2.70	1.29
34.84	0.07	119.79	4.55	0.35	0.22	0.36	0.22
45.42	0.12	142.00	5.21	6.00	3.17	2.83	1.16
30.48	0.06	94.33	4.11	0.42	0.31	0.46	0.25
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
37.00	0.10	224.00	1.00	5.90	3.30	2.60	1.30
49.00	0.20	306.00	13.00	5.90	3.60	2.30	1.60
32.00	0.10	84.00	1.00	5.90	3.70	2.20	1.70
33.00	0.20	256.00	3.00	6.70	3.50	3.20	1.10
37.00	0.10	265.00	1.00	6.10	3.80	2.30	1.70
44.00	0.10	121.00	6.00	7.00	3.60	3.40	1.10
33.00	0.10	155.00	2.00	6.00	3.20	2.80	1.10
29.00	0.10	158.00	1.00	6.40	3.40	3.00	1.10
31.00	0.10	172.00	2.00	5.40	2.90	2.50	1.20
242.00	0.10	450.00	18.00	6.30	3.00	3.30	0.90
17.00	0.10	163.00	4.00	6.60	3.10	3.50	0.90
136.00	0.20	243.00	7.00	6.20	4.00	2.20	1.80
52.00	0.20	87.00	7.00	6.10	3.30	2.80	1.20
63.00	0.20	118.00	9.00	7.00	3.30	3.70	0.90
22.00	0.20	82.00	1.00	6.40	3.20	3.20	1.00
49.00	0.10	85.00	3.00	6.40	3.50	2.90	1.20
26.00	0.10	239.00	6.00	6.00	2.90	3.10	0.90
30.00	0.20	78.00	2.00	5.80	2.60	3.20	0.80
35.00	0.10	331.00	3.00	6.50	3.10	3.40	0.90
50.00	0.10	78.00	4.00	6.60	3.10	3.50	0.90
45.00	0.10	97.00	2.00	6.70	3.50	3.20	1.10
26.00	0.20	53.00	1.00	6.10	3.40	2.70	1.30
.	.	.	.	.	.	.	.
53.00	0.10	140.00	6.00	6.70	3.00	3.70	0.80
50.91	0.13	173.26	4.48	6.29	3.30	2.99	1.15
47.76	0.05	100.82	4.26	0.40	0.33	0.47	0.30
57.04	0.13	176.17	5.08	6.04	3.06	2.98	1.05
84.56	0.06	115.75	5.06	0.46	0.32	0.46	0.23

Appendix B. Blood Biochemistry Parameters for Individual Animals

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
37.00	0.10	102.00	1.00	6.10	3.70	2.40	1.50
19.00	0.10	127.00	8.00	6.50	3.40	3.10	1.10
36.00	0.10	76.00	2.00	5.90	2.90	3.00	1.00
30.00	0.10	223.00	5.00	6.10	3.00	3.10	1.00
36.00	0.10	70.00	1.00	5.70	3.30	2.40	1.40
49.00	0.10	170.00	5.00	5.60	3.20	2.40	1.30
34.00	0.10	107.00	1.00	6.30	3.40	2.90	1.20
30.00	0.10	99.00	1.00	6.60	3.20	3.40	0.90
72.00	0.10	232.00	2.00	6.20	3.20	3.00	1.10
33.00	0.10	71.00	4.00	6.40	3.60	2.80	1.30
21.00	0.10	138.00	2.00	6.70	3.70	3.00	1.20
42.00	0.20	162.00	1.00	6.00	3.50	2.50	1.40
45.00	0.10	495.00	10.00	5.70	2.90	2.80	1.00
187.00	0.20	540.00	38.00	5.70	3.00	2.70	1.10
45.00	0.40	166.00	1.00	5.90	3.20	2.70	1.20
40.00	0.10	158.00	5.00	5.60	3.00	2.60	1.20
39.00	0.10	74.00	9.00	6.10	3.40	2.70	1.30
40.00	0.10	340.00	5.00	5.90	3.30	2.60	1.30
60.00	0.10	147.00	6.00	5.90	3.30	2.60	1.30
38.00	0.10	127.00	8.00	5.80	3.50	2.30	1.50
171.00	0.10	83.00	12.00	6.30	3.40	2.90	1.20
27.00	0.10	218.00	2.00	5.90	3.40	2.50	1.40
52.00	0.10	397.00	1.00	5.70	3.50	2.20	1.60
45.00	0.40	135.00	1.00	5.90	3.30	2.60	1.30
45.00	0.10	495.00	10.00	5.70	2.90	2.80	1.00
50.92	0.13	198.08	5.64	6.01	3.29	2.72	1.23
40.23	0.09	141.95	7.58	0.31	0.24	0.29	0.18
45.42	0.12	142.00	5.21	6.00	3.17	2.83	1.16
30.48	0.06	94.33	4.11	0.42	0.31	0.46	0.25
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
55.00	0.10	92.00	4.00	6.40	3.50	2.90	1.20
36.00	0.20	97.00	4.00	6.90	3.50	3.40	1.00
25.00	0.10	96.00	3.00	6.60	3.40	3.20	1.10
63.00	0.10	78.00	10.00	6.80	3.80	3.00	1.30
25.00	0.10	202.00	4.00	6.30	3.00	3.30	0.90
22.00	0.10	84.00	2.00	6.20	3.20	3.00	1.10
41.00	0.10	560.00	2.00	6.70	3.40	3.30	1.00
54.00	0.10	62.00	1.00	6.60	3.10	3.50	0.90
34.00	0.10	103.00	1.00	6.70	3.40	3.30	1.00
30.00	0.20	41.00	1.00	6.10	3.20	2.90	1.10
.	.	.	.	.	.	.	.
47.00	0.20	67.00	2.00	6.40	2.90	3.50	0.80
33.00	0.10	307.00	1.00	5.90	3.30	2.60	1.30
65.00	0.20	397.00	8.00	5.60	3.00	2.60	1.20
41.00	0.20	84.00	1.00	6.20	3.50	2.70	1.30
38.00	0.10	243.00	1.00	6.60	3.40	3.20	1.10
26.00	0.20	186.00	1.00	5.60	3.40	2.20	1.50
22.00	0.10	79.00	1.00	6.50	3.60	2.90	1.20
29.00	0.10	119.00	4.00	6.10	3.40	2.70	1.30
26.00	0.10	106.00	3.00	5.40	2.90	2.50	1.20
27.00	0.10	181.00	1.00	5.20	2.60	2.60	1.00
134.00	0.10	274.00	7.00	6.20	2.90	3.30	0.90
30.00	0.10	148.00	1.00	6.30	2.80	3.50	0.80
39.27	0.13	134.73	3.09	6.52	3.31	3.21	1.04
24.42	0.05	126.89	2.55	0.46	0.30	0.37	0.18
57.04	0.13	176.17	5.08	6.04	3.06	2.98	1.05
84.56	0.06	115.75	5.06	0.46	0.32	0.46	0.23

Appendix B. Blood Biochemistry Parameters for Individual Animals

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
38.00	0.10	319.00	1.00	5.80	2.80	3.00	0.90
265.00	0.10	592.00	39.00	5.70	3.20	2.50	1.30
42.00	0.10	140.00	7.00	5.80	2.80	3.00	0.90
33.00	0.10	90.00	1.00	5.60	2.90	2.70	1.10
35.00	0.10	62.00	5.00	6.40	3.30	3.10	1.10
39.00	0.10	256.00	4.00	6.10	3.50	2.60	1.30
64.00	0.10	165.00	8.00	6.60	3.60	3.00	1.20
44.00	0.10	96.00	6.00	5.90	3.50	2.40	1.50
72.00	0.10	130.00	1.00	7.00	3.80	3.20	1.20
33.00	0.10	258.00	5.00	6.30	3.60	2.70	1.30
49.00	0.10	285.00	8.00	5.90	3.50	2.40	1.50
26.00	0.20	186.00	1.00	6.70	2.90	3.80	0.80
42.00	0.10	197.00	5.00	6.00	3.10	2.90	1.10
44.00	0.10	109.00	6.00	6.20	2.80	3.40	0.80
61.00	0.10	237.00	1.00	6.00	2.70	3.30	0.80
27.00	0.20	142.00	1.00	6.80	3.40	3.40	1.00
31.00	0.10	45.00	2.00	6.10	3.30	2.80	1.20
45.00	0.10	137.00	1.00	6.20	3.20	3.00	1.10
38.00	0.20	245.00	3.00	5.90	2.70	3.20	0.80
41.00	0.10	73.00	1.00	6.40	3.40	3.00	1.10
26.00	0.20	169.00	1.00	6.30	3.20	3.10	1.00
38.00	0.10	66.00	2.00	5.50	3.00	2.50	1.20
45.00	0.10	106.00	5.00	6.10	3.30	2.80	1.20
42.00	0.10	68.00	1.00	5.20	2.60	2.60	1.00
50.83	0.12	173.88	4.79	6.10	3.17	2.93	1.10
47.00	0.04	118.21	7.70	0.42	0.34	0.35	0.20
45.42	0.12	142.00	5.21	6.00	3.17	2.83	1.16
30.48	0.06	94.33	4.11	0.42	0.31	0.46	0.25
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
24.00	0.20	365.00	1.00	6.00	3.40	2.60	1.30
85.00	0.10	825.00	43.00	5.60	3.10	2.50	1.20
26.00	0.10	49.00	1.00	5.20	2.90	2.30	1.30
42.00	0.10	323.00	5.00	6.60	3.10	3.50	0.90
27.00	0.20	336.00	1.00	5.30	3.40	1.90	1.80
49.00	0.10	105.00	2.00	6.80	3.70	3.10	1.20
27.00	0.10	236.00	4.00	6.20	3.40	2.80	1.20
29.00	0.10	292.00	1.00	6.40	3.20	3.20	1.00
33.00	0.10	163.00	1.00	5.40	3.10	2.30	1.30
77.00	0.10	239.00	12.00	6.30	3.10	3.20	1.00
26.00	0.10	209.00	1.00	6.30	3.10	3.20	1.00
148.00	0.20	512.00	2.00	6.80	4.10	2.70	1.50
.	.	.	.	.	.	.	.
69.00	0.10	68.00	2.00	5.10	1.90	3.20	0.60
47.00	0.10	253.00	1.00	6.00	2.80	3.20	0.90
24.00	0.10	34.00	5.00	6.60	2.90	3.70	0.80
49.00	0.10	92.00	4.00	6.70	3.00	3.70	0.80
39.00	0.10	82.00	1.00	6.60	2.50	4.10	0.60
48.00	0.10	71.00	2.00	5.50	2.40	3.10	0.80
24.00	0.10	76.00	1.00	6.20	3.20	3.00	1.10
47.00	0.20	65.00	2.00	6.40	3.00	3.40	0.90
26.00	0.10	172.00	4.00	5.70	2.60	3.10	0.80
63.00	0.10	82.00	1.00	6.70	3.30	3.40	1.00
56.00	0.10	107.00	9.00	5.90	2.90	3.00	1.00
47.17	0.12	206.78	4.61	6.10	3.05	3.05	1.04
28.36	0.04	183.31	8.83	0.54	0.45	0.51	0.28
57.04	0.13	176.17	5.08	6.04	3.06	2.98	1.05
84.56	0.06	115.75	5.06	0.46	0.32	0.46	0.23

Appendix B. Blood Biochemistry Parameters for Individual Animals

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
43.00	0.10	189.00	5.00	5.60	3.00	2.60	1.20
23.00	0.10	95.00	1.00	6.00	2.70	3.30	0.80
55.00	0.10	257.00	1.00	6.20	2.70	3.50	0.80
36.00	0.10	148.00	2.00	6.50	3.50	3.00	1.20
30.00	0.10	56.00	4.00	5.90	3.20	2.70	1.20
42.00	0.10	161.00	1.00	6.30	3.40	2.90	1.20
31.00	0.10	413.00	1.00	5.90	2.80	3.10	0.90
47.00	0.10	162.00	1.00	5.80	3.30	2.50	1.30
19.00	0.10	179.00	1.00	6.40	3.40	3.00	1.10
30.00	0.10	77.00	1.00	5.90	3.10	2.80	1.10
38.00	0.10	127.00	1.00	5.80	3.30	2.50	1.30
33.00	0.10	48.00	1.00	5.20	2.60	2.60	1.00
40.00	0.10	290.00	5.00	6.00	2.80	3.20	0.90
40.00	0.10	279.00	6.00	5.80	3.20	2.60	1.20
28.00	0.10	135.00	10.00	6.10	3.00	3.10	1.00
30.00	0.10	79.00	3.00	5.40	2.90	2.50	1.20
20.00	0.10	99.00	11.00	4.90	2.00	2.90	0.70
27.00	0.10	259.00	6.00	5.70	3.00	2.70	1.10
55.00	0.10	101.00	4.00	6.30	3.40	2.90	1.20
56.00	0.10	163.00	5.00	6.10	3.50	2.60	1.30
61.00	0.30	136.00	6.00	6.50	3.80	2.70	1.40
28.00	0.10	97.00	4.00	5.10	2.80	2.30	1.20
26.00	0.20	194.00	4.00	6.40	3.50	2.90	1.20
40.00	0.10	168.00	1.00	6.00	3.10	2.90	1.10
36.58	0.11	163.00	3.54	5.91	3.08	2.83	1.11
11.72	0.04	86.26	2.87	0.43	0.39	0.29	0.18
45.42	0.12	142.00	5.21	6.00	3.17	2.83	1.16
30.48	0.06	94.33	4.11	0.42	0.31	0.46	0.25
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
.	.	.	.	.	.	.	.
112.00	0.10	69.00	6.00	6.00	2.70	3.30	0.80
33.00	0.10	554.00	1.00	6.70	3.10	3.60	0.90
.	.	.	.	.	.	.	.
60.00	0.10	81.00	1.00	6.30	3.00	3.30	0.90
32.00	0.10	84.00	6.00	7.00	2.50	4.50	0.60
27.00	0.10	63.00	1.00	6.20	3.00	3.20	0.90
26.00	0.10	76.00	1.00	6.30	3.40	2.90	1.20
75.00	0.10	104.00	1.00	6.20	3.20	3.00	1.10
24.00	0.10	171.00	6.00	5.70	2.60	3.10	0.80
294.00	0.10	160.00	14.00	6.90	3.20	3.70	0.90
65.00	0.10	94.00	17.00	6.40	3.50	2.90	1.20
38.00	0.10	376.00	5.00	6.20	3.40	2.80	1.20
55.00	0.10	277.00	5.00	6.20	3.60	2.60	1.40
28.00	0.10	324.00	6.00	5.70	3.50	2.20	1.60
36.00	0.10	117.00	1.00	6.20	3.30	2.90	1.10
26.00	0.10	179.00	6.00	6.10	3.10	3.00	1.00
36.00	0.10	659.00	4.00	5.40	3.00	2.40	1.30
35.00	0.10	173.00	3.00	5.30	2.90	2.40	1.20
28.00	0.10	105.00	5.00	5.50	2.90	2.60	1.10
176.00	0.10	1292.00	11.00	6.90	4.10	2.80	1.50
63.47	0.10	260.95	5.26	6.17	3.16	3.01	1.09
67.28	0.00	300.84	4.51	0.49	0.38	0.53	0.26
57.04	0.13	176.17	5.08	6.04	3.06	2.98	1.05
84.56	0.06	115.75	5.06	0.46	0.32	0.46	0.23

Appendix B. Blood Biochemistry Parameters for Individual Animals

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
46	0.1	184	4	5.9	3.2	2.7	1.2
33	0.1	137	36	6.4	2.6	3.8	0.7
41	0.1	298	3	5.9	2.7	3.2	0.8
41	0.1	49	4	5.6	3	2.6	1.2
43	0.1	171	5	5.8	3.1	2.7	1.1
29	0.1	305	3	5.7	2.6	3.1	0.8
31	0.1	102	3	5.4	2.8	2.6	1.1
27	0.1	279	4	6.2	3.1	3.1	1
31	0.1	66	1	5.5	3	2.5	1.2
65	0.1	119	7	5.7	2.9	2.8	1
34	0.1	80	6	5	2.4	2.6	0.9
38.27	0.10	162.73	6.91	5.74	2.85	2.88	1.00
10.85	0.00	93.80	9.78	0.38	0.25	0.39	0.18
45.42	0.12	142.00	5.21	6.00	3.17	2.83	1.16
30.48	0.06	94.33	4.11	0.42	0.31	0.46	0.25
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO
36	0.1	54	1	6.3	2.6	3.7	0.7
15	0.1	212	3	4.8	2	2.8	0.7
43	0.1	93	7	6.2	2.9	3.3	0.9
31	0.1	94	56	7.4	2.1	5.3	0.4
29	0.1	69	1	5.4	2.1	3.3	0.6
35	0.1	125	4	5.6	2.5	3.1	0.8
20	0.1	146	4	5.5	2.3	3.2	0.7
42	0.1	92	4	6.8	3.2	3.6	0.9
41	0.1	70	8	5.8	3	2.8	1.1
32.44	0.10	106.11	9.78	5.98	2.52	3.46	0.76
9.80	0.00	48.83	17.49	0.79	0.44	0.76	0.20
57.04	0.13	176.17	5.08	6.04	3.06	2.98	1.05
84.56	0.06	115.75	5.06	0.46	0.32	0.46	0.23

Appendix B. Blood Biochemistry Parameters for Individual Animals

CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
264.00	19.00	1.10	17.00	4.00	8.90	2.20	82.00
224.00	14.00	1.00	14.00	4.30	9.60	2.20	86.00
230.00	17.00	1.10	15.00	4.90	10.40	2.10	83.00
214.00	14.00	1.00	14.00	4.10	10.10	2.50	90.00
217.00	11.00	1.00	11.00	3.00	10.20	3.40	81.00
309.00	20.00	0.90	22.00	3.60	9.40	2.60	86.00
141.00	28.00	1.50	19.00	4.80	9.90	2.10	94.00
175.00	7.00	1.00	7.00	3.00	9.70	3.20	99.00
194.00	13.00	1.10	12.00	5.20	10.30	2.00	93.00
134.00	14.00	0.90	16.00	4.40	9.50	2.20	81.00
348.00	15.00	0.80	19.00	5.10	9.90	1.90	76.00
205.00	12.00	1.30	9.00	3.90	9.30	2.40	76.00
192.00	11.00	0.80	14.00	4.50	9.50	2.10	78.00
205.00	13.00	1.10	12.00	3.90	9.40	2.40	92.00
186.00	12.00	0.90	13.00	4.00	9.20	2.30	74.00
233.00	14.00	1.00	14.00	3.20	9.10	2.80	85.00
386.00	12.00	0.80	15.00	3.70	10.00	2.70	87.00
191.00	16.00	1.20	13.00	4.00	9.30	2.30	84.00
222.00	16.00	1.00	16.00	5.00	9.60	1.90	91.00
200.00	11.00	0.80	14.00	4.20	9.20	2.20	87.00
195.00	13.00	1.00	13.00	4.70	9.00	1.90	89.00
247.00	6.00	0.70	9.00	5.40	11.20	2.10	114.00
335.00	14.00	0.90	16.00	4.90	9.30	1.90	75.00
214.00	11.00	0.80	14.00	4.20	9.10	2.20	92.00
227.54	13.88	0.99	14.08	4.25	9.63	2.32	86.46
61.63	4.37	0.18	3.34	0.67	0.54	0.39	8.83
CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
182.00	10.00	0.90	11.00	4.10	9.30	2.30	79.00
344.00	11.00	0.90	12.00	4.20	9.40	2.20	63.00
202.00	16.00	0.80	20.00	4.80	10.10	2.10	79.00
147.00	17.00	1.10	15.00	4.30	9.70	2.30	81.00
173.00	13.00	1.20	11.00	3.70	9.30	2.50	85.00
236.00	9.00	1.00	9.00	2.50	9.80	3.90	85.00
166.00	10.00	0.90	11.00	4.60	9.40	2.00	76.00
162.00	12.00	0.90	13.00	3.30	9.70	2.90	110.00
281.00	12.00	0.70	17.00	5.30	9.10	1.70	93.00
181.00	11.00	0.60	18.00	4.60	9.80	2.10	75.00
273.00	15.00	1.10	14.00	4.60	9.70	2.10	88.00
208.00	12.00	0.90	13.00	3.70	9.60	2.60	87.00
146.00	13.00	0.90	14.00	3.80	9.50	2.50	79.00
284.00	6.00	0.60	10.00	3.60	9.30	2.60	88.00
212.00	13.00	1.10	12.00	4.40	9.50	2.20	95.00
205.00	20.00	1.20	17.00	5.40	9.70	1.80	87.00
345.00	12.00	1.00	12.00	4.50	9.90	2.20	98.00
239.00	12.00	0.90	13.00	3.60	9.50	2.60	88.00
162.00	10.00	1.00	10.00	3.90	9.20	2.40	91.00
155.00	12.00	0.90	13.00	5.00	9.70	1.90	79.00
283.00	7.00	0.90	8.00	3.40	9.50	2.80	88.00
242.00	9.00	0.70	13.00	4.30	9.80	2.30	92.00
411.00	7.00	0.60	12.00	4.60	8.50	1.80	85.00
284.00	15.00	1.10	14.00	4.50	9.20	2.00	91.00
230.13	11.83	0.91	13.00	4.20	9.51	2.33	85.92
70.84	3.25	0.18	2.86	0.67	0.33	0.46	9.16

Appendix B. Blood Biochemistry Parameters for Individual Animals

CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
270.00	15.00	1.10	14.00	3.20	9.30	2.90	87.00
302.00	17.00	1.10	15.00	3.40	10.30	3.00	114.00
216.00	10.00	0.80	13.00	3.20	9.70	3.00	88.00
217.00	14.00	1.00	14.00	3.60	10.30	2.90	79.00
208.00	10.00	0.90	11.00	3.50	10.00	2.90	92.00
372.00	16.00	0.90	18.00	3.90	9.60	2.50	93.00
344.00	14.00	0.50	28.00	2.70	9.30	3.40	92.00
191.00	10.00	0.50	20.00	3.10	8.80	2.80	84.00
198.00	11.00	0.60	18.00	5.60	9.50	1.70	82.00
154.00	13.00	0.50	26.00	3.60	10.20	2.80	83.00
286.00	14.00	0.50	28.00	3.80	10.60	2.80	87.00
228.00	16.00	1.20	13.00	3.00	10.10	3.40	90.00
224.00	8.00	0.70	11.00	4.10	10.00	2.40	97.00
207.00	11.00	0.90	12.00	3.30	9.30	2.80	101.00
187.00	14.00	0.90	16.00	5.20	9.20	1.80	69.00
223.00	9.00	0.70	13.00	3.90	10.10	2.60	80.00
258.00	7.00	0.50	14.00	4.70	10.00	2.10	82.00
173.00	15.00	1.10	14.00	2.90	8.80	3.00	95.00
175.00	11.00	0.60	18.00	4.20	9.30	2.20	77.00
222.00	9.00	0.50	18.00	2.90	10.10	3.50	75.00
212.00	15.00	0.70	21.00	4.00	9.60	2.40	77.00
279.00	8.00	0.50	16.00	4.90	10.30	2.10	92.00
392.00	13.00	0.50	26.00	5.20	10.60	2.00	73.00
183.00	10.00	0.50	20.00	4.60	9.50	2.10	74.00
238.38	12.08	0.74	17.38	3.85	9.77	2.63	85.96
63.02	2.92	0.24	5.22	0.82	0.52	0.50	10.23
227.54	13.88	0.99	14.08	4.25	9.63	2.32	86.46
61.63	4.37	0.18	3.34	0.67	0.54	0.39	8.83
CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
156.00	10.00	0.90	11.00	4.10	9.50	2.30	81.00
296.00	7.00	0.70	10.00	3.80	9.70	2.60	78.00
168.00	13.00	0.70	19.00	3.40	10.50	3.10	99.00
138.00	9.00	0.80	11.00	3.50	9.90	2.80	93.00
168.00	13.00	0.70	19.00	4.50	10.80	2.40	72.00
250.00	9.00	0.70	13.00	4.40	11.10	2.50	91.00
174.00	11.00	0.90	12.00	3.60	9.00	2.50	89.00
212.00	8.00	0.90	9.00	8.70	10.10	1.20	85.00
154.00	18.00	1.20	15.00	3.40	9.00	2.60	79.00
278.00	10.00	0.60	17.00	4.40	9.50	2.20	86.00
239.00	10.00	0.60	17.00	4.20	9.60	2.30	59.00
221.00	12.00	1.10	11.00	2.70	10.90	4.00	90.00
188.00	13.00	0.60	22.00	3.80	9.40	2.50	94.00
183.00	9.00	0.50	18.00	3.70	9.50	2.60	77.00
187.00	19.00	0.50	38.00	5.50	9.80	1.80	90.00
201.00	14.00	0.50	28.00	2.90	9.70	3.30	98.00
284.00	7.00	0.50	14.00	3.80	8.70	2.30	91.00
272.00	12.00	0.80	15.00	4.20	8.00	1.90	86.00
193.00	9.00	0.50	18.00	4.90	10.10	2.10	90.00
152.00	12.00	0.50	24.00	3.20	9.60	3.00	79.00
252.00	13.00	0.80	16.00	3.70	9.50	2.60	83.00
141.00	11.00	0.50	22.00	3.30	9.90	3.00	90.00
.	.	.	.	.	.	.	.
164.00	16.00	0.50	32.00	3.70	9.20	2.50	94.00
203.09	11.52	0.70	17.87	4.06	9.70	2.53	85.83
49.08	3.16	0.20	7.26	1.19	0.71	0.56	9.08
230.13	11.83	0.91	13.00	4.20	9.51	2.33	85.92
70.84	3.25	0.18	2.86	0.67	0.33	0.46	9.16

Appendix B. Blood Biochemistry Parameters for Individual Animals

CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
188.00	12.00	0.80	15.00	4.30	10.20	2.40	98.00
226.00	8.00	0.90	9.00	3.40	9.70	2.90	97.00
213.00	12.00	0.90	13.00	4.30	9.40	2.20	102.00
285.00	9.00	0.90	10.00	2.70	9.70	3.60	104.00
215.00	8.00	0.70	11.00	4.80	9.40	2.00	104.00
232.00	6.00	0.60	10.00	4.50	9.90	2.20	94.00
207.00	10.00	0.80	13.00	4.30	9.60	2.20	93.00
298.00	12.00	0.80	15.00	4.00	10.00	2.50	88.00
139.00	9.00	0.90	10.00	3.50	9.80	2.80	93.00
203.00	9.00	0.70	13.00	4.70	10.20	2.20	88.00
338.00	12.00	0.70	17.00	4.00	10.20	2.60	97.00
250.00	19.00	1.40	14.00	4.30	10.10	2.30	94.00
310.00	19.00	0.80	24.00	3.10	8.40	2.70	66.00
291.00	18.00	0.70	26.00	5.40	8.80	1.60	71.00
201.00	10.00	0.70	14.00	3.90	9.00	2.30	91.00
206.00	19.00	0.60	32.00	5.50	9.00	1.60	70.00
251.00	13.00	0.60	22.00	4.10	9.10	2.20	81.00
252.00	15.00	0.70	21.00	3.40	9.00	2.60	90.00
229.00	8.00	0.50	16.00	3.10	8.80	2.80	82.00
187.00	12.00	0.70	17.00	3.70	9.20	2.50	86.00
215.00	20.00	0.90	22.00	3.40	9.80	2.90	106.00
171.00	14.00	0.80	18.00	5.80	9.10	1.60	95.00
310.00	19.00	0.70	27.00	2.90	9.30	3.20	73.00
224.00	14.00	0.90	16.00	3.70	9.50	2.60	97.00
310.00	19.00	0.80	24.00	3.10	8.40	2.70	66.00
238.04	13.04	0.78	17.16	4.00	9.42	2.45	89.04
50.33	4.35	0.17	6.10	0.82	0.54	0.48	11.95
227.54	13.88	0.99	14.08	4.25	9.63	2.32	86.46
61.63	4.37	0.18	3.34	0.67	0.54	0.39	8.83
CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
193.00	13.00	0.60	22.00	3.80	9.90	2.60	87.00
160.00	10.00	0.50	20.00	4.50	11.10	2.50	82.00
159.00	10.00	1.00	10.00	3.60	9.70	2.70	103.00
175.00	14.00	0.50	28.00	3.30	10.40	3.20	92.00
305.00	9.00	0.50	18.00	3.30	9.40	2.80	89.00
287.00	9.00	0.70	13.00	3.20	9.70	3.00	96.00
245.00	8.00	0.70	11.00	4.10	10.10	2.50	107.00
168.00	14.00	0.80	18.00	3.20	9.80	3.10	93.00
260.00	10.00	0.90	11.00	3.50	9.70	2.80	97.00
152.00	11.00	0.90	12.00	3.10	10.00	3.20	102.00
.	.	.	.	.	.	.	.
136.00	19.00	0.70	27.00	4.30	9.60	2.20	90.00
204.00	11.00	0.70	16.00	3.90	9.50	2.40	80.00
484.00	10.00	0.50	20.00	4.10	9.20	2.20	78.00
153.00	22.00	0.80	28.00	4.00	10.40	2.60	88.00
144.00	14.00	0.70	20.00	5.30	10.10	1.90	91.00
195.00	12.00	0.70	17.00	4.50	10.10	2.20	88.00
232.00	11.00	0.60	18.00	3.60	10.70	3.00	111.00
157.00	7.00	0.80	9.00	3.90	9.80	2.50	88.00
187.00	12.00	0.90	13.00	3.60	9.80	2.70	87.00
138.00	29.00	1.60	18.00	3.90	8.70	2.20	77.00
276.00	9.00	0.60	15.00	4.00	9.60	2.40	85.00
280.00	11.00	0.50	22.00	5.40	9.60	1.80	93.00
203.64	11.55	0.71	17.27	3.63	9.95	2.78	94.36
80.73	5.04	0.24	5.62	0.62	0.51	0.39	8.89
230.13	11.83	0.91	13.00	4.20	9.51	2.33	85.92
70.84	3.25	0.18	2.86	0.67	0.33	0.46	9.16

Appendix B. Blood Biochemistry Parameters for Individual Animals

CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
268.00	19.00	0.90	21.00	3.40	8.70	.	91.00
360.00	18.00	0.90	20.00	3.20	9.10	.	103.00
202.00	11.00	0.90	12.00	2.90	9.20	.	94.00
203.00	15.00	0.80	19.00	3.90	9.70	.	92.00
279.00	11.00	0.80	14.00	4.10	9.80	.	87.00
236.00	10.00	0.80	13.00	3.10	9.00	.	76.00
282.00	9.00	0.60	15.00	3.50	9.70	.	93.00
221.00	15.00	0.80	19.00	3.30	9.70	.	86.00
262.00	13.00	0.80	16.00	3.50	10.30	.	103.00
164.00	13.00	0.80	16.00	3.20	9.40	.	91.00
335.00	16.00	0.90	18.00	2.70	9.80	.	92.00
186.00	11.00	0.80	14.00	3.40	9.90	.	106.00
251.00	15.00	1.20	13.00	4.70	9.80	2.10	102.00
269.00	12.00	0.60	20.00	6.10	9.30	1.50	85.00
154.00	16.00	0.70	23.00	3.80	8.80	2.30	93.00
284.00	6.00	0.50	12.00	4.30	10.40	2.40	117.00
227.00	12.00	0.70	17.00	4.90	9.50	1.90	91.00
236.00	11.00	0.80	14.00	4.80	9.80	2.00	101.00
290.00	11.00	0.60	18.00	4.00	8.50	2.10	88.00
199.00	12.00	0.80	15.00	5.60	10.30	1.80	98.00
204.00	11.00	0.70	16.00	3.90	9.40	2.40	96.00
227.00	7.00	0.60	12.00	3.90	9.50	2.40	99.00
302.00	8.00	0.60	13.00	5.20	10.10	1.90	89.00
209.00	15.00	0.90	17.00	5.80	8.90	1.50	86.00
243.75	12.38	0.77	16.13	4.05	9.53	2.03	94.13
51.27	3.28	0.15	3.11	0.94	0.52	0.32	8.53
227.54	13.88	0.99	14.08	4.25	9.63	2.32	86.46
61.63	4.37	0.18	3.34	0.67	0.54	0.39	8.83
CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
166.00	11.00	0.70	16.00	4.30	9.30	.	79.00
409.00	12.00	0.80	15.00	3.30	8.80	.	91.00
143.00	21.00	0.80	26.00	4.70	10.80	.	105.00
163.00	10.00	0.70	14.00	3.20	9.70	.	112.00
146.00	14.00	0.80	18.00	3.20	9.50	.	85.00
235.00	11.00	0.70	16.00	3.90	11.10	.	122.00
191.00	10.00	0.90	11.00	2.70	9.80		78.00
240.00	9.00	0.80	11.00	3.80	10.60	.	106.00
151.00	22.00	0.90	24.00	3.70	9.40	.	86.00
275.00	8.00	0.50	16.00	4.00	9.60	.	86.00
295.00	10.00	0.60	17.00	4.80	9.60	.	93.00
308.00	9.00	0.70	13.00	2.60	10.90	.	94.00
.	.	.	.	.	.	.	.
236.00	10.00	0.60	17.00	4.90	8.80	4.80	94.00
222.00	9.00	0.60	15.00	4.60	8.90	1.90	98.00
218.00	12.00	0.70	17.00	5.40	10.30	1.90	77.00
289.00	12.00	0.90	13.00	5.20	9.90	1.90	100.00
147.00	21.00	0.90	23.00	4.80	8.70	1.80	91.00
303.00	11.00	0.60	18.00	5.60	9.50	1.70	95.00
171.00	6.00	0.70	9.00	4.50	9.00	2.00	88.00
192.00	15.00	0.90	17.00	3.80	9.20	2.40	98.00
307.00	7.00	0.50	14.00	3.30	8.60	2.60	90.00
202.00	8.00	0.60	13.00	3.90	9.40	2.40	76.00
192.00	14.00	0.60	23.00	3.70	8.90	2.40	95.00
226.13	11.83	0.72	16.35	4.08	9.58	2.35	93.00
68.81	4.36	0.13	4.30	0.83	0.73	0.87	11.27
230.13	11.83	0.91	13.00	4.20	9.51	2.33	85.92
70.84	3.25	0.18	2.86	0.67	0.33	0.46	9.16

Appendix B. Blood Biochemistry Parameters for Individual Animals

CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
223.00	17.00	1.20	14.00	3.90	10.30	.	96.00
260.00	12.00	0.60	20.00	4.60	9.50	.	75.00
216.00	13.00	0.80	16.00	4.80	9.70	.	109.00
291.00	8.00	0.50	16.00	5.40	11.30	.	94.00
210.00	13.00	0.70	19.00	4.80	10.00	.	87.00
244.00	9.00	0.80	11.00	3.30	10.20	.	112.00
412.00	15.00	0.80	19.00	4.10	9.40	.	91.00
207.00	13.00	0.80	16.00	4.80	10.80	.	86.00
264.00	11.00	0.70	16.00	4.40	9.90	.	85.00
274.00	7.00	0.70	10.00	3.30	9.80	.	113.00
276.00	9.00	0.60	15.00	4.50	9.90	.	103.00
203.00	16.00	0.90	18.00	3.90	8.90	.	115.00
277.00	16.00	1.10	15.00	3.60	9.80		91.00
206.00	20.00	1.00	20.00	3.70	10.50		88.00
191.00	9.00	0.90	10.00	3.10	9.90		98.00
197.00	13.00	0.90	14.00	4.20	10.60		98.00
337.00	13.00	0.80	16.00	4.20	9.20		91.00
219.00	17.00	1.00	17.00	4.20	10.00		89.00
265.00	8.00	0.60	13.00	4.10	10.40		91.00
186.00	14.00	0.80	18.00	4.10	11.20		93.00
215.00	12.00	1.00	12.00	3.20	10.30		94.00
129.00	14.00	0.90	16.00	4.10	9.00		95.00
382.00	12.00	1.00	12.00	3.40	10.60		100.00
198.00	11.00	1.10	10.00	4.10	10.10		93.00
245.08	12.58	0.84	15.13	4.08	10.05	.	95.29
64.02	3.27	0.18	3.13	0.58	0.62	.	9.60
227.54	13.88	0.99	14.08	4.25	9.63	2.32	86.46
61.63	4.37	0.18	3.34	0.67	0.54	0.39	8.83
CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
.	.	.	.	.	.	.	.
202.00	10.00	0.70	14.00	3.30	9.10	.	96.00
237.00	11.00	0.60	18.00	3.60	10.30	.	99.00
.	.	.	.	.	.	.	.
224.00	13.00	0.80	16.00	4.20	9.60	.	105.00
163.00	14.00	1.00	14.00	3.30	9.20	.	81.00
330.00	11.00	0.60	18.00	4.40	9.90	.	86.00
200.00	13.00	0.80	16.00	5.30	10.20	.	87.00
232.00	12.00	0.70	17.00	3.50	9.90	.	89.00
254.00	8.00	0.70	11.00	3.20	9.00	.	86.00
228.00	9.00	0.70	13.00	3.30	10.40	.	59.00
194.00	15.00	0.70	21.00	2.70	9.10	.	101.00
179.00	9.00	0.90	10.00	4.60	10.60		91.00
127.00	10.00	1.00	10.00	4.10	10.30		101.00
167.00	20.00	1.30	15.00	4.90	11.50		92.00
223.00	8.00	1.00	8.00	3.40	10.20		129.00
161.00	10.00	1.00	10.00	4.70	10.20		81.00
192.00	13.00	1.10	12.00	3.50	10.20		99.00
161.00	18.00	1.10	16.00	5.20	10.80		81.00
211.00	8.00	0.80	10.00	3.80	9.90		101.00
329.00	10.00	0.80	13.00	3.90	11.90		96.00
211.26	11.68	0.86	13.79	3.94	10.12	.	92.63
52.61	3.32	0.19	3.47	0.74	0.76	.	13.87
230.13	11.83	0.91	13.00	4.20	9.51	2.33	85.92
70.84	3.25	0.18	2.86	0.67	0.33	0.46	9.16

Appendix B. Blood Biochemistry Parameters for Individual Animals

CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
232	18	1.1	16	5.6	10.3		91
263	12	0.7	17	6.9	9.1		67
180	13	0.8	16	3.9	9.6		83
190	19	0.9	21	4.7	9.1		85
251	14	0.8	18	4.3	9.8		106
282	13	0.8	16	3.8	9.2		86
148	12	0.9	13	4.4	9.3		95
244	12	0.7	17	5.2	9.6		95
226	8	0.6	13	4.1	9.7		102
239	9	0.5	18	5.7	9.9		88
205	16	0.9	18	5.5	9.2		89
223.64	13.27	0.79	16.64	4.92	9.53	#DIV/0!	89.73
39.41	3.38	0.16	2.29	0.95	0.38	#DIV/0!	10.36
227.54	13.88	0.99	14.08	4.25	9.63	2.32	86.46
61.63	4.37	0.18	3.34	0.67	0.54	0.39	8.83
CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM	CA/PO4	GLUCOSE
177	14	0.6	23	4.5	9.9		106
160	12	0.6	20	4.3	8.1		91
254	13	0.9	14	5	9.6		101
150	16	1	16	5.3	9		84
321	18	0.6	30	4.8	8		78
198	11	0.6	18	6	9.1		100
234	13	0.8	16	4.2	9		71
214	13	0.7	19	5.9	9.7		68
182	11	0.6	18	4	9.5		86
210.00	13.44	0.71	19.33	4.89	9.10	#DIV/0!	87.22
53.58	2.30	0.15	4.77	0.73	0.67	#DIV/0!	13.46
230.13	11.83	0.91	13.00	4.20	9.51	2.33	85.92
70.84	3.25	0.18	2.86	0.67	0.33	0.46	9.16

Appendix B. Blood Biochemistry Parameters for Individual Animals

AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
515.00	176.00	146.00	4.20	35.00	115.00	101.00	66.00
490.00	314.00	141.00	4.70	30.00	106.00	172.00	216.00
760.00	340.00	145.00	4.60	32.00	109.00	119.00	300.00
601.00	440.00	144.00	4.10	35.00	116.00	105.00	147.00
888.00	587.00	145.00	4.30	34.00	109.00	125.00	82.00
380.00	190.00	144.00	4.20	34.00	112.00	143.00	71.00
734.00	79.00	151.00	4.60	33.00	119.00	135.00	30.00
689.00	432.00	146.00	4.00	37.00	112.00	138.00	62.00
1211.00	475.00	145.00	4.60	32.00	111.00	94.00	80.00
577.00	257.00	144.00	4.20	34.00	112.00	117.00	25.00
571.00	280.00	142.00	4.50	32.00	109.00	123.00	101.00
580.00	170.00	144.00	4.50	32.00	109.00	243.00	106.00
679.00	210.00	146.00	4.40	33.00	111.00	251.00	68.00
508.00	314.00	143.00	4.20	34.00	112.00	90.00	123.00
826.00	309.00	145.00	4.40	33.00	109.00	76.00	143.00
478.00	394.00	143.00	4.20	34.00	110.00	105.00	80.00
648.00	376.00	144.00	3.90	37.00	107.00	80.00	109.00
856.00	343.00	147.00	4.40	33.00	112.00	138.00	102.00
822.00	207.00	147.00	4.80	31.00	113.00	66.00	98.00
647.00	617.00	149.00	4.60	32.00	118.00	93.00	90.00
686.00	123.00	148.00	4.40	34.00	121.00	89.00	28.00
542.00	509.00	143.00	4.50	32.00	106.00	105.00	633.00
505.00	209.00	141.00	4.10	34.00	105.00	247.00	117.00
760.00	74.00	144.00	4.20	34.00	111.00	115.00	64.00
664.71	309.38	144.88	4.36	33.38	111.42	127.92	122.54
177.19	149.42	2.38	0.23	1.66	4.07	51.85	123.89
AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
407.00	527.00	142.00	4.60	31.00	110.00	110.00	125.00
397.00	398.00	147.00	4.40	33.00	113.00	112.00	127.00
960.00	543.00	144.00	4.30	33.00	107.00	401.00	268.00
648.00	216.00	144.00	4.30	33.00	109.00	244.00	133.00
814.00	563.00	142.00	4.40	32.00	107.00	168.00	94.00
604.00	614.00	143.00	4.10	35.00	108.00	106.00	127.00
626.00	365.00	149.00	4.90	30.00	116.00	159.00	70.00
623.00	365.00	141.00	4.60	31.00	109.00	110.00	78.00
765.00	133.00	144.00	4.20	34.00	113.00	172.00	59.00
867.00	397.00	142.00	4.90	29.00	106.00	144.00	438.00
737.00	101.00	140.00	4.80	29.00	114.00	244.00	96.00
584.00	425.00	143.00	3.70	39.00	110.00	174.00	36.00
539.00	697.00	144.00	4.40	33.00	109.00	118.00	108.00
427.00	372.00	142.00	4.20	34.00	107.00	302.00	155.00
659.00	566.00	145.00	4.30	34.00	111.00	170.00	228.00
767.00	391.00	142.00	4.60	31.00	112.00	94.00	404.00
821.00	483.00	145.00	4.50	32.00	112.00	189.00	136.00
481.00	305.00	146.00	4.60	32.00	111.00	68.00	76.00
498.00	552.00	147.00	4.50	33.00	116.00	121.00	94.00
916.00	299.00	150.00	5.00	30.00	116.00	197.00	44.00
595.00	771.00	144.00	4.60	31.00	112.00	112.00	151.00
740.00	340.00	143.00	4.50	32.00	108.00	148.00	316.00
524.00	43.00	141.00	4.60	31.00	104.00	226.00	130.00
1056.00	125.00	143.00	4.10	35.00	110.00	238.00	65.00
668.96	399.63	143.88	4.46	32.38	110.42	171.96	148.25
178.45	188.44	2.49	0.29	2.20	3.24	75.50	107.40

Appendix B. Blood Biochemistry Parameters for Individual Animals

AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
506.00	129.00	146.00	4.40	33.00	113.00	102.00	48.00
554.00	214.00	146.00	4.40	33.00	107.00	143.00	232.00
631.00	280.00	145.00	4.50	32.00	113.00	115.00	107.00
590.00	399.00	146.00	4.30	34.00	112.00	88.00	53.00
641.00	399.00	149.00	4.20	35.00	114.00	64.00	60.00
370.00	107.00	145.00	4.60	32.00	113.00	151.00	48.00
838.00	688.00	143.00	3.90	37.00	112.00	270.00	71.00
714.00	322.00	143.00	4.50	32.00	107.00	174.00	79.00
962.00	348.00	143.00	4.70	30.00	110.00	118.00	56.00
798.00	238.00	145.00	4.60	32.00	111.00	104.00	38.00
536.00	285.00	142.00	4.50	32.00	111.00	109.00	59.00
648.00	190.00	140.00	4.10	34.00	107.00	139.00	103.00
794.00	165.00	147.00	4.50	33.00	113.00	112.00	72.00
492.00	212.00	145.00	4.40	33.00	113.00	90.00	42.00
794.00	173.00	147.00	4.30	34.00	110.00	152.00	43.00
648.00	331.00	145.00	4.50	32.00	107.00	81.00	75.00
695.00	323.00	146.00	4.20	35.00	109.00	70.00	104.00
786.00	173.00	146.00	4.50	32.00	113.00	164.00	34.00
812.00	202.00	141.00	4.60	31.00	106.00	131.00	120.00
661.00	573.00	142.00	4.30	33.00	110.00	124.00	87.00
760.00	120.00	142.00	4.40	32.00	109.00	252.00	74.00
439.00	438.00	143.00	4.20	34.00	103.00	106.00	162.00
595.00	221.00	140.00	4.40	32.00	105.00	162.00	270.00
849.00	57.00	141.00	4.40	32.00	107.00	184.00	61.00
671.38	274.46	144.08	4.39	32.88	109.79	133.54	87.42
145.32	148.66	2.41	0.18	1.48	3.06	50.78	58.81
664.71	309.38	144.88	4.36	33.38	111.42	127.92	122.54
177.19	149.42	2.38	0.23	1.66	4.07	51.85	123.89
AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
479.00	505.00	147.00	4.60	32.00	112.00	103.00	97.00
347.00	319.00	148.00	4.50	33.00	110.00	112.00	296.00
767.00	452.00	145.00	4.90	30.00	111.00	177.00	153.00
724.00	164.00	146.00	4.50	32.00	111.00	129.00	73.00
548.00	566.00	147.00	4.50	33.00	106.00	104.00	142.00
530.00	308.00	147.00	5.10	29.00	109.00	106.00	258.00
834.00	432.00	145.00	4.80	30.00	110.00	100.00	88.00
657.00	461.00	145.00	4.60	32.00	111.00	231.00	124.00
622.00	313.00	145.00	4.80	30.00	110.00	102.00	155.00
761.00	266.00	145.00	4.50	32.00	110.00	71.00	100.00
547.00	203.00	147.00	4.80	31.00	106.00	217.00	89.00
608.00	579.00	147.00	4.50	33.00	110.00	52.00	73.00
518.00	609.00	141.00	4.20	34.00	106.00	117.00	59.00
606.00	410.00	143.00	4.70	30.00	107.00	83.00	126.00
785.00	296.00	145.00	5.10	28.00	111.00	140.00	74.00
620.00	338.00	144.00	4.90	29.00	108.00	201.00	106.00
774.00	576.00	143.00	4.40	33.00	109.00	104.00	187.00
638.00	114.00	154.00	4.80	32.00	114.00	180.00	72.00
734.00	290.00	145.00	4.60	32.00	110.00	165.00	115.00
1106.00	364.00	146.00	4.70	31.00	109.00	176.00	196.00
897.00	77.00	142.00	4.10	35.00	105.00	283.00	51.00
636.00	350.00	145.00	4.30	34.00	111.00	166.00	41.00
.	.	.	.	.	.	.	.
1108.00	151.00	141.00	4.40	32.00	108.00	176.00	88.00
688.96	354.04	145.35	4.62	31.61	109.30	143.26	120.13
182.09	153.44	2.69	0.26	1.78	2.20	56.55	64.26
668.96	399.63	143.88	4.46	32.38	110.42	171.96	148.25
178.45	188.44	2.49	0.29	2.20	3.24	75.50	107.40

Appendix B. Blood Biochemistry Parameters for Individual Animals

AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
1010.00	215.00	147.00	4.60	32.00	107.00	83.00	109.00
626.00	448.00	140.00	4.00	35.00	118.00	71.00	105.00
712.00	99.00	144.00	4.60	31.00	110.00	151.00	31.00
749.00	537.00	147.00	4.40	33.00	111.00	62.00	125.00
695.00	323.00	147.00	4.30	34.00	110.00	140.00	67.00
456.00	341.00	145.00	4.80	30.00	108.00	120.00	87.00
1007.00	260.00	147.00	4.30	34.00	109.00	94.00	48.00
481.00	135.00	146.00	4.20	35.00	109.00	164.00	129.00
669.00	184.00	137.00	3.60	38.00	117.00	73.00	55.00
845.00	56.00	145.00	4.30	34.00	108.00	127.00	57.00
563.00	283.00	147.00	4.60	32.00	112.00	75.00	93.00
692.00	165.00	147.00	4.30	34.00	109.00	88.00	44.00
484.00	130.00	145.00	4.50	32.00	109.00	157.00	64.00
466.00	212.00	144.00	4.40	33.00	106.00	263.00	60.00
417.00	261.00	146.00	4.40	33.00	111.00	129.00	90.00
811.00	109.00	145.00	4.50	32.00	108.00	110.00	52.00
569.00	325.00	146.00	4.20	35.00	106.00	117.00	51.00
628.00	331.00	144.00	4.40	33.00	112.00	100.00	183.00
606.00	268.00	146.00	3.90	37.00	109.00	133.00	42.00
515.00	427.00	144.00	4.30	33.00	106.00	91.00	44.00
646.00	404.00	146.00	4.00	37.00	100.00	66.00	113.00
901.00	278.00	144.00	4.30	33.00	109.00	84.00	69.00
407.00	156.00	147.00	4.00	37.00	109.00	166.00	66.00
805.00	258.00	147.00	4.20	35.00	105.00	243.00	333.00
484.00	130.00	145.00	4.50	32.00	109.00	157.00	64.00
649.76	253.40	145.12	4.30	33.76	109.08	122.56	87.24
174.37	120.77	2.33	0.26	2.01	3.56	51.03	62.00
664.71	309.38	144.88	4.36	33.38	111.42	127.92	122.54
177.19	149.42	2.38	0.23	1.66	4.07	51.85	123.89
AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
491.00	565.00	145.00	5.10	28.00	111.00	218.00	68.00
604.00	448.00	146.00	5.00	29.00	109.00	166.00	78.00
915.00	282.00	139.00	4.20	33.00	120.00	69.00	91.00
545.00	377.00	147.00	4.70	31.00	111.00	272.00	51.00
815.00	551.00	145.00	4.40	33.00	112.00	158.00	235.00
794.00	122.00	148.00	4.70	31.00	109.00	81.00	108.00
723.00	241.00	146.00	4.80	30.00	107.00	102.00	290.00
1066.00	250.00	146.00	4.40	33.00	108.00	79.00	82.00
791.00	72.00	145.00	4.60	32.00	109.00	177.00	64.00
634.00	320.00	147.00	3.90	38.00	108.00	136.00	39.00
.	.	.	.	.	.	.	.
1277.00	210.00	147.00	4.30	34.00	113.00	67.00	37.00
508.00	514.00	146.00	4.60	32.00	112.00	168.00	81.00
351.00	313.00	146.00	4.50	32.00	106.00	113.00	324.00
940.00	450.00	147.00	4.50	33.00	103.00	158.00	302.00
1042.00	387.00	145.00	4.60	32.00	102.00	200.00	95.00
508.00	521.00	149.00	4.40	34.00	112.00	156.00	61.00
532.00	373.00	145.00	4.50	32.00	102.00	84.00	198.00
759.00	447.00	147.00	4.50	33.00	110.00	155.00	61.00
653.00	427.00	148.00	4.50	33.00	107.00	114.00	85.00
597.00	276.00	147.00	4.80	31.00	113.00	152.00	43.00
740.00	285.00	146.00	4.70	31.00	110.00	146.00	93.00
427.00	199.00	147.00	4.80	31.00	109.00	194.00	75.00
786.82	312.55	145.55	4.55	32.00	110.64	138.64	103.91
230.07	136.32	1.93	0.26	2.00	4.03	52.28	89.95
668.96	399.63	143.88	4.46	32.38	110.42	171.96	148.25
178.45	188.44	2.49	0.29	2.20	3.24	75.50	107.40

Appendix B. Blood Biochemistry Parameters for Individual Animals

AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
580.00	116.00	146.00	4.30	34.00	116.00	244.00	74.00
560.00	232.00	146.00	4.80	30.00	112.00	230.00	175.00
493.00	224.00	146.00	4.80	30.00	115.00	96.00	57.00
882.00	168.00	147.00	4.80	31.00	113.00	77.00	336.00
683.00	331.00	145.00	5.00	29.00	109.00	148.00	61.00
757.00	334.00	145.00	4.90	30.00	113.00	159.00	203.00
936.00	373.00	144.00	4.70	31.00	110.00	67.00	68.00
535.00	388.00	149.00	4.50	33.00	116.00	87.00	52.00
780.00	393.00	150.00	4.20	36.00	116.00	90.00	56.00
1004.00	255.00	147.00	4.60	32.00	113.00	105.00	91.00
421.00	134.00	146.00	4.30	34.00	115.00	126.00	104.00
840.00	162.00	146.00	4.40	33.00	116.00	103.00	47.00
694.00	227.00	143.00	4.10	35.00	110.00	97.00	88.00
511.00	145.00	144.00	4.60	31.00	103.00	295.00	242.00
871.00	153.00	144.00	4.70	31.00	107.00	86.00	83.00
501.00	250.00	143.00	4.90	29.00	104.00	146.00	79.00
906.00	73.00	144.00	4.60	31.00	112.00	89.00	174.00
1164.00	287.00	143.00	4.50	32.00	113.00	110.00	112.00
459.00	595.00	143.00	4.60	31.00	106.00	71.00	170.00
1053.00	252.00	145.00	4.80	30.00	110.00	110.00	234.00
655.00	535.00	146.00	4.30	34.00	110.00	122.00	299.00
702.00	317.00	143.00	4.30	33.00	113.00	136.00	70.00
424.00	402.00	145.00	4.20	35.00	109.00	114.00	216.00
777.00	106.00	147.00	4.40	33.00	111.00	171.00	98.00
716.17	268.83	145.29	4.55	32.00	111.33	128.29	132.88
210.13	133.33	1.88	0.26	1.98	3.71	57.24	83.84
664.71	309.38	144.88	4.36	33.38	111.42	127.92	122.54
177.19	149.42	2.38	0.23	1.66	4.07	51.85	123.89
AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
417.00	380.00	144.00	4.60	31.00	109.00	107.00	48.00
347.00	227.00	143.00	4.70	30.00	106.00	109.00	180.00
934.00	397.00	147.00	5.20	28.00	117.00	151.00	208.00
761.00	227.00	144.00	4.90	29.00	116.00	111.00	93.00
501.00	493.00	146.00	4.50	32.00	112.00	104.00	133.00
549.00	275.00	145.00	5.10	28.00	113.00	86.00	157.00
791.00	391.00	145.00	4.30	34.00	111.00	111.00	51.00
840.00	655.00	142.00	4.60	31.00	113.00	217.00	205.00
602.00	321.00	145.00	5.00	29.00	114.00	120.00	388.00
947.00	312.00	148.00	4.60	32.00	115.00	183.00	91.00
500.00	146.00	144.00	4.70	31.00	106.00	99.00	69.00
840.00	555.00	146.00	4.60	32.00	110.00	207.00	85.00
.	.	.	.	.	.	.	.
651.00	332.00	142.00	5.20	27.00	111.00	236.00	338.00
695.00	280.00	141.00	4.70	30.00	109.00	138.00	324.00
788.00	207.00	143.00	5.00	29.00	111.00	116.00	74.00
852.00	72.00	146.00	5.10	29.00	112.00	200.00	89.00
1228.00	170.00	142.00	4.30	33.00	105.00	79.00	33.00
727.00	150.00	143.00	4.50	32.00	111.00	171.00	93.00
932.00	290.00	144.00	4.70	31.00	112.00	205.00	69.00
728.00	600.00	143.00	4.30	33.00	106.00	157.00	221.00
818.00	497.00	142.00	4.60	31.00	106.00	174.00	256.00
597.00	475.00	143.00	4.90	29.00	106.00	159.00	154.00
550.00	259.00	143.00	4.70	30.00	108.00	211.00	108.00
721.52	335.26	143.96	4.73	30.48	110.39	150.04	150.74
200.84	154.04	1.80	0.28	1.81	3.49	47.31	99.69
668.96	399.63	143.88	4.46	32.38	110.42	171.96	148.25
178.45	188.44	2.49	0.29	2.20	3.24	75.50	107.40

Appendix B. Blood Biochemistry Parameters for Individual Animals

AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
716.00	242.00	145.00	4.60	32.00	111.00	130.00	103.00
778.00	198.00	146.00	4.50	32.00	115.00	307.00	90.00
844.00	89.00	146.00	5.00	29.00	119.00	80.00	43.00
517.00	271.00	145.00	5.30	27.00	111.00	410.00	86.00
951.00	65.00	146.00	4.40	33.00	113.00	112.00	63.00
1306.00	306.00	149.00	4.10	36.00	112.00	136.00	56.00
727.00	628.00	147.00	4.60	32.00	115.00	133.00	269.00
920.00	216.00	145.00	4.50	32.00	111.00	101.00	16.00
679.00	498.00	148.00	4.70	31.00	115.00	140.00	133.00
836.00	426.00	144.00	4.20	34.00	111.00	152.00	112.00
428.00	351.00	144.00	4.60	31.00	109.00	104.00	245.00
840.00	115.00	148.00	4.20	35.00	120.00	176.00	73.00
569.00	163.00	150.00	4.10	37.00	115.00	78.00	61.00
617.00	343.00	145.00	4.50	32.00	109.00	187.00	207.00
534.00	309.00	145.00	4.30	34.00	114.00	82.00	96.00
968.00	235.00	147.00	4.40	33.00	111.00	62.00	366.00
413.00	148.00	143.00	4.90	29.00	107.00	92.00	76.00
1404.00	1109.00	143.00	4.40	33.00	109.00	102.00	65.00
554.00	331.00	146.00	4.20	35.00	110.00	69.00	66.00
546.00	485.00	148.00	4.30	34.00	112.00	60.00	100.00
682.00	410.00	154.00	4.50	34.00	119.00	142.00	59.00
684.00	205.00	147.00	4.20	35.00	112.00	105.00	54.00
359.00	161.00	146.00	4.60	32.00	113.00	210.00	110.00
663.00	211.00	148.00	4.20	35.00	112.00	134.00	135.00
730.63	313.13	146.46	4.47	32.79	112.71	137.67	111.83
254.92	218.98	2.41	0.29	2.34	3.32	79.76	81.81
664.71	309.38	144.88	4.36	33.38	111.42	127.92	122.54
177.19	149.42	2.38	0.23	1.66	4.07	51.85	123.89
AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
.	.	.	.	.	.	.	.
719.00	314.00	148.00	4.80	31.00	112.00	175.00	203.00
811.00	224.00	148.00	4.70	31.00	114.00	175.00	123.00
.	.	.	.	.	.	.	.
765.00	72.00	145.00	5.10	28.00	121.00	149.00	65.00
1067.00	198.00	145.00	4.30	34.00	111.00	124.00	56.00
719.00	107.00	146.00	4.60	32.00	113.00	162.00	130.00
881.00	274.00	148.00	5.40	27.00	115.00	175.00	122.00
449.00	497.00	146.00	4.90	30.00	109.00	197.00	130.00
831.00	382.00	146.00	5.00	29.00	111.00	175.00	197.00
693.00	445.00	146.00	5.20	28.00	109.00	197.00	158.00
654.00	309.00	145.00	4.80	30.00	110.00	233.00	122.00
503.00	491.00	149.00	4.60	32.00	114.00	113.00	168.00
599.00	203.00	152.00	5.20	29.00	118.00	496.00	98.00
657.00	587.00	149.00	4.70	32.00	111.00	85.00	166.00
439.00	286.00	150.00	4.60	33.00	114.00	92.00	368.00
665.00	446.00	150.00	4.70	32.00	113.00	70.00	106.00
700.00	1700.00	153.00	4.80	32.00	119.00	133.00	240.00
565.00	382.00	148.00	4.80	31.00	114.00	64.00	233.00
342.00	220.00	150.00	4.70	32.00	113.00	81.00	51.00
1000.00	506.00	149.00	4.80	31.00	109.00	109.00	100.00
687.32	402.26	148.05	4.83	30.74	113.16	158.16	149.26
185.68	344.77	2.34	0.26	1.85	3.35	95.03	76.11
668.96	399.63	143.88	4.46	32.38	110.42	171.96	148.25
178.45	188.44	2.49	0.29	2.20	3.24	75.50	107.40

Appendix B. Blood Biochemistry Parameters for Individual Animals

AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
713	269	145	4.5	32	109	90	238
641	265	146	4.6	32	107	277	180
833	216	146	4.3	34	111	113	110
941	73	147	4.2	35	113	99	56
1093	363	149	4.2	35	113	86	74
729	826	146	4	37	109	112	124
807	219	145	4.8	30	113	108	78
630	646	146	4.4	33	111	195	182
716	420	147	3.8	39	112	131	83
411	419	146	4.7	31	110	137	215
861	210	145	4.3	34	114	127	47
761.36	356.91	146.18	4.35	33.82	111.09	134.09	126.09
178.70	216.50	1.17	0.30	2.64	2.17	55.95	66.91
664.71	309.38	144.88	4.36	33.38	111.42	127.92	122.54
177.19	149.42	2.38	0.23	1.66	4.07	51.85	123.89
AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO	CHLORIDE	CPK	TRIGLYCERIDE
1052	299	146	4.6	32	109	157	667
563	248	145	4.9	30	115	210	46
838	78	144	4.4	33	110	157	46
1166	197	142	4.8	30	109	95	102
651	116	147	4.1	36	112	157	97
474	470	144	5.2	28	108	181	187
1192	574	148	4.6	32	112	217	51
578	503	145	5.7	25	108	191	217
558	382	145	4.7	31	110	184	103
785.78	318.56	145.11	4.78	30.78	110.33	172.11	168.44
283.56	175.09	1.76	0.46	3.11	2.29	36.50	196.48
668.96	399.63	143.88	4.46	32.38	110.42	171.96	148.25
178.45	188.44	2.49	0.29	2.20	3.24	75.50	107.40

Appendix B. Blood Biochemistry Parameters for Individual Animals

OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
303.00	9.70	.	5.70	5.70	13.20	43.00	76.00
292.00	9.80	.	5.20	7.30	17.90	54.00	73.00
301.00	10.60	.	7.50	6.80	16.10	51.00	75.00
298.00	.	.	5.60	7.40	18.00	54.00	73.00
298.00	.	.	6.20	6.10	14.60	47.00	77.00
300.00	9.60	.	11.70	6.40	16.30	51.00	80.00
317.00	11.00	.	7.70	5.80	13.60	43.00	74.00
300.00	10.00	.	6.90	7.60	17.20	54.00	72.00
300.00	10.90	.	5.60	7.60	17.20	54.00	72.00
298.00	9.90	.	7.70	6.80	15.80	52.00	77.00
294.00	10.50	.	14.80	5.30	12.40	41.00	77.00
297.00	9.70	.	7.60	7.90	18.50	60.00	76.00
300.00	10.00	.	7.10	6.20	14.50	46.00	74.00
296.00	10.00	.	5.80	7.30	16.60	54.00	74.00
298.00	9.40	.	6.40	7.90	18.30	56.00	71.00
296.00	9.40	.	6.20	7.00	16.70	51.00	73.00
297.00	10.10	.	7.40	5.80	13.20	41.00	71.00
304.00	.	.	6.90	6.60	16.60	53.00	80.00
305.00	.	.	6.10	6.40	16.00	50.00	78.00
307.00	9.70	.	6.70	6.70	14.00	46.00	68.00
306.00	9.70	.	6.20	6.80	14.90	48.00	71.00
294.00	.	.	7.70	7.20	16.90	51.00	71.00
291.00	9.60	.	8.50	5.60	13.10	43.00	77.00
297.00	9.30	.	5.20	6.40	14.90	46.00	72.00
299.54	9.94	.	7.18	6.69	15.69	49.54	74.25
5.56	0.49	.	2.12	0.74	1.81	5.10	3.08
OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
292.00	9.60	.	6.30	6.90	15.70	52.00	75.00
301.00	.	.	8.10	7.10	16.80	55.00	78.00
298.00	10.30	.	6.90	7.40	17.70	58.00	78.00
299.00	10.60	.	7.30	7.40	15.40	52.00	71.00
293.00	9.90	.	9.10	6.30	15.50	49.00	77.00
294.00	.	.	7.40	7.00	16.60	55.00	78.00
306.00	10.10	.	11.70	6.50	14.90	47.00	73.00
292.00	10.10	.	6.30	6.40	13.70	43.00	68.00
297.00	9.90	.	6.60	6.90	16.10	52.00	75.00
292.00	10.60	.	10.90	7.10	15.80	49.00	69.00
290.00	10.00	.	6.60	6.90	15.90	52.00	76.00
295.00	9.90	.	6.70	6.60	14.60	48.00	73.00
297.00	9.60	.	7.60	7.50	18.50	57.00	76.00
291.00	.	.	7.30	7.60	18.00	54.00	71.00
300.00	9.80	.	8.40	6.40	15.90	47.00	73.00
296.00	10.20	.	7.90	7.30	17.40	55.00	76.00
300.00	10.30	.	7.60	7.10	16.50	53.00	75.00
301.00	9.90	.	5.20	6.20	13.60	45.00	73.00
303.00	9.60	.	6.00	7.20	17.30	53.00	74.00
309.00	10.10	.	6.40	8.30	19.60	61.00	73.00
295.00	10.50	.	7.40	6.70	16.20	52.00	78.00
294.00	10.10	.	10.70	6.80	15.20	49.00	72.00
289.00	9.70	.	22.20	6.70	12.50	43.00	53.00
296.00	9.60	.	5.90	7.50	16.70	55.00	73.00
296.67	10.02	.	8.19	6.99	16.09	51.50	73.25
5.03	0.32	.	3.39	0.49	1.60	4.58	5.13

Appendix B. Blood Biochemistry Parameters for Individual Animals

OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
302.00	9.90	1.80	4.80	6.80	15.30	47.00	69.00
304.00	.	1.90	6.70	7.10	16.80	51.00	71.00
298.00	.	1.60	7.60	5.80	13.90	42.00	72.00
301.00	.	1.50	5.80	7.40	17.10	52.00	70.00
307.00	.	1.60	7.60	6.00	14.20	43.00	72.00
301.00	9.70	1.90	10.40	5.70	15.00	44.00	77.00
296.00	9.70	1.90	6.10	7.20	15.80	48.00	67.00
294.00	8.90	2.10	7.20	7.40	16.30	50.00	67.00
294.00	9.60	2.30	5.90	8.00	17.40	54.00	68.00
299.00	10.40	1.60	11.70	7.50	17.50	53.00	70.00
294.00	10.70	2.10	9.60	6.40	14.30	44.00	69.00
291.00	10.20	2.10	6.60	7.70	17.80	54.00	71.00
302.00	10.10	1.20	7.90	6.40	14.80	45.00	70.00
300.00	9.70	1.60	6.20	6.70	15.20	46.00	69.00
303.00	9.40	1.60	5.80	6.80	15.20	46.00	68.00
298.00	10.20	1.60	8.60	7.20	16.60	50.00	70.00
299.00	.	1.90	7.70	7.00	15.50	46.00	66.00
303.00	9.00	1.90	7.20	6.30	16.00	47.00	74.00
290.00	.	1.80	6.30	6.10	14.90	45.00	74.00
291.00	.	2.10	4.60	8.80	17.10	53.00	61.00
294.00	9.90	2.00	8.30	6.90	15.50	48.00	70.00
294.00	.	2.40	5.30	8.40	18.10	56.00	67.00
289.00	10.90	2.30	10.30	6.50	14.70	45.00	70.00
290.00	.	2.20	4.70	6.30	14.50	44.00	70.00
297.25	9.89	1.88	7.20	6.93	15.81	48.04	69.67
5.11	0.56	0.30	1.88	0.79	1.23	4.01	3.12
299.54	9.94	.	7.18	6.69	15.69	49.54	74.25
5.56	0.49	.	2.12	0.74	1.81	5.10	3.08
OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
302.00	9.70	1.80	7.30	6.80	15.40	47.00	69.00
303.00	.	1.50	8.80	7.00	16.80	50.00	72.00
300.00	.	1.50	6.00	7.90	18.40	55.00	69.00
300.00	.	1.50	9.60	6.90	16.30	49.00	70.00
303.00	.	1.80	7.70	8.10	19.30	56.00	69.00
302.00	.	1.60	9.40	7.00	16.40	50.00	71.00
299.00	9.30	1.70	8.60	6.10	14.90	44.00	72.00
298.00	10.20	1.70	9.90	6.90	16.30	49.00	71.00
301.00	9.60	1.70	6.00	6.20	14.60	44.00	71.00
298.00	10.00	1.60	10.10	5.60	13.60	40.00	73.00
301.00	10.00	1.80	8.80	6.60	13.90	43.00	64.00
303.00	.	1.60	5.50	6.70	15.40	46.00	69.00
292.00	9.60	.	6.00	7.20	16.60	50.00	69.00
293.00	9.70	.	10.40	7.10	16.30	49.00	70.00
302.00	10.10	2.40	7.70	7.20	16.10	49.00	69.00
298.00	.	.	6.60	6.40	13.90	42.00	67.00
294.00	9.30	.	11.20	7.30	16.90	52.00	71.00
317.00	8.90	1.10	6.60	6.60	14.70	45.00	68.00
298.00	10.50	2.00	10.10	6.50	14.00	44.00	68.00
301.00	10.00	1.40	9.60	7.60	16.40	51.00	67.00
293.00	.	2.20	7.20	6.60	15.60	47.00	72.00
299.00	10.00	2.00	10.60	8.10	16.60	51.00	64.00
.	.	.	.	.	.	.	.
293.00	9.70	.	6.50	6.70	14.70	46.00	68.00
299.57	9.77	1.72	8.27	6.92	15.79	47.78	69.26
5.19	0.41	0.30	1.76	0.62	1.42	4.01	2.34
296.67	10.02	.	8.19	6.99	16.09	51.50	73.25
5.03	0.32	.	3.39	0.49	1.60	4.58	5.13

Appendix B. Blood Biochemistry Parameters for Individual Animals

OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
304.00	.	1.50	7.60	5.70	13.70	41.00	73.00
288.00	9.80	1.90	4.90	7.30	13.40	42.00	58.00
298.00	10.00	1.50	9.30	6.90	15.30	45.00	66.00
303.00	10.20	1.50	8.10	7.20	16.60	49.00	69.00
303.00	9.60	1.80	6.60	7.30	16.40	50.00	68.00
297.00	10.20	1.70	8.50	6.30	14.70	44.00	69.00
303.00	9.70	1.60	6.60	7.70	17.30	52.00	67.00
301.00	10.30	1.90	7.90	6.60	15.10	45.00	68.00
282.00	10.10	1.60	10.90	7.00	16.60	49.00	70.00
298.00	.	1.50	5.60	6.30	14.50	43.00	69.00
304.00	.	1.80	6.70	6.30	14.50	44.00	70.00
306.00	.	1.60	9.70	7.70	18.30	54.00	71.00
300.00	9.00	1.70	4.80	6.90	15.20	47.00	68.00
298.00	9.30	1.60	4.40	6.80	15.30	47.00	70.00
301.00	9.30	1.80	4.40	5.80	12.60	39.00	68.00
301.00	9.50	1.70	5.00	6.60	14.60	45.00	67.00
301.00	9.20	1.30	6.60	6.70	15.10	46.00	68.00
298.00	9.20	1.80	6.00	6.20	14.40	44.00	71.00
299.00	9.00	1.50	4.70	6.30	13.80	42.00	66.00
297.00	.	1.60	3.70	7.00	15.40	48.00	69.00
305.00	9.90	1.70	8.10	6.20	14.40	44.00	70.00
298.00	9.20	2.00	8.00	6.20	14.90	46.00	74.00
305.00	.	1.60	8.40	6.20	15.70	47.00	76.00
304.00	9.70	1.70	6.50	7.50	17.40	50.00	67.00
300.00	9.00	1.70	4.80	6.90	15.20	47.00	68.00
299.76	9.59	1.66	6.71	6.70	15.22	46.00	68.80
5.29	0.44	0.16	1.89	0.55	1.32	3.49	3.28
299.54	9.94	.	7.18	6.69	15.69	49.54	74.25
5.56	0.49	.	2.12	0.74	1.81	5.10	3.08
OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
299.00	.	1.60	5.40	6.70	16.20	48.00	73.00
300.00	.	1.70	9.40	6.90	16.10	49.00	70.00
287.00	9.80	2.00	6.90	8.20	18.20	56.00	68.00
304.00	.	1.40	6.80	6.30	13.70	41.00	66.00
298.00	9.90	1.50	8.60	6.60	15.80	47.00	72.00
305.00	10.00	1.90	14.10	6.60	15.50	46.00	70.00
301.00	10.20	1.60	7.90	6.70	14.90	46.00	68.00
302.00	10.20	1.40	8.50	7.00	15.40	47.00	68.00
299.00	9.80	1.60	8.50	7.40	17.30	51.00	70.00
304.00	10.30	1.60	5.80	8.10	16.90	51.00	63.00
.	.	.	.	.	.	.	.
306.00	10.20	1.50	8.60	6.20	14.00	42.00	68.00
300.00	9.70	1.60	6.70	6.50	14.60	44.00	69.00
300.00	9.70	1.30	8.60	6.80	16.10	49.00	71.00
307.00	.	1.70	6.20	8.40	19.30	57.00	68.00
300.00	10.20	1.90	8.20	7.80	17.80	53.00	67.00
307.00	10.20	1.50	6.20	7.80	16.20	55.00	70.00
300.00	.	1.80	6.10	7.10	16.60	48.00	68.00
301.00	9.90	1.50	10.10	6.60	15.70	47.00	72.00
305.00	10.40	1.50	10.80	6.50	15.20	45.00	69.00
309.00	9.60	1.60	8.00	5.70	13.00	40.00	70.00
300.00	10.20	1.50	7.40	6.00	13.80	42.00	71.00
303.00	10.30	1.60	8.20	5.40	12.00	37.00	68.00
300.45	10.05	1.62	8.23	6.97	15.82	47.64	68.73
4.50	0.25	0.17	1.94	0.80	1.73	5.21	2.21
296.67	10.02	.	8.19	6.99	16.09	51.50	73.25
5.03	0.32	.	3.39	0.49	1.60	4.58	5.13

Appendix B. Blood Biochemistry Parameters for Individual Animals

OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
304.00	9.40	1.90	6.00	7.00	15.90	47.00	68.00
304.00	9.40	1.70	6.10	6.60	15.80	47.00	71.00
301.00	9.90	1.50	7.40	6.90	15.50	46.00	67.00
304.00	10.30	1.90	6.50	6.70	15.80	45.00	68.00
299.00	10.00	1.70	8.30	7.10	16.30	49.00	69.00
298.00	.	1.80	8.70	6.50	15.60	46.00	71.00
296.00	.	1.60	7.20	6.70	15.10	45.00	68.00
308.00	.	1.40	5.70	7.00	15.80	48.00	68.00
310.00	.	1.90	11.50	6.60	15.80	47.00	71.00
304.00	.	1.80	10.60	6.10	15.30	46.00	74.00
303.00	.	1.70	9.10	7.60	18.80	55.00	72.00
302.00	10.50	1.50	10.20	7.30	16.60	48.00	66.00
297.00	10.20	1.60	9.30	7.20	17.20	50.00	69.00
297.00	10.00	1.80	10.10	6.60	14.90	46.00	69.00
299.00	9.60	1.40	10.80	6.10	14.20	42.00	70.00
295.00	10.50	1.70	7.70	6.30	13.80	43.00	69.00
297.00		1.70	6.40	6.10	14.40	42.00	69.00
296.00	10.10	1.60	5.70	8.40	18.20	56.00	67.00
295.00	9.30	1.50	6.00	7.10	15.70	48.00	68.00
300.00	10.40	1.60	6.50	6.10	14.90	44.00	72.00
301.00	9.70	1.70	9.10	8.30	14.90	48.00	58.00
294.00	10.00	1.60	7.30	7.30	15.70	49.00	66.00
298.00	10.30	2.00	5.90	7.80	17.10	54.00	69.00
304.00	9.80	1.60	9.60	7.00	15.10	47.00	67.00
300.25	9.96	1.68	7.99	6.93	15.77	47.42	68.58
4.23	0.39	0.16	1.85	0.64	1.17	3.60	3.01
299.54	9.94	.	7.18	6.69	15.69	49.54	74.25
5.56	0.49	.	2.12	0.74	1.81	5.10	3.08
OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
296.00	9.40	1.70	15.80	6.00	13.80	41.00	68.00
295.00	9.20	1.40	16.70	6.90	16.80	51.00	74.00
307.00	11.40	1.60	10.00	7.30	17.50	50.00	69.00
298.00	10.10	1.20	11.20	7.30	17.20	50.00	68.00
302.00	9.60	1.30	8.00	6.80	16.40	47.00	70.00
301.00	.	1.70	7.10	7.60	17.90	51.00	67.00
298.00	9.90	1.60	14.70	5.70	14.40	41.00	72.00
293.00	10.90	1.30	12.70	6.60	15.40	46.00	69.00
303.00	9.80	1.80	7.30	6.70	15.70	47.00	70.00
304.00	10.00	1.60	11.70	6.20	14.80	44.00	71.00
297.00	10.00	1.40	26.20	5.50	11.70	36.00	66.00
300.00	.	1.30	7.70	6.60	15.20	45.00	67.00
.	.	.	.	.	.	.	.
293.00	10.40	1.40	8.90	5.70	11.90	38.00	67.00
291.00	9.60	1.50	8.00	6.50	14.10	44.00	68.00
295.00	10.90	1.60	11.40	6.70	13.80	43.00	64.00
302.00	10.40	1.50	7.90	6.70	15.20	47.00	70.00
297.00	9.70	1.60	7.70	6.30	14.00	43.00	68.00
295.00	10.60	1.80	4.90	6.30	13.90	43.00	69.00
295.00	9.30	1.80	5.60	8.00	17.30	54.00	68.00
297.00	9.70	1.60	7.60	7.40	17.10	52.00	70.00
292.00	9.50	1.50	7.00	6.30	14.10	44.00	70.00
293.00	9.60	1.50	8.60	7.60	16.70	51.00	68.00
296.00	9.50	1.40	7.60	6.10	12.90	40.00	65.00
297.39	9.98	1.53	10.19	6.64	15.12	45.57	68.61
4.19	0.59	0.17	4.69	0.66	1.78	4.75	2.21
296.67	10.02	.	8.19	6.99	16.09	51.50	73.25
5.03	0.32	.	3.39	0.49	1.60	4.58	5.13

Appendix B. Blood Biochemistry Parameters for Individual Animals

OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
301.00	10.80	1.40	11.10	6.60	16.10	46.00	70.00
300.00	10.30	1.50	11.10	6.50	15.10	44.00	67.00
303.00	10.50	1.50	9.60	6.40	15.40	44.00	69.00
298.00	.	1.70	8.80	5.50	12.20	36.00	66.00
301.00	10.30	1.60	6.90	5.50	13.30	38.00	69.00
307.00	10.30	1.50	5.90	8.50	19.10	56.00	66.00
304.00	10.10	1.70	6.10	6.70	15.30	45.00	67.00
299.00	11.00	1.40	7.10	5.30	13.40	39.00	73.00
305.00	10.00	1.80	7.70	8.70	15.30	48.00	56.00
297.00	10.20	1.60	8.40	7.60	16.80	50.00	65.00
297.00	10.10	1.80	6.30	6.70	15.50	45.00	67.00
308.00	9.80	1.50	9.80	7.40	16.60	48.00	65.00
311.00	10.50	1.70	8.40	6.90	14.90	46.00	66.00
302.00	10.80	1.50	6.70	6.20	14.40	44.00	71.00
299.00	10.40	1.70	8.90	7.20	16.30	49.00	67.00
304.00	11.20	1.60	6.50	7.20	15.60	48.00	67.00
296.00	10.70	1.30	8.20	6.20	11.90	36.00	59.00
297.00	10.50	1.50	7.30	5.60	12.90	39.00	70.00
300.00	10.50	1.60	7.20	6.00	13.20	40.00	66.00
306.00		1.60	5.80	6.80	15.40	47.00	69.00
318.00		1.70	7.80	6.20	14.90	44.00	70.00
304.00	9.70	1.70	8.50	6.00	14.70	44.00	73.00
302.00		1.60	9.60	6.50	15.80	47.00	73.00
305.00	10.50	1.60	5.60	7.10	15.90	47.00	66.00
302.67	10.41	1.59	7.89	6.64	15.00	44.58	67.38
5.06	0.38	0.13	1.59	0.86	1.60	4.72	3.94
299.54	9.94	.	7.18	6.69	15.69	49.54	74.25
5.56	0.49	.	2.12	0.74	1.81	5.10	3.08
OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
.	.	.	.	.	.	.	.
305.00	9.90	1.10	9.60	6.50	13.30	42.00	65.00
305.00	10.70	1.50	10.00	6.40	14.00	43.00	66.00
.	.	.	.	.	.	.	.
300.00	10.10	1.60	9.50	6.80	16.00	47.00	69.00
300.00	10.20	1.70	7.50	5.80	12.80	38.00	66.00
301.00	10.40	1.80	7.60	6.20	14.40	42.00	69.00
305.00	10.30	2.00	6.70	8.10	18.30	54.00	67.00
301.00	10.20	1.60	3.70	7.10	16.60	49.00	69.00
300.00	9.90	2.00	9.50	5.40	12.60	38.00	72.00
298.00	10.70	1.90	10.60	7.30	16.90	50.00	69.00
301.00	.	1.60	10.20	6.10	13.50	40.00	65.00
306.00	10.70	1.90	7.00	7.10	16.10	49.00	68.00
313.00	.	1.60	8.80	7.20	16.70	49.00	69.00
310.00	.	1.70	7.60	7.10	16.50	50.00	70.00
310.00	10.40	1.70	6.40	6.70	15.20	45.00	67.00
308.00	10.60	1.80	12.20	6.50	15.40	46.00	71.00
316.00	10.70	1.70	10.20	6.40	14.80	45.00	70.00
307.00	11.40	1.90	6.50	6.20	14.70	44.00	70.00
308.00	10.50	1.60	5.60	6.10	12.90	39.00	64.00
307.00		1.70	7.10	6.50	14.80	44.00	69.00
305.32	10.45	1.71	8.23	6.61	15.03	44.95	68.16
4.90	0.38	0.21	2.08	0.62	1.60	4.52	2.19
296.67	10.02	.	8.19	6.99	16.09	51.50	73.25
5.03	0.32	.	3.39	0.49	1.60	4.58	5.13

Appendix B. Blood Biochemistry Parameters for Individual Animals

OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
301	10.6	1.9	8.9	7.4	17.9	51	69
300	10	2	12.8	5.9	14	40	67
301	10.4	1.6	11	5.9	14.4	42	71
306	9.6	1.7	6.6	5.1	12.5	35	69
309	10.2	1.5	5.8	8	17.9	52	65
301	10.1	1.6	5.1	6.7	15.4	44	66
300	10	1.3	6.1	5.6	13.9	39	70
302	10	1.9	6.7	8.2	15	46	56
303	10.2	1.8	10.6	6.7	15.1	43	65
300	10.5	1.8	5.6	6.2	14.6	42	68
301	10.3	1.6	10.1	6.3	14.3	42	66
302.18	10.17	1.70	8.12	6.55	15.00	43.27	66.55
2.86	0.28	0.20	2.65	0.98	1.63	4.96	4.03
299.54	9.94	.	7.18	6.69	15.69	49.54	74.25
5.56	0.49	.	2.12	0.74	1.81	5.10	3.08
OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM	WBC	RBC	HGB	PCV	MCV
303	10.8	1.5	12.4	6	14.3	42	70
299	9.6	1.3	7.5	5.3	11.9	35	65
298	10.2	1.6	8.4	5.6	13.4	38	68
294	10.4	1.8	8.3	5.7	13	37	65
305	9.4	1.7	6.8	5.4	12.4	36	67
297	10.1	1.7	7.1	6.2	15.4	43	70
305	10.2	1.6	11.1	5.9	14.4	43	72
298	10	1.9	13.3	5.5	13	37	68
299	10	1.6	7.7	5.9	13	39	66
299.78	10.08	1.63	9.18	5.72	13.42	38.89	67.89
3.77	0.41	0.17	2.43	0.30	1.09	3.06	2.42
296.67	10.02	.	8.19	6.99	16.09	51.50	73.25
5.03	0.32	.	3.39	0.49	1.60	4.58	5.13

Appendix B. Blood Biochemistry Parameters for Individual Animals

MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
23.20	31.00	66.00	3762.00	25.00	1425.00	4.00	228.00
24.40	33.00	68.00	3536.00	22.00	1144.00	5.00	260.00
23.60	31.00	59.00	4425.00	30.00	2250.00	3.00	225.00
24.30	33.00	63.00	3528.00	22.00	1232.00	5.00	280.00
24.20	32.00	75.00	4650.00	14.00	868.00	3.00	186.00
25.70	32.00	75.00	8775.00	16.00	1872.00	3.00	351.00
23.50	32.00	52.00	4004.00	32.00	2464.00	3.00	231.00
22.70	32.00	63.00	4347.00	25.00	1725.00	2.00	138.00
22.70	32.00	51.00	2856.00	34.00	1904.00	7.00	392.00
23.40	31.00	62.00	4774.00	27.00	2079.00	3.00	231.00
23.40	30.00	77.00	11396.00	16.00	2368.00	3.00	444.00
23.40	31.00	59.00	4484.00	29.00	2204.00	3.00	228.00
23.20	31.00	68.00	4828.00	20.00	1420.00	6.00	426.00
22.70	31.00	63.00	3654.00	28.00	1624.00	2.00	116.00
23.30	33.00	63.00	4032.00	30.00	1920.00	2.00	128.00
23.80	33.00	57.00	3534.00	29.00	1798.00	6.00	372.00
22.70	32.00	61.00	4514.00	29.00	2146.00	5.00	370.00
25.20	32.00	68.00	4692.00	23.00	1587.00	4.00	276.00
25.00	32.00	61.00	3721.00	28.00	1708.00	4.00	244.00
20.70	30.00	58.00	3886.00	33.00	2211.00	3.00	201.00
22.10	31.00	54.00	3348.00	37.00	2294.00	4.00	248.00
23.50	33.00	68.00	5236.00	22.00	1694.00	5.00	385.00
23.60	31.00	59.00	5015.00	24.00	2040.00	5.00	425.00
23.30	32.00	51.00	2652.00	36.00	1872.00	5.00	260.00
23.48	31.71	62.54	4568.71	26.29	1827.04	3.96	276.88
1.04	0.91	7.21	1862.17	6.18	407.73	1.37	96.86
MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
22.70	30.00	59.00	3717.00	25.00	1575.00	3.00	189.00
23.70	30.00	75.00	6075.00	21.00	1701.00	3.00	243.00
23.80	31.00	71.00	4899.00	22.00	1518.00	4.00	276.00
20.90	30.00	67.00	4891.00	23.00	1679.00	3.00	219.00
24.60	32.00	62.00	5642.00	27.00	2457.00	3.00	273.00
23.70	30.00	67.00	4958.00	24.00	1776.00	3.00	222.00
23.00	32.00	61.00	7137.00	29.00	3393.00	3.00	351.00
21.60	32.00	60.00	3780.00	31.00	1953.00	5.00	315.00
23.30	31.00	66.00	4356.00	24.00	1584.00	4.00	264.00
22.20	32.00	57.00	6213.00	36.00	3924.00	2.00	218.00
23.20	31.00	56.00	3696.00	35.00	2310.00	5.00	330.00
22.20	30.00	68.00	4556.00	24.00	1608.00	2.00	134.00
24.70	33.00	61.00	4636.00	22.00	1672.00	4.00	304.00
23.60	33.00	64.00	4672.00	22.00	1606.00	6.00	438.00
24.80	34.00	69.00	5796.00	22.00	1848.00	5.00	420.00
23.90	31.00	65.00	5135.00	28.00	2212.00	4.00	316.00
23.20	31.00	64.00	4864.00	24.00	1824.00	4.00	304.00
22.00	30.00	66.00	3432.00	22.00	1144.00	5.00	260.00
23.90	32.00	59.00	3540.00	34.00	2040.00	3.00	180.00
23.50	32.00	58.00	3712.00	26.00	1664.00	3.00	192.00
24.10	31.00	55.00	4070.00	34.00	2516.00	6.00	444.00
22.40	31.00	56.00	5992.00	33.00	3531.00	5.00	535.00
19.00	30.00	85.00	18870.00	9.00	1998.00	5.00	1110.00
22.20	31.00	77.00	4543.00	17.00	1003.00	2.00	118.00
23.01	31.25	64.50	5382.58	25.58	2022.33	3.83	318.96
1.31	1.11	7.30	3029.98	6.23	714.23	1.20	196.36

Appendix B. Blood Biochemistry Parameters for Individual Animals

MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
22.50	33.00	78.00	3744.00	16.00	768.00	6.00	288.00
23.70	33.00	80.00	5360.00	11.00	737.00	9.00	603.00
24.00	33.00	75.00	5700.00	19.00	1444.00	6.00	456.00
22.90	33.00	80.00	4640.00	12.00	696.00	7.00	406.00
23.90	33.00	88.00	6688.00	8.00	608.00	4.00	304.00
26.20	34.00	82.00	8528.00	8.00	832.00	6.00	624.00
21.80	33.00	72.00	4392.00	20.00	1220.00	7.00	427.00
22.00	33.00	73.00	5256.00	22.00	1584.00	5.00	360.00
21.90	32.00	63.00	3717.00	27.00	1593.00	10.00	590.00
23.40	33.00	75.00	8775.00	15.00	1755.00	9.00	1053.00
22.20	33.00	71.00	6816.00	18.00	1728.00	6.00	576.00
23.20	33.00	79.00	5214.00	15.00	990.00	6.00	396.00
23.10	33.00	81.00	6399.00	11.00	869.00	8.00	632.00
22.60	33.00	79.00	4898.00	16.00	992.00	5.00	310.00
22.50	33.00	73.00	4234.00	20.00	1160.00	7.00	406.00
23.20	33.00	69.00	5934.00	21.00	1806.00	9.00	774.00
22.30	34.00	65.00	5005.00	25.00	1925.00	9.00	693.00
25.20	34.00	72.00	5184.00	16.00	1152.00	7.00	504.00
24.40	33.00	74.00	4662.00	17.00	1071.00	8.00	504.00
19.50	32.00	74.00	3404.00	20.00	920.00	6.00	276.00
22.50	32.00	70.00	5810.00	17.00	1411.00	12.00	996.00
21.60	32.00	75.00	3975.00	18.00	954.00	7.00	371.00
22.60	32.00	76.00	7828.00	15.00	1545.00	9.00	927.00
23.00	33.00	70.00	3290.00	21.00	987.00	6.00	282.00
22.93	32.92	74.75	5393.88	17.00	1197.79	7.25	531.58
1.31	0.58	5.59	1508.32	4.79	392.74	1.85	225.45
23.48	31.71	62.54	4568.71	26.29	1827.04	3.96	276.88
1.04	0.91	7.21	1862.17	6.18	407.73	1.37	96.86
MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
22.80	33.00	74.00	5402.00	18.00	1314.00	8.00	584.00
24.20	34.00	61.00	5368.00	36.00	3168.00	2.00	176.00
23.30	34.00	76.00	4560.00	16.00	960.00	8.00	480.00
23.60	34.00	79.00	7584.00	12.00	1152.00	8.00	768.00
23.70	34.00	70.00	5390.00	22.00	1694.00	8.00	616.00
23.60	33.00	69.00	6486.00	25.00	2350.00	6.00	564.00
24.50	34.00	66.00	5676.00	28.00	2408.00	6.00	516.00
23.80	34.00	84.00	8316.00	11.00	1089.00	5.00	495.00
23.70	33.00	71.00	4260.00	22.00	1320.00	7.00	420.00
24.40	34.00	77.00	7777.00	16.00	1616.00	7.00	707.00
21.00	33.00	77.00	6776.00	13.00	1144.00	9.00	792.00
23.00	34.00	71.00	3905.00	23.00	1265.00	6.00	330.00
23.00	33.00	77.00	4620.00	16.00	960.00	5.00	300.00
22.90	33.00	69.00	7176.00	22.00	2288.00	8.00	832.00
22.40	33.00	69.00	5313.00	20.00	1540.00	11.00	847.00
21.90	33.00	74.00	4884.00	17.00	1122.00	9.00	594.00
23.20	33.00	77.00	8624.00	17.00	1904.00	6.00	672.00
22.40	33.00	74.00	4884.00	17.00	1122.00	9.00	594.00
21.60	32.00	71.00	7171.00	21.00	2121.00	8.00	808.00
21.60	32.00	67.00	6432.00	20.00	1920.00	12.00	1152.00
23.80	33.00	73.00	5256.00	18.00	1296.00	8.00	576.00
20.70	33.00	73.00	7738.00	12.00	1272.00	7.00	742.00
.	.	.	.	.	.	.	.
22.00	32.00	71.00	4615.00	21.00	1365.00	6.00	390.00
22.92	33.22	72.61	6009.26	19.26	1582.17	7.35	606.74
1.06	0.67	4.92	1394.43	5.63	567.79	2.08	215.09
23.01	31.25	64.50	5382.58	25.58	2022.33	3.83	318.96
1.31	1.11	7.30	3029.98	6.23	714.23	1.20	196.36

Appendix B. Blood Biochemistry Parameters for Individual Animals

MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
24.20	33.00	78.00	5928.00	15.00	1140.00	7.00	532.00
18.40	32.00	69.00	3381.00	25.00	1225.00	6.00	294.00
22.10	34.00	73.00	6789.00	17.00	1581.00	8.00	744.00
23.00	34.00	77.00	6237.00	16.00	1296.00	7.00	567.00
22.60	33.00	74.00	4884.00	21.00	1386.00	5.00	330.00
23.30	34.00	84.00	7140.00	14.00	1190.00	2.00	170.00
22.40	34.00	75.00	4950.00	17.00	1122.00	6.00	396.00
22.90	34.00	68.00	5372.00	22.00	1738.00	8.00	632.00
23.80	34.00	57.00	6213.00	32.00	3488.00	9.00	981.00
23.20	34.00	72.00	4032.00	18.00	1008.00	9.00	504.00
22.90	33.00	63.00	4221.00	30.00	2010.00	7.00	469.00
23.90	34.00	79.00	7663.00	12.00	1164.00	6.00	582.00
21.90	32.00	79.00	3792.00	15.00	720.00	6.00	288.00
22.60	32.00	82.00	3608.00	11.00	484.00	7.00	308.00
22.00	32.00	85.00	3740.00	12.00	528.00	2.00	88.00
22.10	33.00	70.00	3500.00	21.00	1050.00	8.00	400.00
22.40	33.00	74.00	4884.00	17.00	1122.00	9.00	594.00
23.20	33.00	76.00	4560.00	17.00	1020.00	6.00	360.00
21.80	33.00	70.00	3290.00	20.00	940.00	9.00	423.00
22.10	32.00	76.00	2812.00	16.00	592.00	8.00	296.00
23.20	33.00	86.00	6966.00	7.00	567.00	4.00	324.00
24.10	33.00	76.00	6080.00	16.00	1280.00	6.00	480.00
25.30	34.00	85.00	7140.00	8.00	672.00	7.00	588.00
23.20	35.00	77.00	5005.00	14.00	910.00	5.00	325.00
21.90	32.00	79.00	3792.00	15.00	720.00	6.00	288.00
22.74	33.20	75.36	5039.16	17.12	1158.12	6.52	438.52
1.25	0.87	6.87	1432.35	5.86	614.66	1.92	190.43
23.48	31.71	62.54	4568.71	26.29	1827.04	3.96	276.88
1.04	0.91	7.21	1862.17	6.18	407.73	1.37	96.86
MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
24.30	34.00	71.00	3834.00	19.00	1026.00	8.00	432.00
23.30	35.00	70.00	6580.00	24.00	2256.00	6.00	564.00
22.20	33.00	67.00	4623.00	22.00	1518.00	7.00	483.00
21.90	33.00	78.00	5304.00	16.00	1088.00	6.00	408.00
23.90	33.00	74.00	6364.00	18.00	1548.00	7.00	602.00
23.50	34.00	91.00	12831.00	7.00	987.00	2.00	282.00
22.40	33.00	68.00	5372.00	23.00	1817.00	8.00	632.00
22.10	33.00	67.00	5695.00	24.00	2040.00	7.00	595.00
23.50	34.00	74.00	6290.00	17.00	1445.00	9.00	795.00
20.90	33.00	72.00	4176.00	21.00	1218.00	7.00	406.00
.	.	.	.	.	.	.	.
22.60	33.00	42.00	3612.00	23.00	1978.00	8.00	688.00
22.60	33.00	76.00	5092.00	14.00	938.00	5.00	335.00
23.60	33.00	88.00	7568.00	6.00	516.00	6.00	516.00
22.90	34.00	74.00	4588.00	17.00	1054.00	6.00	372.00
22.90	34.00	72.00	5904.00	17.00	1394.00	7.00	574.00
20.80	30.00	77.00	4774.00	15.00	930.00	8.00	496.00
23.40	34.00	76.00	4636.00	19.00	1159.00	5.00	305.00
23.80	33.00	79.00	7979.00	15.00	1515.00	6.00	606.00
23.50	34.00	82.00	8856.00	12.00	1296.00	3.00	324.00
22.90	33.00	81.00	6480.00	12.00	960.00	7.00	560.00
23.20	33.00	71.00	5254.00	21.00	1554.00	6.00	444.00
22.20	33.00	82.00	6724.00	10.00	820.00	6.00	492.00
22.78	33.45	70.36	5880.09	19.45	1538.27	6.82	535.18
0.91	0.94	9.56	2021.61	5.23	434.38	1.62	132.57
23.01	31.25	64.50	5382.58	25.58	2022.33	3.83	318.96
1.31	1.11	7.30	3029.98	6.23	714.23	1.20	196.36

Appendix B. Blood Biochemistry Parameters for Individual Animals

MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
22.80	34.00	72.00	4320.00	20.00	1200.00	8.00	480.00
23.90	34.00	81.00	4941.00	11.00	671.00	8.00	488.00
22.50	33.00	71.00	5254.00	22.00	1628.00	7.00	518.00
23.60	35.00	80.00	5200.00	12.00	780.00	8.00	520.00
23.00	33.00	69.00	5727.00	22.00	1826.00	9.00	747.00
24.00	34.00	73.00	6351.00	20.00	1740.00	7.00	609.00
22.70	34.00	64.00	4608.00	28.00	2016.00	8.00	576.00
22.70	33.00	74.00	4218.00	18.00	1026.00	8.00	456.00
24.10	34.00	86.00	9890.00	6.00	690.00	7.00	805.00
25.00	34.00	80.00	8480.00	11.00	1166.00	9.00	954.00
24.80	34.00	80.00	7280.00	14.00	1274.00	6.00	546.00
22.90	34.00	84.00	8568.00	7.00	714.00	8.00	816.00
23.80	35.00	63.00	5859.00	19.00	1767.00	2.00	186.00
22.50	32.00	85.00	8585.00	10.00	1010.00	5.00	505.00
23.50	34.00	79.00	8532.00	14.00	1512.00	7.00	756.00
22.00	32.00	81.00	6237.00	15.00	1155.00	4.00	308.00
23.60	34.00	64.00	4096.00	19.00	1216.00	10.00	640.00
21.80	33.00	69.00	3933.00	18.00	1026.00	12.00	684.00
22.10	33.00	72.00	4320.00	19.00	1140.00	9.00	540.00
24.40	34.00	73.00	4745.00	15.00	975.00	7.00	455.00
18.00	31.00	80.00	7280.00	15.00	1365.00	5.00	455.00
21.30	32.00	73.00	5329.00	21.00	1533.00	6.00	438.00
21.80	32.00	76.00	4484.00	17.00	1003.00	6.00	354.00
21.60	32.00	70.00	6720.00	17.00	1632.00	12.00	1152.00
22.85	33.33	74.96	6039.88	16.25	1252.71	7.42	582.83
1.45	1.05	6.66	1748.27	5.12	382.24	2.26	211.49
23.48	31.71	62.54	4568.71	26.29	1827.04	3.96	276.88
1.04	0.91	7.21	1862.17	6.18	407.73	1.37	96.86
MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
23.00	34.00	96.00	15168.00	3.00	474.00	1.00	158.00
24.50	33.00	86.00	14362.00	11.00	1837.00	2.00	334.00
24.00	35.00	72.00	7200.00	16.00	1600.00	11.00	1100.00
23.50	35.00	79.00	8848.00	13.00	1456.00	8.00	896.00
24.10	35.00	74.00	5920.00	15.00	1200.00	11.00	880.00
23.60	35.00	72.00	5112.00	21.00	1491.00	7.00	497.00
25.50	36.00	79.00	11613.00	13.00	1911.00	8.00	1176.00
23.20	34.00	84.00	10668.00	8.00	1016.00	8.00	1016.00
23.60	34.00	74.00	5402.00	20.00	1460.00	6.00	438.00
24.00	34.00	76.00	8892.00	16.00	1872.00	8.00	936.00
21.40	33.00	90.00	23580.00	7.00	1834.00	2.00	524.00
22.80	34.00	72.00	5544.00	18.00	1386.00	6.00	462.00
.	.	.	.	.	.	.	.
20.80	31.00	69.00	6141.00	23.00	2047.00	6.00	534.00
21.70	32.00	66.00	5280.00	24.00	1920.00	9.00	720.00
20.60	32.00	79.00	9006.00	6.00	684.00	10.00	1140.00
22.90	33.00	73.00	5767.00	17.00	1343.00	10.00	790.00
22.40	33.00	75.00	5775.00	15.00	115.00	9.00	693.00
22.30	32.00	72.00	3528.00	21.00	1029.00	5.00	245.00
21.70	32.00	66.00	3696.00	23.00	1288.00	11.00	616.00
23.10	33.00	83.00	6308.00	15.00	1140.00	2.00	152.00
22.50	32.00	71.00	4970.00	25.00	1750.00	4.00	280.00
22.00	33.00	72.00	6192.00	25.00	2150.00	3.00	258.00
21.10	32.00	78.00	5928.00	15.00	1140.00	7.00	532.00
22.80	33.35	76.43	8039.13	16.09	1397.52	6.70	625.09
1.24	1.30	7.41	4583.88	6.24	513.00	3.15	320.02
23.01	31.25	64.50	5382.58	25.58	2022.33	3.83	318.96
1.31	1.11	7.30	3029.98	6.23	714.23	1.20	196.36

Appendix B. Blood Biochemistry Parameters for Individual Animals

MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
24.20	35.00	78.00	8658.00	13.00	143.00	9.00	999.00
23.20	35.00	70.00	7770.00	12.00	1332.00	9.00	999.00
24.00	35.00	61.00	5856.00	27.00	2592.00	9.00	864.00
22.20	34.00	85.00	7480.00	1.00	88.00	14.00	1232.00
24.00	35.00	70.00	4830.00	17.00	1173.00	13.00	897.00
22.60	34.00	82.00	4838.00	6.00	354.00	12.00	708.00
22.80	34.00	81.00	4941.00	11.00	671.00	8.00	488.00
25.10	34.00	77.00	5467.00	16.00	1136.00	7.00	497.00
17.60	32.00	82.00	6314.00	14.00	1078.00	4.00	308.00
22.00	34.00	86.00	7224.00	8.00	672.00	6.00	504.00
23.10	34.00	77.00	4851.00	17.00	1071.00	6.00	378.00
22.40	35.00	78.00	7644.00	13.00	1274.00	9.00	882.00
21.60	33.00	81.00	6804.00	9.00	756.00	9.00	756.00
23.20	33.00	82.00	5494.00	8.00	536.00	10.00	670.00
22.50	33.00	71.00	6319.00	15.00	1335.00	6.00	534.00
21.90	33.00	75.00	4875.00	15.00	975.00	9.00	585.00
19.30	33.00	71.00	5822.00	15.00	1230.00	14.00	1148.00
23.20	33.00	76.00	5548.00	14.00	1022.00	10.00	730.00
21.90	33.00	67.00	4824.00	24.00	1728.00	9.00	648.00
22.60	33.00	69.00	4002.00	14.00	812.00	12.00	696.00
24.00	34.00	84.00	6552.00	10.00	780.00	4.00	312.00
24.40	34.00	73.00	6205.00	19.00	1615.00	8.00	680.00
24.50	34.00	85.00	8160.00	9.00	864.00	6.00	576.00
22.40	34.00	75.00	4200.00	16.00	896.00	9.00	504.00
22.70	33.79	76.50	6028.25	13.46	1005.54	8.83	691.46
1.63	0.83	6.51	1289.88	5.52	529.16	2.78	246.26
23.48	31.71	62.54	4568.71	26.29	1827.04	3.96	276.88
1.04	0.91	7.21	1862.17	6.18	407.73	1.37	96.86
MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
.	.	.	.	.	.	.	.
20.40	32.00	63.00	6048.00	23.00	2208.00	8.00	768.00
21.70	33.00	65.00	6500.00	14.00	1400.00	9.00	900.00
.	.	.	.	.	.	.	.
23.40	34.00	74.00	7030.00	19.00	1805.00	7.00	665.00
22.10	34.00	76.00	5700.00	15.00	1125.00	7.00	525.00
23.40	34.00	72.00	5472.00	14.00	1064.00	13.00	988.00
22.50	34.00	65.00	4355.00	22.00	1474.00	13.00	871.00
23.50	34.00	79.00	2923.00	17.00	629.00	3.00	111.00
23.50	33.00	74.00	7030.00	18.00	1710.00	7.00	665.00
23.20	34.00	70.00	7420.00	23.00	2438.00	7.00	742.00
22.10	34.00	77.00	7854.00	13.00	1326.00	10.00	1020.00
22.50	33.00	69.00	4830.00	17.00	1190.00	10.00	700.00
23.20	34.00	77.00	6776.00	11.00	968.00	6.00	528.00
23.10	33.00	74.00	5624.00	19.00	1444.00	6.00	456.00
22.70	34.00	74.00	4736.00	17.00	1088.00	9.00	576.00
23.70	34.00	78.00	9516.00	13.00	1586.00	9.00	1098.00
23.10	33.00	79.00	8058.00	12.00	1224.00	7.00	714.00
23.60	34.00	76.00	4940.00	16.00	1040.00	8.00	520.00
21.10	33.00	76.00	4256.00	16.00	896.00	6.00	336.00
22.90	33.00	70.00	4970.00	19.00	1349.00	8.00	568.00
22.72	33.53	73.05	6002.00	16.74	1366.53	8.05	671.11
0.90	0.61	4.85	1596.95	3.54	443.77	2.39	243.87
23.01	31.25	64.50	5382.58	25.58	2022.33	3.83	318.96
1.31	1.11	7.30	3029.98	6.23	714.23	1.20	196.36

Appendix B. Blood Biochemistry Parameters for Individual Animals

MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
24.1	35	7031		1068		801	
23.5	35	9984		1408		1280	
24.2	34	8360		1540		990	
24.4	35	5016		990		396	
22.5	35	4350		986		348	
22.9	35	3672		969		408	
24.8	35	4758		854		366	
18.3	33	5427		938		335	
22.6	35	8268		1696		530	
23.5	34	4480		672		448	
22.5	34	7272		1616		1010	
23.03	34.55	6238.00	.	1157.91	.	628.36	.
1.76	0.69	2047.81	.	344.42	.	333.03	.
23.48	31.71	62.54	4568.71	26.29	1827.04	3.96	276.88
1.04	0.91	7.21	1862.17	6.18	407.73	1.37	96.86
MCH	MCHC	NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE
23.7	34	10664		1488		248	
22.4	34	5100		1650		675	
23.9	35	6300		1260		840	
22.9	35	6474		1162		498	
23.3	35	5440		884		340	
24.8	36	5964		710		426	
24.3	34	9435		1221		444	
23.8	35	10108		1995		1197	
22.3	34	5929		847		770	
23.49	34.67	7268.22	.	1246.33	.	604.22	.
0.84	0.71	2162.09	.	413.55	.	296.84	.
23.01	31.25	64.50	5382.58	25.58	2022.33	3.83	318.96
1.31	1.11	7.30	3029.98	6.23	714.23	1.20	196.36

Appendix B. Blood Biochemistry Parameters for Individual Animals

EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
5.00	285.00	0.00	0.00	80.00	1.68	1.69	0.80
5.00	260.00	0.00	0.00	113.00	1.90	1.71	0.80
8.00	600.00	0.00	0.00	109.00	1.91	1.28	1.20
10.00	560.00	0.00	0.00	69.00	1.08	0.92	1.10
8.00	496.00	0.00	0.00	94.00	1.97	1.33	0.80
6.00	702.00	0.00	0.00	113.00	1.67	1.82	1.80
13.00	1001.00	0.00	0.00	99.00	1.98	1.77	1.00
10.00	690.00	0.00	0.00	86.00	1.83	1.47	0.60
8.00	448.00	0.00	0.00	117.00	1.32	1.20	0.70
8.00	616.00	0.00	0.00	88.00	1.18	1.42	0.90
4.00	592.00	0.00	0.00	123.00	2.61	1.56	1.20
9.00	684.00	0.00	0.00	101.00	1.40	1.17	0.90
6.00	426.00	0.00	0.00	91.00	1.97	1.08	0.90
7.00	406.00	0.00	0.00	77.00	1.45	1.50	1.00
5.00	320.00	0.00	0.00	97.00	1.68	1.27	0.80
8.00	496.00	0.00	0.00	60.00	1.78	1.15	0.80
5.00	370.00	0.00	0.00	129.00	2.21	1.02	1.20
5.00	345.00	0.00	0.00	92.00	1.41	1.64	0.80
7.00	427.00	0.00	0.00	62.00	1.55	1.19	0.60
6.00	402.00	0.00	0.00	47.00	1.62	1.06	0.60
5.00	310.00	0.00	0.00	100.00	1.42	1.48	0.70
5.00	385.00	0.00	0.00	95.00	1.89	1.94	0.70
12.00	1020.00	0.00	0.00	92.00	1.15	1.26	2.00
8.00	416.00	0.00	0.00	108.00	2.26	1.23	1.10
7.21	510.71	0.00	0.00	93.42	1.71	1.38	0.96
2.36	201.13	0.00	0.00	20.29	0.37	0.28	0.35
EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
13.00	819.00	0.00	0.00	75.00	1.99	1.60	0.80
1.00	81.00	0.00	0.00	95.00	1.11	1.12	.
3.00	207.00	0.00	0.00	121.00	1.96	1.35	.
7.00	511.00	0.00	0.00	87.00	1.92	1.14	1.10
8.00	728.00	0.00	0.00	84.00	1.50	0.79	1.10
6.00	444.00	0.00	0.00	110.00	2.08	2.11	1.10
7.00	819.00	0.00	0.00	57.00	1.09	0.89	0.70
4.00	252.00	0.00	0.00	66.00	1.44	1.78	0.60
6.00	396.00	0.00	0.00	139.00	1.66	1.65	1.30
5.00	545.00	0.00	0.00	78.00	1.55	1.08	1.20
4.00	264.00	0.00	0.00	108.00	1.06	0.96	1.30
6.00	402.00	0.00	0.00	73.00	2.55	1.71	1.00
13.00	988.00	0.00	0.00	70.00	1.41	1.33	0.80
8.00	584.00	0.00	0.00	104.00	1.43	1.15	0.80
4.00	336.00	0.00	0.00	79.00	1.09	1.01	0.80
3.00	237.00	0.00	0.00	92.00	1.30	1.14	1.00
8.00	608.00	0.00	0.00	115.00	0.79	0.72	1.20
7.00	364.00	0.00	0.00	105.00	1.45	1.23	1.30
4.00	240.00	0.00	0.00	65.00	2.62	1.76	0.70
13.00	832.00	0.00	0.00	83.00	1.49	0.98	0.60
5.00	370.00	0.00	0.00	84.00	1.08	0.87	0.80
6.00	642.00	0.00	0.00	128.00	1.20	1.19	1.20
1.00	222.00	0.00	0.00	119.00	1.50	0.85	1.20
4.00	236.00	0.00	0.00	109.00	1.90	1.09	1.10
6.08	463.63	0.00	0.00	93.58	1.55	1.23	0.99
3.31	243.29	0.00	0.00	21.97	0.46	0.36	0.24

Appendix B. Blood Biochemistry Parameters for Individual Animals

EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	.	74.00	1.25	0.89	1.00
.	.	.	.	116.00	0.77	1.14	1.20
.	.	.	.	94.00	1.38	1.04	1.00
.	.	1.00	58.00	79.00	1.26	0.80	0.80
.	.	.	.	82.00	0.87	1.20	1.00
4.00	416.00	.	.	116.00	1.99	1.37	1.80
.	.	1.00	61.00	130.00	1.42	0.84	1.60
.	.	.	.	108.00	1.23	1.60	0.80
.	.	.	.	105.00	0.72	0.94	1.00
.	.	1.00	117.00	102.00	1.23	0.75	0.90
5.00	480.00	.	.	108.00	1.39	1.01	1.00
.	.	.	.	140.00	1.82	1.58	0.90
.	.	.	.	83.00	1.13	1.05	1.20
.	.	.	0.00	73.00	1.21	0.83	2.20
.	.	.	.	92.00	1.29	0.99	0.90
1.00	86.00	.	.	93.00	2.02	1.27	0.90
1.00	77.00	.	.	112.00	1.49	1.24	1.10
5.00	360.00	.	.	64.00	0.99	0.89	1.00
.	.	1.00	63.00	104.00	1.18	1.23	0.80
.	.	.	.	94.00	1.35	1.09	1.00
.	.	1.00	83.00	100.00	0.99	1.24	1.10
.	.	.	.	134.00	1.38	1.71	0.90
.	.	.	.	139.00	0.70	0.70	2.00
2.00	94.00	1.00	47.00	113.00	0.98	1.19	1.00
3.00	252.17	1.00	61.29	102.29	1.25	1.11	1.13
1.90	186.38	0.00	35.47	20.84	0.35	0.27	0.38
7.21	510.71	0.00	0.00	93.42	1.71	1.38	0.96
2.36	201.13	0.00	0.00	20.29	0.37	0.28	0.35
EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	.	60.00	1.66	1.42	0.90
1.00	88.00	.	.	95.00	0.78	1.02	1.20
.	.	.	.	89.00	1.34	1.39	4.00
1.00	96.00	.	.	75.00	1.27	1.19	1.10
.	.	.	.	110.00	1.61	1.56	0.80
.	.	.	.	78.00	1.29	1.25	1.30
.	.	.	.	71.00	1.23	1.15	1.00
.	.	.	.	58.00	0.78	0.81	1.00
.	.	.	.	68.00	1.27	1.05	0.90
.	.	.	.	91.00	0.69	0.67	1.20
1.00	88.00	.	.	118.00	2.15	1.59	1.50
.	.	.	.	76.00	1.25	1.33	1.10
2.00	120.00	.	.	122.00	2.05	1.34	1.10
.	.	1.00	104.00	73.00	0.90	0.70	1.00
.	.	.	.	91.00	1.22	1.48	0.70
.	.	.	.	102.00	0.75	0.80	1.10
.	.	.	.	121.00	1.54	1.19	1.20
.	.	.	.	144.00	1.85	1.36	0.90
.	.	.	.	132.00	1.50	1.45	0.80
.	.	1.00	96.00	112.00	1.25	1.37	0.80
.	.	1.00	72.00	134.00	1.36	1.12	0.90
8.00	848.00	.	.	78.00	1.35	1.42	.
.	.	.	.	.	.	.	.
2.00	130.00	.	.	110.00	1.75	1.05	1.00
2.50	228.33	1.00	90.67	96.00	1.34	1.20	1.16
2.74	304.07	0.00	16.65	25.03	0.40	0.27	0.66
6.08	463.63	0.00	0.00	93.58	1.55	1.23	0.99
3.31	243.29	0.00	0.00	21.97	0.46	0.36	0.24

Appendix B. Blood Biochemistry Parameters for Individual Animals

EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	.	107.00	1.15	0.95	0.60
.	.	.	.	74.00	0.77	1.14	1.10
2.00	186.00	0.00	0.00	118.00	1.93	1.43	1.20
.	.	.	.	118.00	2.70	1.57	4.50
.	.	.	.	139.00	2.93	2.19	0.60
.	.	.	.	103.00	1.92	1.62	1.10
2.00	132.00	.	.	95.00	1.33	0.82	0.90
2.00	158.00	.	.	111.00	1.21	0.82	1.80
2.00	218.00	.	.	54.00	0.60	0.70	1.10
1.00	56.00	.	.	105.00	1.60	1.41	1.10
.	.	.	.	99.00	2.21	1.65	1.10
3.00	291.00	.	.	112.00	1.31	1.10	0.70
.	.	.	.	120.00	1.80	1.38	1.00
.	.	.	.	131.00	2.06	1.31	1.00
1.00	44.00	.	.	80.00	1.08	1.22	2.10
.	.	1.00	50.00	105.00	1.87	1.55	1.00
.	.	.	.	101.00	1.16	1.25	1.10
1.00	60.00	.	.	110.00	1.51	1.40	0.80
.	.	1.00	47.00	108.00	1.45	1.22	1.10
.	.	.	.	84.00	0.69	0.89	0.70
3.00	243.00	83.00	1.29	83.00	1.29	1.14	1.10
2.00	160.00	0.00	0.00	114.00	2.11	2.01	0.70
.	.	.	.	153.00	2.89	2.27	1.60
4.00	260.00	0.00	0.00	91.00	2.02	1.58	1.00
.	.	.	.	120.00	1.80	1.38	1.00
2.09	164.36	14.17	16.38	105.40	1.66	1.36	1.20
0.94	85.41	33.72	24.90	21.00	0.63	0.40	0.77
7.21	510.71	0.00	0.00	93.42	1.71	1.38	0.96
2.36	201.13	0.00	0.00	20.29	0.37	0.28	0.35
EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
2.00	108.00	.	.	76.00	1.04	1.47	0.90
.	.	.	.	67.00	1.05	1.05	1.00
4.00	276.00	.	.	70.00	1.20	1.17	0.90
.	.	.	.	90.00	2.58	1.49	0.80
.	.	1.00	86.00	135.00	1.83	1.39	1.20
.	.	.	.	93.00	1.47	1.23	1.20
.	.	1.00	79.00	94.00	1.50	1.19	1.10
2.00	170.00	.	.	67.00	1.01	0.93	1.20
.	.	.	.	107.00	0.78	0.82	1.20
.	.	.	.	60.00	1.31	0.88	1.20
.	.	.	.	.	.	.	.
27.00	2322.00	.	.	52.00	1.22	0.92	0.90
5.00	335.00	.	.	98.00	2.32	1.71	0.80
.	.	.	.	113.00	0.78	1.17	1.50
3.00	186.00	0.00	0.00	88.00	2.21	1.24	2.90
4.00	328.00	0.00	0.00	75.00	1.59	1.30	0.70
.	.	.	.	115.00	1.68	1.62	0.80
.	.	.		79.00	1.99	1.22	1.00
.	.	.	.	91.00	1.50	1.43	.
3.00	324.00	0.00	0.00	60.00	1.20	0.87	1.00
.	.	.	.	66.00	1.68	1.49	0.80
2.00	148.00	.	.	85.00	0.56	0.82	1.10
1.00	82.00	1.00	82.00	112.00	2.22	1.88	1.80
8.75	719.00	1.00	82.50	82.82	1.36	1.14	1.05
7.72	672.10	0.55	45.15	21.32	0.54	0.30	0.48
6.08	463.63	0.00	0.00	93.58	1.55	1.23	0.99
3.31	243.29	0.00	0.00	21.97	0.46	0.36	0.24

Appendix B. Blood Biochemistry Parameters for Individual Animals

EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	.	74.00	1.28	0.50	1.00
.	.	.	.	121.00	1.33	0.67	1.30
.	.	.	.	63.00	1.08	0.35	1.40
.	.	.	.	91.00	1.30	0.62	0.80
.	.	.	.	93.00	1.78	0.66	.
.	.	.	.	97.00	1.00	0.59	0.80
.	.	.	.	88.00	1.06	0.55	1.00
.	.	.	.	.	0.96	0.51	0.70
.	.	1.00	115.00	144.00	1.41	0.30	0.70
.	.	.	.	92.00	1.03	0.58	0.70
.	.	.	.	115.00	1.76	0.85	2.80
.	.	1.00	102.00	61.00	1.21	0.38	0.60
16.00	1488.00	.	.	101.00	1.38	0.72	1.00
.	.	.	.	104.00	0.91	1.02	5.90
.	.	0.00	0.00	88.00	1.24	0.84	0.84
.	.	.	.	127.00	2.57	1.42	1.10
6.00	384.00	1.00	64.00	81.00	1.22	0.52	0.90
.	.	1.00	57.00	103.00	1.38	0.53	1.00
.	.	.	.	131.00	2.73	1.69	1.20
4.00	260.00	1.00	65.00	100.00	1.37	0.58	0.90
.	.	.	.	96.00	1.58	1.27	0.80
.	.	.	.	92.00	1.90	0.73	0.90
1.00	59.00	.	.	108.00	2.00	0.93	1.20
.	.	1.00	96.00	85.00	1.39	0.74	0.90
6.75	547.75	0.86	71.29	98.04	1.45	0.73	1.24
6.50	640.98	0.38	38.39	20.25	0.47	0.34	1.11
7.21	510.71	0.00	0.00	93.42	1.71	1.38	0.96
2.36	201.13	0.00	0.00	20.29	0.37	0.28	0.35
EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	.	61.00	0.93	0.63	0.40
1.00	167.00	.	.	82.00	0.48	0.17	1.00
1.00	100.00	.	.	45.00	1.44	0.61	1.80
.	.	.	.	125.00	1.11	0.28	0.60
.	.	.	.	75.00	0.85	0.38	1.20
.	.	.	.	142.00	2.06	0.56	0.70
.	.	.	.	71.00	1.10	0.51	0.50
.	.	.	.	79.00	0.81	0.16	0.70
.	.	.	.	64.00	0.89	0.39	0.50
.	.	.	.	77.00	0.63	0.21	0.70
1.00	262.00	.	.	80.00	0.96	0.57	0.70
4.00	308.00	.	.	128.00	1.77	0.93	0.70
.	.	.	.	.	.	.	.
2.00	178.00	.	.	62.00	1.01	0.29	1.00
.	.	1.00	80.00	94.00	1.64	0.55	1.10
5.00	570.00	0.00	0.00	74.00	1.93	0.59	0.90
.	.	.	.	96.00	1.08	0.38	1.10
.	.	1.00	77.00	65.00	1.42	0.94	0.70
2.00	98.00	0.00	0.00	87.00	0.96	0.47	1.20
.	.	.	.	80.00	1.62	0.53	0.70
.	.	.	.	105.00	1.47	1.40	0.70
.	.	.	.	131.00	1.70	1.37	1.00
.	.	.	.	92.00	0.85	0.85	0.80
.	.	0.00	0.00	67.00	1.32	1.04	0.70
2.29	240.43	0.40	31.40	86.17	1.22	0.60	0.84
1.60	164.79	0.55	43.01	25.12	0.43	0.35	0.31
6.08	463.63	0.00	0.00	93.58	1.55	1.23	0.99
3.31	243.29	0.00	0.00	21.97	0.46	0.36	0.24

Appendix B. Blood Biochemistry Parameters for Individual Animals

EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	.	100.00	0.94	0.35	0.90
9.00	999.00	.	.	99.00	0.51	0.27	2.10
3.00	280.00	.	.	64.00	0.24	0.23	0.90
.	.	.	.	128.00	1.75	0.68	0.90
.	.	.	.	83.00	0.62	0.36	0.90
.	.	.	.	95.00	0.87	0.32	1.30
.	.	.	.	172.00	2.62	0.98	1.20
.	.	.	.	100.00	0.72	0.18	0.90
.	.	.	.	97.00	0.72	0.45	1.00
.	.	.	.	130.00	2.63	0.94	1.30
.	.	.	.	139.00	1.71	0.72	1.30
.	.	.	.	92.00	0.86	0.29	0.70
.	.	1.00	84.00	78.00	1.49	0.62	1.00
.	.	.	.	107.00	1.31	0.66	1.10
8.00	712.00	.	.	74.00	1.00	0.59	1.20
1.00	65.00	.	.	99.00	1.19	0.55	0.80
.	.	.	.	82.00	0.52	0.19	1.20
.	.	.	.	76.00	1.03	0.57	0.80
.	.	.	.	85.00	0.79	0.54	0.90
4.00	232.00	1.00	58.00	94.00	1.53	0.79	1.00
2.00	156.00	.	.	74.00	0.63	0.54	0.90
.	.	.	.	61.00	0.88	0.43	0.60
.	.	.	.	168.00	3.37	1.10	1.70
.	.	.	.	51.00	0.40	0.40	0.70
4.50	407.33	1.00	71.00	97.83	1.18	0.53	1.05
3.27	366.12	0.00	18.38	30.61	0.77	0.25	0.33
7.21	510.71	0.00	0.00	93.42	1.71	1.38	0.96
2.36	201.13	0.00	0.00	20.29	0.37	0.28	0.35
EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	.	.	.	.	.
6.00	576.00	.	.	88.00	0.98	0.42	0.70
11.00	110.00	1.00	100.00	121.00	1.16	0.38	0.80
.	.	.	.	.	.	.	.
.	.	.	.	.	0.49	0.17	0.80
2.00	150.00	.	.	56.00	0.55	0.25	0.80
.	.	1.00	76.00	126.00	1.16	0.52	1.00
.	.	.	.	90.00	0.76	0.41	0.70
1.00	37.00	.	.	82.00	1.32	0.81	0.70
.	.	1.00	95.00	87.00	0.82	0.27	1.20
.	.	.	.	76.00	0.58	0.25	1.00
.	.	.	.	82.00	1.01	0.50	0.70
4.00	280.00	.	.	74.00	1.92	0.82	0.80
6.00	528.00	.	.	64.00	0.99	0.60	0.70
1.00	76.00	.	.	89.00	0.58	0.37	0.70
.	.	.	.	70.00	1.37	0.80	1.00
.	.	.	.	77.00	1.49	0.53	0.90
2.00	204.00	.	.	72.00	0.64	0.37	0.70
.	.	.	.	69.00	1.13	0.41	0.50
1.00	56.00	1.00	56.00	82.00	1.43	0.54	0.80
3.00	213.00	.	.	110.00	1.29	0.56	1..1
3.70	223.00	1.00	81.75	84.17	1.04	0.47	0.81
3.20	189.60	0.00	20.04	18.58	0.39	0.19	0.16
6.08	463.63	0.00	0.00	93.58	1.55	1.23	0.99
3.31	243.29	0.00	0.00	21.97	0.46	0.36	0.24

Appendix B. Blood Biochemistry Parameters for Individual Animals

EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
				117	0.95	0.55	1
		128		87	0.91	0.19	1.8
		110		84	0.5	0.48	1.1
132		66		75	0.6	0.43	0.9
116				85	0.4	0.31	1
		51		116	2.01	0.47	1.1
61		61		66	0.56	0.54	0.8
				101	0.89	0.69	1
106				97	1.28	0.22	0.8
				116	1.66	0.53	0.8
101		101		96	1.52	0.99	0.9
103.20	.	86.17	.	94.55	1.03	0.49	1.02
26.40	.	31.03	.	17.12	0.53	0.22	0.28
7.21	510.71	0.00	0.00	93.42	1.71	1.38	0.96
2.36	201.13	0.00	0.00	20.29	0.37	0.28	0.35
EOSINOPHILS	ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
				85	1.19	0.45	1
		75		65	1.28	0.29	0.9
				111	0.72	0.17	1
166				53	0.72	0.46	1.2
68		68		97	0.62	0.43	1.1
				76	1.05	0.54	0.8
				69	0.78	0.53	1
				93	0.54	0.32	1.1
77		77		77	0.64	0.33	0.7
103.67	.	73.33	.	80.67	0.84	0.39	0.98
54.17	.	4.73	.	17.82	0.27	0.12	0.16
6.08	463.63	0.00	0.00	93.58	1.55	1.23	0.99
3.31	243.29	0.00	0.00	21.97	0.46	0.36	0.24

Appendix B. Blood Biochemistry Parameters for Individual Animals

T4AA	FREE T3	TSH	QUANT. PLATELET
0.80	2.90	0.05	211.00
0.80	5.20	0.22	293.00
0.80	3.00	0.18	330.00
0.90	2.40	0.05	246.00
0.80	3.50	0.05	165*
0.80	4.70	0.45	391.00
0.90	2.70	<.10	317.00
0.80	1.90	<0.10	318.00
0.90	2.90	<0.10	243.00
0.80	3.50	<0.10	302.00
0.90	3.80	0.17	477.00
0.80	2.90	<0.10	191.00
1.10	1.90	0.05	258.00
0.90	2.50	0.05	200.00
0.80	3.40	0.05	313.00
0.80	2.70	0.05	337.00
1.00	2.40	0.15	347.00
0.90	3.20	0.05	253.00
0.80	3.00	0.30	364.00
0.90	1.40	0.16	227.00
0.80	2.30	<.10	268.00
0.70	3.60	0.18	360.00
0.90	3.40	2.77	NA
0.80	3.70	0.76	439.00
0.85	3.04	0.32	303.86
0.08	0.85	0.64	74.93
T4AA	FREE T3	TSH	QUANT. PLATELET
0.90	2.00	0.05	356.00
0.80	2.70	0.80	306.00
0.80	4.30	0.50	310.00
1.20	3.60	0.23	277.00
1.10	2.40	0.20	312.00
0.90	3.40	0.20	322.00
0.90	2.40	2.27	484.00
0.80	2.70	<0.10	353.00
0.80	3.40	<0.10	476.00
1.10	2.90	<0.10	408.00
0.90	3.40	<0.10	258.00
0.80	2.90	<0.10	480.00
0.80	3.30	0.05	179.00
0.90	3.10	0.05	363.00
0.80	2.20	0.05	222.00
0.90	2.20	0.05	380.00
0.90	2.30	0.05	246.00
0.90	3.50	0.05	283.00
0.90	2.70	<0.10	276.00
0.90	2.90	0.28	257.00
0.90	3.00	0.24	293.00
0.80	4.10	0.40	190.00
0.90	2.60	0.27	NA
0.90	3.30	0.22	257.00
0.90	2.97	0.33	316.87
0.10	0.59	0.52	85.83

Appendix B. Blood Biochemistry Parameters for Individual Animals

T4AA	FREE T3	TSH	QUANT. PLATELET
0.90	1.60	0.24	207.00
0.80	3.20	0.26	287.00
0.80	1.90	0.21	194.00
0.80	1.90	0.25	247.00
0.90	3.00	0.10	160.00
0.70	3.90	0.22	427.00
1.10	3.30	0.28	357.00
0.60	3.50	0.16	386.00
0.70	2.50	0.29	308.00
0.90	3.40	0.38	435.00
1.20	2.70	0.49	336.00
0.70	4.10	0.52	180.00
1.00	2.40	1.15	107.00
0.90	1.80	0.33	148.00
0.80	1.40	0.11	209.00
0.80	3.70	<.10	282.00
0.80	4.00	0.16	313.00
0.80	1.70	0.18	230.00
0.70	2.90	1.16	296.00
1.20	2.40	0.32	218.00
0.80	3.20	0.43	323.00
0.70	3.70	0.49	172.00
1.10	3.40	3.55	420.00
0.80	3.00	1.07	347.00
0.85	2.86	0.54	274.54
0.16	0.82	0.73	93.71
0.85	3.04	0.32	303.86
0.08	0.85	0.64	74.93
T4AA	FREE T3	TSH	QUANT. PLATELET
0.80	2.20	<.10	293.00
0.90	1.90	0.88	268.00
0.80	4.40	0.25	98.00
1.00	2.50	0.71	211.00
0.70	4.20	0.23	214.00
0.80	2.30	<.10	359.00
0.80	2.60	0.57	303.00
0.90	2.10	0.11	137.00
0.70	2.30	0.13	251.00
0.90	2.60	<.10	245.00
1.00	3.80	0.13	247.00
0.70	3.10	0.19	240.00
1.00	2.80	0.13	309.00
1.20	1.70	3.62	.
0.90	2.60	0.46	.
1.10	2.20	0.90	464.00
1.00	3.50	0.58	276.00
1.00	3.50	0.25	491.00
0.90	3.60	0.27	121.00
1.00	3.90	0.25	362.00
0.80	4.00	<.1	389.00
1.10	2.20	0.20	332.00
.	.	.	.
1.40	3.10	0.15	330.00
0.93	2.92	0.53	282.86
0.17	0.80	0.79	100.67
0.90	2.97	0.33	316.87
0.10	0.59	0.52	85.83

Appendix B. Blood Biochemistry Parameters for Individual Animals

T4AA	FREE T3	TSH	QUANT. PLATELET
0.40	3.20	0.67	208.00
1.10	2.30	0.50	175.00
0.90	3.30	0.23	264.00
1.10	8.40	0.62	314.00
0.40	4.00	0.21	374.00
0.60	3.10	0.39	181.00
0.80	2.00	0.18	291.00
1.10	2.80	5.24	321.00
1.20	3.30	0.51	231.00
0.70	3.60	1.57	351.00
1.10	4.80	0.21	251.00
0.40	2.80	0.19	158.00
0.80	3.00	0.37	240.00
0.60	2.90	0.36	317.00
0.80	3.10	0.32	44.00
0.80	3.00	<.10	267.00
0.60	3.30	0.64	335.00
0.60	2.80	0.37	352.00
0.60	2.10	0.19	350.00
0.70	1.90	0.33	276.00
0.60	4.70	0.14	231.00
0.80	3.20	0.14	256.00
0.50	4.60	0.68	125.00
0.60	5.30	0.40	306.00
0.80	3.00	0.37	240.00
0.74	3.46	0.62	258.32
0.23	1.34	1.03	79.24
0.85	3.04	0.32	303.86
0.08	0.85	0.64	74.93
T4AA	FREE T3	TSH	QUANT. PLATELET
0.70	2.80	0.12	341.00
0.90	2.20	3.70	475.00
0.90	3.10	0.56	357.00
0.80	2.80	1.09	277.00
0.90	3.40	0.54	251.00
1.00	3.70	1.30	401.00
1.10	4.50	0.51	135.00
1.10	3.20	0.62	175.00
0.80	3.60	0.18	393.00
0.90	3.20	0.38	238.00
.	.	.	.
0.90	1.40	0.11	298.00
0.60	.	0.13	433.00
0.60	6.90	1.22	369.00
0.60	5.50	0.25	358.00
0.70	5.10	0.42	405.00
.	.	0.14	361.00
0.60	4.90	0.17	410.00
.	.	0.44	365.00
0.60	4.40	0.15	280.00
0.60	3.60	0.14	368.00
0.80	5.80	0.12	304.00
0.70	6.60	0.14	484.00
0.91	3.08	0.83	303.73
0.17	1.47	0.79	88.58
0.90	2.97	0.33	316.87
0.10	0.59	0.52	85.83

Appendix B. Blood Biochemistry Parameters for Individual Animals

T4AA	FREE T3	TSH	QUANT. PLATELET
1.10	3.20	0.34	161.00
1.00	4.60	0.81	320.00
1.10	1.90	0.18	150.00
1.00	3.10	<0.1	194.00
.	.	0.14	320.00
0.70	4.10	0.16	283.00
1.00	3.80	<0.1	347.00
1.10	3.30	0.17	176.00
1.10	4.20	0.29	221.00
0.80	4.60	0.11	166.00
2.30	4.70	1.03	183.00
1.10	2.90	0.14	255.00
0.80	6.20	0.17	293.00
0.80	7.60	3.62	362.00
0.80	4.60	0.15	403.00
0.80	5.50	0.11	286.00
0.80	4.60	1.36	462.00
0.80	6.00	0.24	316.00
0.70	5.40	0.16	.
0.70	5.80	0.59	245.00
0.70	4.90	0.30	185.00
0.70	4.80	0.12	294.00
0.70	5.60	0.60	106.00
0.80	5.70	0.32	350.00
0.93	4.66	0.51	264.26
0.34	1.28	0.77	90.42
0.85	3.04	0.32	303.86
0.08	0.85	0.64	74.93
T4AA	FREE T3	TSH	QUANT. PLATELET
0.90	2.00	<0.1	233.00
1.00	2.60	0.58	401.00
1.20	3.10	0.19	309.00
1.00	3.60	0.55	421.00
1.20	1.80	0.10	271.00
0.90	3.20	0.17	465.00
0.90	2.30	0.31	228.00
0.90	2.60	.	202.00
1.00	2.10	<0.1	390.00
1.10	2.40	<0.1	240.00
0.80	2.10	<0.1	498.00
1.00	4.50	0.99	385.00
.	.	.	.
0.90	4.30	0.54	254.00
0.90	5.10	0.28	273.00
0.90	5.00	0.33	469.00
1.00	4.50	<0.10	405.00
0.80	4.50	0.12	377.00
1.00	4.90	0.20	415.00
0.80	4.60	0.32	251.00
0.70	4.70	0.16	298.00
0.70	5.80	0.22	125.00
0.70	4.30	2.90	242.00
0.70	4.70	0.19	490.00
0.91	3.68	0.48	332.26
0.15	1.23	0.66	104.56
0.90	2.97	0.33	316.87
0.10	0.59	0.52	85.83

Appendix B. Blood Biochemistry Parameters for Individual Animals

T4AA	FREE T3	TSH	QUANT. PLATELET
0.90	2.60	0.16	151.00
1.90	3.60	0.96	388.00
1.40	2.20	0.29	328.00
1.40	4.80	0.24	565.00
0.90	2.60	0.57	420.00
0.90	3.00	0.24	311.00
1.70	5.40	0.27	497.00
0.90	2.80	0.73	333.00
1.10	4.10	0.33	229.00
0.60	4.50	0.17	532.00
0.90	3.90	0.51	266.00
.	3.40	0.25	426.00
1.30	2.00	0.65	248.00
1.10	2.80	0.52	409.00
1.50	2.60	0.28	205.00
1.10	3.10	0.20	398.00
1.80	1.80	0.51	712.00
1.10	2.50	0.20	403.00
1.40	2.20	0.10	424.00
1.30	3.10	0.32	279.00
1.10	5.00	0.23	121.00
1.00	1.60	0.26	334.00
2.00	3.80	0.49	474.00
1.00	2.80	0.10	312.00
1.23	3.18	0.36	365.21
0.36	1.03	0.22	135.49
0.85	3.04	0.32	303.86
0.08	0.85	0.64	74.93
T4AA	FREE T3	TSH	QUANT. PLATELET
.	.	.	.
1.30	2.10	0.29	349.00
1.40	4.30	0.54	165.00
.	.	.	.
1.30	3.70	0.12	389.00
1.10	2.60	<0.1	384.00
1.20	5.30	0.50	592.00
0.80	4.00	0.42	442.00
1.00	3.50	0.11	299.00
1.60	2.20	0.19	215.00
0.80	3.20	4.09	519.00
0.80	3.10	0.33	346.00
1.20	2.70	0.21	434.00
0.90	4.00	0.45	417.00
0.80	2.10	0.13	340.00
0.80	4.20	0.10	525.00
0.90	2.10	0.35	326.00
0.80	2.70	0.23	289.00
1.10	2.20	0.28	373.00
0.90	1.60	0.18	260.00
0.90	3.30	1.14	474.00
1.03	3.10	0.54	375.68
0.24	0.98	0.92	108.36
0.90	2.97	0.33	316.87
0.10	0.59	0.52	85.83

Appendix B. Blood Biochemistry Parameters for Individual Animals

T4AA	FREE T3	TSH	QUANT. PLATELET
1	4.7	0.12	208
1.7	4.4	3.03	523
1.2	4.2	0.31	409
1.1	3.3	1.16	422
1.9	4.3	0.2	284
1.5	4.5	0.37	328
1	2.9	0.72	264
1.1	4.6	0.34	248
1.3	4.1	0.12	401
1.1	4.2	0.39	324
1.2	3.7	0.19	379
1.28	4.08	0.63	344.55
0.30	0.56	0.85	92.30
0.85	3.04	0.32	303.86
0.08	0.85	0.64	74.93
T4AA	FREE T3	TSH	QUANT. PLATELET
1.3	3.5	0.37	178
1.2	2.5	0.27	215
1.1	4.2	<0.10	447
1.3	2.7	0.19	369
1.9	3.7	0.2	471
1	3	0.1	280
1.5	3.7	0.51	347
1.2	3.3	3.35	
1.2	3.3	0.2	456
1.30	3.32	0.65	345.38
0.26	0.53	1.10	112.25
0.90	2.97	0.33	316.87
0.10	0.59	0.52	85.83

Appendix C

Reprints and Preprints Resulting from Contract DAMD17-98-1-8622

Running head: LOCOMOTOR ACTIVITY IN AGED CANINES

Age-Dependent Declines in Locomotor Activity in Canines is Environment Specific

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Published in Physiology and Behavior 75(1-2): 65-70, 2002.

Abstract

Motor impairments and declines in motor activity are expected concomitants of normal aging. Age-associated decreases in motor activity have been reported in humans and non-human mammals. We have previously failed to find an age difference in open field activity in beagle dogs. We now report an age-associated decline in motor activity in beagles. Locomotor activity of young and aged dogs was examined in both an open field and their home cage environment. Dogs were given 6 activity tests (2 open field; 2 morning and 2 afternoon home cage tests) every second day. Differences in behavior varied with environment, age, sex and housing conditions. Aged dogs were less active than young dogs in the home cage but no difference was observed in the open field. Behavioral activity is a complex manifestation of many underlying factors, which need to be considered in aging and pharmacological research.

Age-Dependent Declines in Locomotor Activity in Canines is Environment Specific

Motor impairments, decreased motor activity, loss in physical strength, endurance and coordination normally accompany advanced age (Hilleras, Jorm, Herlitz, & Winblad, 1999; LeWitt, 1988; Bassey, 1998). Age-dependent decreases in motor activity have also been demonstrated in non-human mammals. Age-associated decreases in open field activity have been reported for mice (Sprott & Eleftheriou 1974; Elias & Redgate 1975; Goodrick 1975; Elias, Elias, & Eleftheriou, 1975; Dean et al., 1981; Ingram, London, Waller, & Reynolds, 1983; Rosenthal & Morley 1989; Lamberty & Gower, 1990, 1991) and rats (Goodrick 1971; Kametani, Osada, & Inoue, 1984; Dorce & Palermo-Neto, 1994). Emborg et al. (1998) and Gerhardt et al. (1995) have reported decreases in the amount of home cage activity in aged rhesus monkeys.

Rabbits, on the other hand, show an age-associated increase in open field activity (Deyo, Straube, Moyer, & Disterhoft, 1989). Nagahara and Handa (1997) found no difference between young and aged Fischer rats in open field locomotion. The senescent accelerated mouse (SAM) shows increases in activity with age (Miyamoto et al., 1986). Levine, Lloyd, Fisher, Hull, and Buchwald (1987) also found no difference between young and aged cats in levels of activity.

We have been particularly interested in the effects of age on behavioral activity of canines. In two separate studies, we have been unable to demonstrate a statistically significant age-dependent deficit in open field behavior in beagle dogs (Head & Milgram, 1992; Head et al., 1997). This finding was unexpected since pet owners commonly report decreases in activity and affection in older dogs (Houpt & Beaver, 1981).

One factor that may have contributed to the absence of an effect of age on activity was the use of the open field test. This test involves placing animals in a relatively novel environment and because of the novelty, may not provide a pure measure of spontaneous activity. Rosario and Abercrombie (1999) suggest that locomotor responses to a novel open field reflect behavioral reactivity, a stable individual trait, rather than differences in motoric activity.

The present investigation sought to further explore the effect of age on behavioral activity in beagle dogs by comparing locomotor activity in two test situations, the open field and home cage. Open field activity is likely to be influenced by the response to novelty whereas home cage activity is not and represents true spontaneous activity.

Methods

Subjects

Subjects were 36 aged (18 male; 18 female) and 16 young (8 male; 8 female) beagle dogs (Canis familiaris) from the Lovelace Respiratory Research Institute colony in Albuquerque, New Mexico. The aged beagles ranged in age from 9 to 16 years while the young dogs varied in age between 1 to 3 years. The animals were housed either individually (13 aged dogs) or in pairs (23 aged dogs; 16 young dogs) in kennel buildings with indoor/outdoor runs measuring 0.94 m x 6.10 m. They were fed Teklad Lab Dog Diet (W) 8759 once a day in the morning, with water available ad libitum from a wall spout.

Experimental Design

A total of six tests were performed to compare the effect of age on open field and home cage behavior. Each animal was given two open field tests and four home cage

tests. The open field tests were conducted on days 1 and 5. Two daily home cage tests were performed on days 3 and 7.

Behavioral Testing Procedures

The open-field was a 3.81 X 2.35-m test arena sectioned off within a larger room, using a wooden barrier bolted to the floor. A hinged door provided access into the arena. The floor of the arena was marked into 36 rectangles (61.60 X 36.20 cm) with black electrical tape to assist in tracing the behavior patterns of the dogs. Prior to each test, the floor was cleaned with a disinfectant solution to prevent odor cues from having a disruptive effect on other dogs.

All test sessions lasted 10 minutes. The dog was placed inside of the arena and was observed by an experimenter, who recorded the animal's behavior with a video camera. At a later time, a second experimenter analyzed the behaviors from the videotape using dedicated computer software (Head & Milgram, 1992) that provided quantitative measures of locomotion, directed sniffing, urination, inactivity, grooming, rearing, vocalization, and jumping. For locomotion the program provided a measure of total distance. Time measures were taken for grooming and inactivity. The number of times each of the other behaviors occurred (i.e. frequency of that behavior) was recorded. To minimize variability and bias, the same person (CTS) analyzed all of the behavioral observations.

Home-cage testing was performed in each dog's kennel (6.10 X 0.94 m). The experimenter placed a video camera on a tripod in front of the kennel, and behavior was recorded for 10 minutes. The videotape of the home cage was analyzed in the same

manner as the open field videotapes except that interaction was recorded instead of grooming for dogs housed with a kennel mate.

Data Analysis

All statistical analyses were performed using the Statistica software package with the alpha level of 0.05. Results are reported up to the alpha level of .10 if the effects were relevant in the context of the data. The effects of age and test type on the frequency of occurrence of each behavior, other than locomotion and sniffing, were analyzed using chi-square. Yates corrected chi-square values were used due to the presence of small frequencies in some cases. Behaviors that occurred frequently (i.e. almost all dogs exhibited) in the population were subject to analysis of variance (ANOVA). A one-way ANOVA with age as the between subjects factor was performed to analyze the open field locomotion and sniffing behaviors. A two-way ANOVA was used to analyze the home cage locomotion, sniffing, and inactivity behaviors; age was the between subjects factor and test time was the within subjects factor.

Results

Open Field versus Home Cage Tests

Figure 1 illustrates that behavioral profiles in the open field differed from those observed in the home cage. Significantly more of the aged dogs urinated in the open field than the home cage, AM: $\chi^2(1, \underline{N} = 36) = 7.15$, $\underline{p} = .008$, PM: $\chi^2(1, \underline{N} = 36) = 19.20$, $\underline{p} = .0000$. A greater proportion of aged dogs were more inactive in the home cage than the open field, AM: $\chi^2(1, \underline{N} = 36) = 9.20$, $\underline{p} = .002$, PM: $\chi^2(1, \underline{N} = 36) = 21.41$, $\underline{p} = .0000$. More of the aged dogs were inactive in the afternoon home cage test than the morning test, $\chi^2(1, \underline{N} = 36) = 3.44$, $\underline{p} = .06$.

Environmental context also influenced the behaviors displayed by the young dogs. More of the young dogs urinated in the open field test compared to the morning, $\chi^2(1, \underline{N} = 16) = 3.17$, $\underline{p} = .07$, and afternoon $\chi^2(1, \underline{N} = 16) = 6.53$, $\underline{p} = .01$ home cage tests. More of the young dogs were inactive in the afternoon home cage test compared to the open field, $\chi^2(1, \underline{N} = 16) = 3.17$, $\underline{p} = .07$ and morning home cage test $\chi^2(1, \underline{N} = 16) = 4.52$, $\underline{p} = .03$. Vocalization was displayed by more young dogs in the open field than the home cage, AM: $\chi^2(1, \underline{N} = 16) = 8.03$, $\underline{p} = .004$, PM: $\chi^2(1, \underline{N} = 16) = 4.52$, $\underline{p} = .03$.

Behavioral profiles also differed with age as illustrated in Figure 2. Significantly more of the aged dogs urinated than the young group in both the open field, $\chi^2(1, \underline{N} = 52) = 3.41$, $\underline{p} = 0.06$, and home cage environments, AM: $\chi^2(1, \underline{N} = 52) = 3.69$, $\underline{p} = 0.05$. A larger proportion of aged dogs were inactive in the home cage compared to young, AM: $\chi^2(1, \underline{N} = 52) = 13.18$, $\underline{p} = .0003$, PM: $\chi^2(1, \underline{N} = 52) = 6.55$, $\underline{p} = .01$. Significantly more of the young dogs reared, OF and PM: $\chi^2(1, \underline{N} = 52) = 8.29$, $\underline{p} = .004$, AM: $\chi^2(1, \underline{N} = 52) = 3.25$, $\underline{p} = .07$, and jumped, OF: $\chi^2(1, \underline{N} = 52) = 4.01$, $\underline{p} = .05$, AM: $\chi^2(1, \underline{N} = 52) = 4.70$, $\underline{p} = .03$, PM: $\chi^2(1, \underline{N} = 52) = 17.15$, $\underline{p} = .0000$, than the aged dogs in both the open field and home cage tests. Vocalization was more frequent among the old dogs in the morning home cage test $\chi^2(1, \underline{N} = 52) = 7.31$, $\underline{p} = .007$.

Sex differences were also assessed. Open field urination was compared between males and females. In the aged group of dogs there were no significant differences in frequency of urinating in the open field between males and females. Young males, however, were more likely to urinate in the open field than young females $\chi^2(1, \underline{N} = 16) = 13.39$, $\underline{p} = .0003$.

Open Field Locomotion and Sniffing

The ANOVA for open field locomotion revealed no significant difference between the young and old dogs, $F(1, 50) = 0.26$, $p = .61$ (Figure 3A). We did observe, however, a significant main effect of age for sniffing, $F(1, 50) = 19.40$, $p = .00006$. Old dogs sniffed more frequently than the young dogs.

The ages of the old dogs ranged from 9 to 16 years. The absence of a significant age effect may possibly be due to the large proportion of the aged group simply being not old enough. To examine this possibility, we looked at the correlation between activity and age among the aged animals. In all instances, the correlations were small and not significant. For the open field $r = .02$, the AM home cage test $r = .10$, and the PM home cage test $r = .07$.

Home Cage

The analysis of home cage locomotion used only dogs housed in pairs and revealed a significant main effect of age, $F(1, 37) = 12.16$, $p = .001$, and a significant main effect of test time, $F(1, 37) = 5.39$, $p = .03$. The interaction between age and time achieved significance at the 0.1 alpha level, $F(1, 37) = 3.44$, $p = .07$, indicating that locomotor activity in the home cage decreased later in the day in the aged group of dogs (Figure 3B).

A significant main effect of age, $F(1, 37) = 10.10$, $p = .003$, showed that old dogs sniffed more in the home cage than the young dogs. A significant main effect was also obtained for test time, $F(1, 37) = 5.10$, $p = .03$, and indicated that sniffing was less frequent in the afternoon test.

The analysis of home cage inactivity revealed significant main effects of age, $F(1, 37) = 12.64$, $p = .001$, and time, $F(1, 37) = 43.71$, $p = .000$. Aged dogs spent more time

inactive. Dogs were less active later in the day.

Correlation between test types

In the young dog group open field locomotion showed a weak positive correlation with home cage locomotion (OF-HCAM: $r = .51$, $p > .05$; OF-HCPM: $r = .38$, $p > .05$). Home cage locomotion in the morning was highly correlated with home cage locomotion in the afternoon ($r = .88$, $p < .01$).

The aged dogs locomotion in the home cage does not correlate with open field locomotion (OF-HCAM: $r = .004$, $p > .05$; OF-HCPM: $r = .11$, $p > .05$). Morning home cage locomotion is weakly correlated with afternoon locomotion ($r = .56$, $p < .001$).

Habituation

An examination of the mean locomotion scores for each minute of the test reveals differences between open field and home cage locomotion in the pattern of activity over the 10-minute period (Figure 4). In the open field, locomotion is highest at the start of the test and gradually declines over the 10 minutes of the test. In the home cage, locomotion is relatively stable with random peaks over the 10-minute period.

Housing Conditions

An analysis of aged dogs based on their housing condition revealed a significant effect of condition on home cage locomotion, $F(1, 34) = 4.44$, $p = .04$. Aged dogs housed alone ($N = 13$) exhibited higher levels of locomotor activity in the home cage than aged dogs housed with a kennel mate ($N = 23$) (Figure 5). There was a significant effect of time of day on home cage locomotion, $F(1, 34) = 12.29$, $p = .001$. Both groups showed lower levels of locomotion during the afternoon than during the morning test. Sniffing was less frequent in the afternoon test also, $F(1, 34) = 5.46$, $p = .03$.

Housing condition did not affect open field locomotion, $F(1, 34) = .03$, $p = .88$, or sniffing frequency, $F(1, 34) = .00006$, $p = .99$.

Discussion

The present results demonstrate that measures of behavioral activity are critically sensitive to the test situation. We found no significant age-dependent changes in locomotion in a canine open field test, which is consistent with our previous work (Head & Milgram, 1992; Head et al., 1997). When activity recordings were taken of home cage behavior, however, aged animals showed significantly less activity than young dogs and the difference became larger later in the day.

Rosenthal and Morley (1989) suggest that the novelty of the open field environment plays a role in the observed age-related differences in behavior in mice. Differences are apparent in a novel environment but not in a familiar one. The opposite seems to be true for canines. A novel environment masks age differences, which become apparent in a highly familiar environment. The home cage of the dog is a very familiar environment and observations of behavior reflect true spontaneous activity. In the open field the dogs must respond to the change in the environment. The novelty of the situation could arouse the dogs and induce increases in activity that mask any age-associated changes. Giovannini, Bartolini, Kopf, & Pepeu (1998) reported that a novel environment activates the cortical cholinergic system, which presumably is associated with arousal and attention. This could explain why differences in activity with age are not observed in the open field with dogs. The locomotion data per minute of the test supports the contention of novelty being a factor in the absence of age differences in the open field. We found that locomotion decreased over the 10 minutes of the open field test but remained

relatively stable in the home cage. Initially the open field is novel thereby inducing increases in activity but as the situation becomes familiar activity decreases.

Another critically important factor is the environment in which an animal lives. This can have important consequences on behavior (Menich & Baron, 1984). In our aged group of dogs some were housed alone and some were housed with a kennel mate. The reasons for this are due to health (i.e. arthritis) or because their kennel mate was no longer alive. The housing condition had no effect on open field activity. Both alone and paired dogs exhibited similar levels of locomotion in the open field test. In the home cage however, aged dogs housed alone exhibited higher levels of locomotor activity than those housed with a kennel mate. One reason is because of selective bias. The aged dogs housed alone were successful agers (outlived kennel mate of similar age) who had not deteriorated as much as non-successful agers. The dogs housed alone were also not subjected to the complex social relationships present when dogs are housed together. A dominant dog may inhibit activity of the non-dominant dog sharing the same kennel.

Our focus thus far has been on locomotor activity, which provides an incomplete picture of the effects of age on behavior. When a range of behaviors are taken into consideration, aged and young dog clearly show distinct behavioral profiles, which vary with environment. A larger proportion of aged animals urinates but young dogs exhibit higher frequencies of urinating. Aged dogs show longer periods of inactivity and more frequent sniffing than young animals. Young dogs are more likely to rear and jump.

The age differences that we found in sniffing, unlike locomotion, were present in both the open field and home cage environments. Older dogs sniffed more often than the young dogs. Deyo et al. (1989) suggested that old and young rabbits use different

strategies to explore a novel environment. Young rabbits tend to sit and look around the room while aged rabbits wander around. A similar explanation could apply to the canine. The young dogs tended to sit and look at their surroundings while the aged dogs sniffed as they moved about the environment. This could reflect differential utilization of sensory functions with old dogs showing greater dependence on olfaction and young dogs relying more on vision.

Urination frequency consistently produced significant effects. A greater number of the aged dogs urinated than the young dogs in both test situations. This may be due to urinary incontinence, a condition that becomes more common with age (Knoefel, 1994). The aged dogs were more likely to urinate in the open field than the home cage. The young dogs exhibited higher frequencies of urination compared to the aged group in each of the tests. In the open field, young males were more likely to urinate than young females. Urine marking does not appear to have any territorial significance for domestic dogs as dogs will often enter and urinate in an area inhabited by another dog (Dunbar & Carmichael, 1981). Instead, urinary marking in the case of domestic dogs seems to serve the purpose of making an unfamiliar area smell familiar (Dunbar & Carmichael, 1981). This is consistent with the higher levels of urination in the open field, a novel area. The role of environmental context is an important factor to consider when evaluating the behavioral effects of pharmaceuticals. We have previously found that the stimulant adrafinil is much less effective in producing an increase in home cage activity than it is in producing an increase in open field activity (Siwak et al., 2000). Amphetamine, however, produces significant increases in motor activity in both novel and familiar environments (Raskin, 1983). Aging research also needs to consider the influence of the environment.

Studies report results contradictory to the expected age-associated decline in locomotion when the open field is used (Deyo et al., 1989; Nagahara & Handa, 1997; Miyamoto et al., 1986).

The present results indicate that spontaneous behavior in dogs, as in other species, varies as a function of age. The nature of these changes, however, is complex and varies as a function of sex, testing environment and behavioral measure.

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Figure Captions

Figure 1. Behavioral profiles differed between young (n = 16) and aged dogs (n = 36) dogs. The top graph plots the percentage of aged dogs and the bottom the percentage of young dogs that displayed a particular behavior in the open field and home cage environments. Percentages with different subscripts within each behavior differ significantly at $p < .05$ by Yates corrected chi-square test. URI = urination; INACT = inactivity; RER = rearing; VOC = vocalizing; JUM = jumping.

Figure 2. Behavioral profiles differed between the open field and home cage environments. The top graph plots the percentage of dogs displaying a particular behavior in the open field and the bottom graph in the morning (AM) home cage test. Percentages with different subscripts within each behavior differ significantly at $p < .05$ by Yates corrected chi-square test. GRM = grooming; INT = interaction.

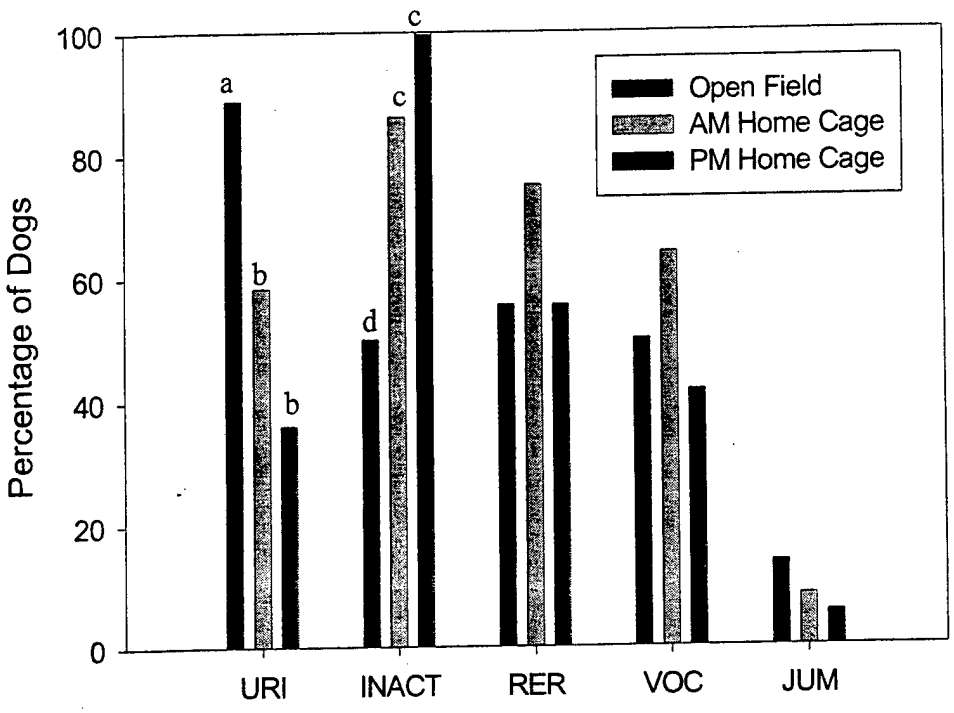
Figure 3. Locomotor activity declined with age in the home cage but not the open field. Graph A plots the mean locomotion (+SE) score for young (n = 16) and aged dogs (n = 36) in the open field. Graph B plots the mean locomotion (+SE) score for young (n = 16) and aged (n = 23) dogs in the home cage for the morning (AM) and afternoon (PM) tests.

Figure 4. Locomotion decreases over the 10 minutes of the open field test but not the home cage test. The mean locomotion score for each minute of the 10-minute test is plotted for young (n = 16) and aged (n = 23) dogs in the open field (OF) and morning (AM) home cage environments.

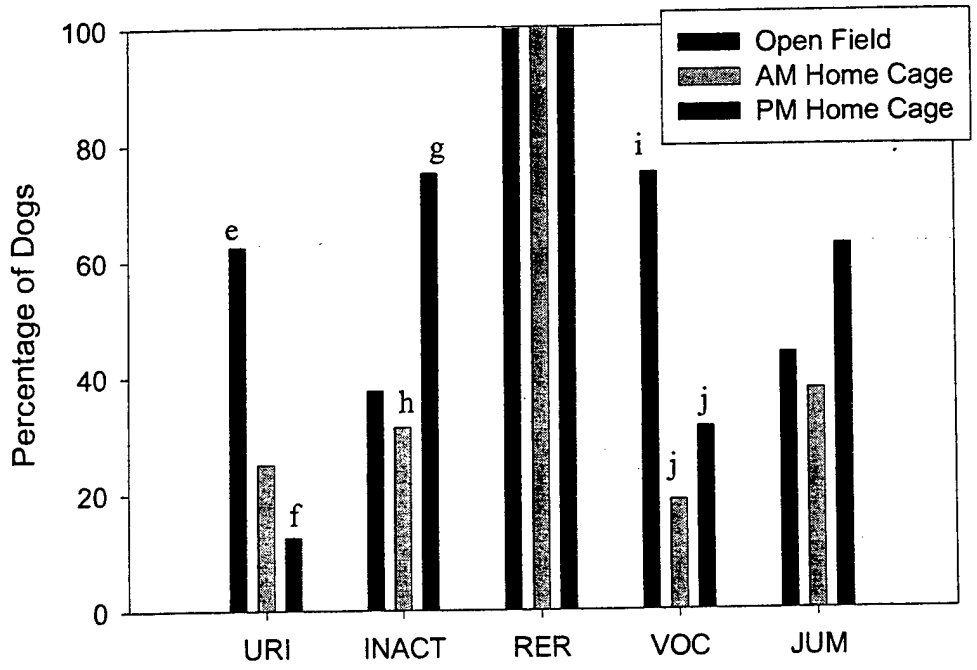
Figure 5. Aged dogs housed alone exhibit higher levels of locomotion than those housed with a kennel mate in the home cage but not the open field. Mean locomotion (+SE)

score for aged dogs housed alone ($\underline{n}$ = 13) and with a kennel mate ($\underline{n}$ = 23) are plotted for the open field (OF) and morning (HCAM) and afternoon (HCPM) home cage tests.

Aged


Percentage of Dogs
100
80
60
40
20
0
Open Field
AM Home Cage
PM Home Cage
a
b
b
d
c
c
URI
INACT
RER
VOC
JUM

Young


Percentage of Dogs
100
80
60
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20
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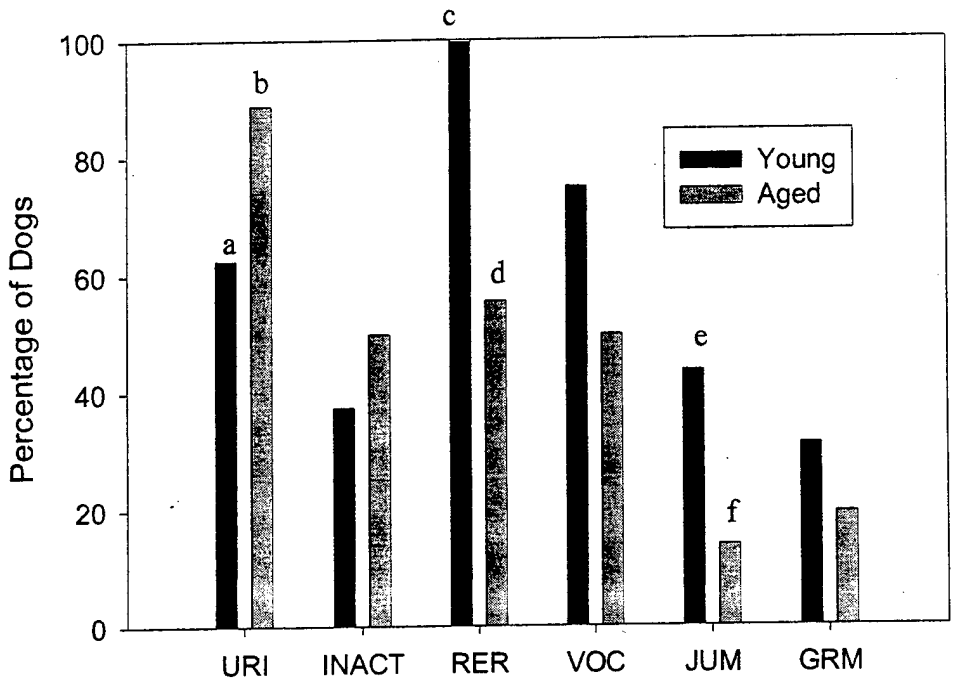
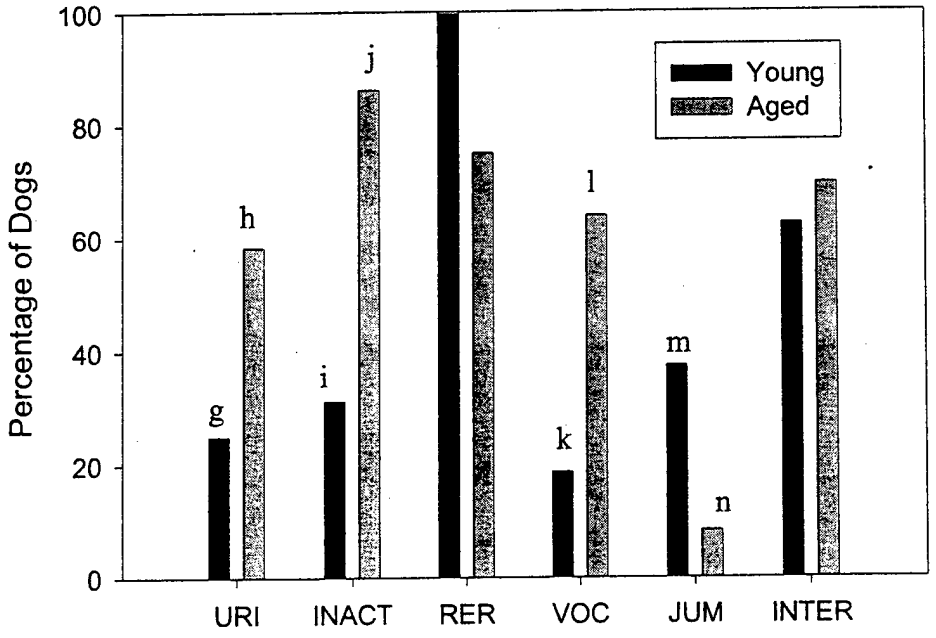
Open Field
AM Home Cage
PM Home Cage
e
f
h
g
i
j
j
URI
INACT
RER
VOC
JUM

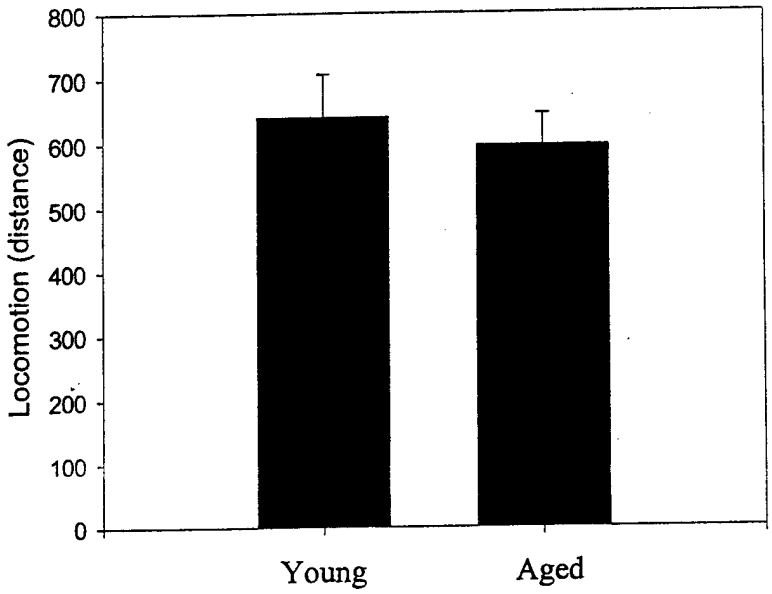
Open Field



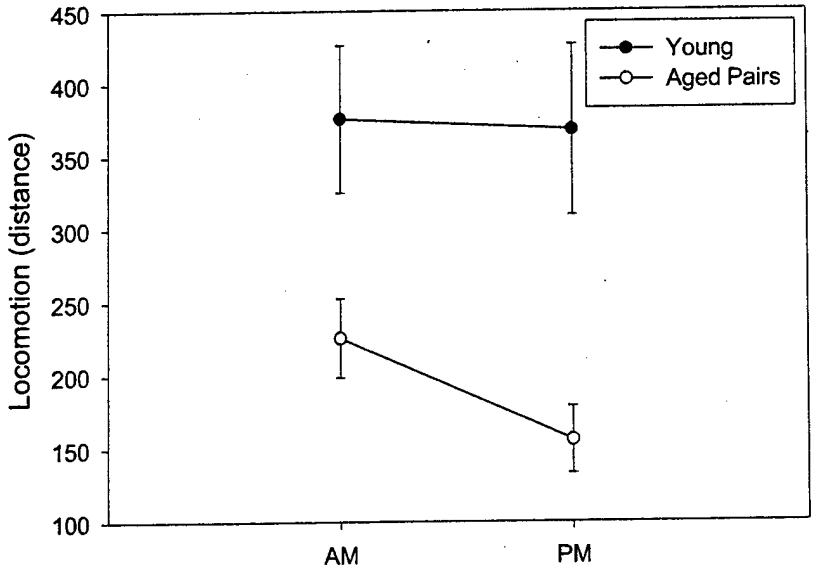
AM Home Cage

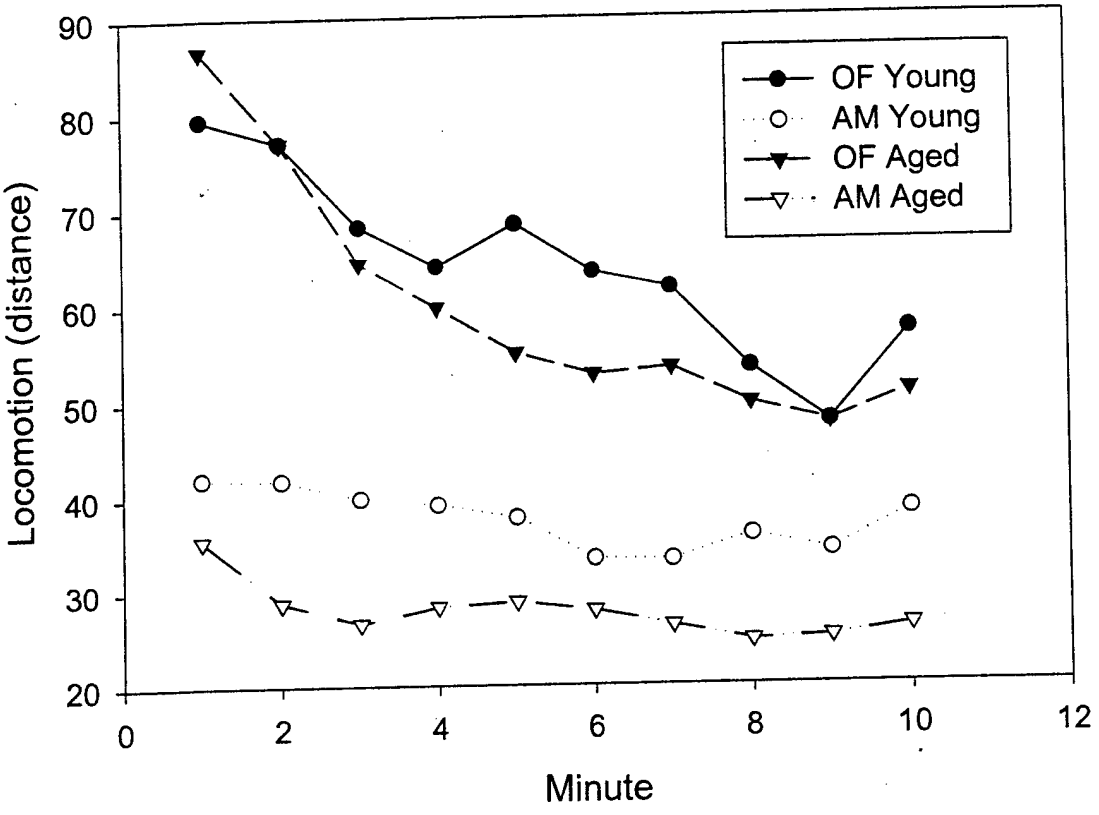


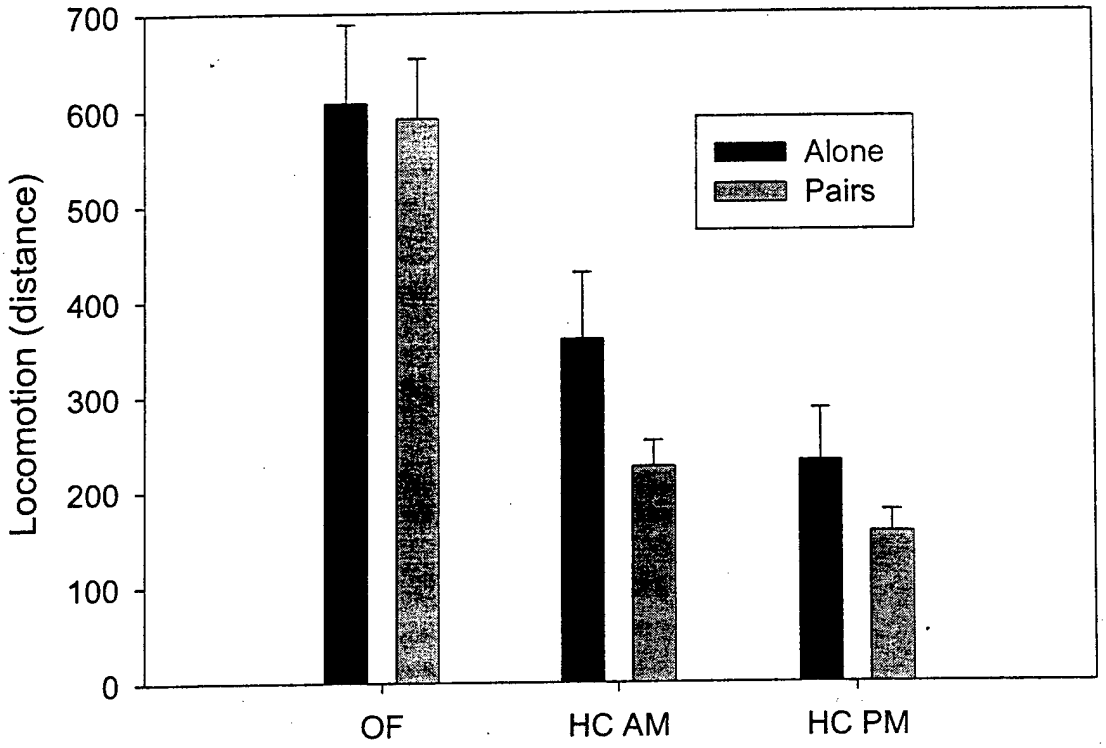
A


800
700
600
500
400
300
200
100
0
Locomotion (distance)
Young
Aged

B


450
400
350
300
250
200
150
100
Locomotion (distance)
Young
Aged Pairs
AM
PM


90
80
70
60
50
40
30
20
Locomotion (distance)
0
2
4
6
8
10
12
Minute
OF Young
AM Young
OF Aged
AM Aged


700
600
500
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300
200
100
0
Locomotion (distance)
Alone
Pairs
OF
HC AM
HC PM

Journal of Neurochemistry, 2002, **82**, 375–381

Oxidative damage increases with age in a canine model of human brain aging

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Abstract

We assayed levels of lipid peroxidation, protein carbonyl formation, glutamine synthetase (GS) activity and both oxidized and reduced glutathione to study the link between oxidative damage, aging and β-amyloid (Aβ) in the canine brain. The aged canine brain, a model of human brain aging, naturally develops extensive diffuse deposits of human-type Aβ. Aβ was measured in immunostained prefrontal cortex from 19 beagle dogs (4–15 years). Increased malondialdehyde (MDA), which indicates increased lipid peroxidation, was observed in the prefrontal cortex and serum but not in cerebrospinal fluid (CSF). Oxidative damage to proteins (carbonyl formation) also increased in brain. An age-dependent decline in GS activity, an enzyme vulnerable to oxidative damage, and in the level of glutathione (GSH) was observed in the prefrontal cortex. MDA level in serum correlated with MDA accumulation in the prefrontal cortex. Although 11/19 animals exhibited Aβ, the extent of deposition did not correlate with any of the oxidative damage measures, suggesting that each form of neuropathology accumulates in parallel with age. This evidence of widespread oxidative damage and Aβ deposition is further justification for using the canine model for studying human brain aging and neurodegenerative diseases.

Keywords: dog, glutathione, glutamine synthetase, malondialdehyde, protein carbonyls, senile plaques.

J. Neurochem. (2002) **82**, 375–381.

The human brain has one of the highest respiratory rates of any tissue and generates oxidative damage that progressively increases over time (Ames *et al.* 1993). Non-dividing cells, such as neurons, are particularly vulnerable to cumulative oxidative damage because they survive for decades. The generation of oxidants leads to damage to proteins, lipids and nucleotides, which may contribute significantly to neuron dysfunction and degeneration associated with aging and neurodegenerative diseases (Liu and Mori 1999; Floyd *et al.* 2001).

Oxidative damage is problematic for a number of reasons. First, lipid peroxidation leading to the formation of malondialdehyde (MDA) can cross-link proteins and form adducts with nucleic acid bases (Esterbauer *et al.* 1991). Second, the accumulation of oxidatively modified proteins disrupts cellular function either by a loss of catalytic ability or by an interruption of regulatory pathways (Stadtman and Levine 2000). Third, oxidatively modified proteins may become cross-linked and resistant to degradation, which can lead to further aggregation within or around neurons (Berlett and Stadtman 1997). Thus, as with the age-associated increases in the production of oxidants, oxidative damage to proteins and lipids also rises with age in rodent and human brain (Beckman and Ames 1998).

Received February 22, 2002; revised manuscript received April 4, 2002; accepted April 8, 2002.

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Abbreviations used: Aβ, amyloid beta; AD, Alzheimer's disease; APP, amyloid precursor protein; CSF, cerebrospinal fluid; DAB, 3,3'-diaminobenzidine; GS, glutamine synthetase; GSH, glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; TBS, Tris-buffered saline.

Oxidative damage may also play a critical role in the development of neuropathology in the age-associated neurodegenerative disease, Alzheimer's disease (AD) (Markesbery 1997). The AD brain, when compared with non-demented elderly controls, shows significantly higher levels of oxidative damage to proteins (Smith *et al.* 1991) and lipids (Markesbery and Lovell 1998). This may be due to the deposition and accumulation of β-amyloid (Aβ) protein in the form of senile plaques, which is one of the hallmark features of the disease (Mirra *et al.* 1991). The amyloid precursor protein (APP), from which Aβ is proteolytically cleaved (Kang *et al.* 1987), is also vulnerable to oxidative damage and exposing APP to metabolic stress favors the production of amyloidogenic fragments (Gabuzda *et al.* 1994; Multhaup 1997). Transgenic mice overexpressing mutant human APP (Tg2576) exhibit a rise in oxidative damage to lipids prior to overt Aβ deposition, which provides further evidence of oxidative damage being an early event (Pratico *et al.* 2001).

Establishing a link between Aβ and oxidative damage in rodent brain is hindered by the lack of natural age-associated Aβ deposition. In human brain, studies are further complicated by the presence of neurofibrillary tangles, which are another potential contributor to AD (Braak and Braak 1991). Like humans, with increasing age, canines naturally accumulate deposits of Aβ in the brain (Wisniewski *et al.* 1990; Head *et al.* 2000). Further, canines and humans share the same Aβ sequence (Johnstone *et al.* 1991), and also first show deposits of the longer Aβx-42 species followed by the deposition of Aβx-40 (Cummings *et al.* 1996c). The extent of Aβ has also been linked to cognitive dysfunction in canines (Cummings *et al.* 1996b; Head *et al.* 1998) but little information concerning oxidative damage to proteins or lipids has been reported. Unlike humans, aged canines develop extensive Aβ in the absence of neurofibrillary tangle formation (Cummings *et al.* 1996a). Thus, the purpose of the current study was to determine whether multiple measures of oxidative damage rise with age and if the presence of Aβ further increased oxidative damage.

Materials and methods

Subjects

The subjects were 19 beagle dogs, from 4.5 to 15.3 years in age, from a colony at the Lovelace Respiratory Research Institute in Albuquerque, New Mexico. All dogs were bred and reared in the same environment throughout their lives and provided with an identical diet. Eight dogs were male, 11 were female and all were reproductively intact. Animals over the age of 6 years had been included in previous non-invasive experiments involving the respiratory system. Animals were maintained with a kennelmate in indoor/outdoor kennels and had free access to water. Dogs were provided with Wayne Mini Laboratory Dog Diet 8759 once daily (Teklad Pioneer Laboratory Diets, Madison, WI, USA). All animals were administered a full physical and neurological examination and none showed neurological, musculoskeletal, or physical abnormalities justifying exclusion from the study. Animals were killed in a method consistent with approved protocols. After removal of the brains, alternating 2-cm thick coronal sections were post-fixed in paraformaldehyde or snap-frozen and stored at – 70°C. CSF and serum samples were collected in red top Vacutainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ, USA), aliquoted and frozen at – 70°C.

Aβ measurements

Paraformaldehdye-fixed tissue blocks from the prefrontal cortex were sectioned at 50 microns using a vibratome. After several washes in 0.1 M Tris-buffered saline (TBS), pH 7.5, sections were pretreated with 90% formic acid for 4 min and then in 3% H_2O_2 in 10% methanol for 30 min to block endogenous peroxidase activity. Sections were subsequently washed in TBS with 0.1% Triton X-100 (TBS-A) and then blocked for 30 min in TBS-A with 3% bovine serum albumin (TBS-B). Samples were incubated overnight at room temperature in anti-Aβ1–16 (6E10; 1 : 5000; Senetek PLC, Maryland Heights, MO, USA). Following two washes in TBS-A and a wash in TBS-B, sections were incubated in biotinylated anti-mouse IgG and then in avidin biotin complex (ABC)(Vector Laboratories, Burlingame, CA, USA). Aβ was visualized using 3,3′-diaminobenzidine (DAB, Vector Laboratories). The extent of Aβ deposition was subsequently quantified using image analysis techniques as described previously (Head *et al.* 1998). Aβ load measures represent the average area occupied by positive Aβ immunostaining from 525 × 410 μm fields in each individual.

MDA assay

Samples from the prefrontal cortex, serum and cerebrospinal fluid (CSF) of 19 canines were used. Frozen prefrontal cortex was prepared in 10 vol of homogenizing buffer [100 mM Tris, pH 8.0, 100 mM NaCl, 20 mM EDTA with proteinase inhibitors (leupeptin 0.5 μg/mL, apoprotonin 0.5 μg/mL, pepstatein 0.7 μg/mL)]. The presence of EDTA was intended to reduce the potential for Fe oxidation. Phenylmethylsulfonyl fluoride was added at 40 μg/mL just before homogenizing. The protein-bound MDA was hydrolyzed with H_2SO_4. MDA was converted to a stable derivative using pentafluorophenyl hydrazine at room temperature. The derivative was extracted with isooctane and detected with a Hewlett Packard 5890 Series II gas chromatograph interfaced to a 5989 mass spectrometry system equipped with a Agilent Technologies (Wilmington, DE, USA) DBWAX capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness) in the negative chemical ionization mode (Liu *et al.* 1997). The results were indexed to protein, which was measured using a microtiter plate assay with bicinchoninic acid (BCA) kit from Pierce (Rockford, IL, USA).

Protein carbonyl assay

Frozen prefrontal cortex homogenates were used to measure the protein carbonyl content by labeling protein hydrazone derivatives using 2,4-dinitrophenylhydrazide (DNPH) according to the method of Levine *et al.* (1994).

GS activity

GS activity in the prefrontal cortex was determined using the technique described by Rowe *et al.* (1970). Corrections were made

for non-specific glutaminase activity by comparing total activity in the presence and absence of adenosine diphosphate and arsenate.

Glutathione analysis

Reduced (GSH) and oxidized (GSSG) glutathione was measured by high-performance liquid chromatography (HPLC) as described by Reed *et al.* (1980). Briefly, tissue was mixed with perchloric acid [10% (w/v), final concentration] and the samples were spun for 1 min at 13 000 rpm in a microcentrifuge to remove denatured debris. An aliquot of the supernatant was added to 100 μL of 1 M Trizma Base buffer (pH 8), followed by the addition of 100 μL of 40 mM fresh aqueous iodoacetic acid (4 μmol). The reaction mixture was brought to pH 8 with $NaHCO_3$ and dinitrophenyl derivatives were made by addition of 500 μL of 2,4-dinitrofluorobenzene (1.5% [v/v] in absolute ethanol) and 100–200 μL of K_2CO_3. The resultant derivatives were separated on a 10-μm Ultrasphere-amine column (4.6 mm × 25 cm) using a Waters HPLC system (Waters, Milford, MA, USA).

Data analysis

Regression analyses, bivariate and partial correlations, and independent *t*-tests were used to determine the role of age and Aβ pathology on the extent of lipid peroxidation, protein oxidation, glutamine synthetase activity, and glutathione levels. All statistics were conducted using SPSS software and an alpha level of 0.05.

Results

Age-dependent increases in oxidative damage

T-tests revealed no significant sex differences in any of the markers of oxidative damage nor Aβ loads. As illustrated in Fig. 1(a and b), MDA levels in serum ($F_{1,16} = 12.10$, $p < 0.003$) and in the prefrontal cortex ($F_{1,17} = 14.16$, $p < 0.002$) progressively increased with age. CSF levels of MDA did not increase with age (Fig. 1c). Protein carbonyl

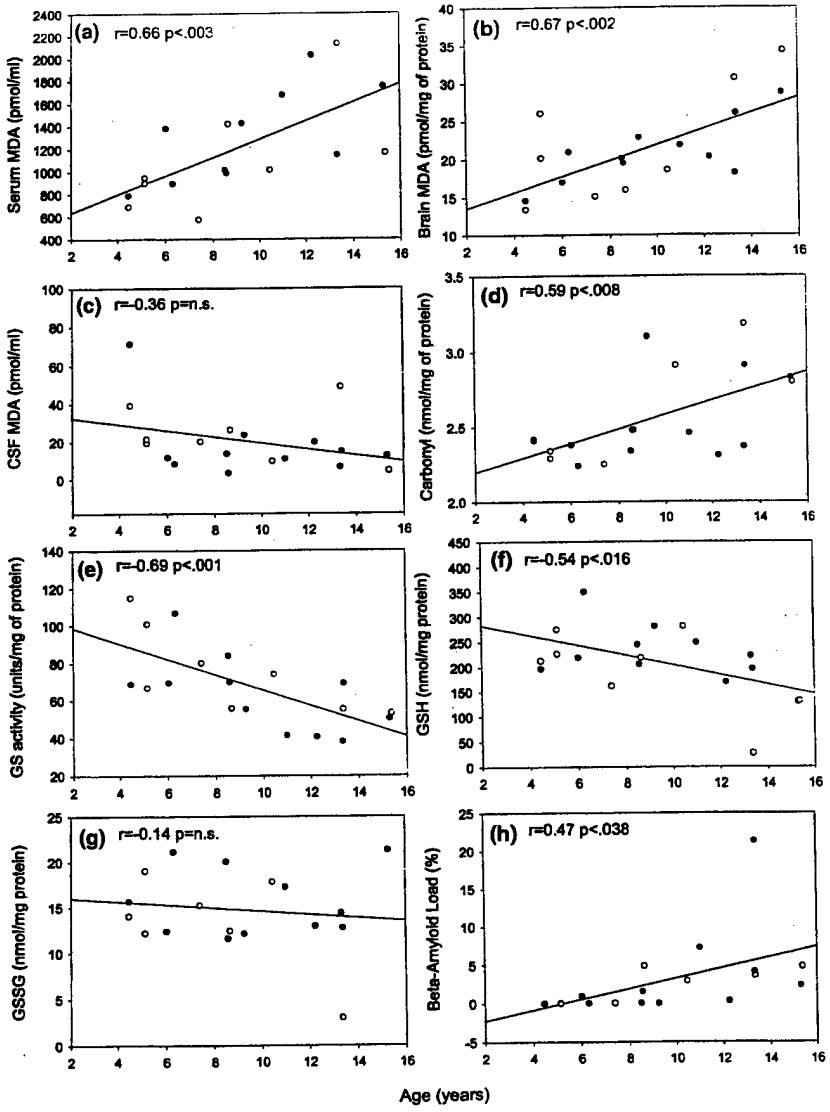

Fig. 1 Individual oxidative damage markers are plotted as a function of age. Progressive and significant increases in serum (a) and brain (b) but not CSF (c) MDA level. A similar parallel increase was observed in protein carbonyl (d) formation. Decreases in GS activity (e) and GSH (f) also occurred with increasing age. However, no consistent age effects were observed in GSSG (g). As reported previously, the extent of Aβ deposition in the prefrontal cortex increased over the age of 8 years (h). ●, Females; ○, males.

formation also increased as a function of age in canines but showed increasing individual variability in older animals ($F_{1,18} = 8.98$, $p < 0.008$; Fig. 1d). The most pronounced increases in individual variability occurred after 8 years of age. In parallel with increased oxidative damage to proteins, glutamine synthetase activity decreased progressively with age ($F_{1,18} = 15.61$, $p < 0.001$; Fig. 1e). The antioxidant GSH was reduced in aged animals ($F_{1,17} = 7.13, p < 0.016$; Fig. 1f) but not GSSG ($F_{1,17} < 1$, $p =$ n.s.; Fig. 1g). The ratio of oxidized GSH to total GSH showed significant age-dependent increases ($r = 0.519$, $p < 0.023$). As shown in Fig. 1(h), increased oxidative damage to lipids and proteins was also accompanied by age-dependent increases in Aβ load ($F_{1,18} = 5.77$, $p < 0.028$). One aged animal had significantly more extensive Aβ deposition than all the other animals (Aβ load = 21.4%). The regression analysis was repeated while eliminating this potential outlier and the regression analysis remained significant ($F_{1,17} = 10.36$, $p < 0.005$). Of all these variables, a multiple stepwise regression analysis that also included sex as a variable indicates that the best predictors of age are either GS alone ($r^2 = 0.48$) or GS and brain MDA ($r^2 = 0.68$). If all variables are included in the multiple regression analysis (GS, MDA, carbonyls, GSH, GSSG, sex and Aβ load) then $r^2 = 0.83$ ($F_{6,12} = 9.58, p < 0.001$), suggesting that Aβ and measures of oxidative damage combine to account for over 83% of the variance in age.

Association between oxidative damage and extent of Aβ

To determine whether animals positive for Aβ ($n = 11$; 8.58–15.37 years) have significantly higher levels of brain oxidative damage than animals without detectable Aβ ($n = 9$; 4.47–9.26 years), a partial correlation coefficient was calculated that corrected for age. The correction was necessary because both Aβ and measures of oxidative damage increased as a function of age. The extent of Aβ did not correlate with any of the measures of oxidative damage nor with endogenous antioxidant levels (Table 1). A second analysis involved selecting only those animals with prefrontal Aβ and these animals also showed no significant association between level of Aβ and oxidative damage.

Inter-sample correlations

Serum level of MDA was significantly correlated with brain level of MDA ($r = 0.51, p < 0.036, n = 17$; Fig. 2). Table 1 also illustrates that many of the different markers of oxidation were intercorrelated. In particular, serum MDA was correlated with brain MDA, protein carbonyl formation and glutamine synthetase activity. Brain MDA levels were correlated with the extent of protein carbonyl formation. GSH and GSSG were highly intercorrelated with GSH levels being associated with protein carbonyl formation. Because age was a significant contributor to almost all of these measures, a second set of correlations was calculated that partialled out the age of individual animals. GSH remained

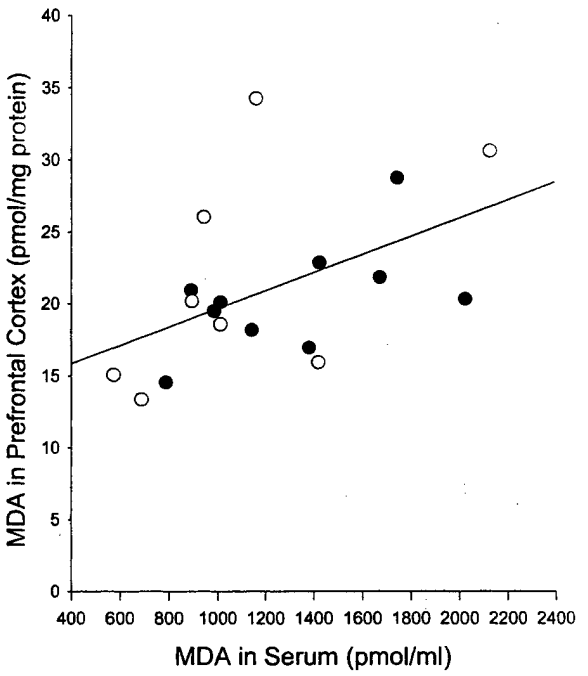

Fig. 2 Individual MDA levels in the serum are plotted as a function of MDA levels in the prefrontal cortex. Higher serum levels of MDA are significantly correlated with higher prefrontal cortex levels of MDA ($r = 0.51$, $p < 0.036$, $n = 17$). ●, Females; ○, males.

Table 1 Oxidative stress marker and Aβ intercorrelations

	CSF MDA	Brain MDA	Brain carbonyls	Brain GS	Brain GSH	Brain GSSG	Brain amyloid
Serum MDA	0.005	0.49*	0.50*	− 0.69**	− 0.47	− 0.38	0.45
CSF MDA	–	− 0.23	0.11	0.12	− 0.30	− 0.32	− 0.18
Brain MDA	–	–	0.60**	− 0.38	− 0.47	− 0.26	− 0.33
Brain carbonyls	–	–	–	− 0.34	− 0.47*	− 0.43	0.40
Brain GS	–	–	–	–	0.43	0.35	− 0.26
Brain GSH	–	–	–	–	–	0.59**	− 0.16
Brain GSSG	–	–	–	–	–	–	− 0.18

significantly correlated with GSSG ($r = -0.64$, $p < 0.006$) but all other correlations were not significant.

Discussion

This is the first report of age-associated increases in oxidative damage to lipids and proteins in the canine model of human brain aging. Second, multiple measures of oxidative damage were found to increase with age in the canine, but only two (GSH and GSSG) were significantly correlated after correcting for age. Last, we also looked at the relationship between Aβ and oxidative damage to determine if animals with significant Aβ also exhibited higher levels of oxidative damage. In contrast to previous reports in humans and in transgenic mice, we observed no significant correlation between the extent of Aβ and oxidative damage.

Lipid peroxidation in the brain progressively rises with age in canines, which is consistent with previous reports in rodents (Beckman and Ames 1998). MDA level may not only reflect the extent of lipid peroxidation but also oxidative susceptibility of high- and low-density lipoproteins in serum (Khalil *et al.* 1998). In addition, age-dependent increases in serum MDA levels may be due to parallel increases in serum protein levels (Lowseth *et al.* 1990). MDA can cross-link protein side-chains, slow protein degradation and subsequently reduce protein turnover (Janero 1990); the latter has been reported in rats and humans (reviewed in Ramsey *et al.* 2000). Thus, the mechanisms underlying the age-dependent increase in MDA in serum may be linked to a number of factors reflecting increased oxidative damage during aging.

The actual source of MDA is difficult to establish because aldehydes can reach targets distant from the original site of oxidation (Esterbauer *et al.* 1991). An alternative, and more likely explanation, is that serum MDA is derived from lipid damage in both central and peripheral systems. Experiments used to modify peripheral oxidative stress with dietary antioxidants result in both improved peripheral and central measures (Cantuti-Castelvetri *et al.* 2000). Further, the link between peripheral and central measures of lipid peroxidation reported in the current study suggests that serum MDA measures may be a useful endpoint measure to monitor antioxidant interventions *in vivo*.

The suggestion of increasing oxidative damage to lipids with age was supported in both the brain and in serum, but not in CSF. The correlation between brain and serum MDA level suggests parallel damage occurs both centrally and systemically. The lack of accumulation of MDA in CSF occurs despite reports that larger proteins accumulate with age due to reduced turnover (Preston 2001). However, a correction for the extent of MDA per mg/protein in CSF was not conducted in this study and could lead to different results. In human CSF studies, lipid peroxidation products also show no age-dependency (Montine *et al.* 1999). Although the number of studies using CSF samples in studies of human aging are limited, the results of the current experiments suggest that CSF samples may not be optimal for studies of oxidative damage.

Canines exhibit an age-dependent increase in protein carbonyl formation and similar results have been reported for aged rodents, humans, and also for patients with AD (Carney *et al.* 1991; Smith *et al.* 1991; Hensley *et al.* 1995). Carbonyls can be formed by the reaction of proteins with aldehdyes, like MDA. The significant correlation between carbonyl formation and MDA suggests that the reaction between MDA and protein side-chains may be a significant source of carbonyls in the aged brain (Berlett and Stadtman 1997). Higher levels of carbonyl formation may also be due to age-dependent changes in the rate of oxidized protein degradation (Stadtman and Levine 2000). This is plausible because cross-linked proteins and lipid peroxidation products are more resistant to proteolysis, which in turn depends upon effective proteosome function (Friguet *et al.* 2000).

A similar series of conclusions can be drawn from the results of assays for GS activity in the current study. GS activity is sensitive to inactivation by oxidizing agents and is frequently used as a measure of oxidative damage (Schor 1988). Reduced GS activity may be linked to alterations in the glutamate cascade and impaired conversion of glutamate into glutamine within astrocytes, thus potentially disrupting both neuronal and glial function (Hertz *et al.* 1999). Further, the reductions in GS activity also suggest that not only are neuron populations but also glial cells are vulnerable to oxidative damage.

The GSH/GSSG ratio is a key parameter of cellular thiol redox status, and provides a measure for the presence of significant oxidative damage. In aged canines, GSH decreased progressively with age, which is consistent with previous reports in rodents (Sohal *et al.* 1995; Ohkuwa *et al.* 1997) and humans (Samiec *et al.* 1998). The altered GSH/GSSG ratio was predominantly due to lower GSH levels; our results further suggest that a loss in GSH synthetic capacity was responsible for the overall decline in the GSH/GSSG ratio. GSH synthesis is governed by cysteine availability as well as by transcriptional control of γ-glutamylcysteine synthetase; therefore, the aged canine brain may be compromised in either one or both of these critical parameters for GSH biosynthesis. Overall, the results of the current study combined with a previous report of decreased levels of other antioxidant enzymes (i.e. superoxide dismutase; Kiatipattanasakul *et al.* 1997) in aged canine brains are consistent, and suggest that antioxidant defenses are reduced with age in the canine.

The link between age and oxidative damage to lipids in other species has been reported to vary as a function of extent of Aβ deposition. Evidence in support of this suggestion derives from the results of a recent study of mice transgenic for mutant human APP (Tg2576) that deposit Aβ as a function of age and show significant increases in lipid

peroxidation with age, which was correlated with the extent of Aβ (Pratico *et al.* 2001). The AD brain is characterized by extensive Aβ deposition and also shows significantly higher lipid peroxidation levels than age-matched control brains that exhibit less Aβ (Markesbery 1997; Pratico and Delanty 2000). Evidence from the current study contrasts with these previous reports because individual levels of Aβ do not correlate with any measure of oxidative damage. The quantification of Aβ did not use stereology-based methods due to the lack of availability of the entire prefrontal cortex for unbiased sampling. Thus, although the absolute Aβ load may be arbitrary, the sampling and imaging techniques were identical for all animals and involved the random capture of fields within the prefrontal cortex. Another potential explanation for the lack of association between Aβ and oxidative damage pertains to the quantification method. Sandwich enzyme-linked immunosorbant assays in future experiments may yield more sensitive measures of both soluble and insoluble Aβ1–42 and Aβ1–40. It is important to note that canine plaques do not develop into thioflavine-S positive β-pleated sheets nor are associated with neuritic elements. This may also be a significant difference leading to the lack of clear association between Aβ and markers of oxidative damage in the current study. However, both oxidative damage and Aβ rise progressively with age suggesting that these events develop in parallel. Oxidative damage and Aβ may be interrelated and one may enhance the other; Aβ can cause oxidative damage and oxidative damage to APP can enhance Aβ production (Gabuzda *et al.* 1994; Multhaup 1997; Behl 1999).

The current study focused upon the prefrontal cortex in canines for several reasons. First, aged canines show deficits on cognitive tasks sensitive to frontal-lobe function (Milgram *et al.* 1994). Second, based upon logistic regression analyses, this region of cortex is the site of the earliest and most predominant Aβ deposition with age (Head *et al.* 2000). Last, prefrontal Aβ is linked to impaired cognitive test scores on the same tasks sensitive to prefrontal aging (Head *et al.* 1998). The result of the multiple regression analysis reported in the current study further suggests that including markers of oxidative damage in addition to the extent of Aβ is a better predictor of age than either measure alone.

In summary, the present study demonstrates progressive age-dependent increases in the level of lipid peroxidation and protein oxidation in canines. GSH measures also indicate a shift in the balance towards lower levels of endogenous antioxidants being available to reduce the impact of free radicals. Further, it may be possible to predict brain levels of lipid peroxidation based upon non-invasive measures of serum levels. Determining whether Aβ deposition or oxidative damage is the first degenerative event in the development of pathological aging is difficult based on correlation studies. To test the hypothesis that oxidative damage leads to, follows from, or is possibly independent of Aβ deposition requires an intervention study that can reduce one or the other form of pathology. The canine model complements existing animal models and may also provide novel insights into the mechanisms underlying brain aging and neurodegenerative diseases in humans.

Acknowledgements

This project was sponsored by funds provided by the National Institute of Aging (Grant AG12694 to CWC) and by the U. S. Department of the Army, Contract No. DAMD17-98-1-8622 (to BAM). The content of the information does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred. The investigators adhered to the 'Guide for the Care and Use of Laboratory Animals' prepared by the Committee on Care and Use of Laboratory Animals of the Laboratory Animal Resources, National Research Council (NIH Publication no. 86–23, Revised 1985. Research was conducted in facilities fully accredited by the AAALACI. Additional funding was provided by the National Institute of Environmental Health Sciences (Grant ES01896), the National Institute on Aging (AG17140), the Ellison Foundation (SS0422-99), the Department of Energy (DE-FG03–00ER6Z943), and the National Foundation for Cancer Research (M2661) to B.N.A and by an NIA grant (AG17141) to T.M.H. The authors appreciate the technical assistance provided by Theresa Dinise.

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Neurobiology of Aging 23 (2002) 809–818

NEUROBIOLOGY OF AGING

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Brain aging in the canine: a diet enriched in antioxidants reduces cognitive dysfunction

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Received 26 February 2002; received in revised form 19 April 2002; accepted 22 April 2002

Abstract

Animal models that simulate various aspects of human brain aging are an essential step in the development of interventions to manage cognitive dysfunction in the elderly. Over the past several years we have been studying cognition and neuropathology in the aged-canine (dog). Like humans, canines naturally accumulate deposits of β-amyloid (Aβ) in the brain with age. Further, canines and humans share the same Aβ sequence and also first show deposits of the longer Aβ1–42 species followed by the deposition of Aβ1–40. Aged canines like humans also show increased oxidative damage. As a function of age, canines show impaired learning and memory on tasks similar to those used in aged primates and humans. The extent of Aβ deposition correlates with the severity of cognitive dysfunction in canines. To test the hypothesis that a cascade of mechanisms centered on oxidative damage and Aβ results in cognitive dysfunction we have evaluated the cognitive effects of an antioxidant diet in aged canines. The diet resulted in a significant improvement in the ability of aged but not young animals to acquire progressively more difficult learning tasks (e.g. oddity discrimination learning). The canine represent a higher animal model to study the earliest declines in the cognitive continuum that includes age associated memory impairments (AAMI) and mild cognitive impairment (MCI) observed in human aging. Thus, studies in the canine model suggest that oxidative damage impairs cognitive function and that antioxidant treatment can result in significant improvements, supporting the need for further human studies.

Keywords: Dog; β-Amyloid (Aβ); Mild cognitive impairment

1. Introduction

The brain progressively accumulates oxidative damage and other types of neuropathology that ultimately result in neuronal dysfunction and cognitive decline. A key challenge is to identify mechanisms underlying pathological aging and to develop therapeutics to prevent or slow disease progression. Animal models, including rodents and nonhuman primates, are critical to the success of this research. Over the past several years we have been investigating a novel animal model of human cognitive aging, the aged canine. The advantages of using canines to study brain aging includes the following: (1) canines share many of the same environmental conditions with humans; (2) canines can perform a sophisticated repertoire of complex cognitive behaviors; (3) the brain in aged canines shows many pathological changes common to humans; and (4) neuropathology is significantly associated with cognitive decline.

Our strategy has been to identify brain and behavioral changes that appear with age and to determine if interventions that target proposed underlying cellular pathological mechanisms can improve cognitive function. The proof of principle to determine whether a specific type of neuropathology contributes to cognitive dysfunction is to show that an intervention targeting the proposed mechanism improves function. Of necessity, studies in humans are primarily correlative but help to establish key pathological mechanisms amenable to manipulation. Over time these studies may lead to clinical trials but even if successful it is difficult to determine if the intervention has an effect on brain pathology. In the canine model it is feasible to test interventions and determine the effect they have on the brain. In this review we present an overview of the progress in characterizing the canine model and the effects of antioxidants on cognitive function. The review has three parts:

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PII: S0197-4580(02)00073-8

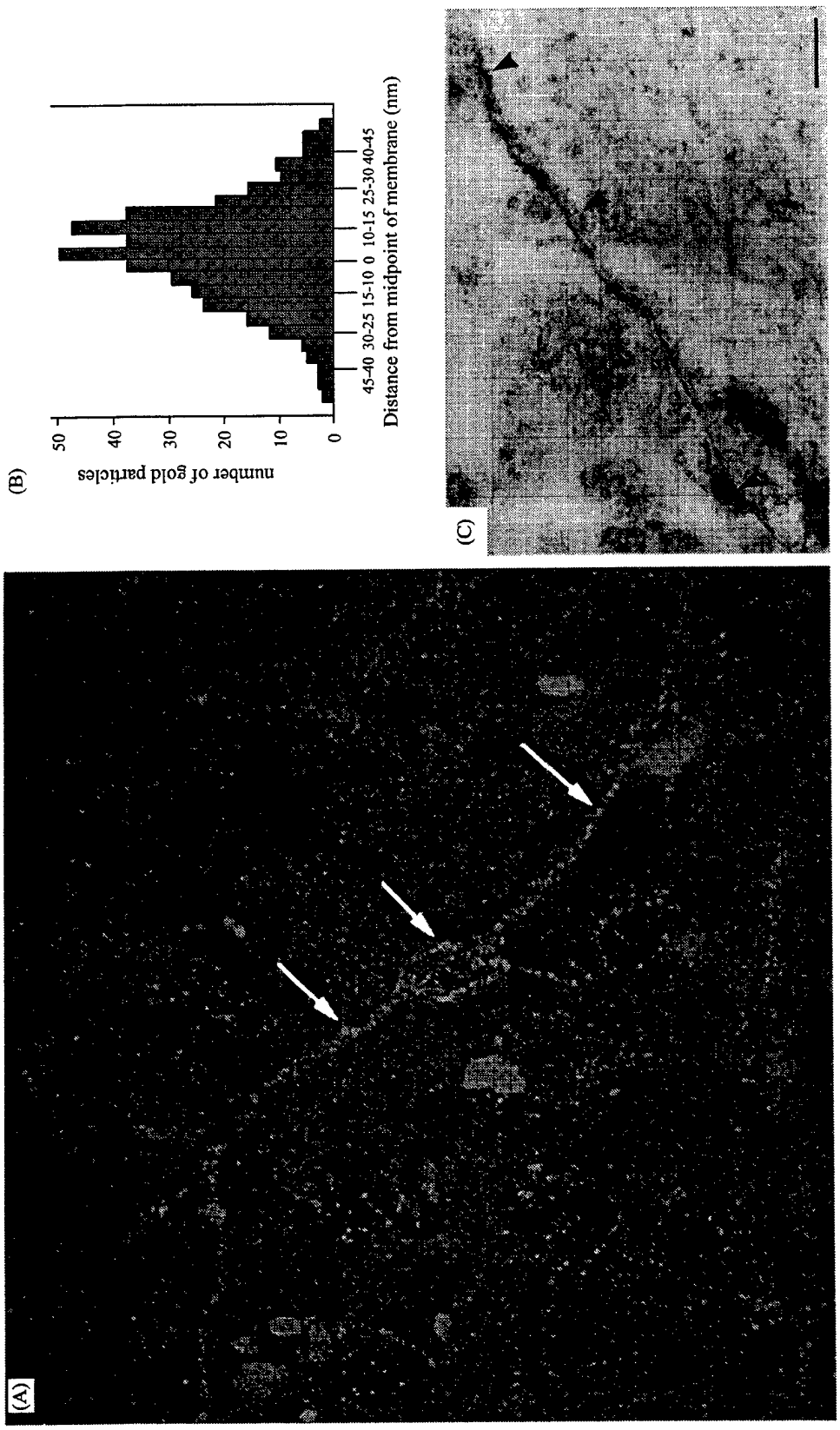

Fig. 1. Aβ is associated with neuronal membranes in the aged canine brain. (A) A confocal image illustrating punctate deposits of Aβ1–42 along the cell body and processes of a neurons. Arrows indicate punctate regions of concentrated Aβ. (B) The distribution of gold particles ($n = 419$) distributed across the neuronal membrane in an axis perpendicular to the dendritic plasma membrane. The peak of the curve represents the maximum particle density and indicates that Aβ is concentrated along the membranes rather than within the extracellular space. (C) An example of labeled dendritic membranes (arrowheads) used for the analysis in B. Bar = 0.3 μm.

(1) an overview of neuropathology in the aged canine brain; (2) the nature of cognitive dysfunction in the aged canine; and (3) recent results demonstrating the effectiveness of an antioxidant intervention in improving cognitive performance on select tasks that decline with age.

2. Neuropathological features of the aged canine brain

A critical issue is to identify neuropathology that has the greatest functional impact on cognitive decline. The canine brain exhibits several key features observed in the aged human brain. Many of these consistent features are associated with early pathology seen in normal human brain aging, in the brains of individuals with mild cognitive impairment (MCI) and in Alzheimer's disease (AD) patients. In the canine, these features do not develop into the full-blown pathology associated with moderate or severe AD. Thus, the canine serves as a model for early stage pathology [37].

One of the first reports of age-associated neuropathology in canines was in 1914 describing abnormal pyramidal neuron sprouting [45]. In the 1950's, other types of neuropathology were reported including "Alzheimer-like" senile plaques [9,21–23,59,79]. Aged canine brains display a number of morphological signatures similar to those observed in aged human brains including cortical atrophy [70], myelin degeneration in the white matter [24], the accumulation of degraded proteins [7], DNA damage [3,42] and a reduction in endogenous antioxidants [43].

Canines naturally accumulate Aβ in the brain with increasing age [16,19,34,66] (Fig. 1) and form a diffuse type of plaque. The amino acid sequence of canine Aβ is identical to that of human Aβ [39]. In addition, there is clear evidence that Aβ accumulation can also be seen in association with neurons at both the light and electron microscopic level [16,72]. Specifically, Aβ appears to be concentrated within microdomains on the plasma membrane identified by immunogold labeling (Fig. 1). These same microdomains also contain presenilin, which is thought to play a role in cleaving the amyloid precursor protein (APP) leading to Aβ production [37]. This membrane localization may cause early functional changes in neurons that may be detectable at the behavioral level of analysis.

Not all brain regions are equally vulnerable to Aβ pathology; pathology develops in the prefrontal cortex at an earlier age and more consistently than other cortical areas studied, such as the entorhinal or parietal cortex [34]. The occipital cortex accumulates Aβ at a much later age than these other brain regions. This pattern of Aβ accumulation with age in canines parallels that seen in humans [8]. Within the prefrontal cortex, Aβ first appears in deep cortical layers and at later ages, the superficial layers are increasingly affected [67]. In studies of over 150 dog brains, Aβ deposition has not been observed in layer I of cortex, which contrasts with clear evidence of Aβ distribution in this layer of the human brain. On the other hand, a diffuse band of Aβ is observed in the outer molecular layer of the canine hippocampus where plaques are also found in the AD brain.

Another common characteristic between canine and human Aβ is that the predominant species of Aβ is the longer, toxic fragment Aβ 1–42 [18,57,80]. At later ages the shorter, more soluble, fragment Aβ1–40 accumulates in plaques and in blood vessel walls. As with human brain aging, Aβ accumulates within the blood vessel walls of the aged canine brain suggesting that the canine may be a useful model to study Aβ angiopathy [64,74,76,78].

Tangles identical to those seen in the human brain are rare in other species and dogs do not develop mature tangles characterized by paired helical filaments [4,20,29,68,79]. However, it is likely that early tangles are present in aged canine brain, since canine tau also becomes hyperphosphorylated as in aged human brain, but they do not mature into the full phenotype [36,44]. Tau phosphorylation, as detected by the AT8 antibody, increases in the aged brain and thus possibly some of the early features of tangles are present in the aged canine brain [60,77]. The canine provides an opportunity to study the role of Aβ pathology on cognition in the absence of overt tangle formation.

Thus, the rationale for using the canine model to understand the role of Aβ in human brain aging include but are not limited to the following: (1) Aβ is normally deposited with increasing age; (2) the distribution of Aβ as a function of age parallels that of humans; and (3) the sequence in which specific fragments of Aβ are deposited is similar and the protein itself is identical to the human. Further, since Aβ deposits remain diffuse in aged dog brain, the model is well-suited for studying early stage pathology of brain aging/Alzheimer's disease prior to the appearance of other complex variables such as tangle formation.

3. Cognitive dysfunction in aged canines

The advanced learning ability of canines is well known, as evidenced by their use as guides for the blind and as military working dogs. Our research has focused on a single breed, beagles, because longevity varies widely with respect to breed as does the age of onset and extent of Aβ [6]. The average life span of a beagle is 13.6 years but animals that live up to 18 years have been observed [67]. Beagles over the age of 8 years are considered old based upon evidence for reduced cerebrovascular function after this age [50]. However, breed differences in lifespan are substantial and larger breeds typically have shorter lifespans [46].

Learning and memory can be tested systematically in dogs using tasks developed for use in nonhuman primates. In parallel with the human and primate literature, tasks are selected that are sensitive to the function of specific cortical circuits and/or brain regions. All testing is conducted using food rewards, which sufficiently motivate dogs to learn each task. The use of deprivation protocols, which are particularly stressful for aged animals, is unnecessary.

Two main conclusions have evolved from these studies: (1) detecting cognitive dysfunction depends on the cognitive processes engaged, the task used and the relative level of difficulty, and (2) variability in the cognitive abilities of dogs increases with age. Aged dogs are able to learn simple skills, on average, to the same extent as younger dogs [54]. However, individual aged dogs can show pronounced impairments. Simple associative learning, such as visual discrimination (learning that one of two objects covers a food reward), typically remains intact with age [27,32,52,54,75]. Significant impairment is seen, however, on more complex discrimination learning problems, such as size and oddity discrimination learning [32,55]. Similar age differences in visual discrimination learning have been reported in primates [73]. On the other hand, prefrontal-dependent tasks are consistently impaired in aged dogs [54]. One of these age-sensitive visual discrimination tasks is a reversal learning problem. Subsequent to successful attainment of a preset criterion level of response on a visual discrimination task the reward contingencies are reversed and animals must shift from responding to one object to the other. Reversal learning involves response inhibition and the ability to shift strategies, functions that are mediated by the prefrontal cortex [27,75].

In addition to learning ability, memory is also compromised in aged canines. Forms of memory that appear to be age-sensitive include spatial memory (the ability to remember the location of a food reward) and object recognition memory (the ability to recognize an object seen 10–120 s previously) [1,12,35]. The variability in performance of these tasks, however, is extensive. Aged dogs can fall into one of three categories: (1) unimpaired or successful agers; (2) age-impaired; (3) severely impaired. These clusters of aged dogs may be analogous to normal aging, MCI and dementia in humans.

The decline in learning and memory in laboratory studies is also consistent with clinical features observed by veterinarians who have identified a canine cognitive dysfunction syndrome (CDS), based on informant-based questionnaires or checklists [13,65]. CDS is characterized by dogs showing signs in one or more categories that include disorientation, disruptions in activity and sleep, changes in housetraining and alterations in interactions with family members. In a survey of 26 owners of aged dogs, common complaints were destructive behaviors, inappropriate urination or defecation and excessive vocalization in older animals. Data from a study at UC Davis Veterinary College involved interviews with owners of 180 dogs aged 11–16 years whose pets had no illnesses that would account for behavioral signs such as altered social interaction with owners, sleep–wake cycles, and activity levels, housesoiling and disorientation. In this study, 28% of dogs between the ages of 11 and 12 and 68% of 15–16-year-olds were positive for at least one category. Ten percent of owners of 11–12-year-old dogs and 36% of owners of 15–16-year-old dogs had signs in two or more categories [58].

4. Relationship between age, pathology and behavior in aged canines

Is cognitive dysfunction associated with Aβ neuropathology? Several studies demonstrate a strong and significant association between the extent of Aβ deposition and the extent of cognitive dysfunction in dogs [16,17,32] similar to that reported in the human brain [15] (Fig. 2). This association can be further refined on a brain region basis: for example, Aβ in the prefrontal cortex is correlated with frontal-dependent learning and memory deficits [32]. A recent paper by Colle et al. showed a significant association between behavioral dysfunction in aged dogs and the extent of Aβ deposition [13]. This recent publication, along with previous reports, supports an association between clinical measures of cognitive dysfunction and pathophysiology in aged canine brain.

While the accumulation of Aβ is part of a series of neuropathological events, it is unlikely to be the only contributing factor to cognitive decline. In our view, the basic molecular events in the aging brain form a cascade involving a sequence of feed-forward and feed-back mechanisms that culminate in neuronal dysfunction and Aβ deposition. Oxidative damage probably plays a central and pivotal role in the evolution of this cascade (Fig. 3).

5. Oxidative damage and brain aging

The brain has among the highest respiratory rate of any tissue and generates oxidative damage that progressively increases over time [2]. Neurons, are particularly vulnerable to cumulative oxidative damage because they are nondividing cells and survive for decades. The generation of oxidants leads to damage to proteins, lipids and nucleotides, which may contribute significantly to neuron dysfunction and degeneration associated with aging and neurodegenerative diseases [25,48]. Oxidative damage may serve as a common mechanism initiating and linking several pathological features of the aging brain. For example, the APP is vulnerable to oxidative damage and metabolic stress favors the production of amyloidogenic fragments [28,56]. Transgenic mice overexpressing mutant human APP (Tg2576) showed increased oxidative damage to lipids prior to overt Aβ deposition, which provides further evidence of oxidative damage being an early event [63]. Aβ is also able to directly generate oxidative damage to lipids and proteins [5,10,11]. According, to this model, antioxidants may have beneficial effects on brain aging at multiple stages.

Oxidative damage to lipids and proteins increase with age in the canine brain [33]. A significant increase in lipid peroxidation, measured by malondialdehyde (MDA) and damage to proteins, measured by carbonyl formation, was observed with age. A significant decline in glutamine synthetase activity, an enzyme vulnerable to oxidative damage and in the level of reduced glutathione (GSH) was observed

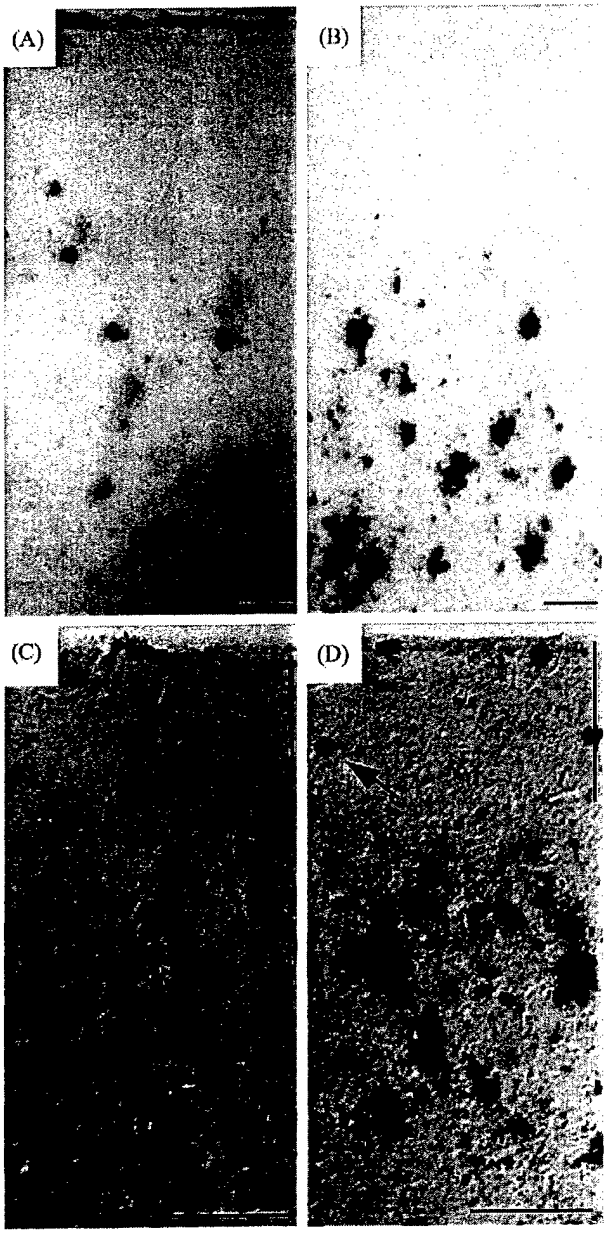

Fig. 2. (A) Aβ immunostaining in the prefrontal cortex of an unimpaired 13-year-old beagle dog takes the form of diffuse senile plaques in layers III–VI. (B) A section from the frontal cortex of a 90-year-old female nondemented control case illustrating a similar pattern of senile plaque deposition as in the aged dog. Note that in both cases, Aβ is distributed in deeper cortical layers. (C) Aβ immunostaining in the prefrontal cortex of a severely impaired 12-year-old beagle dog is extensive and affects layers II–VI. The molecular layer is free of Aβ deposition (indicated by the vertical line). (D) For comparison, a sample of the frontal cortex from an 86-year-old male with Alzheimer's disease shows a parallel extent of Aβ deposition as the dog. Note that diffuse senile plaques are similar in size between the dog and the human. On the other hand, dogs do not develop compact plaques (indicated by arrow in D). Bar = 200 μm.

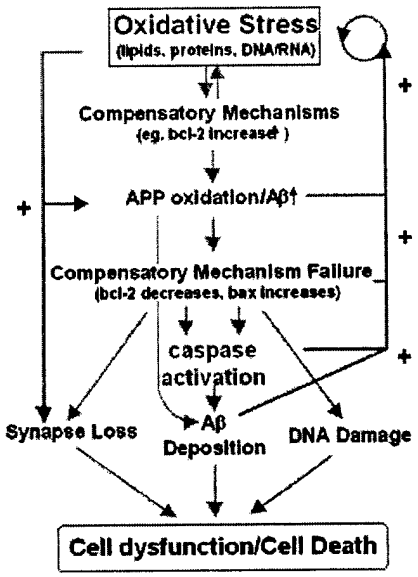

Fig. 3. Oxidation causes damage to lipids, proteins and DNA/RNA. Oxidative stress also induces the expression of APP and can contribute to misprocessing of APP leading to generation of amyloidogenic fragments. The production of Aβ fragments may lead to a loss of compensatory ability (decreased bcl-2, increased bax). All of these factors in turn contribute to more Aβ deposition, possibly synapse loss and DNA damage. Ultimately, the pathways converge and result in neuron dysfunction and/or in some neurons death.

with age. MDA level in serum was a significant predictor of MDA accumulation in the prefrontal cortex (Fig. 4). Thus, the canine brain accumulates oxidative damage and in our model is an early event in the cascade.

Establishing a link between oxidative damage, Aβ and cognitive function in the rodent brain is hindered by the lack of natural age-associated Aβ deposition. In human brain, studies are further complicated by the presence of neurofibrillary tangles. Unlike humans, aged canines develop extensive Aβ in the absence of neurofibrillary tangle formation [18]. The canine brain, therefore, is a simpler model for examining the association between age, oxidative damage, Aβ and cognitive function. Thus, studies in the canine model can complement studies in other animal model systems and provide further insights into human brain aging and neurodegenerative diseases.

6. An antioxidant diet improves learning in the aged canine

Accordingly, we have initiated a series of studies to test the hypothesis that an antioxidant diet can result in improvements in learning and memory and reduce the extent of pathology that accumulates in the aged brain [55]. We have collected extensive data in an ongoing study on learning and memory with treatment but results of the neuropathology

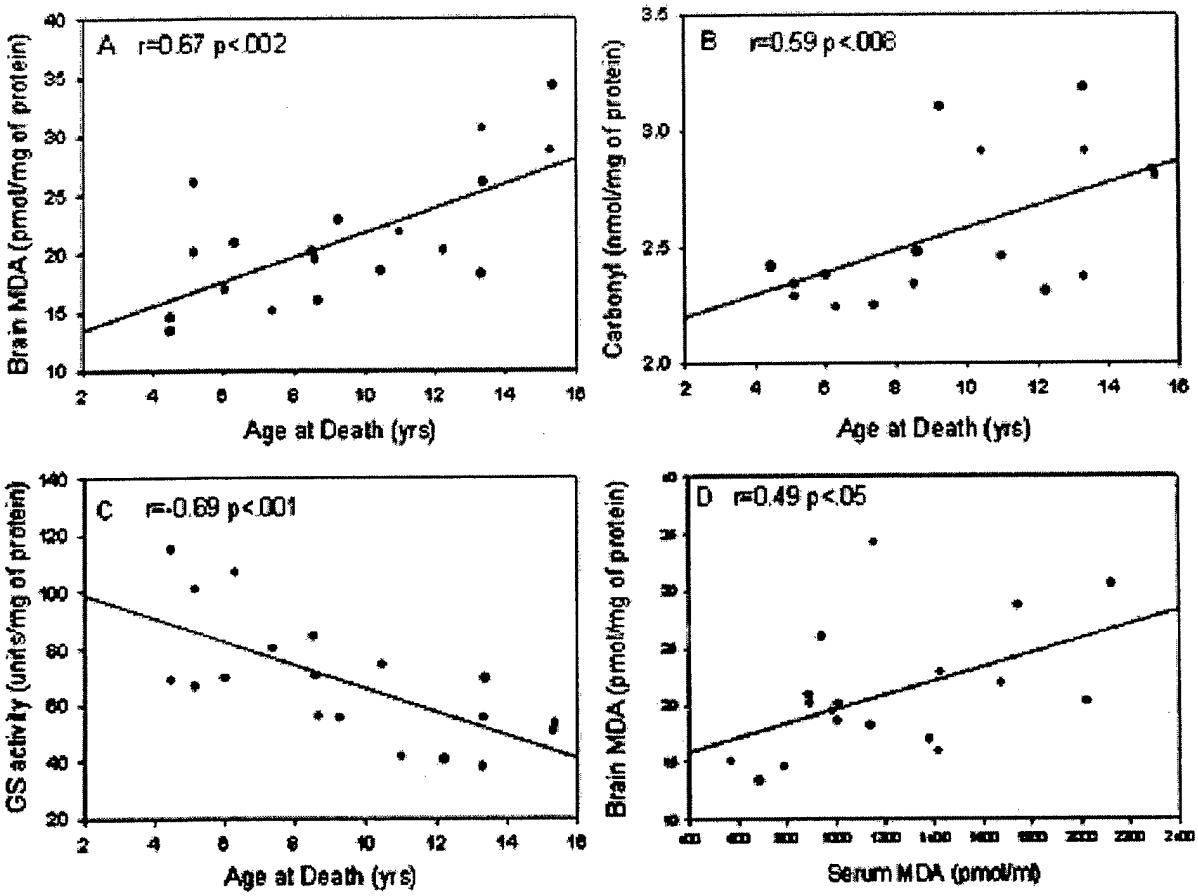

Fig. 4. Individual oxidative damage markers are plotted as a function of age in 19 beagle dogs. Progressive and significant increases in (A) brain malondialdehyde (MDA), and (B) protein carbonyl formation were observed. (C) Decreases in glutamine synthetase (GS) activity were found. (D) Individual MDA levels in the serum are plotted as a function of MDA levels in the prefrontal cortex. Higher serum levels of MDA are significantly correlated with higher prefrontal cortex levels of MDA.

studies are not available at present. The study is being conducted as a random placebo controlled clinical trial. The study involves the selection of animals by rigorous inclusion/exclusion criteria. Throughout the study, data is monitored by an external clinical trials coordinator.

Approximately, 1 year prior to the initiation of this study, old and young dogs were given a series of baseline cognitive tests, which were used to assign animals to cognitively equivalent groups. One of the aged groups and one of the young groups was subsequently changed to a food identical to the control but enriched with a broad spectrum of antioxidants and mitochondrial enzymatic cofactors; the other groups were maintained on the control food. The animals were maintained on the dietary intervention for approximately 6 months prior to scheduled cognitive assessment. The food was supplemented with Vitamins E and C, a mixture of fruits and vegetables, alpha-lipoic acid and L-carnitine (mitochondrial cofactors) to reduce oxidative damage to cells. These agents were selected on the basis of their mechanism of action and preliminary data examining these ingredients singly and in combination on measures of serum and urinary oxidative damage in dogs.

One of the tasks used was an oddity discrimination task, in which the animals were trained on a series of four increasingly more difficult learning problems. Each task involves repeatedly presenting three objects, two of which were identical, and one odd. Using progressively more similar objects for each new problem increases, the difficulty of the task. The animal receives a reward if it selected the odd object. This test protocol provides a series of learning problems of sufficient difficulty to show age sensitivity. The performance of monkeys trained on a similar task also varies as a function of the extent of similarity of the objects used [38,71].

In this task young animals are able to learn the series of tasks without showing a significant increase in error scores whereas the old animals generate additional errors as the task becomes more difficult. For the old animals, performance on the first task did not differ from performance on the second. All other task comparisons were statistically significant; Fig. 5 illustrates that these results

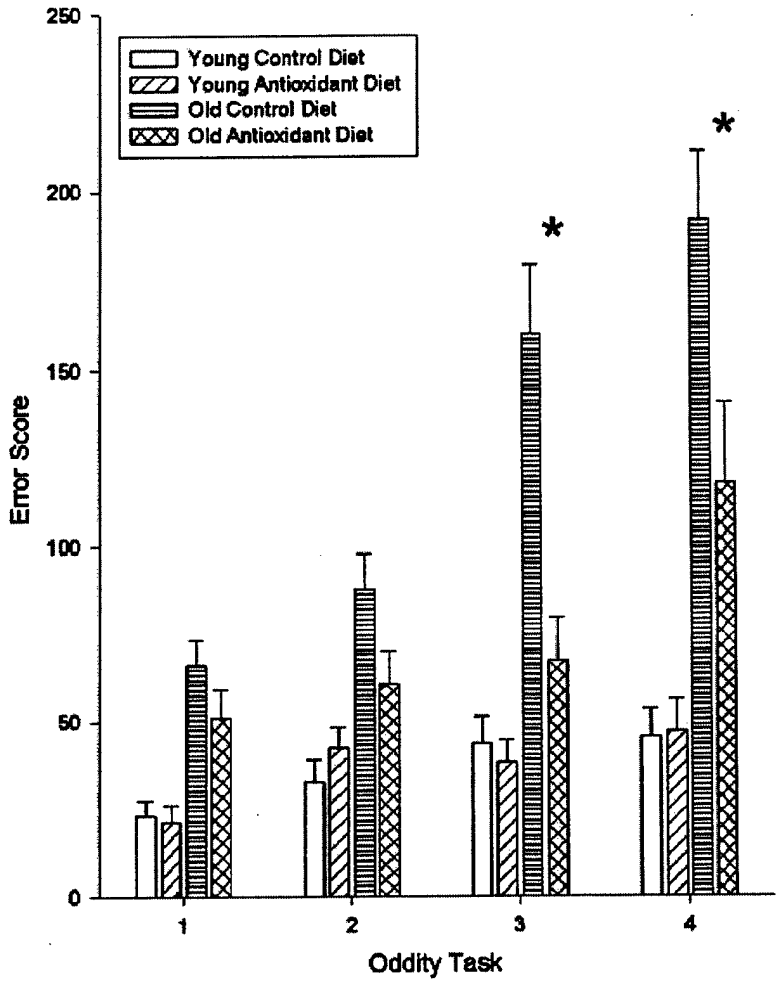

Fig. 5. Effect of age and diet on number of errors made in learning four progressively more difficult oddity discrimination tasks. Aged dogs learned each oddity task with significantly more errors than young dogs as can be seen by comparing young and old animals on the control diet. A diet enriched in antioxidants significantly improved learning in the two most difficult problems, oddity-3 and oddity-4 in aged dogs. Similar improvements were not found in the young dogs provided with the diet.

are due to the animals making more errors on each successively more difficult task than they had on the previous task ($P < 0.025$). The young animals, by contrast, did not show significant differences in performance between any two tasks.

The results of the dietary manipulation are also shown in Fig. 5. The significant overall effect of diet was due exclusively to superior learning shown by the old animals on the antioxidant diet, when compared to the old animals on the control diet. The effect of dietary treatment also varied as a function of task. Diet did not significantly affect performance on oddity 1, the first and simpler task. On the second task, the interaction between age by diet was marginally significant, $F(1, 35) = 3.904$, $P = 0.056$. On task 3, the diet effect was highly significant ($F(1, 34) = 12.32$, $P = 0.0013$) as was the diet by age interaction, $F(1, 34) = 9.715$, $P = 0.004$. Task 4 also had a significant diet effect ($F(1, 34) = 4.78$, $P = 0.035$) and diet by age interaction, $F(1, 34) = 5.118$, $P = 0.030$. Thus, the antioxidant diet produced an improvement in the ability of old dogs to learn a complex task.

The oddity discrimination task provides a sensitive measure of age-dependent cognitive deterioration in dogs, and this age-dependent effect can be at least partially reduced by maintenance on a food fortified with a complex mix of antioxidants and mitochondrial enzymatic cofactors. The use of a series of problems of graded difficulty is an essential design feature of the study and is not commonly used in assessing cognitive interventions in animal models. The protocol revealed that both age and diet effects are amplified by increasing the difficulty of the task. A single level of task difficulty may not have revealed clear effects because of the task being either too easy, or too difficult. Thus, we did not find a significant effect of diet on the first and easiest of the oddity discrimination problems. Similar

results were obtained on landmark discrimination learning, which tests spatial attention [53].

The most important result of this study was the superior performance of the aged animals on the enriched diet compared to controls. A number of factors probably account for the strong dietary effects, including use of aged subjects, 6 months or greater maintenance on the diet, use of a test protocol with progressively more complex problems, and the particular components of the diet. The possibility that the intervention leads to a general, age-independent, improvement in brain function can be excluded since the diet had minimal effects on the young dogs. Thus, oxidative damage is unlikely to induce substantial neuronal dysfunction until relatively late in life.

With respect to dietary constituents, to our knowledge, this is the first study to use combined substances that target enhancement of mitochondrial function with antioxidants that suppress the action of free radicals in a higher animal model. Our results build upon and extend the findings that antioxidants or mitochondrial cofactors alone decrease age related cognitive decline in other species [30,31,40,41,49,69,81]. Our results may be attributable to two different synergistic strategies; first, a complex mixture of antioxidants that supports a network of antioxidants requiring several components to act together for effective function, and; second, improved mitochondrial metabolic function that decreased free-radical production while improving mitochondrial energetics and efficiency.

Alternatively, a reduction in oxidative stress may retard various downstream mechanisms resulting in neuronal dysfunction. Many of the antioxidants utilized in this study also have anti-inflammatory properties [26,47,51]. There has been an association of non-steroidal anti-inflammatory intake and decreased incidence of dementia in humans, which suggests that inflammation is a contributor to neurocognitive decline [30]. As such, the antioxidants included in this dietary fortification may have acted via an anti-inflammatory path, or synergistically, with antioxidant mechanisms to elicit the profound cognitive effects observed.

7. Conclusions

Aged canines, like humans develop age-related neuropathologies, particularly the accumulation of Aβ, develop impaired cognitive function. We hypothesize that cognitive function in canines declines along a "cognitive continuum" that reflects a similar phenomenon in humans [61]. In humans, the continuum is postulated to begin with the development of age associated memory impairment (AAMI) defined as a loss in memory on one or more tests that is 1 S.D. below that of the young population normative values [14]. Probably, because the presence of AAMI is so prevalent in the population of elderly individuals (estimated to be over 50%), the risk for conversion to more advanced stages is relatively low. AAMI is followed by MCI defined by a decline in one or memory functions that is greater than 1.5 S.D. below age-matched norms but is associated with normal activities of daily living [62]. MCI increases the risk for conversion into dementia, particularly AD. Dementia reflects deficits in multiple cognitive domains and the loss of normal activities of daily living. In the aged canine population, the cognitive continuum is primarily associated with the earliest stages, AAMI or MCI though some canines will develop the equivalent of dementia. In the veterinary literature, this latter phase is generally classified as CDS defined as memory impairments and losses in activities of daily living (e.g. social interactions, grooming, disruption of sleep–wake cycles). These features are consistent with the milder expression of neuropathology in canines emphasizing oxidative damage, Aβ accumulation in the form of diffuse plaques and the early stages associated with tangle formation. Thus, the canine represents a higher animal model to study the earliest declines in the cognitive continuum observed in human aging.

We suggest that the combination of antioxidants with mitochondrial enzymatic cofactors may work together synergistically leading to an improvement in learning and memory associated with the progressive decline along the cognitive continuum. Taken together our data supports the hypothesis that oxidative damage and mitochondrial function is a fundamental mechanism contributing to age-associated cognitive dysfunction and underscores the need to conduct similar trials in humans.

Acknowledgments

This project was sponsored by funds provided by the National Institute of Aging (Grant AG12694), the US Department of the Army, Contract No. DAMD17-98-1-8622, and by Hill's Pet Nutrition, Inc. Topeka, KS. The content of the information does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985). Research was conducted in facilities fully accredited by the AAALACI. Additional funding was provided by Science & Technology Center, Hill's Pet Nutrition, Inc., P.O. Box 1658, Topeka, KS. We thank Heather Murphey and Candace Ikeda-Douglas for their assistance in obtaining cognitive data from the longitudinal studies.

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Neurobiology of Aging 5683 (2002) 1–9

NEUROBIOLOGY OF AGING

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Dietary enrichment counteracts age-associated cognitive dysfunction in canines

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Received 20 September 2001; received in revised form 26 November 2001; accepted 24 January 2002

Abstract

Advanced age is accompanied by cognitive decline indicative of central nervous system dysfunction. One possibly critical causal factor is oxidative stress. Accordingly, we studied the effects of dietary antioxidants and age in a canine model of aging that parallels the key features of cognitive decline and neuropathology in humans. Old and young animals were placed on either a standard control food, or a food enriched with a broad spectrum of antioxidants and mitochondrial enzymatic cofactors. After 6 months of treatment; the animals were tested on four increasingly difficult oddity discrimination learning problems. The old animals learned more slowly than the young, making significantly more errors. However, this age-associated decline was reduced in the animals fed the enriched food, particularly on the more difficult tasks. These results indicate that maintenance on foods fortified with complex mixtures of antioxidants can partially counteract the deleterious effects of aging on cognition.

Keywords: Age-dependent cognitive dysfunction; Alpha-tocopherol; Antioxidants; Ascorbic acid; Dogs; L-Carnitine; Lipoic acid; Mitochondrial function; Oddity discrimination learning; Oxidative damage

1. Introduction

Improved nutrition, disease control, and applied biotechnology have prolonged life-span in humans. But enhanced longevity comes at the cost of an increased prevalence of cognitive problems coupled with aging, which range from age-associated memory impairment to the dementia linked to neurodegenerative disorders typified by Alzheimer's disease [8,31,37]. The convergence of increased life-span and increased prevalence of cognitive dysfunction reveals a clear need for identification of mechanisms, models, and testing of interventions for treatment of age-related cognitive dysfunction. The ideal strategy for developing interventions should focus on the underlying pathophysiology in a model system that can be translated to the intended target species, humans.

At the cellular level, the aging process is accompanied by progressive accumulation of oxidative damage, decreased metabolic strategies for mitigating effects of oxidative stress, and decreased efficiency in mitochondrial function, resulting in increased production of cellular oxidants [2,4,17,40]. The consequences are particularly problematic for the nervous system, which exhibits extremely high rates of oxidative metabolism and decreased oxidative defenses, relative to other tissue [16]. A treatment strategy for age-associated cognitive dysfunction and neurodegeneration could include both counteracting the damaging effects of free radicals produced by oxidative stress and enhancing mitochondrial function. We hypothesized that intervention with a complex mixture of antioxidants and mitochondrial enzymatic cofactors should partially reverse, or slow the development of cognitive aging in canines. We chose dogs because these animals develop cognitive dysfunction, beta-amyloid pathology, and oxidative damage that parallel key features of normal and abnormal aging in humans [1,9,18,19,26,33]. We have also found that aged dogs show variability in level of cognitive function that closely resembles the aged human population in the pre-Alzheimer's disease stages, e.g., successful aging, age related memory impairment, and severe cognitive impairments [1].

Alternative models include non-human primates, aged rodents and transgenic mice. Non-human primates are, in many

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0197-4580/02/$ – see front matter
PII: S0197-4580(02)00020-9

respects, the ideal animal model. However, naïve aged primates are expensive, difficult to obtain and often difficult to cognitively test. In addition, the major species of β-amyloid that accumulates in aged non-human primate brain is the shorter, more soluble species [12], which contrasts with reports in human and canine brain [10]. Rodents have a short life-span, absence of neurodegenerative changes, such as amyloid deposition, and limited cognitive abilities [42], which do not clearly model the kinds of complex cognitive deterioration seen in humans. Transgenic mouse models that over-express mutant amyloid precursor protein (APP) deposit B-amyloid, and show cognitive loss but in other respects are limited in their similarities to human brain aging and AD [25].

Previous research with these various models has implicated oxidative damage as a common factor in the development of pathology associated with brain aging. This conclusion is supported by studies indicating antioxidants can delay age-related cognitive decline in humans [36,45] and improve performance in aged rodents [6,22]. These findings, however, remain controversial [32,38,40]. To date the possible role of antioxidant strategies has not been evaluated in a higher animal model than the rodent. Furthermore, the combination of cellular antioxidants and mitochondrial cofactors is novel, and previously been tested.

The present experiment is part of an ongoing longitudinal study of the effects of age, cognitive enrichment, and diet on cognitive decline in beagle dogs. Approximately 1 year prior to the initiation of this study, old and young dogs were given a series of baseline cognitive tests, which were used to assign animals to cognitively equivalent groups. One of the aged groups and one of the young groups were then started on a diet enriched with a broad spectrum of antioxidants and mitochondrial enzymatic cofactors; the other groups were placed on a control diet. The animals were on the dietary intervention for approximately 6 months before starting this study. We tested the subjects on a series of four oddity discrimination learning problems. Each such task involved repeated presentation of three objects, two of which were identical, and providing reward to the subject if it responded to the odd object. We developed this test protocol in an attempt to provide a series of learning problems of sufficient difficulty to show age sensitivity. The performance of monkeys trained on a similar task has been shown to vary as a function of the extent of similarity of object used [21,43].

2. Methods

2.1. Animals

Twenty-four aged and 17 young beagles were acquired from two separate, closed colonies, with known pedigree data. Final enrolled subjects were 23 aged beagles (11 males and 12 females) and 16 young beagles (five males and 11 females). Eleven of the aged beagles and seven of the young beagles were supplied by the Lovelace Respiratory Research Institute colony whereas the rest were from the Hill's Pet Nutrition Colony. At the start of the dietary intervention, the aged dogs ranged from 8.5 to 12.5 years of age and the young beagles ranged in age from 1.95 to 4.9 years of age (see Table 1). The old animals were housed in USDA approved kennels with two dogs per kennel, hand-walked two times per week, and allowed access to toys in their kennels on a rotating basis. The young animals were housed with two to four dogs per kennel. In all other respects, the old and young dogs were treated identically.

Table 1

Age-range[a]	Antioxidant group	Control group
Young dogs	$N = 9$	$N = 7$
<2.00	1	1
2.01–3.99	5	4
4+	3	2
Average age (years)	3.60	3.37
Old dogs	$N = 12$	$N = 11$
8–9.99	3	1
10.0–11.99	8	8
12.0+	1	2
Average age (years)	10.61	10.97

[a] Age taken as subjects age at the start of training on the oddity study.

2.2. Diet

The two foods were formulated to meet the nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs (AAFCO, 1999). Control and test diets were identical in composition, other than inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the test diet. The control and enriched foods had the following differences in formulation on an as fed basis respectively: D,L-alpha-tocopherol acetate (120 ppm vs. 1050 ppm), L-carnitine (<20 ppm vs. 260 ppm), D,L-alpha-lipoic acid (<20 ppm vs. 128 ppm), ascorbic acid as Stay-C (<30 ppm vs. 80 ppm), and 1% inclusions of each of the following (1 to 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 mitochondrial 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 6 months before a new lot was milled.

2.3. Physical exams

All animals were administered a full physical and neurologic examination prior to dietary intervention. Dogs were also examined by slit-lamp for ocular abnormalities that might have impaired visual capabilities of an animal.

2.4. Clinical chemistry

All dogs had complete blood counts, and serum chemistry analysis performed prior to diet intervention. In addition, assessment of endocrine status was performed by way of thyroid panel, and low-dose dexamethasone testing for the presence of Cushing's disease. Concentrations of Vitamin E in serum were determined by HPLC prior to the start of treatment, following 3 months of intervention and following 6 months.

2.5. Cognitive testing apparatus

As described previously [33], the test apparatus was a 0.609 m × 1.15 m × 1.08 m wooden box that was based on a canine adaptation of the Wisconsin General Test Apparatus used in cognitive tests with primates. The box was equipped with a sliding Plexiglas food tray with two lateral wells and a medial food well. Vertical stainless-steel bars cover the front of the box. The height of each bar was adjustable, so that the size of the opening to each food well could be uniquely set for each dog. The experimenter was separate visually from the dog by a screen with a one-way mirror and a hinged door on the bottom. Testing occurred in darkness, except for a light with a 60 W bulb that was attached to the front of the box. The hinged door was opened for the presentation and removal of the food tray.

2.6. Cognitive testing protocol

All subjects underwent a standard pretraining cognitive testing protocol that consisted of reward approach and object approach learning, 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® p/d canned food. This food served as an effective reward for all of the dogs used in the study, in the absence of imposed food deprivation. 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 [33], an object recognition memory task [5] and delayed-non-matching-to-position task (DNMP) [7]. The initial group assignment took into consideration age, sex, and the subjects performance on the reversal learning task, the object recognition task, and the DNMP task. All animals were maintained on the control food during the pretraining period that lasted approximately 6–9 months. Beagles were maintained on dietary intervention for 6 months before behavioral testing was initiated.

After starting the dietary intervention, all of the subjects included in this study were tested on a protocol involving a series of landmark discrimination learning problems [34].

Following 6 months on the food intervention, the animals were tested on a series of oddity discrimination learning tasks. In each such tasks, the animal is presented with three objects, two identical and one different. To obtain reward, the animal is required to respond to the odd object. Every animal was tested on a series of four oddity tasks, referred to as oddity 1–4. The objects were selected based on similarity, with the intent of making each task more difficult than the previous one. The discriminanda used are shown in Fig. 1.

Training on each oddity task started after establishing object preferences. In the preference test session, the animals were given the opportunity of responding to either of the two different objects on 10 successive trials, with both objects associated with reward. Preference was based on the number of times the animal selected each object. If the animal had a preference for one of the objects, the non-preferred object was utilized as the odd-object in the subsequent oddity task. If no preference was determined, a coin toss decided the odd object.

The oddity discrimination testing consisted of 12 daily trials, with an intertrial interval of 30 s. On each trial, the location of the odd object was determined by random generation by the computer with the two identical objects being placed on the remaining two coasters. The computer program also assured that the odd object was located at each of the three positions on exactly four trials each session. The coasters under the two identical objects were scented with the same dog food used for the reward to prevent the animals from using olfactory cues to solve the problem. The tray was presented approximately 25 cm away from the animal for a 2 s period in order for the animal to focus on the object and process the information. The tray was then presented to the animal enabling the subject to respond to one of the three objects.

The animals were tested 6 days per week, and were allowed up to 40 days on each object pair to achieve a predetermined criterion level of accuracy. A two-stage criterion was used for passing the task to assure that animals showed consistently above average performance before learning was assumed. To pass the first phase, the animal had to score at least 11 correct; score 10 correct on two consecutive sessions; or obtain scores of 10, 9, 10 on three consecutive sessions. To successfully complete the second phase, the subject was required to subsequently achieve an average of at least 70% correct over the next three test sessions immediately following phase 1 achievement (e.g., 9, 8, 9). Thus, the minimum number of sessions required to pass the two-phase criterion

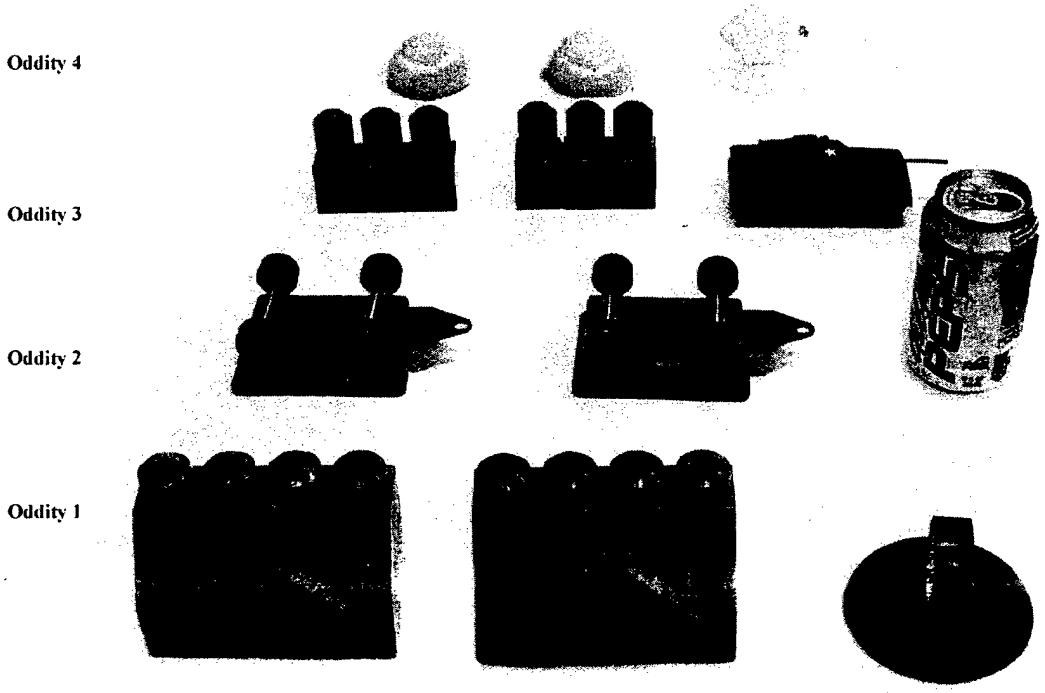

Fig. 1. Objects used in oddity discrimination learning tasks. The objects were selected on the basis of similarity in appearance. The objects used in oddity 1 (large red plastic building blocks and rolls of black hockey tape attached to a blue plastic disk), and oddity 2 (empty diet Pepsi can and bright green plastic toy cart) differed in shape, color and size. The objects used in oddity 3 (dark green plastic toy and small rectangular blue plastic building block) were similar in size, but differed in shape and color. The objects used for oddity 4 (half of yellow tennis balls and a yellow plastic lemon) were similar in size, shape and color.

was 4 days. After completing the task, the animal moved on to next problem, until four such tasks were completed.

2.7. Data acquisition

A customized program controlled all timing and randomization procedures, and indicated the location of each object and reward on every trial. The program also assured that on each trial, the locations of the objects were the same for every 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 were recorded by a key press, which also indicated the end of the trial and signaled the beginning of the inter-trial interval.

2.8. Statistics

Data for cognitive tasks were analyzed by repeated measures ANOVA with respect to source, diet, and age-group using SAS for windows with an alpha level of 0.05 for significance. Since diet and age group each had only two levels, evaluation of main effects and interactions completely explained model variability. Evaluation of the main effects of diets allowed us to detect where the control and antioxidant means were significantly different. Following the initial analysis, separation of means was performed by LSD on SAS for windows with significance set at 0.05. Data for Vitamin E and Vitamin E:triglyceride ratios were analyzed as a repeated measures analysis with respect to food, and age-group. Following initial analysis, separation of means was performed by Tukey's Studentized Range test (HSD) on SAS for windows. Data for clinical bloodwork was analyzed by individual *t*-test for each analyte.

3. Results

3.1. Physical examination

Results of physical examination did not reveal any neurologic, musculoskeletal, ocular or physical abnormalities that would have excluded participation in the study.

3.2. Clinical chemistry

Blood biochemistry profiles revealed that most dogs fell within the range of values considered normal for healthy

Table 2

Group/time	Vitamin E in serum (ug/ml)			Vitamin E:triglyceride in serum (ug/mg)	
	Baseline	3 Month	6 Month	Baseline	6 Month
Control: young	23.3 ± 1.1 a	24.3 ± 1.8 c	25.1 ± 2.9 b	68.2 ± 10.8 a	63.8 ± 9.5 b
Control: old	30 ± 2.2 a	29.4 ± 2.5 b, c	28.9 ± 1.5 b	28.9 ± 1.5 b	26.4 ± 4.5 c
Antiox: young	26.2 ± 1.8 a	40.8 ± 3.7 b	45.4 ± 4.3 a	63.2 ± 6.6 a	109.3 ± 9.6 a
Antiox: old	28.2 ± 2.4 a	49.6 ± 4.9 a	52.8 ± 3.8 a	33.5 ± 9.8 b	66.3 ± 8.8 b

Means of Vitamin E in serum for old ($n = 23$) and young ($n = 16$) dogs in different dietary groups prior to and at 3 and 6 months of feeding test foods. Vitamin E:triglyceride ratio in serum prior to and at 6 months of feeding test foods. Means with different letters are significantly different from each other within that time period.

adult dogs. No significant differences were observed between groups within the young dog category at baseline. There were, however, significant differences between the old and young dogs on baseline measures attributable to age. Total protein, globulin, cholesterol, triglycerides and red blood cells/ul were increased significantly compared to young animals. Conversely, albumin, creatinine, calcium, sodium, and T_3 were decreased in older animals compared to young.

Within the old dog groups, activity of alkaline phosphatase $F(1, 23) = 4.76$, $P = 0.04$ and creatine kinase $F(1, 23) = 4.49$, $P = 0.046$ were significantly higher in the control group compared to the antioxidant group, with both old groups having animals above the normal range. Considering the ages of the older dogs in the study it was anticipated that some measures would not fall within normal ranges established for young healthy dogs. None of the observed changes indicated significant health differences between the groups of old animals. The significant difference in alkaline phosphatase activity was still present at 6 months of time but the creatine kinase difference was no longer significant.

3.3. Serum Vitamin E

There was a significant effect of food $F(1, 35) = 23.07$, $P < 0.0001$ and age-group $F(1, 35) = 5.06$, $P = 0.0308$ over the entire period. There were no significant differences between concentrations of Vitamin E in serum between age or dietary groupings, at the beginning of the study. However, the older dogs had a higher serum concentration of Vitamin E than the young dogs at this and subsequent time points, which resulted in the overall age-group effect. Subsequent analysis of old versus young mean differences did not reveal any significant difference at baseline or 6 months. Following 6 months of dietary intervention, both old and young dogs on

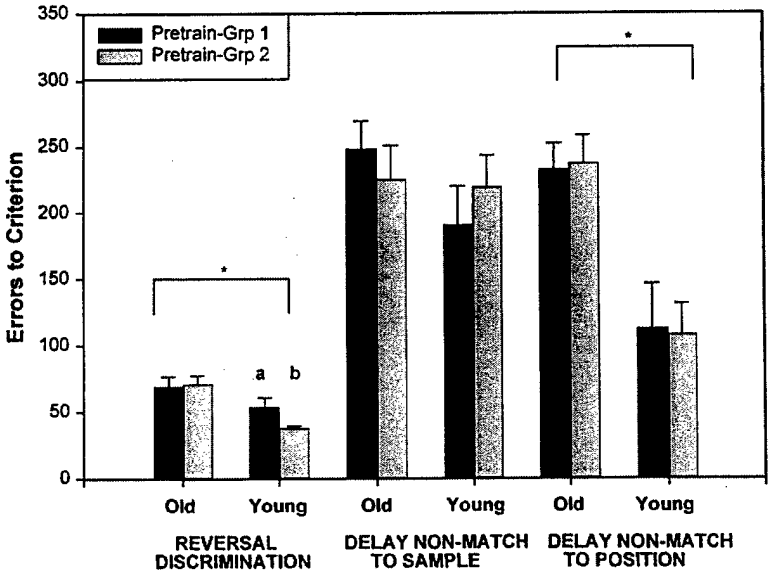

Fig. 2. Baseline cognitive data for aged and young beagles. The aged animals assigned to the antioxidant fortified test food did not differ from the aged animals assigned to the control food on any of the baseline measures, indicating that the groups were cognitively equivalent. On the other hand, we did find significant difference between the old and young groups on the reversal and DNMP tasks (*). A significant effect of treatment group was present for young dogs on the reversal learning task (a vs. b). This difference was not present on the original allocation of young dogs but appeared after one young dog was dropped for motivation concerns as detailed in the testing protocol.

the antioxidant fortified food had significantly higher concentrations of Vitamin E in serum, compared to age-group controls (Table 2).

Since Vitamin E is fat soluble, its concentration in body tissues may be expressed as Vitamin E per unit of fat, such as triglycerides in serum. When serum Vitamin E was expressed in this fashion, young dogs had a much higher concentration of Vitamin E per mg of triglyceride in serum at the start than older dogs. Repeated measures analysis of this ratio revealed significant effects of food $F(1, 35) = 11.69$, $P = 0.0016$ and age-group $F(1, 35) = 35.27$, $P < 0.0001$. Supplementation of Vitamin E in the food resulted in a significant increase in concentration of Vitamin E:triglyceride in both old and young dogs compared to age-group controls. However, supplementation of older dogs with Vitamin E only increased this ratio to an absolute value approximately equal to that observed in young dogs at the start of the study (Table 2).

3.4. Pretraining cognitive results

The baseline performance of the two groups of aged animals was equivalent (Fig. 2), which indicates that the groups were cognitively equivalent before starting the treatment condition. By contrast, the old group differed from the young on baseline measures in two of the three tasks, with young animals performing significantly better than old animals in both the reversal learning $F(1, 37) = 13.74$, $P = 0.0007$ and spatial memory tasks $F(1, 37) = 28.9$, $P < 0.0001$.

3.5. Oddity discrimination results

One aged control animal completed only two of the oddity problems because of time constraints. This animal's data

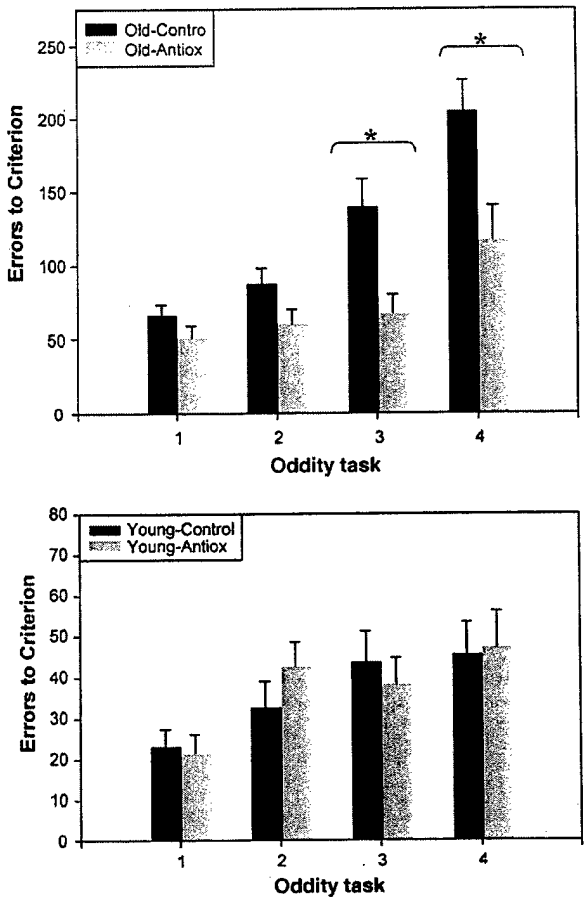

Fig. 3. Effect of age on number of errors made in learning for progressively more difficult oddity discrimination tasks. The data from the control and enriched aged groups were combined, and the data from the control and enriched young groups were combined.

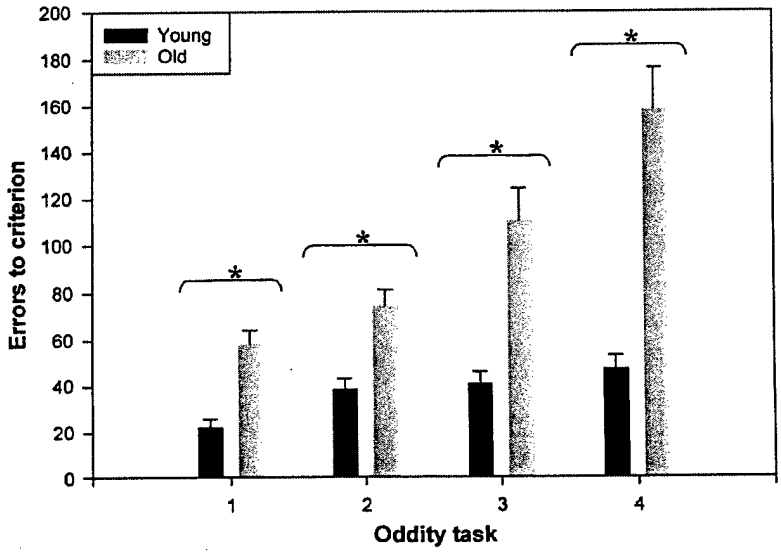

Fig. 4. Effect of food on learning a series of oddity discrimination problems in groups of old (top) and young (bottom) dogs.

were, therefore, excluded from the initial repeated measures ANOVA. To include the data from this dog, subsequent separate analysis were done for each of the four oddity tasks. The initial analysis revealed highly significant effects of age $F(1, 30) = 65.149$, $P < 0.0001$, diet $F(1, 30) = 10.098$, $P = 0.0034$, and task $F(3, 90) = 19.79$, $P < 0.0001$. We also found significant interactions between diet and age $F(1, 30) = 11.09$, $P = 0.002$, task and age $F(3, 90) = 9.257$, $P < 0.0001$, and between task and diet $F(3, 90) = 3.256$, $P = 0.025$.

Fig. 3 illustrates that age effect was due to the young animals committing fewer errors than the old animals. The age differences also varied as a function of task. For the old animals, performance on the first task did not differ from performance on the second. All other task comparisons were statistically significant, Fig. 3 illustrates that these results are due to the animals making more errors on each successive task than they had on the previous task ($P < 0.025$). For the young animals, by contrast, there were no significant differences in performance between any two tasks.

The results of the dietary manipulation are shown in Fig. 4. The top panel shows that the significant overall effect of diet was due exclusively to superior learning shown by the old animals on the antioxidant diet, when compared to the old animals on the control diet. The effect of dietary treatment also varied as a function of task. Diet did not significantly affect performance on oddity 1, the first task. On the second task, the interaction between age by diet was marginally significant $F(1, 35) = 3.904$, $P = 0.056$. On task three the diet effect was highly significant $F(1, 34) = 12.32$, $P = 0.0013$ as was the diet by age interaction $F(1, 34) = 9.715$, $P = 0.004$. Task 4 also had a significant diet effect ($F(1, 34) = 4.78$, $P = 0.035$) and diet by age interaction $F(1, 34) = 5.118$, $P = 0.030$. There was no significant effect of source.

4. Discussion

These results indicate first, that the oddity discrimination task provides a sensitive measure of age-dependent cognitive deterioration in dogs, and second, that this age-dependent effect can be at least partially reduced by maintenance on a food fortified with a complex mix of antioxidants and mitochondrial enzymatic cofactors.

The general utility of any animal model in evaluating the effect of interventions on age-dependent cognition depends on the extent to which the model reflects age-related cognitive dysfunction. The oddity learning task used in the present experiment can be solved by the animals' learning to associate one of two stimuli with reward, which involves visual discrimination learning. Visual discrimination learning is often insensitive to age in animal models [3,27,35,38]. This was not the case in the present experiment: we found highly significant age differences in favor of the young animals. There are two possible reasons why discrimination learning is age-sensitive in some instances only. First, the effect of age may depend on the difficulty of the discrimination. Task difficulty was clearly a factor in the present experiment; the harder the problem, the greater the age-difference. Aged non-human primates are deficient in acquiring some types of visual based discrimination learning, but not others [44]. Second, age differences in discrimination learning could reflect differences in strategies used to solve each of the oddity problems. The subjects could potentially use either an associative (stimulus-reward), or a more cognitive strategy. An associative strategy requires the subject to learn to associate the correct object with reward through repeated pairing of the two, and depends on repetition. A more cognitive strategy involves learning the general rule that only one of the objects is correct—in this case the odd item. Task complexity was manipulated by varying the similarity of the test objects. We assumed that increased difficult would result from increased similarity, and this proved to be the case for the old animals. They showed progressively more errors on each successive task, which is consistent with their using an associative strategy. The young animals performance, by contrast, did not differ significantly on any of the tasks, suggesting the use of a cognitive strategy. In fact, some of the animals learned each successive task progressively faster, despite the increase in task difficulty.

The use of a series of problems of graded difficulty is a novel innovation of the present study, which to our knowledge has not previously been used in assessing cognitive interventions in animal models. The protocol revealed that both age and diet effects are amplified by increasing the difficulty of the task. Had we used only a single level of task difficulty, we may not have seen clear effects because of the task being either too easy, or too difficult. Thus, we did not find a significant effect of diet on the first and easiest of the oddity discrimination problems.

The most important result of this study was clearly the superior performance of the animals on the enriched diet compared to controls. A number of factors probably account for the strong dietary effects seen in this study, including use of aged subjects, 6 month maintenance on the diet, use of a test protocol with progressively more complex problems, and the particular components of the diet. The importance of using aged subjects was illustrated by the absence of any diet effect in the young dogs. Because the young dogs performed at a much higher overall level than the old, creating a possible ceiling effect. But we also would not expect to see an effect of diet on cognition in young dogs for theoretical reasons; namely because oxidative stress is not likely to induce substantial neuronal dysfunction until relatively late in life. The importance of duration of time on the diet is more difficult to evaluate, and needs more systematic study. We have found positive effects of an antioxidant diet after a shorter maintenance period [34], but the effects were less robust.

With respect to dietary constituents, to our knowledge, this is the first study to have combined substances that tar-

get enhancement of mitochondrial function with antioxidants that suppress the action of free radicals. Our results build upon and extend the findings that antioxidants or mitochondrial cofactors alone decrease age related cognitive decline in other species [14,15,23,24,41,46]. Our results may be attributable to two different synergistic strategies: first, a complex mixture of antioxidants that supports a network of antioxidants requiring several components to act together for effective function; and second, improved mitochondrial metabolic function that decreased free-radical production while improving mitochondrial energetics and efficiency.

Alternatively, many of the antioxidants utilized in this study also have anti-inflammatory properties [11,28,29]. There has been an association of non-steroidal anti-inflammatory intake and decreased incidence of dementia in humans, which suggests that inflammation is a contributor to neurocognitive decline [30]. As such, the antioxidants included in this dietary fortification may have acted via an anti-inflammatory path, or synergistically, with antioxidant mechanisms to elicit the profound effect observed.

We suggest that the combination of antioxidants with mitochondrial enzymatic cofactors may work together synergistically to enhance mitochondrial function leading to a decrease in both the production and consequences of reactive oxygen species [13]. Taken together our data supports the hypothesis that oxidative damage and mitochondrial function is a fundamental mechanism contributing to age-associated cognitive dysfunction and underscores the need to conduct similar trials in humans.

Uncited references

[20,39].

Acknowledgment

This project was sponsored by funds provided by Hill's Pet Nutrition, Inc. Topeka, KS, the National Institute of aging (Grant AG12694) and by the US Department of the Army, Contract No. DAMD17-98-1-8622. The content of the information does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985). Research was conducted in facilities fully accredited by the AAALACI. Additional funding was provided by Science & Technology Center, Hill's Pet Nutrition, Inc., P.O. Box 1658 Topeka, KS, 66601, USA. We thank Christina Siwak and Dwight Tapp for assistance in collecting blood samples, Krista Witty for her help in selecting the oddity objects, and Jennifer Lau, Maggie Lau and Joanne Castillo for help in cognitive testing.

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Neurobiology and Behavior
(in press)

Landmark discrimination learning in the dog: effects of age, an antioxidant fortified diet, and cognitive strategy

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Abstract

Cognitively equivalent groups of old and young dogs were placed into either a test group that was maintained on food enriched with a broad-spectrum of antioxidants and mitochondrial cofactors, or a control group maintained on a complete and balanced food formulated for adult dogs. Following a wash-in period, dogs were tested on a series of landmark discrimination problems, all of which required the animals to respond selectively to the object closest to a specific external cue. In experiment 1, dogs were first trained to respond to a landmark placed directly on top of a food well (Landmark-0). They were moved on to the next phase of testing, in which the landmark was 1, 2 or 4 cm (Landmark 1, Landmark 2, Landmark 4) away from the reward object. Learning varied as a function of age group, food group, and task. The young dogs learned all of the tasks more quickly than the old dogs. The aged dogs on the enriched food learned Landmark-0 significantly more rapidly than aged dogs on control food, however this effect did not achieve significance on the subsequent landmark tasks. Experiment 2 showed that accuracy decreased with increased distance between the reward object and landmark, and this effect was greater in old animals. Experiment 3 showed stability of performance, despite using a novel landmark, and new locations, indicating that dogs learned the landmark concept. Experiment 4 found age impaired long-term retention of landmark task. These results both support a role of oxidative damage in the development of age-associated cognitive dysfunction and indicate that short-term administration of a food enriched with supplemental antioxidants and mitochondrial cofactors can partially reverse the deleterious effects of aging on cognition.

Author Keywords: Dog; Discrimination Learning; Cognition; Age; Visuospatial Function; Landmark Discrimination; Antioxidants

Index Terms:learning and memory, antioxidants,

Article Outline

1. General Introduction

We have previously found that dogs have considerable difficulty in learning to use an external landmark as a cue for the location of a food reward. We were initially unsuccessful in training dogs on a landmark discrimination learning task, modified from one first developed for primates [1], in which the dogs were required to respond to one of two identical discriminanda based on the location of a visual landmark. If, however, the landmark was placed on top of one of the objects, dogs readily learned this discrimination and were subsequently able to learn discriminations in which the landmark was spatially displaced from the correct object [2]. Spatial navigation, in general, can be based on either of two kinds of knowledge, allocentric, which is by reference to the position of an external landmark, or egocentric, which is by reference to the observer's body. Landmark discrimination learning is ideally solved using an allocentric strategy. The difficulty originally encountered in training dogs on the landmark task suggests that dogs

prefer using egocentric information in spatial navigation, but are capable of learning to use allocentric information as well. These conclusions are also supported by a recent study by Fiset al., [3], who reported that dogs base their search primarily on an egocentric frame of reference, although they are capable of encoding both allocentric and egocentric information.

The present experiment was intended to further our understanding of the factors affecting acquisition and performance of a landmark discrimination learning task. We specifically focused on the effects of age, food composition, and landmark configuration. Our interest in the effects of age stems from a more general concern with the development of a canine model of human cognitive aging. Dogs show age-related pathology similar to that observed in aging humans, as well as age-dependent visuospatial deficits [4,5]. We have previously used a delayed non-matching- to position task to study the effects of age on visuospatial function. In this task, subjects are first presented with a reward in a particular location on the sample trial. On the subsequent test trial, given after a delay interval, the subjects are required to respond to a new location to obtain food reward [4,6,7]. Compared to young animals, old animals show slower learning and poorer memory [4, 6, 7, 8]. This task can be solved using purely egocentric strategies, such as body orientation. We have not looked at the effect of age on tasks dependent on allocentric strategies. In our previous study on landmark discrimination learning, however, we noted that the older dogs performed more poorly than young dogs, but the sample was too small to statistically support any conclusion. In rodents, tasks requiring allocentric strategies show greater age-dependent disruption than is seen in tasks that can be solved using egocentric strategies [9-10]. Rapp, Kansky and

Roberts [11] found that young monkeys were likely to use external cues, and hence allocentric strategies, to solve a primate version of the radial arm maze task. Aged monkeys, by contrast, tended to use other, non-spatial strategies.

The rationale for studying a dietary intervention stems from evidence implicating free radicals in the aging process. According to the free radical theory of aging, reactive oxygen species formed as by products of cellular metabolism produce cellular damage, and age-dependent neuropathology may be a resultant cumulative response to these alterations [12,13]. This hypothesis predicts that aging should be slowed, and possibly even reversed by appropriately increasing levels of antioxidants in the body.

The central nervous system has a high rate of oxidative metabolism relative to other tissues and is vulnerable to cumulative effects of oxidative damage to proteins, lipids and nucleotides [14]. Such oxidative damage has been observed in aged brains of both humans and rodents [14-21]. We also have found evidence of oxidative damage to lipids and proteins in aged dogs [22], which supports the suggestion that oxidative damage contributes to age-dependent cognitive dysfunction. This hypothesis can be further tested; by conducting intervention studies to reduce oxidative damage in canines as has been reported in rodent models.

One potentially effective way of counteracting oxidative stress is by dietary intervention with antioxidants [23]. Aged rats supplemented with vitamin E, E and C combined, spinach, blueberry or strawberry extracts, exhibited faster learning and better memory retention in a Morris Water maze testing paradigm than animals on a control diet [24, 25]. Motor learning and cerebellar function also are improved in aged rats fed strawberry, blueberry, or spinach extract [26]. In addition, age-related changes in long-

term potentiation and antioxidant defenses are reversed by a diet enriched with the antioxidant alpha-lipoic acid [27].

The rodent model, however, is limited by a short life-span, and the absence of neurodegenerative changes, such as beta amyloid accumulation that occur during human aging. Furthermore, the correspondence is questionable between cognitive test protocols typically used in rodent studies and age-dependent cognitive dysfunction in humans. The present experiment was part of an ongoing longitudinal multiyear study of the effects of dietary enrichment on cognitive aging in dogs. The dietary enrichment was accomplished by supplementing a complete and balanced food with a broad-spectrum of antioxidants and mitochondrial cofactors.

2. General Methods

2.1. General Outline

Aged and young beagle dogs were used as subjects. Approximately 6 months before starting the study, all subjects received extensive baseline cognitive testing. Baseline performance was used to assign the subjects into cognitively equivalent groups – a control group, which was fed a complete and balanced food formulated for adult dogs and a dietary enriched group. After an average wash-in period of 14 days, all subjects were trained on a series of landmark discrimination learning tasks (Experiment 1). Subjects that were able to succesfully learn the first two phases (landmark 0 and landmark 1), were then tested on a variable distance landmark task (Experiment 2). At the completion of experiment 2, the dogs were tested with a novel landmark, and with the landmark at novel locations (Experiment 3). Experiment 4 looked at long-term retention of the task in Experiment 1.

2.2. Subjects

24 aged beagles (12 M, 12 F) and 17 young beagles (6 M, 11F) served as subjects. The young and aged beagles were acquired from two separate, closed colonies, with known pedigree data for at least 2 generations. The old animals were housed in USDA approved kennels at Lovelace Respiratory and Research Institute in Albuquerque New Mexico with 2 dogs per kennel. The young animals were housed with two to four dogs per kennel at the test facility at the University of Toronto.

All animals were administered full physical and neurological examinations prior to diet intervention and at regular intervals, thereafter. Dogs were also examined by slit-lamp for ocular abnormalities that may impair visual capabilities of an animal. We also obtained complete blood counts, and serum chemistry analysis from every subject. Endocrine status was regularly assessed using a thyroid panel, and low-dose dexamethasone testing for the presence of Cushing's disease.

Group placement was based on the subjects combined scores on baseline tests of reversal learning, spatial learning, and object recognition. Twelve of the old animals were placed in the antioxidant group and 12 in the control group. Nine of the young animals were placed in the antioxidant group and 8 in the control group. At the start of the study, the ages of the old dogs ranged from 8.05 to 12.3 years of age and the young beagles ranged in age from 1.95-4.5 years of age. The group distributions are shown in Table 1.

2.3. Testing Apparatus

The test apparatus was a .609-m x 1.15-m x 1.08-m wooden chamber based on a canine adaptation of the Wisconsin General Test Apparatus (Figure 1; for a detailed description see Milgram et al. 1994)[27]. The testing chamber was equipped with a black, sliding Plexiglas food tray with three food wells. Adjustable vertical stainless steel bars at the front of the box provided openings for the animal to obtain food from the food wells, which could be uniquely adjusted for each dog. The experimenter was separated visually from the dog by a one-way mirror with a hinged wooden door below the mirror. Testing was conducted in darkness, except for a light with a 60-watt bulb attached to the front of the box. Each test trial commenced with the hinged door being opened for the presentation of the tray. Approximately 1 gm of Hill's Prescription Diet® p/d canned food was used as the reward.

Data acquisition was acquired using a customized program that controlled all timing and randomization procedures and indicating the location of the reward and the landmark. At the start 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. The dogs' responses were recorded by a key press, which also indicated the end of the trial and signalled the beginning of the inter-trial interval.

2.4 Baseline Behavioral Testing Protocol

Cognitive testing was conducted in the morning and early afternoon. Before initiating this study, every dog was administered a standard pre-training protocol that was intended to familiarize them with the testing apparatus and procedures [28]. The protocol included training on reward and object approach learning, object discrimination learning, and discrimination reversal learning. All subjects from both age groups were trained on both

an object recognition learning task [28, 29] and on a delayed non-matching-to-position task [6] after completing this pre-training protocol.

2.5. Diet Formulation

The foods were formulated to meet the American Association of Feed Control Officials recommendations for adult dogs [30]. Control and enriched foods were identical in composition, other than inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the enriched food. The extra inclusions consisted of the following on a dry matter basis prior to processing losses: l-carnitine (300 ppm), dl-alpha-lipoic acid (150 ppm), dl-alpha tocopherol acetate (1550 ppm), taurine (1095 ppm), ascorbic acid as Stay-C (100 ppm), and 1% inclusions of each of the following (1 to 1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp, Vitamin E is lipid soluble and acts to protect cell membranes from oxidative damage while 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 mitochondrial lipid metabolism.

Blood biochemistry and complete blood counts were obtained prior to dietary intervention. Serum was analyzed by way of HPLC to determine concentration of vitamin E prior to and three months after intervention [31].

2.7. Statistics

Statistical analysis were conducted using Statisica version 6.0 for Windows, with an alpha-level of 5% ($\alpha = .05$). Post-hoc Tukey's HSD tests were used for all pairwise comparisons. We used the Fischer exact probability test (2x2 tables) or chi square test for all comparisons based on category frequencies (nominal data) [32].

3. Experiment 1: effect of age and food on acquisition of a landmark discrimination task

This experiment focused on the effects of both age and the administration of an enriched antioxidant diet on the ability of dogs to acquire a landmark discrimination learning task. Dogs on either control or enriched food were trained on a test protocol consisting of four progressively more difficult versions of a landmark discrimination learning task. Success was set as the ability to meet criterion within the allotted 40 sessions for any given task. Participation in this study was partially constrained by the necessity of passing any given level before going on to the next level. However, dogs that failed the first or second problem were provided with remedial training to enable them to participate in subsequent experiments.

3.1. Experiment 1 Methods

The dietary intervention was started approximately 2 weeks before testing on the landmark discrimination task. Dogs received 10 trials per day, with an inter-trial interval of 0.5 minutes. Testing was carried out once a day, five days per week. We used a partial correction procedure in which the dogs were permitted to correct their response after making an error once each session. Each dog was tested on up to four problems, with each being progressively more difficult than the previous. To move on from one problem

to the next, they had to complete a two-stage criterion. They had to first respond correctly on at least 9 of 10 trials, or on 8 of 10 trials over 2 consecutive days. They then had to respond correctly on at least 70% of the next 30 trials over three consecutive sessions. Dogs that had a non-response in any one session were assigned a score of 0.5, which was assumed to be the response based on random choice, and were given one extra day of testing to complete the 30 trials

The discriminanda were identical white coasters and a thin rectangular 2cm x 2cm x 9 cm block of wood (yellow peg) as the landmark. The white circular coasters wereplaced over the two lateral food wells on the presentation tray. The middle food well was not used in this study. White Velcro tabs 2cm in diameter were glued to the top center of the white coaster and to appropriate loci on the food tray to hold the landmark in place.

A four phase test protocol was used (Figure 2). On the initial test, the landmark was attached to the center of one of the two white coasters. This landmark position was called landmark 0 (L0). On each trial, the experimenter placed the food reward in either the left or right food well and positioned the landmark accordingly. The door was raised and the tray was moved to approximately 25 cm from the dog for 2 seconds, to enable the subject to see the spatial arrangement on the tray. The tray was then presented to the dog, and the dog was allowed to respond. In this and all other tasks, the dogs were required to respond to the discriminanda closest to the landmark to obtain food reward. The correct side was determined randomly by the computer, with the constraint that each side was correct on half of the trials of each test session.

Each dog was allowed a maximum of 40 test sessions (400 trials) to learn to respond to stimulus associated with the landmark for L0. Animals that failed the initial L0 test were given a remedial training program to help teach them the task. This consisted of 5 additional training days, with 15 trials per day. At the start of the remedial training, the animals were presented with a single rewarded stimulus on the majority of the trials. With continued testing, more paired stimulus presentations were given. After completing the remedial learning phase, the animals received additional training, up to a maximum of 20 sessions using the original protocol. This protocol was also used for dogs that failed L1.

Once a dog learned the L0 task (either during the initial training or after the remedial training), the landmark was moved one cm medially and diagonally away from the coaster, which constituted landmark 1 (L1) – see Figure 2. The landmark was attached to the food tray with a black piece of 2 cm wide Velcro. Dogs that did not learn within 40 sessions were given remedial training, as described for L0, and were dropped from the study if they failed after remedial training. Dogs that passed L1 were then tested on landmark 2 (L2), which was identical except that the new landmark position was diagonally one cm away from the previous landmark position – see figure 2. Thus the distance from the new landmark position to the edge of the correct object was 2 cm. Dogs had to pass this to move on to the next phase, and no remedial training was provided. In the final test landmark 4 (L4), the distance between the landmark and object was now 4 cm (see Figure 2), and a maximum of 20 training sessions were allowed.

3.2. Baseline results

No significant differences in learning were found across the treatment groups, within age grouping, prior to intervention for all of the baseline cognitive tests described (Figure 3). However, there were significant differences between age grouping, with the young dogs showing fewer errors on both the reversal learning task (df=1,37, F = 13.74, p=.0007) and the delayed non-matching-to-position task (df=1,37), F = 28.9, p<.0001).

3.3. Effect of age and food on landmark discrimination learning

Because of learning failures, the sample size decreased on successive test phases. Separate ANOVA's were, therefore, used in the analysis of each task. On the first task, L0, there was a significant effect of both food and age (Food df=1,37, F=5.644 P=.0228; Age df=1.37, F=37.42; P=.0001). The food effect was attributable to the old animals on the enriched food making fewer errors than the old animals on the control food (Figure 4). To address the potential confound of dogs being obtained from different sources, old dogs were compared in a separate analysis by source. The antioxidant food improved learning on L0 in both sources and there was no significant effect of source. There was no effect of food on the average number of errors made in learning L1, L2 or L4 within either age group. Learning in subsequent landmark tasks was also markedly affected by age, with the old dogs making significantly more errors than the young in every instance (L1 df=1,36 F=4.41, P=.0266437; L2 df=1,32, F=4.903, p=0.034; L4 df = 1,24; f=4.969; p=.036).

Age and food differences were also manifest in the animals' ability to achieve a criterion level of learning within the allowable time frame. Four of the 12 old dogs in the control group failed L0 within the 40 trials allowed, while all 12 dogs in the antioxidant

group passed the task. Note that the 4 dogs that initially failed to learn L0, did learn when retested after completing the remedial training protocol. Table 2 shows the number of dogs that successfully completed each phase of the protocol. Seven of 12 dogs on the antioxidant food passed L2 compared to only 4 of 12 on the control food. A chi-square analysis of the pass rate for old dogs revealed a significant difference at L0, L1 and L4 (land 0 df=1, F=6.316, P=.012, land1 df=1, F=4.444, P=.035) but not L2 (df=1, F=1.51, P=.219) with dogs on the antioxidant enriched food performing significantly better in all comparisons.

Separate analysis of the younger dogs did not reveal significant effect of food at any of the landmark distances tested. However, the young dogs on the antioxidant food made fewer overall errors on learning the tasks than those on the control food. In addition, 8 of 9 dogs on the antioxidant food passed L2 testing compared to 6 of 8 on the control food.

The aged dogs used in this study ranged from approximately 8 to 12 years at the start of the study. To examine the differences within the age range of the old dogs, we looked at the correlation between age at the time of test and performance on L0. A scatterplot of the data for old dogs displayed a positive correlation, but the correlation did not attain significance (Fig 5).

3.4. Effects of diet on clinical chemistry and serum vitamin E

Blood biochemistry profiles indicated most dogs fell within the range of values considered normal for healthy adult dogs. No significant differences were observed between groups in the young dog category. Activity of alkaline phosphatase and creatine kinase were significantly higher in the old dog control group, compared to antioxidant

group, with both old groups having individual animals with values above the normal range. None of the observed changes were interpreted to indicate significant health differences between the groups of animals.

At the beginning of the study, there was a significant age difference in serum concentrations of vitamin E, with old dogs having higher concentrations than young dogs ($P=.04$), but no differences within age groupings. Following three months of dietary intervention, both old and young dogs on the antioxidant fortified food had significantly higher ($P<.0001$) concentrations of vitamin E in serum than the dogs on the control food (Table 3).

3.5. Discussion Experiment 1

This study demonstrated that both age and food composition affects the ability of dogs to learn a landmark discrimination task. The aged dogs on the enriched diet showed significantly better learning than dogs on the control diet on the initial task, L0, and the second task, L1, by chi square analysis. Furthermore, the design of the study likely led to an underestimation of the size of the food composition effect. The data analyses were based on either errors to achieve a criterion level of performance, or total errors within a maximum of 40 sessions. Four animals on the control diet failed the L0 task, which imposed a ceiling effect on the maximum number of possible errors.

We did not find statistically significant effects of food composition on any of the other landmark distance tasks. However, testing was discontinued for dogs that failed at any level, unless they successfully completed a remedial training protocol. This procedure results in a selective loss of the poorest performing subjects.

Carry-over effects from previous training are another factor that likely affected learning, particularly for the aged dogs. Old dogs that succesfully learned L0 actually learned L1 more rapidly, on average than they had learned L0, suggesting a partial transfer of learning skills. This effect was lost when L2 learning was compared in the same way to L1 in old dogs. Gleason and Rothblat, [33] found that rats trained to use a specific external landmark to obtain food also commit more errors during the initial of training at a distance of 0 cm than at longer distances. We attribute this previous training effect to the animals' having learned to attend to the particular landmark. Clearly, the true landmark is a more difficult task, as we have previously found dogs are generally unable to learn it without having the appropriate pretraining history [2]. The data from the young animals supports this assertion: for the young animals, errors to reach criterion increased with increased distance between the landmark and food well.

4. Experiment 2: effect of landmark distance - performance on the variable distance landmark task

4.1. Introduction experiment 2

Experiment 1 looked at learning as a function of landmark distance, using a protocol where the distance was progressively increased. Based on our previous work, we expected task difficulty to be directly related to landmark distance, [2]. This was the case for the young dogs only. The old animals showed, on the average, more rapid learning of L1 than of L0. This may be due to the old dogs taking close to the maximum number of sessions to acquire L0, a ceiling effect, which limits the possibility of animals doing more poorly on L1. To establish the importance of landmark distance, we tested the dogs on a variable distance version of the landmark task described in experiment 1, in which three

different distances were used in each daily session [2]. We have previously found that performance deteriorates with increasing distance on this task.

4.2. Methods experiment 2

To participate in this study, dogs were required to pass L1 within the allotted time, or after remedial training. Among the aged dogs, a total of 8 control dogs and 11 antioxidant fed dogs were included. Seventeen young dogs were also studied (8 control 9 antioxidant).

In experiment 2, the dogs received 12 trials per day at distances of either 1, 4, or 10 cm, for 20 sessions. The distance and side of the correct response was randomly determined with two constraints. First, each distance was tested on 4 trials on each session. Second, for each distance the correct side was left on half of the trials and right on the other half.

4.3. Results and discussion

The results were analyzed with a repeated measures ANOVA, with landmark distance as a within subject variable and both age and diet as between subject variables. There were significant main effects of distance ($df=2,64$; $F=388.87$; $p=.000$) and age ($df= 1,35$; $F=6.07$; $p=0.19$), and a significant interaction between age and distance ($df=2,64$; $F=3.424$; $p=0.039$). As illustrated in Figure 6a, the results reflected increased errors at longer distances, which was exaggerated in the old dogs. There was no effect of food.

The results presented in Figure 6a were based on all of the animals tested in this experiment. However, some of the animals showed little evidence of having learned the task, and the inclusion of the results from this group could have accounted for the

significant age by distance interaction. To establish a criterion for learning this task, we examined the performance of the animals over the last 10 sessions, and calculated the mean and standard deviation of the corresponding binomial distribution (M=60, variance=15). We then excluded all animals whose performance was within two standard deviation units of the mean. Using this criterion, 4 old and two young animals were removed from the analysis. As indicated in Figure 6b there were still significant effects of age, landmark distance, and a significant age by distance interactions. The multiple comparisons indicated that the old and young animals did not differ at 1 cm, but did differ at 4 and 10. These results, therefore, indicate that age impairs an animals' ability to correctly assess the visuospatial relationship between the landmark and correct object at moderately long distances.

5. Experiment 3: strategies acquired in the landmark task

5.1. Experiment 3: introduction

The landmark task can be solved in either of two ways. The first utilizes a discrimination learning strategy (associative) and the second a relational (cognitive) strategy. The location of the landmark was to the left of the object when a right response was correct, and to the right when a left response was correct. If animals learned to solve this task with an associative strategy, therefore, they would have to learn the correct response to each of these two contingencies.

Alternatively, the use of a relational strategy requires the dog to learn the general rule of approaching the object closest to the landmark. On any given trial, this strategy would require the dog to compare the distance between each object and the landmark, and respond to the closest one.

If an associative strategy were used to acquire the task, then switching to a new landmark would constitute a novel task that would have to be relearned. Dogs that used a relational strategy, by contrast, should readily be able to learn to respond appropriately to a new landmark, independently of its exact location. In the first part of this study, we looked at performance after switching the landmark from a yellow peg to a pink heart shaped object. The second part of the study looked at the effect of moving the landmark to a new location.

5.2. Experiment 4 methods

Subjects were 19 old dogs (8 control food and 11 enriched food) and 17 young dogs (8 control and 9 enriched). Testing on this task commenced one week following completion of the variable landmark task described in experiment 2.

In the first phase of this experiment, the landmark was switched to a pink, wooden, heart-shaped object. We used the same variable-distance procedure described in experiment 2, except that the dogs were only give 10 test sessions.

After completing this test phase, the animals (18 old dogs and 17 young) were given an additional 5 sessions in which three novel landmark positions were used, all of which were a distance of 1 centimeter from the dish (Figure 7).

5.3. Results and discussion experiment 4

The data were first analyzed with a repeated measures analysis of variance with landmark distance as a within subject variable, and both age and diet as between subject variables. There were highly significant effects of age ($df=2,31$; $f=18.53$; $p=.000$). and of distance ($df= 2,62$; $F= 53.74$; $p=.000$ but not of diet. There was also a significant interaction

between age and landmark distance (df=2,62; p=4.57; p=.014). The results, which are summarized in Figure 8 show that the young animals committed fewer errors than the old at the long distances, while the groups did not differ significantly at the 1 cm distances.

The performance of the animals on this task could represent either new learning, or generalization of the landmark principal. Figure 9 shows a scatter plot of the total number of errors by each subject, which indicates that all but three animals in total performed significantly above chance. Figure 10 shows mean performance as a function of session. The absence of any trend towards decreasing errors further suggests that the animals had acquired the landmark concept.

We also found that performance was not disrupted by changing the position of the landmark (Figure 11). We conclude, therefore, that dogs learned to use the relative position of the landmark to determine which stimulus to respond to. These results, suggest a difference between canines and rodents, who show little learning when the position of the landmark is variable rather than fixed [34].

6. Experiment 5: long-term retention

6.1 Experiment 5 introduction

Loss of memory is probably the most common complaint in human aging. However, memory does not represent a unitary process, and some aspects of memory are more sensitive to age than others. Thus, the earliest sign of Alzheimer's disease is a deficit in recall of specific autobiographical events (episodic memory) [35]. Remote memory, which refers to memories established at an earlier period in life, are also sensitive to aging, and age related dementia [36, 37]. There have been few animal models of these

kinds of long-term memory deficits. This study investigated long-term retention of the landmark discrimination task in groups of young and old beagle dogs.

6.2. Experiment 5 methods

The subjects used in this study were a subpopulation of the subjects that had completed experiment 3, and consisted of a total of 20 old dogs (12 on antioxidant food and 8 on control food), and 16 young dogs (9 on antioxidant food and 7 on control food). Four old animals did not participate in this study because they either failed to learn the landmark task, or because they were scheduled for use in another study. One young dog was dropped from the study because of loss of motivation to respond to food rewards.

After completing the earlier landmark testing as described in experiments 1-3, all subjects were administered a series of oddity discrmination learning problems (Milgam et al., in press)[38]. They were then retested on the original landmark discrimination learning task (L0), using the same procedures described in experiment. The interval between the last test on the landmark task, and the first retention task was approximately 7 months.

Because of time constraints, we were unable to complete the training to the criterion levels in several animals. Thus, the analysis used in this study was based on performance over the first 6 retest sessions only.

6.3. Experiment 5 results and discussion

The data were analyzed using a two-way analysis of variance, with age and diet as between subject variables and errors as a within a subject variable. The results indicated a

significant effect of age, with the old animals demonstrating poorer retention than the young, on the average (df=1,29, F=9.966, p=0.004).

The individual scores of the young animals are plotted on Figure 12a, which indicates the origin of the age differences. The young animals showed virtually perfect retention. Eleven of twelve passed the first criterion phase within two sessions. The relearning rate for the old animals was more variable, and could be separated into groups that either showed excellent retention, or required retraining.

We also did a separate comparison on the aged dogs for the effects of food. When the old animals were considered separately, the results were marginally significant using a one tail t-test, (df =19, t = 1.56591, p= 0.0654). As shown in Figure 12b, this reflected better retention in the animals on the enriched food.

7. General discussion

These studies focused on several factors affecting the ability of dogs to learn to and utilize external landmarks to indicate the location of food. We used a landmark discrimination learning task, in which the animals were trained to approach an object based on its proximity to a specific external landmark. The results demonstrated marked effects of age, proximity of the external cue to the correct stimulus, and previous experience with the use of external landmarks. We also found that performance of aged dogs could be improved by a nutritional intervention, consisting of providing food enriched with a broad spectrum of antioxidants and mitochondrial cofactors.

7.1. Effects of age on landmark discrimination learning

The effects of age on allocentric spatial ability was manifested in rate of learning, the ability to accurately assess spatial relations, and in long-term memory of previously acquired tasks.

Experiment 1 demonstrated marked age differences in learning to discriminate between two circular coasters, based on proximity to a yellow peg that served as a landmark. The protocol consisted of 4 separate tasks. In the first, the landmark was attached to the middle of one of the coasters. Because the landmark was in contact with the coaster, this is not a true landmark discrimination task. Rather, the problem involves object discrimination learning, in which the dogs learn to discriminate between the coaster and the coaster – peg combination. However, we have previously found object discrimination learning to be insensitive to age in dogs [28], and similar results have been obtained in studies with primates [39-42]. It is now clear, however, that discrimination learning ability, in general, is sensitive to age. In dogs, we have found age-dependent impairment in both size-discrimination learning [43], and oddity discrimination learning [38]. Even in tasks that do not show age-differences in mean rate of learning, we typically find age differences in variability [28]. Age differences in visual discrimination learning have also been reported in some primate studies [44]. The occurrence of age-dependent deficits in some studies but not others could reflect differences in task difficulty, and also experimental design factors such as sample size. Another potentially important factor is previous test history. The dogs used in this study previously underwent extensive baseline testing, which likely affected the rate of learning of the landmark task. This prior cognitive experience is likely to have facilitated learning, and this effect may possibly be greater in young subjects.

Experiment 1 also tested dogs on a true landmark discrimination learning task, in which the landmark was physically separate from the correct object (L1). We also found age differences in learning, with young animals making significantly fewer errors than old. These differences were also manifest in the proportion of animals from each group that were able to reach our learning criterion.

The young and old groups animals came from the same colonies, but because of space limitations, the groups were housed in different facilities. This is very unlikely to have affected the outcome for two reasons. First, the testing apparatus and testing procedures were identical at both facilities. Second, we have now obtained considerable additional data on old subjects tested at the both facilities with similar results.

Another non-cognitive factor that could have affected the outcome of this study is differences in sensory processing ability. But the existence of such deficits is unlikely to account for the present data. We masked the location of the food reward in order to prevent the dogs from using olfactory cues. The effectiveness of this masking was established by the poor performance of the majority of dogs when the landmark was moved 4 cm away from the correct object. If the animals' were responding based on olfactory cues, distance of the landmark would not have mattered. Regarding the possible impact of deficiencies in visual processing, intact vision based on veterinary examination was a required selection criterion. In addition, all of the subjects had previously learned both an object discrimination and an object reversal learning task, indicating the ability to associate specific visual cues with reward. Furthermore, the present task did not require visual discriminative ability, but rather knowledge of location.

7.2. Effect of age on utilization of allocentric spatial cues

Experiment 2 looked at performance of old and young dogs on a variable distance landmark discrimination learning task. The animals had all previously learned the initial landmark discrimination task with, at a minimum, the landmark 1 cm from the object. This experiment demonstrated first that performance, in general, deteriorated with increasing distance, a result that we previously reported [2], and that also has been reported in rats [33]. This experiment also demonstrated age differences that depended on distance. The young and old groups performed similarly when the landmark was 1 cm from the object; age differences were apparent, however, when the distance was increased to 4 or 10 cm. This represents an age-related deficit in evaluating relative distances, and to our knowledge, this is the first such report of age differences in visuospatial function of animal models. This could represent an age-dependent deficit in peripheral vision. Alternatively, the deficit may relate to an age-dependent deficit in higher level visual processing, reflecting the effect of age on the computational demands necessary to evaluate relative distance.

The results of this experiment indicate age deficits in visuospatial processing, which are specifically linked to the utilization of allocentric cues. We have also found that dogs show age-dependent impairment in performance of a delayed-non matching to position test, which also involves visuospatial function [4, 7] . In this test, old dogs showed slower learning and less efficient memory. Non-human primates tested on delayed response tasks also show deficits in learning [45, 46] and in remembering spatial information when delays are increased [46-51]. These age-related deficits are not

necessarily indicative of global cognitive dysfunction, as visuospatial learning and memory are impaired at an earlier age than object recognition memory [46, 47, 52-54].

Although the landmark task is intended to measure allocentric spatial memory, the task can also be solved with an associative learning strategy. Experiment three was designed to explore this possibility, by using a novel landmark, and by placing it at novel locations. When the landmark was switched from a yellow peg to a wooden heart shaped object, the majority of animals – both old and young showed evidence of generalization of the landmark concept. Indeed, level of performance at the start of training was at, or close to maximal. We also found that performance was maintained when the landmark was switched to a new location. These results strongly suggest first, that the dogs had learned the general concept, and second that this concept readily generalized to the use of a novel landmark.

7.3 Effects of antioxidants and mitochondrial cofactors on discrimination learning

A second major goal of this study was to examine the cognitive effects of providing aged dogs with food enriched with a broad spectrum of antioxidants and mitochondrial cofactors. The strongest evidence of the potential efficacy of nutritional factors came from experiment 1, which showed that subjects on the enriched diet performed significantly better than the control subjects on the initial landmark discrimination task (L0). Significant improvement, however, was seen only in the aged group; there was no significant effect of food on performance of the young animals, although there was a trend in the same direction as the old.

A number of factors probably account for these results, including the use of aged subjects, the selection of tasks, and the components of the food. The primary rationale for

utilizing aged subjects is because in all animals studied to date and in the human literature, progressive increases in oxidative damage are consistently observed with advanced age [55]. Oxidative damage to proteins, lipids and to nucleotides can potentially impair central nervous system function at the level of the single neuron function through a variety of mechanisms. Lipid peroxidation can disrupt membrane function, affecting cellular homeostasis [56]. The accumulation of oxidatively modified proteins disrupts cellular function either by a loss of catalytic ability or by an interruption of regulatory [57]. Oxidatively modified proteins may become cross-linked and resistant to degradation, which can lead to further aggregation or abnormal proteins within or around neurons [58]. In particular, oxidative damage to RNA may interfere with the translation of new proteins][59]. Oxidative events leading to protein dysfunction may in turn, interfere with protein synthesis required for the formation of memories [60, 61]. Reducing oxidative damage to RNA by lipoic acid or a combination of lipoic acid with acetyl-L-carnitine, which are components included in the enriched diet used in this studyhas been reported in aged rats [62] along with significant improvements in spatial memory.

In addition, inclusion of mitochondrial cofactors may have multiple benefits to neuronal cell energetics. Acetyl-carnitine and lipoic acid improve mitochondrial energetics and decrease the production of free radicals [63, 64]. Lipoic acid by itself may improve glucose uptake and utilization in cells, increase GSH:GSSG ratios and act as an antioxidant itself in recycling vitamin E peroxyradicals [64, 65]

With respect to dietary constituents, to our knowledge, this is the first study to have combined substances that target enhancement of mitochondrial function with

antioxidants that suppress the action of free radicals. Our results build upon and extend the findings that antioxidants or mitochondrial cofactors alone decrease age related cognitive decline in other species [63,66,67]. Our results may be attributable to two different synergistic strategies; first, a complex mixture of antioxidants that supports a network of antioxidants requiring several components to act together for effective function, and; second, improved mitochondrial metabolic function that decreased free-radical production while improving mitochondrial efficiency. We suggest that the combination of antioxidants with mitochondrial enzymatic cofactors may work together synergistically to enhance mitochondrial function leading to a decrease in both the production and consequences of reactive oxygen species [64]. Taken together our data supports the hypothesis that oxidative damage and mitochondrial function is a fundamental mechanism contributing to age-associated cognitive dysfunction and underscores the need to conduct similar trials in humans.

FOOTNOTES

[a]Hill's Prescription Diet® p/d, Hill's Pet Nutrition, Inc. Topeka, KS 66601

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Table 1. Age distribution of subjects used in the study.

Age-Range*	Antioxidant Group	Control Group

Young Dogs	N=9	N=8
<2.00	1	1
2.01-3	4	4
3.01-4		1
>4	4	2
Average age (Years)	3.28	2.98
Old Dogs	N=12	N=12
8-9	2	1
9.01-10	3	1
10.01-11	5	7
11.01-12	1	2
12+ -	1	1
Average Age (Years)	10.07	10.51

*Age taken as subjects age at the start of training on the landmark study.

TABLE 2. Number of subjects that successfully completed each phase of Experiment 1.

Group	N	Land-0[1]	Land-1[2]	Land-2	Land-4
Old-Control	12	12 (4)	8 (2)	4	1
Old-Antioxidant	12	12	11 (1)	7	1
Young Control	8	8	8	6	4
Young Antioxidant	9	9	9	8	4

[1] The numbers indicate the number of dogs that successfully passed the task. The number in parenthesis represents the number of dogs that failed initially, but passed after undergoing remedial training protocol. Note that remedial training was only given after training on Land-0 or Land-1.
[2] Testing of one dog in the control group was discontinued during Land-1 because of illness, and this animal's data were excluded from analysis of Land-1.

TABLE 3. Serum levels of vitamin E as a function of age and food. *Indicates value is significantly different from baseline and age matched control within that time period ($p<0.05$)

Group	N	Baseline	3 months
Old-Control	12	30 ± 2.2	29.4 ± 2.5
Old-Antioxidant	12	28.2 ± 2.4	$49.6 \pm 4.9^{*}$
Young Control	8	22.8 ± 1.0	24.4 ± 1.7
Young Antioxidant	9	26.2 ± 1.8	$40.8 \pm 4.9^{*}$

Figure Legends:

Figure 1. Cognitive test apparatus used in testing dogs on landmark discrimination task. The dog is placed in a wooden box with two openings to allow it respond to discriminanda covering food wells.

Figure 2. Landmark and discriminanda used in training dogs on landmark discrimination learning task. The dogs were first trained with the landmark on top of the correct object (land-0). They were then trained with the landmark successively 1, 4 and 10 cm from the object towards the midline.

Figure 3. Performance on baseline cognitive testing.
Figure 4. Effect of age and diet on landmark discrimination learning. A) shows mean errors to reach predetermined two-stage criterion on initial landmark discrimination task (landmark 0), plotted as function of age and diet. B) shows errors to learn true landmark (L1), when the landmark was a distance of 1cm from the correct discriminanda. C) shows errors when the landmark distance was increased to 4 cm (Landmark 2) and D) represents the number of errors committed when the landmark was moved 10 cm away from the coaster(Landmark 4).

Figure 5Errors plotted as a function of chronological age, independent of diet, for the old dogs.
Figure 6. Effect of age and landmark distance on performance of the variable distance landmark task. (A) shows mean errors for the control and enbriched groups of old dogs and for the control and enriched groups of young dogs. (B) shows accuracy as a function of age for those dogs, both young and old, that performed significantly above chance.
Figure 7. Landmark configurations used in Experiment 3.

Figure 8. Effect of age and landmark distance on
performance on the novel landmark task.

Figure 9. Performance of young and old animals on novel landmark. Scores below the dashed line are more than two standard deviation units from the mean, and are therefore unlikely to represent chance performance.

Figure 10. Performance as a function of test session on novel landmark.
The absence of any indication of improvement suggests very
rapid transfer of learning

Figure 11. Performance of young and old animals on novel and modified-location landmark task.

Figure 12. Retention of landmark discrimination learning task as a function Of age and diet. (a) The scatter plot shows the individual data points plotted as a function of age. (b) shows mean errors to criterion and trials to criterion for the old animals, plotted as function of diet.

COGNITIVE TEST APPARATUS

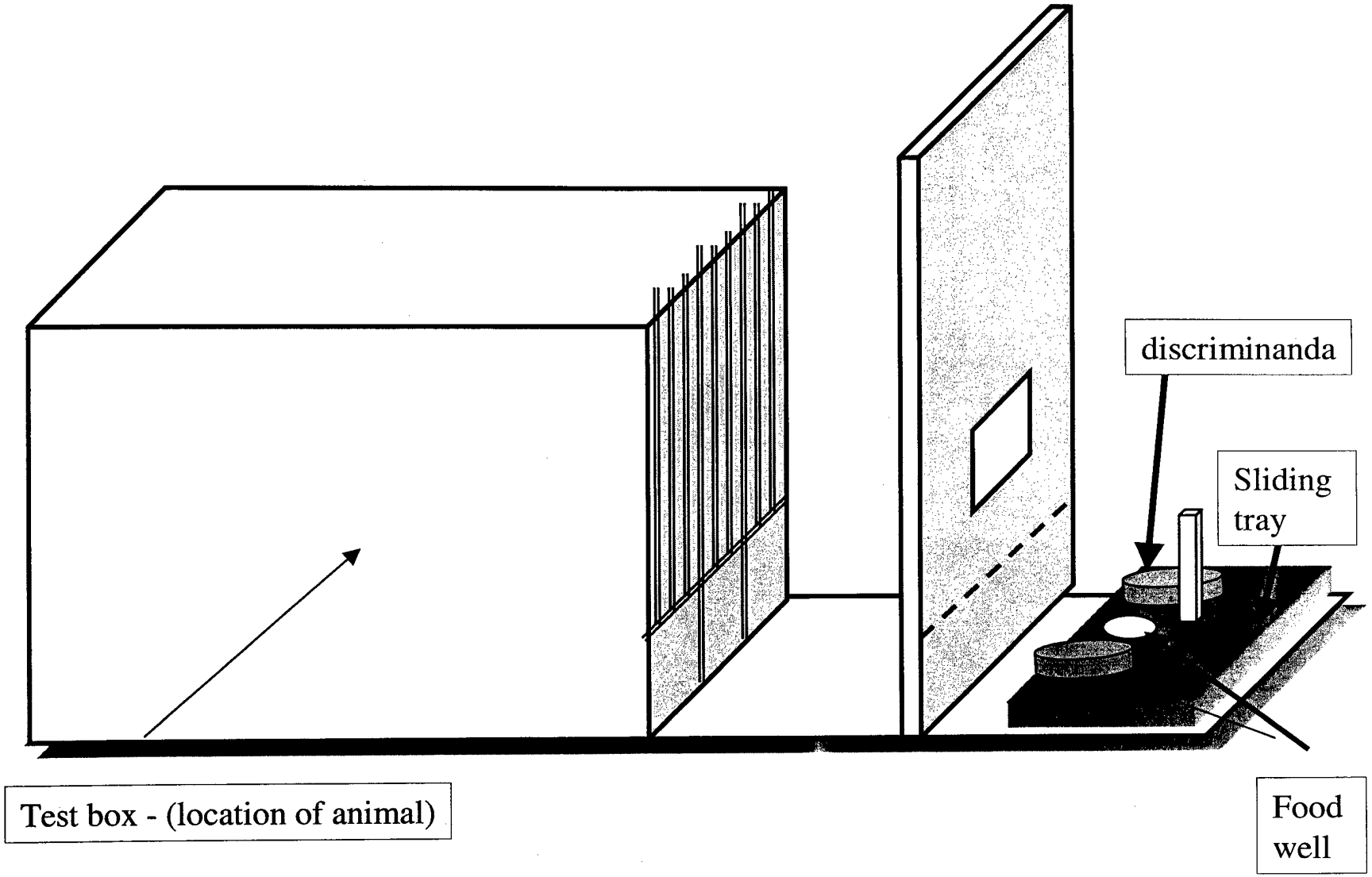


Figure 1. Cognitive test apparatus.

Dog

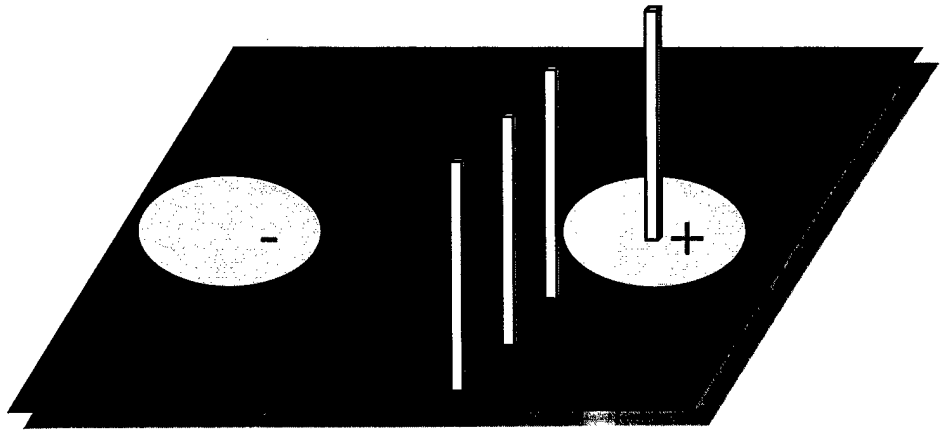
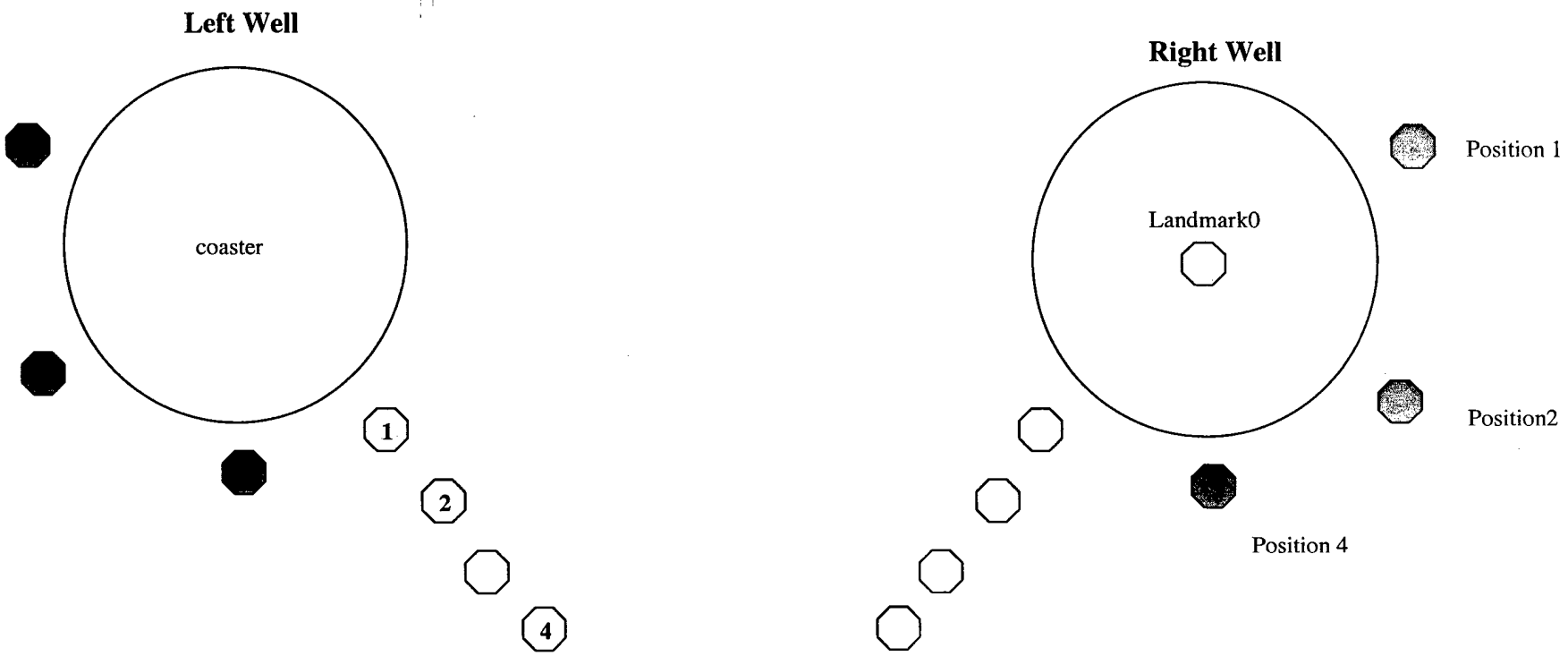


Figure 2

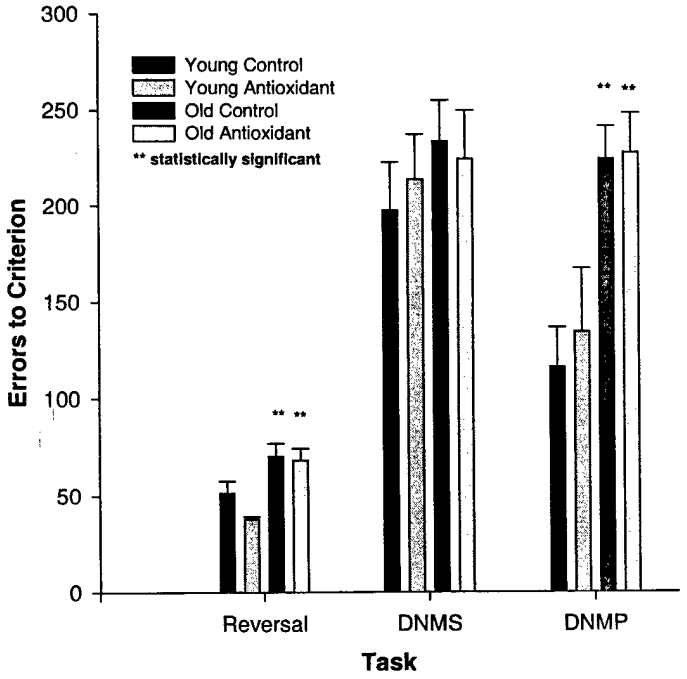
Front of Tray



Birds eye view of the positions for both the original landmark and the modified landmark

Modified Landmark positions

figure 3



USE THE
ABOVE FIGURE

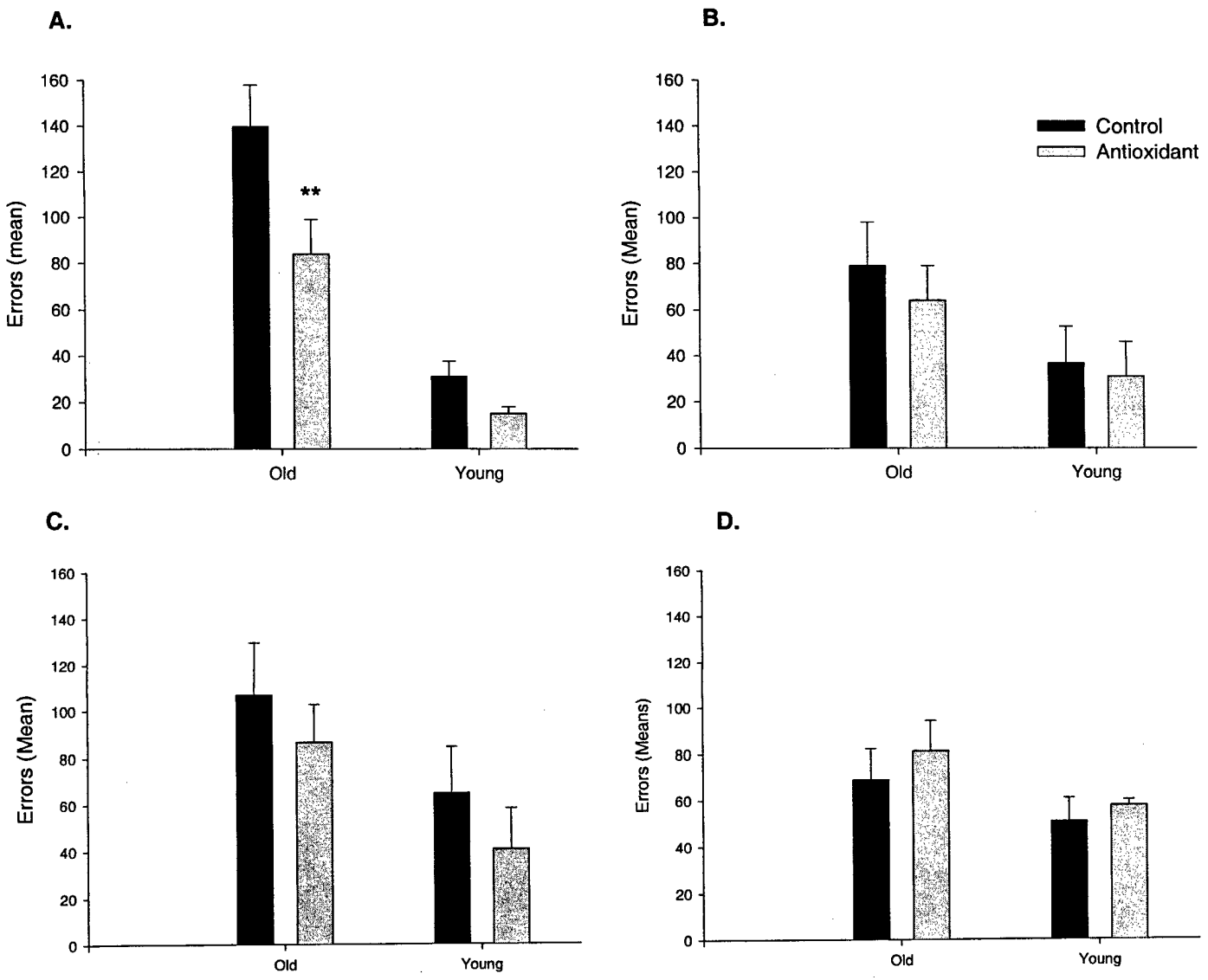


Figure 4 – Effect of age and diet on landmark discrimination learning.

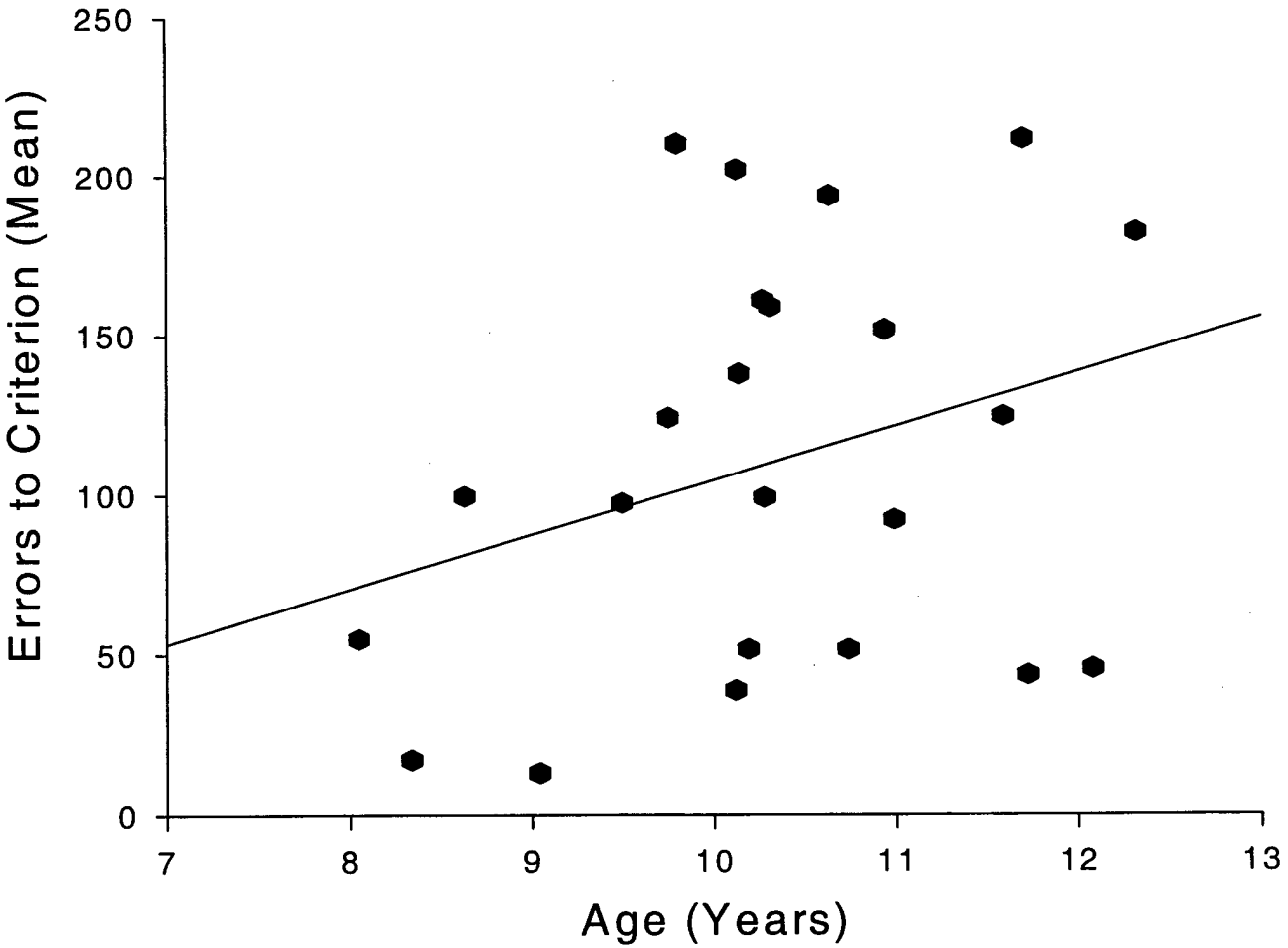


Figure 5. Errors are plotted as a function of age, independent of diet in Land-0 for the old subjects. The correlation was positive, but not significant (r=0.319).

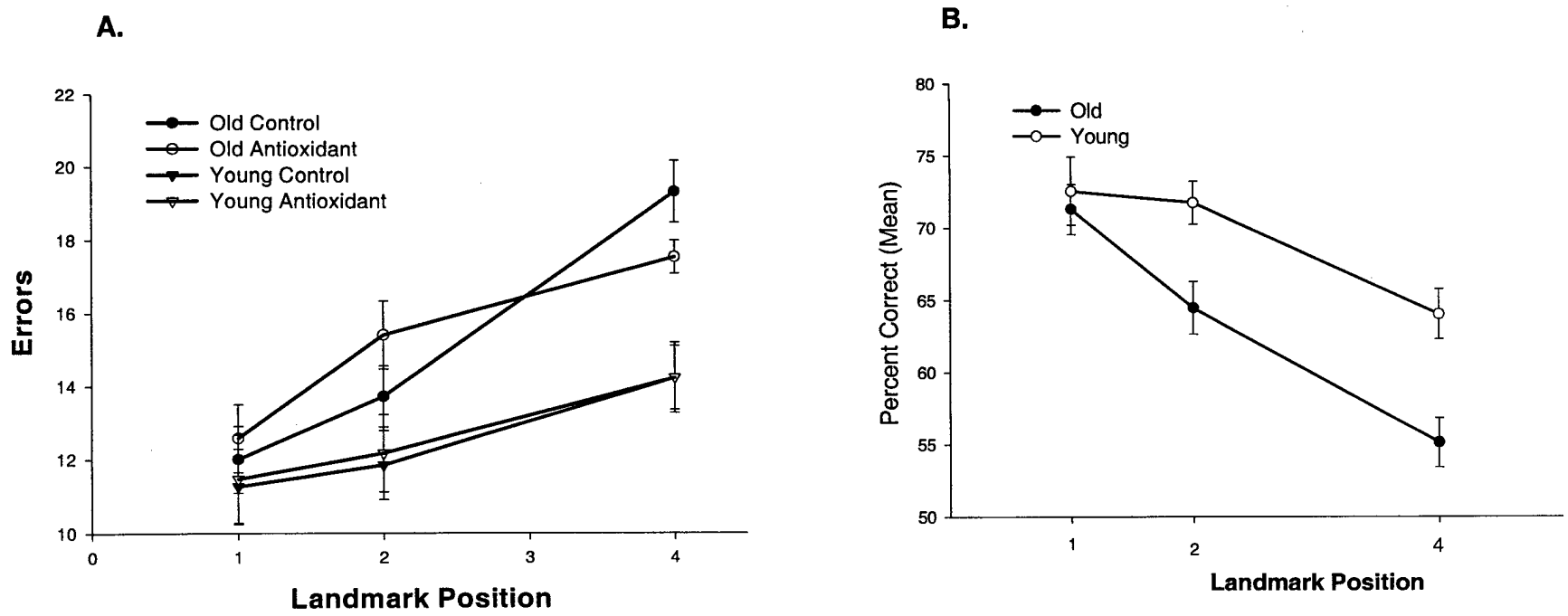


Figure 6. Effect of Age and Distance on Performance of variable distance landmark. (a) shows mean errors for the control and enriched groups of old dogs and for the control and enriched groups of young dogs. (b) shows accuracy as a function of age for those dogs in both the young and groups that performed significantly above chance.

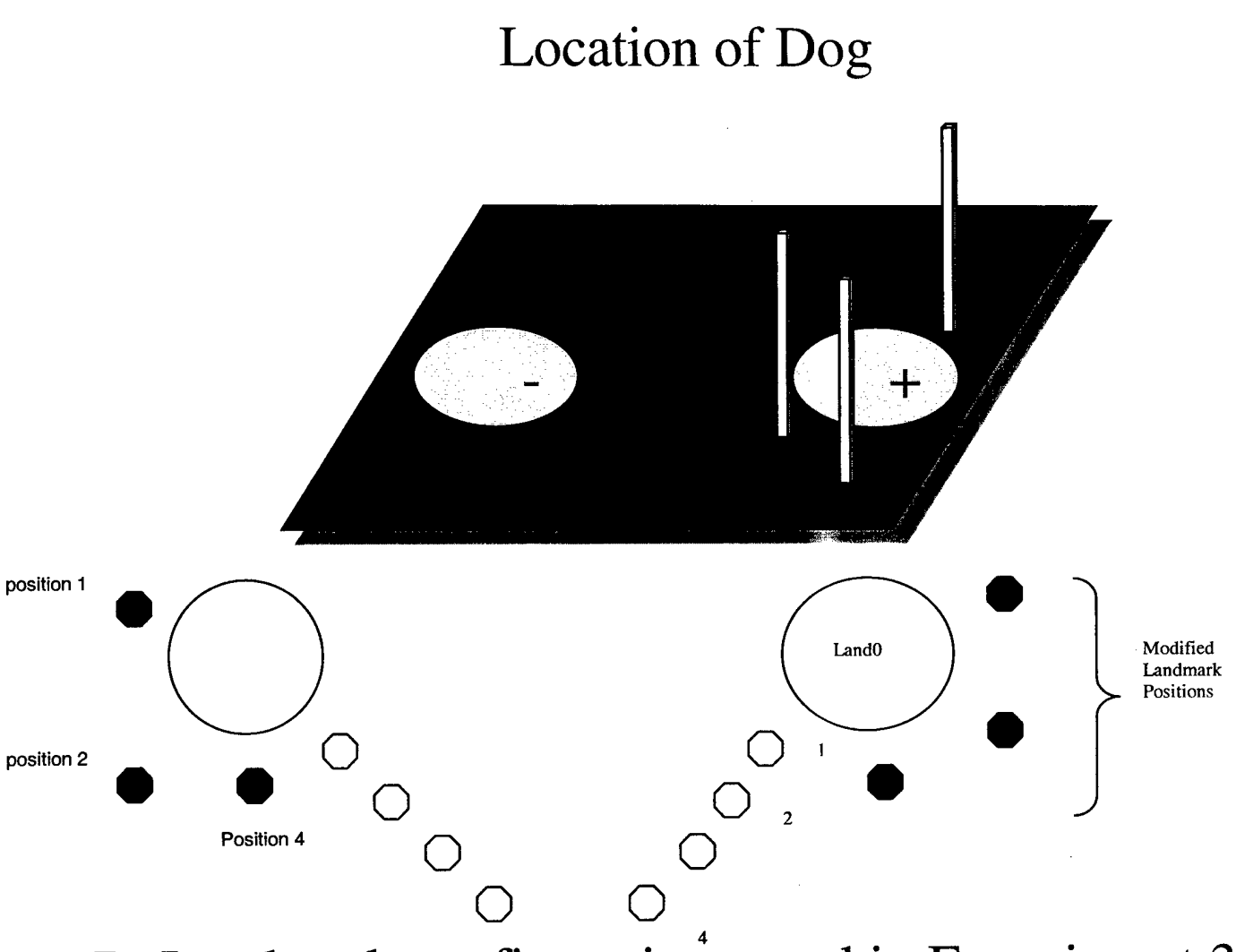


Figure 7. Landmark configurations used in Experiment 3.

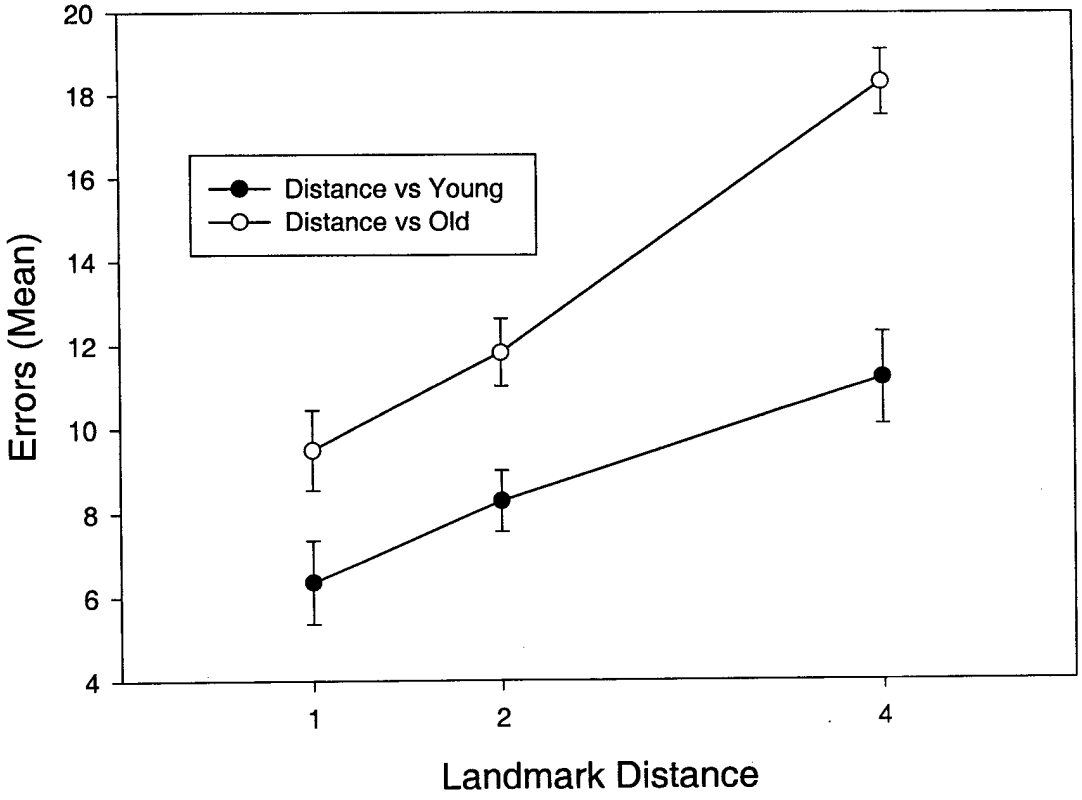


Figure 8. Effect of age and landmark distance on the novel landmark task.

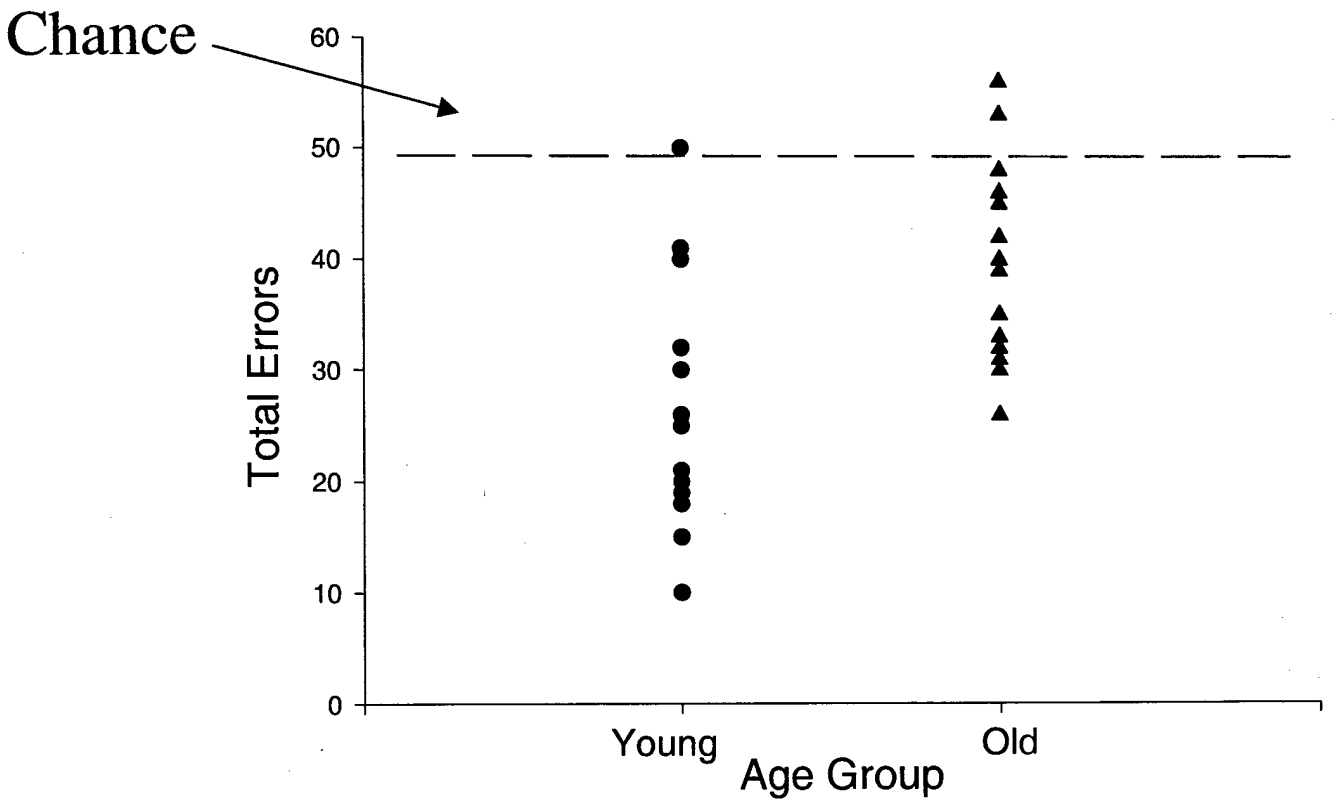


Figure 9. Performance of young and old animals on novel landmark. Scores Below the dashed line are more than two standard deviation units From the mean, and are therefore unlikely to represent chance performance.

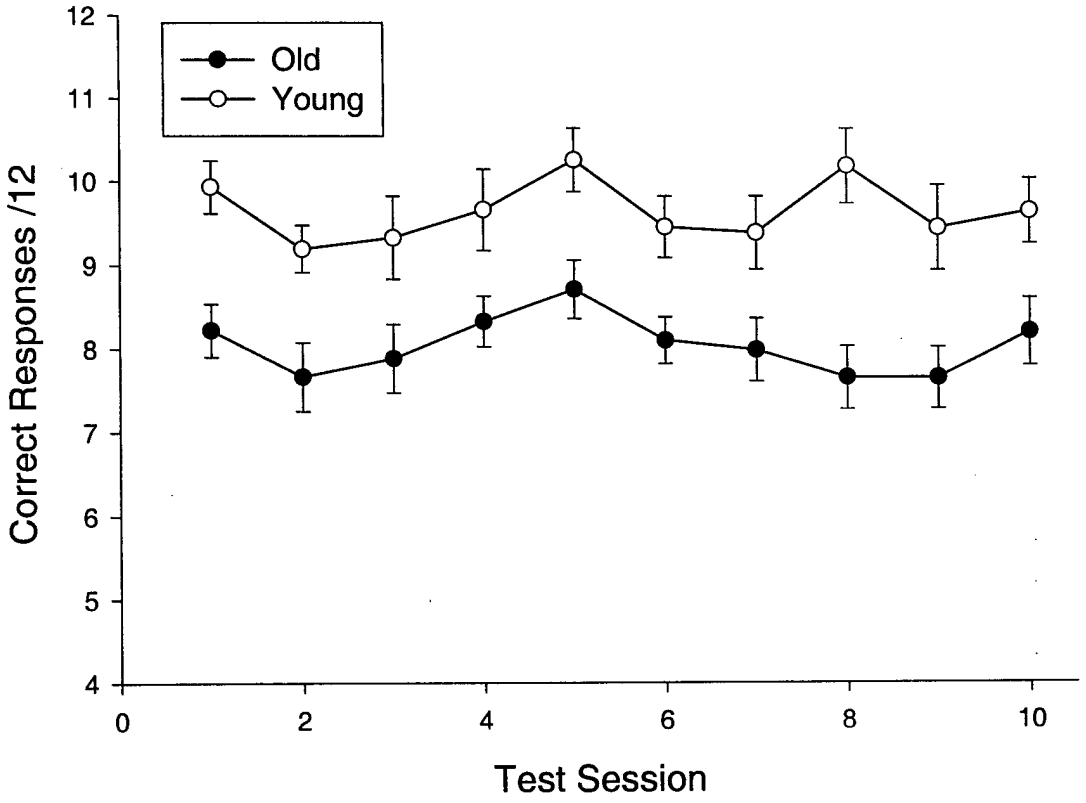


Figure 10. Performance as a function of test session on novel landmark. The absence of any indication of improvement suggests very Rapid transfer of learning

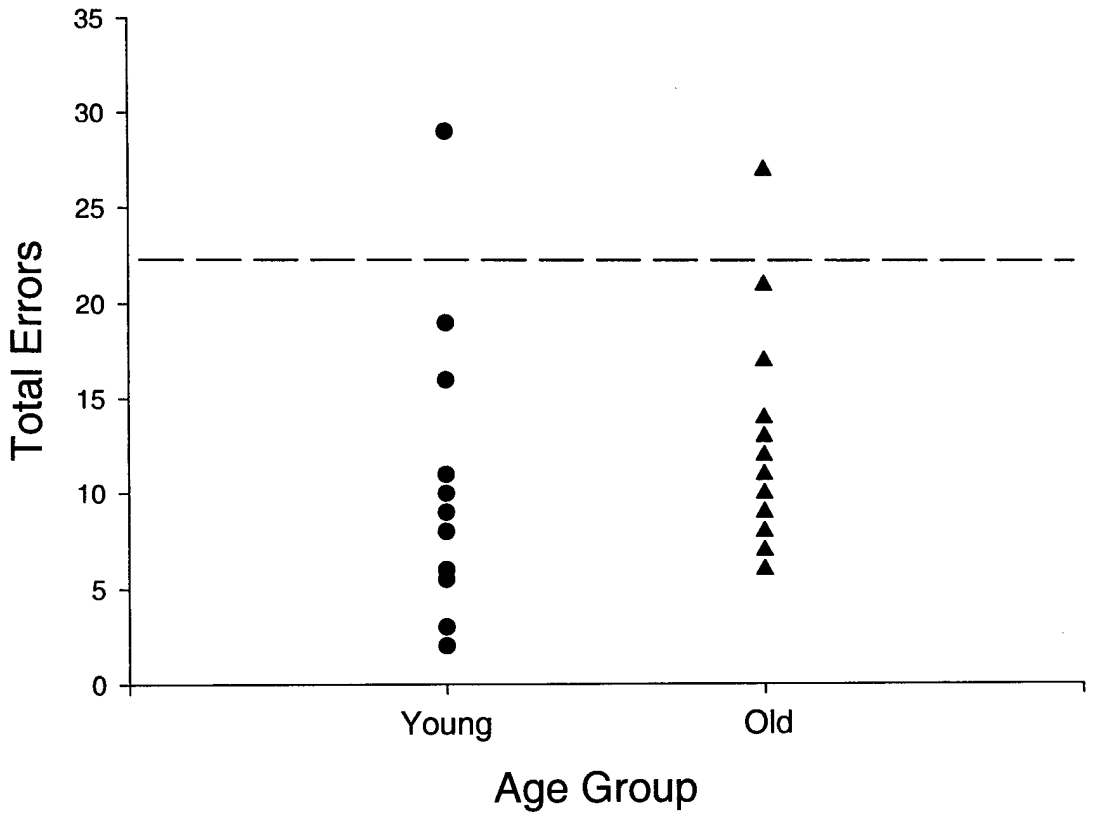


Figure 11. Performance of young and old animals on novel landmark in a modified location. Scores above the dashed line are not significantly different from the chance level of 30 errors.

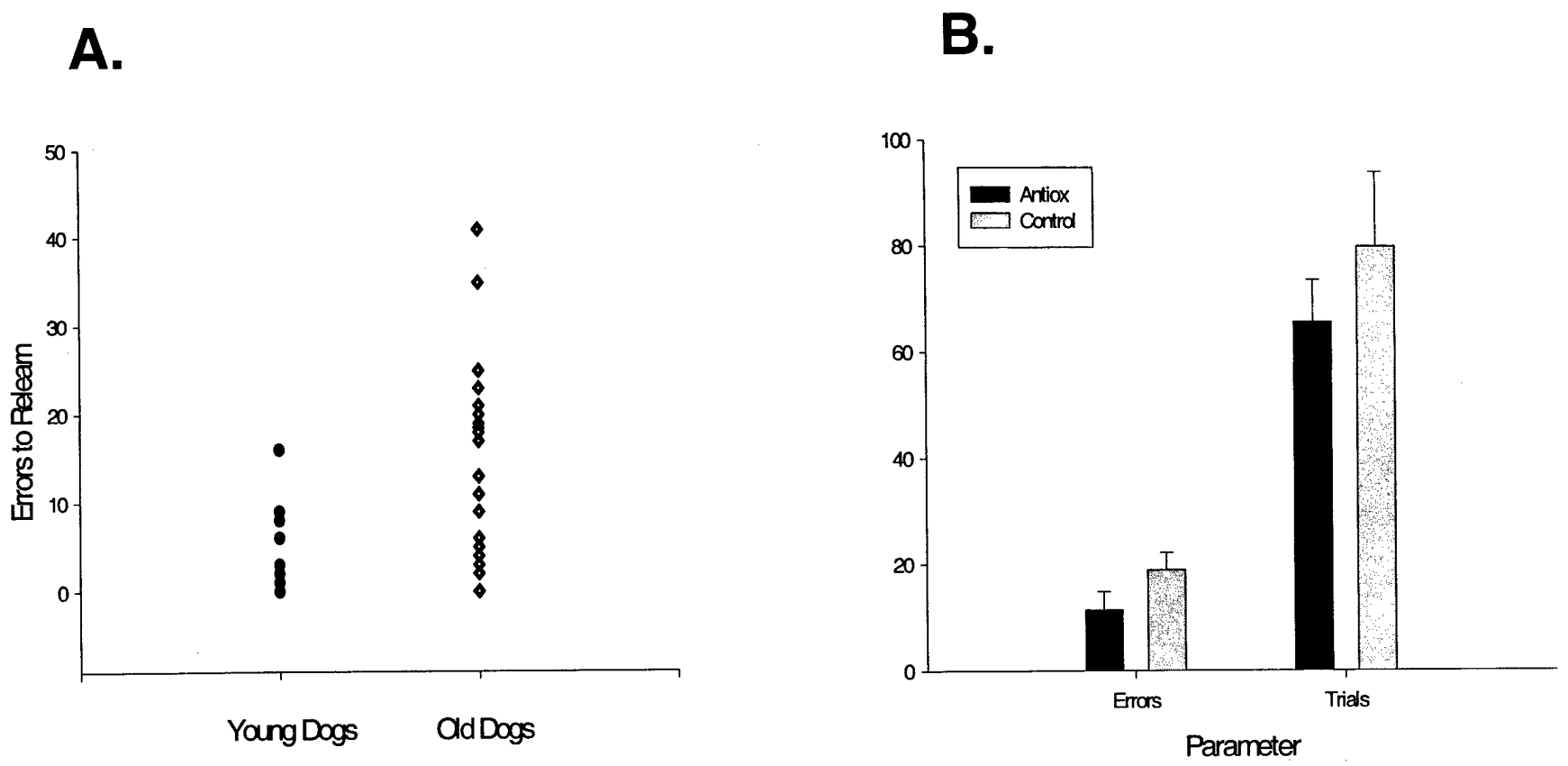


Figure 12. Retention of landmark discrimination learning task as a functio
Of age and diet. (a) The scatter plot shows the individual data points plotted as a function of age. (b) shows mean errors to criterion and trials to criterion for the old animals, plotted as function of diet.

Effect of Age and Level of Cognitive Function on Spontaneous and Exploratory Behaviors in the Beagle Dog

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Cognitively characterized young and aged beagle dogs were administered six different spontaneous behavior tests, which provided measures of locomotion, exploration, and social interaction. Consistent with our previous findings, we obtained no overall effect of age on locomotion. We did find, however, that for the aged dogs locomotion correlated with level of cognitive function, being lowest in age-unimpaired dogs and highest in impaired dogs. Exploratory behavior, as measured by response to novelty, varied with age, with young dogs scoring the highest. Young dogs spent more time with novel toys and a person, responded more to a silhouette of a dog, and interacted more with a model dog compared to aged dogs. Among the aged dogs, age-unimpaired dogs spent the greatest amount of time sitting or standing beside a person whereas age-impaired dogs spent the most time reacting to a reflection in a mirror. The age-impaired dogs show undirected, stereotypical types of behavioral patterns. These differences in activity patterns may be linked to underlying age-associated neuropathology.

Cognitive deficits in aging have been studied extensively in a wide range of species. Typically, however, the variability among aged animals within a species is extensive, and only a limited number show marked behavioral deficits (Ingram 1988; Gallagher and Burwell 1989). Several reports have identified two subgroups of aged animals; one group whose performance on a variety of behavioral tests does not differ from that of young animals and a second group whose performance is dramatically worse compared to young animals (Gallagher and Burwell 1989; Rowe et al. 1998; Adams et al. 2000b). Other aspects of behavior are also affected by age, and these may be independent of cognition (Gage et al. 1984). Age-related deficits on simple tests of motor function (wire-grip test, bridge crossing, locomotor activity, sensorimotor reactivity) were unrelated to impairments in spatial learning using the Morris water maze (Gage et al. 1984).

Gallagher and Burwell (1989), however, reported that performance on other behavioral tasks (i.e., recovery from neophobia) coincided with spatial learning ability in the Morris water maze. Rowe et al. (1998) also found that cognitively impaired and unimpaired aged rats could be discriminated on the basis of behavioral measures distinct from those assessing learning and memory. Impaired animals were less responsive to novel stimuli and exhibited a deficit in habituation to an aversive stimulus.

The majority of this research has used rodent models. The present experiment sought to extend these findings to a higher-level species, the dog (*Canis familiaris*). Dogs and humans share similar cardiopulmonary systems, environmental influences, and brain pathology, and studies using the dog may prove valuable to discern the underlying causes of age-associated ailments in both species (Cummings et al. 1996). We were particularly interested in a possible relationship between behavioral activity, exploratory behavior, and neuropsychological measures of cognitive function in aged dogs.

Exploratory behavior has previously been considered to be a kind of instinctive behavior, necessary for survival. However, researchers now regard exploration to be a high-level aspect of sensory processing involved in investigating novel stimuli (Kelley et al. 1989). A novel environment or novel objects in a familiar environment offer opportunities to learn and explore (Pierce and Courchesne 2001). Exploration generally involves movement or locomotor activity, but locomotion includes behaviors such as spontaneous activity, exercise, or escape that are unrelated to exploration (Archer and Birke 1983). Berlyne (1960) distinguished between specific and diversive exploration. Specific exploration is activity directed towards obtaining selective information, whereas diversive exploration is a more general motor activity elicited by a wide range of internal and external stimuli. Exploratory behavior is likely dependent on intact prefrontal cortical-striatal-pallidal circuitry as well as cerebellar function (Pierce and Courchesne 2001).

One method of studying exploration is with the use of the open field, which involves characterizing an animals

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Article and publication are at http://www.learnmem.org/cgi/doi/10.1101/lm.41701.

LEARNING & MEMORY 8:317–325 © 2001 by Cold Spring Harbor Laboratory Press ISSN1072-0502/01 $5.00

behavior in a standardized environmental chamber. However, the open field test cannot distinguish locomotion per se from stimulus-directed exploratory behavior because of the absence of specific external stimuli (Brennan et al. 1982). In fact, several studies have shown that locomotor activity and exploration can be dissociated. Montgomery (1953) concluded that exploration is independent from general activity when activity deprivation did not lead to an increase in exploratory behavior. Leyland et al. (1976) showed that novel and complex stimulation increased exploration but did not affect locomotor activity in rats. The correlation between the two types of behaviors was close to zero. Finally, amphetamine selectively increases locomotion but decreases exploration (Leyland et al. 1976).

Test situations in which discrete stimuli are present and distinguishable from the general background provide a more appropriate means of studying exploration. Berlyne (1950, 1955) noted that approaches to specific items provide a more informative measure of exploration than nonspecific approaches to areas in the environment.

Exploratory behavior, independent of locomotion, has been found to decline with age in rodents (Furchgott et al. 1961; Williams et al. 1966; Brennan et al. 1982; Handa et al. 1996). In the past we have used locomotor activity as a measure of exploration in dogs. We previously reported that locomotion in the open field is not significantly affected by age in canines (Head and Milgram 1992; Head et al. 1997). The present study attempts to distinguish between exploratory locomotion and stimulus directed exploration and is concerned with the effects of aging on exploratory behavior in beagle dogs. We also sought to study the effect of age on the presence of both artificial conspecifics and people. Accordingly, we developed five novel protocols to provide indices of exploratory and social behaviors. We hypothesized that exploratory behavior would be related to level of cognitive function. Accordingly, we divided the aged dogs into cognitively impaired and unimpaired, based on neuropsychological tests of cognitive function.

RESULTS

Cognitive Characterization

The aged dogs were classified as cognitively impaired or unimpaired based on their performance on the delayed nonmatching to position (DNMP) task at 10-, 20-, and 30-sec delays and on a size discrimination learning task. The combined error score for both tasks was compared to that of the young dog group. An aged dog was considered impaired if its score was greater than two standard deviations from the mean of the young dog group. An aged dog with a score less than two standard deviations from the mean of the young dogs was placed in the unimpaired group. The mean score and standard deviation for the young dog group was 80 ± 59.49. Any aged dog with a score greater than 198.98 was classified as impaired. The distribution of error scores for the combined error score and the ages of the dogs are illustrated in Figure 1. The age-unimpaired group consisted of six males and five females. The age-impaired group had two males and 10 females. The correlation between performance on the DNMP and size discrimination tasks was 0.32.

Open Field Test

A comparison of the old and young dogs' locomotion, without taking into account cognitive status, revealed no significant difference [$F(1, 26) = 1.69$, $P = 0.20$] (Fig. 2). Significant main effects of age [$F(1,26) = 10.11$, $P = 0.0038$] and sex [$F(1,26) = 21.62$, $P = 0.000085$] were obtained for urination frequency. The interaction between age and sex was also significant [$F(1,26) = 18.97$, $P = 0.00018$]. Young males urinated significantly more often than young females ($P = 0.00026$), old females ($P = 0.00018$), and old males ($P = 0.00022$). A main effect of retest on sniffing frequency was obtained [$F(1,26) = 7.14$, $P = 0.013$]. Sniffing frequency increased from open field test 1 to open field test 2. A significant interaction between sex and retest revealed that sniffing frequency increased in the females only [$F(1,26) = 17.25$, $P = 0.00031$]. The interaction between age, sex and retest was also significant [$F(1,26) = 7.77$, $P = 0.0098$], indicating that sniffing frequency increased in young females from open field test 1 to open field test 2.

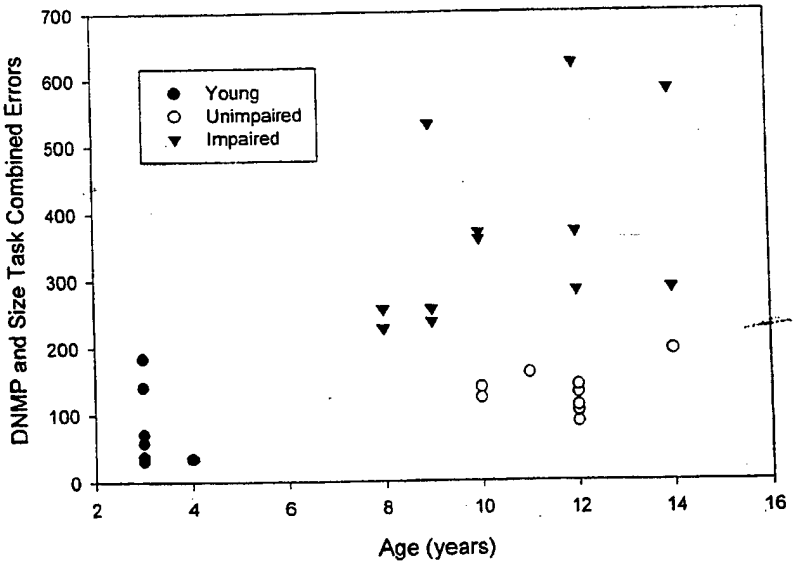

Figure 1 The combined sum of errors required to learn a delayed nonmatching to position task and a size discrimination learning task are plotted as a function of age and presence or absence of cognitive impairment.

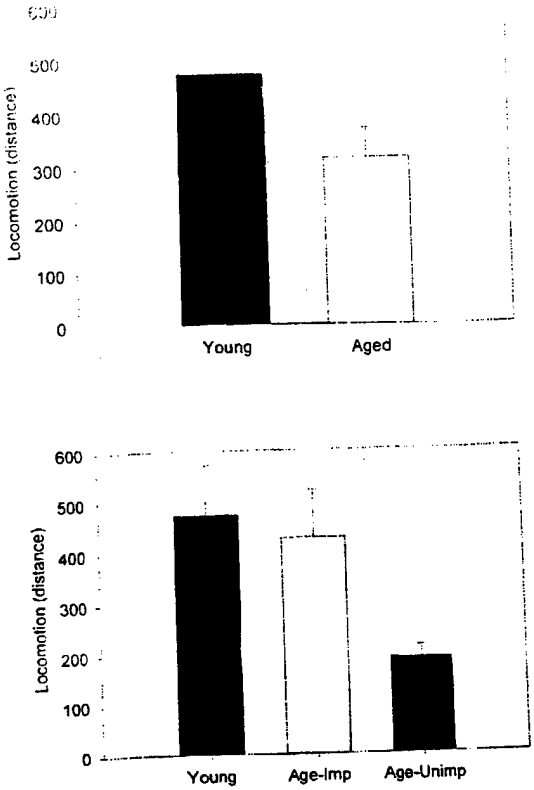

Figure 2 Locomotion in an open field for young, age-unimpaired and age-impaired groups. There is no difference between young and aged dogs in locomotion unless cognitive status is taken into account. The age-unimpaired dogs showed lower levels of locomotion than the young ($P = 0.058$) and age-impaired ($P = 0.067$) groups. Error bars represent standard errors of the mean.

The analysis of time spent inactive also revealed a significant main effect of age [$F(1,26) = 5.31$, $P = 0.029$]. Aged dogs spent more time inactive than young dogs.

When the aged dogs were subdivided into impaired and unimpaired, the ANOVA revealed a significant main effect of group [$F(2,27) = 3.89$; $P = 0.033$] on locomotion (Fig. 2). The age-unimpaired dogs showed less locomotion than the young ($P = 0.058$) and age-impaired ($P = 0.067$) groups.

Mirror Test

The ANOVA revealed a significant main effect of retest [$F(1,26) = 16.48$, $P = 0.0004$] for frequency of sniffing the mirror reflection. Sniffing frequency decreased from mirror test 1 to mirror test 2.

When the analysis considered cognitive status, significant main effects of group [$F(2,27) = 4.32$, $P = 0.024$] and retest [$F(1,27) = 5.16$, $P = 0.031$] appeared for the amount of time spent interacting with the mirror reflection. As shown in Figure 3, the age-impaired dogs spent significantly more time interacting with the reflection than the age-unimpaired dogs ($P = 0.018$). Interaction time decreased from test 1 to test 2.

Human Interaction Test

The analysis of the amount of time spent in physical contact with the person revealed significant main effects of age [$F(1,26) = 7.64$, $P = 0.010$] and retest [$F(1,26) = 10.35$, $P = 0.0034$]. Young dogs spent more time in contact with the person than old dogs, and contact time decreased from human interaction test 1 to test 2. The interaction between age and retest was significant [$F(1,26) = 7.36$, $P = 0.012$], indicating that contact time decreased for the young dogs only from test 1 to test 2, whereas the aged dogs showed no change. The three-way interaction between age, retest, and sex was also significant [$F(1,26) = 4.51$, $P = 0.043$]. Contact time decreased from human interaction test 1 to test 2 in the young female dogs.

The analysis of time spent beside the person revealed a significant main effect of age [$F(1,26) = 4.59$, $P = 0.042$] and an age by sex interaction [$F(1,26) = 4.45$, $P = 0.045$]. Aged males spent significantly more time beside the person than young males ($P = 0.025$) and aged females ($P = 0.017$) No significant effects were obtained for the frequency o sniffing the person in the room.

Cognitive status affected the responses of the dogs t the person. The analysis of the amount of time spent i physical contact with the person revealed significant mai effects of group [$F(2,27) = 4.23$, $P = 0.025$] and rete [$F(1,27) = 5.094$, $P = 0.032$]. The young dogs spent mor time in contact with the person than the age-unimpaire ($P = 0.050$) and the age-impaired ($P = 0.029$) groups.

The analysis of the amount of time that the dogs spe sitting or standing beside the person revealed a significa main effect of group [$F(2,27) = 6.71$, $P = 0.0043$]. The ag

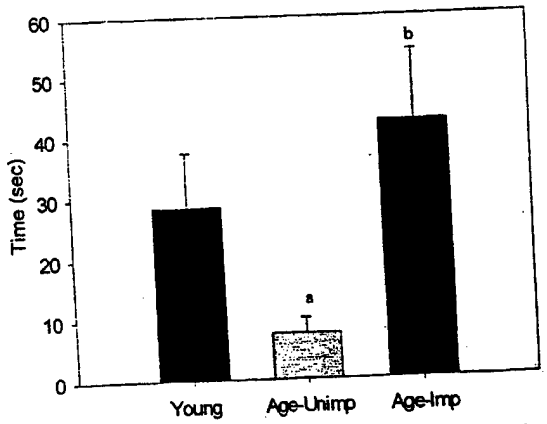

Figure 3 Time in seconds that dogs spent interacting with t reflection in the mirror. Age-impaired dogs spent significantly n time interacting with a mirror reflection than age-unimpa ($P = 0.018$) dogs. The young dogs were intermediate betweer two age groups. "a" is significantly different from "b". Error represent standard errors of the mean.

unimpaired group spent significantly more time near the person than the young ($P = 0.015$) and age-impaired ($P = 0.0095$) groups (Fig. 4).

Curiosity Test

The analysis of time spent exploring the toys in the test room revealed a significant interaction between age and retest [$F(1,26) = 4.25$, $P = 0.049$]. The time spent exploring the objects increased for the young dogs and decreased for the old dogs. No significant effects were obtained for the frequency of sniffing the objects.

Taking into account cognitive status, a significant main effect of group was obtained for amount of time spent in contact with the objects [$F(2,27) = 3.89$, $P = 0.033$]. This was a result of the young dogs spending significantly more time in contact with the objects than the age-impaired group ($P = 0.027$).

Silhouette Test

The analysis of sniffing the head region of the silhouette revealed a significant main effect of retest [$F(1,26) = 4.72$, $P = 0.039$]. The interaction between sex and retest was also significant [$F(1,26) = 6.98$, $P = 0.014$], revealing that sniffing decreased from silhouette test 1 to silhouette test 2 in males only. The analysis of sniffing in the rear region revealed a significant effect of age [$F(1,26) = 8.71$, $P = 0.0066$]. Young dogs sniffed more often than aged dogs.

Cognitive status was related to the animals' response to the silhouette. The analysis of sniffing the head region revealed a significant main effect of retest [$F(1,27) = 4.73$, $P = 0.039$]. The interaction between group and retest was significant at the 0.1 level [$F(2,27) = 2.80$, $P = 0.078$]. The young and age-unimpaired dogs showed less sniffing of the head region from silhouette test 1 to test 2, while the age-impaired dogs showed increased sniffing.

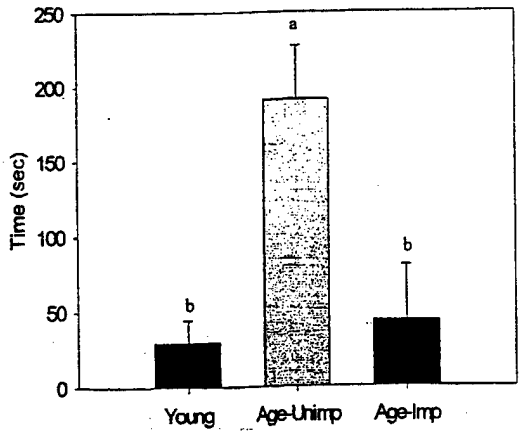

Figure 4 Time in seconds that the dogs spent sitting or standing beside the person in the human interaction test. The age-unimpaired dogs spent significantly more time close to the person than the age-impaired ($P = 0.009$) and young dogs ($P = 0.015$). "a" is significantly different from "b". Error bars represent standard errors of the mean.

Model Dog Test

The analysis of the amount of time spent sniffing the model dog resulted in significant main effects of age [$F(1,26) = 15.65$, $P = 0.00059$] and retest [$F(1,26) = 11.55$, $P = 0.0024$]. Young dogs spent more time sniffing the model than aged dogs, and sniffing time decreased from model dog test 1 to model dog test 2.

The analysis involving cognitive status revealed significant main effects of group [$F(2,27) = 6.18$, $P = 0.0066$] and retest [$F(1,27) = 8.38$, $P = 0.0078$]. The young dogs spent significantly more time sniffing the model dog than the age-unimpaired ($P = 0.017$) and age-impaired ($P = 0.0082$) dogs. Sniffing of the model dog was lower during test 2.

Activity Patterns

To obtain a qualitative assessment of behavior, the activity patterns were examined. Each dog exhibited a characteristic idiosyncratic pattern of activity, which was similar with different types of tests. Thus, some dogs showed frequent jumping in every test, while others showed none. Some dogs urinated frequently while others rarely did. The young and age-unimpaired dogs' path of movements was modified when different stimuli were placed in the test room. The movements of the age-impaired dogs did not change with the various stimuli. These differences are illustrated in Figure 5.

Test-Retest Reliability

To determine the test-retest reliability of the measures, correlation coefficients were determined for each of the measures between the two sessions of the same test for all animals. The results shown in Table 1 illustrate that there were positive correlations for every measure and all but two were significant at the 0.05 level.

Intra-Rater Reliability

The procedures developed used direct observation to sample from a range of behaviors (locomotion, rearing, inactivity, etc.) rather than using automated methods that do not distinguish between the different behaviors, that is, locomotion from rearing. To establish reliability of the measurement procedure, the same person (C.T.S.) analyzed each human interaction test session twice at an 8-month interval. The results shown in Table 2 indicate that there is a high degree of consistency and reliability in the behavioral measures when the same person watches the same test session twice at an 8-month interval.

DISCUSSION

The present experiment demonstrates first that exploratory behavior and locomotion are at least partially distinct in

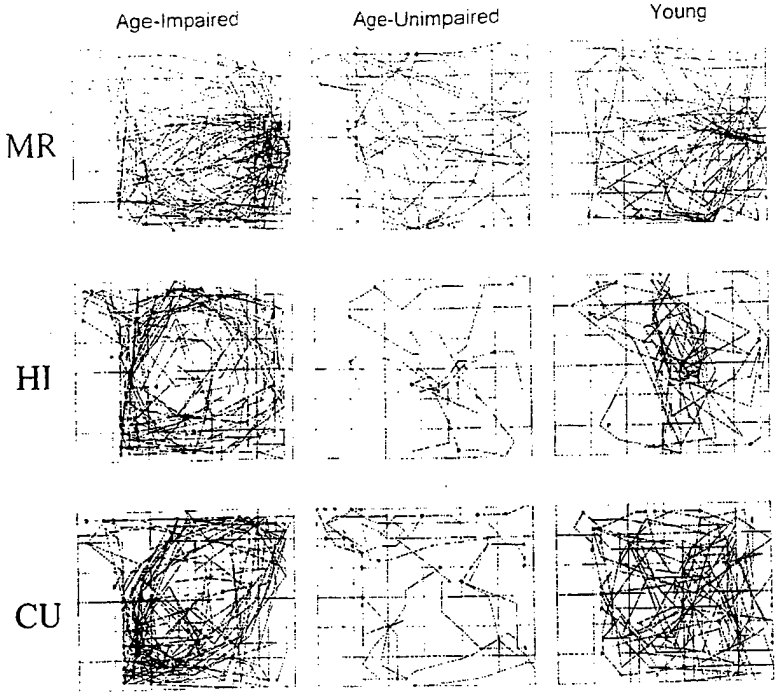

Figure 5 Tracings of the movement patterns of the dogs in the test room. The activity patterns of the age-impaired dogs did not change with the different tests. This group showed the same pattern regardless of stimuli present. The young and age-unimpaired dogs modified their behavior according to the test situation. MR, mirror test; HI, human interaction test; CU, curiosity test.

dogs and second, that both locomotion and exploratory behavior vary as a function of age and level of cognitive function, independently of test environment.

Locomotion

When age was considered independently of level of cognitive function, we found no effect of age on locomotion, consistent with our previous findings (Head and Milgram 1992; Head et al. 1997). However, the aged dogs showed extensive variability. When the aged dogs were divided into cognitively impaired and unimpaired groups, locomotion was lowest in the age-unimpaired dogs compared to the young dogs and age-impaired dogs. These findings support a link between cognitive impairment and behavioral activity.

An age-dependent decrease in behavioral activity is widely assumed to provide an index of canine cognitive dysfunction (Cummings et al. 1996; Ruehl and Hart 1998). The results of the present study suggest a more complex relationship. Age-unimpaired dogs exhibited lower levels of activity than young dogs, but the most severely impaired aged dogs often showed hyperactivity. However, when we consider the human literature, this finding is not surprising. Hyperactivity is often observed in dementia as well as other disorders such as attention deficit disorder and autism (Snowden et al. 1996; Hope et al. 1997; Rubia et al. 1998; Pliszka et al. 2000; Castellanos et al. 2001). As such, increased activity could be one manifestation of the neurodegenerative changes that contribute to cognitive impairment.

Increased activity may result from degeneration of behavioral control mechanisms in the prefrontal cortical-striatal-pallidal circuitry. A disruption in this circuit may release the normal inhibitory controls on behavior that allow for appropriate behavioral responses and can lead to the production of repetitive or stereotypical behaviors (Pierce and Courchesne 2001; Sakagami et al. 2001). The functional interconnection between the frontal lobes and cerebellum also implicates a role for the cerebellum in the maintenance of proper behavioral controls (Pierce and Courchesne 2001). Thus, the normal age-associated decline in physical activity produced by a general deterioration in endurance, strength, and coordination can be disrupted by pathology in the central nervous system that leads to a pathological increase in activity.

Locomotion and Exploration

We operationally defined responses to novel objects (physical contacts and sniffing of objects) in the curiosity test as exploratory behavior. The scores on this measure were independent of locomotion. The age-impaired dogs were very active but almost completely ignored the various toys present. Young dogs contacted the toys significantly more than either of the aged groups. The age-unimpaired dogs played with the toys to a

Table 1. *Test-Retest Correlations*

Test	Behavioral measure	Correlation (r)
Open field	Locomotion	0.85*
	Urination	0.60*
	Sniffing	0.31*
	Grooming	0.06
	Inactivity	0.64*
	Rearing	0.75*
	Vocalization	0.38*
	Jumping	1.00*
Mirror test	Image interaction	0.49*
	Image sniffing	0.63*
Human interaction	Sniffing person	0.69*
	Contacting person	0.65
	Beside person	0.71*
Curiosity test	Contacting objects	0.57*
	Sniffing objects	0.82*
Silhouette test	Sniffing head region	0.33*
	Sniffing rear region	0.28
Model dog test	Sniffing model dog	0.69*

n = 30; *$P < 0.05$ (two-tailed)

Table 2. *Intra-Rater Reliability of All Measures in the Human Interaction Test*

Behavioral measure in human interaction test	Test 1 redo correlations (r)	Test 2 redo correlations (r)
Locomotion	0.99*	0.99*
Urination	0.99*	0.99*
Sniffing	0.96*	0.90*
Grooming	0.97*	0.98*
Inactivity	0.95*	0.99*
Rearing	0.83*	0.88*
Vocalization	0.99*	0.71*
Jumping	n/a	0.99*
Sniffing person	0.95*	0.89*
Contacting person	0.99*	0.99*
Beside person	0.96*	0.99*

n = 30; *$P < 0.05$ (two-tailed).

lesser extent, suggesting that the response to novelty declines with age but that normal aged dogs still like to play.

Behavioral Profiles

Taking all of the tests into consideration, young and aged dogs exhibited distinct behavioral profiles. Young dogs, in general, show greater responsiveness to any type of modification of the test environment than aged dogs. The young dogs spent more time in physical contact with the person and the objects. The young dogs showed greater sniffing of the silhouette and model dog. Finally, the young dogs spent more time reacting to the mirror reflection than the unimpaired aged dogs.

Distinct profiles also existed for the age-unimpaired and age-impaired animals. The age-unimpaired dogs spent the most time beside the person and the least time reacting to the reflection. They were moderate in the time spent in contact with the objects and sniffing the model dog and silhouette. The behavioral profile of the age-unimpaired dogs was similar to that of the young dogs in that they responded to changes in the test environment but to a lesser extent. The unimpaired group displayed appropriate social responses to the human and artificial conspecifics. These findings indicate that with normal aging similar types of responses to various stimuli are present, just diminished. Aged animals have more experience with stimuli and novelty, and a type of desensitization process may occur over the lifespan so that although unimpaired aged animals still respond appropriately, the responses are not as great as those of a young animal that is still learning and exploring.

The age-impaired dogs were generally unresponsive to the person, the objects, the silhouette, and the model dog. The age-impaired dogs' activity patterns were unchanged by modifications in the stimuli present in the test environment. The age-impaired dogs exhibited undirected, random activity that tends to be stereotypical in that it does not change in different situations. This group of dogs just walked around the testing room without reacting to the assortment of novel stimuli in the room. The lack of exploratory behavior exhibited by the age-impaired group may reflect a specific deficit in the frontal lobes or cerebellum, as both of these structures have been implicated in responding to novel stimuli and are functionally interconnected (Daffner et al. 1998; Pierce and Courchesne 2001). Repetitive or stereotypical behaviors are also correlated with cerebellar and frontal lobe measures (Pierce and Courchesne 2001).

This group spent the greatest amount of time reacting to the reflection in the mirror. These dogs would jump at, appear to play with, bark at, and turn and try to catch the dog in the reflection. Some dogs would look behind the mirror in an attempt to find the other dog. The unimpaired dogs, in contrast, showed a rapid habituation. The mirror test is often used as a test of self-recognition in primates (Boysen and Himes 1999) and Alzheimer's patients (Biringer and Anderson 1992; Grewal 1994; Bologna and Camp 1997). Severe degrees of dementia in Alzheimer's disease (GDS > 6) are associated with the inability of patients to recognize themselves. This is one type of misidentification syndrome reported in Alzheimer's patients (Mendez et al. 1992). Misidentification symptoms in Alzheimer's disease may be associated with an accentuated degeneration of the right frontal lobe (Biringer et al. 1989; Forstl et al. 1991; Mendez et al. 1992). The same mechanisms may be disrupted in the dog. The dogs that exhibited the unique reactions to the mirror image are the same ones that showed excessive levels of locomotion. A disruption in the frontal lobe circuitry could produce both effects as well as the lack of response to the novel objects. The frontal lobe in the dog is the site of the earliest and most consistent signs of amyloid deposition with age, and disruption in the behavioral mechanisms mediated by the frontal lobe is consistent with the observations reported here (Head et al. 2000).

Social Responsiveness

The human interaction, silhouette, and model dog tests were designed to examine various aspects of the social behavior of the dogs. The human interaction test was designed to examine the unique social relationship that forms when dogs interact with humans. The silhouette and model dog tests were designed to measure what component stimuli of a conspecific elicit responses. Sniffing of the anal region is a common reaction to meeting a new dog, and preference for the facial region is a purely social greeting (Fox and Weisman 1969). The young dogs were the most socially responsive group. They sought physical contact from the person and sniffed the silhouette and model dog to a greater extent than the aged dogs. The age-unimpaired dogs also showed more human interaction, and sniffed the silhouette more often than the age-impaired group. The age-impaired dogs ignored the person and model dog, and showed an

increase in sniffing the silhouette between tests, the opposite result from the young and age-unimpaired group. The age-impaired dogs appear to have a deficit in social responding and decreased affection consistent with observations of geriatric pet dogs (Ruehl and Hart 1998). Disruption of the behavioral inhibitory mechanisms of the frontal lobes could be responsible for changes in social behavior.

Conclusions

We can distinguish three different types of behavioral activity in dogs: directed, undirected, and stereotypical. Directed activity is oriented toward a goal, that is, exploration of a room, object, or person. Undirected activity is random activity. Stereotypical activity is an organized type of undirected activity, repetitive patterns of behavior. Undirected and stereotypical behaviors are indicative of functional deficiencies in brain systems. Cognitively impaired dogs show more undirected and stereotypical behavior. Brain pathology may disrupt normal control of behavior. Disruption of the frontal or cerebellar regions can release the normal inhibitory controls on behavior leading to nonfunctional repetitive behavior instead of normal directed exploration. It is likely that a dysfunction of the prefrontal cortical-striatal-pallidal circuitry is involved in the production of the abnormal responses observed in our cognitively impaired aged dogs.

MATERIALS AND METHODS

Design

Activity tests were conducted every second day in the following sequence: open field test, mirror test, human interaction test, curiosity test, silhouette test, and model dog test. Each test was given twice. A total of 12 tests was administered for each dog. Dogs were administered a delayed nonmatching to position task prior to activity testing and a size discrimination task following activity assessments.

Subjects

The study was performed using 7 young (4 males; 3 females) and 23 aged (8 males; 15 females) beagles from the colony at the University of Toronto. The aged dogs ranged in age from 9 to 15, and the young dogs ranged from 2 to 4 years old. The dogs were individually housed in 1.07 × 1.22 m pens and maintained on a 12L:12D cycle. Pens were washed daily between 8:00 am and 10:00 am, during which time the animals were exercised for 15 minutes. Water was available ad libitum. Dogs were fed approximately 2 cups of standard laboratory chow daily. All dogs were in good health at the time of behavioral testing.

Behavioral Test Procedures

Cognitive Testing Procedures

The test apparatus, as described previously (Milgram et al. 1994) consisted of a wooden box 0.609m × 1.15m × 1.08m, with vertical aluminum bars at the front, a moveable Plexiglas tray with three food wells, a small overhead incandescent light, and a wooden partition containing a one-way mirror and hinged door to separate the investigator from the animal. The heights of the vertical bars can be adjusted for each dog to allow access to the food placed in the tray wells. A dedicated computer program was used for controlling all timing procedures, for specifying the location of the correct choice, and for capturing data. The test sessions were once daily.

Cognitive characterization was based on the dogs' performance on two neuropsychological tests, a delayed nonmatching to position (DNMP) task and a size discrimination learning task (Head et al. 1995,1998; Adams et al. 2000a,b). Aged dogs were classified as impaired if the combined error score on the size and DNMP tasks was greater than two standard deviations from the mean score of the young dogs.

The size discrimination task used two objects that differed only in size. The tray was presented with the two objects placed over the lateral wells. The dog must displace the object that is associated with the reward, and the choice is based only on the size of the object. The dogs were given ten trials per day with a 30 sec interval between trials. Dogs were tested daily until they passed. The learning measure used was errors made until the criterion was reached.

The DNMP task is more complex. Each trial of the task involves two components. The first is the sample phase, in which the dog was presented with a sample object in one of two lateral wells on the tray. The sample object has a food reward placed beneath it. The tray was then removed for a delay of 10 sec. After the delay, the tray was presented a second time with the sample object covering the same well and a new identical object covering the second well. The dog was required to go to the object in the new location to receive the food reward. The dog was considered to have made an incorrect choice if it came into contact with the sample object that had previously been presented. The dogs were given ten trials per day with a 60-sec interval between trials. When the dog passed the task at the 10-sec delay, it moved on to a 20-sec delay and then a 30-sec delay. The longer delays make the task more difficult. The memory measure used was errors made to reach criterion for each delay.

General Activity Testing Procedures

All testing took place in a 3.66 × 3.66 m room containing a sink and cupboards. There were two large Plexiglas windows and two doors in the walls of the room. The floor was marked into 32 squares 61 × 61 cm with black electrical tape to facilitate localization of the animal's position (two squares were located under the sink). The floor and base of the walls and cupboards were cleaned with a detergent solution prior to each test to prevent odor cues from other dogs affecting the animal's behavior. All windows were covered with black plastic excluding a small area for observing and videotaping the dogs. Test sessions were 10 min in duration. The dog was released into the room through one of the doors, and an observer located outside of one window used a video camera to record behavior. To minimize variability and bias, the same person (C.T.S.) performed all of the behavioral observations. The videotapes were analyzed with a dedicated computer program (see Head and Milgram 1992) that provided quantitative measures of locomotion, directed sniffing, urination, inactivity, grooming, rearing, vocalization, and jumping. The measures recorded were total distance for locomotion, total time for grooming and inactivity, and the frequency of occurrence for sniffing, urination, rearing, vocalizing, and jumping.

Open Field Test

For the open field test, a profile of the animal's total behavior pattern in an empty room over a 10-min period was obtained.

Mirror Test

A mirror was placed against one wall of the test room. The mirror was secured to the wall and cleaned between tests. Additional behavioral measures for this test were time spent reacting to the reflection and frequency of sniffing the reflection. The mirror test was originally developed by Gallup (1968,1970) as a test of self-recognition in primates. We examined the reaction of each dog to the presence of the mirror.

Human Interaction Test

This test assessed the reaction of the dog to the presence of a person and has previously been described (Head et al. 1997). A person sat in the center of the room and was instructed to sit on the floor at a fixed position and avoid responding to the dog. The additional behavioral measures were the total amount of time of physical contact, total amount of time standing or sitting beside the person without making contact, and frequency of sniffing the person.

Curiosity Test

Seven distinct objects were placed in fixed positions in the room. The objects were cleaned with a detergent solution before each test. The objects included a hanging tennis ball, a knotted chew rope for dogs, a large plush squeaky ball, a rubber squeaky fire hydrant, a plush squeaky gerbil, a plush squeaky jack, and a hanging purple plush dinosaur. All of the objects were commercially available from the local pet store. The behavioral measures taken include the total amount of time in physical contact with the objects and frequency of sniffing the objects. Each dog was allowed to freely examine the objects to assess its' reaction to novel objects.

Silhouette Test

A black, laminated cardboard figure of a dog was secured to one wall. The silhouette was cleaned between tests. The behavioral measures included frequency of sniffing the head region and frequency of sniffing the rear region of the silhouette. This test was intended to provide measures of social responsiveness to a conspecific (Fox and Weisman 1969).

Model Dog Test

A life-size sandcast model of a golden retriever was placed in a fixed position in the center of the room. The model was cleaned between tests. The behavioral measures included time spent sniffing the model dog. This test was intended to provide measures of social responsiveness to a conspecific.

Data Analysis

The data were analyzed using a three-way analysis of variance (ANOVA) with age and sex as between-subject factors and retest as a within-subject factor. Tukey's HSD post hoc test was used for multiple comparisons. A subsequent analysis examined the effect of cognitive status of the aged dogs on behavior using a two-way ANOVA with group (young, age-impaired, age-unimpaired) as a between-subject factor and retest as a within-subject factor, because each test was performed twice. Tukey's HSD post hoc test was used to compare the three groups of dogs when a significant main effect of group was obtained. Test-retest and intra-rater reliability were assessed using Pearson product correlation coefficients.

ACKNOWLEDGMENTS

This research was supported by the National Institute of Aging (NIA AG12694) and by a grant from Hill's Pet Nutrition, Topeka, Kansas.

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Size and Reversal Learning in the Beagle Dog as a Measure of Executive Function and Inhibitory Control in Aging

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Running head: Inhibitory Control in Aging Beagles

Key words: executive function, inhibition, reversal learning, aging, beagle,

Abstract

Several studies converge on the idea that executive processes age earlier than other cognitive processes. As part of a larger effort to investigate age-related changes in executive processes in the dog, inhibitory control was measured in young, middle aged, old, and senior dogs using size discrimination Learning and reversal procedures. Compared to young and middle aged dogs, old and senior dogs were impaired on both the initial learning of the size task and the reversal of original reward contingencies. Impaired performance in the two aged groups was characterized as a delay in learning the correct stimulus-reward contingencies and, among the senior dogs in particular, an increase in perseverative responding. Both patterns of impaired cognitive performance on the discrimination tasks reflect deficits in inhibitory control at different levels of complexity and are likely the result of different rates of aging in subregions of the frontal cortex.

Introduction

Inhibitory control and performance monitoring are critical executive functions of the human brain that are linked to frontal lobe function. These neural systems appear to be particularly sensitive to aging, manifested by a decreased efficiency of executive functions during normal aging, especially inhibitory control of attention and behavioral processes. (McDowd et al., 1995; Nielson et al., 2002; Sweeney et al., 2001). Hasher and Zacks (1988) proposed that age-related cognitive deficits result from the failure of separate inhibitory mechanisms to prevent off-goal stimuli and thoughts from entering working memory and interfering with encoding and retrieval processes. Evidence supporting this inhibitory deficit hypothesis has come from studies using go-no/go paradigms (Garavan et al., 1999; Konishi et al., 1999; Menon et al., 2001; Nielson et al., 2002) habituation tasks (McDowd & Filion, 1992),

reading and language comprehension tasks (Dywan & Murphy, 1996; Faust et al., 1997; Hartman & Hasher, 1991; Langley et al., 1998) negative priming tasks (Connelly & Hasher, 1993; Hasher et al., 1991; Kane et al., 1994; McDowd & Filion, 1992) interference paradigms such as the Stroop (1935) task (Hartley, 1993; Kramer et al., 1994; Spieler et al., 1996; West & Alain, 1999; West & Alain, 2000; West & Baylis, 1998) and object-detour reaching tasks (Diamond, 1990; Dias et al., 1996b; Hauser, 1999; Wallis et al., 2001). Reductions in the ability to suppress irrelevant or conflicting information or impulses may underlie patterns of cognitive and executive dysfunction even under relatively optimal aging conditions (Moscovitch & Winocur, 1995).

Reversal learning tasks, which predominantly rely on executive functions (Adams et al., 200b; Lai et al., 1995), provide another measure for assessing inhibitory control in aging. In contrast to conceptual shift paradigms which require inhibition of a single perceptual category and a shift to a new perceptual dimension (e.g. Wisconsin Card Sort Task), discrimination reversals require subjects to inhibit prepotent responses to previously correct stimuli and to shift responses to a new stimulus-reward contingency within the same perceptual dimension. Although discrimination reversal tasks are used primarily to study animal models of aging (Bartus et al., 1979; Beck et al., 1966; Bonney &Wynne, 2002; Buchmann & Grecian, 1974; Coutant & Warren, 1966; Davis, 1978; Freidman & Marshall, 1965; Head et al., 1998; Itoh et al., 2001; Lai et al., 1995; Levine et al., 1987; Means & Holsten, 1992; Milgram et al., 1994; Rapp, 1990; Rahner-Welsch et al., 1995; Tighe, 1964; Voytko, 1999; Warren, 1966) they are easily adapted for tests of inhibitory control in humans (Daum et al., 1991; Freedman & Oscar-Berman, 1989; Kendler & Kendler, 1959; Kendler et al., 1960; Lawrence et al., 1999; Oscar-Berman & Zola-Morgan, 1980).

Discrimination paradigms, when applied to human populations, reveal dissociations between discrimination and reversal learning tasks. Kendler and Kendler (1959) and Kendler et al. (1960), found that young children were impaired on reversal shifts relative to non-reversal shifts. Among older adults however, reversal deficits generally correlate with severity of dementia (Freedman & Oscar-Berman, 1989; Lawrence, et al., 1999; Oscar-Berman & Zola-Morgan, 1980). For example, Freedman and Oscar-Berman (1989), in a study comparing reversal learning performance in groups of Alzheimer's (AD) Parkinson's (PD) and normal aged controls, found that the greatest reversal deficits were from the AD patient group. This was followed by the demented PD group relative to non-demented PD and control subjects. In both dementing conditions, reversal deficits were manifested as perseverative responding.

In nonhuman primates, the research does not reveal a consensus. Some studies have reported robust age-related reversal learning impairments (Davis, 1978; Dean & Bartus, 1988; Lai et al., 1995; Bartus et al., 1979; Itoh et al., 2001; Tsuchida et al., 2002); others have not found differences (Bernstein, 1961; Anderson et al., 1996; Lai, et al., 1995; Herndon et al., 1997; Lacreuse et al., 1999; Rapp, 1990, Voytko, 1993, 1999). Furthermore, attempts to describe the types of errors that occur during reversal learning in aged primates provide mixed results. Although some studies suggest that reversal errors in aged primates result from perseverative responding (Anderson et al., 1996; Lai et al., 1995), others suggest that deficits in forming stimulus reward contingencies underlie reversal impairments (Itoh et al., 2001; Voytko, 1999).

We previously used an object discrimination reversal task as part of an extensive battery of tests to measure age-related cognitive changes in a canine model of aging. Milgram et al. (1994) reported that aged dogs were impaired relative to young dogs on an object reversal learning task, but not on the initial discrimination. In a follow-up study, young and old dogs did

not significantly differ in either initial or reversal learning of the object discrimination task (Head et al., 1998). This study however, did find evidence of age-related differences on a size discrimination learning task, suggesting that size discrimination paradigms are more sensitive to cognitive aging than object discrimination paradigms. Unfortunately, this latter study was limited by small sample sizes (4 young dogs, 7 middle aged dogs, and 4 old dogs), constraints on length of testing (50 trials), and the absence of a size reversal task to assess possible differences in inhibitory control between young and old beagle dogs. These conflicting results and the absence of any measures to asses the nature of errors in the reversal task make it difficult to draw any conclusions about inhibitory control in aging beagle dogs.

Accordingly, the present study re-examined the effects of age on discrimination and reversal learning, using a size discrimination task in young, middle aged, old, and senior beagle dogs. If, as suggested by Head et al. (1998), a size discrimination task is more sensitive to cognitive impairments in age than an object discrimination, then a size reversal task should show even greater age-sensitivity. The present study used errors and trials to criterion to measure discrimination accuracy in young and old dogs. In addition, we also examined the types of errors made during reversal learning to provide a more accurate assessment of deficits in inhibitory control.

Results

Contribution of Object Preference and Different Test Sites

There were no differences between test sites or object preference on either trials or errors to criterion during the original learning and reversal tasks and as such, these results are not reported.

Initial Size Learning

Analysis of size learning, revealed a significant effect of age on both errors [$H(3) = 13.79$, $p = .003$] and trials to criterion [$H(3) = 14.80$, $p = .002$]. Pairwise comparisons of errors and trials to criterion, shown in figure 2, indicated that old and senior dogs made significantly more errors (U 17.0, $p = .003$; $U = 15.5$, $p = .0001$) and required more trials ($U = 16.0$, $p = .002$; $U = 9.0$, $p = .0001$) than the young dogs. Although errors increased progressively with age none of the other group differences were significant.

Backward learning curves are shown in figure 3. The curves, created to illustrate individual differences in size learning, indicate that, relative to the young and middle aged dogs which show rapid, insight-like learning over very few sessions, old and senior dogs show slow but progressive improvement over many more sessions. Analysis of these data indicate that age significantly influenced both the total number of sessions spent at or below 50% correct [$H(3) = 11.26$, $p = .010$] as well as the total number of sessions above 50% correct [$H(3) = 15.88$, $p = .001$]. Between group comparisons indicated that compared to the young dogs, old ($U = 26.5$, $p = .010$) and senior ($U = 26.0$, $p = .002$) spent significantly more sessions performing below chance when learning the size discrimination task. Similarly, both old ($U = 14.0$, $p = .001$) and senior (U

= 7.0, p = .0001) dogs required more sessions responding at above chance to satisfy criterion measures and complete the initial size task. No other comparisons of number of sessions below or above chance performance were significant.

Size Reversal Learning

Analysis of size reversal learning performance of the four groups shown in figure 4 revealed a significant effect of age on both errors [H(3) = 18.56, p = .0001] and trials [H(3) = 19.51, p = .0001] to complete criterion. Separate pair wise comparisons revealed several significant differences between the aged groups First, compared to young dogs, old dogs made significantly more errors (U = 34.50, p = .050) to reach criterion. The number of trials required to reach criterion in the aged group however, did not significantly differ from the young dogs (U = 37.50, p = .074). Second, senior dogs made more errors (U = 11.50, p = .0001) and required more trials to achieve criterion (U = 7.50, p = .0001) compared to the young dogs. Finally, in contrast to the original learning task, senior dogs differed from middle aged dogs on the reversal task with senior dogs making more errors (U = 3.50, p = .001) and requiring more trials (U = 3.50, p = .001) to achieve criterion on the reversal task. No other between group comparisons were significant.

Analysis of the stages of reversal learning (figure 5) revealed a significant effect of age on Stage I [H(3) = 7.58, p = .05], Stage II [H(3) = 19.49, p = .0001], and Stage III [H(3) = 8.43, p = .038]. Group comparisons for each learning stage indicated that relative to young dogs, senior dogs spent significantly more sessions at Stage I (U = 40.00, p = .014). No other groups significantly differed in the number of Stage I errors made. Senior dogs also differed from young (U = 12.50, p = .0001), middle aged (U = 6.00, p = .002) and old (U = 139.00, p =.05) dogs in

the number of sessions spent at Stage II. Similarly, old dogs spent more sessions at Stage II relative to young dogs ($U = 33.00$, $p = .030$). Senior dogs also spent more sessions at Stage III learning compared to young ($U = 46.50$, $p = .029$) and middle aged dogs ($U = 20.00$, $p = .019$). Senior dogs however, did not differ in the number of sessions spent in Stage III ($U = 147.00$, $p = .078$) when compared to old dogs.

Discussion

The primary goal of the present study was to provide evidence of executive dysfunction in aged dogs using a size reversal learning task which examines inhibitory control. Old and young dogs were first trained on a size discrimination learning task. After acquiring the task, the reward contingencies were reversed and the animals were trained on a reversal learning task.

Size Learning.

The size discrimination task is an example of an object discrimination task in which the discriminanda are identical in all respects but one: the height of the objects. As previously discussed, object discrimination is generally insensitive to age in both primates (Anderson et al., 1996; Arnsten & Goldman-Rakic, 1985; Bartus et al., 1979; Dean & Bartus, 1988; Lai et al., 1995; Rapp, 1990; Tsuchida et al., 2002; Walker et al., 1988; Voytko, 1999) and dogs (Head et al., 1998; Milgram et al., 1994). In contrast to the studies which have failed to detect age differences in discrimination learning, age-related impairments in size discrimination learning were observed in the present study. This age effect was greatest among senior and old dogs, which made more errors and required more trials to complete the size discrimination task compared to young and middle aged dogs. According to Mackintosh (1974) and Sutherland &

Mackintosh (1971), successful acquisition of a visual discrimination consists of two stages; 1) attending to the relevant perceptual stimulus dimensions and; 2) associative learning of the correct stimulus reward contingencies. The backward learning curve analyses showed a protracted period during which both old and senior dogs spent significantly more sessions performing below chance when compared to young and middle aged dogs. This period likely represents a delay in attending to the relevant stimulus features (i.e. size) of the discrimination task. We also observed a similar protracted period of responding for both old and senior dogs in the number of sessions responding above chance prior to achieving learning criteria. This lengthy phase of above chance responding likely reflects an age-dependent impairment in acquiring the new stimulus reward contingencies.

Another factor that could account for the notable impairment in learning of the old and senior dogs is task complexity. In primates complex discriminations are likely to be more age sensitive. For example, Voytko (1999) found that initial learning of a pattern discrimination was impaired in both young and old monkeys relative to an easier object discrimination task. Rapp (1990) in contrast, found no age differences in either object or pattern learning, although a subgroup of aged monkeys performed significantly worse on the pattern recognition task relative to the remaining monkeys in the aged group and the young monkeys. Head et al. (1998) observed an effect of age on size discrimination learning but not on an object discrimination task that was acquired more rapidly. The increased difficulty of the size task may reflect intrinsic problems in distinguishing objects on the basis of height. Strong et al. (1968) consistently found that across several species, height is more difficult than color or form to discriminate. Another factor that that may contribute to the difficulty of the size task is the limited number of stimulus dimensions (i.e. the objects were identical in shape, color, and form, but not height). Using a series of oddity

and dimension-abstracted oddity tasks, Thomas and Frost (1983) found that squirrel monkeys learned oddity discriminations more rapidly with two relevant stimulus dimensions compared to one or three stimulus dimensions. Thus, in the present study the prolonged period of responding below chance in the old and senior dogs may reflect a difficulty in attending to an abstract stimulus dimension and the requisite ability to form an important stimulus-reward contingency.

Reversal Learning.

The primary goal of this study was to examine the nature of reversal learning in aging, in an attempt to further our understanding of executive function deficits. Aged dogs were impaired on the size reversal task relative to young animals. Although earlier studies have found age differences in reversal learning (Bartus et al., 1979; Davis, 1978; Dean & Bartus, 1988; Itoh, et al., 2001; Lai, et al., 1995; Milgram et al., 1994; Tsuchida, et al., 2002) this finding is not universal (Anderson et al., 1996; Bernstein, 1961; Head et al., 1998; Herndon et al., 1997; Lacreuse et al., 1999; Lai et al., 1995; Levine et al., 1987; Rapp, 1990; Voytko, 1993, 1999). Several factors can account for these inconsistencies. First, our studies of cognitive aging in beagle dogs have indicated that individual variability in cognitive performance increases with age such that at least two distinct populations of aged dogs can be identified: those that are cognitively impaired and those that perform at levels similar to young dogs (Cummings et al., 1996a, 1996b; Head et al., 1995; Head et al., 2001; Milgram et al., 1994). The present study attempted to capture the heterogeneity of cognitive aging by distinguishing between old and senior dogs. It was the senior dogs, the oldest group of dogs, which showed the greatest impairment on reversal learning. With few exceptions (Voytko, 1999; Itoh, et al., 2001), in most

primate studies the aged subjects do not represent the oldest possible subjects. Thus, differences in age within the aged group are a potentially important confound.

The stage learning analysis revealed the underlying nature of the age differences. The most notable effect was the difference between the senior dogs and the other three groups in Stage I. Perseverative habits result from the inability to suppress prepotent responses to a previously rewarded stimulus following a change in the stimulus reward-contingencies. Only senior dogs in the present study exhibited this behavior indicating that inhibitory control deficits are characteristic of more advanced aging syndromes. Old dogs in contrast, spent more total sessions in Stage II and III learning, suggesting that the nature of reversal impairments in the old dogs largely reflects a deficit in learning new stimulus-reward contingencies. This result is consistent with the impaired learning of stimulus-reward contingencies from the backward learning curve analyses of the original size learning.

Although the old and senior dogs showed a pattern of reversal errors that differed from the middle aged and young dogs, the types of errors made by both the old and senior dogs are likely related to deficits in inhibitory control but at different levels. Inhibitory control is not a unitary construct (Dias et al., 1996a, 1997; Connelly & Hasher, 1993; Hartley, 1993; Kramer et al., 1994; McDowd & Filion, 1995; Wallis et al., 2001). Recently, Roberts and Wallis (2000) suggested that inhibitory mechanisms operate at different levels of cognitive complexity. At higher levels of cognitive functioning, inhibitory processes operate to suppress prepotent behaviors and direct separate attentional systems towards relevant goal-oriented stimuli. At a lower level of cognitive function, inhibitory mechanisms facilitate acquisition of novel stimulus-reward contingencies. A similar distinction regarding separate inhibitory processes was offered by Hauser and his colleagues in a series of behavioral tasks using cotton-top tamarins (Hauser,

1999; Hood et al., 1999; Kralik et al., 2002; Santos et al., 1999). According to Hauser (1999) perseverative habits reflect a failure to inhibit affective prepotent emotional or motivational states while paradigmatic perseveration results from an inability to shift to a new theoretical perspective or stimulus-reward contingency.

Thus, according to the distinctions made by Roberts and Wallice (2000) and Hauser (1999), the increased Stage I errors in the senior dogs likely reflects affective perseverative behaviors resulting from an impairment of higher level inhibitory mechanisms that direct attention to relevant task parameters. In contrast, the higher incidence of Stage II learning in the old dogs suggests that, although old dogs are able to suppress prepotent behaviors towards irrelevant task dimensions, as a group the inhibitory processes that facilitate acquisition of novel stimulus-reward contingencies are impaired.

Functional and Anatomical Implications

The present study is part of an ongoing research program to examine the relationship between executive processes and frontal lobe structure in a canine model of aging. Early studies by Mishkin (1964), Iverson and Mishkin (1970), and Jones and Mishkin (1972) indicated that the inferior prefrontal convexity was essential for inhibitory control. Compared to primates with lesions to the medial orbitofrontal cortex, monkeys with inferior prefrontal convexity lesions exhibited increased perseveration of prepotent behaviors on an object discrimination reversal task. Medial orbitofrontal-lesioned animals, by contrast, were impaired in acquiring the novel stimulus-reward contingency. Recent work indicates that inhibitory control processes are modulated by a large right-lateralized cortical network that includes multiple frontal and parietal regions (Garavan et al., 1999; Kawashima, et al.,1996; Konishi et al., 1999; Liddle et al., 2001;

Menon et al., 2001; Metzler & Parkin, 2000; Nielson et al., 2002; Tsujimoto et al., 1997; Vendrell et al., 1995), which become functionally less active as inhibitory deficits increase with age (Chao & Knight, 1997; Neilson et al., 2002; Rebai et al., 1997; West & Alain, 2000). The results from the present study suggest two provisos regarding frontal lobe aging and inhibitory control in the aging dog. First, since the medial orbitofrontal cortex is commonly associated with stimulus-reward learning (Dias et al., 1997; Wallis et al., 2001; Rolls, 1998, 2000), a senescent orbitofrontal cortex is likely to contribute to age-related cognitive dysfunction in the dog. This hypothesis is consistent with the finding that old and senior animals showed both a protracted period of learning in the initial size discrimination and an increased number of sessions spent at Stage II during the reversal phase. Second, the importance of the inferior frontal gyrus (inferior prefrontal convexity) in sustaining attention and controlling prepotent tendencies (Butter, 1969; Iverson & Mishkin, 1970; Jones & Mishkin, 1972; McEnaney, & Butter, 1969) is consistent with the increased perseverative responding in the senior dogs during the reversal task. Taken together, the observations of perseveration and impaired stimulus-reward learning suggests that frontal lobe aging in the dog may begin in the medial orbitofrontal gyrus of the old dog and extend further to include the lateral inferior frontal gyrus in the oldest of the old dogs.

Although the present results indicate that executive function deficits may underlie age-related reversal learning impairments, clearly, further studies are required to fully characterize executive dysfunction in the aging dog. Additional studies are currently underway to examine a range of executive processes and how age-related changes to these higher-order cognitive processes correlate with subregions of the frontal cortex in the dog.

Methods

Subjects

Subjects consisted of beagle dogs (*Canis familiaris*) that were divided into one of four age groups, young, middle aged, old, and senior. There were eight dogs in the young group (2.91-3.73 years of age; $M = 3.40$, $SD = 0.28$), five in the middle aged group (4.05-5.50 years of age; $M = 4.81$, $SD = 0.66$), 17 in the old group (8.61-10.94 years of age; $M = 9.92$, $SD = 0.87$) and 25 in the senior group (11.10-13.81 years of age; $M = 11.95$, $SD = 0.71$). Dogs were obtained from three different sources (Lovelace Respiratory Research Institute, (LRRI), Albuquerque, New Mexico; Hill's Science and Technology Center, Topeka, Kansas; LBL Kennels, Indianapolis, Indiana) and housed at three different locations (LRRI; Division of Comparative Medicine (DCM), University of Toronto, Toronto, Canada; Scarborough College, University of Toronto). At two of the facilities (DCM and LRRI) dogs were individually housed. All dogs at the third facility (Scarborough) were group housed. Fresh water was provided daily *ad libitum* and subjects were fed approximately 300g of dry dog food in the afternoon following cognitive testing. Animals were visually examined daily by trained veterinary animal personal and research staff. Comprehensive clinical examinations for respiratory, urogential, musculoskeletal, digestive, visual, and auditory functioning were performed biannually. All dogs were in good health at the time of the study and all procedures were conducted in accordance with Canadian Council on Animal Care guidelines.

Apparatus

Testing was conducted in a 0.609-m x 1.15-m x 1.08-m wooden canine-adaptation of the Wisconsin General Test Apparatus as previously described (Milgram et al., 1994). The testing

chamber was equipped with a sliding Plexiglas food tray with three food wells, two lateral and one medial. The front of the box consisted of adjustable vertical stainless steel bars. The experimenter was separated visually from the dog by a screen with a one-way mirror and a hinged door on the bottom. Cognitive testing was conducted in darkness except for a light with a 60-watt bulb attached to the front of the box. The hinged door was opened for the presentation and removal of the food tray. Approximately 1 cm^3 of wet dog food (Hill's® Prescription Diet® p/d®; Hill's Pet Nutrition Inc., Topeka, Kansas) was used as the food reward for each trial. Only the two lateral wells were used for both tasks described in the present study.

Data acquisition was performed using a dedicated computer program developed in the ASYST (ASYST Software Technologies, Rochester, NY) programming language. The program controlled timing, randomization procedures, indicated the location of the reward, and was used to store and backup all data files.

Behavioral Tasks

Pre-training. Dogs at all three research locations received a standard four-phase pre-training protocol (Milgram et al., 1994). This procedure included a phase to expose the dogs to the test apparatus, a phase to teach the dogs that a food reward was always present in one of the food wells (reward approach learning), a phase to manually shape dogs to displace objects, and a final phase to teach dogs to visually locate and approach objects on the sliding tray (object approach learning). All dogs completed each of these four phases before further testing procedures were conducted. Dogs at all three facilities were also tested on a number of other recognition tasks including an object discrimination and reversal task before being tested on the size discrimination and reversal task.

Size Discrimination Learning. Stimuli for this task consisted of three wooden blocks (8.8 x 4 x 2 cm). Two blocks were glued together with epoxy glue to create a single large stimulus. The third block served as the small stimulus. Both objects were identical in color and material, and differed only in apparent size. A small hole (3 cm in diameter, 1 cm deep) was drilled into the bottom of the stimuli so that food could be placed under the incorrect stimulus, hidden from view, to control for odor cues during the task (figure 1).

The first test session of the size learning task was used to establish size preferences. The wooden blocks were placed over the lateral wells both containing the food reward. Locations of each object were randomized across trials with the proviso that each object would not occupy either lateral well more than five times per session. Dogs were required to displace the block to retrieve the reward. A total of 10 trials with both objects baited and presented randomly five times on the left or right side of the tray were administered during the preference test. The block most frequently selected by the animal (i.e. 6 or more times) was deemed the preferred object. If no object preference was established, one of the two blocks was randomly assigned as preferred. Testing began the following day with either the animal's non-preferred (n = 37; 31 aged, 6 young dogs) or preferred (n = 18; 11 aged, 7 young dogs) object as the correct stimulus.

All dogs received 10 daily trials, 7 days per week with a 30 second interval between trials. Each trial began with the placement of food reward in one of the lateral wells and the corresponding positive block placed (i.e. large or small block) over the food well. The remaining lateral well was unbaited and the incorrect block was placed over the food well with food in the bottom of the block to control for odor. After a computer-emitted tone signaled the start of the trial, the hinged door was raised, and the sliding tray extended 1/3 of the way towards the dog. A

3 second inspection interval timed by the computer, allowed the dog to examine both objects before the tray was fully extended for the animal to make a response. A correct response was recorded when dogs approached and displaced the positive block. An error was committed if dogs approached and displaced the negative block. One correction per test session on the first error was permitted and all subsequent errors resulted in the immediate withdrawal of the tray and termination of the trial.

All dogs were required to complete a two-stage criterion procedure to pass the size discrimination learning task. To satisfy the first criterion stage, a score of 9/10 or 10/10 on a single test day or 8/10 on two consecutive test days was required. After completing stage one criterion, a subsequent score of 70% correct or better over three consecutive test days was required to pass criterion. If criterion measures were not met within 40 days, testing was suspended and reversal procedures were not performed.

Size Discrimination Reversal Learning. After the dogs reached criterion measures in the size discrimination learning task, the reward contingencies of the positive and negative block were reversed and the animals were tested on a size reversal task. Testing on this task began the first day after the size learning procedures. In all other respects, testing procedures for the reversal task were identical to those used with the size discrimination learning.

Data Analysis

Total number of errors and trials to complete both the size discrimination and size reversal tasks up to and including criterion days were calculated for each dog. Given the relatively small sample sizes in two of the groups (i.e. $n = 5$ and $n = 8$ for middle aged and young

respectively) and the lack of normal distributions for size and reversal learning errors and trials indicated by the Shapiro-Wilk test of normality, a Kruskal-Wallis one-way ANOVA was used to examine the effects of age on errors and trials to criterion for the size and reversal learning conditions. Post-hoc analyses were performed using the Mann-Whitney U test.

Additional analyses were performed to determine if age selectively affected particular stages of acquisition or reversal learning. For acquisition of the initial size learning task, group averaged backward learning curves were generated according to the method described by Hayes (1953). Unlike conventional learning curves which compare group means, the backward learning curve allows for comparisons among groups of subjects that require different amounts of training before criterion measures are achieved. Using this method, percent correct scores were calculated for each session preceding the attainment of criterion for each dog in the study. Statistical comparisons among the four age groups using the Mann-Whitney U test were performed on the number of sessions at or below 50% correct and the number of sessions above 50% correct.

For reversal learning, a stage learning analysis was performed using the methods described by Jones and Mishkin (1972) and Duel, Mishkin, and Semmes (1971) to categorize the errors into one of three stages and to provide an index of perseveration (i.e. the inability to inhibit responses to a previously reinforced stimulus). We defined Stage I as the occurrence of seven or more errors within a single session of 10 trials. This provided a measure of perseverative responding. Stage II represented chance performance with four to six errors in a block of 10 trials. Stage III was characterized as an above chance level of performance with zero to three errors occurring in a single test session.

Kruskal-Wallis one-way analysis of variance procedures were used to examine the effects of age on each stage of learning during the reversal task. Pairwise comparisons were performed with the Mann-Whitney procedure for nonparametric data. All analyses were performed using SPSS for Windows (version 10.0.5).

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Figure Captions

Figure 1. Wooden blocks used as stimuli for size discrimination and reversal learning tasks. Correct and incorrect blocks are indicated by (+) and (-) respectively. Only responses to the correct stimulus block were rewarded. Large or small stimulus blocks could serve as correct or incorrect stimuli. Location of discriminanda were randomized across trials.

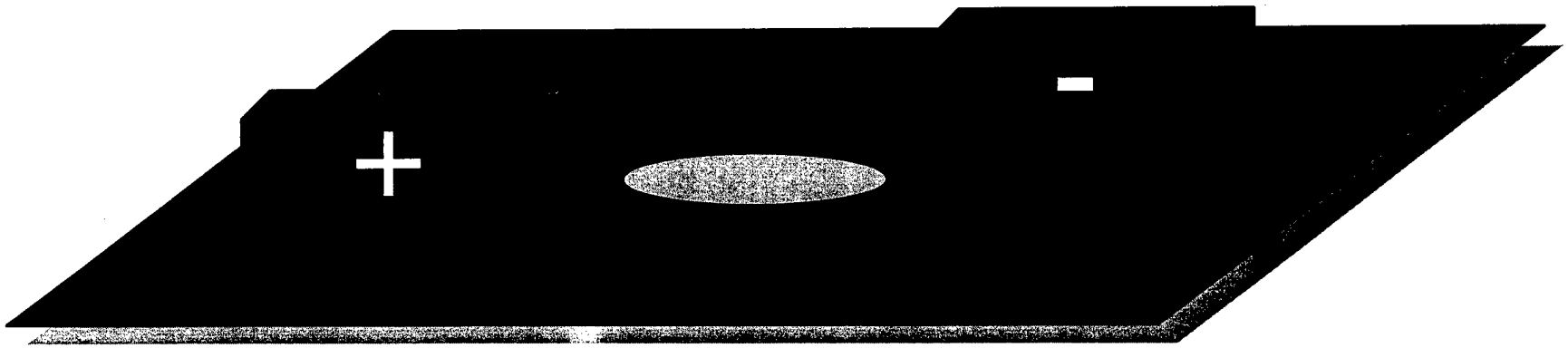
Figure 2. Box-whisker plots of total errors (A) and trials (B) to criterion for young, middle aged, old, and senior dogs on initial size discrimination learning. Median errors and trials to criterion for each group are indicated by a single line inside the box plot. Longer box plots and whiskers indicate greater variability and skewness respectively, within a group. Individual data points indicate outliers within a group.

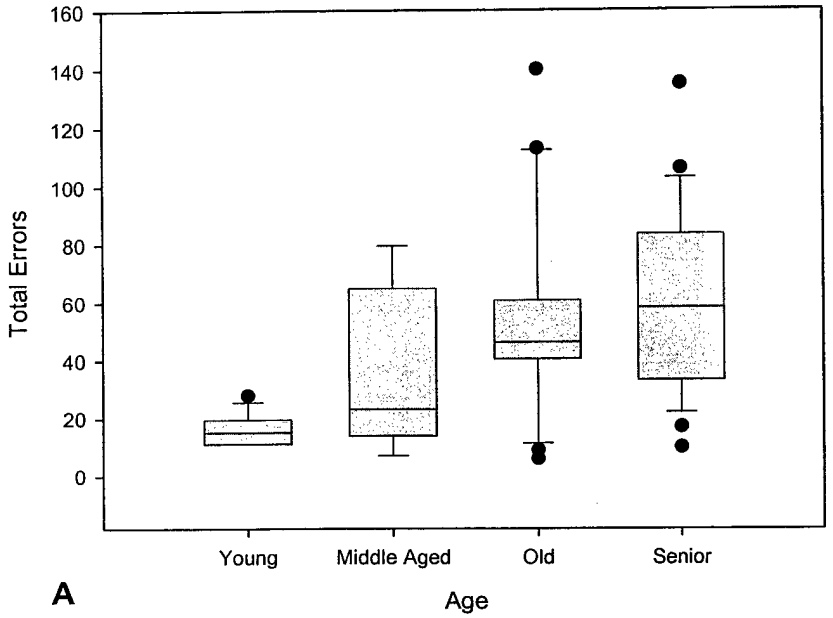
Figure 3. Backward learning curves for the acquisition of the size discrimination task by young (A), middle aged (B), old (C), and senior (D) dogs. Data points represent mean percent correct scores ($\pm$ standard error of the mean) and are plotted backward from the test session in which criterion is reached criterion (i.e. 0). Numbers above the curves indicate the number of dogs represented at each point to the left of the curve.

Figure 4. Box-whisker plots of total errors (A) and trials (B) to criterion for young, middle aged, old, and senior dogs on size reversal discrimination learning. Median errors and trials to criterion for each group are indicated by a single line inside the box plot. Longer box plots and whiskers

indicate greater variability and skewness respectively, within a group. Individual data points indicate outliers within a group.

Figure 5. Median number of sessions spent at Stage I, II, and III during reversal learning by young, middle aged, old, and senior dogs beagle dogs. Variability within each group of dogs is indicated by error bars (± standard error of the mean) in the graph of group means (inset).

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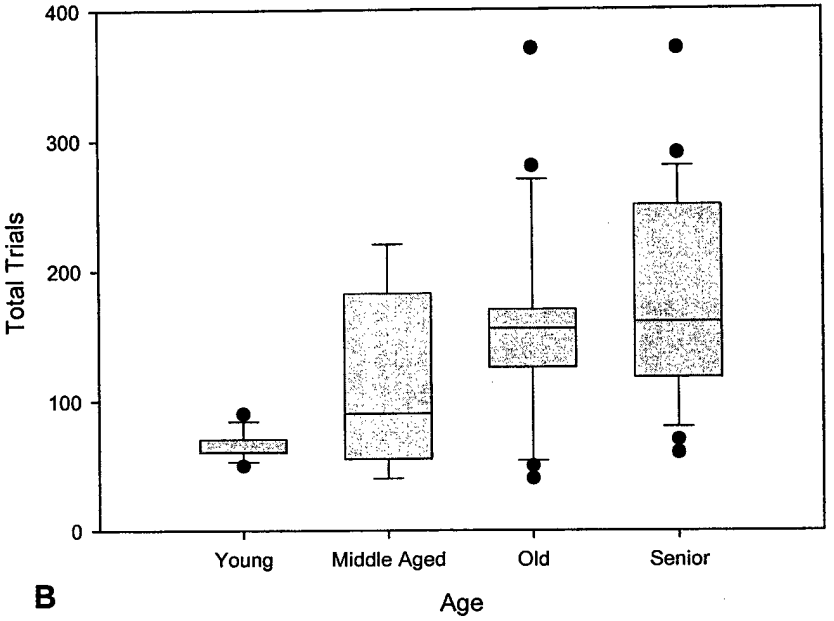
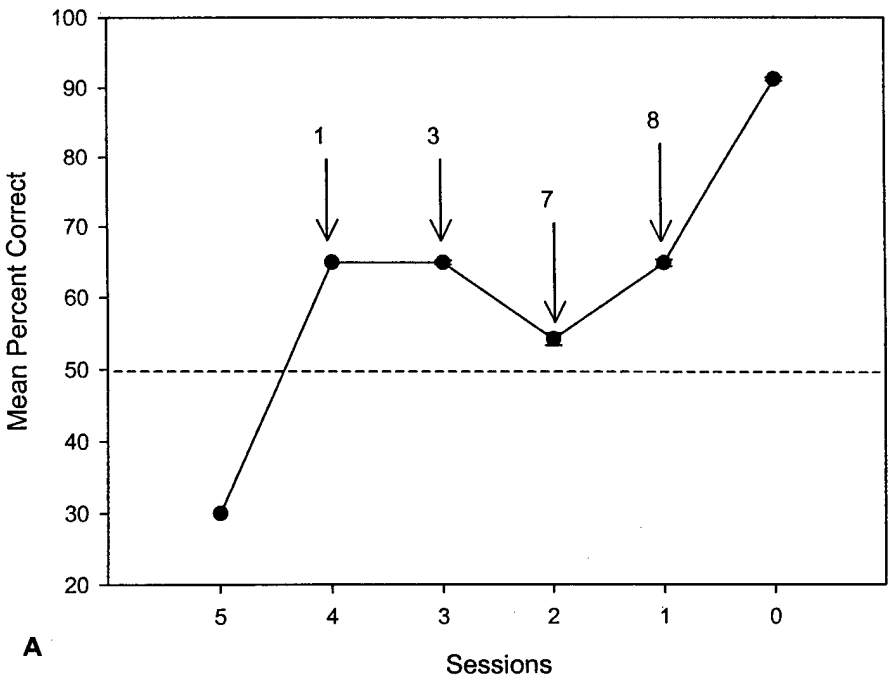
120
100
80
60
40
20
0
Total Errors
Young
Middle Aged
Old
Senior
Age

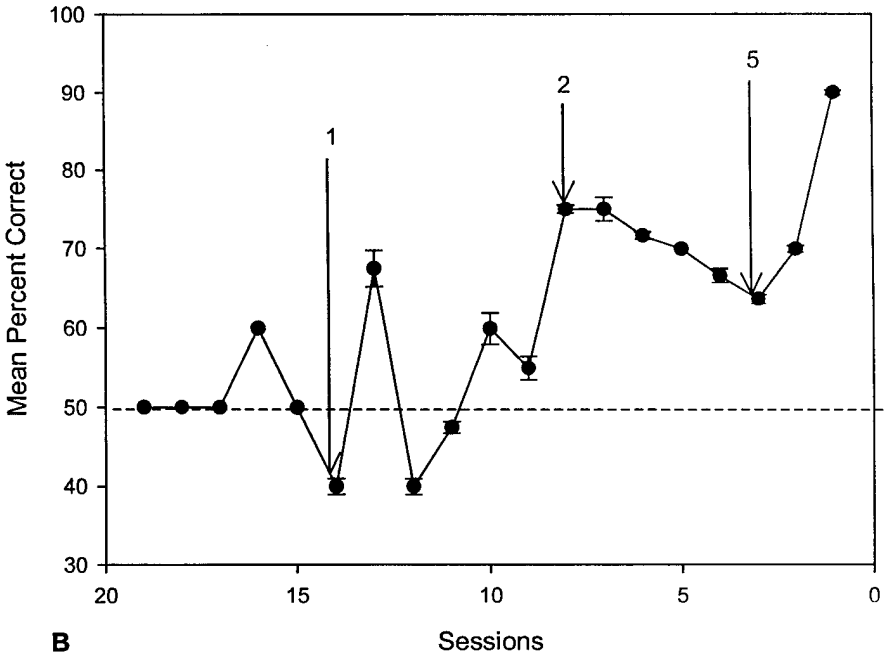
A


400
300
200
100
0
Total Trials
Young
Middle Aged
Old
Senior
Age

B


Mean Percent Correct
100
90
80
70
60
50
40
30
20
1
3
7
8
5
4
3
2
1
0
Sessions

A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5
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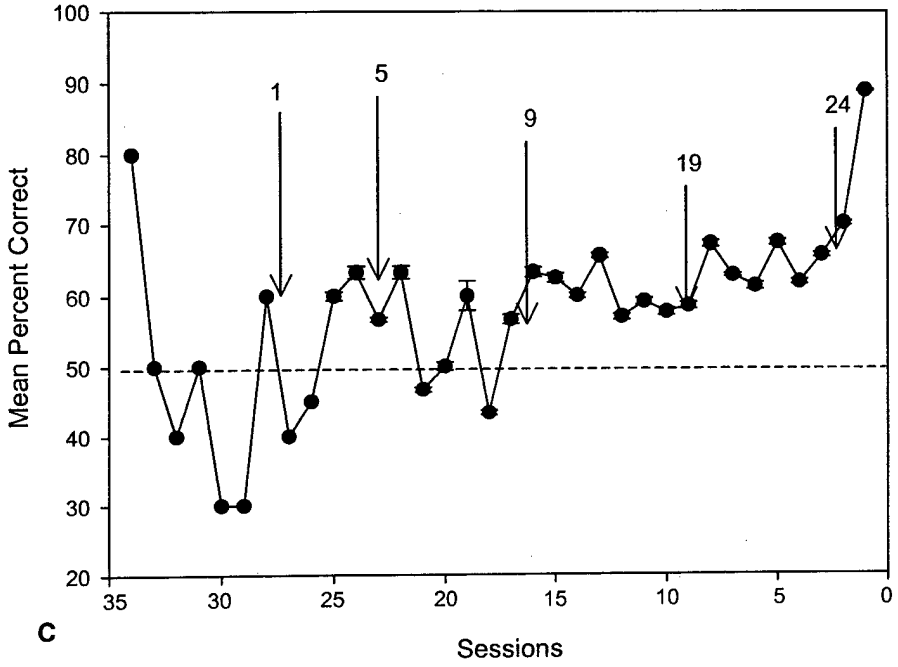
Sessions

B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80
70
60
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24
35
30
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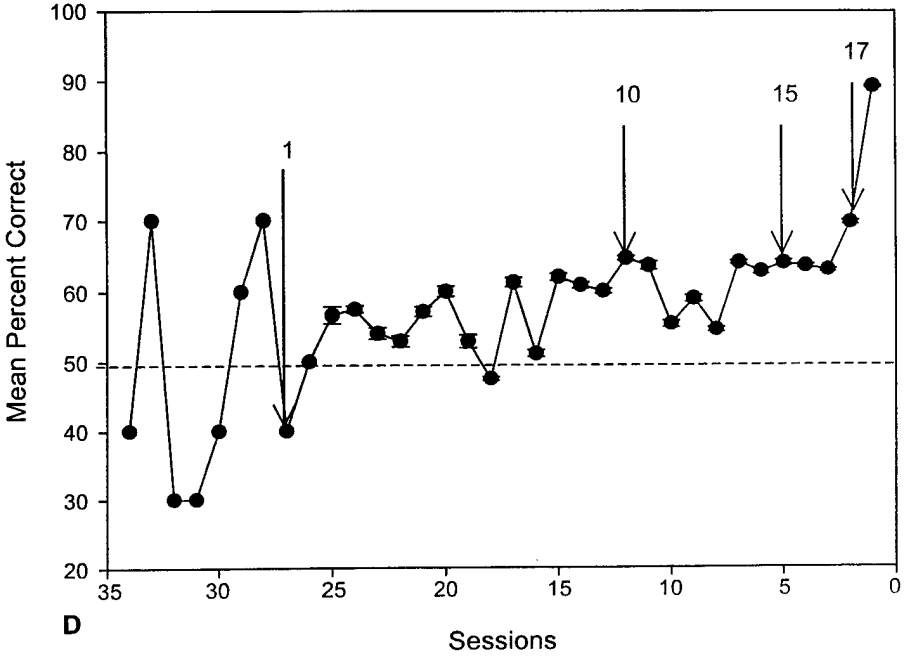
15
10
5
0
Sessions

C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80
70
60
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20
1
10
15
17
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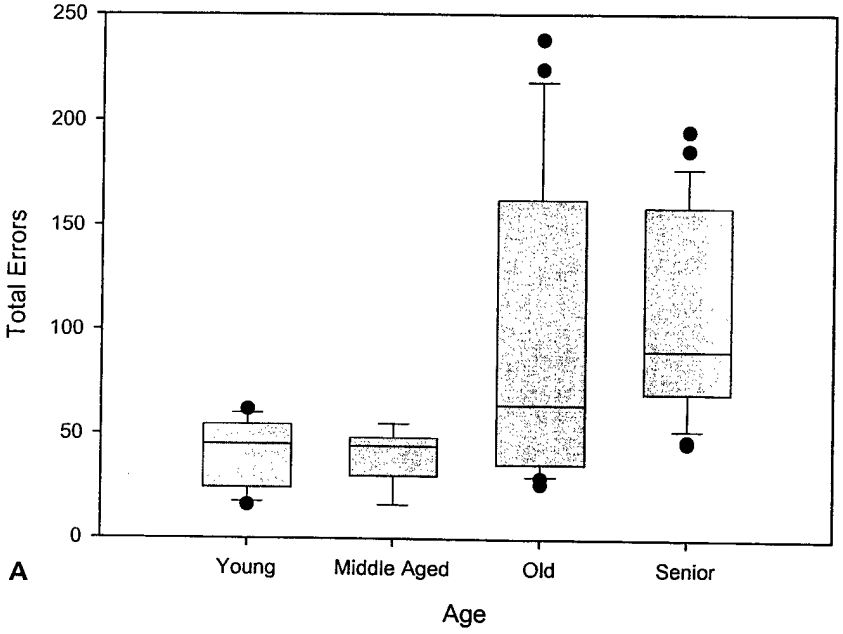
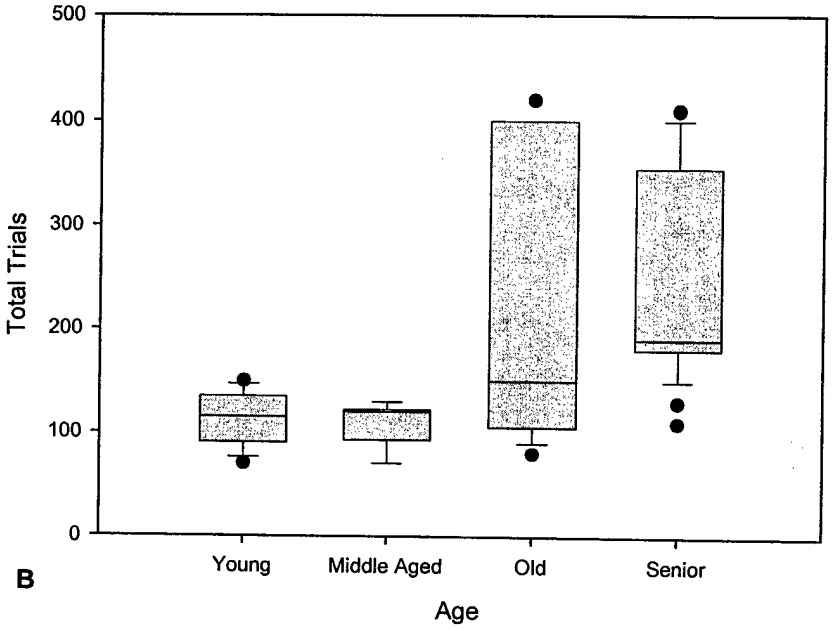
30
25
20
15
10
5
0
Sessions

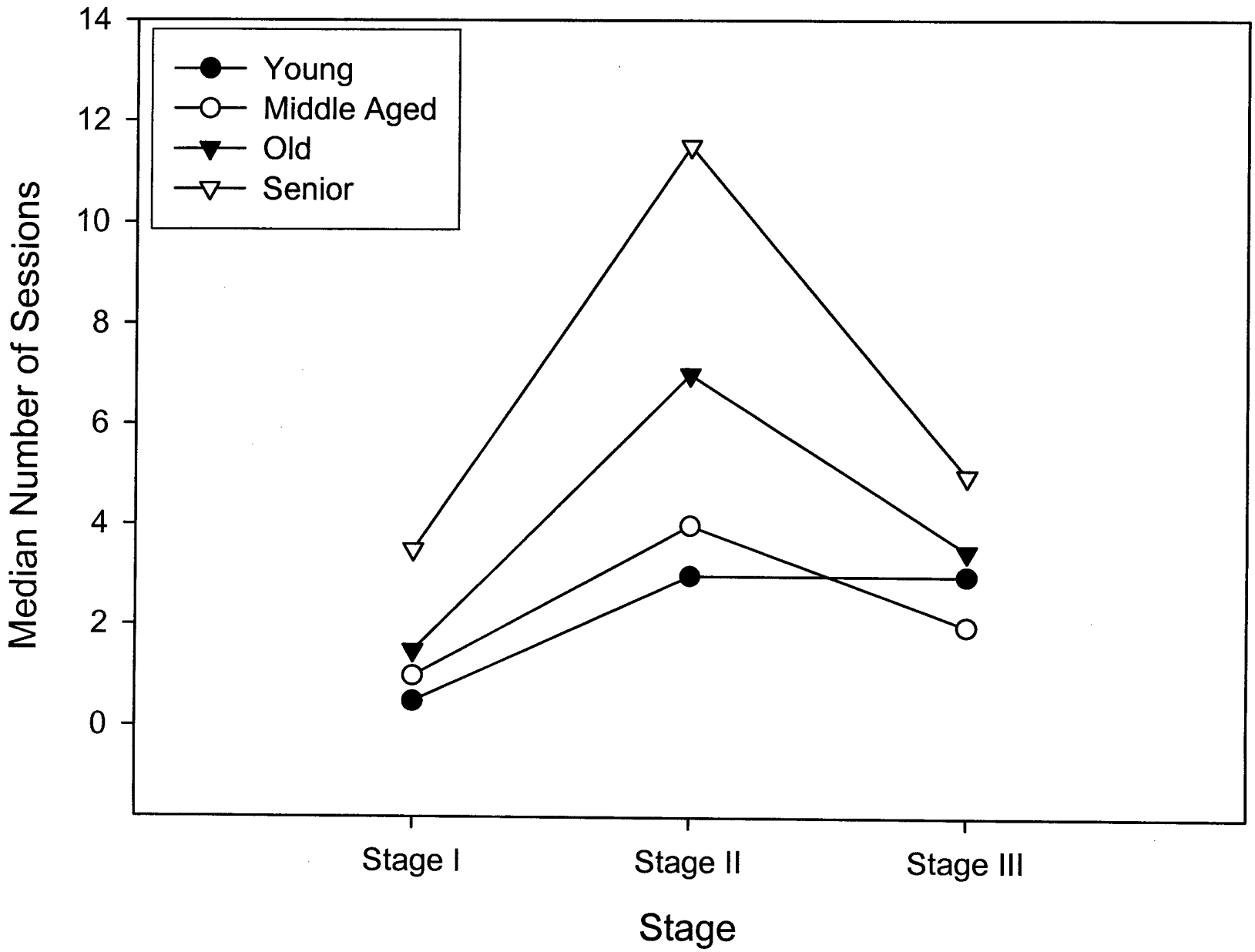
D

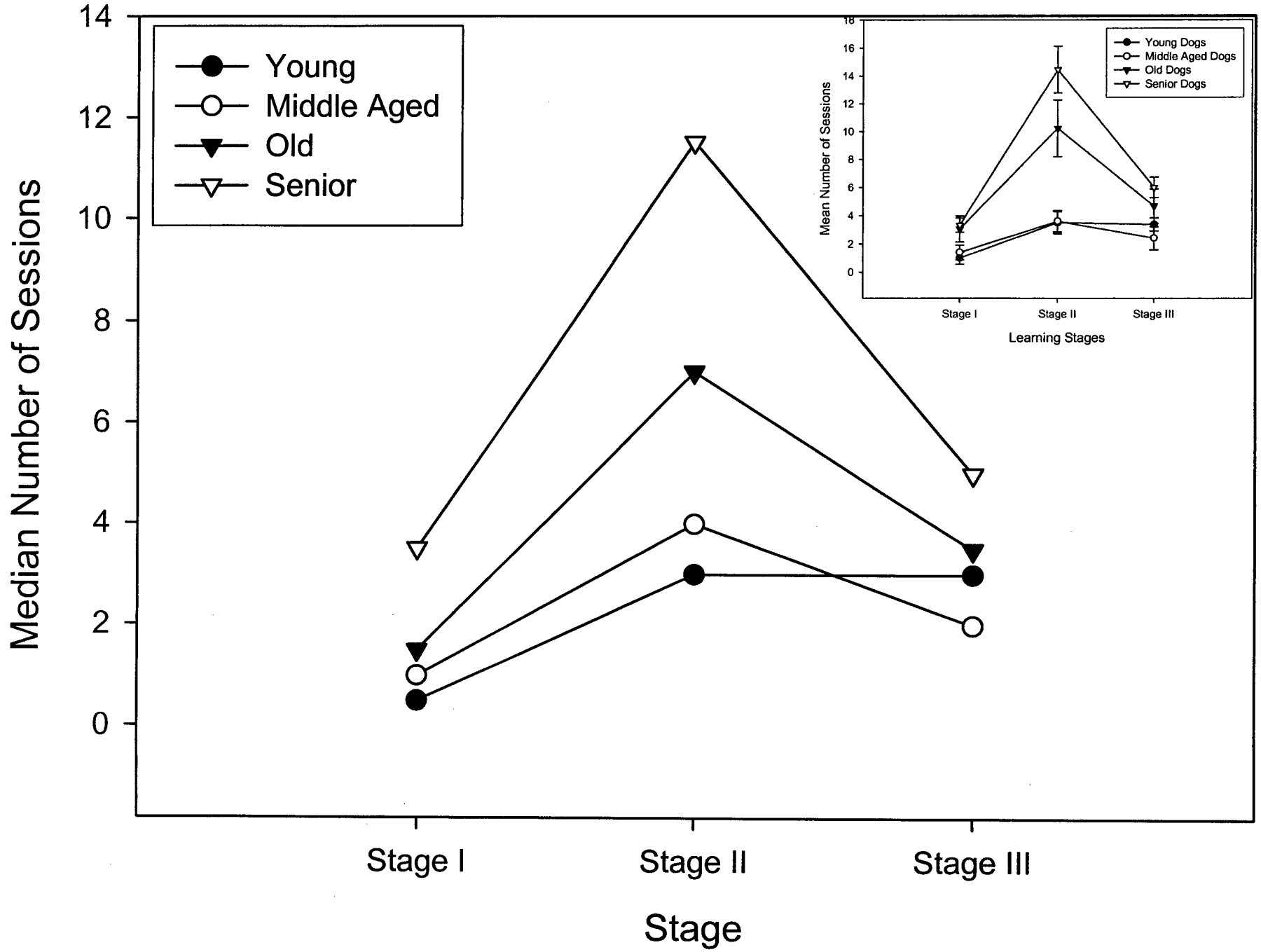

250
200
150
100
50
0
Total Errors
Young
Middle Aged
Old
Senior
Age

A


500
400
300
200
100
0
Total Trials
Young
Middle Aged
Old
Senior
Age

B


Young
Middle Aged
Old
Senior
Median Number of Sessions
14
12
10
8
6
4
2
0
Stage I
Stage II
Stage III
Stage


Young
Middle Aged
Old
Senior
Median Number of Sessions
14
12
10
8
6
4
2
0
Stage I
Stage II
Stage III
Stage
Young Dogs
Middle Aged Dogs
Old Dogs
Senior Dogs
Mean Number of Sessions
18
16
14
12
10
8
6
4
2
0
Stage I
Stage II
Stage III
Learning Stages

Appendix D

Abstract Resulting from Contract DAMD17-98-1-8622

(See p. 24 of the report for a complete listing of the abstracts.)

AN ANTIOXIDANT ENRICHED FOOD IMPROVES LEARNING AND REDUCES LIPID PEROXIDATION IN AGED CANINES: A LONGITUDINAL STUDY

Elizabeth Head, Jiankang Liu, Lydia Su, B.A. Muggenburg, Heather Murphey, Candace Ikeda-Douglas, Steven Zicker, Bruce N. Ames, Carl W. Cotman, Norton W. Milgram

Institute for Brain Aging & Dementia, UCI, Irvine, CA., Children's Hospital of Oakland Research Institute and UC-Berkeley, CA., Dept. Radiology, UCI., Lovelace Respiratory Research Institute, Albuquerque, NM., Div. Life Sciences, University of Toronto, Toronto, Canada, Hill's Pet Nutrition, Topeka, KS.

Background: Oxidative damage to proteins, lipids and nucleotides progressively increases with age both in canine and human brain and is further exacerbated in neurodegenerative conditions such as Alzheimer's disease. Objective: We hypothesized that reducing oxidative damage by a dietary antioxidant intervention (a nutritionally complete senior canine food enriched with vitamins E and C, alpha-lipoic acid, L-carnitine, and fruits and vegetables) may be an effective strategy to reduce cognitive dysfunction in aged dogs, an animal that naturally accumulates human-type β-amyloid. Methods: We have been studying 48 aged and 17 young canines, obtained from two sources, in a longitudinal experiment. In year 1, baseline measures of learning and memory were used to match animals on the basis of cognition and dogs were placed into 1 of 2 treatment groups: (1) Control - CTL; (2) Antioxidant diet - AOX. Dogs have been receiving treatment for 2.5 years. At regular intervals animals have been retested on measures of complex learning tasks. Results: Vitamin E levels in serum are significantly higher and have been maintained in dogs receiving AOX treatment. Serum lipid peroxidation level (malondialdehyde) was significantly lower in AOX dogs from one of the two sources of dogs. Significant improvements in landmark and oddity discrimination were observed in AOX treated dogs relative to CTL animals. Dynamic contrast enhance magnetic resonance imaging indicated that blood brain barrier (BBB) permeability measures from a coronal section including hippocampus, increased at year 1 and year 2 in the CTL animals but was maintained in the AOX animals. Other brain regions including the prefrontal cortex, occipital cortex, thalamus and cerebellum all showed BBB permeability increases that did not vary as a function of treatment. Conclusions: A diet enriched with a broad spectrum of antioxidants can significantly improve cognitive function in aging canines and may be a relatively simple intervention to promote healthy aging in humans.

Funded by U. S. Department of the Army, Contract No. DAMD17-98-1-8622, NIA AG12694, NIA AG17066 and Hill's Pet Nutrition, Topeka, KS.

Program Number: 889.1 Day / Time: Thursday, Nov. 7, 8:00 AM - 9:00 AM A LONGITUDINAL DIETARY ANTIOXIDANT INTERVENTION IN AGED CANINES IMPROVES LEARNING AND REDUCES PERIPHERAL MEASURES OF OXIDATIVE DAMAGE E.Head1*; J.Liu2; B.A.Muggenburg3; H.Murphey3; C.Ikeda-Douglas4; S.Zicker5; B.N.Ames2; N.W.Milgram4; C.W.Cotman1 1. Univ California Irvine, Inst Brain Aging & Dementia, Irvine, CA, USA; 2. UC-Berkeley, Children's Hospital of Oakland Research Institute, Oakland, CA, USA; 3. Dept. Life Sciences, Lovelace Respiratory Research Institute, Albuquerque, NM, USA; 4. University of Toronto, Toronto, ON, Canada; 5. Hill's Pet Nutrition, Topeka, KS, USA Oxidative damage to proteins, lipids and nucleotides progressively increases with age in canine and human brain. We administered 48 old and 17 young beagle dogs a diet rich in antioxidants that included a nutritionally complete senior canine food enriched with vitamins E and C, alpha-lipoic acid, L-carnitine, and fruits and vegetables. Baseline measures of learning and memory were used to match animals obtained from two different sources on the basis of cognition and dogs were placed into 1 of 2 treatment groups: (1) Control - CTL; (2) Antioxidant diet - AOX. Dogs were treated for 2.5 years. At regular intervals animals have been retested on measures of complex learning tasks. Vitamin E levels in serum are significantly higher and have been maintained in dogs receiving AOX treatment. Serum lipid peroxidation level (malondialdehyde) was significantly lower in AOX dogs from one of the two sources of dogs. Improvements in landmark and oddity discrimination were observed in AOX treated dogs relative to CTL animals. A diet enriched with antioxidants can significantly improve cognitive function and reduce peripheral levels of oxidative damage in aged canines. An antioxidant enriched diet may be a relatively simple intervention to promote healthy aging in humans. Supported by: Army, Contract No. DAMD17-98-1-8622, NIA AG12694, and Hills Pet Nutrition, Topeka, KS. Conflict of Interest: Partial funding of laboratory research by a corporation. Citation: E.Head, J.Liu, B.A.Muggenburg, H.Murphey, C.Ikeda-Douglas, S.Zicker, B.N.Ames, N.W.Milgram, C.W.Cotman. A LONGITUDINAL DIETARY ANTIOXIDANT INTERVENTION IN AGED CANINES IMPROVES LEARNING AND REDUCES PERIPHERAL MEASURES OF OXIDATIVE DAMAGE. Program No. 889.1. 2002 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2002. CD-ROM.

Program Number: 374.5 Day / Time: Monday, Nov. 4, 1:00 PM - 2:00 PM LONG TERM MAINTENANCE OF AN ANTIOXIDANT ENRICHED FOOD PLUS BEHAVIORAL ENRICHMENT MARKEDLY DELAYS AGE RELATED COGNITIVE DECLINE IN BEAGLE DOGS C.J.Ikeda-Douglas1; H.Murphey2; B.Muggenburg2; E.Head3; C.W.Cotman3; S.C.Zicker4*; N.W.Milgram1 1. Life Sciences, University of Toronto, Scarborough, ON, Canada; 2. Lovelace Respiratory Research Institute, Albuquerque, NM, USA; 3. Institute for Brain Aging and Dementia, Irvine, CA, USA; 4. Hill's Pet and Nutrition Inc., Topeka, KS, USA
As part of a longitudinal investigation of cognitive aging, we have previously reported that age-related cognitive decline in beagle dogs is delayed by combined environmental enrichment and maintenance on a food fortified with antioxidants and mitochondrial cofactors. This report provides an update of their performance on tests of discrimination learning(DL) and reversal learning(RL) over three years and consisted of: object DL and RL(yr 1), a size DL and RL(yr 2), and an intensity DL and RL(yr 3). Four groups of dogs were used, based on diet and environment: 1) Control food and environment, 2) Control food-control enriched environment, 3) enriched food-control environment, and 4) antioxidant enriched food-enriched environment. Following the 2nd year evaluation, all animals underwent an intensity discrimination task. Young animals performed significantly better than aged animals at all stages of the study. With respect to aged animals, both the food and the environmental enrichment improved performance. The performance was improved even more when the two treatments were combined. These findings indicate when animals are under environmental enrichment and an antioxidant enriched food may help to prevent age-related cognitive decline. Supported by: U.S. Department of the Army, the NIA and Hill's Pet and Nutrition Inc Conflict of Interest: We receive part of our funding from industry. Both N.W. Milgram and E. Head serve as consultants with Hill's Pet Nutrition. Citation: C.J.Ikeda-Douglas, H.Murphey, B.Muggenburg, E.Head, C.W.Cotman, S.C.Zicker, N.W.Milgram. LONG TERM MAINTENANCE OF AN ANTIOXIDANT ENRICHED FOOD PLUS BEHAVIORAL ENRICHMENT MARKEDLY DELAYS AGE RELATED COGNITIVE DECLINE IN BEAGLE DOGS. Program No. 374.5. 2002 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2002. CD-ROM.

Program Number: 286.1 Day / Time: Monday, Nov. 4, 8:00 AM - 9:00 AM
MEASURING COMPLEX WORKING MEMORY PROCESSES USING A SPATIAL LIST LEARNING PARADIGM IN A CANINE MODEL OF AGING C.T.Siwak*; D.P.Tapp; N.W.Milgram Institute of Medical Science, Department of Psychology, University of Toronto, Scarborough, ON, Canada In nature, organisms are often required to learn behavioral sequences, which must occur in a specific temporal order. Little is known, however, about this type of serial pattern learning in animal models of aging. We have developed a delayed spatial list learning (SLL) task, in which beagle dogs were required to remember two spatial locations to obtain a reward, and we have tested both young and old dogs on this task. Compared to young dogs, old dogs made significantly more errors in learning the task at short delays (5 seconds) and no old dog was able to solve the task at delays greater than 10 seconds. Young dogs, by contrast, successfully completed delays of 60 S. Nevertheless, the types of errors were not qualitatively different. Regardless of the delays used, both young and old dogs made significantly more errors to spatial positions that occurred earliest in the learned list as opposed to errors for the most recent spatial position. These results indicate that like aged humans, synchronous working memory processes required for list learning are impaired in aged beagles. Citation: C.T.Siwak, D.P.Tapp, N.W.Milgram. MEASURING COMPLEX WORKING MEMORY PROCESSES USING A SPATIAL LIST LEARNING PARADIGM IN A CANINE MODEL OF AGING. Program No. 286.1. 2002 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2002. CD-ROM.

Program Number: 374.9 Day / Time: Monday, Nov. 4, 1:00 PM - 2:00 PM EFFECTS OF AGE ON FRONTAL AND HEMISPHERIC BRAIN SYMMETRY IN THE CANINE
D.P.Tapp1*; C.T.Siwak2; G.Chiou3; S.E.Black5; S.McCune6; E.Head4; C.W.Cotman4; N.W.Milgram1,2; M.Y.Su3 1. Dept Psychol, Inst Medical Sci, Univ Toronto, Scarborough, ON, Canada; 2. Health Sciences Research Imaging Center, Inst Brain Aging and Dementia, Univ California, Irvine, CA, USA; 3. Sunnbrook and Women's College Health Sciences Centre, Toronto, ON, Canada; 4. Waltham Centre for Pet Nutrition, Leicestershire, United Kingdom
The most common neuroanatomical markers of senescence consist of ventriculomegaly and shrinkage of cerebral parenchyma. Recent neurophysiological and neuropathological studies suggest that brain aging may not be as uniform and non-specific as once implied. The frontal lobes in particular are likely to show age-related changes earlier in life and more severely than other brain areas. We previously reported global cortical atrophy and ventricular dilation in the aged canine, but we did not account for regional and hemispheric variability. In the present study, T1-weighted coronal slices were obtained using a 1.5T General Electric MRI from 24 young (2-5 years)and 23 aged (10-13 years) beagle dogs. Coronal images (1.3 mm thick; spoil gradient sequence; repetition time of 40 msec; echo time of 9 msec) were resliced along the anterior-posterior commissure to correct for differences in head tilt and position. Digital MRI planimetry was performed on each section to examine age-related changes in frontal, total intracranial and hemispheric volumes. We found significant age differences in both right and left frontal volumes, with smaller volumes in aged dogs. We did not find age differences in total hemispheric, intracranial, or frontal ventricular volumes. These results indicate region-specific differences in brain aging in the canine, which likely correlate with patterns of domain-specific cognitive decline. Citation: D.P.Tapp, C.T.Siwak, G.Chiou, S.E.Black, S.McCune, E.Head, C.W.Cotman, N.W.Milgram, M.Y.Su. EFFECTS OF AGE ON FRONTAL AND HEMISPHERIC BRAIN SYMMETRY IN THE CANINE. Program No. 374.9. 2002 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2002. CD-ROM.

INTER-SESSION RETENTION: WHY IT'S HARD FOR AN OLD DOG TO LEARN NEW TRICKS

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Dogs show a similar pattern of age-dependent cognitive decline as humans. Some cognitive functions, such as simple-discrimination learning, remain relatively intact, whereas others, such as reversal learning, are severely impaired with increasing age. The purpose of the present study was to examine the learning pattern of young and aged dogs on a size-discrimination and a reversal-learning task. We hypothesized that aged dogs (> 8 years), but not young dogs (between 1-5 years), would be impaired on inter-session retention of the reversal-learning task, but not the size-discrimination task. Aged dogs (N=22) made more errors in the first half of a session compared to the second half of the previous session on the reversal-learning task only. Analysis of young dog (N=16) data is in progress. The learning pattern seen in aged dogs may be due to changes in attention or consolidation. Nonetheless, this study provides a novel measure for characterizing canine cognition.