AD_____

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

PRINCIPAL INVESTIGATOR: Bruce A. Muggenburg, Ph.D.
Elizabeth Head

CONTRACTING ORGANIZATION: Lovelace Biomedical and Environmental Research Institute
Albuquerque, New Mexico 87185

REPORT DATE: October 2001

TYPE OF REPORT: Annual

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 2001	Annual (15 Sep 00 - 14 Sep 01)

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

6. AUTHOR(S)
Bruce A. Muggenburg, Ph.D.
Elizabeth Head

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lovelace Biomedical and Environmental Research Institute
Albuquerque, New Mexico 87185

E-Mail: bmuggenb@lrri.org

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 9-13 years. Dogs underwent baseline screening of cognitive function and general health evaluation including clinical pathology and physical examinations. Magnetic resonance scans (MRs) 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 group 2) enriched environment 3) dietary enrichment and 4) combined dietary and environmental enrichment. A broad spectrum of antioxidants are added for dietary enrichment. The environmental enrichment condition consists of additional cognitive experience and enriched sensory environment. Cognitive function, physical health and brain MRs will be monitored yearly to establish ongoing effects of the treatment. At the end of the study, detailed histological analysis of brain tissue will be correlated with cognitive function and MR 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, neurotoxin	152
	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

TABLE OF CONTENTS

APPENDICES

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. Dogs have undergone baseline screening of cognitive function and a general health evaluation including clinical pathology and physical examinations. Magnetic resonance scans (MRs) 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) control 2) environmental enrichment 3) dietary enrichment and 4) combined dietary and environmental enrichment. 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 MRs are being monitored annually to establish ongoing effects of the treatment. At the end of the study, detailed histological analysis of brain tissue and biochemical measures will be correlated with cognitive function and MR measures of brain atrophy and cerebrovascular function to establish the effectiveness of the treatments on delaying or preventing the development of age-dependent neuropathologies.

II. BODY OF THE REPORT

In Year 3, we proposed to have completed the second year of dietary and environmental enrichment in the study and to have begun the second treatment year's annual re-evaluation of cognitive ability in all the dogs.

A. Study Status

Twenty-four 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 are supported by the current grant. A second group of 24 beagles ranging in age from 9.5–12.9 years from Hill's Pet Nutrition was added to the study in February 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 individual animals at the time of submitting this progress report is listed in Appendix A, which also provides the length of time individual animals have been on the treatment protocol.

Dogs on the antioxidant-enriched diet are still on study as originally planned. The intervention (either diet or environmental) was started in the LBERI animals between July and October 1999 with animals introduced progressively into the study to distribute the workload of cognitive testing. Hill's dogs were started on treatment between January and February 2000. Dogs in the environmental enrichment group have received additional learning experience on an oddity and landmark discrimination task. In addition, as per the study plan, animals in the environmental enrichment treatment groups are walked outdoors twice a week, 20 minutes each time. Last, environmentally enriched dogs are housed in pairs and provided with play toys that are rotated through the kennels at weekly intervals.

Table 1 summarizes each treatment group and all cognitive tasks completed or in progress for each treatment group. Dogs in the environmental enrichment condition have provided the most cognitive data since they are tested continuously; most of these data were presented in the previous progress report. Dogs in the control condition 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	size discrimination, size reversal, spatial memory, and object recognition memory	landmark discrimination, oddity learning, landmark retention, size discrimination, size reversal, spatial memory, and object recognition memory
Diet	Antioxidant	size discrimination, size reversal, spatial memory, and object recognition memory	landmark discrimination, oddity learning, landmark retention, size discrimination, size reversal, spatial memory, and object recognition memory

Cognitive data from all animals were not available at the time of the last report because animals were still completing their testing for the first annual re-evaluation; thus, the effects of environmental enrichment could not be presented. In this report, we provide the first evidence based upon an evaluation of all animals in the study (not just those within the environmental enrichment groups) and provide comparisons between each of the four treatment groups.

B. Health Status

Medical evaluations of the dogs have been completed through Year 2 of the study for the LBERI dogs and through 1.5 years for the Hill's Pet Nutrition 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 during baseline and at 1 year for all dogs and at 2 years for the LBERI dogs. Three dogs have died. Dog 1492B died on 24 November 1999 from liver degeneration and chronic pancreatitis with atrophy. He was in the environmental enrichment/control diet group and was started on study 15 July 1999, approximately 3 months into the study. The second dog, D058 died on 3 October 2000 from a hemangiosarcoma of the spleen with metastasis to the liver. He was in the enriched environment/antioxidant diet group and was on study for 8 months. The third dog was 1508U. She died on 26 July 2001 from chronic right ventricular heart failure. She was in the control/control group, was started on study 16 July 1999, and was on the study for approximately 2 years.

Some dogs have been treated for medical problems, mostly minor. Three dogs had mammary tumors removed surgically, three had lower urinary tract infections, two had abscessed teeth, and one each had acute back pain, gastroenteritis, acute pancreatitis, surgically removed skin tumor, and surgically removed limbal melanoma of the eye. As these dogs continue to age, we anticipate that additional medical problems will occur over the next year.

C. Size Discrimination and Reversal Learning are Improved in Animals Receiving Environmental Enrichment and a Diet Rich in Antioxidants.

All dogs from all treatment groups from LBERI (n = 21) and Hill's (n = 23) have completed the first year's re-evaluation. Dogs were first given a series of new learning tasks called size discrimination and reversal learning, which were selected because of our previous work indicating that these tasks are age-dependent and sensitive to Aβ neuropathology [1]. We also tested a group of young dogs, which were provided with environmental enrichment, on the same tasks. Size discrimination involves presenting animals with three identical red wooden blocks, a single block on one side and the remaining two blocks stacked upon each other. Dogs are required to select either the smaller or larger stimulus. After reaching criterion on the size discrimination task, the reward contingencies are reversed, and animals must select the object that was previously incorrect.

On the initial size discrimination task, we found a significant effect of diet and environmental enrichment (Figure 1).

On the reversal learning task, we again found a significant interaction between diet and enrichment (Figure 2).

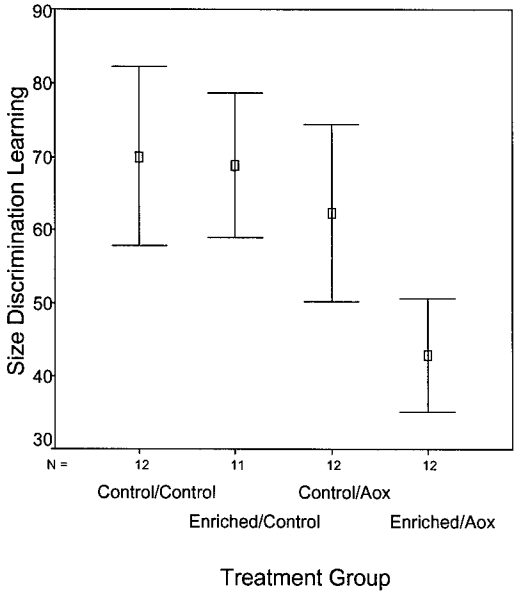


Figure 1. Size discrimination learning is sensitive to treatment condition. Dogs receiving both environmental and dietary enrichment committed fewer errors when learning the task than dogs in other treatment conditions suggesting a synergistic effect of both treatments. Aox = antioxidant diet. Error bars = standard error of the mean.

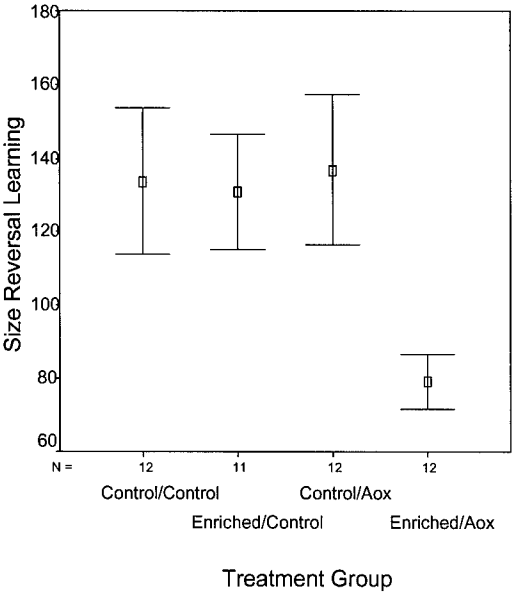


Figure 2. Size reversal learning was significantly improved in dogs receiving both a dietary intervention and environmental enrichment. Single treatments and the control group did not differ significantly from each other. Aox = antioxidant diet. Error bars = standard error of the mean.

These treatment effects reflect superior performance of the animals on the antioxidant diet over all other groups. The results indicate that dietary intervention using antioxidants can either delay or partially reverse the effects of age on cognition in beagle dogs. Furthermore, providing cognitive experience can potentiate these effects.

D. Landmark Long-Term Retention is Unaffected by Treatment Condition: A Dissociation Between Learning and Long-Term Memory

Dogs that were in the environmental enrichment treatment groups were retested for landmark discrimination learning prior to evaluation on the size and size reversal tasks. This includes half of the animals in the study with the second half of the dogs not tested on this problem. In our previous progress report, we described significant improvements in landmark discrimination learning, a measure of spatial attention, in the antioxidant diet group relative to

controls (n = 12 LBERI and n = 12 Hill's for a total of n = 24). After a period of 11-14 months, dogs that could learn the original problem (n = 12 Hill's dogs and n = 11 LBERI dogs for a total of n = 23 with 11 receiving the antioxidant diet) were retested for a maximum of 6 days to reach criterion as a measure of long-term memory ability. There was a trend toward lower error scores in dogs administered the antioxidant diet, but a t-test did not reveal statistical significance ($t(21)<1$ p = n.s.) (Figure 3). Although the diet significantly improved learning ability on the landmark task (data presented in previous report), no significant improvements were observed for long-term memory.

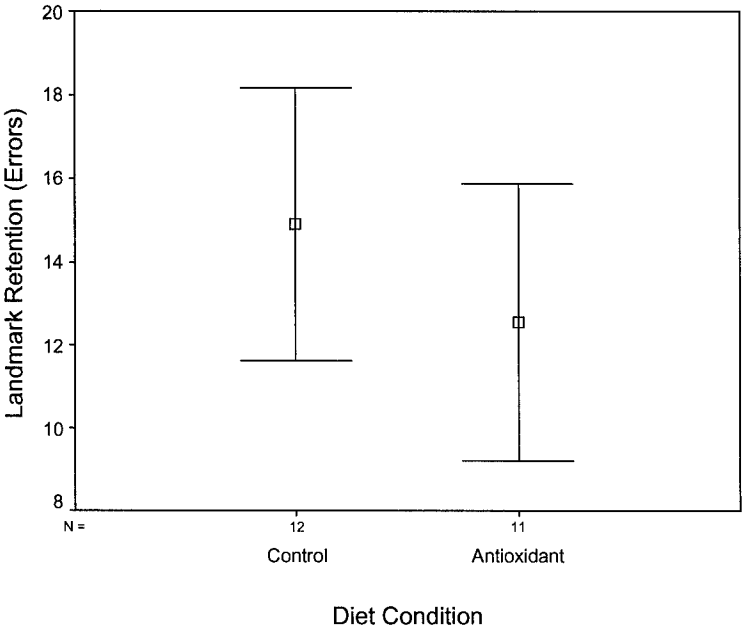


Figure 3. A trend toward improved long-term retention on a test of visual attention was observed in dogs provided with the antioxidant diet. The group differences did not reach statistical significance. Error bars = standard error of the mean.

E. Animals Provided with an Enriched Environment and Antioxidant Diet Display Mild Improvements in Spatial Memory

Spatial memory testing was included in the study design because of our previous studies indicating that this form of memory is sensitive to age in canines [2, 3]. Spatial memory testing in the current study uses a three-choice procedure. Dogs are first shown a single object covering a food reward in one of three recessed food wells (Left, Right, Center). After a delay period of 10 seconds, dogs are shown two identical objects, one of which covers the same well as in the previous presentation and the other covers a new location. The correct response is to select the object covering the well not seen previously (nonmatching procedure). Animals were given a maximum of 600 trials to learn the task during baseline testing, and during Year 1 re-evaluation were retested for another 600 trials to measure long-term memory. In both baseline and Year 1 testing, dogs that could meet criterion levels of responding were subsequently tested on a maximal memory procedure involving an additional 50 days of testing with delay intervals increasing as criterion was met on a shorter delay. For example, dogs were first tested until they reached criterion with a 10-second delay. Subsequently they were tested

with a 20-second delay and then a 30-second delay until a maximum of 500 trials were completed.

During baseline training, no significant differences were noted across the treatment groups prior to the start of intervention. At the 1-year evaluation, there were no significant effects of diet or age among the aged dogs. Eight of 48 dogs achieved the initial stage of learning during baseline. Of these dogs, three were in the control group and five in the antioxidant group. In the Year 1 retest, 15 dogs reached the criterion: (a) enriched antioxidant = 4; (b) control antioxidant = 3; (c) enriched control = 4; and (d) control – control = 4. Animals in the enriched/antioxidant group (Mean = 179.5 errors) on average obtained the lowest error scores when relearning the spatial memory task after a 1-year interval in comparison to the control/control (Mean = 195.7), control/antioxidant (Mean = 220) and the enriched/control group (Mean = 187.4). In addition, animals that could reach criterion levels of responding during the learning phase and had subsequently been tested for maximal memory also showed a trend toward improved memory in the enriched/antioxidant (Mean = 47.5 seconds) group with the control/control group (Mean = 17.5 seconds) showing the poorest memory ability.

The three-choice spatial memory task proved to be quite difficult for the old animals to learn, so we are adding a simpler two-choice task at the end of the Year-2 evaluation to allow more animals to learn the nonmatching procedure. The rationale was to train as many animals as possible during the learning phase in order to increase the number of aged animals from whom we could subsequently obtain memory scores during the longer delay interval procedures. At this time, we will have one time point measure for the simpler spatial memory task where group comparisons can be made, but no longitudinal measures will be possible unless the study is extended to include another full annual evaluation.

F. Rapid Declines in Object Recognition Memory in a 1-Year Interval are Reduced in Animals Receiving Environmental Enrichment with an Antioxidant Diet

Object recognition memory is another task that is sensitive to age in dogs [4, 5]. Object recognition memory involves presenting dogs initially with a single object covering a food reward hidden in the center food well. After a 10-second delay, dogs are shown two different objects, one of which is the same as seen previously. The correct response is to select the novel object (nonmatching procedure). At baseline, dogs were trained on this task for a maximum of 600 trials. Animals that could reach criterion levels of responding were

subsequently tested with longer delays in a maximal memory procedure until a maximum of 50 days had been completed. The maximum delay interval on which individual animals reached criterion was the assigned memory score. Dogs were retested for object recognition memory at the Year-1 evaluation, which involved an additional maximum of 60 days to reach the criterion again. As with the baseline procedure, dogs that could reach criterion during this relearning phase were subsequently tested for maximal memory.

The data have been analyzed for the LBERI dogs with test scores from the Hill's dogs currently being summarized. Thus, the data presented here are only for the LBERI dogs. Twenty-three animals completed baseline testing, and 22 completed the Year-1 evaluation. All but two animals obtained higher error scores during the Year-1 retest than at baseline (one slightly improved, and the other showed no change) suggesting a rapid decline in object recognition memory. However, not all groups were affected equally. Animals in the control/control group showed increases in average error score from baseline to Year 1 of 108.5 errors. The enriched/antioxidant group showed the smallest increase in error scores at 68.5. The control/antioxidant group showed decreases on average of 78.17 errors. Interestingly, the animals receiving only the environmental enrichment showed the largest losses of an average of 140.4 errors. It will be important to replicate these results with data from the Hill's dogs to determine whether these treatment effects are consistent.

G. Open Field Activity Remains Relatively Unaffected by Treatment Condition

Year-1.5 evaluations obtained from LBERI beagles are currently being analyzed. Data evaluations from the open field test, the human interaction test and the curiosity test have been completed. The curiosity test is used as a measure of exploratory behavior, and evidence indicates that treatment with antioxidants reduces exploratory behavior in female rats [6]. Dogs are placed in a room either alone with a nonresponsive person or with dog toys and are observed for 10 minutes.

A significant overall decrease in the amount of time spent playing with objects present within the test room during curiosity testing was observed from baseline to the Year-1 and -1.5 evaluations [$F(2, 38) = 6.39$, $p = .004$]. The largest decrease was in the enriched/antioxidant group, with the enriched/control and control/control groups showing smaller declines, while the levels of the control/antioxidant group remained unchanged. No

other measures of spontaneous behavior were significantly affected, and treatment effects are summarized in Table 2.

Table 2. Changes in Open Field, Human Interaction and Curiosity Testing as a Function of Treatment Group after 1 Year of Intervention.

	Open Field	Human Interaction		Curiosity Test	
LBERI	Locomotion	Contact	Near	Contact	Sniffing
Con/Con	no difference	no difference	no difference	small decrease	no difference
Con/Aox	no difference	no difference	no difference	no difference	no difference
Enr/Con	no difference	no difference	no difference	small decrease	no difference
Enr/Aox	no difference	no difference	no difference	decrease	no difference
HILL'S	Locomotion	Contact	Near	Contact	Sniffing
Con/Con	decrease	no difference	no difference	no difference	no difference
Con/Aox	decrease	no difference	no difference	no difference	no difference
Enr/Con	decrease	no difference	no difference	no difference	no difference
Enr/Aox	decrease	no difference	no difference	no difference	no difference

Data from the human interaction, curiosity and open field tests are complete for the Hill's dogs. The only significant finding among this group of dogs was a decrease in locomotion in the open field test from baseline to the 6-month evaluation point [$F(1, 20) = 28.02$, $p = .000035$]. Locomotor activity decreased in all four treatment groups indicating the effect was not a result of treatment conditions.

H. Blood Biochemistry and Blood Coagulation Studies Suggest No Adverse Consequences of Long-Term Dietary Intervention

In general, all animals had blood biochemistry values within normal limits. The samples obtained at the 1.5-year time point (24 LBERI dogs) and at the 1-year timepoint (47 LBERI and Hill's dogs) measures were not significantly different from baseline values. One dog (1494D) had a fairly low albumin at 1.5 years and will be monitored for signs of ascites/heart disease/protein-losing enteropathy/glomerulopathy. Raw data obtained from samples to date are provided in Appendix B.

As shown in Figure 4, coagulation profiles were obtained after 1 year 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 [7]. Second, this appears to be a problem only when an antagonistic factor to vitamin K is present such as warfarin [8]. As such, we examined the coagulation profiles of older dogs in the study after 1 year of intervention. Significant differences were present between the antioxidant-fed group and the control group, but all levels were well within normal ranges. Interestingly, the antioxidant-fed group had significantly shorter clotting times for activated partial prothrombine time (APTT) and less fibrinogen than the control group ($P < 0.05$). This would argue that the relatively higher doses of vitamin E in the test food had no adverse effects on clotting parameters in dogs as evidenced by these measures.

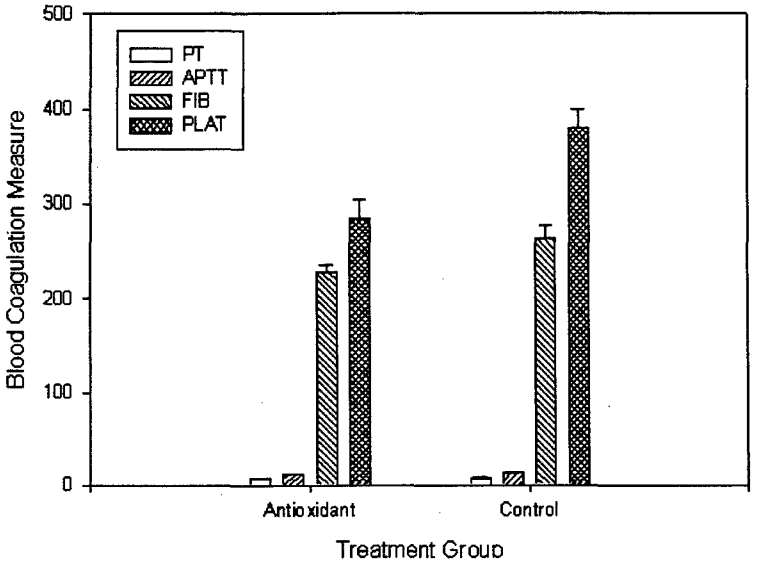


Figure 4. Blood coagulation measures are significantly improved in dogs fed the antioxidant diet, but all measures fall within the normal range. Levels of PT, FIB, and PLAT, but not in APTT, were reduced significantly. Error bars = standard error of the mean. PT = prothrombine time; FIB = fibrinogen; PLAT = platelets; APTT = activated partial prothrombine time.

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

Vitamin E measures are available from dogs in the study for four timepoints including baseline, 3 months, 6 months and 1 year following the start of intervention. All treatment groups were similar at baseline. At 3 months ($F(3,45) = 16.4$ $p<.0001$), 6 months ($F(3,44) = 21.56$ $p<.0001$) and 1 year ($F(3,44) = 26.26$ $p<.0001$), the animals provided with the antioxidant diet showed significant increases in serum levels of vitamin E (Figure 5).

J. Lipid Peroxidation in Plasma Samples is Increased in the Environmental Enrichment Animals but Remains Unaffected in Animals Provided with the Antioxidant Diet

We have obtained peripheral measures of oxidative damage to lipids by measuring malondialdehyde (MDA), a lipid peroxidation marker, in plasma samples. MDA was converted into a stable derivative using pentafluorophenyl hydrazine at room temperature, and the

derivative was detected using gas chromatography-mass spectrometry (GC-MS) in the negative chemical ionization mode [9]. Plasma measures of MDA were obtained in collaboration with Dr. Jiankang Liu at University of California, Berkeley (UC-Berkeley).

In the previous progress report, we described age-dependent increases in MDA levels measured using serum and brain obtained from archived samples. However, MDA levels in plasma from the longitudinal study animals were significantly lower than those measured in the archived serum samples. As with serum measures, plasma measures of MDA were also significantly higher in aged dogs relative to young dogs ($t(54) = 2.25$ $p<.029$). An analysis of variance did not reveal overall treatment effects, but there was a trend for dogs in the environmental enrichment group to exhibit higher MDA levels. In a separate analysis, a t-test revealed that dogs in the environmental enrichment group had significantly higher levels of MDA than control dogs ($t(45) = 2.02$ $p<.049$) (Figure 6, left). A direct comparison of the antioxidant diet group to the control diet group did not show any significant changes; however, there was a trend toward lower MDA levels in the group fed the antioxidant diet (Figure 6, right).

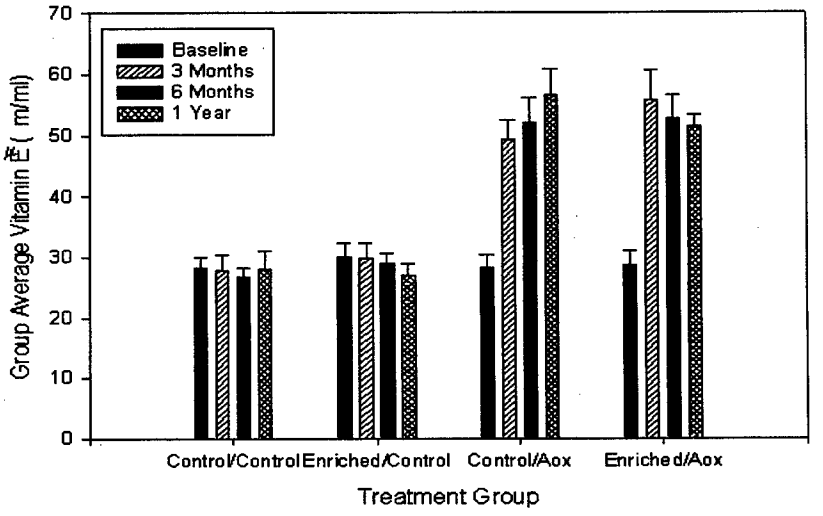


Figure 5. Vitamin E levels are significantly elevated in dogs receiving the antioxidant diet. Baseline levels were not different between each treatment group. Aox = antioxidant. Error bars = standard error of the mean.

K. Plasma Aβ Levels Do not Differentiate Treatment Groups

In collaboration with Dr. Paul Murphy at the Mayo Clinic in Jacksonville, Florida, we have obtained measures of Aβ42 and Aβ40 from plasma using sandwich enyzme-linked immunosorbant assays (ELISAs). No significant differences in plasma measures of Aβ were found as a function of treatment group. This suggests that peripheral Aβ levels remain unaffected by either diet or environmental enrichment.

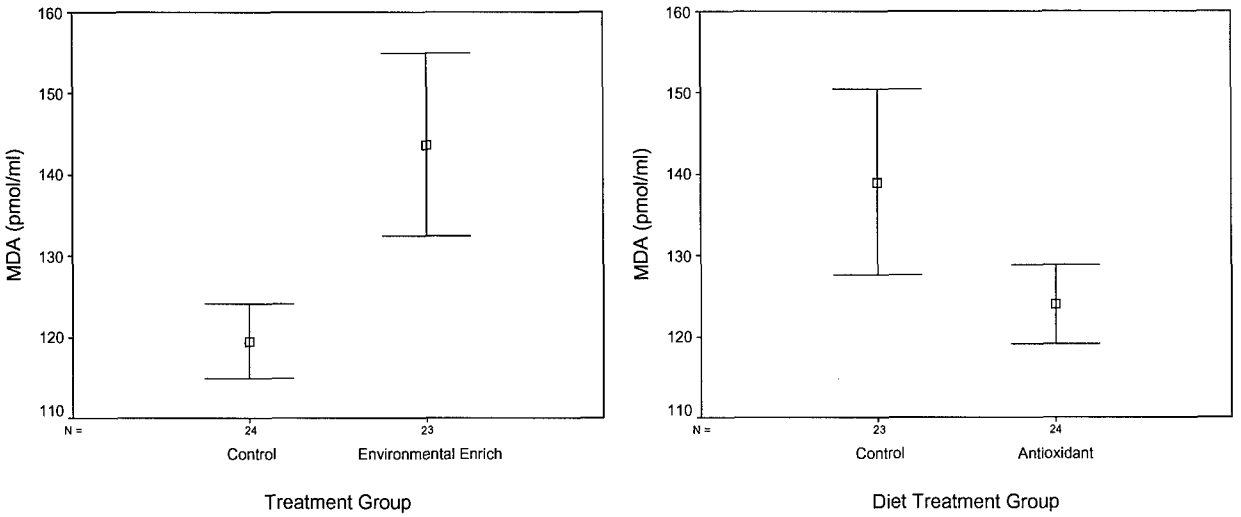


Figure 6. Animals in the environmental enrichment group show a trend toward higher plasma levels of malondialdehyde (MDA), a measure of lipid oxidative damage (left graph). In contrast, the animals on the antioxidant diet show a trend toward decreased lipid oxidative damage compared to dogs on the control diet (right graph). Error bars = standard error of the mean.

L. New Endpoint Measures of Protein Oxidation, Endogeneous Antioxidants and RNA Oxidation are Sensitive to Age in Archived Brain Tissue Samples.

A submitted manuscript reports results from studies in archived tissues analyzing the levels of protein oxidation in prefrontal cortex measured through carbonyl formation [10] and glutamine synthetase activity [11, 12]. These studies were conducted in collaboration with Dr. Jiankang Liu (UC-Berkeley). The extent of protein carbonyl formation increased as a function of age in canines but also showed increasing individual variability in older animals ($F(1,18) = 8.98$ $p<.008$). The most pronounced increases in individual variability occurred after 8 years of age (Figure 7).

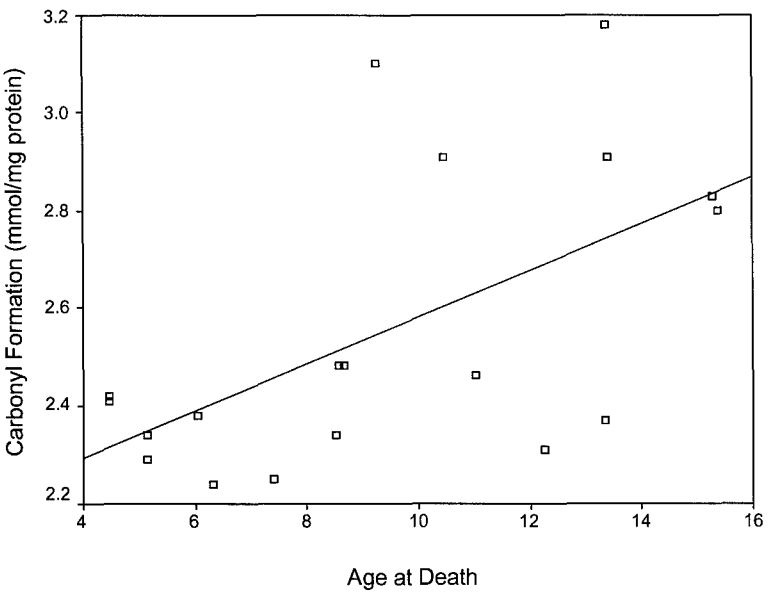


Figure 7. Carbonyl formation, a measure of protein oxidative damage, increases as a function of age in archived samples of canine brain. Note the increasing individual variability after the age of 9 years.

In parallel with increased oxidative damage to proteins, glutamine synthetase activity decreased progressively with age ($F(1,18) = 15.61$ $p<.001$) indicating oxidative damage that interferes with enzyme function as shown in Figure 8.

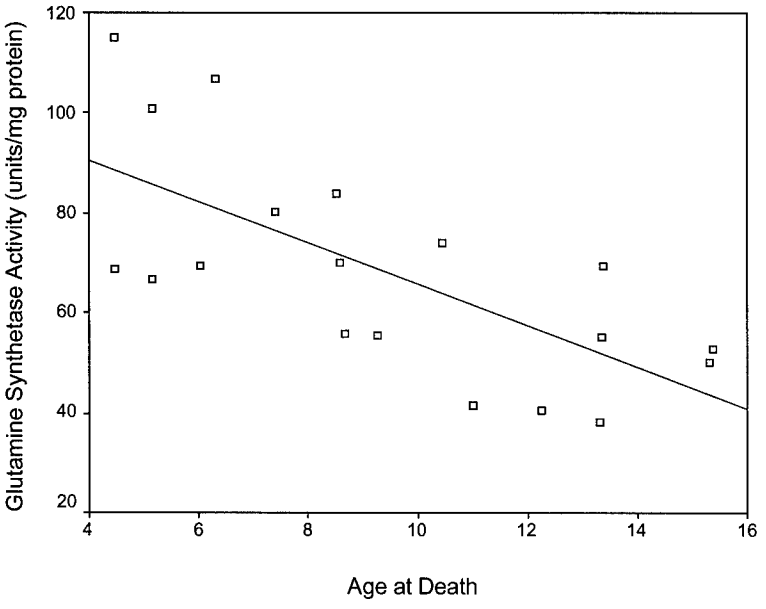


Figure 8. Enzyme dysfunction increases as a function of age with signficant reductions in glutamine synthetase (an astrocyte enzyme necessary for glutamate turnover in neurons) activity in archived samples of canine brain.

In a series of collaborative studies with Dr. Tory Hagan, Linus Pauling Institute, Oregon State University, we have obtained measures of the endogenous antioxidant glutathione (GSH) and its oxidatively reduced form GSSG in archived prefrontal cortex samples [13]. The antioxidant GSH was reduced in aged animals ($F(1,17) = 7.13$ $p<.016$) (Figure 9), but GSSG was not ($F(1,17)<1$ p = n.s.). The ratio of oxidized GSH to total GSH showed significant age-dependent increases ($r = .519$ $p<.023$).

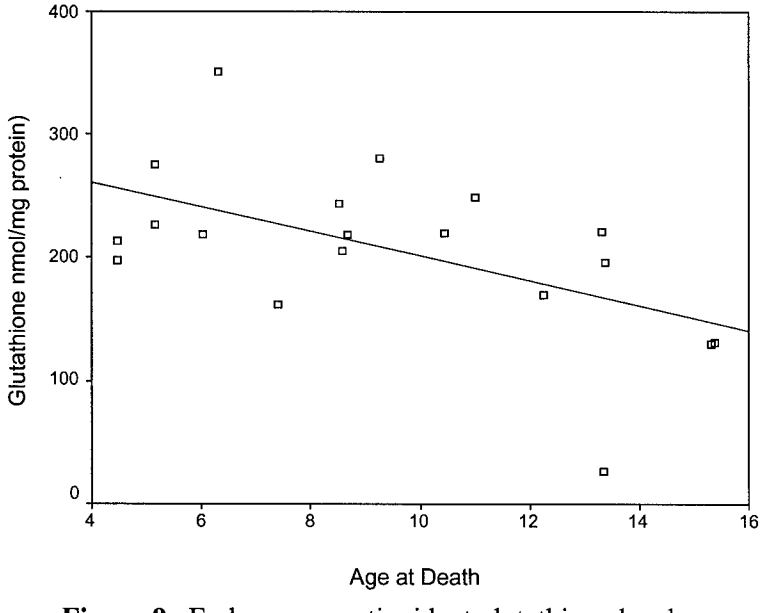


Figure 9. Endogenous antioxidant glutathione levels progressively decrease with age in archived samples of canine brain.

At the University of California, Irvine, we have also obtained preliminary data from a new commercial antibody that detects oxidatively modified nucleotides [14]. To determine whether 8oxodG labeled oxidative damage to DNA or to RNA, a series of control studies were conducted. First, sections were

pretreated with DNAse I to prevent anti-8oxodG from binding DNA, and the extent of 8oxodG remained high. On the other hand, when sections were pretreated with RNAse I, a significant reduction of 8oxodG immunoreactivity occurred. This suggests that 8oxodG immunoreactivity predominantly reflects oxidative damage to RNA (Figure 10).

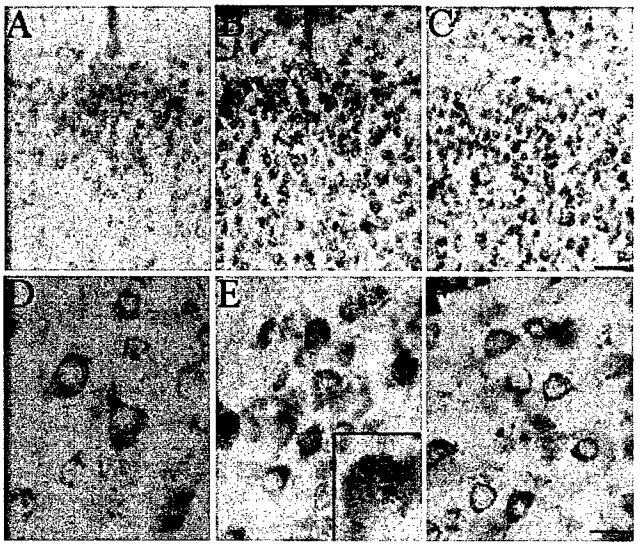


Figure 10. Anti-8oxodG is specific for oxidative damage to RNA. A and D tissue samples were pretreated with RNAse prior to incubation in the primary antibody. B and E illustrate typical staining with the antibody. C and F illustrate that pretreatment with DNAse does not significantly reduce immunoreactivity. The inset on E illustrates the punctate immunoreactivity observed with this antibody suggesting an association between oxidized RNA and intracellular organelles.

The prefrontal cortex of 21 dogs ranging in age from 0.5–17.8 years was used to determine if 8oxodG was associated with age and/or Aβ. A progressive increase in 8oxodG immunoreactivity was observed with age ($F(1,18) = 6.86$ $p<.017$, $r^2 = 0.28$) as shown in Figure 11.

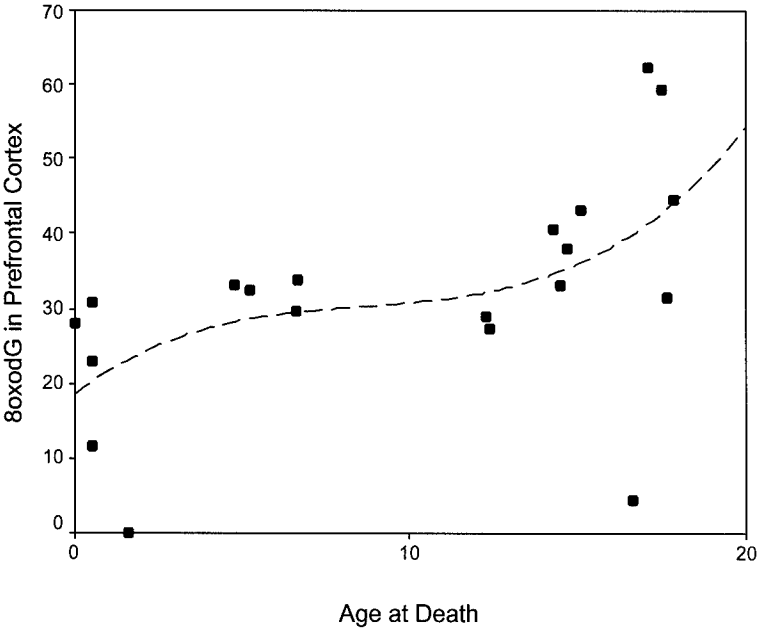


Figure 11. Oxidative damage to RNA increases as a function of age in archived canine brain samples. Note that the relationship between age and oxidative damage was a cubic function suggesting an initial rapid climb in oxidative damage followed by a plateau. In dogs over the age of 14 years, another rapid rise in oxidative damage occurred.

The correlation between the extent of 8oxodG and Aβ was not significant ($r = .22$ $n = 20$ $p =$ n.s.). However, this may reflect the earlier rise in oxidative damage to RNA during middle age (5–10 years) prior to extensive Aβ accumulation (10 years +) as shown in Figure 12.

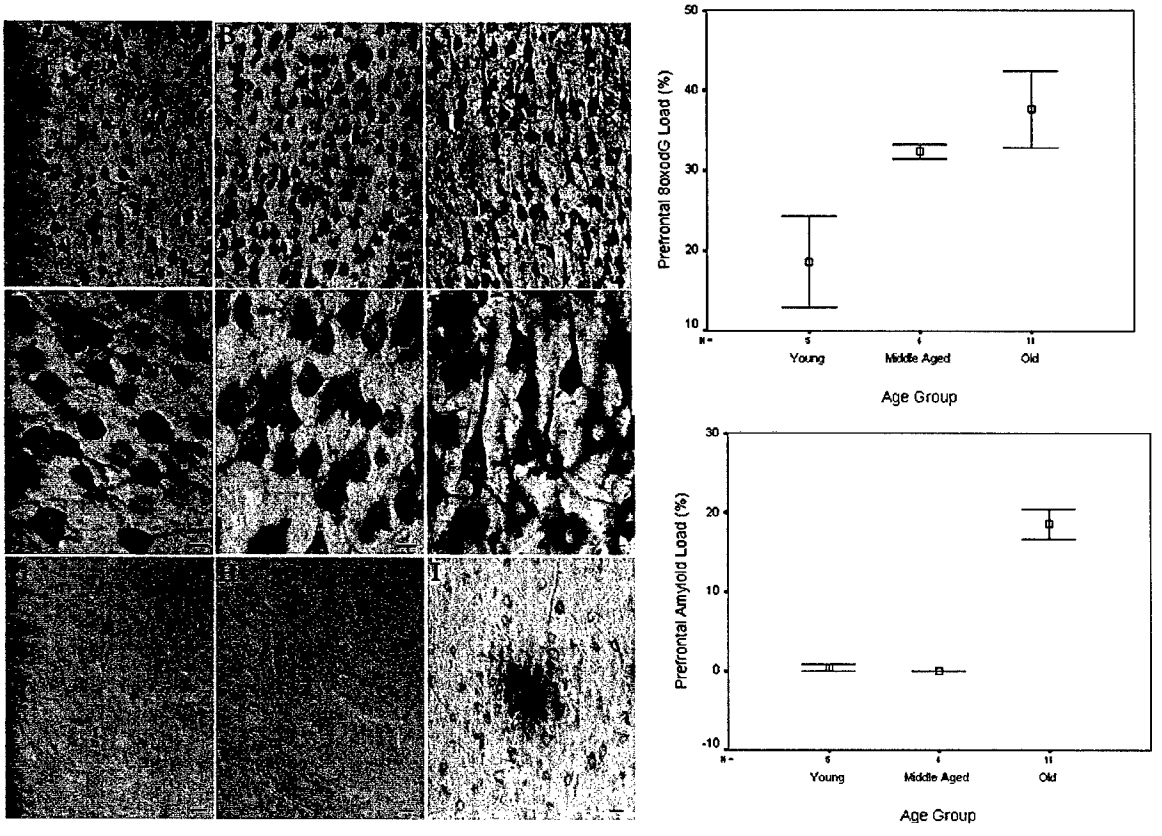


Figure 12. Oxidative damage to RNA precedes accumulation of Aβ during aging in archived samples of prefrontal cortex. Error bars = standard error of the mean.

M. Blood-Brain-Barrier Function is Maintained in Aging Dogs Provided with the Diet Rich in Antioxidants.

The MRI experiments were performed on a GE Signa 1.5 T scanner with a linear head coil as described previously [15]. The dog was anesthetized by inhalation of Isoflurane (1.5-2 %) through the experimental period. A set of 3D images across the whole brain were acquired using a Spoiled Gradient Refocus Pulse Sequence (SPGR) to obtain the detailed anatomic images. The volumes of cerebrum, lateral ventricle, hippocampus and cerebellum were measured. Four slices from the frontal cortex, thalamus, hippocampus and cerebellum were selected for the dynamic contrast enhancement study. A spin echo (SE) pulse sequence (with repetition time/echo time [TR/TE] = 117/14 ms) was applied to acquire T1-weighted images before and after injection of an MR contrast agent, gadopentetate dimeglumine (Gd-DTPA) (0.15 mmol/kg). The enhancement kinetics of Gd-DTPA were measured from the brain tissue by manually drawing a region of interest to cover the brain tissue region. The signal enhancement in the T1-weighted images was proportional to the concentration of the contrast agent in the

tissue, which is dependent on the blood volume and the leakage of agents into the interstitial brain tissue from the damaged blood-brain barrier (BBB). We used the early enhancement in the enhancement kinetics (30-45 seconds) as the vascular volume (VV) parameter. The residual enhancement at the tail of the curve (6.5-7.5 minutes) was used as the BBB permeability indicator. Volumetric and vascular changes in each group of dogs were obtained and compared.

Three MR scanning timepoints have been collected that include the baseline measures, Year 1 and Year 2. In the last progress report, we presented anatomical data that described a significant increase in ventricular volume in all treatment groups but the group receiving the combined treatment. Anatomical information is currently being obtained from MRs for Year 2 and will take several more months to finalize. In the current progress report, we will describe the results of the dynamic contrast enhanced MRI experiments that were used to measure VV and BBB permeability. We predicted a decrease in VV during the longitudinal study that may be reduced or slowed in the treatment groups. A decrease in VV is expected if brain atrophy is occurring, and there is less blood flow to the brain. Aβ also accumulates around blood vessels with age in the canine brain, and this may restrict the amount of blood flowing to the brain. Our second hypothesis is that BBB permeability will increase with age, again due to Aβ pathology associated with blood vessels except in the treatment groups.

A functional MR imaging (MRI) technique called "dynamic contrast enhanced MRI" was used to study vascular function within specific brain regions. By monitoring the kinetics of MR contrast agents in a defined brain region as it is carried in and washed out by the blood stream, it is possible to derive measures of VV and permeability. Five brain slices that were used to collect vascular function parameters include the prefrontal cortex (slice 1), the midbrain at the level of the thalamus (slice 2), the midbrain at the level of the hippocampus (slice 3), the occipital lobe (slice 4) and the cerebellum (slice 5). The rationale for selecting these brain regions was that we expected the prefrontal cortex and the hippocampus to be most vulnerable to aging based upon the extensive Aβ deposition reported in these cortical regions. We also expected that smaller or no age-dependent changes would occur in the occipital lobe, cerebellum and in the midbrain at the level of the thalamus.

To measure longitudinal changes in VV and BBB permeability, we calculated the percent of changes in each measure from baseline to compare the four treatment groups. Each brain region was analyzed separately. A total of 24/24 LBERI dogs were imaged at baseline and

Year 1 with one dog unavailable at the Year-2 timepoint. Twenty of the 24 Hill's dogs were given baseline MRs with one of these animals unavailable for the Year-2 scan. All Hill's dogs were given the Year-1 MR scan. Thus, the data presented here are based upon 43 dogs at baseline, 47 dogs at Year 1 and 45 dogs at Year 2.

Treatment condition was a significant factor in the development of age-associated increases in BBB permeability from baseline to Year 1 ($F(3,39) = 4.18$ $p<.012$) and from baseline to Year 2 ($F(3,39) = 4.6$ $p<.008$). In Year 1, these treatment effects were due to the animals in the control/ control group exhibiting significant increases in BBB permeability relative to the three other treatment groups. The combination treatment group (enriched/antioxidant) exhibited the smallest changes in BBB permeability (Figure 13). The prefrontal cortex exhibited similar increases in BBB permeability as a function of treatment group, but all the groups showed much larger increases in permeability (Mean = 43.9% ± 6.93%) as compared to the hippocampal slice (Mean = 16.2% ± 4.68%).

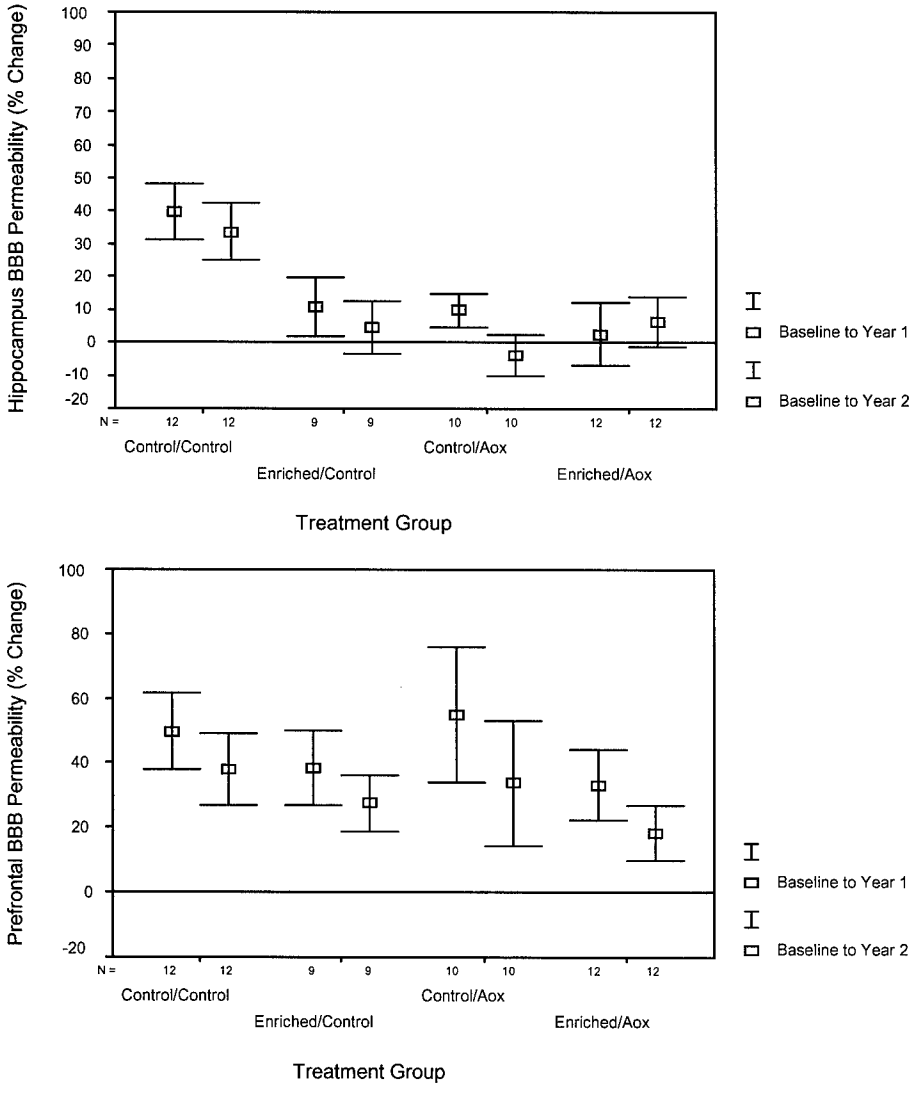


Figure 13. Significant increases in BBB permeability were found in the midbrain region containing the hippocampus of the control/control group (upper graph) but not in the prefrontal cortex (lower graph). Note that the prefrontal cortex overall showed significant increases in BBB leakage (Mean = 43.98% from Baseline to Year 1 and Mean = 29.39% from Baseline to Year 2). Error bars = standard error of the mean.

However, BBB permeability in the prefrontal cortex showed only marginal treatment effects that did not reach statistical significance. Similar trends in the data were also seen in the occipital cortex and in the midbrain at the level of the thalamus, but no trends were apparent within the cerebellum.

No systematic trends in the data were observed for VV in any treatment group in all brain regions sampled. Almost all groups did not show significant reductions in VV with the exception of a weak decrease in VV in the occipital cortex of control/control animals. This observation may be interesting because the occipital cortex is vulnerable to Aβ angiopathy in the aged canine brain (E. Head, unpublished observations). VV changes may not become apparent until the next year of the study.

III. KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 3

- The second year of dietary and environmental enrichment has been completed
- Both the antioxidant diet and environmental enrichment improve associative learning and a prefrontal-cortex sensitive reversal learning task. Further, the combination of both the antioxidant diet and environmental enrichment is additive.
- Long-term memory for a landmark discrimination task appears to be relatively unaffected by the treatment condition.
- Spatial memory remained relatively unaffected by treatment condition, and we propose to introduce a simpler version of the task to provide more data.
- Object recognition rapidly declined over a 1-year period with the combined treatment group showing a nonsignificant slowing in this trend.
- Open field activity, which is a measure of spontaneous behavior, does not show any adverse effects of the diet.
- Vitamin E measures obtained at 1 year into the study verify that the diet rich in antioxidants is maintaining higher vitamin E levels in the treatment groups than in the control groups.

- Blood biochemistry and health examinations indicate no adverse effects of the diet. The antioxidant diet is improving measures of blood coagulation, but all levels remain with normal range.
- Only two of 24 LBERI dogs have been euthanized or died due to age-related health issues.
- Plasma measures of lipid oxidative damage (malondialdehdye) indicate that environmental enrichment is associated with higher levels of oxidative damage, and that the antioxidant diet does not affect oxidative damage. The combination group is not significant from controls.
- Peripheral measures of Aβ do not vary as a function of treatment condition.
- Studies using archived tissue samples indicate that two measures of protein oxidative damage (glutamine synthetase and protein carbonyl formation) increase with age and will be useful endpoint markers for the current study.
- The levels of endogenous antioxidant, glutathione, also decrease with age in archived tissue samples and will be used as another endpoint marker for the current study.
- The extent of oxidative damage to nucleic acids rises with age prior to significant accumulation of Aβ pathology providing further evidence that oxidative damage is an early event in the development of age-associated neuropathology in canines.
- Dynamic contrast enhanced MRI studies reveal that dogs in the control/control groups are showing evidence of increased BBB permeability. In contrast, dogs in the treatment groups and in particular in the combined treatment group are showing preserved BBB permeability. VV remains unchanged with age or with treatment condition.

IV. REPORTABLE OUTCOMES

We have published several abstracts for the Annual Meeting of the Society for Neuroscience. These abstracts are presented in Appendices C through E. Further, three manuscripts have been submitted and are attached in Appendices F through H.

Abstracts:

Appendix C. E. Head, J. Liu, N.W. Milgram, B.A. Muggenburg, B.N. Ames, C.W. Cotman. Age-associated increases in oxidative damage in the prefrontal cortex in a canine model of human brain aging. Soc. Neurosci. Abstr., Vol 27, Program No. 651.19, 2001.

Appendix D. J.T. Rick, C.J. Ikeda-Douglas, H. Murphey, B.A. Muggenburg, S. Zicker, and N.W. Milgram. The effects of experience and antioxidants on size discrimination learning in the dog. Soc. Neurosci. Abstr. Vol 27, Program No. 101.14, 2001.

Appendix E. M.Y. Su, E. Head, J. Wang, J.Y. Chiou, H. Yu, B.A. Muggenburg, C.W. Cotman, O. Nalcioglu. Measurements of anatomic and vascular characteristics in the brain of aging canine with or without environmental enrichment and antioxidant diet, in "Proceedings of the 9th International Society for Magnetic Resonance in Medicine Annual Meeting, Glasgow, UK, 2001," p1477.

Manuscripts in submission:

Appendix F. N.W. Milgram, S.C. Zicker, E. Head, B.A. Muggenburg, H. Murphey, C. Ikeda-Douglas, and C.W. Cotman. Dietary enrichment counteracts age-associated cognitive dysfunction in canines. Submitted to Neurobiology of Aging.

Appendix G. E. Head, J. Liu, T.M. Hagen, B.A. Muggenburg, N.W. Milgram, B.N. Ames and C.W. Cotman. Oxidative damage increases with age and β-amyloid deposition in a canine model of human brain aging. Submitted to the FASEB Journal.

Appendix H. A.D.F. Chan, P.M.D. Nippak, H. Murphey, C.J. Ikeda-Douglas, B.A. Muggenburg, E. Head, C.W. Cotman, N.W. Milgram. Visuospatial impairments in aged canines: The role of cognitive-behavioral flexibility. Submitted to Behavioral Brain Research.

V. CONCLUSIONS

The goals for Year 3 were to complete 2 years of intervention in 24 LBERI dogs using four treatment groups. We have doubled the sample size by including additional dogs (n = 24) from Hill's Pet Nutrition that were introduced 6 months after the LBERI dogs. In total, 12 dogs are serving as controls, 12 are receiving environmental enrichment (physical exercise, play toys, housing with kennel-mate and additional learning experience), 12 are receiving the diet rich in a broad spectrum of antioxidants and 12 are receiving the combined treatment.

High levels of vitamin E are being maintained in animals receiving the antioxidant diet. Plasma measures of oxidative damage to lipids are increased in environmentally enriched dogs relative to the three other treatment conditions. Plasma measures of Aβ show no significant treatment effects, and this may be due to either a lack of effect of the treatments on peripheral Aβ levels or these effects will require an additional year to develop. Although visuo-spatial learning ability was improved in the diet condition, long-term memory for that task was not significantly improved. This suggests that learning and long-term memory are differentially sensitive to dietary and environmental intervention. A new complex learning task called size discrimination learning and a prefrontal-dependent learning task, size reversal, are both sensitive to experimental manipulation. Size reversal, in particular, shows the first evidence of an additive effect of environmental enrichment with an antioxidant diet combining to significantly improve learning in these animals as compared to the other treatment groups. This is the first evidence that our hypothesis, that the combination would be additive, has been observed.

Results of the interventions on memory ability have been relatively mild. There are several possible reasons for this finding. One problem is that the tasks to test treatment effects are proving to be particularly difficult for the aged animals to solve. Second, object recognition memory showed rapid declines over a 1-year period, which is an exciting finding unto itself but precludes our ability to detect further intervention improvements. To counteract these difficulties, we intend to introduce a simpler spatial memory task, one with which we have had past success, in order to obtain additional data on memory ability in response to intervention. Ideally, we would like to obtain two measures with this simpler memory task, but the current study plan prevents this from being possible.

Another aspect to our work was to develop new endpoint markers for anatomical studies to be completed next year. This aspect has yielded three new markers that may be sensitive to the dietary treatment effects. These include measures of protein oxidation (protein carbonyl formation and glutamine synthetase activity) along with measures of oxidative damage to nucleotides (RNA oxidation). These experiments will now be feasible through strong collaborations with two groups, one at UC-Berkeley and at the other at the Linus Pauling Institute at Oregon State University.

The in vivo functional imaging studies have also provided exciting data suggesting that whereas the control group is showing increased BBB permeability, the treatment groups are

showing a much slower development of this pathology. In addition, the combined treatment group is showing the least amount of change over the study period to date. These findings parallel the decreased cognitive abilities of the control dogs and the improved cognitive abilities of the treatment animals. All of these measures will be put together along with the anatomical measures obtained at the end of the study to determine significant intercorrelations.

It is increasingly clear as the study proceeds that the addition of a full 3-year evaluation would be most helpful to strengthen our conclusions regarding the ability of environmental enrichment, antioxidant diet or the combination of both treatments to promote successful cognitive aging. The memory studies and the in vivo imaging studies would benefit most from this additional information. Further, our most dramatic effects on cognitive function to date in the study have been in using complex learning tasks. Confirming and extending these findings with a year added to the study would further strengthen our conclusions. All of these additional measures would contribute significantly to the planned neuroanatomical studies. Nonetheless, the study has provided exciting new data to suggest that dietary and environmental enrichment can significantly improve healthy cognitive aging.

VI. REFERENCES

[1] Head, E., Callahan, H., Muggenburg, B.A., Milgram, N.W. and Cotman, C.W. (1998) Discrimination learning ability and beta amyloid accumulation in the dog. *Neurobiology of Aging*; 19(5):415-425.

[2] Head, E., Mehta, R., Hartley, J., Kameka, A.M., Cummings, B.J., Cotman, C.W., Ruehl, W.W., and Milgram, N.W. (1995) Spatial learning and memory as a function of age in the dog. *Behavioral Neuroscience*; 109:851-858.

[3] Adams, B., Chan, A., Callahan, H., Siwak, C., Tapp, D., Ikeda-Douglas, C., Head, E., Cotman, C.W., and Milgram, N.W. (2000) Spatial learning and memory in the dog as a model of cognitive aging. *Behavioral Brain Research*; 108(1):47-56.

[4] Milgram, N.W., Head, E., Weiner, E. and Thomas, E. (1994) Cognitive functions and aging in the dog: Acquisition of nonspatial visual tasks. *Behavioral Neuroscience*; 108:57-68.

[5] Callahan, H., Ikeda-Douglas, C., Head, E., Cotman, C.W., and Milgram, N.W. (2000) Development of a protocol for studying object recognition memory in the dog. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*; In press.

[6] Dubovicky, M., Ujhazy, E., Kovacovsky, P., Rychlik, I., and Jansak, J. (1999) Evaluation of long-term administration of the antioxidant stobadine on exploratory behaviour in rats of both genders. *Journal of Applied Toxicology*; 19(6):431-436.

[7] March, B.E., Wong, E., Seier, L., Sim, J., and Biely, J. (1973) Hypervitaminosis E in the chick. *The Journal of Nutrition*; 103:371.

[8] Corrigan, J.J. (1979) Coagulation problems relating to vitamin E. *American Journal of Pediatric Hematology/Oncology*; 1:169-173.

[9] Liu, J., Yeo, H.C., Doniger, S.J., and Ames, B.N. (1997) Assay of aldehydes from lipid peroxidation: Gas chromatography-mass spectrometry compared to thiobarbituric acid. *Analytical Biochemistry*; 245:161-166.

[10] Levine, R.L., Williams, J.A., Stadtman, E.R., and Schacter, E. (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology*; 233:346-357.

[11] Miller, R.E., Hadenburg, R., and Gersham, H. (1978) Regulation of glutamine synthetase in cultured 3T3-LI cells by insulin, hydrocortisterone, and dibutyryl cAMP. *Proceedings of the National Academy of Science, USA*; 75:1418-1422.

[12] Rowe, W.B., Remzio, R. A., Wellner, V.P., and Meister, A. (1970) Glutamine synthetase. *Methods in Enzymology*; 17:900-910.

[13] Reed, D.J., Babson, J.R., Beatty, P.W., Brodie, A.E., Ellis, W.W., and Potter, D.W. (1980) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Analytical Biochemistry*; 106(1):55-62.

[14] Nunomura, A., Perry, G., Pappolla, M.A., Wade, R., Hirai, K., Chiba, S., and Smith, M.A. (1999) RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *The Journal of Neuroscience*; 19(6):1959-1964.

[15] Su, M.-Y., Head, E., Brooks, W.M., Wang, Z., Muggenberg, B.A., Adam, G.E., Sutherland, R.J., Cotman, C.W., and Nalcioglu, O. (1998) MR imaging of anatomic and vascular characteristics in a canine model of human aging. *Neurobiology of Aging*; 19(5):479-485.

Appendix A. Status of Individual Animals in the Longitudinal Study

Dog	Date	Intervention Start Date	Birthdate	Age at Start of Study (yrs)	Current Age (yrs)	Time on Intervention (yrs)	Group		Source	Comments
							Diet	Environment		
1532S	10/15/01	08/06/99	02/09/89	10.38	12.69	2.31	Aox	Control	LRRI	On Study
1581S	10/15/01	09/13/99	05/15/91	8.12	10.43	2.31	Aox	Control	LRRI	On Study
1523B	10/15/01	09/13/99	11/26/89	9.58	11.89	2.31	Aox	Control	LRRI	On Study
1508A	10/15/01	08/06/99	02/12/88	11.37	13.68	2.32	Aox	Control	LRRI	On Study
1509U	10/15/01	08/06/99	03/03/88	11.31	13.63	2.32	Aox	Control	LRRI	On Study
1491B	10/15/01	09/13/99	05/13/87	12.12	14.44	2.32	Aox	Control	LRRI	On Study
1541B	10/15/01	09/03/99	05/25/89	10.09	12.4	2.31	Aox	Enriched	LRRI	On Study
1542T	10/15/01	08/06/99	06/03/89	10.07	12.38	2.31	Aox	Enriched	LRRI	On Study
1585A	10/15/01	09/13/99	08/29/91	7.83	10.14	2.31	Aox	Enriched	LRRI	On Study
1581T	10/15/01	09/13/99	05/15/91	8.12	10.43	2.31	Aox	Enriched	LRRI	On Study
1502S	10/15/01	08/06/99	08/16/87	11.86	14.18	2.32	Aox	Enriched	LRRI	On Study
1521B	10/15/01	09/13/99	10/06/88	10.72	13.03	2.32	Aox	Enriched	LRRI	On Study
1543S	10/15/01	07/18/99	06/04/89	10.06	12.37	2.31	Control	Control	LRRI	On Study
B2150	10/15/01	07/18/99	11/12/87	11.62	13.93	2.31	Control	Control	LRRI	On Study
1521S	12/15/00	08/15/99	10/06/88	10.72	12.2	1.48	Control	Control	LRRI	Off Study
1494D	10/15/01	08/15/99	05/27/87	12.08	14.40	2.32	Control	Control	LRRI	On Study
1510A	10/15/01	09/13/99	03/22/88	11.26	13.58	2.32	Control	Control	LRRI	On Study
1508U	07/26/01	08/15/99	02/12/88	11.37	13.46	2.10	Control	Control	LRRI	Dead
1529S	10/15/01	07/18/99	01/23/89	10.42	12.73	2.31	Control	Enriched	LRRI	On Study
1523U	10/15/01	08/15/99	11/26/89	9.58	11.89	2.31	Control	Enriched	LRRI	On Study
1542S	10/15/01	07/18/99	06/03/89	10.07	12.38	2.31	Control	Enriched	LRRI	On Study
1506B	10/15/01	08/15/99	01/04/88	11.47	13.79	2.32	Control	Enriched	LRRI	On Study
1492B	11/24/99	08/15/99	05/23/87	12.09	12.52	0.42	Control	Enriched	LRRI	Dead
1518D	10/15/01	07/18/99	09/18/88	10.77	13.08	2.32	Control	Enriched	LRRI	On Study
D056	10/15/01	01/31/00	12/05/88	10.66	12.87	2.21	Aox	Control	Hills	On Study
D048	10/15/01	01/31/00	09/15/88	10.88	13.09	2.21	Aox	Control	Hills	On Study
D064	10/15/01	01/31/00	08/15/89	9.96	12.18	2.21	Aox	Control	Hills	On Study
D067	10/15/01	01/27/00	10/01/90	8.83	11.05	2.21	Aox	Control	Hills	On Study
D081	10/15/01	02/07/00	02/23/90	9.44	11.65	2.21	Aox	Control	Hills	On Study
D082	10/15/01	01/31/00	09/18/91	7.87	10.08	2.21	Aox	Control	Hills	On Study

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

Dog	Date	Intervention Start Date	Birthdate	Age at Start of Study (yrs)	Current Age (yrs)	Time on Intervention (yrs)	Group		Source	Comments
							Diet	Environment		
D058	10/03/00	02/07/00	09/20/88	10.87	12.04	1.18	Aox	Enriched	Hills	Dead
D060	10/15/01	01/31/00	09/20/89	9.87	12.08	2.21	Aox	Enriched	Hills	On Study
D054	10/15/01	01/27/00	05/15/90	9.22	11.43	2.21	Aox	Enriched	Hills	On Study
D055	10/15/01	01/27/00	10/16/88	10.79	13.01	2.21	Aox	Enriched	Hills	On Study
D065	10/15/01	01/27/00	06/10/89	10.14	12.36	2.21	Aox	Enriched	Hills	On Study
D075	10/15/01	01/27/00	02/08/90	9.48	11.69	2.21	Aox	Enriched	Hills	On Study
D051	10/15/01	01/15/00	08/15/89	9.96	12.18	2.21	Control	Control	Hills	On Study
D059	10/15/01	01/15/00	10/06/90	8.82	11.03	2.21	Control	Control	Hills	On Study
D062	10/15/01	01/15/00	10/01/90	8.83	11.05	2.21	Control	Control	Hills	On Study
D063	10/15/01	01/15/00	04/08/90	9.32	11.53	2.21	Control	Control	Hills	On Study
D066	10/15/01	11/20/99	05/28/90	9.18	11.39	2.21	Control	Control	Hills	On Study
D071	10/15/01	01/15/00	09/24/89	9.85	12.07	2.21	Control	Control	Hills	On Study
D052	10/15/01	02/07/00	07/08/88	11.07	13.28	2.21	Control	Enriched	Hills	On Study
D053	10/15/01	02/07/00	07/19/91	8.04	10.25	2.21	Control	Enriched	Hills	On Study
D080	10/15/01	02/07/00	08/04/89	9.99	12.21	2.21	Control	Enriched	Hills	On Study
D074	10/15/01	02/07/00	09/26/89	9.85	12.06	2.21	Control	Enriched	Hills	On Study
D073	10/15/01	02/07/00	09/21/89	9.86	12.07	2.21	Control	Enriched	Hills	On Study
D072	10/15/01	02/07/00	12/26/89	9.60	11.81	2.21	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	10.65753425	A	C	H	28
D048	07/31/99	Pre	09/15/88	10.87945205	A	C	H	51
D064	07/30/99	Pre	08/15/89	9.961643836	A	C	H	22
D067	07/30/99	Pre	10/01/90	8.832876712	A	C	H	21
D081	07/31/99	Pre	02/23/90	9.438356164	A	C	H	29
D082	07/31/99	Pre	09/18/91	7.871232877	A	C	H	32
D058	07/31/99	Pre	09/20/88	10.86575342	A	E	H	35
D060	07/31/99	Pre	09/20/89	9.865753425	A	E	H	29
D054	07/31/99	Pre	05/15/90	9.216438356	A	E	H	19
D055	07/31/99	Pre	10/16/88	10.79452055	A	E	H	25
D065	07/30/99	Pre	06/10/89	10.14246575	A	E	H	20
D075	07/31/99	Pre	02/08/90	9.479452055	A	E	H	24
1532S	06/25/99	Pre	02/09/89	10.37808219	A	C	L	32
1581S	06/25/99	Pre	05/15/91	8.117808219	A	C	L	21
1523B	06/25/99	Pre	11/26/89	9.583561644	A	C	L	18
1508A	06/23/99	Pre	02/12/88	11.36712329	A	C	L	32
1509U	06/23/99	Pre	03/03/88	11.31232877	A	C	L	19
1491B	06/23/99	Pre	05/13/87	12.12054795	A	C	L	27
1541B	06/25/99	Pre	05/25/89	10.09041096	A	E	L	22
1542T	06/25/99	Pre	06/03/89	10.06575342	A	E	L	20
1585A	06/25/99	Pre	08/29/91	7.82739726	A	E	L	27
1581T	06/25/99	Pre	05/15/91	8.117808219	A	E	L	24
1502S	06/23/99	Pre	08/16/87	11.86027397	A	E	L	34
1521B	06/23/99	Pre	10/06/88	10.71780822	A	E	L	29
AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START								26.666667
								7.2989974
Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D051	07/31/99	Pre	08/15/89	9.964383562	C	C	H	24
D059	07/31/99	Pre	10/06/90	8.821917808	C	C	H	23
D062	07/30/99	Pre	10/01/90	8.832876712	C	C	H	34
D063	07/30/99	Pre	04/08/90	9.315068493	C	C	H	35
D066	07/30/99	Pre	05/28/90	9.178082192	C	C	H	22
D071	07/30/99	Pre	09/24/89	9.852054795	C	C	H	28
D052	07/31/99	Pre	07/08/88	11.06849315	C	E	H	30
D053	07/31/99	Pre	07/19/91	8.038356164	C	E	H	29
D080	07/31/99	Pre	08/04/89	9.994520548	C	E	H	26
D074	07/30/99	Pre	09/26/89	9.846575342	C	E	H	22
D073	07/30/99	Pre	09/21/89	9.860273973	C	E	H	36
D072	07/30/99	Pre	12/26/89	9.597260274	C	E	H	14
1543S	06/25/99	Pre	06/04/89	10.0630137	C	C	L	28
B2150	06/25/99	Pre	11/12/87	11.62465753	C	C	L	27
1521S	06/23/99	Pre	10/06/88	10.71780822	C	C	L	24
1494D	06/23/99	Pre	05/27/87	12.08219178	C	C	L	26
1510A	06/23/99	Pre	03/22/88	11.26027397	C	C	L	34
1508U	06/23/99	Pre	02/12/88	11.36712329	C	C	L	32
1529S	06/25/99	Pre	01/23/89	10.42465753	C	E	L	29
1523U	06/25/99	Pre	11/26/89	9.583561644	C	E	L	32
1542S	06/25/99	Pre	06/03/89	10.06575342	C	E	L	22
1506B	06/23/99	Pre	01/04/88	11.4739726	C	E	L	25
1492B	06/23/99	Pre	05/23/87	12.09315068	C	E	L	27
1518D	06/23/99	Pre	09/18/88	10.76712329	C	E	L	63
AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START								28.833333
								8.8743238

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	11.72328767	A	C	H	30
D048	08/23/00	0.50	09/15/88	11.94520548	A	C	H	47
D064	08/23/00	0.50	08/15/89	11.03013699	A	C	H	22
D067	08/23/00	0.50	10/01/90	9.901369863	A	C	H	19
D081	09/06/00	0.50	02/23/90	10.54246575	A	C	H	22
D082	08/23/00	0.50	09/18/91	8.936986301	A	C	H	22
D058	09/06/00	0.50	09/20/88	11.96986301	A	E	H	30
D060	08/23/00	0.50	09/20/89	10.93150685	A	E	H	34
D054	08/23/00	0.50	05/15/90	10.28219178	A	E	H	29
D055	08/29/00	0.50	10/16/88	11.87671233	A	E	H	24
D065	08/29/00	0.50	06/10/89	11.22739726	A	E	H	17
D075	08/23/00	0.50	02/08/90	10.54520548	A	E	H	27
1532S	02/09/00	0.5	02/09/89	11.00547945	A	C	L	25
1581S	03/21/00	0.5	05/15/91	8.857534247	A	C	L	28
1523B	03/21/00	0.5	11/26/89	10.32328767	A	C	L	24
1508A	02/09/00	0.5	02/12/88	12	A	C	L	35
1509U	02/09/00	0.5	03/03/88	11.94520548	A	C	L	19
1491B	03/21/00	0.5	05/13/87	12.86575342	A	C	L	24
1541B	03/21/00	0.5	05/25/89	10.83013699	A	E	L	27
1542T	02/09/00	0.5	06/03/89	10.69315068	A	E	L	22
1585A	03/21/00	0.5	08/29/91	8.567123288	A	E	L	40
1581T	03/21/00	0.5	05/15/91	8.857534247	A	E	L	25
1502S	02/09/00	0.5	08/16/87	12.49315068	A	E	L	36
1521B	03/21/00	0.5	10/06/88	11.4630137	A	E	L	35
AVERAGE FOR ANTIOXIDANT GROUP AFTER 6 MONTHS ON DIET								27.625
								7.2340529
AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START								26.666667
								7.2989974
Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D051	08/29/00	0.50	08/15/89	11.04657534	C	C	H	26
D059	09/06/00	0.50	10/06/90	9.926027397	C	C	H	19
D062	09/06/00	0.50	10/01/90	9.939726027	C	C	H	28
D063	09/06/00	0.50	04/08/90	10.42191781	C	C	H	26
D066	08/29/00	0.50	05/28/90	10.2630137	C	C	H	22
D071	09/06/00	0.50	09/24/89	10.95890411	C	C	H	22
D052	08/29/00	0.50	07/08/88	12.15068493	C	E	H	32
D053	09/06/00	0.50	07/19/91	9.142465753	C	E	H	22
D080	09/06/00	0.50	08/04/89	11.09863014	C	E	H	36
D074	08/23/00	0.50	09/26/89	10.91506849	C	E	H	22
D073	08/29/00	0.50	09/21/89	10.94520548	C	E	H	27
D072	08/29/00	0.50	12/26/89	10.68219178	C	E	H	23
1543S	01/25/00	0.5	06/04/89	10.64931507	C	C	L	19
B2150	01/25/00	0.5	11/12/87	12.2109589	C	C	L	36
1521S	02/16/00	0.5	10/06/88	11.36986301	C	C	L	28
1494D	02/16/00	0.5	05/27/87	12.73424658	C	C	L	29
1510A	03/21/00	0.5	03/22/88	12.00547945	C	C	L	31
1508U	02/16/00	0.5	02/12/88	12.01917808	C	C	L	32
1529S	01/25/00	0.5	01/23/89	11.0109589	C	E	L	28
1523U	02/16/00	0.5	11/26/89	10.23013699	C	E	L	22
1542S	01/25/00	0.5	06/03/89	10.65205479	C	E	L	27
1506B	02/16/00	0.5	01/04/88	12.1260274	C	E	L	24
1492B	02/16/00	0.5	05/23/87	12.74520548	C	E	L	.
1518D	01/25/00	0.5	09/18/88	11.35890411	C	E	L	38
								26.913043
								5.3759823
AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START								28.833333
								8.8743238

Appendix B. Blood Biochemistry Parameters for Individual Animals

Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D056	01/30/01	1	12/05/88	12.16164384	A	C	H	26
D048	01/30/01	1	08/15/88	12.46849315	A	C	H	47
D064	01/30/01	1	10/15/88	12.30136986	A	C	H	20
D067	01/30/01	1	10/01/90	10.33972603	A	C	H	17
D081	02/06/01	1	02/23/90	10.96164384	A	C	H	24
D082	01/30/01	1	09/18/91	9.375342466	A	C	H	31
D056	01/30/01	1	12/05/88	12.16164384	A	C	H	26
D060	01/30/01	1	09/20/89	11.36986301	A	E	H	37
D054	01/30/01	1	05/15/90	10.72054795	A	E	H	29
D055	01/30/01	1	10/15/90	10.30136986	A	E	H	22
D065	01/30/01	1	06/10/89	11.64931507	A	E	H	19
D075	01/30/01	1	02/08/90	10.98356164	A	E	H	17
D070	02/06/01	1	10/25/90	10.29315068	A	E	H	33
1532S	08/22/00	1	02/09/89	11.53972603	A	C	L	14
1581S	09/20/00	1	05/15/91	9.35890411	A	C	L	23
1523B	09/20/00	1	11/26/89	10.82465753	A	C	L	22
1508A	08/22/00	1	02/12/88	12.53424658	A	C	L	30
1509U	08/22/00	1	03/03/88	12.47945205	A	C	L	18
1491B	09/20/00	1	05/13/87	13.36712329	A	C	L	21
1541B	09/20/00	1	05/25/89	11.33150685	A	E	L	24
1542T	08/22/00	1	06/03/89	11.22739726	A	E	L	21
1585A	09/19/00	1	08/29/91	9.065753425	A	E	L	37
1581T	09/19/00	1	05/15/91	9.356164384	A	E	L	25
1502S	08/22/00	1	08/16/87	13.02739726	A	E	L	28
1521B	09/19/00	1	10/06/88	11.96164384	A	E	L	36
AVERAGE FOR ANTIOXIDANT GROUP AFTER 12 MONTHS ON DIET								25.88
								7.7476878
AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START								26.666667
								7.2989974
Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
D051	01/30/01	1	08/15/88	12.46849315	C	C	H	24
D059	01/31/01	1	10/06/90	10.32876712	C	C	H	18
D062	02/06/01	1	10/01/90	10.35890411	C	C	H	26
D063	02/06/01	1	04/08/90	10.84109589	C	C	H	29
D066	01/31/01	1	05/28/90	10.68767123	C	C	H	25
D052	01/31/01	1	07/15/88	12.55616438	C	E	H	26
D053	02/06/01	1	07/15/91	9.57260274	C	E	H	17
D080	02/06/01	1	08/04/89	11.51780822	C	E	H	29
D074	01/31/01	1	09/26/89	11.35616438	C	E	H	28
D073	01/31/01	1	09/21/89	11.36986301	C	E	H	27
D072	01/31/01	1	12/26/89	11.10684932	C	E	H	24
1543S	07/24/00	1	06/04/89	11.14520548	C	C	L	22
B2150	07/24/00	1	11/12/87	12.70684932	C	C	L	45
1521S	08/22/00	1	10/06/88	11.88493151	C	C	L	22
1494D	08/22/00	1	05/27/87	13.24931507	C	C	L	24
1510A	09/19/00	1	03/22/88	12.50410959	C	C	L	27
1508U	08/22/00	1	02/12/88	12.53424658	C	C	L	32
1529S	07/24/00	1	01/23/89	11.50684932	C	E	L	29
1523U	08/22/00	1	11/26/89	10.74520548	C	E	L	20
1542S	07/24/00	1	06/03/89	11.14794521	C	E	L	29
1506B	08/22/00	1	01/04/88	12.64109589	C	E	L	24
1492B	08/22/00	1	05/23/87	13.26027397	C	E	L	.
1518D	07/24/00	1	09/18/88	11.85479452	C	E	L	32
AVERAGE FOR CONTROL GROUP AFTER 12 MONTHS ON DIET								26.318182
								5.7849259
AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START								28.833333
								8.8743238

Appendix B. Blood Biochemistry Parameters for Individual Animals

Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
1491B	03/10/01	1.5	05/13/87	13.83561644	A	C	L	24
1508A	02/17/01	1.5	02/12/88	13.02465753	A	C	L	36
1509U	02/17/01	1.5	03/03/88	12.96986301	A	C	L	23
1523B	03/10/01	1.5	11/26/89	11.29315068	A	C	L	24
1532S	02/17/01	1.5	02/09/89	12.03013699	A	C	L	17
1581S	03/10/01	1.5	05/15/91	9.82739726	A	C	L	24
1502S	02/20/01	1.5	08/16/87	13.5260274	A	E	L	48
1521B	03/10/01	1.5	10/06/88	12.43287671	A	E	L	31
1541B	03/10/01	1.5	05/25/89	11.8	A	E	L	28
1542T	02/17/01	1.5	06/03/89	11.71780822	A	E	L	28
1581T	03/10/01	1.5	05/15/91	9.82739726	A	E	L	29
1585A	03/10/01	1.5	08/29/91	9.536986301	A	E	L	42
AVERAGE FOR ANTIOXIDANT GROUP AFTER 18 MONTHS ON DIET								29.5
								8.7230103
AVERAGE FOR ANTIOXIDANT GROUP PRIOR TO STUDY START								26.666667
								7.2989974
Animal ID	Date	Period	Birthdate	Age	Diet	Environment	Source	AST (SGOT)
1494D	03/10/01	1.5	05/27/87	13.79726027	C	C	L	35
1508U	03/10/01	1.5	02/12/88	13.08219178	C	C	L	31
1510A	03/10/01	1.5	03/22/88	12.97534247	C	C	L	27
1521S	03/10/01	1.5	10/06/88	12.43287671	C	C	L	30
1543S	02/19/01	1.5	06/04/89	11.72054795	C	C	L	21
B2150	02/18/01	1.5	11/12/87	13.27945205	C	C	L	41
1492B	.	1.5	05/23/87	.	C	E	L	.
1506B	03/10/01	1.5	01/04/88	13.1890411	C	E	L	28
1518D	02/17/01	1.5	09/18/88	12.42465753	C	E	L	29
1523U	03/10/01	1.5	11/26/89	11.29315068	C	E	L	27
1529S	02/18/01	1.5	01/23/89	12.07945205	C	E	L	28
1542S	02/18/01	1.5	06/03/89	11.72054795	C	E	L	28
AVERAGE FOR CONTROL GROUP AFTER 18 MONTHS ON DIET								29.545455
								5.0668262
AVERAGE FOR CONTROL GROUP PRIOR TO STUDY START								28.833333
								8.8743238

Appendix B. Blood Biochemistry Parameters for Individual Animals

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN
40	0.1	226	5	5.2	2.7	2.5
146	0.1	397	19	6.2	3.3	2.9
42	0.2	217	2	6.4	3.3	3.1
48	0.1	93	12	6.1	3.5	2.6
22	0.1	76	4	6.1	3.6	2.5
42	0.1	354	7	5.9	3.3	2.6
50	0.1	63	2	6.7	3	3.7
38	0.1	136	2	5.7	2.9	2.8
23	0.1	81	4	5.7	3.3	2.4
26	0.1	58	7	6.2	3.2	3
56	0.1	171	5	7.1	3.4	3.7
36	0.1	220	4	5.9	3.6	2.3
127	0.1	57	6	6.4	2.4	4
29	0.3	43	6	5.4	3.2	2.2
36	0.3	94	2	6	2.9	3.1
61	.1 (lipemic)	166	7	5.9	3.1	2.8
17	0.1	128	5	5.7	2.9	2.8
51	0.1	139	6	5.7	3.1	2.6
38	0.1	78	1	6.2	3.6	2.6
20	0.1	239	1	6.1	3	3.1
31	0.1	68	1	5.4	2.8	2.6
49	too lipemic	163	1	5.9	3.5	2.4
26	.1 (lipemic)	98	6	6.3	3.2	3.1
36	0.1	43	10	5.7	3.3	2.4
45.41666667	0.123809524	142	5.208333333	5.995833333	3.170833333	2.825
30.47724264	0.062488094	94.32967167	4.107037086	0.422702657	0.30571252	0.462742298
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN
38	0.1	265	4	5.7	3.2	2.5
34	0.1	222	13	6.3	3.6	2.7
29	0.1	99	1	6	3.3	2.7
36	0.1	270	3	6.7	2.6	4.1
31	0.1	125	6	6.1	2.9	3.2
105	0.1	142	5	6	3.5	2.5
38	0.1	358	4	5.4	3.4	2
46	0.2	140	2	6.7	3.5	3.2
25	0.1	150	2	6.1	3.2	2.9
28	0.2	269	1	5.7	3	2.7
444	0.1	472	24	6.4	3.1	3.3
17	0.1	100	5	6.1	3.1	3
52	0.1	105	1	5.8	2.8	3
46	0.2	67	7	5.7	3.1	2.6
32	0.1	48	4	5.7	2.7	3
45	0.1	85	1	6.2	2.7	3.5
61	0.1	96	3	6.4	3.2	3.2
29	0.2	90	7	6.3	3.2	3.1
49	0.2	91	1	5.7	3.1	2.6
29	0.1	79	3	6.1	3.1	3
24	0.3	261	6	5.6	2.5	3.1
35	0.1	402	3	6.4	3.2	3.2
22	0.1	74	11	4.9	2.3	2.6
74	0.1	218	5	7	3.1	3.9
57.04166667	0.129166667	176.1666667	5.083333333	6.041666667	3.058333333	2.983333333
84.56048988	0.055003294	115.7548634	5.055489196	0.461487827	0.324260586	0.456514838

Appendix B. Blood Biochemistry Parameters for Individual Animals

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN
30	0.1	275	7	5.6	2.9	2.7
195	0.2	574	24	6.2	3.5	2.7
27	0.2	239	3	5.9	3.5	2.4
33	0.2	97	4	6.2	3.8	2.4
22	0.1	79	4	6.5	3.9	2.6
24	0.2	255	8	5.9	3.4	2.5
37	0.1	66	2	6.8	3.4	3.4
36	0.2	190	7	6	3.1	2.9
24	0.1	89	6	5.9	3.3	2.6
23	0.1	58	3	6.3	3.4	2.9
36	0.1	161	1	6.4	3.5	2.9
31	0.2	125	6	5.7	3.3	2.4
38	0.3	389	1	6.2	3.1	3.1
61	0.1	72	6	5.4	3.4	2
32	0.1	91	3	6	3.4	2.6
61	0.3	246	1	6.3	3.3	3
17	0.2	134	2	6.3	3.4	2.9
41	0.2	187	5	6	3.4	2.6
29	0.2	91	3	5.7	3.6	2.1
19	0.3	186	4	6.6	3.5	3.1
39	0.1	100	3	5.8	3.2	2.6
38	0.1	139	3	6.2	3.7	2.5
57	0.2	122	6	6.7	3.2	3.5
36	0.1	60	4	6	3.5	2.5
41.08333333	0.166666667	167.7083333	4.833333333	6.108333333	3.404166667	2.704166667
34.83740702	0.070196412	119.794563	4.546060566	0.350051756	0.221612993	0.360530004
45.41666667	0.123809524	142	5.208333333	5.995833333	3.170833333	2.825
30.47724264	0.062488094	94.32967167	4.107037086	0.422702657	0.30571252	0.462742298
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN
37	0.1	224	1	5.9	3.3	2.6
49	0.2	306	13	5.9	3.6	2.3
32	0.1	84	1	5.9	3.7	2.2
33	0.2	256	3	6.7	3.5	3.2
33	0.1	155	2	6	3.2	2.8
136	0.2	243	7	6.2	4	2.2
37	0.1	265	1	6.1	3.8	2.3
44	0.1	121	6	7	3.6	3.4
29	0.1	158	1	6.4	3.4	3
31	0.1	172	2	5.4	2.9	2.5
242	0.1	450	18	6.3	3	3.3
17	0.1	163	4	6.6	3.1	3.5
63	0.2	118	9	7	3.3	3.7
49	0.1	85	3	6.4	3.5	2.9
30	0.2	78	2	5.8	2.6	3.2
50	0.1	78	4	6.6	3.1	3.5
45	0.1	97	2	6.7	3.5	3.2
26	0.2	53	1	6.1	3.4	2.7
52	0.2	87	7	6.1	3.3	2.8
22	0.2	82	1	6.4	3.2	3.2
26	0.1	239	6	6	2.9	3.1
35	0.1	331	3	6.5	3.1	3.4
.	.	.	.	.	.	.
53	0.1	140	6	6.7	3	3.7

50.91304348	0.134782609	173.2608696	4.47826087	6.291304348	3.304347826	2.986956522
47.75782948	0.048698475	100.820821	4.262623661	0.402168038	0.326800274	0.472232943
57.04166667	0.129166667	176.1666667	5.083333333	6.041666667	3.058333333	2.983333333
84.56048988	0.055003294	115.7548634	5.055489196	0.461487827	0.324260586	0.456514838
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN
45	0.1	495	10	5.7	2.9	2.8
187	0.2	540	38	5.7	3	2.7
40	0.1	340	5	5.9	3.3	2.6
38	0.1	127	8	5.8	3.5	2.3
171	0.1	83	12	6.3	3.4	2.9
52	0.1	397	1	5.7	3.5	2.2
45	0.1	495	10	5.7	2.9	2.8
45	0.4	166	1	5.9	3.2	2.7
40	0.1	158	5	5.6	3	2.6
39	0.1	74	9	6.1	3.4	2.7
60	0.1	147	6	5.9	3.3	2.6
27	0.1	218	2	5.9	3.4	2.5
45	0.4	135	1	5.9	3.3	2.6
30	0.1	223	5	6.1	3	3.1
36	0.1	70	1	5.7	3.3	2.4
34	0.1	107	1	6.3	3.4	2.9
72	0.1	232	2	6.2	3.2	3
21	0.1	138	2	6.7	3.7	3
42	0.2	162	1	6	3.5	2.5
37	0.1	102	1	6.1	3.7	2.4
19	0.1	127	8	6.5	3.4	3.1
36	0.1	76	2	5.9	2.9	3
49	0.1	170	5	5.6	3.2	2.4
30	0.1	99	1	6.6	3.2	3.4
33	0.1	71	4	6.4	3.6	2.8
50.92	0.132	198.08	5.64	6.008	3.288	2.72
40.23050252	0.085244746	141.9477369	7.576718727	0.314801525	0.238607069	0.287228132
45.41666667	0.123809524	142	5.208333333	5.995833333	3.170833333	2.825
30.47724264	0.062488094	94.32967167	4.107037086	0.422702657	0.30571252	0.462742298
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN
33	0.1	307	1	5.9	3.3	2.6
65	0.2	397	8	5.6	3	2.6
41	0.2	84	1	6.2	3.5	2.7
38	0.1	243	1	6.6	3.4	3.2
29	0.1	119	4	6.1	3.4	2.7
26	0.2	186	1	5.6	3.4	2.2
22	0.1	79	1	6.5	3.6	2.9
26	0.1	106	3	5.4	2.9	2.5
27	0.1	181	1	5.2	2.6	2.6
134	0.1	274	7	6.2	2.9	3.3
30	0.1	148	1	6.3	2.8	3.5
36	0.2	97	4	6.9	3.5	3.4
63	0.1	78	10	6.8	3.8	3
22	0.1	84	2	6.2	3.2	3
54	0.1	62	1	6.6	3.1	3.5
34	0.1	103	1	6.7	3.4	3.3
30	0.2	41	1	6.1	3.2	2.9
55	0.1	92	4	6.4	3.5	2.9
25	0.1	96	3	6.6	3.4	3.2

25	0.1	202	4	6.3	3	3.3
41	0.1	560	2	6.7	3.4	3.3
.	.	.	.	.	.	.
47	0.2	67	2	6.4	2.9	3.5
42.25	0.133333333	185.0833333	2.75	6.041666667	3.191666667	2.85
24.41989289	0.045584231	126.8928716	2.550358603	0.460495686	0.300072141	0.373499886
57.04166667	0.129166667	176.1666667	5.083333333	6.041666667	3.058333333	2.983333333
84.56048988	0.055003294	115.7548634	5.055489196	0.461487827	0.324260586	0.456514838

ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN
42	0.1	197	5	6	3.1	2.9
61	0.1	237	1	6	2.7	3.3
27	0.2	142	1	6.8	3.4	3.4
45	0.1	137	1	6.2	3.2	3
38	0.2	245	3	5.9	2.7	3.2
38	0.1	66	2	5.5	3	2.5
44	0.1	109	6	6.2	2.8	3.4
31	0.1	45	2	6.1	3.3	2.8
41	0.1	73	1	6.4	3.4	3
26	0.2	169	1	6.3	3.2	3.1
45	0.1	106	5	6.1	3.3	2.8
42	0.1	68	1	5.2	2.6	2.6
40	0.125	132.8333333	2.416666667	6.058333333	3.058333333	3
9.390517461	0.045226702	67.56254117	1.880924982	0.410007391	0.29063671	0.295419578
45.41666667	0.123809524	142	5.208333333	5.995833333	3.170833333	2.825
30.47724264	0.062488094	94.32967167	4.107037086	0.422702657	0.30571252	0.462742298
ALT (SGPT)	T. BILIRUBIN	ALK PHOS	GGT	TOTAL PROTEIN	ALBUMIN	GLOBULIN
69	0.1	68	2	5.1	1.9	3.2
24	0.1	34	5	6.6	2.9	3.7
49	0.1	92	4	6.7	3	3.7
48	0.1	71	2	5.5	2.4	3.1
63	0.1	82	1	6.7	3.3	3.4
56	0.1	107	9	5.9	2.9	3
.	.	.	.	.	.	.
47	0.1	253	1	6	2.8	3.2
39	0.1	82	1	6.6	2.5	4.1
24	0.1	76	1	6.2	3.2	3
47	0.2	65	2	6.4	3	3.4
26	0.1	172	4	5.7	2.6	3.1
44.72727273	0.109090909	100.1818182	2.909090909	6.127272727	2.772727273	3.354545455
15.27148263	0.030151134	61.13561676	2.46797672	0.536825687	0.400227208	0.350324525
57.04166667	0.129166667	176.1666667	5.083333333	6.041666667	3.058333333	2.983333333
84.56048988	0.055003294	115.7548634	5.055489196	0.461487827	0.324260586	0.456514838

Appendix B. Blood Biochemistry Parameters for Individual Animals

A/G RATIO	CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM
1.1	264	19	1.1	17	4	8.9
1.1	224	14	1	14	4.3	9.6
1.1	230	17	1.1	15	4.9	10.4
1.3	214	14	1	14	4.1	10.1
1.4	217	11	1	11	3	10.2
1.3	309	20	0.9	22	3.6	9.4
0.8	192	11	0.8	14	4.5	9.5
1	205	13	1.1	12	3.9	9.4
1.4	186	12	0.9	13	4	9.2
1.1	233	14	1	14	3.2	9.1
0.9	386	12	0.8	15	3.7	10
1.6	191	16	1.2	13	4	9.3
0.6	141	28	1.5	19	4.8	9.9
1.5	175	7	1	7	3	9.7
0.9	194	13	1.1	12	5.2	10.3
1.1	134	14	0.9	16	4.4	9.5
1	348	15	0.8	19	5.1	9.9
1.2	205	12	1.3	9	3.9	9.3
1.4	222	16	1	16	5	9.6
1	200	11	0.8	14	4.2	9.2
1.1	195	13	1	13	4.7	9
1.5	247	6	0.7	9	5.4	11.2
1	335	14	0.9	16	4.9	9.3
1.4	214	11	0.8	14	4.2	9.1
1.158333333	227.5416667	13.875	0.9875	14.08333333	4.25	9.629166667
0.246570682	61.62649005	4.366995784	0.180126767	3.335144436	0.671144383	0.53849966
A/G RATIO	CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM
1.3	182	10	0.9	11	4.1	9.3
1.3	344	11	0.9	12	4.2	9.4
1.2	202	16	0.8	20	4.8	10.1
0.6	147	17	1.1	15	4.3	9.7
0.9	173	13	1.2	11	3.7	9.3
1.4	236	9	1	9	2.5	9.8
1.7	146	13	0.9	14	3.8	9.5
1.1	284	6	0.6	10	3.6	9.3
1.1	212	13	1.1	12	4.4	9.5
1.1	205	20	1.2	17	5.4	9.7
0.9	345	12	1	12	4.5	9.9
1	239	12	0.9	13	3.6	9.5
0.9	166	10	0.9	11	4.6	9.4
1.2	162	12	0.9	13	3.3	9.7
0.9	281	12	0.7	17	5.3	9.1
0.8	181	11	0.6	18	4.6	9.8
1	273	15	1.1	14	4.6	9.7
1	208	12	0.9	13	3.7	9.6
1.2	162	10	1	10	3.9	9.2
1	155	12	0.9	13	5	9.7
0.8	283	7	0.9	8	3.4	9.5
1	242	9	0.7	13	4.3	9.8
0.9	411	7	0.6	12	4.6	8.5
0.8	284	15	1.1	14	4.5	9.2
1.045833333	230.125	11.83333333	0.9125	13	4.195833333	9.508333333
0.234018146	70.83926233	3.252646637	0.177696615	2.859005604	0.67080689	0.32825847

Appendix B. Blood Biochemistry Parameters for Individual Animals

A/G RATIO	CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM
1.1	270	15	1.1	14	3.2	9.3
1.3	302	17	1.1	15	3.4	10.3
1.5	216	10	0.8	13	3.2	9.7
1.6	217	14	1	14	3.6	10.3
1.5	208	10	0.9	11	3.5	10
1.4	372	16	0.9	18	3.9	9.6
1	224	8	0.7	11	4.1	10
1.1	207	11	0.9	12	3.3	9.3
1.3	187	14	0.9	16	5.2	9.2
1.2	223	9	0.7	13	3.9	10.1
1.2	258	7	0.5	14	4.7	10
1.4	173	15	1.1	14	2.9	8.8
1	344	14	0.5	28	2.7	9.3
1.7	191	10	0.5	20	3.1	8.8
1.3	198	11	0.6	18	5.6	9.5
1.1	154	13	0.5	26	3.6	10.2
1.2	286	14	0.5	28	3.8	10.6
1.3	228	16	1.2	13	3	10.1
1.7	175	11	0.6	18	4.2	9.3
1.1	222	9	0.5	18	2.9	10.1
1.2	212	15	0.7	21	4	9.6
1.5	279	8	0.5	16	4.9	10.3
0.9	392	13	0.5	26	5.2	10.6
1.4	183	10	0.5	20	4.6	9.5
1.291666667	238.375	12.08333333	0.7375	17.375	3.854166667	9.770833333
0.218526024	63.01608663	2.917960376	0.241034906	5.21546613	0.816130407	0.518760367
1.158333333	227.5416667	13.875	0.9875	14.08333333	4.25	9.629166667
0.246570682	61.62649005	4.366995784	0.180126767	3.335144436	0.671144383	0.53849966
A/G RATIO	CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM
1.3	156	10	0.9	11	4.1	9.5
1.6	296	7	0.7	10	3.8	9.7
1.7	168	13	0.7	19	3.4	10.5
1.1	138	9	0.8	11	3.5	9.9
1.1	174	11	0.9	12	3.6	9
1.8	221	12	1.1	11	2.7	10.9
1.7	168	13	0.7	19	4.5	10.8
1.1	250	9	0.7	13	4.4	11.1
1.1	212	8	0.9	9	8.7	10.1
1.2	154	18	1.2	15	3.4	9
0.9	278	10	0.6	17	4.4	9.5
0.9	239	10	0.6	17	4.2	9.6
0.9	183	9	0.5	18	3.7	9.5
1.2	201	14	0.5	28	2.9	9.7
0.8	272	12	0.8	15	4.2	8
0.9	152	12	0.5	24	3.2	9.6
1.1	252	13	0.8	16	3.7	9.5
1.3	141	11	0.5	22	3.3	9.9
1.2	188	13	0.6	22	3.8	9.4
1	187	19	0.5	38	5.5	9.8
0.9	284	7	0.5	14	3.8	8.7
0.9	193	9	0.5	18	4.9	10.1
.	.	.	.	.	.	.
0.8	164	16	0.5	32	3.7	9.2

Appendix B. Blood Biochemistry Parameters for Individual Animals

1.152173913	203.0869565	11.52173913	0.695652174	17.86956522	4.060869565	9.695652174
0.296754513	49.07500108	3.160402236	0.203331929	7.256998803	1.192300729	0.708698077
1.045833333	230.125	11.83333333	0.9125	13	4.195833333	9.508333333
0.234018146	70.83926233	3.252646637	0.177696615	2.859005604	0.67080689	0.32825847
A/G RATIO	CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM
1	310	19	0.8	24	3.1	8.4
1.1	291	18	0.7	26	5.4	8.8
1.3	252	15	0.7	21	3.4	9
1.5	187	12	0.7	17	3.7	9.2
1.2	215	20	0.9	22	3.4	9.8
1.6	310	19	0.7	27	2.9	9.3
1	310	19	0.8	24	3.1	8.4
1.2	201	10	0.7	14	3.9	9
1.2	206	19	0.6	32	5.5	9
1.3	251	13	0.6	22	4.1	9.1
1.3	229	8	0.5	16	3.1	8.8
1.4	171	14	0.8	18	5.8	9.1
1.3	224	14	0.9	16	3.7	9.5
1	285	9	0.9	10	2.7	9.7
1.4	215	8	0.7	11	4.8	9.4
1.2	207	10	0.8	13	4.3	9.6
1.1	139	9	0.9	10	3.5	9.8
1.2	338	12	0.7	17	4	10.2
1.4	250	19	1.4	14	4.3	10.1
1.5	188	12	0.8	15	4.3	10.2
1.1	226	8	0.9	9	3.4	9.7
1	213	12	0.9	13	4.3	9.4
1.3	232	6	0.6	10	4.5	9.9
0.9	298	12	0.8	15	4	10
1.3	203	9	0.7	13	4.7	10.2
1.232	238.04	13.04	0.78	17.16	3.996	9.424
0.179629248	50.32765972	4.353925432	0.170782513	6.101092798	0.817761171	0.539505947
1.158333333	227.5416667	13.875	0.9875	14.08333333	4.25	9.629166667
0.246570682	61.62649005	4.366995784	0.180126767	3.335144436	0.671144383	0.53849966
A/G RATIO	CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM
1.3	204	11	0.7	16	3.9	9.5
1.2	484	10	0.5	20	4.1	9.2
1.3	153	22	0.8	28	4	10.4
1.1	144	14	0.7	20	5.3	10.1
1.3	157	7	0.8	9	3.9	9.8
1.5	195	12	0.7	17	4.5	10.1
1.2	232	11	0.6	18	3.6	10.7
1.2	187	12	0.9	13	3.6	9.8
1	138	29	1.6	18	3.9	8.7
0.9	276	9	0.6	15	4	9.6
0.8	280	11	0.5	22	5.4	9.6
1	160	10	0.5	20	4.5	11.1
1.3	175	14	0.5	28	3.3	10.4
1.1	287	9	0.7	13	3.2	9.7
0.9	168	14	0.8	18	3.2	9.8
1	260	10	0.9	11	3.5	9.7
1.1	152	11	0.9	12	3.1	10
1.2	193	13	0.6	22	3.8	9.9
1.1	159	10	1	10	3.6	9.7

0.9	305	9	0.5	18	3.3	9.4
1	245	8	0.7	11	4.1	10.1
.	.	.	.	.	.	.
0.8	136	19	0.7	27	4.3	9.6
1.15	217.5	13.16666667	0.741666667	18	4.225	9.883333333
0.182574186	80.73450691	5.040313671	0.24406372	5.620771619	0.61589691	0.508648153
1.045833333	230.125	11.83333333	0.9125	13	4.195833333	9.508333333
0.234018146	70.83926233	3.252646637	0.177696615	2.859005604	0.67080689	0.32825847

A/G RATIO	CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM
1.1	251	15	1.2	13	4.7	9.8
0.8	154	16	0.7	23	3.8	8.8
1	284	6	0.5	12	4.3	10.4
1.1	236	11	0.8	14	4.8	9.8
0.8	290	11	0.6	18	4	8.5
1.2	227	7	0.6	12	3.9	9.5
0.8	269	12	0.6	20	6.1	9.3
1.2	227	12	0.7	17	4.9	9.5
1.1	199	12	0.8	15	5.6	10.3
1	204	11	0.7	16	3.9	9.4
1.2	302	8	0.6	13	5.2	10.1
1	209	15	0.9	17	5.8	8.9
1.025	237.6666667	11.33333333	0.725	15.83333333	4.75	9.525
0.154478595	43.55212829	3.14305391	0.186474468	3.37997669	0.797154029	0.594099777
1.158333333	227.5416667	13.875	0.9875	14.08333333	4.25	9.629166667
0.246570682	61.62649005	4.366995784	0.180126767	3.335144436	0.671144383	0.53849966
A/G RATIO	CHOLESTEROL	BUN	CREATININE	BUN/CREAT	PHOSPORUS	CALCIUM
0.6	236	10	0.6	17	4.9	8.8
0.8	218	12	0.7	17	5.4	10.3
0.8	289	12	0.9	13	5.2	9.9
0.8	303	11	0.6	18	5.6	9.5
1	202	8	0.6	13	3.9	9.4
1	192	14	0.6	23	3.7	8.9
.	.	.	.	.	.	.
0.9	222	9	0.6	15	4.6	8.9
0.6	147	21	0.9	23	4.8	8.7
1.1	171	6	0.7	9	4.5	9
0.9	192	15	0.9	17	3.8	9.2
0.8	307	7	0.5	14	3.3	8.6
0.845454545	225.3636364	11.36363636	0.690909091	16.27272727	4.518181818	9.2
0.157249079	53.65495826	4.249064068	0.144599761	4.197401794	0.754742581	0.531036722
1.045833333	230.125	11.83333333	0.9125	13	4.195833333	9.508333333
0.234018146	70.83926233	3.252646637	0.177696615	2.859005604	0.67080689	0.32825847

Appendix B. Blood Biochemistry Parameters for Individual Animals

CA/PO4	GLUCOSE	AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO
2.2	82	515	176	146	4.2	35
2.2	86	490	314	141	4.7	30
2.1	83	760	340	145	4.6	32
2.5	90	601	440	144	4.1	35
3.4	81	888	587	145	4.3	34
2.6	86	380	190	144	4.2	34
2.1	78	679	210	146	4.4	33
2.4	92	508	314	143	4.2	34
2.3	74	826	309	145	4.4	33
2.8	85	478	394	143	4.2	34
2.7	87	648	376	144	3.9	37
2.3	84	856	343	147	4.4	33
2.1	94	734	79	151	4.6	33
3.2	99	689	432	146	4	37
2	93	1211	475	145	4.6	32
2.2	81	577	257	144	4.2	34
1.9	76	571	280	142	4.5	32
2.4	76	580	170	144	4.5	32
1.9	91	822	207	147	4.8	31
2.2	87	647	617	149	4.6	32
1.9	89	686	123	148	4.4	34
2.1	114	542	509	143	4.5	32
1.9	75	505	209	141	4.1	34
2.2	92	760	74	144	4.2	34
2.316666667	86.45833333	664.7083333	309.375	144.875	4.358333333	33.375
0.39083708	8.831658304	177.1917358	149.4211476	2.383138231	0.232035729	1.663221076
CA/PO4	GLUCOSE	AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO
2.3	79	407	527	142	4.6	31
2.2	63	397	398	147	4.4	33
2.1	79	960	543	144	4.3	33
2.3	81	648	216	144	4.3	33
2.5	85	814	563	142	4.4	32
3.9	85	604	614	143	4.1	35
2.5	79	539	697	144	4.4	33
2.6	88	427	372	142	4.2	34
2.2	95	659	566	145	4.3	34
1.8	87	767	391	142	4.6	31
2.2	98	821	483	145	4.5	32
2.6	88	481	305	146	4.6	32
2	76	626	365	149	4.9	30
2.9	110	623	365	141	4.6	31
1.7	93	765	133	144	4.2	34
2.1	75	867	397	142	4.9	29
2.1	88	737	101	140	4.8	29
2.6	87	584	425	143	3.7	39
2.4	91	498	552	147	4.5	33
1.9	79	916	299	150	5	30
2.8	88	595	771	144	4.6	31
2.3	92	740	340	143	4.5	32
1.8	85	524	43	141	4.6	31
2	91	1056	125	143	4.1	35
2.325	85.91666667	668.9583333	399.625	143.875	4.4625	32.375
0.459914926	9.155262999	178.4487453	188.4418518	2.490198176	0.29312781	2.203011772

Appendix B. Blood Biochemistry Parameters for Individual Animals

CA/PO4	GLUCOSE	AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO
2.9	87	506	129	146	4.4	33
3	114	554	214	146	4.4	33
3	88	631	280	145	4.5	32
2.9	79	590	399	146	4.3	34
2.9	92	641	399	149	4.2	35
2.5	93	370	107	145	4.6	32
2.4	97	794	165	147	4.5	33
2.8	101	492	212	145	4.4	33
1.8	69	794	173	147	4.3	34
2.6	80	648	331	145	4.5	32
2.1	82	695	323	146	4.2	35
3	95	786	173	146	4.5	32
3.4	92	838	688	143	3.9	37
2.8	84	714	322	143	4.5	32
1.7	82	962	348	143	4.7	30
2.8	83	798	238	145	4.6	32
2.8	87	536	285	142	4.5	32
3.4	90	648	190	140	4.1	34
2.2	77	812	202	141	4.6	31
3.5	75	661	573	142	4.3	33
2.4	77	760	120	142	4.4	32
2.1	92	439	438	143	4.2	34
2	73	595	221	140	4.4	32
2.1	74	849	57	141	4.4	32
2.629166667	85.95833333	671.375	274.4583333	144.0833333	4.391666667	32.875
0.501718063	10.22562496	145.3166894	148.6630211	2.412227307	0.181579224	1.483606065
2.316666667	86.45833333	664.7083333	309.375	144.875	4.358333333	33.375
0.39083708	8.831658304	177.1917358	149.4211476	2.383138231	0.232035729	1.663221076
CA/PO4	GLUCOSE	AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO
2.3	81	479	505	147	4.6	32
2.6	78	347	319	148	4.5	33
3.1	99	767	452	145	4.9	30
2.8	93	724	164	146	4.5	32
2.5	89	834	432	145	4.8	30
4	90	608	579	147	4.5	33
2.4	72	548	566	147	4.5	33
2.5	91	530	308	147	5.1	29
1.2	85	657	461	145	4.6	32
2.6	79	622	313	145	4.8	30
2.2	86	761	266	145	4.5	32
2.3	59	547	203	147	4.8	31
2.6	77	606	410	143	4.7	30
3.3	98	620	338	144	4.9	29
1.9	86	638	114	154	4.8	32
3	79	1106	364	146	4.7	31
2.6	83	897	77	142	4.1	35
3	90	636	350	145	4.3	34
2.5	94	518	609	141	4.2	34
1.8	90	785	296	145	5.1	28
2.3	91	774	576	143	4.4	33
2.1	90	734	290	145	4.6	32
.	.	.	.	.	.	.
2.5	94	1108	151	141	4.4	32

2.526086957	85.82608696	688.9565217	354.0434783	145.3478261	4.62173913	31.60869565
0.555319229	9.083712567	182.0917547	153.4436462	2.690195932	0.259293659	1.777105165
2.325	85.91666667	668.9583333	399.625	143.875	4.4625	32.375
0.459914926	9.155262999	178.4487453	188.4418518	2.490198176	0.29312781	2.203011772
CA/PO4	GLUCOSE	AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO
2.7	66	484	130	145	4.5	32
1.6	71	466	212	144	4.4	33
2.6	90	628	331	144	4.4	33
2.5	86	515	427	144	4.3	33
2.9	106	646	404	146	4	37
3.2	73	407	156	147	4	37
2.7	66	484	130	145	4.5	32
2.3	91	417	261	146	4.4	33
1.6	70	811	109	145	4.5	32
2.2	81	569	325	146	4.2	35
2.8	82	606	268	146	3.9	37
1.6	95	901	278	144	4.3	33
2.6	97	805	258	147	4.2	35
3.6	104	749	537	147	4.4	33
2	104	695	323	147	4.3	34
2.2	93	1007	260	147	4.3	34
2.8	93	669	184	137	3.6	38
2.6	97	563	283	147	4.6	32
2.3	94	692	165	147	4.3	34
2.4	98	1010	215	147	4.6	32
2.9	97	626	448	140	4	35
2.2	102	712	99	144	4.6	31
2.2	94	456	341	145	4.8	30
2.5	88	481	135	146	4.2	35
2.2	88	845	56	145	4.3	34
2.448	89.04	649.76	253.4	145.12	4.304	33.76
0.477074418	11.94947698	174.3660613	120.7676833	2.333095226	0.260576284	2.005824851
2.316666667	86.45833333	664.7083333	309.375	144.875	4.358333333	33.375
0.39083708	8.831658304	177.1917358	149.4211476	2.383138231	0.232035729	1.663221076
CA/PO4	GLUCOSE	AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO
2.4	80	508	514	146	4.6	32
2.2	78	351	313	146	4.5	32
2.6	88	940	450	147	4.5	33
1.9	91	1042	387	145	4.6	32
2.5	88	759	447	147	4.5	33
2.2	88	508	521	149	4.4	34
3	111	532	373	145	4.5	32
2.7	87	653	427	148	4.5	33
2.2	77	597	276	147	4.8	31
2.4	85	740	285	146	4.7	31
1.8	93	427	199	147	4.8	31
2.5	82	604	448	146	5	29
3.2	92	545	377	147	4.7	31
3	96	794	122	148	4.7	31
3.1	93	1066	250	146	4.4	33
2.8	97	791	72	145	4.6	32
3.2	102	634	320	147	3.9	38
2.6	87	491	565	145	5.1	28
2.7	103	915	282	139	4.2	33

2.8	89	815	551	145	4.4	33
2.5	107	723	241	146	4.8	30
.	.	.	.	.	.	.
2.2	90	1277	210	147	4.3	34
2.366666667	87.33333333	638.4166667	386.6666667	146.5833333	4.616666667	31.91666667
0.39326032	8.885028022	230.0703073	136.3212719	1.925000703	0.262563124	1.997834325
2.325	85.91666667	668.9583333	399.625	143.875	4.4625	32.375
0.459914926	9.155262999	178.4487453	188.4418518	2.490198176	0.29312781	2.203011772

CA/PO4	GLUCOSE	AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO
2.1	102	694	227	143	4.1	35
2.3	93	871	153	144	4.7	31
2.4	117	501	250	143	4.9	29
2	101	1164	287	143	4.5	32
2.1	88	459	595	143	4.6	31
2.4	99	702	317	143	4.3	33
1.5	85	511	145	144	4.6	31
1.9	91	906	73	144	4.6	31
1.8	98	1053	252	145	4.8	30
2.4	96	655	535	146	4.3	34
1.9	89	424	402	145	4.2	35
1.5	86	777	106	147	4.4	33
2.025	95.41666667	726.4166667	278.5	144.1666667	4.5	32.08333333
0.322278818	8.938764744	237.5597373	163.0663557	1.337115847	0.244948974	1.928651594
2.316666667	86.45833333	664.7083333	309.375	144.875	4.358333333	33.375
0.39083708	8.831658304	177.1917358	149.4211476	2.383138231	0.232035729	1.663221076
CA/PO4	GLUCOSE	AMYLASE	LIPASE	SODIUM	POTASSIUM	NA/K RATIO
4.8	94	651	332	142	5.2	27
1.9	77	788	207	143	5	29
1.9	100	852	72	146	5.1	29
1.7	95	727	150	143	4.5	32
2.4	76	597	475	143	4.9	29
2.4	95	550	259	143	4.7	30
.	.	.	.	.	.	.
1.9	98	695	280	141	4.7	30
1.8	91	1228	170	142	4.3	33
2	88	932	290	144	4.7	31
2.4	98	728	600	143	4.3	33
2.6	90	818	497	142	4.6	31
2.345454545	91.09090909	778.7272727	302.9090909	142.9090909	4.727272727	30.36363636
0.867598567	8.067893721	186.0779895	162.0428675	1.300349603	0.300302877	1.858640755
2.325	85.91666667	668.9583333	399.625	143.875	4.4625	32.375
0.459914926	9.155262999	178.4487453	188.4418518	2.490198176	0.29312781	2.203011772

Appendix B. Blood Biochemistry Parameters for Individual Animals

CHLORIDE	CPK	TRIGLYCERIDE	OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM
115	101	66	303	9.7	.
106	172	216	292	9.8	.
109	119	300	301	10.6	.
116	105	147	298	.	.
109	125	82	298	.	.
112	143	71	300	9.6	.
111	251	68	300	10	.
112	90	123	296	10	.
109	76	143	298	9.4	.
110	105	80	296	9.4	.
107	80	109	297	10.1	.
112	138	102	304	.	.
119	135	30	317	11	.
112	138	62	300	10	.
111	94	80	300	10.9	.
112	117	25	298	9.9	.
109	123	101	294	10.5	.
109	243	106	297	9.7	.
113	66	98	305	.	.
118	93	90	307	9.7	.
121	89	28	306	9.7	.
106	105	633	294	.	.
105	247	117	291	9.6	.
111	115	64	297	9.3	.
111.4166667	127.9166667	122.5416667	299.5416667	9.942105263	#DIV/0!
4.074487609	51.85424109	123.8944078	5.563695727	0.489121423	#DIV/0!
CHLORIDE	CPK	TRIGLYCERIDE	OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM
110	110	125	292	9.6	.
113	112	127	301	.	.
107	401	268	298	10.3	.
109	244	133	299	10.6	.
107	168	94	293	9.9	.
108	106	127	294	.	.
109	118	108	297	9.6	.
107	302	155	291	.	.
111	170	228	300	9.8	.
112	94	404	296	10.2	.
112	189	136	300	10.3	.
111	68	76	301	9.9	.
116	159	70	306	10.1	.
109	110	78	292	10.1	.
113	172	59	297	9.9	.
106	144	438	292	10.6	.
114	244	96	290	10	.
110	174	36	295	9.9	.
116	121	94	303	9.6	.
116	197	44	309	10.1	.
112	112	151	295	10.5	.
108	148	316	294	10.1	.
104	226	130	289	9.7	.
110	238	65	296	9.6	.
110.4166667	171.9583333	148.25	296.6666667	10.01904762	#DIV/0!
3.242605859	75.50092379	107.398992	5.027460822	0.318777426	#DIV/0!

Appendix B. Blood Biochemistry Parameters for Individual Animals

CHLORIDE	CPK	TRIGLYCERIDE	OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM
113	102	48	302	9.9	1.8
107	143	232	304	.	1.9
113	115	107	298	.	1.6
112	88	53	301	.	1.5
114	64	60	307	.	1.6
113	151	48	301	9.7	1.9
113	112	72	302	10.1	1.2
113	90	42	300	9.7	1.6
110	152	43	303	9.4	1.6
107	81	75	298	10.2	1.6
109	70	104	299	.	1.9
113	164	34	303	9	1.9
112	270	71	296	9.7	1.9
107	174	79	294	8.9	2.1
110	118	56	294	9.6	2.3
111	104	38	299	10.4	1.6
111	109	59	294	10.7	2.1
107	139	103	291	10.2	2.1
106	131	120	290	.	1.8
110	124	87	291	.	2.1
109	252	74	294	9.9	2
103	106	162	294	.	2.4
105	162	270	289	10.9	2.3
107	184	61	290	.	2.2
109.7916667	133.5416667	87.41666667	297.25	9.886666667	1.875
3.064227921	50.78426594	58.80913613	5.109666883	0.559166046	0.296721213
111.4166667	127.9166667	122.5416667	299.5416667	9.942105263	#DIV/0!
4.074487609	51.85424109	123.8944078	5.563695727	0.489121423	#DIV/0!
CHLORIDE	CPK	TRIGLYCERIDE	OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM
112	103	97	302	9.7	1.8
110	112	296	303	.	1.5
111	177	153	300	.	1.5
111	129	73	300	.	1.5
110	100	88	299	9.3	1.7
110	52	73	303	.	1.6
106	104	142	303	.	1.8
109	106	258	302	.	1.6
111	231	124	298	10.2	1.7
110	102	155	301	9.6	1.7
110	71	100	298	10	1.6
106	217	89	301	10	1.8
107	83	126	293	9.7	.
108	201	106	298	.	.
114	180	72	317	8.9	1.1
109	176	196	301	10	1.4
105	283	51	293	.	2.2
111	166	41	299	10	2
106	117	59	292	9.6	.
111	140	74	302	10.1	2.4
109	104	187	294	9.3	.
110	165	115	298	10.5	2
.	.	.	.	.	.
108	176	88	293	9.7	.

109.3043478	143.2608696	120.1304348	299.5652174	9.773333333	1.716666667
2.20402076	56.54701776	64.2631694	5.185873179	0.406143301	0.301467001
110.4166667	171.9583333	148.25	296.6666667	10.01904762	#DIV/0!
3.242605859	75.50092379	107.398992	5.027460822	0.318777426	#DIV/0!
CHLORIDE	CPK	TRIGLYCERIDE	OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM
109	157	64	300	9	1.7
106	263	60	298	9.3	1.6
112	100	183	298	9.2	1.8
106	91	44	297	.	1.6
100	66	113	305	9.9	1.7
109	166	66	305	.	1.6
109	157	64	300	9	1.7
111	129	90	301	9.3	1.8
108	110	52	301	9.5	1.7
106	117	51	301	9.2	1.3
109	133	42	299	9	1.5
109	84	69	298	9.2	2
105	243	333	304	9.7	1.7
111	62	125	303	10.2	1.5
110	140	67	303	9.6	1.8
109	94	48	303	9.7	1.6
117	73	55	282	10.1	1.6
112	75	93	304	.	1.8
109	88	44	306	.	1.6
107	83	109	304	.	1.5
118	71	105	288	9.8	1.9
110	151	31	298	10	1.5
108	120	87	297	10.2	1.7
109	164	129	301	10.3	1.9
108	127	57	298	.	1.5
109.08	122.56	87.24	299.76	9.589473684	1.664
3.558089375	51.02865208	61.99615579	5.285830115	0.442084377	0.157797338
111.4166667	127.9166667	122.5416667	299.5416667	9.942105263	#DIV/0!
4.074487609	51.85424109	123.8944078	5.563695727	0.489121423	#DIV/0!
CHLORIDE	CPK	TRIGLYCERIDE	OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM
112	168	81	300	9.7	1.6
106	113	324	300	9.7	1.3
103	158	302	307	.	1.7
102	200	95	300	10.2	1.9
110	155	61	301	9.9	1.5
112	156	61	307	10.2	1.5
102	84	198	300	.	1.8
107	114	85	305	10.4	1.5
113	152	43	309	9.6	1.6
110	146	93	300	10.2	1.5
109	194	75	303	10.3	1.6
109	166	78	300	.	1.7
111	272	51	304	.	1.4
109	81	108	305	10	1.9
108	79	82	302	10.2	1.4
109	177	64	299	9.8	1.6
108	136	39	304	10.3	1.6
111	218	68	299	.	1.6
120	69	91	287	9.8	2

112	158	235	298	9.9	1.5
107	102	290	301	10.2	1.6
.	.	.	.	.	.
113	67	37	306	10.2	1.5
107.9166667	150.5	124.6666667	302.6666667	10.02222222	1.6
4.034751638	52.28429196	89.95377601	4.497474039	0.249852898	0.173142586
110.4166667	171.9583333	148.25	296.6666667	10.01904762	#DIV/0!
3.242605859	75.50092379	107.398992	5.027460822	0.318777426	#DIV/0!

CHLORIDE	CPK	TRIGLYCERIDE	OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM
110	97	88	297	10.2	1.6
107	86	83	299	9.6	1.4
104	146	79	295	10.5	1.7
113	110	112	296	10.1	1.6
106	71	170	295	9.3	1.5
113	136	70	294	10	1.6
103	295	242	297	10	1.8
112	89	174	297		1.7
110	110	234	300	10.4	1.6
110	122	299	301	9.7	1.7
109	114	216	298	10.3	2
111	171	98	304	9.8	1.6
109	128.9166667	155.4166667	297.75	9.990909091	1.65
3.330301652	59.15381849	77.82434573	2.864357773	0.364566991	0.150755672
111.4166667	127.9166667	122.5416667	299.5416667	9.942105263	.
4.074487609	51.85424109	123.8944078	5.563695727	0.489121423	.
CHLORIDE	CPK	TRIGLYCERIDE	OSMOLALITY- CALC	CORRECTED CA	MAGNESIUM
111	236	338	293	10.4	1.4
111	116	74	295	10.9	1.6
112	200	89	302	10.4	1.5
111	171	93	295	10.6	1.8
106	159	154	293	9.6	1.5
108	211	108	296	9.5	1.4
.	.	.	.	.	.
109	138	324	291	9.6	1.5
105	79	33	297	9.7	1.6
112	205	69	295	9.3	1.8
106	157	221	297	9.7	1.6
106	174	256	292	9.5	1.5
108.8181818	167.8181818	159.9090909	295.0909091	9.927272727	1.563636364
2.713602101	45.42866536	107.5141428	3.015113446	0.540538452	0.136181697
110.4166667	171.9583333	148.25	296.6666667	10.01904762	.
3.242605859	75.50092379	107.398992	5.027460822	0.318777426	.

Appendix B. Blood Biochemistry Parameters for Individual Animals

WBC	RBC	HGB	PCV	MCV	MCH	MCHC
5.7	5.7	13.2	43	76	23.2	31
5.2	7.3	17.9	54	73	24.4	33
7.5	6.8	16.1	51	75	23.6	31
5.6	7.4	18	54	73	24.3	33
6.2	6.1	14.6	47	77	24.2	32
11.7	6.4	16.3	51	80	25.7	32
7.1	6.2	14.5	46	74	23.2	31
5.8	7.3	16.6	54	74	22.7	31
6.4	7.9	18.3	56	71	23.3	33
6.2	7	16.7	51	73	23.8	33
7.4	5.8	13.2	41	71	22.7	32
6.9	6.6	16.6	53	80	25.2	32
7.7	5.8	13.6	43	74	23.5	32
6.9	7.6	17.2	54	72	22.7	32
5.6	7.6	17.2	54	72	22.7	32
7.7	6.8	15.8	52	77	23.4	31
14.8	5.3	12.4	41	77	23.4	30
7.6	7.9	18.5	60	76	23.4	31
6.1	6.4	16	50	78	25	32
6.7	6.7	14	46	68	20.7	30
6.2	6.8	14.9	48	71	22.1	31
7.7	7.2	16.9	51	71	23.5	33
8.5	5.6	13.1	43	77	23.6	31
5.2	6.4	14.9	46	72	23.3	32
7.183333333	6.691666667	15.6875	49.54166667	74.25	23.48333333	31.70833333
2.119816782	0.744788658	1.809230559	5.098841869	3.082207001	1.035738202	0.907896119
WBC	RBC	HGB	PCV	MCV	MCH	MCHC
6.3	6.9	15.7	52	75	22.7	30
8.1	7.1	16.8	55	78	23.7	30
6.9	7.4	17.7	58	78	23.8	31
7.3	7.4	15.4	52	71	20.9	30
9.1	6.3	15.5	49	77	24.6	32
7.4	7	16.6	55	78	23.7	30
7.6	7.5	18.5	57	76	24.7	33
7.3	7.6	18	54	71	23.6	33
8.4	6.4	15.9	47	73	24.8	34
7.9	7.3	17.4	55	76	23.9	31
7.6	7.1	16.5	53	75	23.2	31
5.2	6.2	13.6	45	73	22	30
11.7	6.5	14.9	47	73	23	32
6.3	6.4	13.7	43	68	21.6	32
6.6	6.9	16.1	52	75	23.3	31
10.9	7.1	15.8	49	69	22.2	32
6.6	6.9	15.9	52	76	23.2	31
6.7	6.6	14.6	48	73	22.2	30
6	7.2	17.3	53	74	23.9	32
6.4	8.3	19.6	61	73	23.5	32
7.4	6.7	16.2	52	78	24.1	31
10.7	6.8	15.2	49	72	22.4	31
22.2	6.7	12.5	43	53	19	30
5.9	7.5	16.7	55	73	22.2	31
8.1875	6.991666667	16.0875	51.5	73.25	23.00833333	31.25
3.394408766	0.490710812	1.604697588	4.57782937	5.126656678	1.31311666	1.113162358

Appendix B. Blood Biochemistry Parameters for Individual Animals

WBC	RBC	HGB	PCV	MCV	MCH	MCHC
4.8	6.8	15.3	47	69	22.5	33
6.7	7.1	16.8	51	71	23.7	33
7.6	5.8	13.9	42	72	24	33
5.8	7.4	17.1	52	70	22.9	33
7.6	6	14.2	43	72	23.9	33
10.4	5.7	15	44	77	26.2	34
7.9	6.4	14.8	45	70	23.1	33
6.2	6.7	15.2	46	69	22.6	33
5.8	6.8	15.2	46	68	22.5	33
8.6	7.2	16.6	50	70	23.2	33
7.7	7	15.5	46	66	22.3	34
7.2	6.3	16	47	74	25.2	34
6.1	7.2	15.8	48	67	21.8	33
7.2	7.4	16.3	50	67	22	33
5.9	8	17.4	54	68	21.9	32
11.7	7.5	17.5	53	70	23.4	33
9.6	6.4	14.3	44	69	22.2	33
6.6	7.7	17.8	54	71	23.2	33
6.3	6.1	14.9	45	74	24.4	33
4.6	8.8	17.1	53	61	19.5	32
8.3	6.9	15.5	48	70	22.5	32
5.3	8.4	18.1	56	67	21.6	32
10.3	6.5	14.7	45	70	22.6	32
4.7	6.3	14.5	44	70	23	33
7.204166667	6.933333333	15.8125	48.04166667	69.66666667	22.925	32.91666667
1.884831711	0.788871878	1.232993	4.005204947	3.116110545	1.310907419	0.583592075
7.183333333	6.691666667	15.6875	49.54166667	74.25	23.48333333	31.70833333
2.119816782	0.744788658	1.809230559	5.098841869	3.082207001	1.035738202	0.907896119
WBC	RBC	HGB	PCV	MCV	MCH	MCHC
7.3	6.8	15.4	47	69	22.8	33
8.8	7	16.8	50	72	24.2	34
6	7.9	18.4	55	69	23.3	34
9.6	6.9	16.3	49	70	23.6	34
8.6	6.1	14.9	44	72	24.5	34
5.5	6.7	15.4	46	69	23	34
7.7	8.1	19.3	56	69	23.7	34
9.4	7	16.4	50	71	23.6	33
9.9	6.9	16.3	49	71	23.8	34
6	6.2	14.6	44	71	23.7	33
10.1	5.6	13.6	40	73	24.4	34
8.8	6.6	13.9	43	64	21	33
10.4	7.1	16.3	49	70	22.9	33
6.6	6.4	13.9	42	67	21.9	33
6.6	6.6	14.7	45	68	22.4	33
9.6	7.6	16.4	51	67	21.6	32
7.2	6.6	15.6	47	72	23.8	33
10.6	8.1	16.6	51	64	20.7	33
6	7.2	16.6	50	69	23	33
7.7	7.2	16.1	49	69	22.4	33
11.2	7.3	16.9	52	71	23.2	33
10.1	6.5	14	44	68	21.6	32
.	.	.	.	.	.	.
6.5	6.7	14.7	46	68	22	32

8.269565217	6.917391304	15.78695652	47.7826087	69.26086957	22.9173913	33.2173913
1.757693422	0.61618487	1.420404663	4.010854837	2.33972398	1.061582084	0.671262158
8.1875	6.991666667	16.0875	51.5	73.25	23.00833333	31.25
3.394408766	0.490710812	1.604697588	4.57782937	5.126656678	1.31311666	1.113162358
WBC	RBC	HGB	PCV	MCV	MCH	MCHC
4.8	6.9	15.2	47	68	21.9	32
4.4	6.8	15.3	47	70	22.6	32
6	6.2	14.4	44	71	23.2	33
3.7	7	15.4	48	69	22.1	32
8.1	6.2	14.4	44	70	23.2	33
8.4	6.2	15.7	47	76	25.3	34
4.8	6.9	15.2	47	68	21.9	32
4.4	5.8	12.6	39	68	22	32
5	6.6	14.6	45	67	22.1	33
6.6	6.7	15.1	46	68	22.4	33
4.7	6.3	13.8	42	66	21.8	33
8	6.2	14.9	46	74	24.1	33
6.5	7.5	17.4	50	67	23.2	35
8.1	7.2	16.6	49	69	23	34
6.6	7.3	16.4	50	68	22.6	33
6.6	7.7	17.3	52	67	22.4	34
10.9	7	16.6	49	70	23.8	34
6.7	6.3	14.5	44	70	22.9	33
9.7	7.7	18.3	54	71	23.9	34
7.6	5.7	13.7	41	73	24.2	33
4.9	7.3	13.4	42	58	18.4	32
9.3	6.9	15.3	45	66	22.1	34
8.5	6.3	14.7	44	69	23.3	34
7.9	6.6	15.1	45	68	22.9	34
5.6	6.3	14.5	43	69	23.2	34
6.712	6.704	15.216	46	68.8	22.74	33.2
1.893920097	0.554887376	1.318357564	3.488074923	3.278719262	1.249333155	0.866025404
7.183333333	6.691666667	15.6875	49.54166667	74.25	23.48333333	31.70833333
2.119816782	0.744788658	1.809230559	5.098841869	3.082207001	1.035738202	0.907896119
WBC	RBC	HGB	PCV	MCV	MCH	MCHC
6.7	6.5	14.6	44	69	22.6	33
8.6	6.8	16.1	49	71	23.6	33
6.2	8.4	19.3	57	68	22.9	34
8.2	7.8	17.8	53	67	22.9	34
10.1	6.6	15.7	47	72	23.8	33
6.2	7.8	16.2	55	70	20.8	30
6.1	7.1	16.6	48	68	23.4	34
10.8	6.5	15.2	45	69	23.5	34
8	5.7	13	40	70	22.9	33
7.4	6	13.8	42	71	23.2	33
8.2	5.4	12	37	68	22.2	33
9.4	6.9	16.1	49	70	23.3	35
6.8	6.3	13.7	41	66	21.9	33
14.1	6.6	15.5	46	70	23.5	34
8.5	7	15.4	47	68	22.1	33
8.5	7.4	17.3	51	70	23.5	34
5.8	8.1	16.9	51	63	20.9	33
5.4	6.7	16.2	48	73	24.3	34
6.9	8.2	18.2	56	68	22.2	33

8.6	6.6	15.8	47	72	23.9	33
7.9	6.7	14.9	46	68	22.4	33
.	.	.	.	.	.	.
8.6	6.2	14	42	68	22.6	33
7.991666667	6.791666667	15.53333333	47.16666667	69.41666667	22.925	33.25
1.944890075	0.796379796	1.730881966	5.213410945	2.214180978	0.905299453	0.935125058
8.1875	6.991666667	16.0875	51.5	73.25	23.00833333	31.25
3.394408766	0.490710812	1.604697588	4.57782937	5.126656678	1.31311666	1.113162358

WBC	RBC	HGB	PCV	MCV	MCH	MCHC
9.3	7.2	17.2	50	69	23.8	35
10.8	6.1	14.2	42	70	23.5	34
7.7	6.3	13.8	43	69	22	32
5.7	8.4	18.2	56	67	21.8	33
6	7.1	15.7	48	68	22.1	33
7.3	7.3	15.7	49	66	21.3	32
10.1	6.6	14.9	46	69	22.5	32
6.4	6.1	14.4	42	69	23.6	34
6.5	6.1	14.9	44	72	24.4	34
9.1	8.3	14.9	48	58	18	31
5.9	7.8	17.1	54	69	21.8	32
9.6	7	15.1	47	67	21.6	32
7.866666667	7.025	15.50833333	47.41666667	67.75	22.2	32.83333333
1.823749052	0.825860433	1.343305511	4.461111426	3.441062204	1.660229995	1.193416283
7.183333333	6.691666667	15.6875	49.54166667	74.25	23.48333333	31.70833333
2.119816782	0.744788658	1.809230559	5.098841869	3.082207001	1.035738202	0.907896119
WBC	RBC	HGB	PCV	MCV	MCH	MCHC
8.9	5.7	11.9	38	67	20.8	31
11.4	6.7	13.8	43	64	20.6	32
7.9	6.7	15.2	47	70	22.9	33
4.9	6.3	13.9	43	69	22.3	32
8.6	7.6	16.7	51	68	22	33
7.6	6.1	12.9	40	65	21.1	32
.	.	.	.	.	.	.
8	6.5	14.1	44	68	21.7	32
7.7	6.3	14	43	68	22.4	33
5.6	8	17.3	54	68	21.7	32
7.6	7.4	17.1	52	70	23.1	33
7	6.3	14.1	44	70	22.5	32
7.745454545	6.690909091	14.63636364	45.36363636	67.90909091	21.91818182	32.27272727
1.697270536	0.697788715	1.745435606	5.065031634	1.972538743	0.826823055	0.646669791
8.1875	6.991666667	16.0875	51.5	73.25	23.00833333	31.25
3.394408766	0.490710812	1.604697588	4.57782937	5.126656678	1.31311666	1.113162358

NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE	EOSINOPHILS
66	3762	25	1425	4	228	5
68	3536	22	1144	5	260	5
59	4425	30	2250	3	225	8
63	3528	22	1232	5	280	10
75	4650	14	868	3	186	8
75	8775	16	1872	3	351	6
68	4828	20	1420	6	426	6
63	3654	28	1624	2	116	7
63	4032	30	1920	2	128	5
57	3534	29	1798	6	372	8
61	4514	29	2146	5	370	5
68	4692	23	1587	4	276	5
52	4004	32	2464	3	231	13
63	4347	25	1725	2	138	10
51	2856	34	1904	7	392	8
62	4774	27	2079	3	231	8
77	11396	16	2368	3	444	4
59	4484	29	2204	3	228	9
61	3721	28	1708	4	244	7
58	3886	33	2211	3	201	6
54	3348	37	2294	4	248	5
68	5236	22	1694	5	385	5
59	5015	24	2040	5	425	12
51	2652	36	1872	5	260	8
62.54166667	4568.708333	26.29166667	1827.041667	3.958333333	276.875	7.208333333
7.210976938	1862.17485	6.181875097	407.7332332	1.36665783	96.8613139	2.358687304
NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE	EOSINOPHILS
59	3717	25	1575	3	189	13
75	6075	21	1701	3	243	1
71	4899	22	1518	4	276	3
67	4891	23	1679	3	219	7
62	5642	27	2457	3	273	8
67	4958	24	1776	3	222	6
61	4636	22	1672	4	304	13
64	4672	22	1606	6	438	8
69	5796	22	1848	5	420	4
65	5135	28	2212	4	316	3
64	4864	24	1824	4	304	8
66	3432	22	1144	5	260	7
61	7137	29	3393	3	351	7
60	3780	31	1953	5	315	4
66	4356	24	1584	4	264	6
57	6213	36	3924	2	218	5
56	3696	35	2310	5	330	4
68	4556	24	1608	2	134	6
59	3540	34	2040	3	180	4
58	3712	26	1664	3	192	13
55	4070	34	2516	6	444	5
56	5992	33	3531	5	535	6
85	18870	9	1998	5	1110	1
77	4543	17	1003	2	118	4
64.5	5382.583333	25.58333333	2022.333333	3.833333333	318.9583333	6.083333333
7.295025106	3029.980398	6.233964937	714.234322	1.203858531	196.3555956	3.308968927

Appendix B. Blood Biochemistry Parameters for Individual Animals

NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE	EOSINOPHILS
78	3744	16	768	6	288	.
80	5360	11	737	9	603	.
75	5700	19	1444	6	456	.
80	4640	12	696	7	406	.
88	6688	8	608	4	304	.
82	8528	8	832	6	624	4
81	6399	11	869	8	632	.
79	4898	16	992	5	310	.
73	4234	20	1160	7	406	.
69	5934	21	1806	9	774	1
65	5005	25	1925	9	693	1
72	5184	16	1152	7	504	5
72	4392	20	1220	7	427	.
73	5256	22	1584	5	360	.
63	3717	27	1593	10	590	.
75	8775	15	1755	9	1053	.
71	6816	18	1728	6	576	5
79	5214	15	990	6	396	.
74	4662	17	1071	8	504	.
74	3404	20	920	6	276	.
70	5810	17	1411	12	996	.
75	3975	18	954	7	371	.
76	7828	15	1545	9	927	.
70	3290	21	987	6	282	2
74.75	5393.875	17	1197.791667	7.25	531.5833333	3
5.589197656	1508.318912	4.791296457	392.7379898	1.847442415	225.4473652	1.897366596
62.54166667	4568.708333	26.29166667	1827.041667	3.958333333	276.875	7.208333333
7.210976938	1862.17485	6.181875097	407.7332332	1.36665783	96.8613139	2.358687304
NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE	EOSINOPHILS
74	5402	18	1314	8	584	.
61	5368	36	3168	2	176	1
76	4560	16	960	8	480	.
79	7584	12	1152	8	768	1
66	5676	28	2408	6	516	.
71	3905	23	1265	6	330	.
70	5390	22	1694	8	616	.
69	6486	25	2350	6	564	.
84	8316	11	1089	5	495	.
71	4260	22	1320	7	420	.
77	7777	16	1616	7	707	.
77	6776	13	1144	9	792	1
69	7176	22	2288	8	832	.
74	4884	17	1122	9	594	.
74	4884	17	1122	9	594	.
67	6432	20	1920	12	1152	.
73	5256	18	1296	8	576	.
73	7738	12	1272	7	742	8
77	4620	16	960	5	300	2
69	5313	20	1540	11	847	.
77	8624	17	1904	6	672	.
71	7171	21	2121	8	808	.
.	.	.	.	.	.	.
71	4615	21	1365	6	390	2

72.60869565	6009.26087	19.26086957	1582.173913	7.347826087	606.7391304	2.5
4.915089294	1394.425141	5.626377741	567.792668	2.080399779	215.0850186	2.738612788
64.5	5382.583333	25.58333333	2022.333333	3.833333333	318.9583333	6.083333333
7.295025106	3029.980398	6.233964937	714.234322	1.203858531	196.3555956	3.308968927
NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE	EOSINOPHILS
79	3792	15	720	6	288	.
82	3608	11	484	7	308	.
76	4560	17	1020	6	360	1
76	2812	16	592	8	296	.
86	6966	7	567	4	324	3
85	7140	8	672	7	588	.
79	3792	15	720	6	288	.
85	3740	12	528	2	88	1
70	3500	21	1050	8	400	.
74	4884	17	1122	9	594	.
70	3290	20	940	9	423	.
76	6080	16	1280	6	480	2
77	5005	14	910	5	325	4
77	6237	16	1296	7	567	.
74	4884	21	1386	5	330	.
75	4950	17	1122	6	396	2
57	6213	32	3488	9	981	2
63	4221	30	2010	7	469	.
79	7663	12	1164	6	582	3
78	5928	15	1140	7	532	.
69	3381	25	1225	6	294	.
73	6789	17	1581	8	744	2
84	7140	14	1190	2	170	.
68	5372	22	1738	8	632	2
72	4032	18	1008	9	504	1
75.36	5039.16	17.12	1158.12	6.52	438.52	2.090909091
6.873136111	1432.348208	5.861740356	614.6625985	1.917463602	190.4287093	0.943879807
62.54166667	4568.708333	26.29166667	1827.041667	3.958333333	276.875	7.208333333
7.210976938	1862.17485	6.181875097	407.7332332	1.36665783	96.8613139	2.358687304
NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE	EOSINOPHILS
76	5092	14	938	5	335	5
88	7568	6	516	6	516	.
74	4588	17	1054	6	372	3
72	5904	17	1394	7	574	4
79	7979	15	1515	6	606	.
77	4774	15	930	8	496	.
76	4636	19	1159	5	305	.
82	8856	12	1296	3	324	3
81	6480	12	960	7	560	.
71	5254	21	1554	6	444	2
82	6724	10	820	6	492	1
70	6580	24	2256	6	564	.
78	5304	16	1088	6	408	.
91	12831	7	987	2	282	.
67	5695	24	2040	7	595	2
74	6290	17	1445	9	795	.
72	4176	21	1218	7	406	.
71	3834	19	1026	8	432	2
67	4623	22	1518	7	483	4

74	6364	18	1548	7	602	.
68	5372	23	1817	8	632	.
.	.	.	.	.	.	.
42	3612	23	1978	8	688	27
77.33333333	6202.916667	15.16666667	1199.333333	5.916666667	465.6666667	3
9.560017751	2021.614361	5.22730096	434.3781468	1.619684112	132.573242	7.717944459
64.5	5382.583333	25.58333333	2022.333333	3.833333333	318.9583333	6.083333333
7.295025106	3029.980398	6.233964937	714.234322	1.203858531	196.3555956	3.308968927

NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE	EOSINOPHILS
63	5859	19	1767	2	186	16
79	8532	14	1512	7	756	.
81	6237	15	1155	4	308	.
69	3933	18	1026	12	684	.
72	4320	19	1140	9	540	.
73	5329	21	1533	6	438	.
85	8585	10	1010	5	505	.
64	4096	19	1216	10	640	6
73	4745	15	975	7	455	4
80	7280	15	1365	5	455	.
76	4484	17	1003	6	354	1
70	6720	17	1632	12	1152	.
73.75	5843.333333	16.58333333	1277.833333	7.083333333	539.4166667	6.75
6.757151094	1653.513963	2.968266508	275.1141361	3.117642855	250.7709779	6.5
62.54166667	4568.708333	26.29166667	1827.041667	3.958333333	276.875	7.208333333
7.210976938	1862.17485	6.181875097	407.7332332	1.36665783	96.8613139	2.358687304
NEUTROPHILS	ABSOLUTE	LYMPHOCYTES	ABSOLUTE	MONOCYTES	ABSOLUTE	EOSINOPHILS
69	6141	23	2047	6	534	2
79	9006	6	684	10	1140	5
73	5767	17	1343	10	790	.
72	3528	21	1029	5	245	2
72	6192	25	2150	3	258	.
78	5928	15	1140	7	532	.
.	.	.	.	.	.	.
66	5280	24	1920	9	720	.
75	5775	15	115	9	693	.
66	3696	23	1288	11	616	.
83	6308	15	1140	2	152	.
71	4970	25	1750	4	280	.
73.09090909	5690.090909	19	1327.818182	6.909090909	541.8181818	3
5.337687616	1457.386802	5.949789912	613.9390553	3.113022501	294.7611976	1.732050808
64.5	5382.583333	25.58333333	2022.333333	3.833333333	318.9583333	6.083333333
7.295025106	3029.980398	6.233964937	714.234322	1.203858531	196.3555956	3.308968927

Appendix B. Blood Biochemistry Parameters for Individual Animals

ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
285	0	0	80	1.68	1.69	0.8
260	0	0	113	1.9	1.71	0.8
600	0	0	109	1.91	1.28	1.2
560	0	0	69	1.08	0.92	1.1
496	0	0	94	1.97	1.33	0.8
702	0	0	113	1.67	1.82	1.8
426	0	0	91	1.97	1.08	0.9
406	0	0	77	1.45	1.5	1
320	0	0	97	1.68	1.27	0.8
496	0	0	60	1.78	1.15	0.8
370	0	0	129	2.21	1.02	1.2
345	0	0	92	1.41	1.64	0.8
1001	0	0	99	1.98	1.77	1
690	0	0	86	1.83	1.47	0.6
448	0	0	117	1.32	1.2	0.7
616	0	0	88	1.18	1.42	0.9
592	0	0	123	2.61	1.56	1.2
684	0	0	101	1.4	1.17	0.9
427	0	0	62	1.55	1.19	0.6
402	0	0	47	1.62	1.06	0.6
310	0	0	100	1.42	1.48	0.7
385	0	0	95	1.89	1.94	0.7
1020	0	0	92	1.15	1.26	2
416	0	0	108	2.26	1.23	1.1
510.7083333	0	0	93.41666667	1.705	1.381666667	0.958333333
201.131685	0	0	20.28689161	0.373944892	0.276541791	0.346305553
ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
819	0	0	75	1.99	1.6	0.8
81	0	0	95	1.11	1.12	.
207	0	0	121	1.96	1.35	.
511	0	0	87	1.92	1.14	1.1
728	0	0	84	1.5	0.79	1.1
444	0	0	110	2.08	2.11	1.1
988	0	0	70	1.41	1.33	0.8
584	0	0	104	1.43	1.15	0.8
336	0	0	79	1.09	1.01	0.8
237	0	0	92	1.3	1.14	1
608	0	0	115	0.79	0.72	1.2
364	0	0	105	1.45	1.23	1.3
819	0	0	57	1.09	0.89	0.7
252	0	0	66	1.44	1.78	0.6
396	0	0	139	1.66	1.65	1.3
545	0	0	78	1.55	1.08	1.2
264	0	0	108	1.06	0.96	1.3
402	0	0	73	2.55	1.71	1
240	0	0	65	2.62	1.76	0.7
832	0	0	83	1.49	0.98	0.6
370	0	0	84	1.08	0.87	0.8
642	0	0	128	1.2	1.19	1.2
222	0	0	119	1.5	0.85	1.2
236	0	0	109	1.9	1.09	1.1
463.625	0	0	93.58333333	1.54875	1.229166667	0.986363636
243.288666	0	0	21.96621253	0.464573788	0.363041699	0.235625721

Appendix B. Blood Biochemistry Parameters for Individual Animals

ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	74	1.25	0.89	1
.	.	.	116	0.77	1.14	1.2
.	.	.	94	1.38	1.04	1
.	1	58	79	1.26	0.8	0.8
.	.	.	82	0.87	1.2	1
416	.	.	116	1.99	1.37	1.8
.	.	.	83	1.13	1.05	1.2
.	.	0	73	1.21	0.83	2.2
.	.	.	92	1.29	0.99	0.9
86	.	.	93	2.02	1.27	0.9
77	.	.	112	1.49	1.24	1.1
360	.	.	64	0.99	0.89	1
.	1	61	130	1.42	0.84	1.6
.	.	.	108	1.23	1.6	0.8
.	.	.	105	0.72	0.94	1
.	1	117	102	1.23	0.75	0.9
480	.	.	108	1.39	1.01	1
.	.	.	140	1.82	1.58	0.9
.	1	63	104	1.18	1.23	0.8
.	.	.	94	1.35	1.09	1
.	1	83	100	0.99	1.24	1.1
.	.	.	134	1.38	1.71	0.9
.	.	.	139	0.7	0.7	2
94	1	47	113	0.98	1.19	1
252.1666667	1	61.28571429	102.2916667	1.251666667	1.107916667	1.129166667
186.3807036	0	35.47165199	20.83992649	0.349218589	0.270409981	0.379334849
510.7083333	0	0	93.41666667	1.705	1.381666667	0.958333333
201.131685	0	0	20.28689161	0.373944892	0.276541791	0.346305553
ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	60	1.66	1.42	0.9
88	.	.	95	0.78	1.02	1.2
.	.	.	89	1.34	1.39	4
96	.	.	75	1.27	1.19	1.1
.	.	.	71	1.23	1.15	1
.	.	.	76	1.25	1.33	1.1
.	.	.	110	1.61	1.56	0.8
.	.	.	78	1.29	1.25	1.3
.	.	.	58	0.78	0.81	1
.	.	.	68	1.27	1.05	0.9
.	.	.	91	0.69	0.67	1.2
88	.	.	118	2.15	1.59	1.5
.	1	104	73	0.9	0.7	1
.	.	.	102	0.75	0.8	1.1
.	.	.	144	1.85	1.36	0.9
.	1	96	112	1.25	1.37	0.8
.	1	72	134	1.36	1.12	0.9
848	.	.	78	1.35	1.42	.
120	.	.	122	2.05	1.34	1.1
.	.	.	91	1.22	1.48	0.7
.	.	.	121	1.54	1.19	1.2
.	.	.	132	1.5	1.45	0.8
.	.	.	.	.	.	.
130	.	.	110	1.75	1.05	1

228.3333333	1	90.66666667	96	1.340869565	1.204782609	1.159090909
304.0695096	0	16.653328	25.02725787	0.397502579	0.267817264	0.661642322
463.625	0	0	93.58333333	1.54875	1.229166667	0.986363636
243.288666	0	0	21.96621253	0.464573788	0.363041699	0.235625721
ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
.	.	.	120	1.8	1.38	1
.	.	.	131	2.06	1.31	1
60	.	.	110	1.51	1.4	0.8
.	.	.	84	0.69	0.89	0.7
243	83	1.29	83	1.29	1.14	1.1
.	.	.	153	2.89	2.27	1.6
.	.	.	120	1.8	1.38	1
44	.	.	80	1.08	1.22	2.1
.	1	50	105	1.87	1.55	1
.	.	.	101	1.16	1.25	1.1
.	1	47	108	1.45	1.22	1.1
160	0	0	114	2.11	2.01	0.7
260	0	0	91	2.02	1.58	1
.	.	.	118	2.7	1.57	4.5
.	.	.	139	2.93	2.19	0.6
132	.	.	95	1.33	0.82	0.9
218	.	.	54	0.6	0.7	1.1
.	.	.	99	2.21	1.65	1.1
291	.	.	112	1.31	1.1	0.7
.	.	.	107	1.15	0.95	0.6
.	.	.	74	0.77	1.14	1.1
186	0	0	118	1.93	1.43	1.2
.	.	.	103	1.92	1.62	1.1
158	.	.	111	1.21	0.82	1.8
56	.	.	105	1.6	1.41	1.1
164.3636364	14.16666667	16.38166667	105.4	1.6556	1.36	1.2
85.40523722	33.72486719	24.90184766	21.00396788	0.631447279	0.400811676	0.769740216
510.7083333	0	0	93.41666667	1.705	1.381666667	0.958333333
201.131685	0	0	20.28689161	0.373944892	0.276541791	0.346305553
ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
335	.	.	98	2.32	1.71	0.8
.	.	.	113	0.78	1.17	1.5
186	0	0	88	2.21	1.24	2.9
328	0	0	75	1.59	1.3	0.7
.	.	.	91	1.5	1.43	.
.	.	.	115	1.68	1.62	0.8
.	.		79	1.99	1.22	1
324	0	0	60	1.2	0.87	1
.	.	.	66	1.68	1.49	0.8
148	.	.	85	0.56	0.82	1.1
82	1	82	112	2.22	1.88	1.8
.	.	.	67	1.05	1.05	1
.	.	.	90	2.58	1.49	0.8
.	.	.	93	1.47	1.23	1.2
170	.	.	67	1.01	0.93	1.2
.	.	.	107	0.78	0.82	1.2
.	.	.	60	1.31	0.88	1.2
108	.	.	76	1.04	1.47	0.9
276	.	.	70	1.2	1.17	0.9

.	1	86	135	1.83	1.39	1.2
.	1	79	94	1.5	1.19	1.1
.	.	.	.	.	.	.
2322	.	.	52	1.22	0.92	0.9
233.8333333	0.25	20.5	87.41666667	1.565	1.316666667	1.218181818
672.0986121	0.547722558	45.15048911	21.32174672	0.54114677	0.299340183	0.478091444
463.625	0	0	93.58333333	1.54875	1.229166667	0.986363636
243.288666	0	0	21.96621253	0.464573788	0.363041699	0.235625721

ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
1488	.	.	101	1.38	0.72	1
.	0	0	88	1.24	0.84	0.84
.	.	.	127	2.57	1.42	1.1
.	1	57	103	1.38	0.53	1
.	.	.	131	2.73	1.69	1.2
.	.	.	92	1.9	0.73	0.9
.	.	.	104	0.91	1.02	5.9
384	1	64	81	1.22	0.52	0.9
260	1	65	100	1.37	0.58	0.9
.	.	.	96	1.58	1.27	0.8
59	.	.	108	2	0.93	1.2
.	1	96	85	1.39	0.74	0.9
547.75	0.8	56.4	101.3333333	1.639166667	0.915833333	1.386666667
640.9786138	0.447213595	34.93279262	15.28120255	0.555443448	0.371470257	1.427377336
510.7083333	0	0	93.41666667	1.705	1.381666667	0.958333333
201.131685	0	0	20.28689161	0.373944892	0.276541791	0.346305553
ABSOLUTE	BASOPHILS	ABSOLUTE	T3 (RIA)	T4 (RIA)	FREE T4 (RIA)	T3AA
178	.	.	62	1.01	0.29	1
570	0	0	74	1.93	0.59	0.9
.	.	.	96	1.08	0.38	1.1
98	0	0	87	0.96	0.47	1.2
.	.	.	92	0.85	0.85	0.8
.	0	0	67	1.32	1.04	0.7
.	.	.	.	.	.	.
.	1	80	94	1.64	0.55	1.1
.	1	77	65	1.42	0.94	0.7
.	.	.	80	1.62	0.53	0.7
.	.	.	105	1.47	1.4	0.7
.	.	.	131	1.7	1.37	1
282	0.4	31.4	86.63636364	1.363636364	0.764545455	0.9
252.6024545	0.547722558	43.00930132	20.34833029	0.349950646	0.384144859	0.18973666
463.625	0	0	93.58333333	1.54875	1.229166667	0.986363636
243.288666	0	0	21.96621253	0.464573788	0.363041699	0.235625721

T4AA	FREE T3	TSH	QUANT. PLATELET
0.8	2.9	0.05	211
0.8	5.2	0.22	293
0.8	3	0.18	330
0.9	2.4	0.05	246
0.8	3.5	0.05	165*
0.8	4.7	0.45	391
1.1	1.9	0.05	258
0.9	2.5	0.05	200
0.8	3.4	0.05	313
0.8	2.7	0.05	337
1	2.4	0.15	347
0.9	3.2	0.05	253
0.9	2.7	<.10	317
0.8	1.9	<0.10	318
0.9	2.9	<0.10	243
0.8	3.5	<0.10	302
0.9	3.8	0.17	477
0.8	2.9	<0.10	191
0.8	3	0.3	364
0.9	1.4	0.16	227
0.8	2.3	<.10	268
0.7	3.6	0.18	360
0.9	3.4	2.77	NA
0.8	3.7	0.76	439
0.85	3.0375	0.318888889	303.8636364
0.083405766	0.852011686	0.638139657	74.92999619
T4AA	FREE T3	TSH	QUANT. PLATELET
0.9	2	0.05	356
0.8	2.7	0.8	306
0.8	4.3	0.5	310
1.2	3.6	0.23	277
1.1	2.4	0.2	312
0.9	3.4	0.2	322
0.8	3.3	0.05	179
0.9	3.1	0.05	363
0.8	2.2	0.05	222
0.9	2.2	0.05	380
0.9	2.3	0.05	246
0.9	3.5	0.05	283
0.9	2.4	2.27	484
0.8	2.7	<0.10	353
0.8	3.4	<0.10	476
1.1	2.9	<0.10	408
0.9	3.4	<0.10	258
0.8	2.9	<0.10	480
0.9	2.7	<0.10	276
0.9	2.9	0.28	257
0.9	3	0.24	293
0.8	4.1	0.4	190
0.9	2.6	0.27	NA
0.9	3.3	0.22	257
0.895833333	2.970833333	0.331111111	316.8695652
0.10417029	0.593793381	0.522278818	85.83139092

Appendix B. Blood Biochemistry Parameters for Individual Animals

T4AA	FREE T3	TSH	QUANT. PLATELET
0.9	1.6	0.24	207
0.8	3.2	0.26	287
0.8	1.9	0.21	194
0.8	1.9	0.25	247
0.9	3	0.1	160
0.7	3.9	0.22	427
1	2.4	1.15	107
0.9	1.8	0.33	148
0.8	1.4	0.11	209
0.8	3.7	<.10	282
0.8	4	0.16	313
0.8	1.7	0.18	230
1.1	3.3	0.28	357
0.6	3.5	0.16	386
0.7	2.5	0.29	308
0.9	3.4	0.38	435
1.2	2.7	0.49	336
0.7	4.1	0.52	180
0.7	2.9	1.16	296
1.2	2.4	0.32	218
0.8	3.2	0.43	323
0.7	3.7	0.49	172
1.1	3.4	3.55	420
0.8	3	1.07	347
0.854166667	2.858333333	0.536956522	274.5416667
0.161458479	0.818225368	0.726876473	93.70535652
0.85	3.0375	0.318888889	303.8636364
0.083405766	0.852011686	0.638139657	74.92999619
T4AA	FREE T3	TSH	QUANT. PLATELET
0.8	2.2	<.10	293
0.9	1.9	0.88	268
0.8	4.4	0.25	98
1	2.5	0.71	211
0.8	2.6	0.57	303
0.7	3.1	0.19	240
0.7	4.2	0.23	214
0.8	2.3	<.10	359
0.9	2.1	0.11	137
0.7	2.3	0.13	251
0.9	2.6	<.10	245
1	3.8	0.13	247
1.2	1.7	3.62	.
1.1	2.2	0.9	464
1	3.5	0.25	491
1	3.9	0.25	362
0.8	4	<.1	389
1.1	2.2	0.2	332
1	2.8	0.13	309
0.9	2.6	0.46	.
1	3.5	0.58	276
0.9	3.6	0.27	121
.	.	.	.
1.4	3.1	0.15	330

0.930434783	2.917391304	0.526842105	282.8571429
0.169047553	0.796385114	0.791159856	100.6679123
0.895833333	2.970833333	0.331111111	316.8695652
0.10417029	0.593793381	0.522278818	85.83139092
T4AA	FREE T3	TSH	QUANT. PLATELET
0.8	3	0.37	240
0.6	2.9	0.36	317
0.6	2.8	0.37	352
0.7	1.9	0.33	276
0.6	4.7	0.14	231
0.5	4.6	0.68	125
0.8	3	0.37	240
0.8	3.1	0.32	44
0.8	3	<.10	267
0.6	3.3	0.64	335
0.6	2.1	0.19	350
0.8	3.2	0.14	256
0.6	5.3	0.4	306
1.1	8.4	0.62	314
0.4	4	0.21	374
0.8	2	0.18	291
1.2	3.3	0.51	231
1.1	4.8	0.21	251
0.4	2.8	0.19	158
0.4	3.2	0.67	208
1.1	2.3	0.5	175
0.9	3.3	0.23	264
0.6	3.1	0.39	181
1.1	2.8	5.24	321
0.7	3.6	1.57	351
0.744	3.46	0.617916667	258.32
0.234662879	1.341951316	1.028354444	79.24472643
0.85	3.0375	0.318888889	303.8636364
0.083405766	0.852011686	0.638139657	74.92999619
T4AA	FREE T3	TSH	QUANT. PLATELET
0.6	.	0.13	433
0.6	6.9	1.22	369
0.6	5.5	0.25	358
0.7	5.1	0.42	405
.	.	0.44	365
.	.	0.14	361
0.6	4.9	0.17	410
0.6	4.4	0.15	280
0.6	3.6	0.14	368
0.8	5.8	0.12	304
0.7	6.6	0.14	484
0.9	2.2	3.7	475
0.8	2.8	1.09	277
1	3.7	1.3	401
1.1	3.2	0.62	175
0.8	3.6	0.18	393
0.9	3.2	0.38	238
0.7	2.8	0.12	341
0.9	3.1	0.56	357

0.9	3.4	0.54	251
1.1	4.5	0.51	135
.	.	.	.
0.9	1.4	0.11	298
0.67	5	0.585	384.3333333
0.168272961	1.468260697	0.78862599	88.57975104
0.895833333	2.970833333	0.331111111	316.8695652
0.10417029	0.593793381	0.522278818	85.83139092

T4AA	FREE T3	TSH	QUANT. PLATELET
0.8	6.2	0.17	293
0.8	4.6	0.15	403
0.8	5.5	0.11	286
0.8	6	0.24	316
0.7	5.4	0.16	.
0.7	4.8	0.12	294
0.8	7.6	3.62	362
0.8	4.6	1.36	462
0.7	5.8	0.59	245
0.7	4.9	0.3	185
0.7	5.6	0.6	106
0.8	5.7	0.32	350
0.758333333	5.558333333	0.645	300.1818182
0.051492865	0.838243112	1.001058531	98.80264995
0.85	3.0375	0.318888889	303.8636364
0.083405766	0.852011686	0.638139657	74.92999619
T4AA	FREE T3	TSH	QUANT. PLATELET
0.9	4.3	0.54	254
0.9	5	0.33	469
1	4.5	<0.10	405
1	4.9	0.2	415
0.7	4.3	2.9	242
0.7	4.7	0.19	490
.	.	.	.
0.9	5.1	0.28	273
0.8	4.5	0.12	377
0.8	4.6	0.32	251
0.7	4.7	0.16	298
0.7	5.8	0.22	125
0.827272727	4.763636364	0.526	327.1818182
0.119087439	0.431909081	0.84255168	112.2281767
0.895833333	2.970833333	0.331111111	316.8695652
0.10417029	0.593793381	0.522278818	85.83139092

Appendix C. Age-Associated Increases in Oxidative Damage in the Prefrontal Cortex in a Canine Model of Human Brain Aging

E. Head[1*], J. Liu[2,3], N.W. Milgram[3], B.A. Muggenburg[4], B.N. Ames[2], C.W. Cotman[1]. [1]Institute for Brain Aging and Dementia, Univ. of California, Irvine, CA 92697; [2]Dept of Mol. Cell Biol., [3]Children's Hospital Oakland Research Institute, Oakland, CA 94609; [3]Dept. Psychol., U Toronto, Toronto, Canada; [4]Lovelace Respiratory Research Institute, Albuquerque, NM.

Progressive neuropathology and cognitive dysfunction are features common to both canine and human aging. In humans, age is accompanied by increased oxidative damage and β-amyloid (Aβ) deposition and these effects are exacerbated in Alzheimer's disease. In aged canines, Aβ accumulation occurs early in the prefrontal cortex and is associated with deficits in reversal learning ability. We previously reported that lipid peroxidation increased with age in canine. The present study examined Aβ immunoreactivity, and oxidative damage to proteins (protein carbonyls and glutamine synthetase) and to DNA/RNA (oxo8dG) in the prefrontal cortex of canines (1-17.8 yrs). Oxo8dG increased with age ($r=.412$ $p<.002$). In addition, aged canines with Aβ showed significantly higher oxo8dG than canines without Aβ ($t(40)=2.67$ $p<.011$). Protein carbonyls progressively increased as a function of age ($r=0.59$ $p<.008$) along with parallel decreases in glutamine synthetase activity ($r=.95$ $p<.001$). Thus, like humans, aged canines show progressive increases in oxidative damage to lipids, proteins, and nucleic acids that may result in a positive feedback cycle with Aβ to mutually promote progressive pathology. The combination of increased oxidative damage and Aβ accumulation may account for cognitive deficits on prefrontal tasks observed in aged canines. Supported by NIA AG12694 and U. S. Department of the Army, Contract No. DAMD17-98-1-8622.

Appendix D. The Effects of Experience and Antioxidants on Size Discrimination Learning in the Dog

J.T. Rick*[1], C.J. Ikeda-Douglas[2], H. Murphy[3], B. A. Muggenburg[3], S. Zicker[4], and N.W. Milgram[1,2]

1.Department of Psychology, University of Toronto, Toronto, ON, Canada; 2. Div. Life Sciences, University of Toronto at Scarborough, Scarborough, ON, Canada; 3. Lovelace Respiratory Research Institute, Albuquerque, NM, USA; 4. Hill's Pet Food and Nutrition, Topeka, KS, USA.

Free radicals such as O- are byproducts of cellular metabolism and are thought to play a role in neural degeneration and age-related cognitive impairment. Using a variety of visual tasks, we have examined age-related deficits in a canine model of cognition. Previously, we found cognitive impairments in old dogs to be dependent on task difficulty and previous experience (Milgram et al., 1994, Behav. Neurosci. 108: 57-68). In the present experiment, we studied the effects of environmental enrichment and an antioxidant-rich diet on the learning ability of aged beagles using a size discrimination reversal task (2 between- and 1 within-subjects factors). Using blocks differing only in size, animals were taught to approach one block over the other. On reaching the performance criterion, the reward contingency was reversed and the dogs were required to approach the previously unrewarded block. We found that there was a significant effect of nutrition, with animals on the enriched diet performing better than those on the control diet. The dogs in the control environment committed significantly more errors on the reversal, suggesting that enrichment improve an animal's ability to deal with changes in environmental contingencies. Supported by: Hill's Pet Nutrition and U. S. Department of the Army, Contract No. DAMD17-98-1-8622.

Appendix E. Measurements of Anatomic and Vascular Characteristics in the Brain of Aging Canine with or without Environmental Enrichment and Antioxidant Diet

M. Y. Su[1], E. Head[2], J. Wang[1], J. Y. Chiou[1], H. Yu[1], B. A. Muggenburg[3], C. W. Cotman[2], O. Nalcioglu[1],
[1]Research Imaging Center, University of California, Irvine Hall 164, Irvine, CA USA; [2]Institute for Brain Aging & Dementia, UC Irvine, ;
[3]Lovelace Respiratory Research Institute, Albuquerque, NM ;

Introduction

Deposition of beta-amyloid (A-beta) in brain tissue and cerebral blood vessels is the pathological hallmark of Alzheimer's disease. Evidences have mounted to support that the oxidative stress may lead to these pathological changes. Cellular dysfunction may be a consequence of free radical production resulting from normal cellular function that causes oxidative stress. Normally, cells synthesize antioxidant enzymes that are able to combat the damage caused by free radicals with antioxidant enzymes; the ability to do so effectively diminishes with age. The oxidative stress may also lead to the misprocessing of amyloid precursor protein (APP), which is cleaved to form A-beta. The deposition of A-beta in vessels may have consequences on vascular function. The damaged endothelium may cause disruption of the Blood-Brain-Barrier (BBB), also it may exhibit enhanced vasoconstriction leading to chronic cerebral hypoperfusion. The increased BBB permeability and the decreased vascular volume may be detected by dynamic contrast enhanced MRI.

In this study we measured the anatomic and vascular changes in the aging dog brain with 2 types of interventions, antioxidant diet and environmental enrichment, alone or in combination, compared to the control. It may be possible to reduce the levels of free radicals in the brain with antioxidants to prevent oxidative damage. Promoting neuron growth and improving the survival of existing neurons are other approaches to minimizing the functional impact of cell death. One potential mechanism to reduce apoptotic cell death and promote the survival of existing neurons is by upregulating neurotrophic factors through physical exercise. The anatomic and vascular characteristics in the dog brain before and 1 year after the initiation of intervention were measured. The changes were compared among 4 groups of dogs with or without the antioxidant diet and the environmental enrichment. The results were also correlated with the their cognitive behavioral performance.

Methods

The study was conducted on a group of 48 beagles (11 to 12 years old, from the animal facility at the LRRI, Albuquerque, NM). The dogs were tested for cognitive behavioral performance prior to the assignment into the 4 study groups to ensure that they had a similar baseline to start with. Before the group assignment the baseline MRI study was carried out. Then the dogs were separated into: Group-A with normal diet and no enriched environment; Group-B with normal diet and enriched environment; Group-C with antioxidant diet and no enriched environment; Group-D with both antioxidant diet and enriched environment. The antioxidant diet contained rich Vitamin-E and other nutrients. The enriched environment included regular exercises, companion, and toys to play with. The follow-up MRI was performed one year after the group assignment. Several cognitive tests were repeated after the treatment assignment to investigate the impact of these interventions on the cognitive performance.

The MRI experiments were performed on a GE Signa 1.5 T scanner with a linear head coil. The animal was anesthetized by inhalation of Isoflurane (1.5-2 %) through the experimental period. A set of 3D images across the whole brain were acquired using a SPGR pulse sequence to obtain the detailed anatomic images. The volumes of cerebrum, lateral ventricle, hippocampus and cerebellum were measured. Four slices from the frontal cortex, thalamus, hippocampus, and cerebellum were selected for the dynamic contrast enhancement study. A SE pulse sequence (with TR/TE= 117/14 ms) was applied to acquire T1-weighted images before and after injection of Gd-DTPA (0.15 mmol/kg). The enhancement kinetics of Gd-DTPA were measured from the brain tissue by manually drawing a region of interest to cover the brain tissue region. The signal enhancement in the T1-weighted images was proportional to the concentration of the contrast agent in the tissue, which is dependent on the blood volume and the leakage of agents into the interstitial brain tissue from the damaged blood-brain-barrier. We used the early enhancement in the enhancement kinetics (30-45 sec) as the vascular volume parameter. The residual enhancement at the tail of the curve (6.5-7.5 min) was used as the BBB permeability indicator. The volumetric changes and the vascular changes in each group of dogs were obtained and compared.

Results

The percent lateral ventricle volume (normalized to the total cerebrum volume) showed a significant increase in Groups A and B ($p < 0.0001$), and Group-C ($p < 0.003$), but it was not significant in the combined intervention Group-D ($p < 0.07$). The volume of hippocampus and cerebellum did not show significant changes. The dogs in the 4 groups had a comparable cognitive behavior to start with. After 1 year of intervention the dogs with antioxidant diet (Groups C and D) demonstrated a significantly better performance in a landmark discrimination learning task which tested the spatial attention. The number of errors that the dogs made before meeting the criterion was significant lower in dogs with the antioxidant diet than those with control diet ($p < 0.02$).

The enhancement kinetics from the brain tissues at the mid brain region (through thalamus) was measured. Figure 1a shows the early enhancement ratio (the enhancement in the mean signal intensity at 30-45 sec divided by the pre-contrast intensity) in the 4 groups. This vascular volume index was almost identical among the 4 groups. After one year, the parameter remained unchanged in all 4 groups. Figure 1b shows the late enhancement ratio (the mean enhancement at 6.5-7.5 min divided by the pre-contrast intensity), which reflects the BBB permeability. This parameter was significantly lower in Group-A. After one year, similar to the vascular volume index, they remained unchanged. Group-A still had a significantly lower BBB permeability index after 1 year. The results measured from the brain tissue through hippocampus region had a higher magnitude, but they revealed a very similar pattern as shown in Figure 1.

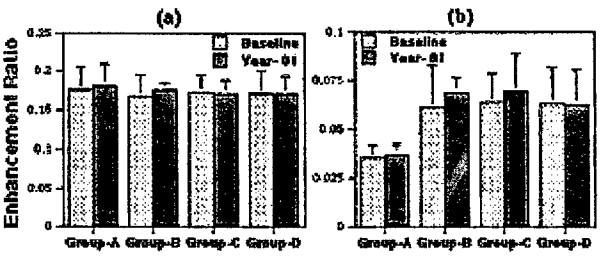


Figure 1

Figure 1: The mean enhancement ratio at an early time 30-45 sec (a) and a late time 6.5 to 7.5 min (b) from the brain tissue of dogs in the 4 groups. The parameters remained unchanged during this year.

Discussion

The canine model is well suited for the assessment of brain aging, including cognitive behavioral performance, neuroimaging, and the final neuropathology. One great advantage of using the animal model is that the decline of functional ability can happen within a short period of time, thus can be easily followed. We found that the ventricle size was increasing significantly, which was also a prominent feature in human aging. The combination of antioxidant diet and environmental enrichment seemed to slow down the ventricular enlargement. The vascular volume and BBB permeability parameters did not reveal much change during this year. We will continue to follow these dogs to investigate the long term effects of the different interventions. With the available information of anatomic and vascular parameters, the relationships between the anatomic and the vascular changes can be investigated.

Appendix F. Dietary enrichment counteracts age-associated cognitive dysfunction in canines

N.W. Milgram,[1*] S.C. Zicker,[2] E. Head,[3] B. A.Muggenburg,[4] H. Murphey,[4] C. Ikeda-Douglas[1], and C.W. Cotman[3]

[1]Life Science Division, University of Toronto at Scarborough, 1265 Military Trail, Scarborough Ontario, M1C 1A4, Canada

[2]Science & Technology Center, Hill's Pet Nutrition, Inc., P.O. Box 1658 Topeka, KS, 66601, USA

[3]Institute for Brain Aging and Dementia, University of California, 1226 Gillespie Neuroscience Research Facility, Irvine, CA, 92697-4540, USA

[4]Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM, 87108, USA

Correspondence should be addressed to:

N.W. Milgram, Ph.D.

Life Science Division, University of Toronto at Scarborough, 1265 Military Trail, Scarborough Ontario, M1C 1A4, Canada

Phone: (416) 287-7402

Fax: (416) 287-7642

E-mail: milgram@psych.utoronto.ca

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 counteracted 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.

Key Words: alpha-tocopherol, antioxidants, ascorbic acid, dogs, l-carnitine, lipoic acid, mitochondrial function, oddity discrimination, oxidative damage

1. Introduction

Improved nutrition, disease control, and applied biotechnology have prolonged life-span in humans. But the enhanced longevity comes at the cost of an increased prevalence of cognitive problems associated with aging, which range from age-related memory impairment and mild cognitive impairment, to the extreme neurodegenerative disorders typified by Alzheimer's disease [8, 24, 28]. 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-associated 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 associated with 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, 16, 31]. 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 [15]. 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 canines (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, 17, 18, 23, 26]. We have also found that aged canines 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 mouse models. Non-human primates are, in many respects, the ideal animal model. However, naïve aged primates are expensive, difficult to obtain, often difficult to cognitively test and are long lived. In addition, the major species of β-amyloid that accumulates in aged nonhuman primate brain is the shorter, more soluble species [11], 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 [33], 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 are still limited in their similarities to human brain aging and AD [22].

Previous research with these various models has implicated oxidative damage as a common factor in driving brain aging. This conclusion is supported by studies indicating antioxidants can delay age-related cognitive decline in humans [27, 35] and improve performance in aged rodents [6, 20]. These findings, however, remain controversial [25, 30, 32]. 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 has not previously been tested.

2. Methods

2.1.Animals. Aged and young beagles were acquired from two separate, closed colonies, with known pedigree data. Subjects were 23 aged beagles (11 males and 12 females) and 16 young beagles (5 males and 11 females). Eleven of the aged beagles and 7 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 7.8 to 12.1 years of age and the young beagles ranged in age from 2.5-4.5 years of age. The old animals were housed in USDA approved kennels with 2 dogs per kennel, hand-walked 2 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.

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: added dl-alpha-tocopherol E acetate, (160ppm vs 1550 ppm), added l-carnitine (0 ppm vs 265 ppm), added dl-alpha-lipoic acid (0 ppm vs 135 ppm), added ascorbic acid as Stay-C (0 ppm vs 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. 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 flavanoids 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 [26], the test apparatus was a 0.609 x 1.15 x 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 food. After completing these, all subjects were trained on an object discrimination learning task which was followed by an object reversal learning task [26], an object recognition memory task [5] and delayed non matching to position task [7]. The latter three of these tasks were used for determination of group allocation. 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.

Following 6 months on different foods the animals were tested on a series of oddity discrimination learning tasks. In each such task, 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 such tasks, of increasing difficulty based on similarity of positive and negative objects. We developed this test protocol in an attempt to provide a series of discrimination learning problems of sufficient difficulty to show age sensitivity. Monkeys require considerable training to learn a similar type of discrimination learning task, with the task difficulty depending on the choice of object pairs used [19]. Training on each oddity task commenced after initial preference testing. On the preference test session, the animals were presented with the two different objects for 10 successive trials, with both objects associated with reward. Preference was based on the number of time 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 a three-choice test. If no preference was determined, a coin toss decided the odd object.

On each testing trial, the animal was presented with the three objects, and allowed to respond to one. 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 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 25 cm away from the animal for a 2 second 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.

Animals had 40 days on each object pair to achieve a predetermined criterion level of accuracy. A two-stage criterion was used for passing the task. Briefly, animals had to have two consecutive days scoring 10 of 12 or 1 day of 11 or 12 correct responses followed by 3 days of testing that achieved at least a 70% average. After completing the task, the animal moved on to next problem, until 4 such tasks were completed.

2.7. Data Acquisition. Data acquisition was controlled using a customised program that controlled all timing and randomization procedures, indicating the location of the reward and the landmark, and stored data in data files. 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 signalled 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. 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 were analyzed as a repeated factorial with subsequent means separation done by LSD on SAS for windows.

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 adult dogs. No significant differences were observed between groups in the young dog category. Activity of alkaline phosphatase and creatine kinase was significantly higher in the old dog control 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. There were significant effects of age for several biochemistry parameters such as total protein, albumin, globulin, cholesterol, triglycerides, creatinine, calcium, sodium, red blood cell/ul, and T_3. None of the observed changes were interpreted to indicate significant health differences between the groups of animals.

3.3. Serum vitamin E

There was a significant effect of time ($p < 0.001$), group ($p < .001$) and a group by time interaction ($p<0.001$) over the entire period. There were no differences between concentrations of vitamin E in serum between dietary groups, within age groupings, at the beginning of the study. However, older dogs had higher concentrations of vitamin E in serum than younger dogs at the beginning of the study ($p<.03$). Following 3 and 6 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, compared to baseline, while dogs on the control food had no such significant effect (Fig. 1).

3.4. Pretraining cognitive results

The baseline performance of the two groups of aged animals was equivalent (Fig. 2). 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 ($p<=.0001$) and spatial memory tasks ($p<.0001$). In both the reversal learning and the DNMP task the old dogs showed significant impairment whereas the young dogs had more difficulty in solving the reward approach task.

3.5. Oddity discrimination results

The test protocol was highly sensitive to age, with old animals performing more poorly than the young (Fig.3). The age differences were most notable on the more difficult problems. As hypothesized, the aged animals on the enriched diet showed marked improvement relative to control animals (Fig. 4). The effect of dietary treatment also varied as a function of task, with treatment having no significant effect on the initial task, and a highly significant effect on the last two tasks. There was no significant effect of source of animals and, in fact, significance of dietary intervention was achieved from both sources of animals when analyzed as replicates.

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.

In general, the utility of any animal model in evaluating the effect of interventions of age-dependent cognition will depend on the extent to which the model reflects age-related cognitive dysfunction. The oddity discrimination learning task used in the present experiment can be solved by the animals' learning to associate one of three stimuli with reward, which involves visual discrimination learning. Visual discrimination learning is often insensitive to age in animal models [3, 29]. 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, but not others. First, the effect of age may depend on the difficulty of the discrimination. Aged non-human primates are deficient in acquiring some types of visual based discrimination learning, but not others [34]. Task difficulty was clearly a factor in the present experiment; the harder the problem, the greater the age-difference. Second, age differences in discrimination learning could relate to the strategies used in solving each oddity tasks. 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. The old animals learned the task progressively more slowly, with increasing task complexity, which is consistent with the use of an associative

strategy. This was not the case, however, with the young animals: their performance did not fall off significantly with increasing task difficulty, 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. This may be one of the mechanisms that the diet counteracts age-related cognitive dysfunction.

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.

The most important result of this study was clearly 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 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.

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 [13, 14, 21]. 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 [12]. 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.

5. Acknowledgements

This project was sponsored by funds provided by the National Institute of aging (Grant AG12694) and by the U. S. 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

6. References

[1] Adams, B., Chan, A., Callahan, H., Siwak, C., Tapp, D., Ikeda-Douglas, C., Head, E., Cotman, C.W., and Milgram, N.W., Spatial learning and memory in the dog as a model of cognitive aging. Behavioral Brain Research, 2000; 108(1):47-56.

[2] Ames, B.N., Shigenaga, M.K., and Hagen, T.M., Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Natl. Acad. Sci. USA, 1993; 90:7915-7922.

[3] Bartus, R.T., Dean, R.L., and Fleming, D.L., Aging in the Rhesus monkey: Effects on visual discrimination learning and reversal learning. J. Gerontol, 1979; 34:209-219.

[4] Beal, M.F., Aging, energy, and oxidative stress in neurodegenerative diseases. Annals of Neurology, 1995; 38(3):357-66.

[5] Callahan, H., Ikeda-Douglas, C., Head, E., Cotman, C.W., and Milgram, N.W., Development of a protocol for studying object recognition memory in the dog. Progress in Neuro-Psychopharmacology & Biological Psychiatry, 2000; In press.

[6] Carney, J.M., Starke-Reed, P.E., Oliver, C.N., Landum, R.W., Cheng, M.S., Wu, J.F., and Floyd, R.A., Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-α-phenylnitrone. Proc. Natl. Acad. Sci., 1991; 88:3633-3636.

[7] Chan, A.D.F., Nippak, P., Murphey, H., Ikeda-Douglas, C., Muggenburg, B.A., Head, E., Cotman, C.W., Milgram, N.W., Visuospatial Impairments in Aged Canines: The Role of Cognitive-Behavioral Flexibility. In submission.

[8] Crook, T.H., 3rd and G.J. Larrabee, Diagnosis, assessment and treatment of age-associated memory impairment. J Neural Transm Suppl, 1991; 33:1-6.

[9] Cummings, B.J., Head, E., Ruehl, W.W., Milgram, N.W., and Cotman, C.W., The canine as an animal model of human aging and dementia. Neurobiology of Aging, 1996; 17:259-268.

[10] Cummings, B.J., Satou, T., Head, E., Milgram, N.W., Cole, G.S., Savage, M.J., Podlisny, M.B., Selkoe, D.J., Siman, R., Greenberg, B.D., and Cotman, C.W., Diffuse plaques contain C-terminal ABeta 1-42 and not ABeta 1-40: Evidence from cats and dogs. Neurobiology of Aging, 1996; 17(4):653-659.

[11] Gearing, M., Tigges, J., Mori, H., and Mirra, S.S., Aβ40 is a major form of β-amyloid in nonhuman primates. Neurobiol Aging, 1996; 17:903-908.

[12] Hagen, T.I., RT; Lykkesfeldt, J; Liu, J; Wehr, CM; Vinarsky, V; Bartholomew, JC; Ames, AB., (R)-alpha-lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increased metabolic rate. FASEB, 1999; 13(2):411-418.

[13] Hagen, T.M., Ingersoll, R.T., Wehr, C.M., Lykkesfeldt, J., Vinarsky, V., Bartholomew, J.C., Song, M-H., and Ames, B.N., Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. Proc. Natl. Acad. Sci. USA, 1998; 95:9562-9566.

[14] Hager, K., *et al.*, Alpha-lipoic acid as a new treatment option for Azheimer type dementia. Arch Gerontol Geriatr, 2001; 32(3):275-282.

[15] Halliwell, B., Reactive oxygen species and the central nervous system. Journal of Neurochemistry, 1992; 59(5):1609-1623.

[16] Harman, D., Aging: a theory based on free radical and radiation chemistry. J Gerontol, 1956; 11:298-300.

[17] Head, E., Thornton, P.L., Tong, L., and Cotman, C.W., Initiation and propagation of molecular cascades in human brain aging: Insight from the canine model to promote successful aging. Progress in Neuro-Psychopharmacology & Biological Psychiatry, 2000; In press.

[18] Head, E., McCleary, R., Hahn, F.F, Milgram, N.W. and Cotman, C.W., Region-specific age at onset of β-amyloid in dogs. Neurobiology of Aging, 2000; 21(1):89-96.

[19] Iversen, S.D. and N.K. Humphrey, Ventral temporal lobe lesions and visual oddity performance. Brain Res, 1971; 30(2):253-63.

[20] Joseph, J.A., *et al.*, Age-related neurodegeneration and oxidative stress: putative nutritional intervention. Neurologic Clinics, 1998; 16(3):747-55.

[21] Joseph, J.A., *et al.*, Oxidative stress protection and vulnerability in aging: putative nutritional implications for intervention. Mech Ageing Dev, 2000; 116(2-3):141-53.

[22] Kawarabayashi, T., Younkin, L.H., Saido, T.C., Shoji, M., Ashe, K.H., Younkin, S.G., Age-dependent changes in brain, CSF, and plasma amyloid β protein in the Tg2576 transgenic mouse model of Alzheimer's disease. J. Neurosci., 2001; 21(2):372-381.

[23] Kiatipattanasakul, W., Nakamura, S., Kuroki, K., Nakayama, H., and Doi, K., Immunohistochemical detection of anti-oxidative stress enzymes in the dog brain. Neuropathology, 1997; 17:307-312.

[24] McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., and Stadlan, E.M., Clinical Diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA work group

under the auspices of Department of Health and Human Services task force on Alzheimer's disease. Neurology, 1984; 34:939-944.

[25] Mendelsohn, A.B., *et al.*, Use of antioxidant supplements and its association with cognitive function in a rural elderly cohort: the MoVIES Project. Monongahela Valley Independent Elders Survey. Am J Epidemiol, 1998; 148(1):38-44.

[26] Milgram, N.W., Head, E., Weiner, E. and Thomas, E., Cognitive functions and aging in the dog: Acquisition of nonspatial visual tasks. Behavioral Neuroscience, 1994; 108:57-68.

[27] Paleologos, M., Cumming, R.G., and Lazarus, R., Cohort study of vitamin C intake and cognitive impairment. American Journal of Epidemiology, 1998; 148:45-50.

[28] Petersen, R.C., Smith, G. E., Waring, S. C., Ivnik, R. J., Tangalos, E. G., Kokmen, E., Mild cognitive impairment: clinical characterization and outcome. Archives of Neurology, 1999; 56(3):303-8.

[29] Rapp, P.R., Visual discrimination and reversal learning in the aged monkey (*Macaca mulatta)*. Behavioral Neuroscience, 1990; 6:876-884.

[30] Sano, M., Ernesto, C., Thomas, R.G., Klauber, M.R., Schafer, K., Grundman, M., Woodbury, P., Growdon, J., Cotman, C.W., Pfeiffer, E., Schneider, L.S. and Thal, L.J., A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The New England Journal of Medicine, 1997; 336:1216-1222.

[31] Shigenaga, M.K., Hagen, T.M., Ames, B.N., Oxidative damage and mitochondrial decay in aging. Proc. Natl. Acad. Sci. USA, 1994; 91:10771-10778.

[32] Socci, D.J.C., B.M. and Arendash, G.W., Chronic antioxidant treatment improves the cognitive perfomance of aged rats. Brain Research, 1995; 693(1-2):88-94.

[33] Thomas, R.K., Investigating cognitive abilities in animals: unrealized potential. Brain Res Cogn Brain Res, 1996; 3(3-4):157-66.

[34] Voytko, M.L., Impairments in acquisition and reversals of two-choice discriminations by aged rhesus monkeys. Neurobiology of Aging, 1999; 20:617-627.

[35] Warsama Jama, J., Launer, L.J., Witteman, J.C.M., den Breeijen, J.H., Breteler, M.M.B., Grobbee, D.E., and Hofman, A., Dietary antioxidants and cognitive function in a population-based sample of older persons. American Journal of Epidemiology, 1996; 144(3):275-280.

7. Figure Legends

Fig. 1. Effectiveness of enriched food in raising concentration of vitamin E in serum. In the enriched diet groups, serum levels of Vitamin E were significantly increased at 3 and 6 months after the start of treatment (*).

Fig. 2. Baseline cognitive data for aged and young beagles. Note the significant effects of age for the reversal and DNMP tasks (*). A significant effect of treatment group was present for young dogs on the reversal learning task (a vs b).

Fig. 3. Effect of age on number of errors made in learning an oddity discrimination task in 23 old dog and 16 young dogs. The effect of age was significant at each level of oddity task (*).

Fig. 4. Effect of food on learning a series of oddity discrimination problems in groups of young and old dogs. Repeated measures analysis revealed a significant effect of diet ($p<.05$), age ($p<.0001$), and diet by age interaction ($p<.0031$). LS means of oddity tasks within the old dog group revealed a significant effect of diet for oddity 3(*) and 4 (*), and a marginally significant effect on oddity 2.

Figure 1.

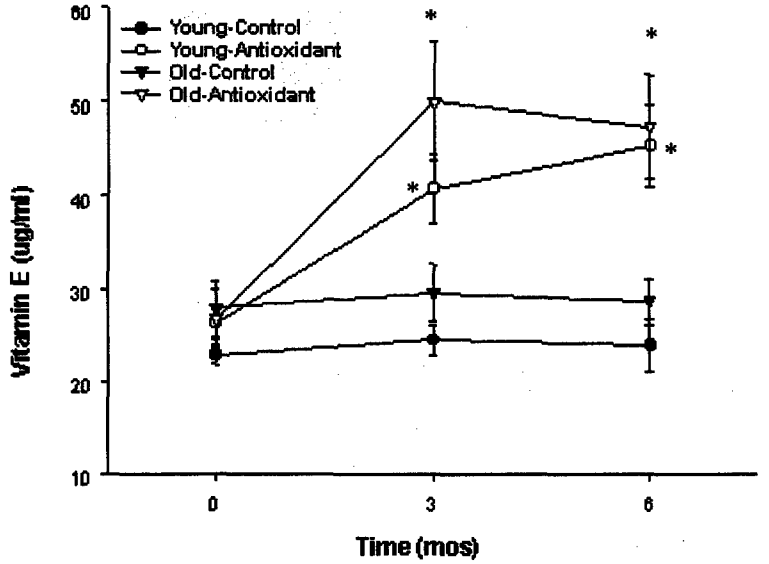


Figure 2.

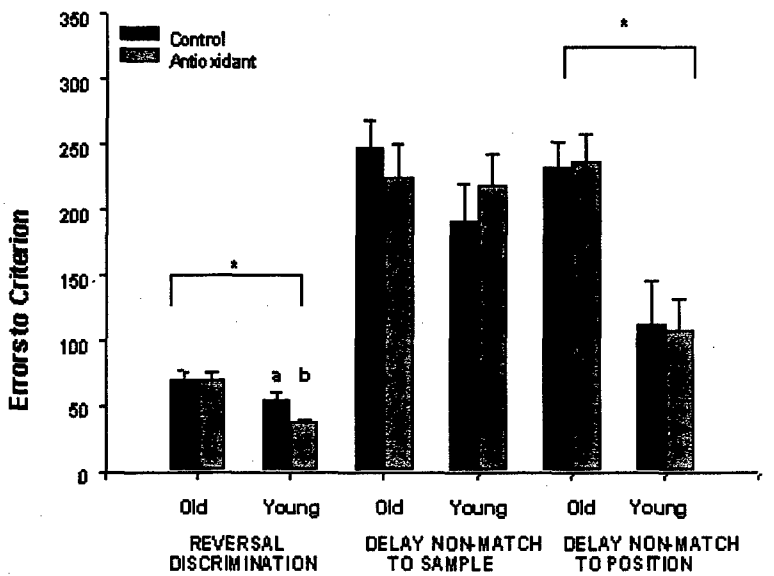


Figure 3.

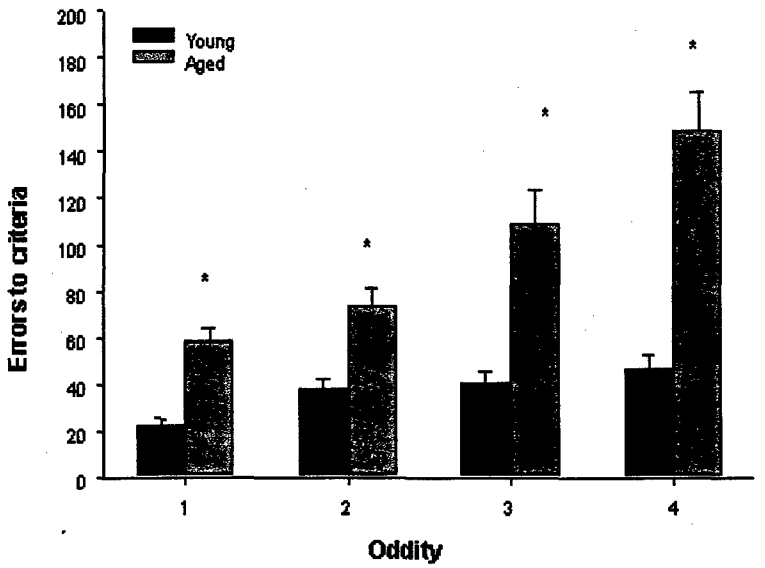
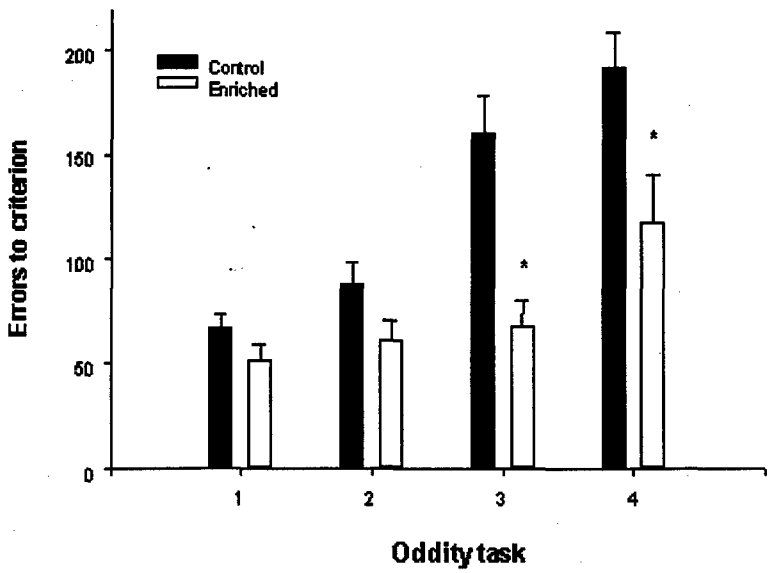


Figure 4.



Appendix G. Oxidative Damage Increases with Age and β-Amyloid Deposition in a Canine Model of Human Brain Aging

E. Head[1], J. Liu[2], T.M Hagen[3], B.A. Muggenburg[4], N.W. Milgram[5], B. N. Ames[2] and C.W. Cotman[1]

[1]Institute for Brain Aging and Dementia, University of California, Irvine, CA, 92697-4540

[2]Division of Biochemistry & Molecular Biology, University of California, Berkeley, CA, 94720 and Children's Hospital Oakland Research Institute, Oakland, CA, 94609

[3] Linus Pauling Institute, Oregon State University, Corvallis, OR 97331

[4]Lovelace Respiratory Research Institute, Albuquerque, NM, 87185

[5]Division of Life Sciences, Scarborough Campus, University of Toronto, Canada, M1C 1A4

Corresponding Author: Elizabeth Head, Ph.D.

Institute for Brain Aging & Dementia

University of California

1226 Gillespie Neuroscience Research Facility

Irvine, CA, 92697-4540

Phone: 949-824-8700

Fax: 949-824-2071

Email: ehead@uci.edu

Running Title: Oxidative Damage in Aged Canine Brain

ABSTRACT

Lipid peroxidation, protein carbonyl formation, glutamine synthetase activity and both oxidized and reduced glutathione levels were measured in the brains of aged canines to establish a link between oxidative stress, aging and β-amyloid (Aβ). The aged canine brain, a model of human brain aging, naturally develops extensive deposits of human-type β-amyloid (Aβ), which is associated with impairments in cognitive function. Aβ deposition was measured in immunostained prefrontal cortex from 19 canines (4-15 years). Oxidative damage to lipids, measured by malondialdehyde (MDA) levels, and damage to proteins, measured by carbonyl formation in the contralateral prefrontal cortex, was significantly elevated with age and with more extensive Aβ deposition. Glutamine synthetase activity (GS), an enzyme vulnerable to oxidative damage, significantly decreased with age and was lower in animals with more extensive Aβ. In parallel, the antioxidant glutathione also decreased with age. MDA levels in serum were also a significant predictor of MDA deposition in the prefrontal cortex. These results suggest an association between oxidative damage to lipids and proteins with age and Aβ neuropathology in canine brain.

Key Words: carbonyls, dog, glutamine synthetase, glutathione, malondialdehyde

Introduction

The proportion of elderly individuals is rapidly rising and a critical issue is the discovery of interventions that will promote successful aging and the maintenance of high cognitive function. The human brain has among the highest respiratory rate of any tissue and generates oxidative damage that progressively increases over time [Ames, 1993 #1096]. Neurons are particularly vulnerable to cumulative oxidative damage because they are post-meitotic cells and survive for decades. The generation of free radicals leads to oxidative damage to proteins and lipids, which may contribute significantly to neuron dysfunction and degeneration [Floyd, 1999 #1028; Liu, 1999 #1030; Beal, 1995 #1042].

Oxidative damage is problematic for a number of reasons. First, oxidative damage to lipids may induce membrane disturbances and a loss of homeostasis within cells [Balazs, 1994 #654]. 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, 2000 #2316]. Third, oxidatively modified proteins may become cross-linked and resistant to degradation, which can lead to further aggregation within or around neurons [Berlett, 1997 #1040]. 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 [Shigenaga, 1994 #2245; Stadtman, 1992 #1995; Carney, 1991 #778; Ames, 1993 #1096; Beal, 1995 #1042].

The development of neuropathology in the age-associated neurodegenerative disease, Alzheimer's disease (AD), may also be associated with oxidative damage [Coyle, 1993 #2317]. Oxidative damage to proteins and lipids appears to be significantly higher in AD brain than in nondemented elderly controls [Smith, 2000 #1021; Floyd, 1999 #1028]. This may be due to the

deposition and accumulation of beta-amyloid (Aβ) protein in the form of senile plaques, which is one of the hallmark features of the disease [Mirra, 1991 #1739][Selkoe, 1996 #577]. The amyloid precursor protein (APP), from which Aβ is proteolytically cleaved, is also vulnerable to oxidative damage [Kang, 1987 #576] and exposing APP to metabolic stress favors the production of amyloidogenic fragments [Gabuzda, 1994 #657; Multhaup, 1997 #660]. 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, 2001 #2259]. Last, Aβ is itself able to generate oxidative damage to lipids [Mark, 1997 #713;Behl, 1992 #32;Pereira, 1999 #711] and proteins [Aksenov, 1997 #2239].

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 disease progression [Braak, 1991 #195]. Like humans, canines naturally accumulate deposits of β-amyloid (Aβ) in the brain with age [Wisniewski, 1970 #81; Head, 2000 #625]. Further, canines and humans share the same Aβ sequence [Johnstone, 1991 #526], and also first show deposits of the longer Aβx-42 species followed by the deposition of Aβx-40 [Cummings, 1996 #152; Wisniewski, 1996 #155; Nakamura, 1997 #544]. The extent of Aβ has also been linked to cognitive dysfunction in canines [Cummings, 1996 #1267; Head, 1998 #1458] 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, 1996 #1266]. The canine brain, therefore, is a simpler model for examining the association between Aβ, age and oxidative damage. Thus,

studies in the canine model can complement studies in other animal model systems and provide further insights into human brain aging.

In the current study, tissue samples from canines were used to determine if oxidative damage to proteins and lipids increases with age. We also measured levels of the endogenous antioxidant, glutathione (GSH), which was hypothesized to decrease as a function of age based on reports from both rats and humans [Hagen, 1998 #2240; Samiec, 1998 #2290]. Since canines naturally accumulate Aβ with age, the association between Aβ and oxidative damage was also examined. The prefrontal cortex was the focus of the current study because this brain region develops extensive Aβ pathology that is linked to cognitive deficits early in the aging process [Head, 2000 #625][Head, 1998 #213]. Most previous studies are restricted by both the model used and also by the number of markers of oxidative damage assessed. This prohibits the identification of the major sources of damage and how it interrelates to other signatures of brain aging such as Aβ accumulation. This is the first study to examine the link between multiple markers of oxidative damage, Aβ and age in the canine model of human brain aging.

Methods

Subjects: The subjects were nineteen beagle dogs, from 4.5 to 15.3 years in age, from a colony at the Lovelace Respiratory Research Institute in Albuquerque, New Mexico. Eight dogs were males and 11 were females and all were reproductively intact. Animals were maintained with a kennelmate in indoor/outdoor kennels and had free access to water. Dogs were provided with Wayne Mini Lab Dog Diet 8759 once daily (Teklad Pioneer Lab Diets, Madison, WI). 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 euthanized 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 degrees. CSF and serum samples were collected in red top Vacutainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ), 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 0.1M Tris buffered saline (TRIS), pH 7.5, sections were pretreated with 90% formic acid for 4 minutes [Kitamoto, 1987 #434] and then in 3% H_2O_2 in 10% methanol for 30 minutes to block endogenous peroxidase activity. Sections were subsequently washed in TRIS with 0.1% Triton X-100 (TRIS A) and then blocked for 30 minutes in TRIS A with 3% bovine serum albumin (TRIS B). Samples were incubated overnight at room temperature in anti-Aβ1-16 (6E10; 1:5000; Senetek PLC, Maryland Heights, Missouri). Following 2 washes in TRIS A and a wash in TRIS B, sections were incubated in biotinylated anti-mouse IgG and then in avidin biotin complex (ABC)(Vector Laboratories, Burlingame, CA). Aβ was visualized using 3,3'-diaminobenzidine (DAB – Vector Laboratories, Burlingame, CA). The extent of Aβ deposition was subsequently quantified using image analysis techniques as described previously [Cummings, 1996 #5; Head, 1998 #213]. Aβ load measures represent the average area occupied by positive Aβ immunostaining from 525 by 410 μm fields in each individual.

Tissue Preparation for MDA, protein carbonyl and GS assays: Samples from the prefrontal cortex of 19 canines were used. Frozen prefrontal cortex was prepared in 10 vol of homogenizing buffer (100mM Tris, pH8.0, 100 mM NaCl, 20 mM EDTA with proteinase inhibitors (Leupeptin 0.5μg/ml, Apoprotonin 0.5μg/ml, Pepstatein 0.7 μg/ml). Phenylmethylsulfonyl fluoride (PMSF) was added at 40 μg/ml just before homogenizing. Samples were centrifuged for 15 min at 4000 rpm. Protein

concentration in the brain was measured using a microtiter plate assay and a bicinchoninic acid (BCA) assay from Pierce (Rockford, IL).

MDA Assay: 250 µl samples of prefrontal cortex, CSF, and serum were used for MDA assays. MDA was converted into a stable derivative using pentafluorophenyl hdyrazine at room temperature and the derivative was detected using GC-MS in the negative chemical ionization mode [Liu, 1997 #770].

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 [Levine, 1994 #746]. Derivatives were sequentially extracted with 10% (vol/vol) trichloroacetic acid followed by treatment with ethanol/ethyl acetate, 1:1 (vol/vol) and reextraction with 10% tricholoracetic acid. The precipitate was dissolved in 6 M guanidine hydrochloride. The difference spectrum between a 2,4-DNPH-protein in guanidine hydrochloride and a guanidine hydrochloride-protein blank was used to calculate nmol of 2,4-DNPH incorporated per mg of protein.

Glutamine Synthetase Activity: Glutamine synthetase activity in the prefrontal cortex was determined using the technique described by Rowe et al. [Rowe, 1970 #2212] and [Miller, 1978 #2213]. Corrections were made for nonspecific 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 HPLC as described by Reed et al. [Reed, 1980 #2241]. Briefly, cells were 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 1M Trizma Base buffer (pH 8), followed by addition of 100 µl of 40 mM fresh aqueous iodoacetic acid (4 µmol). The reaction mixture was brought to pH 8 with NaHCO3 and dinitrophenyl derivatives were made by addition of 500 µl of 2,4-dinitrofluorobenzene (1.5% [v/v] in absolute ethanol) and 100 to 200 µl of K2CO3. The resultant derivatives were separated on a 10 µm Ultrasphere-amine column (4.6 mm x 25 cm) using a Waters HPLC system and solvents as described (same ref as above). GSH and GSSG were quantified relative to standards.

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 damage, protein oxidation and glutathione levels. All statistics were conducted using SPSS software and an alpha level of 0.05.

Results

Age-dependent Increases in Oxidative Damage

As illustrated in Figure 1, MDA levels in serum ($F(1,16)=12.10$ $p<.003$) and in the prefrontal cortex ($F(1, 17)=14.16$ $p<.002$) progressively increased with age. CSF levels of MDA did not increase with age (not shown). Protein carbonyl formation also increased as a function of age in canines but showed increasing individual variability in older animals ($F(1,18)=8.98$ $p<.008$).

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 ($F(1,18)=15.61$ $p<.001$). The antioxidant GSH was reduced in aged animals ($F(1,17)=7.13$ $p<.016$) but not GSSG ($F(1,17)<1$ p=n.s.). The ratio of oxidized GSH to total GSH showed significant age-dependent increases ($r=.519$ $p<.023$). Increased oxidative damage to lipids and proteins was also accompanied by age-dependent increases in Aβ load ($F(1,18)=5.77$ $p<.028$). Of all these variables, a multiple stepwise regression analysis indicates that the best predictors of age are either GS alone ($r^2=.48$) or GS and brain MDA ($r^2=.68$). If all variables are included in the multiple regression analysis (GS, MDA, Carbonyls, GSH, GSSG and Aβ load) then $r^2=.83$ ($F(6,12)=9.58$ $p<.001$) 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 with Aβ had significantly higher levels of brain oxidative damage, animals were placed into one of two groups based on the presence or absence of an Aβ load of greater than 1%. The cutoff of 1% represents background nonspecific immunoreactivity [Head, 2000 #625]. Figure 2 illustrates that brain levels of MDA were marginally higher when significant Aβ was present ($t(17)=1.94$ $p<.069$). Canines with Aβ showed significantly higher protein carbonyl formation than dogs without Aβ ($t(17)=2.5$ $p<.022$). GS activity ($t(17)=2.55$ $p<.021$) was significantly lower and levels of GSH ($t(17)=1.94$ $p<.07$) showed a trend towards lower values in dogs with Aβ than dogs without Aβ. GSSG , by contrast, did not vary with levels of Aβ ($t(17)=1.35$ p=n.s.). Since all of the young dogs exhibited Aβ loads of less than 1%, a

correlation analysis was also used to determine if the level of Aβ deposition was associated with levels of oxidative damage. More extensive prefrontal Aβ pathology was associated with a trend towards higher levels of serum MDA ($r=.45$ $p<.064$) but no other correlations were significant. Finally, peripheral levels of MDA measured in serum did not differentiate animals with Aβ from animals without ($t(16)=1.72$ p=n.s.).

Inter-sample correlations and multiple regression analysis.

Serum levels of MDA predicted brain levels of MDA ($r=.51$ $p<.036$ $n=17$) (Figure 3). 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.

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. Lipid peroxidation in serum and brain, measured by the formation of MDA, increases as a function of age in canines and also predicts the presence of Aβ deposition. MDA in CSF was not associated with age, Aβ, nor with MDA levels in brain and serum. Protein oxidation measured by the formation of carbonyl groups increased with age with a parallel decline in GS activity. The endogenous antioxidant glutathione (GSH) also declined with

age, which also promotes oxidative damage. Peripheral measures of MDA also predicted brain levels of MDA suggesting a possible endpoint marker for evaluating antioxidant interventions. By combining a battery of markers of oxidative damage in the same sample of animals, we were also able to determine that increases in lipid oxidation were associated with similar increases in protein oxidation. Thus, oxidative damage to lipids and proteins with parallel declines in endogenous antioxidants suggests that the aged canine prefrontal cortex is vulnerable to widespread oxidative damage.

Oxidative Stress to Lipids

Lipid peroxidation in the brain, measured by the formation of MDA progressively rises with age in canines, which is consistent with previous reports in mice [Mo, 1995 #25][de Haan, 1992 #1053]. In contrast are other reports in mice of a lack of age-dependent increases in either MDA formation [Cini, 1995 #1047], or another sensitive and specific measure of lipid peroxidation, isoprostane [Pratico, 2001 #2259][Pratico, 1999 #2322].

The age-dependent increase in serum MDA levels is due, in part, to an accumulation of lipid peroxidation products. In addition, serum levels of MDA may not also reflect the extent of lipid peroxidation but also oxidative susceptibility of high and low-density lipoproteins in serum [Khalil, 1996 #170]. In addition, age-dependent increases in serum MDA levels may be due to age-dependent increases in serum protein levels [Lowseth, 1990 #2292]. Protein levels may increase with age because MDA can cross-link protein side chains, slow protein degradation and subsequently reduce protein turnover [Janero, 1990 #1526]; the latter has been reported in rats and humans (reviewed in [Ramsey, 2000 #2293]). 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 finding that serum and brain MDA are significantly correlated suggests that serum MDA originates, at least in part, in the brain. The actual source of MDA is difficult to establish with certainty, however, because aldehydes can reach targets distant from the original site of oxidation [Esterbauer, 1991 #1332]. An alternative, and more likely explanation, is serum MDA is derived from lipid damage in both central and peripheral systems. Serum levels of MDA probably reflect oxidative stress as a general phenomenon present in many organs. Both suggestions are supported by experiments used to modify peripheral oxidative stress with dietary antioxidants resulting in both improved peripheral and central measures [Joseph, 1996 #47; Joseph, 1998 #1036; Joseph, 1999 #648]. Further, the link between peripheral and central measures of lipid peroxidation suggests that serum MDA measures may be a useful endpoint measure to monitor antioxidant interventions *in vivo*.

The hypothesis of increasing oxidative damage to lipids with age was supported in both the brain and in serum, but not in CSF. The lack of accumulation of MDA in CSF occurs despite reports that larger proteins accumulate in with age due to reduced turnover [Preston, 2001 #2294]. In human CSF studies, lipid peroxidation products also show no age-dependency [Montine, 1999 #2325]. Although the number of studies using CSF samples in studies of aging are limited, the results of the current experiments suggest that these tissue samples may not be optimal for studies of oxidative damage.

Oxidative Damage to Proteins and Enzyme Dysfunction

Canines exhibit an age-dependent increase in protein carbonyl formation with a parallel decline in the levels of glutamine synthetase activity. Similar results have been reported for aged rodents, humans and also for patients with Alzheimer's disease [Smith, 1991 #2236; Carney, 1991 #778; Sohal, 1995 #26; Hensley, 1995 #2285]. Carbonyls may 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, 1997 #1040]. Higher levels of carbonyl formation may also be due to age-dependent changes in the rate of oxidized protein degradation [Stadtman, 2000 #2316]. 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, 2000 #2281].

A similar series of conclusions can be drawn from the results of assays for GS 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 #2286]. Reduced glutamine synthetase 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 [Tansey, 1991 #2282; Hertz, 1999 #2283]. Further, the reductions in GS activity also suggest that not only are neuron populations vulnerable to oxidative damage but also glial cells.

The GSH/GSSG ratio is a key parameter of cellular thiol redox status, and also 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, 1995 #26; Hazelton, 1985 #2289] [Ohkuwa, 1997 #21] and humans [Samiec, 1998 #2290]. However,

oxidized glutathione (GSSG) was not age-dependent. This suggests that the ability to maintain glutathione in a reduced state is not related to age, and that either GSSG reductase activity and/or the ability to maintain cellular pyridine nucleotide levels does not change with age. Since 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 activity of other antioxidant enzymes (i.e. superoxide dismutase) in aged canine brains are consistent, and suggest that antioxidant defenses are reduced with age [Kiatipattanasakul, 1997 #545].

Oxidative Damage and Aβ

The link between age and oxidative damage to lipids in aged canines is likely to be mediated, at least in part, by the deposition of Aβ. 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. These transgenic animals showed significant increases in lipid peroxidation with age correlated with the extent of Aβ [Pratico, 2001 #2259]. 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 [Pratico, 2000 #2321; Markesbery, 1997 #1680]. The canine model more closely parallels transgenic mice and possibly AD brain because both exhibit Aβ deposition. Thus, the results of the current study in combination with data from

humans and transgenic mice provides further support that not only is aging a significant factor in the accumulation of lipid peroxidation products but also the presence of significant Aβ pathology.

Aβ itself can stimulate the production of free radicals and consequently cause lipid oxidative damage. Application of Aβ to cell cultures leads to the formation of free radicals, which leads, in turn, to lipid peroxidation -- measured by the formation of HNE [Mark, 1997 #713]. Aβ application to cell-free systems and to PC12 culture also increases the formation of MDA [Avdulov, 1997 #1116; Xiao, 2000 #2161; Zambrzycka, 2000 #2187]. Prior application of free radical scavengers, such as vitamin E, can reduce these toxic effects in vitro [Koppal, 1998 #1051]. The results of the current study provide the first in vivo evidence of a similar link between Aβ and lipid peroxidation in canines and extends the results reported in transgenic mice.

We also found that animals with more extensive Aβ deposition showed lower levels of GS than was seen in animals with minimal levels of Aβ. GS can be directly inactivated by Aβ [Askenov, 1997 #2287]. Recent immunocytochemical evidence also demonstrates that astrocytes in the vicinity of Aβ deposits show lower levels of GS reactivity [Robinson, 2000 #2288]. Thus, reductions in GS activity in the aged canine brain may be due to both increased levels of oxidative damage to proteins in addition to further inactivation due to the presence of Aβ. Of all the oxidative damage markers included in this study, GS activity appeared to be the most sensitive to both age and Aβ deposition. Indeed, a multiple regression analysis suggests that GS activity alone is the best predictor of age over that of other oxidative markers and of Aβ combined.

Aβ may not only cause oxidative damage but evidence from another series of studies suggest that oxidative damage can lead to the production of more Aβ. In vitro experiments suggest that energy-related metabolic stress leads to reduced levels of secreted APP mediated by α-secretase and

in fact, may lead to increased production of amyloidogenic fragments [Gabuzda, 1994 #657; Gasparini, 1997 #658; Multhaup, 1997 #660]. In addition, oxidative stress increases the production of both APP and Aβ [Frederikse, 1996 #659]. This potential role of oxidative damage leading to Aβ accumulation is supported by recent evidence from Tg2576 transgenic mice where lipid peroxidation products measured by the formation of isoprostanes increased prior to the accumulation of Aβ [Pratico, 2001 #2259]. The generation of free radicals, oxidative damage and Aβ combine to form a feedback loop that without parallel increases in compensatory mechanisms can lead to significant neuron dysfunction and degeneration.

The current study focused upon the prefrontal cortex in canines for several reasons. The first is that aged canines show deficits on cognitive tasks sensitive to frontal-lobe function [Milgram, 1994 #9]. Second, based upon logistic regression analyses, this region of cortex is the site of the earliest and most predominant Aβ deposition with age [Head, 2000 #625]. Last, prefrontal Aβ is linked to impaired cognitive test scores on the same tasks sensitive to prefrontal aging [Head, 1998 #1458]. The results of the multiple regression analysis 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 levels of lipid and protein oxidation in canines. Glutathione measures also indicate a shift in the balance towards lower levels of endogenous antioxidants being available to reduce the impact of free radicals. The presence of Aβ is also associated with more oxidative damage, with the strongest associations being with GS and carbonyl formation. Of all the markers used to measure oxidative damage, GS activity appeared to be the most sensitive to age and to Aβ deposition but many of these markers were significantly intercorrelated. Another promising finding of the current study is

that it may be possible to predict brain levels of lipid peroxidation based upon noninvasive 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, or follows from Aβ deposition requires an intervention study that can reduce one or the other form of pathology. An antioxidant intervention trial is currently underway in canines to address this issue. Preliminary evidence indicates that antioxidant support can reduce age-associated learning impairments [Milgram, 2000 #1735]. The canine model complements existing animal models and may also provide novel insights into the mechanisms underlying brain aging in humans.

References

[1] Ames, B.N., Shigenaga, M.K., and Hagen, T.M., (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA*; 90:7915-7922.

[2] Floyd, R.A., (1999) Antioxidants, oxidative stress, and degenerative neurological disorders. *Proceedings of the Society for Experimental Biology and Medicine*; 222(3):236-45.

[3] Liu, J. and A. Mori, (1999) Stress, aging, and brain oxidative damage. *Neurochemical Research*; 24(11):1479-97.

[4] Beal, M.F., (1995) Aging, energy, and oxidative stress in neurodegenerative diseases. *Annals of Neurology*; 38(3):357-66.

[5] Balazs, L., and Leon, M., (1994) Evidence of an oxidative challenge in the Alzheimer's disease brain. *Neurochem Res*; 19(9):1131-1137.

[6] Shigenaga, M.K., Hagen, T.M., Ames, B.N., (1994) Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci. USA*; 91:10771-10778.

[7] Stadtman, E.R., (1992) Protein oxidation and aging. *Science*; 257:1220-1224.

[8] Carney, J.M., Starke-Reed, P.E., Oliver, C.N., Landum, R.W., Cheng, M.S., Wu, J.F., and Floyd, R.A., (1991) Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-α-phenylnitrone. *Proc. Natl. Acad. Sci.*; 88:3633-3636.

[9] Sohal, R.S., Agarwal, S., Candas, M., Forster, M.J. and Lal, H., (1994) Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. *Mechanisms of Ageing and Development*; 76:215-224.

[10] Nunomura, A., Perry, G., Pappolla, M.A., Wade, R., Hirai, K., Chiba, S., and Smith, M.A., (1999) RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *The Journal of Neuroscience.*; 19(6):1959-1964.

[11] Nunomura, A., Perry, G., Hirai, K., Aliev, G., Takeda, A., Chiba, S., and Smith, M.A., (1999) Neuronal RNA oxidation in Alzheimer's Disease and Down's syndrome. *Annals of NYAS*; 893:362-364.

[12] Davis, D.G., *et al.*, (1999) Alzheimer neuropathologic alterations in aged cognitively normal subjects. *J Neuropathol Exp Neurol*; 58(4):376-88.

[13] Head, E., McCleary, R., Hahn, F.F, Milgram, N.W. and Cotman, C.W., (2000) Region-specific age at onset of β-amyloid in dogs. *Neurobiology of Aging*; 21(1):89-96.

[14] Cummings, B.J., Satou, T., Head, E., Milgram, N.W., Cole, G.S., Savage, M.J., Podlisny, M.B., Selkoe, D.J., Siman, R., Greenberg, B.D., and Cotman, C.W., (1996) Diffuse plaques contain C-terminal ABeta 1-42 and not ABeta 1-40: Evidence from cats and dogs. *Neurobiology of Aging*; 17(4):653-659.

[15] Wisniewski, T., Lalowski, M., Bobik, M., Russell, M., Strosznajder, J. and Frangione, B., (1996) Amyloid Beta 1-42 deposits do not lead to Alzheimer's neuritic plaques in aged dogs. *Biochem J.*; 313:575-580.

[16] Nakamura, S., Tamaoka, A., Sawamura, N., Kiatipattanasakul, W., Nakayama, H., Shoji, S., Yoshikawa, Y., and Doi, K., (1997) Deposition of amyloid β protein (Aβ) subtypes [Aβ40 and Aβ42(43)] in canine senile plaques and cerebral amyloid angiopathy. *Acta Neuropathol*; 94:323-328.

[17] Mark, R.J., Pang, Z., Geddes, J.W., Uchida, K., and Mattson, M.P., (1997) Amyloid β–peptide impairs glucose transport in hippocampal and cortical neurons: Involvement of membrane lipid peroxidation. *The Journal of Neuroscience*; 17(3):1046-1054.

[18] Behl, C., Davis, J., Cole, G.M., Schubert, D., (1992) Vitamin E protects nerve cells from amyloid-beta protein toxicity. *Biochem Biophys Res Commun*; 186:944-950.

[19] Pereira, C., Santos, M.S., and Oliveira, C., (1999) Involvement of oxidative stress on the impairment of energy metabolism induced by Aβ peptides on PC12 cells: Protection by antioxidants. *Neurobiology of Disease*; 6:209-219.

[20] Aksenov, M.Y., Aksenova, M.V., Carney, J.M, and Butterfield, D.A., (1997) Oxidative modification of glutamine synthetase by amyloid beta peptide. *Free Radical Research*; 27(3):267-281.

[21] Hagen, T.M., Ingersoll, R.T., Wehr, C.M., Lykkesfeldt, J., Vinarsky, V., Bartholomew, J.C., Song, M-H., and Ames, B.N., (1998) Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. *Proc. Natl. Acad. Sci. USA*; 95:9562-9566.

[22] Samiec, P.S., Drews-Botsch, C., Flagg, E.W., Kurtz, J.C., Sternberg, Jr., P., Reed, R.L. and Jones, D.P., (1998) Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes. *Free Radical Biology & Medicine*; 24(5):699-704.

[23] Kitamoto, T., Ogomori, K., Tateishi, J., and Prusiner, S.B., (1987) Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. *Laboratory Investigation*; 57(2):230-236.

[24] Cummings, B.J., Head, E., Ruehl, W.W.,Milgram, N.W., and Cotman, C.W., (1996) Beta-amyloid accumulation correlates with cognitive dysfunction in the aged canine. *Neurobiology of Learning & Memory*; 66:11-23.

[25] Head, E., Callahan, H., Muggenburg, B.A., Milgram, N.W. and Cotman, C.W., (1998) Discrimination learning ability and beta amyloid accumulation in the dog. *Neurobiology of Aging*; 19(5):415-425.

[26] Liu, J., Yeo, H.C., Doniger, S.J., and Ames, B.N., (1997) Assay of aldehydes from lipid peroxidation: Gas chromatography-mass spectrometry compared to thiobarbituric acid. *Analytical Biochemistry*; 245:161-166.

[27] Levine, R.L., et al., (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymology*; 233:346-357.

[28] Rowe, W.B., Remzio, R. A., Wellner, V.P., and Meister, A., (1970) Glutamine synthetase. *Methods Enzymology*; 17:900-910.

[29] Miller, R.E., Hadenburg, R., and Gersham, H., (1978) Regulation of glutamine synthetase in cultured 3T3-LI cells by insulin, hydrocortisterone, and dibutyryl cAMP. *Proc. Natl. Acad. Sci. USA*; 75:1418-1422.

[30] Reed, D.J., Babson, J.R., Beatty, P.W., Brodie, A.E., Ellis, W.W., and Potter, D.W., (1980) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Analytical Biochemistry*; 106(1):55-62.

[31] Khalil, Z., Chen, H.B. and Helme, R.D., (1996) Mechanisms underlying the vascular activity of beta-amyloid protein fragment at the level of skin microvasculature. *Brain Research*; 736:206-216.

[32] Lowseth, L.A., Gillett, N.A., Gerlach, R.F., Muggenburg, B.A., (1990) The effects of aging on hematology and serum chemistry values in the beagle dog. *Veterinary Clinical Pathology*; 19(1):13-19.

[33] Janero, D.R., (1990) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology & Medicine*; 9:515-540.

[34] Ramsey, J.J., M.E. Harper, and R. Weindruch, (2000) Restriction of energy intake, energy expenditure, and aging. *Free Radic Biol Med*; 29(10):946-68.

[35] Esterbauer, H., Schaur, R.J., and Zollner, H., (1991) Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. *Free Radical Biology & Medicine*; 11:81-128.

[36] Joseph, J.A., Denisova, N., Villalobos-Molina, R., Erat, S. and Strain, J., (1996) Oxidative stress and age-related neuronal deficits. *Molecular and Chemical Neuropathology*; 28:35-40.

[37] Joseph, J.A., *et al.*, (1998) Age-related neurodegeneration and oxidative stress: putative nutritional intervention. *Neurologic Clinics*; 16(3):747-55.

[38] Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., Bickford, P.C., (1999) Reversals of age-related declines in neuronal signal transduction, cognitive and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *The Journal of Neuroscience.*; 19(18):8114-8121.

[39] Preston, J.E., (2001) Ageing choroid plexus-cerebrospinal fluid system. *Microsc Res Tech*; 52(1):31-7.

[40] Smith, C.D., Carney, J.M., Starke-Reed, P.E., Oliver, C.N., Stadtman, E.R., Floyd, R.A., and Markesbery, W.R., (1991) Excess brain protein oxidation and enzyme dysfunction in normal aging and Alzheimer's disease. *PNAS*; 88(23):10540-10543.

[41] Sohal, R.S., Agarwal, S. and Sohal, B.H., (1995) Oxidative stress and aging in the Mongolian gerbil(Meriones unguiculatus). *Mechanisms of Ageing and Development*; 81:15-25.

[42] Hensley, K., Hall, N., Subramaniam, R., Cole, P., Harris, M., Aksenov, M., Aksenova, M., Gabbita, S.P., Wu, J.F., Carney, J.M., Lovell, M., Markesbery, W.R., and Butterfield, D.A., (1995) Brain regional corespondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. *J. Neurochem.*; 65:2146-2156.

[43] Berlett, B.S. and E.R. Stadtman, (1997) Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry*; 272(33):20313-6.

[44] Friguet, B., *et al.*, (2000) Protein degradation by the proteasome and its implications in aging. *Ann N Y Acad Sci*; 908:143-54.

[45] Schor, N.F., (1988) Inactivation of mammalian brain glutamine synthetase by oxygen radicals. *Brain Research*; 456:17-21.

[46] Tansey, F.A., Farooq, M., Cammer, W., (1991) Glutamine synthetase in oligodendrocytes and astrocytes: New biochemical and immunocytochemical evidence. *J. Neurochem.*; 56:266-272.

[47] Hertz, L., Dringen, R., Schousboe, A., and Robinson, S.R., (1999) Astrocytes: Glutamate producers for neurons. *Journal of Neuroscience Research*; 57:417-428.

[48] Gibbs, M.E., O'Dowd, B.S., Hertz, L., Robinson, S.R., Sedman, G.R., Ng, K.T., (1996) Inhibition of glutamine synthetase activity prevents memory consolidation. *Cogn Brain Res*; 4:57-64.

[49] Hazelton, G.A., and Lang, C.A., (1985) Glutathione peroxidase and reductase activities in the aging mouse. *Mech Aging Dev*; 29:71-81.

[50] Ohkuwa, T., Sato, Y. nd Naoi, M., (1997) Glutathione status and reactive oxygen generation in tissues of young and old exercised rats. *Acta Physiol Scand*; 159:237-244.

[51] Kiatipattanasakul, W., Nakamura, S., Kuroki, K., Nakayama, H., and Doi, K., (1997) Immunohistochemical detection of anti-oxidative stress enzymes in the dog brain. *Neuropathology*; 17:307-312.

[52] Avdulov, N.A., Chochina, S.V., Igbavboa, U., O'Hare, E.O., Schroeder, F., Cleary, J.P., and Wood, W.G., (1997) Amyloid beta-peptides increase annular and bulk fluidity and induce lipid peroxidation in brain synaptic plasma membranes. *Journal of Neurochemistry*; 68(5):2086-2091.

[53] Xiao, X.Q., Wang, R., Han, Y.F., and Tang, X.C., (2000) Protective effects of huperzine A on β-amyloid$_{25-35}$ induced oxidative injury in rat pheochromocytoma cells. *Neuroscience Letters*; 286:155-158.

[54] Zambrzycka, A., Strosznajder, R.P., and Strosznajder, J.B., (2000) Aggregated beta amyloid peptide 1-40 decreases Ca^{2+} - and cholinergic receptor mediated phosphoinositide degradation by alteration of membrane and cytosolic phospholipase C in brain cortex. *Neurochemical Research*; 25(2):189-196.

[55] Koppal, T., Subramaniam, R., Drake, J., Prasad, M.R., and Butterfield, D.A., (1998) *Brain Research.*

[56] Askenov, M.Y., Aksenova, M.V., Carney, J.M., Butterfield, D.A., (1997) Oxidative modification of glutamine synthetase by amyloid beta peptide. *Free Radical Research*; 27:267-281.

[57] Robinson, S.R., (2000) Neuronal expression of glutamine synthetase in Alzheimer's disease indicates a profound impairment of metabolic interactions with astrocytes. *Neurochemistry International*; 36:471-482.

[58] Gabuzda, D., Busciglio, J., Chen, L.B., Matsudaira, P., and Yankner, B.A., (1994) Inhibition of energy metabolism alters the processing of amyloid precursor protein and induces a potentially amyloidogenic derivative. *Journal of Biological Chemistry*; 269(18):13623-13628.

[59] Gasparini, L., Racchi, M., Benussi, L., Curti, D., Binetti, G., Bianchetti, A., Trabucchi, M., and Govoni, S., (1997) Effect energy shortage and oxidative stress on amyloid precursor protein metabolism in COS cells. *Neuroscience Letters*; 231:113-117.

[60] Multhaup, G., Ruppert, T., Schlicksupp, A., Hesse, L., Beher, D., Masters, C.L., and Beyreuther, K., (1997) Reactive oxygen species and Alzheimer's disease. *Biochmical Pharmacology*; 54:533-539.

[61] Frederikse, P.H., Garland, D., Zigler, J.S., Piatigorsky, J., (1996) Oxidative stress increases production of β–amyloid precursor protein and β–amyloid (Aβ) in mammalian lenses, and Aβ has toxic effects on lens epithelial cells. *The Journal of Biological Chemistry*; 271(17):10169-10174.

[62] Pratico, D., *et al.*, (2001) Increased Lipid Peroxidation Precedes Amyloid Plaque Formation in an Animal Model of Alzheimer Amyloidosis. *J Neurosci*; 21(12):4183-7.

[63] Milgram, N.W., Estrada, J., Ikeda-Douglas, C., Castillo, J., Head, E., Cotman, C.W., Murphey, H., Holowachuk, D., Muggenburg, B., and Zicker, S., (2000) Landmark discrimination learning in aged dogs is improved by treatment with an antioxidant enriched diet. *Society for Neuroscience Abstracts*; 193.9.

Figure Legends

Figure 1. Individual oxidative damage markers are plotted as a function of age. Progressive and significant increases in (A) serum and (B) brain malondialdehyde (MDA), and (C) protein carbonyl formation were observed. Decreases in (D) glutamine synthetase (GS) activity and (E) glutathione (GSH) also occurred with increasing age. As reported previously, (F) the extent of Aβ deposition in the prefrontal cortex increased over the age of 8 years.

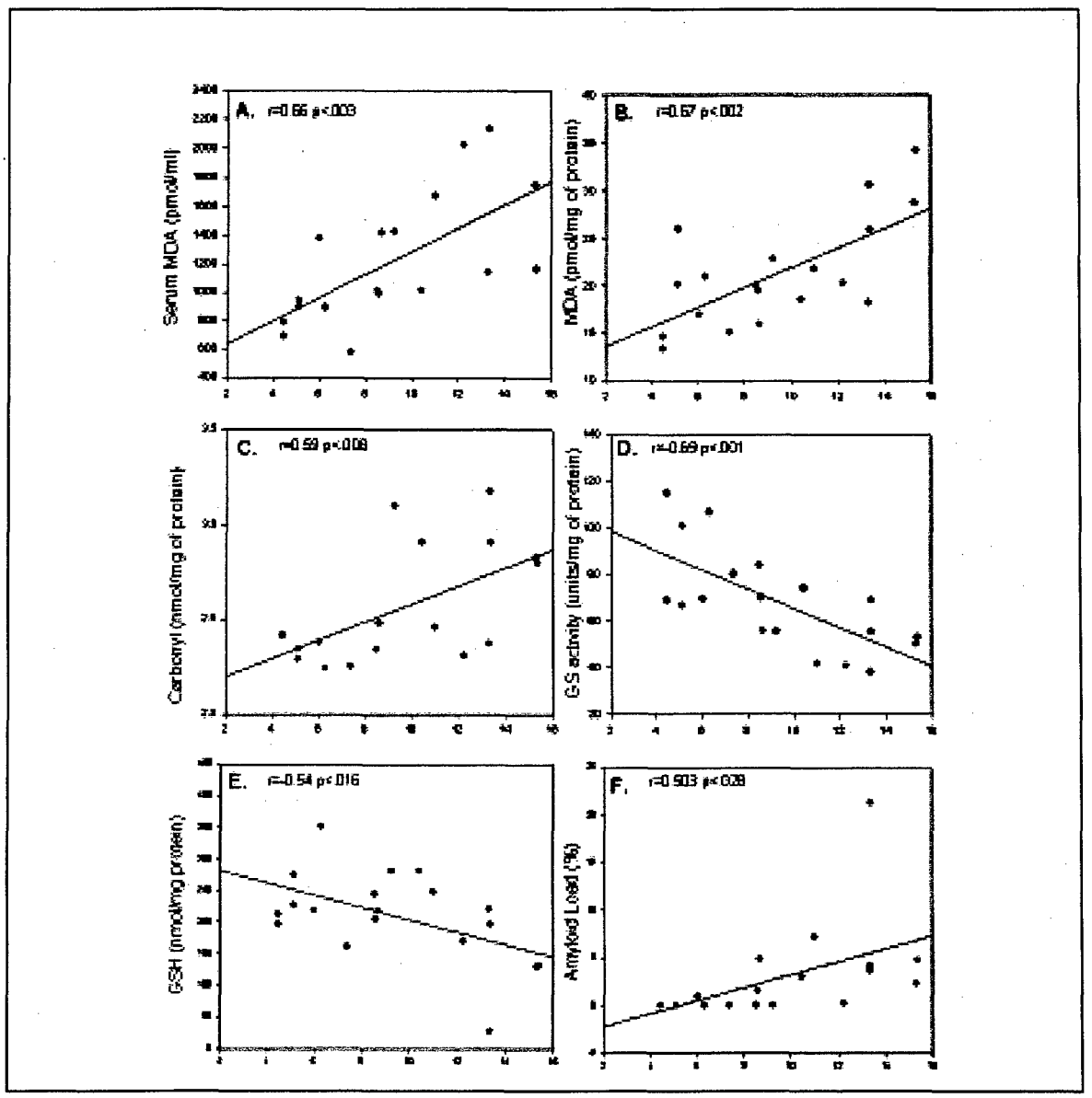


Figure 2. The relationship between the presence and absence of Aβ deposition in the prefrontal cortex and the amount of (A) MDA in samples from the contralateral prefrontal cortex (B) protein carbonyl formation, (C) glutamine synthetase activity and (D) glutathione. Data are represented as group means ± SEM. Ten dogs exhibited Aβ deposition in the prefrontal cortex and 9 dogs were negative. *p<.05

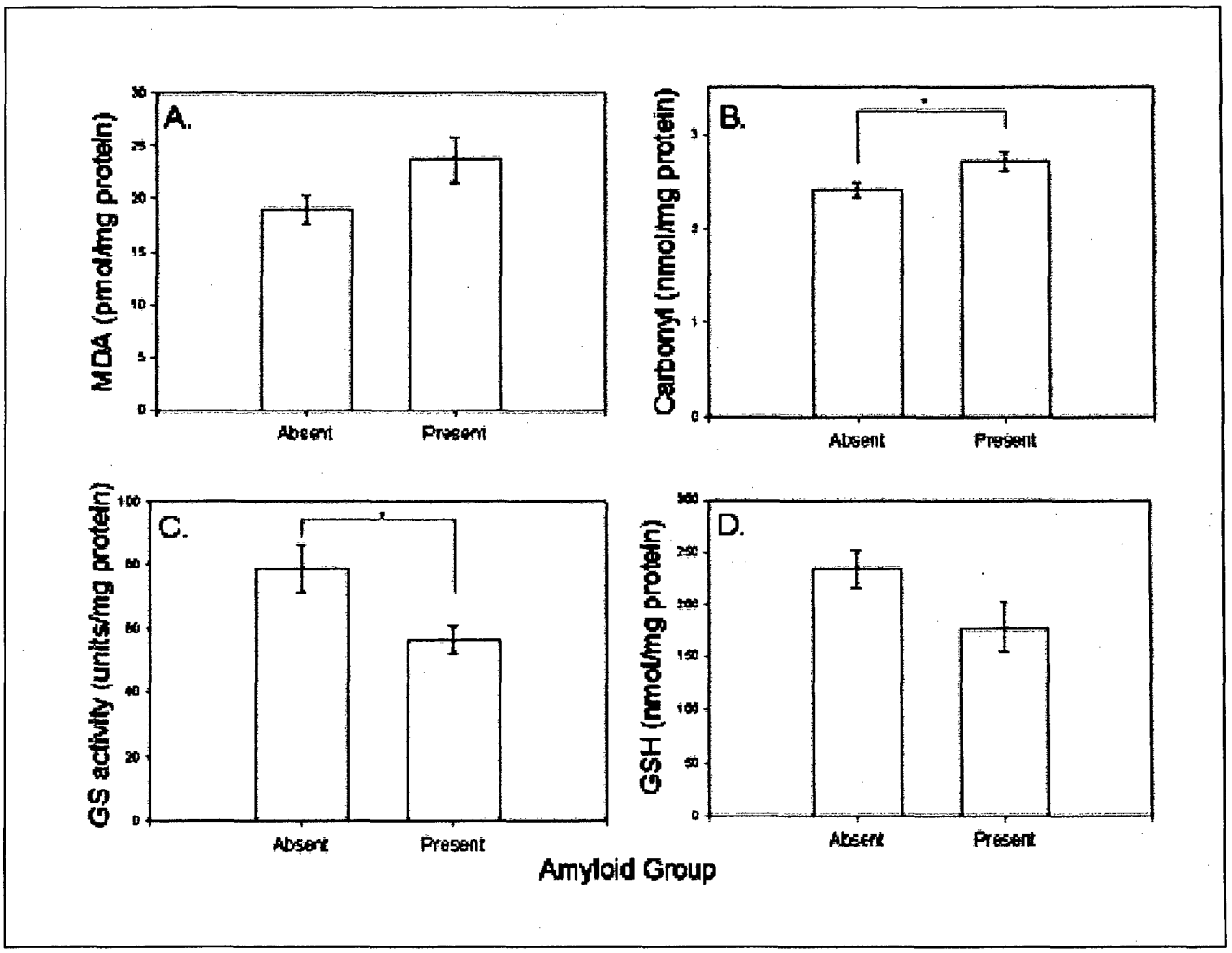


Figure 3. Individual MDA levels in the serum are plotted as a function of MDA levels in the prefrontal cortex. Higher serum levels of MDA are significantly correlated (r=.51 p<.036 n=17) with higher prefrontal cortex levels of MDA.

Acknowledgements

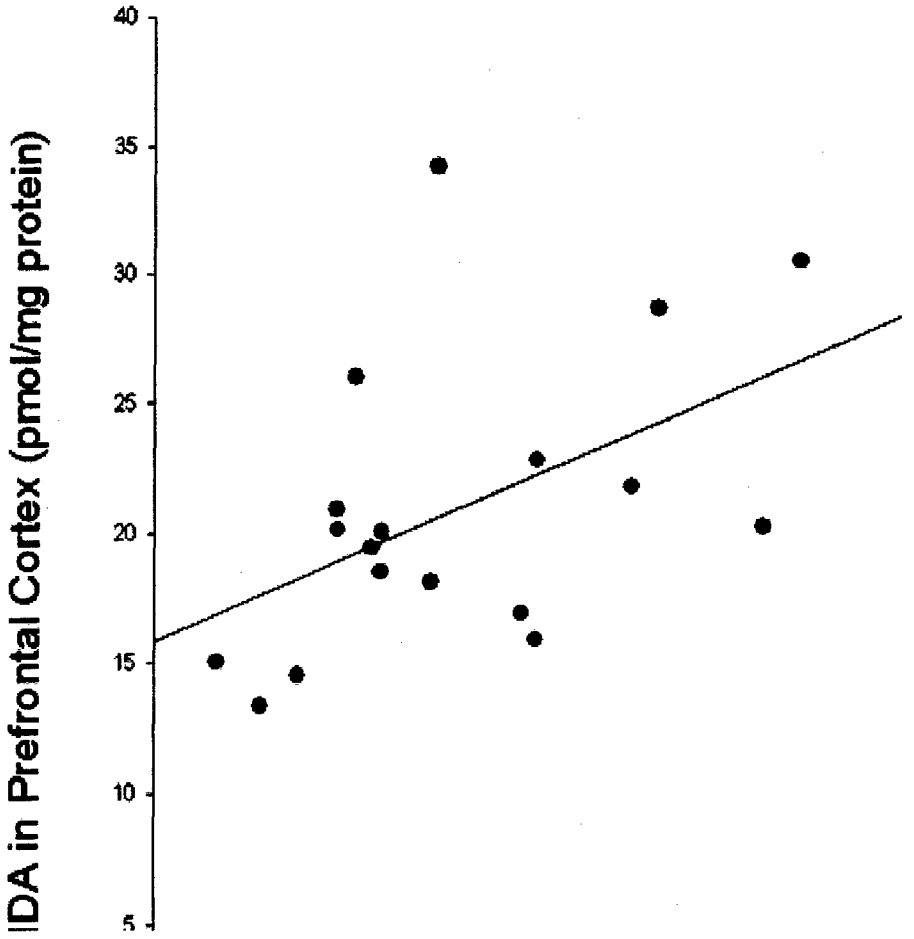
This project was sponsored by funds provided by the National Institute of Aging (Grant AG12694 to CWC) and by the U. S. Department of the Army, Contract No. DAMD17-98-1-8622 (to BAM). The content of the information does not necessarily reflect the position or the policy of the government, and no official 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.

Table 1. Oxidative Stress Marker Intercorrelations

	CSF MDA	Brain MDA	Brain Carbonyls	Brain GS	Brain GSH	Brain GSSG
Serum MDA	-0.005	0.49*	0.50*	-0.69**	-0.47	-0.38
CSF MDA		-0.23	0.11	0.12	-0.30	-0.32
Brain MDA			0.60**	-0.38	-0.45	-0.26
Brain Carbonyls				-0.34	-0.47*	-0.43
Brain GS					0.43	0.35
Brain GSH						0.59**

*p<.05

**p<.01



Appendix H. Age Differences in Strategy Selection

Visuospatial Impairments in Aged Canines: The Role of Cognitive-Behavioral Flexibility

Alan D.F. Chan and Pria M.D. Nippak

University of Toronto

Heather Murphey
Lovelace Respiratory Research Institute

Candace J. Ikeda-Douglas
University of Toronto

Bruce Muggenburg
Lovelace Respiratory Research Institute

Elizabeth Head and Carl W. Cotman
University of California at Irvine

Norton W. Milgram
University of Toronto

Abstract

This study used a novel delayed non-matching-to-position task to compare visuospatial learning and memory in young and aged beagle dogs. The task used three, rather than two, spatial locations, which markedly increased the difficulty. There were striking age differences in acquisition. Most of the aged canines did not learn the task and those that did showed impaired learning when compared to the young canines. The aged dogs also showed reduced maximal working memory capacity compared to the young. Analysis of the response patterns of individual canines indicated that the deficits were related to the use of ineffective strategies and inflexibility in strategy modification, both of which are probably independent of visuospatial ability.

Visuospatial Impairments in Aged Canines: The Role of Cognitive-Behavioral Flexibility

Spatial learning and memory ability is impaired in aged humans (Barnes, 1988; Olton, 1988; Rutledge, Hancock, & LaJuana, 1997; Sharps & Gollin, 1987; Uttl & Graf, 1993; Weber, Brown, & Weldon, 1978) and is a prominent feature of age-related neurobiological disorders including Alzheimer's and Parkinson's disease (Freedman & Oscar-Berman, 1989). Visuospatial function is also age-sensitive in non-human primates (Bachevalier et al., 1991; Bartus, Fleming, & Johnson, 1978; Moss, 1993; Rapp & Amaral, 1991), and rodents (Barnes, 1979; Barnes, Nadel, & Honig, 1980; Colombo & Gallagher, 1998; Dunnett, Martel, & Iversen, 1990; Frick, Baxter, Markowska, Olton, & Price, 1995; Gage, Dunnet, & Bjorklund, 1989; Dunnett, Evenden, & Iversen, 1988; Gallagher & Burwell, 1989; Gallagher, Burwell, & Burchinal, 1993; Gallagher & Pelleymounter, 1988; Rapp, Rosenberg, & Gallagher 1987). Aged primates are deficient compared to younger animals in learning delayed response tasks (Moss, 1993; Rapp & Amaral, 1991) and are also deficient in remembering spatial information when delays are increased (Bachevalier et al., 1991; Bartus et al., 1978; Marriott & Abelson, 1980; Medin, 1969; Rapp & Amaral, 1989; Voytko, 1993). 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 (Bachevalier, 1993; Bachevalier et al, 1991; Herndon, Moss, Rosene, & Killiany, 1997; Rapp & Amaral, 1989; Rapp, Kansky, & Roberts, 1997).

We previously described age-dependent deficits in visuospatial function in a canine model of human aging using a delayed non-matching-to-position (DNMP) task (Adams et al., 2000a; Adams et al., 2000b; Head et al., 1995). In this task, dogs are presented with a sample stimulus at one of two spatial locations. Following a delay, the sample and an identical stimulus are placed at both spatial locations. To obtain reward, the animal must respond to the location that was not

presented in the sample phase. Aged dogs show slower learning and impaired performance at long-delays, when compared to young animals. Using this data, we have been able to cognitively characterize different subsets of dogs as being successful, impaired, or severely impaired agers (Adams et al., 2000a; Adams et al., 2000b; Head et al., 1995; Milgram, Head, Weiner, & Thomas, 1994).

The DNMP task can be solved using either of two strategies: (1) remembering where the sample was, and acquiring the general rule of avoiding responding to the sample or (2) remembering which direction to respond to, which can be determined from the sample location. Because the location of the correct response can be deduced from the location of the sample, this task can be solved by a non-mnemonic strategy, such as maintaining a fixed posture and orienting towards the correct location over the delay period. Orienting strategies have been observed in rodents performing on a similar DNMP task (Chudasama & Muir, 1997). As possible evidence of dogs learning to use orientation strategies to solve this task, we have found that performance of both young and aged animals can improve markedly with extensive repeated testing, and that dogs of all ages become adept at very long delays. To decrease the likelihood of animals using a non-mnemonic solution to solve this visuospatial task, we have developed a new version of the DNMP task, the three-position delayed non-matching-to-position (3-DNMP) task.

The modified 3-DNMP task uses three, rather than two spatial positions. The addition of a third location makes it impossible for the subject to know the correct location before being presented with the test stimuli. Successful performance requires that the subjects remember the location of the sample and learn the general rule of avoiding the responding to the sample location in the comparison phase. The present report compares both acquisition and performance at progressively increasing delays on this novel task in a group of young and old dogs.

Methods

Subjects

Two groups of beagle dogs (Canis familiaris) served as subjects. The first group consisted of 17 young dogs 3-5 years old (8 males and 9 females). The second group consisted of 48 aged dogs 9-12 years old (24 males and 24 females). The justification for using the age of 9 as a cutoff was based on partly on survival data, indicating that 9 years is the point at which mortality begins to accelerate in beagle dogs at the test facility (Muggenburg, Hahn & Benjamin, 2001). Almost 90%of the population is still alive, however, so that the dogs will become successful, impaired, and severely impaired agers are still in the population. In addition, 9 is approximately the age of appearance beta amyloid protein in the beagle brain (Head, McCleary, Hahn, Milgram & Cotman, 2000). Both groups of dogs came from colonies at the Lovelace Respiratory Research Institute (LRRI) in Albuquerque, New Mexico (8 young and 24 old), and Hill's Pet Food Inc. (9 young and 24 old). All dogs were fed approximately 300g of dry dog food once daily, and were given periodic clinical examinations over the course of the study to ensure that cognitive performance was not affected by deficits in physical, sensory, or motor functioning.

The aged dogs were housed, either singly or in pairs, in pens with continual access to fresh water at the Lovelace Respiratory Research Institute, Albuquerque, New Mexico. The young dogs were housed at the animal facility at the University of Toronto at Scarborough, 2 to 4 per room. In all other respects, the animals were treated identically.

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). Briefly, the testing chamber was equipped with a 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. The height of the bars was uniquely set 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 one cm^3 of Hill's Pet Food p/d pediatric diet was used as the food reward.

Behavioral Testing Protocols

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 (Milgram et al., 1994). The protocol included training on reward and object approach learning, object discrimination learning, and discrimination reversal learning. Half of the animals in both groups were trained on the 3-DNMP test after completing this pre-training protocol. The other half was first trained on an object recognition task (Callahan, Ikeda-Douglas, Head, Cotman, & Milgram, 2000; Milgram et al., 1994).

In the 3-DNMP task, each trial begins with a sample phase in which a small red block covering a food well is presented at one of three positions, the left, center or right. After the dog displaces the block and obtains the reward, the tray is withdrawn. Following a delay, the test phase starts with the presentation of both the sample and the non-match (an identical red block in one of the two other locations). The dog must now displace the non-match to obtain reward. If the dog makes contact with the sample (incorrect response), the tray is immediately withdrawn and an error is recorded. To prevent the animals from using olfactory cues to solve the task, a

quantity of food approximately equal to that associated with the non-match was stuck to the bottom of the incorrect stimulus. After a 60s inter-trial interval, the sample phase of the next trial is initiated. The memory demands of this task are manipulated by varying the length of delay between the sample and test phase.

Data was manually collected using a customized computer program that controlled timing, randomization procedures, location of sample and non-match position, and recorded choice-reaction times. Before the start of each trial, the computer emits a tone that serves as a cue for the subject and instructs the experimenter to deliver the food tray. Each trial is initiated when the experimenter presses a key and simultaneously presents the tray. Each response was recorded by a key press, which also indicates the end of the trial and signals the beginning of the inter-trial interval.

During the actual training, each animal received 12 trials per day. We used a correction procedure in which the subject was allowed to correct its response after making its first error only on each session. The dogs were initially trained at a 10s delay until they either completed 600 trials (50 sessions) or passed a two-stage criterion. The first phase involved correctly responding on 11/12 trials or better on one day, on 10/12 trials or better over two consecutive days, or on 10/12, 9/12, and 10/12 over three successive sessions. To successfully complete the 2nd stage of criterion, they had to respond correctly on at least 70% of the next 36 completed trials (over three consecutive sessions). Thus, a minimum of 4 test days was required to achieve the criterion level. The subjects that passed the criterion at the 10s delay were then tested at progressively longer delays over 40sessions. The successive delays used were 20s, 30s, 50s, 70s, 110s & 150s. To advance to a higher delay, the dogs were required to pass the two-stage criterion at the present delay. The last delay a dog was successfully able to pass criterion on was considered that dog's

maximal memory capacity, i.e., the longest interval the animal was able to accurately retain spatial information.

Strategies Analysis

Dogs frequently develop a preference for responding to one location when presented with a new task, which we defined as a positional response bias (Milgram et al., 1994). A pure positional bias was not possible in this task because of the use of three possible positions. However, we noted that some animals showed either a position preference (i.e., responded to a particular position whenever possible), or a position avoidance (i.e., never responded to a particular position when given the opportunity). To quantify this type of positional strategy, we developed the following position bias index (PBI):

$$PBI = |4 - (\#\text{ right-side choices})| + |4 - (\#\text{ center-choices})| + |4 - (\#\text{ left-side choices})|$$

Each spatial location is an optional response a total of 8 times per session (12 trials) but is the correct location on only 4 of these. Thus, the range of possible scores on this index varies from 0 (four responses to each of the three locations) to 8 (complete avoidance of a particular spatial location).

We also noted that the difficulty of the task varies as a function of the position of the sample. When presented to either the far left or far right, the correct response is always to the animals opposite side. When the sample is presented in the center, however, the correct response is to the left of the sample half of the time, and to the right the other half. We characterized these alternatives in terms of three separate problems or subtests, based on the sample-non-match configurations (see Figure 2). In the first, the center-non-option, the sample is presented to either the left or right position and the other lateral well is used as the non-match. This configuration is identical to that used in the 2-DNMP task. The second, the center-correct, involves presenting the

sample to one of the two lateral wells and the non-match in the center well. The third, the center-incorrect, involves all trials in which the sample is presented in the center food well.

We distinguished between these subtests because of the possibility that the dogs learned some of the subtests only. For example, when the sample is presented to the animals' right, the animal will always be rewarded if it responds towards the opposite side (center or left) on the comparison trial. However, this strategy will not help solve the center-incorrect subtest.

On two of the subtests the location of the correct response is indicated by the location of the sample. The dogs can solve both of these by learning to orient away from the sample. Since this solution can be acquired using associative learning, we will use the term S-R strategy to describe this type of solution based, based on terminology used by Toates (1998). By contrast, we will use the term cognitive strategy to describe the situation in which knowledge of the correct response is linked to a general rule (Toates, 1998), rather than a particular stimulus. From this perspective, S-R strategies can be used to solve both the center-nonoption and center-correct subtests as the correct response is to one-side of the sample, e.g., if the sample appears at the animal's left, the correct response is toward the animal's right, to either the center or right position. A cognitive strategy can also be used to solve these two subtests, but must be used to solve the center-incorrect subtest. In the center-incorrect subtest the nonmatch can appear in either the left or right position. Thus, a general non-matching rule must be used to guide choice response, as the correct response cannot be anticipated from the sample position.

To determine the type of strategy used to solve the 3-DNMP task, we calculated percent accuracy scores for each subtest over the final five sessions of acquisition training, and the results were compared against random chance performance. Chance performance was determined as a binomial probability function with a 50% probability of selecting one of the two locations in the

comparison phase. An animal was classified as using an S-R strategy if they performed better than chance on the center-nonoption and center-correct subtest but no better than chance on the center-incorrect subtest. An animal was classified as using a cognitive strategy if it performed better than chance on all three subtests. An animal was classified as using a position bias if any other subtest performance pattern was present. The specific pattern would be a function of the particular bias an animal possessed.

Statistical Analysis

All statistical analyses were conducted using SPSS for Windows 10.0.7 software package with an alpha-level of 5% ($\alpha = .05$). All univariate repeated measures are Greenhouse-Geisser Epsilon corrected. 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). Finally, we used the Mann Whitney U test for comparisons of maximal memory because of unequal distances between delays (ordinal data).

Results

3-DNMP Acquisition

Figure 3 illustrates that the age effect was due to poorer performance by the old animals. In fact, only 8 of 48 aged dogs passed the two-phase criterion at the 10s delay. By contrast, 15 of 17 of the young dogs learned the task. A Fisher's Exact test indicated that this difference was highly significant ($p < .001$).

We also compared the groups with the use of a 3-way repeated measure ANOVA based on the number of errors committed during training at the 10s delay. Age (young or old) and sex (male or female) were between-subject variables and subtest (center-nonoption, center-correct

and center-incorrect) was a within-subject variable. There were significant main effects of age, F (1, 61) = 61.99, $p < .001$, and subtest, F (1.37, 83.55) = 68.25, $p < .001$, but no effect of sex.

We also found a significant age by subtest interaction, F (1.37, 83.55) = 22.10, $p < .001$ (Figure 4.a - All Subjects). Separate post-hoc one-way ANOVA's were conducted on the young and old groups. For the aged dogs, the post-hoc comparisons revealed significant differences amongst all the three subtests ($p < .001$) with the most errors committed on the center-incorrect subtest and the least on the center-correct subtest. For the young dogs, post-hoc comparisons revealed significantly more errors on the center-incorrect subtest than the center-nonoption (p = .016 and center-correct (p = .021) subtests, which did not significantly differ.

Figure 4.b (Successful-subjects) also shows the results when the analysis was restricted to only those animals that successfully passed the initial criterion (N = 15 young; N = 8 aged). A 3-way repeated measures ANOVA was conducted on the number of errors committed by those who passed the preset criterion at a 10s delay with age and sex as a between-subjects variable and subtest as a within-subject variable. There was a main effect of age, F (1, 21) = 7.267, p = .014, with aged dogs committing more errors during acquisition than the young. There was also a main effect of subtest, F (1.76, 33.44) = 23.84, $p < .001$ and a significant age by subtest interaction, F (1.76, 33.44) = 8.28, p = .002. Separate post-hoc one-way ANOVA's were conducted on the young and old groups. For the aged dogs, post-hoc comparisons revealed significant differences amongst all the three subtests ($p < .002$) with the most errors committed on the center-incorrect subtest and the least on the center-correct subtest. For the young dogs, post-hoc comparisons revealed significantly more errors on the center-incorrect subtest than the center-nonoption (p = .019) and center-correct (p = .034) subtests, which did not significantly differ.

Finally, we looked at the effect of prior training on the object recognition task on 3-DNMP task acquisition. A Fisher's exact test was conducted on both the young and aged group. Prior test experience did not effect 3-DNMP acquisition for either the young ($p = .21$) or the old ($p = .99$) dogs.

Position Bias

At the start of training, the majority of subjects showed a preference for one location, yielding high position bias scores. Many of the old animals maintained this bias throughout the period (Figure 5). To compare the groups statistically, a 2-way repeated measures ANOVA was conducted on block averages (five sessions) of the PBI scores. Age (young or old) was a between-subject variable and block (10 test sessions) was a within-subjects variable. Statistically significant main effects were obtained for age, $F (1, 58) = 20.06$, $p < .001$, and test block, $F (9, 522) = 6.24$, $p < .001$, and there was a significant age by block interaction, $F (9, 522) = 3.25$, $p < .001$. As illustrated in Figure 6.a, these results are due to the PBI scores of aged dogs showing slower decline during training than the young dogs.

When the same analysis was conducted on only the dogs that acquired the task, significant effects were obtained for age, $F (1, 17) = 6.57$, $p < .02$, and block, $F (9, 153) = 5.05$, $p < .001$, but the age by block interaction was not significant. Overall, these aged dogs still possessed higher PBI scores than the young dogs. However, over the course of training, PBI scores for both age groups decreased at similar rate (Figure 6.b).

Behavioral Strategies

A qualitative analysis was then conducted to examine the strategies used by young and old dogs. Some animals displayed performance curves that averaged to chance levels of accuracy

throughout training (Figure 7.a), while others were unable to move beyond stage one learning and performance on the center-incorrect subtest remained poor (Figure 7.b).

The animals that acquired the task tended to do so in two stages where (1) performance on the center-nonoption and center-correct subtests improved first and (2) accuracy on the center-incorrect subtest increased later (Figure 7.c and d). However, there were exceptions with four young dogs appearing to learn the entire task in one stage; their performance accuracy on all three subtests improved coincidentally. This pattern of learning was not seen in any of the aged animals. Figure 7.e shows the most dramatic illustration of this pattern. This dog performed at a chance level over the first 32 successive sessions. On the 33rd session, the subject's performance was perfect. As illustrated in Figure 7.e, after initially learning, the subject maintained a high level of performance.

To further examine whether the dogs had (1) not acquired, (2) partially acquired or (3) completely acquired the task solution, we examined performance at asymptotic levels of the initial 10s training. A 3-way ANOVA was conducted on the percent accuracy scores over the final five sessions of training at the 10s delay. Age (young or old) and sex (male or female) was a between-subjects variable and subtest (center-nonoption, center-correct, and center-incorrect) was a within-subject variable. There was a main effect of age, $\underline{F}$ (1, 61) = 48.33, $\underline{p}$ = .001, with the performance accuracy of young dogs being better than the aged dogs. There was also a main effect of subtest, $\underline{F}$ (1.97, 119.91) = 32.66, $\underline{p}$ = .001 (Figure 5.b-All Subjects). Post-hoc comparisons revealed poorer performance accuracy on the center-incorrect subtest relative to the center-nonoption ($\underline{p}$ = .001) and center-correct ($\underline{p}$ = .001) subtests, which were not different.

Similar results are obtained even when analysis was restricted to only those animals that successfully passed the initial criterion. There was a main effect of age, $\underline{F}$ (1, 19) = 4.80, $\underline{p}$ = .041

and subtest, F (1.93, 36.70) = 24.33, p = .001 (Figure 5.b-Successful Learners). Post-hoc comparisons revealed poorer performance accuracy on the center-incorrect subtest relative to the center-nonoption (p = .001) and center-correct (p = .001) subtests, which were not different.

To determine whether young dogs favoured a specific strategy over that of old dogs –the number of dogs using each of the three task solutions (positional, S-R or cognitive) was counted and compared using a Chi-square test. As illustrated in Figure 8.a, there was a significant effect of age on the type of strategy, χ^2 (5) = 24.04, $p < .001$, at the 10s delay. A higher percentage of aged dogs used position bias strategy than young dogs, which employed an S-R or cognitive strategy. Figure 8.b compares strategies used by those animals that acquired the task. In these groups, a Fisher Exact test revealed no significant age differences.

Maximal Memory Capacity

The successful animals were ranked according to the longest delay they were able to pass within the 40 days after acquiring the task at 10s. A Mann-Whitney U test of age by ranking revealed significant difference, U (8, 15) = 17.50, $p < .005$. Young dogs were able to perform more accurately at longer delays than the aged dogs (Figure 9).

DNMP Task Comparison using Historical Data

We compared acquisition on the 3-DNMP task by the young dogs to that of a similar group of young dogs on the 2-DNMP task, from our historical database previously described by Adams et al. (2000b). Briefly, the historical sample consists of 15 young (1-3 years old) and 50 aged (8-12 years old) dogs. The dogs were all administered a standard training protocol prior to this study to familiarize them with the testing apparatus and procedures (Milgram et al., 1994). The standard pre-training protocol included training on reward and object approach learning, object discrimination learning, and discrimination reversal learning.

The failure rate of aged dogs was 83% on the 3-DNMP task compared to only 18% on the 2-DNMP task. Of the young dogs, 12% failed to acquire the 3-DNMP where none have been found to fail to acquire the 2-DNMP task (Adams et al., 2000b).

A 2-way ANOVA of sessions to pass criterion was conducted with task (2-DNMP and 3-DNMP) as a between-subjects variable and delay (10s, 20s, and 30s) as a within-subject variable. There was a significant effect of task, $\underline{F}$ (1, 21) = 11.32, $\underline{p} < .003$, with young animals acquiring the 2-DNMP in fewer sessions than the 3-DNMP. There was also a significant effect of delay, $\underline{F}$ (2, 42) = 5.60, $\underline{p} < .007$, with post-hoc comparisons revealing that more errors are committed during acquisition of the 10s delay than the 20s ($\underline{p} < .019$) or 30s ($\underline{p} < .010$) delays. Both groups were then ranked according to their maximal memory capacity. A Mann-Whitney U test of task by ranking revealed significant difference, $\underline{U}$ (5, 9) = 1.50, $\underline{p} < .005$. The dogs were able to perform more accurately at longer delays on the 2-DNMP than on the 3-DNMP.

Discussion

This study re-examined spatial learning and memory deficits in aged dogs with a novel DNMP task that uses three rather than two spatial positions. We found striking age differences in acquisition and in performance following learning; when compared to young dogs, aged dogs committed more errors, required a longer training period, and showed reduced memory capacity. The young and old groups 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 three reasons. First, the testing apparatus and testing procedures were identical at both facilities. Second, these results are comparable to findings on the 2-DNMP task obtained at the University of Toronto facility, in which the failure rate for old beagles was 18% and none of the young beagles tested failed. This contrasts with a failure rate of 12% for the young beagles on the more

difficult 3-DNMP task. Finally, we have now tested additional dogs, both young and old, on the 3-DNMP task at the same facility (University of Toronto), and have obtained a high success rate for young dogs and a poor success rate for old dogs.

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 fact that the task was difficult for all of the dogs, and that difficulty increased at long delays strongly suggests that the masking was effective, and dogs were not using olfactory cues. 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 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 discriminative ability, but rather knowledge of location.

The basic finding of age-dependent deficits in spatial learning and memory is consistent with results from the 2-DNMP task (Adams et al., 2000a; Adams et al., 2000b; Head et al., 1995). The magnitude of the age effect, however, was markedly larger in this study. The majority (83%) of aged dogs failed to acquire the 3-DNMP task after 50 sessions of training. By contrast, only 18% of aged dogs failed to acquire the 2-DNMP task.

Age and Behavioral Strategies

Although the 3-DNMP task provides an index of visuospatial function, analysis of response patterns and errors suggested differences between the young and aged animals in the use of behavioral strategies. Individuals of both age groups initially approached the task with distinct position preferences. This strategy results in reward on 50% of the trials. Despite this low level

of reinforcement, the majority of the aged dogs maintained a position bias strategy throughout the acquisition phase (50-sessions). We have previously noted that age was related to the persistent use of a positional bias strategy on other tasks, e.g., discrimination reversal learning (Milgram et al, 1994).

The young and aged animals that did acquire the task tended to shift first from a position strategy to an S-R strategy, and subsequently from a S-R to a cognitive strategy. The S-R strategy involved responding in a given direction depending on the location of the sample. This type of anticipatory strategy is only successful whenever the sample appears at either the left or right position, i.e., the center-nonoption and center-correct subtests. To perform with maximal success, the subjects had to learn the cognitive strategy, the more general rule of avoiding the sample position.

Evidence for this two-stage learning comes from an error analysis. The center-incorrect subtest was typically the last subtest to improve in performance. The aged animals took longer to solve the center-incorrect subtest and made proportionately more errors on this subtest than the young animals. Both age groups showed differential performance accuracy on the subtests even over the criterion days, when they were close to asymptotic levels. Performance on the center-incorrect subtest was always poorer than on the center-nonoption and center-correct subtests. Moreover, some animals never became proficient in solving the center-incorrect subtest and maintained the use of an S-R strategy.

The subtest performance differences also indicate that animals were not solving the task by orienting to and then avoiding the sample position. This type of strategy would have resulted in equivalent performance on all subtests.

The persistent utilization of inefficient strategies by the aged animals represents an age-dependent decline in cognitive flexibility, which can be defined as the ability to shift between problem-solving strategies. Reductions in cognitive flexibility occur with advanced age in humans (Botwinick, 1978; Daigneault, Braun, & Whitaker, 1992), rats (Stephens, Weidmann, Quartermain, & Sarter, 1985), primates (Lai et al., 1995; Voytko, 1993; Voytko, 1999) and dogs (Milgram et al., 1994). Cognitive flexibility is generally regarded as an executive function that depends on the integrity of the prefrontal cortex (Daigneault et al., 1992; Dias, Robbins, & Roberts, 1996; Gansler, Covall, McGrath, & Oscar-Berman, 1996). We have recently found that deposition of β-amyloid protein in aged dogs occurs in a brain region-dependent pattern, with the prefrontal cortex being the earliest and most consistent area affected (Head et al., 2000). Thus, we hypothesize that executive function will be particularly age-sensitive in beagle dogs. While we do not currently possess direct evidence as to the neural circuitry underlying the 3-DNMP task, previous studies with dogs have shown that auditory based delayed response performance is markedly disrupted by prefrontal cortex lesions (Lawicka & Konorski, 1959; Lawicka, Mishkin, Kreiner, & Brutkowski, 1966).

Age Differences in Maximal Memory Capacity

As previously mentioned, age also affects ability to perform accurately at long delays. We have described similar results with the 2-DNMP task (Adams et al., 2000b; Head et al, 1995). Age-dependent spatial memory deficits have also been reported in non-human primates. Non-human primates appear to differ from canines, however, in their ability to respond correctly at long delays; compared to the dog, accurate performance of primates falls off far more rapidly as delays are increased. Age-differences are noted at a delays as short as 5s (Presty et al., 1987) and performance drops to chance levels at a 30s delay (Bartus et al., 1978; Marriot & Abelson, 1980).

Such species differences may reflect differences in testing protocols. In the non-human primate studies, spatial memory is tested using a matching-to-sample procedure. Some evidence suggests that monkeys learn non-matching tasks more rapidly than matching-to-sample tasks (Mishkin & Delacour, 1975). In contrast, the current study used a non-matching paradigm. Furthermore, the dogs were allowed to obtain reward by responding to the sample whereas in primate tasks, subjects are often simply shown the location of the sample.

The DNMP task is generally utilized as a tool for assessing visuospatial function, and the existence of age differences in learning suggests age-related spatial deficits. However, analysis of the types of errors indicates that age-related differences in strategy are likely to contribute to the age differences in performance as well.

The aged animals that were able to acquire the task demonstrated reduced memory capacity. The existence of age-dependent deficits in visuospatial memory may help account for the deficits we obtained in acquisition. Aged monkeys (Bartus et al., 1978; Rapp & Amaral, 1989) and rats (Dunnet et al., 1988) are not impaired on a delayed response task when the delay is very short (<1s). Our relatively long 10s training delay may have contributed significantly to the age-related acquisition deficits. To the extent that dogs have to learn both the general rule and remember the location of the sample, the 3-DNMP can be considered to involve working memory, in which information is temporarily stored for the use in the performance of other tasks and operations. A deficit in working memory could result from an inability to store the location of sample over the delay period. Working memory deficits could impair recall of (1) the subtest configuration (sample-non-match pairing) and/or (2) the reward-based feedback information. Research with concept learning tasks in humans indicates that older adults possess less reliable memories for

previous outcomes trials than young adults do and that young adults are more accurate in recalling their previously employed strategy (Fristoe, Salthouse, & Woodard, 1997).

The choice of 10s as a training delay originates from our previous experience with the 2-DNMP task. Both young and old dogs readily acquire this simpler task at a 10s delay. Furthermore, aged dogs are typically able to perform accurately on the 2-DNMP task at delays of 50s (Adams et al., 2000). However, the 3-DNMP task is more difficult to learn than the 2-DNMP task. In the DNMP procedure, subjects must first learn to discriminate between the two phases within a trial (sample and comparison). This may require attention to additional discriminative stimuli and response contingencies etc. Moreover, the subject must learn to remember events (hence to non-match) only within a given trial. This determination is relatively simple in the 2-DNMP task because there are only two unique configurations (L-R & R-L). However, the 3-DNMP is comprised of six unique sample-non-match configurations (L-R, L-C, R-L, R-C, C-R, & C-L). Trying to determine the correct relationship amongst these many configurations may overload the memory capacity of the aged group, e.g., the amount of data that can be considered at any one time.

Individual Differences

As expected, marked individual differences were seen in both the young and old dogs. What was striking though, were the four young dogs who acquired the cognitive strategy in a single stage, rather than two. This very rapid learning may reflect a higher order type of rule learning, in so far as the non-match rule was not induced out of an associatively learned behavior. The absence of this type of learning in any aged animal may be indicative of an age-related decline in higher-order cognitive abilities. Aging is associated with a decline in executive function in both humans (Grigsby, Kaye, & Robbins, 1995) and primates (Lai et al., 1995).

Conclusions and Future Utility

We have described a novel modification of a DNMP task, in which the use of an orientation strategy will not lead to maximal performance. Microanalysis of choice responses of the task proved to be extremely powerful in uncovering age-related differences in cognition by identifying various behavioral strategies. Notably, the aged dogs were markedly deficient in learning the task, and also showed extensive spatial memory impairments. A number of factors may contribute to the age differences found in this study, other than those directly related to visuospatial function. These include age-related decreases behavioral flexibility and working memory. These deficits may also account for the qualitative age-differences in the learning process as some young dogs expressed higher-order learning that was not detected amongst the aged dogs. The large majority of aged animals did not achieve the preset criterion. But this does not mean they are incapable of learning the task. First, aged dogs should be capable of more proficient learning if the initial delay is shortened. We have preliminary evidence indicating that a higher proportion of aged animals can learn the task if they are trained initially at delay of 5s. In addition, we have found that aged dogs are capable of proficient learning if they are previously trained on the 2-DNMP task (Chan, Tapp & Milgram, submitted). Finally, in this study we limited the number of acquisition sessions to 50, and it's very likely that more extensive training would lead to a higher success rate.

In future studies it would be of considerable interest to include an age group intermediate between the old and young used here. It is notable that the two young dogs that failed the task were among the 6 oldest young dogs. It would also be of considerable interest to test both rodents and primates on a similar task. As potential use for orienting strategies is reduced in this

task, it may be particularly useful in rodent studies where the validity of the traditional 2-DNMP task has been questioned (Chudasama & Muir, 1997).

References

Adams, B., Chan, A.D.F., Callahan, H., & Milgram, N.W. (2000a). The canine as a model of human cognitive aging: Recent developments. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 24, 675-692.

Adams, B., Chan, A.D.F., Callahan, H., Siwak, C., Tapp, D., Ikeda-Douglas, C., Atkinson, P., Head, E., Cotman, C.W., & Milgram N.W. (2000b). Use of a delayed non-matching to position task to model age-dependent cognitive decline in the dog. Behavioral Brain Research, 108(1), 47-56.

Andrews, J.S., Jansen, J.M.H., Linders, J.S., & Pincen, A. (1994). Effects of disrupting the cholinergic system of short-term spatial memory in rats. Psychopharmacology, 115, 485-494.

Bachevalier, J. (1993). Behavioral changes in aged rhesus monkeys. Neurobiology of Aging, 14, 619-621.

Bachevalier, J., Landis, L.S., Walker, L.C., Brickson, M., Mishkin, M., Price, D.L., & Cork, L.C. (1991). Age monkeys exhibit behavioral deficits indicative of widespread cerebral dysfunction. Neurobiology of Aging, 12, 99-111.

Barnes, C.A. (1979). Memory deficits associated with senescence: A neurophysiological and behavioral study in the rat. Journal of Comparative & Physiological Psychology, 93(1), 74-104.

Barnes, C.A. (1988). Aging and the physiology of spatial memory. Neurobiology of Aging, 9, 563-568.

Barnes, C.A., Green, E.J., Baldwin, J., & Johnson, W.E. (1987). Behavioral and neurophysiological examples of functional sparing in senescent rat. Canadian Journal of Psychology, 41, 131-140.

Barnes, C.A., Nadel, L., & Honig, W.K. (1980). Spatial memory deficit in senescent rats. Canadian Journal of Psychology, 34(1), 29-39.

Bartus, R.T., Fleming, D.L., & Johnson, H.R. (1978). Aging in the rhesus monkey: debilitating effects on short-term memory. Journal of Gerontology, 33, 858-871.

Bartus, R.T., Dean, R.L., & Fleming, D.L. (1979). Aging in the rhesus monkey: Effects on visual discrimination learning and reversal learning. Journal of Gerontology, 34, 209-219.

Botwinick, J. (1978). Aging and behavior. New York: Springer Publishing Co.

Callahan, H., Ikeda-Douglas, C., Head, E., Cotman, C.W., & Milgram, N.W. (2000). Development of a protocol for studying object recognition memory in the dog. Progress in Neuro-Psychopharmacology & Biological Psychiatry, 24(5), 693-707.

Chan, A.D.F., Tapp, P.D., & Milgram N.W. (2001). Cognitive Test Experience Can Improve Visuospatial Test Performance In Aged Beagle Dogs. In submission

Chudasama, Y., & Muir, J.L. (1997). A behavioral analysis of the delayed non-matching to position task: The effects of scopolamine, lesions of the fornix and of the prelimbic region on mediating behaviors by rats. Psychopharmacology, 134, 73-82.

Columbo, P.J., & Gallagher, M. (1998). Individual differences in spatial memory and striatal ChAT activity among young and aged rats. Neurobiology of Learning and Memory, 70(3), 314-327.

Craik, F.I.M., & Jennings, J.M. (1992). Human memory. In: F.I.M. Craik & T.A. Salthouse (Eds.), Handbook of aging and cognition (pp. 51-110). New Jersey: Lawrence Erlbaum Assoc.

Daigneault, S., Braun, C.M.J., Whitaker, H.A. (1992). Early effects of normal aging on perseverative and non-perseverative prefrontal measures. Developmental Neuropsychology, 8, 99-114.

Dias, R., Robbins, T.W., & Roberts, A.C. (1996). Primate analogue of the Wisconsin card sorting test: Effects of excitotoxic lesions of the prefrontal cortex in the marmoset. Behavioral Neuroscience, 110(5), 872-886.

Dunnett, S.B., Evenden, J.L., & Iversen, S.D. (1988). Delay-dependent short-term memory deficits in aged rats. Psychopharmacology, 96, 174-180.

Dunnett, S.B., Martel, F.L., & Iversen, S.D. (1990). Proactive interference effects on short-term memory in rats II. Effects in young and aged rats. Behavioral Neuroscience, 104, 666-670.

Fischer, W., Chen, K.S., Gage, F.H., & Bjorklund, A. (1992). Progressive decline in spatial learning and integrity of forebrain cholingergic neurons in rats during aging. Neurobiology of Aging, 13, 9-23.

Flicker, C., Ferris, S.F., Bartus, R.T., & Crook, T. (1984). Effects of aging and dementia upon recent visuospatial memory. Neurobiology of Aging, 5, 75-83.

Fox, M.W. (1971). Integrative development of brain and behavior in the canine. Chicago: University of Chicago Press.

Freedman, M, & Oscar-Berman, M. (1989). Spatial and visual deficits in Alzheimer's and Parkinson's disease. Brain and Cognition, 11, 114-126.

French, G.M. (1959). Performance of squirrel monkeys on variants of delayed response. Journal of Comparative & Physiological Psychology, 52, 741-745.

Frick, K.M., Baxter, M.G., Markowska, A.L., Olton, S.D., & Price, D.L. (1995). Age-related spatial reference and working memory deficits assessed in the water maze. Neurobiology of Aging, 16(2), 149-160.

Fristoe, N.M., Salthouse, T.A., & Woodard, J.L. (1997). Examination of age-related deficits on the Wisconsin Card Sorting Test. Neuropsychology, 11(3), 428-436.

Gage, F.H., Dunnet, S.B., & Bjorklund, A. (1989). Age-related impairments in spatial memory are independent of those in sensorimotor skills. Neurobiology of Aging, 10, 347-352.

Gallagher, M., Burwell, R., & Burchinal, M. (1993). Severity of spatial learning impairment in aging: Development of a learning index for performance in the Morris water maze. Behavioral Neuroscience, 107, 618-627.

Gallagher, M., & Burwell, R.D. (1989). Relationship of age-related decline across several behavioral domains. Neurobiology of Aging, 10, 691-708.

Gallagher, M., & Pelleymounter, M.A. (1988). Spatial learning deficits in old rats: A model for memory decline in the aged. Neurobiology of Aging, 9, 549-556.

Gansler, D.A., Covall, S., McGrath, N., & Oscar-Berman, M. (1996). Measures of prefrontal dysfunction after closed head injury. Brain and Cognition, 30(2), 194-204.

Grigsby, J., Kaye, K., & Robbins, L.J. (1995). Behavioral disturbance and impairment of executive functions among the elderly. Archives of Gerontology and Geriatrics, 21, 167-177.

Head, E., Mehta, R., Hartley, J., Kameka, M., Cummings, B.J., Cotman, C.W., Rhuel, W.W., & Milgram, N.W. (1995). Spatial learning and memory as a function of age in the dog. Behavioral Neuroscience, 109(5), 851-858.

Head, E., McCleary, R., Hahn, F.F., Milgram, N.W., & Cotman, C.W. (2000). Region-specific age at onset of β-amyloid in dogs. Neurobiology of Aging, 21, 89-96.

Herndon, J.G., Moss, M.B., Rosene, D.L., & Killiany, R.J. (1997). Patterns of cognitive decline in aged rhesus monkeys. Behavioral Brain Research, 87, 25-34.

Hunter, W.S. (1913). Delayed reaction. Behavioral Monographs, 2, 1-86.

Kesner, R.P., Bierley, R.A., & Pebbles, P. (1981). Short-term memory: The role of *d*-amphetamine. Pharmacology, Biochemistry & Behavior, 15, 673-676.

King, J.E., & Michels, R.R. (1989). Error analysis of delayed response in aged squirrel monkeys. Animal Learning & Behavior, 17(2), 157-162.

Lacreuse, A., Herndon, J.G., Killiany, R.J.M., Rosene, D.L., & Moss, M.B. (1999). Spatial cognition in rhesus monkeys: Male superiority declines with age. Hormone & Behavior, 36, 70-76.

Lai, Z.C., Moss, M.B., Killiany, R.J., Rosene, D.L., & Herndon, J.G. (1995). Executive system dysfunction in the aged monkey: spatial and object reversal learning. Neurobiology of Aging, 16, 947-954.

Lawicka, W., & Konorski, J. (1959), The physiological mechanism of delayed reactions: III The effects of prefrontal ablations on delayed reactions in dogs. Acta Biologica Exp (Warsz), 19, 221-231.

Lawicka, W., Mishkin, M., Kreiner, J., & Brutkowski, S. (1966). Delayed response deficit in dogs after selective ablation of the proreal gyrus. Acta Biologica Exp (Warsz), 26(3), 309-322.

Marriott, J.G., & Abelson, J.S. (1980). Age differences in short-term memory in test-sophisticated rhesus monkeys. Age, 3, 7-9.

Medin, D.L. (1969). Form perception and pattern reproduction in monkeys. Journal of Comparative and Physiological Psychology, 68, 412-419.

Milgram, N.W., Adams, B., Callahan, H., Head, E., Mackay, B., Thirlwell, C., & Cotman, C.W. (1999). Landmark discrimination learning in the dog. Learning and Memory, 6, 54-61.

Milgram, N.W., Head, E., Weiner, E., & Thomas, E. (1994). Cognitive functions and aging in the dog: Acquisition of nonspatial visual tasks. Behavioral Neuroscience, 108(1), 57-68.

Mishkin, M., & Delacour, J. (1975). An analysis of short-term visual memory in the monkey. Journal of Experimental Psychology: Animal Behavior Processes. 1(4), 326-334.

Moss, M.B. (1993). The longitudinal assessment of recognition memory in aged rhesus monkeys. Neurobiology of Aging, 14, 635-636.

Moss, M.B., Rosene, D.L., & Peters, A. (1988). Effects of aging on visual recognition memory in the rhesus monkey. Neurobiology of Aging, 9, 495-502.

Muggenburg, B.A., Hahn , F., & Benjamin, S.A. (2001). Causes of mortality in laboratory Beagle colonies. In: In: U. Mohr, W W Carlton, D L Dungworht, S A Benjamin C C Capen, & F. F Hahn (Eds), Pathobiology of the Aging Dog (pp. 25-33 Iowa State University press.

Olton, D.S. (1988). Aging and spatial cognition. Neurobiology of Aging, 9, 569-570.

Paule, M.G., Bushnell, P.J., Maurissen, J.P.J., Wenger, G.R., Buccafusco, J.J., Chelonis, J.J., & Elliott, R. (1998). Symposium Overview: The use of delayed matching-to-sample procedures in studies of short-term memory in animals and humans. Neurotoxicology & Teratology, 20(5), 493-502.

Parasuraman, R., & Nestor, P.G. (1993). Preserved cognitive operations in early Alzheimer's disease. In: J. Cerella, J. Rybash, W. Hoyer, & M.L. Commons (Eds.), Adult information processing: Limits on loss (pp. 77-115). San Diego: Academic Press Inc.

Pontecorvo, M.J., Sahgal, A., & Steckler, T. (1996). Further developments in the measurement of working memory in rodents. Cognitive Brain Research, 3, 205-213.

Presty, S.K., Bachevalier, J., Walker, L.C., Struble, R.G., Price, D.L., Mishkin, M., & Cork, L.C. (1987). Age differences in recognition memory of the rhesus monkeys (Macaca mulatta). Neurobiology of Aging, 8, 435-440.

Rapp, P.R., & Amaral, D.G. (1989). Evidence for task-dependent memory dysfunction in the aged monkey. Journal of Neuroscience, 9, 3568-3576.

Rapp, P.R., Kansky, M.T., & Roberts, J.A. (1997). Impaired spatial information processing in aged monkeys with preserved recognition memory. Neuroreport, 8, 1923-1928.

Rapp, P.R., Rosenberg, R.A., & Gallagher, M. (1987). An evaluation of spatial information processing in aged rats. Behavioral Neuroscience, 101, 3-12.

Rupniak, N.M.J., Samson, N.A., Stevenston, M.J., Iversen, S.D. (1990). Induction of cognitive impairment by scopolamine and noncholinergic agents in rhesus monkeys. Life Science, 48, 893-899.

Rutledge, P.C., Hancock, R.A., & LaJuana, W. (1997). Effects of retention interval length on young and elderly adults' memory for spatial information. Experimental Aging Research, 23, 163-177.

Savage, L.M., Pitkin, S.R., & Careri, J.M. (1999). Memory enhancement in aged rats: The differential outcome effect. Development Psychobiology, 35, 318-327.

Sharps, M.J., & Gollin, E.S. (1987). Memory for object locations in young and elderly adults. Journal of Gerontology, 42, 336-341.

Shukitt-Hale, B., Mouzakis, G., & Joseph, J.A. (1998). Psychomotor and spatial memory performance in aging male Fischer 344 rats. Experimental Gerontology, 33(6), 615-624.

Squire, L.R. (1992). Memory and hippocampus: A synthesis from findings with rats, monkeys, and humans. Psychological Review, 99(2), 195-231.

Stephens, D.N., Weidmann, R., Quartermain, D., & Sarter, M. (1985). Reversal learning in senescent rats. Behavioral Brain Research, 17(3), 193-202.

Toates, F. (1998). The interaction of cognitive and stimulus-response processes in the control of behavior. Neuroscience and Biobehavioral Review, 22(1), 59-83.

Uttl, B., & Graf, P. (1993). Episodic spatial memory in adults. Psychological Aging, 8, 257-73.

van Haaren, F., & van Hest, A. (1989). Spatial matching and nonmatching in male and female Wistar rats: Effects of delay-interval duration. Animal Learning and Behavior, 17, 355-360.

van Hest, A., & Stecklar, T. (1996). Effects of procedural parameters on response accuracy: Lessons from delayed (non-)matching procedures in animals. Cognitive Brain Research, 3, 193-203.

Voytko, M.L. (1993). Cognitive chances during normal aging in monkeys assessed with an automated test apparatus. Neurobiology of Aging, 14, 634-644.

Voytko, M.L. (1999). Impairments in acquisition and reversals of two-choice discriminations by aged rhesus monkeys. Neurobiology of Aging, 20, 617-627.

Weber, R.J., Brown, L.T., & Weldon, J.K. (1978). Cognitive maps of environmental knowledge and preference in nursing home patients. Experimental Aging Research, 4, 157-174.

Author Note

Alan D.F. Chan, Pria Nippak, Institute of Medical Science, University of Toronto; Heather Murphey, Bruce Muggenburg, Lovelace Respiratory Research Institute, Albuquerque, New Mexico; Candace J. Ikeda-Douglas, Norton W. Milgram, Division of Life Sciences, University of Toronto at Scarborough; Elizabeth Head, Carl W. Cotman, Institute for Brain Aging and Dementia, University of California at Irvine.

This research was supported by the National Institute of Aging (NIA AG12694) and by the U.S. Army Medical Research and Materiel Command under Contract No. DAMD17-98-1-8622. The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation. In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985). Additional support was provided in the form of postgraduate scholarships to Alan Chan and Pria Nippak from the National Science and Engineering Research Council of Canada.

We thank Shelly Brethouwer, Joanna Castillo, Joanna Drozdz, Dan Holowachuk, and Antonio Salinas for their professional help in the behavioral testing. Special thanks are also due Christina Siwak and Dwight Tapp for their helpful comments in preparing this manuscript.

Correspondence concerning this article should be addressed to Norton W. Milgram, who is at the Division of Life Sciences, University of Toronto at Scarborough, 1265 Military Trail, Scarborough, Ontario, Canada, M1C 1A4. Electronic mail may be sent via Internet to milgram@psych.utoronto.ca.

Figure Captions

Figure 1. Apparatus used in cognitive testing with dogs. The apparatus contains a rectangular box where the dog resides (a); stainless steel bars of adjustable heights that provide three openings (b); a screen with a one-way mirror separating the experimenter and animal (c); and a black Plexiglas presentation tray with three food wells, two lateral and one medial (d).

Figure 2. Three-position delayed non-matching-to-position (3-DNMP) paradigm and the three associated subtests. Note that two configurations exist for each of he subtests.

Figure 3. Scatter plots of errors made by individual subjects during the acquisition phase for the young and old groups. The dashed line shows the group averages.

Figure 4. Acquisition errors as a function of age, subtest, and success in learning. (a) shows the total errors during acquisition at the 10 second delay plotted as a function of age and subtest. The left hand side is for all subjects and right hand side shows the results the errors made by those animals that learned the task. Note that the aged dogs made more errors relative to young dogs and disproportionately more on the center-incorrect subtest. (b) shows percent accuracy scores over the last 5 training sessions for both age groups.

Figure 5. Position preference responding for three aged animals over the course of training. (a) shows an animal that started with and maintained a strong right position bias and a strong left side avoidance (b) is from an animal that showed a consistent right side avoidance. (c) shows another aged animal that initially responded to all three locations, but over the course of training developed a very strong center position bias.

Figure 6. Age-differences in position bias scores. (a) compares PBI scores for aged and young dogs over the 50 training sessions, separated into 5-session blocks. Only the young dogs showed

progressive decline in PBI scores. (b) compares PBI scores of only the aged and young dogs that successfully acquired the task. Both age groups showed a progressive decline in PBI scores.

Figure 7. Shifting of strategies during learning of the 3-DNMP. (a) shows an aged canine who retained a position preference through the course of testing, and performed at chance on all subtests. (b) shows an aged canine who shifted from a positional bias to an S-R strategy, in which performance on the center-nonoption and center-correct subtests became proficient. (c) is an aged canine who shifted from an S-R strategy to a cognitive strategy at about the 14^{th} training block, as evidenced by equal above chance performance on all subtests. (d) shows a young canine who shifted to a cognitive strategy on the 4^{th} training block. (e) is from a young canine who acquired the cognitive strategy in a single session.on all subtests.

Figure 8. Age-differences in the use of strategies. (a) shows the percentage of dogs using a particular strategy for both age groups. The majority of aged dogs employ a positional bias strategy, which is reflected in their high failure rate. (b) shows the percentage of dogs that acquired the task, using a particular strategy.

Figure 9. Age-differences in maximal memory capacity. (a) shows the percent of animals that were able to complete each delay for both age groups. Note that the majority of aged dogs did not pass (DNP) the 10s delay. (b) shows the average maximal delay completed for the dogs that learned the task from both the old and young groups.

Figure 1A and B

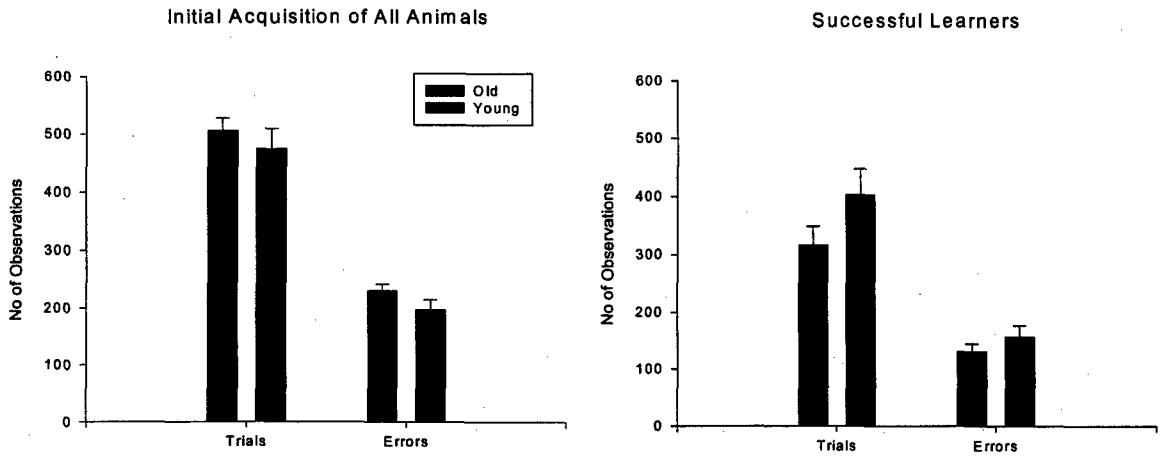
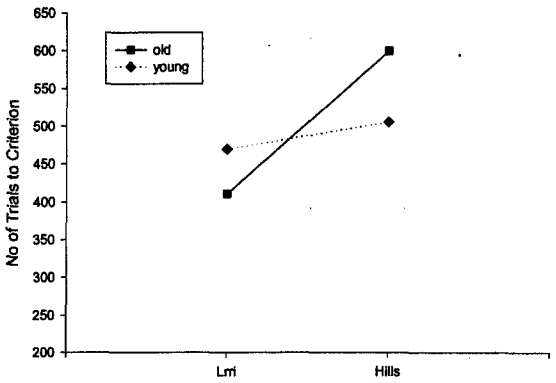


figure 2 - source and age interaction
f(1,61) = 4.27, p<0.0430)



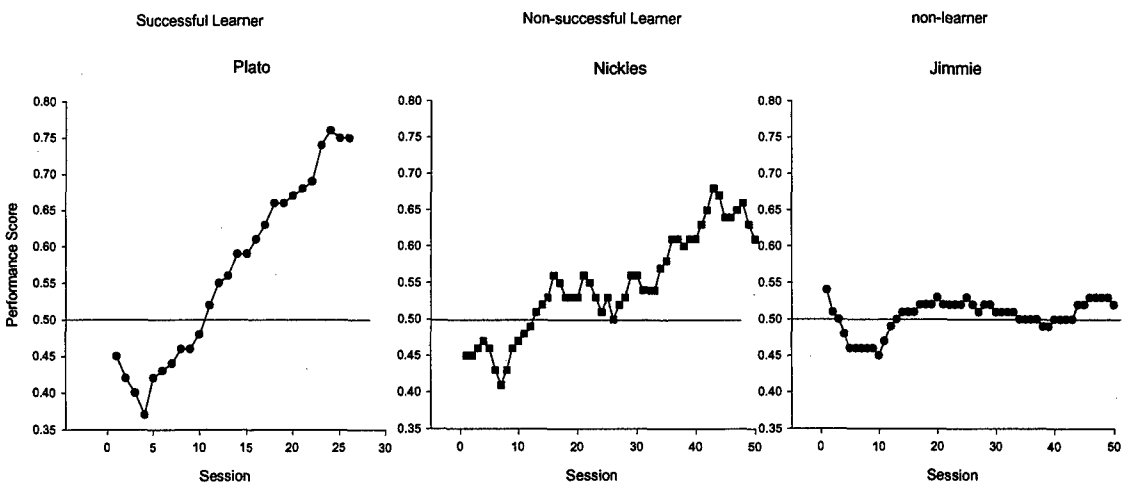


Figure 4 f(1,27=8.30; p<0.0077)

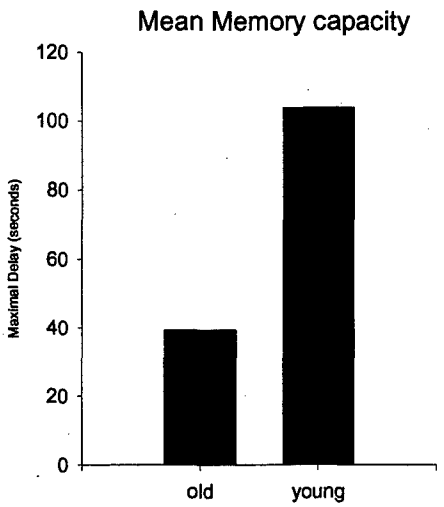
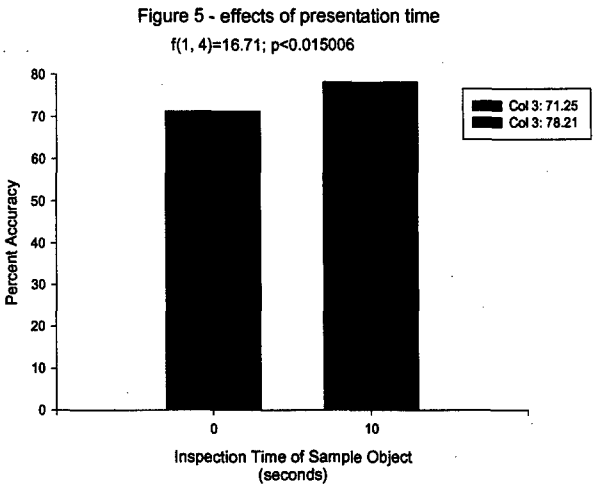


Figure 5 – effects on inspection time



Lovelace

Louise - Please substitute these pages in report at DTIC

January 28, 2002

Commander
U. S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, MD 21702-5012

Dear Commander:

Enclosed are the original and two copies of replacement Appendices A and B of the annual report for the third year of the project entitled, "The Effects of Antioxidants and Experience on the Development of Age Dependent Cognitive Dysfunction and Neuropathology in Canines, " Award No. DAMD17-98-1-8622.

No data affecting the study have changed, only the columns Date, Intervention Start Date, and Birth Date in Appendix A and Date and Birth Date in Appendix B. These appendices were originally done using Excel software, and when the tables were formatted in the word processing software, those columns did not translate for some reason. We did not notice the mistake when the report was originally submitted in October 2001. We apologize for the error.

If there are questions on the appendices, please contact me by phone at 505-348-9441, by fax at 505-348-4980, or by email: bmuggenb@lrri.org.

Sincerely,

Bruce A. Muggenburg, DVM, PhD
Principal Investigator

Encl. A/S
XC: Co-Investigators
Dr. Charles Hobbs
Ms. Kathleen Aragon, Grants & Contracts

A398089