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Mid-Life Onset of Dietary Restriction Extends Life and Prolongs Cognitive Functioning

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MEANS, L. W., J. L. HIGGINS AND T. J. FERNANDEZ. *Mid-life onset of dietary restriction extends life and prolongs cognitive functioning.* PHYSIOL BEHAV **54**(3) 503–508, 1993.—Fourteen-month-old C57BL/6 (NIA) mice were placed on a nutritionally complete diet providing 139.4 kcal/week. Over a 2-month period the food ration of experimental mice (AE) was reduced to 85 kcal/week, where it remained for the duration of the study. An aged control group (AC) continued with the higher calorie diet. At age 22 months, AC mice and half of the AE mice (AE22) were given a battery of behavioral tests. The remaining AE mice (AE25) were given the test battery at age 25 months. Also, a middle-aged control group (MC) was tested at age 13 months. Mid-life onset caloric restriction (CR) increased longevity and preserved strength, coordination, and spontaneous alternation behavior, and altered responses to enclosed alleys. A spatial discrimination in the Morris water maze and a spatial delayed matching-to-sample water-escape task were insensitive to age and diet. The aged mice were adversely affected by testing.

Caloric restriction Cognition Longevity Spontaneous alternation Strength Coordination

RESEARCH over the past 15 years has consistently demonstrated that animals given a restricted diet (CR), one that is nutritionally equivalent but calorically reduced (25% to 65%) compared to the diet consumed by animals being fed ad lib, have maximum life spans that are as much as 65% longer than those of animals fed ad lib (3,25,26,28). Contributing to the increased longevity is a reduced incidence of senescence-associated diseases and deceleration of age-related changes in biochemical, physiological, and immunological processes (6,13–15,19,23,29). Finally, some age-related behavioral and cognitive changes are decelerated by CR. A CR initiated at weaning decreases age-related declines in stereotyped motor responses (12), motor coordination (rotorod) (11), and learning in complex (9,11) and radial arm (10) mazes. Rats maintained on restricted diets that do not control for nutrition show normal age-related deficits on cognitive tasks (1,2).

When CR is initiated at mid-life, it produces many of the same effects as CR begun at weaning. For example, CR initiated in mice at 12 months results in increased maximum life span, inhibited spontaneous lymphoma, increased percentage of T-cells, and increased lymphocytotoxic response to alloantigens (20,27,28). We are not aware of any studies evaluating the effects of mid-life onset CR on cognitive functioning.

The purpose of the present experiment is to examine the effects of CR initiated at mid-life on longevity and behavior. Thus, mice placed on CR at 14 months of age were tested on a battery of behavioral tests beginning at either 22 or 25 months. They were compared with mice maintained on a control diet that were tested at either 13 or 22 months.

METHOD

Subjects

Eighty 11-month-old, male C57BL/6 mice (NIA) that had been maintained on standard laboratory chow were obtained from Charles River Labs. The mice were individually housed in plastic cages in a room maintained at 22 ± 2°C and a 16/8, light/dark cycle (lights on at 0700 h). The mice had free access to water. Within 2 months, 28 mice were euthanized following spontaneous development of skin lesions (8,30). Fifty male C57BL/6 mice (NIA) cohorts of the previous mice were obtained when the mice were 13 months old. Throughout this study each mouse that developed a skin lesion was euthanized.

When the mice described above were 21 months old, 15 11-month-old mice from the same source and strain (NIA) were obtained to be used as middle-aged controls.

Diet Conditions

Two diets (11) were used. The diets are approximately isocaloric by weight and nutritionally equivalent except for amount of carbohydrate calories. The high-calorie control diet provided 3.44 kcal/g, and the low-calorie experimental diet provided 3.54 kcal/g. When the mice were 14 months old, two random groups, each containing 47 mice, were formed, and the control diet was administered to all mice on an ad lib feeding schedule. The amount eaten by each animal was monitored for 1 week. Mean food consumption was 42.63 g/week. Subsequently, the control diet administered to a group of mice designated AC was 95% (40.5 g/week) of the amount eaten during the ad lib feeding period. A group of mice designated AE was changed from the control diet to the experimental diet, and the amount of diet

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was reduced in two steps. For 2 months, AE mice received 80% (34 g/week) of the amount eaten during the ad lib feeding period, and then the experimental diet was reduced to 60% (26 g/week) of the amount eaten during the ad lib feeding period.

When AC and AE mice were 21 months old, 15 11-month-old mice, designated MC, were placed on the diet administered to AC mice.

When AC and AE mice were 22 months old, and MC mice were 12 months old, behavioral testing began for all AC and MC mice and a group of AE mice designated AE22. The AE mice not tested when 22 months old were designated AE25 and were tested when 25 months old.

Behavioral Tests

The mice were compared on a battery of behavioral tests that included wire hanging and platform walking (day 1), spontaneous alternation (SAB) in a Y-maze (days 2–4), plus maze escape (days 5–7), Morris water maze (days 8–14), and win-stay water-escape in an M-shaped maze (days 15–35). To reduce emotional responsiveness, each subject was handled for 2 min each day for 3 days (days 36–38) and then the mice were retested for SAB in the Y-maze (days 39–41). Mice that survived behavioral testing and remained healthy were maintained on their respective diets to enable continued measurement of longevity.

Platform Time. Time on platform (to a maximum of 120 s) before falling or escaping was measured. The platform was a long (60 cm), narrow (2.0, 1.3, or 0.7 cm wide), rectangular piece of wood between a wall and a wide escape platform (9 cm × 28 cm). The platform was 30 cm above a foam cushion. Testing consisted of placing the subject on the end of the narrow platform opposite the escape platform with the subject's head facing the escape platform.Each subject was tested, respectively, on 2.0-, 1.3-, and 0.7-cm platforms. The interval between successive tests was approximately 5 min.

Wire Hang. An 18-ga wire 30 cm above a foam cushion was used to measure length of time a subject hanged from the wire with forepaws before falling. Holding a subject's skin at the back of the neck between the thumb and index finger, the experimenter picked up and held the subject in front of the wire until the subject held the wire with its forepaws. The experimenter then released the subject and measured hang time. Hang time was measured three times with an intertrial interval (ITI) of approximately 60 s.

Spontaneous Alternation. A Y-maze having sides 20 cm high and arms 8 cm wide and 45 cm long was used to measure SAB and amount of locomotor activity. The maze was painted gray and rested on newspaper. The location and orientation of the maze remained constant throughout all testing. Testing consisted of placing a mouse at the distal end of one arm (the same arm was used for all tests) and noting the pattern of arm changes. An arm change was recorded when the subject left one arm and entered another arm with all four feet; an alternation was recorded each time three consecutive arm changes included each of the three Y-maze arms. Subjects were tested 5 min/day for 3 consecutive days.

Plus Maze. A plus maze with two open arms painted white intersecting with two enclosed arms painted black was elevated 50 cm above a foam cushion. The arms were 20 cm long and 8 cm wide, and the sides of the enclosed arms were 18 cm high. Testing consisted of placing a mouse at the distal end of an open arm (the same arm was used for all tests) with the mouse facing away from the center of the maze. The interval between placement of the mouse on open arm and the mouse entering a closed arm with all four feet was recorded as escape latency, and the interval between entering and exiting a closed arm was recorded as dark duration. The maximum dark duration before removing a subject from the apparatus was 300 s. The mice were tested one trial/day for 3 days.

Morris Water Maze. A circular metal tank, 61 cm in diameter by 27.5 cm high, was filled with water to a depth of 14.75 cm. The tank was subdivided into quadrants by small marks placed on the perimeter. An escape platform, 9 cm in diameter and 0.25 cm below the water surface, was located in the center of one quadrant. The location and orientation of the tank and the location of the escape platform remained the same for all tests. Nontoxic white paint was added to the water to make the platform invisible. Each test trial consisted of placing a subject on the periphery of a randomly determined quadrant not containing the escape platform. The interval between placement in water and escape onto platform was recorded as escape latency. Subjects that did not escape were removed from the water at the end of 120 s. Subjects were tested three trials/day for 6 consecutive days. The ITI was approximately 5 min.

M Maze. An M-shaped maze, described previously (17), was used to test win-stay, water-escape behavior. The M shape resulted from a start section (22 cm × 12.5 cm) joined at a T-junction to two L-shaped choice sections. Each choice section consisted of a 54 cm × 8 cm choice alley originating at the end of the start section and a 22-cm × 12.5-cm escape section that was parallel to the start section. A circular escape platform, 9 cm in diameter and 0.25 cm below the surface of the water, was placed at the terminus of the escape section of the appropriate choice section. For a mouse to escape in the right choice section, he had to make two 90° right turns. Likewise, to escape in the left choice section he had to make two 90° left turns. The maze was placed in a dark room with a 7.5-W bulb mounted above the left choice section.

The mice were first given two adaptation trials per day for 2 days. On the first adaptation trial each escape section contained an escape platform. On the second adaptation trial the escape platform was removed from the escape section chosen on the first trial. When a mouse failed to escape within 120 s, the mouse was manually guided toward the nearest escape platform. Daily testing consisted of one information trial followed by three test trials. The escape platform remained in the same choice section. For the information trial a barrier was used to prevent the subject from entering the choice section of the maze not containing the escape platform. The barrier was removed during test trials. Choice and escape latency were recorded. When an animal climbed onto the escape platform or failed to escape within 120 s, the animal was removed from the maze. The ITI was approximately 5 min. The day-to-day and subject-to-subject location of the escape platform was semirandomly changed to interfere with place learning and use of odor trails (16).

Statistical Analyses

Unless otherwise stated, one-way independent-factors analyses of variance (ANOVA) were used to compare the groups on each of the measures. Significant main effects were further examined with *t*-tests.

RESULTS

Longevity

The CR initiated at mid-life increased longevity. Figure 1 shows longevity curves for groups AE and AC. Note that the initial change in diet resulted in the loss of more AE mice than AC mice, but that after 19 months, the survival rate of the AE

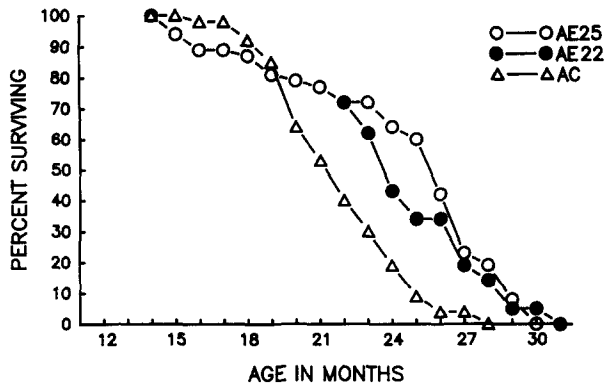

FIG. 1. Percentage of mice on reduced calorie and high-calorie diets surviving at the end of each month. At 22 months the reduced calorie mice (AE) were divided into two groups for subsequent behavioral testing beginning at either age 22 months (AE22) or 25 months (AE25). As is apparent in the figure, behavioral testing resulted in an accelerated death rate for all groups.

mice began to increasingly exceed that of the AC mice. Also, note that whenever mice were being behaviorally tested the percentage of survivors dropped rapidly. Percentage of survivors decreased rapidly in groups AE22 and AC between ages 22 and 24 months, and in group AE25 between ages 25 and 27 months. In fact, between ages 22 and 24 months, when the AC and AE22 mice were being behaviorally tested, six of 15 (40%) of AE22 and 10 of 19 (53%) of AC mice alive at the beginning of testing died, whereas only two of 19 (11%) of the AE25 mice, who were not yet being tested, died. A higher percentage of mice in the two groups being tested combined (AE22 and AC) died than in the group not being tested (AE25), $\chi^2(1) = 5.72$, $p < 0.025$. Later, during the 2 months that AE25 mice were being tested and AE22 and AC mice were no longer being tested, 10 of 16 (62%) of the AE25 mice died, whereas only three of seven (43%) and two of four (50%) of the AE22 and AC mice died, respectively. At this age, there were so few subjects still alive and the death rates were so high in all groups the difference in percentage of mice dying in the tested group vs. the two groups not being tested was not significant, $\chi^2(1) = 0.23$, $p > 0.05$.

The longest surviving AC mouse died at age 877 days, whereas the longest surviving AE mouse was sacrificed at age 953 days, when he became moribund. Complete necropsies were not performed. However, the circumstance of each death was noted (see Table 1). Animals were sacrificed whenever they developed the aforementioned skin lesions, developed middle-ear disease, or became moribund. A greater proportion of the AC deaths was due to skin lesions than was the AE deaths, $\chi^2(1) = 11.43$, $p < 0.01$.

Behavior

General Observations. Whenever an experimenter entered the vivarium, usually to feed or weigh the subjects, it was apparent that the AE animals were more active. Whereas the control animals showed little activity, the AE mice were observed to run around the cage and climb onto and hang from the wire cage tops throughout their life spans. In fact, the longest surviving AE mouse was observed hanging from the top of his cage only 3 days before he became moribund. Never was an AC mouse observed to hang from the cage tops.

Wire Hanging. Because hang time is affected by weight of the subject, a regression equation was used to adjust the mean hang times. Figure 2 shows the original and weight-adjusted mean hang times over the three trials for all four groups of mice. The original hang times differed, $F(3, 56) = 6.40$, $p < 0.01$. Groups AE22 ($p < 0.01$), AE25 ($p < 0.01$), and MC ($p < 0.05$) had greater mean hang times than AC. The AE22 mice also had a greater mean hang time than MC ($p < 0.05$). Thus, the AE mice had longer hang times than the AC mice, and the AE22 mice even had longer hang times than the MC mice. An analysis of covariance of the same data using weight as the covariate failed to produce a significant effect, $F(3, 55) = 2.08$, $p > 0.10$. Together, the two analyses suggest that the increased hang time of the AE mice was largely due to their weight loss.

TABLE 1

CIRCUMSTANCE OF DEATH OF AE AND AC MICE

	AC		AE	
Death Circumstance	*n*	*p*	*n*	*p*
Found dead in cage	19	0.40	32	0.68
Sacrificed				
Lesion	20	0.42	4	0.09
Middle ear	1	0.02	1	0.02
Moribund	6	0.13	9	0.19
Other	1	0.02	1	0.02

Platform Time. The narrow (0.7 cm) platform proved to be the most sensitive test of balance and coordination. Figure 3 shows the mean time that each group stayed on the narrow platform. The statistical comparison revealed a significant group effect, $F(3, 54) = 3.49$, $p < 0.05$. Group AC had a shorter platform time than groups MC ($p < 0.01$), AE25 ($p < 0.05$), and AE22 ($p < 0.05$), which did not differ significantly from one another. Thus, the combination of age and the control diet resulted in less time on the platform.

Spontaneous Alternation. The mean percentage of spontaneous alternation (percentage of times that any three successive choices involved entering all three alleys of the Y-maze) for all groups on the tests given before and after water maze training is shown in Fig. 4. Because animals in each group died between the two tests, the tests were analyzed separately. An analysis of the premaze training tests revealed no significant differences

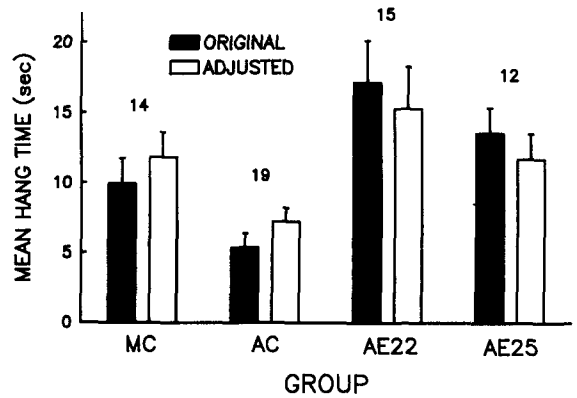

FIG. 2. Mean ± SEM original and weight-adjusted hang times for the four groups. Numbers above the bars are group *n*s. Groups AE22 ($p < 0.01$), AE25 ($p < 0.01$), and MC ($p < 0.05$) had greater mean original hang times than group AC.

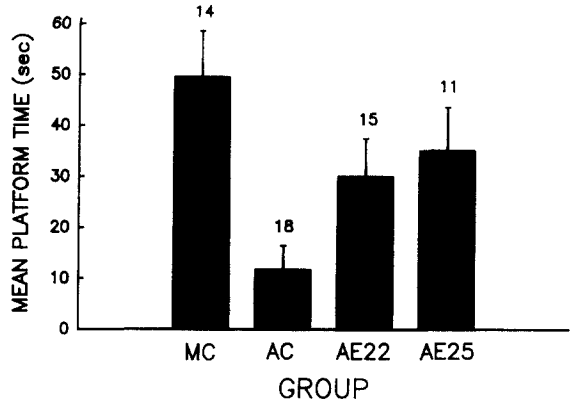

FIG. 3. Mean ± SEM time that each group remained on the 0.7-cm wide platform. Groups MC ($p < 0.01$), AE25 ($p < 0.05$), and AE22 ($p < 0.05$), which did not differ significantly from one another, stayed on the platform longer than group AC.

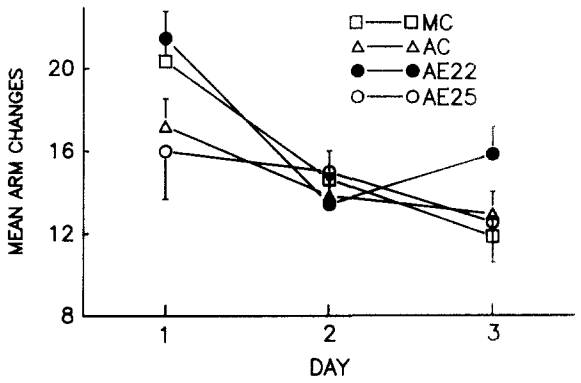

FIG. 5. Mean ± SEM daily arm changes made by mice in each of the groups on the first 3 days of testing in the Y-maze. On the first day AE22 made more arm changes than AC ($p < 0.05$) and AE ($p < 0.05$).

among the groups, $F(3, 55) = 0.98$, $p > 0.05$. Because in the second test group AE25 had only five surviving members, including one mouse that appeared ill, and had such high variability, group AE25 was not included in the postmaze training analysis. The remaining three groups differed from one another, $F(2, 31) = 4.59$, $p < 0.05$. Group AE22 had a higher rate of spontaneous alternation than either groups MC ($p = 0.051$) or AC ($p < 0.05$). From Fig. 4, it appears that the significant difference on the second test was due to the AE22 mice increasing their rate of spontaneous alternation following water maze testing, whereas the MC and AC mice did not.

Figure 5 shows the mean number of arm changes made by each of the groups on each of the 3 days of testing. Analysis revealed a significant group × day interaction, $F(6, 106) = 3.455$, $p < 0.01$. On the first day of testing, the mean number of arm changes was greater for AE22 than for AC ($p < 0.05$) and AE25 ($p < 0.05$) mice. Other differences were not significant.

Plus Maze. The mean ± SEM latencies across the 3 days of testing for the MC, AC, AE22, and AE25 mice to escape the open white arms of the plus maze were 25.1 ± 5.0, 28.8 ± 2.7, 27.1 ± 3.3, and 23.3 ± 4.0 s, respectively. These means are not significantly different, $F(3, 53) = 0.386$, $p > 0.05$. Figure 6 shows the mean log duration that the mice stayed in an enclosed black maze alley once they had escaped the open white alleys on each of the 3 days of testing. A two-way (group × day) mixed-factors ANOVA resulted in significant group, $F(3, 53) = 3.62$, $p < 0.05$, and day, $F(2, 106) = 14.89$, $p < 0.001$, main effects and a significant interaction, $F(6, 106) = 3.19$, $p < 0.01$. Group AE25 remained in the dark alley for a shorter duration than groups MC ($p < 0.01$) and AC ($p < 0.05$). Although group AE22 had a shorter dark alley duration than did the two control groups, the differences were not significant.

Morris Water Maze

Group AE25 was not tested in the Morris water maze. Several of the mice in each group (the proportion was nearly the same in all groups) stopped swimming, remaining nearly motionless, before reaching the escape platform. Data from mice that failed to reach the platform within the allotted 120 s on 2 or more days were excluded from further analysis. Also, to reduce within-cell variance, the daily latencies were converted to common log scores. Figure 7 shows the mean log latencies of the remaining mice in each of the three groups. A two-way mixed-factors ANOVA (group × day) resulted in significant group, $F(2, 24) = 5.77$, $p < 0.01$, and day, $F(5, 120) = 14.81$, $p < 0.001$, main

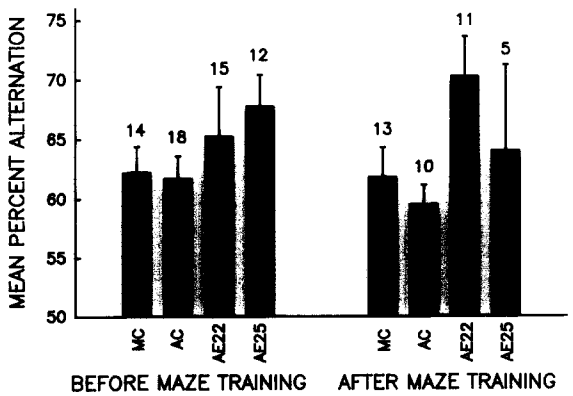

FIG. 4. Mean ± SEM percentage spontaneous alternation responses in Y-maze on tests given before and after maze training. On the postmaze training test, group AE22 had a higher rate of spontaneous alternation than either groups MC ($p = 0.051$) or AC ($p < 0.05$).

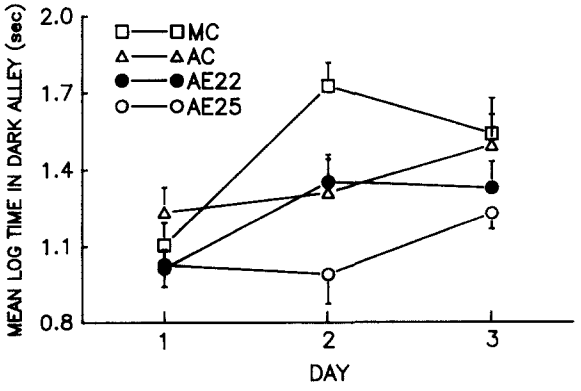

FIG. 6. Mean ± SEM time (s) that mice in each of the groups remained in the black enclosed alley of the plus maze after having escaped the open white alleys. Group AE25 remained in the dark alley for a shorter duration than groups MC ($p < 0.01$) and AC ($p < 0.05$).

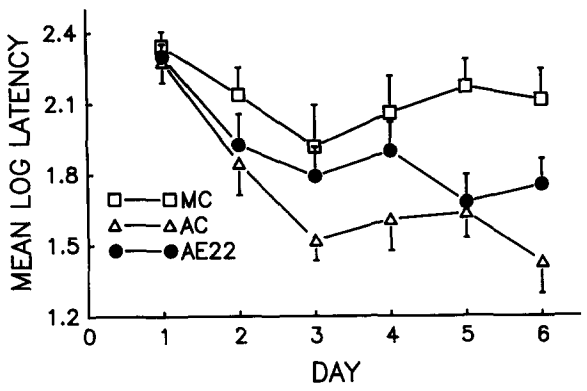

FIG. 7. Mean ± SEM log of latency to escape across the six trials in the Morris water maze. Mice that failed to escape within the allotted 2 min on two or more trials were excluded. Group AC has a shorter mean log latency than did group MC ($p < 0.01$).

effects. The mean ± SEM log latencies over all days for MC, AC, and AE were 2.12 ± 0.09, 1.72 ± 0.07, and 1.89 ± 0.08, respectively. Group AC had a shorter mean log latency than did group MC ($p < 0.01$). Group AE did not differ from either of the control groups. As is apparent from Fig. 7, the day effect was due to the fact that collectively the mice had decreasing mean log latencies over days.

Win-Stay Water-Escape. Four of nine AE25 mice appeared weak and/or ill during acquisition training and died before retention testing could be completed. All nine AE25 mice were included in the acquisition analysis, but the subject-depleted group AE25 was not included in the retention analysis. Figure 8 shows the mean percentage of correct responses made by each of the four groups during acquisition training and by MC, AC, and AE22 during retention testing on the win-stay water-escape task. There was a significant group effect on acquisition, $F(3, 45) = 8.25$, $p < 0.001$. Group AE25 made a lower percentage of correct responses than each of the other three groups ($p < 0.05$ in each case), which did not differ from one another. The poor performance of the AE25 mice is almost certainly related to the poor health of many the subjects in the group. The group effect was not significant on the 15-min retention tests.

DISCUSSION

Consistent with previous research, CR increased longevity. Throughout their life span, AE mice appeared to sleep less and be more active when awake than AC mice. The AE mice had fewer skin lesions than AC mice, and AE22 mice survived the stress of behavioral tests better than AC mice. Death rate during behavioral testing was high for AE25 mice.

The AE22 and AE25 mice had a longer hang time than AC mice, and AE22 mice had a longer hang time than MC mice. There are at least two possible reasons for the longer hang time of the AE mice:

1. Ratio of lean body weight to fat weight. The AE, AC, and MC mice may have been similar in lean body weight, with the heavier body weight of AC and MC mice, as compared to AE mice, being due, primarily, to amount of body fat.
2. Exercise. The AE mice were observed to frequently hang from the wire top of their home cage; neither AC nor MC mice were ever observed to hang from the wire top of the home cage.

Balance time on a narrow platform was longer for AE and MC mice than AC mice; the balance time of AE mice did not differ from the balance time of MC mice. These results clearly suggest that the balance and coordination necessary for this task were preserved by the low-calorie, nutritious diet.

Two tests of spontaneous alternation (SAB) were made. The percentage of SAB was greater for AE mice than for AC and MC mice on both tests, but the difference was statistically significant only for the second test, where AE22 mice had a higher percentage SAB than the other groups. The AE25 mice were extremely variable on the second test (a common characteristic of aged subjects), and they were not significantly different from AC and MC mice. Lack of statistical significance on the first test may have been due to the emotionality of the subjects (5). The day before the first SAB test the mice were subjected to wire hanging and platform tests without previous handling and gentling. The mice were handled and gentled before the second SAB test.

In the plus maze test, AE, AC, and MC mice did not differ on escape latency from an open lighted arm to a closed dark arm; however, the time that the mice stayed in the closed dark arm was shorter for AE mice than for MC and AC mice. The shorter dark duration of AE mice may have been due to the AE mice having a lower level emotionality and/or a higher level of activity.

The AE mice did not perform better than AC or MC mice on the water maze tasks. The AC and MC mice weighed more and appeared to have a greater amount of body fat than AE mice. If MC and AC mice did have more body fat than AE mice, MC and AC mice would have greater buoyancy and thermal insulation in the water mazes than AE mice. The high death rate among the aged mice during testing in the water maze indicated that these tests were stressful for the aged mice.

Forster and Lal (7) have discussed a possible pitfall in experiments designed to study the long-term effects of CR upon aging. These authors state that the short-term (i.e., immediate) effects of CR (e.g., change in arousal, exploratory behavior, learning, and memory processes) may persist through a long period of CR. Thus, behavioral differences between calorically restricted and nonrestricted animals in a longitudinal study may be due to the short-term (i.e., immediate), rather than long-

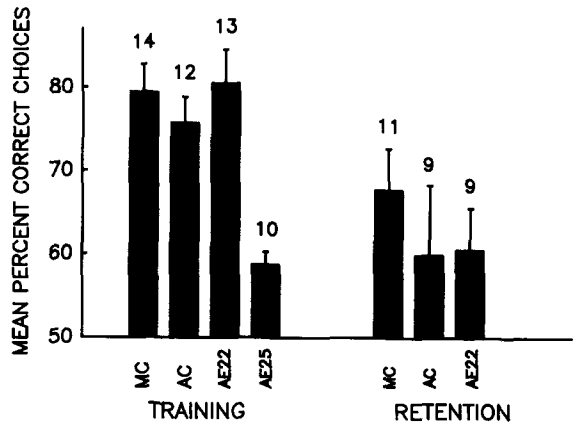

FIG. 8. Mean ± SEM percentage of correct test run choices during the original 18 acquisition trials and the six 15-min retention trials on the win-stay water-escape task. Due to poor health and a small n, group AE25 was not included in retention. On acquisition, group AE25 had a lower percentage of correct choices ($p < 0.05$ in each case) than all other groups, which did not differ from one another.

term, effects of CR. Since several studies suggest an inverse relationship between drive level and SAB (4,18,21,22), this criticism is probably not relevant with respect to SAB. Since SAB requires sensing, perceiving, storage, and retrieval of information about the environment (22), the results of the SAB tests indicate that these important cognitive functions were preserved by the low-calorie, nutritious diet.

While the present study introduced CR in the mid-life of mice, most previous studies have introduced CR early in life—usually at the time animals are weaned. There are two important reasons for continued study of the effects of mid-life onset CR. First, while a nutritious but restricted calorie diet introduced at weaning does not reduce adult brain weight, it does reduce adult body weight, including lean body weight (24). Should CR be shown to prolong human life and cognitive functioning, many parents would be reluctant to initiate a diet in infants that would result in a reduced adult size. Second, many of us who may wish to extend our lives and cognitive abilities are well beyond the age of weaning.

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