THE EFFECTS OF AGING ON NON-ASSOCIATIVE LEARNING
IN THE NEMATODE \textit{CAENORHABDITIS ELEGANS}

by

CHRISTINE DAILY O'BRIEN BECK

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Department of Psychology

The University of British Columbia
Vancouver, Canada

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With the advantages of simplicity and a well-understood anatomy, development and genome, *C. elegans* may be an effective model of the role of the genome in the effects of aging on learning and memory. The purpose of this thesis is to begin this research by describing the effects of aging in *C. elegans* on a simple form of learning, habituation, in the tap withdrawal reflex.

First, the effects of aging on the spontaneous locomotor behavior and simple reflexive behavior of *C. elegans* were examined. Worms were tested at 4, 7 and 12 days post-hatching. The average life-span of worms raised in the conditions of this laboratory (solid medium, 21° C) was 14 to 16 days. The amount of spontaneous activity did not change with age, but the nature of that activity did change. Worms moved more slowly and both spontaneous and reflexive reversals decreased in magnitude at day 12. Worms at all ages exhibited graded responses to taps of different intensities.

The effects of aging on habituation and dishabituation were then examined. There appeared to be a dissociation of response frequency and magnitude: all ages tested (4, 7 and 12 days post-hatching) showed similar changes in magnitude of reversals due to habituation and dishabituation. However at day 7 the proportion of worms reversing did not decrease during habituation training as it did at the other ages (days 4 and 12) tested. There was also an age-related change in the recovery from habituation; day 12 worms did not recover within 30 min of the
last habituation stimulus, unlike worms tested at day 4 and 7 which recovered back to baseline levels by 30 min.

Finally the effects of tail-touch habituation training on inhibition of the reversal response to tap was examined at the three test ages. At all ages tail-touch habituation training decreased the inhibition of reversal to tap by tail-touch. Clearly, even day 12 worms are capable of habituation independent of fatigue effects. The age-related changes seen may be produced by

From these experiments it is clear that although the behavior of C. elegans does change with age, aged worms are capable of the simple form of learning, habituation. Further behavioral tests with normal and mutant worms may help elucidate the nature of the age-related changes in learning and memory in C. elegans and the genetic mechanisms which underlie them.
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Introduction

The loss of learning and memory abilities with advanced age has been the subject of study in many species. The objective of this research is to understand the effects of aging in one species at behavioral, neural and genetic levels. Changes in behavior as an organism ages must reflect changes in cell physiology. Much research to this point has been concerned with the link between changes in specific behaviors and the changes in neuronal function which may produce them. However, as work in molecular biology has indicated, changes in the function of cells such as neurons must in turn reflect changes in gene expression. Thus a model of the effects of aging on learning and memory in which as many of these levels may be approached at once is desirable.

The complexity of the mammalian nervous system, containing many millions of neurons, makes it difficult to understand the effects of aging at a neuronal level. One solution to this problem of nervous system complexity is to use a model system. The key to this type of research is investigating the relationship between behavior and identified neurons in an organism with a simple nervous system. An example of this approach is the marine mollusc Aplysia californica, in which the neurophysiological function of identified neurons in circuits controlling the behaviors of gill and siphon withdrawal are known. Studies on aging in Aplysia have found losses in learning and memory. Rattan and Peretz (1981) showed that the threshold for behavioral response to gill
stimulation was significantly higher in old Aplysia than in young, mature animals. Furthermore they found that old Aplysia habituated more rapidly than the younger animals. In addition, these old animals did not exhibit dishabituation when a neuron (L7) identified as one that produces dishabituation in younger animals was stimulated. Based on these results, Peretz (1989) suggested that individual neurons age differentially, affecting the behaviors they underlie in different ways. Thus he proposed that some behavioral processes might be expected to change with age and others not.

The studies with Aplysia have advanced our understanding of some of the physiological processes underlying aging. However, if we are to understand how aging is controlled at the level of the genome we must use models in which the genetics can be understood as in as much detail as possible.

*Caenorhabditis elegans*, a small free-living (non-parasitic) nematode has been widely used as a genetic model in the study of anatomy, development and behavior. Because the neuroanatomy of *C. elegans* is simple, containing only 302 neurons, the relationship between gene and neuron function may be narrowly determined. The neuroanatomical map of this nematode has been described completely (White, Southgate, Thompson, & Brenner, 1986; Chalfie, 1984). Furthermore, the functions of neural circuits underlying certain behaviors such as the touch withdrawal circuit have been demonstrated, establishing the link between behavior and anatomy (Chalfie, Sulston, White, Thompson, Southgate, & Brenner, 1985;
C. elegans is a very simple multicellular organism: at maturation, about 3 days after hatching, the worm has approximately 1000 somatic cells. The complete developmental lineage of each somatic cell has been mapped using Nomarski microscopy on living worms (Sulston, Schierenberg, White, & Thompson, 1983). In addition, the genetics of this nematode are relatively simple, with only $8 \times 10^7$ nucleotide pairs (approximately half the size of the genome of Drosophila) in six haploid chromosomes (Sulston & Brenner, 1974; Nigon, 1949). The combined efforts of many laboratories using a variety of classical and molecular genetic techniques have produced a map of over 95% of the C. elegans genome (Coulson, Sulston, Brenner, & Karn, 1986; Hodgkin, Edgley, Riddle, & Albertson, 1988). C. elegans exists primarily as a hermaphrodite, producing both eggs and sperm and reproducing by self-fertilization. This mode of reproduction allows true breeding of mutants. However, mutants with only male reproductive systems occur at a low rate (Hodgkin, Horvitz, & Brenner, 1979; Rose & Baillie, 1979); these males mate with the hermaphrodites and fertilize the hermaphrodites' eggs. Thus both homozygous and heterozygous offspring can be produced. Many mutants have been isolated. These mutant lines are preserved by freezing the larva in liquid nitrogen. Characterized mutant worms are available from a central library of mutants in the Caenorhabditis Genetics Center at the University of Missouri, Columbia (Hodgkin, Edgley, Riddle, & Albertson, 1988).
This body of background knowledge on the anatomy, development and genetics of *C. elegans* make it an excellent candidate model system for the study of biological mechanisms underlying any process. However, if *C. elegans* is to be used as a model system for the study of age-related deficits in learning and memory, there must be evidence that *C. elegans* expresses a variety of observable behaviors and learning processes.

Like its anatomy, the behavior of *C. elegans* is simple yet varied enough to provide a broad range of possible areas of study. *C. elegans* locomotes by producing rhythmic coordinated contractions of the ventral and dorsal muscles. These contractions cause undulatory movements down the length of the body in the dorsoventral plane. When moving on a firm surface such as agar, the worms lie on their sides. They can move their heads laterally and dorsoventrally and can rotate their bodies over 180 degrees. The worm responds to a number of stimuli including touch and vibration by changing direction and swimming backward for some distance (Sulston et al., 1975; Chalfie & Sulston, 1981; Rankin, Beck, & Chiba, 1990). *C. elegans* can also move up or down gradients of chemical concentration, osmolarity, and temperature (Ward, 1973; Hedgecock & Russel, 1975). Mature worms of each sex have specific sexual behaviors: the hermaphrodites lay eggs (already fertilized with their own sperm), whereas the males (which produce only sperm) engage in a complex set of mating behaviors when they come in contact with an adult hermaphrodite (Wood, 1988).
Sensory-motor behaviors in *C. elegans* mediated by the touch withdrawal reflex circuit are particularly well understood (Sulston et al., 1975; Chalfie & Sulston, 1981). When touched on the tail with a small hair, the worm responds by moving forward; to a light touch across the head, it moves backward. On the basis of electron microscopic reconstructions and the analysis of laser ablations and nervous system mutants, Chalfie and colleagues (1985) proposed a simple reflex model for tail touch induced forward locomotion and for head touch induced backward locomotion. They determined that the touch circuits consist of touch receptors connecting to interneurons which in turn connect to motorneurons.

Recent work on the behavioral plasticity expressed by this organism has confirmed that *C. elegans* has a rich repertoire of learning processes (Rankin & Chiba, 1988; Rankin, Beck, & Chiba, 1990; Kumar, Williams, Culotti, & van der Kooy, 1989). Rankin, Beck and Chiba (1990) showed that the tap withdrawal reflex in *C. elegans* exhibits the major forms of non-associative learning: habituation, dishabituation, and sensitization. The advantage of working with the tap withdrawal reflex is that the touch withdrawal circuit, defined by Chalfie and colleagues (1985), has been shown to be responsible for the tap withdrawal reflex as well (Rankin & Chalfie, 1989). With repeated presentations of a single tactile stimulus such as a single tap or train of taps, the reversal response habituates. After habituation training, the reversal response recovers over about 20 to 30 min to baseline levels (Rankin & Broster, 1990). The presentation of a novel or
noxious stimulus such as a 60 V shock immediately after the habituation of the reversal response produces dishabituation or a partial recovery to approximately 50% of the baseline response rate (Rankin, Beck, & Chiba, 1990; Rankin & Broster, in press). In a naive worm, presentation of a strong stimulus (a train of taps) produces a higher than baseline response to a single tap; the response to tap is sensitized (Rankin, Beck, & Chiba, 1990). Finally, memory for extensive habituation training lasts for at least 24 h, showing that *C. elegans* is capable of long-term memory (Rankin, Beck, & Chiba, 1990). Work by van der Kooy and his colleagues (Kumar et al., 1989) indicates that *C. elegans* may be capable of associative learning in a taste-approach/avoidance paradigm. However, the neural circuit underlying taste-related behaviors has not yet been defined.

Thus it is clear that *C. elegans* possesses a range of learning and memory capabilities, making it a promising candidate for the investigation of the biological mechanisms underlying age-related deficits in learning and memory.

The first step in the behavioral analysis of aging in *C. elegans* is to determine the normal effects of aging on the simple non-associative forms of learning already described in *C. elegans*. Thus, the purpose of the experiments described here was to define the effects of age first, on baseline activity levels, second, on the tap withdrawal reflex and finally, on two forms of non-associative learning, habituation and dishabituation, expressed by the tap withdrawal reflex. In studying habituation
and dishabituation, I attempted to isolate the age-related changes by approaching the question of the effects of habituation training from several different perspectives. First, I examined the dynamics of habituation itself, and the appearance and extent of dishabituation. Next, I examined how recovery from habituation changes during aging. Finally, I examined how aging affects the way that habituation training interacts with response competition or inhibition.

General Methods

Subjects

Mature hermaphroditic *C. elegans* (strain N2 from Bristol, England) were used throughout these studies. When grown in liquid culture at 20°C, the life-cycle of *C. elegans* is approximately 21 days (Woods, 1988); however, in the conditions maintained during these experiments (solid medium, 20°C) the average lifespan was 14 to 16 days. Tests of behavior were performed at 4, 7 and 12 days post-hatching. At 4 days post-hatching worms are at the peak of egg-laying, at 7 days egg-laying is complete, and by 12 days post-hatching worms are well into the post-reproductive period.

Materials

Worms were maintained on Nematode Growth Medium (NGM) agar-filled Petri plates (5 cm diameter) and fed *Escherichia coli* (strain OP50) which was streaked or spotted on the surface of the agar. Subjects were maintained and tested with the methods and apparatus used in previous behavioral studies (Rankin, Beck, &
Behavioral observations were made through a stereomicroscope with attached videorecording equipment. Mechanical and electrical stimulation were controlled by a Grass S88 stimulator. Vibrational stimuli were produced by a mechanical tapper controlled by an electromagnetic relay; the mechanical tapper tapped the side of the Petri plate holding the worm. Electrical shocks were produced using a spanning electrode; the two wires were placed on either side of the animal on the surface of the agar approximately 2 mm apart. Each shock stimulus consisted of a train of shocks (each shock was 10 ms in duration) delivered over 600 ms at a frequency of 10 Hz.

**Procedure**

The hermaphroditic *C. elegans* that were used in these studies were hatched synchronously. To ensure synchrony, mature egg-laying worms were selected from the general population and were placed on an agar plate streaked with *E. coli*. These worms were permitted to lay eggs for 3 to 4 h and were then removed from the plate. The eggs hatched in 9 to 11 h. At 3 days post-hatching (72 to 84 hours) the maturing worms were placed individually on numbered agar plates spotted with *E. coli*. In experiments where individual worms were followed throughout post-reproductive development, the worms were tested at three chosen ages: 4, 7 and 12 days. Tests were performed on plates without food. After each test, the worms were individually placed on new plates with fresh *E. coli* to ensure a consistent food source and to prevent confusing the subjects with their offspring.
In experiments where worms were tested at only one of the test ages, worms were plated individually and raised to that test age. Worms were replated approximately every 2 days to maintain isolation.

Behavioral responses were scored in several ways depending on the experiment. The reversal response (swimming backward) to vibrational stimuli (taps) was the chief dependent measure in these experiments. In order to have been considered a reversal to tap, the response must have occurred within 1 s of the stimulus. Any visible backward movement (≥ .02 mm) was considered a reversal and was scored. Both the frequency and the magnitude of these reversals were scored. The magnitude of the reversal responses were quantified by tracing the reversal paths on acetate sheets. These tracings were then digitized using a Bit Pad Plus digitizing tablet, and the digitized tracings were measured using MacMeasure software on a Macintosh SE personal computer. Statistical analysis of proportion data was performed with a Cochran Q test when the design involved repeated measures; otherwise proportion data were analyzed with chi-squared tests. Magnitude data were analyzed using ANOVAs with Fisher's post-hoc comparisons when statistical significance was achieved. The level of significance was set at alpha = .05 unless the performance of multiple tests made it necessary to adjust the alpha level downward.
Experiment I. Spontaneous locomotor activity

In order to determine whether there were changes in the baseline activity during aging that might affect the expression of plasticity by the tap withdrawal reflex, the level of spontaneous locomotor activity of the worms during late (post-reproductive) development was measured. The measures of spontaneous locomotor activity were 1) time spent active (any forward or backward swimming movement was considered activity) over a 10 min observation period 2) the velocity of spontaneous swimming and 3) the number and magnitude of spontaneous reversals within the 10-min observation period.

Subjects

The same 21 hermaphroditic worms were tested for spontaneous activity three times, once at each of the test ages: 4, 7 and 12 days after hatching. As the average life-span of the subjects was roughly 14 to 16 days under these conditions and many worms die before this age, 45 worms had to be tested at day 4 (35 at day 7) to obtain data for 21 animals on day 12. These data allowed an investigation of behavioral differences between worms that died young (between day 4 and day 7 or between day 7 and day 12) and those that survived 12 days.

Procedure

Spontaneous locomotor activity was observed over a 10 min period; the time active was determined by measuring the amount of time the worm spent swimming (any visible forward or backward movement). The velocity of movement, measured as the distance
travelled over a specific 10-s interval (from 5 min 0 s to 5 min 10 s in each observation period) was also calculated. The number of spontaneous reversals (swimming backward in the absence of an obvious external stimulus) was scored over the same period. The magnitude of these spontaneous reversals was determined by video stop-frame analysis and computer-aided digitizing of reversal length.

Results and Discussion

A measure of worm length at each test age was made by tracing the worms' magnified image (50 X) from the video screen (see Figure 1). A repeated-measures ANOVA with post-hoc comparisons showed that the length of the worms significantly increased between day 4 and day 7 but not between day 7 and 12 ($F(2,40) = 12.062, p = .0001$). Because of this age-dependent change in size, the reversal magnitudes were standardized by dividing them by the worm's own length at that age so that responses by worms at different ages could be directly compared (Chiba & Rankin, 1990).

The spontaneous velocity and the mean magnitude of spontaneous reversals were found to change with age. However, time active and the number of spontaneous reversals did not change with age (see Figures 2A and B). The time spent active, measured over a 10-min period for each worm at each test age, was analyzed with a repeated-measures ANOVA; no significant difference was found among the test ages ($F(2,40) = 2.675, p = .0812$). However, spontaneous velocity, or the speed with which the worms moved, did change with age. Spontaneous velocity was measured as the total distance
Fig. 1. Worm length +/- SEM (n = 21) magnified 50x and measured from a video screen at 4 days, 7 days and 12 days post-hatching.
WORM LENGTH (mm)

DAY 4

DAY 7

DAY 12
Fig. 2A. The percent time spent active +/- SEM measured over a 10 min observation period at each of the three test ages, 4, 7 and 12 days of age (n = 21).

B. The velocity of spontaneous movement (mm/s) +/- SEM was measured as the distance travelled both backward and forward during 10 s in the 10 min observation period at each of the three test ages, 4, 7 and 12 days post-hatching (n = 21).
Fig. 3A. The number of spontaneous reversals (swimming backward in the absence of any obvious external stimuli) during the 10 min observation period at each of the three test ages, 4, 7 and 12 days post-hatching.

B. Mean spontaneous reversal magnitude was calculated for each worm at each age by taking the mean of the magnitude of all the spontaneous reversals that worm exhibited during the 10 min observation period and dividing it by the worm's body length.
REVERSAL MAGNITUDE / WORM LENGTH

NUMBER OF REVERSALS

4 DAY
7 DAY
12 DAY
travelled in a specific 10 s period (from 5 min 0 s to 5 min 10 s) during the 10 min observation period. Velocity is expressed as distance travelled (mm) / time (s). It was found that at day 12 worms swam significantly more slowly than at day 4 or 7 ($F(2,40) = 23.158; p = .0001$).

Spontaneous reversals were analyzed both in terms of the number of spontaneous reversals each worm expressed and the mean magnitude of the reversals each worm expressed (see Figures 3A and B). The number of spontaneous reversals during the 10 min observation period did not change with age ($F(2,40) = 1.634, p = .2037$). However, the mean magnitude of spontaneous reversals at day 12 were significantly smaller than at day 4 or 7 ($F(2,40) = 16.407, p = .0001$).

Thus there was a decrease in the velocity of spontaneous movement and in the magnitude of spontaneous reversals with age. The possibility that these changes in spontaneous movement are also reflected in the reversal response to vibrational stimulation will be examined in the next experiment.

Experiment 2. Response to tap and head touch.

In addition to the spontaneous reversals described above, worms also show reflexive reversals in response to a variety of stimuli. In previous experiments we have shown that in 4 day old adults, a tap to the dish elicits a withdrawal response of swimming backward called a reversal (Rankin, Beck, & Chiba, 1990). In this experiment, age-related changes in the tap withdrawal reflex were
examined. The proportion and magnitude of the reversal responses to tap were measured; in addition the frequencies of other types of response to tap (accelerations and pauses) were scored. Finally each worm's response to a light touch to the head with a hair was tested. In this context, the head-touch test was administered after the tap test to determine whether the worm was capable of a normal reversal, as all healthy worms reverse to head-touch (Chalfie et al., 1985). Thus, if a worm did not respond to a head-touch with a reversal, it was assumed to be incapacitated and was not included in the results. Although the magnitude of the reversal to head-touch might also change with age it was not scored because the strength of the head-touch could not be controlled because the stimulus was hand-delivered with a fine hair.

Subjects

At 4, 7 and 12 days post-hatching, following the recording of spontaneous activity, worms were tested for their response to tap and head-touch.

Procedure

The single tap and the head-touch were administered immediately following the observation period for spontaneous activity with a 3 min interval between tap and head touch. Head-touch was administered with a fine hair to the head (the region of the pharynx) of the worm. Enough pressure was exerted to bend the hair as it touched the subject's head; pressure from the hair did not damage the worms.
Results and Discussion

Both the frequency and the magnitude of the reversal response to tap were scored (see Figures 4A and B). When the frequency of reversal response was analyzed with a Cochran Q test, no change with age was evident \( Q(2) = 2.8, \ p > .05 \). However, the magnitude of reversal responses to tap, standardized by each worm's length, was significantly smaller at day 12 than at day 4 (there was no significant difference between day 7 and either day 4 or 12; \( F(2,38) = 9.923, \ p = .0003 \)).

The observation that reversals to tap were smaller at day 12 than at day 4 is similar to the finding in Experiment 1 that spontaneous reversals were smaller at day 12 than at day 4. In both cases the magnitude of the reversals changed while the frequency of reversals did not. The decrease in velocity of spontaneous movement, in magnitude of spontaneous and reflexive reversal at day 12 may have been related to these worms' approaching deaths. A number of worms died during these experiments (22% died between day 4 and 7; 31% died between day 7 and 12). By comparing the behavior of worms which died earlier to those that survived to the day 12 test, we may test this hypothesis.

To determine whether deterioration related to an individual's life-span affected the spontaneous and reflexive activity of the worms, the behavior at day 4 of worms that survived to 12 days was compared with the behavior of those that died between day 4 and 7 and with the behavior of those that died between day 7 and 12.
Fig. 4A. The number of worms responding to tap with a reversal at 4, 7 and 12 days post-hatching (n = 21).

B. The magnitude of reversals to tap (including only those worms that responded with a reversal) divided by each worm's body length.
MAGNITUDE REVERSAL /WORM LENGTH

NUMBER OF WORMS REVERSING

DAY 4
DAY 7
DAY 12

DAY 4
DAY 7
DAY 12
In addition, the behavior at day 7 of worms that survived to 12 days was compared with the behavior of those that died between day 7 and 12. Only those behaviors that changed with age (the spontaneous velocity of movement, see Figure 5A; the mean magnitude of spontaneous reversals, see Figure 5B; and the magnitude of the reversal response to tap, see Figure 5C) were examined.

At day 7, worms that died before day 12 moved more slowly than worms that survived to day 12 (day 7: $t(32) = -3.399, p = .0019$). There was no apparent relationship between spontaneous velocity and life-span at the day 4 test (day 4: $F(2,42) = 1.269, p = .2917$). Neither the mean magnitude of spontaneous reversals (day 4: $F(2,42) = 0.355, p = .703$; day 7: $t(33) = 1.656, p = .1072$), nor the magnitude of reversal response to tap was related to time of death at either day 4 or day 7 (day 4: $F(2,42) = 0.355, p = .703$; day 7: $t(33) = 1.656, p = .1072$). Whether the change in spontaneous velocity occurred because of approaching death is not clear; however this possibility must be considered.

One possible explanation for the decrease in response magnitude in 12 day old worms is that there might be a change in sensory ability with age; a lower sensitivity in older worms could produce a decrease in the size of the responses to tactile stimulation. An examination of the reversal reflex in response to a series of taps graded in intensity (beginning with a very weak stimulus) at each of the three test ages might help to clarify this issue.
Fig. 5. The performance of worms that survived until day 12 ( > D12; n = 21) compared with the performance of worms that died between day 4 and day 7 (4D - 7D; n = 10) and day 7 and day 12 (7D - 12D; n = 14). D4 TEST = performance at day 4; D7 TEST = performance at day 7.
A. Spontaneous velocity +/- SEM shown comparing worms with different life-spans.
B. Mean magnitude of spontaneous reversals +/- SEM (divided by worm length) shown comparing worms with different life-spans.
C. Magnitude of reversal response to tap +/- SEM (divided by worm length) shown comparing worms with different life-spans.
Experiment 3. Graded response.

In this experiment, the effects of aging on the responses to taps of different intensities were examined. The tap intensities chosen were ones which, during pilot studies, induced reversals of different magnitudes in 4 day old worms.

Subjects

Twenty naive worms were tested at each age for a total of 60 worms.

Procedure

Worms were placed individually on test plates with a small amount of *E. coli* (strain OP50) 24 h before testing. Tap intensity was altered by changing the voltage from the Grass S-88 stimulator to the electromagnetic relay which controlled the tapper. The objective in selecting the stimuli intensities was to choose ones that consistently evoked reversals, yet covered a range of intensities from the strong tap used in other experiments in this thesis to much weaker taps that might evoke smaller reversal responses. The lowest intensity voltage (38 V) produced a barely perceptable tap, while the highest intensity voltage (60 V) produced a strong tap and was the intensity used in the other experiments in this thesis. An intermediate voltage of 40 V produced a tap of intermediate intensity. The taps were administered individually to worms at 10 min intervals. In studies of recovery from habituation, Rankin and Broster (in press) showed that taps administered at 10 min intervals did not produce significant response decrement in worms that were 4 days
old. Of the 20 worms tested at each age (4, 7 and 12 days post-hatching), 10 worms received the stimuli in ascending order of intensity (38, 40 and 60 V) and 10 worms received the stimuli in descending order of intensity (60, 40 and 38 V). Intensity of stimulation, not order of presentation, was the manipulation of interest here; therefore if possible it would be appropriate to pool the data across order of presentation within each age. However, if the order of presentation (ascending or descending) had an effect on the responses, that would indicate that the data from the ascending and descending orders of presentation should be considered separately. At each age, the reversal responses were compared with a two-factor (Order x Intensity) repeated-measures ANOVA to test for an effect of the order of presentation or an interaction between the order of presentation and stimulus intensity. At the ages at which there was no effect of the order of presentation and no interaction between order and intensity, the data from the ascending and descending orders of presentation were pooled.

The magnitude of the reversals at each age for different intensities were analyzed with a repeated-measures ANOVA. Only those responses that were reversals were included in the analysis of magnitude because the objective of this experiment was specifically to reveal the effect of intensity on the magnitude of reversal response. Non-reversals (pauses, accelerations, no change in behavior or no response: approximately 5% of all responses) were replaced with the mean of the group for the
purposes of the statistical analysis. At each test age, 4, 7 and 12 days post-hatching, the numbers of reversals to taps of different intensities was analyzed with a Cochran Q test.

Results and Discussion

To determine whether the data from the groups which received the stimuli in ascending and descending orders could be pooled together, the response magnitudes from worms that received the stimuli in ascending order were compared with the response magnitudes from those worms that received the stimuli in descending order across the three stimulus intensities at each test age (4, 7 and 12 days post-hatching). To control experiment-wise error rate, the alpha level (.05) was divided by the number of tests that might be performed on the data from each age (3 tests: one initial comparison of the orders of stimulation, and possibly two follow-up tests, one for each order); .05 / 3 = .016. At day 4 and day 7, there was an effect of stimulus intensity, but no effect of stimulus order nor any interaction between stimulus order and intensity (day 4: Order: $F(1,18) = 1.855, p < .19$; Intensity: $F(2,36) = 5.725, p = .0069$; Order x Intensity: $F(2,36) = .65, p = .5283$; day 7: Order: $F(1,18) = .922, p = .3497$; Intensity: $F(2,36) = 5.511, p = .0082$; Order x Intensity: $F(2,36) = .367, p = .6955$). However, at 12 days the order of stimulation affected the response of the worms to stimuli of different intensities in addition to a significant effect of intensity (day 12: Order: $F(1,18) = 7.152, p = .0155$; Intensity: $F(2,36) = 11.601, p = .0001$; Order x Intensity:}
Therefore, while data from ascending and descending orders of presentation from day 4 and 7 worms were pooled within each age, the data from day 12 were not. At day 12, the data from ascending and descending orders of presentation were analyzed separately.

**Response Frequency.** As seen in Figures 6A and B, at days 4 and 7 no change was evident in number of worms responding with different stimulus intensities (day 7: $Q(2) = 2.4; p > .05$). At day 12, the data from worms that received the stimuli in ascending and descending orders were considered separately (see Figure 6C). No change in the number of worms responding was seen in either group (ascending: $Q(2) = 1.2, p > .05$; descending: $Q(2) = 2.0, p > .05$). Since these stimuli were selected because they all consistently produced reversal responses, it is not surprising that no change in frequency of response with intensity was observed. It is possible that with the addition of lower intensity stimuli nearer to the sensory threshold, more of a decrease in the frequency of reversal responses would be seen at all ages.

**Response Magnitude.** At day 4 (see Figure 7A), worms responded with significantly smaller reversals to a 38 V tap than to either a 40 or 60 V tap ($F(2,38) = 5.833, p = .0062$). At day 7 (see Figure 7B), worms responded with significantly larger reversals to a 60 V tap than either a 40 or 38 V tap ($F(2,38) = 7.791, p = .0015$). At day 12, the data from worms that received the stimuli in ascending and descending orders were considered separately.
Fig. 6. The number of worms that responded to taps of different intensities with reversals at 4, 7 and 12 days post-hatching (n = 20 at each age). At days 4 and 7, the data from worms that received the stimuli in different orders were pooled, while at day 12 the data from worms that received the stimuli in ascending order (ASCENDING; n = 10) and the data from worms that received the stimuli in descending order (DESCENDING; n = 10) are shown separately.
Fig. 7. The magnitude of reversal responses +/- SEM to taps of different intensities at 4, 7 and 12 days post-hatching (n = 20 at each age). The magnitude of reversals was divided by each worm's body length. Only those responses that were reversals were included in the analysis. At days 4 and 7, the data from worms that received the stimuli in different orders were pooled, while at day 12 the data from worms that received the stimuli in ascending order (ASCENDING; n = 10) and the data from worms that received the stimuli in descending order (DESCENDING; n = 10) are shown separately.
Day 12 worms (see Figure 7C) that received stimulation in ascending order (38, 40 and 60 V) did not show a graded response to stimuli of different intensities ($F(2,18) = 1.77, p = .1986$). However, day 12 worms that received the stimuli in descending order (60, 40 and 38 V) did respond with significantly larger reversals to the 60 V tap than to either the 38 or 40 V taps ($F(2,18) = 17.713, p = .0004$). This finding suggests that day 12 worms, like day 4 and 7 worms, are capable of responding to stimuli of different intensities with graded responses, but that the effect of repeated stimulation may mask the effect of stimulus intensity even when the stimuli are administered at 10-min intervals. This response decrement was evident only in the ascending order of stimulation, possibly because the response magnitude to the last stimulus of the descending order (38 V tap) was already so small that any response decrement was lost in a floor effect.

It may be that older worms are more susceptible to habituation training than younger worms. If so, day 12 worms might be expected to exhibit a faster rate of response decrement during habituation training.

Experiment 4. Habituation and dishabituation.

The effects of aging on habituation and dishabituation in *C. elegans* were examined at each of the three test ages, 4 days, 7 days and 12 days post-hatching.
Subjects
20 naive worms were used at each of the three test ages (4, 7 and 12 days) for a total of 60 worms.

Procedure
Trains of taps (1 train = 6 taps at a frequency of 10 Hz) were the stimuli used in both the habituation/dishabituation experiment and the recovery from habituation experiment because this stimulus was found to produce larger responses than the single tap (Chiba & Rankin, 1990) and thus afforded a large response range in which changes in plasticity might be observed. In the present study, at day 4 the mean reversal response to tap was 1.275 ± .141 (± SEM) while the mean response to a train of taps was 2.005 ± .157. Trains of taps have been used in our other studies of non-associative learning (Rankin, Beck, & Chiba, 1990; Rankin & Broster, in press).

In this experiment, 60 trains of taps were administered at a 10 s interstimulus interval (ISI). Ten seconds after the last habituating stimulus, a 60 V train of shocks (a stimulus that produces dishabituation in 4 day olds; Rankin, Beck, & Chiba, 1990) was administered. Within 20 s after the dishabituating stimulus, 12 more trains of taps was administered at a 10 s ISI to test for dishabituation.

Results and Discussion
The analysis of the frequency and the magnitude of the reversal response during habituation and dishabituation showed different patterns of results across ages.
**Response frequency.** The frequency of reversals, scored as the proportion of worms responding to each stimulus with a reversal, showed that both habituation and dishabituation were evident at 4, 7 and 12 days of age (see Figure 8 and 10A). Accelerations (approximately 3% of the responses) were omitted from the analysis and treated as missing data. Cochran Q tests at each age (4, 7 and 12 days of age) confirmed that at each age there were significant changes across the initial response, habituation (last response of the habituation training) and dishabituation (first response after the disabituating stimulus) (day 4: \( Q(2) = 10.64, p < .02 \); day 7: \( Q(2) = 7.625, p < .05 \); day 12: \( Q(2) = 21.375, p < .001 \)). However, as seen in Figure 8, the pattern of responding during habituation training did appear different at day 7. At 4 and 12 days of age, the number of worms responding decreased early in the habituation training and remained low throughout. At 7 days of age, the worms appeared to stop responding early in habituation training then begin responding again as the habituation training continued. This finding was reflected in a change in the frequency of response averaged across all 60 habituation trials with age; the mean frequency of response was significantly greater at day 7 than at day 4 or day 12 (day 4: \( \bar{X} = .265 \pm .026 \); day 7: \( .606 \pm .032 \); day 12: \( .329 \pm .034 \); \( F(2,57) = 33.667, p = .0001 \)).

**Response magnitude.** In order to compare response magnitude across ages the reversal magnitudes were standardized by expressing each response as a percent of the initial reversal
Fig. 8. The proportion of worms responding to stimuli with reversals during habituation training to trains of taps (10 s ISI, 60 stim; n = 20 at each age).
Fig. 9. The magnitude of reversal responses +/- SEM during habituation training with trains of taps (10 s ISI, 60 stimuli). The magnitude of reversal responses was expressed as a percent of each worm's response to the initial stimulus in habituation training.
A DAY 4

PERCENT INITIAL RESPONSE

TRIALS

B DAY 7

PERCENT INITIAL RESPONSE

TRIALS

C DAY 12

PERCENT INITIAL RESPONSE

TRIALS
Fig. 10. Reversal responses before and after habituation training with trains of taps (10 s ISI, 60 stimuli) and after dishabituation with electric shock at 4, 7 and 12 days post-hatching (n = 20 at each age). INIT = initial response of the habituation training; HAB = final response of the habituation training; DIS = first response after the dishabituating stimulus.

A. Proportion of worms responding to stimuli with reversals before and after habituation training and following the dishabituating stimulus.

B. Magnitude of the reversal response +/- SEM before and after habituation training and following the dishabituating stimulus. Magnitude of reversal responses was expressed as a percent of each worm's reversal response to the initial stimulus in habituation training.
response (which was set at 100%) for each worm (see Figures 9A, B and C). Acceleration responses were not included in the analysis because they represent discrete motor responses that cannot be compared to reversals; however, the absence of a response or a pause were included as scores of zero (Rankin, Beck, & Chiba, 1990). Missing data points (approximately 3% of the responses) in repeated measures analyses were replaced by the group and condition mean (Glass & Hopkins, 1984).

Worms at all ages showed significant habituation (see Figure 9A, B and C) and dishabituation (see Figure 10B) in the magnitude of reversal response. Repeated measures ANOVA's were performed on each of the three ages. To control family-wise error rate, the alpha level was reduced from .05 to .05/3 = .016 (Glass and Hopkins, 1984). As seen in Figure 10B, at all ages the initial response was significantly greater than the habituated response and the dishabituated response, and the dishabituated response was significantly greater than the habituated response (4 days: $F(2,38) = 134.358$, $p < .0001$; 7 days: $F(2,38) = 280.389$, $p < .0001$; 12 days: $F(2,38) = 786.631$, $p < .0001$). It was not possible to compare the degree of habituation or dishabituation across the ages tested because the habituated response of worms at all ages were small enough that the characteristics of any change measured against that low level of responding may have been lost in a floor effect.

The rate of response decrement during habituation training was analyzed by calculating the slope of the regression lines for
reversal responses to the first five stimuli in the habituation training for each worm and then taking the mean of these slopes for each age (day 4 $\bar{X} = -22.125 \pm 2.375$; day 7 $\bar{X} = -25.512 \pm 1.695$; day 12 $\bar{X} = -19.721 \pm 2.317$). A comparison of the slopes showed that there was no significant change in the rate of habituation as a function of age ($F(2,56) = 1.786, p = .177$).

While the findings from this experiment seemed to indicate that there was no change with age in the reversal response magnitude in habituation and dishabituation, there was a change with age in response frequency during habituation. A dissociation of the response magnitude and response frequency was apparent at day 7, where worms continued to respond to stimuli during habituation training but responded with small reversals. This dissociation may reflect some change in the underlying memory mechanisms at that age.

Experiment 5. Recovery from habituation

Recovery from habituation may be thought of a form of short-term memory; the stronger the memory, the slower the recovery from habituation might be. Thus, if short-term memory changes with age, the rate of recovery from habituation might also change with age.

Subjects

Twenty worms were tested at each of the test ages 4, 7 and 12 days post-hatching for a total of 60 worms.
Subjects

Twenty worms were tested at each of the test ages 4, 7 and 12 days post-hatching for a total of 60 worms.

Procedure

Habituation was established by delivering 60 trains of taps at a 10 s ISI; at the end of habituation training, single trains of taps at 30 s, 10 min, 20 min and 30 min were delivered to test for recovery from habituation. This procedure has been used to demonstrate recovery from habituation 20 to 30 min after the last habituating stimulus in 4 day old worms (Rankin & Broster, 1990, in press).

Results and Discussion

In the analysis of the recovery of the magnitude of the reversal response after habituation training, reversal magnitude was standardized as percent initial response as in the previous experiment. Only the frequency and magnitude data from the 30 min recovery test were statistically analyzed because the variance in the intermediate tests (at 30 s, 10 min and 20 min) was too great to be usefully included. The data from all tests are depicted in Figures 11 and 12A, B and C.

Response frequency. The frequency of reversals, scored as the proportion of worms responding to each stimulus with a reversal, showed that both habituation and recovery from habituation were evident at 4, 7 and 12 days of age (see Figure 11). Cochran Q tests at each age comparing the initial, habituated and recovered (30 min post-habituation) response frequency confirmed that at
Fig. 11. Proportion of worms responding with reversals during recovery from response decrement from habituation training (10 s ISI, 60 stimuli; n = 20 at each age). Tests of recovery were given 30 s, 10 min, 20 min, and 30 min post-habituation. INIT = the response to the first habituation stimulus; HAB = the response to the last habituation stimulus.
Fig. 12. The magnitude of reversal responses +/- SEM during recovery from habituation (n = 20 at each age). The data are expressed as percent initial response which for each worm was set at 100%. The solid horizontal line at 100% represents this initial response during habituation training. HAB = the reversal response to the last habituation stimulus in the training. Recovery was tested at 30 s, 10 min, 20 min and 30 min post-habituation.
each age there were significant changes with the treatments (day 4: $Q(2) = 17.231, p < .001$; day 7: $Q(2) = 13.0, p < .01$; day 12: $Q(2) = 19.0, p < .001$).

Response magnitude. A different pattern is seen in the analysis of the magnitude of reversal responses (see Figures 12A, B and C). All ages showed significant habituation (day 4: $F(2,38) = 23.563, p < .0001$; day 7: $F(2,38) = 16.953, p < .0001$; day 12: $F(2,38) = 3.426, p < .0001$). By 30 minutes after habituation training, 4 day and 7 day worms showed significant recovery of response magnitude over habituated levels. However, worms tested at 12 days post-hatching did not show recovery over habituated levels.

The effects of recovery on the response magnitude were further examined by comparing the difference between habituated response levels and 30 min post-habituation response levels across ages.

Experiment 6. Inhibition

As a final test of the effects of aging on behavior, the inhibition of one antagonistic reflex by another as described by Rankin (in press) was examined. First, the response to tap alone was tested. Second, the inhibition of the reversal withdrawal in response to tap by tail-touch was examined. Third, the inhibiting stimulus, tail-touch, was habituated and the interaction between the reflexes was examined again. The effect of habituation training on response competition was then compared at the three test ages.
Subjects

Twenty worms at each age (4, 7 and 12 days) were tested for a total of 60 worms.

Procedure

There were three treatments in this procedure. All animals received a tap alone, a tap preceded within 1 s by a tail-touch, and tail-touch habituation training (2 s ISI; 50 stimuli) immediately followed by a tap preceded within 1 s by a tail-touch. The treatments were given in the same order for all worms so that the habituation to tail-touch did not interfere with the other responses. There was a 20 to 30 min interval between all tests. In order to determine the effects of the tail-touch on the reversal to tap, worms that did not respond to the single tap with a reversal were eliminated from the experiment (approximately 10% of worms tested). The reversal responses to the tap were traced and digitized for all groups.

Results and Discussion

Response frequency. At each age there was a marked inhibition of the frequency of the reversal response to tap when tap was preceded by tail-touch and a decrease in the inhibition of the frequency of response from that level when the tail-touch was preceded by habituation training with tail-touch (see Figure 13A).

Response magnitude. The magnitude of reversal responses were standardized by expressing them as a percent of each worm's reversal response to the single tap which was set at 100%. In measuring the effects of tail-touch and tail-touch habituation on
Fig. 13. The effect of habituation training on inhibition of the reversal response to tap by tail-touch (n = 20 at each age).

A. The number of worms reversing to tap alone (TAP), tail-touch followed within 1 s by tap (TT), and tail-touch habituation training (2 s ISI, 50 stimuli) followed within 2 s by tail-touch/tap.

B. The magnitude of reversals +/- SEM to tap alone (TAP), tail-touch followed within 1 s by tap (TT), and tail-touch habituation training (2 s ISI, 50 stimuli) followed within 2 s by tail-touch/tap. The magnitude of the reversals that occurred were expressed as a percent of each worm's response to the tap alone which was set at 100%.
the magnitude of reversals (see Figure 13B), only animals that reversed under these conditions were included in the analysis. Repeated measures ANOVAs at each age showed that the reversal magnitude was significantly lower in the tail-touch/tap condition than the tap alone condition and significantly higher in the habituated tail-touch/tap condition than in the tail-touch/tap condition (day 4: \( F(2,38) = 48.679, p = .0001 \); day 7: \( F(2,38) = 11.027, p = .0002 \); day 12: \( F(2,38) = 19.618, p = .0001 \)).

Clearly, tail-touch inhibited both the frequency of reversal response and the magnitude of the reversal response. Habituation training with the tail-touch diminished the amount of inhibition; thus there was an increase in both the frequency and the magnitude of the reversal response to tap. The observation that worms showed reversals to tap following tail-touch habituation suggests that the habituation did not simply produce fatigue. From the results of this experiment, one can conclude that even aged worms are capable of habituation independent of fatigue effects.

General Discussion

These experiments have demonstrated that in *C. elegans* the effects of aging may be seen in spontaneous and reflexive behaviors, and more importantly in changes in learning and memory. Velocity of spontaneous movement diminished with age, as did the magnitude of spontaneous reversals and the reversal response to tap. In addition spontaneous velocity of locomotion tested at 7
days of age was lower in worms that died before the day 12 test than worms that survived until day 12. However, the mean magnitude of spontaneous reversals and the magnitude of response to single taps was not related to time of death. Clearly aging did have an effect on the spontaneous and reflexive behaviors. These changes must be kept in mind when considering the effects of aging in behavioral plasticity.

To test the sensitivity to tactile stimuli of the aged worms, stimuli of different intensities were administered. Worms at all ages tested showed graded responses to taps of different intensities. Interestingly, the response magnitude of day 12 worms appeared to decrease during the administration of the three stimuli given at a 10 min ISI. Rankin and Broster (in press) found no evidence of habituation when they administered taps at a 10 min ISI to day 4 worms. It may be that older worms are particularly vulnerable to habituation.

Habituation to repeated stimulation at a 10 s ISI and dishabituation to 60 V shock were present in worms at all ages and did not appear to change greatly during post-reproductive development. However, at day 7 there appeared to be a dissociation of the probability of response and the magnitude of response. At day 4 and day 12 these measures appeared to follow the same pattern, however in day 7 worms the probability of response stayed high while response magnitude diminished. It is not clear why this change with age in response frequency during habituation training occurred; however a closer examination of the
behavior of worms between the ages tested (4, 7 and 12 days post-hatching) might provide further information on this phenomenon.

The rate of habituation to stimulation at a 10 s ISI did not appear to change with age. This finding does not support the suggestion arising from Experiment 4 (Graded response) that day 12 worms may be particularly vulnerable to habituation. However it may be that the interstimulus interval used in the habituation training (10 s ISI) was short enough that worms of all ages habituated quickly, masking any difference in rate of habituation. During the later part of habituation training (trials 50 through 60), the reversal magnitude to vibrational stimuli when exhibited appears to be greater than the reversal magnitude to the low intensity tap (38 V) whether the low intensity tap was given first or last (day 12 habituation of reversal magnitude from trials 50 to 60: \( \bar{x} = 0.339 \pm 0.073 \); day 12 reversal magnitude to 38 V tap \( \bar{x} = 0.096 \pm 0.036 \)); thus it may be that little further habituation of reversal magnitude could be exhibited. An examination of the effects of aging on the rate of habituation to longer ISI's might be help clarify this question.

Worms tested at 4 and 7 days of age showed recovery from habituation of both the frequency and response magnitude 30 min post-habituation training, but worms tested at 12 days did not. This deficit in recovery might reflect a persistence of habituation in the older worms. This persistence is unlikely to be explained by a factor such as more rapid exhaustion since day
12 worms with the same habituation training showed dishabituation (facilitation of the habituated response) immediately after a mild electric shock. In addition, habituation of tail-touch prior to tests of inhibition helped establish that habituation can be distinguished from simple fatigue effects in worms of all ages. Because habituation training to tail-touch increased the reversal response to tap by releasing it from tail-touch inhibition at each test age, clearly even aged worms are capable of habituation independent of fatigue or response diminishment related to age. Further studies on the dynamics of habituation and recovery from habituation and on long-term memory in aged worms may help the understanding of mechanisms underlying the age-related changes in habituation.

An assumption underlying the use of simple-system models of learning and memory is that these simple forms of learning, habituation and dishabituation, seen in so many species, must share some common biological mechanisms. If the underlying mechanisms are conserved across species, it may be possible to make viable predictions about the patterns of learning in one species based on the patterns of learning in another. Work in other species on the effects of aging on habituation has not been extensive and findings have been mixed. Eisenstein and colleagues (1990) found that in human males the galvanic skin response habituated more quickly in younger subjects (early 20's) than in older ones (late 20's). They related this deficit with the loss of dopamine receptors in the caudate nucleus observed in PET
studies between age 20 and 30. Fraley and Springer (1981) found that middle-aged and older mice (12 to 24 mo) did not retain habituation training for as long as 2 month old mice. Parsons, Fagan and Spear (1973) found no change in short-term retention of habituation training in old age in rats. As discussed earlier, Rattan and Peretz (1987) found more rapid habituation in older Aplysia and an absence of dishabituation.

The results from the present experiments offer little support for any generalities that might be drawn from the above studies. It is possible that aged C. elegans habituate more rapidly than younger adults; however this suggestion has not yet been confirmed. Aged C. elegans showed dishabituation after electric shock; this finding does not support the assertion of Rattan and Peretz (1987) that dishabituation is specifically disabled in older organisms (although a recent personal communication from Peretz (April, 1991) indicated that behavioral experiments on Aplysia have shown that dishabituation may be exhibited by older Aplysia; the deficit seen formerly appears to have been dependent on the specific habituation training procedure used). From Experiment 6 (Recovery from habituation) there is evidence of a longer retention of habituation which seems to contradict the findings of Fraley and Springer (1981) that middle-aged and aged mice showed shorter retention of habituation training than young mature adults. Clearly there is as yet no single description of the effects of aging on habituation. One of the difficulties with comparing these studies is that methodologies used are
diverse and full parametric examinations of the learning phenomena studied have not been done. Further development of a model for the effects of aging on simple forms of learning such as habituation in *C. elegans* may help to clarify the role of aging effects in other species.

The studies described here provide a groundwork for further research on the effects of aging on learning and memory in *C. elegans*. There are several directions in which this research could continue. First, the mechanisms underlying the age-related changes observed in habituation and recovery from habituation might be investigated with studies focussing on the parameters of those changes. Second, known mutant strains with effects on certain types of learning or responses might be examined. For example the use of a strain of worms which is incapable of locomotion yet still feeds may aid in the development of alternative response measures that may be used in studies of learning such as pharyngeal bulb pumping. Finally genetists interested in investigating the biological mechanisms of aging have begun work on the genetic controls of aging mechanisms in *C. elegans*. Johnson and his colleagues (Johnson, Friedman, Foltz, Fitzpatrick, & Shoemaker, 1989) chose to focus on searching for a mutant with an extended life span, with the logic that there are many reasons why a mutant may have a shortened life span, but a mutation producing an extended life span is more likely to be directly related to the mechanisms underlying aging. Johnson and his colleagues (1989) isolated and mapped a single gene mutation
that produces a 60-110% increase in life span; attempts are underway to clone this gene. In this strain of worms, the extended life span is not due to the extension of any early developmental stage, but to a lengthening of the post-reproductive life span. A series of recombinant mutation experiments focussing on genes that modify development have shown that early development, reproduction and life span are all under independent genetic control (Johnson, 1987). However, in all strains, decreased motor activity correlates with life span; longer lived strains undergo slower motor activity decay than wild-type strains. Thus it may be that aging and motor activity decay share a common genetic mechanism. Finally mild food deprivation causes an increase in the lifespan with a decrease in fertility. This effect is seen in both wild-type and mutant strains of *C. elegans*. Thus, the effect of food deprivation on life-span seems to be independent of the extended life span produced by the aging mutation (Johnson, 1987). The work of Johnson and his colleagues not only gives us candidate aging mechanisms, but also a means of testing the proposed mechanisms behaviorally. By comparing aging in normal and mutant worms it may be possible to define some of the mechanisms underlying learning and memory deficits resulting from the aging process.

Clearly a model of aging effects on learning and memory in *C. elegans* provides a rich set of possible research directions. By establishing in this set of experiments the patterns of change with aging in these simple forms of learning, habituation and
dishabituation, we may begin to develop a simple-systems model of the aging effects on learning and memory. Using *C. elegans*, such a model may provide us with a unique opportunity to investigate the genetic control of changes in learning and memory in senescence.
Bibliography


