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Comparison of survival rates in four inbred mouse strains under different housing conditions: effects of environmental enrichment

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Abstract: Housing conditions can affect the well-being of laboratory animals and thereby affect the outcomes of experiments. The appropriate environment is essential for the expression of natural behavior in animals. Here, we compared survival rates in four inbred mouse strains maintained under three different environmental conditions. Three mouse strains (C57BL/6J, C3H/HeN, and DBA/2J) housed under environmental enrichment (EE) conditions showed improved survival; however, EE did not alter the survival rate of the fourth strain, BALB/c. None of the strains showed significant differences in body weights or plasma corticosterone levels in the three environmental conditions. For BALB/c mice, the rates of debility were higher in the EE group. Interestingly, for C57BL/6J and C3H/HeN mice, the incidence of animals with alopecia was significantly lower in the EE groups than in the control group. It is possible that the enriched environment provided greater opportunities for sheltering in a secure location in which to avoid interactions with other mice. The cloth mat flooring used for the EE group was bitten and chewed by the mice. Our findings suggest that depending on the mouse strains different responses to EE are caused with regard to health and survival rates. The results of this study provide basic data for further studies on EE.

Key words: cloth mat flooring, environmental enrichment, inbred strain, lifespan, mice

Introduction

Human life expectancy is increasing globally due to improved healthcare infrastructures and to the early diagnosis of disease. Age-related decreases in physiological functions can have a significant and adverse influence on daily life and quality of life in elderly populations [1]. The mechanisms of aging are currently the focus of a wide range of investigations. Animal models are commonly used in aging research because they allow researchers to obtain data that are difficult or impossible to obtain from humans. Laboratory mice are widely used for aging and senile disease research because they are

genetically and physiologically similar to humans, easy to breed and to produce genetically modified animals, have a well-developed database, and are relatively short-lived with an average lifespan of about 3 years [2].

Advances in our understanding of the optimal housing conditions for animals in biomedical research have led to the increased use of “environmental enrichment (EE)” for rodents. EE can be defined as any modification in the environment of captive animals that seeks to enhance physical and psychological well-being by providing stimuli that meet the specific needs of the animals [3]. It is important to evaluate EE in terms of health benefit and lifespan of the animals; assessment of the effects on

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behavior and on physiological parameters can also be used to investigate the potential benefits of EE.

In general, two types of experiment are performed to evaluate the potential improvement in rodent welfare by EE: (1) experiments that examine the preference of animals for specific objects or environments (preference testing); and (2) experiments that examine the effects of enrichment on behavioral or physiological parameters [4]. Research on the effects of EE on mice has demonstrated significant differences in behavior: mice kept under enriched conditions show increased locomotory and exploratory activities [5], object exploration [6], learning ability [7], and decreased anxiety [8]. These differences in behavior reflect differences in brain anatomy and chemistry, including brain size, brain weight, density of spines, levels of dendritic branching, number of synapses per neuron, and activity of neurotransmitters [9]. The influence of EE on cognition and memory has been well documented [10–13].

In preference testing experiments, mice are provided with a choice of different conditions, and the environment most frequently chosen is assumed to be that most suitable [14, 15]. In a previous study, we showed that mice remained longer in cages with cloth bedding materials [16]; cloth bedding materials offer both a significant reduction in waste and an improvement in the habitat of the mice [17]. Mice prefer an environment that matches their fur color as it provides greater protection [18]; mice also show a clear preference for cages containing a wooden quadrangle [19].

Particularly relevant to investigations of aging are studies examining enrichment-induced alterations in middle-aged and aged rodents. The majority of studies on aged mice generally provide an enriched environment for only a short time period [20–23] although one study on rats examined enriched versus standard housing conditions over a 12 month period [24]. To date, however, no study has evaluated the effects of EE on lifespans and survival rates. The present study was initiated to examine the effect of three different environmental conditions on the survival rates of four inbred mouse strains.

Materials and Methods

Animals and housing conditions

Six-week-old female mice from four inbred strains (BALB/c, C57BL/6J, C3H/HeN, DBA/2J) were used (CLEA Japan, Inc., Tokyo, Japan). To eliminate the influence of the rearing environment, mice were housed in TPX plastic cages (220 × 320 × 135 mm, Natsune Seisakusho, Co., Ltd., Tokyo, Japan) with wire mesh floors for a 2-week acclimatization period. All animals

were kept in an animal room at a constant temperature ($23 \pm 2^\circ\text{C}$), humidity ($55 \pm 10\%$), and 12 h light/12 h dark cycle (lights on at 07:00 and off at 19:00). The mice were fed a plain commercial diet (NMF, Oriental Yeast Co., Ltd., Tokyo, Japan) and had tap water *ad libitum*. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at Shimane University and approved by the Institutional Review Board of the University (Approved number: IZ21-127).

Experimental procedure (housing conditions of test animals)

Experiments were designed to compare the effects of three housing conditions on survival rates in the four strains of mice (Fig. 1). For each strain, 75 female mice were used: 5 × 5 animals were maintained in the three housing conditions. Groups of mice were composed on the basis of body weight of the animals at Six weeks of age, so that groups had comparable mean body weights.

Standard housing

Mice were housed in cages containing standard wood shavings (Clean-chip™, Shimizu, Inc., Kyoto, Japan). Approximately 65 g of flooring material was placed in each cage and this was replaced once a week. These mice served as the control group.

AGMAT housing

The second group of mice was housed in cages containing a cloth mat flooring (AGMAT group, Agrebe-mat™, 160 × 270 mm, about 50 g, CLEA Japan, Inc.). The mice were rehoused once per week. AGMAT is composed of meth acrylic acid graft copolymerized cellulosic knit fiber; this material has been reported to have the property of adsorbing ammonia [17]. In addition, unlike conventional flooring materials, it can be reused after washing [25]. The amount of dust in the cages is significantly reduced using this material compared to conventional flooring materials.

Enriched housing

The third group of mice was housed in cages with cloth mat flooring (Agrebe-mat™, 160 × 270 mm, about 50 g, CLEA Japan, Inc.), and containing a quadrangular wooden block with four small holes (110 × 110 × 75 mm, one small hole (φ40), CLEA Japan, Inc.) [19] and a piece of cloth (Agrebe™, 450 × 650 mm, CLEA Japan, Inc.).

Housing materials of similar color to the mouse fur were used: the housing materials for BALB/c and DBA/2J mice were white, while those for C57BL/6J and C3H/HeN mice were black [18]. Cloths were dyed black

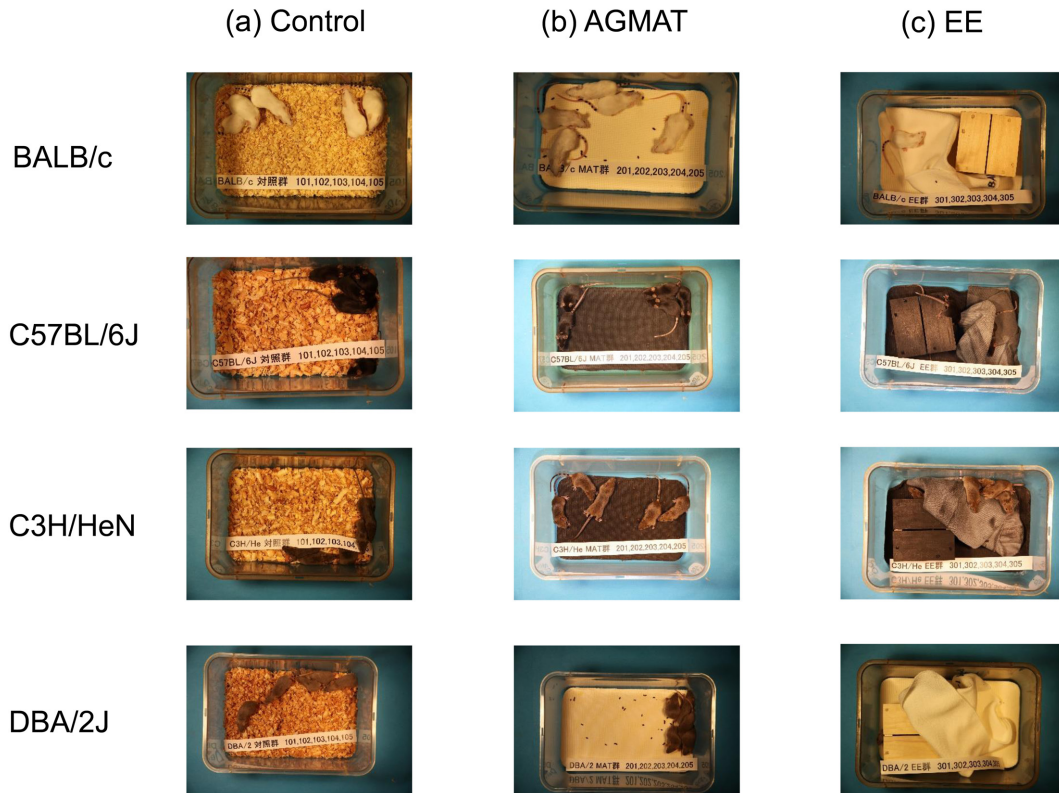


Fig. 1. The three housing conditions evaluated here: (a) standard wood shaving cages (Control group); (b) cloth mat flooring cages (AGMAT group); (c) cloth mat flooring cages containing a quadrangular wooden block with four small holes; the cloth was color matched to the mouse fur color (Environmental enrichment (EE) group).

using India ink (calligraphy liquid, Juntendo Co., Ltd., Masuda, Japan) and sterilized in an autoclave.

Observation scores

We monitored the status of the mice daily. Body weight, food consumption, and water consumption were measured monthly using an electronic balance (PE-1600, Mettler Ltd., Tokyo, Japan). Food consumption per animal was determined by placing a known weight and weighing the amount of food left after 7 days. Water consumption was measured in the same way. Survival rates were assessed over the entire lifespans of the animals. Deaths due to natural causes were recorded. Animals were euthanized under anesthesia with isoflurane (Escain, Mylan Seiyaku, Tokyo, Japan) using an anesthetic instrument (KN-1071-I, Natsune Seisakusho, Co., Ltd.) if they were too debilitated to reach food and water and/or had a palpable mass of ≥ 2.0 cm in diameter [26]. Autopsies were performed on all mice, other than that rotten carcass or partly eaten and all organs were subjected to macroscopic examination. Debility in the mice was assigned to various categories: abnormal behavior; skin lesions; increased lordosis and kyphosis; alopecia, which could develop in a diffuse manner or affected circular areas on the dorsal surface; tumors, which in-

cluded various skin tumor types and cancers; ascites; hepatic cirrhosis, which included hepatic failure and hepatic fibrosis; lesions of the spleen, including inflammatory and necrotic fibrosis; spleen atrophy; cataracts; anal prolapse, including symptoms of intestinal inflammation such as perianal inflammation and bleeding.

Corticosterone (CORT) assay

After 12 weeks, five mice from each group of all four strains were moved from the animal room to the laboratory and euthanized in their cages using CO₂ supplied through a safety glass lid. All animals lost consciousness within 30 s and stopped breathing within 2–3 min. To minimize the effect of sampling order on the levels of CORT, the five mice were euthanized together in their home cage. The treatment groups were sampled as one cage per group in the order: control, AGMAT, and EE. This sampling order was maintained until all of the animals had been euthanized. Blood samples were collected by heart puncture directly after euthanasia. Blood plasma was separated by centrifugation ($1,530 \times g$ for 10 min) and deep frozen (-80°C) until assayed. CORT levels were determined using an ELISA kit according to the manufacturer's instructions (AssayPro, Funakoshi Co., Ltd., Tokyo, Japan).

Cloth mat flooring

The weights of the cloth mat flooring in the AGMAT and EE groups were compared before and after use (for 1 week) using an electronic balance (PE-1600, Mettler Ltd., Tokyo, Japan). During cage cleaning, the condition of each mat was also subjectively assessed. The mats were then washed, reprocessed, sterilized, and weighed, and reused over about 4 months.

Statistical analysis

Data are expressed as means \pm SD. Differences were tested by analysis of variance (ANOVA) and Dunnett's PLSD post hoc test. The data from the post-mortem analyses of animals euthanized during the experimental period were analyzed using the chi-square test. Survival rates were analyzed using the Kaplan–Meier survival test and the log-rank test. Cloth mat flooring weights were analyzed using the student's *t*-test of unpaired comparisons. Analyses were performed using StatView (SAS Institute Inc., Cary, NC, USA). A *P* value less than 0.05 was considered statistically significant.

Results

Survival

Figure 2 shows the survival curves for BALB/c,

C57BL/6J, C3H/HeN, and DBA/2J mice during this experiment. In BALB/c mice, the numbers of mice dying increased slightly earlier in the AGMAT and EE groups compared with the control group, but the difference was not significant. All of the BALB/c mice died before 900 days. BALB/c mice showed a mean lifespan of 660.3 ± 22.2 days in the control, 615.1 ± 40.8 days in the AGMAT group ($P=0.32$), and 615.6 ± 29.2 days in the EE group ($P=0.32$). C57BL/6J mice in the EE group showed significantly longer survival than the control group: mean lifespan was 723.5 ± 39.2 days in the control group, 802.4 ± 30.2 days in the AGMAT group ($P=0.08$), and 840.9 ± 24.1 days in the EE group ($P<0.05$). The 900-day survival rate for the C57BL/6J mice was 15% in the control group, 25% in the AGMAT group, and 25% in the EE group. In C3H/HeN mice, survival in the EE group was significantly longer than the control group: mean lifespan was 814.4 ± 25.9 days in the control group, 842.8 ± 11.9 days in the AGMAT group ($P=0.30$), and 906.6 ± 16.6 days in the EE group ($P<0.01$). The 900-day survival rate for the C3H/HeN mice was 30% in the control group, 15% in the AGMAT group, and 60% in the EE group. In DBA/2J mice, survival was significantly longer in the EE group than the control group. DBA/2J mice also tended to have a shorter lifespan than the other three strains: the mean lifespan of the DBA/2J

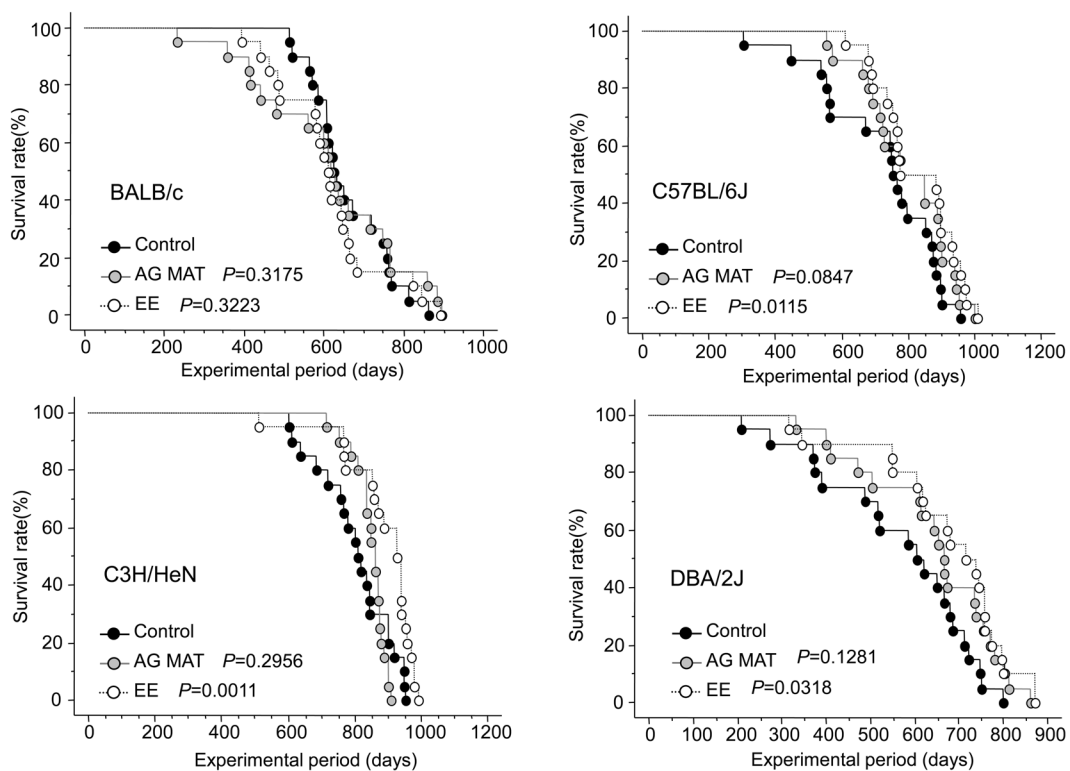


Fig. 2. Survival curves for the four mouse strains in each housing condition. Survival rates were analyzed using the Kaplan–Meier survival test and the log rank test. A *P* value less than 0.05 was considered statistically significant differences from the control group.

control group was 567.8 ± 37.9 days, that of the AGMAT group was 644.4 ± 33.6 days ($P=0.13$), and that of the EE group was 677.0 ± 33.6 days ($P<0.05$). All of the DBA/2J mice had died before 900 days.

Body weight

Figure 3 shows the changes in body weight in all four mouse strains and in all three housing conditions during the period of the experiment. Measurements of body weight and food consumption are useful methods for early detection of the onset of disease or debilitation in animals. BALB/c, C57BL/6J, and C3H/HeN mice showed no significant differences in body weight at 26 months among the three groups. DBA/2J mice in the AGMAT and EE groups had slightly larger body weight increases compared with that of the control group at 10–16 months, but these were not statistically significant.

Food and water consumption

As shown in Fig. 4, food consumption in BALB/c mice did not differ significantly among the three groups. Food consumption of C57BL/6J mice decreased slightly in the AGMAT and EE groups during the experimental period but there were no significant differences in food consumption among the three groups. Food con-

sumption of C3H/HeN and DBA/2J mice did not differ significantly among the three groups. In all four mouse strains, the type of housing did not have a significant effect on food consumption, although food consumption varied from week to week.

As shown in Fig. 5, BALB/c mice showed no significant differences in water consumption among the three groups, although there was a remarkable drop in the EE group at 22 months. In C57BL/6J mice, daily water consumption varied widely in the control group throughout the experiment but, overall, there were no significant differences among the three groups. C3H/HeN and DBA/2J mice showed no significant differences among the three groups.

Plasma CORT

To measure the stress response, plasma CORT levels were assessed. Figure 6 shows the plasma CORT levels in the four strains after 12 weeks. Plasma CORT of BALB/c mice represented the basal levels: control, 69.3 ± 63.9 ng/ml; AGMAT group, 70.3 ± 87.1 ng/ml; EE group, 72.9 ± 43.6 ng/ml. There were no significant differences among the groups. Housing conditions did not influence CORT levels in C57BL/6J or DBA/2J mice; these strains showed similar levels of CORT as BALB/c

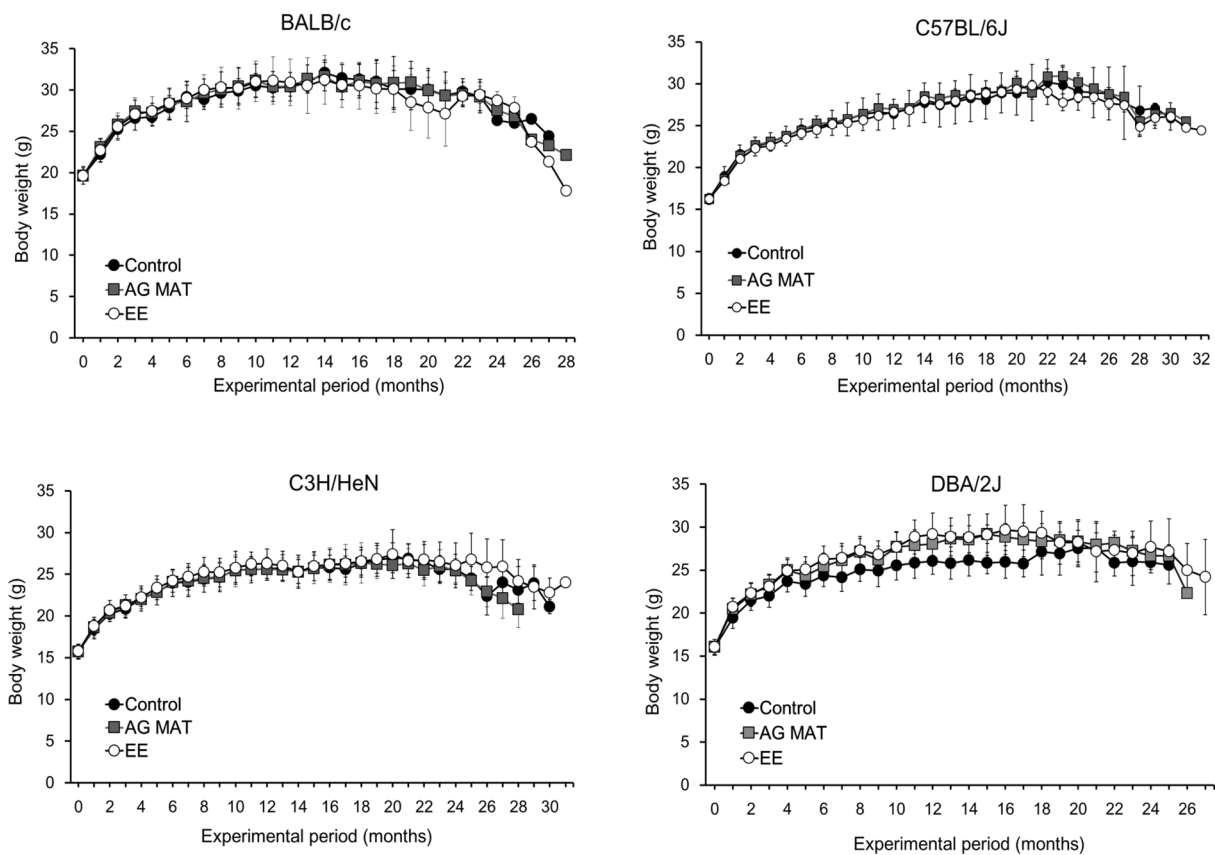


Fig. 3. Average body weights for all mouse strains in each housing condition. Each value represents the mean \pm SD.

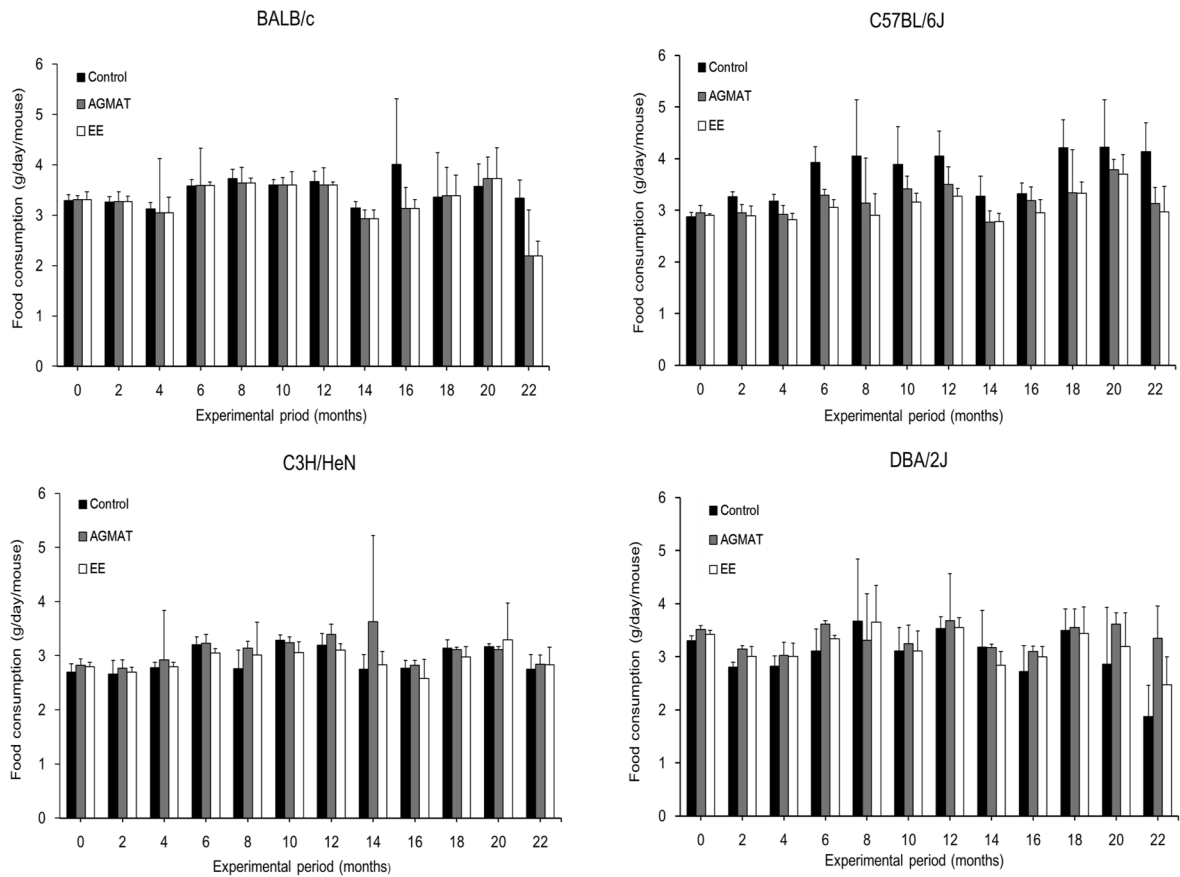


Fig. 4. Food consumption in four mouse strains in each housing condition. Each value represents the mean \pm SD.

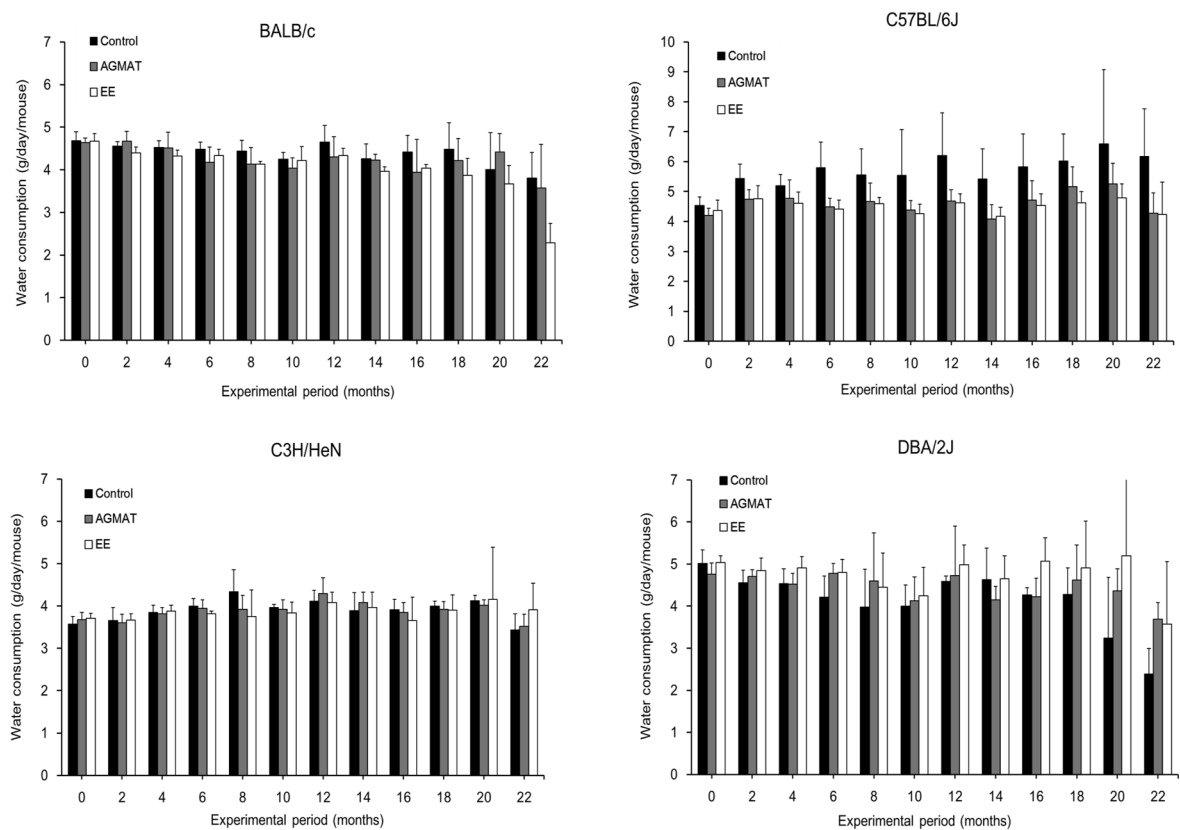


Fig. 5. Water consumption in four mouse strains in each housing condition. Each value represents the mean \pm SD.

mice. By contrast, plasma CORT levels were up to four times as high in C3H/HeN mice as in the other strains.

Post-mortem observations

The results of the necropsic analyses of mice from the four strains are shown in Table 1. In BALB/c, increases in neoplastic lesions, ascitic fluid, and splenomegaly were observed. The rates of debility in the EE groups were significantly higher ($P<0.05$) compared with the control group. In C57BL/6J mice, the rates of alopecia in the AGMAT and EE groups were significantly lower ($P<0.01$ and $P<0.05$, respectively) compared with the control group. The date of onset of alopecia was also significantly later in the AGMAT group ($n=5$, $457.0 \pm$

96.1 days) compared with the control group ($n=15$, 248.4 ± 37.6 days). Similarly, the onset of alopecia was significantly later in the EE group ($n=8$, 451.5 ± 45.1 days) compared with the control group. In C3H/HeN mice the rate of alopecia in the EE group was lower than in the control group ($P<0.05$). However, there were no differences in timing of onset of alopecia among the three groups: control, 473.1 ± 43.2 days; AGMAT, 533.6 ± 72.3 ; EE, 500.5 ± 70.6 days.

Condition of cloth mat flooring

Figure 7A shows representative images of cloth mat flooring before and after use. In the AGMAT group and the EE group, the mat displayed scratches and tooth

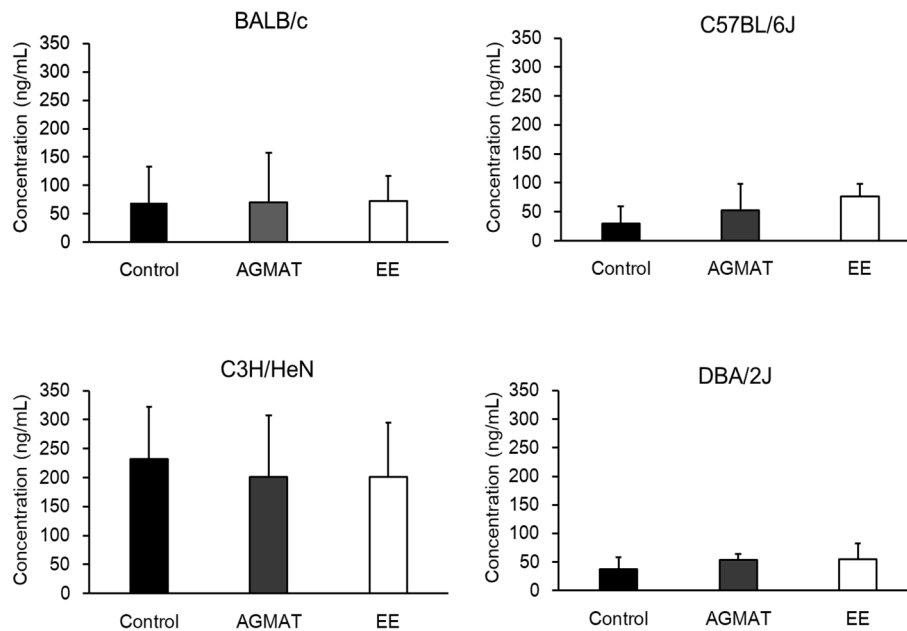


Fig. 6. Corticosterone levels in four mouse strains in each housing condition. Data are presented as the mean \pm SD ($n=5$) and were analyzed using a variance (ANOVA) and Dunnet's PLSD post hoc test.

Table 1. Necropsy findings in four mouse strains in each housing condition

Strain	Group	Debility	Alopecia	Tumor	Ascites	Hepatic cirrhosis	Spleen hypertrophy	Spleen atrophy	Cataract	Anal prolapse
BALB/c	Control	0/20	7/20	10/20	10/20	4/20	10/20	0/20	1/20	2/20
	AGMAT	0/17	2/17	5/17	11/17	3/17	8/17	0/17	0/17	2/17
	EE	6/18*	0/18	3/18	4/18	0/18	5/18	0/18	0/18	0/18
C57BL/6J	Control	2/19	15/19	2/19	13/19	3/19	4/19	0/19	5/19	1/19
	AGMAT	4/18	5/18**	5/18	6/18	3/18	8/18	0/18	4/18	3/18
	EE	7/20	8/20*	6/20	10/20	3/20	1/20	0/20	4/20	1/20
C3H/HeN	Control	7/20	12/20	4/20	8/20	4/20	1/20	8/20	1/20	0/20
	AGMAT	10/20	5/20	6/20	5/20	0/20	0/20	11/20	0/20	1/20
	EE	7/19	4/19*	2/19	5/19	1/19	1/19	11/19	2/19	0/19
DBA/2J	Control	1/18	0/18	8/18	7/18	2/18	4/18	0/18	3/18	0/18
	AGMAT	1/20	0/20	12/20	8/20	4/20	6/20	0/20	3/20	0/20
	EE	2/18	1/18	11/18	10/18	6/18	5/18	2/18	2/18	0/18

The data from the post-mortem analyses of animals euthanized during the experimental period were analyzed using the chi-square test. **: $P<0.01$ vs Control, *: $P<0.05$ vs Control.

marks; these were observed for mats used in all strains. The matting was replaced after about 4 months when it became too tattered for use. Figure 7B shows the changes of average weights of the floor mats for the four strains in AGMAT and EE groups. In both the AGMAT and EE group of BALB/c mice, a 45% rate of damage to the floor

mat was observed after 4 months; there was no significant difference between the two housing groups. In the C57BL/6J mice, the floor mat weight in the AGMAT group was significantly lower than in the EE group ($P < 0.05$) after two and four months. In the C3H/HeN mice, floor mat weight in the AGMAT group was sig-

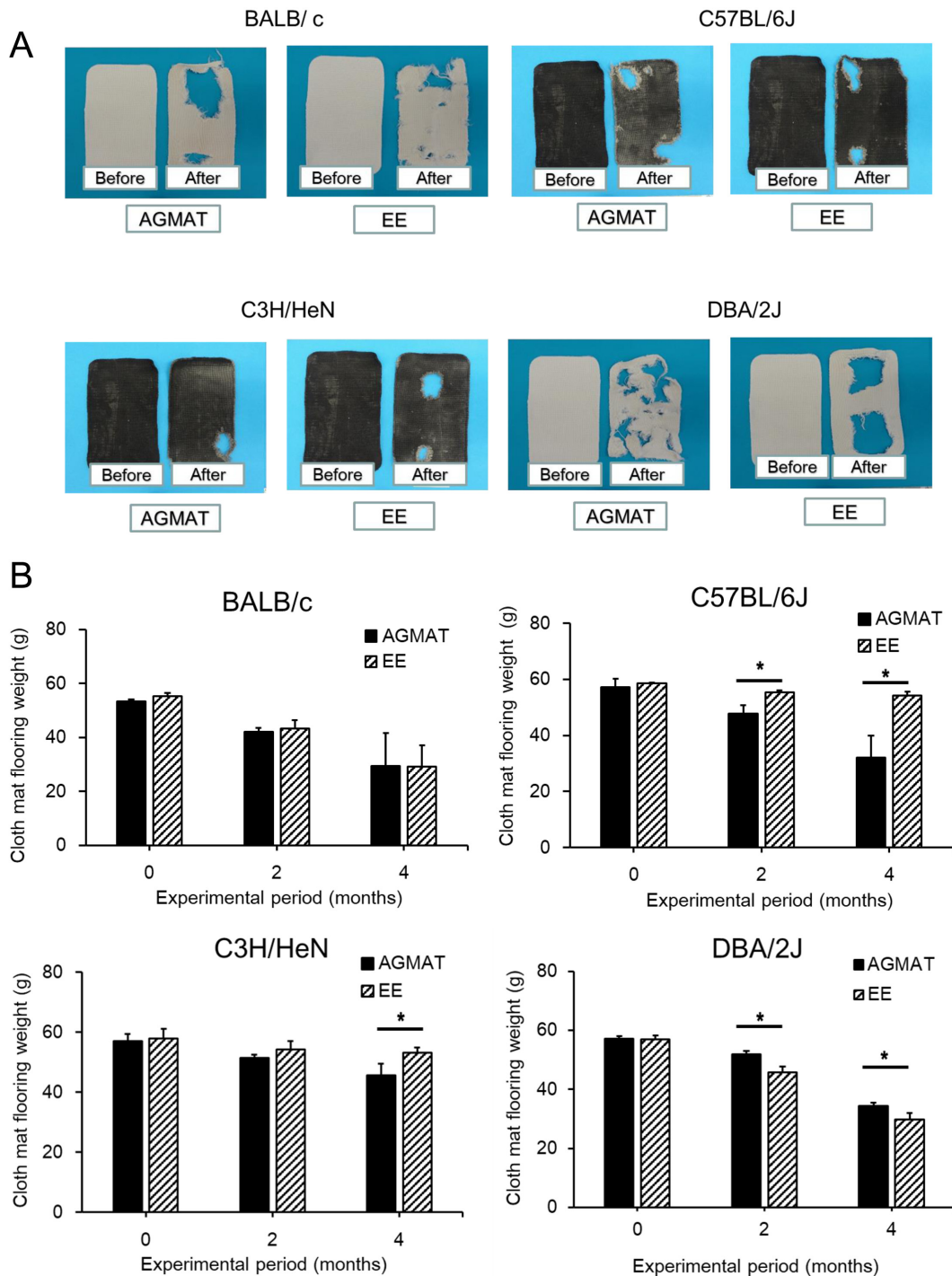


Fig. 7. Comparison of the condition of the cage mat flooring for each mouse strain in AGMAT and EE housing conditions. A: Representative images of the cloth mat before and after use. Each mat was used for about 4 months. B: Changes in weight of cage mat flooring before and after use. Four mats were assessed in the AGMAT and EE groups of each strain. Each value represents the mean \pm SD and were analyzed using the student's *t*-test of unpaired comparisons. *: $P < 0.05$.

nificantly lower than in the EE group ($P < 0.05$) at four months. In the DBA/2J mice, floor mat weight in the AGMAT group was significantly larger at two and four months than in the EE group ($P < 0.05$).

Discussion

In the present study, we examined the effects of enrichment of housing conditions on health and survival of four inbred mouse strains. The EE condition represents specific measures to improve the psychological well-being of laboratory animals from an animal welfare viewpoint [3, 15]. To date, the effects of EE on brain structure and function [27], behavior [5], immune responses [28], pup mortality [29], physiological parameters [22], and aggression [30] have been investigated; however, the effects of enrichment on lifespan and survival rates have not been evaluated. The results of our study provide fundamental data for other studies seeking to improve housing conditions as part of EE.

In general, when male mice are fostered in groups, the frequency of external wounds is high due to fighting [15]; however, female mice rarely fight. To avoid aggressive behavior among cage mates, we examined the effects of complex EE on female mice. The survival times of three of the mouse strains examined (C57BL/6J, C3H/HeN, and DBA/2J) were longer in the EE group; however, no effect was observed in BALB/c mice. In the former three strains, functional behavioral activity improved; the presence of places where they were secure from attack may have been one cause [31, 32]. Tsai *et al.* [33] reported that EE stimulated physiological function and behavior in mice. For instance, although it is not possible to escape from the cage, shelters or visual barriers could allow mice to hide from a threatening conspecific. However, in BALB/c mice, the survival rate was not influenced by EE. BALB/c mice have been reported to be adversely affected by EE, female BALB/c mice kept in an enriched environment have fewer immature thymocytes [34]. BALB/c female mice tended to have lower mature body weights when kept under enriched conditions, and a smaller number of litters were produced by female BALB/c mice housed in an enriched environment [4]. Even among studies using the same mouse strain, both increases and decreases in locomotor activity have been reported for mice in EE conditions [35–38]. The reasons for these conflicting results are unclear.

The use of an AGMAT cloth as a bedding material is a new concept in housing laboratory mice. The use of the cloth can improve the habitat of the animals. The AGMAT bedding was bitten and chewed by the mice.

The mat floors in both the AGMAT and EE groups were damaged in all the strains used here. The mice may have chewed the mat flooring to provide nesting material. The goal of EE is to provide animals with opportunities to express their full range of species-typical behavioral patterns [4]. Also, response-contingent enrichment objects (i.e., items the animals themselves can alter) are often more effective at eliciting novel interactive responses. Therefore, AGMAT is considered to be a beneficial alternative to wood shavings.

CORT, the main glucocorticoid produced by rodent, is a highly sensitive and reliable indicator of stress [39, 40]. In our study, CORT levels in the EE groups were similar to those in control mice. CORT levels were similar to those previously reported that standard housing and enriched housing were no significant difference [33, 41, 42]. Presumably, the lack of any change here among housing conditions indicates that the experiment did not load extreme stress on the animals during the experimental period. The C3H/HeN mice had slightly higher CORT levels than the other strains. Marashi *et al.* [43] reported CORT levels of 80–100 ng/ml in C3H/HeN mice, suggesting that slightly higher CORT levels are characteristic of this strain. CORT levels have previously been examined in mice kept under EE conditions [44, 45]. EE may decrease emotional reactivity by lowering the levels of stress hormones such as ACTH and CORT [46, 47]. While some studies found that EE decreased resting CORT levels [39] or following exposure to stressors [11, 44], others have found that resting levels in EE animals did not differ from those of non-enriched controls [24, 42, 48] and that animals kept under EE conditions had higher basal levels of CORT [41]. Possibly, the increases in CORT levels may be attributable to increased physical activity of animals in EE. Future studies comparing the effects of various levels in enrichment settings on stress response are needed [49].

In our study, the incidence of C57BL/6J and C3H/HeN mice with alopecia was significantly lower in the EE group than in the control group. As C57BL/6J mice age, they can develop a mild punctuate ulcerative dermatitis [50]. Continuous grooming is an abnormal behavior that can cause alopecia in mice; it has been particularly noted in C57BL/6J mice [51]. Skin lesions are common in mice that pull or pluck hair fibers from the follicles [52]. This behavior is a symptom of brain dysfunction and frequently occurs in a stressful environment [53]. Sundberg *et al.* [54] reported that the relative incidence of alopecia areata in one production colony of C3H/HeJ mice was 0.25% for female mice. The frequency in an aging colony selectively bred reached 4.7% for mice over 18 months of age, suggesting that this might be a

common aging change in C3H/HeJ mice. The EE used in this experiment appears to have alleviated this hair loss symptom associated with aging phenomenon and excessive grooming.

In conclusion, three mouse strains housed under EE conditions showed improved survival; however, EE did not alter the survival rate of the BALB/c mice. We suggest that there are inter-strain differences in response to changes in environmental conditions. Variations in the preferences of different species, strains, age groups, and sex require further evaluation. It is possible that the EE provided greater opportunities for sheltering in a secure location in which to avoid interactions with other mice. Our findings here suggest that depending on the mouse strains different responses to EE are caused with regard to health and survival rates. The results of this study provide basic data for further studies on EE.

Conflict of Interests

The authors declare that have no conflict of interests.

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