

## Effects of Germfree Status and Food Restriction on Longevity and Growth of Mice

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An investigation was undertaken to study the effects of germfree (GF) status and mild food restriction on life span in GF and specific pathogen-free (SPF) male ICR mice either full-fed (*ad libitum*) or on a restricted diet of 4.5 grams per day (equivalent to approximately 80% of full-fed intake) from five-week-old. The mean life span of the full-fed SPF and GF mice was 75.9 and 88.9 weeks respectively, while the mean life span of the food-restricted SPF and GF mice was 117.5 and 109.6 weeks, respectively. Mice in both GF and SPF food-restricted groups were characterized by lower body weight and increased survival. These findings suggest that the cessation of growth may be importantly and perhaps causally related to longevity. The GF mice survived longer than the SPF mice, but the combination of GF status with food restriction did not seem to extend life span more than food restriction alone. — KEY WORDS : aging, food restriction, germfree, growth, life span

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We are currently conducting a long-term study on aging in germfree (GF) mice together with study on the life-extending effect of restricted diet intake. Gordon *et al.* [6] reported that GF mice lived longer than conventional (CV) mice. Pollard and Wostmann [12] showed that full-fed GF Lobund-wistar (L-W) rats lived longer than full-fed CV L-W rats. From these studies, it has been deduced that the life extension in GF animals may be due to unique characteristics of the GF state.

On the other hand, food restriction has been of particular interest in aging research as the most effective means known for increasing life span in rodents. McCay *et al.* [8] has reported that restricting the food intake of rats markedly increased life span. Since then, many repetitions of similarly designed experiments have confirmed this fact [1, 2, 5, 10, 13, 14, 16, 19, 21-23]. It therefore appears that food restriction influences basic mechanisms of the aging process common to these animals.

GF animals live substantially longer than their CV counterparts. Food restriction

increases life span even further. However, little is known about aging processes in GF animals subjected to food restriction from weaning through their usual or extended life spans [12, 17, 18]. Hence, the present investigation was designed to examine both the effects of GF status and food restriction on the longevity and growth of mice.

### Materials and Methods

Mice : Four-week-old GF and specific pathogen-free (SPF) male ICR mice were purchased from CLEA Japan Inc., Tokyo, Japan. GF mice, reproduced and maintained under GF conditions in plastic isolator systems were free of detectable microflora. The SPF mice were maintained in clean standard animal room quarters. Room temperature for all mice was kept at  $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$  humidity at  $60\% \pm 5\%$ . Full-fed mice (GF-F and SPF-F) were housed three to a commercial plastic cage (approximately  $22 \times 15 \times 12$  cm). Food-restricted mice (GF-R and SPF-R) were housed individually in small

steel cages (approximately  $22 \times 8 \times 9$  cm).

**Diets:** All animals were provided with laboratory diet (CL-2, CLEA Japan), tap water, bedding, and cages sterilized by autoclave. Until the start of experimentation, all mice were full-fed on the sterilized diet. At 5 weeks of age, mice were assigned randomly to experimental groups. Intake was restricted to 4.5 grams of food per day beginning at 5 weeks of age in the food restricted animals. This is equivalent to approximately 80% of full-fed intake.

**General observation:** The mice were observed daily to ensure that food and water were available and to check for deaths. Starting at 5 weeks of age, each mouse was weighed every week until death. Organ wet weights were measured in mice from each group up sacrifices at 25, 40, 70 and 90 weeks of age.

**Statistical analysis:** The Wilcoxon rank sum test was employed to test the difference in the mean life span and maximum life span between experimental groups. Significance of differences between mean values was tested using Student's *t*-test.

## Results

**Longevity:** The mean life span and age of the longest surviving 10% are presented in Table 1. In both GF and SPF mice, the mean life span of the food-restricted mice was significantly longer than that of their full-fed counterparts ( $p < 0.01$ ). The mean age of the longest 10% survivors was also significantly higher for the groups of food-restricted mice than for groups of full-fed mice ( $p < 0.05$ ). Survival curves for all groups are shown in Figure 1. The first death occurred in week 38 in group SPF-F, week 70 in SPF-R, week 54 in GF-F, and week 80 in GF-R. Fifty-percent mortality was recorded in week 74 in SPF-F, week 122 in SPF-R, week 90 in GF-F, and week 109 in GF-R.

**Body weights:** The body weights of the GF and SPF mice used in this study are shown in Figure 2. The weights of mice in group SPF-F increased up to 70 weeks of age with some decline in weight at more advanced ages. The weights of mice in group GF-F was similar to those of group SPF-F through 70 weeks of age, but in contrast to group SPF-F, their weights showed no decline until the end of the experi-

Table 1. Summary of longevity findings

Group	n	Mean life span	Maximum life span*
SPF-F	49	75.9 ± 3.6	113.4 ± 1.3
SPF-R	28	117.5 ± 5.6	156.8 ± 4.8
GF-F	21	88.9 ± 3.8	116.0 ± 1.4
GF-R	22	109.6 ± 3.1	136.0 ± 1.4

Note. Entries are in weeks. Values are expressed as mean ± S.E. \* Mean survival time for longest 10% survivors of each population

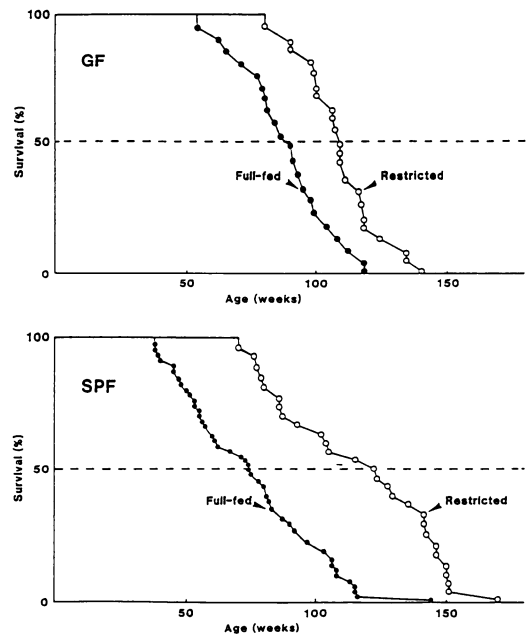


Fig. 1. Survival of GF and SPF mice fed full-fed or restricted diets. Each symbol represents onemouse

ment. Both groups of food-restricted mice showed slower weight gain for 16 weeks and then remained at this lower weight throughout, gaining significantly less body weight than their full-fed counterparts ( $p < 0.01$ ).

**Organ weights:** Liver, kidneys, heart and lung weights expressed as percentage of body weight as well as actual weights are given in Table 2. Actual weights of these organs were significantly less in both food-restricted GF and SPF mice at all ages ( $p < 0.05$ ). The weight of liver and kidneys increased with age, whereas the weight of heart and lung were constant

Table 2. Effect of food restriction on organ weights (in milligram) of GF and SPF mice at various ages

Group	n	Liver	Kidney*	Heart	Lung
25 weeks					
SPF-F	6	2,524 ± 95 (5.37)**	836 ± 31 (1.78)	217 ± 10 (0.46)	243 ± 15 (0.52)
SPF-R	6	1,568 ± 82 (4.53)	588 ± 23 (1.70)	185 ± 4 (0.53)	195 ± 5 (0.56)
GF-F	6	1,695 ± 53 (4.10)	678 ± 10 (1.64)	166 ± 4 (0.40)	180 ± 9 (0.44)
GF-R	6	1,318 ± 46 (3.72)	558 ± 10 (1.57)	150 ± 6 (0.42)	170 ± 4 (0.48)
40 weeks					
SPF-F	6	2,668 ± 102 (5.56)	878 ± 48 (1.87)	216 ± 6 (0.45)	233 ± 15 (0.49)
SPF-R	6	1,894 ± 45 (5.12)	619 ± 12 (1.78)	177 ± 3 (0.48)	195 ± 12 (0.53)
GF-F	6	2,255 ± 106 (5.28)	759 ± 36 (1.78)	220 ± 17 (0.51)	201 ± 17 (0.47)
GF-R	6	1,641 ± 57 (4.51)	605 ± 20 (1.66)	195 ± 6 (0.54)	183 ± 8 (0.50)
70 weeks					
SPF-F	3	2,866 ± 88 (5.72)	915 ± 13 (1.83)	215 ± 4 (0.43)	238 ± 8 (0.48)
SPF-R	3	1,805 ± 60 (4.20)	642 ± 24 (1.70)	182 ± 5 (0.48)	205 ± 15 (0.54)
GF-F	3	2,334 ± 116 (5.12)	819 ± 21 (1.80)	214 ± 15 (0.47)	218 ± 5 (0.48)
GF-R	3	1,705 ± 84 (4.51)	632 ± 24 (1.67)	187 ± 6 (0.49)	203 ± 13 (0.53)
90 weeks					
SPF-F	3	3,387 ± 131 (6.78)	999 ± 55 (2.03)	215 ± 10 (0.44)	276 ± 15 (0.56)
SPF-R	3	2,010 ± 62 (5.79)	662 ± 26 (1.91)	184 ± 8 (0.53)	203 ± 11 (0.59)
GF-F	2	2,653 (5.16)	963 (1.87)	222 (0.43)	214 (0.42)
GF-R	3	1,703 ± 65 (4.40)	687 ± 22 (1.76)	186 ± 2 (0.48)	191 ± 7 (0.49)

Note. Values are expressed as mean ± S.E. \* Weights of right plus left kidney

\*\* The percentage of organ weight to body weight are in parentheses.

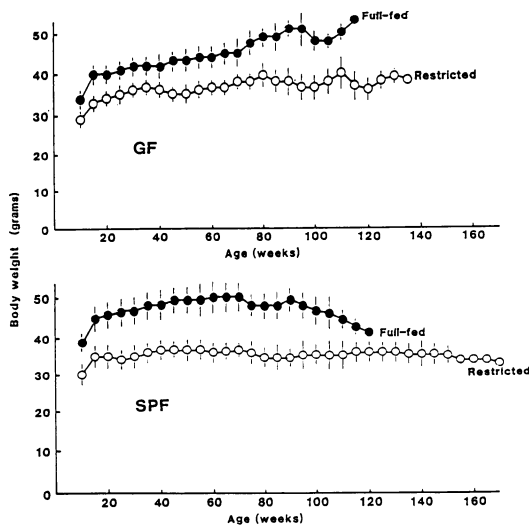


Fig. 2. Effect of diet on body weight. Values represent mean ± S. E. body weight in grams for all mice alive in each group at the indicated age.

up to 90 weeks of age in all animals. In both food-restricted groups, mice had lower liver weight compared to their full-fed counterparts ( $p < 0.05$ ). By relative weight, the kidneys were also lighter in the food-restricted groups, although the differences were not significant. On the contrary, the relative weights of both the heart and lung were higher in the food-restricted mice than the full-fed mice, but this increase was not significant.

## Discussion

In this report, we have presented data on the effects of mild food restriction and GF environment on the aging process. In our study, mean and maximum life spans were shortest for SPF-F mice, longer for GF-F mice, even longer for GF-R mice, and longest for SPF-R mice, indicating longer survival of the GF-F mice in comparison to their SPF counterparts, with both greater mean life span and maximum life span. These results are in agreement with the studies of Gordon *et al.* [6] and Pollard

and Westmann [12] noting longer survival for GF than CV mice and rats. From these experiments, it was believed that life extension in these GF animals may be characterized by a infectious disease-free status.

Data from the second aspect of our experiment agrees with other studies indicating that appropriate food restriction in rodents can increase mean and maximum life spans [1, 2, 5, 8, 10, 12-14, 16, 19, 21-23].

Improved survival observed in the food-restricted mice of both GF and SPF groups in our experiments occurred as delayed onset of age-dependent increases in mortality, appearing as significantly longer survival. It is noteworthy that mean and maximum life spans of the GF-R mice were shorter than the SPF-R mice. Analysis of the survival data indicates that under the present experimental conditions, the combination of GF status with food restriction did not extend life span beyond that of food restriction alone.

Brain and Benton [3] have reviewed the effects of differential housing on rats and concluded that the individual housing imposes a stress on the animals which affects their physiological parameters. The full-fed mice (GF-F and SPF-F) in the present study were housed three to a cage while the food-restricted mice (GF-R and SPF-R) were individually housed. This difference in housing may have differing influences in life span. However, as food-restricted animals are individually housed it would appear that food restriction overrides any possible detrimental effects of individual housing in respect of life span.

It is not difficult to imagine food intake to have significant effects on body weight. As expected, it was found that body weight gain was suppressed by restricting food supply. Body weights of both food-restricted groups of mice were lower than those of their full-fed counterparts. The lower body weight and longer life span observed in relation to food restriction from weaning agree with the results of previous investigator [8, 16-18, 23]. The importance of low body weight in longevity is indicated by the long mean life span of the food-restricted mice. The results suggest that the effects of food restriction on the aging processes may be due to delayed maturation or slowed growth, perhaps causally related to longevity.

Actual weights of all organs were significantly lower in both food-restricted GF and SPF mice. These smaller organs of the food-restricted animals are probably constructed of smaller cells. Enesco and Samborsky [4] have demonstrated that food restriction reduces the rate of cell-size increase and growth. In both GF and SPF mice, the relative weights of liver and kidneys on the food-restricted animals were less than those of organs from the full-fed animals. The lower liver-and kidneys-weights of the food-restricted groups observed in this study are in agreement with the results reported by other investigators [11, 15, 20]. However, the relative weights of the heart and lung were observed to be higher by 5-15% in both GF and SPF food-restricted mice, indicating that the organ weights do not develop in parallel with the body weight.

As another aspect of aging with relation to longevity, it is known that the normal immune functions decline with advancing age in both men and rodents [9]. Age-associated malignancies which contribute to early death have been correlated with decreased immune activity [7]. Weindruch *et al.* [24, 25] have suggested that immunological consequences of food restriction may contribute to effectson longevity and late-life spontaneous cancer. Hence, we have examined the effects of food restriction on immune system function and prolongation of life span in GF and SPF mice, yielding the following information on immunological function. In both GF and SPF mice, food-restricted mice exhibited elevated immune function in comparison to full-fed mice. Additionally, the immunological responses were lower in GF-R mice than in SPF-R, which may explain why the GF status in this case did not benefit their life spans of beyond that of their SPF counterparts. Details on immunological functions will follow in later publications.

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## マウスの寿命および成長に及ぼす無菌状態と 制限食の影響

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マウスの寿命に及ぼす無菌状態と食餌制限の影響について検討した。実験動物は雄の ICR 系無菌および SPF マウスを用いた。制限食の開始時期は生後 5 週とし、自由摂取群と制限食群とに分けた。制限食群の食餌は自由摂取群の摂取量の 80% (4.5g/日) を毎日与えた。平均寿命は自由摂取群の SPF マウスでは 75.9 週、無菌マウスでは 88.9 週、制限食群の SPF マウスでは 117.5 週、無菌マウスでは 109.6 週であった。また、体重を計測した結果、制限食群は自由摂取群と比べて、SPF および無

菌マウス共に低値の成績が得られた。この結果、離乳直後からの食餌制限は成熟を遅らせ、成長期間が長くなり、寿命が延びている可能性が考えられる。一方、無菌マウスの平均寿命は SPF マウスの平均寿命と比べて、自由摂取群では長く、制限食群では短かった。この成績から、無菌状態と食餌制限の組合せでは顕著な延命効果は認められなかったが、各々単独では平均寿命の延長に影響を及ぼしている可能性が示唆された。