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Life Span, Morphology, and Pathology of Diet-Restricted Germ-Free and Conventional Lobund-Wistar Rats

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The effect of germ-free life and dietary restriction (DR) on life span and pathology was investigated in isolator housed germ-free (GF) and conventional (CV) Lobund-Wistar rats fed either ad libitum or restricted to 12 grams per day (70% of adult ad libitum intake) of a natural ingredient diet from weaning. The median length of life of ad libitum CV and GF rats was 31.0 and 33.6 months respectively, while DR increased the median length of life of CV and GF rats to 38.6 and 37.8 months respectively. DR reduced the frequency or postponed the occurrence of diseases which eventually lead to death in the Lobund-Wistar rat. This was especially true of prostate adenocarcinoma, prostatitis, and mammary fibroma. The reduced early food intake and smaller body weight of adult GF rats may be the reason ad libitum fed GF rats live slightly longer than their CV counterparts, but GF life was without additional effect on life span when food intake was restricted.

THE use of germ-free (GF) animals in aging research was begun at Lobund Laboratory in 1958 with the belief that protection from the deleterious effects of microbes on the aging host would permit a better view of the endogenous aging process. Gordon et al. (1966b) clearly showed that GF mice lived at least eight months longer than conventionally housed (CV) mice (24 vs 16). The leading causes of death among the CV mice were respiratory infection (38%) and kidney lesions (14%), while GF mice died of intestinal atonia (36%), related to enlarged and twisted cecums, and of kidney lesions (12%). Later research also suggested that GF rats had superior longevity when compared to CV rats (Pollard, 1971; Pollard, 1973; Pollard and Luckert, 1979).

Young and young adult GF rats and mice have lower resting oxygen consumption, cardiac output, and reduced heart size (Wostmann, 1975). Therefore, according to the free radical theory of aging (Harman, 1986), the lower resting metabolic rate should extend the life span of the GF rat over that of the CV rat. The growing GF Lobund-Wistar (L-W) rat also has a reduced food intake (Wostmann et al., 1987; Ratcliffe, 1988), and the adult has a reduced body size (Snyder and Wostmann, 1987) when compared to CV L-W rats. The extended life span, reduced body size, and reduced food intake of the GF rat are conditions similar to those reported for rats on dietary restriction (DR) (Holehan and Merry, 1986). Therefore, life extension in GF animals may be due to a natural early DR in the GF state. The effect of GF life and DR on longevity in L-W rats has been reported by Pollard and Wostmann (1985). This pilot study used 10 GF L-W rats restricted to 12 grams of diet a day from weaning. The approximately 30% reduction in food intake from adult levels resulted in all 10 rats living to 37 months of age and having few of the pathologic changes of ad libitum fed GF L-W rats aged 30 months or more.

The results of this early study initiated the much larger Lobund Aging Study of more than 500 L-W rats. This study was designed to test the hypothesis that life in a germ-free environment will extend longevity beyond that of a pathogen-free conventional environment for both ad libitum and diet-restricted rats. This report focuses on the growth, longevity, and prominent pathology of isolator housed ad libitum and restricted fed CV and GF L-W rats up to 50 months of age.

METHODS

The L-W strain of rats originated at Notre Dame in 1958 with the creation of a GF breeding colony. The closed colony is now in its 56th generation. The CV breeding colony was derived from the GF colony, and at regular intervals GF males and females are added to the CV colony to maintain close genetic proximity. CV and GF L-W rats accept reciprocal skin transplants. All L-W rats are free of pathogenic microorganisms including viruses as defined by Pollard (1971). Based on assays for serum antibodies, L-W rats are negative for a broad spectrum of viral contaminants. The Lobund animal facility is AAALAC accredited.

Housing. — Only male rats were used in this study. The GF rats were housed in plastic or steel isolators and were maintained using routine gnotobiotic procedures (Subcommittee on Standards for Gnotobiotics, 1970). The CV rats were housed in plastic isolators to limit exposure to the local environment. CV isolators were opened only for introducing feed and water and to clean the cages. CV rats were weighed outside the isolator. All rats were weighed each week from age 6 to 10 weeks and thereafter once every 4 weeks. Due to the limitation of available isolator space, ad libitum fed rats (GF-F and CV-F) were housed 4 to a cage (commercial plastic boxes), which measured approximately 46 × 24 × 20 cm, allowing 276 cm^2 per rat. There were two cages per isolator. Diet-restricted rats (GF-R and CV-R) were taken

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from their respective breeding colonies at 6 weeks of age and housed individually in plastic cages measuring 36 × 20 × 18 cm, which allowed 720 cm² per rat. All rats were kept on sterilized Sani Cell corncob bedding and given sterilized untreated tap water. The rooms containing the isolators were air- and humidity-controlled with 12-hour light/dark cycles.

Diet. — All rats were fed steam-sterilized natural ingredient diet L-485, our colony diet since 1968 (Kellogg and Wostmann, 1969). L-485 is composed of ground corn, soybean meal, alfalfa meal, and corn oil to give 20% protein, 5% fat, and 3% fiber. Additional lysine, dl-methionine, and vitamins are included in the diet to compensate for losses due to sterilization. L-485 contains no casein. A pesticide and heavy metal analysis of the diet showed that all contaminants measured were below accepted safety limits or less than the detection limits. The restricted rats were never allowed more than 12 g of diet per day. This method of feeding becomes restrictive at about 8 weeks of age and results in a 30% reduction in feed intake in adult rats (Snyder and Wostmann, 1987).

Necropsy. — Rats were sacrificed for one of three reasons. One, the rats were in a moribund condition due to large palpable prostate adenocarcinomas or prostatitis, a mammary fibroma one-half the total body weight, or severe weight loss over several months. Two, ex-GF rats less than 30 months of age from microbe-contaminated isolators were sacrificed soon after the contaminations were discovered. Three, rats were selected at various ages from 3 to 30 months for measurement of physiological parameters related to aging. Each rat was anesthetized with halothane and blood was removed from the exposed heart with a needle and syringe. Rats were examined for tumors, and tissue samples were fixed for histological examination. A complete description of the pathological examination of the rats in this study has been published (Pollard et al., 1989).

Data organization and survival analysis. — Information on date of birth, date of death, and physical condition at time of death (selected or moribund/dead) was organized for use in the LIFETEST program of the Statistical Analysis System (SAS Institute Inc., 1985). A survival distribution function (SDF) was calculated for each experimental group using the LIFETEST program. The SDF is used to determine the probability that an individual rat will have a life span exceeding a particular age. Since many of the rats were removed prematurely from the study due to scheduled sacrifices or because of germ-free isolator contamination, any calculation of the SDF without considering these rats would be biased. In order to avoid this, the Kaplan-Meier product limit estimator was used to calculate the SDF (Cox and Oakes, 1984). This nonparametric survival model utilizes the age of selected rats which were prematurely removed from the study and compensates for their early removal when determining the SDF. 100 CV-F, 88 CV-R, 96 GF-F and 127 GF-R rats were used in the survival analysis. The hypothesis that the SDFs of each of the experimental groups were equal was tested by using the Wilcoxon rank test to compare each combination of experimental groups. Mos and Hollander (1987) have tested the effect of removing animals on survival parameters. Up to 70% removal of animals had no significant effect upon the estimated median survival even when the majority of removals occurred prior to the median age. An incomplete cohort of at least 80 animals was necessary to accurately estimate survival parameters. Based on these requirements, our estimate of the median survival of L-W rats in the four experimental groups is accurate.

Table 1. Survival Characteristics of Male Lobund-Wistar Rats[a]

Group	*N*	Median	10th Percentile	Maximum
CV-F	101 (45)[b]	31.0 (28.5–32.5)	36.3 (34.7–37.7)	38.9
CV-R	88 (43)	38.6 (36.4–40.0)	43.9 (41.2–45.5)	50.9
GF-F	96 (70)	33.6 (30.1–37.1)	39.0 (37.4–40.2)	40.5
GF-R	127 (75)	37.8 (36.3–40.4)	44.8 (43.0–46.8)	47.4

[a]Ages are given in months with 95% confidence levels in parentheses.
[b]The number of rats prematurely removed from the study but used in the calculation of the survival characteristics is in parentheses.

Analysis of pathologic changes. — Only moribund rats were used to test the hypothesis that tumor frequencies and the mean age of occurrence of tumors were equivalent among experimental groups. Contingency tables and chi-square analysis were used to make multiple comparisons between experimental groups to analyze tumor frequencies. The nonparametric Mann-Whitney test was used to make multiple comparisons between experimental groups to analyze the mean age of tumor occurrence (Steel and Torrie, 1980). A nonparametric test was used because the age of tumor occurrence was not expected to follow a normal distribution. Statistical significance for all tests was set at $p < .05$.

RESULTS

Survival characteristics. — Table 1 gives the median and 10 percent survival as determined by the SDFs, and the maximum life span of each of the experimental groups. Figure 1 is a graphic depiction of the SDFs for each experimental group. The test of equality between each of the SDFs showed that the survival of each of the restricted groups was different from that of the corresponding full-fed groups, that the survival of the GF-F rats was different from that of the CV-F rats, and that the survival of the GF-R and CV-R rats was equivalent.

Body weight. — As shown in Figure 2, each of the experimental groups has a characteristically different pattern of growth. Up to 4 months of age the GF-F rats grow at the same rate as the CV-F rats. After 5 months the GF-F rats grow at a slightly slower rate. By 18 months the average weight of CV-F rats is just over 480 grams, but the GF-F rats average just over 450 grams. Because the cecum of GF rats is 20 grams larger than that of CV rats even at a young age, the corrected difference in body weights between GF-F and CV-F rats may be considered closer to 50 grams in adults and as much as 30 grams at 5 months. Feed intake per cage was

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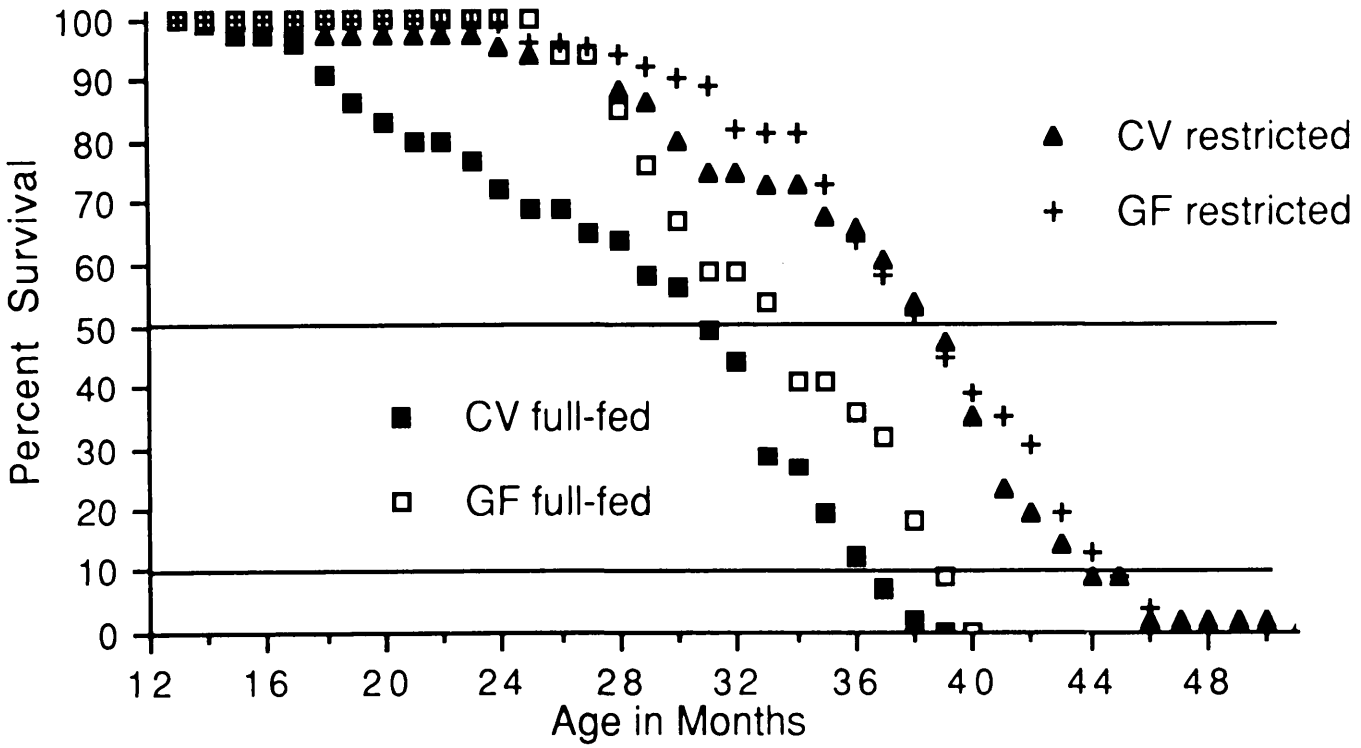

Figure 1. The survival distribution curves calculated for each experimental group of Lobund-Wistar rats. CV = conventional, GF = germ-free.

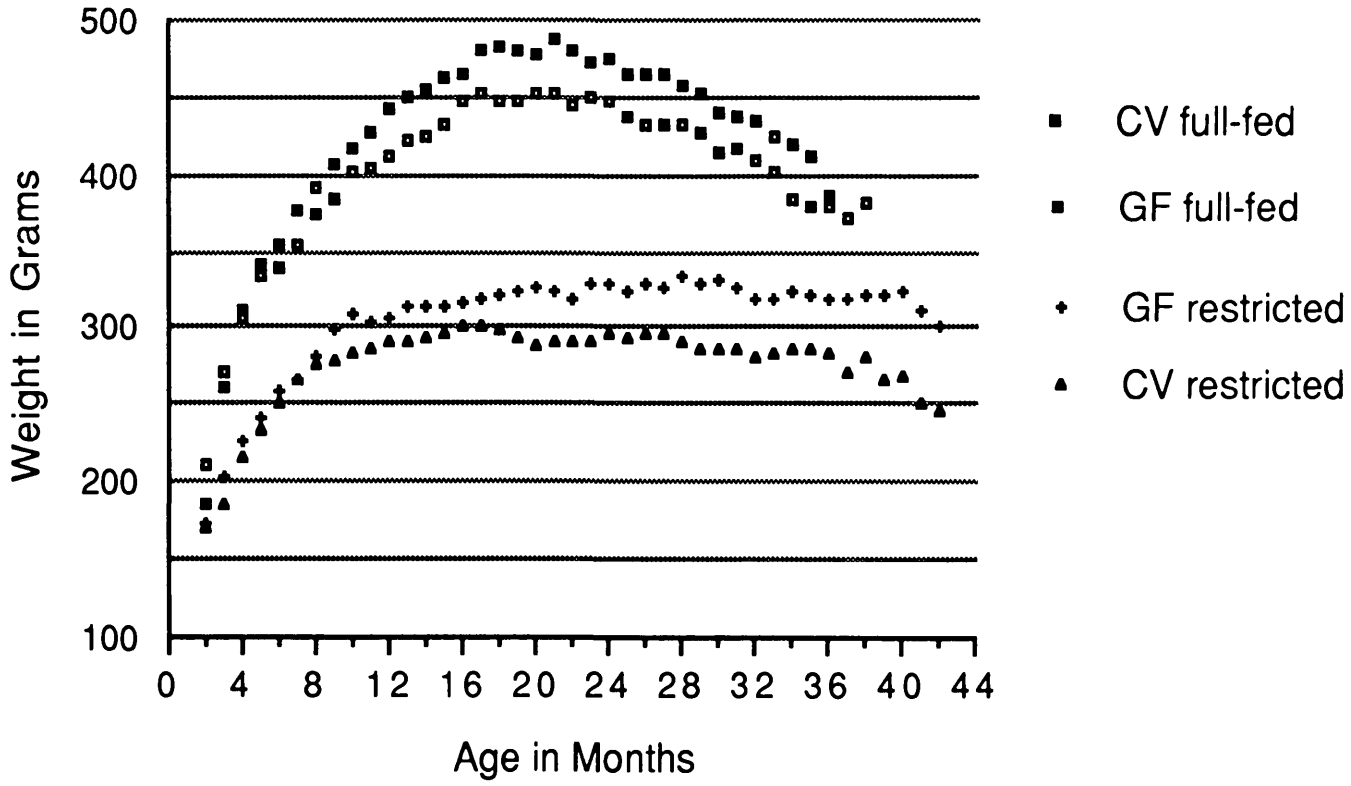

Figure 2. Growth curves of each experimental group of Lobund-Wistar rats. CV = conventional, GF = germ-free.

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measured in a few CV-F and GF-F rat cages between 2 and 18 months. Calculated as g intake/100 g body weight, the intake of CV-F rats declined from 9 g to 5 g from 2 to 7 months of age. During the same time period the intake of GF-F rats declined from 8 g to 5 g. At 18 months of age both ad libitum fed groups consumed 3 g per 100 g body weight.

The CV-R and GF-R rats grew at similar rates. The GF-R rats eventually outweighed the CV-R rats, but after compensating for the 20 gram cecum the difference was only 10 grams. Feed intake as grams/100 grams body weight in the R rats fell from 7 g at 2 months of age to 4.5 g at 7 months of age. By 18 months both CV-R and GF-R rats were consuming about 4 g per 100 g body weight. Further details on the growth of GF and CV L-W rats are available in Snyder and Wostmann (1987).

Organ weights. — Table 2 gives the weights of various organs from 3 to 30 months of age for each of the four experimental groups. Young and adult GF rats are known to have smaller livers and hearts when compared to CV rats (Gordon et al., 1966a; Wostmann et al., 1968). Data from our study confirm this relationship from 3 to 30 months of age both for absolute weight and as a percent of body weight. The DR rats had higher liver, heart, kidney, and brain weights as a percent of body weight at all ages when compared to the full-fed rats. Absolute gastrocnemius muscle weight declined in the CV-F and GF-F rats between 6 and 30 months but not in the CV-R and GF-R rats. The epididymal fat pad increased in absolute weight between 6 and 18 months in all groups but then declined in weight between 18 and 30 months. As a percent of body weight the DR rats had less epididymal fat pad and more gastrocnemius at each age when compared to the full-fed rats.

Pathology. — Table 3 gives a summary of the prominent pathologic changes found in moribund rats during this study. Tumors first appear in the L-W rat at about 18 months of age, but most tumors are not found until 30 months of age. The majority of these tumors develop spontaneously in the liver (hepatomas and adenocarcinomas) and several endocrine-related organs, specifically the adrenal medulla (hyperplasia), the prostate gland (adenocarcinoma), and the mammary gland (fibroma). Prostatitis is common in the CV-F rats between 18 and 24 months. Nephrosis was not common in any of the experimental groups. When it occurred, the kidney lesions never exceeded grade 1 as described by Maeda et al. (1985). A detailed description of the pathological findings from the present study has been published (Pollard et al., 1989).

Although the frequencies for each of the pathologies listed in Table 3 were smaller in the CV-R, GF-F, and GF-R rats when compared to the CV-F rats, these differences were not statistically significant. The mean age of occurrence of most pathologic changes, however, was significantly delayed in CV-R, GF-F, and GF-R rats when compared to the CV-F rats. There were no significant differences in mean age of occurrence of pathologic changes when CV-R, GF-F, and GF-R rats were compared with each other.

Table 2. Absolute Organ Weights (in Grams) of Lobund-Wistar Rats at Various Ages, $n = 8$

Group	Liver	Heart	Kidney	Muscle[a]	Fat Pad[b]	Brain
3 months						
CV-F	9.79	1.06	1.86	1.45	3.49	1.84
CV-R	6.70	0.73	1.32	0.93	1.68	1.54
GF-F	6.99	0.94	1.80	1.40	3.16	1.70
GF-R	5.88	0.62	1.29	0.92	1.98	1.63
6 months						
CV-F	9.89	1.27	2.07	1.98	4.97	1.76
CV-R	8.50	1.00	1.60	1.48	2.45	1.73
GF-F	7.09	1.05	2.01	1.83	4.47	1.85
GF-R	6.36	0.77	1.51	1.29	2.52	1.74
18 months						
CV-F	11.12	1.61	2.51	1.73	7.46	1.81
CV-R	8.42	1.31	1.84	1.51	3.67	1.76
GF-F	8.39	1.22	2.29	1.39	6.29	1.85
GF-R	7.80	0.99	1.86	1.39	3.48	1.75
30 months						
CV-F	12.17	1.80	2.63	1.48	6.19	1.85
CV-R	8.86	1.29	1.78	1.48	2.74	1.79
GF-F	9.87	1.43	2.61	1.13	4.74	1.92
GF-R	8.54	1.07	2.08	1.40	2.62	1.87

[a]Single gastrocnemius muscle.
[b]Both epididymal fat pads.

DISCUSSION

Our study confirms the ability of DR to extend life span in the rat by showing that rats which are virtually free of kidney disease (due to consumption of a casein-free diet) and free of infectious disease (from living in a germ-free environment) still receive considerable benefit from a reduction of dietary intake of only 30%. DR reduced the incidence or postponed the occurrence of the diseases that eventually lead to death in the L-W rat. This was particularly true of prostate tumors, prostatitis, and mammary fibroma. As a result, this restriction extended life span by approximately 8 months in CV L-W rats and 4 months in GF L-W rats.

The GF state increased the median length of life of full-fed L-W rats by 8 percent. The increase in percent survival of GF-F rats at all ages when compared to CV-F rats is due partly to the lack of prostate infections in GF rats. Reduced early feed intake and a smaller adult body weight may also have helped to extend the life span of the GF-F rats. They had a greater 10% survival (39.0 and 36.3 months) and maximum survival (38.9 and 40.5 months) ages when compared to CV-F rats. Previous research with young growing GF L-W rats fed a casein starch diet has shown a distinctively lower food intake and growth in the GF rats compared to CV rats (Wostmann et al., 1987, Coburn et al., 1989). A recent report using a different strain of GF rats confirms the reduced feed intake of young GF rats (Ratcliffe, 1988). The effect of a natural DR in the germ-free state is indirectly supported by the similarity in life spans between CV-R and GF-R rats that have the same feed intake and similar adult body weights.

Due to the constraints of available germ-free isolators, the

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Table 3. Pathology of Moribund Lobund-Wistar Rats Sacrificed Just Prior to Natural Death

Group		Adrenal Medulla Tumors	Hepatomas	Liver Adeno-carcinomas	Prostatitis	Prostate Adeno-carcinomas	Mammary Fibromas	Thymomas	Nephrosis
CV-F (N = 36)									
mean age ± SE	29.5 ± 1.0	31.1 ± 0.8	32.5 ± 0.6	32.4 ± 1.0	23.3 ± 2.3	28.1 ± 2.8	29.9 ± 2.3	33.6 ± 1.1	29.7 ± 2.8
range	11–37	19–37	26–37	28–37	18–31	11–34	17–35	28–37	19–37
percent		81	58	25	17	22	22	19	17
CV-R (N = 29)									
Mean age ± SE	37.5 ± 1.2	39.8 ± 0.8[a]	41.1 ± 1.3[a]	42.0 ± 2.1[a]	41.0 ± 9.0	40.0 ± 5.0[a]	41.0 ± 4.0[a]	36.5 ± 8.5	33.5 ± 16.5
range	17–50	32–50	35–50	38–45	32–50	35–45	37–45	28–45	17–50
percent		79	34	10	7	7	7	7	7
GF-F (N = 15)									
mean age ± SE	33.5 ± 1.5	35.4 ± 1.4[a]	37.3 ± 1.0[a]	36.7 ± 2.0	—	22.0 ± 0.0	35.0 ± 2.4	34.5 ± 5.5	34.3 ± 4.1
range	22–40	22–40	33–40	33–40		22	24–40	29–40	22–40
percent		80	47	20		7	40	13	27
GF-R (N = 31)									
mean age ± SE	37.7 ± 0.9	38.3 ± 1.3[a]	38.4 ± 1.2[a]	42.2 ± 1.7[a]	—	37.0 ± 5.0	35.8 ± 3.1	43.0 ± 0.0	35.3 ± 4.2
range	27–48	27–48	30–43	35–48		32–42	29–44	43	27–43
percent		61	42	19		6	13	3	13

Note. The mean age ± the standard error and the age range are listed for the entire group of moribund rats and for those rats in which the respective pathologies were found. Individual pathology occurrence was not significantly ($p < .05$) different between any of the experimental groups as determined by contingency tables and chi-square analysis.

[a]Mean age of pathology occurrence was significantly ($p < .05$) different from the CV-F group as determined by the nonparametric Mann-Whitney test.

ad libitum fed rats in the present study were housed four to a cage while the diet-restricted rats were individually housed. When this study began in 1983, the NIH guideline for cage space per rat was 258 cm² for all rats over 300 g. In 1985, however, the NIH space requirement changed to 387 cm² per rat between 400 g and 500 g (ILAR, 1985). We were unable to change the caging arrangement in the middle of our study, which ended in 1988. Therefore two potential housing variables — stress from individual housing in the restricted rats and stress from crowding in the ad libitum rats — may have influenced the outcome of the study. Brain and Benton (1979) have reviewed the effects of differential housing on rats and concluded that although there was considerable research on the subject, there was no conclusive evidence on how housing may influence physiological parameters. Charpenet et al. (1982) have shown that stress can alter testicular function and reduce serum testosterone levels. If housing stress had occurred during the present study, then a reduction in serum testosterone may be expected when the ad libitum fed rats between 8 and 12 months old reach 400 g. Our previously published findings on serum testosterone (Snyder et al., 1988) and as yet unpublished data show that serum testosterone levels increase rapidly through 7 months of age, decrease slightly between 12 and 18 months of age, and decline more rapidly after 24 months. R rats generally maintain higher serum testosterone levels when compared to F rats at all ages. Other parameters such as brain size, adrenal cortex histology (Pollard et al., 1989), blood chemistry (Snyder and Towne, 1989), white blood cell counts (Eberly and Bruckner-Kardoss, 1989), and the longer median length of life of the L-W rat (31 months) when compared to the 23-month Fischer 344 rat (Yu et al., 1985), the 24-month Sprague-Dawley rat (Nolen, 1972), and the 29-month Wistar rat (Kroes et al., 1981) also indicate that there was no undue influence of differential housing on animal health during our study.

It is unclear how the reduced oxygen consumption and metabolic rate of the young and young adult GF rat influence life span. If there is an inverse relationship between basal metabolic rate and life span, GF rats should live significantly longer than their conventional counterparts. Although no information is available on the oxygen consumption of middle-aged and old GF rats, the reduced liver and heart size (both absolute and relative) of GF-F and GF-R rats suggests that their metabolic rates are reduced. If this is true, the reduced metabolic rate of GF rats had only a small effect on life span. The reduction in food intake, relative heart size, cardiac output, and resting oxygen consumption of GF animals has been related to bioactive compounds produced in the enlarged cecum (Wostmann et al., 1968; Snyder and Wostmann, 1987). These conditions may reflect a greater metabolic efficiency in the young GF-F rat and be responsible in part for the lower adult body size of the GF-F rats; they were without effect on life span, however, when dietary intake was limited.

Masoro (1980) has reported that in most of the rat strains used in aging research the median length of life is between 24 and 29 months, and that diet and housing can have considerable influence on life span. The extended median length of life of CV-F L-W rats, when compared to other rat strains (31 vs 25 months), seems to be due primarily to the lack of kidney disease in the L-W rat. Prior to the present study, the average life span of the L-W rat had been estimated at 25 months (Pollard and Wostmann, 1985). Because this estimate was based on the survival of rats raised in an open animal colony, the limited isolator protection of the CV

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rats in the present study may also have contributed to the extended survival of L-W rats compared to other rat strains. Kidney disease is the major cause of death in most of the rat strains used in aging research. Nephrosis is a major problem in the use of rats for studying alterations in the aging process, especially when distinguishing age-related from drug-related effects during chronic toxicity studies (Goldstein et al., 1988). In the present study, nephrosis was uncommon and never severe enough to be the cause of death. The development of this disease can be altered by DR (Maeda et al., 1985) or by the use of soy protein instead of casein as a protein source in the diet (Iwasaki et al., 1988). Prior to the introduction of L-485 as the colony diet at Lobund Laboratory in 1969, Lobund-Wistar rats older than 24 months did develop severe nephritis on a semirefined diet containing casein (Pollard, 1971).

The usefulness of natural ingredient diet L-485 in long-term studies instead of a semi-synthetic diet containing casein seems to be without question. Environmental contamination of natural ingredient diets, however, may introduce unknown factors into a long-term study. Cadmium, arsenic, and lead were detected in low levels in L-485, but these appear to be below chronically toxic levels (Underwood, 1977). Aflatoxin and pesticide residues in L-485 were below detectable levels, but the possible influence of these contaminants on liver tumors in L-W rats cannot be discarded at this time. Further study is needed to clarify the association between liver tumors and exposure to the low levels of contaminants in L-485.

The GF animal has been suggested as an ideal model for aging research because of the freedom from microbial interference during aging, but the cost of maintaining GF animals for extended periods is prohibitive. Our study has shown that CV L-W rats derived from a clean breeding colony, housed in minimal barrier isolation, and fed L-485 will live almost as long as GF L-W rats. Mos and Hollander (1987) have stated that a rectangular shape to the survival curve and the presence of multiple pathological lesions are prerequisites for an animal model of aging that resembles the situation in man. The CV L-W rat may fit this ideal and serve as a model for examining the interaction of nutrition and the aging process. True age-related changes in physiology, cellular biochemistry, and pathology can be monitored in the L-W rat between 18 and 30 months without interference from kidney disease or prominent neoplastic disease.

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