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INFLUENCE OF FOOD INTAKE AND SEXUAL SEGREGATION ON LONGEVITY, ORGAN WEIGHTS AND THE INCIDENCE OF NON-NEOPLASTIC AND NEOPLASTIC DISEASES IN RATS

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Abstract—In a small-scale experiment the effects of four variables were investigated in male and female Wistar rats fed on a standard laboratory chow and not deliberately exposed to any carcinogen. The variables investigated were (i) diet restriction by limiting access to food to 6.5 hr/day instead of 24 hr/day; (ii) housing males in a single-sex room as distinct from a mixed-sex room; (iii) life-long segregation of males from females, as distinct from access to one virgin female for 5 days during each alternate week; and (iv) uniparity in females versus life-long virginity. The endpoints compared were body-weight gain, longevity, visible and palpable swellings, absolute and relative organ weights, microscopically confirmed malignant neoplasms that contributed to death before the end of the study at 2 yr, incidence of other neoplasms in decedents and rats killed at the end of the study, and the incidence of various non-neoplastic conditions including myocardial inflammation and fibrosis, chronic progressive nephropathy, liver glycogen storage and fatty degeneration, mammary gland hyperplasia and secretory activity, pancreatic polyarteritis, radiculoneuropathy and certain testicular changes. The results indicated major beneficial effects of dietary restriction on most of the endpoints. By comparison the other variables had only marginal effects. Leydig-cell hyperplasia and neoplasia occurred at significantly higher incidences in males housed intermittently with females than in permanently segregated males. No convincing differences were seen between females that littered once and those that remained virgins. The relevance of these findings to the prediction of cancer mortality risk in man and to the design of rodent carcinogenicity studies is discussed.

INTRODUCTION

It has long been evident that, just as in humans (Doll and Peto, 1981; Higginson and Muir, 1979; Lew and Garfinkel, 1979), caloric intake and other variables relating to animal husbandry influence the longevity and risk of cancer development in untreated laboratory rodents. It is already clear that overnutrition is a major factor in so far as mild dietary restriction significantly prolongs life and reduces cancer incidence in both rats and mice (Conybeare, 1980; Tucker, 1979). In the case of rats, the effect of dietary restriction on longevity is partly explained by reductions in the incidence and severity of chronic progressive nephropathy and of polyarteritis (Berg, 1967; Berg and Sims, 1965; Turnbull *et al.*, 1985). The beneficial effect of diet restriction on tumour incidence both in rats and mice is evident for many different kinds of neoplasm. However in rats, reductions in the incidences of tumours of endocrine tissues (pituitary, pancreatic islets, adrenal cortex, adrenal medulla, thyroid follicular, thyroid C-cell) and of hormone-controlled tissues (mammary gland) are particularly evident (Roe, 1981 and 1987). In association with these reductions there is a reduced incidence of hyperplastic lesions in the same tissues.

The first aim of the small-scale study reported in the present paper was to see whether caloric intake and certain other variables influence the incidence of 'fatal' neoplasms in rats since 'fatal' neoplasms are much more relevant to cancer mortality data for humans than are tumour incidence data (Roe, 1989a). For this purpose 'fatal' cancer was defined as a histologically malignant tumour that either actually caused the death of an animal or necessitated its killing on humane grounds.

The second aim was to explore the possible role of certain factors other than dietary restriction on tumour incidence in rats and to compare such effects, if any, with the effect of restricting access to food from 24 hr/day to 6.5 hr/day. The variables investigated were:

(i) Diet restriction (tested in males and females): 6.5 hr/day feeding *v.* 24 hr/day feeding.
(ii) Isolation (tested in males only): being housed in a single-sex room *v.* being housed in a mixed-sex room.
(iii) Sexual segregation of males: no access to females *v.* access to one virgin female during 5 days of each alternate week.

(iv) Parity of females: uniparity *v.* life-long virginal status.

MATERIALS AND METHODS

Rats, caging, general husbandry and composition of diet. The study design is summarized in Table 1. ICI Wistar-derived SPF rats aged 4 wk were obtained from the Animal Services Department of Smith Kline and French Research Ltd, Welwyn, UK in whose laboratories the study was conducted. They were individually housed (except in the case of group 3 males and group 5 females; see Table 1) in grid-bottomed cages in a room containing animals of both sexes or containing males only. Cages were arranged in five rows of four on racks, each group occupying one rack. In each of the two rooms racks were moved around at regular intervals in an attempt to control for variations in local environmental conditions. However, the arrangement of cages on individual racks remained unaltered throughout the study. Both rooms were maintained at a temperature of 21 ± 2 C and a relative humidity of $50 \pm 10\%$. A 12-hr light/dark cycle was maintained throughout the study with the light period starting each day at 07.00 hr. All animals had free access to tap-water and, with the exception of those in group 2, all had free access to food at all times; group 2 animals had free access to food for only 6.5 hr/day. The same pelleted PRD diet (Labsure Ltd, Manea, Cambs., UK) was given to rats in all groups. The diet has the following formula (based on the analysis of 38 batches): crude protein, 20%; crude oil, 3%; total carbohydrate, 57.5% (crude fibre, 4.6%, starch, 53.5%); water, 13%; ash, 5%; calcium, 0.62%; magnesium, 0.17%; phosphorus, 0.70%; metabolizable energy, 13 kJ/kg. The females in group 5 (see Table 1) were housed until aged 7–8 wk in the room containing animals of both sexes. They were then transferred to another room, mated and allowed to litter. Their progeny were weaned 3 wk after birth and the now uniparous females were returned to their original room for the remainder of the study.

Observations during life. Food consumption was recorded over a period of 1 wk at 6-weekly intervals during the first 3 months of the study and thereafter at 3-monthly intervals. Body weights were recorded every 4 wk and animals were observed twice daily for their general state of health and examined once a week for the presence of skin lesions and visible or palpable masses. Moribund animals were killed using diethyl ether vapour followed by exsanguination.

Termination of the study and autopsy procedure. After 24 months survivors were killed with diethyl ether vapour followed by exsanguination. The following organs were weighed: adrenals, brain, gonads, seminal vesicles, heart, kidneys, liver and prostate. An extensive list of tissues was fixed in formal saline for histological processing and examination. A full histopathological evaluation was performed on rats found dead during the study. This entailed a systematic post-mortem examination, the recording of all macroscopically visible abnormalities, and the sampling, fixation and sectioning of the following tissues: adrenals (both cortex and medulla); aorta; bone marrow; brain (four levels); colon; duodenum; epididymis (both); eyes (both); Harderian gland; heart (3 sections); ileum; jejunum; kidneys (both); lacrimal gland; larynx; liver (2 pieces); lung (both); mammary gland; mandibular lymph node; mesenteric lymph node; nasal cavity; oesophagus; optic nerve (both); pancreas; parathyroids (both); pituitary; prostate; salivary glands (parotid, submaxillary and sublingual); sciatic nerve; caecum; seminal vesicles (both); skeletal muscle; skin; spinal cord (three levels); spleen; stifle joint; stomach (fore and glandular); testes (both); thymus; thyroids (both); tongue; trachea; urinary bladder; other tissues noted to be abnormal macroscopically. Sections ($5\,\mu$) of these tissues were stained with haematoxylin and eosin.

Statistical methods. Parametric analysis of variance was used for between-group comparison of food consumption and body weight, with equivalent non-parametric rank tests used for analysis of organ weights. The incidences of premature deaths, tumours and non-neoplastic conditions were analysed using the method of Peto *et al.* (1980). Tumours were regarded as not contributing to death except for malignant tumours in decedents, which were considered fatal. Non-neoplastic conditions considered to have contributed to death were moderate, severe or very severe nephropathy and those conditions listed in the footnote to Table 4. In the case of commonly occurring non-neoplastic conditions scored as 0 = absent, 1 = minimal, 2 = slight, 3 = moderate, 4 = severe, 5 = very severe, analyses were carried out of overall incidence and of incidence above appropriate grades. Probability (P) values, two-tailed, were expressed as $P < 0.001$, $P < 0.01$, $P < 0.05$, $P < 0.1$, or NS (not significant, $P \geq 0.1$). No formal correction of P values was carried out in respect of multiple endpoints, time points or between-group comparisons. Interpretation of significant P values, in terms of a true effect of the variable under study, takes into account level of significance, biological plausibility, consistency of results between the sexes, and multiple tests.

Table 1. Experimental design

Group no.	Sex	No. of rats	Access to food (hr/day)	Room type	Sexual status
1	Male	20	24	Mixed sex	No access to females
	Female	20	24	Mixed sex	Life-long virgin
2	Male	20	6.5	Male only	No access to females
	Female	20	6.5	Mixed sex	Life-long virgin
3	Male	20	24	Male only	No access to females
4	Male	20	24	Mixed sex	Sexually active*
5	Female	20	24	Mixed sex	Uniparous

*Housed with a fresh virgin female for 5 days during each alternate week.

Table 2. Food consumption of rats maintained under various conditions of food intake, sexual segregation and parity for 2 yr

Week no.	Food consumption (g/rat/wk)				
	Group 1	Group 2	Group 3	Group 4	Group 5
			Males		
6	191 ± 13	148 ± 17***	192 ± 22	188 ± 17	—
25	174 ± 14	135 ± 18***	177 ± 15	186 ± 12**	—
38	178 ± 14	120 ± 10***	177 ± 19	199 ± 14***	—
54	182 ± 15	143 ± 18***	181 ± 21	201 ± 14***	—
64	181 ± 18	155 ± 18***	179 ± 18	195 ± 17**	—
78	159 ± 16	131 ± 13***	167 ± 25	176 ± 22**	—
91	166 ± 15	128 ± 15***	161 ± 33	178 ± 19*	—
104	159 ± 18	145 ± 14*	179 ± 34	164 ± 47	—
			Females		
6	127 ± 22	113 ± 9**	—	—	128 ± 17
25	129 ± 11	98 ± 23***	—	—	132 ± 8
38	135 ± 10	96 ± 10***	—	—	136 ± 13
54	130 ± 9	103 ± 8***	—	—	134 ± 15
64	153 ± 16	98 ± 10***	—	—	145 ± 18
78	129 ± 16	107 ± 9***	—	—	130 ± 17
91	147 ± 18	105 ± 13***	—	—	141 ± 27
104	149 ± 22	129 ± 13**	—	—	155 ± 34

group 1 = controls group 2 = restricted access to food
group 3 = in room with males only group 4 = sexually active
group 5 = uniparous (See Table 1 for full details.)
Values are means ± SD; those marked with asterisks differ significantly (parametric analysis of variance) from the corresponding control (group 1) values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

RESULTS

Food consumption

In both sexes, food consumption in group 2 averaged about 80% of that in other groups throughout the study and this difference was highly significant (Table 2). In females, food consumption was similar in groups 1 and 5 except during wk 13–17 (not shown in Table 2) when group 5 rats were pregnant. In males, food consumption in group 3 was similar to that in group 1 at all times, but that in group 4 was significantly higher than that in group 1 during wk 25, 38, 54, 64, 78 and 91.

Body-weight gain

Throughout the study male and female rats in group 2 weighed significantly less ($P < 0.001$) than those in other groups (Fig. 1). The only significant differences between group 1 and group 5 females in body weight were between wk 13 and 17 when group 5 females were pregnant. For males, the only significant differences between groups 1 and 3 in mean body weight were between wk 72 and 88 ($P < 0.05$). Similarly significant differences were seen between groups 1 and 4 during the same period. There is no obvious explanation of these differences other than that the possibility that intercurrent disease was on average affecting the mean body weights of group 1 males more than those of group 3 or group 4 males.

Organ weights

Dietary restriction had very clear effects in both sexes on absolute and relative organ weights. Table 3 compares group 2 with the appropriate comparison groups for those organs most clearly affected. Compared with group 3 males or group 1 females, absolute heart, liver and kidney weights were significantly ($P < 0.001$) reduced in both sexes in group 2. Reductions in absolute brain weight were seen in males only ($P < 0.01$), but there was an increase in absolute

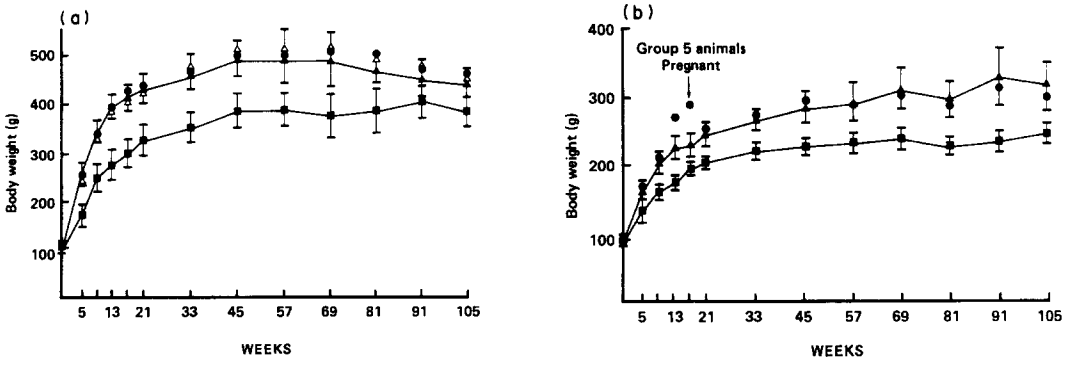

Fig. 1. Group mean body weights in a 2-yr study in rats. (a) Males of group 1 (controls, ▲), group 2 (restricted access to food, ■), group 3 (in room with males only, ●) and group 4 (sexually active, △). (b) Females of group 1 (▲), group 2 (■) and group 5 (uniparous, ●). For clarity curves and standard deviations are only shown for groups 1 and 2. See Table 1 for details of the groups.

Table 3. Effect of dietary restriction on organ weights in terminally killed rats

Organ	Sex	24 hr/day access to food* (mean ± SD)	6.5 hr/day access to food** (mean ± SD)	Significance
		Absolute weight (g)		
Body	Male	437 (31)	384 (38)	$P < 0.01$
	Female	311 (33)	244 (15)	$P < 0.001$
Heart	Male	1.45 (0.22)	1.16 (0.11)	$P < 0.001$
	Female	1.17 (0.10)	0.85 (0.08)	$P < 0.001$
Liver	Male	19.2 (5.1)	12.3 (1.6)	$P < 0.001$
	Female	14.3 (1.5)	8.6 (0.9)	$P < 0.001$
Kidney	Male	1.89 (0.34)	1.45 (0.19)	$P < 0.001$
	Female	1.39 (0.16)	1.05 (0.12)	$P < 0.001$
Brain	Male	2.53 (0.08)	2.39 (0.13)	$P < 0.01$
	Female	2.27 (0.09)	2.22 (0.08)	NS
Gonads	Male	2.32 (0.43)	2.84 (0.66)	$P < 0.05$
	Female	0.082 (0.030)	0.081 (0.019)	NS
		Weight relative to body weight %		
Heart	Male	0.332 (0.046)	0.303 (0.032)	NS
	Female	0.380 (0.055)	0.350 (0.036)	NS
Liver	Male	4.36 (0.84)	3.21 (0.31)	$P < 0.001$
	Female	4.64 (0.74)	3.51 (0.33)	$P < 0.001$
Kidney	Male	0.432 (0.053)	0.379 (0.049)	$P < 0.05$
	Female	0.451 (0.076)	0.431 (0.032)	NS
Brain	Male	0.582 (0.049)	0.630 (0.70)	NS
	Female	0.736 (0.073)	0.916 (0.067)	$P < 0.001$
Gonads	Male	0.533 (0.111)	0.745 (0.178)	$P < 0.01$
	Female	0.027 (0.011)	0.033 (0.008)	$P < 0.05$

Key: *group 3 (males) or group 1 (females); **group 2

gonad weight ($P < 0.05$). The reduction in liver weight was also significant ($P < 0.0001$) in both sexes when measured relative to body weight. Other organs showing a reduction in absolute weight showed no significant reduction in relative weight with the exception of the kidney in males ($P < 0.05$). Significant increases in relative weight were seen in the gonads ($P < 0.01$), in the ovary ($P < 0.05$) and, in females, in the brain ($P < 0.001$).

Females in group 5 and males in group 4 showed no significant difference from group 1 for any organ. The only significant differences between males in group 3 and group 1 were an increase in absolute brain weight ($P < 0.05$) and a decrease in relative gonad weight ($P < 0.05$) in group 3. There was also some reduction in absolute testis weight ($0.05 < P < 0.1$).

Premature death and the predominant pathology involved

Only two animals died in the first year, both group 1 females, one after 4 wk from an accident and one after 33 wk from an imperforate vagina. Up to wk 78, no differences in survival were evident in either sex. Between then and the end of the study, more males in groups 1 and 3 died or had to be killed than in groups 2 and 4. Compared with group 1 males, the number of survivors was significantly increased in group 2 ($P < 0.01$) and in group 4 ($P < 0.05$). In the case of females, no significant differences in survival were seen between groups.

Malignant neoplasms, of a wide variety of types, were seen in 17 of the 45 premature decedents, and all or virtually all of them contributed to the deaths. Malignant neoplasms were seen in four group 1, four group 3, and two group 4 decedent males; none of the diet-restricted males of group 2 died prematurely with a malignant neoplasm. In the case of the female decedents, three group 1 and three group 5 animals died with malignant tumours compared with only one group 2 female.

Similarly striking were the differences in numbers of premature deaths in which pituitary adenomas and other benign tumours were found. It is possible that some of the pituitary tumours, though benign, contributed to death through hormonal disturbance. However, it is unlikely that any of the other benign tumours contributed to the deaths of rats.

Table 4 also gives details of non-neoplastic conditions considered to have contributed to death alone or in combination with other conditions. By far the most common of these was moderate, severe, or very severe nephropathy, seen in 27 of the decedents. Only one prematurely dying group 2 male died because of this, as against seven group 1 decedent males, seven group 3 decedent males and all four group 4 decedent males. The picture in females was similar, with no cases in group 2 females, compared with three in group 1 females and five in group 5 females.

Incidence of neoplasms

No indication of the effects of isolation (group 3 compared with group 1 males) or of parity (group 5 compared with group 1 females) were seen in any analysis (Table 5). A possible effect of sexual segregation was only evident in respect of Leydig-cell tumours of the testis; when their incidence in group 4 males was compared with that in other males fed *ad lib.* (groups 1 and 3), a significant increase was seen in group 4 males.

Given the lack of effect of other factors (except in the testis) a more sensitive test of a possible effect of dietary restriction could be gained by comparing group 2 with all the other groups combined. When results for the two sexes were also combined, a significant reduction was seen in group 2 in respect of incidence of fatal tumours of any site, malignant tumours of any site, benign or malignant tumours

Table 4. Premature deaths and the predominant pathologies involved in a 2-yr study in rats of the effects of food intake, sexual segregation and parity

	No. of animals affected*						
	Males				Females		
Parameter Group . . .	1	2	3	4	1	2	5
Total deaths	10	2	12	4	6	4	7
Deaths							
to wk 52	0	0	0	0	2	0	0
to wk 78	3	1	2	0	2	2	1
to wk 91	5	1	4	0	2	2	3
to wk 100	9	2	10	3	5	4	4
Accident/died during bleed	0	0	1	0	1	0	0
Malignant tumours†	4	0	4	2	3	1	3
Benign tumours							
Any	5	1	5	2	3	1	6
Pituitary	4	1	2	0	3	1	6
Testis	1	0	2	2	0	0	0
Other‡	1	0	4	1	0	0	0
Moderate, severe or very severe nephropathy	7	1	7	4	3	0	5
Other non-neoplastic conditions contributing to death§	1	1	6	0	2	2	2

group 1 = controls group 2 = restricted access to food group 3 = in room with males only
group 4 = sexually active group 5 = uniparous (See Table 1 for full details.)

*In a few animals, multiple factors were considered to be contributory.

†Renal pelvis (1M), Zymbal gland (1M, 3M, 4M), abdominal myxosarcoma (1M), sarcoma next to spine (1M), brain ependymoma (3M, 1F), generalized lymphosarcoma (3M), brain glioma (3M), thyroid follicular (4M), thymus sarcoma (1F), mammary adenocarcinoma (1F, 5F), liver cholangiocarcinoma (2F), tongue squamous carcinoma (5F), cheek squamous carcinoma (5F). Numbers in parentheses are group numbers.

‡Lung (1M, 3M), pancreas islet-cell (3M), adrenal medulla (3M, 3M), parathyroid (3M). Numbers in parentheses are group numbers.

§Cerebral oedema (1M), convulsed when handled (2M), injury to paws (3M), hind leg paralysis (3M), swollen leg and eye (3M, 3M), severe polyarteritis (3M, 3M, 3M), blind vagina (1F), thrombus in atrium (1F), uterine haemorrhage (2F), severe uterine distension (2F), dystrophic osteofibrosis (5F), swollen feet and jaw (5F). Numbers in parentheses are group numbers.

of any site, and benign or malignant tumours of multiple sites. The reduction was evident in both males and females in each of these analyses, though the differences did not always reach statistical significance. Similarly, group 2 showed a lower than expected incidence in the analyses shown in Table 5 of pituitary pars anterior tumours, lung tumours, and mammary gland tumours, though none of the differences reached statistical significance. Tumours at other sites (listed in Tables 4 and 5) were of insufficient incidence for statistical analysis. If the number of tumours per rat was considered, the reduction in diet-restricted groups was evident both in males (0.55 in group 2 compared with 0.95, 0.95 and 1.05 in groups 1, 3 and 4, respectively), and in females (0.40 in group 2 compared with 1.05 and 0.75 in groups 1 and 5, respectively), despite the better survival in group 2.

Non-neoplastic conditions

A wide range of conditions was seen, results being presented only for those showing a possible effect of the four variables under study (Tables 6 to 8). The incidences of these conditions are shown in Table 6, while Table 7 gives the results of age-adjusted significance tests in relation to isolation, sexual segregation and parity, and Table 8 gives the results of such tests in relation to dietary restriction.

When group 3 males were compared with group 1 males, no very clear effects of isolation were apparent. Only three significant differences were seen (Table 7), and these may all have been due to chance given the level of significance ($0.01 < P < 0.05$) and the number of endpoints analysed.

Similarly, when group 5 females were compared with group 1 females, no convincing evidence of an effect of parity was seen. The increased incidence of round-cell infiltration of the liver in group 5 females may well have been a chance finding due to an unusually low incidence in group 1 females, since group 1 females also showed a significant reduction in the incidence of round-cell infiltration compared with group 2 females, that was not evident for males (see Table 6). The overall pattern of response for inflammation of olfactory epithelium of the nose is difficult to explain, with the incidences in group 1 males and females substantially exceeding the incidences in all other groups in both sexes. The only other statistically significant effect of parity was a reduced incidence of the higher grades of mammary gland hyperplasia in group 5 females.

Because of the lack of significant effect of isolation, tests for the effect of sexual segregation were normally made by comparing group 4 males with males in groups 1 and 3 combined. Of the five endpoints listed in Table 7 as showing a significant difference, those in the thymus, trachea and urinary bladder are considered unlikely to represent true effects, bearing in mind the significance level ($0.01 < P < 0.05$), the number of comparisons tested, and the lack of biological rationale. The significant increases in atrophy of the epididymis ($P < 0.05$) and in Leydig-cell hyperplasia of the testis ($P < 0.01$) seem likely to represent true effects. The evidence of an effect in the testis is strengthened by the fact, noted above, that males in group 4 also had a significant increase in Leydig-cell tumours of the testis. An additional analysis of the

Table 5. Incidence of neoplasms in a 2-yr study in rats of the effects of food intake, sexual segregation and parity

Tumour type*		No. of animals affected					Results of principal significance tests†		
	Group . . . 1	2	3	4	5	O	E	P
Tumours of any site— fatal	Males	4	0	4	2	—	0	2.79	<0.05
	Females	3	1	—	—	3	1	2.40	NS
	Combined						1	5.19	<0.05
Tumours of any site— malignant	Males	4	0	5	4	—	0	3.83	<0.05
	Females	4	1	—	—	5	1	3.52	<0.1
	Combined						1	7.35	<0.01
Tumours of any site— benign or malignant	Males	14	9	13	17	—	9	14.95	<0.01
	Females	13	7	—	—	9	7	10.14	NS
	Combined						16	25.10	<0.01
Tumours of more than one site— benign or malignant	Males	4	2	4	4	—	2	3.10	NS
	Females	7	1	—	—	5	1	3.73	<0.1
	Combined						3	6.83	<0.05
Pituitary pars anterior tumours— benign	Males	6	4	5	6	—	4	5.43	NS
	Females	11	5	—	—	9	5	7.75	NS
	Combined						9	13.18	<0.1
Testis Leydig-cell tumours— benign	Males	4	3	2	9	—	9	5.71	<0.05
Lung tumours— benign adenomas	Males	3	0	2	0	—	0	1.37	NS
Mammary gland tumours— malignant	Females	2	0	—	—	1	0	1.06	NS
Mammary gland tumours— benign or malignant	Females	4	0	—	—	2	0	2.18	<0.1

group 1 = controls group 2 = restricted access to food group 3 = in room with males only group 4 = sexually active group 5 = uniparous (See Table 1 for full details of groups.) O = observed E = expected

*Table shows the major tumour categories only. Other tumours seen in decedents are listed in Table 4. Those seen in terminally killed animals are: epididymis mesothelioma (4M), forestomach squamous papilloma (2M), liver adenoma (2F), mesenteric node haemangioma (1M, 2M, 3M, 4M), nose squamous carcinoma (5F), pancreas islet-cell adenoma (1F), parathyroid adenoma (1M, 2M, 2M), pituitary pars intermedia adenoma (1F), skin benign basal cell tumour (5F), skin squamous papilloma (2F), spleen haemangioma (1F), uterus adenocarcinoma (1F), hindlimb anaplastic carcinoma (4M), jaw squamous carcinoma (3M).

†The effects of restricted access to diet were tested in all cases (by comparing group 2 with all the other groups) except in the case of the Leydig-cell tumours for which the effect of sexual activity was tested, by comparing group 4 with groups 1 and 3. Statistical analysis was by the method of Peto *et al.* (1980).

incidence of Leydig-cell tumour or hyperplasia (O = 13, E = 7.62, for group 4; O = 7, E = 12.38, for groups 1 and 3 combined) showed a difference that was almost significant at the $P < 0.001$ level.

Except where relationships with parity or sexual segregation had already been established, tests for the effect of dietary restriction were carried out by comparing incidences in group 2 with those in all other groups combined. Table 8 presents the results for statistically significant relationships found for males, for females or for both sexes combined. Diet restriction dramatically reduced the incidence of a wide range of non-neoplastic conditions. The most striking reductions seen, all highly significant, were in respect of inflammation and focal fibrosis in the heart, chronic progressive nephropathy and pelvic epithelial hyperplasia in the kidney, glycogen in the liver and (in males only) agglomeration of colloid in the thyroid. It is also considered likely that dietary restriction reduced the incidence of fatty degeneration in the liver, hyperplasia and secretory activity in the mammary gland, polyarteritis affecting the pancreatic artery, hyperplasia in the parathyroid, radiculoneuropathy in the spinal cord, and atrophy and arteriolitis in the testis. Other conditions listed in Table 8 may well be chance findings, with the possible exception of inflammation of oral epithelium of the nose, discussed above. It is notable that there were no clear adverse effects of dietary restriction.

DISCUSSION

Although the original purpose of this small study was to investigate the effect of four different variables, namely diet restriction, isolation, sexual segregation and parity, on longevity and non-neoplastic and neoplastic disease, it is clear that, compared with the other variables, diet restriction had a very large effect. Consequently, in the light of preliminary analyses of the data as a whole, it was deemed generally valid to compare diet-restricted animals (group 2) with those in all other groups ignoring the other variables. Secondly, for the purpose of looking for possible effects of the other variables, it was necessary to eliminate group 2 rats from the calculation because of the overwhelming effect of diet restriction. The main conclusions in respect of the four variables studied are as follows.

Sexually active males in group 4 with repeated access to virgin females ate more food than segregated males during most of the study period. They

Table 6. Incidence of selected non-neoplastic conditions in a 2-yr study in rats of the effects of food intake, sexual segregation and parity

	No. of animals affected						
	Males				Females		
Condition / Group...	1	2	3	4	1	2	5
Epididymis—atrophy	4	5	3	8			
Heart—inflammation	12	5	9	11	11	0	6
—focal fibrosis	9	6	13	13	11	6	11
—focal fibrosis (3+)*	5	0	3	8	3	0	4
Kidney—chronic progressive nephropathy	20	13	20	20	18	4	19
—chronic progressive nephropathy (3+)*	15	1	13	14	12	0	12
Kidney—pelvic epithelial hyperplasia	14	10	11	10	11	3	12
—pelvic epithelial hyperplasia (2+)*	10	1	6	8	9	1	5
Liver—glycogen	8	1	9	12	13	0	11
—round cell infiltration	16	16	14	14	10	20	19
—round cell infiltration (2+)*	4	3	6	4	0	9	5
—fatty degeneration	4	0	3	6	2	0	2
Lung—peri-airway lymphocytic infiltration (2+)*	17	11	11	14	13	8	8
—interstitial pneumonitis (3+)*	1	0	2	4	4	2	4
Mammary gland—hyperplasia	10	3	6	8	16	11	14
—hyperplasia (3+)*	0	0	0	1	11	3	4
—secretory activity	4	2	3	6	12	6	15
Nose—infected tooth socket(s)	1	6	0	4	0	4	3
—inflammation of olfactory epithelium	14	6	8	8	13	3	3
Pancreas—polyarteritis	2	0	3	2	3	0	2
Parathyroid—hyperplasia	9	2	9	9	4	3	1
Pituitary—intermediate lobe cyst	10	4	4	11	2	5	4
Spinal cord—radiculoneuropathy	11	8	13	12	7	7	9
Testis—atrophy	11	10	15	17	—	—	—
—arteriolitis	5	1	7	6	—	—	—
—oedema	14	17	7	7	—	—	—
—Leydig-cell hyperplasia	4	7	0	10	—	—	—
Thymus—cyst(s)	2	2	7	9	5	2	2
Thyroid—agglomeration of colloid	10	2	9	13	3	3	1
Trachea—inflammation	8	11	11	6	6	7	8
Urinary bladder—distension	5	1	2	0	0	0	1

group 1 = controls group 2 = restricted access to food group 3 = in room with males only group 4 = sexually active group 5 = uniparous (See Table 1 for full details.)

*Many conditions were scored on the scale 1 = minimal, 2 = slight, 3 = moderate, 4 = severe, 5 = very severe. 2+ indicates at least grade 2; 3+ at least grade 3.

also had a significantly increased incidence of Leydig-cell tumours, Leydig-cell hyperplasia, and atrophy of the epididymis compared with sexually segregated males in groups 1 and 3. Despite this, males in this group showed increased survival. Ten cases of Leydig-cell hyperplasia were seen in the sexually

Table 7. Statistically significant relationships of sexual isolation, parity and sexual activity to the incidence of certain non-neoplastic conditions in a 2-yr study in rats

	No. of animals affected		
Condition	Observed (O)	Expected (E)	*P*
		Isolation*	
Lung—peri-airway lymphocytic infiltration (2+)	11	14.00	<0.05
Pituitary—intermediate lobe cyst	4	6.99	<0.05
Thymus—cyst(s)	7	4.06	<0.05
		Parity*	
Liver—round cell infiltration	19	14.98	<0.01
Mammary gland—hyperplasia (3+)	4	8.02	<0.05
Nose—inflammation of olfactory epithelium	3	7.63	<0.01
		Sexual activity*	
Epididymis—atrophy	8	5.01	<0.05
Testis—Leydig-cell hyperplasia	10	5.71	<0.01
Thymus—cyst(s)	9	6.44	<0.05
Trachea—inflammation	6	9.75	<0.05
Urinary bladder—distension	0	2.85	<0.05

*For isolation, O and E relate to an age-adjusted comparison of group 3 males with group 1 males; for parity group 5 females are compared with group 1 females; for sexual activity group 4 males are compared with groups 1 + 3 males combined (except for thymus cyst(s) where group 1 is the base for comparison). See Table 1 for details of the groups.

Table 8. Statistically significant relationships of dietary restriction to the incidence of certain non-neoplastic conditions in a 2-yr study in rats

Condition	Males* No. of animals affected O	E	*P*	Females* No. of animals affected O	E	*P*	Combined* *P*
Heart—inflammation	5	9.15	<0.05	0	5.71	<0.001	<0.001
—focal fibrosis	6	10.03	<0.05	6	9.56	<0.05	<0.01
—focal fibrosis (3+)	0	3.63	<0.05	0	2.48	<0.05	<0.001
Kidney—chronic progressive nephropathy	13	17.76	<0.001	4	13.33	<0.001	<0.001
—chronic progressive nephropathy (3+)	1	9.14	<0.001	0	7.37	<0.001	<0.001
—pelvic epithelial hyperplasia	10	10.18	NS	3	8.20	<0.01	<0.05
—pelvic epithelial hyperplasia (2+)	1	5.00	<0.05	1	4.60	<0.05	<0.001
Liver—glycogen	1	9.15	<0.001	0	8.93	<0.001	<0.001
—round cell infiltration	16	15.71	NS	20	16.77	<0.05	<0.1
—round cell infiltration (2+)	3	4.49	NS	9	4.89	<0.01	NS
—fatty degeneration	0	2.24	<0.1	0	1.41	<0.1	<0.05
Mammary gland—hyperplasia	3	6.40	<0.05	11	13.51	NS	<0.05
—hyperplasia (3+)	0	0.00	NS	3	6.34	<0.05	<0.05
—secretory activity	2	3.79	NS	6	10.35	<0.05	<0.01
Nose—infected tooth socket(s)	6	3.46	<0.1	4	2.60	NS	<0.05
—inflammation of olfactory epithelium	6	9.47	<0.1	3	5.71	<0.1	<0.05
Pancreas—polyarteritis	0	1.04	NS	0	1.86	<0.1	<0.05
Parathyroid—hyperplasia	2	6.03	<0.05	3	2.87	NS	<0.1
Pituitary—intermediate lobe cyst	4	7.53	<0.05	5	3.72	NS	NS
Spinal cord—radiculoneuropathy	8	11.81	<0.05	7	8.56	NS	<0.05
Testis—atrophy	10	12.77	<0.05	—	—	—	—
—arteriolitis	1	2.77	<0.05	—	—	—	—
—oedema	17	14.15	<0.05	—	—	—	—
Thyroid—agglomeration of colloid	2	8.12	<0.001	3	2.48	NS	<0.05

O = observed E = expected

*Generally O and E relate to the comparison of group 2 with all the other groups combined. For the testis, the comparison is of group 2 with group 3. For the mammary gland, group 5 is excluded. See Table 1 for details of the groups.

group compared with four in the sexually segregated group housed in the room with animals of both sexes and 0 in the sexually segregated rats housed in the room containing only males. This suggests that the condition was related to sexual stimulation and/or activity which was presumably least in the male-only room where even olfactory sexual stimuli were absent.

No very clear effects of isolation were seen. The only significant differences seen (increased absolute brain weight, reduced relative testis weight, reduced incidence of pituitary cysts and of higher grades of peri-airway lymphocytic infiltration in the lung, and increased incidence of thymic cysts) were all marginal ($0.01 < P < 0.05$) and probably due to chance.

No very convincing evidence of any effect of parity was seen. An increased incidence of round-cell infiltration of the liver in group 5 females is considered a chance finding due to a low incidence in group 1 females. A reduced incidence of inflammation of the olfactory epithelium of the nose is also difficult to interpret as a clear effect of parity when the data are considered as a whole. Higher grades of mammary gland hyperplasia were seen less frequently in group 5 females but the level of significance ($0.01 < P < 0.05$) did not exclude a chance explanation.

A number of effects of diet restriction were evident. These included reduced body weight, reduced weight of heart, liver and kidneys in both sexes and increased gonad weight in males as well as increased survival in males and reduced early deaths from malignant neoplasms. The incidences of malignant neoplasms and benign neoplasms were significantly reduced and, as shown in Table 8, the incidence of a wide range of nonneoplastic conditions was reduced dramatically. Higher grades of chronic progressive nephropathy were virtually totally eliminated, as was polyarteritis of the pancreatic artery and fatty degeneration and glycogen in the liver.

The data concerning specific endpoints are discussed below.

Increased survival was seen in the diet-restricted males and in the males with access to a virgin female. Isolation, parity, or, in females, diet restriction, had no apparent effect on longevity. Diet restriction was associated with reduced body-weight gain in both sexes but other factors had no obvious effect on body-weight gain.

Diet restriction eliminated or virtually eliminated the higher grades of chronic progressive nephropathy, and of pelvic epithelial hyperplasia, as well as liver glycogen and fatty degeneration, and polyarteritis of the pancreatic artery. Diet restriction also reduced the incidence of inflammation and focal fibrosis of the heart, hyperplasia and secretory activity of the mammary gland, parathyroid hyperplasia, agglomeration of colloid in the thyroid, radiculoneuropathy in the spinal cord as well as atrophy and arteriolitis in the testis. Intermittent access to females was associated with an increased incidence of Leydig-cell hyperplasia and atrophy of the epididymis but no clear effects of isolation or parity were seen.

As shown in Table 3, in both sexes diet restriction was associated with highly significant reductions in the absolute weights of the heart, liver and kidneys ($P < 0.001$). In males absolute brain weight was also decreased ($P < 0.01$) but absolute gonad weight was increased ($P < 0.05$). The other variables studied probably had no real effect on absolute organ weights. Marginally reduced absolute gonadal weight ($P < 0.1$) and increased absolute brain weight ($P < 0.05$) in group 3 males were probably chance findings.

More surprisingly, perhaps, and of more potential toxicological interest, are the significantly lower organ weights relative to body weight in the diet-restricted group (see Table 3). In the case of the liver, this effect was highly significant in both sexes ($P < 0.001$). In males but not females relative kidney weights were also significantly reduced by diet restriction ($P < 0.05$). The increased relative brain weight in females ($P < 0.001$) merely reflects the fact that diet restriction affects body weight without affecting absolute brain weight. The same explanation holds for relative gonadal weights. None of the other variables studied had any effect on relative organ weight except that relative gonadal weight was lower in group 3 males than in controls (group 1) ($P < 0.05$).

Diet restriction reduced the overall tumour incidence with evidence of a reduction in fatal malignant tumours as well as in pituitary and mammary tumours. Intermittent access to females was associated with an increased incidence of Leydig-cell tumours of the testis. Again, no evidence of an effect of isolation or parity was seen. The data presented in Table 5 for malignant tumours contributing to the deaths of animals before 2 years are arguably much more comparable with human cancer mortality data than are tumour incidence data from rodent studies. Of special interest is that many of the malignant tumours (although not the three carcinomas of the Zymbal gland, which is not present in humans) are of kinds that afflict humans, and very few of them were of endocrine tissues.

What are the implications of these findings in relation to the design and interpretation of oncogenicity studies in rodents? The common practice has been to regard any enhancement of the incidence of any kind of neoplasia at any site as indicating that the test substance is a carcinogen and to ignore any reduction in the incidence of neoplasia of any kind, at any site. At the same time, little attention has been paid to the fact that animals in control groups exhibit high incidences of both neoplastic and non-neoplastic (mainly age-related degenerative) conditions. Enhancement of tumours of endocrine or sex hormone-controlled tissues has, in general, been regarded as, perhaps, less predictive of cancer risk to man but the unwritten rule has been to suspect any enhancement of tumours of non-endocrine epithelial tissues as being indicative of genotoxic carcinogenic risk. It is in relation to this unwritten rule that the present results are so important. Of the malignant neoplasms that killed 16 out of 100 rats fed *ad lib.* 24 hr/day before the end of 2 yr, no less than 13 were of non-endocrine tissues whilst there was only one fatal non-endocrine neoplasm before 2 yr among the 40 rats fed for only 6.5 hr/day ($P < 0.01$). In addition, when the study was ended at 2 yr, a further three malignant non-endocrine tumours were found among the 61 rats fed for 24 hr/day killed at that time, compared with none among the 34 rats on restricted feeding.

Compared with the effects of diet restriction, none of the other variables we studied had much effect either on longevity or on disease incidence. The males of group 4 that were fed *ad lib.* and had intermittent access to females survived much better than sexually segregated males of groups 1 and 3 that were also fed *ad lib.* Both this finding and the finding that males of group 4 were at a significantly increased risk of developing Leydig-cell tumours of the testis require confirmation. Allowing females to litter once had no obvious effect either on longevity or on the risk of disease. It should, however, be emphasized that studies on variables other than the daily period of access to food were all conducted on rats fed for 24 hr/day. Before these variables are dismissed as unimportant, it will be necessary to conduct similar studies in animals on restricted diets.

The observed effects of diet restriction on liver and kidney weights relative to body weight have many implications not only in relation to the testing of chemicals for carcinogenicity but also for the general pharmacological, toxicological and pharmacokinetic evaluation of potential drugs and other agents. Conybeare (1988) reported that the phenobarbitone sleeping time of diet-restricted female rats is significantly prolonged compared with that of animals fed *ad lib.* This suggests that food intake levels influence hepatic drug-metabolizing enzyme activity.

Do these results in rats have any direct relevance for man? There are American Cancer Society data that are consistent with there being an adverse effect of overnutrition on cancer risk in humans (Lew and Garfinkel, 1979). However, these rely on obesity relatively late in life for their index of food intake and this is clearly not ideal. In the studies reported in the present paper, the mean body weight of the diet-restricted males relative to that of the unrestricted males rose during the study (males 9 wk, 73.4%; 24 wk, 76.4%; 52 wk, 77.3%; 76 wk, 81.4% 105 wk, 84.4%) with a similar but less marked trend in females. More important is the point that although, in theory, humans can eat as much as they like at any time they like, in practice most people have other things to do besides just wandering around a small cage and eating. They have worries, fears, anxieties, duties, responsibilities for others, etc. and, although doubtless many humans overeat, they probably do not do so to the same extent as a bored caged rat. The fact that rats fed *ad lib.* develop all manner of endocrine changes (Roe, 1981 and 1987) from which humans are largely free, is consistent with the view that overeating is a quantitatively more serious problem in caged rats than in humans. Nevertheless, simple overeating may be a risk factor for many kinds of cancer in man. The huge effect of overnutrition on cancer incidence in rats has until now been largely overlooked because in most carcinogenicity tests all the rats in control as well as treated groups are grossly overfed. It may well be that a major contribution of overeating to cancer risk in man is similarly being overlooked because virtually all Westernized humans overeat.

The effect of diet restriction on the overall incidence of neoplasia in the present study was large compared with treatment-related differences in tumour incidence that spark off regulatory action against chemicals subjected to formal oncogenicity testing. This is particularly obvious if the findings for all the groups fed *ad lib.* are combined and compared with those for the single group on the restricted diet (Table 8). A larger experiment would be needed to demonstrate significant differences in the incidences of tumours at particular sites. An experiment involving a total of 1200 rats (the "Labsure 1200-Rat Study") is presently in progress. Preliminary findings in this larger study, like those of the present study, suggest that diet restriction reduces the incidence of benign and malignant tumours of virtually all kinds. In the case of the commoner kinds of neoplasm statistically significant differences are apparent in the larger study but for less common tumours significance is only demonstrable if tumours of different sites and kinds are aggregated. It seems that the incidence of many kinds of neoplasm that have hitherto been regarded as specifically associated with exposure to particular chemicals or other agents may be influenced non-specifically by calorie intake. If this is so then test substances that influence calorie intake in either direction may also non-specifically influence the risk of development of neoplasia in either direction. This has important implications for the design and interpretation of carcinogenicity tests, and these have hitherto not been adequately taken into account. Undoubtedly the first important step that needs to be taken is to try to find a way of maintaining untreated rats and mice such that they can live into old age without becoming unduly obese and without developing prematurely all manner of endocrine disturbances and ageing-related diseases that interfere with the assessment of response to possible carcinogens. To achieve this it will certainly be necessary to reconsider dietary formulation and it will probably be necessary to introduce one or other form of dietary restriction for all animals. Restriction by rationing is time consuming and costly. Restriction by limiting the daily period of access to food can be inexpensive if cages are so designed that access to food can be withdrawn simply by swivelling the food hoppers from positions of access to positions of non-access. This method has the added advantage that effects of test substances on food consumption can still be monitored.

At present the philosophy underlying the testing of chemicals for carcinogenicity demands that substances should as far as possible be tested at maximum tolerated dose levels. Whether or not this in itself is sensible is arguable. However, what is undoubtedly important is that in the interpretation of the results of exposure to doses that disturb physiological status and/or nutritional status, consideration should be given to the fact that these disturbances *per se* may non-specifically influence the risk of tumour development (Roe, 1989b).

Another issue for discussion is the possible mechanisms by which restricted access to food influences cancer risk in rats. At present, we can only speculate about this. The absolute and relative weights of the liver of the restricted rats were markedly lower than those of the rats fed 24 hr/day. This suggests that the restricted animals were much less metabolically active. We speculate quite simply that this results in reduced cell turnover and reduced cellular metabolic activity generally throughout the body and that these reductions are associated with a reduction in the endogenous generation of mutagenic electrophiles that predispose both to degenerative diseases and to neoplasia (Roe, 1989b). It is very difficult to test this hypothesis in man because it is virtually impossible to control or measure how much people eat throughout their lives. Consequently although one can compare obese and lean people, one cannot construct "overfed" and "restricted" groups for comparison of survival, neoplastic and non-neoplastic diseases. Perhaps our best hope in relation to humans is to discover the mechanism by which diet-restriction reduces cancer risk in rats. If we knew this, then we might be able to see whether it is operating in man.

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