Onset of puberty and duration of fertility in rats fed a restricted diet

B. J. Merry and Anne M. Holehan

Wolfson Laboratory for Research in Gerontology, University of Hull, Hull HU6 7RX, U.K.

Summary. Chronic dietary restriction such that the body weight of CFY Sprague–Dawley female rats was 50% that of animals fed *ad libitum* resulted in enhanced longevity (LD₅₀ of 1090 days compared to 704 days). All the experimental females had reached puberty by 227 days and 80% were able to conceive and wean young at 510 days, an age beyond that at which the control rats had ceased to breed (450 days). Some (25%) of the experimental rats were able to breed at over 800 days of age.

Introduction

Reduction of dietary intake remains one of the most effective methods of modifying early development and the apparent rate of rodent senescence. Although animals fed a restricted diet live longer and are relatively free from the pathologies which affect control animals fed *ad libitum* they remain sexually immature (Asdell & Crowell, 1935; Kennedy & Mitra, 1963). After dietary restriction for periods of up to 1000 days, survivors were reported capable of resuming growth and achieving sexual maturity when returned to *ad libitum* feeding (McCay, 1942).

That general and specific diet deficiencies cause suppression of reproductive functions is well known, and several authors confirm the cessation of oestrous cycles in adult female rats fed a restricted diet in adulthood after normal feeding in early life (Rinaldini, 1949; Piacsek & Meites, 1967). Carr, King & Visscher (1949) studied the increase in both total and reproductive lifespan in chronic calorie-restricted mice. On a diet containing half the calories of a standard mouse diet, C₃H females, which are normally sterile at 11–12 months of age, were still fertile at 21 months when returned to *ad libitum* feeding, but were unable to wean their litters. Similar results were recorded for the A strain of mice by Ball, Barnes & Visscher (1947). Berg (1960) restricted the food intake of female Sprague–Dawley rats to 67% of the control group fed *ad libitum*. Between 730 and 790 days fertility was assessed in 24 animals after return to *ad libitum* feeding; 67% of the animals were fertile but the litters were small and the weaning quotient was low.

Glass, Harrison & Swerdloff (1976) demonstrated a highly significant negative linear relationship between the age of puberty and the growth rate in underfed amino acid-deficient rats. Long–Evans rats in which growth had been suspended for 2 years by maintenance on a tryptophan-deficient diet were able to reproduce at 17–28 months of age when growth was resumed after returning to *ad libitum* feeding (Segall & Timiras, 1975). Control animals of this age were infertile because of senescence.

The attainment of sexual maturity in rats fed a restricted diet from weaning has only been achieved through the resumption or acceleration of growth resulting from a return to *ad libitum* feeding. It was decided to re-investigate the reported sexual immaturity of aged rats fed a restricted diet without enhancing growth rate by return to *ad libitum* feeding.

Materials and Methods

Animals

All the animals were CFY Sprague–Dawley rats from the Departmental colony. They were housed individually and maintained in a constant temperature ($22 \pm 1^\circ\text{C}$), a humidity range of 50–70% and a light regimen of 12 h light/24 h. All the animals had unrestricted access to water.

Control animals were virgin females taken randomly from the colony at 21 days of age and allowed to feed *ad libitum* on a pelleted diet (Rat breeder diet, E. B. Bradshaw & Sons Ltd, Driffield). Mortality studies, monitoring of body weight and food intake were carried out on 200 animals. A further 200 animals were used for puberty and fertility studies.

In a group of 45 experimental animals retarded growth was achieved by limiting intake of the pelleted diet from 21 days of age such that body weight was 50% that of the control animals. This reduced both carbohydrate and protein intake (Text-figs 1b and 1c) whilst supplying adequate mineral and vitamin supplements to support maximal growth (National Research Council, Committee on Animal Nutrition, 1962). Daily total food intake was adjusted at 49, 402, 438, 579 and 730 days such that total protein and carbohydrate intake was as indicated in Text-figs 1(b) and 1(c). Probit transformation of mortality data gave an LD_{50} of 1090 days for the animals on the restricted diet, compared to 704 days for the controls (Text-fig. 1d). The 300 experimental animals used for puberty and fertility studies were matched to these body weight curves by continuous monitoring of body weight and adjustment of diet according to the schedule.

Observations

Control rats. The onset of puberty, i.e. the first oestrus, was determined in 45 animals by inspection for vaginal canalization and cornification each day at 09:00 h from 30 to 50 days of age. These animals (Group A) were then used for breeding studies when 150 days old. The vaginal cytology of other animals was examined daily for 20 days when 180 days old (Group B, 100 animals) and at 390 and 540 days (Group C, 55 animals) to determine age-related deviations in vaginal cytology at oestrus. The rats in Group B were randomly subdivided at 200 days of age into groups of 20, 20, 30 and 30 and their breeding performance was assessed at 210, 270, 420 and 450 days of age respectively.

Experimental rats. The onset of puberty was determined in 100 animals (Group E) examined daily between 21 and 227 days for evidence of vaginal canalization and cornification, and 60 of these animals were used in breeding studies when 300 days old. Vaginal cytology was examined for 20 days in 150 animals (Group F) when 180 and 240 days old. At 330 and 510 days of age, 2 subgroups of 50 animals each were randomly taken from Group F and used for breeding studies. The vaginal cytology of the 39 animals that were still alive at 730 days was examined as before and those animals exhibiting Stage 1 or 2 cytology (see below) were used to assess fertility between 840 and 930 days. The vaginal cytology of a third group of 50 animals (Group G) was examined for 20 days when they were 500 and 650 days old.

Vaginal smears were taken by means of a glass rod and cells were dispersed into a 1% solution of methylene blue for immediate examination. The cell patterns were classified according to the scheme of Mandl (1961). Animals exhibiting mostly cornified cells (oestrus) for >4 days were classed as having Stage 1 cytology. Those showing a dioestrous smear (leucocytic infiltration) for >3 days were Stage 2, and those with sporadic mucification and irregular appearance of epithelial cells were Stage 3. Any animal showing a Stage 3 smear was not used in the breeding studies. To avoid any effects of parity only first litters were considered and animals were discarded after an infertile mating or bearing one litter.

For the breeding studies, females were randomly paired with proven males for a period of 19 days. To facilitate feeding of the restricted diet, males were removed from the experimental

Text-fig. 1. Comparisons of (a) growth curves, (b) protein intake, (c) carbohydrate intake and (d) mortality of rats fed *ad libitum* (●) with those of rats fed a restricted diet (○). Initial group numbers, *ad libitum* = 200, restricted = 45. The plots for curves (a), (b) and (c) were terminated when 20 *ad libitum* fed (10%) and 11 dietary restricted (24%) rats still survived. The s.e.m. is shown when it exceeds the width of the symbol. In (b) and (c) the total protein and carbohydrate intakes per day of the dietary restricted animals were, respectively, 1.42 and 3.14 g up to 48 days, 1.89 and 4.18 g from 49 to 401 days, 2.30 and 5.23 g from 402 to 437 days, 2.83 and 6.28 g from 438 to 578 days, 3.30 and 7.32 g from 579 to 729 days, and 3.77 and 8.37 g after 730 days.

females between 09:00 and 16:00 h each day. For experimental females <500 days old it was necessary to allow feeding *ad libitum* after parturition so that the litters could be raised and weaned.

Analysis of results

The significance of the differences between means was tested by using Student's *t* test. Nested analysis of variance for unequal sample size utilizing the procedure of Student–Newman–Keuls for *a posteriori* comparison of means was used to analyse data from the breeding studies (Sokal & Rohlf, 1969). Homogeneity of two or more percentages was tested using the approach of Brandt and Snedecor (Snedecor, 1962).

Results

Vaginal cytology

Vaginal opening and the first oestrus was observed in 75% of the control animals between the ages of 38 and 42 days (age range for all animals 36–45 days), and most of these then experienced regular 5-day oestrous cycles. In the experimental animals, all 100 in Group E had shown vaginal perforation and first oestrus by 227 days, the numbers being 86, 55, 45 and 10 at 178, 143, 115 and 80 days of age respectively. After the first oestrus >70% of control and experimental animals demonstrated a normal 5-day oestrous cycle up to 200 days of age, but the older control rats then had more irregular cycles (Table 1). In contrast, there was no obvious change in the regularity of the cycles in the experimental rats, even at 730 days of age (Table 1).

Table 1. Effect of chronic dietary restriction on oestrous cycles (mean $\pm$ s.d.) and vaginal cytology of rats of different ages

Age (days)	No. of rats	Length of oestrous cycle (days)	% of females:			
			Cycling	Stage 1	Stage 2	Stage 3
<i>Control rats</i>						
180-200	100	4.8 ± 0.4*	73	22	5	0
390-410	55	6.2 ± 1.6*	15	51	34	0
540-560	47‡	—†	9	0	36	55
<i>Experimental rats</i>						
180-200	150	5.1 ± 0.7	76	0	0	0
240-260	150	4.8 ± 0.4	69	21	10	0
500-520	50	5.0 ± 0.1	66	24	10	0
650-670	50	4.9 ± 0.5	64	20	16	0
730-750	39	5.0 ± 0.7	61	13	18	8

* Significantly different (Student's *t* test), $P < 0.001$.

† Cycles too irregular to determine.

‡ 8 animals died from Group C (normal mortality loss) before observations began at 540 days.

The most common abnormality in the oestrous cycle in both groups of animals was an extension of the period of cornification (Stage 1). An increased frequency of Stage 2 vaginal cytology occurred with advancing age but was later in the experimental than in the control rats. More than half of the control females were showing Stage 3 cytology by 560 days of age but <10% of the experimental animals showed this characteristic of senescence by 750 days.

Fertility

The results of the breeding studies are shown in Table 2. There was a rapid decline in the fertility of the control animals after 270 days, but although fewer young were born to the older females those young which were successfully weaned did not appear to be undernourished. Some of the control females (5) which conceived after 1 year of age experienced extended pregnancies and a protracted parturition in which partial or complete retention of the fetuses was common.

Table 2. Effect of chronic dietary restriction on breeding performance in rats of different ages

Age (days)	No. of rats	% Pregnant	Litter size (mean $\pm$ s.e.m.)	Mean $\pm$ s.e.m. wt of young at weaning	No. weaned/no. born live
<i>Control rats</i>					
150	45	100	12.5 $\pm$ 0.4	43.1 $\pm$ 1.1	0.88
210	20	100	8.3 $\pm$ 0.5*	42.3 $\pm$ 1.5	1.00
270	20	75†	6.3 $\pm$ 0.5*	40.1 $\pm$ 2.8	0.68
420	30	20†	1.0 $\pm$ 0.4*	—	0.00
450	28‡	0	—	—	—
<i>Experimental rats</i>					
300	60	13	6.0 $\pm$ 0.7	39.0 $\pm$ 0.8	0.67
330	50	30†	10.0 $\pm$ 0.2	38.8 $\pm$ 1.5	0.68
510	50	80†	5.0 $\pm$ 0.5	36.5 $\pm$ 0.6	0.89
840–930	32	25†	4.5 $\pm$ 0.8	35.0 $\pm$ 0.6	0.86

* Significantly different from value at 150 days, $P < 0.01$ (Student–Newman–Keuls multiple range test following a nested analysis of variance).

† Significant nonhomogeneity of percentages, $P < 0.01$ (method of Brandt & Snedecor).

‡ 2 animals died before the breeding study could be undertaken.

Although all the experimental females had reached puberty by 300 days of age, conception rate was low except for those breeding at 510 days (Table 2). Litter sizes did not change with age but were less than those for control rats. Weight at weaning also remained constant.

Nested analysis of variance of weaning weight with age revealed a significant ($P < 0.001$) variance amongst mothers in any one age range in both control and experimental animals.

Discussion

These results show that a restriction of food intake to maintain body weight at 50% that of control littermates in the CFY strain delays but does not inhibit sexual maturation. Although puberty was delayed for as long as 180 days by restricted feeding, the onset of the reproductive decline was also markedly delayed, thereby extending the reproductive life-span in these animals. However, there was a discrepancy between the time of first vaginal opening and time of maximum conception. Asdell, Bogart & Sperling (1941) reported a delay of approximately 6 days between the opening of the vagina and the ability to conceive in rats fed *ad libitum* and this period may well be greatly exaggerated in the dietary restricted animal. Because of the short period of overlap of the reproductive life-spans of the two groups of animals direct comparisons are difficult but the experimental females were clearly still fertile at an age twice that at which the controls had ceased to breed. However, only first litters were considered and the effects of multiple litters on the reproductive life-spans of rats fed a restricted or unrestricted diet may be different.

In the present study the older animals showed adaptation to the reduced food intake: part of

the daily ration was frequently left uneaten and supplementation of the allotted diet at 2 years of age did not result in increased food consumption. The higher daily protein and carbohydrate intake per 100 g body weight of the experimental animals after 430 days of age (Text-figs 1b and 1c) could be due to their need to assimilate protein for continued body growth, whilst the weight gain shown by the control rats is mainly attributable to the deposition of lipid. Because of these higher intakes at 510 and 840 days, experimental females which conceived were not returned to *ad libitum* feeding after parturition. Although this might be expected to have affected adversely these weaning data, 80% of the young born to the experimental females at these ages were successfully reared (Table 1).

Asdell & Crowell (1935) and McCay (1942) achieved an extension of life-span in rats by restricting them to a maintenance diet on which the animals were held, as far as possible, at a constant weight after weaning until they began to fail, and then the diet was supplemented to allow a prescribed increase in body weight. This procedure was repeated throughout the first 900–1000 days of life. Such an extreme of dietary restriction results in sexually immature animals. The work of Berg (1960) and Ross (1969) demonstrated that far less severe regimens of dietary restriction which allowed a slow rate of growth were equally effective in increasing life-span. Berg (1960) restricted Sprague–Dawley rats to 66% of the *ad libitum* food intake and assessed fertility at 730–790 days after a return to *ad libitum* feeding. Of 24 animals used, 67% conceived, but litters were small, death rate of the newborn was high and cannibalism of the young was common. No attempt was made to assess fertility of animals maintained on the restricted diet or of animals on the restricted diet and younger than 730 days. The animals described as of the Sprague–Dawley strain by Berg (1960) were smaller and slower growing than the CFY Sprague–Dawley rats used in the present study and more like rats of the CD Sprague–Dawley strain. The CD and CFY Sprague–Dawley strains are not genetically identical since the growth rate of the CFY stock far exceeds that of the CD strain and, up to 9 weeks of age, CFY females grow faster than do CD males fed the same diet and kept under identical conditions of husbandry (Festing, 1978). In the Berg (1960) study, females restricted to 67% of the food intake recorded for the control animals showed a rapid growth to 160 g over the first 100 days of life, followed by a plateau such that, when assessed for fertility at 730 days, mean body weight was only 197 g whereas the CFY experimental females in the present study reached this weight at 300 days (Text-fig. 1a). Glass *et al.* (1976) demonstrated in undernourished rats that the age of puberty (vaginal opening or first oestrus) was a negative linear function of the growth rate. In these same rats, the weight at puberty was not constant but was a quadratic function of the growth rate which was therefore considered to be more important in the timing of puberty than the arrival at a fixed body weight. Thus strain differences and the growth allowed by the degree of dietary restriction may well explain the differences recorded by authors for fertility in rats fed a restricted diet.

This work was supported by a grant from the Nuffield Foundation to Professor J. G. Phillips. Acknowledgement is also made to the Medical Research Council for the receipt of a Research Studentship (A.M.H.)

References

- Asdell, S.A. & Crowell, M.F. (1935) The effect of retarded growth upon the sexual development of rats. *J. Nutr.* **10**, 13–24.
- Asdell, S.A., Bogart, R. & Sperling, G. (1941) The influence of age and rate of breeding upon the ability of the female rat to reproduce and raise young. *Mem. Cornell. Univ. agric. Exp. Stn* **238**, 3–26.
- Ball, Z.B., Barnes, R.H. & Visscher, M.B. (1947) The effects of dietary calorie restriction on maturity and senescence, with particular reference to fertility and longevity. *Am. J. Physiol.* **15**, 511–519.
- Berg, B.N. (1960) Nutrition and longevity in the rat. I. Food intake in relation to size, health and fertility. *J. Nutr.* **71**, 242–254.

- Carr, C.J., King, J.T. & Visscher, M.B.** (1949) Delay of senescence infertility by dietary restriction. *Fedn Proc. Fedn Am. Socs exp. Biol.* **8**, 22.
- Festing, M.F.W.** (1978) When is a Sprague-Dawley not a Sprague-Dawley? *Lab. Anim. Cent. Newsl.* **54**, 13-15.
- Glass, A.R., Harrison, R. & Swerdloff, R.S.** (1976) Effect of undernutrition and amino acid deficiency on the timing of puberty in rats. *Pediat. Res.* **10**, 951-955.
- Kennedy, G.C. & Mitra, J.** (1963) Body weight and food intake as initiating factors for puberty in the rat. *J. Physiol., Lond.* **166**, 408-418.
- Mandl, A.M.** (1961) Cyclical changes in the vaginal smear of senile nulliparous and multiparous rats. *J. Endocr.* **22**, 257-268.
- McCay, C.M.** (1942) Chemical aspects and the effect of diet upon ageing. In *Problems of Ageing*, 2nd edn, pp. 680-727. Ed. E. V. Cowdry. Williams and Wilkins, Baltimore.
- National Research Council, Committee on Animal Nutrition** (1962) Natl. Acad. Sci. Natl. Res. Council Publ. No. 990.
- Piacsek, B.E. & Meites, J.** (1967) Reinitiation of gonadotropin release in underfed rats by constant light or epinephrine. *Endocrinology* **81**, 535-541.
- Rinaldini, L.M.** (1949) Effects of chronic inanition on the gonadotrophic content of the pituitary gland. *J. Endocr.* **6**, 54-61.
- Ross, M.H.** (1969) Ageing, nutrition and hepatic enzyme activity patterns in the rat. *J. Nutr.* **97**, 563-602.
- Segall, P.E. & Timiras, P.S.** (1975) Age related changes in thermoregulatory capacity of tryptophan-deficient rats. In *Biology of Ageing and Development*, pp. 161-166. Ed. C. J. Thorbecke. Plenum Press, London.
- Snedecor, G.W.** (1962) *Statistical Methods*. The Iowa State University Press, Ames, Iowa.
- Sokal, R.R. & Rohlf, R.J.** (1969) *Biometry*. Freeman and Company, San Francisco.

Received 2 October 1978