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Effect of Dietary Restriction on Estrous Cyclicity and Follicular Reserves in Aging C57BL/6J Mice[1]

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ABSTRACT

Restricting the food intake of female mice by alternating days of feeding and fasting delayed the age-related loss of estrous cycling potential and retarded the rate of follicular depletion, as determined after reinstatement of ad libitum (AL) feeding. During the period of food restriction (FR; 3.5–10.5 mo), food intake and body weight were about 80% of AL values. Mice were acyclic and predominantly in a state of diestrus during FR, but after reinstatement of an AL diet at 10.5 mo all FR mice resumed cycling regularly. By contrast, 80% of AL controls had become acyclic by this age, and the cycles of the remaining mice were significantly longer than those of the reinstated FR mice. Follicular reserves of 12.5-mo-old FR mice were twice those of age-matched AL controls. Cycling performance of reinstated FR mice, measured by cycle length and the proportion of mice still cycling, was equivalent to that of AL mice when the latter were 2–5 mo younger. Ovarian age, measured by the size of the follicular reserve, was similarly retarded in FR mice. Based on these data and previous evidence that follicular depletion plays a major role in the cessation of cyclicity in this strain, we hypothesize that the delayed loss of estrous cyclicity in aging FR mice is mediated at least in part by the retarding effect of dietary restriction on the rate of follicular depletion.

INTRODUCTION

Restricting caloric intake of laboratory rodents markedly prolongs life (Young, 1979) and delays the age-related loss of many physiologic functions, including fertility in females (Ball et al., 1947; Berg, 1960; Segall and Timiras, 1976; Merry and Holehan, 1979). Although dietary restriction can delay the loss of estrous cyclicity in Sprague-Dawley rats (Merry and Holehan, 1979), it was not known whether a comparable effect occurs in other strains or species of rodent.

The purpose of this study was twofold: first, to determine whether dietary restriction initiated early in adult life would delay the loss of estrous cyclicity in aging C57BL/6J mice, and second, to determine whether the rate of declining follicular reserves showed a correlative change. Because follicular depletion seems to play an important role in the cessation of cyclicity of this strain (Felicio et al., 1983; Gosden et al., 1983), it seemed probable that a retarding effect of dietary restriction on the loss of cyclicity might be mediated by a corresponding effect on the age-related loss of follicular reserves. Previous studies showed a

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retarding effect of dietary restriction on oocyte depletion in prepubertal rats (Lintern-Moore and Everitt, 1978), but whether this effect occurred in other species or persisted in older animals had not been firmly established.

MATERIALS AND METHODS

Animal Husbandry

Virgin 3-mo-old female C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in a limited-access, 23-m^2 room restricted to this strain. The total number of females and males in the colony during the experiment averaged 163 ± 20 and 12 ± 5, respectively. The temperature in the animal room averaged between 20° and 23°C, and the colony was maintained on a 12L:12D schedule (lights on at 0700 h). Mice were randomly assigned to food restricted (FR) and ad libitum-fed (AL) groups and were housed singly in transparent polycarbonate cages (19 × 25 cm; 15 cm high) with Beta Hardwood Chips (Northeastern Products, Warrensburg, NY). Animals had free access to tap water and food (Purina Rodent Laboratory Chow #5001), except for FR mice, whose access to food was restricted to every other day. Previous studies have shown that a similar regimen of intermittent fasting prolongs the average lifespan of this strain of mouse (Cheney et al., 1980; Talan and Ingram, 1983) as well as of rats (Goodrick et al., 1982). Because FR mice adapted to the initial every-other-day regimen (50% fasting) by overeating on feeding days relative to AL controls, and consequently approaching the food intake of AL mice (data not shown), more stringent regimens (62–67% fasting) were adopted beginning when mice reached 6 months of age (Table 1). Body weight and food intake were monitored biweekly. The difference in weight of food remaining in the cage hopper at successive weighings was taken as an index of food consumption. Although pieces of food found on the cage floor were collected and weighed, this measure of food intake is probably an overestimate since not all spillage could be accounted for. Body weight was measured on two consecutive days, first after the maximal period of fasting (24 or 48 h, depending on the food restriction regimen; see Table 1) and again 24 h after initiation of refeeding. These times were chosen to obtain the minimum and maximum weights of FR mice. AL mice were weighed at the same times, but the two consecutive measurements were averaged for each animal.

Experimental Design

Estrous cyclicity was monitored by daily vaginal lavage. Stages of the estrous cycle were determined from Giemsa-stained vaginal smears by one individual without knowledge of treatment group. Valid estrous cycles and cycle lengths were determined by programs written for a microcomputer (Nelson et al., 1982). Onset of acyclicity was defined as the day of the first cycle or acyclic interval >9 days in length, unless such an interval was followed by more than 3 consecutive cycles ≤9 days long. Although the age of cessation of cyclicity is criterion dependent, we have shown that relative differences in onset of acyclicity between groups are maintained across widely varying criteria (Felicio et al., 1984). Restriction of food intake began at 3.5 mo of age and continued until 10.5 mo, when FR mice were returned to an AL diet. Vaginal lavage continued until mice were 12.5 mo old, when they were ovariectomized under anesthesia (2,2,2-tribromoethanol; BDH Chemicals, Ville St. Laurent, Quebec) (Nelson et al., 1981). Ovaries were fixed in Bouin's solution, embedded in paraffin, serially sectioned at 7 μ and stained in hematoxylin and eosin (Gosden et al., 1983). The total number of primordial follicles was estimated based on counts of every tenth section. When estimated values of follicular reserves were <150, additional sections (every second) were counted to obtain a more accurate estimate.

Statistical Analysis

All interval data are expressed as the mean ± standard error. Significance of differences among groups

TABLE 1. Feeding schedules of food-restricted mice.

Interval	Sun	Mon	Tue	Wed	Thu	Fri	Sat	Time fasted (%)
7/12–10/6/82	RM[1]	RT[2]	RM	RT	RM	RT	RM	50
10/7–11/7/82	RM	RT	RM	RTam[3] RMpm	RM	RT	RM	67
11/8–2/16/83	RM	–[4]	RT	RM	–	RT	RM	
	RT	RM	–	RT	RM	–	RT	
	RM	RT	(etc. . . .)					62

[1] Food removed in the morning (RM) of this day.

[2] Food returned in the morning (RT) of this day.

[3] Food returned in the morning and removed 8 h later (RT am or RM pm).

[4] Food not returned (–).

was determined by t test or analysis of variance (ANOVA) followed, when appropriate, by Duncan's Multiple Range Test using the GLM procedure of the SAS statistical package (Helwig and Council, 1979). The percentage of acyclic mice and the frequency distribution of cycle lengths were analyzed by the Chi-square and G tests for independence; Mann-Whitney U tests were employed to evaluate differences in follicular reserves (Sokal and Rohlf, 1969). Differences were considered significant at $P<0.05$.

RESULTS

Food Consumption and Body Weight

Average food intake of FR mice was 79 ± 2% of control values ($P<0.001$, t test). Although food intake on the every-other-day feeding regimen (fasting 50% of the time) was initially at 70% of control values, it increased by the third month on this regimen to 95% of AL intake. On the subsequent, more restricted regimens, food intake of FR mice never exceeded 80% of the control values. Body weight of control mice increased gradually during the course of the experiment from 21 to 25 g (Fig. 1). The weight of FR mice, whether measured after fasting or refeeding, was significantly less than that of controls (80.4 ± 4.1% or 93.5 ± 1.3% of AL weights, respectively; $P<0.001$, ANOVA). After FR mice were returned to an AL diet, their body weight rapidly increased to a plateau significantly greater ($P<0.05$, t test) than the 24-h-fed value of the last weighing obtained during FR. However, this plateau weight (24.3 ± 0.1 g) remained significantly less than that of AL controls (25.1 ± 0.1 g) ($P<0.01$, t test).

Estrous Cyclicity

Food restriction markedly suppressed estrous cyclicity (Figs. 2 and 3). By 5 mo of age, 77% (7/9) of FR mice were acyclic, and cycle lengths of the remainder were significantly longer than those of AL controls ($P<0.005$, G test). Age of onset of acyclicity in FR mice was directly proportional to their body weight (average of fed and fasted values; $r=.68$, $P<0.05$), indicating a protective effect of body weight against the acyclic effect of FR. Over 70% of the vaginal lavages from acyclic mice were leukocytic (i.e., diestrus or metestrus-2). After the more stringent FR regimen was instituted, all FR mice became acyclic. Although the proportion of AL mice still cycling began to decline by 7 mo of age, this value remained significantly greater than that of FR mice throughout the period of FR ($P<0.05$, G test). By 11 mo of age 80% of the AL mice were acyclic. By contrast all 11-mo-old FR mice, which had been reinstated to an AL diet at 10.5 mo, were cycling regularly. This level of cyclicity was equivalent to that of AL mice when they were 2–5 mo younger (Fig. 2). The age-related lengthening of cycles was also attenuated in FR mice (Fig. 4). Cycle lengths of 11-mo-old AL-reinstated FR mice were equivalent to those of 6- to 9-mo-old controls. Although mice were ovariectomized before they completed their 12th month of life, 45% (4/9) had ceased cycling at the time of ovariectomy.

Follicular Reserves

The number of primordial follicles in 12.5-mo-old FR mice (2 mo after returning to an AL diet) was twice that of AL controls ($P<0.05$, Mann-Whitney U test; Fig. 5). Based upon the more rapid rate of follicular depletion estimated for the AL mice (see Fig. 4), it can be inferred that the number of primordial follicles present in the 12.5-mo-old FR mice was equivalent to that present 2 mo earlier in AL mice.

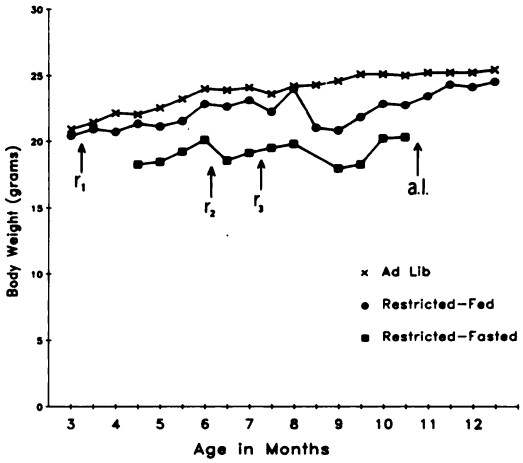

FIG. 1. Effect of age and food restriction on average body weight of 10 AL-fed and 9 FR female C57BL/6J mice. Because food restriction involved intermittent feeding, mice were weighed on two consecutive days, first after the maximal period of fasting (24 or 48 h, depending on the food restriction regimen; see Table 1) and again 24 h after initiation of refeeding (Restricted-Fasted and Restricted-Fed, respectively) to obtain their minimum and maximum weight during the food restriction regimen. "r_1", "r_2", and "r_3" refer to the times of onset of the 3 different dietary regimens (50%, 67%, and 62% fasting, respectively). "*a.l.*" indicates the age of reinstatement of the ad libitum diet to the food-restricted mice.

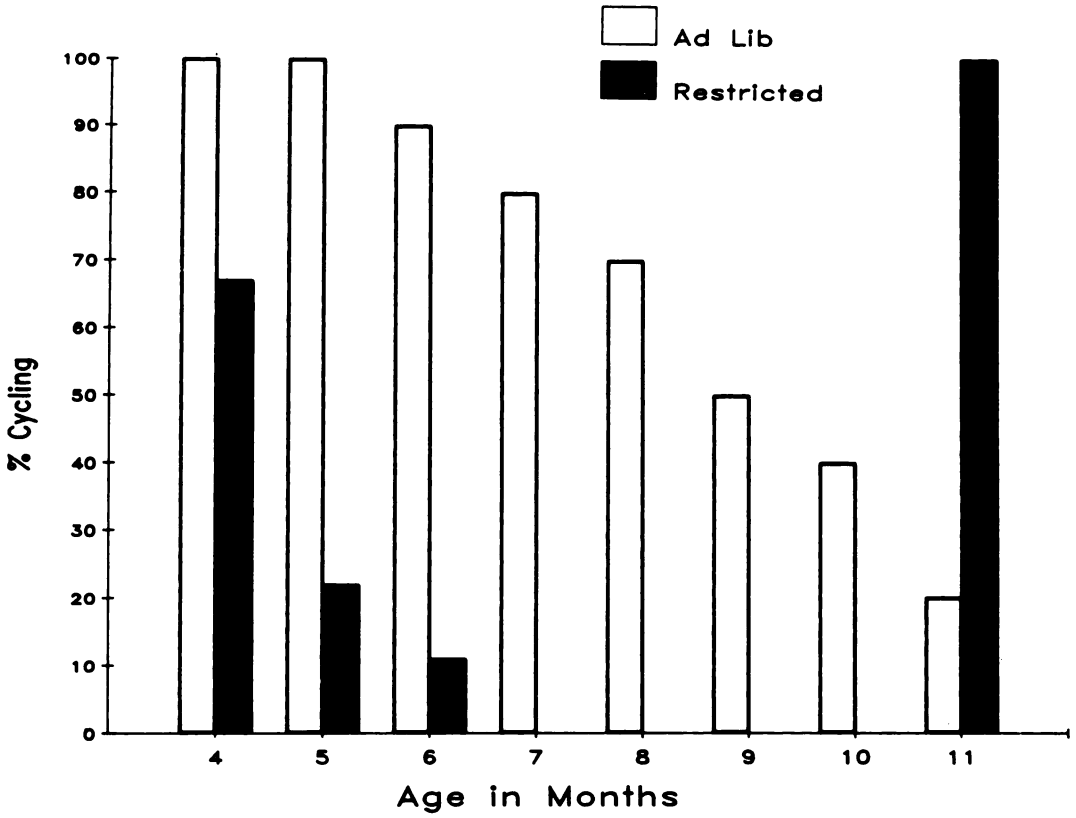

FIG. 2. Effect of age and food restriction on the percentage of the population still cycling (see *Materials and Methods* for determination of cycling status). Note the marked increase in the percentage cycling in the FR group at 11 mo (2 wk after they were returned to an AL diet).

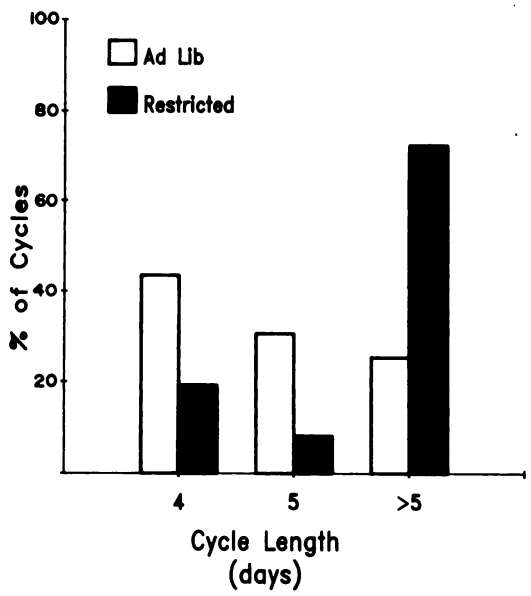

FIG. 3. Effect of food restriction between 3.5 and 10.5 mo of age on cycle length. Data are based on the total number of cycles during this time interval: 36 and 279 cycles in the FR and AL groups, respectively. Effect of group on the distribution of cycle lengths is significant ($P<0.005$, G test).

DISCUSSION

Three major observations were made in this study. First, intermittent feeding (fasting 50–67% of the time) markedly suppressed estrous cyclicity of mice. Second, this dietary regimen delayed the age-related loss of capacity to cycle and the age-related increase in cycle length, as determined after reinstatement of ad libitum feeding. Third, dietary restriction resulted in a corresponding retardation of the age-related loss of primordial follicles.

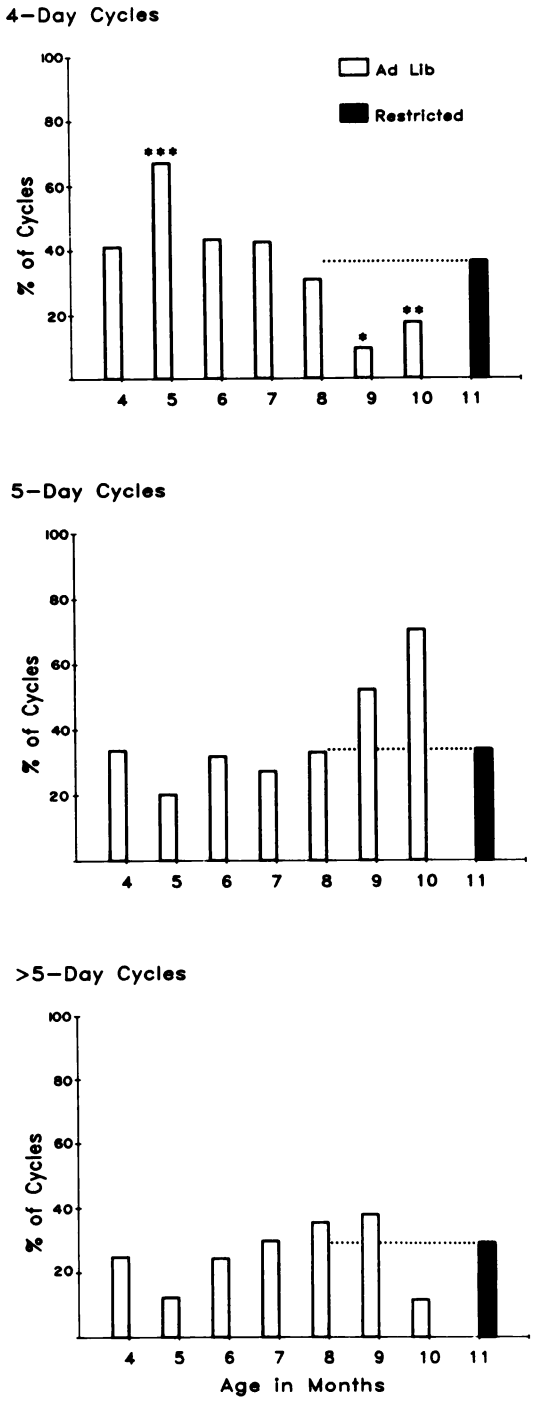

FIG. 4. Cycle lengths in 4- to 10-mo-old AL-fed mice compared to those of FR mice at 11 mo of age, 2 wk after reinstatement of an AL diet. Ages at which the distribution of cycle lengths differ significantly between AL-fed mice and 11-mo-old, formerly FR mice are indicated with asterisks (***, $P<0.01$; **, $P<0.05$; *, $P<0.1$, G test). The dotted lines intercept the first age of AL-fed mice that is not distinguishable from the 11-mo-old formerly FR mice.

That dietary restriction can suppress estrous cyclicity in mice has been previously reported (Lee et al., 1952). Whether this effect is the consequence of generalized caloric restriction or the reduction of some specific nutrient cannot be ascertained from the available data. Although marked caloric restriction, using a wide range of dietary regimens, suppresses cyclicity and fertility in animal models (Leathem, 1966), even small modifications of the dietary constituents of nearly isocaloric diets can have marked effects on fertility (Pryor and Bronson, 1981) and estrous cyclicity (Nelson and Felicio, 1984).

After reinstatement on an AL diet at 10.5 mo of age, all FR mice resumed cycling, and their cycle lengths were equivalent to those of their AL counterparts when the latter were 2–5 mo younger. Moreover, whereas all FR mice

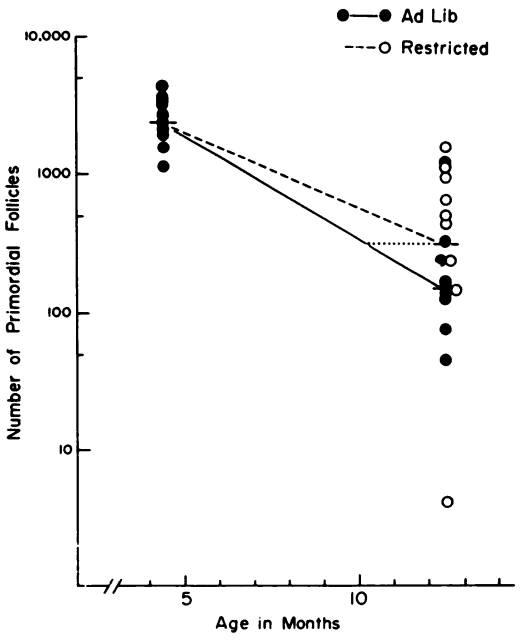

FIG. 5. Effect of age and food restriction on the primordial follicular reserves. Estimates of the rates of follicular depletion (FR: $\log y = 3.89 - 0.11x$; AL: $\log y = 4.07 - 0.15x$, where y is the number of primordial follicles at x months of age) are based on the evidence that follicular depletion occurs exponentially (Jones and Krohn, 1961; Faddy et al., 1983). At 12.5 mo of age, FR mice (food restricted between 3.5 and 10.5 mo) had twice the follicles of AL-fed mice ($P<0.05$, Mann-Whitney U test). The dotted line intercepts the estimated regression of follicular depletion for the AL-fed mice at the age when their follicular reserves would match those of 12.5-mo-old FR mice.

were cycling at this age, 80% of the AL controls had become acyclic. This result extends previous studies showing that FR mice returned to an AL diet remained fertile for a much longer duration than AL mice (Huseby et al., 1945; Ball et al., 1947). The underlying mechanisms involved in the delay of age-related infertility by food restriction remain unknown. However, the FR-mediated delays in oocyte depletion and in the loss of regular cycles may be contributing factors, since the size of the follicular reserve (Thung, 1961; Brook et al., 1984) and the regularity of estrous cyclicity (Fugo and Butcher, 1971; Barkley and Bradford, 1981) can affect fertility.

The retardation by FR of the rate of oocyte depletion may also explain the prolonged cycling potential of FR mice. In C57BL/6J mice, oocyte reserves are nearly exhausted when cyclicity ceases, and anovulatory mice have half the remaining oocytes of age-matched mice that are still ovulating (Gosden et al., 1983). Moreover, young ovaries partially restore cyclicity when grafted into old anovulatory mice (Felicio et al., 1983), and fully restore cyclicity in mice about to become acyclic (Felicio, Nelson, and Finch, unpublished data). These observations implicate ovarian aging—follicular depletion, in particular—in the cessation of cyclicity in this strain, and support the view that the FR-mediated retardation of the rate of follicular depletion contributes to the delayed loss of cycling potential in FR mice. Consistent with this hypothesis is the finding that the functional ovarian age of FR mice, as measured by the number of remaining follicles, was retarded by an amount of time similar to that of the functional cycling age of FR mice.

Although ovarian aging appears to be the primary determinant of the cycling lifespan of the C57BL/6J mouse, a modulatory role of hypothalamic-pituitary dysfunction seems probable. In both rats and mice, the size of the preovulatory LH surge begins to deteriorate well before the cessation of ovulatory cycles (Wise, 1983). That these reduced LH surges reflect a primary impairment of the hypothalamic-pituitary axis is indicated by the reduced ability of exogenous estradiol to elicit an LH surge in middle-aged rats and mice (Gray and Wexler, 1980; Gee et al., 1984). It was beyond the scope of the present study to determine whether dietary restriction delays the age-related decline of this positive feedback sensitivity to estradiol.

Neural effects of dietary restriction have not been extensively studied. Segall et al. (1978) reported that a 30-day period of tryptophan deficiency suppressed serotonin levels in brains of adolescent rats. However, generalized food restriction did not have a comparable effect, nor did it alter catecholamine levels. Food restriction delayed the age-related loss of striatal dopamine receptors in rats (Roth et al., 1984) and retarded age-associated losses of choline acetyltransferase activity in several brain regions of male rats, although other neurochemical age changes were not affected (London et al., 1983). Although the effects of dietary restriction on neural activity appear to be selective, given the pervasive influence of dietary restriction on aging processes (Young, 1979), an effect of dietary restriction on neuroendocrine mechanisms governing estrous cyclicity seems probable and deserves study.

In the present study, postpubertal dietary restriction resulted in a marked retardation of the age-related loss of primordial follicles. Food restriction can also delay the loss of follicles in prepubertal rats (Lintern-Moore and Everitt, 1978), and a similar effect was reported for adult C3H mice (Huseby et al., 1945), but this observation was not quantitative. Although FR can thus retard follicular depletion in adulthood as well as prepubertally, the mechanism(s) mediating this effect are unknown. Depletion of primordial follicles can occur via two routes: oocyte death or transition to a growing stage. Both hypophysectomy (Jones and Krohn, 1961; Faddy et al., 1983) and food restriction (Lintern-Moore and Everitt, 1978) appear to reduce the rate of transition from primordial to growing follicles. Whether there is a corresponding reduction in the spontaneous rate of cell death in hypophysectomized and FR animals has not been examined, although hypophysectomy appears to protect specifically against oocyte death induced experimentally by ionizing radiation (Beaumont, 1969).

Because both FR and hypophysectomy can retard oocyte depletion, it is of interest that FR has been proposed in another context to be the functional equivalent of hypophysectomy (Mulinos and Pomerantz, 1940). Although the effects of chronic FR on pituitary tropic hormone secretion are largely unknown, evidence indicates that FR is not the equivalent of hypophysectomy. In chronically FR male rats, neither LH nor androgen levels were reduced

(Merry and Holehan, 1981). Even during acute FR or starvation, not all circulating levels of pituitary hormones are reduced (Campbell et al., 1977; Rattner et al., 1978). Although hormone concentrations were not measured in this study, the absence of estrous cycles and the persistent leukocytosis of FR mice are consistent with low concentrations of estradiol and suppressed gonadotropins. Previous studies have shown that acute FR in pregnant mice suppresses LH but not FSH concentrations; in fact, FSH may be elevated (Rattner et al., 1978). Thus, it seems probable that at least the circulating levels of LH were reduced in the FR mice of this study. It is therefore possible that altered levels or patterns of pituitary hormones retard the rate of follicular depletion in FR mice; however, there is presently little basis for speculating as to which hormones may be involved.

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