Onset of genetic obesity in the absence of hyperphagia during the first week of life in the Zucker rat (*fa/fa*)

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Abstract The aim of this study was to discover which of three major abnormalities of the genetically obese Zucker rat (*fa/fa*), namely hyperphagia, excess adiposity, and hyperlipidemia, is the first to appear prior to manifest obesity, i.e., before weaning. Suckling *fa/fa* rats, bred from heterozygous parents, were detected by sizing fat cells obtained from an inguinal fat pad biopsy. Cell hypertrophy was observed in *fa/fa* rats, compared to *Fa/–* littermates of the same sex, as soon as 5–7 days after birth. Prediction of *fa/fa* genotype at this age by this method was assessed using a series of 80 pups and proved to be totally successful. The identity of the "predicted" obese pups was confirmed morphologically at 6 weeks of age. Food (milk) intake was estimated from water turnover rates determined on 86 pups aged 2–8 days using tritiated water. The results show that 7-day-old *fa/fa* rats had heavier inguinal fat pads with larger adipocytes and higher lipoprotein lipase activity than their lean controls. There was no genotype effect on water intake adjusted to body weight during the first week of life. Moreover weight of stomach contents and triglyceridemia were similar in all animals at 7 days. These results show that excess adiposity develops in the *fa/fa* rat during the first week of life, before hypertriglyceridemia and hyperphagia, and raises the question of whether this adiposity results from a defect in energy expenditure or an abnormality of fat cell storage capacity, or both.—**Boulangé, A., E. Planche, and P. de Gasquet.** Onset of genetic obesity in the absence of hyperphagia during the first week of life in the Zucker rat (*fa/fa*). *J. Lipid Res.* 1979. **20:** 857–864.

Supplementary key words early prediction of *fa/fa* genotype · lipoprotein lipase activity · triglyceridemia · water turnover rate

The genetically obese Zucker rat 'Fatty' (*fa/fa*) develops massive obesity after weaning (1), which is associated in the young adult with hyperphagia (2, 3), increased fatty acid synthesis in liver and adipose tissue (4, 5), high fat storage capacity (6), hypertriglyceridemia (2, 3), and hyperinsulinemia (7, 8). Any of these associated abnormalities may contribute to the development of obesity, but it is not clear as to which are present at its onset.

Zucker and Antoniades (7) succeeded in classifying progenies from *fa/fa* × *Fa/fa* matings into two groups differing in body fat content as early as 2 weeks after birth. They also showed that hyperinsulinemia first appeared in the *fa/fa* rat at 3 weeks of age, which suggests that hyperinsulinemia is not involved in the onset of this obesity. Normally, de novo lipogenesis in suckling rats is inhibited in liver and adipose tissue (9–12) by the high fat content of the mother's milk (13), but this may not be the case in *fa/fa* pups. It is likely however that the fat stores in rat pups are largely derived from circulating triacylglycerols of dietary origin. Transport of triacylglycerol fatty acid moieties into adipose tissue depends on the enzyme lipoprotein lipase (LPL) (14), which probably plays a major role in the development of this tissue in the newborn (15) and growing (16) nonobese rat. Thus the activity of this enzyme may be an important factor in the development of obesity in the suckling *fa/fa* rat. Hypertriglyceridemia in the adult fatty results from overproduction of triacylglycerol-rich lipoproteins (17) due in part to hyperphagia, hyperlipogenesis, and hyperinsulinemia. For the reasons mentioned above, the two latter abnormalities may not be as important in the suckling *fa/fa* rat as they are in the adult. Obviously, hyperphagia, if present, would be a determinant in the appearance of obesity and hypertriglyceridemia. Increased ingestion of solid food by the obese pups has been detected from day 16–17 onwards (18), but it has also been reported that obese and lean pups ingest comparable amounts of milk from 13 to 21 days (19).

Investigations concerning onset of obesity in the Zucker rat have been blocked by the lack of a suitable method for early detection of the *fa/fa* genotype. In this study, such a method is described, based on the

Abbreviation: LPL, lipoprotein lipase.

presence of fat cell hypertrophy in the *fa/fa* pup. Using this method we were able to characterize the overdevelopment of adipose tissue in 1-week-old *fa/fa* pups, and to answer the question: is onset of obesity associated with hyperphagia, increased fat storage capacity in adipose tissue, and hypertriglyceridemia?

EXPERIMENTAL

Animals

Obese Zucker rats (*fa/fa*) and their lean littermates (*Fa/–*) were bred in our laboratory from *Fa/fa* parents originally supplied by L. M. Zucker. The pups and their mothers were housed in polypropylene cages at an ambient temperature of 23°C with lighting from 7 AM to 7 PM. Animals had free access to laboratory biscuits and tap water.

Biopsy sample for prediction of *fa/fa* genotype at 1 week

At 5–7 days of age, 80 pups of both sexes (11 litters) were anesthetized with ether, and a 20-mg piece of one inguinal fat pad was removed from each. Fat cells were isolated with collagenase (20) and at least 100 cells were sized by photomicrography (21). The frequency distribution of fat cell diameters was then plotted. On the basis of this distribution, the rats were classified tentatively as *fa/fa* or Fa/– (Fig. 1). The pups were numbered and left undisturbed for 5–6 weeks after which identification as *fa/fa* or *Fa/–* was verified. The prediction of *fa/fa* genotype at 1 week was reliable, provided that the following rules were strictly observed: the pups had to be *1*) littermates, *2*) of similar body weight (10 g or more at 6 days), and *3*) of the same sex.

Adipose tissue and serum determinations

At 7 days of age, one day after initial biopsy for genotypic prediction, 8 male *fa/fa* and 15 male *Fa/–* (seven litters) were killed. In order to study all animals in a comparable state of nutrition, the pups were separated from their mothers for 3 hr and then allowed to suckle for 3 hr before killing. They were killed by decapitation, and weights of stomach contents were recorded. The intact contralateral inguinal fat pad was weighed; half was used for determination of cellularity and half was used for determination of LPL activity.

Cellularity. Fat cell size was determined on histological preparations essentially as described by Lemonnier (22). Fat cell number was calculated by dividing pad lipid content by cell weight derived from cell volume and triolein density (0.915). Lipid content of fat pads in these rats was calculated using an equation determined in a separate experiment (16 litters, 84 males aged 1–2 weeks) in which adipose tissue lipids were extracted (23) and determined gravimetrically. In 8 of the 16 litters used, it was possible to separate two subgroups differing in fat pad weight relative to body weight. The relationship between the natural logarithms (ln) of lipid weight (Y) and tissue weight (X) was similar in both groups: $\ln Y = (1.90 \pm 0.13)\ln X - (5.11 \pm 0.54)$ in the 11 fatter pups ($r = 0.981$); and $\ln Y = (2.12 \pm 0.09)\ln X - (6.01 \pm 0.37)$ in the 37 thinner littermates ($r = 0.969$). Therefore the data from the 84 pups were pooled, and the resulting regression line equation ($r = 0.956$):

$$\ln Y = (1.96 \pm 0.07)\ln X - (5.43 \pm 0.29)$$

was used to determine fat pad lipid content for the "predicted" *fa/fa* and *Fa/–* animals killed at 7 days of age.

As it can be seen from the above, adipose cell size was determined twice on the same rat: *1*) on day 6 using the biopsy sample (collagenase method) for prediction of genotype, and *2*) on day 7 when killed (histological method). Both methods correlated well ($r = 0.878$), as already stated (24). The data obtained with the histological method were used for cellularity determination, as a much larger number of cells could be counted (2000).

LPL assay. Tissue preparations and LPL assay were as previously described (25). Briefly, acetone–diethyl ether-dried powders were prepared from homogenates of adipose tissue in 2 ml of rat serum containing 4 IU/ml heparin. The powders were extracted with 4 ml of 50 mM NH_4Cl (pH 8.1), and the extracts were assayed for lipase activity using Intralipid activated by rat serum as the substrate. Enzyme activity (μmol of fatty acids released per hr at 37°C) was expressed per fat pad and per 10^6 cells.

Serum triacylglycerols. Serum triacylglycerols were extracted in chloroform–methanol 2:1 (v/v) according to Folch, Lees, and Sloane Stanley (23) and were determined by an enzymatic assay (26).

Water turnover studies in vivo

Water turnover was estimated during the first week of life according to Romero, Canas, and Baldwin (27) in 86 pups of both sexes (13 litters). All but two pups per litter were injected subcutaneously with 30 μl of tritiated water (1.5 mCi/ml) at 2 days of age. All pups were numbered and maintained for 5–6 weeks until identification of the obese could be made morphologically. Urine samples (30–100 μl) were collected (19) daily for 6 days; they were immediately delivered into 10 ml of Bray scintillation mixture (28) and counted for radioactivity in a liquid scintillation

counter (Packard Tricarb 3375). The two uninjected pups per litter served as controls, since radioactivity in the urine of the pups was recycled through their mother. At each sampling time, their urine specific radioactivity (dpm/ml) was subtracted from that of the injected littermates. Urine specific radioactivity declined logarithmically with time (the correlation coefficient always being above 0.996) and was used to calculate the fractional turnover rate. Daily water intake was calculated on day 5 (middle of the observation period) from the product of fractional turnover rate (day^{-1}) and body water content (ml).

Body water content could only be determined in the injected pups at 2 days of age by extrapolating the regression line of urine specific radioactivity on time at zero time (2 days). As no significant effect of genotype on body water content at 2 days could be detected, it was assumed that the difference in body water content between the fatty and the lean, which is manifest during the third week of life (19), was small during the first week. Consequently body water content of all injected pups at 5 days was calculated using the equation:

$$\text{Body water (\%)} = (86.9 \pm 0.5) - (0.68 \pm 0.05)\text{body weight (g)}; \; r = 0.915;$$

which was computed from data obtained by oven desiccation at 70°C using a separate group of 31 rats of both sexes 2–8 days old.

In estimating water intake, no correction was made for metabolic water (27).

RESULTS

Prediction of the *fa/fa* genotype at 1 week

When isolated adipocytes obtained by biopsy were sized and distributed in frequency classes, there were in general three types of distribution per litter: *1*) small, *2*) medium, and *3*) large cells (**Fig. 1**). The rats with larger cells always became obese by 5–6 weeks of age, while the rats with small and medium sized cells remained lean, corresponding to either *Fa/Fa* or *Fa/fa* genotype.[1] Early prediction of the *fa/fa* genotype could not be made with confidence using *mean* cell size as a basis, due to the not infrequent asymmetry of the distribution curves, probably resulting from the limited number of sized cells (100–300). It was the *median* of frequency distribution that proved to be the discriminating factor (see rats 1, 2, and 3 in Fig. 1). In some litters, only two types of cell distribution were apparent, and prediction could not be made because the phenotypic identity of the missing type was not known.

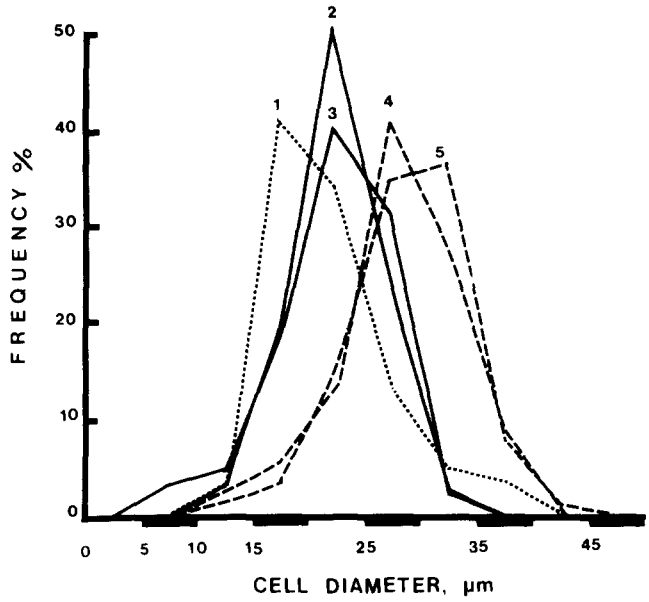

Fig. 1. Frequency distribution of cell diameters from inguinal adipose tissue from five male (1-week-old) Zucker siblings. The class width was 5 μm. For clarity, the curves have been drawn through class midpoints. After weaning, only rats 4 and 5 were obese. Mean and median of cell diameters of the pups at 1 week were:

Rat	Mean	Median
1	21.73	20.86
2	22.66	22.73
3	22.17	22.75
4	28.22	28.47
5	28.84	29.29

When the rules set out in the experimental section were adhered to, and provided that there were three types of cell distribution, prediction of *fa/fa* genotype was unmistakable; out of a total of 80 pups from 11 litters biopsied on days 5–7, 14 were identified as fatties and only these became overtly obese at 6–7 weeks of age. Prediction of *fa/fa* genotype at 3 days was unsuccessful, as the shift in fat cell size distribution from one type to another was indistinguishable. After day 7 however, prediction of genotype was facilitated, because the difference in median cell size between obese and lean littermates increased with advancing age.[2]

Cellularity and LPL activity in adipose tissue

Pups from seven litters, identified as described above, were killed at 7 days for tissue studies. One or two pups from each of the three types of cell distribution were taken from each litter. Adipose

[1] By analogy with the results of Joosten and van der Kroon (29) using the *ob/ob* mouse, it is presumed that rats with small (see rat 1 in Fig. 1) and medium (see rats 2 and 3 in Fig. 1) cells correspond to lean genotypes *Fa/Fa* and *Fa/fa*, respectively.

[2] Boulangé, A. Unpublished results.

TABLE 1. Inguinal adipose tissue weight and cellularity in the 1-week-old Zucker rat

Size of Fat Cells[a] / Genotype (Number of Pups)	Small *Fa/–* (7)	Medium *Fa/–* (8)	Large *fa/fa* (8)	Probability of Significance[c]: *1* vs. *2*	Probability of Significance[c]: (*1* + *2*) vs. *3*
	1	*2*	*3*	*4*	*5*
Body weight, g	12.32 ± 0.45[b]	12.48 ± 0.35	13.70 ± 0.38	N.S.	<0.05
Fat pad weight, mg	35.5 ± 9.8	41.4 ± 5.7	69.1 ± 10.9	N.S.	<0.01
Fat pad weight, mg / Body weight, g	2.85 ± 0.66	3.28 ± 0.40	4.95 ± 0.69	N.S.	<0.01
Lipid content of fat pad, mg	12.8 ± 5.8	14.2 ± 4.0	35.0 ± 8.8	N.S.	<0.01
Adipocyte diameter, μm	27.8 ± 1.9	30.7 ± 1.9	35.6 ± 1.8	<0.05	<0.01
Adipocyte number, 10^6 per 1 pad	0.94 ± 0.43	0.99 ± 0.12	1.44 ± 0.23	N.S.	N.S.

[a] As described in text and Fig. 1.
[b] Mean ± SEM.
[c] A two-way analysis of variance of unweighted means was performed (30) and showed no significant interaction. The main effect of genotype was partitioned as indicated in columns *4* and *5*, which give the significance of the corresponding *F* ratios.

tissue characteristics of these pups are shown in **Table 1.** As expected, the two types of lean pups did not differ significantly from each other except in cell size. The *fa/fa* pups were significantly heavier and fatter than the lean. Inguinal tissue in the fatty was 66% heavier than in the lean, and relative to their body weight, this increase amounted to 51%. Cell hypertrophy in this tissue accounted for this increase in weight, as there was no significant difference in cell number between obese and lean.

Inguinal adipose tissue LPL activity, serum triacylglycerol concentrations, and weights of stomach contents are given in **Table 2.** LPL activity per g of tissue (not shown) was similar in all pups, but the activity per one pad was higher in the fatties. When expressed per cell, however, the enzyme activity was not significantly higher in the obese.

Triglyceridemia was similar in all rats. This is not in contradiction with the finding of a higher LPL activity in adipose tissue of the obese; in fact, LPL activity in white adipose tissue of 7-day-old Zucker rat, either lean or obese, amounts to only a small percentage of total activity in other tissues, such as muscle and brown adipose tissue.[2] Thus differences in LPL activity in white adipose tissue alone would not be large enough to interfere with triglyceridemia.

The weights of the stomach contents were slightly, but not significantly, higher in the fatties, suggesting that the 1-week-old fatty does not ingest significantly more milk than the lean.

Water intake

The average body weight of the fatties used in the water turnover study was slightly less than that of the lean on day 5 (**Table 3**) and on all other experimental days, i.e., days 2–8 (not shown). This is at variance with the body weights of pups killed for tissue study (Table 1). Possibly the strict criteria applied in detecting the *fa/fa* genotype resulted in selection of heavier animals. However unselected 1–2-week-old fatties have been reported to be either heavier or lighter than lean littermates (19, 31, 32). Therefore

TABLE 2. Plasma triacylglycerol, adipose tissue LPL activity, and weight of stomach contents in the 1-week-old Zucker rat

Size of Fat Cells[a] / Genotype (Number of Pups)	Small *Fa/–* (6)	Medium *Fa/–* (7)	Large *fa/fa* (8)	Probability of Significance[c]: *1* vs. *2*	Probability of Significance[c]: (*1* + *2*) vs. *3*
	1	*2*	*3*	*4*	*5*
LPL activity per one pad	0.94 ± 0.43[b]	1.72 ± 0.65	3.70 ± 1.10	N.S.	<0.05
LPL activity per 10^6 cells	1.48 ± 0.68	1.53 ± 0.50	2.42 ± 0.43	N.S.	N.S.
Plasma triacylglycerols, mg/dl	105.0 ± 20.0[d]	95.7 ± 16.6	103.6 ± 13.4	N.S.	N.S.
Stomach contents, mg	290 ± 19	300 ± 37[e]	316 ± 44	N.S.	N.S.

[a,b,c] See corresponding footnotes in Table 1.
[d] There were five pups in this group.
[e] There were eight pups in this group.

TABLE 3. Water intake in the 5-day-old Zucker rat[a]

Sex	Males		Females	
Genotype	*Fa/fa*	*fa/fa*	*Fa/fa*	*fa/fa*
Number of Animals	20	23	28	15
Body weight, g	9.74 ± 0.23	9.58 ± 0.15	9.56 ± 0.15	9.30 ± 0.22
Body weight gain[b], g/day	0.989 ± 0.036	0.941 ± 0.032	0.947 ± 0.028	0.874 ± 0.040
Water intake, ml/day	2.725 ± 0.084	2.582 ± 0.064	2.665 ± 0.067	2.447 ± 0.084
Adjusted values of water intake[c], ml/day	2.678	2.576	2.665	2.519
Correlation between water intake (Y) and body weight (X)				
Slope	0.280 ± 0.055	0.243 ± 0.074	0.256 ± 0.067	0.271 ± 0.078
Intercept	−0.03 ± 0.54	0.25 ± 0.71	0.20 ± 0.65	−0.07 ± 0.73
r	0.770	0.713	0.598	0.726
P	<0.001	<0.005	<0.001	<0.005

[a] In this experiment the pups were bred from ♂*fa/fa* × ♀*Fa/fa* matings.
[b] The pups grew in a linear fashion during the experimental period. Individual regression coefficients of body weight on time were ≥0.9. Daily weight gain was calculated for each pup from the corresponding regression line equation.
[c] Water intake values adjusted for mean body weight of all animals (9.564 g).

the relatively large variability in body weight of the Zucker rat may have been responsible for the discrepancy between the two studies.

Data concerning water intake estimated at 5 days are shown in Table 3. Unexpectedly, water intake was significantly lower ($P < 0.05$) in the fatty rats than in the controls. This result does not necessarily indicate that the obese are hypophagic, since they were lighter and grew more slowly, although not significantly. As indicated in Table 3, water intake and body weight are dependent variables, being significantly and similarly correlated in each of the four groups of pups. When both parameters were analyzed together (covariance analysis), no significant effect of genotype was detected. This is illustrated in Table 3 by the similarity of the values of water intake adjusted to mean body weight. The data therefore demonstrate that the Zucker rat is not hyperphagic during the first week of life.

DISCUSSION

Adipose cell hypertrophy in the newborn fatty

The main difficulty when studying onset of genetic obesity in rodents, such as *fa/fa* rats or *ob/ob* mice, is early detection of the obese genotype during the preobese phase, i.e., before weaning. Methods of early detection of the obese, based on the presence of anomalies associated with the overdevelopment of adipose tissue, have been described. For instance, oxygen consumption in preobese *ob/ob* mice is decreased (33–35), as are body temperature (34, 36), response to cold (36), and body weight loss during starvation (34). As these methods are only applicable to animals over 2 weeks of age, a method was devised in the present study, based on the determination of adipocyte size, that could reliably detect genotype of pups at 5–7 days after birth. Fat cell hypertrophy appears to be an early feature of the genetically obese Zucker rat. A similar finding has been reported for the *ob/ob* mouse (29).

Fat cell hypertrophy alone apparently accounted for the overdevelopment of inguinal adipose tissue in the fatties, since cell number remained unchanged. Johnson et al. (37) reported hyperplasia in the adult, but these authors also reported "a tendency for the very young fatty to have fewer fat cells." It should be stressed, though, that cell number in adipose tissue at an early stage of development is likely to be underestimated for the following reasons: *1*) incomplete removal of the pad will lead to underestimation of its lipid content and therefore the cell number, *2*) unsized fat cells neither large enough to float after collagenase digestion or to be visible as "holes" on the histological preparation may lead to overestimation of mean cell size and consequently to underestimation of cell number. Obviously, for both these reasons, underestimation of cell number will tend to be greater in the lean than in the obese. Owing to the limits of the methods presently available for determination of cellularity in the developing fat pad, no firm conclusion can be drawn as to the presence or absence of hyperplasia in the 7-day-old fatty rat.

During the course of this study it became evident that the pups that had been identified as fatties, using the method described above, could be characterized by the overdevelopment of their inguinal adipose

tissue. The ratio of inguinal pad weight to body weight was constantly higher in fatties than in lean littermates (range +25% to +97%). This observation could constitute the basis for a rapid method of detection of 1-week-old fatties. However the presence of two types of lean siblings with different patterns of adipose tissue development, probably corresponding to the two lean genotypes,[1] may be confusing. It is likely that this difficulty could be overcome by using litters originating from ♂*fa/fa* × ♀*Fa/fa* matings.

Fat storage in adipose tissue

As stated in the introduction, de novo lipogenesis is repressed in normal suckling rats by the high fat content of milk. Recently it has been shown that dietary fat does not suppress fat synthesis in the adult *ob/ob* mouse (38), which may indicate that the overdevelopment of fat stores in the suckling *ob/ob* pup possibly results from an extralipogenesis. However, de novo lipogenesis appears to be equally inhibited in the suckling *mdb/mdb* mouse and its lean littermate (39). Therefore it would be of interest to know what contribution to fat stores is made by de novo lipogenesis in the Zucker pup.

In normal pups, a large part of the fat in adipocytes is probably derived from plasma triacylglycerols, following hydrolysis by LPL. The fact that LPL activity per inguinal pad is higher in the 7-day-old fatty than in the lean may thus explain why fat deposition is more rapid in this tissue in the fatty. Failure to show increased LPL activity per cell in the fatty may not be conclusive since, as discussed above, there was some doubt concerning the cell count. The reason why LPL activity per tissue is elevated in the obese is not clear. Insulin is known to increase the activity of this enzyme in adipose tissue (40), and hyperinsulinemic animals are known to have a high LPL activity in this tissue (6, 41). As the young fatty does not become hyperinsulinemic before 3 weeks of age (7), the increased LPL activity at 1 week cannot be ascribed to hyperinsulinemia. However the effect of insulin on a tissue does not depend only on the level of circulating insulin. It also depends on the response of the tissue to the hormone. Clearly more information on insulin sensitivity of adipose cell in suckling *fa/fa* rats is needed.

Absence of hyperphagia at onset of obesity

Milk intake in suckling pups was estimated by means of an isotope dilution technique. The results clearly indicated that water turnover in a pup was related to body weight regardless of sex or genotype, and that the *fa/fa* rat is not hyperphagic during the first week of life; all animals ingested similar volumes of milk when intake was adjusted for body weight. Also the weights of stomach contents on day 7 were similar in both obese and lean when the pups were killed in the fed state. Under the same experimental conditions, heavier stomach contents were found in the fatties only at weaning (21 days)[2] i.e., after appearance of hyperphagia (18, 19). Lin, Romsos, and Leveille (42) recently reported work on weights of stomach contents of suckling *ob/ob* mice. Similarly, they concluded that the *ob/ob* pups aged 7–21 days were not hyperphagic. The absence of hyperphagia in the suckling *fa/fa* rat does not exclude the possibility of an increased efficiency in fat absorption from the gut. It is also possible that patterns of food intake differ in the *fa/fa* pup and its lean control as they do in the adult (43). This could influence the rate of conversion of nutrients into fat.

The nutritional status of suckling *fa/fa* pups, with food intake comparable to that of the lean pups, resembles a "pair-feeding" situation. Weaned *fa/fa* rats are known to develop excess adiposity even when pair-fed to lean controls (44–46). It would seem therefore that excess energy intake is not the cause of overdevelopment of adipose tissue in the Zucker rat during the first week of life. The results imply that the *fa/fa* rat suffers from an abnormal partition of ingested energy, favoring energy storage probably at the expense of energy expenditure. The hormonal and metabolic factors responsible for such a disturbance in the Zucker rat are not known. In more general terms, the results imply that the partition of ingested energy between storage and expenditure is genetically determined.

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