Hepatic lipogenesis gene expression in two experimental egg-laying lines divergently selected on residual food consumption

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Hepatic lipogenesis gene expression in two experimental egg-laying lines divergently selected on residual food consumption

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Abstract – Two Rhode Island Red egg-laying lines have been divergently selected on residual food intake (low intake R^- line, high intake R^+ line) for 19 generations. In addition to direct response, correlated responses have altered several other traits such as carcass adiposity and lipid contents of several tissues, the R^+ animals being leaner than the R^- ones. In a search for the biological origin of the differences observed in fat deposit, the hepatic mRNA amounts of genes involved in lipid metabolism were investigated. No difference was found between lines for mRNA levels of ATP citrate-lyase, acetyl-CoA carboxylase, fatty acid synthase, malic enzyme and CCAAT/enhancer binding protein α, a transcription factor acting on several lipogenesis genes. The genes coding for stearoyl-CoA desaturase and apolipoprotein A1 displayed significantly lower mRNA levels in the R^+ cockerels compared to the R^-. All together these mRNA levels explained 40% of the overall variability of abdominal adipose tissue weight, suggesting an important role of both genes in the fatness variability.

laying fowl / food efficiency / fatness / mRNA / liver

Résumé – Expression hépatique de gènes de la lipogénèse chez des lignées divergentes de poules pondeuses sélectionnées sur la consommation alimentaire résiduelle. Deux lignées de poules pondeuses Rhode Island Red ont été sélectionnées de façon divergente sur la consommation alimentaire résiduelle (R^-/R^+) pendant

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19 générations. En plus de la réponse directe à la sélection, des réponses corrélées ont été observées sur d'autres caractères comme l'adiposité de la carcasse et le taux de lipides de plusieurs tissus, les animaux de la lignée R^+ étant plus maigres que ceux de la lignée R^-. Afin d'identifier l'origine biologique des différences observées dans l'état d'engraissement, les niveaux d'ARNm de gènes impliqués dans le métabolisme des lipides ont été étudiés. Aucune différence d'accumulation n'a été trouvée entre lignées concernant les ARNm de l'ATP citrate-lyase, l'acétyl-CoA carboxylase, la synthase des acides gras, l'enzyme malique et le facteur de transcription CCAAT/enhancer binding protein α qui agit sur plusieurs gènes de la lipogénèse. Les niveaux d'ARNm des gènes codant la stéaroyl-CoA désaturase et l'apolipoprotéine A1 sont significativement plus faibles chez les animaux de la lignée R^+ que chez ceux de la lignée R^-. Ces deux taux d'ARNm expliquent ensemble 40 % de la variabilité du poids de tissu adipeux abdominal entre les deux lignées, suggérant un rôle important de ces deux gènes dans la variabilité de l'état d'engraissement.

poule pondeuse / efficacité alimentaire / engraissement / ARNm / foie

1. INTRODUCTION

Since 1975, two R^- and R^+ lines have been divergently selected on residual food intake from a Rhode Island Red egg-laying population (low level R^-, high level R^+) [4, 6]. This criterion has been the food consumption adjusted for body weight, egg mass and body weight change by multiple linear regression [3]. In addition to direct selection response, correlated responses have been also obtained on carcass composition [14, 38, 41]. Actually, the R^+ animals became leaner than the R^- and this difference has occurred progressively throughout the course of selection. The abdominal fat pad to liver weight ratio has evolved from 6.4% and 5.5% for R^+ and R^- adult hens, respectively, at the first generation [31], to 5.7% and 8.2% at generation 17 [14], with consistent changes observed at generations 7 [41] and 14 [5]. The leanness has been even more marked in the R^+ males in which the ratio was 1.1% against 5.4% in the R^- males at generation 17 [14]. Besides, lipid contents of various tissues, including skin and breast muscle, have been significantly lowered in the R^+ line compared to the R^- [14]. Thus, the difference in the amount of energy accumulated as lipids cannot account for the difference in food intake between the two lines. Furthermore, in unrestricted fed conditions, the R^+ animals have exhibited a significantly higher diet-induced thermogenesis compared to the R^-, in both sexes [16, 17], which could allow a higher dissipation of ingested energy. These results suggested that the high fatness divergence between the R^- and R^+ lines could be mainly dependent on the fatty acid metabolism.

The aim of the present study is to investigate the genetic origin of the difference observed in fat deposit between the two lines. Although rather complex, fatty acid metabolism in birds can be summarized as follows: hepatic fatty acid synthesis, esterification into triglycerides, and secretion into the blood stream in the form of lipoproteins. Then, triglycerides can be taken up by various tissues and either stored in adipose tissue or catabolized for energy requirements. The hepatic mRNA levels of genes coding for enzymes and protein involved in hepatic fatty acid synthesis and triglyceride secretion were quantified and compared between the R^- and R^+ lines. The lipogenic enzymes were acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS),

malic enzyme (ME), providing the lipogenic NADPH cofactor, ATP citrate-lyase (ACL), synthesizing the lipogenesis precursor acetyl-CoA, and stearoyl-CoA desaturase (SCD1), catalysing the first fatty acid desaturation step. The mRNA of apolipoprotein A1 (APOA1), involved in lipid transport, and of the transcription factor C/EBPα (CCAAT / enhancer binding protein) were also analysed. This transcription factor, mainly expressed in hepatocytes and adipocytes, is known to be an essential regulator of lipid metabolism [10, 30, 39].

As a result, most of the genes studied presented higher mRNA levels in the fatter R^- line compared to the R^+, suggesting that they could be related to the fatness variability.

2. ANIMALS, MATERIALS AND METHODS

2.1. Animals and diets

Fifteen cockerels from each R^+ and R^- laying line were studied at the 19th generation of selection on residual food intake [4]. Birds were housed in individual cages between 18 and 55 weeks of age and were fed ad libitum a diet containing 11.2 MJ of metabolizable energy per kg with 16.41% crude protein, 3.43% lipids, 42.46% carbohydrates and 12.52% moisture. The lighting regimen was 14 hours light per day. The individual residual food consumption was calculated from mean body weight, weight gain and food intake, all recorded over a 28-day period between 33 and 37 weeks of age. At 55 weeks of age, birds were slaughtered, and body weights were recorded after slaughter and exsanguination. The liver was removed, weighed, frozen in liquid nitrogen and kept at $-80\,^{\circ}C$ until RNA extraction. Abdominal fat tissue was removed according to Ricard and Rouvier [35] and weighed.

2.2. Reagents and chemicals

Hybond-N nylon membrane, random priming kit, [α^{32}P]dCTP, Kodak X-Omat AR film and intensifying screens were purchased from Amersham (Orsay, France). Primers and Taq polymerase were obtained from Life Technologies (Cergy Pontoise, France), M-MLV reverse transcriptase from Promega (Charbonnières, France) and 100 bp ladder from Pharmacia (Orsay, France). Thermal Cycler 480 was from Perkin Elmer (Courtaboeuf, France). Chroma-spin-100 column was purchased from Clontech (Montigny-le-Bretonneux, France). Phosphorscreen and Storm system were from Molecular Dynamics (Bondoufle, France), CCD camera from Appligene (Illkirch, France) and Densylab^{TM} 2.6.6 software from Microvision Instruments (Evry, France).

2.3. Northern blot and hybridisation signal analyses

Total RNA was isolated from individual livers according to the guanidium thiocyanate method [9]. Electrophoresis and northern blots were performed with 10 µg of liver total RNA from each bird, as previously described [13]. Each northern blot carrying all the samples, i.e. liver RNA from the 15 R^- and 15 R^+ cockerels, was probed successively with one of the studied probes and then with the 515 bp probe coding for a part of the mouse 18S ribosomal RNA used as a control of RNA loading [34].

The probes were cloned from the chicken. The full length cDNA for apolipoprotein A1 (1 kb) and malic enzyme (2 kb) and the 1.3 kb partial cDNA for C/EBPα were kindly given by Ferrari et al. [15], Back et al. [1] and Calkhoven et al. [7], respectively. The partial cDNA of the stearoyl-CoA desaturase (1.2 kb) and ATP citrate-lyase (620 bp) were cloned in the Rennes laboratory. All the probes were labelled by random priming with [α^{32}P]dCTP (3000 Ci·mmol^{-1}), except for the 18S probe which was labelled by polymerase chain reaction (PCR). In that case, 10 pg of the 18S plasmid was amplified in the following PCR mixture: 1×PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 50 µM of each dATP, dTTP, dGTP, 10 µM of dCTP, 1.5 mM $MgCl_2$, 200 ng of each specific primer (forward: 5'CGTCC-CTATCAACTTTCG3'; reverse: 5'CATTATTCCTAGCTGCGG3'), 30 µCi of [α^{32}P]dCTP (3000 Ci·mmol^{-1}) and 2.5 units of Taq polymerase. PCR amplification was performed by 35 cycles (40 s 95 °C / 40 s 54 °C / 40 s 72 °C). The PCR product was then purified using a Chromaspin-100 column, and 6.5 pmol of non-radiolabelled 18S plasmid were added in order to ensure an excess of probe molecules compared to the target.

Ribonucleic acid blots were prehybridised for at least 4 h and hybridised for 18 h at 42 °C in 50% formamide, 5×SSPE, 0.1% SDS, 5×Denhardt buffer (0.2% bovine serum albumin fraction V, 0.2% Ficoll, 0.2% polyvinylpyrrolidone), 50 mM NaH_2PO_4 pH 6.5 and 100 µg·mL^{-1} denatured herring sperm DNA. Membranes were then washed in 3×SSPE with 0.1% SDS at 50 °C and twice in 1×SSPE with 0.1% SDS at 50 °C.

Hybridisation was revealed by autoradiography at −80 °C using Kodak X-Omat AR film and intensifying screens. The hybridisation signals were quantified on digitalized images using a CCD camera and the Densylab^{TM} 2.6.6 package. Quantification was done on 256 grey levels. For some experiments, the hybridisation signals were directly quantified by the Storm instrument on a 10^5 grey level scale. Then, the mRNA amounts were expressed as arbitrary grey level units relative to 10 µg of liver total RNA, corrected for the possible variation of the quantity loaded onto the membrane by the 18S intensity (mRNA to 18S signal ratio).

2.4. Reverse transcription - polymerase chain reaction (RT-PCR) analyses

Quantification of ACC and FAS mRNA was achieved by RT-PCR. The analysis of ACC mRNA needed an amplification because of its low hepatic level which was hardly detectable by northern blot. RT-PCR was also relevant to the study of FAS mRNA, whose large size often gave rise to poor hybridisation signals. 500 ng of total denaturated RNA were reverse-transcribed by 200 units of M-MLV reverse transcriptase in the presence of 100 ng of downstream primer, 200 µM of each dNTP, 50 mM Tris pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT. The downstream primer sequences were 5'AA-CAACAAGCGAAGCTGAAG3' for FAS and 5'GAAAGGGAATCCGAGCA-GACA3' for ACC. Reverse transcription was carried out at 60 °C for 15 min and at 45 °C for 30 min for ACC and FAS mRNA, respectively. The reverse transcribed products (20 µL) were amplified in a final PCR reaction volume of 80 µL. The PCR mixture consisted of 1×PCR buffer (20 mM Tris-HCl

pH 8.4, 50 mM KCl), 100 ng of upstream primer (5'TTCAGAGATGGAA-GACGTGG3' and 5'CTCCACCGCGCCCGGCACCCT3' for FAS and ACC, respectively), 100 μM of each dNTP, 2.5 units of Taq polymerase and 1.5 mM or 3 mM $MgCl_2$, for ACC and FAS, respectively. PCR amplifications were performed in sequential cycles at 30 s 95 °C / 30 s 60 °C / 30 s 72 °C. The numbers of cycles were determined in order to perform the analysis during the exponential amplification phase. They were 30 for ACC and 25 for FAS.

Finally, the PCR products of the studied animals were all together submitted to electrophoresis on 1% agarose gel with 1 μg of 100 base-pair ladder, visualized with ethidium bromide (EtBr) and quantified on image using CDD camera and Densylab^{TM} 2.6.6 package.

2.5. Experimental design and statistical analyses

For each type of mRNA, the whole northern blot and RT-PCR analysis was repeated two or three times. Therefore, the between strain differences were tested by analyses of variance using the "GLM" procedure of the SAS package [36] with the "repeated time" option to take the repetitions into account.

3. RESULTS

3.1. Animal traits

Mean values of traits recorded in both lines are given in Table I, along with the statistical significance of the between line comparisons. As expected, the divergence in the R selection criterion was highly significant. So was the difference in abdominal adipose tissue weight, the R^+ birds being leaner than the R^- (2% versus 7% when expressed as a ratio to body weight). For both traits, the R^+ line displayed a higher variability (expressed as standard deviation to mean ratio) than the R^-.

Table I. Means and standard errors of means of traits of 55-week-old cockerels from the R^- and R^+ lines.

Traits	R^- line (15)	R^+ line (15)	P
R	-669 ± 39	790 ± 86	$\mathbf{2 \times 10^{-11}}$
Observed consumption (g)	2586 ± 60	3948 ± 97	$\mathbf{2 \times 10^{-11}}$
Body weight (g)	3512 ± 60	3549 ± 97	0.75
Abdominal adipose tissue weight (g)	245 ± 22	79 ± 18	$\mathbf{4 \times 10^{-6}}$
(g of abdominal adipose tissue per kg of body weight)	(69.8 g·kg^{-1})	(22.2 g·kg^{-1})	
Liver weight (g)	34.2 ± 3.2	37.2 ± 3.4	0.38

R is the residual food intake in accordance with [4], the number of cockerels is given in brackets; P = Probability of the F value related to the line effect.

3.2. Liver mRNA Levels

A sample of individual data of mRNA hybridisation signals and EtBr stained RT-PCR products is displayed in Figure 1. The APOA1, SCD1, ME, ACL and C/EBP(probes identified mRNA species of the expected sizes. The RT-PCR of FAS and ACC mRNA gave unique bands of the expected size, 255 and 273 bp, respectively.

For each quantified mRNA, the line mean values +/− standard error recorded in each experiment, and the probabilities of mean identity are reported in Table II. Like the abdominal fat weight, the levels of hepatic APOA1 and SCD1 transcripts were significantly higher in the R^- line compared to the R^+ (2.5 to 3.5 times differences). For these traits also, the R^+ line displayed a higher variability than the R^-. As regards ACL mRNA amounts, the between line difference was close to significance (F test probability = 0.07). No significant difference was found for the ACC, ME, FAS and C/EBPα mRNA levels, although the values were higher again in the R^- line.

Table II. Mean and standard errors of means values of liver mRNA contents of 55-weeks-old cockerels from the R^- and R^+ lines.

mRNA	R^- line (15)	R^+ line (15)	R^- / R^+	P
ATP-citrate-lyase	888820 ± 133638	605555 ± 55762	1.5	**0.07**
	67268 ± 7984	45266 ± 4294	1.5	
	116469 ± 17720	78854 ± 10789	1.5	
acetyl-CoA	51.9 ± 1.9	48.7 ± 1.4	1.1	0.49
carboxylase	49.4 ± 2.3	47.6 ± 3.1	1.0	
fatty acid synthase	51.2 ± 3.5	42.0 ± 3.0	1.2	0.10
	53.6 ± 2.0	46.5 ± 2.5	1.2	
malic enzyme	101227 ± 13670	74176 ± 4764	1.4	0.20
	327908 ± 43231	248665 ± 13600	1.3	
	133816 ± 15486	108042 ± 4507	1.2	
stearoyl-CoA	62.3 ± 2.7	24.5 ± 2.6	2.5	**0.03**
desaturase	85.9 ± 3.1	37.9 ± 3.8	2.3	
	931873 ± 284271	261037 ± 38296	3.6	
apolipoprotein A1	52.6 ± 9.3	17.8 ± 2.0	3.0	**0.002**
	51.6 ± 6.7	39.1 ± 4.3	1.3	
	34.5 ± 5.8	15.5 ± 2.4	2.2	
C/EBPα	72.8 ± 3.4	66.0 ± 3.4	1.1	0.18
	85.3 ± 13.1	65.8 ± 10.7	1.3	

The number of cockerels is given in brackets. mRNA were quantified after hybridisation experiments, except for ACC and FAS which were estimated by RT-PCR (see Materials and methods). Means and SEM of each experiment are presented (arbitrary units); R^- / R^+ = ratio of mRNA level mean in the R^- line to that in the R^+ line; statistical significance includes all the repetitions; P = probability of the between lines F value.

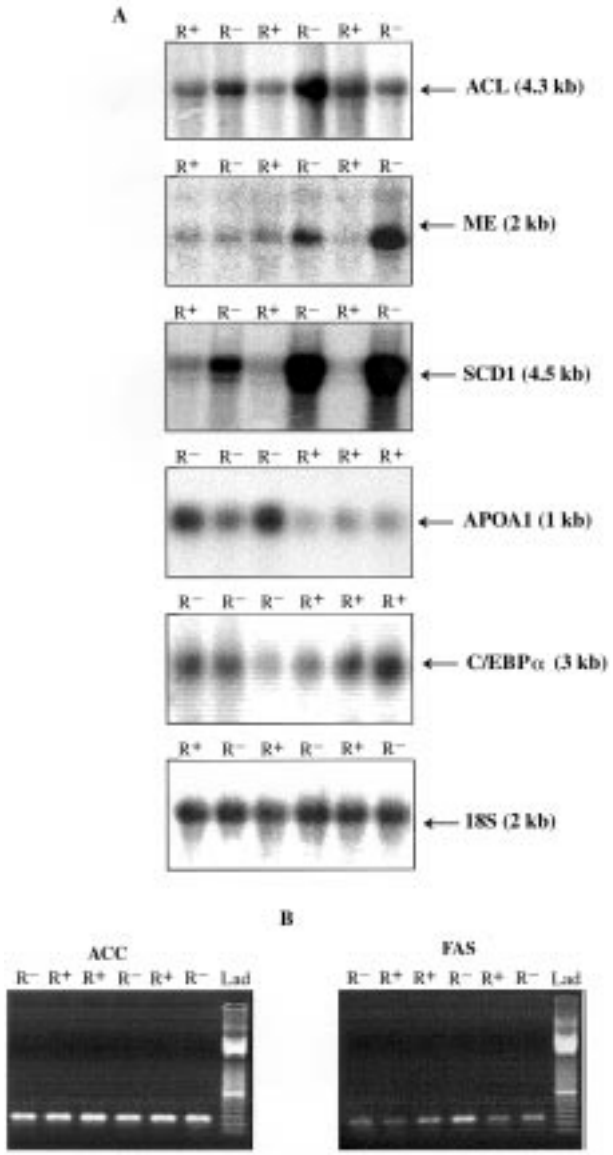

Figure 1. mRNA analyses. **A.** Northern blot analyses, as described in Section Materials and methods, of liver RNA of cockerels from the R^+ and R^- lines, hybridised with different probes: ACL = ATP Citrate-Lyase; ME = Malic Enzyme; SCD1 = Stearoyl-CoA Desaturase; APOA1 = Apolipoprotein A1; C/EBPα = CCAAT/Enhancer Binding Protein α; 18S = rRNA 18S. The sizes of the specific RNA are indicated on the left. **B.** RT-PCR analyses (as described in Materials and Methods) of liver FAS and ACC mRNA of cockerels from the R^+ and R^- lines: ACC = Acetyl-CoA Carboxylase; FAS = Fatty Acid Synthase; Lad = 100 base-pair ladder (Pharmacia, Orsay, France). Different sets of R^+ and R^- animals are displayed for each mRNA analysed.

4. DISCUSSION

In the present experiment, the R^- cockerels showed a larger abdominal fat pad than the R^+ in agreement with previous reports [4, 14, 38], and confirmed the correlated response in fatness coming with selection on residual food intake.

In the investigation on genes whose liver expression is related to the variability of fat deposit in the R^+ and R^- laying lines, ACC and ME mRNA amounts did not differ between lines, although the latter was slightly higher in the fatter R^- line (1.3 times). In the same way, the FAS and ACL mRNA amounts were not significantly different at the 5% level while the higher values were always in the R^- line (1.2 and 1.5 times, respectively). The activities of all these enzymes are known to be regulated mainly at the transcriptional level and to follow the variations of the mRNA amounts [18, 19, 20, 24], even if short-term changes mediated by allosteric modifications can occur, particularly for ACC [26]. Therefore, altogether these mRNA level differences could suggest a higher fatty acid synthesis in the R^- line with a predominant role for ACL. Similarly, in other animal models like Wistar obese rats [24] or broiler lines divergently selected on plasma very low density lipoprotein (VLDL) concentration [2] or on abdominal adipose tissue [11, 12], higher hepatic activities and/or mRNA levels of ACL were observed in the fat lines compared to the lean lines.

A significant difference between SCD1 mRNA amounts was found between the R^- and R^+ birds, the higher level being observed in the R^- fatter line again. The SCD1 gene is known to be regulated mainly at the transcriptional level in rodents [32] and chickens [27]. Hence, the SCD1 transcript level could be considered as an indicator of the SCD1 enzyme activity. Moreover, in the chicken, several studies have shown that hepatic SCD1 enzyme and the subsequent availability of endogenous oleic acid have an enhancing effect on the hepatic triglyceride secretion [28, 29, Diot, Laboratoire de génétique animale, INRA, Rennes, France, personal communication]. Mono-unsaturated fatty acids would be incorporated more easily into triglycerides and subsequently into VLDL, which would facilitate their hepatic secretion [25, 37]. In the light of these reports, the difference in SCD1 mRNA levels could indicate a difference in triglyceride secretion between both lines. This hypothesis is consistent with results of El-Kazzi et al. [14] who have reported higher plasma triglyceride concentration and higher lipid contents in various tissues such as skin and muscle, in the R^- line when compared to the R^+. Moreover, the R^- birds have exhibited higher proportions of mono-unsaturated fatty acids in abdominal adipose tissue and liver, whereas the proportions of saturated fatty acids in adipose tissue were equal in both lines, and lower in the R^- liver compared to the R^+.

The fatter line R^- exhibited significantly higher hepatic APOA1 mRNA amounts compared to the R^+. That could also be related to the difference in plasma triglyceride concentrations previously reported [14, 38], assuming that liver APOA1 mRNA amount reflects that of APOA1 protein. APOA1 is the major protein of the high density lipoproteins (HDL), which are mainly considered for their role in cholesterol reverse transport, although, in chicken plasma, the HDL account for a significant part of the circulating triglycerides [22]. Besides, in chickens, contrary to mammals, APOA1 has also been found in the VLDL, primarily devoted to triglyceride transport from the liver to other tissues [23]. The chicken APOA1 sequence has been partly similar to that of

the apolipoprotein E, present in mammal lipoproteins but not yet evidenced in birds. The similarity includes the part of apolipoprotein E which mediates the interaction with lipoprotein cellular receptors, suggesting that chicken APOA1 could have the same functions as apolipoprotein E in mammals [33] and be implied in the catabolism of VLDL and triglyceride storage in adipose tissue as already suggested [21]. Other studies, carried on divergently selected lean and fat broiler lines, and on goose strains differing in hepatic steatosis ability, have also shown significantly higher amounts of hepatic APOA1 mRNA levels in the fatter lines, compared to the lean ones [8, 11, 12, 13]. These results increased the interest in this gene whose product seems to play a role in triglyceride transport and storage, and further fatness, in birds.

The present results on the SCD1 and APOA1 mRNA suggested that these genes could play large parts in the fatness difference observed between the R^- and R^+ lines. Multiple linear regression analyses showed that 40% of the overall variability (both lines together) of the abdominal adipose tissue weight were explained by APOA1 and SCD1 mRNA levels (33% explained by APOA1 alone and 27% by SCD1 alone). However, the genetic origin of the variation of the mRNA amounts remains to be elucidated. It could result from either gene structure differences or differences in activity of a common transcription regulator. As regards the latter hypothesis, C/EBPα mRNA amounts were investigated because of the C/EBPα role in lipid metabolism [10, 30, 39]. Moreover, transcription has been reported as the main determinant of C/EBPα gene expression [40]. No difference in C/EBPα mRNA levels was observed between the two lines, suggesting that this transcription factor was neither involved in the variation of SCD1 and APOA1 gene expression nor in the phenotypic fatness variability. Further metabolic and genetic investigations like co-segregation analyses between genes and fatness should be performed to answer the question of the genetic origin of the observed variations in APOA1 and SCD1 mRNA and in fatness, between the R^- and R^+ egg-laying lines.

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