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Effects of thyroid hormone deficiency on mice selected for increased and decreased body weight and fatness

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Summary

A study was undertaken to test whether the elimination of metabolic pathways strongly involved in growth and fatness, comprising thyroid hormones (TH) and growth hormone (GH), is responsible for a substantial part of the genetic change produced by selection. Lines used in this study have been selected for about 50 generations for high (PH) and low (PL) body weight at 10 weeks and for high (F) and low fat content (L) at 14 weeks, producing a 3-fold difference in body weights and a 5-fold difference in fat content. Thyroid ablation was achieved by repeated backcrossing into the four selection lines of a transgene comprising the *HSV1-tk* gene coupled to the promoter of the thyroglobulin gene. Hemizygous pregnant dams were treated with ganciclovir leading to thyroid-ablated dams and offspring and therefore to a lack of TH and subsequently of GH. In the absence of TH and GH, lines still differ in body weight over the period studied (10 d to about 100 d; e.g. at the end PH = 32·1 g vs PL = 10·2 g) and in fat content (F = 16·2% vs L = 3·8%); the corresponding values for the wild-type controls were PH = 49·9 g vs PL = 17·4 g and F = 27·5% vs L = 4·8%. The effect of the transgene depended on the genetic background for body weights at most ages and for relative gonadal fat pad weights, but less for fat content. The L line showed the lowest growth depression. The *lit* gene, which causes GH but not TH deficiency, was also transferred by repeated backcrosses into three of these lines (PH, PL, F). The combined deficiency of TH and GH had bigger effects on body weights at earlier ages than did GH deprivation. The data show that changes in the TH- and GH-systems are not the only cause of line differences in growth and fatness resulting from long-term selection, but both are involved to a significant extent. The interactions between the effects of the transgene and of the *lit* gene and the genetic background were, nevertheless, relatively small and therefore these results support a polygenic model of selection response.

1. Introduction

Selected lines of mice provide a model for the analysis of the genetic basis of quantitative traits in animals. Whilst the responses of quantitative traits such as body size and fatness are likely to be dependent on changes at many loci, the contributions of particular candidate loci or metabolic or hormonal pathways can be investigated to find out whether they are responsible for a substantial part of the genetic change. Their contribution to genetic variation in the trait in the segregating base population from which the selected lines were taken can then be assessed, with the aim of understanding the basis of quantitative genetic variation. In this laboratory we have lines of mice that have been selected divergently from the same base population for body weight and for fatness for about 50 generations. At the age of selection, 10 weeks, the high body weight line is three times as heavy as the low body weight line, but these lines differ little in proportion of fat (Hastings *et al.*, 1993).

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The fat line has 5- to 6-fold higher fat percentage of body weight than the lean line at an age of 14 weeks, this ratio increasing with age, but the lines differ little in fat-free body weight (Hastings *et al*., 1991). These lines therefore provide very suitable material for experiments to investigate the role of candidate genes and pathways.

This paper describes an investigation of the roles of thyroid hormone (TH) and growth hormone (GH) in the response to selection, carried out by manipulating the relevant pathways on the genetic backgrounds of the different selection lines.

A functional thyroid is critical for normal growth and also influences body composition (e.g. Nantosalonen & Rosenfeld, 1992). It comprises three hormone-secreting cell types – C-cells, parathyroid gland cells and follicle cells – the last secreting thyroxine (T_4) and triiodothyronine (T_3). Thyroid hormone is known to induce GH mRNA in rat pituitary somatotrophs (Seo *et al*., 1978; Dobner *et al*., 1981) and T_3 response elements were found in the promoter region and in the third intron of the rat GH gene (Crone *et al*., 1990; Glass *et al*., 1987; Koenig *et al*., 1987). As a consequence of thyrocyte ablation in mice, the GH level fell to a plateau value close to that observed in GH-deficient *lit*/*lit* homozygous mice (Wallace *et al*., 1994) because a principal effect of T_4 is to enable the transcription of the GH gene (Evans *et al*., 1982; Spindler *et al*., 1982).

Thyroid ablation can be achieved by using a transgenic stock that has a construct comprising the herpes simplex type I virus thymidine kinase gene coupled to the promoter of the bovine thyroglobulin gene (Wallace *et al*., 1991). Treatment of these animals with the antiherpetic agent ganciclovir leads to ablation of the thyroid, such that thyroid hormone is not produced. In this experiment the transgene was backcrossed into each of four selected lines, so that the performance of thyroid-deficient animals could be assessed. In particular, if the thyroid pathway was contributing significantly to the genetic change in growth or fatness it would be expected that the effect of ablation would differ greatly between the high and the low lines. Ganciclovir treatment of pregnant females hemizygous for the transgene leads to ablation both in the mother and in transgenic embryos (Wallace *et al*., 1995), so it was possible to compare the effects of thyroid ablation combined with GH deprivation among littermates of young mice.

GH deprivation leads to reduced growth, partly due to its deficiency and partly due to consequential deficiency of insulin-like growth factor-I (IGF-I) (e.g. Nantosalonen *et al*., 1993). It is likely that, for example, some of the response obtained in body weight is associated with this axis: animals either releasing less or being less responsive to the hormone or others in the pathway leading to growth.

The recessive *lit* mutation and its effects are described in detail by Doolittle *et al*. (1996). In brief, it is known to cause a GH deprivation, due to a defect in the growth hormone releasing factor receptor gene (Lin *et al*., 1993). Homozygotes fail to release significant levels of GH in response to the growth hormone releasing factor and are consequently dwarfs. Homozygotes are smaller than normal from about 2 weeks of age, with adult weights about 50–65% that of controls (Eicher & Beamer, 1976; Phillips *et al*., 1982; Jansson *et al*., 1986; Green, 1989; Hastings *et al*., 1993).

By repeated backcrosses the *lit* gene was introduced into the selection lines (Bootland *et al*., 1991). In previous experiments it has been shown that growth of *lit*/*lit* homozygotes of the high and low body weight lines is reduced by a similar proportion of the wild-type of these lines, although the absolute difference is larger in the high selected lines. Further, exogenous GH administration to wild-type and to *lit*/*lit* mice leads to similar proportionate responses in weight gain in the high and low lines, but much larger absolute responses in the *lit*/*lit* (Hastings *et al*., 1993). Whilst these results suggest that the response to body weight selection is not greatly associated with either production of or receptors to GH, it does not address whether stimulation of GH is affected.

The objective of the present study was therefore to determine whether differences between selection lines in growth and body composition are caused in a major way by TH and whether there is any significant interaction between the hypothyroid/euthyroid condition and the genetic background. A further aim of the experiment was to compare the effects of ablation of the thyroid follicle cells, which leads to both TH and GH deficiency, with those of the homozygous *lit* gene, which causes GH deficiency alone.

2. Materials and methods

(i) *Mouse lines*

Selection lines were initiated in this laboratory from a three-way cross (two inbred and one outbred line) base (Sharp *et al*., 1984). Lines (P, or protein lines) were divergently selected for high (PH) and low (PL) lean mass, estimated from an index of body weight and gonadal fat pad weight in males, and in subsequent generations for body weight in both sexes at 10 weeks of age. From the same base population lines were selected for increased and decreased fat content, resulting in fat (F) and lean (L) lines, with selection for the first 20 generations based on the ratio of gonadal fat pad weight to body weight, and subsequently on dry matter content of males at 14 weeks of age, which is strongly correlated with fat content (Hastings & Veerkamp, 1993).

Table 1. *Numbers of litters, offspring born and recorded, classified by whether wild-type (wt) or transgenic (tr), and numbers for which thyroid sections were made*

Line	No. of litters	Animals[a] Total born	Born alive	With record	Females tr	Females wt	Males tr	Males wt	Sectioned[b] ab	ma	na	Total
PH(igh)	5	54	54	45	8 (6)[c]	9	10 (4)[c]	18	2	3	3	8
PL(ow)	7	47	46	40	5 (1)	10	11 (2)	14	5	3	7	15
F(at)	4	45	45	38	12 (1)	7	11 (3)	8	2	4	5	11
L(ean)	3	27	27	26	5 (3)	9	7 (1)	5	2	2	4	8
Total	19	173	172	149	30 (11)	35	39 (10)	45	11	12	19	42

[a] The difference in numbers born alive and with PCR record (172–149) results mostly from losses between birth and the start of the experiment at 10 d. A very few animals were subsequently lost – they either escaped or more probably were cannibalized.
[b] ab, ablated thyroid (all transgenic animals); ma, mostly ablated (all transgenic animals); na, not ablated (all non-transgenic control animals).
[c] Number of losses during the experiment are given in parentheses.

(ii) *Experimental animals*

By repeated backcrossing two sets of animals in each line were produced: transgenic/non-transgenic for *HSV1-tk* and homozygous/non-homozygous for the *lit* gene.

(a) *HSV1-tk-transgenic/non-transgenic animals*

Males from generation 51 of the selection lines were used for the initial cross (= backcross generation 0) with the original transgenic stock carrying the *HSV1-tk* construct (Wallace *et al*., 1991). Female offspring were chosen for mating if they were found to be hemizygous by polymerase chain reaction (PCR) analysis of tail or ear cut DNA, using primers directed against the bacterial *supF* gene, which comprises part of the transgene. Such females were backcrossed to contemporary males of the corresponding selected line. The same procedure was followed at each backcross generation. Ganciclovir was not administered during the time that the lines were developed.

Transgenic females of each line from the seventh backcross were mated to males of the same selected line, which was by then at generation 60, such that embryos had an expected proportion of 99·6% of their genotype from the selected line, with most coming from the last few generations of selection. Ganciclovir was administered to pregnant females by intraperitoneal injections of 10 μg/g body weight on each of eight occasions, on days 14 (one injection, 4 p.m.), 15–17 (two injections per day, 9 a.m. and 4 p.m.) and 18 (one injection 9 a.m.) of gestation. Numbers treated and giving birth are shown in Table 1. These ranged from three to seven litters per line and differed mainly because numbers of transgenic females were limited.

Mice were fed a standard expanded breeding diet (Rat and Mouse No. 3, Special Diet Services, Witham, Essex, UK). They were weaned at 21–28 d, but many of the pups with ablated thyroids were very small and were left longer with their dam. These very small pups were weaned when the dam gave birth to a second litter, which was necessary for line propagation, but some additional food (a mixture made from soft cheese and honey and some pellet powder mixed with water) was provided on a Petri dish on the cage floor until they gained body weight. This additional food source was necessary because these mice might have been unable to reach food in the standard food hoppers and were not able to eat the usual hard pellets.

Pups were usually weighed at 10, 13, 17, 21 and 24 d of age and then weekly from 28 to 98 d of age, but a small proportion were weighed 1 or, rarely, 2 d earlier or later to reduce the number of days on which records were taken (see caption to Fig. 1 for mean ages). Some of the transgenic mice died during the experiment but the body weights obtained before they died were included in the data analysis (Table 1). None of the non-transgenic mice died.

Ear clips were taken on all surviving animals at an age of about 60–70 d, and on any that died as soon as they were observed, and the PCR test for the transgene was undertaken on each. At 97 d $\pm$ 1·7 d (SD) all animals were killed by cervical dislocation and blood samples were collected. Thyroid glands were removed from about one-third of the animals and examined histologically as described by Wallace *et al*. (1994).

Gonadal fat pads of males were removed, weighed and returned to the carcass. The digestive tract (stomach and intestine) of all animals (in total 128 animals) was then removed and the weight of the remaining body was recorded as wet weight (WW). These carcasses were freeze-dried and dry weight

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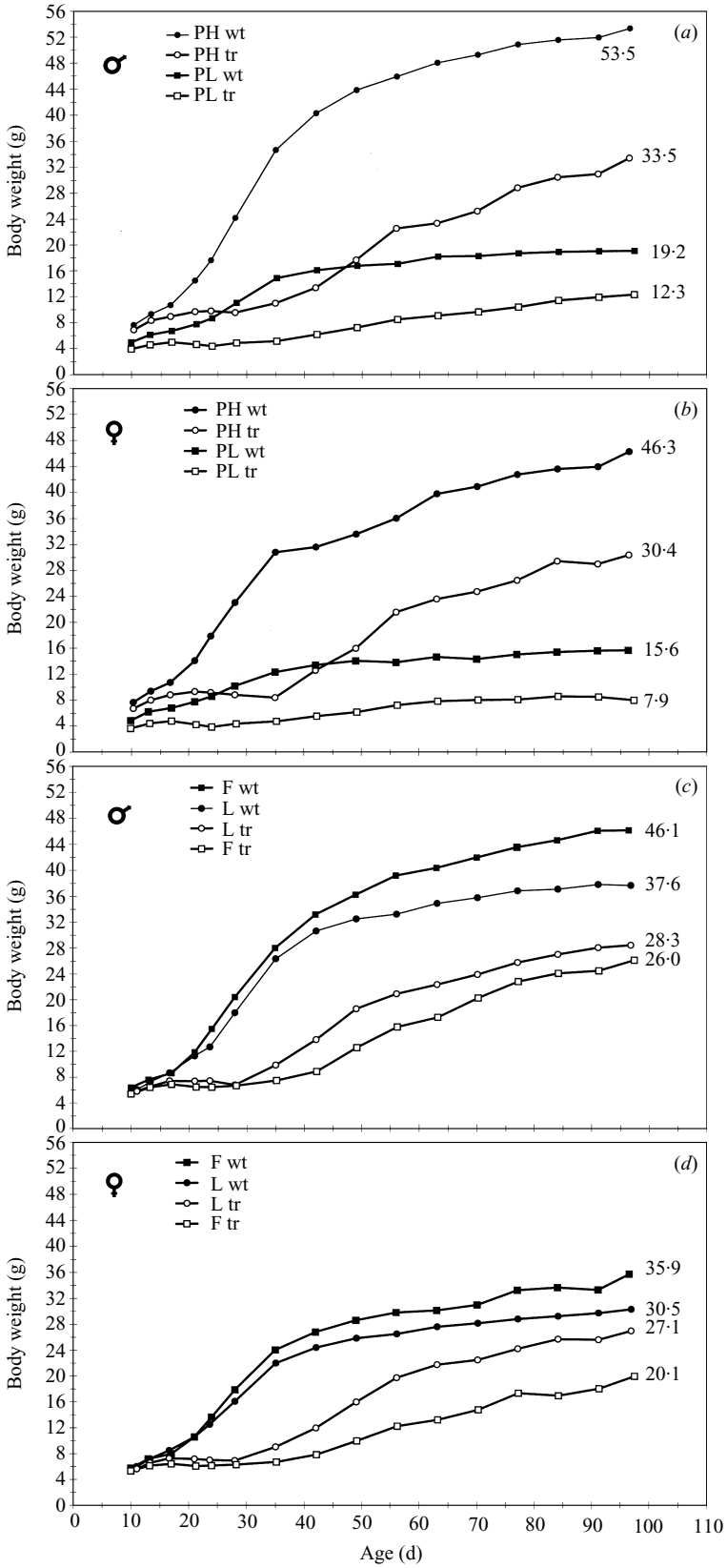

Fig. 1. Body weights for wild-type (wt) and transgenic (tr) mice from all four selection lines: PH, PL, selected for high and low body weight [males (*a*), females (*b*)]. F, L, selected for fatness and leanness [males (*c*), females (*d*)]. The mean (over lines) ages (days) when weights were taken were: 10, 13, 17, 21, 24, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91 and 97, with standard deviations of about 0·8 d at the beginning, then mostly about 0·4 and at the last day of the experiment 1·7.

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Table 2. *Number of homozygote* (lit/lit) *and wild-type* (*wt*) *animals from the backcross lines derived from the high* (*PH*) *and low* (*PL*) *body weight lines and from the fat* (*F*) *line*

	*l*PH (bc 7)[a]		*l*PL (bc 7)[a]		*l*F (bc 5)[a]		Total	
	42 d	70 d	42 d	70 d	42 d	70 d	42 d	70 d
Females								
lit/lit	14	9	12	7	10	8	36	24
wt	44	26	68	37	32	33	144	96
Males								
lit/lit	14	7	16	9	7	9	37	25
wt	54	27	51	27	34	20	139	74
Total homozygotes (%)[b]	29		24		26		26	

wt, either *lit*+ or +/+ animals.
[a] Number of backcross generations (bc); data obtained from F_2s.
[b] Percentage of homozygous animals is given at 42 d, because not all were weighed at 70 d.

(DW) recorded. The data from one batch of 40 animals had to be excluded from the analysis because of a technical fault during freeze-drying. Prediction of individual fat content (fat %) values on the remaining 88 animals was from the dry matter content (DW/WW) using a regression equation (fat % = DW/WW × 140 − 39·41) given by Bünger & Hill (1997) for mice from an unselected control and from the fat line of a similar age, but starved for about 18 h.

(b) *Homozygous/non-homozygous* lit *animals*

For comparison of the effects of thyroid ablation with GH deprivation alone due to the *lit* mutation, data on 42 d and 70 d body weight obtained from another subset of lines were available. These were derived from three (PH, PL and F) of the four main selection lines and denoted *l*PH, *l*PL and *l*F. The *lit* gene was introduced into these lines by repeated backcrosses with progeny testing (Bootland *et al*., 1991). (The introduction of this gene into the L line had recently been started again and insufficient backcross generations had been made to allow inclusion of these animals in the analysis.) The *l*PH and *l*PL lines were used previously to examine the involvement of GH in the differences between these selection lines (Hastings *et al*., 1993).

The last cross with the selection lines used mice from generation 57 of the PH and PL lines and from generation 58 of the F line. The numbers of animals used are given in Table 2. About 25% of F_2 animals after backcrossing are expected to be homozygous for the *lit* gene. These animals can easily be distinguished at the phenotypic level from their wild-type (wt, comprising *lit*/+ and +/+) littermates, because of a 40–50% reduction in body weight at 6 weeks (Hastings *et al*., 1993).

(iii) *Data analysis*

Data on body weights at each age were analysed using the following model:

$$Y = M + T + X + S + GX + TS + XS + TSX + F(S) + e,$$

where M is an overall mean, T (1–2) is the effect of thyroid status (ablation/non-ablation [= transgenic/wild-type] or homozygous/heterozygous for *lit*-backcrosses), X is a sex effect (1–2), S is a selection line effect (1–4 or 1–3), TS, TX, SX and TXS are interactions, $F(S)$ is a family within-line effect, and e is residual error. All effects were fitted as fixed except $F(S)$ and e, fitted as random. The line effect was tested against $F(S)$ and all other effects were tested against the error term.

ANOVA was undertaken using the GLMprocedure of the SAS System for Windows Release 6.08 (SAS Institute, Cary, NC 27513, USA).

Where data were available only on males (e.g. gonadal fat pad weights) no sex effect was fitted. Data were analysed using non-transformed data and also, where there was a clear relation between mean and standard deviation, using a natural log transformation.

3. Results

(i) *Transgenic and wild-type animals*

Numbers for each line are given in Table 1 of animals born, of those born alive and of those, ranging among lines from 26 to 45, which survived long enough to provide tail cuts and at least some weight records. There was a small, but non-significant ($P > 0{\cdot}05$), excess of wild-type (wt) animals over transgenic (tr) animals, and of males over females. All 19 dissected wild-type animals showed normal thyroid glands. Of

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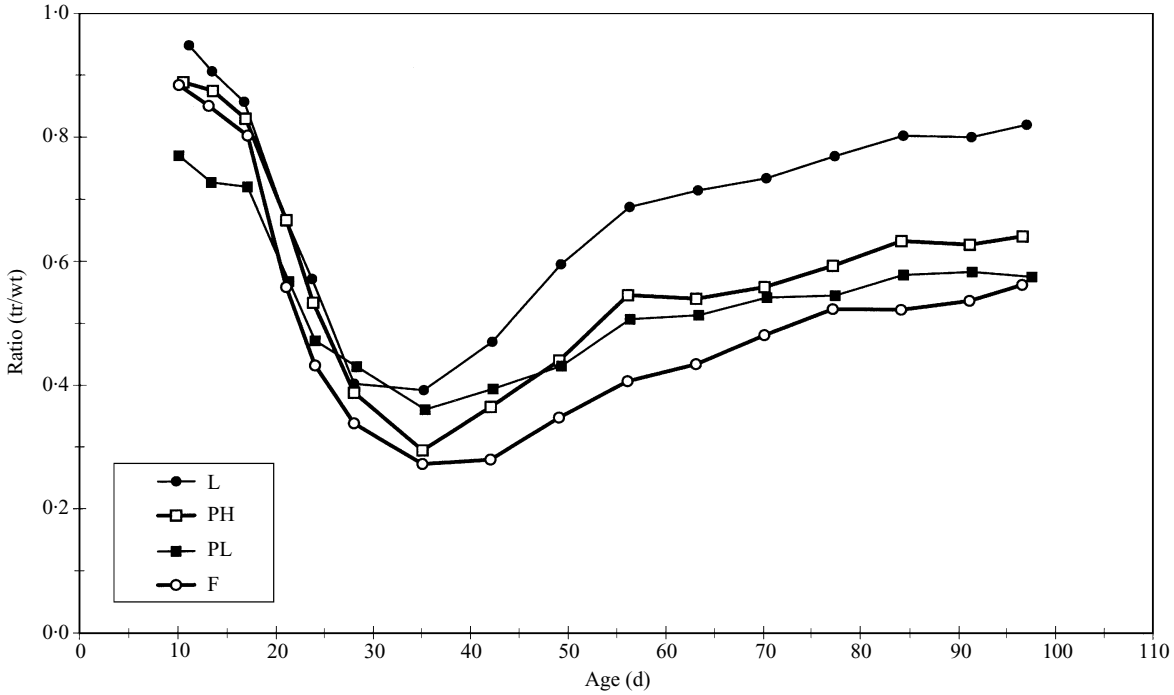

Fig. 2. Body weight of transgenic animals expressed as a ratio of wild-type animals for all selection lines. Data were averaged over sexes.

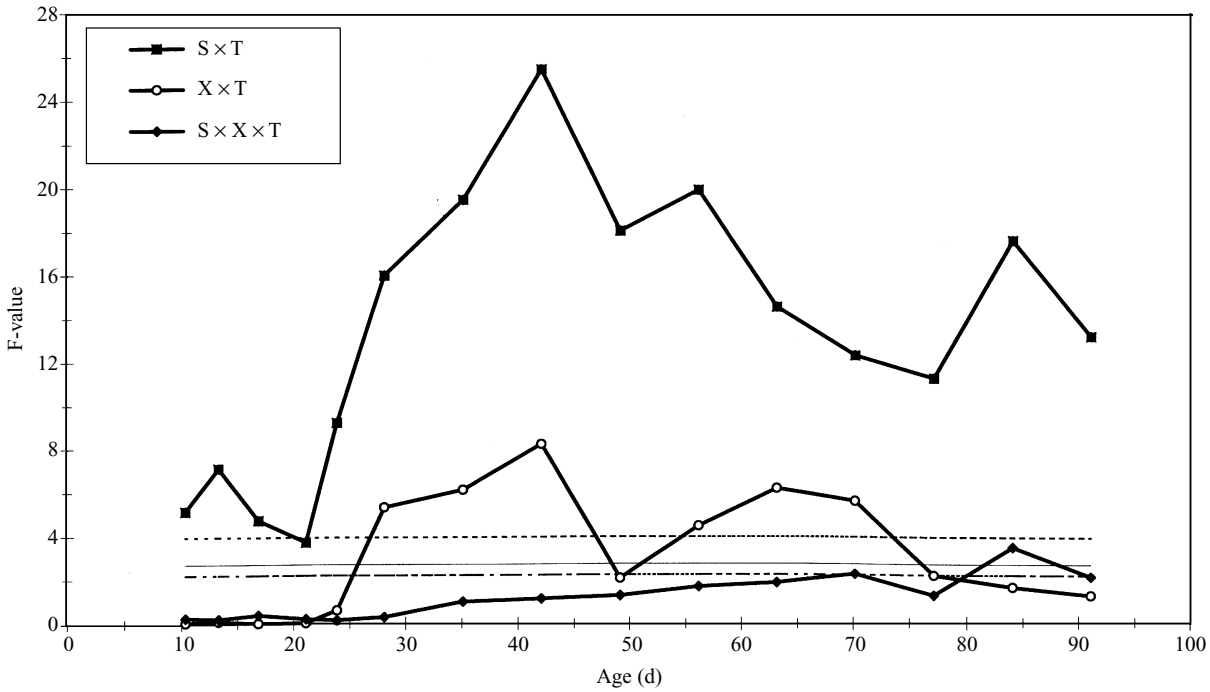

Fig. 3. Summary of ANOVA, showing *F* values for the interaction terms: line × thyroid status (L × T), sex × thyroid status (X × T) and line × sex × thyroid status (S × X × T) using log-transformed data. Critical *F*-values ($P < 0{\cdot}05$) for S × T, X × T, and L × X × T) are 2·7, 3·9 and 2·2, respectively.

the 23 transgenic animals sectioned, 11 showed complete ablation of the thyroid gland and 12 had small residual numbers of TH-producing cells. In previous experiments, such animals have been shown not to have any detectable TH in their blood (Wallace *et al.*, 1994). There was no significant association between the level of ablation and sex or line, but numbers were small.

In total 21 animals of 149 died during the experiment (Table 1). All were transgenic, comprising 30 % of the transgenic animals. The ages at which losses occurred varied considerably, but most were observed between

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Table 3. *Least-squares means for body composition traits of transgenic (tr) and wild-type (wt) mice, and their differences with standard errors (SE)*

		CW97 (g)	DM (%)	Predicted fat (%)	ffBW (g)	GFPWr (mg/g)
Effects in body weight lines averaged over sexes						
PH	tr	26·8	34·76	9·3	24·3	16·3
	wt	41·4	34·68	9·2	37·4	17·0
	tr − wt[a]	−14·6	0·08	0·1	−13·0	−0·7
	SE	1·2	1·03	1·44	1·13	3·0
PL	tr	8·3	33·18	7·1	7·5	5·3
	wt	14·7	34·77	9·3	13·3	13·4
	tr − wt[a]	−6·4	−1·59	−2·2	−5·8	−8·1
	SE	0·95	0·87	1·22	0·96	2·9
PH (tr − wt) − PL (tr − wt)[b]		−8·2	1·67	2·3	−7·2	7·4
	SE	1·53	1·35	1·89	1·48	4·2
Effects in fat and lean line averaged over sexes						
F	tr	19·2	39·70	16·2	15·6	19·8
	wt	34·6	47·75	27·5	25·0	41·7
	tr − wt[a]	−15·4	−8·05	−11·3	−9·4	−21·9
	SE	0·87	0·86	1·20	0·94	3·3
L	tr	22·8	30·83	3·8	21·6	6·7
	wt	28·5	31·60	4·8	26·9	7·5
	tr − wt[a]	−5·7	−0·77	−1·1	−5·2	−0·8
	SE	1·26	1·08	1·51	1·18	3·6
F	(tr − wt) − L(tr − wt)[b]	−9·7	−7·28	−10·2	−4·2	−21·1
	SE	1·53	1·38	1·93	1·51	4·9
Effects in sexes averaged over lines						
♀	tr	17·5	34·61	9·1	16·0	
♀	wt	26·4	37·28	12·8	22·8	
♀	tr − ♀ wt[a]	−8·9	−2·67	−3·7	−6·7	
SE		0·87	0·74	1·04	0·82	
♂	tr	21·1	34·63	9·1	18·5	12·0
♂	wt	33·2	37·13	12·6	28·5	19·9
♂	tr − ♂[a]	−12·1	−2·5	−3·5	−10·0	−7·9
SE		0·66	0·63	0·88	0·69	1·6
Effects of thyroid status averaged over lines						
tr		19·3	34·62	9·1	17·3	12·0
wt		29·8	37·2	12·7	25·6	19·9
tr − wt[a]		−10·5	−2·58	−3·6	−8·4	−7·9
SE		0·55	0·48	0·67	0·53	1·6

[a] Differences between groups (e.g. transgenic vs wild-type) with SE calculated from the SE of the involved groups.
[b] Comparison between the effect in one line versus the effect in the corresponding line. CW 97, carcass weight at 97 d; DM, dry matter; both measured on all animals; ffBW, fat-free body weight, one freeze-drying batch was excluded (cf. Section 2); GFPWr, relative gonadal fat pad weight, measured on all males only.

weaning and 5 weeks of age. The mean ages (±SD) at which the last body weights were taken from these animals alive in PH, PL, F and L lines were 28±3, 63±24, 31±11 and 28±1 d, respectively. The number of losses differed among litters (e.g. in PH 6 of the 10 animals were from one litter).

(a) *Body weight development in transgenic and wild-type animals*

Body weights of wild-type and transgenic males and females for the four lines are shown in Fig. 1. In all lines thyroid ablation had a dramatic effect on growth from about 10 d of age, there being little growth to about 35 d. Subsequently weights increased in all lines, but earliest and most rapidly in the L line. Transgenic mice of all lines were still growing when the experiment was terminated at almost 100 d. Nevertheless, females of the PL line weighed only about 8 g and males 12 g at this age, compared with wild-type weights of 16 and 19 g, respectively, mainly attained by 40 d. Whereas wild-type F animals were heavier than L animals, the opposite was found for ablated animals.

The ratios of the mean weights of transgenic to wild-type animals for the four lines are shown in Fig. 2. Whereas transgenic animals at 10 d weighed 77–96% as much as non-transgenic animals, this ratio

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Table 4. *ANOVA results for predicted fat content, fat amount and fat-free body weight on a metric and log scale*

		Metric scale		Log scale	
Source	d.f.	Fat content (%) MS	ffBW (g) MS	Fat content (%) MS	ffBW (g) MS
Line	3	1025***	1310***	7·57***	4·04***
Family (line)	15	10·19^{ns}	15·64***	0·22^{ns}	0·04***
Sex	1	0·11^{ns}	233·1***	0·01^{ns}	0·61***
Thyroid status	1	204·9***	1098***	1·39**	2·88***
Line × thyroid status	3	123·2***	45·99***	0·32^{ns}	0·08***
Sex × thyroid status	1	0·26^{ns}	45·74***	0·04^{ns}	0·01^{ns}
Line × sex × thyroid status	6	5·98^{ns}	5·94^{ns}	0·07^{ns}	0·03*
Error	57	7·3	4·52	0·12	0·01

ffBW, fat-free body weight.
* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$; ns, non-significant, $P > 0·05$.

decreased to 27–39 % at about 35 d. Animals from the L line showed the highest ratios, e.g. the least effect of ablation, the ratio falling in both sexes to about 40 % at 28–35 d, but increased at a younger age and more rapidly than in the other lines. By the end of the experiment at about 97 d transgenic animals from the L line had reached about 82 % and transgenic F animals had reached about 56 % of the weight of their wild-type control. The corresponding values for the transgenic PH and PL animals were similar: 64 % and 58 %, respectively.

An analysis of variance (ANOVA) of the log-transformed body weights revealed significant interaction between line and thyroid status (hypothyroid/euthyroid) throughout the experiment (Fig. 3), but this was smaller than the main effect of either line or thyroid status. The line × thyroid status had a maximum effect at around 40 d and seemed to be due mainly to the lower response of the L animals. Sex × thyroid status effects were small, but mostly significant ($P < 0·05$) between 28 and 70 d.

(b) *Fatness of transgenic and wild-type animals*

Effects in selection lines. Fatness as predicted from dry matter for all available animals and estimated from gonadal fat pads for males is given together with the final weight, carcass weight and fat-free body weight in Table 3. The predicted fat content (fat %) of PH and PL mice was nearly independent of thyroid status and very similar, varying between about 7 % and 9 % with the lowest value in the transgenic PL line. Higher differences were seen in the F line, where transgenic mice had 16 % fat and wild-types had 27 %. The corresponding values for the L line were about 4 % and 5 % for the transgenic and wild-type animals, respectively. The effect of the ablation was therefore much higher in the F line than in the L line (−11 % vs −1 %). This strong reaction of the F line seemed to cause the significant line × thyroid status interaction when measured on the absolute scale, which is, however, not significant when data were log-transformed (Table 4).

Table 5. *ANOVA results for relative gonadal fat pad weight (GFPWr; in mg per g of carcass) on a metric and log scale*

Source	d.f.	Metric scale MS	Log scale MS
Line	3	1170***	3·86***
Family (line)	15	49·5^{ns}	0·35***
Thyroid status	1	804***	2·75***
Line × thyroid status	3	336***	0·68**
Error	51	31·24	0·14

* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$; ns, non-significant, $P > 0·05$.

A similar picture can be derived from the other indicator of fatness: the relative gonadal fat pad weight (GFPWr) (Table 5). However, there was a greater but not significant ($P > 0·05$) effect of thyroid status in the PL line compared with the PH line. The ratio of GFPW to total fat was 0·09 in the PL transgenic mice, which was much lower than in all other groups where it varied between about 0·15 and 0·2, indicating a high reduction especially in GFPW in PL when ablated (Table 3).

Whereas the difference between the F line transgenic and wild-type mice for GFPWr was about 22 mg/g carcass weight, the corresponding difference in the L line was only −0·8 and not significant ($P > 0·05$). The S × T interaction was significant for both data sets: non-transformed and log-transformed (Table 5).

Effects in sexes. Wild-type males and females, calculated over all lines, had a very similar fat content (about 13 %). The fat content of transgenic males and

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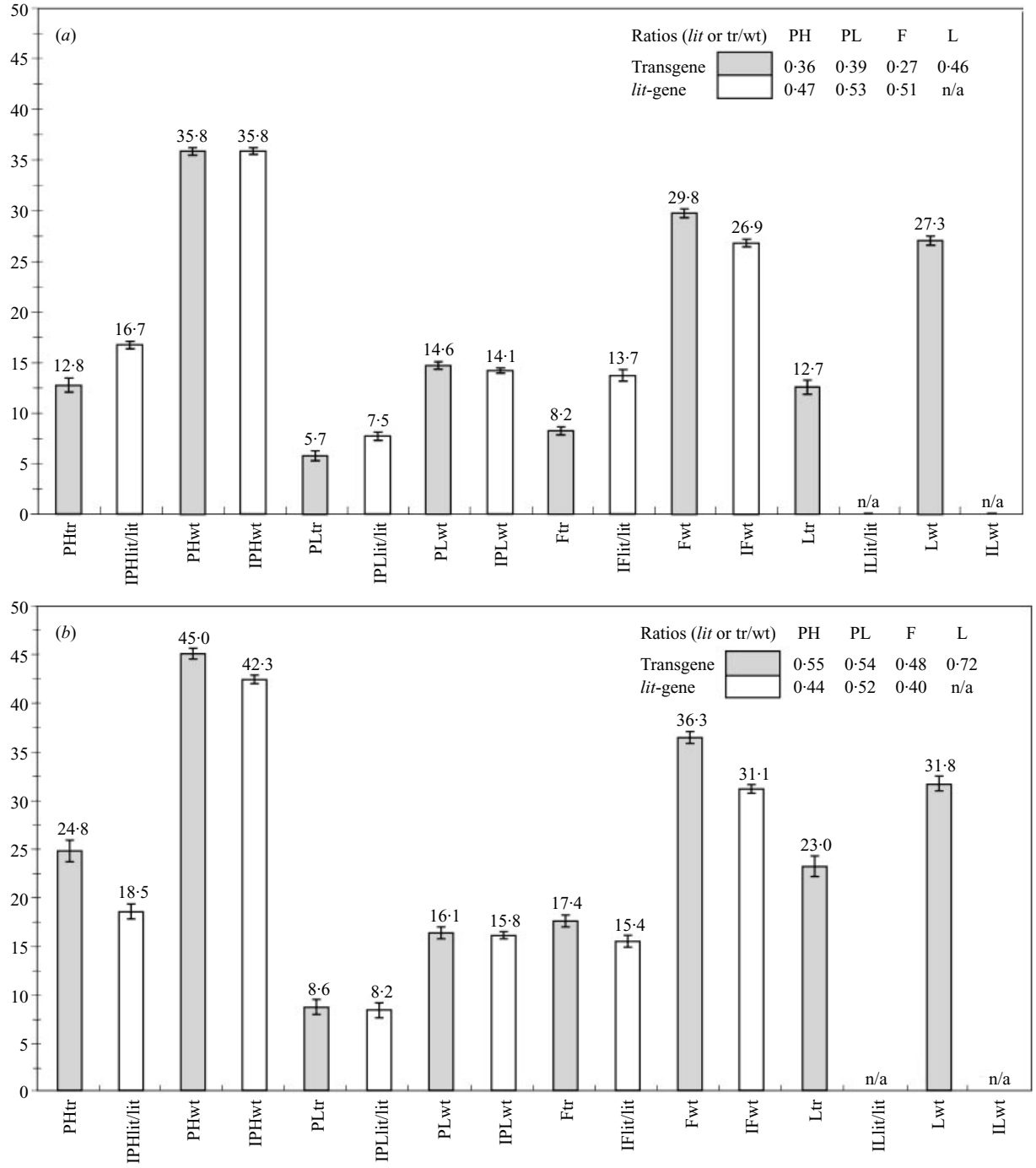

Fig. 4. Least-squares means (line × thyroid status) and their standard errors for body weights at 42 (*a*) and 70 days of age (*b*). Grey columns (backcross of the transgene), transgenic (tr) and wild-type (wt) animals of lines PH, PL, F and L. White columns (backcross of the *lit* gene, homozygous (*lit*/*lit*) vs wild-type animals from lines *l*PH, *l*PL and *l*F. Ratios: transgenic and homozygous (*lit*/*lit*) over wild-type are given.

females was reduced in a similar way by about 3·5% and 3·7% respectively, indicating no significant interaction between sex and thyroid ablation.

Effects of thyroid ablation. In general there was a large effect of the thyroid status on fat%, the transgenic animals being leaner as shown by the estimated fat content (9·1 vs 12·7%) and GFPW (12 vs 20 mg/g carcass weight).

(c) *Fat-free body weights in transgenic and wild-type animals*

Effects in selection lines. In all lines the fat-free body weight (ffBW) of the transgenic animals was significantly lower (Table 3). In the PH line the difference between transgenic and wild-type animals was 13 g (35%); that in the PL line was lower in absolute terms (5·8 g) but slightly higher in relative terms (44%).

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Table 6. *ANOVA results for body weight at 42 at 70 days in the* lit-*backcross lines on a linear and a log scale*

		Metric scale		Log scale	
		BW 42 (g)	BW 70 (g)	BW 42 (g)	BW 70 (g)
Source	d.f.	MS	MS	MS	MS
Line	2	4383***	3588***	14·76***	9·05***
Family (line)	48	27·70***	34·9***	0·0426***	0·0403***
Sex	1	358·0***	604·6***	0·984***	1·314***
Genetic status	1	7638***	7820***	21·35***	16·36***
Line × sex	2	7·888^{ns}	37·62**	0·0117^{ns}	0·008^{ns}
Line × genetic status	2	734·3***	694·1***	0·0678***	0·0786***
Line × sex × genetic status	3	81·90***	24·90*	0·0429**	0·015^{ns}
Error	296	4·72	7·96	0·00863	0·00932

BW 42, body weight at 42 d; BW 70, body weight at 70 d.
* $P < 0·05$, ** $P < 0·01$; *** $P < 0·001$; ns, non-significant, $P > 0·05$.

The reduction of ffBW in transgenic F animals (9·4 g, 38 %) was greater than in L animals (5·2 g, 20 %) and the S × T interactions were significant for both the transformed and non-transformed data (Table 4).

The difference between wild-type animals from the F and L lines was small (1·9 g) compared with that for transgenic animals (6 g), with a higher ffBW in the L line.

Sex effects. The ffBW of transgenic females and males was on average 6·7 g (30 %) and 10 g (35 %) respectively, lower than for wild-type animals, such that the X × T interaction was significant for the untransformed, but not the log-transformed data (Table 4).

Line effects. The average ffBW of PH animals was about 3-fold higher than that of PL animals. The difference between the F and L lines was 4 g and significant, with a higher ffBW in the L line, particularly of transgenic animals.

(ii) *Lit-backcrosses*

The body weights for homozygous (*lit*/*lit*) and wild-type (*lit*/+ and +/+) animals from lines *l*PH, *l*PL and *l*F at 42 d and 70 d are shown in Fig. 4. The corresponding values for the transgenic (tr) animals and their non-transgenic (ntr = wt) controls are included for purposes of comparison.

Homozygous *lit* animals weigh on average only about one-half as much as their wild-type littermates at both 42 d and 70 d and the effects of thyroid status and line were highly significant on both linear and log scales (Table 6).

At 42 d *lit*/*lit* PH, PL and F animals weighed 10·1 g (53 %), 6·6 g (47 %) and 13 g (49 %) less than their wild-type (*lit*/+ and +/+) littermates, with similar proportional changes at 70 d: 23·8 g (56 %), 7·6 g (48 %) and 15·8 g (51 %), respectively.

The interaction of line × genetic status (S × T), was highly significant for body weights at both ages, whether or not data were log-transformed (Table 6), but still small compared with the effects of other factors. The highest effect was in the PH line, followed by the F and PL lines.

(iii) *Comparison of body weights of* lit-*backcrosses and transgenic animals*

At 42 d the transgenic animals of lines PH, PL and F were 24–40 % lighter than the homozygous *lit* animals (Fig. 4*a*). Their wild-type controls had very similar body weights; however, the *l*Fwt animals were 2·9 g (10 %) lighter than Fwt animals. The ratios (*lit*/*lit* or tr over wild-type) were lower for transgenic animals (0·27–0·46) than for *lit*/*lit* animals (0·47–0·53). Whereas the highest relative effects of the *lit* gene at 42 d was found in the PH line (−53 %), the F line showed the highest relative effect of the transgene (−63 %).

At 70 d transgenic animals seem to be heavier than *lit*/*lit* animals (Fig. 4*b*). The differences on the line background of PH, PL and F were 5–25 %. However, the controls (wt vs *l*wt) were in general also lighter by 2–13 %. The ratios were very similar at 70 d in the transgenic and *lit* groups in lines PL (0·54 vs 0·52) and F (0·48 vs 0·49) (Fig. 4), but higher in the PH (0·55) than in the *l*PH line (0·44), suggesting a greater effect of the *lit* gene than of the transgene.

4. Discussion

There are several ways in which TH and GH might contribute to the observed differences in body weights and fatness of the selected lines. The amount of circulating hormone might differ and the first question

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then is whether the selected lines will still differ in growth or in fatness without these hormones. If they do, it would demonstrate that TH plus GH or GH could not be the sole cause of the differences between them. If these metabolic regulators are not involved, the transgenic mice and homozygous (*lit*/*lit*) mice would be expected to be identical to their wild-type littermates. If these hormones were involved to some extent in the differences between lines, their effects would depend on the genetic background, indicated by a significant interaction between line and transgenic or *lit* status. A comparison of the two sets of groups should show whether the effects of the transgene and the *lit* gene on body weights differ.

(i) *Transgenic animals*

(a) *Body weight development*

The lack of both TH and GH decreased body weights at 10 d on average by 12% and the effect increased with age to 60–70% at about 6 weeks. As a result of a 'catch-up growth' this effect decreased to about 35–40% when animals were 12–14 weeks old and transgenic mice of all lines were still growing when the experiment was terminated at about 14 weeks.

Significant interactions between genetic background (line) and the thyroid status (T: transgenic/wild-type) for non-transformed body weights (linear scale) are not surprising and indicate a higher growth depression in absolute terms, e.g. in ablated PH animals compared with PL animals. To ask whether the transgene has similar or different *proportionate* effects in the four genetic backgrounds, an ANOVA was undertaken at each of the 16 ages using log-transformed data to reduce scale effects for body weights differing 6-fold.

The S × T interaction was significant throughout the experiment, although not at 21 d (Fig. 3), but was small compared with the effects of line, thyroid status and sex. This interaction reflected the smaller effect on weight of ablation in the L line and the strong response of the F line (Fig. 2), resulting in a changed order of lines (F vs L) when transgenic. The relative growth depression of the body weight lines (PH, PL) was intermediate.

This implies that the effects of TH and its stimulatory effect on GH have been altered by selection and contribute especially to the difference between the L and F lines. The question arises as to what makes the ablated L mice grow and how they reach this relative 'independence' from TH and GH.

Compensatory growth seems to coincide with attaining sexual maturity, suggesting that sex hormones may be important and perhaps play different roles in the F and L lines. Future experiments should focus especially on these two lines at this stage of development.

(b) *Body composition*

As expected, both the line (genetic background) and the thyroid status had a great effect on fat content, with the F line animals being the fattest (predicted fat % = 22%) and the L line animals the leanest (4·3%). Wild-type animals had on average 13% fat, but transgenic animals were leaner, having 9% fat; even hypothyroidism is usually accompanied by obesity in mice (e.g. Oh & Kaplan, 1994) and rats (Katzeff & Selgrad, 1993; Chomard *et al*., 1994).

The lines reacted differently to the lack of TH (Table 5) and the interaction between thyroid status and genetic background was significant for GFPWr on both the linear and log scales. This seemed to be primarily due to the strong reaction of the F line when ablated, but also to the response of the PL line, which reacted much stronger than the PH line (Table 3) and showed an obviously stronger reaction in GFPWr than in fat%.

Whereas ablated F animals had 16% fat, wild-type F animals contained nearly 28% fat. This reduction was much higher compared with the reduction of fat by 1%, 0·1% and 2·2% in the L, PH and PL lines, respectively. The large response of the F line was reflected in a significant interaction between line and thyroid status on a linear scale, but not on the log scale, suggesting that the lines reacted in a similar proportional way. This implies that the effects of TH and GH on fat content have not been altered by selection.

Analysis of ffBW allows the effects of ablation on fat and on 'non-fat body weight' to be disentangled. The significance of the main effects, line and thyroid status, was not surprising (Table 4). The interaction between thyroid status and line for ffBW was significant on both the linear and log scales, indicating a different reaction of the lines to ablation. The ffBW of transgenic animals of lines PH, PL, F and L line was 20–44% lower than that of their wild-type littermates. This indicates that the PL line showed the highest response to a lack of both TH and GH while the L line showed the lowest, and suggests an involvement of these hormones in the genetic difference between these lines.

(ii) Lit-*backcrosses*

Attempts to introduce the *lit* gene into line L have failed up to now. The small response to the lack of both TH and GH suggests one possible reason for this failure. If the lack of GH, caused by the *lit* gene, results in a similar low body weight depression of about 20% in the L line as does the transgene, it would be difficult to differentiate between the phenotype of homozygous *lit*/*lit* animals and wild-type 'runts' in each F_2 during the repeated backcross.

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Table 7. *Comparison of some male body weights with an earlier study using the same selection lines* (*PH*, *PL*) *and the* lit *gene*

	PH			PL			
Age (d)	Body weight (g)		Ratio	Body weight (g)		Ratio	Reference
	wt	tr		wt	tr		
28	24·1	9·5	0·39	11·0	4·8	0·44	This experiment[a]
49	43·7	17·6	0·40	16·7	7·1	0·42	
	wt	*lit*/*lit*		wt	*lit*/*lit*		
42	35·8	16·7	0·47	14·1	7·5	0·53	This experiment[a]
70	46·5	20·4	0·44	17·6	8·9	0·50	
28	21·0	10·6	0·50	11·6	5·9	0·51	Hastings *et al.* (1993)[b]
49	39·5	17·6	0·45	17·2	8·4	0·49	

[a] wt animals comprise *lit*/+ and +/+ littermates of *lit*/*lit* animals, resulting from *inter se* matings of heterozygous backcross animals. The last backcross was to animals of generation 57 of the selection lines. Litters were brought up by heterozygous 'line typical sized' females. Numbers are given in Table 2.
[b] wt animals were pure line homozygous +/+ males from generation 43. There were about 15 animals in each group. *Lit*/*lit* animals were offspring of *lit*/*lit* mothers.

As expected, genetic background and homozygosity for the *lit* gene affected body weights significantly on both the linear and log scales (Table 6). As for the transgenic animals lacking both TH and GH, the GH-deficient homozygous *lit* animals of all three lines had different growth rates, indicating that GH cannot be the sole cause of line differentiation (Fig. 4). On average, homozygous *lit* animals weighed only about half as much as their wild-type littermates at both 42 and 70 d. However, there were apparent interactions between thyroid status and line on both measurement scales, with the highest absolute and proportional effect in the PH line. This implies that the GH axis has been altered to some extent by selection, making the PH mice more dependent than the PL mice upon GH.

Eisen *et al.* (1993) analysed the effect of *dwarf mutant bovine growth hormone transgene*, acting as a GH antagonist on two genetic backgrounds, by a single cross between male mice hemizygous for the dwarf mutant and females of high-growth selected and control lines. The dwarf mutant transgene had a slightly but significantly greater effect in the selection line (30%) than in the control (26%) on 70 d body weights.

Pidduck & Falconer (1978) backcrossed the Snell dwarf gene (*dw*) into two lines divergently selected on body weight and an unselected control line. At 3 weeks the dwarfs were about 70% of the weight of the wild-types in all three strains; its effect then increased sharply until it stabilized at 6 weeks, when the dwarfs were 25–40% the weight of wild-types. The order of the effects of the homozygous *dw* gene was High > Control > Low, suggesting that GH played some part but was not the sole cause for the observed body weight differences, in agreement with our findings. The relative depression of body weights in the experiment of Pidduck and Falconer was larger than in the present experiment, possibly because the *dw*/*dw* genotype has a defective anterior pituitary (mutation in the pituitary transcription factor gene, Pit 1) so not only GH, as in our experiment, but also prolactin, thyrotropic hormone and probably also corticotrophin are deficient (for references see Doolittle *et al.*, 1996).

Using earlier backcross generations of the *lit* lines (*l*PH, *l*PL), Hastings *et al.* (1993) found that there was a stronger weight reduction on the PH than the PL background for body weights from 42 to 70 d and that the interaction between the genetic status and line was small and non-significant in log-transformed data. To enable a comparison with results of this experiment, the data are jointly presented in Table 7. Although there were some small differences in methodology (Table 7), the ratios of weights in the two experiments were similar, showing a reduction of body weights by about 54% and 49% in the homozygous PH and PL lines, respectively. Therefore, it seems reasonable to conclude that differences in the GH axis are not the sole cause of the observed divergence in body weight produced by selection, although the presence of a small interaction between gene effect and line implies that GH is partly involved in line differentiation.

(iii) *Comparison of body weights of transgenic animals and* lit*-backcrosses*

Comparison between a lack of both TH and GH and a lack of only GH is restricted to the weights at these two ages and to lines PH, PL and F (Fig. 4). By chance the wild-type animals in the backcross lines were mostly lighter than the controls for the transgenic

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animals, so comparisons were made between the ratios of *lit*/*lit* to wt and tr to wt.

The lack of both TH and GH resulted in a depression of growth by 56% in an average at 42 d, whereas the absence of only GH produced a reduction of 50%. Growth depression in the transgenic animals was greater than in the homozygous *lit*/*lit* animals in all three lines, being most in the F line. At 70 d the effects of the transgene or the homozygous *lit* gene were a little less different (48% vs 52%, respectively), indicating that the absence of TH affects weights more at younger ages.

There was only negligible growth or even weight loss in the transgenic animals from about 15 to 35 days. There were no body weight records on the *lit*/*lit* animals at these young ages in this experiment or in that by Hastings *et al*. (1993) starting at 28 d of age, but the early growth of the *lit*/*lit* mice seemed to be depressed less than that of mice lacking TH and GH. The body weight depression due to the *lit* gene was 49% and 50% in the PL and PH lines, respectively, at 28 d (Hastings *et al*., 1993, Table 7), whereas the reduction in transgenic mice was about 56% and 61%, respectively.

Therefore growth near weaning (15–30 d) should be looked at in more detail to find out whether the observed effects are due solely to the lack of the effect of the hormones (i.e. hormones and their receptors) or whether other factors also contribute. At 21 d (usual weaning age) the average body weights of ablated animals were, depending on line, only about 56–66% that of their normal littermates, indicating an immature stage of development at this time. Despite special treatment (see Section 2) about 30% of the transgenic animals died, mostly in the postweaning period.

This divergence in the perinatal and preweaning period between transgenic and wild-type animals seems not to be caused by an effect of the ablation of the mothers on their milk output, because transgenic and wild-type animals were littermates, but wild-type animals could be better in getting pelleted food when dams give less milk. Wallace *et al*. (1995) found that non-transgenic pups grew normally in the postnatal period when brought up by ablated dams and concluded that T_4 deprivation did not affect the volume or composition of milk to a degree detrimental to the pups, but milk output was not measured. Recent results from Capuco *et al*. (in preparation) showed a reduced weight at 10 d (−4 g, 11%) of litters brought up by dams that were thyrocyte-ablated before mating. Transgenic dams treated as in this experiment are expected to become completely T_4-deficient around the time of birth (Wallace *et al*., 1994). This could enhance the energetically critical situation at weaning when mice have to change from feeding on milk to feeding on solid pelleted food.

It cannot be excluded, however, that the immature stage of transgenic animals could have reduced their competitive ability in a litter and contributed to the divergence between transgenic and wild-type animals – an effect that would be amplified by the difficulty that immature transgenic pups might encounter in coping with weaning, as they were more dependent upon the milk supply of the mother. Intra-litter competition and unfavourable conditions, because of immaturity at weaning (change of diets, etc.), in litters of heterozygous *lit* dams comprising *lit*/*lit* animals and wild-type littermates should be very similar, however. No 'special treatment' was necessary to secure the survival of *lit*/*lit* animals and the frequency of *lit*/*lit* animals at 42 d was about one-quarter (Table 2), as expected if there is no increased mortality of *lit*/*lit* animals.

Therefore it may be concluded that a lack of TH and GH has a strong effect on early growth, whereas the later effects on body growth seem to be very similar to those of a lack of GH alone.

In this study no body composition data were available on *lit*/*lit* animals, but Hastings *et al*. (1993) found they were much fatter than wild-types at 7 weeks of age in the P lines, with an increase in fat content (estimated from dry matter content) from 24% to 50% in the PH background and from 26% to 38% in PL. In contrast, transgenic animals that lacked both TH and GH were leaner on average, with no change of fatness in the PH line, a small reduction in the PL and L lines and a strong reduction in the F line (Tale 3). Although it is not surprising to find an effect of hypothyroidism on fat content, because TH levels are a major determinant of energy balance and are thought to modify body composition by their effects on metabolism of lipids, carbohydrate and protein, hypothyroidism usually is accompanied by obesity in mice (e.g. Oh & Kaplan, 1994) and rats (Katzeff & Selgrad, 1993; Chromard *et al*., 1994). This difference in results remains unexplained.

It is difficult to be sure whether lines reacted differently to deficiency of both TH and GH compared with deficiency of GH alone, because body weight data for the *lit* mice were available only for two age points and effects are clearly age-dependent. At later ages (70 d) it seemed that growth in the transgenic PL mice was reduced more than in the PH mice (Fig. 2) whereas for homozygous *lit*/*lit* the PH line showed a higher response to the lack of GH than the PL line (Fig. 5*b*). Transgenic F line animals showed the highest depression in growth due to the lack of GH and TH (Fig. 2), but at 70 d the effects of the transgene and the *lit* gene seemed to be very similar (Fig. 5*b*).

Because there were significant interactions between a lack of TH and GH and the line for log-transformed body weights, the fat-free body weight and partly also

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for fatness (GFPWr), it has to be concluded that changes in Th and GH metabolism contributed to the divergence of selected lines. However, this interaction was relatively small, so the results support a model of many genes with diverse metabolic roles contributing to the divergence between selected lines. Further work is needed to determine whether the changes in TH effects were due to differences in the amount of hormone or in tissue sensitivity. Treating the transgenic and homozygous animals with either TH and/or GH would allow tissue sensitivities of the selected lines to the two hormones to be tested, free of background hormones as in wild-type animals.

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