Investigation into the Involvement of Growth Hormone in Genetic Differences in Growth and Size

By

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Declaration

I declare that the work presented in this thesis is my own, unless otherwise stated and that this thesis has been composed by me.

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Abstract

Lines selected high or low on lean body mass (P-Lines) or on carcass fat (F-Lines) for more than 30 generations were treated with recombinant bovine growth hormone (GH) to obtain information about the effects of selection on GH levels and responsiveness. All lines responded to GH with an increase in final body weight. In the lean mass selected lines the high line increased more than the low, but the increase relative to initial weight was similar in both lines (10-15%). GH did not have a significant effect on gonadal fat percentage.

Further investigations involved the lean mass selected lines only. Weights were recorded on a daily basis from birth to weaning at 21 days, to investigate growth before mice become responsive to GH at 14 days. The high line had a mean litter size and approximately twice that of the low line, and a mean birth weight approximately 50% greater than the low line. The difference in total litter weight at birth is greater (3 to 4 fold) than the difference in adult body weight (approximately 3 fold) suggesting that there may be between line differences in individual effects on *in utero* growth rates as well as differences due to maternal effects. Also an increase in the rate of gain was observed for both line at approximately 18 days of age. An increase was also observed in GH deficient *little* mice from the high P-Line, and hence is not due to GH. The increase in the rate of weight gain is greater in the wild type mice than in the *little* mice suggesting GH acts to magnify the effects of whatever causes the increase.

Plasma GH levels were assayed, the high P-line had lower levels of GH at 4, 5, and 7 weeks of age then the low P-line. As IGF-I levels become GH inducible at about 14 days of age and the lines experience an increase in the rate of weight gain, which is greater in the high line than in the low line, at about 18 days it appeared

possible that IGF-I levels differed between the lines. However IGF-I levels did not differ between the lines at 5 weeks of age, during the period of rapid growth (McKnight & Goddard, 1991). Hence IGF-I does not appear to be responsible for the differences between the high and low P-lines in growth rate.

The *little* gene was backcrossed into both P-Lines and then *little* and wild type P-Line mice treated with GH. The dwarfs were approximately half the body weight of wild type mice from the same line. *Little* mice from both lines responded to the recombinant bovine GH with an increase in final body weight. The response was proportional to body weight, again suggesting that GH and responsiveness to GH have not been altered by selection. Preweaning weights of little mice were also recorded.

Finally P-Line mice were treated with antiserum to GH. It appeared to reduce the rate of weight gain. The reduction in the high and low lines was proportional to body weight, and similar in magnitude to the increase induced by treatment with exogenous GH. This again suggests that GH is directly involved in the differences between the lines.

In conclusion the proportionate effects of all attempts to manipulate GH levels indicate that growth hormone and responsiveness to GH are not directly responsible for the differences in growth rate and body weight between the P-Lines, although GH levels have been altered. Further investigation into the levels of GH mediators such as IGF-I, IGF-II, and the IGF binding proteins may be informative. Also, given the differences in birth weight, investigation of prenatal growth effects may be informative.

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Chapter 1
Introduction

Several factors influence the profitability of livestock production for meat. These include how fast the animals reach slaughter weight, how much food they consume while growing, what proportion of the carcass can be sold and how healthy they are. These can be manipulated by a variety of means, selection of lines, and alteration of diet for example. As it is more feed efficient to lay down muscle than fat, producers will prefer leaner animals, providing the meat is not too lean for customer taste.

In the wealthy 'First' world better nutrition and hygiene and, to a lesser extent, medicine have changed the principle causes of death from infectious diseases to non-infectious illness, for example cancer and heart disease (World Health Organisation, 1990). There is increasing evidence that some at least of these conditions are linked to diet. Indeed there are recommendations limiting the maximum daily intake of saturated fats, as high intakes of these are epidemiologically linked with high levels of cardiovascular disease and certain types of cancer (World Health Organisation 1990). Thus lean meat is preferred by consumers on health grounds, making lean animals more economically desirable.

1.1 Selection

Selection is a well established method of generating animals with particular characteristics. The effects are passed onto subsequent generations, and the process does not involve the treatment of animals with exogenous substances, which can arouse protest from consumers. Breeding experiments in farm animals are slow and expensive due to their size and long generation interval. Small mammals such as mice display many of the same traits, have a shorter generation interval, and require

less space. Not being the same species only general conclusions can be drawn, but this is usually sufficient (Roberts, 1965). For example in mice recurrent selection (where selection is carried out in one line on the basis of the performance of progeny from a cross between that line and an inbred line), and reciprocal recurrent selection (where both parental lines are selected on the offspring of the cross) was less effective in altering body weight than mass or within family selection, obviating the need for costly experiments in farm animals (Roberts, 1965).

One trait that has frequently been subject to selection is rate of growth. Differences in growth rates of pigs have been induced by selection (Baird, Nalbandov and Norton, 1952). Fast growing (Broiler) lines of chickens have been produced which have an adult weight more than twice that of unselected lines (Burke and Marks, 1982) or than lines selected for egg-laying ability (Stewart and Washburn, 1983, Goddard, Wilkie and Dunn, 1988) . Many lines of mice differing in adult body weight or gain have been produced (McCarthy, 1982). Lines selected for high adult weight, or high post weaning gain can have an adult body weight which is two to three times that of a line selected for low weight (Timon and Eisen, 1969, Pidduck and Falconer, 1978, Sharp, Hill and Robertson, 1984). The divergence from the unselected control is usually assymetric on an arithmetic scale, with the increased body weight line showing a greater response than the decreased body weight line (McCarthy, 1982). Similar results have been obtained in the rat (Baker, Chapman and Wardell, 1975, O'Sullivan, Millard *et al*, 1986).

Selection on the rate of gain of weight over a particular age range, or on final weight has correlated effects on other production traits such as carcass fat and feed efficiency. These latter traits have also been subjected to selection, and correlated effects were noted on rate of gain and weight. Rats were selected on an index for the rate and efficiency of lean gain and divergence was found (Notter, Dickerson and Deshazer, 1976). The faster growing, more efficient animals ate more and were less

fat than the slower growing line (Rios, Nielsen, Dickerson and Deshazer, 1986). Selection on an index for increased lean mass in mice also resulted in greater feed efficiency (Bishop and Hill, 1985). Another study in mice showed that selection to increase any one of, feed intake, rate of gain or efficiency also increased the other traits (Sutherland, Biondini *et al*, 1970). This correlation suggests that to improve all these traits by selection the simplest method would be selection on rate of gain, as that only requires the recording of body weight at different times. Perhaps selection could be carried out on body weight which would be simpler still as it only requires one measurement.

However work on selection on body weight at various ages in mice indicates that the age at which selection is carried out can affect the final weight, growth rate and fat composition in the selected lines (McCarthy and Bakker, 1979). For example analysis of the proportion of carcass fat at several ages in mice selected for increased weight at 5 and 10 weeks of age showed that selection at 5 weeks had no effect on carcass fat at any age. However selection at 10 weeks resulted in lower fat deposition at all ages analysed (Hayes and McCarthy, 1976).

Fat and lean percentages are negatively correlated and at least one study has indicated that selection for high fat percentage may have the same effects on carcass composition as selection for low lean percentage at the same age, and vice versa (Prasetyo and Eisen, 1989). This differs from earlier work in which lines were selected for high or low carcass fat (estimated as 8 times gonadal fat pad weight) or on an index which estimated lean mass. The lines selected on lean mass differed little in carcass composition, unlike the fat lines which had a two to three fold difference in fat, but little in lean mass (Bishop and Hill, 1985). The difference between the results of the studies may be due to selecting on lean or fat mass, as opposed to lean or fat percentage.

It would appear that selection can produce substantial alterations in the desired trait providing the appropriate selection point and criterion are chosen. However selection is a long term project, and may have disadvantages, for example there is evidence of infertility in lines of mice selected for long periods for high body weight (McCarthy, 1982). Investigation has also proceeded into methods of altering rates of gain and carcass composition with more immediate effects. These include attempts to manipulate levels of growth hormone (GH), which is necessary for normal growth (Phillips, 1987).

1.2 The growth hormone axis

The control of GH levels in the plasma is complex, and, while the general pattern is similar across a range of mammalian species, details vary. Growth hormone is produced in the anterior pituitary and released into the blood (Karin, Castrillo and Theill, 1990). Transcription of the GH gene, and release of GH from somatotrophs is increased by thyroid hormone, glucocorticoids and GH-releasing Factor (GHRF) (Phillips, 1987). Indeed in some species normal development of somatotrophs is dependent on GHRF (Karin *et al*, 1990). Release is inhibited by somatostatin, which is produced by the hypothalamus (Brazeau, Vale, *et al*, 1973). Somatostatin and GHRF levels appear to be affected by plasma levels of GH and IGF-1 and by environmental stimuli.

Plasma GH levels vary over the long and the short term. GH secretion differs between the sexes in both periodicity and concentration. In males GH secretion shows more pronounced and regular (every three hours in rats) but less frequent peaks than in females, which have lower levels of GH both at peak and trough (Eden, Jansson, and Oscarsson, 1987). Periodicity appears to be influenced by the steroid sex hormones (Eden *et al*, 1987). These hormones also act to increase GH secretion

(Reeds, 1987). Injection of GH into females so as to mimic male secretory patterns results in greater growth than injections which reproduce female patterns (Robinson and Clark, 1987). Also plasma levels of GH rise during sleep (Phillips, 1987). These effects are in addition to the changes in GH secretion during development (Slabaugh, Leiberman, Rutledge and Gorski, 1982).

Mediation of growth hormone effects is equally complex. Growth hormone is necessary, but not sufficient, for normal growth and development as some forms of dwarfism exist in which GH is produced, and treatment with exogenous GH is ineffective (Phillips, 1987). Also GH treatment of thyroidectomised-hypophysectomised rats induced skeletal and muscle growth, but thyroxine was necessary for maturation, for example for closure of the epiphyses. Thyroxine appeared to have some growth promoting effects, but primarily acted to induce differentiation of the cells resulting from the increased rate of division induced by GH (Scow, Simpson *et al*, 1949, Scow, 1959). The effects on growth may also be site specific: in human males suffering from growth hormone deficiency, treatment with GH during puberty increased standing height, but not sitting height, implying that GH affected mainly growth of the long bones of the legs (Tanner, Whitehouse, Hughes and Carter, 1976).

Growth hormone binds to receptor sites on many tissues, including adipose cells. Displacement experiments on rat adipocytes using radioactive GH showed that it has its own, specific receptors on these cells (Grichting, Levy and Goodman, 1983). Earlier studies had suggested this (Fagin, Lackey, Reagan, and Di-Girolamo, 1980, Gavin, Saltman and Tollefsen, 1982). The existence of such receptors strongly suggests that GH acts directly on rat adipocytes. GH rarely affected fresh adipocytes from normal rats, but did those from hypophysectomised rats. This suggested that the lack of response was not primarily a consequence of cell surface damage occurring during the isolation of the adipocytes, but was at least partly due to the GH levels in

the pre-excision environment. It was found that culturing adipocytes from normal rats in GH free medium for 3-4 hours caused them to become responsive to GH (Herington, Stevenson, and Andrew, 1985). Similar treatment did not induce responsiveness in rabbit adipose tissue segments or isolated adipocytes (Richter and Schwandt, 1985, Barenton, Batifol *et al*, 1989). A study on rat and sheep adipose tissues showed that GH increased lipolysis in the rat tissue, but not in sheep tissue. Both were lipolytically competent and demonstrated β-agonist induced lipolysis. Anti-GH immunoglobulins blocked the response of the rat tissue (Duquette, Scanes and Muir, 1984). This suggests species specific differences.

Puromycin, which blocks protein synthesis, does not affect the increase in free fatty acid release induced in adipocytes by GH (Dawson and Beck, 1965). This suggests that GH does not induce this release by increasing enzyme production. Grichting, Levy and Goodman, (1983) observed that GH stimulated glucose utilization, and induced refractoriness in adipose cells. It also antagonised the lipolytic activity of adrenaline. GH treatment of adipose tissue of hypophysectomised rats increased glucose oxidation in the short term and lipolysis in the long term (Goodman, 1984). Earlier work had shown that a chronic high dose of GH reduced fat storage and the synthetic ability of adipose tissue in hypophysectomised rats (Goodman, 1963). However adipocytes from GH deficient *little* mice which had been given short term or chronic treatment with GH showed a reduction in fatty acid synthesis regardless of length of treatment (Ng, Adamafio and Graystone, 1990). This may reflect differences in other pituitary hormones, as the *little* mutants appear to only lack GH, unlike hypophysectomised mice. Some of the difference between long and short term effects on adipocytes of GH treatment may be due to modification of the GH receptors which are rapidly taken into the cell after binding, and have to be resynthesised (Gorin and Goodman, 1985). In pigs at least the long term insulin antagonist effects do not appear to be due to decreases in insulin

binding or insulin receptor kinase activity (Magri, Adamo, Leroith and Etherton, 1990).

The liver and cartilage release insulin-like growth factor (IGF) into the plasma in response to GH stimulation (Phillips, 1987). It is thought that these and other tissues also produce locally acting IGFs in response to GH exposure (Bomboy and Salmon, 1975, Etherton, 1989). Evidence for the induction of IGF-I release by GH stimulation includes work showing that injection of exogenous GH increases IGF-I levels in two different strains of pigs (Owens, Johnson, Campbell and Ballard, 1990). Also inhibition of GH levels in the neonatal rat by an antiserum to rat GH lead to a reduction in circulating levels of IGF-I (Flint and Gardner, 1989, Gardner and Flint, 1990). Further evidences that IGF-I mediates the effects of GH on growth are that injection of IGF-I induced growth in growth hormone deficient mice (Woodall, Breier, O'Sullivan, and Gluckman, 1991) and that measurements of IGF-I levels in transgenic mice with high levels of GH show that high growth occurs only after IGF-I levels have become GH inducible (Mathews, Hammer, Brinster and Palmiter, 1988). Similarly mice transgenic both for GH-diphtheria toxin (which is only expressed in somatotrophs and kills cells which express it) and for Metallothionien-IGF-I express IGF-I and grow normally. Mice transgenic for the toxin alone are dwarfs (D'Ercole, 1990). Further reinforcement is given by the effects of the frequency of IGF-I injections. IGF-1 had a greater effect on growth if four injections were given in 24 hours, rather than if one or two were, which is similar to the effects of the frequency of GH injection (Woodall *et al*, 1991).

Not only do IGF-I and thyroxine appear to mediate growth hormone's effects on growth there is evidence that insulin and GH may interactively modify fat metabolism. In hypophysectomised rats, which have very low insulin levels, adding glucose to the diet, or injecting the animals with insulin increased adipose tissue responsiveness to GH (Gause, Eden, Jansson and Isaksson, 1983, Gause, Isaksson,

Lindahl, and Eden, 1985). The maintenance of fatty acid synthesis by insulin in sheep adipose tissue slices and cell cultures was blocked by growth hormone (Vernon, 1982). In rats injection of Anti-GH immunoglobulins shortly before excision of the fat pad did not affect insulin binding. It did alter the dose-response curve for insulin (Schwartz and Eden, 1985). This suggests that GH alters post receptor responses to insulin. GH also blocked insulin-induced increases in lactation levels in sheep (Vernon and Finley, 1986). In mutant *little* mice, which lack GH, acute GH treatment had insulin-like activities, chronic treatment had insulin antagonist effects (Ng and Veis, 1990). Again complex interactions were occurring between GH and another hormone.

There is evidence that the effects of GH on fat metabolism are, at least in part, controlled by post translational modification of the protein (Reeds, 1987). In rats, recombinant bovine GH (bGH) had lower lipolytic and insulin antagonising activities than pituitary bGH *in vitro*, while their effects were virtually indistinguishable *in vivo* (Hart, Chadwick *et al*, 1984). Also two electrophoretic fractions of pure pituitary derived growth hormone were equally effective *in vivo*, but not *in vitro* (Salaman 1972). These differences may have been the result of contamination but it has been shown that a bacterially produced GH was more lipolytically active *in vitro* after a limited protease treatment (Baumann, Eisemann, and Currie, 1982). Also the secretory pattern of GH has measurable effects on growth, at least in the rat (Robinson and Clark, 1987). Despite uncertainties as to its precise mode of action GH has a clear effect in decreasing fat percentage and increasing efficiency of gain. This makes treatment with exogenous GH potentially valuable for commercial use. Also it is apparent that there are several points at which manipulation of the growth hormone axis could be attempted. Such manipulation could involve treatment with exogenous hormone, selection on levels of a hormone, or production of transgenic animals, for example.

1.3 Effects of growth hormone manipulation on production traits

Production traits are those traits which affect the profitability of animal production. These include rate of increase of body weight, mature body weight, feed efficiency and carcass fat percentage. Manipulation of the growth hormone axis would only be useful if it affects some or all of these, and improves profitability.

1.3.1 Injection of exogenous Growth Hormone

Injection of exogenous growth hormone has been studied in a variety of species. In four studies in pigs it was found that GH increased growth rate and feed efficiency when injected, whether porcine (Chung, Etherton, and Wiggins, 1985, Evock, Etherton, Chang and Ivy, 1988, Mclaren, Easter,*et al*, 1988) or human growth hormone was used (Baile, Della-Fera, and McLaughlin, 1983). The study using human growth hormone found no significant effects on carcass quality (assessed as the product of several traits including composition and muscle tenderness) (Baile *et al*, 1983). In contrast, with porcine GH fat percentage decreased and muscle area increased regardless of whether recombinant or pituitary GH was used (Evock *et al*, 1988). Fat percentage was reduced even when weight of fat in the carcass was not, due to increased lean mass (Chung *et al*, 1985). Similarly, in another study using porcine GH a considerable effect on carcass composition was observed (Novakofski, McKeith *et al*, 1988). The difference may be due to the differences in growth hormone or it may be due to other factors, such as diet.

Several workers have observed decreased fat percentage and improved muscle mass in lambs after administering GH (Wagner and Veenhuizen, 1978, Muir, Wien *et al*, 1983, Johnsson, Hart, Butler-Hogg, 1985, Butler-Hogg and Johnsson, 1987, Johnsson, Hathorn *et al*, 1987). There was little or no effect on absolute weight.

Others have found no significant effects (Sinnet-Smith *et al*, 1989). One study, on intact male lambs, rather than females or castrates, did note significant differences in body weight, and the rate of increase of body weight (Pullar, Johnsson and Chadwick, 1986). This may indicate a sex difference in response. The varied results of these studies were reflected by another which showed equivocal effects on growth (Pell, Blake *et al*, 1987). GH increased the rate of synthesis of mRNA in muscle, and the efficiency of protein production, hence the rate of protein synthesis increased (Pell and Bates, 1987), as was implied by an earlier study which showed that GH increased nitrogen retention (Davis, Garrigus and Hinds, 1970). Protein accumulation was specific to the muscles, in cartilage there was increased synthesis and breakdown (Pell and Bates, 1987). The general increase in muscle mass found in sheep, and similar results in pigs (Chung *et al*, 1985) indicate that the weight of carcass fat is not necessarily decreased by GH treatment, muscle weight increases, and carcass fat percentage consequently declines. In sheep, unlike pigs, GH does not generally increase feed efficiency as, although an increase was noted in one study (Johnsson, *et al*, 1985), it was not apparent in a later one (Johnsson, Hathorn, *et al*, 1987). Also Butler-Hogg and Johnsson (1987), and Johnsson, Hathorn *et al* (1987) both showed that GH increased non-carcass components such as wool.

In hypophysectomised rats, exogenous growth hormone increased growth by increasing anabolism, without affecting catabolism. In contrast, work-induced hypertrophy acted by also reducing catabolism. These processes affected the two muscle fiber types in different ways, and the resulting muscles had disparate fiber compositions (Goldberg, 1969). In intact rats a study showed that exogenous GH did not appear to effect muscle growth by increasing satellite cell proliferation and myonuclei accumulation (Beerman, Liboff, Wilson, and Hood, 1983). Long term administration of GH to lambs increased muscle RNA and activity per unit of RNA. Protein synthesis also increased, more so in red muscle than white, for a given gain

(Pell and Bates, 1987). GH in fed lambs increased nitrogen retention. This, with accompanying alterations in other traits, suggested increased protein production (Davis *et al*, 1970). In contrast a study in fasting lambs showed that GH reduced protein synthesis (Crompton and Lomax, 1987). Other studies have shown that GH in fasting ruminants acts to maintain protein gain (Hart and Johnsson, 1986). It is generally accepted that GH acts, directly or indirectly, to increase protein synthesis (Hart and Johnsson, 1986).

As well as its effects on meat production, emphasised by Goodman and Grichting (1983), and Reeds (1987), GH has been shown to increase the efficiency of milk production (Baumann *et al*, 1982). Indeed three breeds of cattle all had increased milk and fat yields when treated with recombinant bGH, with no breed by treatment interaction effects (Oldenbroek, Garssen, Forbes and Jonker, 1989).

The sequence of GH is highly conserved across mammals and it has been shown that injection of foreign GH can induce an increased rate of gain in many species, for example human growth hormone into rats (Baile *et al*, 1983), and bovine GH into lambs (Johnsson *et al*, 1985, Johnsson, *et al*, 1987). This cross reactivity is not universal, humans appear to respond only to human GH (Phillips, 1987), consequently experimentation may be needed to identify a GH which is active in the species being investigated.

Although GH treatment appears promising it may not always be effective, if only because treatment could lead to down regulation of endogenous GH levels. Exogenous GH has been shown to reduce the plasma levels of endogenous GH and the number of somatotrophs in the pituitary in pigs (Chung *et al*, 1985). Similarly, GH-Metallothionien transgenic mice completely lacked somatotrophs and pituitary GH production (Palmiter, Norstedt *et al*, 1983).

1.3.2 Immunoneutralization

Growth hormone levels have been manipulated by injection of animals with antibodies to growth hormone. Such treatment reduces growth in young postweaning rats (Gause *et al*, 1983) and in neonatal rats (Flint and Gardner, 1989). Despite the effects of GH in increasing milk yield (Baumann *et al*, 1982) injection of antiserum to rat GH into nursing rats did not reduce lactation (Madon, Ensor, Knight and Flint, 1986). Removing prolactin reduced milk yield to approximately 50 percent, and then treatment with anti-rat GH completely blocked milk yield (Madon, *et al*, 1986). This suggests that although GH elicits some milk production, possibly by binding to prolactin receptors, the increase in yield produced by GH treatment may be due primarily to alterations in repartitioning, making more energy available for milk production. Reduction of growth hormone levels is unlikely to be commercially viable, although it may be useful to minimise adverse side effects of high GH levels, such as infertility, in breeding stock. For such a purpose it would be preferable to find a long lasting treatment. It may be of interest that some rats show a long term reduction in rate of gain after treatment with anti-rat GH (Gardner and Flint, 1990). However this appears to be due to some rats mounting an inadequate immune response to the antiserum to the GH, and may not be practicable to reproduce (Madon, Parton and Flint, 1991). Improvement of growth by treatment with antiserum to somatostatin seems a reasonable prospect as such treatment has been shown to increase GH levels (Ferland, Labrie, *et al*, 1976)

1.3.3 Production of Transgenics

It has been clearly shown that the introduction of growth hormone genes into fertilised eggs by microinjection can produce larger animals, up to twice the size in mice (Palmiter, Brinster *et al*, 1982). The improvement is passed on to the offspring

(Palmiter *et al*, 1983). These results are comparable to those obtainable by selection, but were effectively obtained in a single generation. This would suggest that the production of transgenic animals might be a major innovation, if the method can be repeated in meat producing animals. Transgenic rabbits, sheep , pigs (Hammer, Pursel *et al*, 1985) have been produced, as well as cows, (Hammer *et al*, 1986). Not all those which incorporated growth hormone genes were larger than their non-transgenic litter mates. This depended in part on the site of insertion, some inserted genes were inserted into an inactive part of the chromosome, and in part on the promoter used. Pigs carrying a prolactin-Growth hormone gene construct were only larger than their wild type littermates if given prolactin inducers (Polge, Barton *et al*, 1989). On the other hand pigs with a metallothionien-GH construct grew faster with just the normal dietary levels of zinc as inducer (Hammer *et al*, 1985). The quality of the diet may also be important, GH transgenic pigs showed increased daily gain when given protein supplements, but did not on the normal diet (Pursel, Pinkert *et al*, 1989).

GH transgenic animals have a greater final weight than non transgenics of similar background. This seems to be due to an increased growth rate. In mice the period of rapid growth is of similar length in wild type and in transgenics, but transgenics grow faster (Brem, Wanke *et al*, 1989). Transgenic animals also differ from wild type mice in body composition. Dwarf *little* mice transgenic for GH were similar in size to, or larger than, non-transgenic wild type mice of similar genetic background, but body proportions were not restored to normal (Hammer, Palmiter and Brinster, 1984). Mice selected for high eight week body weight were similar in size to those transgenic for GH. When compared to unselected control animals, transgenics had longer bones than the controls, whereas the selected animals did not. However control animals had longer bones than transgenic or selected animals when the comparison was of bone length relative to the cube root of maximum body mass

(Wolf, Rapp *et al*, 1991). Earlier work indicated that liver weight was a greater fraction of body weight in transgenics than in controls (Brem *et al*, 1989). Clearly GH does not just increase all growth.

GH transgenics tend to have reduced fertility and mobility (Jaenisch, 1988). Even mutant *little* mice, which lack endogenous GH production, suffer a reduction in female fertility when made transgenic for metallothionien-GH constructs (Hammer *et al*, 1984). Ovary transplants between GH transgenic mice and non transgenic litter mates showed that ovaries from transgenic mice produced normal eggs in a normal physiological environment. Ovaries from non transgenic mice did not function in transgenic females (Brem *et al*, 1989). This suggests that the excessive plasma levels of GH are interfering with ovulation. This is supported by investigation of hormone levels in female mice transgenic for GH. The transgenics were less responsive to gonadotrophin releasing hormone than the controls, and were also hypoprolactinaemic. The latter may be due to the effects of GH in mimicking prolactin, and down regulating its secretion (Chandrashekar, Bartke and Wagner, 1990).

The mobility and other health problems may also be a function of excess GH, as injection of high levels of exogenous GH resulted in reduced mobility and in osteochondrosis in pigs (Evock *et al*, 1988) and caused kidney and liver degeneration, also in pigs (Machlin, 1972). Comparisons have been made of mice transgenic for growth hormone releasing factor (GRF), GH, and IGF-I. The first two groups have high GH levels, the latter does not. Animals with high GH levels showed progressive glomerulosclerosis, similar to that observed in human diabetics. Transgenics for IGF-I had enlarged glomeruli but no sclerosis, suggesting growth inducing effects of IGF-I on the glomeruli (Doi, Striker *et al*, 1988). Also high GH levels were associated with high blood cholesterol and an enlarged liver (Quaife, Mathews *et al*, 1989). Some health effects such as abnormal skin development and high serum

triglycerides were found in all three groups (Quaife *et al*, 1989). These suggest that it may be possible to avoid some health problems by altering parts of the GH axis other than GH. The growth increase of transgenics over controls may be smaller, but if fertility and health are maintained the animals may be more efficient than GH transgenics.

Alternatively consideration may be given to blocking production of one of the down regulators of the GH axis. It is possible to block hormone production by use of transgenics, GH levels have been reduced by ablation of GH producing cells either by the insertion of a GH-Diphtheria toxin construct (Behringer, Mathews, Palmiter and Brinster, 1988), or by use of a GH-herpes virus thymidine kinase construct. It may be possible to use such a technique to block, say, somatostatin production.

Another consideration with transgenic mice is the efficiency of transmission of the improvement to their offspring. Transmission from founder animals expressing the transgene to their offspring ranges from 0 to 50 percent, depending on what fraction of the germ line carries the gene (Hammer *et al*, 1986). Then the offspring may be infertile. However once past the first generation rates of transmission within a line are usually close to fifty percent (Hammer *et al*, 1986). However a study on the effects of crossing of mice transgenic for rat GH with a line not congenic with their background strain found that the frequency of the transgene in the population dropped. This happened even in a line which was selected for high 42 day body weight after the initial cross, despite transgenic mice being significantly heavier at that age than non-transgenics (Sabour, Ramsey and Nagai, 1991). This suggests that the change in genetic background caused the transgene to become unstable in the population, possibly by increasing the infertility or death rates in the transgenics. This could be a disadvantage if crossing a GH transgene into commercial strains. It is also possible that animals homozygous for the transgene could be produced, if the problem of female infertility is solved. In mice such animals were produced after

ovary transfer. The animals homozygous for the GH transgene had more than twice the basal plasma levels of GH of the hemizygous animals. There was also a two fold or greater difference in inducible GH (Yun, Li *et al*, 1990). If this resulted in a comparable increase in growth, but not in health problems, such homozygous animals could be valuable.

1.4 Changes in the growth hormone axis and growth after selection.

There is considerable data to show that selection on growth rate or final weight can affect growth hormone levels. Growth hormone differences between genetically large and small rats were correlated with growth, with large animals having significantly higher peak and basal plasma GH concentrations than the small ones (O'Sullivan *et al*, 1986). Similarly comparison of two lines of Hampshire swine, one fast gaining, one slow, showed that the faster line had a larger pituitary. This line also had more GH activity per unit weight of dried pituitary, suggesting more GH secretion (Baird *et al*, 1952). There is also evidence that larger breeds of cattle have increased levels of GH (Hart and Johnsson, 1986). However work in which the Snell Dwarf gene was backcrossed into strains of mice selected for large and small size, thereby depleting them of endogenous GH, showed that GH secretion was only partially responsible for differences in growth rate and that the smaller mice had reduced sensitivity to GH (Pidduck and Falconer, 1978).

The correlation between components of the growth hormone axis can be altered by selection. Mice expressing a major gene for high growth rate have lower levels of GH but higher levels of IGF-I than unselected controls, this may indicate IGF-I levels influence the down regulation of GH levels (Medrano, Pomp *et al*, 1989). A similar reduction in GH levels associated with growth has also been observed in chickens, broiler lines of chickens selected for faster growth rate have lower plasma levels of

GH than slower growing layer lines (Burke and Marks, 1982). Three broiler strains differing in growth rate also showed a negative correlation between growth rate and GH levels, although high GH levels reduced carcass fat (Stewart and Washburn, 1983). This may be due to an alteration in the induction of IGF-I, as appears to be the case in the mice. However IGF-I need not be affected, lines of mice divergently selected for high or low protein or fat body mass ratios did not differ in circulating IGF-I levels at 5 weeks of age, during a period of rapid growth. The high body weight line did have significantly higher plasma concentrations of IGF-I at 10 weeks of age (McKnight and Goddard, 1989). Similarly other lines of broiler chickens which showed reduced plasma GH levels showed no difference in IGF-I levels (Goddard *et al*, 1988). Selection has also been carried out on plasma concentrations of IGF-I. Correlated differences in size were obtained, although the differences were less than those obtainable by direct selection on body weight (Blair, McCutcheon *et al*, 1989).

In general studies in a variety of species indicate that the relationship, within species, between endogenous levels of growth hormone and several growth parameters is poor (Hart and Johnsson 1986). There is also the problem of the pulsatility of release which appears to influence effects on growth, at least for GH (Eden *et al*, 1987) and IGF-I (Woodall *et al*, 1991). Such factors would tend to reduce the efficiency of selection on components of the GH axis. The components also need not diverge in a correlated manner, as shown by lines of mice in which the high body weight line has higher IGF-I and lower GH levels than unselected controls (Medrano *et al*, 1989). In addition there is evidence for differences in GH sequence, different restriction fragment length polymorphisms were fixed in a line selected for high body weight and in unselected controls (Salmon, Berg, Yeh and Hodgetts, 1988). In a backcrossing experiment the haplotype fixed in the high line was associated with low 42 day body weight however, which suggests the effect of this

haplotype may be specific to the genetic background (Winkelman, Querengesser and Hodgetts, 1990). This suggestion was supported by further selection on body weight, using the F2 from the backcross as the base population, which increased the frequency of this haplotype in lines selected for high body weight, and decreased it in lines selected for low body weight (Winkelman and Hodgetts, 1992). Also other hormones or enzymes may be affected, broiler lines of chickens have up to 20 times the levels of ornithine decarboxylase levels in breast muscle as layers (Bulfield, Isaacson and Middleton, 1988). The system is complex, and assays expensive, which suggest that, for the moment at least, selection is best carried out on physical traits such as weight. The differences in components of the GH axis also suggest that responses to exogenous treatments, such as GH injection, may also differ between selected lines, with some not responding at all. Investigation of such differences could give useful information as to differing ways of managing different strains.

1.5 Justification

Growth and fat control are complex and need further investigation. I am interested in the involvement of growth hormone on both these traits, in particular how selection on either trait alters the growth hormone axis. Several possible sites of alteration exist, for example, plasma GH levels, responsiveness to GH or changes in the activity of GH due to sequence alterations. There is no reason to assume that only one of these potential alterations has occurred or been selected for during the selection process.

Lines of mice selected from the same base population which differ two to three fold in lean mass with no change in fat content or two to three fold in carcass fat with no change in lean mass (Bishop and Hill, 1985) were available. These have not been investigated for differences in growth hormone levels, activity, or responsiveness.

Such investigation could throw light on the differences between the lines in question, and would add to the information on the growth hormone system.

1.6 Animals

The mice used throughout the experimental work were taken or derived from the F and P lines of mice maintained at the University of Edinburgh, Genetics mouse house. The background of the lines is included in the introduction because they are of such importance to the thesis.

The F and P line mice were taken from selected lines which descended from a common base population, the 'G' strain. This was established in 1980 (Sharp *et al*, 1984). Two inbred lines, JU and CBA were crossed and the F1 crossed to the outbred strain CFPL, which was obtained from the Carnworth laboratory in 1976. There followed one generation of random mating, the next being generation 0 of the selection experiment (Sharp *et al*, 1984). Three replicates of each direction, high, low and control of the selection criteria were established. Selection was applied within family on 10 week old males. The F-Line was selected on the ratio of gonadal fat pad weight to body weight, the P-Line on an index (body weight (g -[8 x gonadal fat pad weight])) which was a predictor of fat free mass (Sharp *et al*, 1984).

In generation 20 the selection criteria were modified such that the P-Lines were selected on 10 week weight in both sexes and the F-Lines were selected on the ratio of dry to wet weight in 14 week old males. The three replicates of each direction were crossed at this stage to give new, unreplicated, lines with lower inbreeding. The generation interval was also increased from 12 to 15 weeks, for ease of management. The P-Lines differed two to three fold in lean mass, but little in carcass composition (Bishop and Hill, 1985). The F-lines differed two to three fold in carcass fat percentage but little in lean mass (Bishop and Hill, 1985). This divergence in carcass

fat was maintained even after the selection index was changed to the ratio of dry weight to body weight instead of gonadal fat pad (Hastings and Hill, 1989).

Litters were culled to a maximum of 12. Care was taken to leave as near as possible to equal numbers of males and females. The mice were fed B.P. expanded mouse diet number one food *ad libitum*, with the same food available as mash around weaning if required. The presence of mash ensures that even small mice have access to food and water. Pregnant and nursing females of the F and P Lines received number 3 food *ad libitum*. Number 1 food contains 14.8 MJ/ Kg gross energy and 14.6 % by weight crude protein, number 3 contains 15.4 MJ/ Kg gross energy and 22.7 % crude protein (Manufacturers specifications). The temperature in the animal house was maintained at above 20 Celsius. Stock and breeding animals were not kept in a controlled light environment and were exposed to normal light period variation for this latitude.

Chapter 2
Preliminary Trial of Growth Hormone

2.1 Introduction

It seems plausible, given the known effects of GH on growth and composition that GH is involved in the differences between the high and low body weight P-Lines, and in the differences between the fat and lean F-Lines. One line of investigation is to study the different responses of the lines to exogenous GH. If sensitivity, measured as proportional increases in growth, had altered, the existence of a difference in the number of receptors could be inferred. It is also possible that a given tissue, for example fat or liver, would be more affected in one line than another, suggesting changes in the metabolic control mechanisms.

Males and females have different mature weights and growth rates, which seem to be linked to differences in the pattern of pulsatile release of growth hormone (GH) (Robinson and Clark, 1987). This suggests that the sexes would differ, at least quantitively, in their response to injected GH. However in transgenic mice males and females usually have similar mature weights and growth rates (Hammer *et al*, 1986) in response to high plasma levels of GH. In transgenic animals GH levels are continuously high, which is not the case in injected animals (Robinson and Clark, 1987). Only one sex was used in the experiments, removing a potential source of variability.

2.2 Materials and Methods

F-Line mice were assigned randomly within family to a treatment group after weaning at 3 weeks of age. They were transferred to a controlled light room, 14

hours light, 10 hours dark, at this stage. Treatment commenced seven days later in order to reduce maternal influences, the major source of variability in body weight and composition at weaning (Eisen, 1974). Consequently treatment started when the mice were 4 weeks of age and continued until they were 7 weeks of age, thus covering the period of rapid growth. BP expanded diet number one (special diets services, Witham, Essex, UK.), and water were available *ad libitum* throughout the period from weaning until the end of the experiment. The same food was available as mash until all the mice in a cage weighed 10g, at which weight the animals appeared to be able to reach the pelleted food and the water bottle (personal observation).

The recombinant bovine growth hormone (rbGH) used was kindly donated by the American Cyanamid Co., New Jersey. It was given as a sterile solution prepared in isotonic bicarbonate buffer (pH 9.4) or an isotonic saline placebo. All animals were injected within the two hours before the start of the dark period with 5 µl fluid/ gram body weight/ day.

For this preliminary trial three concentrations of GH were used, 1.8mg/ml, 0.3mg/ml and 0.05mg/ml. These gave daily doses per gram body weight as shown in Table 2.1. All mice used were males from the fat F-Line, 11 generations after the cross, that is after 31 generations of selection.

Three *lit/lit* mice were used to ensure that the recombinant bovine GH (rbGH) used was active in mice. They were drawn from *lit/lit* stock in a C57/BL6J background maintained in the same mouse house as the F and P lines, on the same *ad libitum* diet. One received saline at 5µl /g /day, one 0.25µg GH /g /day and one 9µg GH /g /day. They were injected in the same way as the F-Line mice. Their weights were recorded on a graph on a daily basis. The injections were discontinued after 15 days as it was clear that the mouse on the high dose of GH was growing faster (See Figure 2.1) and hence the preparation used probably had biological activity in the

Table 2.1

Preliminary trial of the effects of GH on male mice from the fat F-line from 4 to 7 weeks of age. Treatments given and number of mice in each treatment group. Animals were kept in a 14hr light, 10 hour dark cycle with food (SDS number 1 food) and water available *ad libitum*.. Injections were given daily for 21 days in the two hours before the start of the dark period.

Group	Treatment	Number of mice
control	5 µl saline/g/day	9
Low Dose	0.25 µg GH/g/day	8
Medium Dose	1.5 µg GH/g/day	6 *
High Dose	9 µg GH/g/day	9

* This group started with 9 mice , but 3 escaped

mouse.

Weights were taken daily, immediately before the injection. Mice were weighed and then killed by cervical dislocation on the day after the final injection during the 4 hours before the start of the dark period. The liver, kidney, spleen and gonadal fat pad (GFP) were dissected out and weighed. These organs were chosen to monitor changes in fat content (GFP), general metabolism (liver and kidney), and to monitor immune stress caused by the injection of a foreign protein (spleen). Spleens vary in shape and weight within and between lines (Dunn, 1954) so a crude comparison of weights was unlikely to give unambiguous information. However dissection and visual inspection ensured that they were checked for gross abnormalities which might indicate immune stress.

2.2.1 Method of Analysis

The means and standard errors of means plotted in Figures 2.1 and 2.2 were simply calculated for each treatment group. No allowance for family effects was made. The statistical analysis was carried out using a mixed model least squares and maximum likelihood computer program (Harvey, 1985). All analyses were carried out on natural log transformations of the weights to avoid scale effects that would otherwise be present in subsequent analyses of the P-Lines due to the size differences between those lines. Scale effects were unlikely to be important in this trial as only one line was investigated.

2.3 Results

Figure 2.1, which shows the weights of *lit/lit* mice over an eighteen day period, suggests that mice respond to the rbGH used in this trial, at least at the highest dose. Only one mouse was tested at each dose, but the animal given 9μg GH/ g body

Figure 2.1

Weights of individual *little* mice after treatment with GH or saline. The animals were injected daily for 15 days, at which point the injections were discontinued as GH appeared to be having a definite effect .

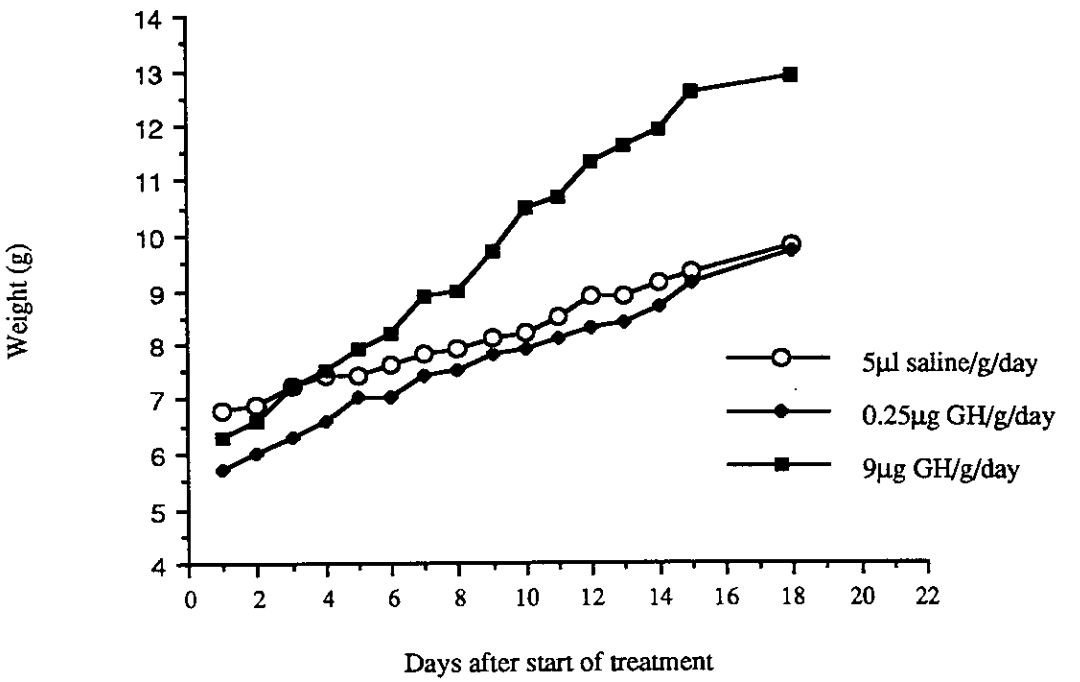
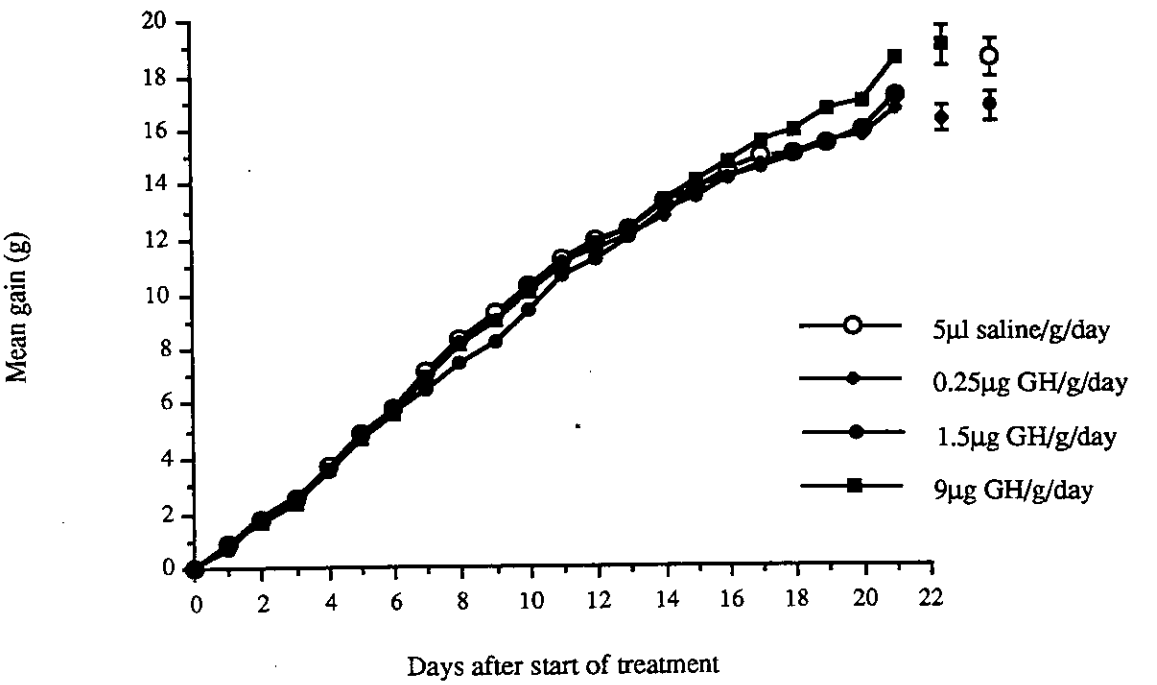


Figure 2.2

Mean daily gains of male Fat F-Line mice injected daily with saline or recombinant bovine GH from 4 to 7 weeks of age. Doses were as given in the key. The error bars shown give the maximum standard error for each treatment group.



weight per day gained approximately twice as much as the animals given either saline or the lowest dose of rbGH. This was however only 3 to 4 g more than the latter animals. The *little* stock is inbred and it is unlikely that inbred mice would show this level of variation in growth rate purely by chance. Effects of similar absolute, as opposed to proportional magnitude would be less noticeable in normal mice with their much higher rate of gain over the period of study. The mean gains over the period of the Fat line mice (Figure 2.2), do not show such a clear effect, although it appears that the high dose group has gained more over the treatment period than the control or lower doses.

No significant effects of treatment were noted on body weight or gain (Table 2.2). However when the three groups which were treated with rbGH were compared there appeared to be a trend of increasing gain with increasing dose. This in conjunction with the results from Figures 2.1, 2.2 suggested that the rbGH has increased growth. Although the sample size used was too small to detect the effect it suggested that further experiments should be conducted. There does not appear to have been any effect on gonadal fat pad weight as a proportion of body weight.

The significant effect recorded on kidney weight may be due to the treatment, in that kidney weight seemed to decline as a proportion of carcass weight with increasing dose. However the significant effects observed for spleen and liver weights as a percentage of carcass weight did not appear to follow any trend. This suggests that the results may have been a statistical quirk, resulting in part from the small sample sizes used (Table 2.2)

2.4 Discussion

The results suggest that these mice responded to the recombinant bovine GH used. Consequently it would be suitable for investigating differences between the

Table 2.2

Body and organ weights of mice from the fat F-line

Male mice were injected daily for 21 days from 4 to 7 weeks of age with one of three concentrations of rbGH or with saline. Organ weights given are as a percentage of final body weight. All traits other than initial weight are regressed onto Ln(initial weight) The data were analysed by the analysis of varience and a mixed model least square and maximum likelihood computer program.

	Control (Saline)	0.25µg GH/ g body wt /day	1.5µg GH/ g body wt /day	9µg GH/ g body wt /day	Sign treat	S.E. Means
Ln(init wt)	2.38	2.33	2.3	2.34	NS	±0.05
(initial wt (g))	(10.8)	(10.3)	(10.0)	(10.4)		
Ln(final wt)	3.34	3.30	3.32	3.34	NS	±0.029
(final wt (g))	(28.2)	(27.1)	(27.7)	(28.2)		
RG(overall)	0.99	0.95	0.97	1.00	NS	±0.03
GFP weight (% body weight)	0.70	0.60	0.63	0.63	NS	±0.05
Liver weight (% body weight)	2.59	2.53	2.28	2.34	*	±0.088
Kidney weight (% body weight)	0.62	0.59	0.58	0.55	*	±0.25
Spleen weight	0.12	0.14	0.16	0.14	*	±0.008

Ln natural logarithm

RG relative gain, calculated as the difference between the natural logs of the initial and final weights of the treatment period

NS no significant difference between the groups being compared

* $P<0.05$, ** $P<0.01$ for line treatment or interaction effects

The data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985).

lines in response to exogenous GH. It is likely that the non-significant difference between the control and GH treated groups in this trial is due to the small sample sizes used. The data gathered was used to provide parameters to calculate the number of mice needed by the following method (Chapter 6, Snedecor and Cochran, 1980)

Assuming a mean gain of 20g for untreated mice between 4 and seven weeks of age from the data

To identify a difference between the means, δ of 2g, (or 10%), with a probability, P_1=0.95, at a significance level 0.99 and a standard error between the means, σ of 1g, (or 5%) using a two tailed test a sample size, n, was needed, where

$$n = \frac{M \times 2 \times \sigma^2}{\delta^2}$$

where M is a multiplier related to the significance level and probability of detection required. In this case M=17.8 (table 6.14.1, p104, Snedecor and Cochran).

so $n = (17.8 \times 2 \times 5^2)/(10)^2$

$n = 8.9$

Rounding up, and adding two to correct for the approximate formula used gave

$$n = 11$$

that is, 11 mice per treatment group were needed.

This confirms that the samples used were too small to reliably detect an effect due to GH. Further work should be carried out on samples of at least 11 mice per treatment group, preferably a few more to allow for such events as illness, escaping, and incorrect determination of sex.

Chapter 3
Effects of GH on F and P-Line mice.

3.1 Introduction

The data from the preliminary trial indicated that the rbGH used was growth promoting in normal mice. This suggested that it would be practical to use the rbGH to identify and analyse any differences in the reactions of the lines differing in fat composition and those differing in body weight to exogenous GH. The preliminary trial had indicated that the highest dose, 9μg/g body weight/day, was the only one tested to have induced an increase in growth, consequently this dose was used for the treatment of the four lines.

Traits not analysed in the preliminary trial were studied in this experiment. Pectoralis muscle weight was recorded as the pectoralis is an easily dissected muscle, and tibia length as it is a large identifiable bone. These provide information about changes in muscle mass and skeletal growth respectively. Measuring a small bone would have limited utility as the measurement error would have been a greater fraction of the bone length, and possibly have masked differences due to the GH. These measurements would hopefully give further information on any differences between the line in the partitioning effects of GH. The carcasses were also freeze dried to obtain another estimate of percentage fat in the body as initial selection was based on gonadal fat pad weight so the drying method gave a non-biased estimate of overall body composition (Hastings and Hill, 1989).

3.2 Materials and methods

3.2.1 Animals

The mice used in this experiment were taken from the line which resulted from the crossing of the original three replicates. The F-Line came from generation 12, and the P-Line generation 13, after the cross. That is after a total of 32 or 33 generations of selection respectively (section 1.6). The F and P lines were non-contemporaneous. They were housed from weaning in controlled light conditions (14h light, 10h dark) and fed the same *ad libitum* diet as the animals in the preliminary trial. Only males were used to avoid the confounding effects of sex based differences.

Mice were assigned randomly within family to the control or treatment group when weaned at 21 (±2) days. Treatment commenced 7 days later in order to reduce maternal influences, the major source of variability in weight at weaning (Eisen, 1974). Animals were treated with recombinant bovine growth hormone at 9μg/g body weight/day, given as a 1.8mg/ml sterile solution prepared in isotonic bicarbonate buffer (pH 9.4) or with a saline placebo at (0.1ml/20g body weight) daily from 4 to 7 weeks of age. Injections were given subcutaneously within the 2 hours before the start of the dark period and the mice were weighed immediately before injection.

On the 21st day after the treatment started the mice were weighed and then killed by cervical dislocation. The liver, spleen, kidneys, gonadal fat pad (GFP) and pectoralis muscle were dissected out and weighed. The organs were then restored to the carcass which was freeze dried and weighed. Finally the hind legs were taken and boiled to remove the flesh, enabling tibia length to be measured. The length did not include the terminal epiphyses.

Three *lit/lit* mice were used to confirm that the rbGH had not lost its biological activity. Two were treated with rbGH, and one with saline. The dose and regimen of administration were as described for the F and P-Line mice. Weights were recorded, but the animals were not dissected at the end of the treatment period.

3.2.2 Statistical Analysis

The results were analysed using a mixed model least squares and maximum likelihood computer program (Harvey, 1985). Analyses were carried out using a model with family as random effect, nested within line, with line and treatment as fixed, crossclassified effects. The model also tested for line by treatment interactions. All analyses used natural log transforms of weight to remove scaling effects due to the substantial difference in size between the high and low selected P-line mice. Traits, other than initial weight, were also regressed on the natural log of initial weight to remove the effects of initial body weight differences between animals. This regression reduced the standard errors of the means in the analyses. (Log final weight showed a correlation with initial weight of over 0.80 for all lines other that one selected for high body fat, in which the correlation exceeded 0.65). Relative gain was also analysed as its logarithm, calculated for each animal as (natural log final weight) -(natural log initial weight).

3.3 Results

All the lines of mice in this study responded to rbGH by increasing their growth rate (Tables 3.1 and 3.2). At 4 weeks of age, before treatment, there was no significant difference in body weight between the lines selected for high and low fat percentage (F-Lines) (Table 3.1). The line selected for high body weight was significantly ($p<0.01$) heavier, by about 9g, at the start of treatment than the line

Table 3.1

Body weights of mice selected for high and low body fat content (F-Lines). Male mice from the fat and lean lines were injected daily from 4 to 7 weeks of age with 9μg rbGH/g body weight/day, or with 0.1 ml saline/20g body weight/day. All traits other than initial weight were regressed on Ln(initial weight)

	Fat		Lean		Significance Treatment	Significance Line	Significance Interaction	S.E. Mean
	Saline	GH	Saline	GH				
Number of animals	18	17	18	18				
Ln Body Weight at 4 weeks	2.94	2.89	2.93	2.84	*	NS	*	±0.084
(initial body wt (g))	(18.9)	(18.0)	(18.5)	(16.9)				±(1.09)
Ln Body Weight at 7 weeks	3.39	3.47	3.32	3.40	**	**	NS	±0.017
(Final body wt (g))	(29.7)	(32.0)	(27.0)	(30.2)				±(1.02)
RG(overall)	0.49	0.57	0.42	0.51	**	**	NS	±0.017

Ln natural logarithm

RG relative gain, calculated as the difference between the natural logs of the initial and final weights of the treatment period

NS no significant difference between the groups being compared

* $P<0.05$, ** $P<0.01$ for line treatment or interaction effects

The data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985).

Table 3.2

Body weights of mice selected for high and low whole body weight (P-Lines). Male mice were injected daily from 4 to 7 weeks of age with 9μg rbGH/g body weight/day, or with 0.1 ml saline/20g body weight/day. All traits other than initial weight were regressed on Ln(initial weight)

	High		Low		Significance Treatment	Significance Line	Significance Interaction	S.E. Mean
	Saline	GH	Saline	GH				
Number of animals	30	30	30	30				
Ln Body Weight at 4 weeks	3.13	3.13	2.68	2.53	*	**	**	±0.067
(initial body wt (g))	(22.9)	(22.9)	(14.6)	(12.6)				±(1.07)
Ln Body Weight at 7 weeks	3.63	3.71	3.16	3.23	**	**	NS	±0.021
(Final body wt (g))	(38.1)	(40.0)	(23.6)	(25.3)				±(1.02)
RG(overall)	0.83	0.90	0.33	0.40	**	**	NS	±0.049

Ln natural logarithm

RG relative gain, calculated as the difference between the natural logs of the initial and final weights of the treatment period

NS no significant difference between the groups being compared

* $P<0.05$, ** $P<0.01$ for line treatment or interaction effects

The data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985).

Figure 3.1

Mean daily gains of mice selected for high and low body fat content (F-Lines). Male mice from the lines were injected daily from 4 to 7 weeks of age (groups as in the key). Error bars show the maximum standard error for each line and treatment group

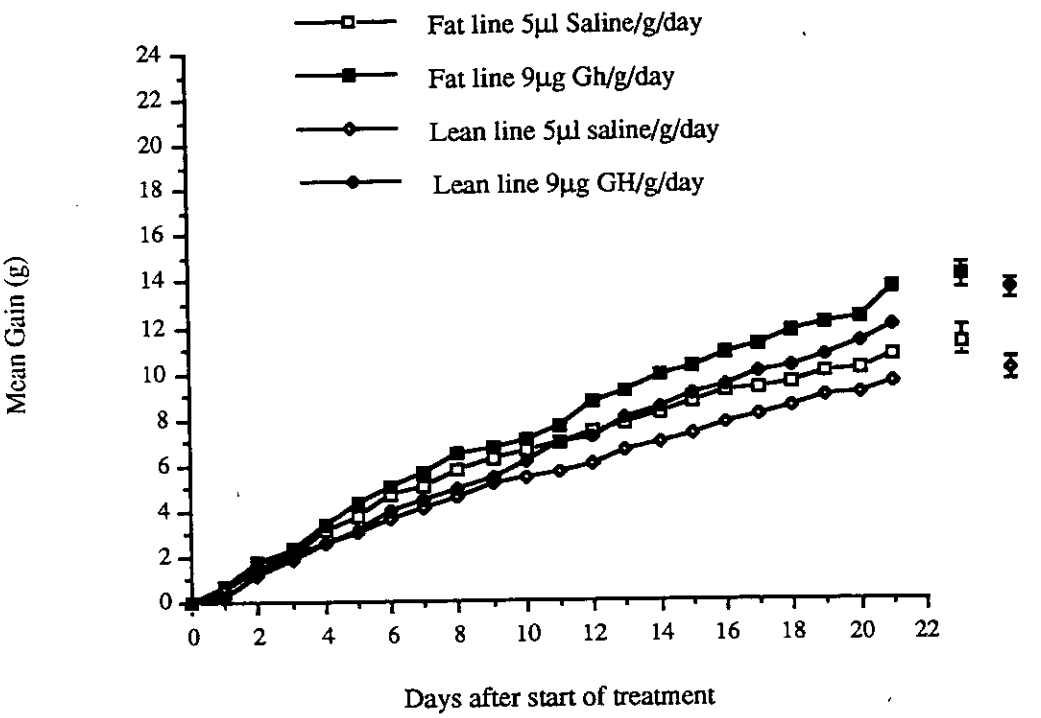
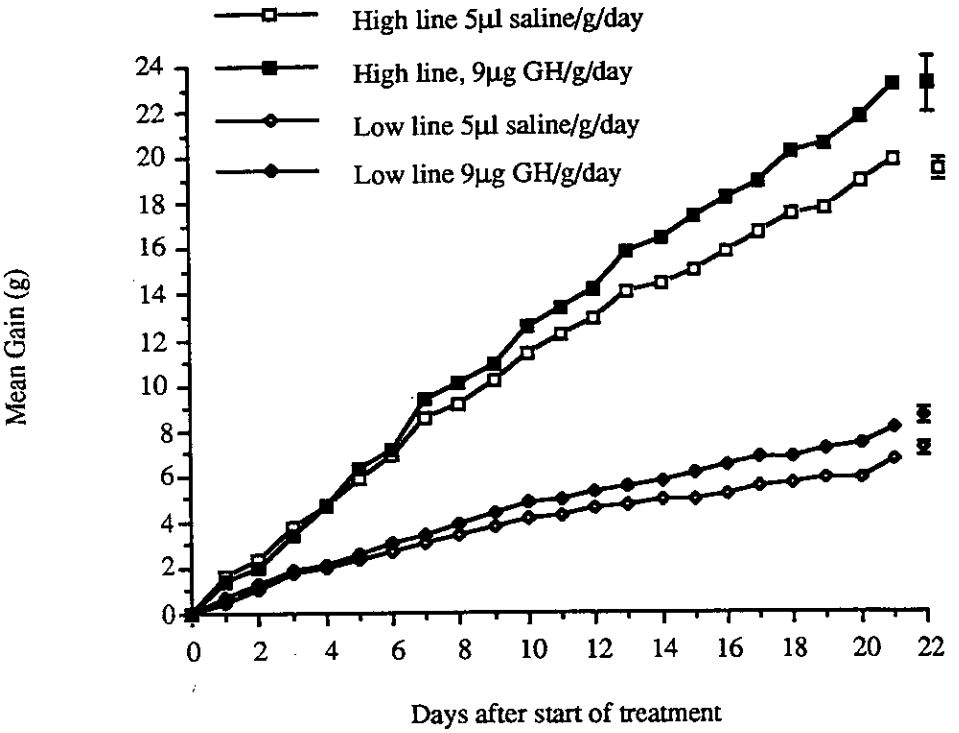


Figure 3.2

Mean daily gains of mice selected for high and low body mass (P-Lines). Male mice from both lines were injected daily from 4 to 7 weeks of age (For groups see key) Error bars show the maximum standard error for each line and treatment group



selected for low body weight and gained twice as much weight over the test period (Table 3.2). In both body weight lines the gain of the treated mice was a factor of 1.15 greater than that of the untreated mice (Figure 3.2). Mice selected for high and low fat percentage grew at similar rates throughout the treatment period (Figure 3.1) and both lines responded with an increase in weight gain of 3 to 4 g when treated with rbGH. This is a factor of approximately 1.1, similar to the 1.15 noted for the P-Lines and to that noted in chapter 6. Cumulative gains were plotted rather than weights as the mice assigned to be injected with rbGH were significantly lighter than mice assigned to the group to be given saline in the line selected for low body weight and the line selected for low body fat percentage, by chance (Tables 3.1 and 3.2).

From figures 3.1 and 3.2 it appears that the rbGH has caused the maximal rate of weight gain to be maintained for a longer period than in untreated mice, resulting in greater weight gain over the whole period and greater final weights. The rbGH also appeared to induce growth in the GH deficient *little* mice (Figure 3.3).

There is an apparent discrepancy between the values of the relative gain in the tables, and that which can be calculated from the initial and final weights given. The initial weight given has not been regressed onto itself, while final weight has been regressed onto initial weight. The relative weights have also been regressed onto initial weight. The method of calculating the relative gain involves the initial weight as well as final weight, so there will be different correction factor applied to relative gain, than to final weight, because of their regression on initial weight. The discrepancy is greater in the body weight lines due to the greater difference in weight between these lines (Tables 3.1 and 3.2).

Gonadal fat pad and dry matter percentages are indicators of the amount of fat in the carcass as expected. Both were significantly ($p<0.01$) greater in the high fat line than in the low fat line (Table 3.3). Gonadal fat pads were proportionately smaller in

Table 3.3

Organ weights of mice selected for high and low body fat content (F-Lines). Male mice from the fat and lean lines were injected daily from 4 to 7 weeks of age with 9μg rbGH/g body weight/day, or with 0.1 ml saline/20g body weight/day. Organ weights are as a percentage of final body weight. All traits were regressed on Ln(initial weight)

	Fat		Lean		Significance Treatment	Significance Line	Significance Interaction	S.E. Mean
	Saline	GH	Saline	GH				
Gonadal Fat Pad (% body weight)	1.09	1.06	0.41	0.36	NS	**	NS	±0.044
Dry body weight (% Wet body weight)	38.6	37.9	31.8	29.9	*	**	NS	±0.62
Pectoralis muscle (% body weight)	0.35	0.29	0.30	0.38	NS	NS	**	±0.017
Tibia bone length (mm)	16.5	16.6	16.6	16.8	NS	NS	NS	±0.10
Liver weight (% body weight)	5.5	5.5	5.4	5.2	NS	NS	NS	±0.12
Kidney weight (% body weight)	1.43	1.37	1.58	1.53	*	**	NS	±0.042
Spleen weight (% body weight)	0.31	0.32	0.40	0.41	NS	*	NS	±0.027

Ln natural logarithm
RG relative gain, calculated as the difference between the natural logs of the initial and final weights of the treatment period
NS no significant difference between the groups being compared
* $P<0.05$, ** $P<0.01$ for line treatment or interaction effects
The data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985).

Table 3.4

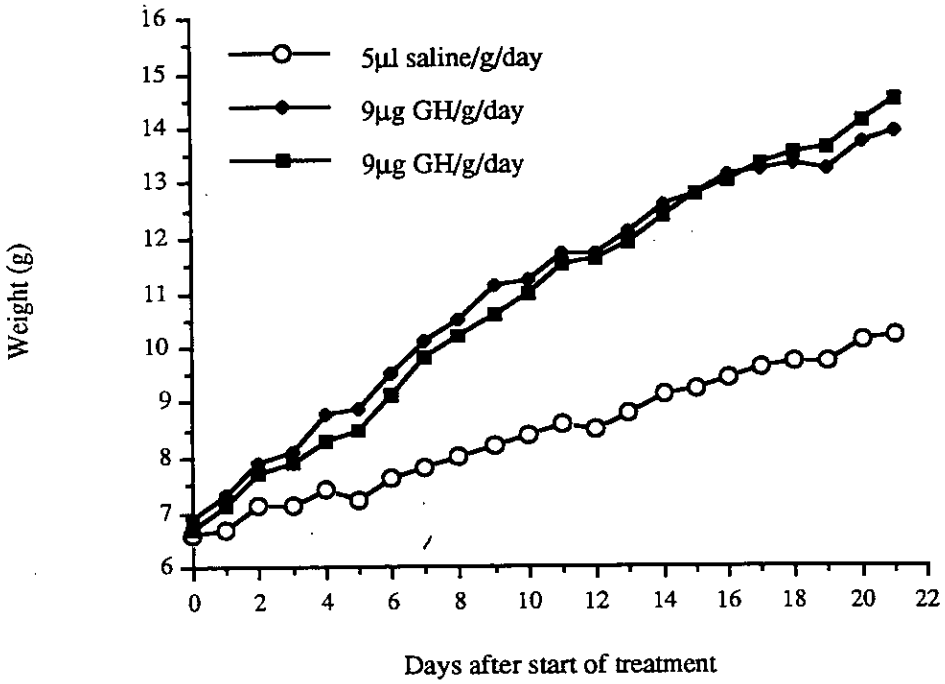
Organ weights of mice selected for high and low whole body weight (P-Lines). Male mice were injected daily from 4 to 7 weeks of age with 9μg rbGH/g body weight/day, or with 0.1 ml saline/20g body weight/day. Organ weights are as a percentage of final body weight. All traits were regressed on Ln(initial weight)

	High		Low		Significance Treatment	Significance Line	Significance Interaction	S.E. Mean
	Saline	GH	Saline	GH				
Gonadal Fat Pad (% body weight)	0.47	0.50	0.95	0.56	NS	**	NS	±0.088
Dry body weight (% Wetbody weight)	33.1	36.0	36.0	35.2	NS	NS	NS	±1.63
Pectoralis muscle (% body weight)	0.36	0.35	0.34	0.34	NS	NS	NS	±0.028
Tibia bone length (mm)	17.2	17.6	15.8	16.0	NS	**	NS	±0.23
Liver weight (% body weight)	5.8	5.5	5.4	5.3	NS	NS	NS	±0.17
Kidney weight (% body weight)	1.47	1.38	1.55	1.49	*	NS	NS	±0.069
Spleen weight (% body weight)	0.38	0.44	0.22	0.21	NS	**	*	±0.023

Ln natural logarithm
RG relative gain, calculated as the difference between the natural logs of the initial and final weights of the treatment period
NS no significant difference between the groups being compared
* $P<0.05$, ** $P<0.01$ for line treatment or interaction effects
The data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985).

Figure 3.3

Weights of dwarf *little* mice injected daily for 15 days with GH or saline (see key).



the high P-line mice ($p<0.01$) (Table 3.4). This may be due to the original selection criterion being fat free mass, predicted from gonadal fat pad weight and body weight. Treatment with rbGH had no significant effect on gonadal fat pad weight as a percentage of body weight in the P or F-Lines or on dry weight as a percentage of body weight in the P-Line. An effect of treatment on dry body weight in the F-Lines was observed (Tables 3.3 and 3.4). Also when all four lines were analysed together there was a significant ($p<0.05$) line by treatment interaction for dry weight percentage. These may be chance effects or indicate between line differences in the involvement of GH in fat regulation.

The pectoralis was chosen as a representative, easily dissected muscle which could be used to estimate the effects of GH on muscle mass. GH treatment did not affect pectoralis weight as a percentage of final weight in the lines selected for body weight (Table 3.4). GH treatment reduced pectoralis weight as a percentage of final weight in the high fat mice but increased it in the low fat mice (Table 3.3). This was apparent as significant line by treatment interactions ($p<0.05$), and suggests that selection in the high fat mice has altered the interaction of GH with metabolism.

The high body weight line had a significantly ($p<0.01$) greater tibia length than the low body weight line (Table 3.4). Tibia length was consistently, but not significantly, greater in treated animals than in the control group in each of the lines of mice (Tables 3.3 and 3.4).

There were no significant line or treatment effects on liver weight as a percentage of body weight. The significant ($p<0.05$) reduction in kidney weight as a percentage of body weight (Tables 3.3 and 3.4) after treatment implies that the kidney is less susceptible to GH than other tissues. This may be due to kidneys reaching their final size before the rest of the body, or reflect distribution of GH receptors. A significant ($p<0.05$) difference between lines in spleen weight as a percentage of body weight

was noted in these experiments, as was a significant ($p<0.05$) interaction between line and treatment in the body weight lines (Tables 3.3, 3.4). Previous studies have shown that spleen size and shape vary considerably between inbred strains (Dunn, 1954). This suggests that the differences may be due to drift following the long separation of the lines. No pathological changes were noted.

3.4 Discussion

GH treatment increased the rate of weight gain and final weight in mice of all lines. The similarity of the response between lines in terms of the increase of weight gain and final weight relative to initial weight suggest that selection has not altered the ability to respond to GH. The effect of selection on response to GH might have been expected to be greatest in the lines selected for high and low body weight. Studies in other lines of mice selected for high and low body size indicated that the mice selected for smaller size were also less responsive to GH (Pidduck & Falconer, 1978). Other research has shown that GH concentration often does not show obvious correlations with differences in rates of weight gain and body size. Broiler chickens have lower levels of plasma GH than slower growing layer lines (Burke & Marks, 1982, Stewart & Washburn, 1983).

The general increase in tibia bone length in mice treated with rbGH indicates a long lasting effect of the GH on growth, as it increased bone growth. Some of the greater body weight observed in treated animals may be accounted for by their increased bone size and bone weight. In sheep a substantial increase in non-carcass components of the body, such as skin, fleece and gut was observed after GH treatment (Johnsson *et al*, 1987). Skin and gut may account for part of the greater weight observed in these treated mice.

Treatment with rbGH did not significantly affect fat content as measured by

gonadal fat pad percentage and dry weight percentage although there was generally a slight decrease. The results in this study may be due to species specificity with rbGH having a lesser effect on fat deposition in mice than it does in lambs (as human GH had no significant effect in pigs (Baile *et al*, 1983)). It might have been expected that the fat mice, having the greatest fat percentage, and hence potential for reduction, would have shown the greatest reduction in body fat after treatment with rbGH but this was not the case. This may be due to selection having altered the way GH affects growth and maintenance of fat deposits in the fat line.

The principal conclusion from these experiments is that mice from all the lines studied respond to treatment with rbGH by gaining weight fractionally, i.e. around 10 to 15 percent, faster. This growth is not solely due to an increased rate of gain of protein and is probably also due to greater growth of the bones, gut and skin. As the lines all reacted very similarly in terms of increase of final weight relative to initial weight, and of gain relative to initial weight it can be concluded that there is no difference between them in their response to exogenous GH. The mice also showed some evidence of a reduction in fat after treatment, which supports previous work showing that exogenous GH improves carcass quality by reducing fat percentage. It appears therefore that the differences in growth rate observed in these lines are possibly not due to differences in responsiveness to GH. Any involvement of GH in these genetic differences may lie in differences in synthesis or secretion.

The major effects were observed in the P-Lines. This suggests that further investigations could most profitably be carried out in the P-Lines. Possible lines of investigation include assaying GH levels in both the high and the low line, and an investigation of the effect of removing GH. It is possible that plasma levels of GH regulate the number of receptors available, in which case an increase in plasma GH levels would result in an increase in receptor levels. Thus the proportion of receptors available to respond to endogenous GH would be similar, regardless of plasma levels

of GH. If that is the case, removing GH could cause disproportionate effects.

Chapter 4
GH Assay and investigation of preweaning growth

4.1 Introduction

The high and low P-Lines do not differ in their response to injection of rbGH. This implies that either both the levels of endogenous GH and of its receptors have changed to the same extent, possibly due to GH regulation of receptor levels, or that the size difference between the lines is not due to differences in GH or its receptors. One way to distinguish these possibilities would be to assay GH in both lines at several ages. GH is produced in the pituitary and released into the blood for transport, thus GH concentrations in the blood or the pituitary could be assayed. Blood is easier to obtain, but GH levels in serum are cyclical in rats (Robinson and Clark, 1987) and humans (Phillips, 1987) and can vary considerably, e.g. by a factor of at least 10 in the female rat (Robinson and Clark, 1987). Similar variability may well occur in the mouse. This suggests that between line comparisons of single samples of serum would yield little useful information. Repeated samples would need to be taken over a period, then the resultant secretory patterns could be compared. However mice are too small to readily permit repeat sampling. It is possible that GH levels in the pituitary are more stable and analysis of these could give more information about differences between the lines. A large number of blood samples might identify substantial differences in GH levels between the lines.

The collection of pituitaries involves removing the head, so animals were killed by decapitation, which enabled collection of a terminal blood sample. Four age groups were sampled, 4 and 7 weeks of age, that is, the beginning and end of the age range for treatment with rbGH, and 5 and 10 weeks old, as other analyses have been carried out on these lines at those ages (Sharp *et al*, 1984, McKnight and Goddard,

1989). Indeed selection was based on measurements made on ten week old mice (Sharp *et al*, 1984, Bootland *et al*, 1991). Only males were investigated, as in the previous experiments with rbGH, to avoid the complications of sex linked differences.

Although mice generate GH from day 16 of foetal life (Slabaugh *et al*, 1982) GH does not seem to be necessary for body weight gain until 14 days after birth (Borrelli *et al*, 1989). Investigation of growth rate of the P-Lines from birth to weaning might identify differences in growth rate, which would imply GH was not involved in those differences. The average litter size at birth in the high P-Line is approximately twice that in the low line (Brien *et al*, 1984), that is in proportion to maternal weight. Any differences in birth weight suggests there may be *in utero* differences in growth rate due to individual effects, not maternal effects. It would be simple to weigh the litters which are being produced to provide mice for the GH assay but identifying individual pre-weaning mice is difficult, especially before the growth of fur. The methods available, such as toe clipping, involve injuring the animals and are not suitable in this case as the mice are to be used for further investigation later and toe clipping. However weighing a whole litter at once was possible, and was used to calculate the average weight of mouse as litter size was recorded.

There may also be a limiting effect on growth due to limits on the dam's supply of milk. In mice selected on body weight artificial adjustments in litter size found litter size was inversely correlated to weaning weight of pups (Hayes and Eisen, 1979). Any limitations of maternal milk production on preweaning growth in the low line should be relieved by fostering onto high line dams, which normally nurse larger litters. The crossfostering would not be expected to yield useful information about maternal effects on preweaning growth of high line mice as there are too many effects being altered.

4.2 Preweaning growth

4.2.1 Materials and Methods

4.2.1.1 Animals

The mice used were the offspring of matings of surplus P-Line stock. Preweaning weights were recorded on whole litters, including both males and females for the first and second litters. All second litters studied were carried while the dam was nursing the first litter. Third litters were produced from the same pairs and used in the crossfostering. For the first two litters, litters with more than 12 pups were culled to 12 at birth.

For crossfostering litters were standardised to 8 pups, the average for the low line. There were not enough litters born on any one day to cross foster litters born on the same day. Every other day the litters were taken. Eight pups were selected randomly from the low line, and rolled in sawdust from the sleeping area of a dam from the high line. She was then given the pups to foster. The rolling in sawdust was to add the scent of the foster mother's pups to the pups' own scent and hopefully improve the chances of successful fostering. This continued until all high line dams had a litter. The procedure was then repeated with high line pups and low line dams. Any high line pups remaining were killed.

All the mice in each litter were weighed together every morning, from birth (or fostering) to weaning at twenty one days of age. Any deaths that had occurred since the previous day were recorded. At weaning the mice were separated from the dam, identified by ear clips and moved into single sex cages. Mice from the second litters, and the crossfostered litters were then weighed daily until they reached 28 days of age.

4.2.1.2 Statistical Analysis

Mean mouse weight at birth for each litter was plotted against number of mice in that litter for each line separately and then birth weight was regressed onto litter size.

The weight of each pre-weaning litter was divided by the number of mice in the litter to find the average weight. Means and standard errors of these were calculated and plotted. Mean mouse weight was used rather than mean litter weight, because of the differences in litter size. The high line dams have a mean litter size of more than twice that of the low line dams, before culling to a maximum of 12 (Brien *et al*, 1984) for the figures. Means and standard errors were calculated using individual mouse weights after weaning. No allowance for line or treatment effects was made when calculating the means and standard errors.

Statistical analysis was performed on the preweaning weights using a mixed model least squares and maximum likelihood computer program (Harvey 1985), using a model with dam as random effect, nested within line, and line and litter status (first, second or crossfostered), as fixed, crossclassified effects. Line by treatment interactions were also tested for. Analyses of initial and final weights were carried out on their natural log transformations to remove scaling effects due to the substantial difference in size between the lines. Traits, other than initial weight, were also regressed on the natural log of initial weight. Relative gain was analysed as its logarithm, calculated for each animal as (natural log final weight) -(natural log initial weight) for the period in question (As in chapters 2 and 3)

4.2.2 Results

The results were obtained from ten families per line for the first litter and the second low line litter in the low line. Due to parental deaths, the results for the

second high line litter and for the litters which were crossfostered were obtained from eight families per line.

The regression of mean mouse weight at birth on litter size at birth was not significantly different from zero for either line. From Figures 4.1 and 4.2 it appears that most of the litters born fell within a limited range for litter size of 6 to 8 pups in the low line, 14 to 18 pups in the high line.

From Table 4.1 it appears that high line mice are approximately 1.4 times as heavy as low line mice at birth and there is a difference of almost a factor of two between the lines in litter size. High line mice are significantly heavier than low line mice at birth. At weaning there is no significant difference in weight between the lines, although the high line is still heavier (Table 4.1). Nor do the high and low line differ significantly in relative gain during preweaning growth (Table 4.1). This is also reflected in Figure 4.3 with the lines gaining at similar rates until three or four days before weaning. The increased rate of gain of body weight observed could be temporary, to investigate this the weights of the second litters and crossfostered litters were recorded for a week after weaning, until they reached four weeks of age. The increased rate of gain was observable throughout the extra week analysed (Figures 4.4, 4.5).

There is an apparent discrepancy between the values of the relative gain in Tables 4.1, and that which can be calculated from the initial and final weights given. The initial weight given has not been regressed onto itself, while final weight has been regressed onto initial weight. The relative weights have also been regressed onto initial weight. The method of calculating the relative gain involves the initial weight as well as final weight, so there will be different correction factor applied to relative gain, than to final weight, because of their regression on initial weight. This is similar to effects noted in chapter 3.

Figure 4.1

Plot of mean weight at birth of mice in a litter against the number of mice in a litter for the high P-Line. The regression of birth weight on litter size was not significantly different from zero.

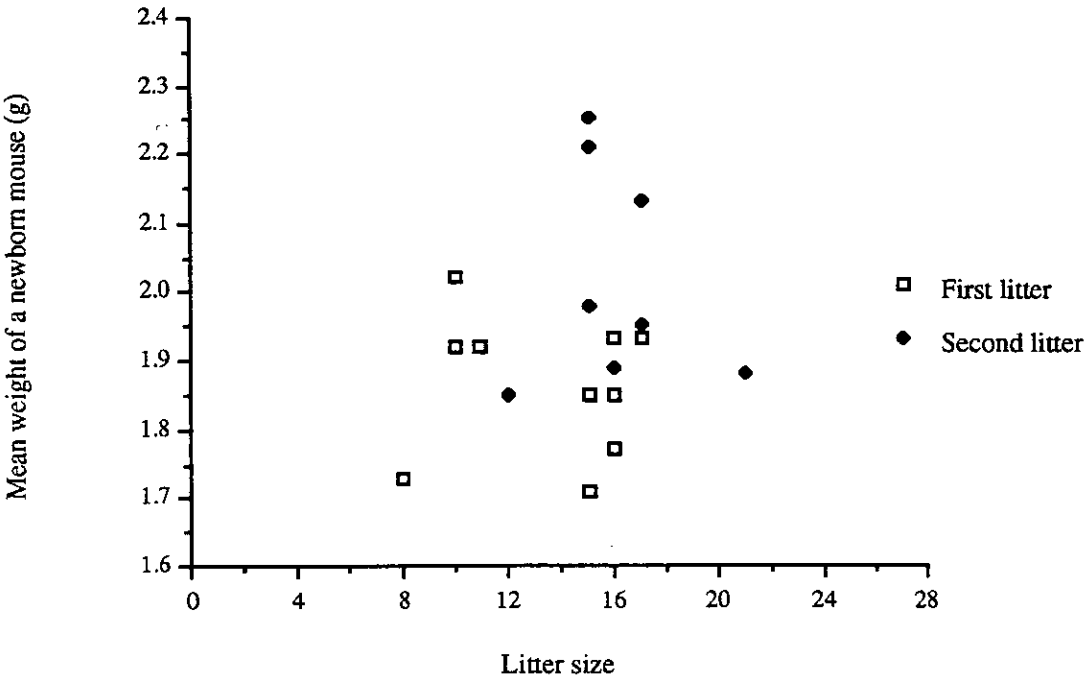


Figure 4.2

Plot of mean weight at birth of mice in a litter against the number of mice in a litter for the low P-Line. The regression of birth weight on litter size was not significantly different from zero.

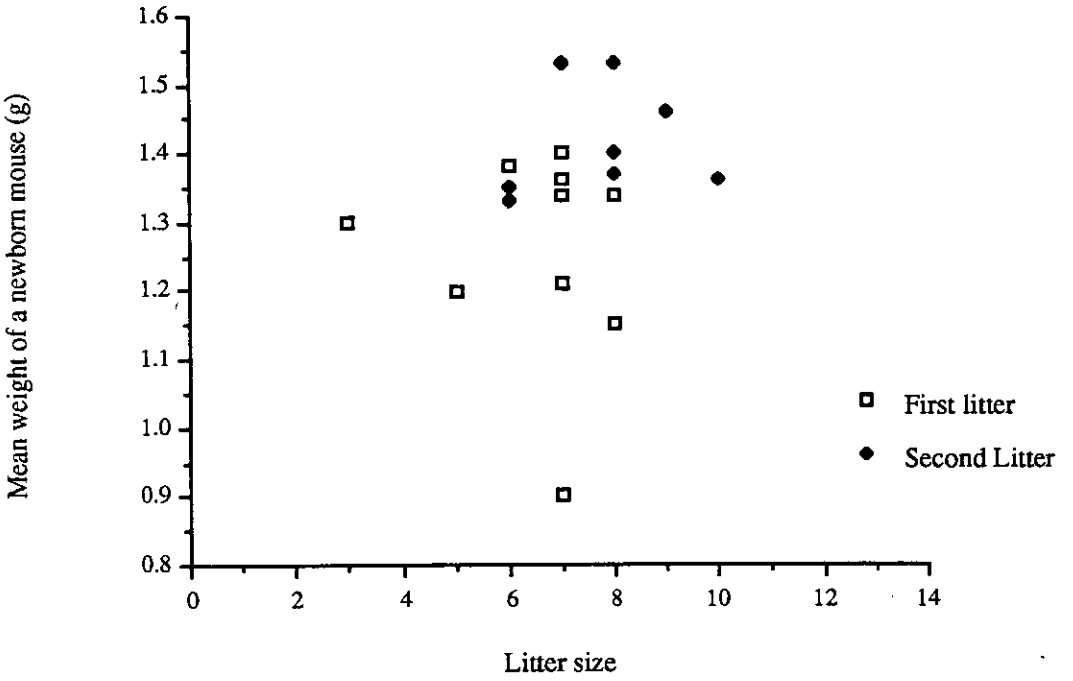


Table 4.1

Preweaning weights and gains of P-Line mice

	High line			Low Line					
	litter 1	litter 2	cross foster	litter 1	litter 2	cross foster	Significance line effects	Significance litter effects	S.E. means
(mean litter size)	(11.1)	(12.0)	(7.7)	(6.5)	(7.7)	(8.0)			
Ln(initial wt) (init wt (g))	0.609 (1.84)	0.709 (2.03)	0.711 (2.04)	0.236 (1.27)	0.342 (1.41)	0.391 (1.48)	**	**	±0.044
Ln(3 Week wt) (3 Week wt (g))	2.331 (10.29)	2.075 (7.96)	2.407 (11.10)	2.108 (8.23)	2.047 (7.74)	2.345 (10.45)	NS	**	±0.094
RG(week 1)	1.067	1.039	0.995	0.915	0.911	1.124	NS	NS	±0.064
RG(week 2)	0.488	0.324	0.533	0.385	0.349	0.423	NS	**	±0.052
RG(week 3)	0.292	0.231	0.395	0.325	0.303	0.316	NS	NS	±0.025
RG(Preweaning)	1.847	1.591	1.923	1.624	1.563	1.863	NS	**	±0.094

Ln, Natural Logarithm
RG, Relative gain, calculated as the difference between the natural logs of the initial and final weights of the period in question
NS, no significant difference between the groups being compared.
* $P<0.05$, ** $P<0.01$)

Figure 4.3

First litter. Mean daily weights of mice from birth to weaning. The mean weight of mice in a litter was found by dividing litter weight by the number of mice in a litter. Then the mean of those means was calculated and plotted. The error bars give the maximum standard error of the final mean.

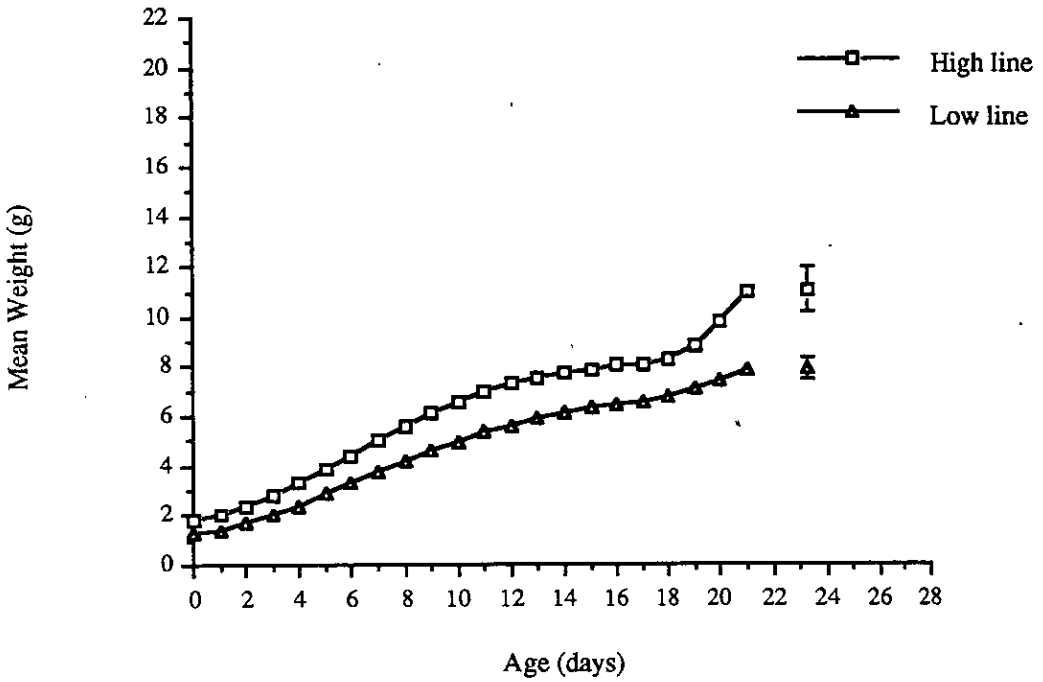


Figure 4.4

Second litter. Mean daily weights of mice from birth to 28 days. The mean weight of mice in a litter was found by dividing litter weight by the number of mice in a litter. Then the mean of those means was calculated and plotted. The error bars give the maximum standard error of the final mean.

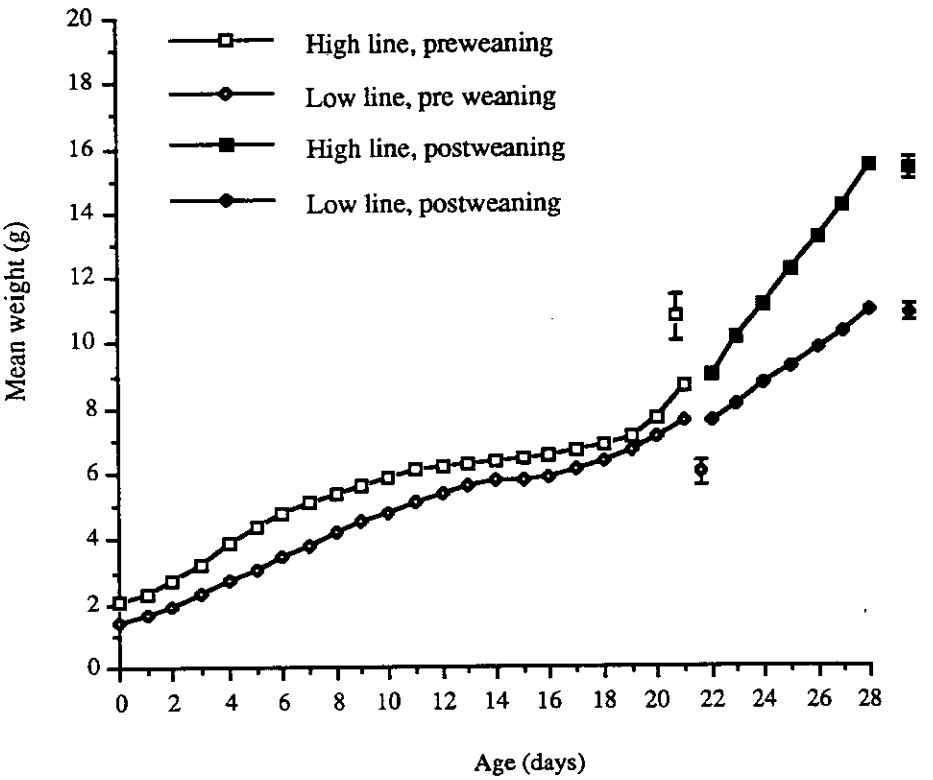
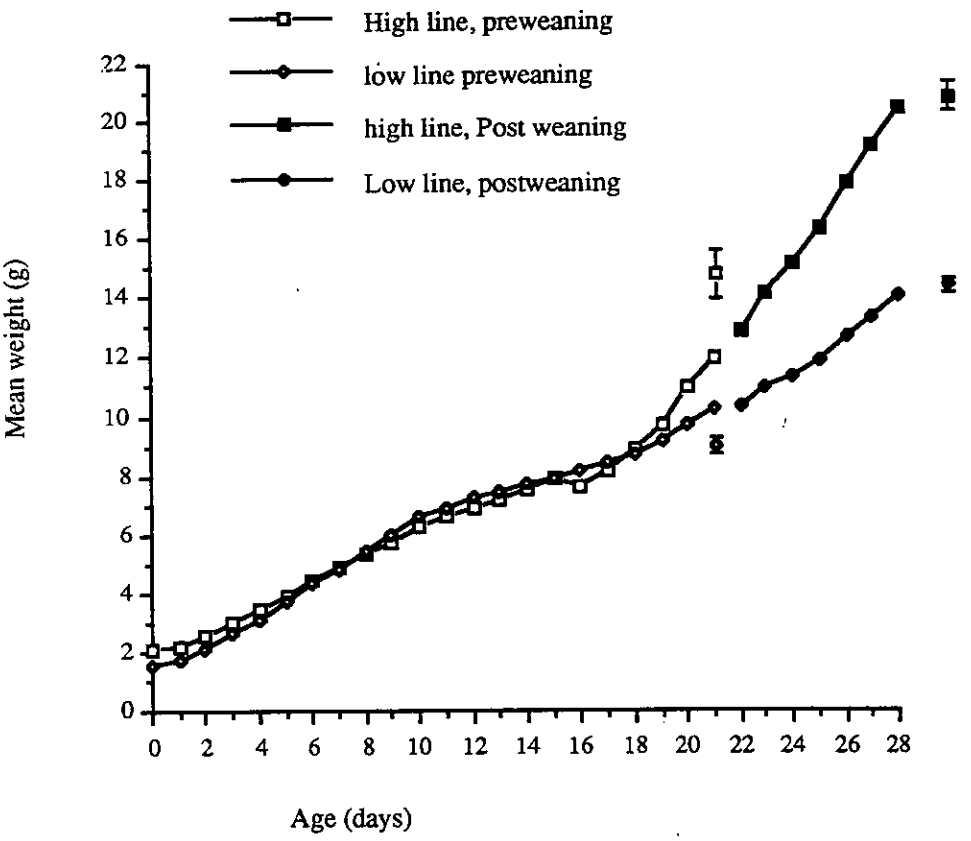


Figure 4.5

Crossfostered litter. Mean daily weights of mice from birth to 28 days. The mean weight of mice in a litter was found by dividing litter weight by the number of mice in a litter. Then the mean of those means was calculated and plotted. The error bars give the maximum standard error of the final mean. The line specified in the key is the genotype of the pups, not their foster mother.



There is a significant litter effect on three week weight, and on relative gain for the whole preweaning period. The second litter of both lines have a lower three week weight than the first litters or crossfostered litters, and a lower relative gain for every period except the first week (Table 4.1). Temperatures in the animal house were over 32°C during this period and the reduced growth may reflect the effects of heat stress on the pups or dams. Thus second litters should not be used as control to detect any effects of fostering low-line pups onto high line dams and vice versa. Low line pups fostered onto high line dams weighed approximately 26% more at three weeks of age (weaning), and have a relative gain for the the whole preweaning period approximately 15% greater, than low line pups from the first low line litter (which were nursed by low line dams) (Table 4.1). High line pups suckled by low line dams have a final weight approximately 8% greater, and an overall preweaning relative gain approximately 4% greater than that of the the first high line litter, which were nursed by high line dams. This suggests that low line pups grow faster when suckled by high line dams than low line dams. As the low line mice still increased the rate of weight gain at 18 days (Figure 4.3) food supply cannot be the only factor responsible for this increase in weight gain.

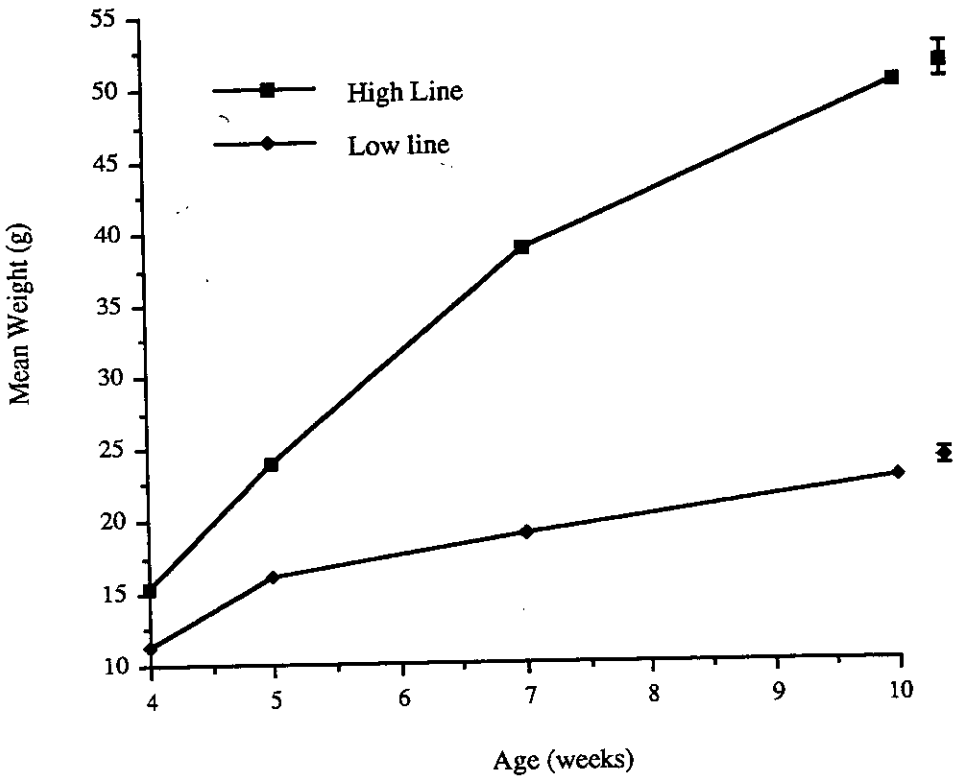
The difference in rate of gain between the lines continues until at least ten weeks of age (Figure 4.6). Low line mice weigh less at all these ages, and the rate of gain of weight of low line mice appears to show a greater reduction between 5 and seven weeks of age than that of the high line.

4.2.3 Discussion

The regression of birth weight on litter size at birth was not significantly different from zero for both lines which suggests that within a line factors other than litter size have more effect on birth weight than litter size (Figures 4.1, 4.2).

Figure 4.6

Mean weights of male P-line mice from 4 to 10 weeks of age. Error bars indicate the maximum standard error of the mean.



Only tentative conclusions can be drawn from these experiments as the second litter was *in utero* while the first was being suckled. The increased demands on maternal metabolism of simultaneous milk production and pregnancy maintenance may have reduced growth in the first or second litters, or in both. Also there were problems with regulating the temperature in the mouse house during the preweaning period of the second litter. This litter had a lower rate of gain during this period than the first litter, possibly due to maternal heat stress.

There was no significant difference between the lines in body weight at 3 weeks of age, or in relative gain at any point analysed, that is during the preweaning period. Given the significant difference between the P-Lines in four week weight noted in the previous chapter (Table 3.1) this is unexpected. The lines appear to start to diverge in body weight, at around 18 days of age, (Figures 4.3, 4.4, 4.5). The increased rate of gain of weight observed in both lines at this point is maintained after weaning. It is greater in the high line than the low line (Figures 4.4 and 4.5). GH appears to begin to affect the rate of gain at about 14 days of age in the mouse (Borrelli *et al*, 1989), and could be responsible for the observed increase in the rate of gain. However high line GH deficient *little* mice also show this growth spurt (Figures 5.6, 5.7) suggesting that GH is not primarily responsible for the increase in the rate of weight gain, although it is involved, wild type mice had a greater increase than *little* mice.

Mice appear to start to feed themselves at around 18 days of age, in that litters whose dam dies after that age are likely to survive, whereas before then they will die unless they can be fostered (Personal observation). It is possible that mice are limited in their growth by the food supply before weaning, and that growth increases once they are no longer dependent on milk from the dam. The crossfostering should check this, if food supply is limiting growth, low line mice being suckled by a high line dam should have a greater weight at the later preweaning ages than those suckled by a low line dam. This does appear to have happened, if the low line mice fostered by high

line dams are compared to the first litter of low line pups, which were suckled by low line dams (Table 4.1). Hence food restrictions appear to have some limiting effect on growth, at least in the low line. Differences between the corresponding litters in the high line are smaller (Table 4.1). As the size of litters of high line pups was reduced in the cross fostering experiment as well as the pups being nursed by a low line dam instead of a high line dam it is not practicable to isolate the effects of cross fostering. Hayes and Eisen (1979) altered litter sizes in lines of mice selected on body weight by culling and by within line fostering. They found that weight at weaning was inversely correlated with litter size. Similar manipulations of litter size in the P-Lines should identify effects of food supply on preweaning weight gain in the high line.

From Table 4.1 it appears that high line mice are approximately 1.4 times as heavy as low line mice at birth. The average litter size in the high line is approximately twice that in the low line (Brien *et al*, 1984). Thus the average total litter weight at birth in the high line is approximately three times that of the low line. Adult mice differ in body weight by a factor of approximately two (Sharp *et al*, 1984). This suggests that the difference in litter weights at birth may not be explained entirely by factors correlated to maternal body weight and that the pups differ in feotal growth rate. This could only be tested by embryo transfer to put embryos of different genotypes in the same maternal environment.

4.3 Assaying Plasma GH levels

4.3.1 Materials and Methods

4.3.1.1 Animals

The mice used for the assays were the males from the first and second litters described in the growth measurements above. Mice from the first litter were assigned

randomly within family to be killed at either 5 or 10 weeks of age. Similarly, mice from the second litter were assigned randomly within family to be killed at either 4 or 7 weeks of age. This particular division was chosen for reasons of time and space. Unfortunately the mice killed at 5 weeks of age were not weighed prior to death due to an oversight. Their litter mates were weighed before being killed at 10 weeks of age. The males from the second litter were all weighed at 4 weeks of age and then the survivors were weighed at 5 and 7 weeks old.

4.3.1.2 Collection of samples

The mice were weighed and then killed, on the day they reached the relevant age, with a pair of dog tail clipping scissors, by decapitation. The blood from the head and body was passed through a funnel to a eppendorf on ice. The funnel had been dipped into a solution of heparin and excess solution drained off before use. The heparin solution was 100 I. U. /ml in 0.9% saline, using grade 1 heparin sodium salt extracted from porcine intestinal mucosa, obtained from Sigma. The head was then cut open using suitable dissecting scissors along a line passing just below the ears to the eyes. The upper half of the skull was then lifted with a pair of blunt forceps. This generally also lifted the brain. Otherwise the brain was then carefully lifted using blunt forceps. The pituitary could then be removed from its location in a small depression in the base of the skull and placed on ice. The pituitaries from all the males of one family were placed in the same container. Once all the males from one family had been dissected the pituitaries were dropped into liquid nitrogen. The blood was then spun down and the plasma transferred to fresh eppendorfs before both plasma and pituitaries were stored in a -60 freezer. Plasma from full sib litter mates was pooled for low line mice at 4 and 5 weeks of age due to the small body weight, and hence blood volume, of these mice. Due to limitations in time and equipment only the plasma samples were assayed.

4.3.1.3 Growth Hormone Assay

The assay was carried out at the A.F.R.C, Institute of animal production and genetic research, Roslin by Dr C. Goddard.

The Radioactive GH used as label was prepared at Roslin, by the method shown (Mouse GH Iodination, Table 4.2) . After iodination the immunologically active radioactive GH was purified by being run through a PD10 fractionation column. The fractions taken to be purified in the next column are shown in Figure 4.7 . These fractions were run through a G100 column, and those fractions from this column which made up the third peak (Figure 4.8) were taken for the assay as peaks one and two represent trimeric and dimeric GH respectively.

The Immunoassay is as shown in Table 4.3 , Mouse GH immunoassay. The principal of the assay was to take a known volume of the sample, dilute it with assay buffer to a constant volume and mix with antiserum to GH. After incubation radioactive iodinated standard GH was added, and incubated. This bound to immunoglobulins which had not yet reacted with GH. A second antibody, this time to the anti-GH, was added to cross link the antiserum-GH complexes. This caused them to precipitate out and the resultant suspension could then be centrifuged, to give a pellet of antibody-antiserum-GH complexes and leave the unbound GH in the supernatant. The number of molecules of radioactive GH bound was inversely proportional to the concentration of GH in the sample. As only bound GH was precipitated out the amount of radioactive GH, and hence counts per minute, in the pellet at the end of the procedure was inversely proportional to GH concentration in the sample. A standard curve was generated using samples of known concentration, and used to calculate the GH concentrations in the unknown samples.

Table 4.2

Mouse GH Iodination

1) 85 µl PO_4 Buffer (300 mm) / l
2) 10µl NaI^{125} (Amersham)
3) 1 Iodobead (P.S.Wariner)
4) Incubate for 5 seconds at room temperature
5) Add 5µg (10 µl) mGHdissolved in a further 20µl PO_4
6) Incubate for 10 minutes at room temperature
7) Add 400 µl PBS/T20/azide PBS 10mm pH7 T20 0.1% v.v, Na azide 0.02% v.v.
8) Run on a PD10 column and collect 30 fractions
9) run the peak through a G100 column
10) store at -20° C in aliquots containing 1.55 x 10^6 cpm / 500µl

Columns from NIADDK, NIH, Bethesda, Maryland, USA.

mGH from Dr A. S. Parlow, Pituitary hormones and antisera centre, Torrance, California, USA.

Figure 4.7

Counts per minute found in each fraction of the first purification stage in the preparation of radioactive iodinated GH. The solution is eluted through a PD10 gel filtration column. The first peak is the peak containing iodinated GH, the second is unbound iodine washing out.

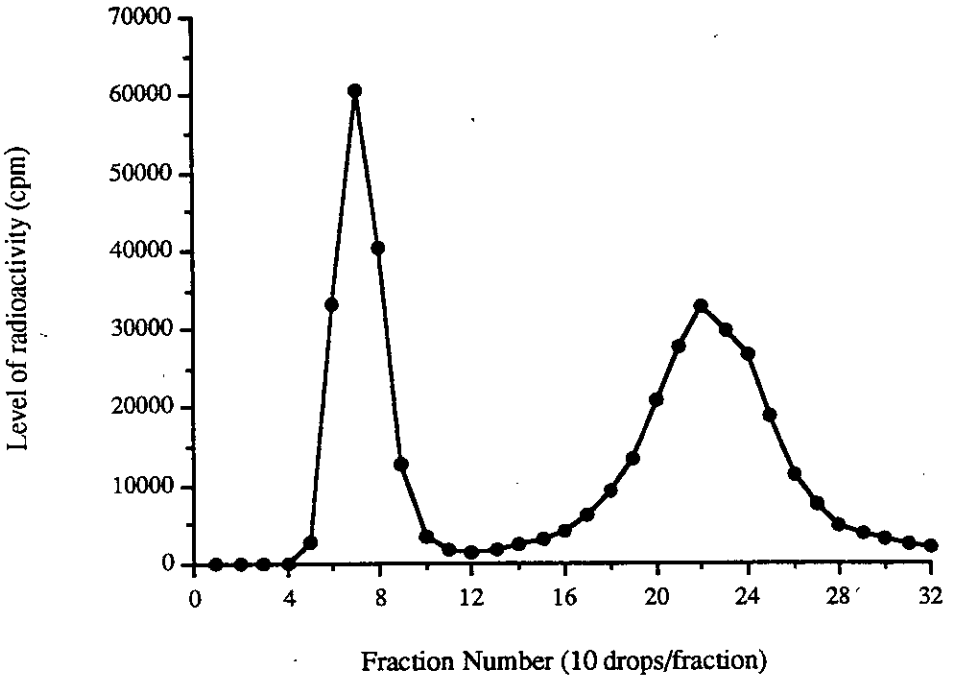


Figure 4.8

Counts per minute found in each fraction of the second purification stage in the preparation of radioactive iodinated GH. The solution is eluted through a G100 gel filtration column. The first peak contains GH trimers, the second GH dimers, and the third GH monomers, which are the only ones useable in the assay.

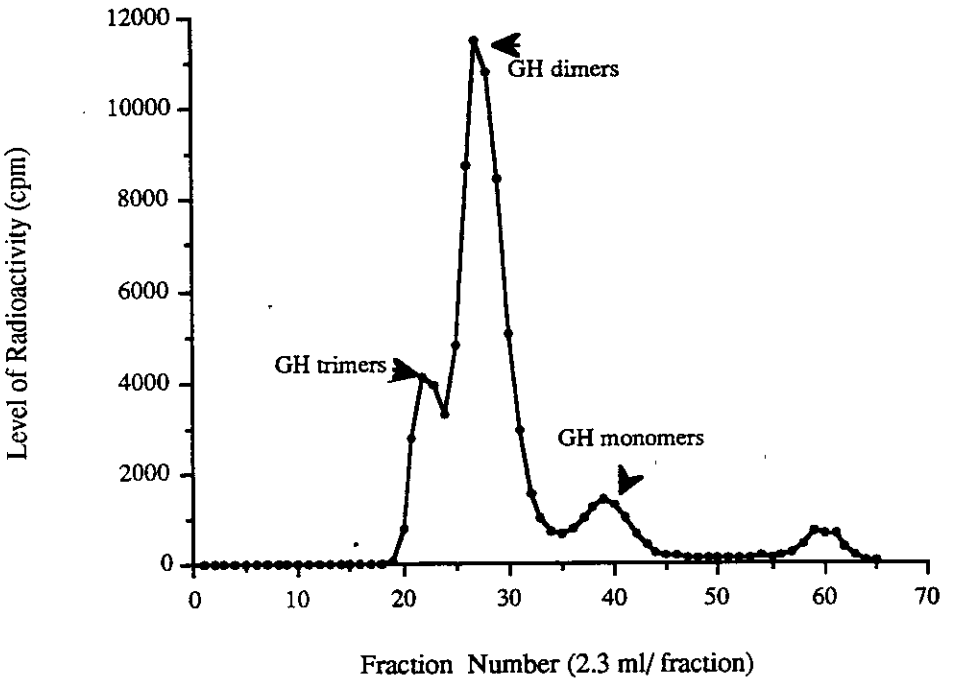


Table 4.3

Mouse GH Immunoassay

Day 1

Assay Buffer	PBS tablets	10
	2% Na azide	10 ml
	Tween 20	1ml

Dissolve these in 985ml distilled water. store at 4 C

Assay tubes	Total Counts (TC)
	Non Specific Binding (NSB)
	Binding at zero dose (B_0)
	Standards
	QC high
	QC Low
	samples

These are usually in duplicate or triplicate.

Antiserum

Monkey anti-rat GH serum (NIADDK-rGH-S-5) (From NIADDK, NIH, Bethseda, Maryland). This is stored in 100µl aliquots (in assay buffer) in the -80°C freezer. Each Aliquot is a 1:500 dilution of the original serum.

(a) Make up 1 aliquot to 3.2ml,(6.4ml or 12.8 ml) with assay buffer to give an initial dilution of 1:16 000 (1:32 000 or 1:64 000) (of the original)

(b) Add 100 µl of the dilute antiserum to all tubes except TC and NSB

(c) add 200 µl of assay buffer to the NSB tubes

Quality Controls

High and low quality controls are stored in the -80°C freezer.

Standards

The stock solution is stored in the -80°C freezer. This was made from an aliquot of 5µg/10µl by making up to 2.5ml. The sloution is 2µg/ml and is stored in 25 aliquots each containing 200ng/100µl. Prepare solutions for the standard curve as follows.

Table 4.3 cont

1) Take 1 aliquot of 200ng/100µl and add 0.9ml assay buffer.
This is **solution A** at a concentration of 200ng/ml

2) Take 100µl of solution A and Add 0.9ml assay buffer.
This is **solution B** at a concentration of 20ng/ml

3) Prepare all the standards according to the following table

Volume of A (µl)	Assay Buffer (µl)	Final Standard (ng/tube)
250	nil	20.0
125	125	10.0
93.75	156.26	7.5
62.5	187.5	5.0
31.25	218.75	2.5

Volume of B (µl)	Assay Buffer (µl)	Final Standard (ng/tube)
250	nil	2.0
125	125	1.0
62.5	187.5	0.5
31.25	218.75	0.25
15.625	234.375	0.125

Put 100µl of each dilution into each of two standard assay tubes to give duplicates of the final concentration as above.

Samples

Samples should ideally be mouse serum, plasma, or clarified pituitary homogenate.

Add 100µl of sample/buffer mixture to the appropriate tubes.

The assay should now be as follows:-

	Antibody	Assay Buffer	Sample	Standard
TC	0	0	0	0
NSB	0	200	0	0
B_0	100	100	0	0
STDS	100	0	0	100
QCH	100	0	100	0
QCL	100	0	100	0
SPLS	100	0	100	0

Mix the tubes carefully and incubate for 24hr at 4°C

Table 4.3 cont

Day 2

Add the [^{125}I]-mGH
When a new batch of label is made the amount required to give 40% binding with 0 dose of exogenous GH is determined and noted.
Dilute the label in assay buffer to give the concentration which gives 40% binding and add 100µl to all the tubes.

Mix the tubes carefully and incubate for 24h at 4°C.

Day 3

Second antibody
Sheep anti-Human IgG (SAPU) 1ml aliquots (From the Scottish Antibody Production Unit, Law Hospital, Carluke)

Make up a solution in assay buffer containing anti-human IgG (1:20). Add 100µl to each tube except TC, mix, and incubate overnight at 4°C.

Day 4

Centrifuge at 2500 - 3500 rmp for 15 minutes

Make up 10% starch solution and add to each tube except TC, and centrifuge at 2500-3500 rmp for 15 minutes

Aspirate the supernatant very carefully and count the pellet on the gamma counter linked to Assay ZAP

4.3.1.4 Statistical Analysis

Estimates of likely GH levels in the plasma were taken from the literature and concentrations for the standard curves chosen to enable measurement of values in that range. This range underestimated the actual concentrations, so many values for which all that was known was that they were greater than 400ng/ml were found. This cut off in values gave a sample distribution that was not normal. Consequently it was decided to analyse the data using the Mann-Whitney rank order test which compares the rank order distribution of two sample populations. First the median of each is found, in a normally distributed populations the median would be the same as the mean. However the median is less influenced by the presence or absence of extreme values than the mean. Then both sets of data are ranked together and a t-test performed on the rank values of the two populations, to determine if distribution of rank values is the same in both populations. Values which tie are all given the mean rank value, for example if there three identical values which could be given ranks 3,4, or 5, they would all be given rank 4. The test was carried out using the Minitab statistical package. For the purpose of analysis values for which only the minimum of a range was known were recorded as that minimum. The results of the analysis are shown in Table 4.4.

Some samples were lower in volume than required for the assay. A known volume of these was taken and diluted up to the same volume as the rest of the samples. This meant that concentrations, in the sample, of greater than 400ng/ml could be accurately measured because a diluted specimen was being assayed. Low line mice are smaller than high line mice, and have a lower blood volume. Thus the low line had a higher proportion of small sample volumes than the high line. This will have distorted the analysis by giving the low line more high rank values than would have been case if all samples had been the same size. To check the importance

of this possible bias the Mann-Whitney test was repeated with all values greater than 400ng/ml replaced by 400ng/ml. The results of this analysis are shown in table 4.5.

GH concentration was also plotted against rank number to give a visual indication of the differences between the lines and the different ages. For the purpose of the figures rank numbers were assigned randomly in the event of a tie (Figures 4.9 to 4.12).

4.3.2 Results

The between line comparisons show that Low line mice had a higher median concentration of Plasma GH at all ages sampled except for 10 weeks (Table 4.4) . This difference is significant even after all values greater than 400ng/ml have been replaced by 400ng/ml to avoid upwards bias for the low line because more of its samples were assayed after dilution (Table 4.5) . The difference between the lines appears to reduce with age (Table 4.4, 4.5).

The proportion of samples with concentrations greater than 400ng/ml (known or otherwise unknown) rose with age. The fractions of samples with greater than 400ng/ml were approximately, 15%, for four week old mice, 40% for 5 and 7 week old mice, and 57% for 10 week old mice (Figure 4.7 to 4.10) . This would suggest that GH concentrations increase with age.

The large number of samples with GH concentrations greater than or equal to 400 ng/ml explains why this is the commonest median value found (Tables 4.4, 4.5).

4.3.3 Discussion

The results indicated that Plasma GH concentrations are lower in the mice selected for high body weight than in those selected for low body weight. This would

Table 4.4

Median GH concentrations in ng/ml for the High and Low P-Line mice at several ages. The Mann -Whitney rank order test (minitab) was used to test the null hypothesis that the two populations did not differ in Plasma GH concentration. Concentrations measured as '≥' a value recorded as the value, other concentrations as measured.

	Low Line median (ng/ml)	High line median (ng/ml)	Significance of difference
4 week old	233.8	94.2	**
(number of samples)	(12)	(35)	
5 week old	486.0	156.4	**
(number of samples)	(13)	(31)	
7 week old	≥400.0	219.0	**
(number of samples)	(17)	(29)	
10 week old	≥400.0	≥400.0	NS
(number of samples)	(16)	(29)	

** $p < 0.01$, * $p < 0.05$, NS non significant

Table 4.5

Median GH concentrations in ng/ml for the High and Low P-Line mice at several ages. The Mann -Whitney rank order test (minitab) was used to test the null hypothesis that the two populations did not differ in Plasma GH concentration. All concentrations measured as over 400 ng/ml altered to 400ng/ml.

	Low Line median (ng/ml)	High line median (ng/ml)	Significance of difference
4 week old	233.8	94.2	**
(number of samples)	(12)	(35)	
5 week old	≥400.0	156.4	*
(number of samples)	(13)	(31)	
7 week old	≥400.0	219.0	*
(number of samples)	(17)	(29)	
10 week old	≥400.0	≥400.0	NS
(number of samples)	(16)	(29)	

** $p < 0.01$, * $p < 0.05$, NS non significant

Figure 4.9

GH concentration in plasma samples from 4 week old high and low P-Line mice. The assay could not measure concentrations greater than 400 ng/ml, hence this is a minimum value. Some values higher than this were recorded from samples with lower volumes than the 200 µl required, which were consequently diluted to give a sufficient volume. Samples from both lines were placed in rank order of increasing concentration. Where samples from different lines had the same recorded concentration, rank orders were chosen at random.

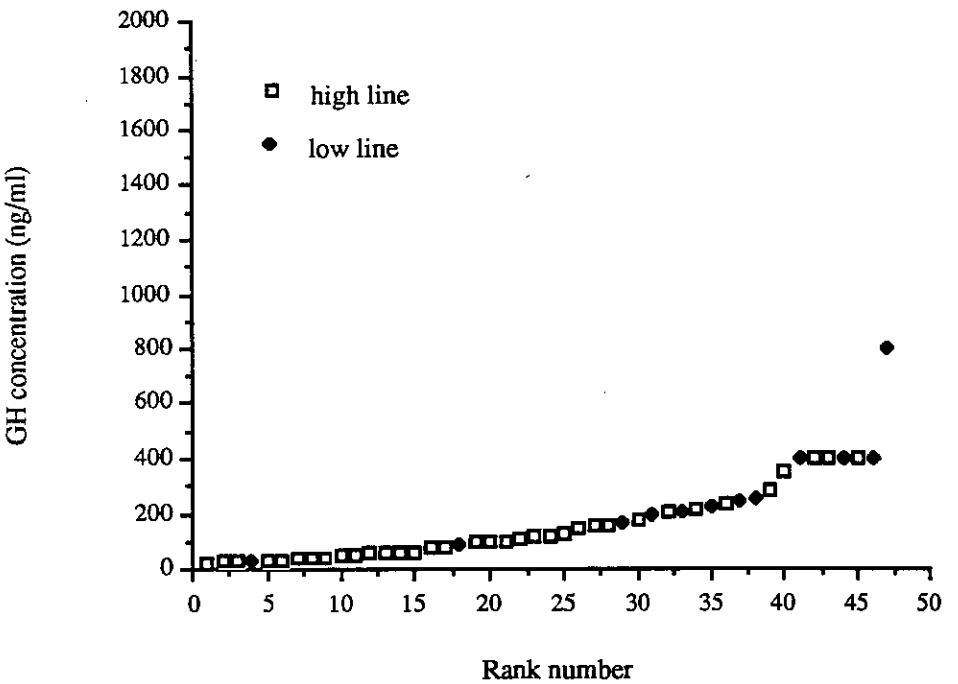


Figure 4.10

GH concentration in plasma samples from 5 week old high and low P-Line mice. The assay could not measure concentrations greater than 400 ng/ml, hence this is a minimum value. Some values higher than this were recorded from samples with lower volumes than the 200 µl required, which were consequently diluted to give a sufficient volume. Samples from both lines were placed in rank order of increasing concentration. Where samples from different lines had the same recorded concentration, rank orders were chosen at random.

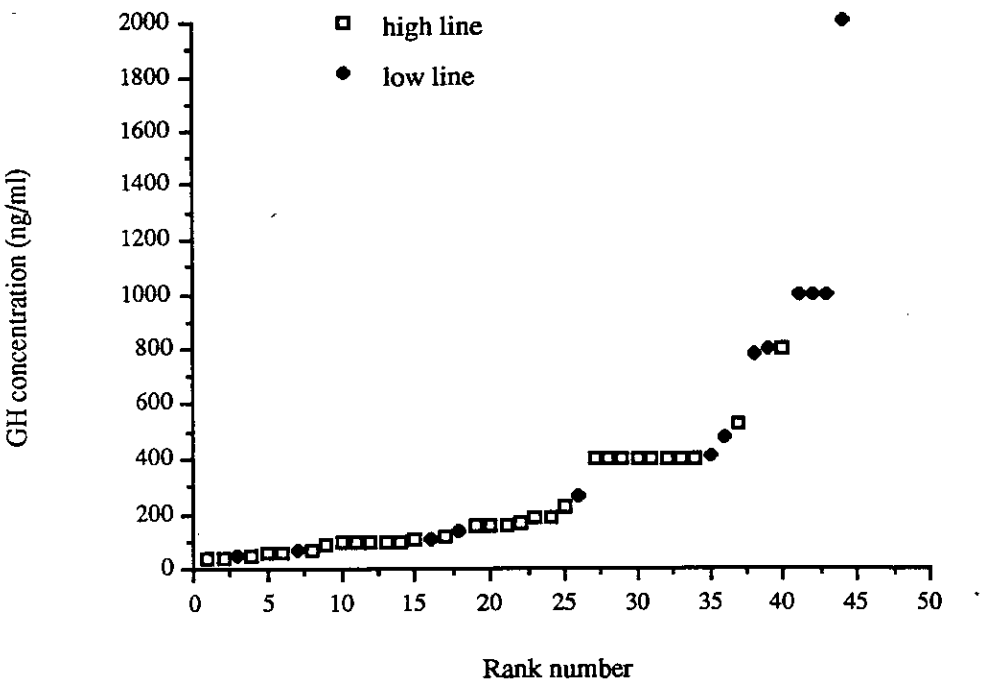


Figure 4.11

GH concentration in plasma samples from 7 week old high and low P-Line mice. The assay could not measure concentrations greater than 400 ng/ml, hence this is a minimum value. Some values higher than this were recorded from samples with lower volumes than the 200 µl required, which were consequently diluted to give a sufficient volume. Samples from both lines were placed in rank order of increasing concentration. Where samples from different lines had the same recorded concentration, rank orders were chosen at random.

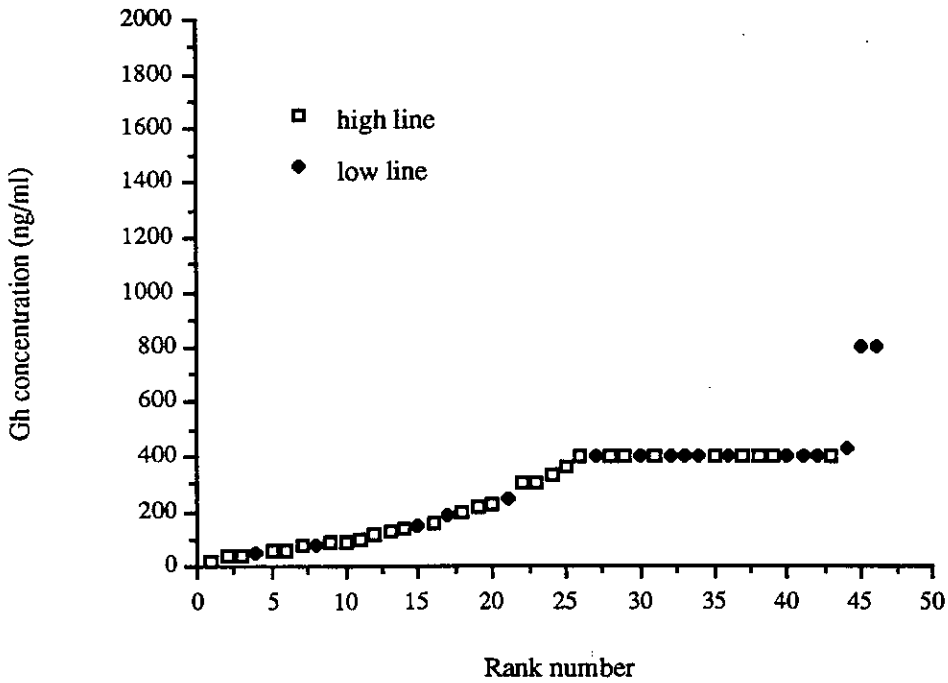
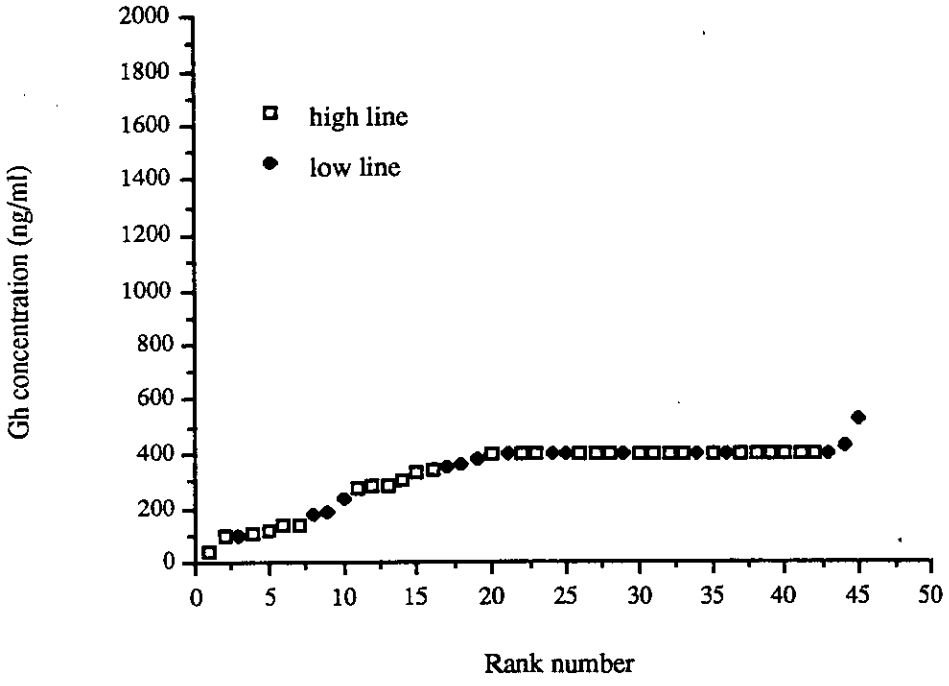


Figure 4.12

GH concentration in plasma samples from 10 week old high and low P-Line mice. The assay could not measure concentrations greater than 400 ng/ml, hence this is a minimum value. Some values higher than this were recorded from samples with lower volumes than the 200 µl required, which were consequently diluted to give a sufficient volume. Samples from both lines were placed in rank order of increasing concentration. Where samples from different lines had the same recorded concentration, rank orders were chosen at random.



agree with work in high growth mice (due to a major gene) which found that they had lower levels of plasma GH and higher levels of plasma IGF-I than unselected controls (Medrano, *et al*, 1991) and from work in chickens which found that broiler chickens had lower plasma GH levels than egg layers (Burke and Marks, 1982). As the lines show divergent growth from shortly after the point at which they become responsive to GH, and this is the age at which IGF-I secretion becomes GH inducible (Mathews, *et al*, 1988) the growth curves shown in Figures 4.4 and 4.5 are consistent with the suggestion that the high P-Line mice have higher levels of IGF-I than the low line. It is not conclusive since the increased growth at day 18 could also be explained by mice feeding directly on pellets rather than on milk. However work on these lines has already indicated that they do not differ significantly in basal IGF-I levels at 5 weeks old, that is, during the period of rapid growth. Basal levels do differ significantly between the lines at 10 weeks of age, with the high line having higher IGF-I levels. (McKnight and Goddard, 1989). This suggests that selection in the P-Lines has not produced a situation analogous to that found by Medrano *et al* (1991).

Although the results are suggestive, no concrete conclusions can be drawn from these results, primarily because the assay as used did not measure high enough concentrations. Also the samples had been stored in a -60°C freezer for three years waiting for the assay to be developed which may have affected the samples. Apparently the main problem in developing the assay was using the material from the first and second elution peaks from the G100 column in the assay. These contained trimers and dimers of radioactive GH, which did not bind to the antiserum (Chris Goddard, Personal Communication). It would be useful to repeat the experiment with a different range of dilutions to discover if similar results can be obtained. Such a repeat assay might also show whether GH levels drop with age as is normally the case (Slabaugh *et al*, 1982).

Chapter 5
Introduction of *little* dwarfism into the P-Lines

5.1 Introduction

The high and low body weight P-Line mice responded similarly to injections of rbGH (chapter 3) but appeared to differ in plasma concentrations of GH with the high line having lower concentrations than the low line at 4, 5 and 7 weeks of age (Chapter 4). Plasma IGF-I levels are not significantly different at 5 weeks, although the high line has more IGF-I at 10 weeks (McKnight and Goddard, 1989). It is possible that that the lines differ in responsiveness to IGF-I levels. Transgenic animals with high concentrations of GH only show high rates of growth after IGF-I has become GH inducible (Mathews *et al*, 1988) and mice become responsive to exogenous GH at approximately 14 days of age (Borrelli *et al*, 1989). This suggests that the increase in rate of weight gain observable in both lines at approximately 18 days of age (Figures 4.3 to 4.5) may be due to IGF-I release becoming GH inducible. The difference in the magnitude of the increase between the lines may be related to differences in responsiveness to IGF-I. This could lead to the proportional response to exogenous GH noted earlier.

GH release is pulsatile and the difference in the pattern of release between males and females appears to be responsible for part of the sex related differences in growth rates and adult body weights (Robinson and Clark, 1987). Such differences in secretory pattern might be involved in the size difference between the lines but would not necessarily be observable with the assay, as only one sample was taken per mouse. Neither would response to exogenous rbGH in the trials I carried out earlier necessarily differ between the lines as the timing of the injections did not mimic a natural pattern of secretion.

Removal of GH would block that fraction of IGF-I secretion which is GH inducible. It would also prevent the expression of differences in the pattern of GH release. Consequently if either, or both, are involved in the difference in growth and final body weight between the lines removal of GH would reduce the difference.

One way of removing growth hormone would be to backcross a gene for disrupting GH metabolism into the lines. This has already been successfully carried out with the backcrossing of hypopituitary dwarfism (*dw*) into lines of mice selected for high or low body weight (Pidduck and Falconer 1978); animals with this mutation lacks GH and prolactin and have reduced levels of thyrotrophic hormone. Their study found mice selected for high growth rate grew faster than those selected for low growth rate even in the absence of GH, i.e. in the dwarfs. However the reduction in growth rate as a result of substituting the *dw* gene was greater in the large strain than in the control or unselected strains. This indicated that GH was involved in the differences but was not the sole factor (Pidduck and Falconer, 1978). Similar results might be obtained with the P-Line mice. The dwarfs would also provide a GH free stock in which to test further the effects of rbGH. It is possible, for example, that a difference in responsiveness would be detectable without the masking effects of endogenous GH.

5.1.1 Assessment of the sample size required

The number of mice needed were calculated by the method used previously in chapter 2. The following assumptions were made. injection of GH into *lit/lit* mice, would give increases in final weight similar to those from previous work, that is 2 or 3 g. It was also assumed that untreated *little* mice would have a final weight at the end of the experiment of 10g. From previous work the standard deviation of the mean male mouse weight was 0.4g (with n=15, Table 3.2). If the number of *lit/lit* bred were small and females had to be used as well as males the variability would be

greater, so a population standard error of 1g was assumed.

Thus to identify a difference between the means, δ of 2g (or 20%) with probability , P=0.95, at a significance level 0.95 and a standard error between population means, σ, of 1g (or 10%) using a two tailed test a sample size, n, was needed where

$$n = \frac{M \times 2 \times \sigma^2}{\delta^2}$$

where M is a multiplier related to the significance level and probability of detection required. In this case M=13.0 (table 6.14.1, p104, Snedecor and Cochran).

so $n = (13 \times 2 \times 10^2)/20^2$
$n = 6.5$

Rounding up, and adding one for safety gave a sample size of 8 per treatment group.

To generate this number of mice a mean litter size equivalent to that of the low line was assumed. This was reasonable, as although the number of pups in live born litters was greater in the high line, the high line had greater problems of infertility. This gave an average litter size of six pups. Heterozygotes would have been mated to produce the *lit/lit* mice for experiment, giving the probability of any one pup being *lit/lit* as 1/4. Thus the minimum number of matings required to produce 16 mice was 16/(6 times 0.25), which rounded up to 11 matings.

The males to be used had already been test-mated to identify which were heterozygotes, the females were of unknown genotype. These factors had to be included in the calculation of the number of matings needed. The assumption of a litter size of six pups was made, as above. A female, sired by a known heterozygote onto a wild type dam, had a 50% chance of being a heterozygote. However testing

would not have identified even all females which produce litter for by chance there may have been no *lit/lit* pups born to a heterozygous dam. The chance that a pup born to a pair of heterozygotes was of normal phenotype is 3/4. The chance that all six in a "standard" litter were phenotypically normal was $(3/4)^6$. Thus the chance that an unknown female was shown after mating to be *lit/+* was

$$C_h * (1-C_f)$$
$$= 0.5 * (1-(3/4)^6)$$
$$= 0.41$$

Where C_h is the probability that the female is a heterozygote and C_f is the probability that the female was a heterozygote and was not identified as such.

Thus in order to have 11 heterozygote by heterozygote matings to produce the *lit/lit* mice for measurement and GH treatment, 11/0.41= 27 (after rounding) matings had to be set up between heterozygous males and unknown females. This assumed no infertility problems, and, so far as possible, more matings were used. Assuming half of the mice born were female the 27 females needed for these mice could be generated from 9 matings with average litter size of 6 mice. Thus each backcross generation should have contained at least 9 usable matings where possible.

5.2 Materials and Methods

The backcrossing was started using 6 *lit/lit* mice on a C57/BL6J background, from the stocks normally maintained in the mouse house. They were 3 adult females, which had already had one litter each, and 3 adult males which had not previously been mated.. The P-Line mice used throughout the backcrossing program were surplus stock that had failed to be selected. Infertility was known to be common in *lit/lit* males (Eicher and Beamer, 1976). This infertility was not present in *lit/lit* mice expressing a growth hormone transgene (Hammer, Palmiter and Brinster, 1984) which suggested that treatment with GH might improve fertility. There was also a

three to four fold size difference between *lit/lit* males and high P-Line females. Female *lit/lit* mice grow with pregnancy, so the difference was less pronounced (Beamer and Eicher, 1976). To increase the size and, hopefully, improve the fertility of the *lit/lit* males they were treated with rbGH at 9μg rbGH/ g body weight/ day, injected subcutaneously shortly before the start of the dark period, for 21 days. This was the same regimen as that used to treat normal P and F Line mice.

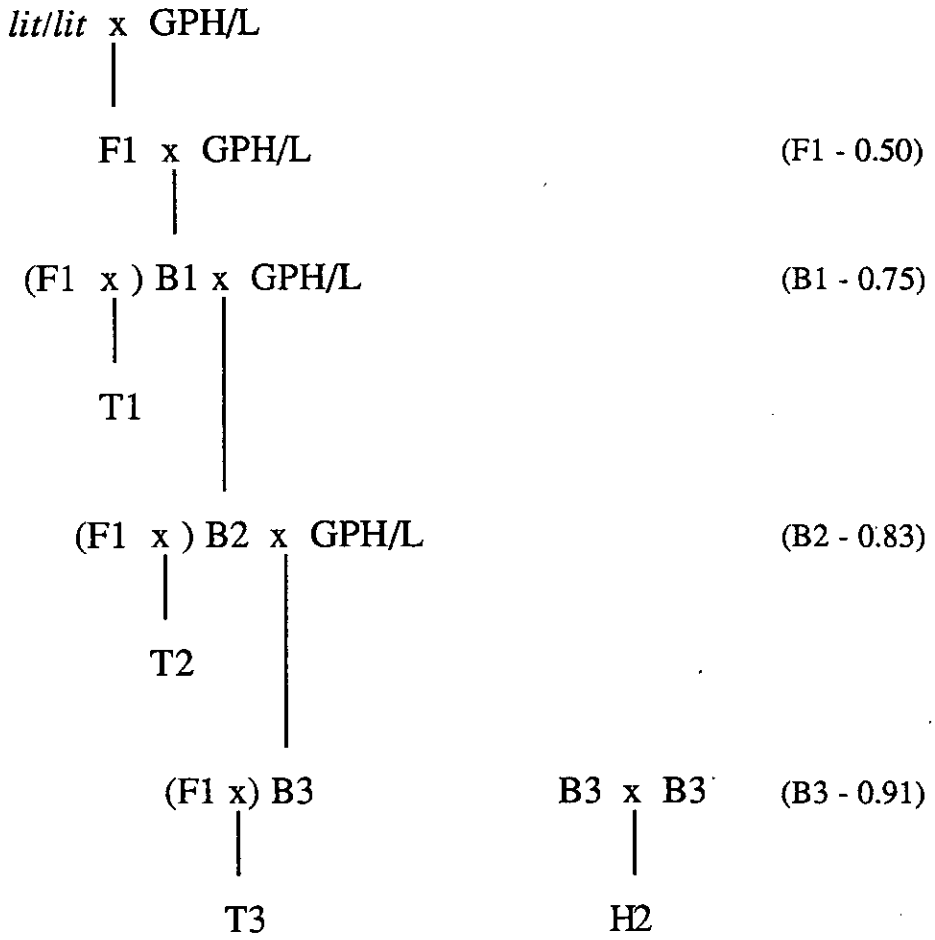
Two *lit/lit* females were mated to a high P-Line male, the third to a low P-Line male, while two *lit/lit* males were mated to a low P-Line female, the third to a high P-Line female. The largest, after GH treatment, of the 3 males was chosen to be mated to the high line female, however he was still less than half her size. All low line matings successfully produced offspring as did the two high line male by *lit/lit* female matings. The high line female by *lit/lit* male mating did not. Consequently there will be Y chromosome material from the C57 black 6J background in the low line, but not in the high line. This is unlikely to have affected results due to the limited amount of genetic information coded on the Y chromosome.

All offspring from the first litters of these matings were used in the first backcross matings. Second and subsequent litters were kept to provide a source of known heterozygous females for test-matings. For the backcross each female was mated to one P-Line male and each male harem mated to two or three P-Line females. This maximised the number of litters. There were 10 matings for each line, all produced a litter in the high line, one proved infertile in the low line.

The general pattern of the backcrossing, up to the third generation, is shown in Figure 5.1 . Males were chosen randomly within family to be test-mated to a known heterozygous female, at between 6 and 7 weeks of age. All test-matings were set up at the same time. The number of males tested was limited to between 12 and 15 per line, equal numbers in each, by the number of heterozygous females available. The

Figure 5.1

Backcrossing of *lit/lit* dwarfism into P-lines



The numbers in brackets indicate the theorectical proportion of P-Line genetic background at sites unlinked to the *little* gene.

T1, T2, and T3 are testcrosses

H2 is the mating between lit/lit offspring of B3 to produce mice for the trial of the effects of GH, as described in section 5.2.1

males were removed after 18 days and each was harem mated to 2 or 3 of the appropriate P-Line females. This resulted in between 30 and 35 matings per line, numbers again being limited by the availability of females.

BP expanded diet number one (special diets services, Witham, Essex, UK.), and water were available *ad libitum* as in the previous experiments. Pups were identified individually by toe clipping, and weighed daily throughout the nursing period. Litters from all matings were weaned at 21 days of age, mash was provided if any pups were particularly small. This ensured that small mice had access to food and water even if they could not reach the normal food and water containers. The sexes were segregated at weaning.

Pups in litters from test-matings were weighed daily from weaning at 21 days until 24 days, to record any growth check at weaning. They were then weighed weekly from 4 to 6 weeks of age. Mice that were clearly ill at any stage were killed to prevent false identification as *lit/lit*, which resulted in large losses in backcrosses 2 and 3 when rheo virus and sendai virus infected the mouse house. Any litters containing mice noticeably smaller than their sibs were analysed further. The small mice were segregated into one group and their sibs into another. No distinction between the sexes was made. Analysis of variance was then carried out on the two groups to determine if they were significantly different at the 5% level. Litters in which a significant result was obtained were deemed to have been sired by a heterozygote. If no *lit/lit* mice were apparent at six weeks of age the sire was deemed homozygous wild type and he, and the litters he had sired on P-Line females, were culled. This method of distinguishing *lit/lit* mice is biased as only litters which appear to contain small mice are analysed. The bias is not important providing mice identified as *lit/lit* are *little*. The accuracy of this method was checked in the third backcross generation test cross by ranking the mice by increasing order of 42 day weight within line by genotype and sex groups (for example Wild type high line

females). Weight was then plotted against ranking.

The mice used could not be treated with GH from 4 to 7 weeks of age, unlike the mice in the previous trials, as it is often not possible to distinguish *lit/lit* mice from heterozygous litter mates until they reach 6 weeks of age. A period to adapt to the controlled light regime was necessary and consequently the treatment period used was from 7 to 10 weeks of age.

5.2.1 Replacement matings

Due to the deaths from the rheo and sendai viruses there were too few *lit/lit* mice from the first litters of matings of the third backcross generation to give the sample sizes required. In an attempt to remedy this the *lit/lit* offspring were mated together within line (the H2 matings). These mating were set up at the same time as the heterozygote matings were set up again. Very small sample sizes resulted (Table 5.1) but the experiment was performed in the hope that enough information would be obtained to indicate whether or not to maintain the lines in the hope of repeating the experiment with reasonable sample sizes. This was subsequently carried out (chapter 6).

Lit/lit mice were assigned randomly within family to treatment groups at 6 weeks of age. Mice from one sex, line and treatment group were caged together. This confounded any cage effects with treatment effects but reduced the risk of a mouse being accidentally injected with saline instead of GH, or vice versa. Wild type males from the heterozygote by heterozygote matings (that is full sibs of some of the *lit/lit* mice) were chosen as controls. The experimental animals were maintained in controlled light conditions (14 hours light, 10 hours dark) from 6 weeks of age. Treatment started at 7 weeks, to allow time to recover from the transfer and adjust to the controlled light conditions. Food and water were freely available *ad libitum*. The

Table 5.1

Preliminary trial of GH on *lit/lit* mice
Numbers of mice per group

	0.1 ml Saline/20g/day	9µg GH/g/day
Low line males	3	3
Low line females	4	4
High line males	3	3
High line females	3	4

lit/lit mice were injected daily from seven to ten weeks of age with GH at 9μg/g/day or saline at 0.5μl/g/day in the two hours before the start of the dark period. They were weighed immediately before injection. After all the *lit/lit* mice had been injected the wild type mice were weighed. Dissection and freeze drying were not carried out, due to the small sample sizes.

5.3 Results

Examination of six week weights suggest that each group of putative wild type mice contains one or more mice which are substantially smaller than the others (Figure 5.2, 5.3) . This is particularly apparent for the high line males with weights of less than 12g (Figure 5.2), and the low line female with a weight of less than 8g (figure 5.3). These animals may have shown reduced growth due to ill health or maternal effects, but may also be *lit/lit* mice that were not identified by the classification used. All the putative *lit/lit* mice fall within a weight range of 4g (Figures 5.4, 5.5) and at least 2g less than the minimum weight of the main block of wild type values for each line and sex group (Figures 5.2 to 5.5). This suggest no wild type mice have been identified as dwarfs.

No mice were removed from the calculation of mean daily preweaning weights because of doubts about their correct genotype. All line and genotype and sex groups appear to show similar growth rates until weaning, at which point wild type mice from both lines show a definite increase in growth rate as do the high line *littles* (figures 5.6, 5.7) . Such an increase is less apparent in the Low Line *littles*, if indeed it exists. The growth of *littles* from the high line is similar to the growth of wild type mice from the low body weight line (Figure 5.6, 5.7). Indeed, in females the curves are almost superimposed (Figure 5.7).

The injected rbGH has clearly affected the growth of the *little* mice as the animals

Figure 5.2

6 week weights of high P-Line putative wild type mice plotted against the rank number. The mice were the offspring of the test mating of the third backcross generation and were ranked in increasing order of weight.

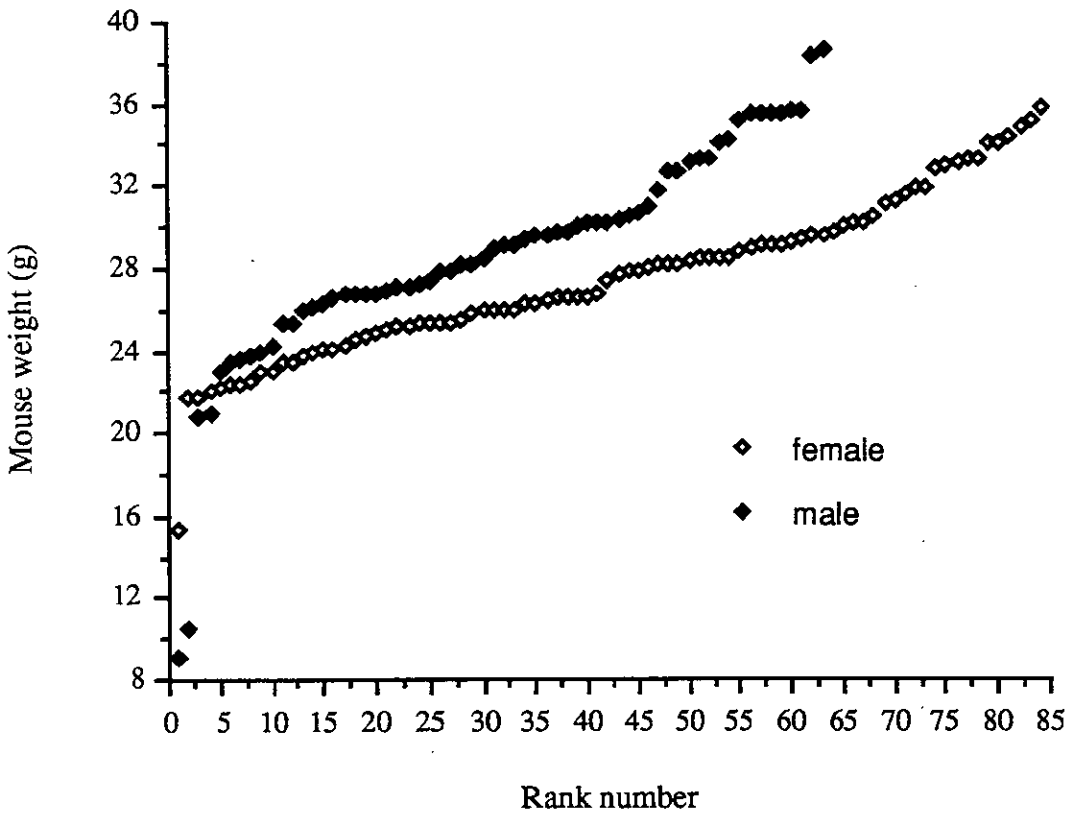


Figure 5.3

6 week weights of Low P-Line putative wild type mice plotted against the rank number. The mice were the offspring of the test mating of the third backcross generation and were ranked in increasing order of weight.

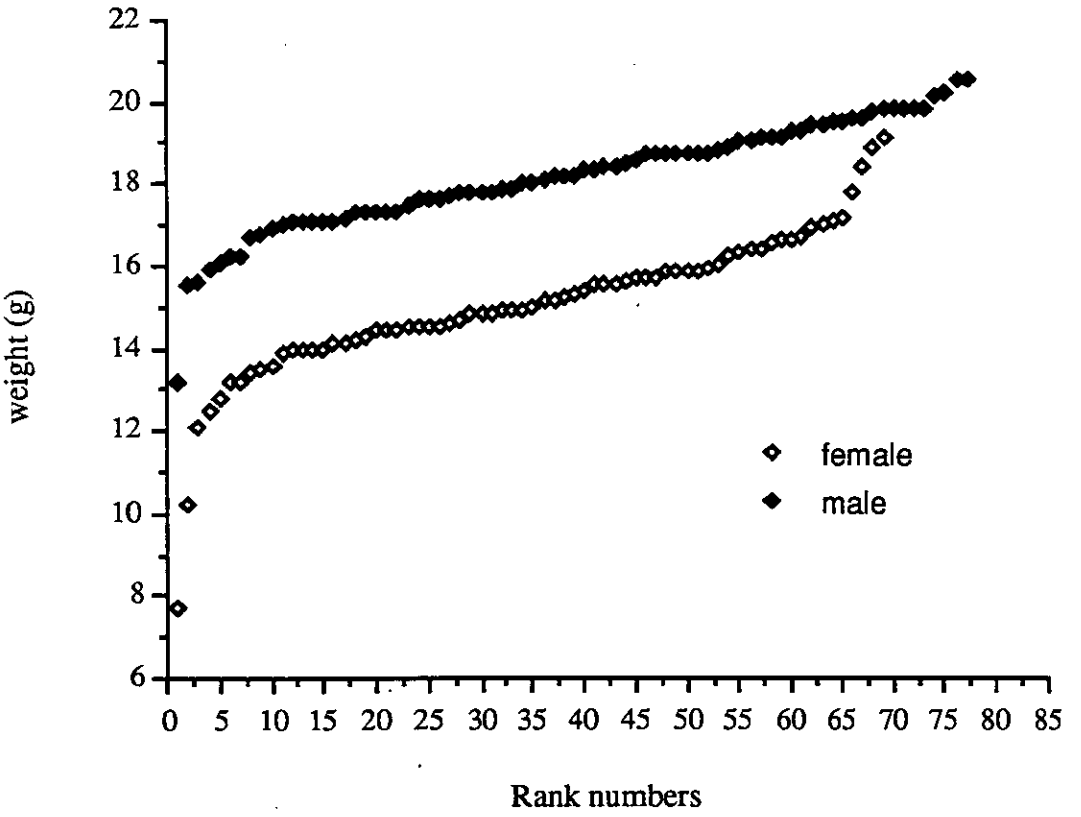


Figure 5.4

6 week weights of high P-Line putative *lit/lit* mice plotted against the rank number. The mice were the offspring of the test mating of the third backcross generation and were ranked in increasing order of weight.

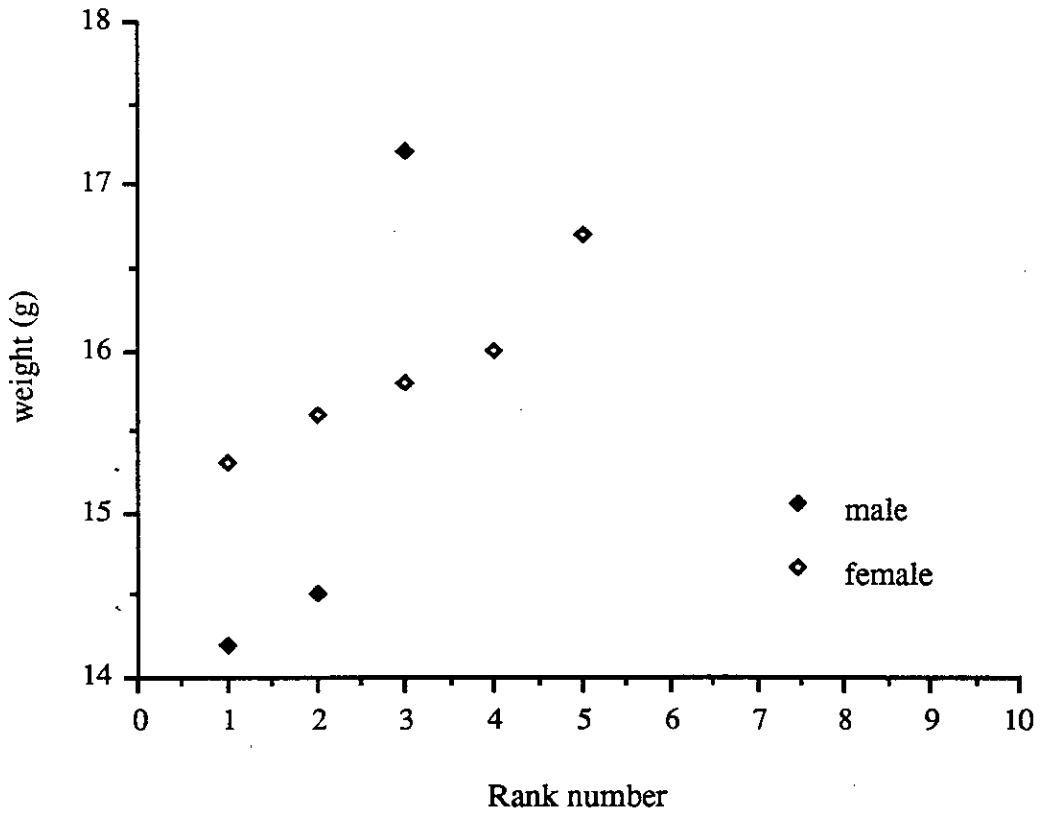


Figure 5.5

6 week weights of low P-Line putative *lit/lit* mice plotted against the rank number. The mice were the offspring of the test mating of the third backcross generation and were ranked in increasing order of weight.

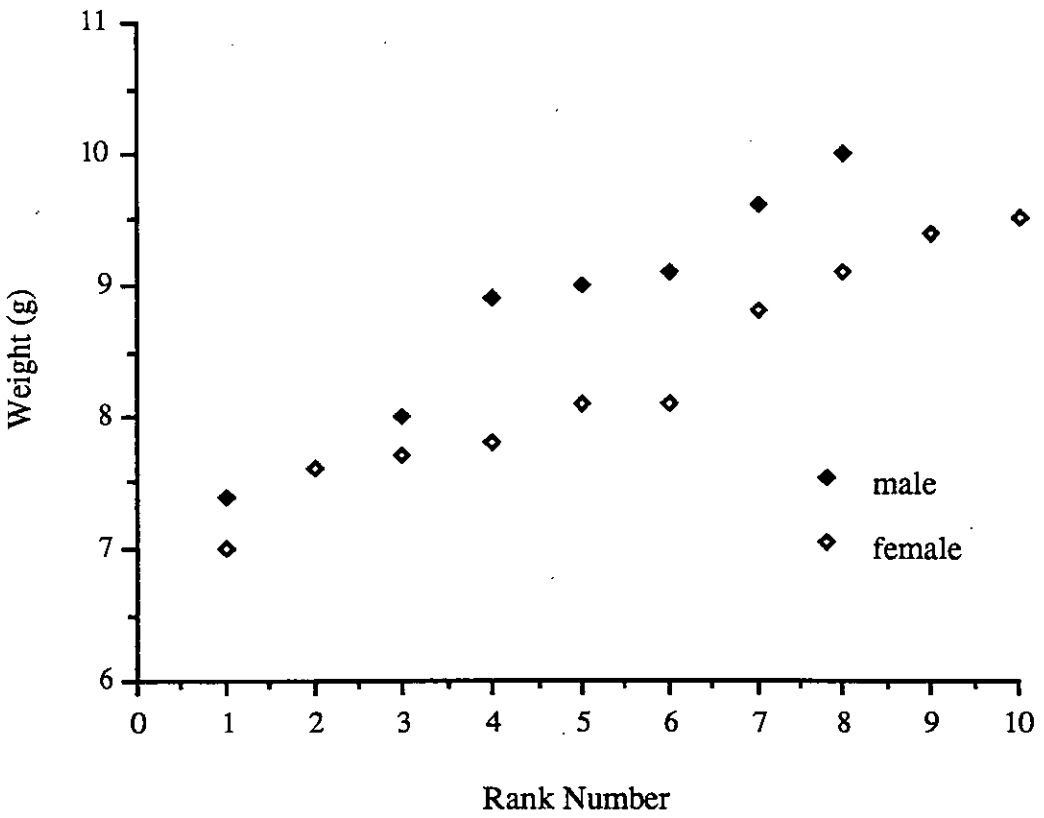


Figure 5.6

Mean daily weights of male putative wild type and *lit/lit* P-line mice from birth to 42 days of age. The mice are the offspring of the test matings of the third generation backcross. The error bars give the maximum standard errors of the means.

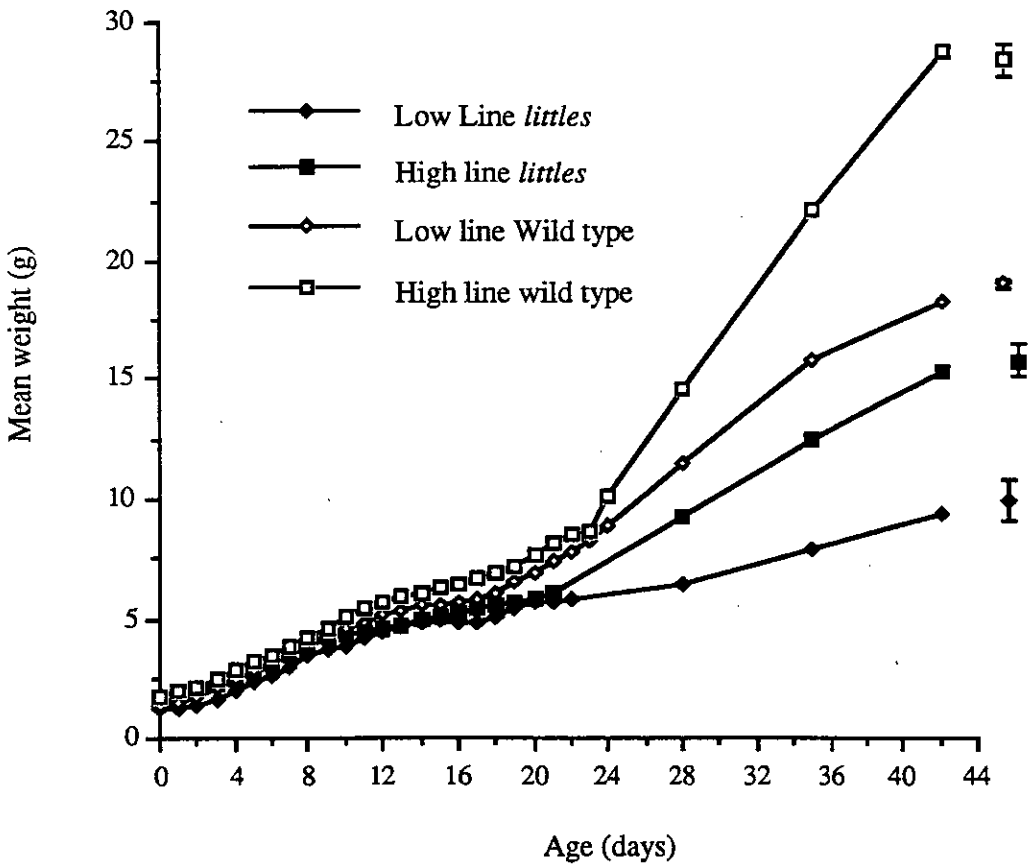
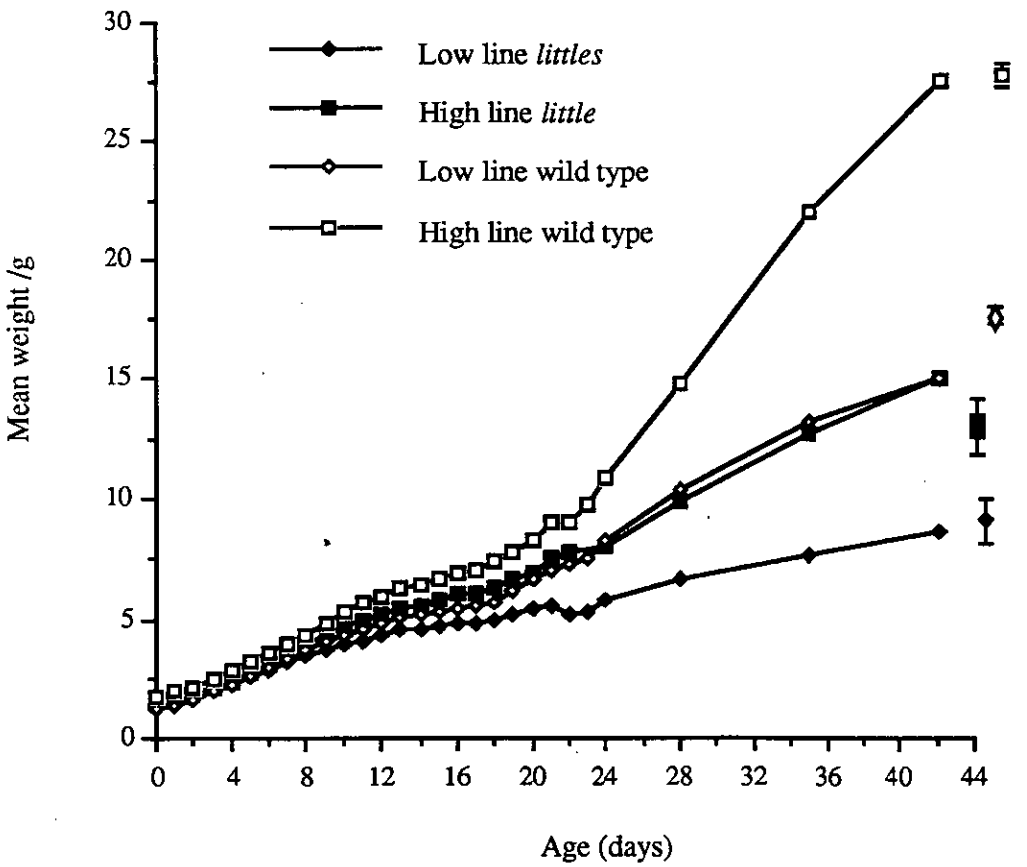


Figure 5.7

Mean daily weights of female putative wild type and *lit/lit* P-line mice from birth to 42 days of age. The mice are the offspring of the test matings of the third generation backcross. The error bars give the maximum standard errors of the means.



from both lines which were injected with rbGH have gained more weight over the treatment period than those injected with saline (Figure 5.8, 5.9) . Also the weight gain of treated mice was substantially higher than that of the uninjected wild type mice over the same age range. In the High line *lit/lit* mice gained approximately 11g, untreated wild type mice approximately 5g. For the low line the figures were approximately 5g and approximately 1g respectively (Figures 5.8 to 5.11). High line *littles* appeared to gain two to three times as much weight as low line *littles* over the treatment period, regardless of treatment (figures 5.8, 5.9). There appears to be a difference between the sexes in response of *lit/lit* mice to rbGH, this may be an artifact due to the small sample sizes (Figures 5.8, 5.9). The growth data of the *little* mice over the period of treatment with GH or saline lacks error bars because the sample sizes were too small to enable meaningful errors to be calculated (Figures 5.8, 5.9) due to the deaths caused by the rheo and sendai virus infections. For this reason no statistical analysis was performed on the results.

5.4 Discussion

The unusually small mice in the wild type type group may be misclassified *littles* or small as a result of an unidentified infection. Some misclassification would have been expected to happen, regardless of the method chosen to distinguish between the two types of mice, as no method is error free. In this experiment it was more important to be sure that every mouse identified as *lit/lit* was indeed a dwarf than it was to identify every such animal. This conservative policy ensured sires used in the backcrossing carried the *lit* gene and that sick animals, which might respond differently to treatment, were not used in the investigation of the effects of exogenous GH. Although the sample sizes are smaller, the limited range of weights observed, and the absence of overlap between *little* weights and the main group of wild type weights (Figures 5.4 and 5.5) suggest that all mice identified as *littles* were indeed

Figure 5.8

Mean daily gains of male *lit/lit* mice injected with 9µg GH/g body weight /day or with 5µl saline/ g body weight/ day for 21 days from 7 to ten weeks of age.

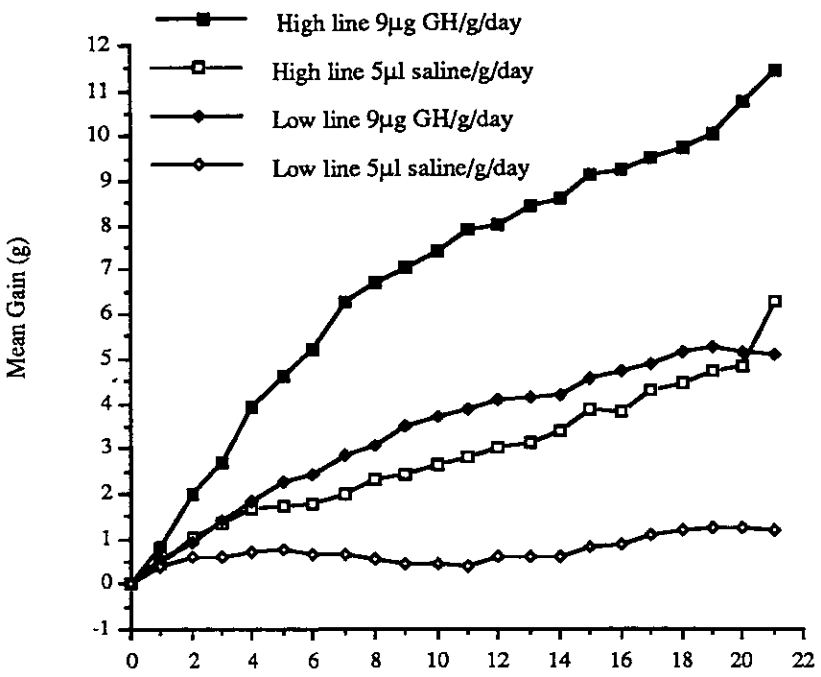


Figure 5.9

Mean daily gains of female *lit/lit* mice injected with 9µg GH/g body weight /day or with 5µl saline/ g body weight/ day for 21 days from 7 to ten weeks of age.

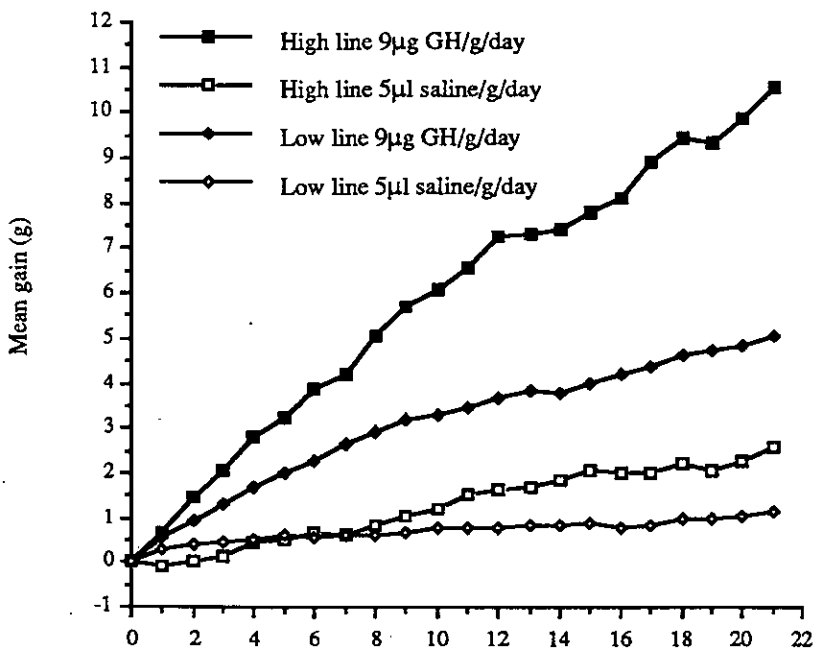


Figure 5.10

Mean daily gains of male P-Line mice from 7 to ten weeks of age. The error bars give the maximum standard errors of the means.

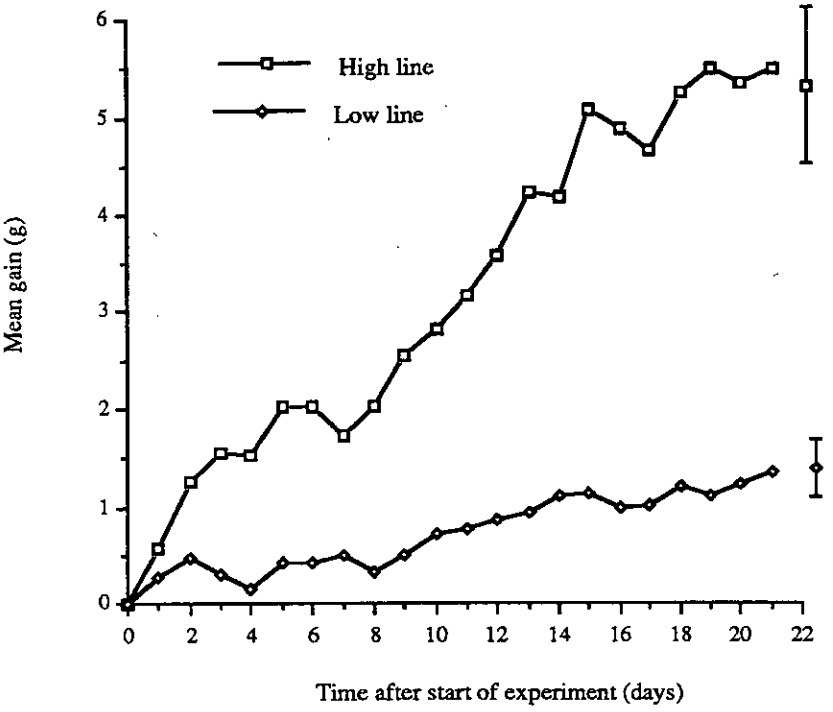
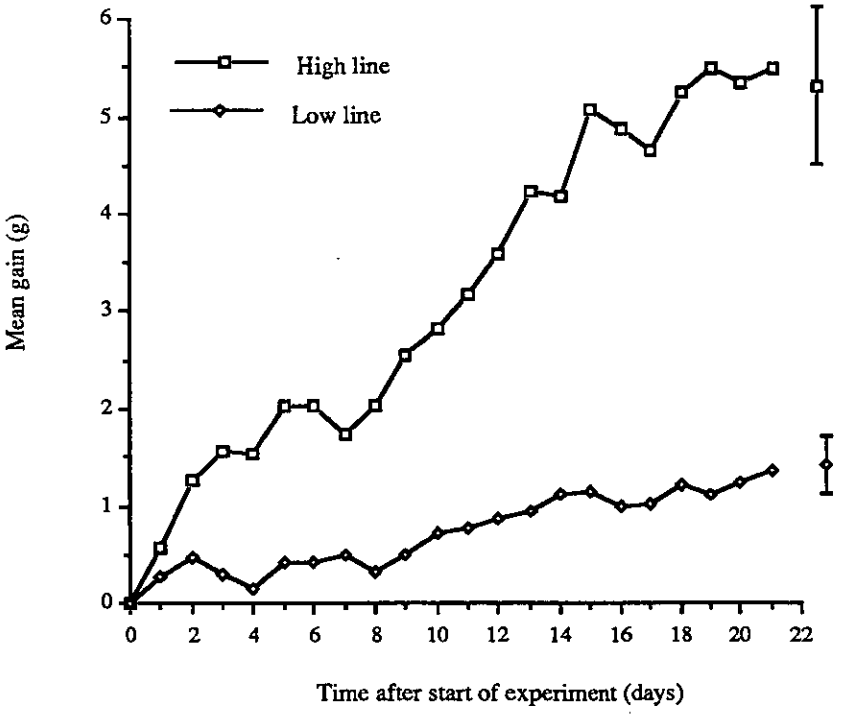


Figure 5.11

Mean daily gains of female P-Line mice from 7 to ten weeks of age. The error bars give the maximum standard errors of the means.



dwarfs. The faster absolute growth rate of *littles* from the high lines suggests that selection has not acted on responsiveness to GH, but to some other factor. Due to scale effects the relative proportionate increase would be more informative, this was calculated in chapter 6 but was inappropriate here due to the large standard errors.

The previous plot of mouse weight against age (Figure 4.3 to 4.5) showed an increase in growth rate in wild type mice from both lines, starting from about 18 days of age. As mice appear to become responsive to GH shortly before this age (Borrelli *et al*, 1989) it appeared possible that this induced the increase in growth rate. If this was the case then *lit/lit* mice should not show such an increase in growth rate at 18 days old, regardless of line. However, *lit/lit* mice from the high line clearly show such an increase in growth rate (Figures 5.6, 5.7). The evidence is less clear for the low line *littles*. This suggests that GH, or response to it, is not solely responsible for the increased rate of gain. However the increase in the *little* mice is not as substantial as that observed in wild type mice which suggests that GH has some effect, possibly amplifying the effect of some other factor. This factor could have been increased food intake as mice begin to eat the pelleted food available at about 18 days of age (personal observation). Certainly low line pups crossfostered onto high line dams showed greater preweaning growth, suggesting that for the low line at least, food supply has a limiting effect on growth (Figure 4.5). Given that high line mice were larger at birth than low line mice the factor responsible for the difference in weights may be an *in utero* effect.

After removal of GH by backcrossing in the *little* gene the mice responded to exogenous GH with increased growth. The difference in the absolute weight gained between *littles* treated with GH and controls was greater in the high line than in the low line but given the small sample sizes the analysis of proportionate gain was delayed until larger sample sizes were available (Chapter 6). As high line *lit/lit* mice were also heavier than low line *lit/lit* mice at the start of treatment this suggests that

the proportional difference in response noted in chapter 3 exists despite the removal of GH. (A difference in body weight at the start of the experiment between *little* mice from high and low body weight lines was not noted in chapter 6, which covered an earlier age range). Also saline controls from the high line *littles* grew faster than the those from the low line, suggesting differences between the lines are not related solely to GH. Due to the sample sizes no firm conclusions could be drawn, but the results indicate that it would be useful to maintain the lines and repeat the experiment with larger samples. This would enable statistical analysis of the results and investigation of the effects on carcass composition. In the wild type mice, the greater gain of the high line mice may indicate that they continued to grow over a longer period than the low line mice.

Chapter 6
Effects of exogenous GH in GH deficient *little* mice

6.1 Introduction

The growth of high and low body weight mice suggested that the removal of GH affected both lines in proportion to their body weight. Ability to respond to exogenous rbGH also remained similar between the lines. However the sample sizes used were too small to enable conclusions to be drawn with any degree of certainty, due to the high number of deaths after rheo and sendai virus entered the animal house. Thus it was decided to repeat the treatment with larger sample sizes including wild type P-Line mice for comparison purposes.

I was not able to continue breeding the mice, or perform the experiment because of time constraints. I gave assistance with the design of the experiment, performed some of the injections and weighing and ran the resultant data through the "Harvey" statistics program to check the analysis. I also made some contributions to the interpretation. However most of the work was performed by Dr Ian Hastings.

6.2 Materials and Methods

The maintenance of the *Little* lines of mice was taken over by Ian Hastings. The breeding program was altered, primarily for ease of handling. As the new schedule involved large numbers of *lit/lit* by *lit/lit* matings it was also easier to generate animals of known genotype for experimental purposes. In the new program 16 families were set up per line in each generation. One male and one female were taken from each family as parents of the next generation. Each backcross generation was followed by four generations of within line mating before the next backcross carried

out. The backcrossing transmitted alterations in gene frequency, and any new mutations from the P-Lines to the *little* lines. The subsequent within line matings meant that only one in 5 generations produced a mixture of wild type and *little* mice. This reduced the number of mice that had to be maintained, and also meant that only one generation had to be weighed to distinguish between wild type and *little* mice.

The offspring of the first within line mating after each backcross, that is the offspring of two heterozygous parents, were weighed at 6 weeks to enable identification of *lit/lit* mice. The weights also provided useful data for the comparison of wild type and *little* mice. Wild type and *lit/lit* mice were distinguished by using the weights of mice obtained in the generations previous to the change in breeding protocol. These indicated that at 6 weeks of age, High Line *lit/lit* mice should weigh less than 18g, and Low Line *lit/lit*s less than 10g (Figures 5.4, 5.5). All ill mice were assumed to be wild type to avoid misclassifying a wild type runt as a dwarf.

The *little* mice used were taken from litters produced from homozygous *lit/lit* matings set up after the third backcross generation, that is with an average of 93% parental line alleles at loci unlinked to the *lit* gene. The wild type P-Line mice had been selected for 44 generations. Contemporaneous wild type and *little* matings were set up in a fixed light environment (14hour light, 10 hour dark). BP expanded diet no. 1 was available *ad libitum* both as pellets and as mash. Water was also freely available. All mice were weaned on the same day, at age 26 (±2) days. Mice were assigned randomly within family to a treatment group (GH or saline), and cage at weaning. All mice in a cage were given the same treatment to reduce the chance of a mouse receiving the wrong injection. Each cage contained mice from all four lines. At weaning there were 15 mice in each group listed in Table 6.1. Two mice were removed from the trial after being discovered to be misclassified females, one after being bitten by its cage mates, and one died. There were no apparent adverse effects

from the injections.

Mice were injected subcutaneously, daily from 28 (±2) days of age for 21 days. Injections were either 9μg/g body weight/day (as a 1.8 mg/ml solution in isotonic bicarbonate saline) or a corresponding volume of the same saline as in chapters 2 and 3 of this thesis, and were given between 130 and 45 minutes before the start of the dark period. Animals were weighed on the first day, and on mondays, wednesdays, and fridays thereafter. The amount to be injected was calculated from the last recorded weight. Saline and GH cages were injected alternately.

After the injections the mice were killed and freeze dried. Each group was sorted into four sets of three individuals split across family and cage. The fat, protein (estimated as 6.25 times nitrogen) and ash contents of each set was determined chemically, by S.A.C., Edinburgh. The results were then analysed by restricted maximum likelihood (REML) using the Genstat statistics package. The model fitted high or low line genetic background, *little* genotype, and treatment (GH or saline) as fixed effects, and family and cage number. Batch of freeze drying was also included as a random effect where appropriate.

I also ran the data through a mixed model least squares and maximum likelihood computer program (Harvey 1985), as had been used in the analysis of previous experiments. The results of the analysis supported those obtained from the Genstat package and are not shown.

6.3 Results

It can be seen from both Table 6.1, and Table 6.2 that there are large differences in body weight between the groups investigated. The analysis of main and interaction effects was thus carried out on both untransformed and natural log transformed data,

Table 6.1

Weights and carcass composition for male wild type and *little* mice from the high and low P-Lines. treated with 9μg GH/g/day, or with 0.1ml saline/20g/day. Means (± standard deviation) of body weight at 4 and 7 weeks of age, weight gain from 4 to 7 weeks expressed in grams (g) and as a percentage of weight at 4 weeks (%). Means of carcass traits at 7 weeks of age expressed in grams (g) or percentage of dry weight (%). Standard errors are average standard errors of group means.

	High				Low				
	+/+		*lit/lit*		+/+		*lit/lit*		
	GH	saline	GH	saline	GH	saline	GH	saline	s.e
n*	14	15	14	15	14	14	15	15	
4 week weight (g)	21.6	21.0	10.6	10.6	11.6	11.6	5.7	5.9	0.58
	±5.6	±4.9	±1.4	±1.0	±1.8	±1.5	±0.8	±0.9	-
7 week weight (g)	44.2	39.5	24.1	17.6	18.3	17.2	11.0	8.4	0.51
	±3.3	±3.4	±2.7	±1.7	±1.5	±1.0	±1.1	±1.0	-
gain (g)	22.5	18.6	13.5	7.0	6.7	5.5	5.3	2.5	0.41
	±2.8	±3.5	±1.9	±1.1	±1.0	±0.8	±0.8	±0.5	-
gain (%)	114	95	129	66	60	49	95	45	5.8
	±43	±34	±20	±12	±16	±13	±21	±18	
water (g)	29.1	26.2	14.7	9.7	11.9	11.2	7.0	5.1	0.32
fat (g)	3.6	3.1	3.3	3.7	1.4	1.5	1.0	1.2	0.17
protein (g)	8.5	7.8	4.2	2.9	3.6	3.4	2.1	1.5	0.07
ash (g)	1.4	1.3	0.8	0.6	0.6	0.6	0.4	0.3	0.16
fat (%)	26	24	39	50	24	26	28	38	1.3
protein (%)	60	61	49	39	62	62	57	49	1.0
ash (%)	10	11	9	8	11	11	11	10	0.2

* number of animals and records, of body weights, water weights and gains.

For other traits there were four samples of pooled mice within each group

Table 6.2

Main effects and interactions (± standard errors) for body weight (BW), gain, lean mass, and % fat at 7 weeks of age.

	Untransformed			
	BW (g)	gain (g)	lean mass(g)	% fat
Main effects				
Background (High - Low)	17.9±0.6***	10.2±0.5***	3.2±0.06***	5.5±1.1***
lit gene (*lit*/*lit* - +/+)	-14.7±0.6***	-5.9±0.5***	-3.15±0.06***	13.8±1.1***
treatment (GH - Saline)	3.7±0.3***	3.6±0.2***	0.68±0.06***	-5.5±1.1***
Interactions				
lit x background	-6.7±0.9***	-3.8±0.7***	-1.42±0.08***	5.9±1.5***
lit x treatment	1.0±0.6	1.1±0.5*	0.23±0.08**	-5.0±1.5**
background x treatment	1.8±0.6**	1.6±0.5***	0.32±0.08***	0.4±1.5
lit x background x treatment	0.3±0.9	0.3±0.8	0.04±0.12	-1.3±2.1

	Natural log. transformed			
	BW	gain	lean mass	% fat
Main effects				
Background (High - Low)	0.82±0.03***	1.08±0.05***	0.76±0..02***	0.14±0.04***
lit gene (*lit*/*lit* - +/+)	-0.67±0.03***	-0.60±0.05***	-0.77±0.02***	0.42±0.44***
treatment (GH - Saline)	0.19±0.01***	0.51±0.07***	0.20±0.02***	-0.14±0.04***
Interactions				
lit x background	-0.05±0.04	-0.11±0.07	-0.07±0.03**	0.15±0.06**
lit x treatment	0.10±0.03***	0.26±0.07***	0.13±0.03***	-0.13±0.06*
background x treatment	0.02±0.03	-0.02±0.07	0.02±0.03	0.04±0.06
lit x background x treatment	1.00±0.04	-0.02±0.08	0.00±0.04	-0.05±0.08

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

to provide information on the scale effects

At 4 weeks of age the line by background groups differed in body weight, with the high line being just under twice the size of the low line, whether *lit/lit* or wild type (Table 6.1). Also the *little* mice were approximately half the weight of the wild type mice. There were no significant differences in weight between the groups assigned to different treatments. Supporting the results from the previous chapter, wild type low line mice were of similar size to *lit/lit* high line mice (Table 6.1).

All three factors investigated had affected body weight and gain (Table 6.2). High line mice were heavier than low line mice by 17.9g, and wild type mice were heavier than *lit/lit* mice by 14.7g, on average. Treatment with GH increased body weight by an average of 3.7g. (Table 6.2, untransformed data). The effects of all these traits remained significant after correction for scale effects by natural logarithm transformation (Table 6.2, transformed data). Significant interactions were observed between the *little* gene and genetic background, and between the genetic background and treatment for 7 week weight, and for gain in grams, on the untransformed data (Table 6.2). However these were non significant in the transformed data, indicating that the lines respond proportionately to the removal, or addition, of GH (Table 6.2). The interaction between *little* genotype and GH treatment was significant only in the transformed data suggesting that *little* mice respond to GH treatment with a greater increase in weight, for their size, than wild type mice.

Lean mass and fat percentage were also affected by all three traits investigated, and significant effects were observed in the transformed and in the untransformed data. Averaging over wild type and *lit/lit* mice the high line had a lean mass 3.2g heavier than the low line, and were also 5.5% fatter (table 6.2, untransformed data). This suggests that the selection procedure used has not been entirely successful in the goal of increasing lean mass, while maintaining body composition unchanged.

There were also significant interactions which remained after transformation, between the *little* gene and genetic background, and between the *little* gene and GH treatment for both lean mass and fat percentage (Table 6.2), although the interaction with treatment for the fat percentage becomes smaller after transformation. Protein content (g) measures lean mass, and high line mice lose a greater proportion of their protein when GH is removed by backcrossing the *little* gene (approximately two thirds) than do low line mice (approximately half) (table 6.1, comparing untreated mice). Fat percentage in high line littles was approximately double that in wild types, while in the low line it was only approximately 50% greater. *Lit/lit* mice had a smaller lean mass and greater fat % than wild type mice. Treatment with GH had greater effects on increasing lean mass and decreasing fat content in littles than in wild type mice (Table 6.1)

6.4 Discussion

Selection on high and low body weight in these lines has not solely affected the GH axis. Indeed it appears that as far as final weight and weight gain are concerned the growth hormone axis has not been affected, as there is no interaction between genetic background and *little* genotype, or between genetic background and treatment. This is in contrast to the results obtained by Pidduck and Falconer (1978) who found that when the *dw* dwarfism gene was backcrossed into lines selected for high and low growth rate the reduction in growth rate was greater in the high selected line than the low line. Also *dw/dw* mice from the low selected line were less responsive to exogenous GH suggesting that GH levels and responsiveness to GH had been altered by selection (Pidduck and Falconer, 1978). There is a suggestion that low P-Line mice stop growing at a younger age than High P-Line mice (Figure 4.6), which may indicate differences between the lines at later ages.

There does however appear to have been some effect on partitioning. The effects on fat percentage of the removal of GH, and its exogenous administration were probably explicable by the lipolytic effects of GH (Fielder and Talamantes, 1992). Why there should be an interaction with line genetic background is unclear.

Chapter 7
Effects of Treatment with antiserum to rat GH

7.1 Introduction

Backcrossing the *little* gene into the P-Lines of mice was designed to repress GH production and release, by preventing somatotrophs from responding to GHRF (Jansson *et al*, 1986). Consequently in *lit/lit* mice GH was effectively absent throughout development, whereas in normal mice it is expressed from embryonic day 16 (Slabaugh *et al*, 1982). This absence may well have effects on the development of other parts of the endocrine system. Support for this idea was given by the loss of first litters of *lit/lit* females, and the poor performance of *lit/lit* males in siring litters (Eicher and Beamer, 1976). It seemed possible that further information on the activities of growth hormone itself might be obtained if the GH could be selectively removed for a defined period of growth.

A possible way of removing the GH was by use of an antiserum to GH. In rats, treatment of 35-40 day old males with goat anti-rat GH (anti-rGH) over a 6 day period resulted in reduced body weight gain (Gause, Eden, Jansson, and Isaksson, 1983). A single injection of sheep anti-rGH into 28 day rats temporarily reduced the rate of body weight gain, an effect which was prevented by simultaneous injection of ovine GH (Flint and Gardner, 1989). Later work showed that 35 day male rats injected with sheep anti-rGH serum could be separated into two categories. One group rapidly recovered from the anti-rGH, and their subsequent growth curve paralleled that of the untreated control groups. The other group had a very low weight gain for several weeks, before recovering. Again the subsequent growth curve paralleled that of the controls of the same age, there was no catch up growth (Madon, Parton and Flint, 1991). The difference appears to be due to differences in the rate of

removal of the injected sheep antisera from the blood stream (Madon *et al*, 1991). The experiments described in this chapter were designed to investigate if similar effects could be discerned in the mouse and furthermore to investigate any interactions with line genetic background.

Rats given a single injection of sheep anti-rGH at 2, 10, or 20 days old continued to grow at the same rate as control rats injected with normal sheep serum or saline (Flint and Gardner, 1989). In addition, rats given normal sheep serum and ovine GH at 2, 10, or 20 days of age continued to grow at the same rate as saline controls. Rats given the same treatment at 28 days had an increased rate of body weight gain (Flint and Gardner, 1989). These results suggested that, although GH is present in the rat from before birth (Walker, Dussault, Alvardo-Urbine and Dupont, 1977), it may have limited effects on growth before day 20. However, repeated injections of 2 day old rats with sheep anti-rGH resulted in long term reductions of growth (Flint and Gardner, 1989). This reduction was temporarily reversible by injections of ovine GH (Flint and Gardner, 1989), and appeared to be due to destruction of somatotrophs in the pituitary as there was a reduction in pituitary size and in the release of GH in response to exogenous GH releasing factor (Gardner and Flint, 1990). This suggests that antiserum administration in the mouse should be carried out after weaning to avoid such complications as limited effects of reduction in GH levels prior to this age, or to the destruction of the somatotrophs.

The sera available were sheep anti-rat GH (anti-rGH), therefore a preliminary trial was carried out to determine whether they were active in mice.

7.2 Preliminary Trial

7.2.1 Materials and Methods

Excess mice from the backcrossing programme were used in this preliminary trial as there were mice of a suitable age available. Males had been mated to females of the appropriate P-Line before their genotype was known, some were not shown to be heterozygous *lit/+*. Offspring of such males, from generation B1 of the backcross, were used in this preliminary trial. On average their genomes were 83% P-Line. Test matings would not identify all *lit/+* males, and thus there may have been *lit/+* males in the test groups. For a preliminary trial this seemed unlikely to be important, in particular as the *little* allele is recessive to the wild type in its effects on growth (Eicher and Beamer, 1976)

The antisera used, sheep anti-rGH, were kindly donated by D. Flint, R. Madon and D. Parton (Hannah Research Institute, Ayr, Scotland) as dried whole serum and diluted with distilled water before use. They had been raised by injecting rat growth hormone into five different sheep and had been prepared as described by Madon *et al* (1986). The sera obtained were numbered with the same code as the sheep from which they came. They were ranked by *in vivo* tests in rats as 9 >>> 854 > 817> 879 > 880, that is the serum from sheep, code 9, was much the most effective in rats (Flint, Madon and Parton, personal communication.). All the sera were used, for this preliminary trial, as it was possible they would differ considerably in effectiveness in mice.

The mice were 35 days old at the start of treatment. For simplicity it was assumed that all the mice weighed approximately 20g, and doses were given per mouse rather than per gram body weight. Two doses were used in low line mice, one of antiserum at a dose per mouse similar to that given to the rats, and one at twice that concentration. Insufficient high line mice were available for both dose levels to be tested, the higher dose was tested in this line as the mice were bigger than the 20

grams assumed. There was also a control group given normal sheep serum at the same concentration as the higher dose in both lines. Serum was used as control rather than saline as it appeared possible that the injection of large quantities of foreign proteins could in itself affect the growth of the mice. Amount of serum given and number of mice per group are shown in Table 7.1.

The animals were maintained as specified in chapter 2, although they were not maintained in controlled light conditions. Insufficient animals were available to assign one from each family to each treatment group. Consequently they were randomly assigned to treatment group, with the proviso that animals from the same family were not in the same treatment group. Animals were housed together by treatment group. Injections were given subcutaneously into the back of the neck at 9am and 5pm, when the mouse reached 35 days old. This ensured that, despite a range of 10 days in dates of birth, the animals were of comparable ages when studied. Weights were recorded on a daily basis, shortly before 5pm, from the day before the injections until 7 days after the injections.

7.2.2 Results

The effects of the antiserum were not clear in the low line mice treated with sera at a concentration of 25μg/150μl (Figure (7.1) . The group injected with normal sheep serum did display less of a reduction of growth on the day after the injection than the other groups (Figure 7.1). Effects were even less clear in the high line mice which had been injected with antiserum at 50μg/μl in that some of the sera appeared to have had no more growth reducing activity than did the control serum (Figure 7.2) . As the high line mice weighed approximately twice as much as the low line mice, these two groups received comparable doses on a per gram of body weight basis. Low line mice treated with 50μ/μl did appear to react to the antisera, in that every group showed a temporary reduction in growth after the injection (Figure 7.3) . There

Table 7.1

Numbers of mice used in preliminary anti serum trial

	Serum 817	Serum 879	Serum 880	Serum 854	Serum 9	Control Serum
High Line 50µg/150µl	3	3	2	3	3	3
Low line 50µg/150µl	4	4	4	4	4	6
Low line 25µg/150µl	4	4	4	4	4	--

Table 7.2

Numbers of mice used in second anti serum trial

	Anti Serum	Control Serum	Saline Control
Low line Only	10	10	10

Table 7.3

Numbers ofmice used in final anti serum trial

	Anti Serum	Control Serum	Saline Control
Low line	7	6	6
High line	11	11	11

Figure 7.1

Mean daily gains of the male offspring of male mice from generation B1 of the backcrossing program, and low P-Line females, genotype 83% low P-Line They recieved two identical injections, at 9am and 5pm on day one totalling 150µl solution containing either 25µg antiserum, or 50µg normal sheep serum (controls). 5 different antisera were tested. Error bars give the maximum standard errors of the means

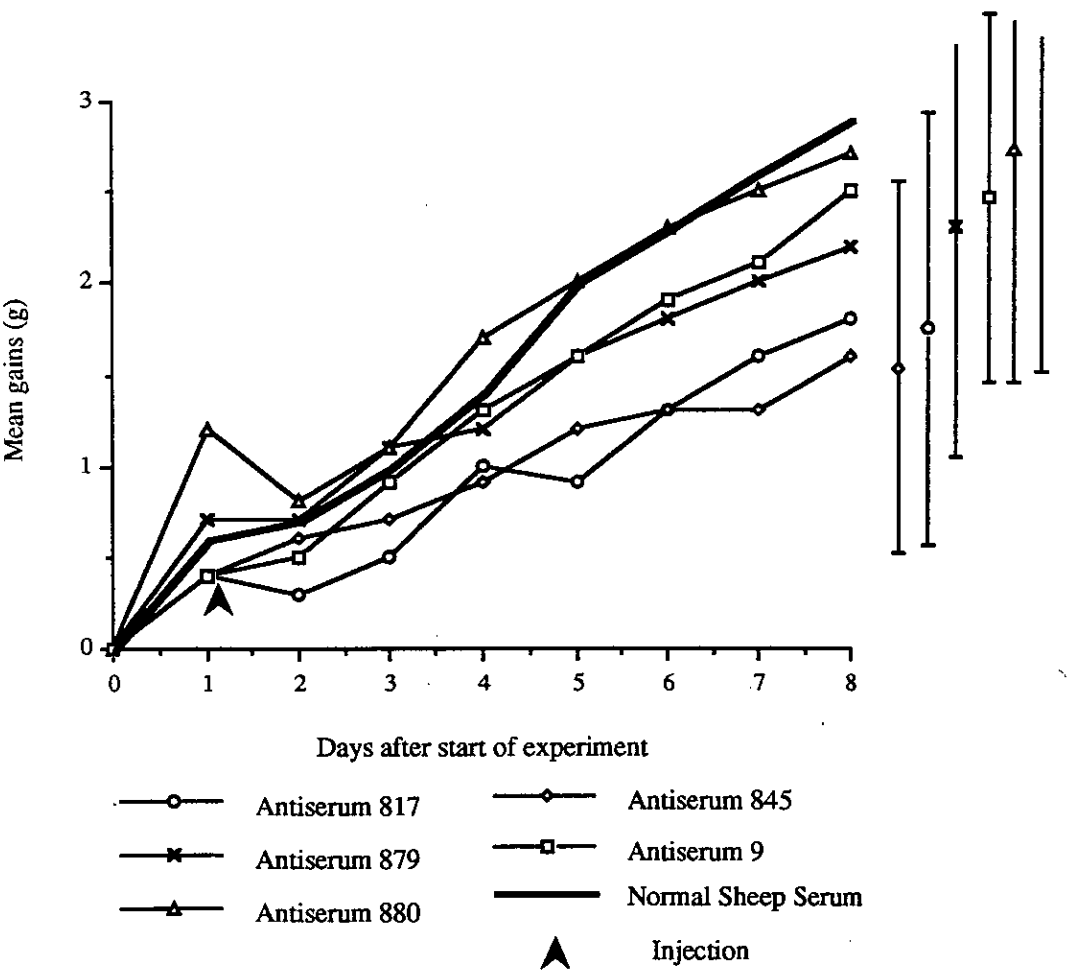


Figure 7.2

Mean daily gains of the male offspring of male mice from generation B1 of the backcrossing program, and high P-Line females, genotype 83% high P-Line They recieved two identical injections, at 9am and 5pm on day one totalling 150µl solution containing either 50µg antiserum, or 50µg normal sheep serum (controls). 5 different antisera were tested. Error bars give the maximum standard errors of the means

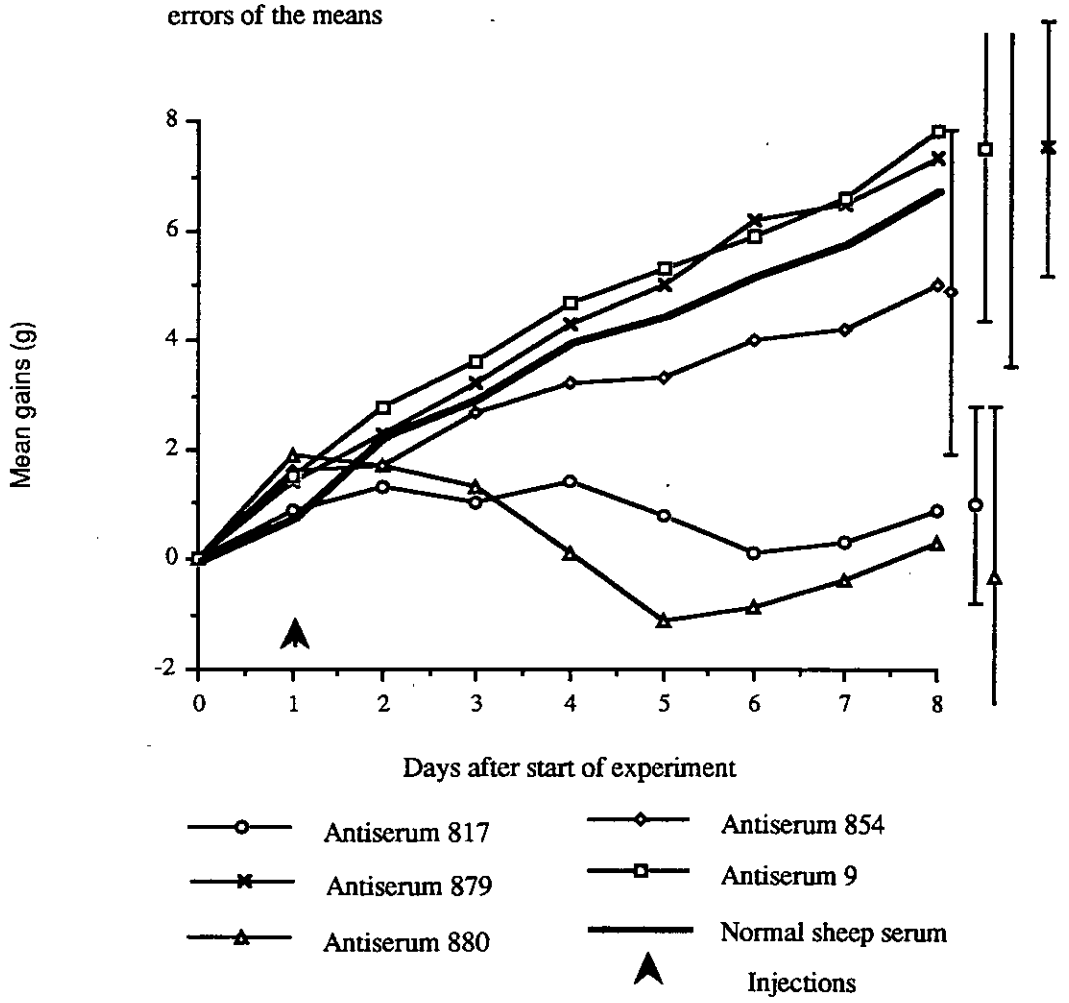
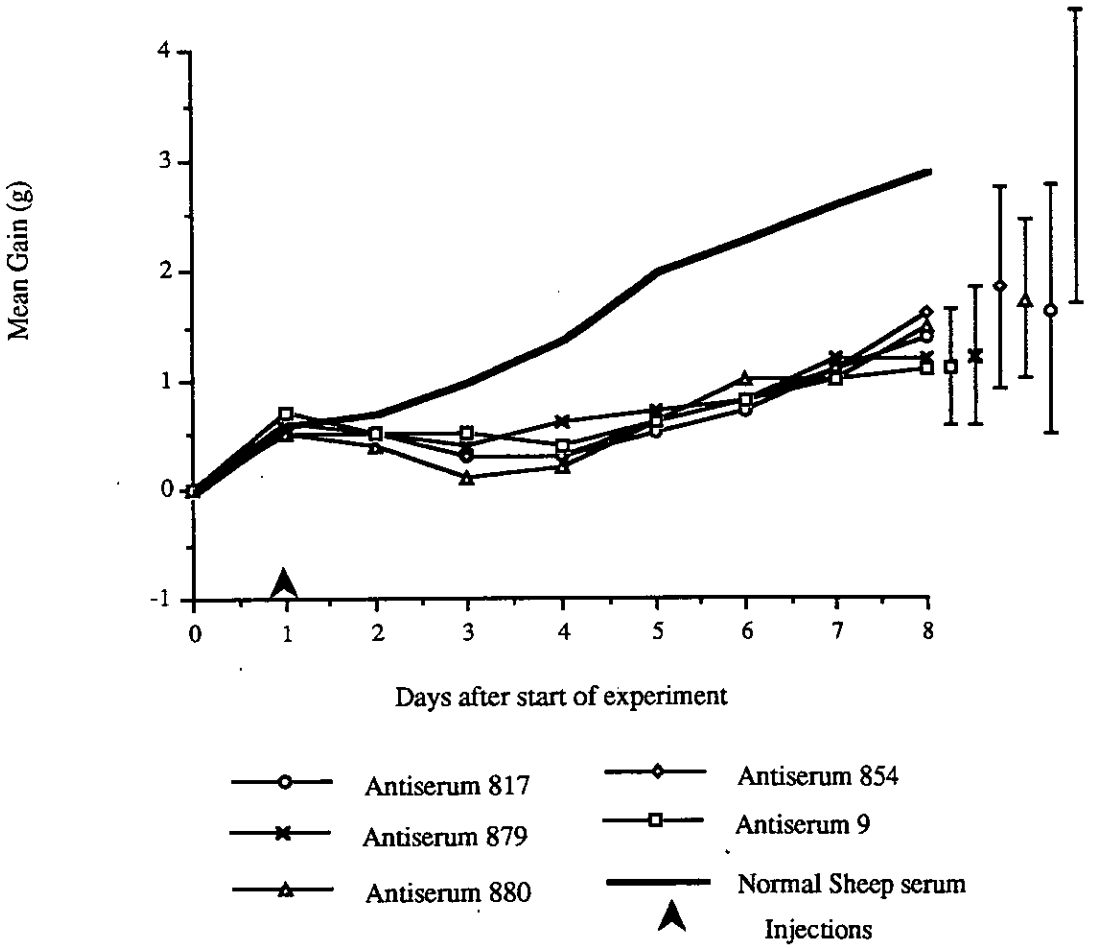


Figure 7.3

Mean daily gains of the male offspring of male mice from generation B1 of the backcrossing program, and low P-Line females, genotype 83% low P-Line They recieved two identical injections, at 9am and 5pm on day one totalling 150µl solution containing either 50µg antiserum, or 50µg normal sheep serum (controls). 5 different antisera were tested. Error bars give the maximum standard errors of the means



was an apparent check in growth in growth in the group given the control serum, although this was smaller than than that observed in the groups given anti-sera (Figure 7.3). These results were obtained from extremely small sample sizes, and are therefore suggestive rather than conclusive. On the basis of this evidence it was concluded that the anti-sera reduced growth in mice, at least at the higher dose. This suggested that a further trial, injecting mice with serum on a μg/g of body weight basis would be useful.

Due to limitations in the numbers of mice available and in the time required to carry out the weighings and injections it seemed advisable to only use one serum for further work. Serum 880 caused a noticeable reduction in growth in all three line and treatment combinations (Figures 7.1, 7.2, 7.3) although it was the least effective serum in rats. This probably reflects a species specific difference and serum 880 was consequently chosen.

Low line mice given 50μg/μl showed reduced rate of increase of body weight throughout the experiment (Figure 7.3). If this difference in gain between treated and untreated mice was maintained for a prolonged period the magnitude of the difference in final weights between the lines should increase. This would increase the probability of identifying any differences between the lines. Consequently it was decided to continue weighing the mice for 21 days after the first injection. Shifting the first injection to when the mice were 4 weeks of age, to cover the period of maximum growth, and hence maximum potential to reduce growth would also increase the probability of finding any effects. A further way of increasing the effects on growth would be to give injections on more than one day.

7.3 Second Preliminary Trial

7.3.1 Materials and Methods

In the preliminary trial the effective dose had been 50μg in 150 μl, on low line mice, of average weight 15g, so the average dose was 3.3μg/g body weight in 10 μl solution/ g body weight at each injection. For simplicity of dilution a standard solution of 3μg/10μl was used and the mice were injected with 11 μl of solution/ g body weight. A control group injected with saline as in chapters 2, 3, and 6 was also added to enable identification of effects due to normal sheep serum.

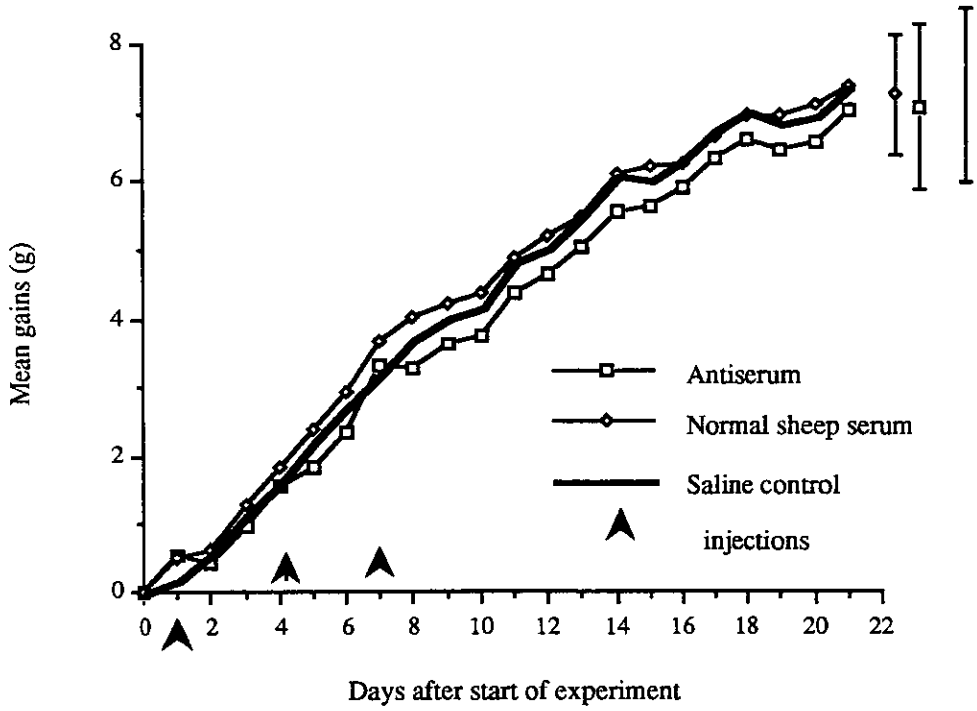
Matings were set up using mice from the P-lines, Generation 17, to provided contemporaneous animals from both lines for treatment with the antiserum. Due to disease problems in the animal house insufficient high line mice were produced by the matings. The low line mice were used in a second preliminary trial in order to observe the effects of repeating the injections. They were 28 days (±2 days) old at the start of the experiments. Animals were assigned within family to a treatment group at weaning, numbers as given in Table 7.2, but caged with their sibs to reduce the risk of transmission of infection. They were otherwise maintained as specified in chapter 2, although not in controlled light conditions. Injections were given and daily weights recorded as specified in the previous experiment. The mice were injected on days one, four, and seven of the experiment.

7.3.2 Results

The daily gains were as shown in Figure 7.4 . Both the groups given sheep serum, either normal sheep serum or anti rat GH, showed some flattening of the growth curve on the days following the injections. These apparent reductions in growth were neither clear nor conclusive, given that fluctuations of similar magnitude in

Figure 7.4

Mean daily gains of male low P-Line mice from 28 to 35 days old. They recieved two injections, at 9 am and 5pm on days 1, 4 and 7 after the start of the experiment, of 11µl/ g body weight each time. They recieved 3µg/ml of antiserum, 3µg/ml of normal sheep serum or saline. Error bars give the maximum standard errors of the means.



cumulative daily gain were observable towards the end of the experiment, when no treatment was being given (Figure 7.4). This could mean that the effects observed in the preliminary trial were an experimental artifact resulting from the small sample sizes involved. However, the occurrence of such a reduction in the group treated with antiserum to GH, after every set of injections supported the idea that the antiserum is interfering with the GH pathway and that a large scale trial should be carried out. No adverse effects of repeated antiserum injections on the mice were detected.

7.4 Final Trial

7.4.1 Materials and Methods

The mice used were the offspring of mice from the P6 Lines at generation 39. They were treated as in the preceding experiment, with the exception that only one injection was given on day 7, in the morning, due to a shortage of serum. Animals were not weighed on all days, due to time limitations. Their age at the start of the experiment was 28 (±3) days. The numbers per group were as shown in Table 7.3. These group sizes were not enough to permit satisfactory statistical analysis. However analysis was carried out to check for interactions between line and treatment effects. Any effects found would have to be interpreted with caution.

Means and standard errors were calculated, and plotted. Weights and relative gains were analysed using a mixed model least squares and maximum likelihood computer program, as in chapter 2 (Harvey, 1985). Relative gains were calculated as natural log of final weight minus natural log of initial weight for the period in question. Analyses were carried out using a model with line and treatment as fixed cross classified effects. Family was included as a random effect. The model also tested for interactions between lines and treatments. The final weight, and all relative gains were regressed onto the natural log of initial weight to remove scaling effects

due to the substantial differences in size between the high and low lines.

Relative gains on days two, five, and eight, were analysed as being the days after one, two, or three courses of injection respectively, and hence likely times to observe effects. The longevity of the effects on continued gain was analysed by the relative gain at days fifteen and twenty-two.

7.4.2 Results

Significant effects of treatment on final body weight and on relative gain for the period of the experiment, RG(day 22), ($P<0.01$) were found, also on relative gain for the period from day 0 until day 15 ($P<0.05$) (Table 7.4) . Animals treated with antiserum had lower final body weights and lower overall relative gains than animals treated with either saline or normal sheep serum. Indeed in the high line animals injected with normal sheep serum had greater overall relative gains and final weights than animals injected with saline (Table 7.4). No significant interaction effects were observed (Table 7.4).

There was no clearly discernible check in the growth of the high line mice after any of the injections (Figure 7.5) . However overall the group treated with the antiserum to rat-GH appeared to have a lower growth rate. Effects were even less clear in the low line, where daily variation in growth rate appeared to be greater (Figure 7.6) .

7.5 Discussion

Sheep antiserum to rat growth hormone appears to have reduced growth in mice. The effects of the antiserum on growth appear to have been greatest in the first preliminary trial but this may reflect the small sample sizes used in all the trials. Also

Table 7.4

Weights and gains of P-Line mice treated with antiserum to GH

	Low line			High Line			Significance line effects	Significance treatment effects	Significance interaction effects	S.E. means
	saline	normal serum	antiserum	saline	normal serum	antiserum				
Ln(initial wt) (init wt (g))	2.517 (12.39)	2.506 (12.26)	2.494 (12.11)	2.895 (18.08)	2.852 (17.32)	2.862 (17.50)	**	NS	NS	±0.044 (1.04)
Ln(Final wt) (Final wt (g))	2.991 (19.91)	2.941 (18.93)	2.908 (18.32)	3.593 (36.34)	3.617 (37.22)	3.542 (34.53)	**	**	NS	±0.039 (1.04)
RG(day 2)	0.036	0.041	0.025	0.127	0.144	0.134	**	NS	NS	±0.018
RG(day 5)	0.060	0.070	0.034	0.283	0.326	0.288	**	NS	NS	±0.029
RG(day 8)	0.126	0.113	0.093	0.468	0.512	0.435	**	NS	NS	±0.030
RG(day 15)	0.195	0.153	0.132	0.701	0.727	0.648	**	*	NS	±0.034
RG(day 22)	0.255	0.205	0.172	0.856	0.880	0.806	**	**	NS	±0.039

Ln, Natural Logarithm
RG, Relative gain, calculated as the difference between the natural logs of the initial weight and the weight on the day stated.
NS, no significant difference between the groups being compared.
* P<0.05, ** P<0.01)

Figure 7.5

Mean daily gains of male high P-Line mice from 28 to 35 days old. They recieved two injections, at 9am and 5pm on days 1, 4 and one injection at 9 am on day 7 after the start of the experiment, of 11µl/ g body weight each time. They recieved 3µg/ml of antiserum, 3µg/ml of normal sheep serum or saline. Error bars give the maximum standard errors of the means

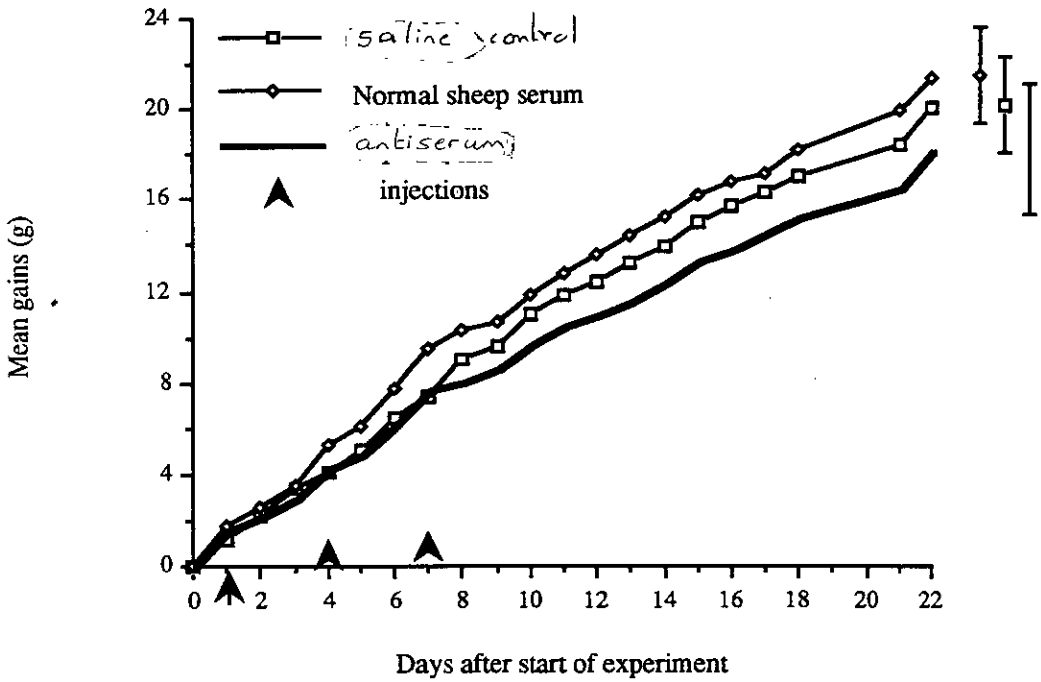
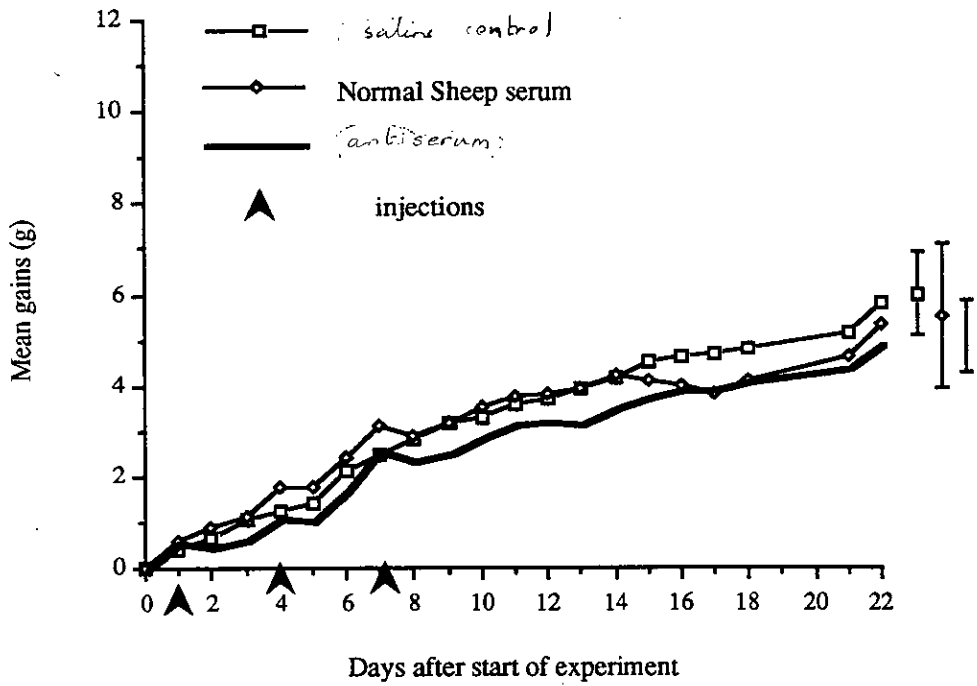


Figure 7.6

Mean daily gains of male low P-Line mice from 28 to 35 days old. They recieved two injections, at 9am and 5pm on days 1, 4 and one injection at 9 am on day 7 after the start of the experiment, of 11µl/ g body weight each time. They recieved 3µg/ml of antiserum, 3µg/ml of normal sheep serum or saline.Error bars give the maximum standard errors of the means.



the mice used used in the first trial were only 83% P-Line, and it is possible that the difference in genotype caused the difference in response to the injected antiserum. Low line mice from the backcross did show a reduction in growth rate for the duration of the first trial (Figure 7.3) which was not apparent in either of the later trials using pure bred P-Line mice. Differences in long term responses to antiserum were noted in rats and attributed to differences in ability to remove the foreign proteins from the blood (Madon *et al*, 1991)

In the final trial no significant interactions between line and treatment effects (Table 7.4) were found, which indicated that the lines have shown response to the removal of GH in proportion to their weight, which supports the result of Chapter 6 in which no difference between the line in response to the removal of GH by backcrossing the *little* gene into the P-Lines was found.

It was possible that serum to rat growth hormone would not bind to mouse GH, and that the effects observed were non-specific reactions to the sera. The differences between the effects of normal sheep serum and the antiserum, in particular the increased final weight of high line mice injected with normal sheep serum compared to those injected with saline (Table 7.4) do not support this. It appears that the sheep antiserum to rat growth hormone serum has indeed reduced growth by removing GH, the effect is not just due to illness from the large amounts of foreign protein injected.

Although all conclusions from the chapter must be treated with caution due to the small sample sizes used it appears that treatment of growing mice with sheep antiserum to rat growth hormone has reduced growth although the magnitude of the effect was less than when GH was removed by mutation (Chapters 5 and 6 this thesis, Pidduck and Falconer, 1978). The magnitude of the decrease due to the putative removal of serum GH was similar to the increase noted when serum GH was elevated by the injection of exogenous GH, i.e. approximately 10-15% (Figure 3.2 compared

to figures 7.5 and 7.6). The results in combination suggest that direct manipulation of serum GH levels is not an effective method of modulating growth rate. The lines do not appear to have differed in their response, which supports the results of the previous chapters which attempted to alter the GH axis.

Chapter 8
Discussion

Several lines of investigation have been carried out to determine if selection for lean mass, or for fat mass, had affected the growth hormone axis. The mice investigated were selected on indices similar to those used in livestock, and the information obtained may be applicable to such animals. Investigation attempted, directly or indirectly, to determine if the levels of growth hormone had altered, and if sensitivity to GH had altered.

8.1 Growth Hormone Treatment in selected lines

Mice selected for high or low lean mass, and mice selected for high or low fat mass all showed an increase in the rate of weight gain over the treatment period, and in their final weight after treatment with rbGH, compared to mice injected with saline. No significant differences between the lines in the increase relative to initial weight were observed (Chapter 3 and 6). This suggests that selection has not altered the ability of the lines to respond to exogenous GH, unlike earlier work in other lines of mice selected for high and low body size in which differences in growth implied that changes in GH metabolism had occurred as a result of selection on growth rate (Pidduck and Falconer, 1978). This difference might have been explained by a masking effect of endogenous GH in the P-Lines as Pidduck and Falconer, (1978) used GH deficient *dw* mice. However the *little* gene has been backcrossed into the P-Lines, and when GH deficient *lit/lit* P-Line mice were treated with rbGH they responded to exogenous GH in proportion to their body weight (Chapter 6) suggesting that there is no difference between the P-Lines in responsiveness to GH. This suggests that there have been no effects of selection on GH receptors in the

P-Lines.

Plasma GH concentrations appear to be lower in the mice selected for high body weight than in those selected for low body weight at 4, 5, and 7 weeks of age (Tables 4.4, 4.5). As the lines do not differ in responsiveness to GH wild type (with respect to dwarfism) high line mice might have been expected to show a greater response to exogenous GH than low line animals. Such a difference was not detected (Chapter 3), which may indicate no difference or that the difference was too small to be detected. Certainly the additional increase of weight gain in response to exogenous GH was small compared to the total gain over the same period (10 to 15 percent, Chapter 3) and any difference between the lines in response is likely to have a smaller effect than this.

The general increase in tibia bone length in mice treated with rbGH indicates a long lasting effect of the GH on growth, as it increased bone growth. Some of the greater body weight observed in treated animals may be accounted for by their increased bone size and bone weight. Such an increase in bone size would be consistent with other studies, for example the greater bone length noted in mice transgenic for GH (Wolf *et al*, 1991). Also long bone length in pubescent humans suffering from isolated growth hormone deficiency increased after treatment with GH (Tanner *et al*, 1976). Alternatively the increased weight may have been due to carcass components that were not analysed, for example, in sheep a substantial increase in non-carcass components of the body, such as skin, fleece and gut has been observed after GH treatment (Johnsson *et al*, 1987).

Treatment with rbGH did not significantly affect fat content as measured by gonadal fat pad percentage and dry weight percentage although there was generally a slight decrease. The results in this study may be due to species specificity with rbGH not affecting fat percentage in mice. Bovine GH can affect carcass composition,

exogenous bGH reduced carcass fat in female lambs (Butler-Hogg and Johnsson, 1987) so this may reflect a species specific difference. The effects of GH from one species can vary across species, for example, ovine GH increased lipolysis in rat, but not in ovine, adipose tissue (Duquette *et al*, 1984).

Taken together the effects on body weight, and on carcass fat suggest that responsiveness to exogenous GH has not been affected by selection on lean mass, or on total fat. In addition the effects of GH on dwarf *little* P-Line mice suggest that selection on lean mass has not altered responsiveness to GH, which suggests that the selection has not altered receptor levels or sensitivity.

Removal of GH for short periods was attempted by treatment with antiserum to GH (Chapter 7), a strategy that had had dramatic effects on growth in rats (Madon *et al*, 1981). This treatment appeared to reduce growth rates more than treatment with normal sheep serum, which suggested that the antiserum had reduced plasma levels of GH (Table 7.4) although the effects were generally small, similar in magnitude to the effects of treatment with exogenous GH. No significant interaction between line and treatment effects were noted which suggests that the lines have responded in a proportional manner to the treatment, as they did to treatment with GH, again suggesting that selection has not altered the response to GH.

The magnitude of the decrease due to the putative removal of serum GH was similar to the increase noted when serum GH was elevated by the injection of exogenous GH, i.e. approximately 10-15% (Figure 3.2 compared to figures 7.5 and 7.6). The results in combination suggest that direct manipulation of serum GH levels is not an effective method of modulating growth rate.

8.2 Preweaning growth

The lines of mice differed in weight at birth, which indicated prenatal differences in growth. This may be a maternal effect, high line females were two to three times the size of low line females (Sharp,*et al*, 1984, Hastings and Hill, 1989). However not only were the high line pups approximately twice the weight of low line pups at birth, nearly twice as many were born, on average. Thus the average litter weight of high line females was approximately four times that of low line females, which suggests the differences may not be entirely due to maternal effects.

The lines studied here showed similar absolute, not relative, rates of gain over the period to 18 days of age (Figures 4.3, 4.4). At this age the rate of gain increased in both lines, but the increase was greater for the high line than the low line (Chapter 4). Responsiveness to growth hormone begins at approximately 14 days of age in the mouse, (Borrelli, *et al*, 1989). Thus the increase in the rate of gain at 18 days may be due to the animals having become responsive to GH and the effect being detectable. However this does not explain the greater increase in the high line (Figures 4.3, 4.4, and 4.5) which has lower plasma levels of GH, particularly as an increase in the rate of weight gain was also observed in high line GH deficient *little* mice (Figures 5.6, 5.7). Low line *little* mice had a much smaller increase than high line *littles* and wild type low line mice. In both lines the increase in the rate of weight gain was greater in the wild type animals than in the *little* dwarfs (Figures 5.6, 5.7). This suggests that the increased rate of weight gain is not primarily due to growth hormone, but to some other factor and that GH acts to amplify this effect.

Mice start to be weaned and eat solid food at approximately 18 days of age (Personal observation). It is possible that maternal milk supply may be limiting growth before weaning and that the increase in growth rate may be in response to an

increased food supply. The crossfostering experiment (Chapter 4) suggests that food supply does limit growth before weaning, at least in the low P-Line, but an increase in rate of weight gain occurs at 18 days old in low line pups suckled by high line dams (Figure 4.3) suggesting that food supply is not the only factor involved.

This all suggests that some factor other than GH is responsible for the differences between the lines, probably some other component of the GH axis such as the insulin like growth factors.

8.3 GH levels

Plasma GH concentrations appear to be lower in the mice selected for high body weight than in those selected for low body weight at 4, 5, and 7 weeks of age (Tables 4.4, 4.5). This would agree with work in high growth mice which found that they had lower levels of plasma GH and higher levels of plasma IGF-I than unselected controls (Medrano *et al*, 1991) and from work in chickens which found that broiler chickens had lower plasma GH levels than egg layers (Burke and Marks, 1982). As the lines show divergent growth from the point at which they become responsive to GH, and this is the age at which IGF-I secretion becomes GH inducible (Mathews, *et al*, 1988) it appears possible that the high P-Line mice have higher levels of IGF-I than the low line. However work on these lines has already indicated that they do not differ significantly in basal IGF-I levels at 5 weeks old, that is, during the period of rapid growth. Basal levels do differ significantly between the lines at 10 weeks of age, with the high line having higher IGF-I levels (McKnight and Goddard, 1989).

Growth in older mice from 4 to 10 weeks of age, appears to show differences between the lines (Figure 4.6). It appears that Low line mice have a reduction in the rate of gain between 5 and 7 weeks of age which does not occur until later in the high line mice. Such a check is not visible in wild type control animals from 4 to 7 weeks

old (Figure 3.2). It may be an artifact due to weights only being recorded at 4 points during the period from 4 to 10 weeks of age. However it does suggest that selection may have altered the length of the growth period, especially when the changes in plasma levels of GH and IGF-I with age are considered.

8.4 Future directions

The lines differ in body weight at birth. This may be due solely to maternal effects, or to a combination of maternal and individual *in utero* effects. Reciprocal crosses between the lines would give pups of identical genotype in different uterine environments which should enable separation of maternal and individual effects on body weight before and at birth. (This experiment is presently being carried out, Guneren and Hastings, personal communication). If no individual effect was identified then factors which could only act *in utero* are less likely to be important in differences between the lines; indeed since selection was within family, *in utero* effects common to the whole litter would not be subject to selection.

There is evidence for individual *in utero* differences between the lines. In the original replicates, P1, P2, and P3, which were crossed to give the P6 lines (Bootland *et al*, 1991) the high lines had two to three times higher levels of ornithine decarboxylase than the low lines at embryonic days 11-13 (Gray, Tait and Bulfield, Personal communication). Ornithine decarboxylase is involved in cell growth and proliferation, so may be responsible for the differences between the high and low P-Lines. Investigation to determine the age at which embryos start to differ in size may help determine whether this difference is related to the difference in final body weight between the high and low P-Lines.

IGF-II, which has not been investigated in the P-Lines, is necessary for animals to reach normal adult body weight. Mice heterozygous for an IGF-II gene which had

been disrupted by homologous recombination were only 60% of the body weight of homozygous wild type littermates (Dechiara, Efstratiadis and Robertson, 1990). The difference in body weight was observable by embryonic day 16, and appeared to be due to effects on the placenta (Dechiara *et al*, 1990). IGF-I and IGF-II activities are modulated by binding proteins which have been shown, at least for IGF-II, to inhibit their activity (McGuire, Jackson *et al*, 1992)). Thus IGF-II, or growth factor binding proteins may be involved in the differences between the lines. Investigation of levels of IGF-II in plasma, and various tissues at different stages of embryonic development accompanying measures of body size would indicate whether IGF-II could be involved in the differences between the lines. If no differences in IGF-II levels between the lines were found but the time at which it were expressed is the same, or shortly before, the time at which differences in weight appear, investigation into the expression of IGF-II binding proteins may be informative.

The induction of IGF-I and IGF-II may also have been altered by selection, and could be investigated by monitoring plasma levels of these hormones after treatment of GH deficient *little* mice with GH.

8.5 Conclusion

GH levels between the lines have been altered by selection, with the high P-Line having lower plasma levels of GH than the low line, whether this is due to an increase in levels in the low line, a decrease in the high line, or both, cannot be ascertained at present. Responsiveness to GH does not appear to have been altered by selection, despite this alteration in GH levels. Alterations in GH are clearly not solely responsible for the differences between the lines in growth, as shown by the differences between the high and low *lit/lit* mice. Given the differences in body weight and litter size at birth it is likely that an important component in the

differences between the lines acts before birth.

Taken together the results presented in this thesis suggest that the dramatic (three fold) difference in body weight observed in the P-Lines is not a consequence of large scale alterations in GH metabolism. The smaller number of investigations of the F-Lines also suggest that the three fold difference in fat content is not due to alterations in GH.

This all suggests that some factor other than GH is responsible for the differences between the lines, probably some other component of the GH axis such as the insulin like growth factors. It is therefore anticipated that future work on these lines will investigate the mediators of GH activity such as IGF-I, IGF-II and the IGF binding proteins. Also given the difference in weights at birth it is suggested that regulation of *in utero* growth would reward further study.

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Appendix 1

Bootland L. H., Hill W.G. and Sinnet Smith P.A., (1991). Effects of exogenous growth hormone on growth and body composition in genetically selected mice. *Journal of Endocrinology* **131:** 19-24

Effects of exogenous growth hormone on growth and body composition in genetically selected mice

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ABSTRACT

The effects of exogenous GH on growth and body composition were investigated in lines of mice selected for high or low body weight (P-lines) or high or low body fat (F-lines). Mice from all lines were given daily injections of recombinant bovine GH or a placebo for 21 days from 4 weeks old. They were killed and various organ weights measured. There was no consistent effect of GH on organ weights. In all lines of mice the rate of weight gain and final weight increased in response to GH. In both lines selected for body fat, GH treatment decreased fat content. The low body weight mice also became less fat, but in the high body weight mice GH treatment increased fat percentage. The results indicate that the differences in growth rate and body composition observed in these lines are not due to differences in responsiveness to GH.
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INTRODUCTION

Investigations of the role of growth hormone (GH) in the differences between genetic lines of animals differing in body weight, lean mass or rate of growth have produced conflicting observations. Broiler lines of chickens selected for faster growth rate have lower plasma levels of GH than slower growing layer lines (Burke & Marks, 1982; Stewart & Washburn, 1983). An experiment in which the Snell Dwarf gene was backcrossed into strains of mice selected for large and small size, thereby depleting them of endogenous GH, showed that GH was only partially responsible for differences in growth rate and that the smaller mice had reduced sensitivity to GH (Pidduck & Falconer, 1978). In addition, studies on a variety of species indicate that the relationship, within species, between endogenous levels of GH and several growth parameters is poor (Hart & Johnsson, 1986). There is, however, some evidence that larger breeds of cattle have increased levels of GH (Hart & Johnsson, 1986).

Experiments involving GH treatment in a number of species have produced a variety of results. Treatment of pigs with recombinant DNA-derived human GH (Baile, Della-Fera & McLaughlin, 1983) or with pituitary-derived porcine GH (Chung, Etherton & Wiggins, 1985; McLaren, Easter, Novakofski *et al.* 1988) increased growth rate. In lambs some studies have shown an effect on weight gain after treatment with pituitary bovine GH (Johnsson, Hart & Butler-Hogg, 1985) or with recombinant bovine GH (rbGH) (Pullar, Johnsson & Chadwick, 1986). However, other studies by the same group and others found no significant effect of rbGH on growth rates (Johnsson, Hathorn, Wilde *et al.* 1987; Sinnett-Smith, Woolliams, Warriss & Enser, 1989). Similarly, treatment with ovine GH did not significantly affect weight gain in lambs (Muir, Wein, Duquette *et al.* 1983). These studies indicate that while changes in plasma GH may be correlated with increased size and growth rate of some genetically different lines, it is probably not the only growth factor involved.

Investigations into effects of GH on carcass composition have consistently shown a reduction in the proportion of fat in the carcass. This has been demonstrated using porcine GH in pigs (Chung *et al.* 1985; Novakofski, McKeith, Grebner *et al.* 1988) although the weight of fat is not always decreased (Chung *et al.* 1985). A study using human GH in pigs, however, found no significant effect on body fat (Baile *et al.* 1983). All studies in lambs have shown a reduction in carcass fat in GH-treated animals as compared with controls (Wagner & Veenhuizen, 1978; Muir *et al.* 1983; Johnsson *et al.* 1985, 1987; Pullar *et al.* 1986;

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Butler-Hogg & Johnsson, 1987; Sinnett-Smith *et al.* 1989).

Treatment of pigs with porcine GH increased carcass protein (Chung *et al.* 1985), and in a study using bovine GH in lambs, significant increases were obtained in muscle mass after treatment (Butler-Hogg & Johnsson, 1987), whereas another study using intact male lambs showed no significant increase in the proportion of protein in the carcass after bovine GH treatment despite a significant increase in growth rate (Pullar *et al.* 1986). Others have found only a small effect on muscle gain in lambs (Muir *et al.* 1983; Johnsson *et al.* 1987).

These results indicate that effects of GH on body composition are likely to be greater than on body weight and rates of weight gain. Consequently any differences between divergent stocks in responsiveness to GH are more likely to affect composition. In this study we have investigated the effects of rbGH on growth differences in lines of mice selected for differences in growth and body composition. The selected lines of mice in this study differ by a factor of 1·5 in body weight at 10 weeks (P-lines), or by a factor of 2·5 in gonadal fat pad weight as a proportion of body weight at 10 weeks (F-lines) (Hastings & Hill, 1989).

MATERIALS AND METHODS

Animals

The mice were taken from lines established in 1980 (Sharp, Hill & Robertson, 1984). Mice were drawn from a common base population to give three replicates of each of three selection treatments: high, low and control for each selected trait. Selection was within litter on 10-week-old males. The F-line was selected on the ratio of gonadal fat pad weight to body weight; the P-line on fat-free mass predicted from body weight and gonadal fat pad weight (Sharp *et al.* 1984). In generation 20 the selection criteria were modified such that the P-lines were selected on 10-week weight in both sexes and the F-lines were selected on the ratio of dry to wet weight in 14-week-old males. The three replicates of each direction were crossed at this stage to give a single, unreplicated line with lower inbreeding. The mice used were the offspring of mice from generation 12 (F-line) or 13 (P-line) after the cross, i.e. after a total of 32 or 33 generations of selection respectively.

The animals were kept in controlled light conditions (14 h light : 10 h darkness) from weaning at 3 weeks until the end of the treatment period. They were given free access to BP expanded mouse diet number 1 food (Special Diet Services, Witham, Essex, U.K.), with the same food available as mash until the smallest mouse in a cage reached 10 g in weight. Only males were used, to avoid the confounding effects of sex-based differences. The F- and P-lines were non-contemporaneous.

Experimental design and treatments

Mice were assigned randomly within family to the control or treatment group when weaned at 21 (±2) days. Treatment commenced 7 days later in order to reduce maternal influences, the major source of variability in weight at weaning (Eisen, 1974). Animals were treated daily from 4 to 7 weeks of age with rbGH (kindly donated by the American Cyanamid Co., Princeton, NJ, U.S.A.) at 9 μg/g body weight per day given as 1·8 mg/ml sterile solution prepared in isotonic bicarbonate buffer (pH 9·4) or with a saline placebo (0·1 ml/20 g body weight). Injections were given s.c. within the 2 h before the start of the dark period and the mice were weighed immediately before injection. On day 21 after the treatment started the mice were weighed and then killed by cervical dislocation. The liver, spleen, kidneys, gonadal fat pad (GFP) and pectoralis muscle were dissected out and weighed. These components were then restored to the carcass which was freeze-dried and weighed. Finally the hind legs were taken and boiled to remove the flesh, enabling tibia length to be measured. The length did not include the terminal epiphyses.

In a preliminary experiment (not shown), the growth-promoting ability of rbGH was confirmed in GH-deficient Little (*lit/lit*) mice which lack circulating GH and respond to GH treatment by increased growth (Beamer & Eicher, 1976).

Statistical analysis

The results were analysed using a mixed model least squares and maximum likelihood computer program (Harvey, 1985). Analyses were carried out using a model with line and treatment as fixed cross-classified effects. Family was included as a random effect, nested within line. The model also tested for interactions between line and treatment. All analyses used natural logarithms of weight to remove scaling effects due to the substantial difference in size between the high and low selected P-line mice. Traits, other than initial weight, were also regressed on the natural log of initial weight to remove the effects of initial body weight differences between animals. This regression reduced the S.E.M. values in the analyses (log final weight showed a correlation with initial weight of over 0·80 for all lines other than the one selected for high body fat, in which the correlation exceeded 0·65). Relative gain was also analysed as its logarithm, calculated for each animal as natural log of final weight minus natural log of initial weight.

RESULTS

All the lines of mice in this study responded to rbGH by increasing their growth rate (Tables 1 and 2).

TABLE 1. Body weights of mice selected for high and low body fat content (F-lines). Male mice from the fat and lean lines were injected daily from 4 to 7 weeks of age with 9 μg recombinant bovine GH (rbGH)/g body weight per day, or with 0·1 ml saline/20 g body weight per day. Organ weights are as a percentage of final body weight. All traits other than initial weight were regressed on Ln (initial weight)

	Fat		Lean		Significance treatment	Significance line	S.E.M
	Saline	GH	Saline	GH			
No. of animals	18	17	18	18			
Ln (body weight (g)) at 4 weeks	2·94	2·89	2·93	2·84	*	NS	±0·084
(initial body wt (g))	(18·9)	(18·0)	(18·5)	(16·9)			±(1·09)
Ln (body weight (g)) at 7 weeks	3·39	3·47	3·32	3·40	**	**	±0·017
(final body wt (g))	(29·7)	(32·0)	(27·0)	(30·2)			±(1·02)
RG (overall)	0·49	0·57	0·42	0·51	**	**	±0·017

Ln, natural logarithm; RG, relative gain, calculated as the difference between the natural logs of the initial and final weights of the period in question. NS, no significant difference between the groups being compared. *$P<0·05$, **$P<0·01$ for line or treatment effects (the data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985)).

TABLE 2. Body weights of mice selected for high and low whole body weight (P-lines). Male mice were injected daily from 4 to 7 weeks of age with 9 μg recombinant bovine GH (rbGH)/g body weight per day, or with 0·1 ml saline/20 g body weight per day. All traits other than initial weight were regressed on Ln (initial weight)

	High		Low		Significance treatment	Significance line	Significance interaction	S.E.M.
	Saline	GH	Saline	GH				
No. of animals	30	30	30	30				
Ln (body weight (g)) at 4 weeks	3·13	3·13	2·68	2·53	*	**	**	±0·067
(initial wt (g))	(22·9)	(22·9)	(14·6)	(12·6)				(±1·07)
Ln (body weight (g)) at 7 weeks	3·63	3·71	3·16	3·23	**	**	NS	±0·021
(final body wt (g))	(38·1)	(40·0)	(23·6)	(25·3)				(±1·02)
RG (overall)	0·83	0·90	0·33	0·40	**	**	NS	±0·049

Ln, natural logarithm; RG, relative gain calculated as the difference between the natural logs of the initial and final weights of the period in question.
NS, no significant difference between the groups being compared. *$P<0·05$, **$P<0·01$ for line or treatment effects (the data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985)).

At 4 weeks of age, before treatment, there was no significant difference in body weight between the lines selected for high and low fat content (F-lines) (Table 1). Mice selected for high fat had a final weight 1·14 times that of the lean line. The line selected for high body weight was significantly ($P<0·01$) heavier, by about 9 g, than the line selected for low body weight, irrespective of treatment, and gained twice as much weight over the test period (Table 2). Averaged over both fat lines, the gain of the treated mice was a factor of 1·9 (3–4 g) greater than that of the untreated mice. Averaged over both body weight lines the gain of the treated mice was a factor of 1·15 greater than that of the untreated mice. There is an apparent discrepancy between the values of the relative gain in Tables 1 and 2, and that calculated from the initial and final weights given. This is a consequence of the different correction factors applied to relative gain, and to final weight, by their regression on initial weight. The discrepancy is greater in the body weight lines due to the greater difference in weight between these lines.

In all lines it appeared that the rbGH had caused the maximal rate of weight gain to be maintained for a longer period than in untreated mice, resulting in greater weight gain over the whole period, and greater final weights. Gonadal fat pad and dry matter percentages are indicators of the amount of fat in the carcass (see Materials and Methods). Both were significantly ($P<0·01$) greater in the high fat line than in the low fat line (Table 3). Gonadal fat pads were proportionately smaller in the high P-line mice ($P<0·01$) (Table 4). This may be due to the original selection criterion being fat-free mass, predicted from gonadal fat pad weight and body weight. Treatment with rbGH had no significant effect on gonadal fat pad weight as a percentage of body weight in any line or

TABLE 3. Organ weights of mice selected for high and low body fat content (F-lines). Male mice from the fat and lean lines were injected daily from 4 to 7 weeks of age with 9 μg recombinant bovine GH (rbGH)/g body weight per day, or with 0·1 ml saline/20 g body weight per day. Organ weights are expressed as a percentage of final body weight. All traits other than initial weight were regressed on Ln (initial weight)

	Fat		Lean		Significance treatment	Significance line	Significance interaction	S.E.M.
	Saline	GH	Saline	GH				
Gonadal fat pad (% body weight)	1·09	1·06	0·41	0·36	NS	**	NS	±0·044
Dry body weight (% wet body weight)	38·6	37·9	31·8	29·9	*	**	NS	±0·62
Pectoralis muscle (% body weight)	0·35	0·29	0·30	0·38	NS	NS	**	±0·017
Tibia bone length (mm)	16·5	16·6	16·6	16·8	NS	NS	NS	±0·10
Liver weight (% body weight)	5·5	5·5	5·4	5·2	NS	NS	NS	±0·12
Kidney weight (% body weight)	1·43	1·37	1·58	1·53	*	**	NS	±0·042
Spleen weight (% body weight)	0·31	0·32	0·40	0·41	NS	*	NS	±0·027

Ln, natural logarithm; RG, relative gain, calculated as the difference between the natural logs of the initial and final weights of the period in question.
NS, no significant difference between the groups being compared. *$P<0·05$, **$P<0·01$ for line or treatment effects (the data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985)).

TABLE 4. Organ weights of mice selected for high and low body weight (P-lines). Male mice were injected daily from 4 to 7 weeks of age with 9 μg recombinant bovine GH (rbGH)/g body weight per day, or with 0·1 ml saline/20 g body weight per day. Organ weights are expressed as a percentage of final body weight. All traits other than initial weight were regressed on Ln (initial weight).

	High		Low		Significance treatment	Significance line	Significance interaction	S.E.M.
	Saline	GH	Saline	GH				
Gonadal fat pad (% body weight)	0·47	0·50	0·95	0·86	NS	**	NS	±0·088
Dry body weight (% wet body weight)	33·1	36·0	36·0	35·2	NS	NS	NS	±1·63
Pectoralis muscle (% body weight)	0·36	0·35	0·34	0·34	NS	NS	NS	±0·028
Tibia bone length (mm)	17·2	17·6	15·8	16·0	NS	**	NS	±0·23
Liver weight (% body weight)	5·8	5·5	5·4	5·3	NS	NS	NS	±0·17
Kidney weight (% body weight)	1·47	1·38	1·55	1·49	*	NS	NS	±0·069
Spleen weight (% body weight)	0·38	0·44	0·22	0·21	NS	**	*	±0·023

Ln, natural logarithm; RG, relative gain calculated as the difference between the natural logs of the initial and final weights of the period in question.
NS, no significant difference between the groups being compared. *$P<0·05$, **$P<0·01$ for line or treatment effects (the data were analysed by analysis of variance with a mixed model least squares and maximum likelihood computer program (Harvey, 1985)).

on dry weight as a percentage of body weight in the body weight lines (Tables 3 and 4). There was a significant reduction of dry weight as a percentage of body weight in the rbGH-treated fat lines. When all four lines were analysed together there was a significant ($P<0·05$) line-by-treatment interaction for dry weight percentage. This suggests a between-line difference in the involvement of GH in fat regulation.

The pectoralis was chosen as an easily dissected muscle which could be used to estimate the effects of GH on muscle mass. GH treatment did not affect pectoralis weight as a percentage of final weight in the lines selected for body weight (Table 4). GH treatment reduced pectoralis weight as a percentage of final weight in the high fat mice but increased it in the low fat mice (Table 2). This was apparent as a significant line-by-

treatment interaction ($P<0{\cdot}05$), and suggests that selection in the high fat mice has altered the interaction of GH with metabolism.

The high body weight line had a significantly ($P<0{\cdot}01$) greater tibia length than the low body weight line (Table 4). Tibia length was consistently, but not significantly, greater in treated animals than in the control group in each of the four lines of mice (Tables 3 and 4).

There were no significant line or treatment effects on liver weight as a percentage of body weight. The significant ($P<0{\cdot}05$) reduction in kidney weight as a percentage of body weight (Tables 3 and 4) after treatment implies that the kidney is less susceptible to GH than other tissues. This may be due to kidneys reaching their final size before the rest of the body. A significant ($P<0{\cdot}05$) difference in spleen weight as a percentage of body weight between lines was noted in these experiments, as was a significant ($P<0{\cdot}05$) interaction between line and treatment in the body weight lines (Tables 3 and 4). Previous studies have shown that spleen size and shape vary considerably between inbred strains (Dunn, 1954). This suggests that the differences may be due to drift following the long separation of the lines. No pathological changes were noted.

DISCUSSION

Treatment with GH increased the rate of weight gain and final weight in mice of all lines. The similarity of the response between lines in terms of the increase of weight gain and final weight relative to initial weight suggest that selection has not altered the ability to respond to GH. The effect of selection on response to GH might have been expected to be greatest in the lines selected for high and low body weight. Studies in other lines of mice selected for high and low body size indicated that the mice selected for smaller size were also less responsive to GH (Pidduck & Falconer, 1978). However, other research has shown that GH often does not show obvious correlations with differences in rates of weight gain and body size. Broiler chickens have lower levels of plasma GH than slower growing layer lines (Burke & Marks, 1982; Stewart & Washburn, 1983).

Assigning mice by weight within family to treatment group would have prevented the occurrence of significantly lower weights in the mice assigned to the treatment group in the low body weight and low body fat lines (Tables 1 and 2). However, the problem was not observed until the start of the treatment. Carcass analysis of 4-week-old mice would also have been useful in giving a base line to which comparisons could be referred. This was prevented by limitations in the number of mice available.

The general increase in tibia bone length in mice treated with rbGH indicates a long-lasting effect of GH on growth, following increased skeletal size. Some of the greater body weight observed in treated animals may be accounted for by their increased bone size and bone weight. In sheep a substantial increase in non-carcass components of the body, such as skin, fleece and gut, was observed after GH treatment (Johnsson *et al.* 1987). Skin and gut may account for part of the greater weight observed in these treated mice.

Treatment with rbGH did not consistently affect fat content as measured by gonadal fat pad weight as a percentage of body weight and dry weight as a percentage of body weight although there was generally a slight decrease. There may be species specificity with rbGH having a lesser effect on fat deposition in mice than it does in lambs. Similarly human GH had no significant effect in pigs (Baile *et al.* 1983). It might have been expected that the fat mice, having the greatest fat content, would have shown the greatest reduction in body fat after treatment with rbGH but this was not the case. This may be due to the limited effects on fat observed in this study.

The principal conclusion from these experiments is that mice from all the lines studied respond to treatment with rbGH by gaining weight faster. This growth is not solely due to an increased rate of gain of protein, as indicated by the relative size of the pectoralis muscle, and is probably due to greater growth of the bones, gut and skin. As the lines reacted very similarly in terms of increase of final weight relative to initial weight, and of gain relative to initial weight it can be concluded that there is no difference between them in their response to exogenous GH. The mice also showed some evidence of a reduction in fat after treatment, which supports previous work showing that exogenous GH improves carcass quality by reducing fat percentage. It appears therefore that the differences in growth rate observed in these lines are possibly not due to differences in responsiveness to GH. Any involvement of GH in these genetic differences may lie in differences in synthesis or secretion.

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Appendix 2

Bootland L. H., Hill W.G. and Hastings I. M., (1991). Production of high and low body weight *lit/lit* dwarves. *Mouse genome* **89**: 564

PRODUCTION OF HIGH AND LOW BODY WEIGHT *lit/lit* DWARVES

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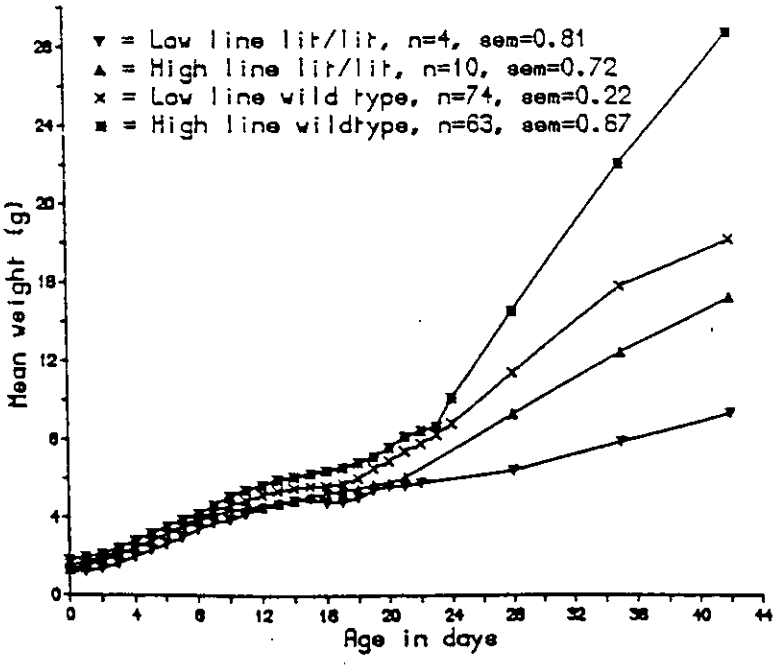
Lines for high and low body weight were established to investigate the consequences of selection on fat free body mass, (P-Lines) (1). After 20 generations the initial replicates were crossed and selection changed to 10 week weight in both sexes (2). At the start of this experiment 34 generations of selection had been carried out in total and adult body weight in the high line was about twice that in the low line.

As part of an investigation into the involvement of growth hormone (GH) in the differences between the P-Lines *lit* dwarfism was backcrossed into them. *lit/lit* dwarves on a C57BL/J background were mated to unselected mice from the lines. Test matings were carried out in each generation to ensure that the *lit* gene was being propagated. Three generations of backcrossing have been carried out giving a background which is 93% P-Line on average. The backcrossing program is continuing. *lit/lit* dwarves which differ in body size might be useful for studies into growth.

Effects on body weights of males from birth to 6 weeks of age can be seen below. Clearly GH is not the sole factor in the difference between the high and low P-Lines. This accords with the results of backcrossing *dw* dwarfism, which has greater pituitary effects than *lit* into the lines selected to diverge in growth rate (3).



Growth of male offspring of lit/+ matings from birth to 42 days old

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