GENETIC AND BIOCHEMICAL ANALYSES OF GROWTH

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.

October,

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ABSTRACT

Mouse lines were available which had been divergently selected for twenty generations on three traits of economic importance in commercial species: (i) food intake (Appetite, "A" lines); (ii) lean ~~mass~~ WEIGHT (Protein, "P" lines), and (iii) fat content (Fat, "F" lines). An analysis of carcass composition was undertaken at generation 20. The "F" lines had diverged in composition with only slight changes in lean ~~mass~~ WEIGHT. The "P" lines had diverged in lean ~~mass~~ WEIGHT but not composition. The "A" lines had diverged in lean ~~mass~~ WEIGHT and the high intake lines were slightly leaner than their low intake counterparts. Selection in these lines was continued up to generation 34 and the responses which occurred during this period were similar to those obtained during the first 20 generations: the type of correlated responses were the same and the heritability of the traits did not appear to have declined. The "F" and "P" lines showed little phenotypic reconvergence after nine generations of relaxed selection.

The "P" lines were examined to determine the contribution of "major genes" to their response; the X chromosome appeared to have a disproportionately large effect on body weight, but whether this was due to a single sex-linked "major gene" or due to polygenic divergence of the X chromosome remained unresolved. Growth and reproduction was investigated in the "F" lines. The Fat line accumulated fat over the entire period of study (26 weeks) whereas the (slower) rate of accretion in the Lean lines had effectively ceased by eight weeks of age. Males from the Fat line had smaller testes than those from the Lean line, but this did not appear to affect their fertility. The Fat line appeared to have slightly higher ovulation and pre-weaning survival rates.

The structure of the lines (replicated with control lines and all derived from a single base population) makes them ideal for investigating physiological changes which may be associated with the changed phenotype. The activities of six enzymes involved in *de novo* lipogenesis were measured, four of which were found to have diverged in the "F" lines; the magnitude of their divergence is similar to that observed in fat content and lipogenic flux.

A consideration of alleles affecting individual enzyme activities stimulated an attempt to asses their significance in natural populations, in particular whether small variations in activity are likely to be subjected to natural selection. An earlier model was extended to overcome some of its restrictive assumptions and the results suggested that selection pressures acting on small variation in

activity are likely to be much higher than previously predicted. Further consideration of selection on biochemical pathways led to the conclusion that it is likely to be more intense in the germline stage of the life cycle. Such selection depends on the processes of mutation, mitotic crossing over, and mitotic gene conversion to create diversity within diploid germline cells. A model is developed to describe this process and its wider implications for evolutionary theory are discussed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

ACC Acetyl CoenzymeA carboxylase

ACL ATP-citrate lyase

CoA CoenzymeA

$^{14}CO_3$ Carbon-14 labelled carbonate

EDTA Ethylene diamine tetra acetic acid

FAS Fatty acid synthetase

HCl Hydrochloric acid

MDH Malate dehydrogenase

ME Malic enzyme

NAD Nicotinamide adenine dinucleotide

NADH Nicotinamide adenine dinucleotide (reduced)

NADP Nicotinamide adenine dinucleotide phosphate

NADPH Nicotinamide adenine dinucleotide phosphate (reduced)

PK Pyruvate kinase

TRIS Tris(hydroxymethyl)aminomethane

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Publications arising from this thesis

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Hastings, I. M. and Hill, W. G., 1989. A note on the effect of different selection criteria on carcass composition in mice. *Animal Production* **48**:229–233.

Hastings, I. M. and Hill, W. G., 1989. Analysis of mice selected for fat content. 2. Correlated responses in the activities of enzymes involved in lipogenesis. *Genetical Research*, in press.

These correspond to Chapters 9, 3 and 7 respectively.

CHAPTER 1

GENERAL INTRODUCTION

Selection experiments on laboratory animals produce detailed pedigree and selection records under carefully controlled and defined conditions. These are important models for animal breeding where such detailed records of the selection used to in establish breeds are generally unknown. An additional advantage of laboratory species is that replicated lines can be easily derived from the same base population and used to estimate the effects of random genetic drift. Such estimates are a prerequisite for detailed genetic and biochemical analyses of the lines (Falconer, 1973; Hill, 1980). The advantages of large population sizes, carefully defined husbandry, short generation time, large litter size and a physiology similar to commercially important species (chiefly mammals) make mice a useful model species. Murine physiology and genetics are known in detail and mice have been the basis of numerous selection experiments on growth traits. Unfortunately their size has made them less suitable for biochemical research where larger amounts of tissue are desirable. Rats are the favoured species for such studies but the biochemistry and physiology of both species are similar and the techniques developed on rats can be successfully transferred to mice with little modification. This enables a wide range of assays to be utilized when investigating the biochemical and physiological changes associated with the altered phenotypes produced by selection experiments.

Basic metabolism and its physiological control appear similar in all mammalian species (Prosser, 1973), so the results of mouse experiments should be applicable to commercial species. It is important to understand the physiological basis of divergent phenotypes produced in selection experiments since the technology now exists for direct germline manipulation of genetic material. Successful application of such technology to commercial livestock production has enormous potential economic rewards. Its application is prevented not by technical limitations, but by the lack of any identified critical metabolic sites capable of altering the phenotype. Attempts to manipulate obvious physiological traits such as hormone levels have been hampered by deleterious pleiotropic effects; in the case of growth hormone in pigs these include morbidity, lameness, susceptibility to stress, peptic ulcers, and decreased fertility (Pursel *et*

al. 1989; Polge *et. al.* 1989). A detailed metabolic analysis of divergent phenotypes is necessary to identify specific sites in metabolism suitable for manipulation, or at least to identify the *type* of changes which may underlie a changed phenotype. The selection intensity applied in conventional breeding schemes will increase with the application of techniques such as artificial insemination, multiple ovulation and embryo transfer (MOET), and embryo splitting and cloning (Smith, 1988a). This raises the question of whether a phenotypic limit in response to selection will be reached and if so, how fast. The prediction of future responses may be aided by identifying the biochemical parameters which determine the phenotype and investigating their genetic control. The application of molecular techniques to livestock improvement both directly (by germline manipulation), and indirectly (by allowing greater selection intensities) will be enhanced by understanding the biochemical basis of altered phenotype.

The stimulus for the work described in this thesis was provided by the availability of lines of mice which had been selected for 20 generations on three traits of economic importance in commercial species: fat content, lean ~~mass~~ WEIGHT and food intake. It was deemed useful to continue these selection lines for three main reasons. Firstly, to discover if the lines continue to respond to selection. Secondly, to identify any changes in genetic parameters which had occurred over the course of selection such as heritability and any alterations in the type and extent of correlated responses, and to asses the contribution of major genes. Thirdly, to create lines of highly divergent phenotype as a resource for studies designed to elucidate their physiological basis.

(i) Continued response to selection.

The first part of this study will analyse the phenotypic responses to selection up to 34 generations. Of particular interest to commercial breeders are: is response maintained, what are the correlated responses, and do the nature or extent of these correlated changes alter over the course of selection? Increased public awareness and concern over the possible health implications of excessive intake of animal fats have resulted in consumer pressure for leaner meat, and as lean requires less feed energy to deposit than fat, there is a strong financial incentive to reduce the amount of fat in the carcass. One strategy is to alter these components by appropriate selection; for example, the fat content of pigs has approximately halved over the last twenty years as a result of selection (Webb, 1986). It is important to know the feasibility and limitations of this approach and the possibility of predicting how different selection criteria may be expected to

change meat composition. Carcass compositions of the mouse lines were determined at generation 20 and compared to previous results obtained at generations 7 and 14 to find how these traits had changed. Reversion of phenotypic traits after selection is discontinued is important in commercial contexts and occurs when natural selection acts against the changed phenotype. Changes in growth traits which had occurred during 9 generations without selection were measured in lines selected on the basis of fat content and lean ~~mass~~ WEIGHT.

(ii) Genetic basis of response.

Genetic variance is lost over the course of selection by random genetic drift and by fixation of selected alleles. Random gene loss by drift can be estimated but fixation by selection cannot be quantified. The cumulative loss of additive genetic variance should slow the rate of response as measured by the heritability of the trait. The heritability of each trait will be estimated for different periods of the selection history to ascertain if a significant reduction had occurred. Recent work has attempted to assess the contribution of major genes (i.e. single alleles with large effect on phenotype), in quantitative traits. The presence of such genes can be detected statistically (Hill and Knott, 1989) and have been implicated in several morphological traits (Stewart and Elston, 1973). Several major genes affecting growth traits have been identified in commercial species (Pirchner, 1988) and there are major genes in mice affecting two of the traits selected in this study: fat content (obesity; Festing, 1979) and growth rate (Bradford and Famula, 1984).

(iii) Physiological basis of changed phenotype.

There are two branches of genetics concerned with investigating the response to selection. Quantitative genetics attributes the response to the differential survival of alleles at loci affecting the trait and their interaction through linkage, epistasis and dominance; no attempt is made to identify the gene product of these loci. Physiological genetics relegates the role of DNA to that of a passive carrier of information and concentrates on the biochemical differences which determine the phenotype. Historically there has been little interaction between the two approaches, but potential genetic manipulation of livestock has added impetus to a joint approach (e.g. Bulfield, 1980). The lines studied here have been the subject of several physiological studies (Brien *et al*, 1984; Bishop and Hill, 1985; Brien and Hill, 1986; Parker, 1988; Asante, 1989; Asante, Hill and Bulfield, 1989; M^{c}Knight and Goddard, 1989; Moruppa, Hill and Sinnett-Smith,

1989), they are replicated with control lines and their genetic and selective history is well documented (Sharp, Hill and Robertson, 1984; Hill and Bishop, 1986). They therefore provide a suitable resource for attempts to elucidate the biochemical basis of divergence in quantitative traits. Identification of such biochemical changes may provide a starting point for direct genetic manipulation or provide markers, correlated to a desirable trait, upon which selection could be based. Such markers may be of commercial interest if they allow selection on sex-limited traits (e.g. lactation in dairy cattle), predict performance at an earlier age thereby reducing the generation interval, are cheaper, easier or faster than direct measurement of the desired trait, or are related to a variable physiological trait underlying a threshold character (e.g. litter size). A suitable biochemical model trait for investigation should be simple, well characterised and commercially important. The *de novo* synthesis of fatty acids is an appropriate biochemical pathway which satisfies these criteria. By investigating the activities of six enzymes involved in lipogenesis it was hoped to draw inferences about the genetic control of their activity and to examine their phenotypic effects. This study is aided by the presence of replicates and controls. The data obtained should illustrate the importance of enzyme activities as indirect indicators of fat content in the mouse. The activities of four enzymes producing NADPH (a cofactor necessary for fat synthesis) and the rate of lipogenesis were determined in the same tissues from the same lines in approximately the same generations (Asante, 1989; Asante *et al*, 1989). The two studies in conjunction should provide a detailed study of the enzymes affecting lipogenesis.

Theoretical considerations of enzyme activity variants in populations were undertaken in parallel with these studies. Enzymes are unique in that both their function *in vivo* and their population genetics have been extensively investigated. They function in complex biochemical pathways and their influence on the phenotype depends on the properties of such pathways. These properties have been attracting increasing attention (Kacser and Burns, 1973, 1979, 1981; Heinrich and Rapoport, 1974; Groen *et al.* 1982; Crabtree and Newsholme, 1985, 1987; Keightley and Kacser, 1987; Kacser and Porteous, 1987). A method is developed to predict their effects *in vivo* from data obtained in studies of their population genetics, without the restrictive assumptions of previous analysis. The results confirm previous predictions that even relatively large changes in activity have very small effects *in vivo* and are likely to be associated with equally small selective differences. It is difficult to explain how such small selective differences acting on biochemical pathways in adults can maintain the very high levels of catalytic efficiency observed in enzymes. This led to the novel

hypothesis that selection may act on biochemical pathways in the germline stage of the life cycle. Mutation, mitotic crossing over and mitotic gene conversion are known to occur in eukaryotic cells and will create diversity within diploid germline cell lineages. If selection occurs between lineages on the basis of their biochemical characteristics, selection pressures on enzyme variants will be increased. A model is developed to describe this process and its implications for population genetics are discussed.

The study therefore has three main aims: to describe, analyse and interpret the responses that occurred during selection between generations 20 and 35; to try to identify physiological traits correlated with the changed phenotype, in this case between fat content and enzymes involved in lipogenesis; and thirdly, to make inferences on the type of biochemical changes that caused the changed phenotype, and investigate the population genetics of enzymes acting in complex metabolic environments.

CHAPTER 2

DIRECT AND CORRELATED RESPONSES IN LINES OF MICE SELECTED ON CARCASS FAT CONTENT, LEAN ~~MASS~~ WEIGHT AND FOOD INTAKE

2.1. A brief introduction to the lines.

The selection lines studied in this thesis were designed to investigate the consequences of selection on a range of traits related to growth, composition and appetite, namely food intake corrected for body weight (appetite, A), fat content (F) and lean ~~mass~~ WEIGHT (protein, P). They were initiated from a base population derived from the following strains: JU (inbred), CBA (inbred) and CFLP (outbred). The offspring of reciprocal JU/CBA matings were crossed with CFLP, followed by one generation of random mating; the next generation was designated as generation 0. A more comprehensive description of their origin and maintenance can be found in Sharp (1983) and Sharp et al. (1984). Precise definitions of the selection criteria together with means of the selected traits at generation 20 are given in Table 2.1. Initial investigations had shown that gonadal fat pad weight (GFPW) was highly correlated to total fat and consisted of approximately one-eigth of the total fat. Thus in the F lines selection was essentially on the proportion of fat in the carcass, and in the P lines selection was essentially on lean ~~mass~~ WEIGHT. Selection was practised high and low for each criterion, together with an unselected control with three replicates of each, hence 27 lines were established in total. Within-family selection was practised on a mating scheme similar to that described by Falconer (1973); this scheme was designed to minimize short-term inbreeding by mating together the least-related individuals in each generation,

2.2. Changes in husbandry and reorganisation of the lines

Selection on adjusted food intake in the A lines had resulted in correlated changes in body weight making it difficult to disentagle the effects of changes in food intake from those of body weight. Therefore a new selection criterion was used to establish lines selected on the basis of food intake between ages 8 and 10 weeks, the maintenance (M) lines. It was hoped to alter food intake in these lines without correlated changes in body weight, so food intake was adjusted for body weight at both ages. The lines were established during reorganisation of the F and P lines at generation 20 (described later). Replicates numbered 1 in

Table 2.1. Selection criteria and mean replicate response of selected traits at generation 20.

Line	Criterion	High H	Control C	Low L	Divergence (H-L)/C
A (Appetite)	Food intake between ages 4 and 6 weeks corrected for 4 week body weight* (g)	77.5	62.7	58.8	0.28
F (Fat)	Ratio of gonadal fatpad weight to body weight in males at age 10 weeks (mg/g)	27.8	13.8	6.7	1.53
P (protein)	Body weight minus eight times gonadal fatpad weight in males at age 10 weeks (g)	38.1	27.5	23.1	0.55

*Exact criteria are

Males: 2.21x(17.755 - 4 week weight) + food intake

Females: 1.65x(16.132 - 4 week weight) + food intake

These criteria were calculated in the base population from the within-family phenotypic regression of food intake on four week body weight. The regression was 1.65 grams of food per gram body weight in the females, and 2.21 g/g in the males. Their mean four week weights were 16.132g for females and 17.755g for males. These observations were combined to give "A" selection criteria designed to minimize correlated changes in four week body weight (Sharp, Hill and Robertson, 1984).

the M lines were established from a cross between control replicates numbered 1 in the F and P lines, similarly M replicates 2 and 3 were derived from crosses of F and P control replicates numbers 2 and 3 respectively. Each of the three replicates was divided into three contemporaneous lines which were selected High and Low with an unselected Control; therefore nine new lines were formed. The regression of food intake on the mean of 8 and 10 week body weight was calculated in the base populations which enabled selection indices for each sex to be designed to minimize changes in body weight. These criteria are given in Table 2.2. Food intake between eight and ten weeks of age was chosen as mice typically reach mature weight over this period and have reached chemical maturity (Sutherland, Biondini and Ward, 1974). It was therefore hoped to select on the underlying 'maintenance' costs of metabolism.

Gonadal fatpad weight formed part of the selection index in the F and P lines and became increasingly small in the Low F lines, presumably resulting in a relative increase in environmental variance in their excision and weighing. The selection criteria in the F lines was changed at generation 20 to try and overcome this while still selecting on the same underlying physiological trait of fat content. Fat content is known to be correlated with carcass water content (Eisen and Leatherwood, 1981; Rogers and Webb, 1980), a relationship confirmed in these lines by analysis of carcass components at generation 20 (see Chapter 3 and Figure 3.1 on page 42). The selection criterion in the F lines was therefore changed to the ratio of dry weight to body weight in 14 week old males, the change in age of selection being a consequence of the increased generation interval introduced at generation 20. The index used in the P lines to select on lean ~~mass~~ WEIGHT is closely correlated with body weight (r=0.94 Sharp et al., 1984), so the P selection criterion was changed to selection simply on body weight at age 10 weeks in both sexes. The selection criterion in the A lines remained unchanged.

The P and F lines were becoming increasingly inbred by generation 20 (between 21% and 27%) and it was possible that their decreased genetic variance would result in decreased responses. The three replicates within each criterion/direction (i.e high fat, low fat, high lean ~~mass~~ WEIGHT, and low lean ~~mass~~ WEIGHT), were crossed to form new base populations for subsequent selection on the new criteria. These populations are the basis of the replicate 6 lines (so named because rep1 + rep2 + rep3 = rep 6), and the original High and Low selected lines within the F and P lines were maintained without selection. A similar reorganisation occurred in the A lines at generation 24 (inbred by 28% to 32%) with no change in selection criterion. The three control replicates from the A

Table 2.2. Selection indices used to create new lines after reorganisation at generation 20 and summary of changes in the number of families per generation and changes in generation interval which occurred over the course of selection.

(i) *Selection criteria.*

M: food intake of both sexes between ages 8 and 10 weeks. This is corrected for sex and body weight effects by phenotypic regressions determined on the base population. The exact criteria are as follows.

females: food intake - mean body weight

males: food intake - 1.4 x mean body weight

where: mean body weight = (8 week weight + 10 week weight)/2

F6: ratio of dry weight to body weight in males at age 14 weeks.

P6: 10 week body weight in both sexes.

(ii) *Changes in the number of families per generation.*

Generation	Lines				
	A,F,P	A6	F6	P6	M
0-7	16	-	-	-	8
8-20	8	-	-	-	8
21-34	8	12	12	16	-

(iii) *Changes in generation interval (weeks)*

Generation	Line				
	A,F,P	A6	F6	P6	M
0-20	12	-	-	-	15
21-34	15	15	15	15	-

lines were maintained to act as control lines for the original A, F and P lines.

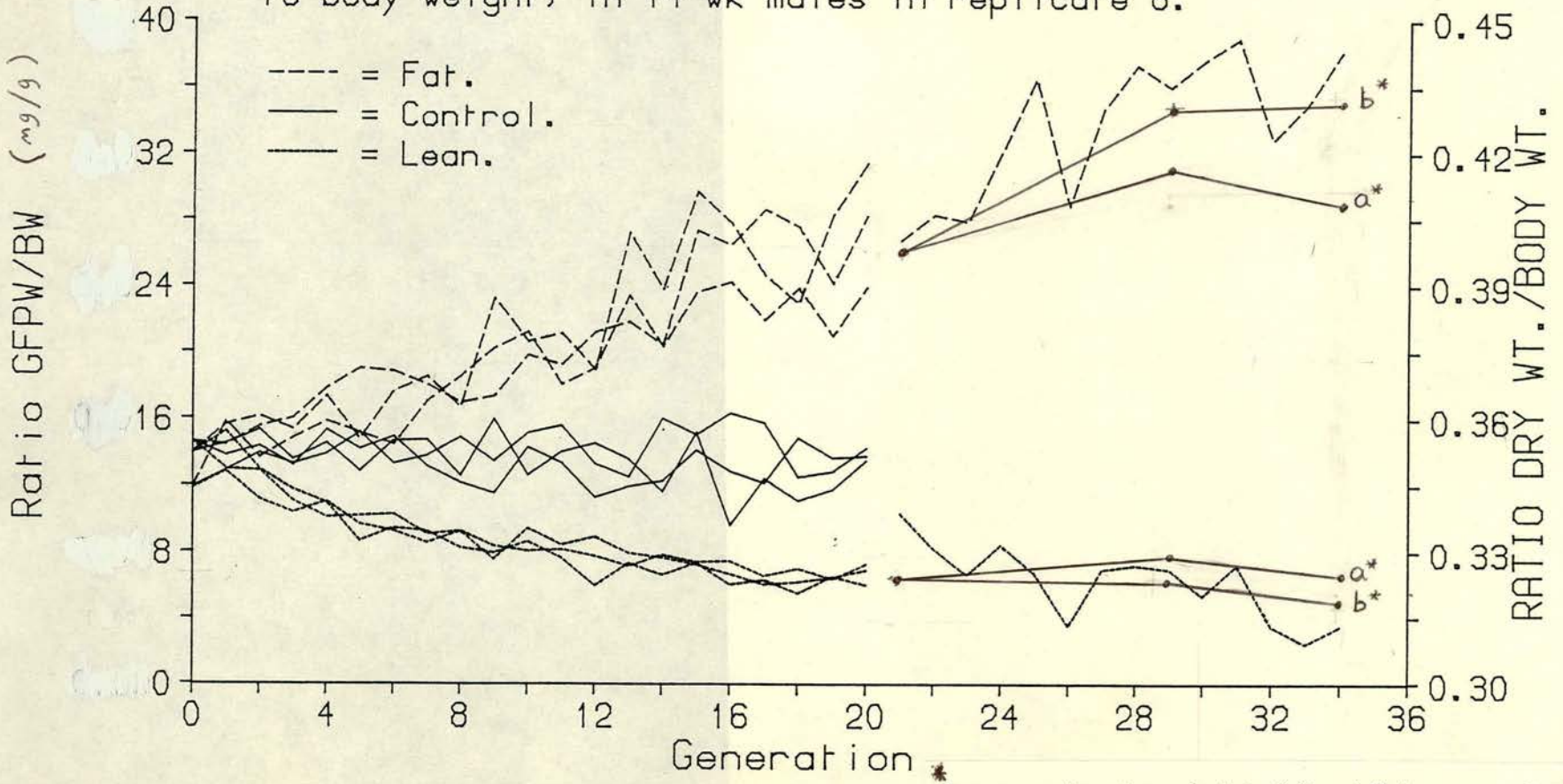
The new selection indices in the F6 and P6 lines are shown in Table 2.2, together with other changes in husbandry which occurred in the lines. As will be seen, large scale heterosis did not occur as a result of crossing the individual replicates.

2.3 Responses to selection

(i) F LINES

Large responses have resulted from selection on both the criteria designed to alter fat content, and are shown in Figure 2.1. The slight decrease in response noted in later generations of the Lean lines can be attributed to the decreased selection differential applied to the lines (see later calculations of their realized heritabilities). Correlated changes in body weight at the age of selection are shown in Figure 2.2. These can be largely explained by differential deposition of fat on a similar underlying lean ~~mass~~ WEIGHT (Sharp et al., 1984; Bishop and Hill, 1985; Chapters 3 and 6, this thesis). Correlated changes in litter size (litter size in this thesis refers to the number of living offspring in the primiparous litter) are shown on Figure 2.3. There was little divergence prior to generation 28, after which a slight difference became established, the Fat line having on average one or two more offspring per litter. The underlying physiology of reproduction in these lines has been studied previously (Brien *et al*, 1984; Brien and Hill, 1986) and is discussed in more detail in Chapter 6. Lines of mice selected on fat content were established by Eisen (1987a, 1987b) who selected within-family for 10 generations on the ratio of gonadal fatpad weight to body weight (the same criteria used to establish the F lines) at age 12 weeks, or the ratio of hind carcass weight to body weight at age 12 weeks. He reported similar direct responses, but larger correlated responses at generation 10 in body weight at age 6 weeks (the Fat lines were 3 to 5 grams heavier) and litter size when mated between 8 and 10 weeks of age (The Fat lines produced 0.3 to 1.5 more offspring per litter); these responses were estimated from regression of response on generation. Given the relationship between body size and litter size (see later) it is not possible to separate the two effects on the basis of his data. The lines examined in this thesis therefore have several advantages over other lines selected on fat content: they have undergone between 20 and 34 generations of divergent selection, are replicated with contemporaneous control lines, are all derived from the same base population and exhibit only small correlated responses, all of which makes interpretation of their underlying physiology easier.

Figure 2.1. Direct response to "F" selection: Mean ratio (Gonadal Fatpad Weight/Body Weight) in 10 week old males in replicates 1,2,3 and ratio (dry weight to body weight) in 14 wk males in replicate 6.



For an explanation of plots "a" and "b", see overleaf.

The F6 line had not undergone a chemical carcass analysis so divergence in the ratio of dry weight to body weight had not formally been shown to be associated with further divergence in fat content. Data on the ratio of gonadal fatpad weight (GFPW) to body weight (BW) is available from Figures 6.1 and 6.2 (pp 69,70) and Table 7.1 (p79) for generations 34 and 29 respectively. These data are plotted as line "a" on Figure 2.1. The data for generation 21 was the mean of the three replicates over the last three generations of "F" selection (Table 4.2, p48) and was 26.0mg/g and 6.4mg/g for the Fat and Lean lines respectively; this assumes that no heterosis occurred at crossing. These data suggest that little further divergence occurred in the ratio GFPW/BW after crossing. However in this instance we are interested in the ratio GFPW/BW as an indicator of fat content. Selection on the ratio of dry weight to body weight removed direct selection on GFPW and Table 4.2 suggests that by generation 29, the proportion of fat content in the gonadal fatpad had fallen from 17.8% to 15.6% in the Fat lines, and risen from 6.4% to 7.6% in the Lean. In order to continue using GFPW/BW as an indicator of fat content, the ratio should be increased by a factor of 1.14 (=17.8/15.6) in the Fat line and decreased by a factor of 0.84 in the Lean line at generation 29. Assuming this phenotypic reversion to be a constant process, the scaling factors at generation 34 should be 1.22 (=1+ 0.14x14/9) and 0.75 for the Fat and Lean lines respectively. These are shown as lines "b" on Figure 2.1 and represent the best available estimates of how the relative fat content has changed during selection in the F6 lines. It is interesting to note that the rate of relative change in fat content was similar to that observed prior to crossing.

Figure 2.2. Correlated responses to "F" selection: mean body weight in males at age of selection (10 weeks in replicates 1,2 & 3 and 14 weeks in replicate 6).

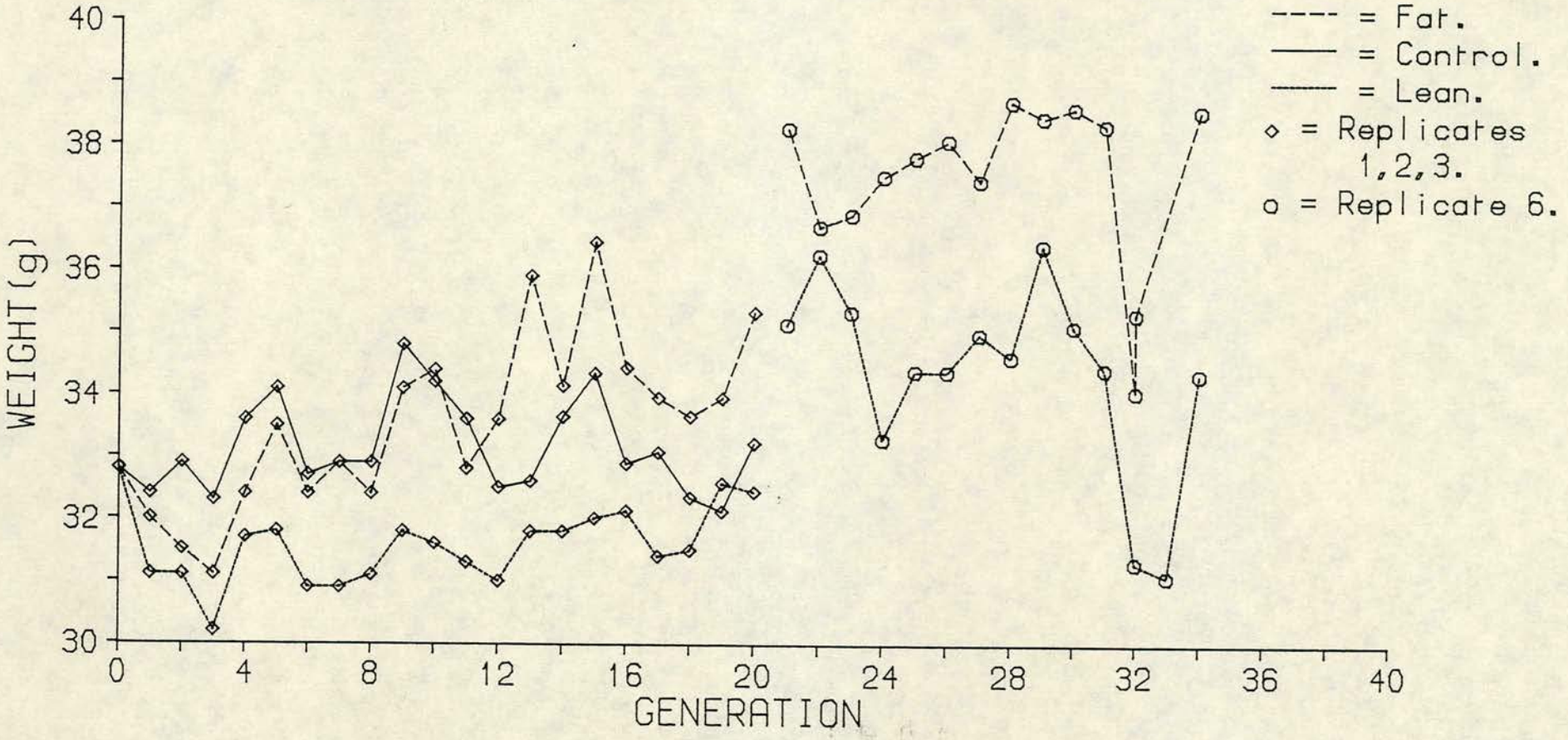
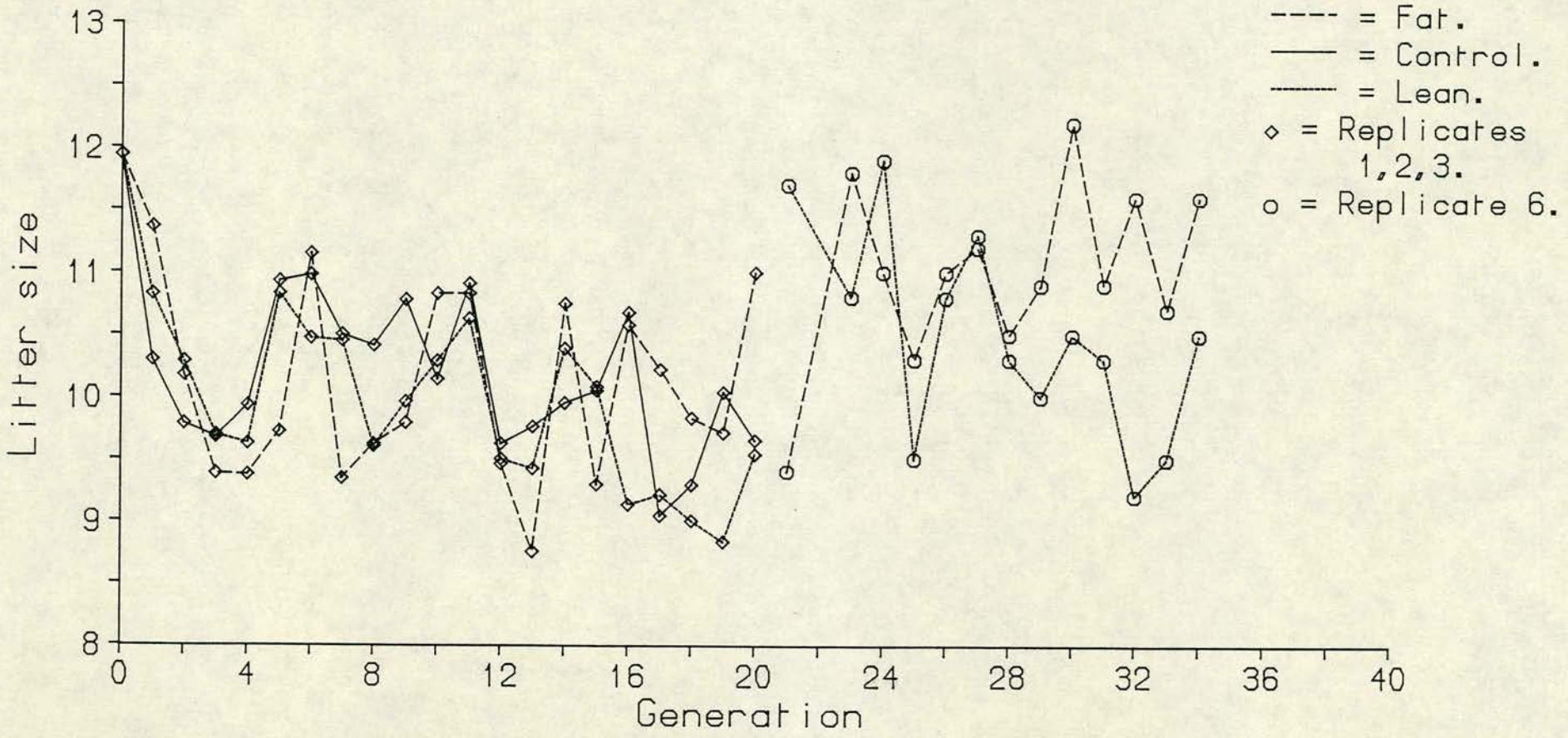


Figure 2.3. Correlated response to "F" selection: mean litter size.



(ii) P LINES

The direct response to selection on the index body weight less eight times gonadal fatpad weight in males at age 10 weeks is shown on Figure 2.4. The decreasing response to selection in the low lines can be explained as a combination of scale effects and decreasing selection differential; response plotted on a logarithmic scale or against units of cumulated selection differential on the horizontal axis restores much of the symmetry. Correlated response in male 10 week body weight is shown in Figure 2.5, together with the direct response to selection on 10 week body weight in both sexes after generation 20. Similar responses were observed in six week body weight (data not shown). Figure 2.6 shows there has been a small but consistent correlated response in fat content, as estimated by the ratio of gonadal fatpad weight to body weight; however this was obscured by replicate differences in analysis of variance of any single generation and explains why chemical carcass composition studies in generations 7, 14 and 20 (op. cit.), failed to reveal any significant difference in fat content between the High and Low lines. Large correlated responses occurred in litter size and are shown in Figure 2.7. The larger fluctuations in mean litter size observed after generation 20 are due to the absence of reserve matings in replicate 6; this resulted in families of small litter size being weaned whereas in other lines such families were discarded and replaced by a reserve mating. There have been numerous selection experiments on body size (reviewed by M^{c}Carthy, 1982, and Eisen, 1989) and the results appear similar to those reported here, i.e. a large response in body weight with little change in fat content at the age of selection. Large correlated changes in litter size invariably occur and are generally attributed to increased ovulation rate in heavier lines (Land and Falconer, 1969; Land, 1970).

(iii) A lines

The direct response to selection on adjusted 4 to 6 week food intake is shown on Figure 2.8. A large correlated response occurred in 6 week body weight and a slightly smaller one occurred in 4 week weight; these are shown on Figure 2.9. The selection index adjusted only for body weight at 4 weeks of age and animals in the high intake lines converted their increased intake into weight gain. This resulted in increased gross efficiency (weight gain per unit food intake) between ages 4 and 6 weeks in the High lines as compared to the Low lines (High=15.4%, Control=14.5%, Low=12.2% at generation 20, High=16.25%, Low=12.5% at generation 30). These results are similar to those obtained when selecting directly on weight gain (Falconer, 1960; Fowler, 1962; Rahnefeld *et al*, 1965; Lang

Figure 2.4. Direct response to "P" selection: index (body weight less eight times gonadal fatpad weight) in males at age 10 weeks in replicates 1, 2 & 3.

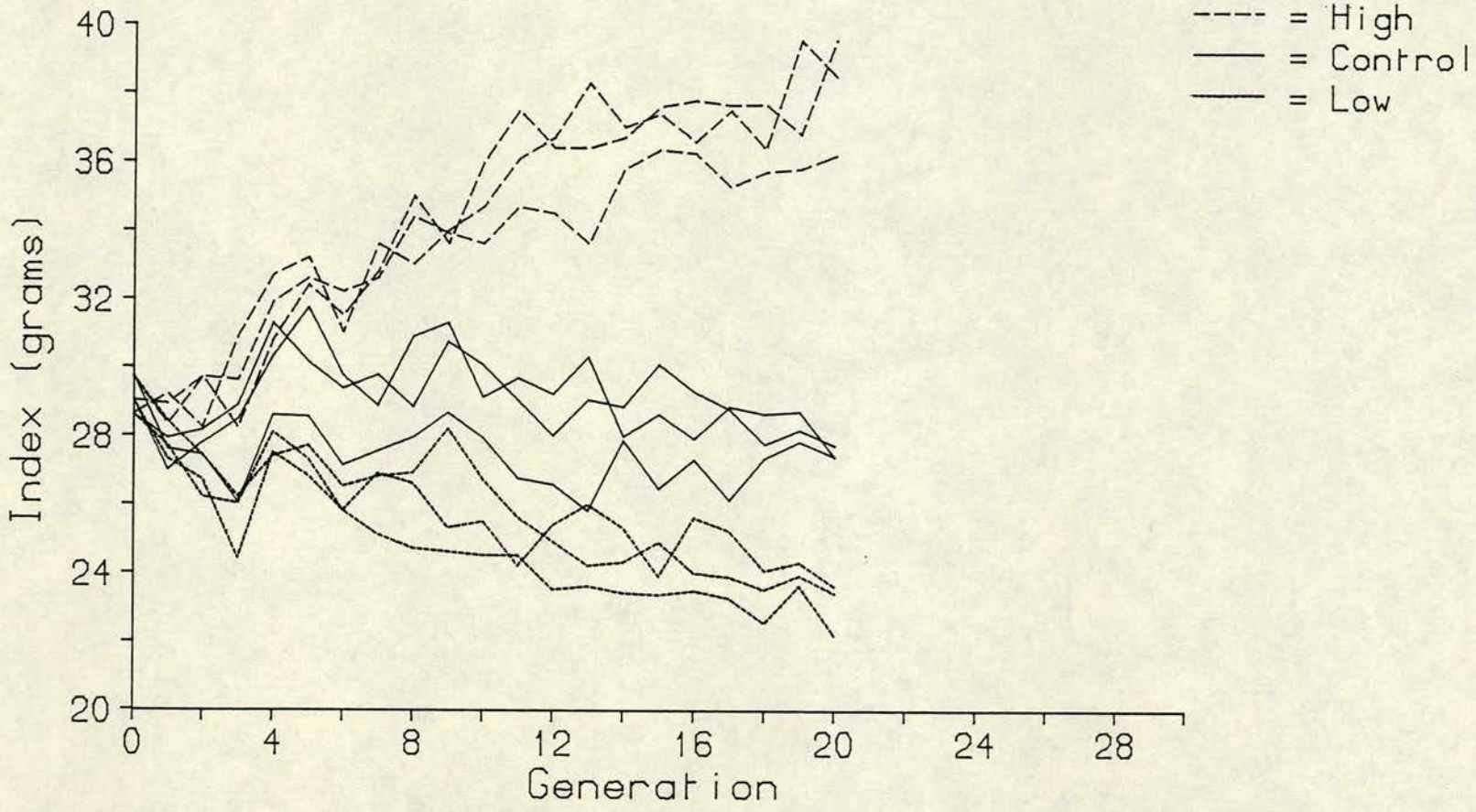


Figure 2.5. Responses to "P" selection:
correlated response in male 10 week body weight in replicates 1, 2, & 3
and direct response in 10 week body weight in replicate 6.

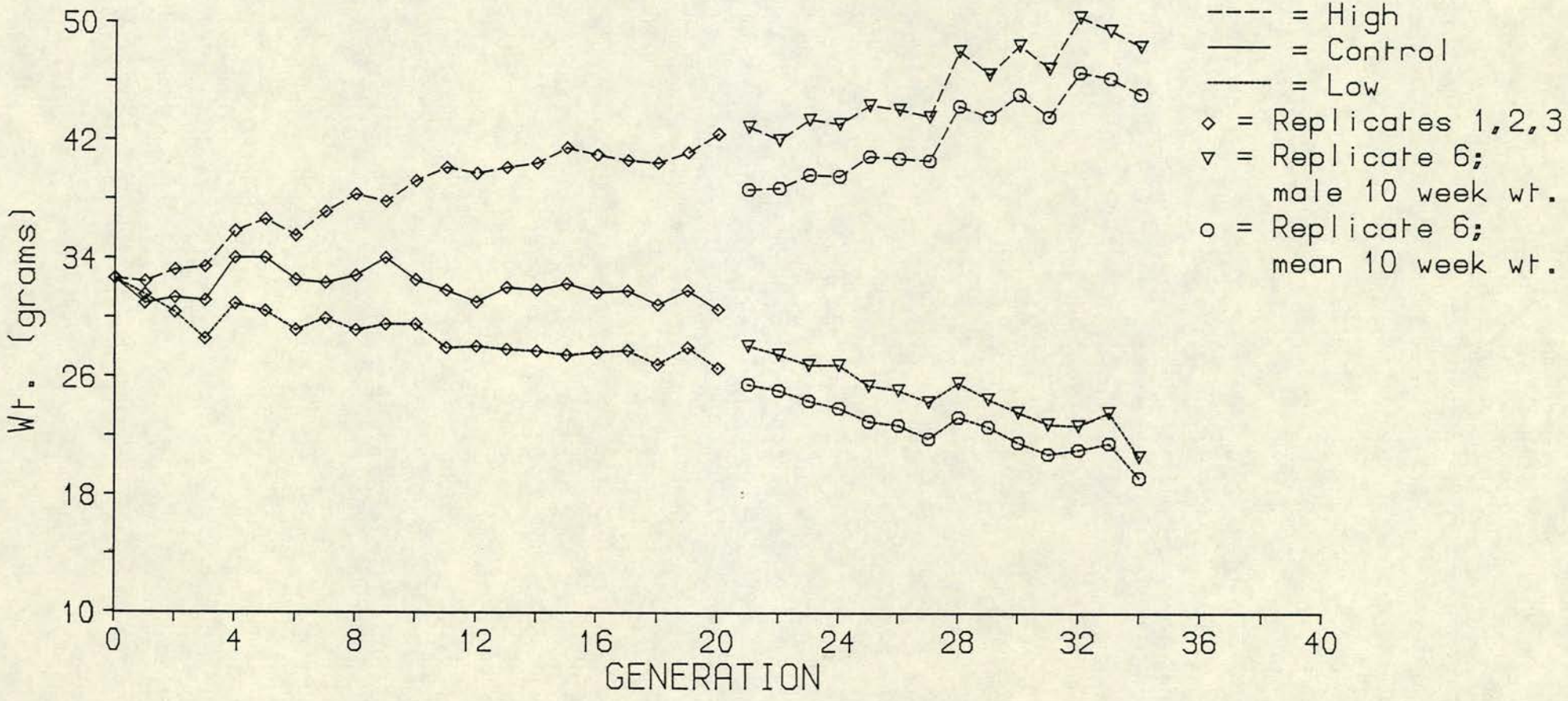


Figure 2.6. Correlated response to ""P"" selection: Fat content (estimated as the ratio of gonadal fatpad weight to body weight) in 10 week old males in replicates 1, 2, & 3.

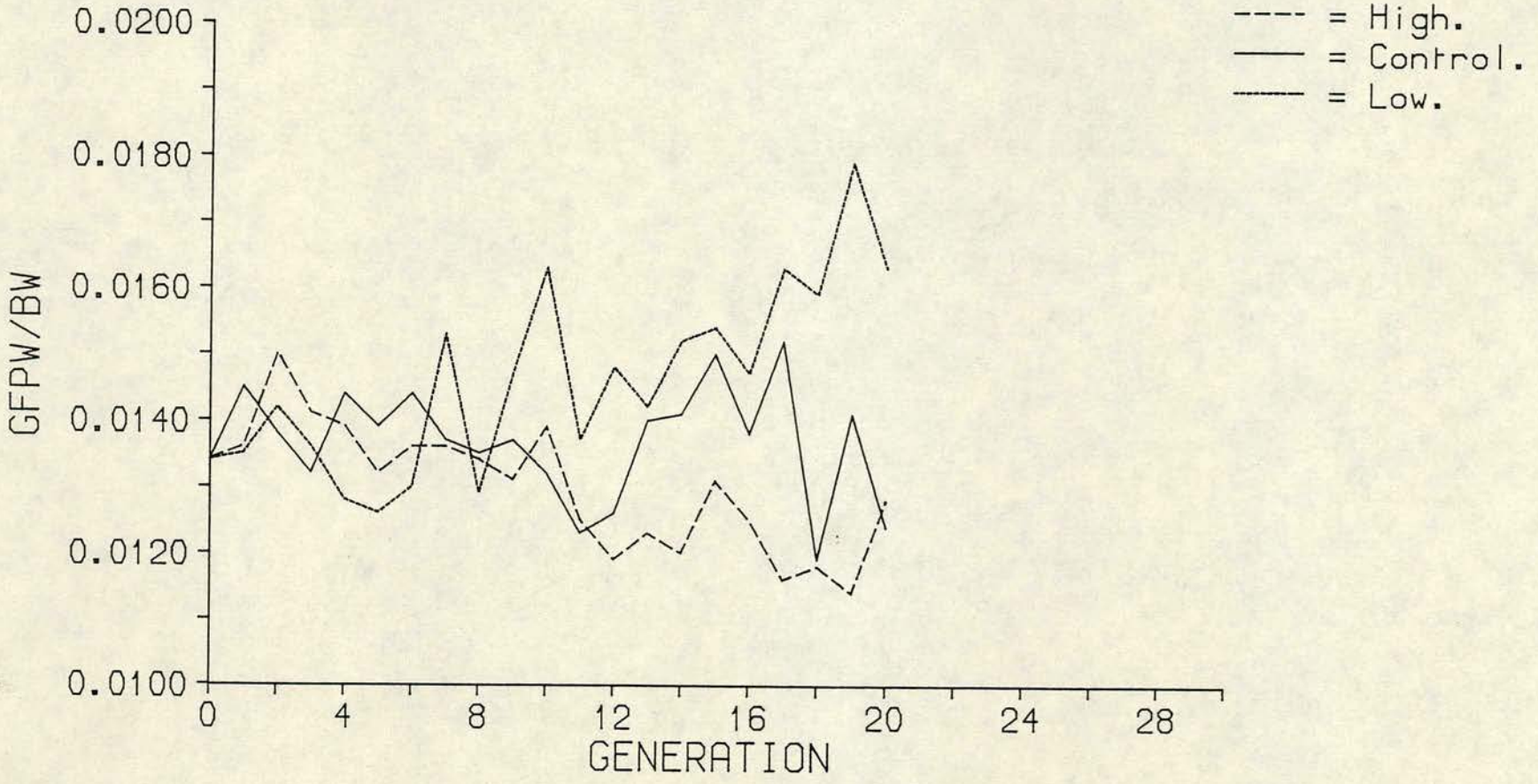


Figure 2.7. Correlated response to "P" selection: mean litter size.

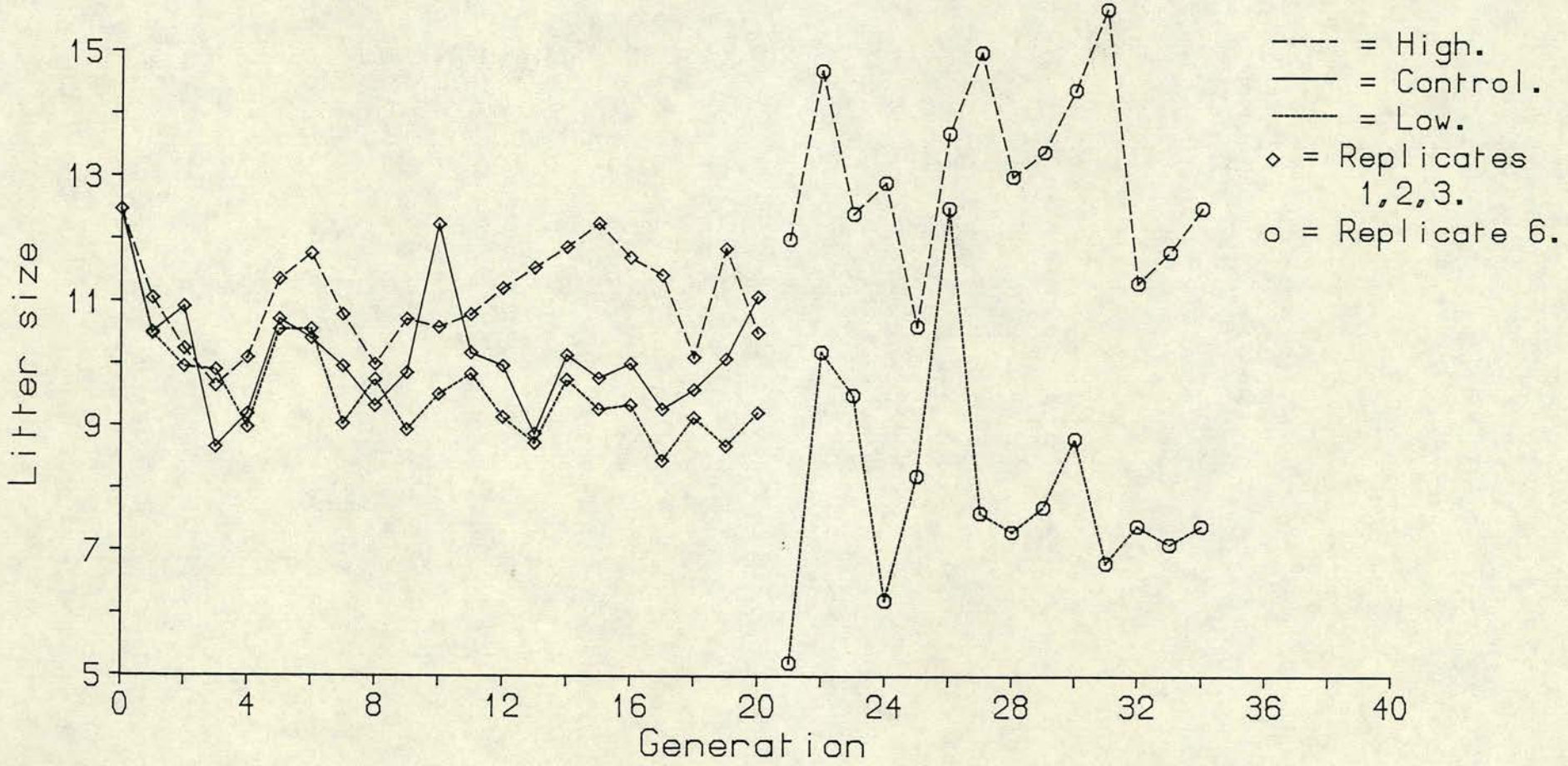


Figure 2.8. Direct response to "A" selection: adjusted food intake between ages 4 and 6 weeks.

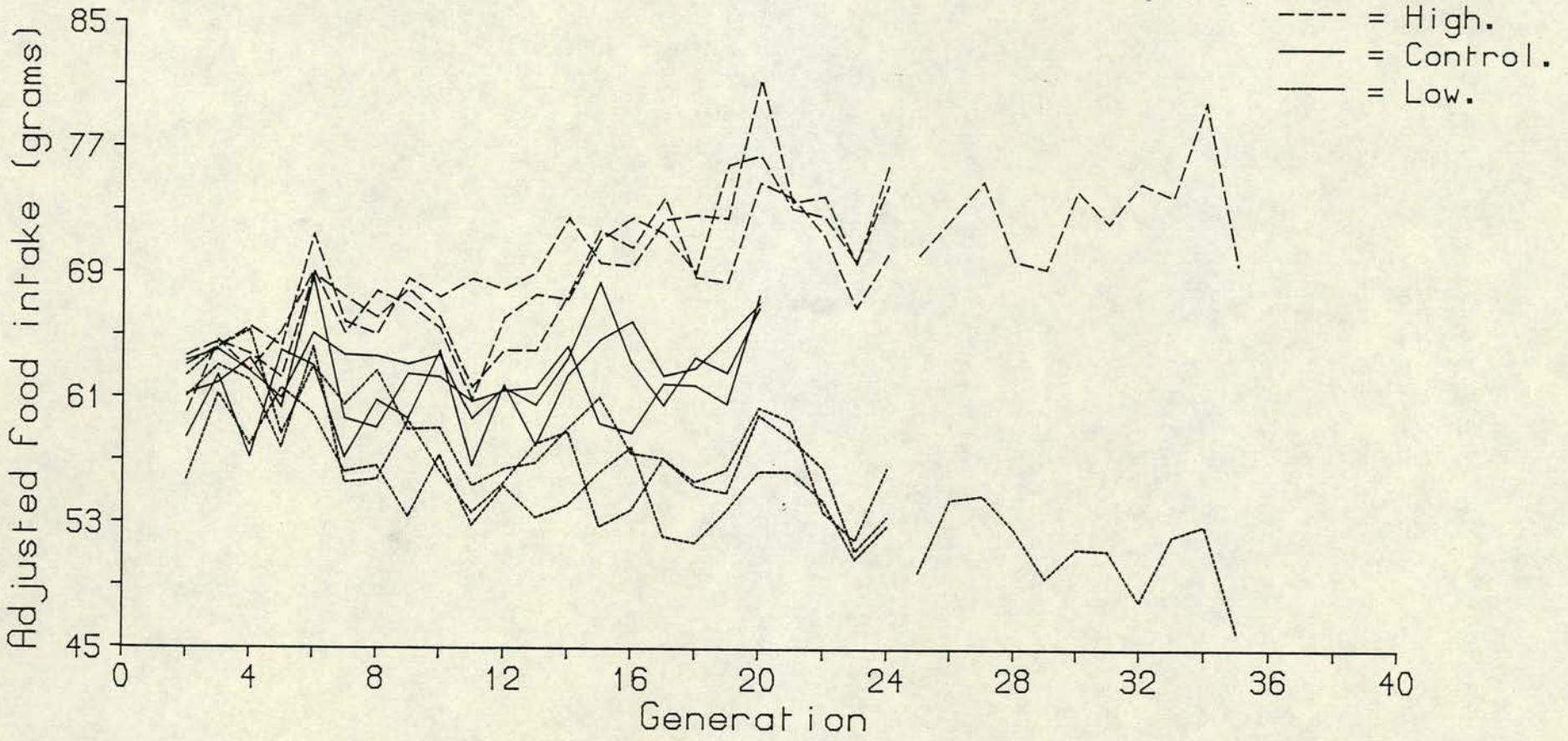
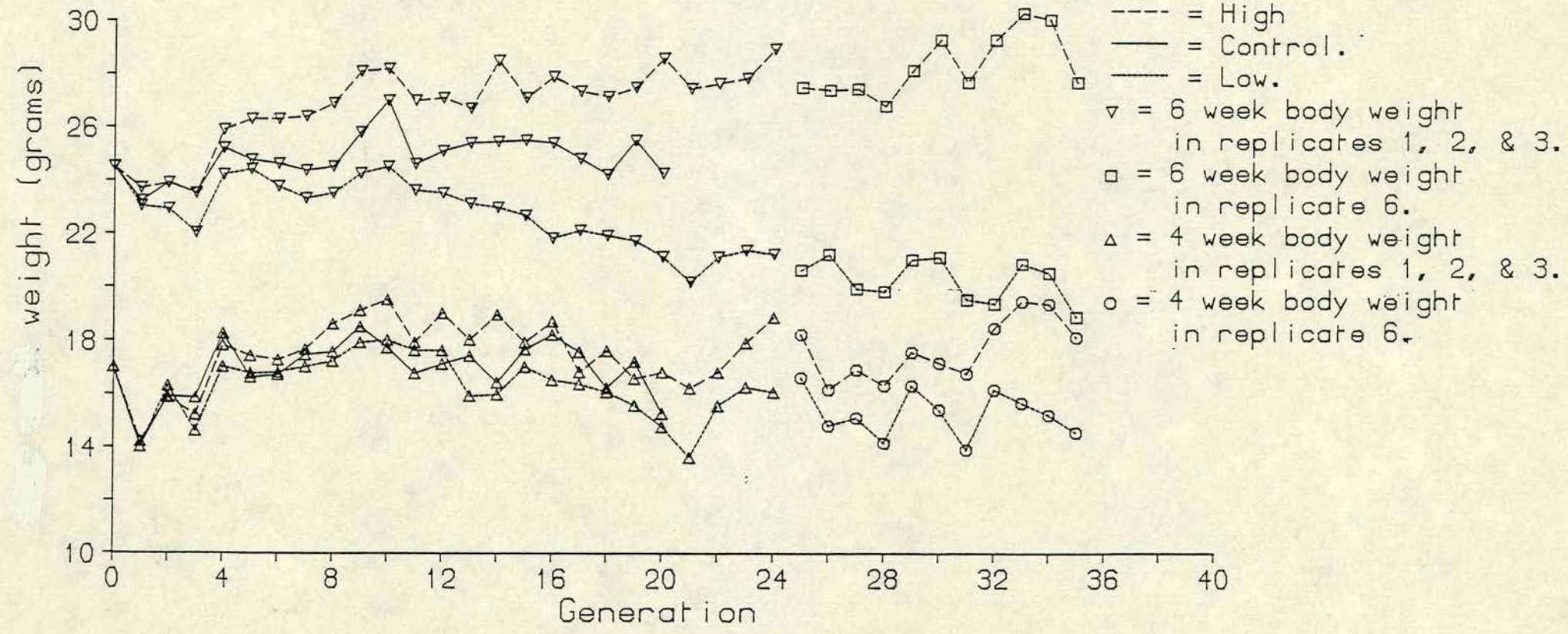


Figure 2.9. Correlated responses to ""A"" selection: body weight at ages 4 and 6 weeks.



and Legates, 1969; Sutherland *et al*, 1970; Timon and Eisen, 1970). Correlated changes in litter size occurred and are shown in Figure 2.10 but it is difficult to separate the effects of increased food intake from the effects of increased body size discussed earlier.

(iv) M LINES

Direct responses are shown in Figure 2.11 and correlated changes in body weight at ages 8 and 10 weeks in Figure 2.12. The similar body weights in both lines indicate that the response is largely due to divergence in food intake and the graph of unadjusted food intake against generation (not shown) is very similar to Figure 2.11. Selection in these lines appears to have resulted in divergence in food intake without the large changes in body weight observed in the A lines, although Figure 2.12 reveals a consistent difference in weight gain between the lines, the Low lines gaining less weight and even losing weight in generations 2 and 13. This change in growth led to a gradual divergence in gross efficiency, the High line being slightly more efficient (data not shown). There was no apparent change in litter size, Figure 2.13. These lines appear to be unique in differing greatly in food intake (by a factor of 1.6 at generation 13), with only marginal changes in body weight and gain. The effects of these traits are usually confounded in selected lines and diminish their usefulness (Timon and Eisen, 1970; Sutherland *et al*, 1974). Analysis of these lines may therefore help to identify physiological traits specifically associated with altered food intake.

(V) Summary of responses.

A summary of direct and correlated changes in the F6, P6, A6 and M lines is given in Table 2.3. A critical economic parameter of livestock production is the partition of food intake (appetite) into growth (lean ~~mass~~ WEIGHT), fat and maintenance. This selection experiment has succeeded in deriving lines of mice from the same base population which diverge dramatically in these four physiological characteristics. The F lines have altered fat content while their underlying lean ~~mass~~ WEIGHT appears unchanged, the A and P lines have altered lean ~~mass~~ WEIGHT with only marginal changes in fat content and the M lines differ significantly in food intake but only slightly in weight gain.

2.4. Changes in genetic parameters over the course of selection.

Direct and correlated responses appear generally constant over the period of selection despite the changes which occurred in the underlying genotype. The

Figure 2.10. Correlated response to "A" selection: mean litter size.

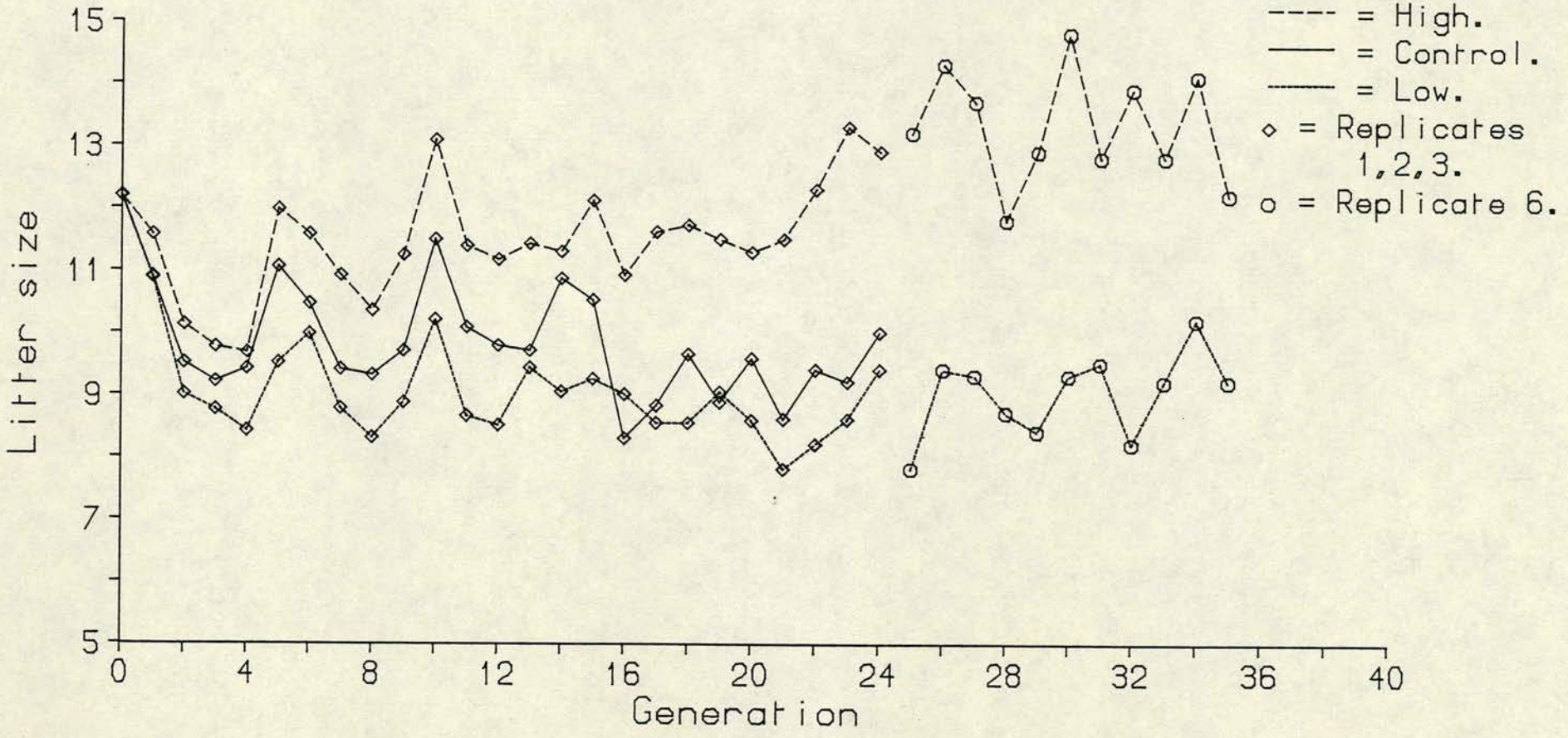


Figure 2.11. Direct response to ""M"" selection: adjusted food intake between 8 and 10 weeks of age.

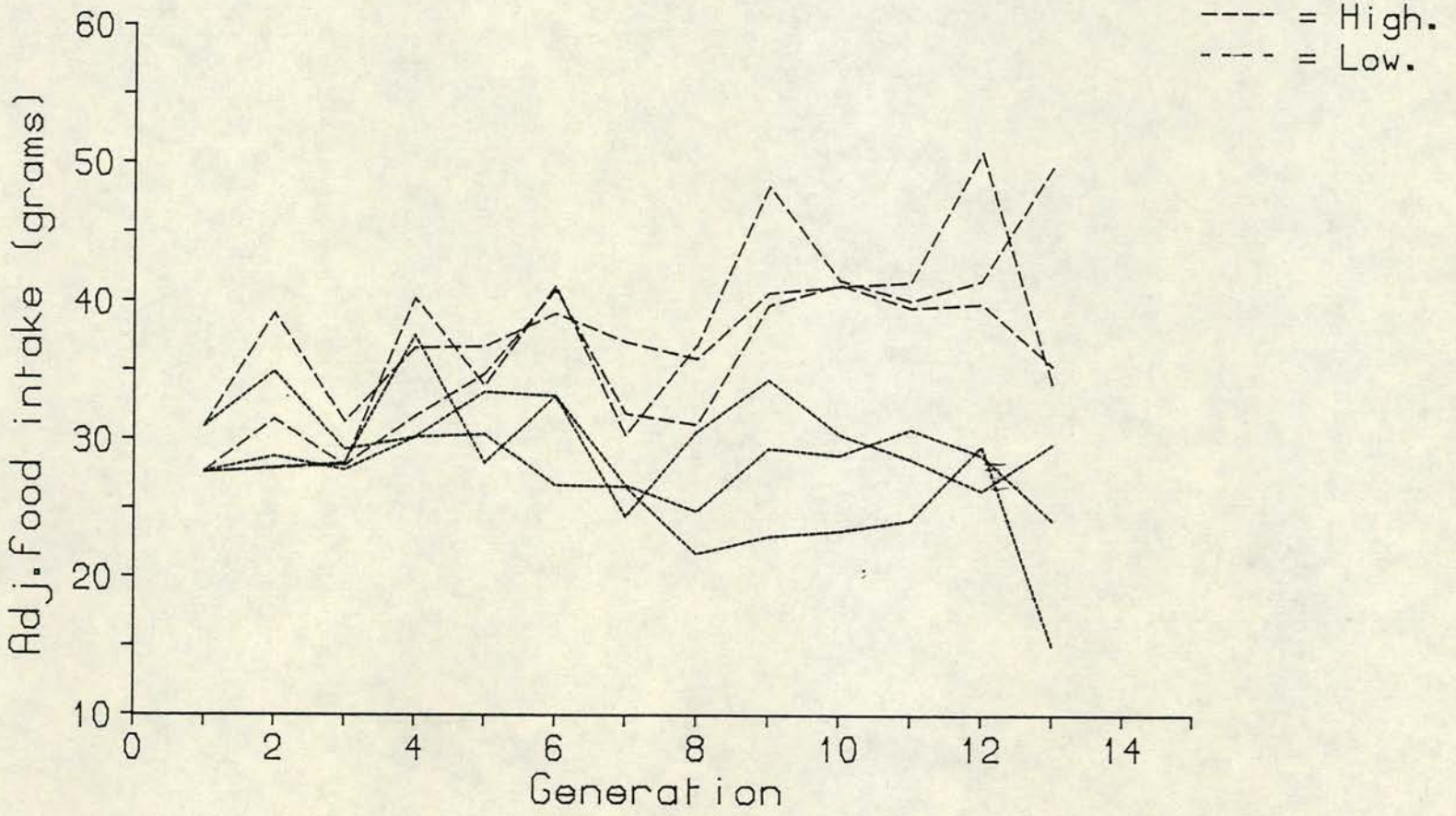


Figure 2.12. Correlated responses to ""M"" selection: body weight (BW) at ages 8 and 10 weeks.

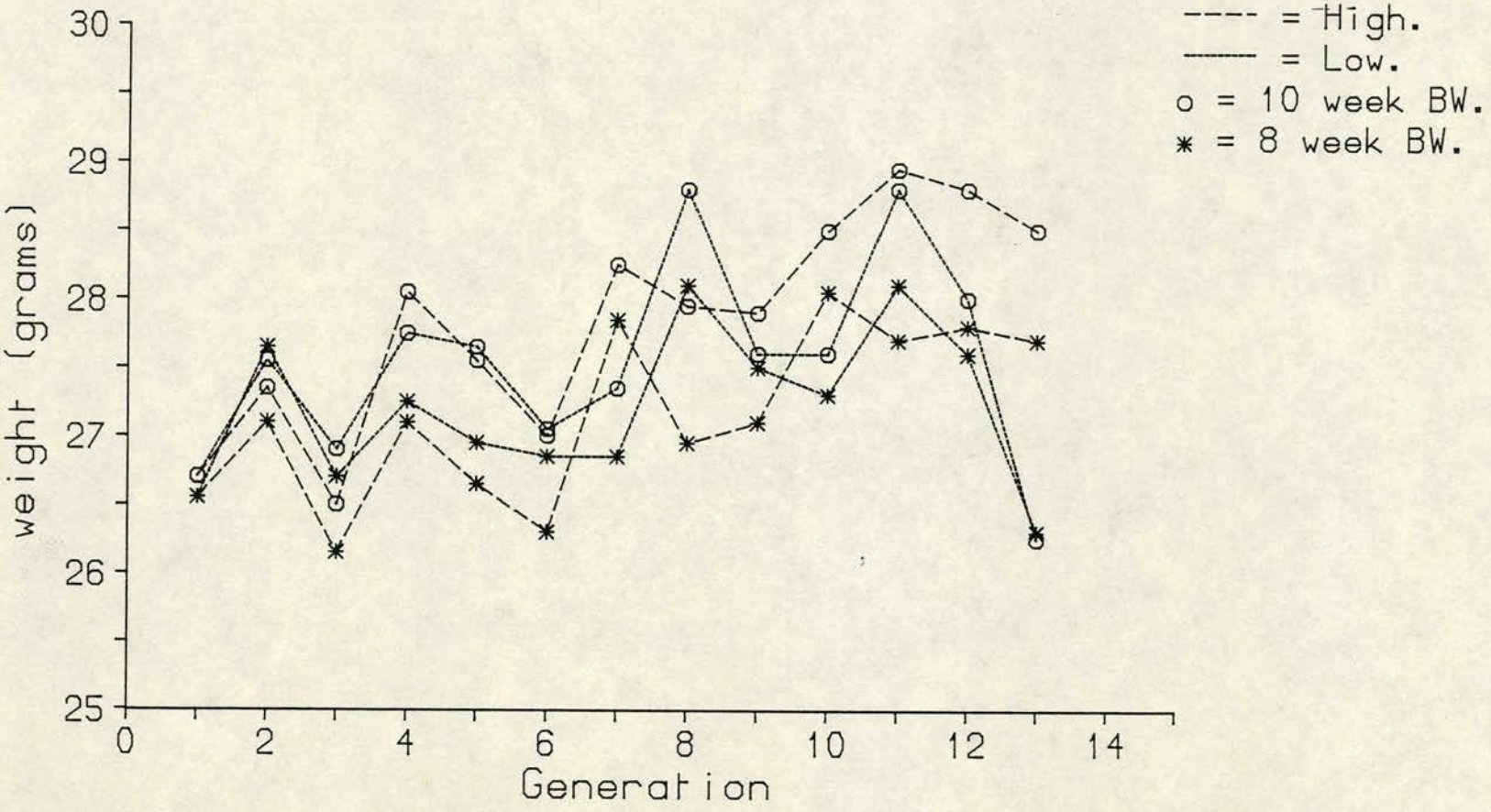


Figure 2.13. Correlated responses to ""M"" selection: mean litter size.

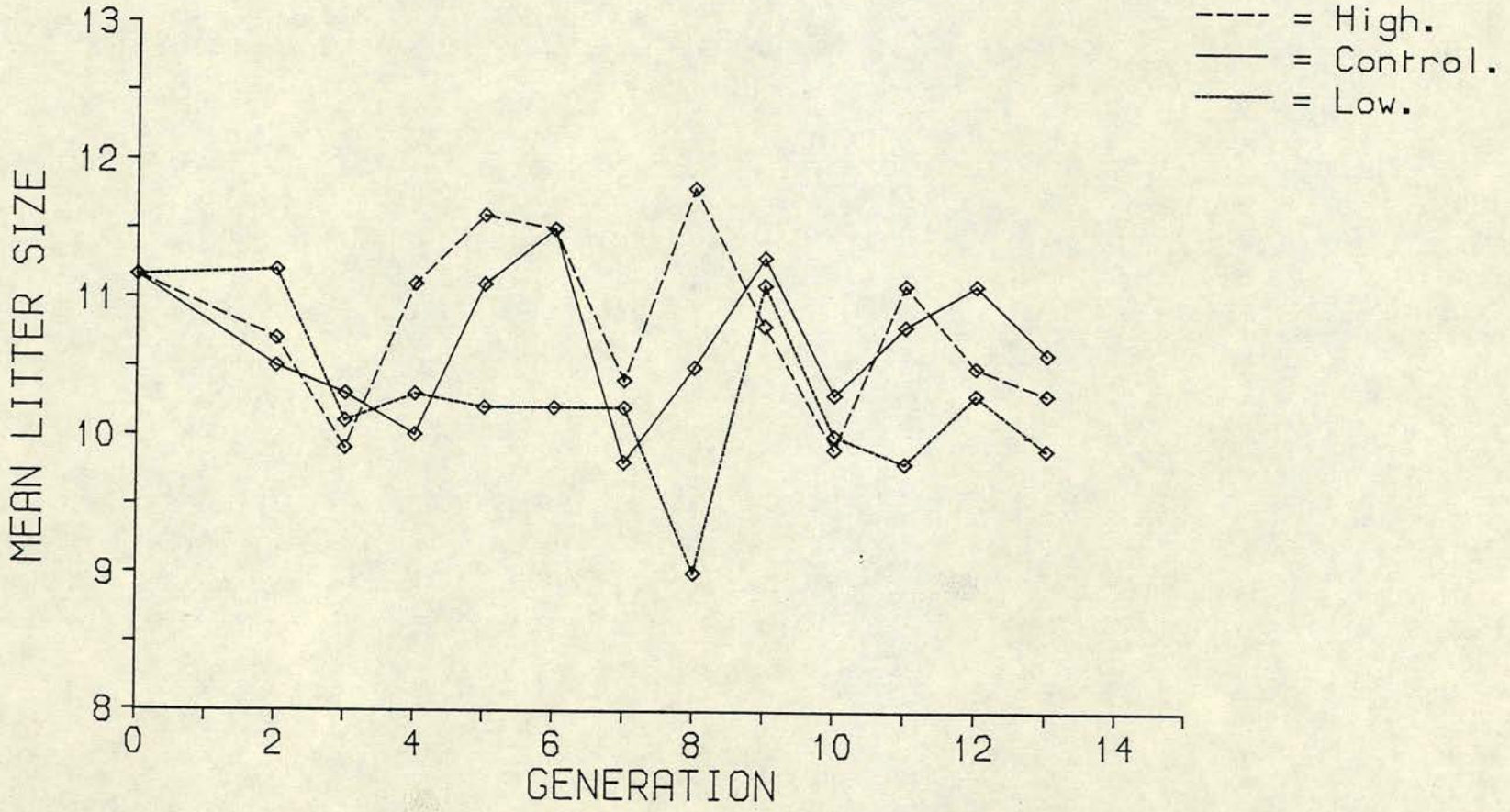


Table 2.3. Direct and correlated responses to selection: mean values over the last three generations (generations 11, 12, and 13 in the M lines and generations 32, 33, and 34 in the A6, F6 and P6 lines).

Line	Trait	High (H)	Low (L)	Ratio (H/L)
F6 (Fat)	Ratio dry weight to body weight in males aged 14 weeks (% DM)	43.2	31.9	1.39
	Estimated % fat content (from DM)	18.6	5.1	3.68
	14 week body weight of males (g)	36.0	32.2	1.12
	Primiparous litter size	11.3	9.7	1.16
P6 (Protein)	10 week body weight (g)	46.0	20.7	2.22
	Primiparous litter size	11.9	7.3	1.63
A6 (Appetite)	Adjusted 4 to 6 week food intake (g)	74.5	50.4	1.48
	4 week weight (g)	19.0	15.1	1.25
	6 week weight (g)	29.3	20.1	1.46
	4 to 6 week food intake (g)	78.3	46.8	1.67
	Primiparous litter size	13.0	9.5	1.37
M (Maintenance)	Adjusted 8 to 10 week food intake (g)	41.3	26.3	1.57
	8 week weight (g)	27.7	27.3	1.01
	10 week weight (g)	28.7	27.7	1.04
	8 to 10 week food intake (g)	73.9	58.7	1.26
	Primiparous litter size	10.6	10.0	1.06

most easily predicted change in genotype composition is the decrease in genetic variance attributable to random loss of alleles by drift and inbreeding, although the loss of variation due to fixation of selected alleles is unknown. Such changes would be expected to result in decreased heritabilities and possibly a decrease in phenotypic variance. The remainder of this chapter will be concerned with estimating the magnitude of any such changes.

Inbreeding and drift coefficients were computed for each line using the coancestry matrix method (Cruden, 1949). These averaged 0.07 and 0.09 respectively at generation 10, 0.23 and 0.26 at generation 20 and 0.14 and 0.17 at generation 30 (the decrease was due to crossing the lines at generation 20). In the M lines the values were 0.25 and 0.28 at generation 10. Inbreeding coefficients lag several generations behind drift coefficients because animals were mated to those individuals least related. Heritability estimates are realized within-family heritabilities defined as the regression of response (deviation of selected line from contemporaneous control or deviation from mean H/L value where no controls were maintained) on cumulative selection differential. The selection differential each generation is defined as the mean deviation of the selected animal from the mean of its sibs of the same sex. This differs from the methodology of Sharp (1983) and Sharp et al. (1984) as it does not weight selection differential by the number of offspring used to estimate response in the subsequent generation. The method used here is more accurate for periods exceeding a few generations as the mating scheme ensures equal representation of each family in the next generation rather than allowing random mating to occur. Mass selection heritability can be estimated by multiplying the within-family heritability by $2(1-t)$, where t is the full sib within-line correlation calculated for the base population. The values of t were calculated by Sharp *et al.* (1984) to be 0.43, 0.43 and 0.48 for the A, F and P lines respectively.

The estimates of within-family heritability are shown in tables 2.4 to 2.7 and do not show any consistent reduction over the course of selection. There is little change in the coefficient of variation (standard deviation over mean) of the selected traits or within-family heritability over the period of selection in the F and P lines. This suggests that the asymmetry of response shown in Figures 2.1 and 2.4 is largely due to scaling effects; the transformation of response onto a log scale resulted in a symmetrical response in the F lines and a more symmetrical divergence in the P lines (Figures 2.14 and 2.15).

Selection experiments on growth traits have been performed by several groups. The heritability (h^2) estimates and correlated changes are similar to those

Table 2.4. F lines. Changes in within-family heritability over the course of selection on male fat content; calculated as twice the regression coefficient of response (deviation from contemporaneous control line) on cumulated selection differential (CSD) in replicates 1, 2 & 3, and twice the regression of response (deviation from High+Low/2) on CSD in replicate 6. Note the change in selection criterion at generation 20 from selection on the ratio of gonadal fatpad weight to body weight to selection on carcass water content.

Replicate	Generation			
	0-20	0-11	12-20	21-34
		High		
1	0.527	0.377	0.415	-
2	0.600	0.475	0.792	-
3	0.369	0.315	0.228	-
6	-	-	-	0.202
mean	0.499	0.389	0.478	-
s.e.*	0.056	0.038	0.135	-
		Low		
1	0.400	0.666	1.131	-
2	0.536	0.766	0.632	-
3	0.531	0.725	0.874	-
6	-	-	-	0.086
mean	0.489	0.719	0.879	-
s.e.*	0.036	0.024	0.118	-

*between-replicate standard error of mean.

Table 2.5. P lines. Changes in within-family heritability over the course of selection on lean ~~mass~~ WEIGHT calculated as twice the regression coefficient of response (deviation from contemporaneous control line) on cumulated selection differential (CSD) in replicates 1, 2 &3, and the regression of response (deviation from High+Low/2) on CSD in replicate 6. Note the change in selection criterion at generation 20 from selection on the index body weight minus eight times gonadal fatpad weight in males to selection on body weight in both sexes.

Replicate	Generation			
	0-20	0-11	12-20	21-34
		High		
1	0.582	0.739	0.151	-
2	0.468	0.387	0.496	-
3	0.543	0.512	0.287	-
6	-	-	-	0.230
mean	0.531	0.546	0.311	-
s.e.*	0.027	0.084	0.082	-
		Low		
1	0.293	0.163	0.530	-
2	0.364	0.783	0.124	-
3	0.283	0.503	0.341	-
6	-	-	-	0.496
mean	0.313	0.483	0.332	-
s.e.*	0.021	0.146	0.096	-

*between-replicate standard error of mean.

Table 2.6. A lines. Changes in within-family heritability over the course of selection on four to six week food intake corrected for four week body weight; calculated as the regression coefficient of response on cumulated selection differential. Response defined either as (a) deviation from contemporaneous control line or (b) deviation from High+Low/2 in each replicate depending on the availability of data. Commences at generation 2 as food type was changed at this point.

(a) response defined as deviation from control line.

Replicate	Generation		
	2-20	2-11	12-20
		High	
1	0.195	0.206	0.271
2	0.139	0.105	0.241
3	0.105	0.083	0.153
mean	0.146	0.131	0.222
s.e.*	0.021	0.031	0.029
		Low	
1	0.120	0.263	0.085
2	0.133	0.198	0.083
3	0.212	0.168	0.298
mean	0.155	0.210	0.155
s.e.*	0.023	0.023	0.058

*between-replicate standard error of mean.

Table 2.6. continued.

(b) response defined as deviation from High+Low/2.

Replicate	Generation			
	2-24	2-11	12-24	24-34
		High		
1	0.130	0.222	0.113	-
2	0.126	0.153	0.115	-
3	0.132	0.122	0.118	-
6	-	-	-	0.127
mean	0.129	0.166	0.115	-
s.e.*	0.001	0.024	0.001	-
		Low		
1	0.148	0.249	0.124	-
2	0.121	0.152	0.104	-
3	0.156	0.133	0.178	-
6	-	-	-	0.231
mean	0.142	0.178	0.135	-
s.e.*	0.009	0.029	0.018	-

*between-replicate standard error of mean.

Table 2.7. M lines. Within-family heritability obtained from 12 generations of selection on food intake between ages 8 and 10 weeks, corrected for body weight at each age; calculated as the regression coefficient of response (defined as deviation from High+Low/2) on cumulated selection differential.

Replicate	Direction	
	High	Low
1	0.274	0.318
2	0.168	0.169
3	0.216	0.300
mean	0.219	0.262
s.e.*	0.025	0.038

*between-replicate standard error of mean.

Figure 2.14. Direct response to "F" selection: log of mean ratio (gonadal fatpad weight to body weight) in 10 week males in replicates 1, 2, & 3.

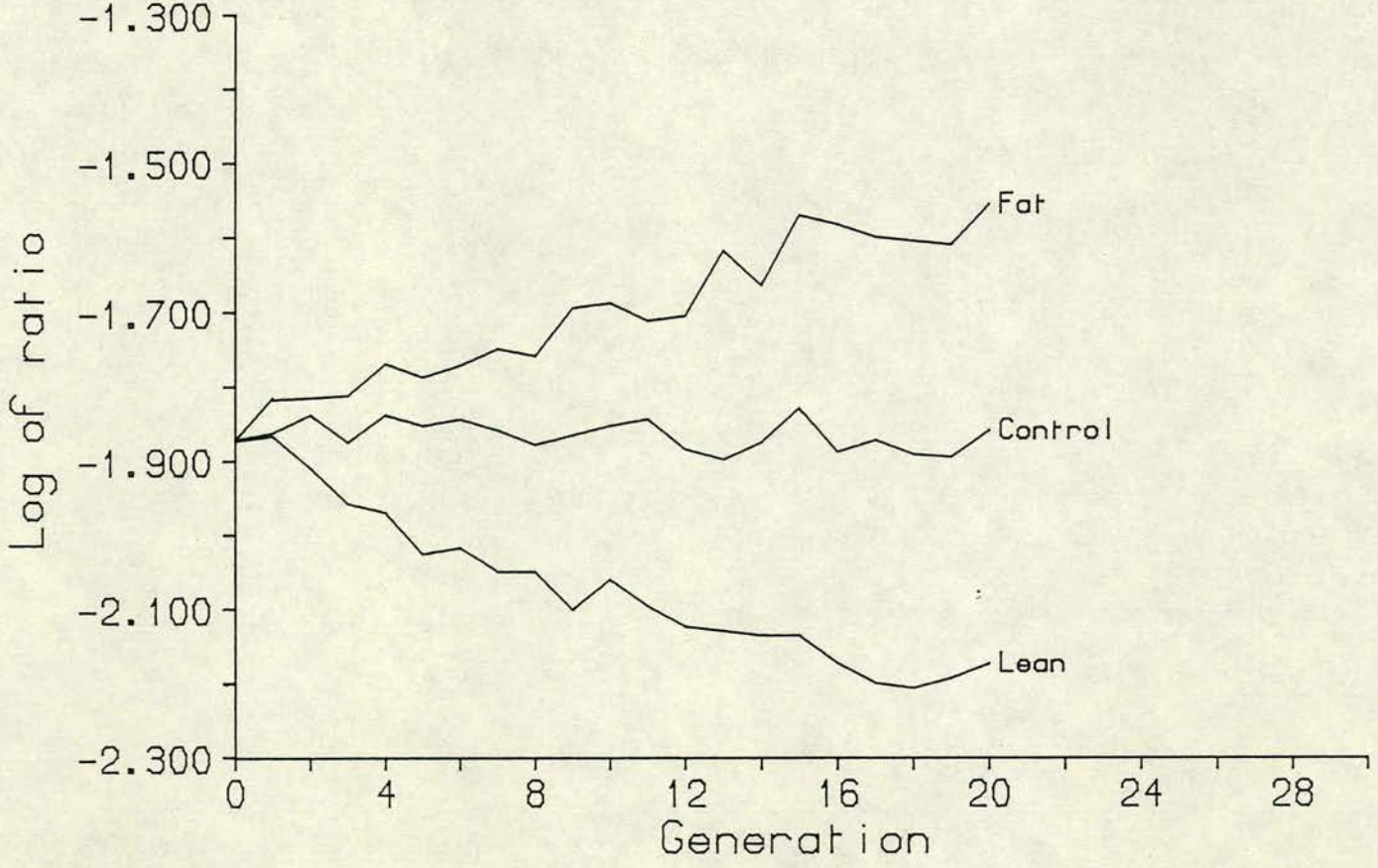
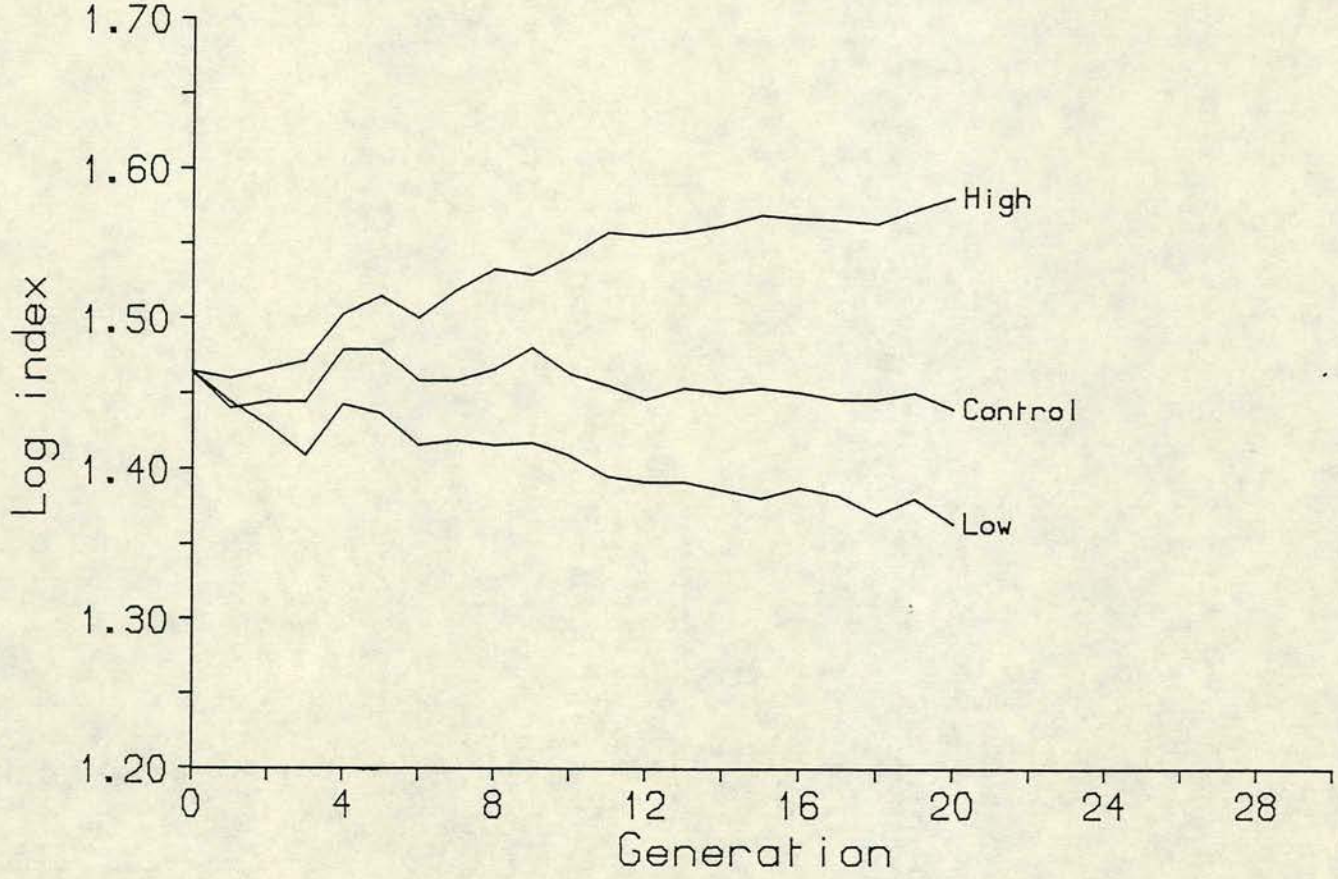


Figure 2.15. Direct response to "P" selection: log index (body weight less eight times gonadal fatpad weight) in 10 week males in replicates 1, 2, & 3.



reported here. Eisen (1987a) selected for fat content on the basis of the ratios gonadal fatpad weight to body weight (h^2=0.6) and hind carcass weight to body weight (h^2=0.32) at age 12 weeks. Selection on percentage fat at ages 14 to 18 weeks gave h^2=0.32 (Horstgen, quoted in Eisen, 1987a). These values in mice are similar to those obtained in other species e.g. h^2=0.4 for backfat thickness in pigs (Hetzer and Harvey, 1967); h^2=0.40 for plasma triglyceride concentration (fat being transported from liver to adipose tissue) in broilers (Whitehead and Griffin, 1984). The values of h^2 obtained for selection on a lean ~~mass~~ WEIGHT index are similar to those obtained selecting on body weight or weight gain alone (M^{c}Carthy, 1982). This is due to the large positive genetic and phenotypic correlation between this index and body weight at age of selection (M^{c}Lellan and Frahm, 1973; Sharp et al., 1984). Eisen and Prasetyo (1988) concluded from genetic and phenotypic parameters of mice at 12 weeks of age, that the index was equivalent to selection on body weight with unchanging gonadal fatpad weight; such a prediction appears accurate given the results of chemical carcass composition at generations 7, 14 and 20 (Sharp et al., 1984; Bishop and Hill, 1985; Chapter 3, this thesis). There have been fewer selection experiments on food intake. Sutherland *et al.* (1970), selected replicate lines on the basis of food intake between 4 and 11 wks and reported a realized h^2 of 0.2.

These differences in realized heritabilities can be attributed to sampling error, differences in the base populations and differing genetic correlations between the selection indices and the underlying physiological traits of fat metabolism, lean growth and appetite control. Of more importance is the observation in our lines that within-family heritabilities appear not to have declined over the 34 generations of selection despite loss of genetic variability by drift and fixation. The estimates of h^2 given above come from selection experiments of less than 15 generations duration (except Sutherland *et al* who selected for 22 generations) and have been unable to provide the information on long term responses given here.

2.5 Further analyses of the lines.

This completes a description of responses obtained over 34 generations of selection in the A, F and P lines, and over 13 generations of selection in the M lines. Subsequent chapters of this thesis will investigate the lines in more detail to determine changes in chemical carcass composition in the A, F and P lines (Chapter 3), reconvergence of F and P lines after nine generations of relaxed selection (Chapter 4), the contribution of major genes to response in the P6 line (Chapter 5), growth and reproduction in the F6 line (Chapter 6) and changes in

the activity of enzymes associated with fat synthesis in the F6 line (Chapter 7). The desire to understand the biochemical basis of these responses stimulated theoretical investigations into the quantitative genetics of enzyme variants (Chapter 8), and the possibility that selection on basic metabolism may occur in the germline stage of the life cycle (Chapter 9).

CHAPTER 3

CARCASS COMPOSITION OF MICE FROM THE A, F, AND P LINES AT GENERATION 20

3.1 Introduction

Analyses of body composition of mice from the A, F, and P lines had been undertaken at generations 7 by Sharp *et al.* (1984) and at generation 14 by Bishop and Hill (1985). Response to selection had continued up to generation 20 at which point it was decided to cross the replicates (see previous chapter), and all the lines were re-analysed at this time. In particular it was deemed necessary to investigate: (i) the effects of selection for food intake corrected for body weight on fat content since data from earlier generations had suggested that the High intake lines were becoming leaner; (ii) the effects of indirect selection for fat through gonadal fatpad weight; (iii) whether the lines selected on lean ~~mass~~ WEIGHT still exhibited little change in composition.

3.2 Methods

Ten week old males were taken from each line, weighed, and killed by cervical dislocation. The gonadal fatpads were removed, weighed, and reinserted into the carcass which was subsequently freeze dried and ground to a fine powder in a centrifugal mill, sieve size 2mm. Three samples were taken per line, each sample consisting of four carcasses. Chemical determination of fat, ash and nitrogen content was undertaken by the East of Scotland College of Agriculture by standard proximate analysis procedures.

Analyses of variance utilized the following nested model:

$$Y_{ijkl} = \mu + F_i + D_{ij} + R_{ik} + L_{ijk} + e_{ijkl},$$

where: Y_{ijkl} is the observation on the l^{th} sample of the k^{th} replicate of the j^{th} direction of the i^{th} selection criterion; μ is the overall mean; F_i is the effect of the i^{th} selection criterion (i = 1,2,3 for A, F, P); D_{ij} is the effect of the j^{th} direction of selection (j = 1,2,3 for H, L, C) within the i^{th} selection criterion; R_{ik} is the effect of the k^{th} replicate (k = 1, 2, 3) within the i^{th} selection criterion; L_{ijk} is the effect of the individual line; and e_{ijkl} is the residual error. The effect of direction of selection within criterion (D_{ij}) was tested against individual lines (L_{ijk}) to take account of drift variation between lines. Replicates within criteria (R_{ik}) were also tested against individual lines to reveal any fixed effect of replicates. To test for

replicate differences within each criterion, a subset of the model was applied in which the term R_{ik} was not fitted.

3.3 Results and discussion

The mean values of the traits in each line are shown on Table 3.1 and the corresponding analysis of variance shown on Table 3.2. The results show that the effect of P selection was to change body weight rather than composition. Selection on body weight alone is usually associated with only marginal changes in fat content at the age of selection (Hayes and McCarthy, 1976; Roberts, 1979; McCarthy, 1982). The P line mice were examined at age 10 weeks (the age of selection) so the results of selection on lean ~~mass~~ WEIGHT practised here are similar to those obtained from selection simply on body ~~mass~~ WEIGHT. It is possible that selection on lean ~~mass~~ WEIGHT has circumvented the problem of increased fat deposition associated with selection of size alone but this putative benefit would not become apparent until later ages when size-selected animals tend to accumulate fat.

Selection for food intake corrected for body weight was also found to have only a small effect on body composition. The high intake lines were slightly, but insignificantly, leaner than their low intake counterparts as previously noted in generations 7 and 14 (Sharp *et al*., 1984; Bishop and Hill, 1985). This contrasts with results of Sutherland *et al.* (1974), who selected on 4 to 11 week food intake, uncorrected for body weight, and whose high intake mice were fatter. The restriction on body weight in the "A" selection index meant that the high appetite mice were also selected to dispose of their increased calorific intake in a manner which minimized increases in body weight. This can be achieved by increasing maintenance requirements per unit body weight and appears to have occurred in mice from the high appetite lines (Hill and Bishop, 1986). Lean meat is an inefficient energy store since protein turns over rapidly and therefore has a higher cost of maintenance than fat. This protein turnover cost is estimated to be a fraction of 0.15 to 0.20 of total basal metabolic rate in rats (Millard, Garlick and Reeds, 1976). If a similar fraction occurs in mice then small changes in the proportion of lean ~~mass~~ WEIGHT can create significant differences in maintenance requirements and may explain why the high appetite lines appear to have become proportionally leaner.

The only selection which led to any substantial change in composition was that on the ratio of gonadal fat pad to body weight (F). The lean body ~~mass~~ WEIGHT of mice from these lines appear to be similar (averaging 29.6, 29.7 and 30.3 grams for

Table 3.1. Body weight (BW), carcass composition on a wet weight basis and proportion (prop) of total fat in the gonadal fatpad of 10 week old males of each replicate at generation 20.

Criterion	A					F					P				
Rep	BW	Fat	Ash	N	Prop	BW	Fat	Ash	N	Prop	BW	Fat	Ash	N	Prop
	(g)		(g/100g)			(g)		(g/100g)			(g)		(g/100g)		
			High					Fat					High		
1	38.50	9.80	3.75	3.16	13.76	35.85	14.58	3.49	2.90	19.31	44.85	11.22	3.41	2.99	14.23
2	39.86	8.94	3.66	3.24	14.54	37.02	16.54	3.42	2.85	19.77	40.03	9.17	3.76	3.03	12.77
3	39.37	11.49	3.38	2.99	13.39	33.08	16.86	3.19	2.86	14.29	42.37	7.54	3.51	3.12	14.50
Mean	39.19	10.08	3.60	3.13	13.90	35.27	16.00	3.37	2.87	17.79	42.36	9.31	3.56	3.05	13.83
			Control					Control					Control		
1	33.66	9.52	3.75	3.12	12.76	34.38	10.40	3.43	2.98	13.83	30.05	8.16	3.44	3.00	13.37
2	31.75	8.92	3.78	3.17	12.68	31.13	10.01	3.55	2.96	13.33	30.18	8.49	3.65	3.09	12.83
3	31.36	13.48	3.50	3.00	11.49	34.17	10.82	3.35	2.97	12.51	31.34	10.08	3.54	3.04	13.68
Mean	32.27	10.64	3.68	3.10	12.31	33.20	10.41	3.45	2.97	13.22	30.49	8.91	3.55	3.04	13.29
			Low					Lean					Low		
1	30.68	12.31	3.75	3.03	12.86	32.33	6.42	3.85	3.07	11.57	26.43	8.78	3.73	3.01	16.13
2	30.49	9.40	3.73	3.11	11.36	33.48	5.74	3.93	3.14	10.77	26.36	12.68	3.73	2.99	15.64
3	25.79	10.96	3.73	3.05	11.67	31.52	7.11	3.84	3.10	9.61	26.80	10.26	3.61	2.95	14.80
Mean	29.13	10.89	3.74	3.06	11.96	32.42	6.42	3.87	3.10	10.65	26.53	10.57	3.69	2.98	15.53

Table 3.2. Analysis of variance of carcass composition data from Table 3.1.

Source	d.f.	m.s.			
		Fat	Ash	N	Prop
A (H vs L)	1	2.952	0.0882	0.0220	16.94*
(Sym)	1	0.144	0.0006	0.0001	2.31
F (H vs L)	1	412.994***	1.1250***	0.2380***	229.41***
(Sym)	1	3.840	0.1734*	0.0013	6.00
P (H vs L)	1	7.144	0.0760	0.0220	13.00*
(Sym)	1	6.365	0.0337	0.0037	11.59
Rep/criterion	6	7.905	0.0945	0.0217	8.22
Lines	12	6.255***	0.0255	0.0074*	2.71
A (replicates)	6	9.94***	0.0597**	0.0266***	1.49
F (")	6	2.16*	0.0365*	0.0022	10.64***
P (")	6	8.32***	0.0495**	0.0076	1.51
Remainder	54	0.862	0.0142	0.0035	1.66

H vs L is High vs Low, tested against lines
Sym is (H+L)/2 - Control, tested against lines
Replicates averaged over directions within criterion, tested against lines

Replicate lines within direction within criterion, tested against remainder, using reduced model in which pooled replicates not fitted.

Lines tested against remainder

the Fat, Control and Lean lines respectively at age 10 weeks, derived from Table 3.1) so the differences in body weight can be attributed largely, if not exclusively, to the differing amounts of fat tissue. There were no consistent differences in nitrogen or ash if expressed as a proportion of fat-free tissue.

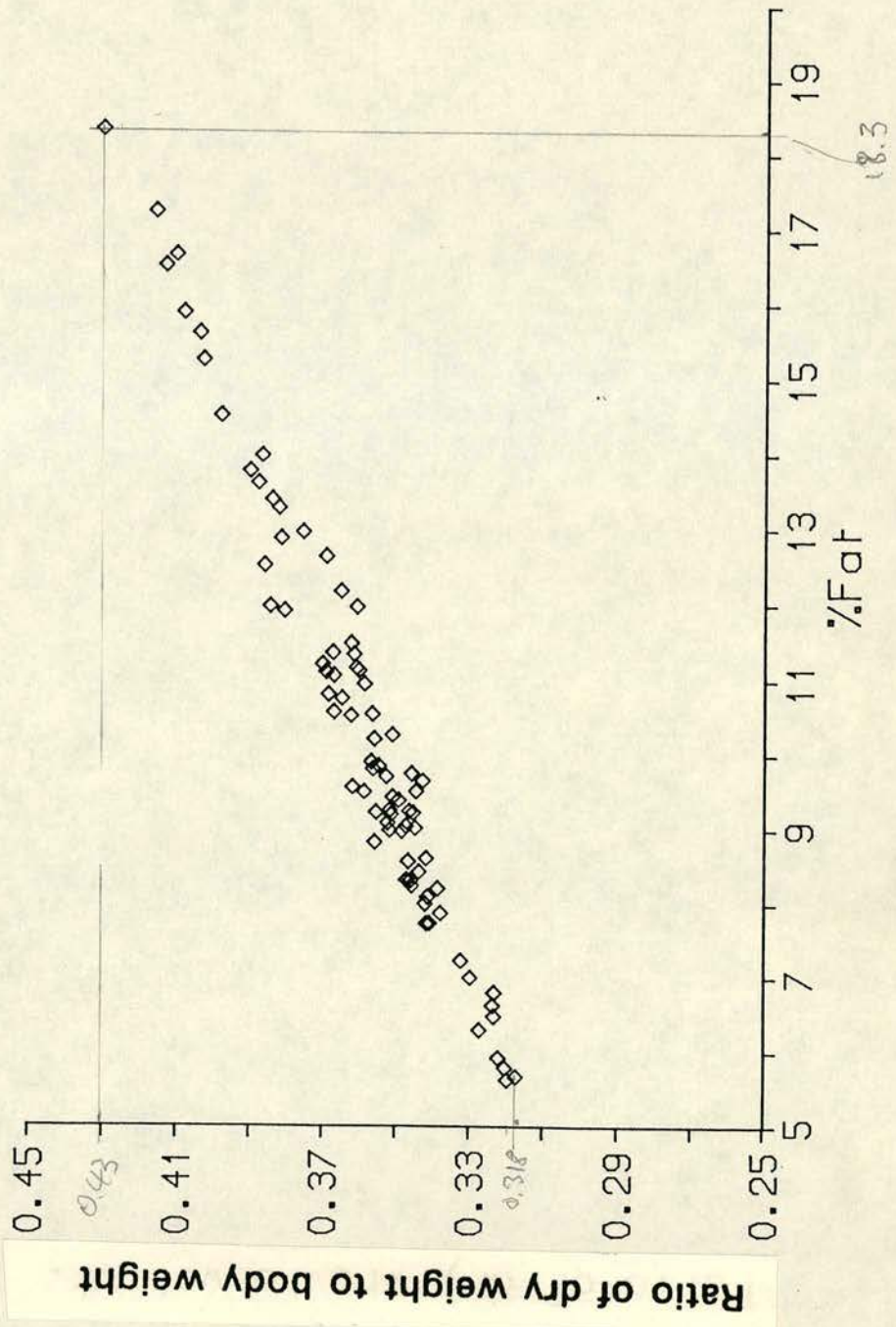
The proportion of total fat contained within the gonadal fat pad has also been altered to some extent in all treatments (Table 3.2). In the F and P lines this proportion has changed according to the direction of selection; for example, selection for an increased ratio of gonadal fatpad weight to body weight in the High fat lines has resulted in a disproportionally large increase in gonadal fatpad weight compared to growth in overall fat content. Similar changes in fat distribution have been observed in lines of chickens where abdominal fat pads formed part of the selection index (Ricard, Leclercq and Touraille, 1983) and in pigs where subcutaneous backfat formed part of a selection index (Rook *et al*, 1987). The distribution of fat within a carcass is in itself an important economic trait; for example meat that is too lean may be less palatable and more difficult to process (Webb, 1986).

Replicate line differences within treatments may occur as a result of the random drift of alleles underlying a trait or be a consequence of sampling from the base population. In this instance the replicate lines were all derived from random samples of the base population and by the time of this study had undergone 20 generations in small population size. Drift is therefore likely to be the main source of replicate differences compared to that due to sampling among animals within replicates. These differences (Tables 3.1 and 3.2) illustrate the risks of conclusions drawn from unreplicated lines and emphasize the advantage of using replicated model systems such as mice to investigate physiological traits of importance in other animals.

The relationship between water content and fat content within the carcass was also investigated. Lipids are strongly hydrophobic and fat tissue is known to contain significantly less water than other tissues, so it should be possible to predict fat content of a carcass from its water content (e.g. Blaxter, 1962; Rogers and Webb, 1980). The results are shown in Figure 3.1. This enables fat content (Y = fat weight/~~wet~~ Body weight) to be predicted from dry matter content (X = dry weight/~~wet~~ Body weight) using the linear relationship, $Y = a + bX$. The least squares estimates of these parameters are $a = -0.302 \pm 0.009$ and $b = 1.13 \pm 0.03$; the correlation between water content and fat content is 0.98.

This regression analysis using samples from all lines places most emphasis on

Figure 3.1. Fat content plotted against dry matter for individual samples from all lines.



points at the extremes of the range of fat content. To investigate whether the relationship still applies to samples in the middle of the range and within lines, a term for the partial regression on dry matter content was added to the model used in the analysis of variance described above. If this regression is a consistently good predictor of fat content then its inclusion should remove all differences in fat content between lines. The results (Table 3.3) show that although some differences in the relationship remain between individual lines, these differences are not associated with selection criteria, direction of selection or replicates, so it seems reasonable to conclude that water content is a robust indicator of fat content.

3.4 Conclusions

It is known that the lines of mice have diverged in food intake, the High A, F, and P replicates all consuming more than their equivalent Control and Low replicates (Bishop and Hill, 1985). The results obtained in this chapter reveal the extent to which the different selection criteria have altered the way this extra intake is partitioned: selection in the A and P lines have altered body weights with only marginal changes in body composition, while selection in the F lines has elicited large responses in carcass composition with only marginal changes in body weight. The next chapter will investigate whether these responses were subject to natural selection over the 9 generations following this analysis, during which selection was discontinued.

Table 3.3. Analysis of variance of fat content of 10 week old male mice at generation 20 before and after fitting regression on dry matter content.

Source	Before regression d.f.	Before regression m.s.	After regression d.f.	After regression m.s.
Direction/criterion	6	72.233***	6	0.595
Replicate/criterion	6	7.905	6	0.834
Lines	12	6.255***	12	0.471**
Regression on dry matter content	-	-	1	37.496***
Remainder	54	0.862	53	0.1704

CHAPTER 4

RECONVERGENCE OF RESPONSES DURING NINE GENERATIONS OF RELAXED SELECTION IN LINES SELECTED ON FAT CONTENT AND LEAN ~~MASS~~ WEIGHT.

4.1 Introduction.

Characters changed by selection pressures typically revert towards their original values after selection is discontinued (Falconer, 1981). These changes have important implications for commercial species where strains with desirable characteristics produced by selection may suffer a decline in performance after removal of selection. Lean ~~mass~~ WEIGHT and fat content are two such characters frequently subjected to selection, so it may be informative to determine the extent of any reconvergence in the murine model system. It was also necessary to confirm that no major reconvergence of traits had occurred before using animals from later generations of these lines in the biochemical/physiological studies of Asante (1989), Asante *et al.* (1989), Morrupa *et al.* (1989), and the study described in Chapter 7 of this thesis.

4.2 Methods

Selection was discontinued at generation 20 in the original High and Low selected F and P lines which were subsequently maintained without selection (Chapter 2). Changes in carcass composition at this time were determined in the previous chapter and serve as a baseline when investigating the F and P lines in later generations.

The mating scheme of the F and P lines at generation 29 produced eight families per replicate per generation. All the offspring from each family in each line were weighed at ages six and ten weeks. All male mice (except two per family randomly chosen as parents of the next generation) were starved overnight, weighed and killed by cervical dislocation at age 10 weeks. Their gonadal fatpads were excised and weighed before being reinserted into the carcass which was then freeze dried. Additional information on the F lines at generation 31 became available when dissecting mice to obtain tissue for the measurement of enzyme activities described in Chapter 7.

The data on the F and P lines were analysed separately by analysis of variance using the following model:

$$Y_{ijk} = \mu + D_i + R_j + G_k + L_{ij} + K_{ik} + e_{ijk}$$

where Y_{ijk} is the value in the k^{th} generation of the j^{th} replicate in the i^{th} direction of selection; μ is the overall mean; D_i is the effect of the direction of selection; R_j is the effect of the j^{th} replicate and represents the effects of contemporaneity; G_k is the effect of the k^{th} generation; L_{ij} is the effect of individual lines; K_{ik} is the interaction between direction of selection and generation; and e_{ijk} is the residual error. K_{ik} represents the effects of reconvergence of the trait during relaxed selection plus the effects of continued response to selection in generations 18 to 20 and was tested against residual error. This term can be partitioned into the effects of selection (applied or absent, corresponding to before and after generation 20), and re-tested against residual error to distinguish the two effects. Sample sizes are shown on Table 4.1.

4.3 Results

The results of chemical carcass analysis in the previous chapter show that water content is strongly correlated with fat content and can be used to estimate the carcass fat content. In conjunction with the other data, this allows us to estimate the following carcass traits: mean six and ten week body weight, the ratio of gonadal fatpad weight to body weight in 10 week old males (the F selection criterion), the index body weight minus eight times gonadal fatpad weight in 10 week old males (the P selection criterion), fat content (estimated from water content) and the proportion of estimated fat in the gonadal fatpad. The results obtained for the F lines are shown in Table 4.2 and for the P line on Table 4.3. The analysis of variance of the F lines is shown in Table 4.4 and that of the P lines on Table 4.5; no significant reconvergence occurred in any of the traits in either line.

4.4 Discussion

Reconvergence after relaxation of selection pressures is a widely recognised phenomenon (see for example Lerner, 1954 & 1958 and Falconer, 1981) and is usually attributed to the effects of natural selection acting on the altered phenotype. The data presented here suggest that reconvergence has been insignificant in the F and P lines. This may be due to either the absence of natural selection pressures acting on the trait or, alternatively, a lack of additive genetic variance in the trait which prevents response. Response in the lines had continued up to generation 20 (when selection was discontinued) which suggests that sufficient additive variance existed upon which natural selection

Table 4.1. Sample size (number of animals) used to estimate mean line values of 6 week weight, 10 week weight, ratio gonadal fatpad weight to body weight (F lines), index body weight less eight times gonadal fatpad weight (P lines), estimated fat content (est. fat) and proportion of estimated fat in the gonadal fatpad (prop) in F and P lines generations 18, 19, 20, 29 and 31.

Trait	Gen.	F lines						P lines					
		Fat			Lean			High			Low		
		replicate			replicate			replicate			replicate		
		1	2	3	1	2	3	1	2	3	1	2	3
6 week wt.	18	57	82	69	67	78	54	83	71	62	66	66	67
	19	70	70	80	68	64	72	84	76	75	56	68	74
	20	77	76	82	63	83	69	64	64	79	75	78	57
	29	77	76	62	75	79	76	86	86	77	70	68	54
10 week wt.	29	37	38	34	35	34	32	40	41	37	37	36	28
	18, 19, 20, 31	as in following table											
Ratio, index, est. fat, and prop.	18	16	16	17	13	16	16	28	25	23	25	24	29
	19	16	15	16	16	16	16	25	27	27	24	23	26
	20	16	15	16	15	15	16	15	16	16	14	16	15
	29	21	24	18	18	18	16	24	25	21	21	20	13
	31	15	20	15	15	20	20	-	-	-	-	-	-

Table 4.2. changes in growth traits of the F lines before and after cessation of selection at generation 20. Means of six and 10 week body weights, ratio of gonadal fatpad weight to body weight at age 10 weeks (the selection criterion), estimated fat content at age 10 weeks (from water content) and the proportion of estimated fat in the gonadal fatpad at age 10 weeks (prop) in generations (Gen.) 18, 19, 20, 29 and 31. Divergence is the mean value of the trait in the Fat lines minus the mean value in the Lean in each generation.

Trait	Gen.	Line								Divergence
		Fat replicate				Lean replicate				High-Low
		1	2	3	mean	1	2	3	mean	
6 Wk Wt (g)	18	26.2	24.2	23.3	24.6	24.2	22.5	22.5	23.1	1.5
	19	25.8	25.8	22.6	24.8	22.0	25.3	22.4	23.2	1.6
	20	27.2	26.2	23.4	25.6	25.2	24.4	22.1	23.9	1.7
	29	25.4	24.5	24.6	24.8	21.8	23.8	22.8	22.8	2.0
10 Wk wt (g)	18	35.6	32.5	32.7	33.6	31.5	32.1	31.2	31.5	2.1
	19	35.1	35.8	30.9	34.0	31.8	34.5	31.4	32.6	1.4
	20	35.8	37.0	33.1	35.3	32.3	33.5	31.5	32.4	2.9
	29	34.5	33.8	32.6	33.6	31.7	33.4	32.4	32.5	1.1
	31	35.0	34.1	32.0	33.7	30.7	30.5	30.5	30.6	3.1
Ratio (mg/g)	18	27.5	22.9	23.9	25.0	7.0	5.5	6.1	6.2	18.8
	19	24.1	28.3	21.0	24.9	6.3	6.5	6.4	6.4	18.5
	20	28.2	31.4	23.9	28.2	7.2	6.0	6.9	6.7	21.5
	29	23.8	29.3	22.2	25.1	8.1	7.1	7.5	7.6	18.5
	31	25.3	25.4	21.7	24.1	8.7	6.6	6.7	7.3	16.8
Est. fat (%)	20	14.6	16.5	16.9	16.0	6.4	5.7	7.1	6.4	9.6
	29	15.9	16.7	13.3	15.3	6.6	6.6	9.4	7.6	7.7
Prop. (%)	20	19.3	19.8	14.3	17.8	11.6	10.8	9.6	10.6	7.2
	29	15.0	18.4	13.6	15.6	11.8	11.0	11.5	11.4	4.2

Table 4.3. Changes in growth traits in P lines before and after cessation of selection at generation 20. Means of six and ten week body weights, selection index (body weight less eight times gonadal fatpad weight at age 10 weeks), estimated fat content at age 10 weeks (from water content), and the proportion of estimated fat in the gonadal fatpad (prop) at age 10 weeks in generations (Gen.) 18, 19, 20 and 29. Divergence is the mean value of the trait in the High lines minus the mean value in the Low in each generation.

Trait	Gen.	Line								
		High replicate				Low replicate				Divergence
		1	2	3	mean	1	2	3	mean	High-Low
6 Wk. Wt. (g)	18	30.5	28.5	27.4	28.8	20.4	19.2	20.2	19.9	8.9
	19	28.5	28.8	30.9	29.4	21.6	20.2	21.6	21.2	8.2
	20	31.8	28.2	28.2	29.4	19.9	19.8	20.0	19.9	9.5
	29	30.4	27.7	30.6	29.6	19.8	20.4	20.7	20.3	9.3
10 Wk wt (g)	18	42.0	39.4	39.9	40.4	26.8	26.6	26.9	26.8	13.6
	19	40.7	39.1	43.7	41.2	27.5	28.7	27.7	28.0	13.2
	20	44.8	40.0	42.4	42.4	26.4	26.4	26.8	26.5	15.9
	29	42.4	37.1	43.0	40.8	25.6	26.6	27.8	26.7	14.1
Index (g)	18	37.7	35.7	36.4	36.6	23.5	22.5	24.1	23.4	13.2
	19	36.8	35.8	39.6	37.4	23.9	23.6	24.3	24.0	13.4
	20	39.6	36.2	38.5	38.1	23.4	22.2	23.6	23.1	15.0
	29	38.2	32.8	38.6	36.5	20.9	22.0	24.7	22.6	13.9
Est. Fat (%)	20	11.2	9.2	7.5	9.3	8.8	12.7	10.3	10.6	-1.3
	29	9.4	9.3	7.2	8.6	13.3	15.0	10.6	12.9	-4.3
Prop. (%)	20	14.2	12.8	14.5	13.8	16.1	15.6	14.8	15.5	-1.7
	29	13.7	17.5	18.0	16.4	16.8	14.3	13.2	14.7	1.7

Table 4.4. Analysis of variance of carcass composition of F lines using data from Table 4.2.

Source†	d.f.‡	mean squares				
		6 wk wt	10 wk wt	ratio	est. fat	prop§
Direction	1	17.00	33.08	2544**	226*	96.90
replicate	2	7.69	10.23	15.32	0.65	8.42
generation	3,4,4,1,1	1.19	2.59	3.54	0.14	1.40
dir*rep	2	2.05	4.82*	17.57**	3.24	5.80
dir*gen	3,4,4,1,1	0.09	1.18	4.09	2.52	6.31
(selection	1	0.18	0.00	7.94)		
error	12,16,16,4,4	1.30	0.88	2.69	1.94	1.15

†: effects of direction (dir) and replicate (rep) tested against individual lines (dir*rep). Effects of generation, individual lines (dir*rep), and reconvergence (dir*rep or selection) tested against residual error.

‡: where this varies, the value of each column is given.

§: for abbreviations see Table 4.2.

* $p<0.05$; ** $p<0.01$; ***$p<0.001$

Table 4.5. Analysis of variance of carcass composition of P lines using data from Table 4.3.

Source†	d.f.‡	mean squares				
		6 wk wt	10 wk wt	index	est. fat	prop§
Direction	1	483**	1214**	1164***	23.80	0.00
replicate	2	6.63	7.50	11.85	7.29	0.02
generation	3,3,3,1,1	2.67	1.45	1.71	2.17	2.52
dir*rep	2	2.49	9.09*	3.42	3.77	5.80
dir*gen	3,3,3,1,1	1.31	2.03	0.96	6.90	8.17
(selection	1	0.18	0.01	0.00)		
error	12,12,12,4,4	15.7	1.46	1.39	1.35	2.24

†,‡: see Table 4.4

§: see Table 4.3.

* $p<0.05$; ** $p<0.01$; ***$p<0.001$

could act. The inference must therefore be that natural selection pressures were insignificant or absent. Natural selection can act directly on a character, for instance by preventing fat content falling below a physiologically necessary level (Leenstra, 1986), or may act indirectly on detrimental correlated responses such as the small litter sizes associated with low body weight in the P lines. The pleiotropic effects of genetic changes may be clearly recognisable as correlated traits, such as changes in litter size, but some effects may be less well defined. For example, Yoo (1980a) selected for more than 86 generations on increased abdominal bristle number in *Drosophila melanogaster*, a character generally regarded as "near-neutral" in terms of natural selection, and obtained responses of between 3 and 4 times the original bristle number. Substantial reconvergence occurred after selection was discontinued, and in one replicate 40% of the response gained in the 88 generations of selection was lost during the first 7 generations of relaxed selection. Yoo (1980b) attributed this to the presence of recessive lethal alleles with pleiotropic effects on bristle number, rather than the effects of natural selection acting directly on bristle number. The data presented in this chapter suggest there is little direct selection in the laboratory against fat content or lean ~~mass~~ WEIGHT, and that few alleles with detrimental pleiotropic effects appear to be associated with these characters. This latter conclusion may be relevant to selection for these characters in commercial species since the same basic biochemistry and physiology occurs in all mammalian species.

Previous selection experiments on mouse body weight at 6 weeks of age also reported only a gradual reconvergence of the selected trait after cessation of selection (Falconer, 1955; Roberts, 1966) but there appears to be no similar reports in the literature on reconvergence in mouse lines after selection for fat content.

4.5 Conclusions.

The conclusions are straightforward: no significant degree of reconvergence occurred following relaxation of selection in replicated lines of mice selected on fat content or lean ~~mass~~ WEIGHT. This was apparently due to a lack of natural selection pressures rather than an absence of additive genetic variance associated with the traits.

CHAPTER 5

EXAMINATION OF THE P6 LINE FOR THE PRESENCE OF SEGREGATING ALLELES WITH LARGE EFFECTS ON BODY WEIGHT.

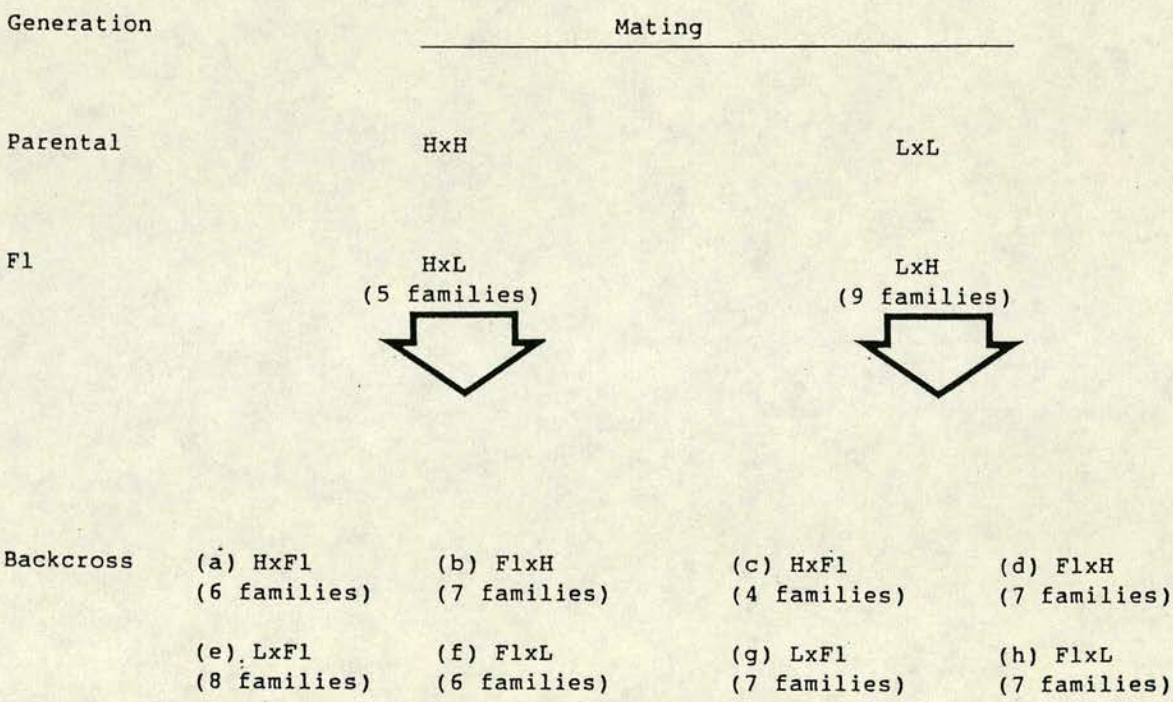
5.1 Introduction.

The P6 lines were chosen to assess the possible contribution of major genes to their response for three main reasons. Firstly, a group of genes (oncogenes) are known to have dramatic effects on cell growth and may have large effects on body weight. Secondly, investigations into the gene coding for ornithine decarboxylase (ODC) in the original P lines suggested that a segregating RFLP may be associated with body weight (A. Tait, pers. comm.). If this acts as a major gene it should be detected statistically and the DNA extracted from backcross generations will enable further testing of the putative relationship between this RFLP and body weight. Thirdly, major mutations affecting body weight in mice have occurred spontaneously (Bradford and Famula, 1984) and by deliberate manipulation (Palmiter *et al*, 1982). The gene identified by Bradford and Famula occurred spontaneously in a line of mice selected for rapid weight gain and appeared to be autosomal and recessive.

5.2 Methods.

Several methods are available to detect the presence of genes with large effects within a population (Hill and Knott, 1989). The protocol used here was to backcross the progeny of a cross between the High and Low P6 lines to both parental lines. The data obtained in the experiment can then be analysed by the methodology of Elston and Stewart (1973) and Elston (1979 and 1984) if this later proved necessary. This protocol also measures heterosis in the F1. The mating scheme is shown on Figure 5.1. Each family from the P6 parental lines was, as nearly as possible, equally represented in the F1. Care was taken to ensure equal representation of the reciprocal halves of the F1 in the backcross and equal representation of each family within this restriction. The backcross was designed to minimize matings between related individuals and in practice no individuals more closely related than uncle/niece or aunt/nephew were mated together. All individuals from the parental lines and F1 cross were weighed at 6 and 10 weeks of age. In the backcross all individuals were weighed at 3 weeks. However due to space restrictions the number of families in each group of the

Figure 5.1. Mating scheme used to test for segregation of major genes in the P6 lines. Female parent represented first in the crosses e.g. F1xH is an F1 female crossed with a High male. The number of families is the number of fertile matings within each cross. The arrows between the F1 and backcross generations indicate which reciprocal half of the F1 was used in the backcross. The backcross matings are designated (a) to (h) to simplify reference to this figure in the text and in subsequent tables of data.



backcross was reduced to five. Eight individuals from these five families were kept and weighed at six and ten weeks. Where possible four of each sex were chosen at random within this restriction. In group (c) (see Figure 5.1) only four families survived so six families were kept from group (d) to equilibrate the number of families at higher levels of subdivision. After removal from the mating scheme the animals were killed by cervical dislocation and their livers removed and kept temporarily on dry ice before being stored intact at -70°C. This created a resource for groups attempting to identify major genes in these lines either directly by RFLP or by their products on 2D electrophoresis. Putative major genes or products identified in such studies can be tested for correlation with body weight. The number of animals used in each group is shown on Table 5.1.

5.3 Results.

Table 5.2 shows data obtained from the parental P6 lines in generations 31, 32 and 33. Generation 31 was the parental stock and generations 32 and 33 were contemporaneous with the F1 and backcross generations respectively. Any environmental fluctuations affecting body weight (such as food batch differences or disease epidemics) should have been revealed by these data. After allowing for the continued response to selection applied on generations 31 and 32 there appeared to be no effects of contemporaneity.

Table 5.3 shows the results of the F1 cross. The larger litter size associated with the "High" body weight mother is presumably a maternal effect. There appeared to be no effect of heterosis (discussed in more detail later). There were sex differences in body weight in the F1 where the weight of males was biased towards that of the female parent. If this was a maternal effect it would be expected to affect both sexes equally but the weight of females is midway between that of the parental lines in both halves of the reciprocal cross. A major difference in genotype between the sexes is that males receive only a maternal X chromosome, so initial inspection of the data suggested that this chromosome may have a significant effect on body weight.

Tables 5.4 and 5.5 can be studied in detail to test predictions based on the possible modes of inheritance i.e. polygenic, autosomal major gene, sex-linked major gene, Y-chromosome effects, and maternal (cytoplasmic) inheritance. For reasons which will become obvious, the sex- linked major gene hypothesis will be considered first. This hypothesis generates four predictions in the backcross based on the fact that males receive only one X chromosome which is transmitted maternally;

Table 5.1. Number of animals used to estimate body weights in P6 parental lines and subsequent crosses.

	Parental P6 lines					
	High generation			Low generation		
	31	32	33	31	32	33
Female six week weight	58	60	67	41	59	49
Male six week weight	49	70	72	40	48	52
Female ten week weight	58	46	51	41	51	47
Male ten week weight	49	56	57	38	45	46

	Crosses									
	F1		Backcross groups							
	HxL	LxH	a	b	c	d	e	f	g	h
Female three week weight	-	-	22	36	18	32	44	28	25	34
Male three week weight	-	-	20	37	20	32	32	29	27	35
Female six week weight	22	30	17	20	15	25	20	20	21	20
Male six week weight	19	31	19	20	14	23	20	20	18	20
Female ten week weight	22	30	17	20	15	25	20	20	21	20
Male ten week weight	19	31	19	20	14	23	20	20	18	20

Table 5.2. Means and standard errors of litter size, body weights at ages six and ten weeks, and proportion of fertile matings in the P6 parental lines (generation 31) and P6 lines generations 32 and 33 (contemporaneous with the F1 and backcross generations respectively). Standard errors are given in preference to standard deviations as the data are presented to reveal any changes in mean values attributable to environmental fluctuations.

	High			Low		
	Generation					
	31	32	33	31	32	33
Litter size	15.7±0.9	11.3±1.0	11.8±1.2	6.8±0.4	7.4±0.4	7.1±0.4
Female six week weight	31.9±0.4	33.3±0.6	33.6±0.4	16.5±0.3	16.3±0.2	17.1±0.2
Male six week weight	35.9±0.5	38.6±0.6	38.6±0.5	19.7±0.4	18.9±0.2	20.7±0.3
Female ten week weight	40.3±0.4	42.9±0.7	43.0±0.5	18.7±0.3	19.4±0.2	19.4±0.2
Male ten week weight	46.9±0.4	50.4±0.6	49.5±0.6	22.8±0.4	22.7±0.3	23.6±0.4
Proportion of fertile matings	10/16	15/16	15/16	13/16	15/16	15/16

Table 5.3. Means and standard deviations of litter size, body weights at ages six and ten weeks, and proportion of fertile matings in P6 parental (generation 31) and F1 crosses (female parent represented first).

Trait	HxH	F1 cross		LxL
		HxL	LxH	
Litter size	15.7±2.75	10.8±2.73	7.1±2.89	6.8±1.46
female six week weight	31.9±2.73	23.7±3.67	24.6±2.29	16.5±1.77
male six week weight	35.9±3.35	30.4±3.36	25.6±2.38	19.7±2.71
female ten week weight	40.3±3.25	28.6±3.12	29.9±3.22	18.7±1.98
Male ten week weight	46.9±2.87	36.6±3.85	31.1±2.54	22.8±2.83
Proportion of fertile matings	10/16	5/10	9/10	13/16

Table 5.4. High Backcross: Means and standard deviations of litter size, body weights at ages three, six and ten weeks, and the proportion of fertile matings.

Cross:	HxFl		FlxH	
Source of Fl:	HxL (group a)	LxH (group c)	HxL (group b)	LxH (group a)
Litter size	14.3±4.64	11.8±5.74	12.1±1.86	13.3±2.63
Female three week weight	10.6±3.03	11.1±3.06	10.0±1.90	9.4±2.04
Male three week weight	11.4±3.36	10.9±3.75	9.4±1.94	9.2±1.93
Female six week weight	28.8±2.45	26.6±1.52	26.3±2.00	26.2±2.06
Male six week weight	34.1±3.37	33.4±2.46	29.7±4.23	30.1±2.40
Female ten week weight	36.0±3.45	33.3±1.76	33.2±3.67	33.2±2.83
Male ten week weight	42.6±2.71	42.0±2.93	37.3±4.50	39.8±3.43
Proportion of fertile matings	6/8	4/7	7/7	7/8

Cross:	HxFl	FlxH
Source of Fl:	pooled (groups a+c)	pooled (groups b+d)
Litter size	13.3±4.88	12.7±2.22
Female three week weight	10.8±3.05	9.7±2.00
Male three week weight	11.2±3.55	9.3±1.94
Female six week weight	27.8±2.33	26.3±2.03
Male six week weight	33.8±3.04	29.9±3.38
Female ten week weight	34.7±3.10	33.2±3.22
Male ten week weight	42.3±2.82	38.7±4.15
Proportion of fertile matings	10/15	14/15

Cross:	pooled
Source of Fl:	pooled (groups a+b+c+d)
Litter size	13.0±3.57
Female three week weight	10.2±2.53
Male three week weight	10.0±2.84
Female six week weight	26.9±2.29
Male six week weight	31.6±3.76
Female ten week weight	33.9±3.25
Male ten week weight	40.3±4.06
Proportion of fertile matings	24/30

Table 5.5. Low Backcross: Means and standard deviations of litter size, body weights at ages three, six and ten weeks, and the proportion of fertile matings.

Cross:	LxF1		F1xL	
Source of F1	HxL (group e)	LxH (group g)	HxL (group f)	LxH (group h)
Litter size	9.0±1.69	9.0±1.29	13.3±1.97	11.1±3.58
Female three week weight	10.0±1.73	8.9±1.92	8.0±1.73	8.5±1.61
Male three week weight	9.6±2.06	9.6±1.77	8.3±2.19	9.0±1.97
Female six week weight	20.7±2.38	19.7±1.48	19.3±2.72	19.9±1.64
Male six week weight	21.7±2.70	22.7±2.62	24.8±2.79	24.4±2.18
Female ten week weight	24.6±2.99	23.1±1.97	23.0±3.07	23.1±2.45
Male ten week weight	26.8±2.68	27.6±2.88	30.4±2.34	29.0±2.82
Proportion of fertile matings	8/8	7/8	6/7	7/8

Cross:	LxF1	F1xL
Source of F1:	pooled (groups e+g)	pooled (groups f+h)
Litter size	9.0±1.46	12.2±3.08
Female three week weight	9.5±1.90	8.3±1.69
Male three week weight	9.6±1.92	8.7±2.10
Female six week weight	20.2±2.04	19.6±2.27
Male six week weight	22.2±2.72	24.6±2.51
Female ten week weight	23.8±2.62	23.0±2.78
Male ten week weight	27.2±2.81	29.7±2.68
Proportion of fertile matings	15/15	13/15

Cross:	pooled
Source of F1:	pooled (groups e+f+g+h)
Litter size	10.5±2.84
Female three week weight	8.9±1.91
Male three week weight	9.1±2.07
Female six week weight	19.9±2.18
Male six week weight	23.4±2.89
Female ten week weight	23.4±2.73
Male ten week weight	28.5±3.01
Proportion of fertile matings	28/30

- In the High backcross (Table 5.4) males from "F1 females x High males" (groups b+d) should be lighter than those from the reciprocal "High female x F1 male" (groups a+c) since 50% of them inherit a Low X chromosome. Their phenotypic standard deviation (SD) should be higher since both types of X chromosome will be present.

- In the Low backcross (Table 5.5), males from "F1 females x Low males" (groups f+h) should be heavier than their reciprocal counterparts (groups e+g) since 50% of them should have inherited the High X chromosome. Their SD should also be higher

- In the High backcross (Table 5.4) the females from "High female x F1 male" should be heavier in matings where the father carries the High X chromosome (group a) since they will be homozygous "High X" rather than heterozygous (group c). The homozygous group (a) should also have a lower SD.

- A similar argument applies in the equivalent Low backcross "Low females x F1 males"; females in group (e) should be heavier since they are heterozygous for X chromosomes rather than homozygous "Low X" in group (g); the homozygous group (g) should also have a lower SD.

The data appear consistent with these predictions. The predicted differences in body weights are all observed. The differences in S.D. are largely fulfilled, the only noticeable exception being the fourth prediction where the heterozygous group appears to have a smaller S.D. than the homozygous.

The data suggest that the X chromosome has a semi-dominant effect on body weight in the order of 5g when hemizygous or homozygous and 2.5g when heterozygous at age 10 weeks. This represents 22% and 11% of the divergence in body weight. The effect was also apparent at age 6 weeks where the equivalent figures are 4g (hemi- or homozygous) and 2g (heterozygous) which represent 25% and 13% respectively of the divergence in body weight.

Allowing for the possible presence of X-linked effects we can make predictions to detect the presence of autosomal major genes. Body weights in females from the F1 are midway between the parental values which is consistent both with a polygenic or autosomal semi-dominant gene. The presence of a major gene segregating in the backcross can be detected by increases in the coefficient of variation (C.V., standard deviation/mean) relative to the F1. The presence of possible sex linked effects restricts the comparison to groups within which each individual has the same X chromosome genotype namely females within groups (a) ,(c), (g) and (h) and males within group (c) and (e). No increase in C.V. was

evident so it is doubtful if an autosomal gene with a large phenotypic effect could have been present.

The data can also be investigated to detect major effects on body weight associated with the Y chromosome. In the F1 cross the effects of the X and Y chromosomes cannot be separated. Males in groups (a) and (c) of the High backcross (Table 5.4) should have similar genotypes except that the Y chromosome was derived from the High and Low lines respectively. Similarly in the Low backcross, males in groups (e) and (g) will contain Y chromosomes derived from the High and Low lines respectively. The lack of discrepancy in body weight between groups (a) and (c) and between groups (e) and (g) suggest that significant effects on body weight were not associated with the Y chromosome.

There appears to be no maternal *in utero* effects or indications of cytoplasmic or mitochondrial inheritance. Consideration of these effects yield three predictions which are not fulfilled:

- The females in the F1 contain a X chromosome inherited from each parental line. However the females from "High female x Low male" half of the cross will also inherit the High cytotype and should be larger.

- In the High backcross there should be differences in "F1 female x High male" depending on whether the F1 female inherited the High (group b) or Low (group d) cytotype.

- Similarly in the Low backcross there should be differences in "F1 female x Low male" depending on whether a High maternal cytotype (group f) or Low maternal cytotype (group h) was inherited

The data are incapable of distinguishing whether the difference associated with the X chromosome is a major mutation or the result of a series of segregated alleles at sex-linked loci affecting body weight. It may be possible to distinguish the two hypotheses by repeated backcrossing to the Low line (Wright, 1952) in the following manner. A High female can be mated to a Low male, the heaviest female offspring selected and mated to another Low male and so on. Selection through the female line ensures that recombination breaks up the High X chromosome as well as the autosomes and the genetic distance of the backcross line from the Low line decreases by 50% each generation. The body weight of the backcross line will either decline to that of the Low line (if polygenic) or stabilize at 2.5 grams above (in females) of 5g above (in males) if

due to the putative major gene. The reason being that any individual "High" gene has a 50% chance of being lost each generation but selection for body weight will ensure its survival if it is of sufficient effect to overcome this random loss. This methodology requires a phenotypic effect in excess of two standard deviations which entails the experiment running at the limit of its resolution.

An alternative method of testing whether the sex- linked effect is due to a major gene is to use a tester X chromosome containing a number of phenotypic markers, such as coat colour, along its length. Mating males with this chromosome with a female from the P6 lines will produce female offspring heterozygous for the X chromosomes. Backcrossing to P6 males will produce male offspring with recombinant X chromosomes. The absence of the putative major gene will be indicated by the presence of one of the phenotypic markers in the recombinant X chromosome, which in turn should be correlated with a decrease in body weight. A search is underway to obtain a suitable tester chromosome.

5.4 Discussion

On the basis of the data shown on Tables 5.3, 5.4 and 5.5, it is impossible to distinguish whether there is a single sex- linked major gene or whether the X chromosome contains a number of segregating alleles at loci affecting body weight. The X chromosome is associated with approximately 25% of the high/low divergence at 6 and 10 weeks although it accounts for only around 5% to 6% of the haploid DNA in mice (Ohno, 1967). Sex- linked genes are dominant in the males so an increased selection pressure on these alleles may account for the skewed distribution of effects towards the X chromosome, particularly since selection was practised only on males during the first 20 generations. Thus consideration of a polygenic response in body weight may explain the possible bias towards the sex chromosome.

The bias which appears to exist towards sex-linked alleles has an interesting methodological implication. The heritability of a trait is a property of a population in a specific environment and most researchers do not explicitly acknowledge that its value may differ between sexes; for example, when selection is applied on one sex, realized heritability is calculated as twice the regression of response on cumulated selection differential (Falconer, 1981). However sex-linked genes are dominant in the heterogametic sex (males in mammals, females in birds) so selection on recessive or semi- dominant alleles

on the sex chromosomes will be more effective, and their contribution to response proportionally larger than, autosomal alleles. Extending the results to both sexes will therefore overestimate the heritability. The proportion of DNA in the sex chromosome and its gene content is similar in all mammalian species (Ohno, 1967; Lalley and M^{c}Kusick, 1985) so differences noted in mice lines may be significant in other, commercially important species. The magnitude of this difference in heritability can be estimated, assuming that alleles influencing growth traits are spread at random throughout the genome i.e. there is no bias in these loci towards autosomal or sex chromosomes. Assuming the sex chromosome contains 6% of the genome but contributes 25% to the response predicts that realized heritability will be 19% higher in males, a result with significant commercial implications.

A related bias in the effects of the sex chromosome was reported by Stonaker (1963) who crossed unselected strains of cattle, turkeys and broilers and measured heterosis in body weight. By comparing sexes he found that the sex chromosome accounted for 50%, 75% and 40% respectively of heterosis in these species. He coined the term *homogametic heterosis* to describe the phenomena. It is plausible that sex-linked alleles were more liable than autosomal alleles to be fixed by selection during the establishment of the breeds and that the phenomena is the result of the same process as that postulated above. Subsequent work has been inconclusive, some reports confirming his observations in mice and *Drosophila melanogaster* (Kidwell and Nash, 1964), some finding no effect in pigs (Cox, 1960), rats (Kidwell and Nash, 1964) and *Drosophila melanogaster* (Kidwell, 1963). White, Eisen and Legates (1970) reported higher heterosis in male mice selected for 6 week weight; however their study had a methodological drawback apparently not recognized by the authors. If "heterosis" occurs in the selected trait, such as six week body weight, it does not necessarily indicate an increase in general fitness since it also measures the relative dominance of the alleles which segregated to cause the response to selection. True heterosis, the general increase in fitness components attributable to increased heterozygosity, should be measured on a character not correlated with the selected trait, for example in mice selected on body weight heterosis could be measured as pre- weaning survival rate or resistance to infection.

Bhuvanakumar (1981) and Bhuvanakumar *et al.* (1985a) crossed lines of mice divergently selected for 6 week body weight. The high/low divergence in body weight at 6 weeks was by a factor of 1.9, exactly that of the P6 lines although their lines were slightly lighter (approximately 2g). They found reciprocal

differences of 1.6 gram or 12% of divergence at 6weeks. Unfortunately the sexes were not treated separately so any differences between males from the two reciprocal halves would have been obscured. However, if we assume maternal effects to be negligible (as in this study) the only difference between the reciprocal halves was the X chromosome content of the males. Under this assumption, the X chromosome then accounts for 24% of the divergence, exactly the estimate obtained earlier in the P6 lines.

Heterosis was defined as the value of F1 less the mean of the parental lines expressed as a percentage of the mean parental value. It was determined from the female offspring to avoid the complications associated with the X chromosome and the values obtained were - 0.2% (6 weeks) and -0.8% (10 weeks) on an arithmetic scale, and 1.63% and 1.91% on a scale of natural logarithms. The same calculation including males gave corresponding values of 0.2% and -1.9% for 6 weeks and 10 weeks, (1.54% and 1.39% on a log. scale), and for males alone 0.7% and -2.9% (1.46% and 1.39% on a log scale), all data derived from Table 5.3. The inbreeding coefficients of the parental lines were 0.18 in the high and 0.21 in the low. The value obtained by Bhuvanakumar *et al.* (1985a) for heterosis of 6 week body weight was 1.7% (arithmetic) of the mean between line value for crosses between divergent lines and the inbreeding coefficients of the lines was 0.6. They also reported higher heterosis for six week weight in males ($p<0.01$). The magnitude of heterosis seems to vary between studies e.g. Bakker, Nagai and Eisen, (1976), reported heterosis of 5% (arithmetic) in a mouse line selected for 6 week body weight crossed with an unselected control line (divergence in 6 week weight was by a factor of 1.3). The type of genetic changes underlying a response in body weight appears to be varied. For instance White *et al.* (1970) investigated lines differing in 6 week body weight by a factor of 1.7, due to divergent selection, and reported three findings in direct contrast to those reported here: that significant heterosis occurs, that it is higher in males (7% in females 21% in males on an arithmetic scale of 6 week weight) and that there was no significant effect of sex- linked genes.

5.5 Conclusions.

The contribution of sex-linked alleles to the response to selection may have significant economic implications which would reward further work. The suggestion that heritability may be 19% higher in the heterogametic sex is of obvious economic interest. The P6 lines are important irrespective of the type of genetic change; the presence of a sex-linked major gene allows biochemical studies with the possible identification of a locus suitable for direct

manipulation, whereas changes in the frequencies of alleles at several sex-linked loci implies that differences in heritability and heterosis are likely to occur between the sexes of mice and possibly also in commercially important species.

CHAPTER 6

GROWTH AND REPRODUCTION IN THE F6 LINES.

6.1 Introduction.

Substantial responses to selection on fat content have occurred in the F lines with little apparent difference in other growth characteristics (Chapters 2 and 3). Fat growth and distribution are important parameters in livestock production so some growth characteristics of these lines were investigated in more detail. The characters under examination were (i) changes in body weight and internal organ growth, (ii) changes in lean ~~mass~~ WEIGHT and carcass fat content and (iii) relative growth rates of fat deposits. The growth of fat deposits also provides background information for the biochemical studies of fat synthesis in these lines (Chapter 7, this thesis; Asante, 1989; Asante *et al*., 1989) and and studies on the growth of adipocytes in these lines (presently under way in this laboratory and by P. Sinnet-Smith).

The Fat lines have slightly larger litter sizes than the Lean lines whereas lines of mice with major-gene induced obesity tend to have reduced fertility. Similarly, other lines with high fat content as a correlated response to selection on body size, also tend to have reduced fertility. For these reasons the following traits associated with fertility were also investigated: (i) ovulation rate, (ii) pre-natal survival of embryos, (iii) the effects of embryo genotype.

6.2 Methods.

(i) Growth characteristics.

Male mice from generation 32 were investigated at ages 4, 5, 6, 8, 10, 14, 18, 22 and 26 weeks. Due to fertility differences between the lines, 8 individuals from the Fat line and 6 from the Lean line were examined at each age except 14 weeks. Males mated in the F6 lines were sacrificed at age 14 weeks (the age of selection) allowing examination of 48 from the Fat line and 42 from the Lean. Individual mice were killed by inhalation of diethyl ether, weighed and the following organs dissected and weighed: testes, kidneys, liver, lungs, heart and spleen. Three fat deposits were dissected and weighed: gonadal fatpad, hindleg fatpad and shoulder fatpad. The organs and fatpads were re-inserted into the body cavity and the carcass freeze dried, allowing estimation of carcass fat content as described in Chapter 3. The lean ~~mass~~ WEIGHT was calculated as the

difference between body weight and estimated fat content.

(ii) Fertility traits.

Animals were obtained from the F6 line at generation 34, and control animals from the contemporaneous unselected replicate 1 of the M lines at generation 13. These were mated at age ten weeks, and a complete reciprocal diallele cross made with six matings in each of the nine combinations. Females were sacrificed 17 days after appearance of the last vaginal plug. The number of corpora lutea on the ovaries was noted as was the number of moles, resorptions and live embryos present in the uterus. The number of corpora lutea indicate the number of eggs released from each ovary, and the number of moles and resorptions indicate the number of prenatal deaths. These data were collected for each side of the reproductive tract but have been pooled in the interests of clarity.

6.3 Results and discussion.

Changes in body weight, estimated lean ~~mass~~ WEIGHT and the ratio dry weight to body weight are shown in Figure 6.1. Changes in the weight of fat deposits are shown in Figure 6.2, and changes in organ weights in Figure 6.3. Results from the diallele cross used to investigate components of fertility are shown in Table 6.1. One Lean female crossed with a Control male ~~released~~ CONTAINED thirteen corpora lutea of which twelve were resorbed and one was unaccounted for. This female was regarded as atypical and omitted from the analysis.

(i) Growth.

The formula used to estimate fat content from dry weight was derived from data on ten week old mice in Chapter 3. This may result in slight biases at earlier ages when chemical maturity may not have been attained, but will still be appropriate when considering the relative differences between the lines. The lines show divergence in body weight and the ratio of dry weight to body weight at all ages whereas predicted lean ~~mass~~ WEIGHT appears similar in both lines. The slight tendency towards a lower estimated lean ~~mass~~ WEIGHT in the Fat line may be explained as follows: mice selected on water content may respond by means other than changes in fat content, alteration in bladder size being one bizarre but plausible response. This will result in an overestimated fat content (and hence underestimated lean ~~mass~~ WEIGHT) in the Fat line, and *vice versa* in the Lean line. Given the small relative differences in estimated lean ~~mass~~ WEIGHT, the fact that this is reversed at age 26 weeks, and the possible bias in its estimation, it seems

Figure 6.1. Body weight (g), predicted lean ~~mass~~ WEIGHT (g), and the ratio of dry weight to body weight against age in F6 lines.

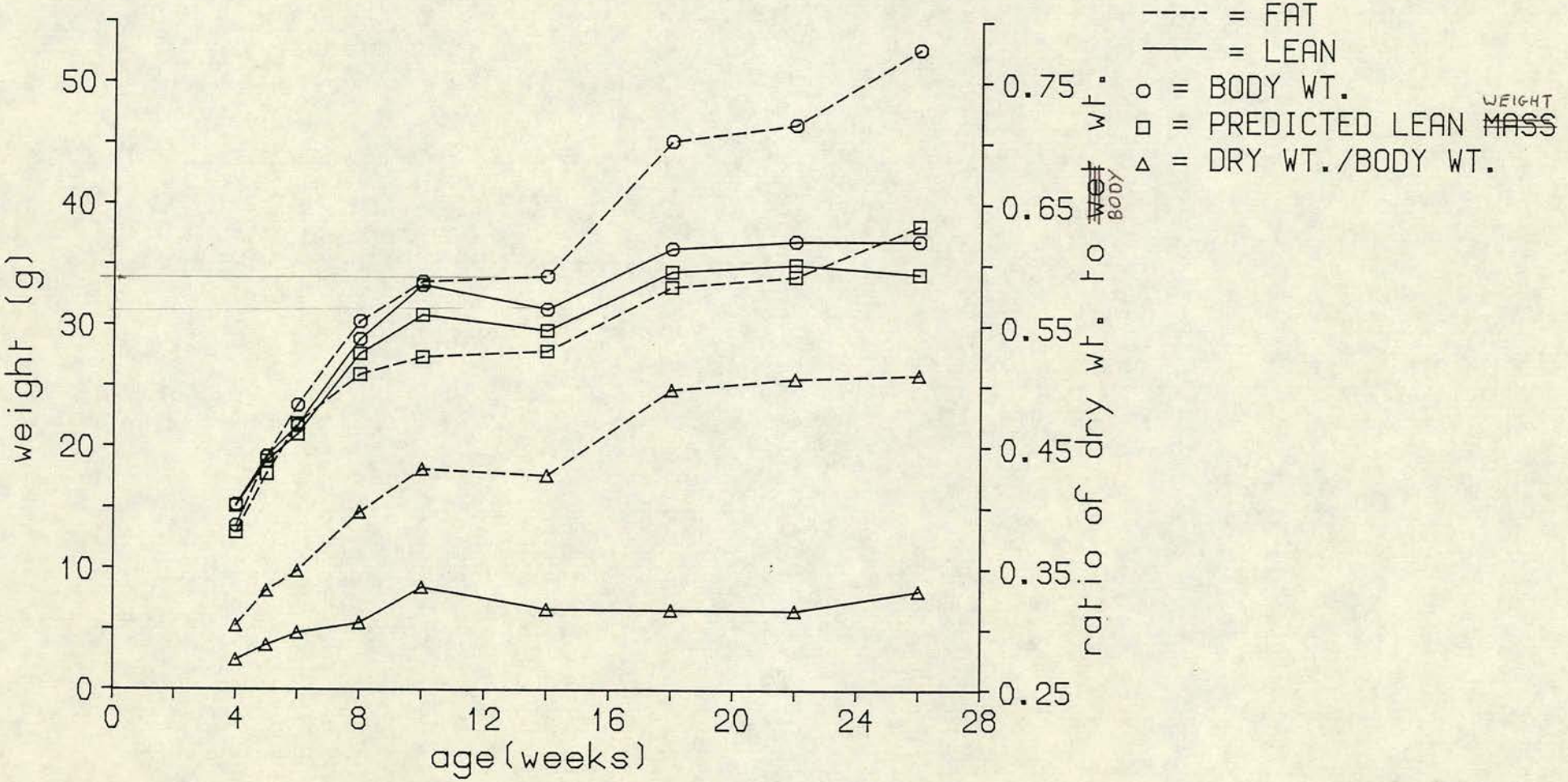


Figure 6.2. Weight (g) of gonadal fatpad (GFP), hindleg fatpad (HFP) and shoulder fatpad (SFP) against age in F6 lines.

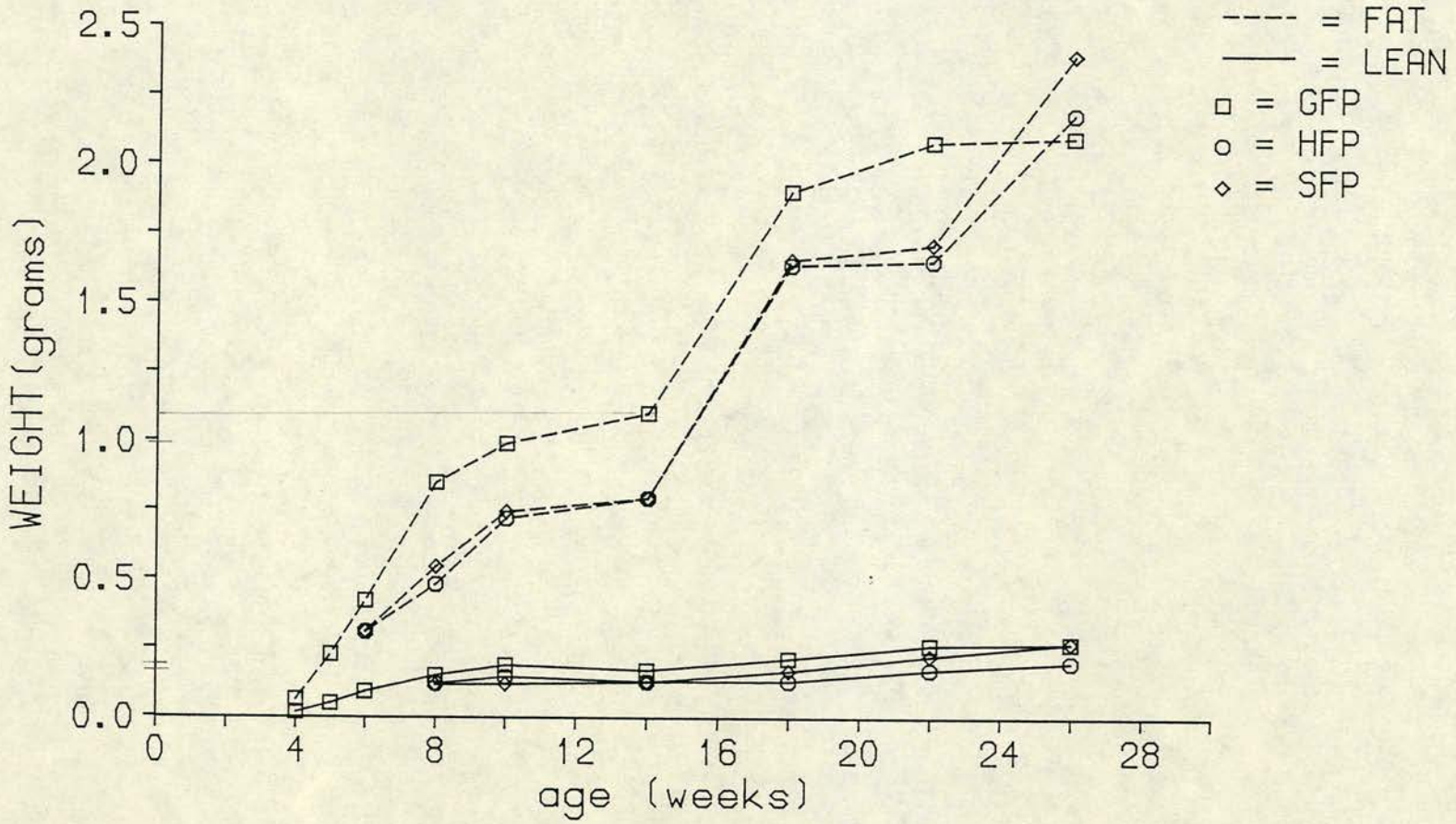


Figure 6.3. Weight (g) of testes, kidney, liver, lung, heart and spleen against age in F6 lines (testes scaled x4, liver by x0.4).

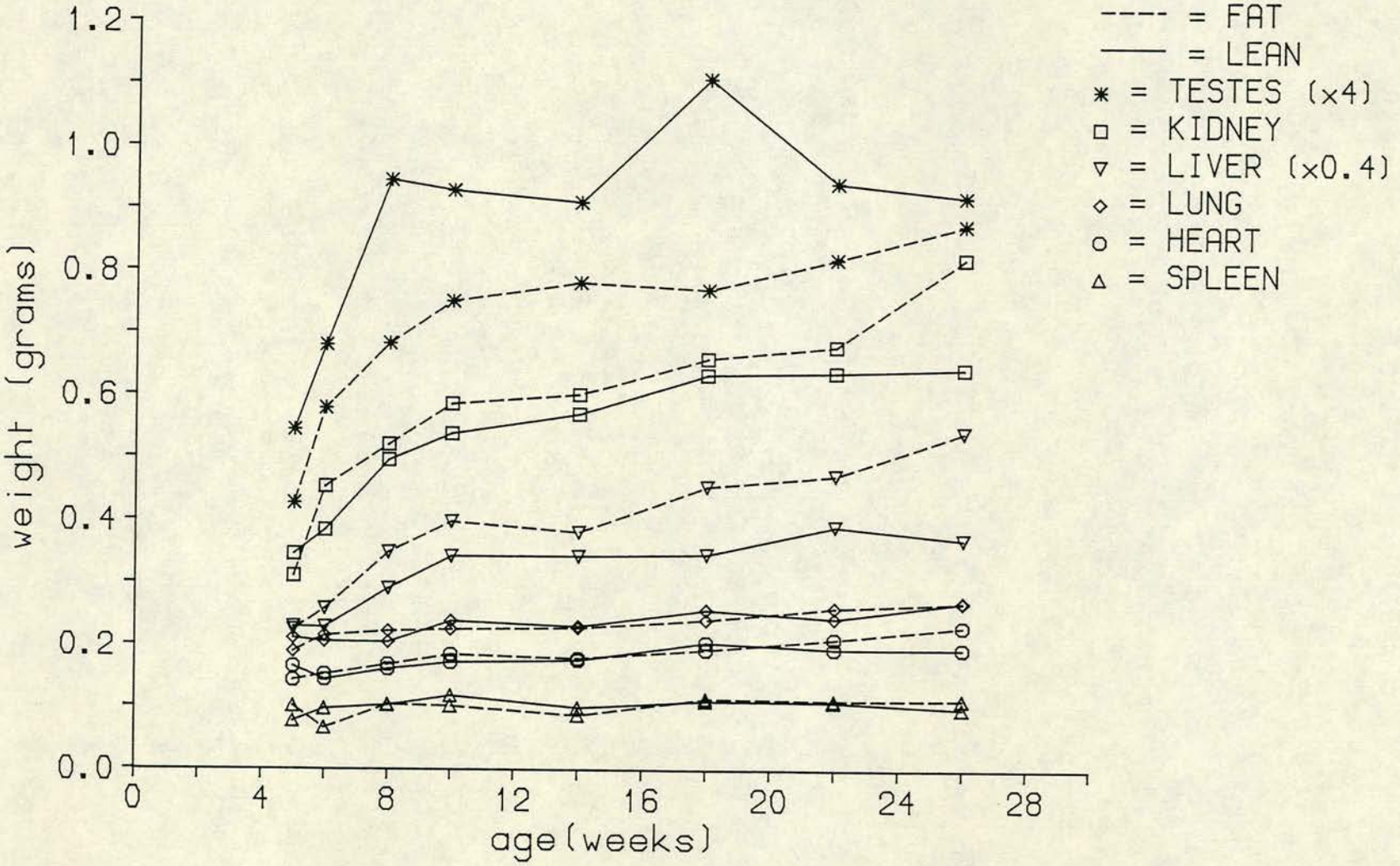


Table 6.1. Fertility traits in the diallele cross of Fat, Control and Lean lines. Numbers of families and mean numbers of corpora lutea, moles, readsorptions, embryos alive at day 17 and prenatal survival from corpus lutea to live embryo at day 17, and standard error (s.e.) of cell means. Heterosis is defined as the mean of the cross-bred cells minus that of the pure-bred cells.

	Sire					
Dam	Fat	Control	Lean		s.e.	Heterosis
	No. of families			Total		
Fat	6	6	5	17	-	-
Control	6	6	6	18		
Lean	6	5	5	16		
Total	18	17	16	51		
	Corpora Lutea			Mean		
Fat	12.00	10.67	11.20	11.29	0.777	-0.46
Control	10.00	10.00	10.83	10.28		
Lean	11.50	10.60	11.80	11.30		
Mean	11.17	10.42	11.28	11.00		
	Moles					
Fat	0.17	0.17	0.60	0.31	0.32	-0.68
Control	0.33	1.00	0.00	0.44		
Lean	0.33	0.80	2.00	1.04		
Mean	0.28	0.66	0.87	0.58		
	Resorbtions					
Fat	0	0	0	0	0.12	-0.04
Control	0	0	0.17	0.06		
Lean	0	0.40	0.40	0.27		
Mean	0	0.13	0.19	0.32		
	Alive at day 17					
Fat	10.17	10.33	10.20	10.23	0.89	0.83
Control	9.50	8.50	10.50	9.50		
Lean	11.00	8.80	9.00	9.60		
Mean	10.22	9.21	9.90	9.61		
	Prenatal survival					
Fat	0.84	0.97	0.92	0.91	0.05	0.12
Control	0.95	0.85	0.97	0.92		
Lean	0.96	0.83	0.76	0.85		
Mean	0.92	0.88	0.88	0.88		

reasonable to conclude that there is not a large difference between the two lines. The growth of lean ~~mass~~ WEIGHT with age appears similar in both lines.

Murine growth typically exhibits increases in body weight, lean ~~mass~~ WEIGHT and fat content up to maturity at about 10 weeks of age. These characteristics are noted in the Lean lines. In the Fat lines, lean growth appears typical but body weight and the ratio dry weight to body weight continue to increase after age 10 weeks. The simplest explanation for these characteristics is the continued deposition of fat on an near-constant "mature" lean ~~mass~~ WEIGHT as suggested by Figures 6.1 and 6.2. This accords with previous studies on carcass composition in generations 7, 14 and 20 (Sharp *et al.* (1984), Bishop and Hill (1985), Chapter 3 of this thesis), indicating that the differences between the lines at 10 weeks was almost entirely attributable to differential deposition of fat on a similar lean ~~mass~~ WEIGHT. The data also fits that of Asante (1989) who observed significantly higher rates of lipogenesis in the liver and gonadal fatpads from the High F6 line compared to the Low at ages 5 and 10 weeks.

The lines show large differences in growth rates of fat deposits. Deposition was more rapid in the Fat line and continued over the whole study period (26 weeks) whereas in the Lean line it had effectively ceased by age 8 weeks. The relative growth of the three fat deposits appear unchanged, an interesting result as the lines were selected on the ratio of gonadal fatpad to body weight during the first 20 generations of selection and analysis of the original lines at generations 20 and 29 (Chapters 3 and 4) had suggested that the proportion of total fat contained within the gonadal fatpad had been altered. The data presented here shows that growth in the three fat pads are similar which suggests that their growth is regulated by the same set of genes, distinct from those which regulate growth in other sites such as subcutaneous deposits.

Growth of lung, spleen and heart are similar in both lines. The increased weight of liver and kidneys in the Fat line may be a result of increased food intake since these organs represent important sites of metabolism and waste excretion. Liver is also a major site of lipogenesis in mice (22%, Holland and Cawthorne, 1981) so indirect selection on its weight cannot be discounted. One unexpected result is that mice from the Fat lines appear to have smaller testes. One explanation may be that mice selected for high fat content may have been indirectly selected to repartition energy from reproductive effort to deposition of fat. The results of the fertility study carried out under these conditions, see later, failed to reveal any decrease in fertility associated with males from the Fat line.

An interesting result was the decrease in body weight, lean ~~mass~~ WEIGHT and ratio of dry weight to body weight observed at age 14 weeks in both lines (Figure 6.1). There also appeared to be a decrease in the growth of the fat deposits at this age (Figure 6.2). This was presumably an environmental effect since the mice examined at this age had been mated over the previous 3 weeks while kept in male/female pairs whereas those examined at other ages had been kept in stock cages each of which contained six males. These changes in growth may therefore be a result of stress or increased reproductive effort over the mating period.

(ii) Reproduction.

There appeared to be no significant difference between lines in the number of corpora lutea observed on the ovaries (Table 6.1). Males and females from the Lean line are associated with the largest number of moles and resorptions and consequently have the lowest prenatal survival rate. In contrast, males and females from the Fat line are associated with the lowest numbers of moles and resorbtions and therefore have the highest prenatal survival rate. This suggested that the slight differences observed in litter size were due to differential survival of eggs and zygotes. The lower testes size in the Fat mice had no apparent effect on their fertility.

The results are similar to those reported for the replicated F lines at generation 10 (Brien et al, 1984). They obtained values for the Fat, Control and Lean lines of 13.4, 13.5 and 13.4 respectively for corpora lutea, 11.0, 10.8 and 10.4 respectively for numbers alive in the primiparous litter and 0.83, 0.80 and 0.79 respectively for prenatal survival rate. Similar results were noted in the same lines at generation 13 (Brien and Hill, 1986). The slight differences in the values between lines can be explained by the effects of drift within the replicates. Eisen (1987b) selected on carcass fat content using two different criteria: one was the ratio of gonadal fatpad weight to body weight (as used during the first twenty generations of selection on these lines), the other was the ratio of hind carcass weight to body weight. He reported a similar small decrease in litter size in the lean line but did not attempt to identify the underlying reasons.

Land (1973) suggested that testis size may be an indirect indicator of ovulation rate as the same range of hormones are involved in gonad development in both sexes. Islam, Land and Hill (1976) divergently selected replicated mouse lines for 5 generations on tesis weight at age 11 weeks. They obtained a divergence of 60% of the original mean weight and reported that females from the "High

testes" line released approximately two more eggs than females from the Low line; no divergence in litter size occurred which was presumably a result of increased pre-natal mortality in the High lines. Marks (1988) studied similar lines of mice, in this case divergently selected on testis weight at age 5 weeks; after 11 generations the divergence was 65% of the original mean weight. She reported a positive correlated divergence of 20% in mean litter size and attributed this to a changed ovulation rate. The F lines studied in this chapter therefore appear atypical in not having a change in ovulation rate associated with their divergence in testis weight.

Heterosis was defined as the mean crossbred value minus the mean purebred value. Its effects are small despite inbreeding coefficients of 0.19 in each line. The lack of heterosis is not unexpected since fertility are chiefly determined by the dam, and the effects of heterosis become more apparent in later generations when the dams are heterozygous (Roberts, 1960; Bhuvanakumar *et al*, 1985b). Small differences in survival of zygotes may have occurred as a result of heterosis since the number of moles and resorptions is smaller in crossbred families and prenatal survival higher. The differences in litter size are therefore probably a result both of maternal effects (mothers selected for low fat have increased zygote mortality) and of the zygotes own genotype (hybrid zygotes have the highest zygote survival).

6.4 Conclusions.

The Fat and Lean F6 lines appear to have a similar underlying lean ~~mass~~ WEIGHT upon which the Fat line accumulates fat at a faster rate. This accumulation continued unabated in the Fat lines up to age 26 weeks (the maximum age studied) but had effectively ceased by age 8 weeks in the Lean. Testis size in the Fat line was consistently lower than in the Lean but had no apparent effect on fertility. On the contrary, the Fat line was slightly more fertile, apparently due to a higher prenatal survival rate.

CHAPTER 7

THE ACTIVITIES OF SIX ENZYMES INVOLVED IN DE NOVO LIPOGENESIS IN LINES OF MICE DIVERGENTLY SELECTED FOR CARCASS FAT CONTENT.

7.1. Introduction

The lines of mice divergently selected for fat content differ by factors of between two and three in estimated fat content with negligible changes in lean ~~mass~~ WEIGHT (Chapters 3 and 6), and are known to differ in their rates of lipogenesis (Asante, 1989). The fat synthesis pathway is a short, well characterised pathway with the advantage for analysis of having a well defined end point (Figure 7.1). Its biochemistry and physiology has been studied in depth, partly due to its commercial importance in animal production and partly due to its implications in human health. This pathway is therefore a suitable model for studying the type and magnitude of enzymatic changes associated with the changed phenotype.

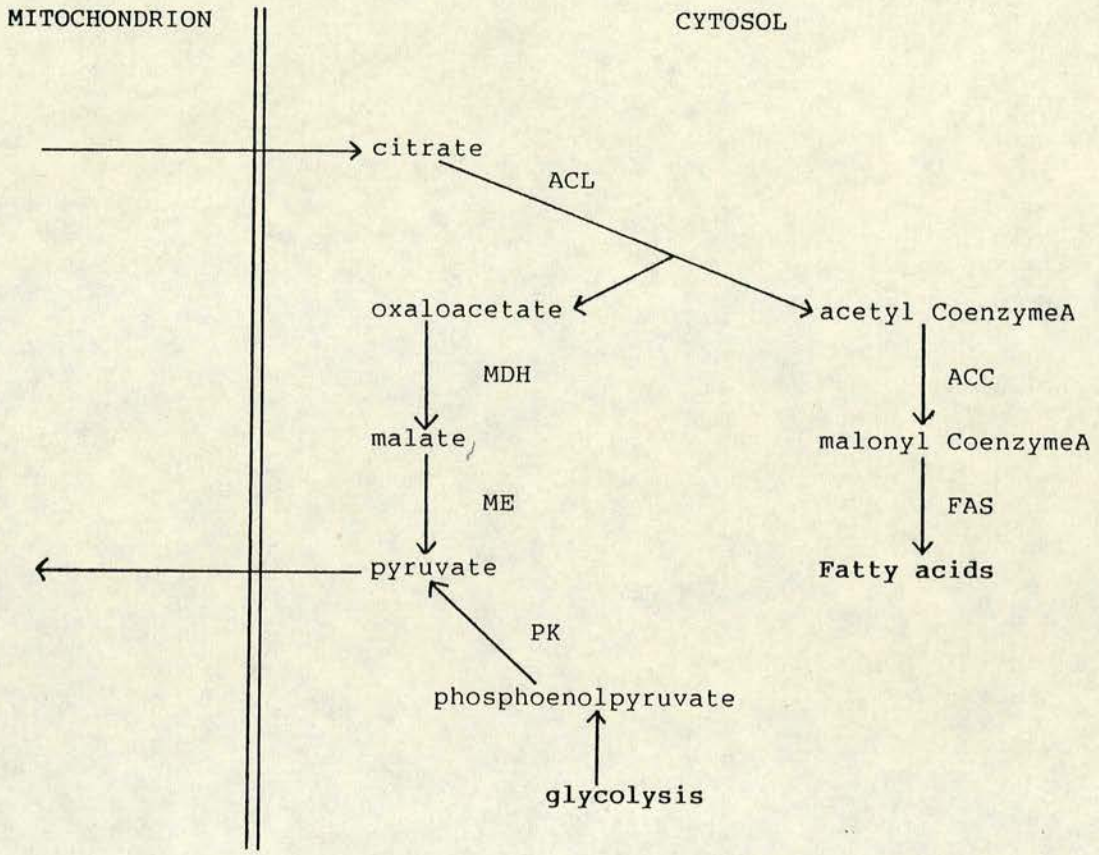
This study determined the activities (V_{max}) *in vitro* of the following enzymes involved in *de novo* lipogenesis: ATP-citrate lyase (ACL; EC 4.1.3.8), acetyl coenzymeA carboxylase (ACC; EC 6.4.1.2) , fatty acid synthetase (FAS), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40) and pyruvate kinase (PK; EC 2.7.1.40). The pathway contains enzymes exhibiting a range of functional features typical of metabolic enzymes such as hormonal regulation of activity (ACC), non-hormonally regulated enzymes (FAS), and isozyme variation between tissues (PK, which is hormonally regulated in liver but not adipose tissue). It was hoped to identify some of the metabolic changes which contribute to the changed phenotype.

7.2. Materials and Methods

(i) *Mouse stocks and preparation of samples*

The following brief resume is derived from Chapters 2, 3, and 4. Three replicates of mice (named F1, F2 and F3) were divergently selected for 20 generations on the basis of fat content in 10 week old males, estimated by the ratio of gonadal fatpad weight to body weight. Unselected Control lines were maintained giving nine lines in all, three each of Fat, Lean and Control. This resulted in an average 2.5 fold difference in fat content at age 10 weeks (Fat=16.0%, Control=10.5%, Lean=6.4%). Eleven generations of relaxed selection

Figure 7.1. Schematic diagram of *de novo* fat synthesis.



Key: ACL, ATP-citrate lyase; ACC, acetyl coenzymeA carboxylase, FAS, fatty acid synthetase; MDH, malate dehydrogenase; ME, malic enzyme; PK, pyruvate kinase.

These enzymes may be regulated or expressed differently in the two tissues under examination i.e. liver and gonadal fatpad (GFP), and fat synthesis may be more accurately portrayed as occuring within two compartments linked by an arrow representing triglyceride transport from the liver to the GFP. Since GFP weight formed part of the selection index, it may be expected that selection would favour a shift in synthesising resources from liver to GFP tissue. The proportion of total fat contained in the GFP has been showed to change (Chapter 3) and, as we shall see, the enzyme profiles of the two tissues have also been differentially altered.

occurred prior to this experiment but the lines continued to show divergence in fatpad weight (Tables 4.2 and 7.1). Carcass fat content estimated by the phenotypic correlations with water content obtained in Chapter 3 were 13.7%, 11.5% and 6.9% in the Fat, Control and Lean lines respectively. The F6 lines were a product of crossing these replicates at generation 20 to produce the F6 Fat line (a product of the three Fat replicates) and the F6 Lean line (a product of the three Lean replicates). These crosses restored much of the genetic variability lost through random drift over the initial 20 generations but were unreplicated and no control line was established. At the time of this experiment they had undergone a further 8 generations of selection on dry matter content in 14 week old males resulting in a 3.9 fold divergence in the ratio of gonadal fatpad weight to body weight at 10 weeks of age (Table 7.1), corresponding to estimated fat contents of 17.2% and 7.3%. The data in Table 7.1 were obtained during removal of liver and fatpad tissue for use in subsequent biochemical assays and hence describe the experimental population.

The mice were maintained *ad libitum* on Beta Diets' Rat and Mouse Diet No. 1. The lines are known to have diverged in food intake (Bishop and Hill, 1985) but were not artificially restricted to the same intake as the primary concern was to understand how the additional intake in the Fat lines is partitioned into fat in preference to lean tissue (as happens in the analogous set of P lines derived from the same base population).

Male mice were used as they are less prone to variation caused by steroid hormones and selection on fat content was applied only on this sex. Two ages were used, 5 weeks which represents the time of high growth and 10 weeks which represents the onset of maturity and was the age of selection in the original F1,2,3 lines. The mice were killed by cervical dislocation between 10 and 11 a.m. to reduce any circadian variation in enzyme activity. Families of Fat, Lean and Control were killed alternatively for the same reason. Body weight was measured and the liver and gonadal fatpads immediately removed , weighed and placed in ice-cold homogenisation buffer in the ratio 1 gram of tissue to 3 ml buffer. This was homogenised and stored on ice. The samples were centrifuged at 17,000G for 40 minutes at 4°C, the supernatant aliquoted into Eppendorf tubes and stored at -70°C until use; preliminary studies of tissue extracts from both high and low lines had indicated that the enzyme activities were stable at this temperature. The homogenisation medium consisted of 0.25M sucrose, 0.05M TRIS/HCl pH7.4 , 1mM EDTA and 1mM dithiothreitol. Liver and adipose tissue were taken as they represent major sources of lipogenesis in the normal mouse (22% and 7% respectively, Holland and Cawthorn, 1981). Gonadal fat pad was

Table 7.1. Body weights (BW), liver weights (LW), gonadal fatpad weights and ratio of gonadal fatpad weight to body weight of mouse lines used to determine the activities of six lipogenic enzymes.

	BW (g)	LW (g)	GFPW (g)	GFPW/BW	BW (g)	LW (g)	GFPW (g)	GFPW/BW
	F6 lines				Mean F1, F2, F3 replicate lines			
				5 weeks				
Fat	24.8	1.54	0.336	13.2	21.5	1.36	0.185	8.3
Control	-	-	-	-	21.9	1.45	0.152	6.7
Lean	24.7	1.47	0.154	6.1	21.3	1.33	0.096	4.4
				10 weeks				
Fat	36.0	1.99	1.10	30.5	33.7	1.86	0.833	24.3
Control	-	-	-	-	32.0	1.77	0.487	15.1
Lean	35.6	2.09	0.28	7.8	30.6	1.72	0.220	7.2

the chosen source of adipose tissue because it is easily removed and was used to estimate total fat content during the first 20 generations of selection.

In the F6 lines 40 males from each direction were chosen representing 7 families from the Fat line and 8 from the Lean. Half of each family was sacrificed at 5 weeks and the other half at 10 weeks of age. There was insufficient volume of fatpad tissue in several mice, particularly at age 5 weeks to enable all the subsequent assays to be performed. In these cases gonadal fatpads were pooled with others from the same line, wherever possible with members of the same family. This resulted in 20 samples for 10 week Fat, 12 for 10 week Lean, 15 for 5 week Fat and 8 for 5 week Lean. The samples were assigned arbitrary numbers and all the subsequent storage and assays were performed blind.

In the F1,2,3 lines, 5 samples were prepared from each replicate giving 45 in total. Each sample contained pooled material from between two and five mice, no more closely related than cousins, depending on numbers available. The same number being pooled for each of the five samples within each line. Where possible sibs were balanced so that half were killed at age 5 weeks and the remainder at 10 weeks. These were sorted into cages of five individuals, one individual from each cage going to each pool. The samples were therefore balanced for family effects, cage effects and age of sacrifice.

(ii) *Biochemical methods*

Activities corresponding to each age (5 or 10 weeks) were determined on separate days using freshly made reagents. The samples from the F1,2,3 lines were collected and assayed several months after the initial F6 assays so some variation should be expected between assays. Each sample was measured three times, with the exception of FAS which was measured twice. Where necessary, dilutions of samples were made in standard homogenisation buffer. ACL was assayed by the production of oxaloacetate from citrate (Linn and Srere, 1979), FAS by the production of NADP from NADPH (Carey and Dils, 1970), MDH by the production of NAD from NADH (Mosbach and Mattiasson,1976), ME by the production of NADPH from NADP (Wise and Ball, 1964) and PK by the production of pyruvate from phosphoenolpyruvate (Fitton, 1989). ACC was assayed by the incorporation of $^{14}CO_3$ into malonyl CoenzymeA (Browsey *et al*, 1979). Determination of soluble protein in the homogenate was by a bicinchonic acid assay kit supplied by Pierce Chemical Company, Illinois.

(iii) *Statistical analysis*

In the F6 lines a simple t-test using between sample error was used. For the F1,2,3 lines analysis of variance was represented by the model

$$Y_{ijk} = \mu + D_i + R_j + L_{ij} + e_{ijk}$$

where Y_{ijk} is the observation on the k^{th} sample in the ij^{th} line of the j^{th} replicate in the i^{th} direction; μ is the overall mean; D_i is the effect of direction of selection (H,L,C); R_j is the effect of replicate (1,2,3 equivalent to contemporaneous effects of animal husbandry, batch of homogenisation medium used, time stored at -70°C, etc.); L_{ij} is the effect of individual lines (1-9, attributable to drift); e_{ijk} is residual error . In practice the effect of replicate was negligible and was omitted to gain an increase in degrees of freedom. The term D_i, representing the direction of selection, can be partitioned into two effects each with one degree of freedom: (i) due to divergence between the Fat and Lean lines and (ii) due to the asymmetry of divergence compared to the control lines. The effect of direction of selection and its two subdivisions were tested against L_{ij} to take account of drift variation between lines.

Within line correlations between enzyme activities were obtained from the same model or one omitting R_j and L_{ij} for the F6 lines. These models eliminate differences due to direction of selection or line drift and the activities are compared on the basis of their deviation from the line mean. In the F6 line gonadal fatpad tissue from several individuals was pooled so was tested for correlation with liver activities by comparing with the mean liver activity of those animals contributing to the pool. A methodological factor should be noted when considering these within-line correlations: errors in estimating the soluble protein content of a sample will cause systematic errors when calculating activities per mg protein and false positive correlations will occur. This potential error cannot be avoided since expression of activity per sample volume will result in the same dangers, in this case due to the much larger errors involved in measuring weights and volumes when excising and homogenising the tissues and variance attributable to random factors such as blood content of the liver caused by damage to the hepatic portal vein.

7.3 Results.

Mean line activities (V_{max}) per mg soluble protein in the F6 lines are given in Table 7.2, and those of the individual replicates of the F1,2,3 lines in Tables 7.3 and 7.4. The significance of differences in the F6 lines are shown on Table 7.2. Appropriate parameters extracted from the analysis of variance of F1, F2 and F3 given in table 7.5 which gives the significance of divergence, asymmetry and line

Table 7.2. Enzyme activities (nmol/min/mg soluble protein) in F6 lines, residual standard deviations (SD), standard error (SE) of difference estimated from residual SD, and significance (SIG) of the difference.

Tissue	Liver						Gonadal fat pad					
Enzyme	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
Line					5 weeks							
Fat	68.9	344	18.8	13180	148	1980	62.8	2250	84.8	5910	612	1380
Lean	45.4	307	15.5	13270	114	1790	17.8	948	33.7	5400	174	998
SD	16.2	71	3.6	2748	34.0	439	17.8	525	15.8	1565	118	312
SE	5.1	22	1.1	869	10.7	139	7.8	230	6.9	685	51.6	137
SIG	***	ns	**	ns	**	ns	***	***	***	ns	***	*
					10 weeks							
Fat	51.8	344	13.4	7530	154	1980	50.1	631	33.6	3250	154	615
Lean	49.5	355	12.9	7390	137	1930	32.8	416	23.0	4010	98	722
SD	12.9	83	2.0	567	49.2	489	17.4	226	7.6	440	43.1	184
SE	4.07	26.2	1.58	179	15.6	155	6.4	82.5	2.8	161	15.8	67.2
SIG	ns	ns	ns	ns	ns	ns	*	*	***	***	**	ns

ns, no significant difference
* P < .05; ** P < .01; *** P < .001

Table 7.3. Enzyme activities (nmol/min/mg soluble protein) at age 5 weeks for each replicate (F1, F2, F3) and their means, residual standard deviations (SD), and standard error (SE) of the difference between means estimated from the between replicate mean square.

Tissue	Liver						Gonadal fat pad					
Enzyme	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
Rep.						Fat lines						
1	46.0	609	15.6	3750	124	1142	174	1660	49.4	4980	505	751
2	43.1	544	14.7	3530	103	1095	161	2360	45.0	4870	590	666
3	41.3	487	14.9	3920	129	1390	176	1560	55.8	4990	657	591
$\bar{X}$	43.5	547	15.0	3740	119	1209	170	1860	50.0	4950	584	670
						Control lines						
1	41.2	433	15.6	3840	124	1223	136	2280	43.6	4770	554	649
2	49.5	599	16.0	3470	112	1050	142	2500	50.3	4660	525	557
3	48.9	531	13.7	3450	111	1062	147	1470	47.2	5510	602	581
$\bar{X}$	46.5	521	15.1	3590	116	1112	142	2080	47.0	4980	560	596
						Lean lines						
1	35.6	457	12.2	3770	95	1381	128	1310	42.4	4460	381	619
2	31.3	387	13.1	3610	80	1176	117	1570	38.0	4540	418	671
3	45.3	497	15.0	3660	132	1353	128	1960	38.6	4610	445	672
$\bar{X}$	37.4	447	13.4	3680	102	1304	124	1614	39.7	4540	415	654
SD	5.4	68	1.90	287	15.1	153	20.0	460	5.74	605	71	141
SE	4.16	55.4	0.89	145	14.5	101.9	5.6	362	3.22	207	43.1	46.4

Table 7.4. Enzyme activities (nmol/min/mg soluble protein) at age 10 weeks for each replicate (F1, F2, F3); otherwise as Table 7.3

Tissue	Liver						Gonadal fat pad					
Enzyme	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
Rep.						Fat lines						
1	42.1	392	8.90	5060	133.7	366	42.3	975	33.6	3390	178	94
2	49.8	480	12.17	4910	151.9	196	36.9	970	30.9	3330	206	92
3	32.3	289	8.48	5080	109.3	315	34.2	1044	31.4	3510	193	96
$\bar{X}$	41.4	387	9.85	5020	131.6	293	37.8	996	32.0	3410	192	94
						Control lines						
1	29.8	297	8.77	5210	74.1	285	27.6	679	24.0	3980	134	90
2	33.1	330	8.89	4870	89.0	203	24.5	709	23.0	4030	141	121
3	39.1	323	7.63	4820	86.4	177	49.1	1056	35.9	4270	240	119
$\bar{X}$	34.0	316	8.43	4970	83.2	221	33.7	814	27.6	4090	172	110
						Lean lines						
1	29.5	297	8.23	5150	89.3	277	35.5	606	25.1	4320	167	116
2	35.6	350	9.41	4640	95.9	263	24.6	439	20.4	4180	122	137
3	37.8	325	8.09	4750	100.2	289	32.6	584	23.1	4080	142	125
$\bar{X}$	34.4	324	8.57	4850	95.1	276	30.9	531	22.9	4190	144	126
SD	3.6	34	1.73	308	15.1	60	11.0	267	5.2	296	35	45
SE	5.1	47.5	1.03	166	11.1	49.3	7.1	108.4	3.61	101	30.6	9.7

Table 7.5. Extract from the analysis of variance of enzyme activities of replicates F1,2,3. Ratios of mean squares of direction (with 1 d.f.) (H-L), asymmetry (1 d.f.) and individual lines (6 d.f.) to residual mean square (36 d.f.). Direction and asymmetry tested against lines; lines tested against remainder.

Tissue	Liver						Gonadal fat pad					
Enzyme	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
					5 weeks							
H-L	9.57	16.29	4.75	2.00	8.69	2.85	39.83***	2.24	24.38*	3.45	42.30***	0.09
Asym	12.88	1.26	1.85	0.03	1.27	8.87	0.79	5.68	1.39	1.00	7.40	2.22
Lines	4.48**	4.98***	1.70	1.92	6.97***	3.31**	0.58	4.66**	2.36*	0.88	2.76*	0.82
					10 week							
H-L	29.15	26.02	4.07	2.32	43.78*	0.56	2.97	22.83**	23.07*	56.66***	14.30	3.76*
Asym	11.42	13.45	2.04	0.14	39.97*	11.08	0.03	0.36	0.01	10.40*	0.09	0.00
Lines	14.87***	14.89***	3.38**	2.18	4.02**	5.09***	3.16*	1.24	3.61**	0.94	5.70***	0.35

(drift) effects. The ratio of activities are summarized on Table 7.6.

Some differences are apparent in activities between the F1,2,3 and F6 assays i.e. at age 5 weeks adipose ACL is higher in F1,2,3 than in F6 by a factor of between three and eight , liver MDH is higher in F6 than in F1,2,3 by a factor of four and fatpad PK is higher in F6 than in F1,2,3 by about 75% (Tables 7.2 *vs.* Table 7.3). Similarly at age 10 weeks liver and fatpad PK is higher in the F6 than F1,2,3 by a factor of 6 to 7 (Table 7.2 *vs.* Table 7.4). Except in the case of the 10 week PK assays these differences cannot be attributed to variation in the assays since adipose and liver samples were run concurrently using the same assay reagents. In effect each tissue was a control for the other and any changes in assay sensitivity would be reflected in both tissues. The differences are therefore probably due to genetic factors such as drift, (unlikely to be of this magnitude) or environmental fluctuations such as food batch differences which may alter energy intake. Changes in dietary intake or composition are known to cause altered activities in these enzymes (Grigor & Gain; 1983) and may have occurred here. For these reasons the relative differences between lines are more informative and are given in Table 7.6.

The activities of ACL, ACC, FAS and ME were significantly higher in the Fat than the Lean lines, and the differences were more pronounced at the earlier age and in the gonadal fatpad, where activities in the F6 fat line were higher by factors of 3.5, 2.4, 2.5 and 3.5 respectively (Table 7.6). The activity of PK was unchanged in each tissue. MDH activity was significantly lower in the Fat lines in gonadal fatpad tissue at age 10 weeks but not at age 5 weeks nor in liver tissue. There are significant effects of random genetic drift ("lines" component in Table 7.5), but little asymmetry of response. The activities of each enzyme were measured for each individual mouse or pool of fatpad tissue so within-line correlations could be calculated between enzyme activities and estimated fat content. There appeared to be no consistent correlation between the activity of any one enzyme and fat content (Tables 7.7 and 7.8).

7.4 Discussion

The largest and most consistent differences occur in the three enzymes which constitute the pathway of *de novo* synthesis of fatty acids, i.e. ACL, ACC and FAS. Among the three enzymes indirectly associated with fat synthesis, only ME exhibits the same degree of elevation, possibly due to its importance in generating NADPH, a substrate of FAS necessary for fatty acid synthesis. Levels of PK and liver MDH had not diverged which may indicate a lesser influence on

Table 7.6. Ratios of enzyme activities in Fat to Lean lines.

Age (wks)	Liver						Gonadal fat pad					
	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
						F6 lines						
5	1.52	1.12	1.21	0.99	1.29	1.11	3.53	2.37	2.52	1.09	3.51	1.38
10	1.05	0.97	1.04	1.02	1.13	1.03	1.53	1.52	1.46	0.81	1.57	0.85
						Mean F1, F2, F3 replicate lines						
5	1.16	1.22	1.12	1.02	1.16	0.93	1.37	1.16	1.26	1.10	1.41	1.02
10	1.21	1.19	1.15	1.04	1.38	1.06	1.22	1.88	1.40	0.81	1.33	0.75

The differences in enzyme profiles between the two tissues are presumably a result of the processes described in Figure 7.1 (page 77) and on page 90.

Table 7.7. Within line correlations (x100) between estimated %fat and enzyme activities (µmol/min/mg soluble protein) in liver (L) and gonadal fatpad (A) tissues of mice from the F6 lines at ages 5 weeks (above diagonal) and 10 weeks (below diagonal).

	%fat	ACL		ACC		FAS		MDH		ME		PK	
		L	A	L	A	L	A	L	A	L	A	L	A
%fat	-	15	25	-10	43	11	48	10	-16	25	51	13	09
ACL L	01	-	58	33	80	84	66	-12	-08	78	76	77	42
A	-07	-24	-	25	76	33	74	-36	07	37	79	17	63
ACC L	-38	61	-27	-	51	21	25	-24	05	01	35	44	42
A	-20	-23	85	-21	-	60	84	-32	00	59	90	40	72
FAS L	03	90	-20	63	-29	-	47	08	-15	86	54	72	18
A	-11	-25	57	-37	54	-19	-	-04	33	54	95	26	71
MDH L	-11	39	-07	23	04	37	-09	-	20	03	-14	07	-23
A	-19	27	41	16	45	15	33	14	-	-07	13	04	32
ME L	30	78	-40	40	-41	76	-46	26	-03	-	60	64	19
A	05	-25	92	-25	71	-15	58	-16	26	-37	-	33	72
PK L	46	53	-50	18	-57	49	-31	16	-19	67	-45	-	12
A	04	-10	28	-10	33	-06	39	-16	44	-17	18	-06	-

All liver/liver correlations have 38 d.f. hence p = 0.05 when r =±0.31, p = 0.01 when r =±0.41 and p = 0.001 when r =±0.50.

All 5 week correlations involving adipose tissue have 21 d.f. hence p = 0.05 when r =±0.42, p = 0.01 when r =±0.54 and p = 0.001 when r =±0.65.

All 10 week correlations involving adipose tissue have 30 d.f. hence p = 0.05 when r =±0.35, p = 0.01 when r =±0.45 and p = 0.001 when r =±0.55.

Table 7.8. Within line correlations (x100) between estimated %fat and enzyme activities (µmol/min/mg soluble protein) in liver (L) and gonadal fatpad (A) tissues of mice from the F1, F2 and F3 lines at ages 5 weeks (above diagonal) and 10 weeks (below diagonal).

	%fat	ACL		ACC		FAS		MDH		ME		PK	
		L	A	L	A	L	A	L	A	L	A	L	A
%fat	-	-10	-05	-01	-23	03	47	25	-43	05	-32	19	-02
ACL L	-26	-	23	44	10	65	10	02	19	57	36	29	02
A	-46	44	-	-14	54	02	34	-06	-06	03	77	-11	34
ACC L	-10	34	11	-	06	52	03	-01	-14	26	01	50	-08
A	-01	18	62	03	-	-23	20	-11	-19	-08	37	-14	-01
FAS L	-20	40	43	29	22	-	05	-03	31	45	15	28	03
A	-26	28	77	04	90	28	-	34	-30	06	16	16	-05
MDH L	-09	03	11	28	17	44	15	-	-13	-15	-03	23	15
A	-40	17	47	-01	13	10	28	-08	-	03	43	11	40
ME L	04	56	35	32	08	28	15	09	21	-	07	24	02
A	-29	47	88	13	72	39	80	18	42	41	-	09	47
PK L	32	07	-16	-02	-08	15	-18	18	-06	08	-10	-	02
A	-03	-03	20	-17	01	01	-07	-03	10	13	09	47	-

All correlations have 36 d.f. hence p = 0.05 when r = ±0.32, p = 0.01 when r = ±0.42 and p = 0.001 when r = ±0.52.

fat synthesis or possibly a greater degree of constraint imposed by their more diverse metabolic significance. A similar argument may explain why differences are more apparent in fatpad tissue. Liver has more diverse metabolic functions and may be constrained by the changes it can tolerate whereas the gonadal fatpads appear to have little metabolic significance beyond the synthesis, release and storage of fats so may not be under the same degree of constraint. The reason why MDH activity was lower in gonadal fatpad tissue from the Fat lines is unclear. One explanation is as follows: activities were expressed per mg soluble protein extracted from the tissue so if the concentration of ACL, ACC, FAS and ME had increased in the Fat lines (as suggested by the data), this would lower the specific activity of MDH.

Gonadal fatpad may not be representative of all adipose tissue since metabolic differences occur between different anatomical sites (Anderson, Kauffman and Kastenschmidt, 1972). It was used to estimate total fat in the selection index in the F1,2,3 lines so may have been selected for self enlargement in the Fat lines i.e. an increased capacity for lipogenesis with a decreased capacity for lipid release, or conversely decreased lipogenesis with increased mobilization in the Lean lines. Such changes make it less typical of adipose tissue in general.

A criticism sometimes levelled at attempts to relate changed physiological traits with altered phenotype is that such experiments frequently use unreplicated strains of animals (Eisen, 1989). The presence of replicates is a prerequisite for investigating correlated responses to selection as they reveal the extent of random genetic drift (Hill, 1980). The results obtained here suggest that random genetic drift had significant effects on enzyme activities but when incorporated into the analysis of variance did not alter the conclusion that significant changes in activity had occurred as a consequence of the type of selection used to establish the lines.

Asante (1989) measured the relative rates of lipogenesis *in vivo* in the same tissue, age and mouse lines, by the incorporation of tritiated water into lipids. Lipogenesis was elevated in the Fat lines relative to the Lean lines by factors of 1.14 and 2.15 at age 5 weeks, and factors of 1.22 and 1.45 at age 10 weeks for liver and fatpad respectively. These ratios are similar to those obtained in this study for the relative *in vitro* activities of lipogenic enzymes. Asante *et al.* (1989) measured the activities (V_{max}) of four NADPH-producing enzymes at ages 5 and 10 weeks in liver and gonadal fatpad tissue from the F1, F2 and F3 lines. The enzymes were glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase and malic enzyme (ME). The largest

differences were apparent in fatpad tissue and at age 5 weeks where activities were higher in the Fat lines by factors of 1.31, 1.11 1.27 and 2.25 respectively. The degree of divergence in ME activity is similar to that obtained in this study and is the only one of the four where the divergence in *in vitro* activity is similar to the divergence in *in vivo* lipogenesis. They also reported significant drift effects in the activities of these enzymes.

The enzyme activity changes reported in this study are similar to those associated with increased fat deposition in major mutations and a phenocopy strain of mice. Chang *et al* (1967) found elevated activities of hepatic ACC and FAS in obese ob/ob mice compared to their normal littermates. Bulfield (1972) found elevated levels of hepatic ACL, ME and PK in murine obesity caused by the ob gene, the db^{ad} (previously known as the Ad gene) or an aurothioglucose induced phenocopy; MDH activities were unaltered. Kaplan and Fried (1973) reported elevated activity of ME in adipose tissue of ob/ob mice. It appears that quantitative genetic selection , major gene mutations, and an environmentally-induced phenocopy have similar effects on the enzyme activities underlying the increased accumulation of fat.

Since basic metabolism is common to all animal species, it is informative to consider work on other species. Bannister *et al* (1984) investigated lines of domestic broilers divergently selected for plasma very-low-density lipoprotein levels which had resulted in a 34% difference in body fat content between birds from the high and low lines. They reported higher activities of hepatic ACL, FAS and ME in the high line while PK remained unchanged (ACC and MDH were not assayed). Basic metabolism is highly conserved so it may be that similar enzyme changes not only occur with major gene and polygenic phenotypic equivalents but may also be reflected across species.

A range of enzymes were elevated rather than just one critical "rate-limiting" enzyme, as expected from recent analyses which stress that each constituent enzyme has an effect or potential effect on the overall rate of any biochemical pathway (Kacser & Burns, 1981). In particular the hormone-regulated enzymes ACC and liver PK do not appear to have diverged to a greater amount than the others. The results presented here show that all enzymes directly involved in lipogenesis show similar divergence in activity irrespective of their temporal role in regulating lipogenesis; for instance, the activity of ACC is modulated by several hormones *in vivo* to regulate the rate of lipogenesis but is no more divergent in activity between the Fat and Lean lines than FAS which is not modulated. The view that attempts to improve livestock production by genetic

manipulation should concentrate on classical "rate-limiting" enzymes should therefore be regarded with some scepticism on the evidence reported here. Similarly evolutionary change may be expected to proceed via a series of enzyme activity changes rather than by a series of mutations at critical "rate-limiting" sites.

Theoretical aspects of the population genetics of alleles affecting enzyme activity in complex biochemical pathways will be considered in the next chapter.

CHAPTER 8

THE POPULATION GENETICS OF ALLELES AFFECTING ENZYME ACTIVITY

8.1 Introduction

The discovery of widespread polymorphisms at enzyme loci in 1966 has been followed by more than two decades of speculation on their evolutionary significance, in particular whether they constitute a source of genetic variance which can be utilized by natural selection. Enzymes are the main entities in the organism which inter-convert metabolites throughout the body and most higher levels of metabolic regulation produce their effects by altering the activities of enzymes; for example insulin stimulates lipogenesis partly by altering the activity of acetyl Coenzyme A carboxylase. Changes in enzyme activity appear to play a part in the response to artificial selection (see previous chapter), and if enzyme activity polymophisms can be shown to be subject to the effects of natural selection it seems reasonable to suppose that artificial selection may also utilize these polymorphisms

One of the more useful predictions of quantitative genetics is based on the assumption that deleterious alleles occur in any population at an equilibrium frequency determined by their rate of input (by mutation) and their rate of removal (by natural selection). Thus if any two of these parameters are known, the third can be inferred. This has enabled researchers to estimate the mutation rate at single "disease" loci in humans and also the mutation frequency of polygenic traits (Charlesworth, 1989) from the equilibrium frequencies of the deleterious (lethal) trait. The selection pressure acting against deleterious alleles at single loci can similarly be inferred when their equilibrium frequency and mutation rates are known.

This chapter will investigate deleterious mutations at loci affecting the activities of individual enzymes. In this situation there will be three potential genotypes present in the population: homozygous wild-type, heterozygous wild-type/deleterious, and homozygous deleterious. If their fitnesses can be ascertained and positioned on a "fitness curve" relating fitness to enzyme activity then in principal it should be possible to predict the fitness associated with any level of enzyme activity.

The "fitness curve" most frequently used to predict changes in fitness as a

consequence of changes in gene dosage or enzyme activity is shown on Figure 8.1. This type of curve was inferred from consideration of dominance indices by Wright (1934) and Gillespie (1976). Its biochemical basis was elucidated by Kacser and Burns (1973, 1981) from a consideration of the properties of individual enzymes embedded in biochemical pathways. When considering the changes in fitness caused by variation in the activity of individual enzymes, it is convenient to assume that fitness is proportional to flux through a biochemical pathway (Hartl, Dykhuizen and Dean, 1985; Dean, Dykhuizen and Hartl, 1988). All subsequent arguments in this chapter are based on this assumption and utilize the type of fitness curve shown of Figure 8.1. This type of curve may be approximated by the equation:

$$F = F_{max}\, a / (K_a + a) \qquad (8.1),$$

where F is fitness and a is the activity of the enzyme. *Fmax* and K_a are parameters of the enzyme system, determined by the joint activities of the other enzymes in the pathway, and have the following properties: *Fmax* is the maximal flux through the pathway as a tends to infinity, and K_a is the value of a at which half maximal flux occurs (Hartl *et al*., 1985; Torres *et al.* 1986).

8.2 The importance of "bypass" fluxes.

An important feature of Figure 8.1 is that the curve passes through the origin; this implies that a complete lack of activity in any enzyme is lethal. As we are assuming that fitness is proportional to flux, it also implies that flux through a pathway will be completely eliminated when the activity of any enzyme falls to zero. The question arises as to whether this is a realistic assumption for all enzymes. Figure 8.2 shows a pathway carrying a flux from metabolite X to metabolite Y through enzymes e_1 to e_6 and substrate pools s_1 to s_5. In this linear pathway it is inevitable that elimination of activity in any of these enzymes will completely eliminate the flux. However a feature of biochemical pathways is that "bypass fluxes" may be present and an example is shown in Figure 8.2 which is catalysed by enzymes b_1 to b_4 and converts s_2 to s_5. The bypass flux means that elimination of activity in enzymes e_3, e_4, or e_5 will not completely eliminate flux from *X* to *Y*, implying that in the case of these enzymes the curve shown on Figure 8.1 should not pass through the origin.

These *bypass fluxes* have been observed in *E. coli*. Fraenkel and Livisohn (1967) examined a strain lacking phosphoglucose isomerase activity: when grown with glucose as a sole nutrient it grew at approximately one third the rate of the normal strain. The bypass flux utilizing glucose apparently occurred chiefly

Figure 8.1 The effect on flux through a linear biochemical pathway due to changing the activity of one constituent enzyme (after Kacser and Burns, 1973).

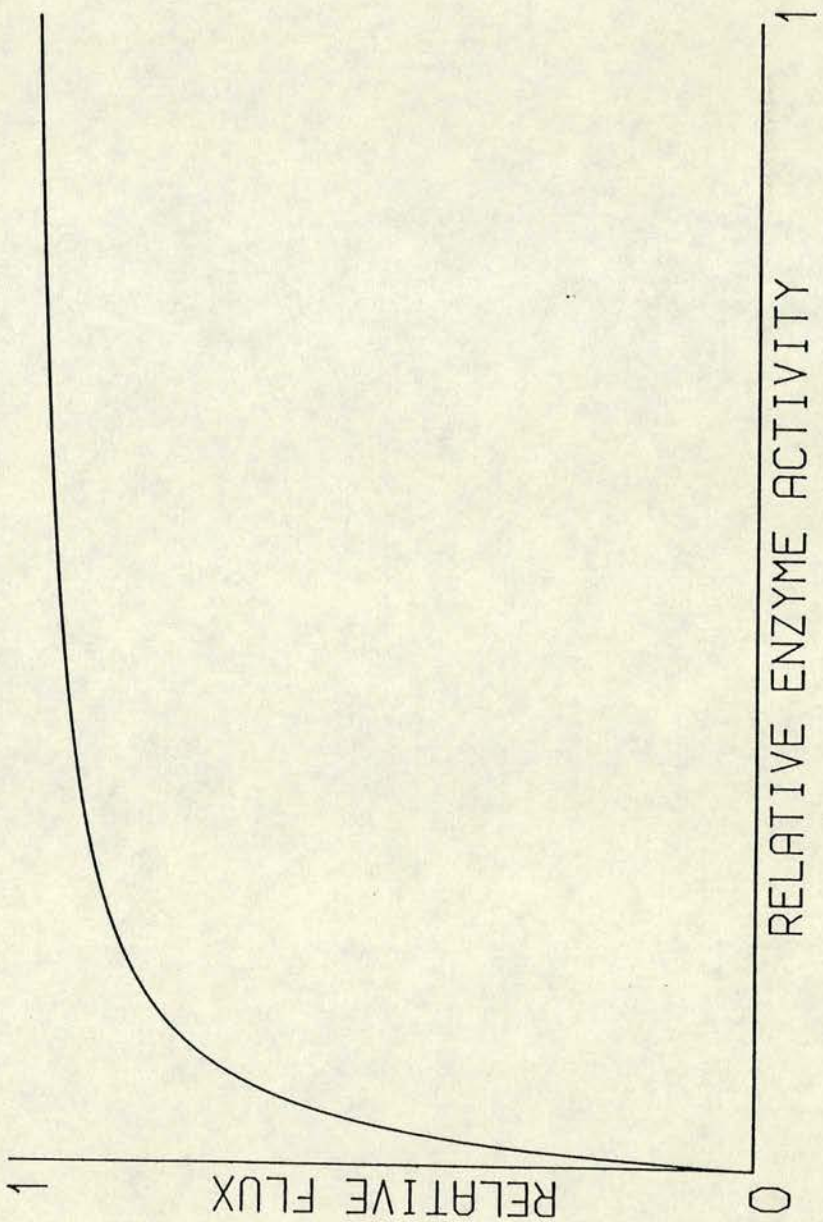
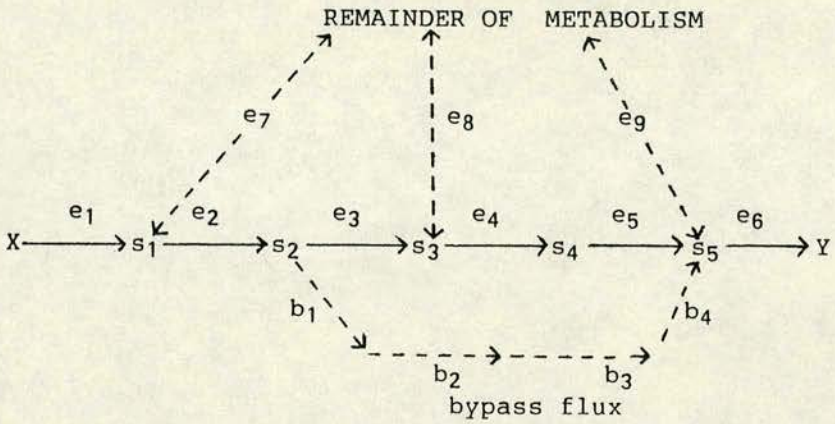


Figure 8.2. The pathway from X to Y via substrates s_1 to s_5 and constituting enzymes e_1 to e_6. A "bypass flux" is also shown and the complex nature of metabolic pathways is represented by additional enzymes e_7, e_8 and e_9 which utilize or produce substrates s_1, s_3 and s_5.



through the hexose monophosphate shunt and the Entner-Doudoroff pathway. Zablotny and Fraenkel (1967) similarly examined a strain lacking gluconate-6-phosphate dehydrase activity: when grown with gluconate as the limiting nutrient it also grew at around one third the rate of the normal strain, in this case the bypass flux occurred chiefly via the hexose monophosphate shunt. Dykhuizen and Hartl (1980) measured the growth rates of strains with natural occurring variants of 6-phosphogluconate dehydrogenase activity: differences only became apparent in the presence of a mutation removing phosphogluconate dehydratase activity which blocks an alternative metabolic pathway.

These particular bypass fluxes are well characterised as they appear to have a specific biochemical function. However less well defined bypass fluxes may also occur as a consequence of the branched nature of biochemical pathways. Linear pathways assume that each substrate is unique to a pathway i.e. that substrates s_1 to s_5 in Figure 8.2 are not involved in any other metabolic pathways. Metabolic maps and biochemical textbooks emphasize the branched nature of metabolism, in particular that many substrates are used or produced by more than one enzyme. These additional enzymes are represented in Figure 8.2 by reversible enzymes e_7, e_8 and e_9 which may produce or utilize substrates s_1, s_3 and s_5. In this context null enzyme activity no longer predicts a complete elimination of flux to Y. Taking e_2 as an example and assuming a linear pathway: null activity will result in the non-production of s_2 which in turn eliminates production of s_3, s_4, s_5 and ultimately Y, resulting in zero flux. In contrast, taking e_2 as embedded in the same pathway with the addition of enzymes e_7 to e_9, lack of activity does not completely eliminate flux. Null activity in e_2 will result in the non-production of s_2 but this does not result in a complete loss of s_3 since a substrate pool of s_3 will be maintained by enzyme e_8. Some s_4 will still be produced which in turn is converted to s_5 (further supplemented by e_9), which in turn is converted to the product Y. In this example, a bypass flux occurs despite the lack of activity in e_2. Similar bypass fluxes to Y occur despite null activities in any of the enzymes e_1 to e_5; e_6 is the only enzyme in the pathway which eliminates production of Y by an absence of activity, although e_1 may do so if X is the sole source of substrate entering the metabolic pathways (such as occurs in many chemostat experiments where a "limiting nutrient" is the sole source of substrate).

The possible occurrence of bypass fluxes suggests that the curve shown on Figure 8.1 should not necessarily pass through the origin, but should join the vertical axis at a value corresponding to the magnitude of these bypass fluxes (obviously if bypass fluxes are absent their magnitude is zero and the curve will

pass through the origin).

8.3 The model.

Before developing an appropriate model it is necessary to make a distinction between the various types of mutations affecting enzyme activities.

The first distinction is between "null" mutations which completely eliminate activity, and "non-null" mutations which merely reduce activity. In this model, an arbitrary scale of enzyme activity is used where the wild-type homozygote has activity of $a=1$, the null homozygote has $a=0$, and the non-null homozygote has $a=a_0$ The heterozygotes are assumed to have activities midway between the wild-type and mutant homozygotes i.e $a_h=0.5$ for null mutations and $a_h=(1+a_0)/2$ for non-nulls. The relative fitnesses of the wild-type homozygote, heterozygote, and deleterious homozygote genotypes are defined as 1, $1-hs$ and $1-s$ respectively, where s is selection against the deleterious homozygote and h is a dominance index.

The second distinction is between structural and regulatory mutations. Structural mutations lack activity due to a disruption of their catalytic capability and all three genotypes produce equal concentrations of enzyme molecules. Regulatory mutations disrupt the production of the enzyme and in this case the three genotypes produce different concentrations of enzyme molecules. The synthesis of enzyme molecules entails a metabolic cost to the organism which reduces fitness, and which appears to be a linear function of the concentration of enzyme produced (R. K. Koehn, pers. comm.). This cost can be incorporated into equation 8.1 as follows:

$$F = F_{max} a/(K_a + a) - ca \qquad (8.2),$$

where c is the linear cost function. If we assume that natural selection has optimized this fitness, this will occur when $dF/da = 0$ and it can be shown that

$$c = F_{max} K_a/(K_a+1)^2 \qquad (8.3)$$

Equation 8.2 now becomes our standard "fitness curve" and can also be used to describe structural mutations be setting c to zero (in which case equations 8.1 and 8.2 are equivalent).

The third distinction is made between mutations affecting enzyme activity depending on whether or not bypass fluxes can occur. As explained earlier, if a bypass flux occurs, the curve shown on Figure 8.1 should meet the vertical axis

at the value of this bypass flux; since fitness is assumed to be proportional to flux their relative values are identical and the magnitude of this bypass flux equals the fitness of the deleterious homozygote i.e. bypass flux = $1-s$. If we assume the magnitude of this bypass flux to be constant in all genotypes (an unlikely situation, discussed in more detail later), the flux through the linear part of the pathway in the wild-type homozygote is $1-(1-s) = s$, and in the heterozygote is $1-hs-(1-s) = s-sh$.

These three distinctions enable us to describe the fluxes for the three types of mutation: null, non-null, and null in the presence of bypass fluxes. The equations are shown on Table 8.1 together with the solutions to their parameters solved by substitution. Note that it is impossible to solve the situation of a non-null in the presence of bypass fluxes as we cannot estimate the magnitude of the bypass flux.

8.4 Choice of parameters for the model and results.

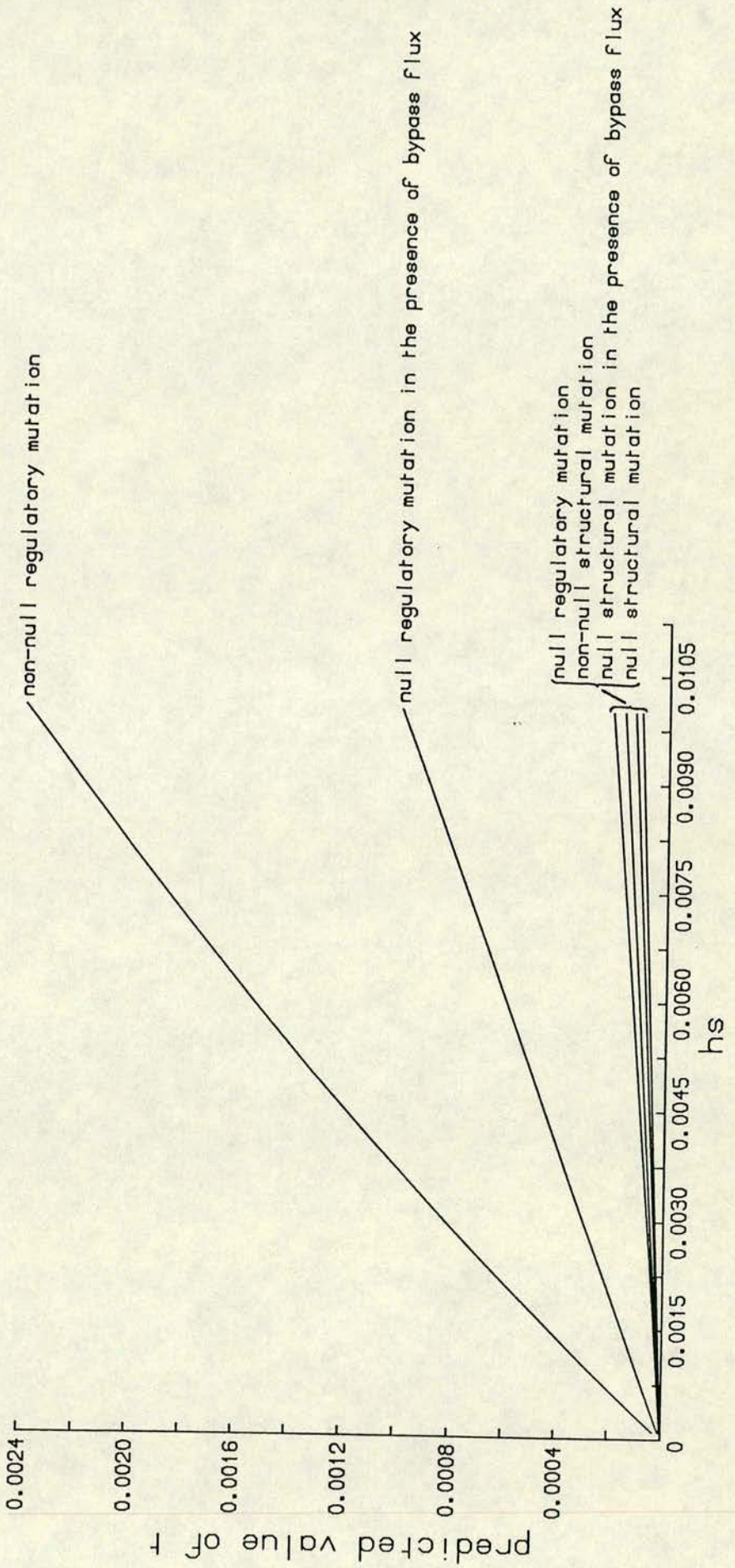
The equilibrium frequency of deleterious mutations maintained in a diploid population as a result of a mutation/selection balance is given by the formula $q = \mu/(hs)$, where q is the equilibrium frequency and μ is the mutation rate (Crow, 1986). The magnitude of the selection product hs can be calculated if q and μ are known. When considering the situations of non-null alleles or bypass fluxes, the product hs must be split into its two constituent factors h and s. In these two situations the mutant alleles are deleterious rather than lethal and Mukai *et al* (1972) and Ohnishi (1977) have estimated that dominance indices (h) for these types of alleles are typically in the region 0.3 to 0.5 in *Drosophila melanogaster*. The choice of an appropriate value of h allows s to be estimated from its product hs. The value of h used here is $h = 0.2$ which was chosen for two reasons: firstly, it is impossible to solve the equations under the assumption of bypass fluxes when $h>0.25$ (K_a becomes negative, see Table 8.1); secondly, this value of h errs on the side of caution by underestimating the value of the selective differences associated with variation in enzyme activity.

Following the terminology of Hartl *et al.* (1985), the selection coefficient is expressed as that acting on a structural mutation increasing enzyme activity by 1% and is given the symbol t. The value of t was obtained by substituting the values of F_{max}, K_a, and a_0 obtained from Table 8.1 into equation 8.2 and finding the difference in fitness between the wild-type activity (a =1) and the mutant activity (a =1.01). The values obtained over a range of values of hs and under the six sets of assumptions are shown of Figure 8.3. This shows that dramatic

Table 8.1. Equations describing fluxes in the three potential genotypes (wild-type homozygote, heterozygote, and deleterious homozygote) under each of the three assumptions: (a) nulls in the absence of bypass fluxes (b) nulls in the presence of bypass fluxes (c) non-null in the absence of bypass fluxes. The solutions to the parameters of these equations were obtained by substitution.

Genotype	Null mutations: no bypass fluxes	Null mutations: bypass fluxes present	Non-null mutations, no bypass fluxes
Wild-type:	$F_{max}/(K_a + 1) - c = 1$	$F_{max}/(K_a + 1) - c = s$	$F_{max}/(K_a + 1) - c = 1$
Heterozygote:	$(F_{max}/2)/(K_a + 0.5) - c/2 = 1-hs$	$(F_{max}/2)/(K_a + 0.5) - c/2 = s-hs$	$(0.5 + a_0/2)F_{max}/(K_a + 0.5 + a_0/2) - (0.5 + a_0/2)c = 1-hs$
Deleterious:	0	Bypass flux $= 1-s$	$a_0 F_{max}/(K_a + a_0) - a_0 c = 1- s$
	solution for structural mutations		
	$K_a = hs / (1 - 2hs)$	$K_a = h/(1-2h)$	$K_a = (a_0+1)hs/(1-a_0-2hs)$
	$F_{max} = K_a + 1$	$F_{max} = (K_a + 1)s$	$F_{max} = K_a+1$
			$a_0 = (s - 1)h/(h-1)$
	solution for regulatory mutations		
	$K_a = 2hs/(1 - 4hs)$	$K_a = 2h/(1-4h)$	$K_a = [2(a_0+1)hs] / [a_0^2-2a_0-4hs+1]$
	$F_{max} = (K_a + 1)^2$	$F_{max} = (K_a + 1)^2 s$	$F_{max} =(K_a+1)^2$
			$a_0 = (\sqrt{(1-16h^2s+16h^2+8hs-8h)}+1) / 2(1-2h)$

Figure 8.3 The value of t (the selection coefficient acting on an allele increasing wild-type activity by 1%) as a function of hs. In the analyses assuming non-null mutations or the presence of bypass fluxes, h is taken as 0.2.



differences in the magnitude of the predicted selection coefficient are obtained under the different sets of assumptions. It emphasises that the nature of the mutation and its position in the metabolic map (which determines whether bypass fluxes occur) should be ascertained before conclusions are drawn on the fitness differences likely to be associated with the different genotypes. An interesting result is that, all other parameters being equal, the fitness differences predicted from the equilibrium frequency of regulatory mutations are much higher (by factors of between 2 and 12) than predicted from the equilibrium frequency of structural mutations. The recent work of R.K. Koehn suggesting that a metabolic cost is associated with enzyme synthesis may therefore have important implications for the population genetics of allozymes.

The best estimates of hs for a range of loci in natural populations comes from the data of Langley *et al.* (1981). They reported frequencies (assumed to be equilibrium frequencies) of "null" mutations at 14 loci in an English population of *Drosophila melanogaster* (the same group had also reported similar frequencies in a North American population; Voelker *et al*, 1980a). The mutation rate to "null" mutations (μ) was estimated by Voelker, Shaffer and Mukai (1980b) to be 4×10^{-6} per locus , which allows the value of hs to be estimated from the formula $q = \mu/hs$ given earlier. The frequencies (q), the estimated values of hs , and the predicted values of t under the six sets of assumptions are given in Table 8.2.

The values of t obtained under the assumption of structural null mutation with no bypass fluxes are approximately twice that previously predicted by Hartl *et al.* (1985) from the same data under the same assumptions. Their methodology appears to overestimate the fitness difference between the wild-type homozygote and wild-type/deleterious heterozygotes and a minor adjustment to correct for this gives values of t identical to those obtained here. They used a parameter-free version of equation (8.1):

$$f = a/(a+1) = \text{fitness} \qquad (8.4)$$

where f is flux/$fmax$ and a in this case is activity/K_a.

By assuming that f in the heterozygote is $1-hs$, the value of a in the heterozygote (a_{het}), was calculated. The value of the wild-type activity (a_{wt}) is assumed to be twice a_{het} and it was shown that

$$a_{wt} = 2[(1/hs)-1] \qquad (8.5)$$

An implicit assumption is that the fitness of the wild-type is unity; however

Table 8.2. Frequencies of "null enzymes" (q) and inferred values of hs of a range of enzyme loci of *Drosophila melanogaster* from the data of Langley *et al.* (1981). Also shown are the estimated values of t (the selection coefficient acting on a structural mutation increasing activity to 1% above the wild-type level) obtained by Hartl *et al.* (1985) (HDD) and by substituting the solutions given in Table 8.1 into equation 8.2 under the following sets of assumptions: (i) that the mutations were nulls and no bypass fluxes were present, (ii) that the mutations were nulls and bypass fluxes occurred, and (iii) that the mutations were non-nulls and no bypass fluxes were present.

Locus*			$t \times 10^5$						
				Structural			Regulatory		
	q	hs	HDD	(i)	(ii)	(iii)	(i)	(ii)	(iii)
Got-2	2.63	1.50	0.74	1.49	1.86	2.47	2.99	14.9	42.7
α*Gpdh*	8.33	0.46	0.23	0.46	0.57	0.76	0.91	4.58	13.5
cMdh	1.64	2.36	1.2	2.34	2.93	3.88	4.72	23.5	65.8
Adh	0.85	4.52	2.2	4.50	5.61	7.40	9.12	45.0	120
Dip-A	4.36	0.89	0.44	0.88	1.10	1.47	1.77	8.87	25.8
Pgi	0.87	4.45	2.2	4.43	5.52	7.29	8.97	44.4	118
Hex-c	1.72	2.25	1.1	2.23	2.79	3.70	4.50	22.4	62.9
Idh	2.20	1.76	0.87	1.75	2.18	2.90	3.51	17.5	49.8
Pgm	0.74	5.19	2.6	5.17	6.44	8.49	10.5	51.7	136
Est-c	5.15	0.75	0.37	0.74	0.93	1.24	1.49	7.47	21.8
Odh	0.85	4.53	2.2	4.51	5.62	7.42	9.14	45.1	120
Men	6.10	0.63	0.31	0.62	0.78	1.04	1.25	6.28	18.4
Aldox	11.3	0.34	0.17	0.34	0.42	0.56	0.67	3.39	10.0
Acph-1	2.47	1.57	0.78	1.56	1.95	2.58	3.13	15.6	44.6

* The loci code for the following enzymes: Glutamate-oxaloacetate transaminase (*Got-2*), α-Glycerolphosphate dehydrogenase (α*Gpdh*), cytoplasmic Malate dehydrogenase (*c-Mdh*), Alcohol dehydrogenase (*Adh*), Dipeptidase A (*Dip-A*), Phosphoglucose isomerase (*Pgi*), Hexokinase C (*Hex-C*), Isocitrate dehydrogenase (*Idh*), Phosphoglucomutase (*Pgm*), Esterase C (*Est-c*), Octanol dehydrogenase (*Odh*), Malic enzyme (*Men*), Aldehyde oxidase (*Aldox*) and Acid phoshatase-1 (*Acph-1*).

equation 8.4 shows that this only occurs when a is infinity so the analysis overestimates the fitness difference between wild-type and heterozygote. This can be avoided by using the relative values of fitness i.e.

$$[a_{het}/(1+a_{het})]/[a_{wt}/(1+a_{wt})] = (1-hs)/1$$

Since $a_{wt} = 2a_{het}$, it can be shown that

$$a_{wt} = 1/hs-2.$$

For small values of hs this method gives values of a_{wt} half that predicted by equation (8.5). The value of t is approximately $10^{-2}/(1+a_{wt})$ which explains why its value differs by a factor of two between the two analyses.

The question remains as to which is the most appropriate set of assumptions to use when analysing the data of Langley *et al* (1981). The authors state that the mutations may not all be true null mutations as some may have retained a low level of catalytic activity below the resolution of their assays i.e. could have been non-null. The values of a_0, the magnitude of this low level activity, was calculated as 25% and 63% of wild-type level for structural and regulatory mutations respectively (from the formulae on Table 8.1). It is unlikely that such high activities could have been below the resolution of their assays so the non-null assumption can probably be ignored. All their alleles were made homozygous in their *Drosophila* stocks and all such genotypes were viable and fertile with the exception of one *Pgi* allele; this weakens the assumption of null alleles with no bypass fluxes as these alleles would be recessive lethals. This leaves the assumption of null alleles in the presence of bypass fluxes as the most plausible analysis. The predicted values of t obtained under this assumption differ by a factor of eight depending on whether the alleles were assumed to structural or regulatory; unfortunately Langley *et al.* did not characterise the alleles so we can only conclude that these are the probable limits within which the true values lie.

Two assumptions were made in these analyses which warrant further consideration. Firstly that the magnitude of any bypass flux is the same in each of the three genotypes. This may occur if enzyme b_1 (Figure 8.2) is saturated by its substrate s_2, or if a feedback control operates somewhere in the bypass flux. However it is more likely that the bypass flux decreases as a null enzyme activity becomes non-zero: as enzyme activity increases and a linear flux develops, the substrate pools s_1 to s_5 are supplemented and the input of enzymes e_7 to e_9 is likely to be reduced and may even divert substrates away

from the pathway. If this behaviour can be predicted, the value "residual flux = $1-hs$" can be replaced by $f(a)$, where f is the known function, and a more accurate estimate of t would be obtained. The assumption that bypass fluxes are constant means that the relative differences in flux between the three genotypes will be under-estimated, as will the magnitude of t. The second assumption to warrant further consideration is that the activity of the heterozygote is midway between that of the two homozygotes. As mentioned in the previous chapter, enzyme activity levels are frequently induced or repressed according to metabolic requirements and the example cited was lipogenic enzymes where the activity within an individual may vary up to three fold depending on its nutritional status. If enzyme activity in the heterozygote is induced to a level greater than 50% of the homozygous wild-type, the analyses will underestimate the differences in "basal" flux between the two genotypes, which will result in an underestimated value of t. Thus both assumptions tend to result in a underestimated value of t and the values given in Table 8.2 should be interpreted with this proviso in mind.

8.5 Discussion

The reader will have noted the number of assumptions made while analysing the data of Langley *et al.* (1981): (i) that the null enzyme frequencies were at equilibrium, (ii) that the mutation rate of 4×10^{-6} was appropriate, (iii) that the enzyme activity in the heterozygote was midway between that of the two homozygotes, (iv) that the magnitude of bypass fluxes remain constant, (v) that a cost in fitness terms is associated with the synthesis of enzyme molecules and that natural selection has optimized equation 8.2, (vi) that the value $h = 0.2$ was appropriate. The previous analysis of Hartl *et al.* (1985), which stimulated this study, also made assumptions (i), (ii) and (iii) but also made three other assumptions which were as follows. Firstly, they used the type of curve shown on Figure 8.1 which passed through the origin (they did not recognise the possibility of bypass fluxes); this predicted that all "null" alleles would be recessive lethals, a prediction contradicted by Langley *et al.* (1981) who reported that all except one were viable and fertile when homozygous. Secondly they did not recognize a fitness cost associated with enzyme synthesis. A large amount of genetic variation is associated with the regulation of enzyme concentration (Paigen, 1979, 1986; Laurie-Ahlberg *et al.* 1980; Laurie-Ahlberg, 1985), and the simple assumption that flux equals fitness might be expected to cause selection for ever increasing enzyme concentrations if not constrained by some metabolic cost of enzyme synthesis. Thirdly, their methodology was unable to deal with non-null mutations. For these reasons it was decided to instigate this more

detailed analysis of the population genetics of enzyme activity variants. Figure 8.3 shows that large differences in the predicted value of t may occur depending upon the type of mutation investigated and suggests that future researchers will need to carefully characterize such mutations before drawing conclusions on their selective differences.

The methodology developed here and applied to data from natural population suggested that the selection coefficients acting on structural mutations raising enzyme activity to 1% above wild-type level are likely to be between 2 and 16 times higher than previously predicted (Table 8.2); moreover these values are likely to be underestimates. Hartl *et al.* (1985) used the theories of Kimura (1983) to test whether such differences are likely to be effectively neutral in natural populations. Kimura's theory states that the fate of such alleles (loss or fixation) will be determined primarily by selection when $4Ns > 10$, primarily by random drift when $4Ns < 0.1$, and by both processes when $0.1 < 4Ns < 10$, where N is the effective population size and s is the selective difference between the alleles. Taking a value of $N = 10^4$ as an approximation of a *Drosophila* deme size, Hartl *et al.* (1985) calculated these boundary values as 2.5×10^{-6} and 2.5×10^{-4}; the values obtained for t in Table 8.2 suggest that selection plays a more important role than was previously thought in determining the fate of alleles deviating only slightly (1%) from wild-type levels.

There are several objections to the assumption that fitness is proportional to flux. The first one, why the genetic variation associated with enzyme concentrations is not utilized to provide ever higher enzyme activities and hence flux, has been countered by the argument that a fitness cost is associated with enzyme synthesis. This argument also has important implications for features such as heterosis (R. K. Koehn, pers. comm) and is one reason why a more sophisticated analysis of the population genetics of enzyme activity variants was undertaken (for example, the value of the cost gradient can be calculated from equation 8.3). The second objection is that substrate concentrations affect fitness and are altered by variation in enzyme activities (Burton and Place, 1986); this is a valid point but unfortunately could not be incorporated into the simple model developed here. A third objection is that selection on multicellular organisms is unlikely to act merely on maximum flux, but is more likely to favour those individuals with fluxes acting effectively at appropriate metabolic states. This is presumably the evolutionary force behind the sophisticated systems of metabolic regulation seen in living organisms. One consequence of these regulatory mechanisms is that selection on metabolic pathways may be more intense in the germline stage of the life cycle as germline cells appear to be

critically dependant on maximum fluxes through their basic biochemical pathways. The implications of selection on biochemical pathways during this stage of the life cycle are discussed in the next chapter.

CHAPTER 9

POTENTIAL GERMLINE COMPETITION IN ANIMALS AND ITS EVOLUTIONARY IMPLICATIONS

9.1 Introduction

The analysis of Hartl *et al.* (1985) suggested that moderate variation in enzyme activity is likely to be effectively neutral in natural populations (but see the discussion in the previous chapter of this thesis). This raises a dilemma (discussed in more detail later) of how enzymes are maintained at such high levels of catalytic efficiency despite constant mutation pressure disrupting their structure. One possible resolution is to look for some stage in the life cycle where numerous genotypes compete on the basis of their basic metabolic pathways. The widespread occurrence of sperm competition in higher animals stimulated the following investigation as to whether significant selection pressures could act on biochemical pathways in this stage of the life cycle.

The phenomenon of gamete competition in plants has been considered and documented (Mulcahy, 1975; but see Charlesworth, Schemske and Stork, 1987). This occurs because plant gametes express their haploid genotype. One adult plant produces a population of different haploid phenotypes with the potential for competition to occur between them. In contrast animal germline cells appear only to transcribe their diploid genotype. The haploid gametes appear transcriptionally silent and function using the metabolic machinery inherited from their diploid progenitor (Sivinski, 1984). Thus animal germline cells must be functionally identical, all being a product of the adult diploid genotype. This is demonstrated by the ability of *Drosophila melanogaster* sperm to function normally even when nearly devoid of DNA (Lindsley and Grell, 1969). Competition between (identical) animal gametes derived from a single individual therefore cannot occur and its potential has been largely ignored.

However this line of reasoning ignores the molecular processes that create diversity in diploid cell lineages through mutation and genetic exchange by mitotic crossing over and mitotic gene conversion. The analysis presented here will investigate whether these processes can create sufficient diversity for selection between *diploid* genotypes to occur.

9.2 Description of model

This section develops a model describing diploid cell lineage diversification and selection.

The meiotic products of diploid cells, the gametes, are considered to be metabolically passive copies of their diploid progenitor cell and to contain a random haplotype, i.e. a heterozygous progenitor will produce a 1:1 ratio of gametes containing each allele. The model will consider selection in only one sex, the male, by sperm competition, while the female germline is not considered to undergo selection. This is the simplest, most rigorous case and allows widespread applicability even to large mammals were millions of sperm may compete for only one egg. A simple two allele model will be considered where A and a represent the two alleles. The molecular processes can be represented as follows: μ is the mutation rate from A to a, μ_r is the reverse mutation rate from a to A and X is the rate of unbiased mitotic crossing over and unbiased mitotic gene conversion. Note that X represents not only mitotic crossing over plus gene conversion but any molecular process that results in reciprocal exchange between homologous chromosomes. This creates diversity in germline cells as shown in Table 9.1.

The relative fitness of the germline cells is defined as $AA = 1$, $Aa = 1-hs$ and $aa = 1-s$, where s is the selection coefficient against the homozygote aa, and h is a measure of dominance. If we define the initial frequencies of A as p and a as q, the initial adult genotype frequency of AA is p^2, Aa is $2pq$ and aa is q^2.

The frequency of A gametes derived from males in the next generation is calculated from Table 9.1 as follows:

$$\begin{aligned}\text{freq.} = {} & p^2\{(e_{11})+0.5(e_{21})(1-hs)\}/\{(e_{11})+(e_{21})(1-hs)\} \\ & + 2pq\{(e_{12})+0.5(e_{22})(1-hs)\}/\{(e_{12})+(e_{22})(1-hs)+(e_{32})(1-s)\} \\ & + q^2\{0.5(e_{23})(1-hs)\}/\{e_{23}(1-hs)+(e_{33})(1-s)\}\end{aligned} \qquad (9.1)$$

and the frequency of gametes derived from females is as in (9.1) above except that $s = 0$.

This equation gives the proportion of A gametes produced by each adult genotype and weights them by the relative frequency of their adult genotypes. It therefore assumes no differences in fertility between the three adult genotypes.

In the absence of a precise solution for this equation the equilibrium values of A

Table 9.1. Conditional genotype probabilities produced within parental genotypes by mutation from *A* to *a* (μ), mutation from *a* to *A* (μ_r) and genetic exchange between homologous chromosomes (X), assuming that μ, μ_r and X are sufficiently small that second order terms can be ignored. The probabilities can be regarded as components of a matrix, designated $\mathbf{e}_{11}$ to $\mathbf{e}_{33}$, and used to clarify equation (9.1) in the text.

GERM CELLS	PARENTAL GENOTYPE		
	AA	*Aa*	*aa*
AA	$1-2\mu$ (e_{11})	$\mu_r+0.5X$ (e_{12})	0 (e_{13})
Aa	2μ (e_{21})	$1-\mu_r-X-\mu$ (e_{22})	$2\mu_r$ (e_{23})
aa	0 (e_{31})	$\mu+0.5X$ (e_{32})	$1-2\mu_r$ (e_{33})

These processes are effective in germline lineages until they differentiate into spermatogonia. These type of cells undergo a further five or six mitotic divisions prior to meiosis, but all cells produced by these divisions develop within a single syncytium. The members of each syncytium appear to share the contents of their cytoplasm and are therefore likely to be metabolically identical (ref. Braun *et al*, 1989, *Nature* **337**: 373-376.

and a were calculated using an iterative computer program incorporating the chosen values for the parameters. Such a program also yields information on the dynamics of the process, which was assumed to have reached equilibrium when the proportionate change in gene frequency over one generation was less than $2x10^{-8}$. The program calculated gene frequencies transmitted through each sex and used them to calculate adult genotype frequencies in the next generation. This avoids problems inherent in assuming Hardy-Weinburg equilibrium when the sexes have differing gene frequencies.

9.3 Results

The program was run for different parameter sets and typical results are illustrated in Figures 9.1 and 9.2. Values of X below 10^{-4} were not considered since the process is slow, large amounts of computer time are involved, and the equilibrium levels of the favourable allele are low.

The process can be visualized as acting in three stages: mutations (μ) create new variants within the population and/or individual, DNA exchange and conversion mechanisms (X) generate a range of diploid genotypes within the germline and subsequent competition and selection (h and s) operates to eliminate the less beneficial allele. The end product of the process is therefore the result of the interplay between these four parameters μ, X, h and s. Simulation studies (Table 9.2, Figures 9.1 and 9.2) suggest that X is the critical parameter, since the diversity generated by X is directly responsible for the subsequent competition. The model appears relatively insensitive to changes in h and s at levels of X greater than 10^{-3}, and particularly insensitive to changes in h, an interesting result when applied to selection on biochemical pathways (see later). Figure 9.2 illustrates the effect of changing X and μ while holding h and s constant and is a typical result over a range of h and s values. The mutation rate μ appears influential at lower levels of X but becomes increasing less important at higher levels when sufficient germline diversity is generated to allow effective selection to operate against the unfavourable allele. Although there appears to be no simple algebraic solution for the equation, it is possible to make some assumptions and derive approximate expressions to describe the process (see appendix). The formulae support these results by showing that at high values of X, the equilibrium value of A will also be large; μ and s affect the equilibrium but at the levels of X considered here their contributions are swamped in absolute (but not relative) terms.

The dynamics of the process are illustrated graphically for a range of parameter

Figure 9.1. The effects of changing parameters s and h on the equilibrium frequency of A. X is 10^{-3}, μ is 4×10^{-6} and μ_r is 4×10^{-9}. The value of s is varied from 0 at the front to 1 at the rear in increments of 0.1. The value of h is varied from 1 on the left to 0 on the right, in increments of 0.1.

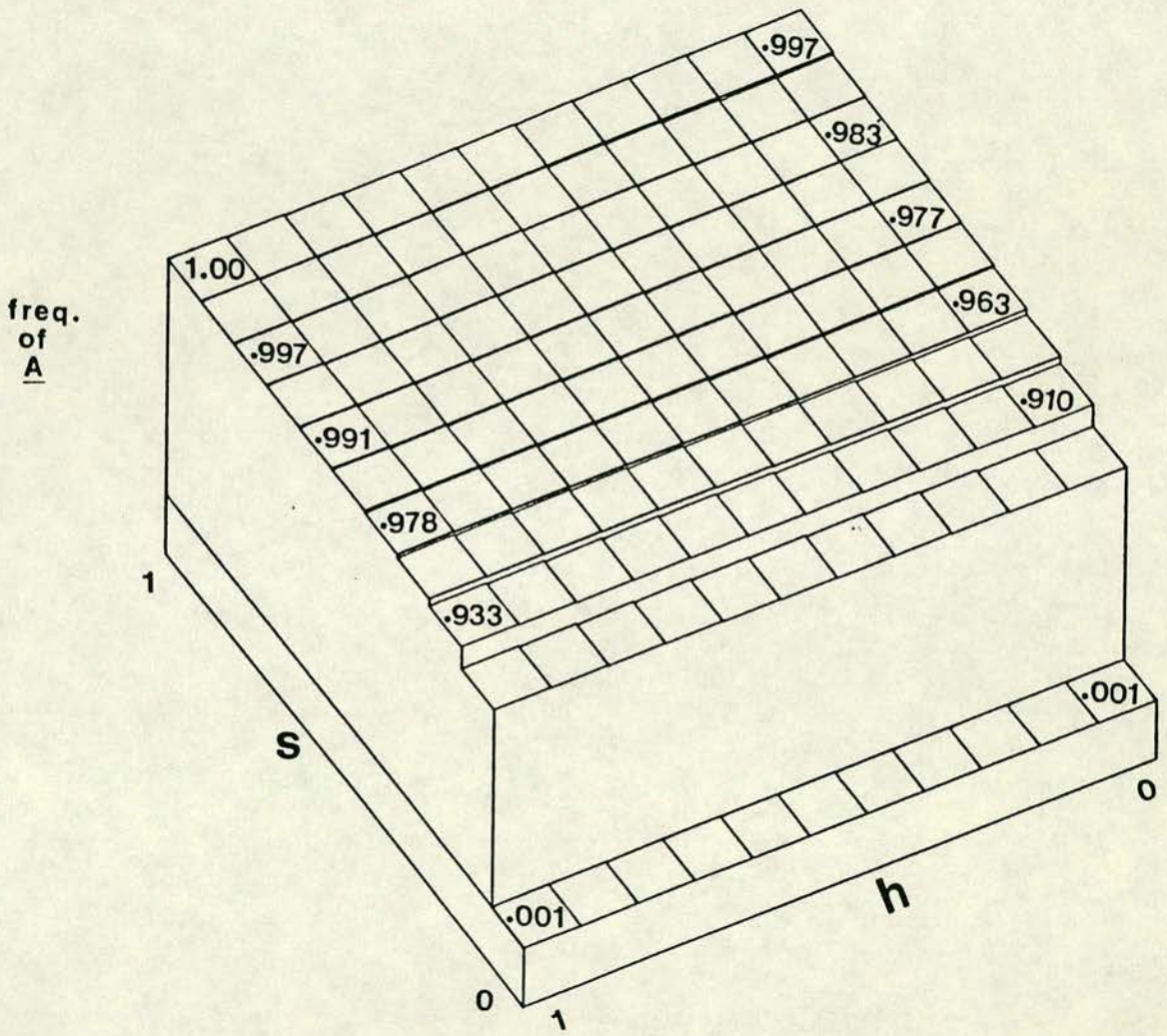


Figure 9.2. The effects of varying the parameters X and μ on the equilibrium frequency of A when s is 0.5 and h is 0.02. The value of X is varied from 10^{-4} at the front to 1.01×10^{-2} at the rear in increments of 10^{-3}. The value of μ is varied from 1.01×10^{-4} at the left to 10^{-6} on the right in increments of 10^{-5}; μ_r is $\mu \times 10^{-3}$.

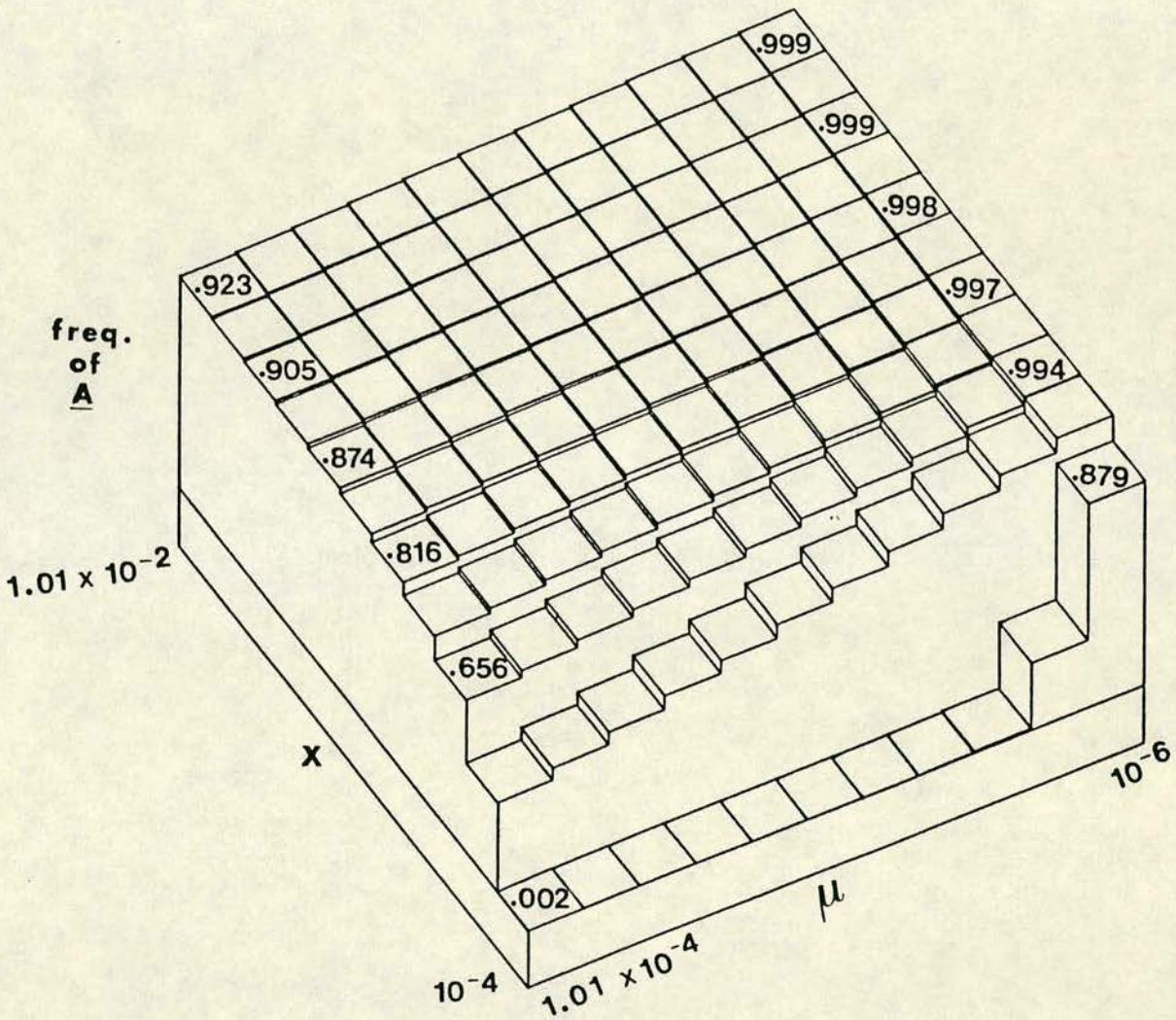


Table 9.2 Equilibrium frequency of *A* due to selection on germline or adult phenotype, assuming $\mu = 4 \times 10^{-6}$ and $\mu_r = 4 \times 10^{-9}$.

		Germline Selection				Adult Selection
s	h	x				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	
0.9	0.50	1.000	0.999	0.991	0.992	1.000
	0.02	1.000	0.998	0.981	0.814	1.000
	0.00	1.000	0.998	0.980	0.810	0.998
0.5	0.50	1.000	0.997	0.976	0.753	1.000
	0.02	1.000	0.996	0.965	0.652	1.000
	0.00	1.000	0.996	0.964	0.647	0.997
0.1	0.50	0.998	0.983	0.828	0.006	1.000
	0.02	0.998	0.982	0.818	0.005	0.998
	0.00	0.998	0.982	0.817	0.005	0.994

values in Figure 9.3. Although X is the critical equilibrium-determining factor, h and s influence the speed at which this equilibrium is regained after perturbation.

The slow rate of change may make the process vulnerable to the effect of random genetic drift in small populations when the adult stage of the life cycle acts as a bottleneck. The magnitude of drift effects will depend on the adult effective population size and subsequent recovery from perturbations will depend on the size of parameters X, h, μ and s. An approximate expression for this rate of recovery is derived in the appendix and appears to fit the observations shown in Figure 9.3.

When compared with selection against adult phenotypes ($p = 1-2\mu/s$ for $h = 0.5$, $p = 1-(\mu/hs)$ for $h = 0.02$, $p = 1-(\mu/s)^{0.5}$ for $h = 0$, Crow (1986)), the model was less effective at screening out the unfavourable allele; see Table 9.2. There is of course no reason why the two processes cannot act simultaneously. Their relative contributions would depend on the magnitude of the relevant parameters.

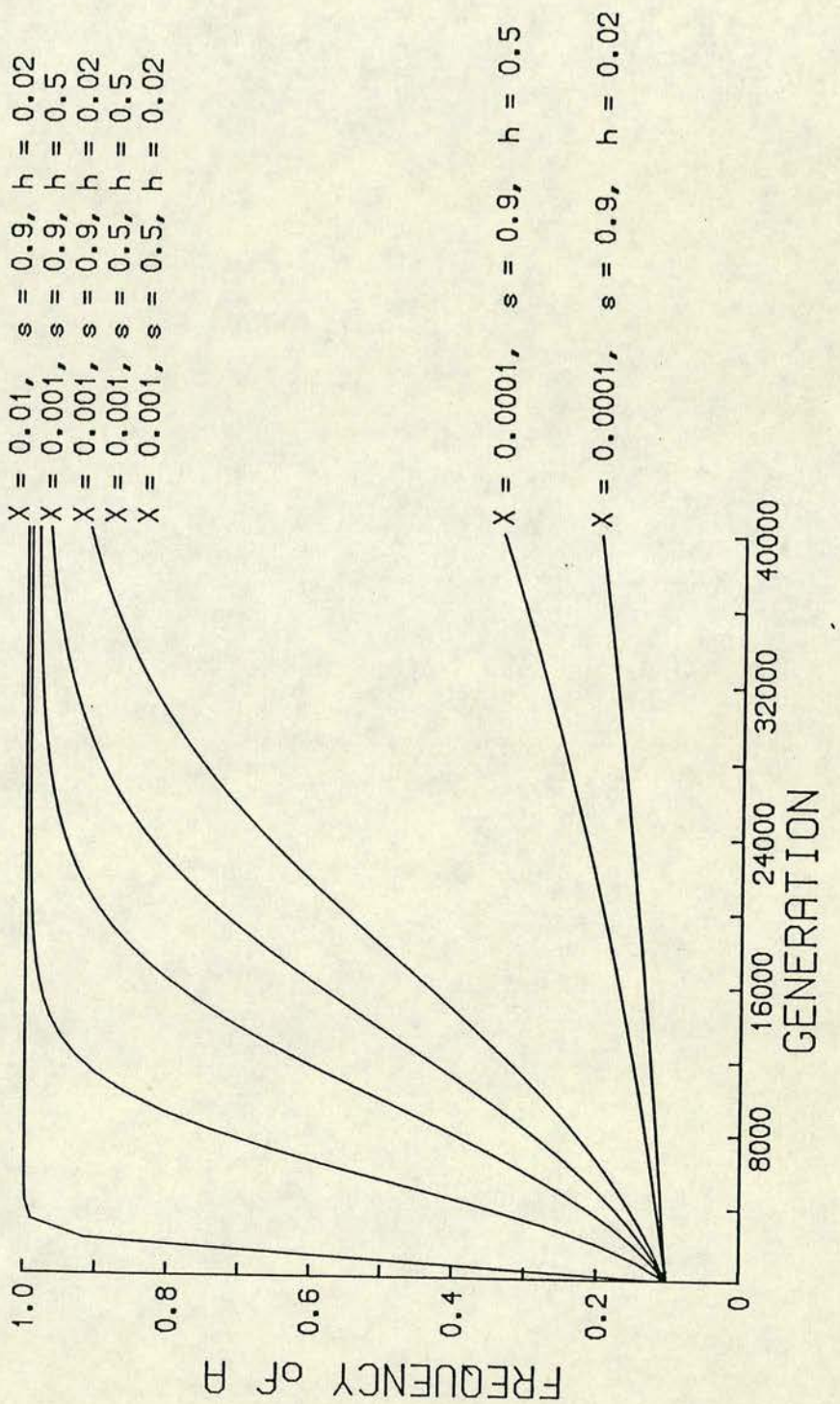
9.4 Discussion

(i) Limitations of the model

The model does not apply to genes coded by the mitochondrial genome which are transmitted maternally and are governed by the processes of clonal selection and drift. Enzymes that function in the mitochondria but are coded by the nuclear genome (e.g. enzymes of the Krebs cycle or electron transport chain) are described by this model, since their site of expression is immaterial. In many species with differentiated sex chromosomes, genetic exchange does not occur between the two sex chromosomes. When males are the heterogametic sex, no opportunity exists for genetic exchange between sex-linked loci during sperm formation and the value for X is zero. Sex-linked genes may be subject to a mutation/selection balance within the germline but the value of s would have to be considerably higher to achieve an equilibrium favouring allele A. The model only considers selection in one sex, but if selection occurs in both sexes, genetic exchange (X) in the homogametic will result in the same equilibrium frequencies obtained above. Selection in the adult phase of the life cycle may be sufficient to eliminate deleterious sex-linked genes since by definition these loci will be dominant in the heterogametic sex.

(ii) Choice of parameter values in the model

Figure 9.3. The effects of changing parameters *X, s* and *h* on the dynamics of gene frequency change attributable to germline competition. μ is $4x10^{-6}$ and μ_r is $4x10^{-9}$.



The parameters X, μ and μ_r describe diversity within germline cells immediately prior to meiosis. They represent cumulative totals of these processes over the cell divisions prior to meiosis. Mitotic crossing over is a well documented phenomenon (Whitehouse, 1982) but there are no reliable estimates of its frequency. Mitotic gene conversion has been observed in yeasts but its frequency in higher organisms is unknown (John and Miklos, 1988). Any mechanism of reciprocal exchange between homologous chromosomes is represented by the term X The value of X is essentially guesswork but testis is a rapidly dividing tissue and gametes are the product of a larger number of cell divisions than somatic tissue. The value of X in germline tissue may therefore be much higher than that estimated for the soma. Mitotic crossing over appears to occur even in the absence of meiotic crossing over, for example in males of higher Diptera. Gethmann (1988) concluded that "mitotic, or somatic crossing over probably occurs at a low frequency in all Diptera"; the estimates of its frequency were in the region $2x10^{-4}$ to $1x10^{-3}$. Mutation rate per enzyme coding region per gamete is usually regarded as around 10^{-5} although the best estimate for *Drosophila melanogaster* is $4x10^{-6}$, (Voelker *et al*,1980b). Mutations restoring enzyme activity are estimated to be 1000 times less frequent than those destroying activity in bacteria (Freifelder, 1987) . Since enzyme structure and function are similar in all organisms it is possible to estimate μ_r as $\mu \times 10^{-3}$. The magnitude of these parameters suggest that biases in the transmission frequency are likely to be small. Assuming $X = 10^{-3}$, $\mu = 4x10^{-6}$, $\mu_r = 4x10^{-9}$, h = 0.02 and s = 0.9 (resulting in an equilibrium frequency of A =0.981; Table 9.2), the frequency of A gametes transmitted by an Aa individual will be 0.50023; such small biases will be invisible in most experimental protocols.

Estimates of sperm competitive ability are available for several mammalian species. Cohen and M^{c}Naughton (1974) mated rabbits and later recovered sperm from several regions of the reproductive tract. The recovered sperm were used to inseminate other rabbits and the number required to produce a pregnancy was estimated. On average 100 sperm from the oviduct, 80,000 from the uterus or $4x10^6$ fresh sperm were sufficient to cause pregnancy; giving rise to a potential value of s as high as 0.999975. This may also reflect differences in the physiological maturation of the sperm but the experimental conditions were designed to favour the fresh sperm. In any case it demonstrates the enormous potential for sperm competition which exists *within* an individual irrespective of its biochemical or physiological basis. Beatty (1975) cites experiments designed to measure sperm competitive ability between strains of mice, rabbits and cattle. Fertilizing ability differed by a range of factors up to 12, 12 and 27 respectively

but these represent differences between genotypes rather than differences generated within an individual. Although these reports cannot give definitive figures for the value of s, the selection differentials of sperm within an individual, they provide a guide as to their potential magnitude.

(iii) Germline cell metabolism

The process of germline competition predicted above can maintain a high frequency of advantageous alleles in the face of persistent mutation pressure. The question is whether these alleles are specific to sperm function or of more general metabolic interest.

Biochemical studies of sperm are relatively recent and mainly concerned with mammalian systems (Blum 1986; Zaneveld and Chatterton, 1982). Sperm express the enzymes necessary for the oxidation of fatty acids, glycolysis, Krebs cycle, electron transport, oxidative phosphorylation, and possibly the hexose-monophosphate shunt. Evidence also points to their synthesis of lipids and glycogen while stored in the testes. Thus all the major biochemical pathways appear to play a role in sperm metabolism. During internal fertilization the ability of the sperm to reach the egg is dependent largely on its ability to convert metabolites into motility. The metabolites come either from its surrounding fluids which contain sugars, amino acids etc. (selection on the degradative pathways) or from its internal stores of lipid and glycogen (retrospective selection on the synthetic pathways). Although passive transport of sperm may occur in some mammalian species by smooth muscle contractions of the uterus etc., their own motility still plays a large part. It is possible that sperm must move to avoid depletion of nutrients within its immediate vicinity (Peterson, 1982), this would add an amplification step to the process in the form of positive feedback i.e. the more motile sperm gain more nutrients which further increases their motility.

Sperm motility is the most obvious physiological process affecting fitness but is not the only one. Any processes acting in the cell lineage which affect viability generate selection pressures on enzymes not normally expressed in sperm such as DNA synthesising and repair enzymes, ribosomal proteins etc. For example, mutation of an enzyme involved in DNA replication may increase a cell's doubling time, reducing its fitness in an environment of clonal expansion and selection. This situation is analogous to bacteria competing in a chemostat except that diploid cells are considered and diversity is created not only by mutation but also by mitotic crossing over and gene conversion. The model can

describe this process simply by altering the definition of the selection coefficient s. Diversification within the germline described earlier assumed that no selection occurred before the final meiotic division, biases in transmission frequency being a result of subsequent differences in sperm competitive ability as described by the parameter s. In the case of germline clonal competition the bias arises *before* the final meiotic division as a result of differential survival of lineages. This differential survival can also be represented by the parameter s, which in this case describes the selection coefficient accumulated over the cell generations prior to meiosis. This type of competition can occur in the diploid lineages of both sexes, resulting in much higher equilibrium frequencies of the favoured allele (but note that in mammalian females the final meiotic division occurs very early in development). It is therefore possible that germline competition plays a role in the maintenance of efficient metabolic machinery, even before the meiotic division forms the haploid germ cells. This makes the process applicable to mating systems where direct sperm competition is likely to be of minimal importance compared to chance events, such as the mass release of eggs and sperm into water. In these organisms the ability of diploid cell lineages to survive and flourish in the reproductive sacs prior to release will result in the biases predicted above. Once such a process has started even in a rudimentary form, it has the impetus to persist and develop given the marginal cost to the adult of within-germline competition and the benefits of passing on a more efficient basic metabolism.

(iv) Metabolic pathways in germline cells.

The model derives circumstantial support from recent work examining the influence of individual enzymes on biochemical pathways. These theoretical and empirical investigations found a law of diminishing returns as shown in Figure 8.1 (page 95). This suggests that the activity of an enzyme may fluctuate widely with a negligible effect on flux; even the 50% reduction in a wild type / null heterozygote may have a negligible effect on phenotype (Kacser and Burns, 1981). Hartl *et al.* (1985) summarized these studies and applied them to population genetics. Using data from *Drosophila melanogaster* they calculated that most metabolic enzymes were located along the plateau of Figure 8.1. They concluded that enzyme variants spread along this plateau are effectively neutral since the value of $4N_e s$ is considerably less than one, where N_e is effective population size and s is the relative selective disadvantage of an allele, in this case taken as equivalent to its effect on flux. In effect they are below the level of resolution possible by natural selection (but see previous chapter). An appraisal of these results led to a dilemma not usually acknowledged in

evolutionary theory. Enzymes are exceedingly efficient and in some cases approach "catalytic perfection", the stage at which the rate of catalysis is limited by the rate of diffusion bringing substrates into contact with the enzyme molecule. This efficiency is maintained across species and through generations in the face of repeated mutations. The vast majority of mutations will result in decreased catalytic efficiency and it is obvious that some selective pressure(s) must be maintaining them at this level of efficiency. However as pointed out, natural selection as usually envisaged lacks the degree of resolution necessary to achieve this.

A critical assumption in this analysis is that flux equals fitness. In the adult multicellular phenotype, complex physiological systems have evolved to regulate biochemical pathways and flux is determined not only by individual enzymes but also by higher processes of homeostasis such as hormonal regulation which modulate their activity. In this situation, maximum fluxes are less likely to be critical parameters affecting fitness. In contrast, germline cells appear critically dependant on their basal metabolic pathways and maximum fluxes determine fitness to a larger extent than in adults. Selection pressures acting on individual enzyme activities will therefore be higher in this stage of the life cycle. The problem remains as to the ability of germline competition to discriminate between enzyme variants with only a marginal effect on flux. This problem exists only if we continue to regard flux as equivalent to fitness. As stated earlier, sperm motility may have a positive feedback effect since more motile sperm avoid local depletion of nutrients; this phenomenon will generate larger differences in fitness between genotypes than predicted by consideration of flux alone. In the case of competition between different germline lineages it is possible that flux equals fitness at the level of individual cells, but small differences in fitness act cumulatively to create increasingly large fitness differences over a number of cell generations (as, for example, in chemostat experiments). These mechanisms amplify the small fitness differences associated with flux to the extent that selective pressures may be able to discriminate between enzyme variants with only minor changes in activity.

A feature of this process of germline competition is that selection occurs as an endogenous property and, unlike normal selection/mutation models, does not require differences in fertility or viability between adult phenotypes, a feature more in line with empirical observations. Given the assumptions that germline cells are critically dependent on their basic metabolic machinery, that molecular processes create diversity in the germline and that competition occurs between gametes or lineages, it is possible to generate large selection pressures acting

on biochemical pathways. It offers a solution to the problems raised by recent studies on the effects of enzyme mutations on biochemical pathways and their apparently negligible effects on adult phenotypes.

(v) Evolutionary implications of germline competition.

If the model accurately represents processes occurring in the germline it has implications for evolutionary theory beyond the maintenance of enzyme efficiency. It implies that some enzyme variants are selected to compete in the internal conditions of the reproductive tract and are effectively buffered from the external environment. Attempts to correlate certain enzyme variants with clines of environmental variables are likely to be unrewarding with the possible exception of temperature clines in poikilotherms. It predicts a possible conflict of interests between germline and adult phenotypes in the maintenance of enzyme efficiency. Advantageous phenotypic changes in the adult may be achieved by decreasing the efficiency of enzymes, e.g. decreased adult fat content by mutations in the fat synthesising pathway, but such enzymatic changes might be selected against in the germline. This conflict suggests that evolutionary change may occur not by modulation of enzyme activity but by mutations in genes involved at higher levels of physiological control in adults, such as hormones or hormone receptors, and explains why enzymes are functionally so similar even between species of widely divergent phenotype.

Differences in competitive ability of alleles in the germline can be observed directly as the meiotic drive systems known in many species, such as the *t* locus in mice or *SD* locus in *Drosophila*. These transmission biases appear to result from physiological disruption of meiotic divisions eliminating the sensitive alleles rather than by a biochemically more efficient metabolism. They are prevented from spreading to fixation by their deleterious effect when homozygous, i.e. are genes of large phenotypic effect. Dawkins (1982) suggested that segregation disorders may be more common than realized since only those with large phenotypic effects will be noticed; those involving small quantitative effects of the type envisaged here are largely invisible. It is tempting to speculate that such genes first arose by eliminating those lineages in the germline which did not contain a copy i.e in a heterozygote *Aa* individual, where *A* is the driven gene, lineages of type *aa* would be eliminated with a subsequent bias in the transmission frequency of *A*. Once started evolution would increase their segregating ability and their frequency would increase until fixation (if no deleterious effects) or until balanced by selection against the homozygote. Considerations of segregation disorders must be speculative because of their

different underlying physiology but it is encouraging to note that biases in transmission frequency do occur in nature as predicted by the model, albeit under different circumstances.

9.5 Conclusions.

Given that somatic mutation and DNA exchange occur within the germline, and that germline cells depend on their basic metabolic pathways, it is plausible (perhaps even inevitable) that the processes described above will occur. However it is not yet possible to calculate the magnitude of these effects due to the lack of data on the frequencies of the molecular events and the degree of competition in the germline.

SEMINAR 10

GENERAL DISCUSSION

It is appropriate to reiterate the three main aims of this study outlined in the introduction. Firstly to analyse the response to selection in the mouse lines up to generation 34; secondly to attempt to identify physiological traits correlated with a changed phenotype, specifically the activities of six lipogenic enzymes with fat content; thirdly to speculate on the type of biochemical changes likely to cause an altered phenotype.

Selection experiments using mice as a model species have several advantages over the use of commercial species. Mice have a short generation interval with a comparatively large litter size and are cheaper to maintain allowing larger population sizes and replicated selection lines. These features enable replicated divergent lines to be rapidly produced from a common base population and used to investigate genetic, physiological and biochemical aspects of growth; no such lines at present exist in commercial mammalian species. The presence of replicates was important to indicate whether the responses are repeatable and whether the same correlated biochemical changes occur in all lines. One disadvantage of using mice as a model system is their relatively inefficient growth due to high metabolic maintenance costs. These costs are larger than in commercial mammalian species but may have the advantage of making maintenance requirements more susceptible to the effects of artificial selection; the results can be extrapolated to commercial species assuming the underlying genetic basis is similar.

Growth rate, carcass composition and food intake are parameters which largely determine profits in commercial livestock production. Selection in these mouse lines has succeeded in producing four sets of lines which differ significantly in the partition of food intake: A and P lines differ in size but not composition, F lines differ in composition but only marginally in lean ~~mass~~ WEIGHT, and the M lines differ little in size or composition but have diverged in food intake by a factor of 1.5. The clear inference is that significant additive genetic variance is associated with food intake, food intake above maintenance requirements and the subsequent partition of this excess intake into lean growth and fat deposition. The similarity of mammalian physiologies makes these results encouraging for

breeders of commercial species contemplating selection experiments to alter size or composition.

The F lines are particularly interesting as they differ up to three fold in the amount of fat deposited but are associated with small changes in growth of internal organs or lean ~~mass.~~ WEIGHT These traits combined with the extensive knowledge of the physiology and biochemistry of fat metabolism, its economic importance in livestock production and its implications for human health make the F lines a useful model of an altered phenotype and have been investigated by several other groups, both biochemically (Asante *et al*, 1989) and physiologically by manipulation of energy intake (Morrupa *et al*, 1989) and by administration of exogenous bovine growth hormone (associated with slightly increased weight gain and decreased fat content; L. Bootland, Pers. Comm.).

The M lines are important as they indicated that intake may be *directly* altered by selection rather than being *indirectly* determined by metabolic requirements (as may be the case in the A lines where changes in food intake are correlated with changes in weight gain). A small changes in gain has become apparent in the M lines (Figure 2.12, page 24) but is insufficient to account for the divergence in intake (S. Moruppa, pers. comm.). This slight divergence in growth may require a minor adjustment in the selection indices to reverse the trend.

Responses in the A, F and P lines have continued from generation 20 (when last reported) to generation 34. This suggests that fixation of alleles influencing the selected phenotype have not yet become a factor limiting response. This is encouraging for investigators intending to increase selection intensity by technology such as MOET, artificial insemination and embryo splitting. There were no changes in the type of characters showing correlated responses over the period of selection, with the possible exception of litter size in the F6 replicates which may have diverged slightly in later generations. This suggested that selection was still acting on alleles and loci with similar pleiotropic effects to those in the initial stages of response. The records generated in the establishment of these lines can be used as a resource for testing and evaluating the application of new statistical techniques in complex pedigree structures; for instance Hill and Meyer (1988) analysed the early generations of the "A" lines by restricted maximum likelyhood (REML). These methods when applied to the analysis of extended pedigrees will greatly improve the accuracy of estimates of genetic parameters in the population (such as heritability or genetic correlations between traits), and how these parameters may have altered during the period of selection.

The lack of phenotypic reconvergence during nine generations of relaxed selection in the F and P lines indicated that little natural selection acted against the altered phenotype. Similar changes produced by selection in commercial species may also be stable and would not require continual selection to maintain the phenotype.

The indication that the X chromosome may have a large effect on body weight in the P6 line was interesting, irrespective of its genetic basis. The presence of a sex-linked major gene represents a site which, if identified, may constitute a suitable site for attempts at direct manipulation. If the X chromosome resembles a major gene due to an abnormally high polygenic divergence, it has interesting implications for selection experiments. It predicts that selection on the heterogametic sex (males in mammals, females in birds) will be more effective than the equivalent selection effort applied to both sexes or on the homogametic sex alone. The role of the X chromosome in selection and heterosis was a source of debate in the early 1960s but subsequently has been largely ignored. However the recent publication of a theoretical paper on its quantitative genetic effects (Grossman and Eisen, 1989) suggests that it may once again be a source of interest. If the observed bias that 25% of divergence is attributable to the 5% of sex-linked DNA is representative of other species it has considerable commercial implications by predicting that selection will be most effective when concentrated on the heterogametic sex.

The use of selection to produce changes in phenotypic traits of interest has resulted in a 1% to 3% improvement per year in most commercial species (Smith, 1988b) and will be enhanced by technologies mentioned above such as MOET. These improvements are cumulative and do not appear to have reached a plateau so selection will continue to play an important part in livestock production in the foreseeable future, especially as there are several problem associated with the two main alternative strategies. The first alternative strategy is the direct manipulation of animal physiology by administration of exogenous metabolic agents such as growth hormone, growth hormone releasing factor, oestradiol, B-agonists or by immunological destruction of tissues or cells such as adipocytes. Widespread public concern and possible legislation on this type of manipulation limits its application. The second alternative strategy is the direct genetic manipulation of animals to produce transgenic strains with desirable production traits. Its application is currently limited by difficulties in identifying trait-specific genes suitable for manipulation. Even when suitable sites have been identified there are still likely to be practical problems associated with its application. The introduction of exogenous DNA results in insertional

mutagenesis at its site of integration which may be deleterious or lethal (about 7% of integrations in mice; Palmiter and Brinster, 1986). Smith (1988b) has estimated that an effect in excess of 5% to 10% for any one transgene will be necessary to justify the screening, characterisation of pleiotropic and epistatic effects, out-crossing and utilization of resources which would otherwise be spent on continued selection. This also presupposes that public opinion and patent laws are condusive to the production of transgenic strains.

The production of replicated lines of mice divergently selected for growth traits therefore has two applications for commercial livestock production. Firstly, they allow us to study responses to selection on these traits in terms of magnitude, repeatability, correlated responses, selection limits, and reconvergence after selection is discontinued; on the supposition that mammalian physiologies are similar, these results provide the best predictions of long-term responses to similar selection in commercial species. Secondly, they provide an ideal resource for studies attempting to identify trait-specific genes controlling aspects of mammalian growth which may constitute suitable sites for transgenic manipulation. These two applications represent the twin aims of this study.

The changes in activity of lipogenic enzymes associated with divergence in fat content in the F6 lines were similar to those associated with obesity caused by major gene mutations. This highlights a critical problem associated with this type of study: how to separate the cause and effect relationship. Individual activities may have been altered by changes in allele frequency at loci which directly determine their activities and alter fat content in the adult (the causal relationship), or may merely have been induced or repressed as a consequence of an altered metabolic or nutritional status determined by other genetic changes such as the the presence of obesity major genes (the effect relationship). Alterations of activity by the induction, repression and stabilization of enzyme molecules are known mechanisms of metabolic control so may have resulted in the altered activities noted here. For this reason it is often preferable to refer to the activities as "markers" to avoid the problems associated with cause and effect; attempts to separate this relationship can be made later if the activities serve as useful markers.

The effectiveness of these enzyme activities as markers of fat content is inconclusive. There were clear differences (up to 3.5 fold) in enzyme activities between Fat and Lean lines but no consistent correlations between activity and fat content within lines. Between-line differences were more evident at five weeks than ten weeks so may have an advantage in allowing selection to take

place at an earlier age. The economic potential of selection at an earlier age will be determined by the correlation between activity at this earlier age and fat content at the desired age, and should be calculated for commercial species before assessing its potential value. The change in fat content which would occur as a correlated response to selection on individual enzyme activities can be crudely predicted in the following manner. Assuming an equal selection intensity could be practised it can be shown that $CR_e/\sigma_e = CR_f/\sigma_f$ where CR_e and CR_f are the correlated responses in enzyme activity and fat content (gonadal fatpad weight / body weight) respectively, and σ_e and σ_f are their corresponding phenotypic standard deviations (Asante *et al*, 1989). Using activities in gonadal fatpad tissue at age 5 weeks in the F6 lines as the "markers" with the greatest divergence, the ratios CR_e/σ_e can be calculated from Table 7.2 for the enzymes ACL, ACC, FAS and ME. In ACL the divergence CR_e is 45 nmol/min/mg soluble protein with a phenotypic standard deviation of 17.8 nmol/min/mg soluble protein, giving a value for the ratio CR_e/σ_e of 2.53; corresponding values for ACC, FAS and ME were 2.48, 3.23 and 3.71 respectively. The correlated response in fat content at age 10 weeks, as measured by the ratio of gonadal fatpad weight to body weight, was 22.7 mg/g, obtained from Table 7.1. Sharp *et. al.* (1984) estimated the phenotypic standard deviation in the base population to be 4.09 mg/g, giving a value of 5.55 for the ratio CR_f/σ_f. Since the two ratios should be equal, this implies that selection on enzyme activity will half to two-thirds as effective as direct selection on the ratio of gonadal fatpad weight to body weight. This result must be qualified as selection at the earlier age may have decreased the generation interval and husbandry costs, measurement on biopsy samples would have allowed selection on both sexes rather than retrospective selection only on males, the bias in fat distribution noted in the gonadal fatpads in Chapter 3 may have been reduced. In any case, the values of these genetic parameters may be different in commercial species and would have to be evaluated and the practical costs of husbandry incorporated into an appropriate strategy; the calculations outlined above can provide no more than a rough outline of how such a strategy could be assessed.

The genes coding the three enzymes of the lipogenic pathway have all be cloned i.e. ACL (murine; Sul *et al*, 1984), ACC (rat; Bai *et al*, 1986) and FAS (rat; Braddock and Hardie, 1988). Introduction of this cloned DNA into the germline of recipient strains could be a starting point to investigate the biochemical control of lipogenesis in different genetic backgrounds. Measurement of lipogenic flux is relatively straightforward either in the whole animal (Asante, 1989), or in isolated adipocytes *in vitro* (P. Sinnet-Smith, pers. comm.). When inserted with a

different promoter such as metalothionine, the enzymes should escape the pre-transcriptional control which is known to be a mechanism of modulating their activity. One potential problem with this approach is that transcription may occur in inappropriate tissues (such as lens or brain) or at inappropriate stages of development with possible deleterious effects. This drawback is common to all transgenics, including those with sequences encoding hormones. Recent developments in promoter technology suggest that promoters may soon become available which allow transcription of the inserted sequence to be switched on or off as required by the investigator and may also confer a greater degree of tissue specificity. The ability to modulate the activity of individual enzymes allows the investigator to asses its importance in controlling lipogenic flux. Analysis of biochemical pathways suggests that the control coefficients (defined as the proportional change in flux caused by a proportional change in individual enzyme activity) of the enzymes in a pathway should sum to unity (Kacser and Burns, 1973). The distribution of control over the three enzymes will reveal their relative importance in controlling flux in differing genetic backgrounds (i.e. the Fat, Lean and Control lines). An alternative approach is to decrease the activity of these enzymes in cultured adipocytes by means of specific inhibitors: given certain properties of the inhibitor such as non-toxicity, known kinetic affinities, and ability to freely cross the cell membrane, this enables the control coefficients of each enzyme to be calculated from the response of flux to changes in inhibitor concentration (Groen *et. al.,* 1982. These two types of investigation were beyond the scope of the present study but their implementation are currently under consideration.

Consideration of enzymes acting in complex metabolism led to the prediction that bypass fluxes may occur across many enzymatic "steps" of biochemical pathways. When the cost of enzyme synthesis was considered, this led to predictions that selection coefficients on enzyme structural variants could be much higher than previously thought.

The role of germline selection in evolution has several intriguing possibilities, in particular its role in the maintenance of enzyme catalytic efficiency. The dynamics of the process (Figure 9.3, page 116) suggested that it is unlikely to have any significant short-term effects on strains of animals selected for divergent phenotypes. A quantitative assessment of its effects in natural populations must await better estimates of parameters such as the frequency of mitotic crossing over and mitotic gene conversion in diploids.

Future technical developments are likely to make the production of transgenic

animals easier and therefore more widespread. A recent report claims to have produced transgenic animals by introducing exogenous DNA into sperm which were subsequently used to fertilize eggs (Lavitrano *et. al*, 1989). This remains to be confirmed but strengthens the assertion that techniques are likely to become more straightforward. As this trend continues it will highlight the lack of knowledge regarding which DNA sequences it is appropriate to transfer rather than how best to achieve the transfer. The answer to such a question may be resolved by an increased understanding of changes in metabolism that have produced the altered phenotypes in selection experiments such as the one described here.

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APPENDIX

SIMPLIFIED GERMLINE COMPETITION EQUATIONS

The following equations were obtained by W. G. Hill.

(1) Derivation of some simple approximations for equation 9.1.

In the following it is assumed that the frequency q of the mutant type a is sufficiently small that terms in q^2 can be ignored relative to q. The frequency of a gametes transmitted from AA males is:

$$q'_{AA} = \mu(1-hs)/(1-2\mu hs) \simeq \mu(1-hs).$$

Similarly for Aa males:

$$q'_{Aa} = [(1-\mu-X)(1-hs)/2 + (\mu+X/2)(1-s)]/[(1-hs)(1-\mu-X)-s(\mu+X/2)]$$

which if we also consider s to be small and disregard higher order terms reduces to:

$$q'_{Aa} \simeq (1+\mu-\mu s-Xs/2)/2.$$

For q small we can approximate the adult genotype frequencies of AA as $1-2q$ and Aa as $2q$. Thus:

$$q' = (1-2q)\mu(1-hs)+q(1+\mu-\mu s-Xs/2)$$

$$= q(1-\mu-\mu s+2\mu sh-Xs/2)+\mu-\mu hs.$$

In females the same equation applies but with $s = 0$. Thus the mean frequency transmitted through both sexes is:

$$q' = q+q(2\mu sh-2\mu-\mu s-Xs/2)/2 + \mu-\mu hs/2.$$

At equilibrium $q' \simeq q$ and, ignoring deviations from Hardy-Weinburg equilibrium the equilibrium frequency of a, represented by q_e is:

$$q_e = \mu(1-hs/2)/[s(X/2+\mu-2\mu h)/2+\mu].$$

Consider some special cases:

for $h = 0$: $q_e = \mu/[s(X/2+\mu)/2+\mu]$

For $X >> \mu$: $q_e = \mu(1-hs)/(sX/4)$

for $X >> \mu$ and h or s small: $q_e = 4\mu/sX$

which is analogous to selection against the adult ($q = \mu/hs$ for partial dominance) since the equation can be rewritten as $q = (2\mu/s)/(X/2)$

(2) The effects of drift.

From the previous section, the expected change in terms of the equilibrium frequency can be shown to be:

$$\delta q = -(q-q_e)/(\mu - \mu hs + \mu s/2 + Xs/4)$$

which if s is small and $X >> \mu$ reduces to:

$$\delta q = -(q-q_e)/(\mu + Xs/4).$$

The variance among lines due to drift is given by $q(1-q)/2N$ approximately, where N is the effective population size, which for small q approximates to $q/2N$. Thus the final variance about the equilibrium value will depend on the relative values of $1/N$ for the adult population and the terms X, μ, h and s operating in the germline; it can be shown that when q is small this variance will equal q_e/Nsx.