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Molecular Genetic Analysis of Rodent Non-Insulin Dependent Diabetes Mellitus

A Thesis Submitted to the Trustees of the Rockefeller University in Partial Fulfillment of
the Requirements for the Degree Doctor of Philosophy

by Wendy Chung

March, 1996
New York, NY 10021

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Dedication

To the poet and his muses for teaching me the most important lesson of all.

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This body of work really represents the work of all of the above individuals. Some day I hope that it will result in a better understanding and treatment of a disease from which too many people currently suffer.

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Abbreviations

ABI: Applied Biosystems Incorporated
ADA: adenosine deaminase
ANOVA: analysis of variance
ASP: agouti signaling protein
ATP: adenosine triphosphate
A^y: agouti, lethal yellow
B6: C57BL/6J
BKS:C57BL/KsJ
BMI: Body Mass Index
BN: brown norway
bp: base pair
CAST: CAST/Ei
cDNA: complementary deoxyribonucleic acid
Ci: curie
c-Jun: c *Jun* proto-oncogene
Ckc: SM/Ckc
cM: centimorgan
cm: centimeters
CO₂: carbon dioxide
Cpe:Carboxypeptidase E
DAB: diaminobenzidine
db: *diabetes*
DBA: DBA/2J
DHEA: dihydroepiandrostenedione
dl: deciliter
EDTA: ethylenediaminetetraacetic
F1: first intercross
F2: second intercross
F3: third intercross
fa: *fatty*
Fabp2: fatty acid binding protein 2
fat: *fat*
g: grams
Glp: glucagon like peptide
GLUT 1: glucose transporter 1
GLUT 2: glucose transporter 2
GLUT 4: glucose transporter 4
HbA1c: hemoglobin A1c
IRS-1: insulin receptor substrate-1

LOD: log of the odds
mg: milligrams
ml: milliliters
mm: millimeters
mM: millimole
MODY: maturity onset diabetes of the young
Mom-1: Multiple intestinal neoplasias
N2: second backcross
N2F1: second backcross, first intercross
ng: nanogram
NIDDM: non insulin dependent diabetes mellitus
ob: obese
Obr: *obese* receptor
PCR: polymerase chain reaction
PEPCK: phosphoenolpyruvate
pg: picogram
QTL: quantitative trait locus
RAD: ras gene association with diabetes
RFLP: restriction fragment length polymorphism
RIA: radioimmunoassay
SSR: simple sequence repeat
Tnf: tumor necrosis factor alpha
Tnfr: tumor necrosis factor receptor
tRNA: transfer ribonucleic acid
tub: *tubby*
WHO: world health organization
ZDF: zucker diabetic fatty

Abstract

Non-insulin dependent diabetes mellitus (NIDDM) is perhaps the most common metabolic disorder in humans. In its essence, NIDDM is a disorder of glucose homeostasis which results from a relative deficiency of circulating insulin. Approximately 15% of all Americans over the age of 60 and 100 million individuals worldwide are affected by this disease whose micro- and macrovascular complications include blindness, nephropathy, neuropathy, and atherosclerosis. There is a close association between obesity and NIDDM, due in part to the increased insulin resistance imposed by increased body fat. The large genetic contribution to susceptibility to NIDDM is evidenced by the nearly 100% concordance rate for NIDDM in identical twins. The disease endpoint resolves (in a phenotypic sense) complex interactions of genes and environmental factors affecting glucose transport, production and pancreatic beta cell function. Due to these complexities, only a small number of the genes, usually in rare sub-types of NIDDM, have been identified in humans. Because inbred rodent strains are inherently more tractable genetically and can be employed in cases controlled for relevant environment, they have been used as a model system for studying the underlying genetic basis of NIDDM. Intercrosses of mouse (and rat strains) with divergent inherent diabetes susceptibilities in matings simultaneously segregating for one of four mouse obesity mutations (*ob*, *db*, *fat*, or *tub*) or the rat mutation *fa* were used. Obese F2 progeny, as expected, demonstrated widely varying degrees of obesity and diabetes. The obese progeny were characterized at approximately 100-120 days of age for body weight, body mass index, fasting plasma [glucose] and [insulin], HbA1c, pancreatic hormone

content and pancreatic islet morphology using sections immunohistochemically stained for glucagon or insulin. These phenotypes intentionally emphasized the identification of genes which ultimately affect pancreatic beta cell function because primary beta cell defects have been associated with single gene mutations causing diabetes in humans. By selectively genotyping the most extremely affected 5% of the progeny with markers spaced approximately 20 cM apart throughout the genome, regions of the genome demonstrating deviation from the expected Mendelian ratios were identified and subsequently tested in all obese F2 progeny for association with obesity and diabetes phenotypes. When possible, additional genetic markers were used to more precisely position the quantitative trait loci (QTLs). QTLs identified in one set of F2 progeny were tested for replicability either in F3 or N2F1 progeny of the same cross or in other F2 progeny segregating for the same or different obesity mutations between the same of other parental strains. Using this technique in seven sets of obese F2 progeny and two set of obese F3 progeny, 23 separate QTLs on 18 mouse autosomes and two QTLs on two rat autosomes relating either to obesity and/or diabetes have been identified and (for the most part) replicated in multiple crosses. Candidate genes for the QTLs have been identified and in some cases directly evaluated by screening for mutations or allelic variants. Developmental studies have indicated the likely times of expression of four of the QTLs as well as likely points in NIDDM pathogenesis at which the QTLs act. Introgression techniques and strategies for cloning the genes underlying the QTLs and testing their relevance in human populations are described. Finally, evidence for the

potential role of mitochondrial genes and the unanticipated differences of obesity penetrance in *ob/ob* and *db/db* mice are presented.

Chapter 1

Introduction

Diabetes mellitus is a metabolic disorder associated with relative or absolute deficiencies of insulin. Untreated, the disease is characterized by fasting hyperglycemia, glycosuria, polyuria and polydipsia. With the development of the radioimmunoassay, it was possible to distinguish two subtypes of diabetes based upon absolute circulating insulin concentrations. Type I, or insulin dependent diabetes mellitus (IDDM), is characterized by absolute insufficiency of insulin; patients are ketosis prone and dependent upon exogenous insulin for survival. Classically, IDDM is of juvenile onset and associated with autoimmune destruction specifically of pancreatic beta cells. Genetic predisposition to IDDM is largely mediated by genes in the major histocompatibility (HLA) region. Type II, or non-insulin dependent diabetes mellitus, NIDDM, is not associated with ketosis; affected individuals are not absolutely dependent upon exogenous insulin although many patients are treated with supplemental insulin, especially later in the course of the disease. Classically, NIDDM onset is in adulthood (middle age or older) and is associated with glucose intolerance. Because NIDDM is the end point of a continuous process of metabolic change, definitions of affectation may vary. The World Health Organization (WHO) defines NIDDM as present in any individual meeting one or more of the following diagnostic criteria:

- 1). Fasting venous plasma glucose > 140 mg/dl
- 2). Plasma glucose concentration > 200 mg/dl at two hours of 75g dextrose oral glucose tolerance test
- 3). Random venous plasma glucose > 200 mg/dl (Seltzer 1970).

NIDDM is estimated to affect 15% of Americans over 60 years of age. Worldwide, nearly 100 million individuals are affected (World Health Organization 1994). NIDDM produces the majority of its morbidity and mortality via complications attributable to hyperglycemia: retinopathy, nephropathy, neuropathy, dyslipidemias, and coronary artery disease. Treatment of NIDDM and its complications accounts for 10% of all health care expenditures in the US (Huse et al. 1989).

There is clearly a large genetic component to NIDDM susceptibility, but the genetics are complex. Obesity is associated with 60-70% of all instances of NIDDM, and may predispose to diabetes by increasing peripheral insulin resistance (Campbell and Carlson 1993). However, the specific biologic mechanisms by which obesity conveys diabetes risk and the genes which increase susceptibility to diabetes are not well understood. The object of this dissertation is to identify the regions of the mouse and rat genomes responsible for obesity-associated predisposition or resistance to NIDDM in the context of obesity. Identification of the genes responsible for diabetes-susceptibility in rodents can hopefully provide valuable information about the etiology of NIDDM in humans, and could lead to more rational treatment and/or prevention of a major cause of morbidity and mortality in the United States and elsewhere.

Pathophysiology of NIDDM

Approximately 60-70% of all NIDDM patients (as defined by WHO criteria) are also obese (West and Kalbfleisch 1971). Obesity *per se* confers a baseline of relative insulin resistance due to alterations in both oxidative and nonoxidative glucose disposal (DeFronzo 1988, Groop et al. 1991). In obese individuals, increased fat mass is associated with increased release (by lipolysis) of free fatty acids and glycerol from adipose tissue into plasma (Groop and Saloranta 1991). With increased free fatty acid oxidation, glucose oxidation and storage (glucose disposal) in muscle may be reduced due to inhibition of enzymes in the glycolytic cascade ("Randle effect"). In the liver, increased glycerol released by triglyceride hydrolysis in fat provides a substrate for gluconeogenesis, while increased ambient free fatty acid concentrations promote gluconeogenesis and hepatic glucose production. When ambient free fatty acid concentrations are high, hepatic energy requirements are derived from these free fatty acids, sparing the glucose produced by gluconeogenesis for release into the circulation.

The most striking evidence for a link between obesity and NIDDM is the amelioration of diabetes with modest weight reduction. After weight loss, glucose homeostasis is improved by coordinate effects on hepatic glucose production, post-prandial glucose excursion, and insulin production (Henry et al. 1986). Conversely, if normal-weight subjects are overfed to increase body weight by 15-20%, moderate degrees of insulin resistance result (Sims 1973, Olefsky et al. 1974). However, obesity-associated insulin resistance alone is not enough to cause diabetes; a substantial fraction of obese patients

never advance beyond the stage of impaired glucose tolerance (West and Kalbfleisch 1971).

The precise biological mechanisms by which humans develop overt NIDDM are difficult to discern for two reasons: 1) The mechanism or sequence of biological events by which different individuals develop overt NIDDM may not be the same and 2) unless the disease can be studied longitudinally in susceptible individuals before they become overtly diabetic, it is impossible to distinguish the primary and secondary cellular and systemic events. Such studies are difficult since absolutely reliable predictors of NIDDM are not yet available. Therefore, although there are experimental data to support the pathogenic mechanism proposed below, the exact sequence and relative importance of a particular step may vary among individuals.

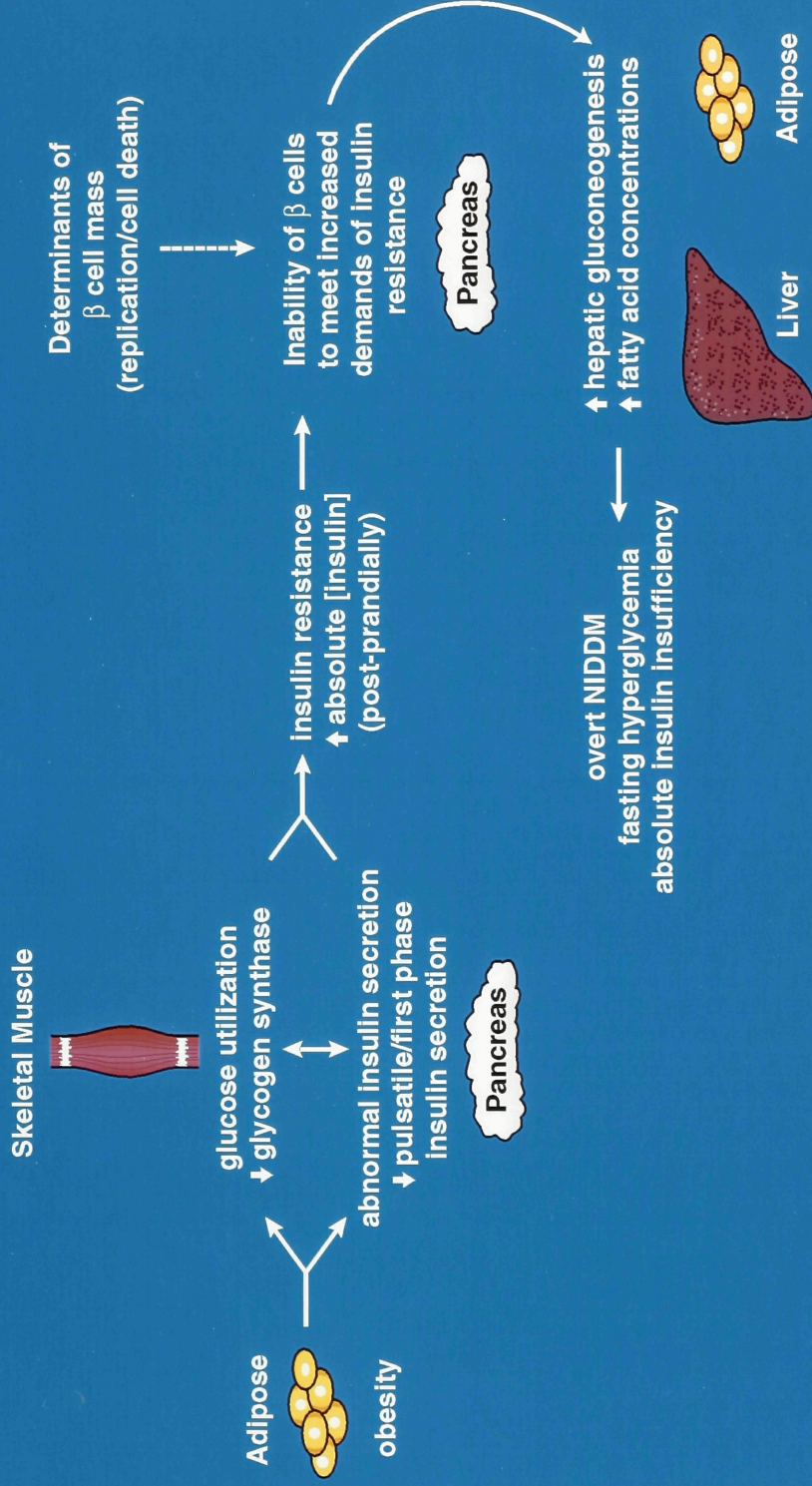
Epidemiological studies designed to identify the number of diabetes susceptibility genes indicate that there is a nonlinear relationship between the risk of developing NIDDM and genetic relatedness, suggesting that NIDDM is a multigenic disorder with a threshold effect (Rich 1990). Thus, the disease becomes manifest when a sufficient number of diabetes-susceptibility genes interacting with the “environment” (including obesity) exceed a threshold which results in the appearance of clinical NIDDM.

In the model proposed (Figure 1), three tissues are predominantly responsible for disease progression through insulin resistance and impaired glucose tolerance to overt NIDDM.

Skeletal muscle is involved in peripheral glucose utilization, liver in both glucose utilization and glucose production, and the pancreas in producing the hormones insulin and glucagon which regulate glucose homeostasis.

Figure 1. Pathogenesis of NIDDM.

Pathogenesis of NIDDM



Glucose uptake is regulated in skeletal muscle, cardiac muscle, adipose tissue and the liver (as compared to the brain and erythrocytes) by the insulin responsive glucose transporter 4 (GLUT4) in these tissues. Insulin increases glucose disposal via translocation of the transporters to the plasma membrane from intracellular vesicles (Marette et al. 1992). Once glucose is transported into the cells, it can either be oxidized or stored in the form of glycogen, or, in adipocytes or liver, converted to fatty acids. Reductions in glucose oxidation have only been noted in the latest stages of NIDDM (Golay et al. 1988), but defects in glycogen synthase have been reported in human studies of NIDDM up to two decades before the development of frank diabetes (Young et al. 1988, Bogardus et al. 1984, Beck-Nielsen et al. 1992). Decreased peripheral glucose utilization, a manifestation of insulin resistance, is one of the earliest detectable events in diabetes disease progression (Warram et al. 1990). Many patients demonstrate decreased glucose clearance in a glucose tolerance test and in the postprandial state before they develop NIDDM (DeFronzo 1988).

Insulin secretion is stimulated by glucokinase-mediated glucose sensing in the beta cell. Glucokinase catalyzes the first step in glycolysis (glucose phosphorylation) allowing extracellular glucose to continue entering the cell by facilitated transport. Defects in glucokinase activity have been associated with maturity onset diabetes of the young (MODY), an autosomal dominant form of NIDDM (Froguel et al. 1992). The mild glucose intolerance noted early in the development of NIDDM, is accompanied by abnormally low first phase-insulin secretion while second phase release and total release

in response to a glucose load are normal (Seltzer et al. 1967). As described later, it is possible that these abnormalities in insulin secretion are primary events in the pathogenesis of NIDDM.

There is considerable debate about the primary event in the pathogenesis of NIDDM; that is, whether the defect lies in insulin secretion or in insulin resistance. Longitudinal studies indicate that insulin resistance is present long before overt manifestations of NIDDM (Eriksson et al. 1989); however, these studies are not able to precisely time the onset of insulin resistance relative to a beta cell defect. It is possible that pulsatile insulin secretion primes the peripheral tissues for sensitivity to insulin when a large bolus of insulin is released. Oscillations in insulin secretion are disrupted not only in NIDDM patients but also in patients with impaired glucose tolerance (O'Meara et al. 1993). This abnormality in insulin secretion could itself be responsible for the resulting insulin insensitivity (Granner and O'Brien 1992). In addition, prolonged excessive second phase insulin secretion might desensitize peripheral tissue to the effects of insulin, providing an additional or alternative mechanism by which a beta cell defect might influence insulin resistance. Alternatively, if proinsulin were incompletely converted to insulin, what might appear to be peripheral insulin resistance could actually be an artifact of a defect within the beta cell if sensitive radioimmunoassays distinguishing the two molecular species were not used (Olefsky 1982).

Hepatic dysregulation is apparently one of the latest events in the pathogenesis of NIDDM based on: 1) Longitudinal studies in the Pima Indians in whom both insulin resistance and pancreatic beta cell defects precede increased hepatic glucose production; and 2) The linear correlation between hepatic glucose production and fasting blood glucose only when the latter is > 140 mg/dl (DeFronzo 1988). Although hepatic glucose production is the sum of glycogenolysis and gluconeogenesis, glycogenolysis is thought to be normally regulated in NIDDM (Consoli et al. 1989). Although a large part of hepatic gluconeogenic activity is hormonally regulated by reciprocal actions of insulin and glucagon, 10-30% is reflective only of substrate delivery to the liver and would mirror dysregulation (e.g. increased glycerol delivery from increased adipose tissue lipolysis) in other tissues (Consoli 1992). Normally, the combination of high insulin, low glucagon/catecholamine/glucocorticoid and growth hormone levels inhibits hepatic gluconeogenesis by reducing the activity of the rate limiting step, phosphoenolpyruvate carboxykinase (PEPCK). The combination of increased circulating concentrations of glycerol and fatty acids, and insufficient insulin action on the liver, results in dysregulated gluconeogenesis. Hepatic glucose production probably only becomes unrestrained once plasma insulin concentrations begin to decline because hepatic gluconeogenesis remains relatively responsive to insulin in early NIDDM (Granner and O'Brien 1992). Poorly regulated gluconeogenesis, the last major step in the progression to NIDDM, results in fasting hyperglycemia.

Once hyperglycemia exists, NIDDM may be exacerbated in all three major tissues by glucose-mediated down regulation of its own transport through glucose toxicity (Unger and Grundy 1985).

Although controversy persists about the exact primary causes and the secondary effects, a number of different combinations of defects in the above triad are probably sufficient to surpass the threshold for the development of overt NIDDM.

Complex Genetic Basis for NIDDM in Humans-A Geneticist's Nightmare

There is a strong genetic component to NIDDM as indicated by 1) different prevalences of disease in various ethnic groups (Zimmet et al. 1982) and in populations with varying admixture of ethnicity-based susceptibilities (Chakraborty et al. 1986); 2) familial clustering of disease, and 3) by the nearly 100% concordance in monozygotic twins followed long enough to assure full ascertainment of disease (Barnett et al. 1981, Newman et al. 1987). Although there is clearly a genetic component to NIDDM, the nature of the genes involved has, for the most part, evaded geneticists. NIDDM has been termed a “geneticist’s nightmare” both because of the complexity of the disease phenotype and the complex genetics that surely underlie the phenotype. Because NIDDM generally has its onset relatively late in life, once probands are identified, it may be too late to study the parents of the proband who are often deceased, especially if they too were affected. In addition, it is often too early to study the progeny or even the siblings of the proband

who might manifest disease only at some future point. NIDDM is likely not to be a single disease with a single etiology but rather a common, convergent endpoint to many otherwise subclinical metabolic derangements. Both peripheral insulin resistance (either in uptake of glucose by muscle or in suppression of hepatic glucose production) and failure of the insulin producing beta cells in the pancreatic islets are involved in the pathophysiology of the disease, but the relative weight or chronological order of the two is likely to differ between individuals within populations as suggested by earlier salience of the beta cell defect in Caucasians and the insulin resistance in African-Americans and Mexican Americans (Joffe et al. 1992, Haffner et al. 1986). In addition, although genes clearly play a role in the etiology of the disease, environmental factors such as increased caloric intake or reduced physical activity are additional factors which contribute independently of genetics to diabetes-susceptibility.

There is strong familiarity to NIDDM. Major gene effects have been demonstrated for circulating insulin concentrations (Lillioja et al. 1987) and tissue insulin action within sibships (Schumacher et al. 1992). The concordance rates of NIDDM for monozygous twins approaches 100% (Barnett and Eff 1981, Newman and Selby 1987). In contrast, concordance between monozygous twins for insulin dependent diabetes mellitus is only 25-50% (Schumacher and Hasstedt 1992). In segregation analyses, a major recessive locus accounts for approximately 33% of NIDDM-susceptibility, leaving an undetermined number of polygenes to account for the remaining susceptibility (Prochazka et al. 1992).

With few exceptions, the inheritance of NIDDM is complex and non-Mendelian (Cox et al. 1992). Although NIDDM is often considered to be one disease, NIDDM more likely represents a phenotype with several etiologies (phenocopies), reflecting the interactions of genes, age, body weight and environment (DeFronzo et al. 1992). Even the most clearly defined variant of the disease, maturity onset diabetes of the young (MODY) which is inherited as an autosomal dominant, has gradations of phenotype within that group of individuals whose diabetes is related to glucokinase mutations (Froguel and Vionnet 1995, Froguel and Velho 1994). MODY phenocopies also occur in individuals without glucokinase mutations (Froguel and Vaxillaire 1992, Vionnet et al. 1992, Hattersley et al. 1992). For example MODY1 in the RW Michigan family is incompletely penetrant and segregates with other non-MODY forms of NIDDM in this family (Cox and Xiang 1992). With such genetic complexity in a clearly defined subset of NIDDM, the more common forms, which are phenotypically heterogeneous, are likely to be even more genetically complex.

Molecular Mapping of NIDDM in Humans

The genetic heterogeneity of NIDDM, the frequent occurrence of more than one form of the disease in a single family, and the late onset of the disease make it impractical (although not impossible) to use approaches such as a whole genome scans for linked markers in humans, with the exception of phenotypically distinct forms of NIDDM such as MODY (Weissenbach et al. 1992). Instead, the more practical "candidate gene"

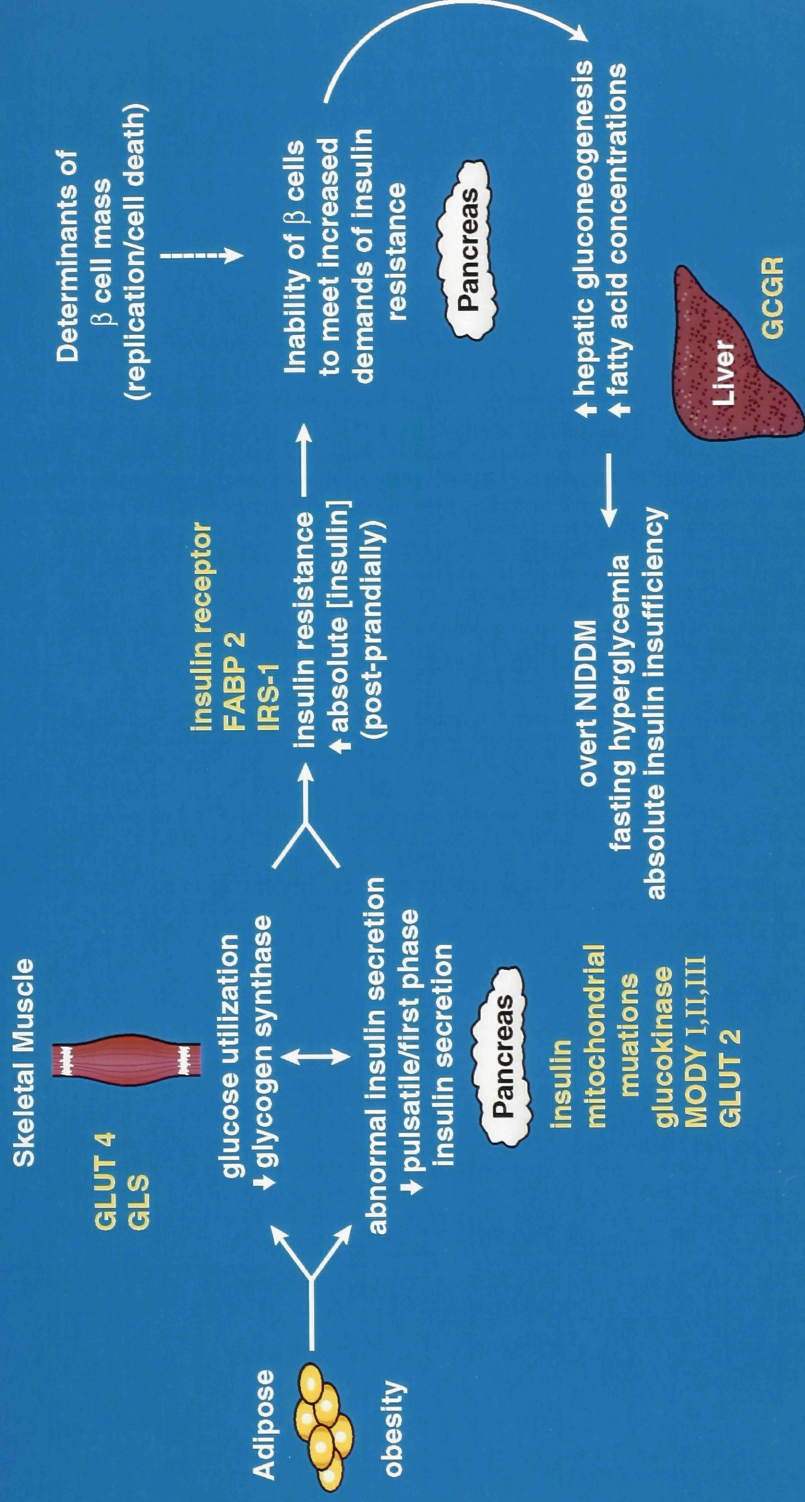
approach has been employed based on the current understanding of glucose and insulin metabolism and homeostasis (Figure 2).

MODY

The most successful application of the candidate gene approach to NIDDM has been the identification of 16 French families in whom missense, stop, or splice site mutations of the glucokinase gene (7p15-p13) result in maturity onset diabetes of the young (MODY II) (Froguel, 1993; Vionnet, 1992). Glucokinase is a hexokinase which is expressed exclusively in the liver and beta cells, and its activity regulates glucose uptake and therefore metabolism in these tissues. Because of this unique role in the beta cell, it acts as the “glucosensor” of ambient glucose concentrations (Matschinsky 1990). MODY patients who are heterozygous for glucokinase mutations have reduced insulin secretory responses to continuous infusions of glucose in a hyperglycemic clamp. Additionally, during hyperinsulinemic euglycemic clamps, endogenous insulin secretion is suppressed in affecteds but not controls (Polonsky 1995). Glucokinase mutations probably result in the synthesis of sub-normal amounts of enzyme which in turn results in chronic hyperglycemia due to the increased threshold required for the beta cell to initiate insulin secretion. This was the first demonstration of a primary beta cell defect causing NIDDM. Similar studies have now associated glucokinase mutations with non-MODY NIDDM in Mauritian Creoles (Chiu et al. 1992), and African American populations (Chiu et al. 1992) but not in French, Finnish, British, Swedish, Japanese, or Hispanic

Figure 2. Candidate genes which have been associated with the pathogenesis of NIDDM.

Genes Identified in the Pathogenesis of NIDDM



(Cook et al. 1992) populations segregating non-MODY forms of NIDDM. Screening women with gestational diabetes who also had first degree relatives with NIDDM identified 2/40 women with glucokinase mutations which by extrapolation would suggest that 1 in 2,500 American NIDDM patients have glucokinase mutations (Stoffel et al. 1993).

Bell et al. have demonstrated linkage of adenosine deaminase (20q13) to the MODY I phenotype in a Michigan family. This phenotype includes abnormal delayed insulin secretory oscillations and decreased amplitude of insulin pulses in response to glucose, favoring a primary beta cell defect (Bell et al. 1991). However, the penetrance of the putative mutation in this region is still not 100%. Sib-pair analysis of markers tightly linked to ADA on 20q failed to demonstrate linkage in a group of Italian and British patients with late onset NIDDM (Baroni et al. 1992).

MODY II (glucokinase mutations) only accounted for 56% of the French families with early onset NIDDM. Genome scanning of the remaining 12 families, indicated linkage of markers on 12q22-qter in six of the families (Vaxillaire et al. 1995). Linkage analysis of three additional MODY families from Denmark, Germany and the US has further refined the location of this third MODY locus (MODY III) to 12q24.1-q24.32 between D12S86 and D12S807 (Menzel et al. 1995). The phenotype associated with MODY III is hyperglycemia with a severe insulin secretory defect, again suggestive of a primary beta

cell defect. The MODY III locus does not appear to play a major role in late onset NIDDM in 172 French families (Lesage et al. 1995).

Candidate Genes for NIDDM

Several candidate genes for obesity and NIDDM have been tested either by linkage analysis or by direct mutation screening of candidate genes (Figure 2). Many of the candidate genes have been implicated in select populations or individuals although an equal number have been rejected as genetic mechanisms for obesity or NIDDM.

Mutations in the coding sequence for insulin (11p15.5) can result in high concentrations of pro-insulin and may be, but are not necessarily, associated with hyperglycemia (Gabbay et al. 1976, Shoelson et al. 1983, Haneda et al. 1983, Gruppuso et al. 1984). However, mutations within the coding sequence of insulin are rare and cannot account for a significant proportion of NIDDM. 5' of the insulin gene, polymorphisms potentially affecting the regulatory sequence have been associated with NIDDM (Rotwein et al. 1981). A variant promoter present in African Americans and Mauritius Creoles, but absent in Caucasians, reduces insulin expression *in vitro* (Olansky et al. 1992).

By analogy, mutations in the insulin receptor (19p13.2) could be responsible for insulin resistance. Depending on the exact mutation, phenotypes including Leprechaunism and Rabson-Mendenhall syndrome, insulin resistant acanthosis nigricans with or without polycystic ovaries, and insulin-resistant diabetes mellitus can result from insulin receptor

mutations (Kahn et al. 1976). However, most of the characterized mutations result in more severe phenotypes than are observed in classical NIDDM patients; and the majority of NIDDM patients do not have mutations in the insulin receptor gene (Elbein et al. 1992, Elbein et al. 1992).

Allelic variation or mutation in the genes responsible for post-receptor insulin signalling could account for some aspects of the NIDDM phenotype. Three variants in insulin receptor substrate 1 (IRS1-2q36) were discovered in Finnish NIDDM patients (Gly81Arg, Ser892Gly, Gly971Arg) (Laakso et al. 1994). Two polymorphisms (Ala512Pro and Gly971Arg) were common in Danish NIDDM patients (Almind et al. 1993). The functional consequences of these sequence variants have not yet been determined.

Patients with NIDDM are said to more likely have affected mothers than fathers (Alcolado and Alcolado 1991). Some cases of maternally transmitted NIDDM (often associated with deafness) have been associated with mitochondrial mutations including a mitochondrial leucyl tRNA gene mutation at nucleotide 3243. Presumably, such associations are due to the fact that mitochondria are essential to beta cell function as evidenced by the ability of streptozotocin (an inhibitor of mitochondrial DNA replication, transcription, and oxidative phosphorylation) to cause diabetes (vandenOuweland et al. 1992).

Glucose transporters (GLUT) which differ in their tissue specificity have been popular candidate genes because they can influence glucose disposal and/or glucose sensing in the beta cell. GLUT2 (3q26.1-q26.1), the liver/beta cell glucose transporter, was associated with NIDDM Caucasian patients with a strong family history of NIDDM (Alcolado et al. 1991), but was not associated with NIDDM in an affected-pedigree member analysis (Baroni et al. 1992), in Caucasian or West Indian populations (Li et al. 1991), or in 18 large Mormon pedigrees (Elbein and Hoffman 1992). A Val197Ile mutation in GLUT2 in a single NIDDM patient abolished transport activity in *Xenopus* oocytes (Tanizawa et al. 1994, Mueckler et al. 1994). A single mutation (Val383Ile) in GLUT4, the insulin-responsive glucose transporter, was detected in 1/30 NIDDM patients examined, but was determined to be an uncommon cause of NIDDM on the basis of its low population frequency (Kusari et al. 1991). Increased allele frequency of the X1 allele of GLUT1 (1p35-p31.3), the ubiquitous transporter, has been associated with NIDDM in 89 patients from 3 different ethnic populations (Li et al. 1988). However, no association of this allele with NIDDM was found in Caucasians (Elbein and Hoffman 1992, Elbein and Sorensen 1992). The molecular rationale for effects of GLUT1 on diabetes phenotypes are tenuous since this is not an insulin sensitive transporter.

The effects of the insulin counter regulatory hormone glucagon are mediated through the glucagon receptor (GCGR, 17q25). Association of a Gly40Ser variant in the glucagon receptor has been associated with late onset NIDDM in the French and Sardinians (Hager

et al. 1995). Receptor binding studies with the mutation indicated that the mutation receptor binds glucagon with a three fold lower affinity compared to the wild type receptor. Decreased binding could therefore result in decreased ability of glucagon to counterbalance the effects of insulin.

One allele for glycogen synthase (19q13.3) was associated with NIDDM, a stronger family history and a more severe defect in insulin-stimulated glucose storage in 107 Finnish patients (Groop et al. 1993). The concentrations of glycogen synthase in skeletal muscle in these patients were normal suggesting that gene regulation was normal. The association was not reproducible in French (Zouali et al. 1993) or Japanese (Kadowaki et al. 1993) populations.

Intestinal fatty acid binding protein (FABP2:4q28-31) binds and transports long chain fatty acids from the luminal surface of enterocytes. In the Pima Indians, linkage was found between markers on 4q and fasting insulin concentrations and maximum insulin-stimulated glucose uptake (Prochazka et al. 1993). Subsequent mutation analysis of this population revealed alanine and threonine polymorphisms at codon 54. The threonine allele was dominantly associated with higher fasting plasma insulin, lower insulin-stimulated glucose uptake, higher insulin response to oral glucose, and a higher rate of systemic fat oxidation (Baier et al. 1995). Purified recombinant threonine FABP2 demonstrated a two fold higher affinity for long chain fatty acids and may alter the

kinetics of fatty acid absorption to increase absorption rate to reduce insulin secretion post-prandially.

Many more candidate genes have failed to show either linkage or association with NIDDM, or failed to demonstrate functional effects of sequence variants. These candidate include hexokinase (2) in the Pimans (Ardehali et al. 1996), inwardly rectifying potassium channel (21q22.1-q22.2) in the Japanese (Yasuda et al. 1995), Ras gene associated with diabetes (RAD) (Reynet and Kahn 1993), islet transcription factor 1 (5q) in African Americans (Tanizawa, Riggs 1994, Tanizawa et al. 1994) and glucagon, phosphoenolpyruvate carboxykinase, pyruvate kinase, phosphofructokinase, apolipoprotein B, apolipoprotein A2, lipoprotein lipase, hepatic triglyceride lipase, very low density lipoprotein receptor, and cholesterol ester transfer in Caucasians (Elbein et al. 1995).

Although the candidate gene approach has yielded fruitful results in selected families, the number of candidate genes based on the known physiology remaining to be tested are few; and none of the candidate genes tested to date can account for more than 2% of the common forms of NIDDM.

Rodent Models of Obesity and NIDDM

For the reasons given above, it is extremely likely that we do not yet know the identity of all of the molecules and genes playing a role in the pathogenesis of NIDDM. One

efficient means of identifying new genes important in the etiology of NIDDM is to study the molecular genetics of NIDDM in a simplified genetic system such as the rat or mouse. Rodents have previously been useful in studying the molecular physiology of many other genetic human diseases (Darling and Abbott 1992). Rodent models of genetically determined obesity associated with NIDDM are plentiful and well characterized physiologically, facilitating a systematic search for susceptibility and resistance genes for NIDDM. Rodent models provide several experimental advantages including :

- 1) comparable metabolic physiology to humans
- 2) potential to control the environment
- 3) short generation time (21 days) and large litter sizes (8-10)
- 4) several, well characterized single gene mutations resulting in obesity (with varying degrees of associated diabetes)
- 5) availability of many different inbred strains with differing susceptibilities to the diabetogenic effects of the individual obesity mutations
- 6) high degree of synteny between the rodent and human genomes which can be used to identify corresponding human loci for those identified in rodents contributing to diabetes susceptibility or resistance
- 7) availability of systems for gene insertions and knockouts and
- 8) availability of tissue at any stage of development to facilitate the study of the ontogenesis of NIDDM.

The most thoroughly studied obesity/diabetes mutations in rodents, the mutated genes (where known) and their genetic locations are given below (Table 1) (Friedman and Leibel 1992):

Table 1 Autosomal rodent obesity mutations

Mouse	Gene	Inheritance	Gene	Chromosome location
yellow	<i>A^y</i>	dominant	agouti signalling protein	2
diabetes	<i>db</i> (<i>Lepr^{db}</i>)	recessive	leptin receptor	4
obese	<i>ob</i> (<i>Lep^{ob}</i>)	recessive	leptin	6
tub	<i>tub</i>	recessive	?	7
fat	<i>fat</i> (<i>Cpe^{fat}</i>)	recessive	carboxypeptidase E	8

Rat				
fatty	<i>fa</i> (<i>Lepr^{fa}</i>)	recessive	leptin receptor	5

The dominant *A^y* mutation results in deregulated overexpression of agouti signalling protein (ASP) (Bultman et al. 1992). The gene product bears homology to mollusc toxins (Manne et al. 1995). The mechanism by which overexpression of ASP produces obesity has not yet been determined, but ASP may bind centrally to interfere with the action of melanocyte stimulating hormone or a related ligand. *Fat* mice have a single amino acid change (Ser202Pro) which abolishes carboxypeptidase E activity (Naggert et al. 1995).

Defective processing of an as yet undetermined prohormone or neuropeptide may explain the mechanism by which *fat* mice become obese. *Ob^{l/l}* is the result of a premature stop in the leptin gene, a hormone which is secreted from adipose tissue (Zhang et al. 1994). Peripheral or central administration of recombinant leptin corrects all of the phenotypes in *obese* mice including hyperphagia, reduced energy expenditure and infertility (Halaas et al. 1995, Campfield et al. 1995, Pelleymounter et al. 1995). *Db* and *fa* are mutations in the Ob receptor of which there are at least six different naturally occurring splice variants. Mutation of the long form which includes an intracytoplasmic tail presumably required for signal transduction in the *db^{l/l}* mouse produces obesity. The *db^{l/l}* mutation suggests that a defective long splice variant is sufficient to produce obesity (Lee et al. 1996). Leptin is secreted from adipose and presumably binds to receptors in the hypothalamus to decrease food intake and increase energy expenditure. Leptin, *Obr* and *Cpe* have human homologues which may be involved in the pathogenesis of human obesity. It is also likely that rodent genes mediating NIDDM susceptibility will have human homologues.

Strain-Related Differences in Diabetes Susceptibility

Leiter and his associates demonstrated that the diabetes phenotype of mice segregating obesity mutations is dependent upon the genetic background strain upon which the mutation is bred (Leiter et al. 1989). For example, when either *ob* or *db* is carried on a diabetes-resistant strain such as C57BL/6J, 129/J, or MA/MyJ, the result is obesity with hyperinsulinemia (>1000 μ U/ml vs. 100 μ U/ml in non-obese) and glucose intolerance but

not overt diabetes. The pancreatic beta cells are able to meet the increased demands for insulin by responding with either hypertrophy or hyperplasia (Leiter and Chapman 1989). The identical mutation on a diabetes-susceptible background strain such as C57BL/KsJ, DBA/2J, SWR/J, and C3H.SW/SnJ results initially in insulin resistance with compensatory hyperinsulinemia followed by pancreatic beta cell loss and overt diabetes (Coleman 1982). The diabetes in these genetically obese animals is not autoimmune in origin (Leiter 1989) and is absolutely dependent upon the prerequisite obese phenotype since lean animals are metabolically euglycemic with normal plasma insulin concentrations (Leiter 1989).

Homozygosity for *db* on the diabetes susceptible BKS background, regardless of sex, results in severe hyperglycemia (>400 mg/dL), and glycosuria, and extreme hypoinsulinemia (10-100 μ U/ml). However, in other susceptible strains, sexual dimorphism is observed in diabetes susceptibility: variable degrees of protection are conferred by the female sex. In contrast to males, expression of the obesity mutations in females results in profound hyperinsulinemia (1000-2000 μ U/ml) which compensates for the peripheral insulin resistance to maintain a mild hyperglycemia (200 mg/dL) without glycosuria. The etiology of the increased insulin secretory capacity differs with background strain. Hyperplasia occurs in C57BL/6J animals while beta cell hypertrophy is the predominant mechanism in MA/MyJ (Leiter 1981, Leiter et al. 1981).

Leiter has shown that gonadal steroids play a role in modifying the diabetes-susceptibility in the murine models of genetic obesity (Leiter and Chapman 1989). Two enzymes determine the balance of activity of estrogenic and androgenic sex steroids: 1) sex steroid sulfotransferase which removes androgen prehormones, androgens and estrogens from the receptor active pools by sulfurylation (Leiter et al. 1991) and 2) a sex-linked steroid sulfatase which can activate these steroids by desulfation. In general, androgens increase NIDDM expression while estrogens are protective although the mechanisms for these effects have not yet been identified (Leiter and Chapman 1989). As noted, males are more susceptible to the diabetogenic effect of the obesity mutations than females, with inter-strain differences in diabetes-susceptibility due in part to reciprocal effects of strain-specific sex steroid sulfotransferase. In diabetes-susceptible BKS females segregating for the *db* mutation, Leiter has shown abnormally high levels of expression of the estrogen sulfotransferase and negligible expression of the DHEA sulfotransferase in females (a male-like pattern) which parallels the diabetes susceptibility of BKS females (Leiter and Chapman 1993, Leiter 1993). In contrast, B6 mice have four fold lower rates of estrogen sulfurylation, tilting the hepatic hormonal balance in favor of estrogenic over androgenic. In males, castration or estrogen administration prevents hyperglycemia and decreases beta cell loss without modifying the expression of obesity in C3HeB/FeChp but not BKS *db/db* mice (Leiter and Chapman 1989, Leiter 1989, Leiter et al. 1987, Leiter 1987). Similarly, ovariectomy eliminates the diabetes resistance of *db/db* C3HeB/FeChp females (Leiter and Chapman 1989).

Analogies can be made to the findings of increased concentrations of free testosterone in NIDDM-prone women (Andersson et al. 1994). Androgens increase insulin resistance, decrease glycogen synthase activity, and are associated with an increased incidence of NIDDM in women (Lindstedt et al. 1991). Androgen receptors are not required to mediate the diabetogenic effects of increased androgenicity as shown by the overt NIDDM which occurs in *db/db* XY mice simultaneously lacking functional androgen receptors (testicular feminization) but with normal concentrations of circulating androgens (Prochazka and Leiter 1991).

There are also interactions of obesity mutations with background strain for diabetes expression which may also be a result of altered hepatic steroid sulfotransferases. Unlike *ob/ob* and *db/db* mice on BKS, *fat/fat* mice on BKS are not diabetic. Absence of Cpe activity protects against the virilizing changes in hepatic sulfotransferase activity observed with expression of *ob* and *db* (Leiter and Chapman 1991). A^{vy} is associated with sexually dimorphic expression of diabetes on an VY/WfL background. When females are treated with dexamethasone, hyperglycemia occurs due to an induction of hepatic estrogen sulfotransferase with resultant “androgenization” of the female (Gill et al. 1994).

Obesity is not absolutely required to produce glucose intolerance in rodents. Transient hyperglycemia has also been demonstrated in nonobese C3H.SW/SnJ males which were stressed by group housing. This mild hyperglycemia which occurred between 4 and 8

months of age was accompanied by hyperinsulinemia and beta cell hypertrophy. The hyperglycemia could be blocked by castration, adrenalectomy or replacement of the C3H.SW/SnJ Y chromosome carrying steroid sulfohydrolase with the diabetes resistant C3H.SW/SnJ Y chromosome. Thus, glucose intolerance may be dependent in this model on adrenal secretions (glucocorticoids), testicular secretions (testosterone), and a Y linked gene such as steroid sulfolhydrolase (Leiter et al. 1988, Leiter 1988).

There are also complex genetic and environmental interactions of the obesity mutations. *db/db* BKS mice of both sexes fed a high protein diet free of refined carbohydrates or starches, demonstrated dramatic improvements in glycemia and islet morphology (Leiter et al. 1983). Diet composition rather than total caloric intake was the primary variable responsible for the phenotypic difference (Leiter et al. 1981). Quantification of islet cell type and hormone content in mice fed the high protein-no carbohydrate diet demonstrated doubling of the volume of alpha cells (glucagon producing) and almost 50% reduction in beta cells with similar changes in pancreatic hormone content (Morley et al. 1982). Therefore, the relief of glucose mediated stress on the beta cells with the no-carbohydrate diet may prevent diabetes development in the *db/db* mice. Non-mutant B6 mice fed a high fat, high simple carbohydrate diet become obese and demonstrate more diabetes and glucose intolerance than similarly treated A/J mice, suggesting that the B6 is not a completely diabetes-resistant mouse strain (Surwit et al. 1988) (see chapter 14).

There are also complex interactions of glucose homeostasis with aging. Non-mutant two year old B6 mice demonstrate increased islet size, pancreatic insulin content and glucose stimulated insulin release relative to 5 month old mice (Leiter and Premdas 1988). These rodents are useful models to study NIDDM because there are age and environmental modulators of diabetes expression, and each of these factors can be precisely controlled and altered as necessary.

Diabetes susceptibility has also been described in rat models of genetic obesity. The Zucker fatty rat (*fa/fa* on 13M strain) demonstrates extreme obesity, hyperinsulinemia (500-1000 μ U/ml) and glucose intolerance, yet remains euglycemic (Zucker and Antoniades 1972). The *fa* gene was backcrossed onto outbred carbohydrate-intolerant Wistar stock (Kyoto University). The male *fa/fa* WKY (WDF/Ta-*fa*) are phenotypically obese, hyperinsulinemic, and eventually hyperglycemic. Males develop moderate hyperglycemia (400 mg/dl) and extreme hyperinsulinemia (1200 μ U/ml) by 3-4 months of age (Kava et al. 1990, Ikeda et al. 1981). Urinary glucose excretion is over 3 g/day by 12 weeks of age. Plasma glucose concentrations continue to rise with age in the WKY rats (Koh et al. 1990). Increased insulin resistance/decreased glucose tolerance and decreased ability of WKY beta cells to survive the stress of insulin resistance and hyperglycemia probably account for the increased diabetes susceptibility of this strain. The underlying cause of the sexual dimorphism is unknown, but ovariectomy does not make females diabetic (Kava et al. 1992).

Zucker and WKY *fa/fa* rats also respond differently to adrenalectomy. Adrenalectomy ameliorates the severity of obesity in both Zucker and WKY *fa/fa* rats, and the glucose intolerance and hyperinsulinemia in the Zucker but not WKY rats (Turkenkope et al. 1991).

Clark and Palmer identified several Zucker *fa/fa* animals with unusually high concentrations of plasma glucose (Clark et al. 1983). From an initially outbred colony, Peterson inbred for this trait, producing a colony of rats (ZDF/Drt-*fa/fa*) segregating for *fa* on a 13M background in which most *fa/fa* males develop hyperglycemia (500 mg/dl) in the presence of relatively low (50-200 μ U/ml) concentrations of insulin (Peterson et al. 1990). The inbreeding regimen has apparently fixed diabetes-promoting alleles in this usually diabetes-resistant strain (13M). The *fa/fa* ZDF demonstrate more marked insulin resistance and decreased pancreatic insulin content than the standard *fa/fa* 13M animals. Thus expression of *fa* on three different background strains (13M, WKY, and ZDF) results in three different diabetes phenotypes in the setting of obesity.

Molecular Dissection of Quantitative Traits

The conflict between Mendelian particulate theory and the continuous variation observed for most biological traits was initially resolved by Johanssen's analysis of bean weight in various inbred lines (Johanssen and Uber 1903). He proposed a theory which included inheritance of multiple genetic factors, each with small effects and interactions with the

environment or each other to influence phenotype. Experiments on the assortment of oat and wheat seed color by Nilsson-Ehle supported the notion of multiple factors influencing quantitative variation of a single phenotypic trait (Nilsson-Ehle 1909). East performed the first intercross between two phenotypically divergent parental strains (inbred varieties of *Nicotiana longiflora* which differed in flower length). Each parental variety as well as the F1 progeny had a narrow distribution for the phenotype. The F1 progeny were intermediate between the two parental varieties. Intercrossing of the F1 progeny produced wide variation of flower lengths with much greater variance than that seen in the F1 progeny, indicating that the variation was not due to environmental factors alone. Selfing of the F2 progeny produced F3 progeny whose flower length was greatly dependent on that of the F2 (East 1969). Sewall Wright, using crosses between inbred strains of guinea pigs differing in number of digits, demonstrated that polygenes could act in an additive manner to influence discontinuous traits once thresholds had been surpassed due to cumulative susceptibility from multiple genes (Wright 1934). Wright also developed the mathematics required to estimate the number of factors likely to be responsible for a given trait in crosses such as those studied by East. Although Wright studied polydactyly in guinea pigs, the same principles are applicable to susceptibility for other traits.

For many years, little progress was made in quantitative genetics other than biometric analyses. With the development of restriction fragment length polymorphisms (RFLPs) and framework maps, accurate mapping of quantitative trait became feasible. Loci for

fruit mass, soluble solids and fruit pH in tomatoes was the first demonstration of systematic resolution of quantitative traits into Mendelian factors (Paterson et al. 1988). Until recently, the density of the genetic maps in rodents was not sufficient to allow identification or accurate localization of QTLs. The discovery of microsatellites of dinucleotide repeats (Saiki et al. 1988) and the ability to amplify these regions from genomic DNA using Taq polymerase (Saiki and Gelfand 1988) quickly expanded the number of highly informative genetic markers. The availability of 6000 polymorphic PCR based markers in the mouse (Dietrich et al. 1996) and 432 markers in the rat (Serikawa et al. 1992, Jacob et al. 1995) has made QTL mapping experiments feasible. In parallel, analytical techniques for interval mapping using maximum-likelihood linkage analysis, analyzing multiple pedigree structures, and non-parametric traits have been developed (Lander and Botstein 1989, Kruglyak and Lander 1995). With these advances, QTLs for many complex traits with relevance to human disease including obesity (Warden et al. 1993, Andersson et al. 1994, Horvat and Medrano 1995), hypertension (Jacob et al. 1991), insulin dependent diabetes mellitus (Ghosh 1993), seizure susceptibility (Rise et al. 1991), and substance abuse (Crabbe et al. 1994, Quock et al. 1994) have been mapped in other mammals. The likely genomic regions conveying susceptibility to multiple intestinal neoplasias (Mim-1) was identified first by QTL mapping (Dietrich et al. 1993) and the responsible gene (secretory phospholipase A2) then identified by the candidate gene approach (MacPhee et al. 1995).

In parallel with the development of genetic maps in rodents, the human genetic and physical maps have become increasingly dense, enabling more precise definition of homologous segments between mouse and man. Therefore, QTLs identified in rodents can immediately be tested for linkage in human pedigrees and genes or diseases mapped either in the rodent or human genomes can be quickly evaluated as potential candidates. Such mouse-human homologies allowed the evaluation of angiotensin converting enzyme in essential hypertension in humans based on QTL mapping of hypertension in rats (Jacob and Brown 1995, Jacob and Lindpaintner 1991). With the increasing number of anonymous cDNA's being mapped in the human genome (Matsubara and Okubo 1993), a rich source of genes with unknown functions will be available for evaluation based on their position within the genome.

The frontier of QTL mapping is actually cloning the genes responsible for the phenotypes observed. Because cosegregation of genotype with phenotype is not likely to be perfect, single meiotic recombination events cannot provide the same absolute resolving power as in qualitative single gene mutations. Rather, map resolution becomes a statistical argument requiring many more progeny and precise phenotypic assessment of the quantitative phenotype. Because any single QTL is responsible for only a fraction of the phenotypic variance, it is necessary to reduce to a minimum other interacting loci and environmental variation to facilitate cloning of the responsible gene. Progeny testing is the most definitive method for determining the genotype associated with a given phenotype; however, this approach is technically difficult if the rodents are infertile (as

obese mice often are). Even with such manipulations, the highest resolution to which a QTL has currently been mapped is 3 cM (Paterson 1995), an interval an order of magnitude larger than is readily and routinely attained for mapping a single gene affecting a qualitative phenotype. However, advances in the genomics infrastructure, including projections for completed human and mouse physical maps by the year 2000 and mapping of an increasing number of cDNAs in both species should greatly facilitate reverse genetic approaches to the molecular cloning of both simple and complex traits.

Chapter 2

Strategies for Molecular Mapping of Diabetes-Susceptibility Loci in Rodents

Development of Rodent Crosses

We have used the strain-related differences in diabetes susceptibility among genetically obese animals to identify the strain-specific genes responsible for diabetes-susceptibility or resistance. Parental strains widely divergent for susceptibility to obesity-related non-insulin dependent diabetes mellitus (NIDDM) were intercrossed, and the F2 animals at the most extreme phenotypes scanned with markers evenly spaced throughout the genome (Dietrich and Miller 1996). Initially, crosses previously generated for the purposes of fine mapping of *ob* and *db*, were used to identify diabetes-susceptibility QTL's. However, few data were available about the diabetes phenotypes of these animals other than fasting plasma [glucose] and [insulin] obtained at the time of sacrifice. Additional phenotypic data including pancreatic histology and pancreatic hormone content have provided a more complete clinical picture with clues about disease etiology, thus providing additional, quantitative subphenotypes for which responsible loci have been identified by molecular mapping techniques. Use of other strains in addition to B6, DBA, and CAST, the strains used in the intercrosses used to map and ultimately clone *ob* and *db*, provided additional genetic resources from which different loci for diabetes-susceptibility and resistance were identified based on the varying strain-related effects on phenotypes of interest. Use of multiple mouse and rat interstrain intercrosses segregating for different obesity mutations enabled confirmation of genome region specific effects on any phenotype.

The mouse crosses segregated either of four obesity mutations, *ob*, or *db*, *tub*, or *fat* on several inbred background strains (CAST/Ei, C57BL/KsJ, DBA/2J, C57BL/6J, and SM/Ckc) (Table 2). In each cross, the two background strains were selected to be widely divergent in susceptibility to NIDDM in the setting of obesity. The diabetes-susceptible strains were CAST/Ei (CAST), C57BL/KsJ (BKS), and DBA/2J (DBA), and the diabetes-resistant strains were C57BL/6J (B6), and SM/Ckc (Ckc) (Leiter 1981, Leiter, Coleman 1981).

Table 2 Genetic crosses for analysis of genes contributing to diabetes in genetically obese mice

Obesity mutation	NIDDM Resistant strain	NIDDM Susceptible strain
<i>db</i>	Ckc	BKS
<i>db</i>	B6	CAST
<i>db</i>	B6	DBA
<i>ob</i>	B6	DBA
<i>ob</i>	B6	CAST
<i>ob</i> ^{2j}	Ckc	BKS
<i>fa</i>	13M	WKY
<i>fat</i>	B6	CAST
<i>tub</i>	B6	CAST

The rat cross segregated the obesity mutation *fa* in a breeding scheme similar to the mice using diabetes-susceptible (WKY/N) or diabetes-resistant (13M) strains (Table 2). The fatty mutation originally arose on the 13M background (Zucker and Antoniades 1972), and obese animals are hyperinsulinemic and normoglycemic (Peterson and Shaw 1990). Animals homozygous for *fa* on the diabetes-susceptible strain WKY display sexually dimorphic diabetes phenotypes in which only the males are hyperglycemic and glycosuric. WKY males, paradoxically, have high plasma insulin concentrations in the presence of pathological hyperglycemia (Koh and Seino 1990).

Each mouse cross comprised two reciprocal crosses where possible to enable detection of any strain-specific effects of the sex chromosomes or maternally transmitted mitochondrial genes on diabetes expression. Male and female heterozygotes for a given obesity mutation were mated to opposite sex mice of the appropriate counterstrain to generate F1 animals. F1 progeny were randomly intercrossed to determine which animals were heterozygous for the obesity mutation by virtue of the production of phenotypically obese progeny.

The rat breeding scheme did not include reciprocal crosses because the most efficient method of generating F1 breeding pairs entailed the use of homozygous *fa/fa* parental males (*fa/fa* females are infertile). A fertile *fa/fa* male for the 13M strain was mated to

+/+ females of the appropriate counterstrain to produce F1 obligate heterozygotes. Intercrossing of obligate *fa*/+ F1 animals produced obese F2 progeny.

Classification of Obese F2 Progeny as NIDDM

Phenotypic characterization included weight, body mass index (weight/nasoanal length²), dipstick tests for glycosuria and ketonuria, fasting plasma glucose and insulin concentrations, red cell HbA1c, pancreatic islet morphology judged qualitatively by two experienced observers and quantitatively using image analysis of islet number and area, and pancreatic hormone content of insulin and glucagon normalized to pancreatic protein content. In the homozygous state, the obesity mutations *ob*, *db*, and *fatty* when expressed on a homogeneous genetic (strain) background produce a visually discernible obese phenotype by three weeks of age. However, in interstrain crosses segregating these obesity mutations, there was considerable variability in the development and degree of obesity within and between crosses. This variable degree of obesity was also scored as a quantitative trait. In this context, a particular QTL might have a direct effect on accumulation of fat or an indirect effect on weight due to weight loss secondary to glycosuria. Fasting plasma [insulin] and [glucose] are measures of insulin resistance and severity of diabetes, respectively. HbA1c is a measurement of glycosylated hemoglobin used to assess degree and duration of hyperglycemia over extended periods of time based on the non-enzymatic glycosylation of hemoglobin in the red blood cell. Longitudinal pancreatic sections were stained immunohistochemically for insulin and glucagon. Pancreatic morphology (islet size, number, and anatomical integrity) was scored using a 5-

point scale in order to quantify the homogeneity of functional beta cells within islets and degrees of hypertrophy and hyperplasia of the islets. Pancreatic hormone quantities were normalized to pancreatic protein content and were used to quantify the potential reserve of insulin in the pancreas and to quantify the amount of glucagon, an indicator of the number of alpha cells. The pancreatic studies in particular have provided additional quantitative traits for analysis and have elucidated part of the mechanism by which the pancreas is able to match the increased demands for insulin production, by either beta cell hyperplasia or hypertrophy.

Quantitative trait locus mapping

To minimize the number of animals which were genotyped in the initial screens of the crosses, phenotypic criteria were established independently in each cross to identify a small number of animals with the most extreme diabetes-related phenotypes for “selective genotyping.” A small subset of animals at least one standard deviation above or below various phenotypic means was identified for each cross. In most crosses, three groups of animals comprising 5-10% of the total progeny with extreme phenotypes were identified:

1. “Insulin-resistant, nondiabetic”: a non-diabetic, insulin resistant group with high fasting plasma [insulin], normoglycemia, and normal HbA1c; 2. “Diabetic”: a diabetic group with extremely low fasting plasma [insulin], hyperglycemia, and elevated HbA1c (>14%); and
3. “Protected pancreas”: a group with compensated pancreatic phenotypes of intact, hyperplastic, hypertrophic islets. A genome scanning experiment (outlined below) was then performed on this subset of animals at extremes of affection to identify regions of

the genome with increased sharing of alleles within any particular sub-group of extremely affected animals. This strategy of selective genotyping limited the number of genotypes which were performed and somewhat protected the data from false positive results because positive results from the genome scanning experiments were tested for replicability within the same cross. Loci with statistically significant deviations from the expected Mendelian 1:2:1 ratio of alleles were treated as quantitative trait loci and used to genotype all obese F2 progeny in the cross to detect correlations between genotype at the locus and phenotypic characteristic of diabetes susceptibility or resistance. This strategy identified loci involved in the pathogenesis of NIDDM within the setting of obesity, including genes mediating resistance to insulin-mediated effects on glucose transport and hepatic gluconeogenesis, and beta cell sufficiency in the face of increased demands imposed by insulin resistance. Using a similar approach, Coleman identified co-dominant alleles on mouse chromosome 9 near the *Mod-1* locus which influence diabetes-susceptibility in a C57BL/6J X C57BL/KsJ intercross segregating for the *db* mutation (Coleman 1992).

The resources to thoroughly scan the mouse and rat genomes are now readily available with over 6000 loci mapped in the mouse (Dietrich and Miller 1996) and 432 loci mapped in the rat (Serikawa and Kuramoto 1992, Jacob and Brown 1995). Microsatellite maps with markers spaced throughout the genome and highly polymorphic between rodent strains are commercially available. Gene order is highly conserved between mouse and rat, permitting rapid initial positioning of rat genes based on the mouse map. The large

mapping resource has already been utilized to identify susceptibility loci for autoimmune diabetes in the non-obese diabetic mouse (Todd 1992), epilepsy in mice (Rise and Frankel 1991), and hypertension in rats (Jacob and Lindpaintner 1991). The high degree of synteny homology among the mouse and rat and human genomes (Nadeau et al. 1992) has facilitated identification of loci which are being tested for linkage in human pedigrees segregating NIDDM.

Using commercially available primer pairs (MapPairs, Research Genetics) which amplify polymorphic simple sequence repeats (SSR's) in the mouse or rat genome when used in the polymerase chain reaction, genomic "scans" of the animals identified in the diabetic, insulin resistant non-diabetic, and protected pancreas groups of each cross were performed. The primer pairs were selected to be polymorphic between the two parental strains and spaced approximately 15-20cM apart for a total of 80-120 markers to encompass the entire genome (~1500 cM). The size polymorphisms in the PCR products are known for the B6, CAST, and DBA strains, but unpublished for the other strains which were employed. Therefore, the first step was to develop a set of primer pairs for each cross which was polymorphic between the two inbred parental strains for both the mice and the rats.

Once candidate intervals were identified in the initial genome scan, a finer map of the region was generated utilizing all primer pairs polymorphic within the interval to locate the locus with the most significant deviation of genotypic frequencies from those

expected based upon Mendelian segregation of parental alleles. Markers which demonstrated the most significant deviations were used to test for segregation distortion by genotyping 50 lean progeny from the same cross. Candidate regions were analyzed by chi-square analysis for deviations from expected genotypic frequencies (based upon lean progeny) in both the diabetic, insulin resistant/non-diabetic groups, and protected pancreatic groups. The loci which had the most significant influence on a phenotype might be expected to demonstrate non-Mendelian segregation in more than one of the phenotypically extreme groups. The most influential loci demonstrated enrichment of the respective parental alleles in the diabetic versus the insulin resistant groups. Although the crosses were designed on the premise that the diabetic progeny would be enriched for alleles from the diabetes susceptible parental strain and the non-diabetic/insulin resistant group alleles from the diabetes-resistant strain, such an association was not always observed and indicated that other loci within the genome mask the effects of these loci within the inbred parental strains. The phenotype of the animals then appears to “transgress” its genotype at a particular locus. Such apparent discrepancies are resolvable once sufficient data are available regarding the molecular mechanisms by which the interacting genes produce the phenotype under study.

Diabetes phenotype-associated loci with the greatest deviation from expected Mendelian ratios without segregation distortion in the leans were used to genotype all obese F2 animals. These candidate loci were treated as quantitative trait loci with continuous variation in multiple phenotypes. Analysis of variance was used to compare measures of

plasma glucose, plasma insulin, HbA1c, pancreatic hormone content, and beta cell morphology between the three genotypic classes at each candidate locus. Gender, age, bmi, and genotype at other loci were treated as covariates in the ANOVA. MapMaker QTL was also used to determine the most likely interval containing the QTL, to eliminate unlikely genetic models of inheritance (dominant, recessive, and additive), and to estimate the percentage of the variance attributable to any single QTL.

For some intervals containing genes which strongly influence a particular diabetes phenotype in multiple crosses, regions of human synteny have been identified and are now being analyzed in linkage (sib pair) studies of the Pima Indians. The phenotypes being examined for linkage include acute insulin release, insulin sensitivity, onset of NIDDM, BMI, and body fat content.

Support Intervals for NIDDM Loci which Integrates Genes

For candidate loci which demonstrated significant association between genotype and NIDDM phenotype, the support interval originally consisting of only SSR's was in some cases expanded to include genes, especially those which might be considered candidate genes in the pathogenesis of NIDDM. Such genes were mapped relative to the SSR's and also the NIDDM phenotype. Polymorphisms between progenitor strains were assessed using single stranded conformational polymorphism (SSCP), by restriction digestion of PCR products generated from introns and 3' untranslated regions, and in some cases by

direct sequencing. Mapping anonymous DNA sequences relative to flanking genes enabled localization of candidate loci in other species of rodents and ultimately in humans.

Introgression of diabetes-susceptibility intervals

As the first step toward molecular cloning a quantitative trait locus, it was necessary to define the genetic interval in which the locus resided. The interval containing the QTL was initially defined as the interval ± 1 LOD score from the highest peak of any marker analyzed by QTL analysis. By a series of backcrosses to the diabetes-resistant parental strain, the obesity mutation (*ob* and *db*) and the QTL were maintained in the heterozygous state. Markers at 5 cM intervals spanning the interval were genotyped on all animals to ensure homogeneity of heterozygosity through the interval (i.e. absence of intercurrent meiotic recombination). After a series of five backcrosses (N6) to the recurrent strain, the genome is 98.5% homogeneous for alleles from the recurrent strain with the exception of regions around the obesity mutation and the QTL which have been maintained heterozygous. Breeders were intercrossed at each backcross generation beginning with the third backcross to follow the diabetes phenotypes with progressive elimination of other parts of the genome which might introduce additional phenotypic variance. Breeders from the N6 generation are being bred to produce obese progeny. If the QTL interval has been correctly identified and acts largely or totally independently of other loci to influence phenotype, the obese N6F1 progeny should segregate a specific diabetes phenotype in a Mendelian fashion. The mode of inheritance and the phenotype of interest may be predicted from studies of the F2 and F3 progeny, but may not confirm

expectations if the QTL's effect was influenced by independently segregating loci which were not accounted for in the original analysis. Extensive phenotypic characterization of the N6F1 progeny may be necessary to identify a phenotype which would then segregate as a qualitative trait on a relatively homogenous genetic background rather than a quantitative trait on a heterogenous genetic background. If precise phenotypes can be identified which meet these criteria, cloning the gene responsible for the quantitative trait will be no more difficult than cloning any other single gene mutation which produces a reproducible, highly penetrant phenotype. Large intercrosses would then be established to narrow the interval containing the quantitative trait gene (now segregating as a qualitative trait) until the interval is sufficiently narrow to identify and test candidate genes or to construct a series of overlapping genomic clones across the interval for reverse genetic techniques to identify the gene such as exon trapping or cDNA selection, especially if the phenotype for the trait indicates a defect in a specific tissue.

Chapter 3

Materials and methods

Animal Breeding and Care

Parental B6, DBA, BKS, CAST, and Ckc mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). The strain upon which the obesity mutation was carried in the various intercrosses is indicated below:

Obesity Mutation	Parental strain carrying the obesity mutation	Counterstrain
<i>ob</i>	B6	DBA
<i>db</i>	B6	DBA
<i>ob</i>	B6	CAST
<i>db</i>	B6	CAST
<i>ob^{2J}</i>	Ckc	BKS
<i>db</i>	BKS	Ckc
<i>fat</i>	BKS	CAST
<i>tub</i>	B6	CAST
<i>fa</i>	13M	WKY

The animal crosses were begun before the specific mutations for *ob* and *db* had been characterized and therefore relied heavily on test crosses. Lean mice heterozygous for the appropriate obesity mutation were crossed to +/+ mice from the appropriate

counterstrain. F1 progeny were test crossed to identify heterozygotes. F1 mice which had produced obese progeny were then intercrossed to generate F2 progeny.

The alternate breeding scheme for the rats was discussed in the previous chapter.

Markers closely flanking the obesity mutations were used to identify heterozygous F2 breeders to generate F3 progeny.

Contemporaneous controls of *ob/ob* on B6, *db/db* on BKS, and *ob^{2J}/ob^{2J}* on Ckc were produced by mating the appropriate heterozygotes. These *ob/ob* B6, *db/db*, BKS, and *ob^{2J}/ob^{2J}* Ckc controls were housed and treated in a manner identical to the outcrossed animals and provided an internal control for environmental factors which may differ among animal facilities or within the Rockefeller animals facility over time. Phenotypic data on the contemporaneous controls are provided below:

Strain	Obesity phenotype	Sex	Age (days)	Plasma [glucose] mg/dl	Plasma [insulin] μ U/ml	HbA1c (%)	Glycosuria
B6	+/+		92	219	6.0		
DBA	+/+		94	204	15.4		
F1 B6DBA	+/+		95	208	15.5		
KSJ	<i>db/db</i>	female	~16 weeks			13.5	ketonuric, glucosuric
KSJ	<i>db/db</i>	male	~16 weeks			13.8	ketonuric, glucosuric
KSJ	<i>db[?]/+</i>	female	~16 weeks			2.7	non-glycosuric
KSJ	<i>db[?]/+</i>	male	~16 weeks			2.8	non-glycosuric
B6	<i>ob/ob</i>		~16 weeks			7.0	non-glycosuric
B6	<i>db/db</i>		~16 weeks			6.2	non-glycosuric
Ckc	<i>ob^{2J}/ob^{2J}</i>	female	~16 weeks			3.3	non-glycosuric
Ckc	<i>ob^{2J}/ob^{2J}</i>	male	~16 weeks			5.3	non-glycosuric

The F2 offspring were weaned at 3 weeks of age. Males and females were separated and raised in sibling groups. Rodent chow (Pico Lab mouse breeding chow 5058 [9% fat] Purina Mills St. Louis, Missouri) and water were provided ad libitum; light-dark cycle was 12 on, 12 off; ambient temperature 25°C. The colony was consistently negative for MHV, Mycoplasma, and parasitic infections as indicated by sentinel mice. The obese phenotype could be discerned at approximately the time of weaning; therefore, at four weeks of age obesity phenotype was scored and only the obese progeny and a single same sex lean littermate control were retained.

Animals were sacrificed by carbon dioxide asphyxiation at approximately 120 days of age. Sacrifice was performed between 1000 and 1200 hours after a 3 hour fast. Following sacrifice, one lean animal and all obese littermates had approximately 1.0 ml of whole blood withdrawn by cardiac puncture into a micro-centrifuge tube containing 50 µl of 82 µM EDTA as anti-coagulant and 15-30 trypsin inhibitory units/ml aprotinin as a protease inhibitor. The plasma was decanted and frozen at -80°C for subsequent radioimmunoassay of insulin (Herbert et al. 1965) and glucose oxidase determination of glucose (Kadish et al. 1968). The red blood cell pellet was used for quantifying glycohemoglobins using the Glyc-Affin GHb assay (Isolab, Inc, Akron, Ohio) (Abraham et al. 1983, Fairbanks 1983, Willey et al. 1984). Urine samples were tested for degree of glycosuria using Chemstrip uGK urine test strips for glucose and ketones (Boehringer Mannheim; Indianapolis, Indiana). Glucose was detected on the strip using the glucose

oxidase/oxidase method (Keston 1956, Comer 1956, Free et al. 1957). Ketones were detected when sodium nitroferricyanide and glycine reacted with acetoacetate and acetone to form a violet dye complex which was visualized on the strip (Chertack, 1958). For all progeny, sex, weight and nasoanal length were determined. Body mass index (BMI) $[(\text{wt(g)}/\text{length (cm)}^2)]$ was used to estimate the degree of adiposity. The pancreas was removed and split longitudinally. Half of the pancreas was immediately frozen at -80°C for subsequent determination of pancreatic hormone and protein content. The other half was fixed in a modified Bouin's solution (750 ml saturated aqueous picric acid, 250 ml glacial acetic acid, and 50 ml 40% formalin) for 6 hours. Liver, spleen, kidney, tail, and thorax contents were immediately removed and frozen at -80°C as a source of genomic DNA.

Pancreatic phenotypic characterization

Pancreata for histological studies were washed with 70% ethanol following fixation with picric acid until leaching of picric acid from the tissue was minimal. Tissues were processed through the following solutions for 25 minutes each: 80% ethanol X 1, 95% ethanol X 3, 100% ethanol X 3, and xylene X 3 before saturation with paraffin. Tissues were then embedded in blocks of paraffin, and 5 mm sections were mounted on adhesive slides. Tissue sections were processed by removing the paraffin with xylene and rehydration through a series of decreasing ethanol solutions. Sections were immunohistochemically stained using the avidin-biotin immunoperoxidase technique (Hsu et al. 1981, Hsu et al. 1981). Endogenous peroxidases were inhibited by treating the

sections with 0.3% hydrogen peroxide in methanol for 30 minutes. To minimize non-specific staining, sections were incubated for 20 minutes with diluted normal serum for the species from which the secondary antibody was derived. Sections were incubated overnight with a 1:3000 dilution of primary polyclonal guinea pig anti-porcine insulin antibody (DAKO, Carpinteria, California) or a 1:1500 dilution of polyclonal rabbit anti-human glucagon antibody (DAKO, Carpinteria, California) followed by incubation with either an anti-guinea pig or anti-rabbit secondary antibody bound to biotin. An avidin/horseradish peroxidase complex was then allowed to bind to the secondary antibody. Diaminobenzidine tetrahydrochloride (DAB) was then reacted with the peroxidase to form an insoluble brown substance specifically at the sites at which the primary antibody originally bound. Hematoxylin was used as a counterstain.

Each pancreatic section was independently graded by two observers into one of five grades using the following criteria (Figure 3):

Grade 1: No evidence of β cell destruction, completely intact islets. Presence of dramatic b cell hypertrophy and islet hyperplasia.

Grade 2: Dramatic β cell hypertrophy and islet hyperplasia and some evidence of islet destruction (<10% of the islets) with a minority of the constituent β cell or affected islets lacking insulin immunostaining.

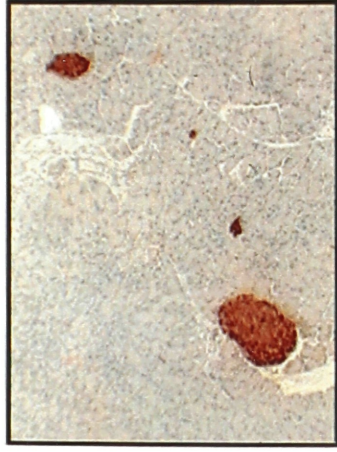
Grade 3: Moderate hypertrophy and/or hyperplasia. Significant but not severe destruction of islet architecture (10-75% of islets affected).

Grade 4: Greater than 75% of the islets demonstrating fragmentation with destruction of at least 30% of the β cell in these islets. Hypertrophy and/or hyperplasia not necessarily present.

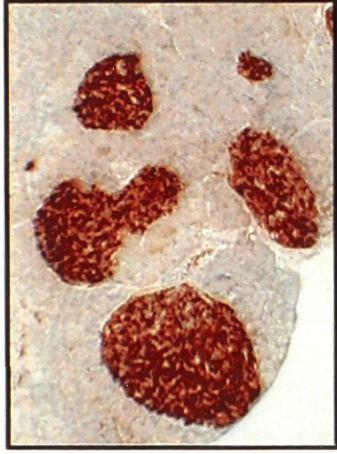
Grade 5: Complete islet destruction. Only scattered single β cell remaining. Fibrosis and neovascularization may be present.

Figure 3. Pancreatic grade in *ob/ob* and *db/db* mice. Sections immunohistocheically stained for insulin.

Pancreatic Grading in Genetically Obese Mice



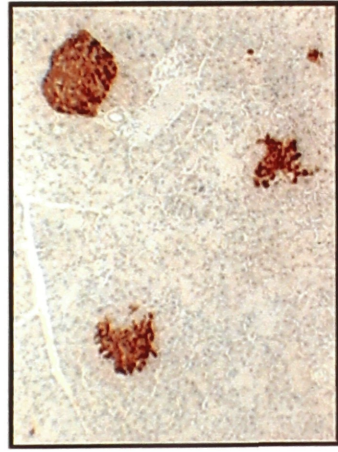
Lean



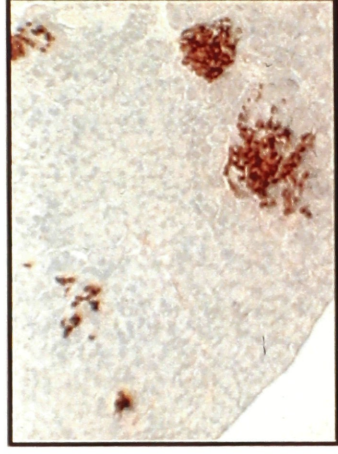
Obese Grade 1



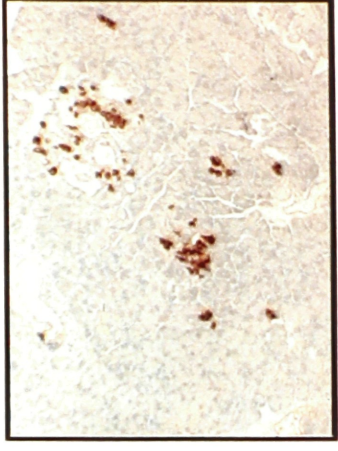
Obese Grade 2



Obese Grade 3



Obese Grade 4



Obese Grade 5

In addition, pancreatic histology was quantitatively analyzed using an image analysis system running Image Pro Plus (version 1.3). Single frames encompassing an entire pancreatic section were captured on the screen and converted to a gray scale enabling rapid quantitation of the number of islets (defined as any collection of DAB positive cells occupying an area greater than $60\ \mu\text{m}^2$), the area of each islet, and the total area of the pancreatic section.

Pancreatic hormones were extracted with acid-ethanol and precipitated with alcohol ether (Karam and Grodosky 1962). Protein content of the pancreatic extracts was determined by the Lowry procedure using a modified biuret reagent (Ohnishi and Barr 1978). Pancreatic insulin and glucagon concentrations were then determined by radioimmunoassay (Herbert and Lau 1965).

Genetic characterization

Genomic DNA was isolated using standard techniques in which homogenized tissue (usually kidney) was digested with proteinase K and subsequently extracted with phenol and chloroform to remove proteinaceous material (Amar et al. 1985). All animals were originally genotyped with markers flanking *ob* to ensure that animals which had been identified as phenotypically obese were in fact genotypically *ob/ob*. Once the mutation in *ob^{lj}* had been identified (Zhang and Proenca 1994), a mutation-specific assay was used to confirm that all phenotypically obese animals were *ob/ob* and all lean animals were either *+/+* or *ob/+* (Chung et al. 1995).

Polymerase chain reaction (PCR) was used to detect allele size differences for simple sequence repeats (SSRs). PCR reactions were performed in 20 μ l reaction volumes containing 5 ng/ μ l genomic DNA, 0.2 μ M of both forward and reverse primers, 200 mM dNTPs, 1x reaction buffer consisting of 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1 mg/ml gelatin, and 1 unit Taq polymerase. Thermocycling conditions, unless indicated otherwise, consisted of 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Polymorphic molecular markers and their positions were identified by inspection of microsatellite maps available from the Whitehead Institute (Copeland et al. 1993, Dietrich et al. 1994, Lander and Research 1995). Alleles for B6 and DBA were distinguished by size variation on 3% agarose/ 2% low melt agarose gels stained with ethidium bromide if the polymorphism was larger than 12 base pairs (bp). For SSR's requiring greater resolution than attainable in an agarose medium, 100 pmole of both forward and reverse primer were end-labeled in a 25 μ l reaction volume containing 10 μ Ci γ ^{32}P ATP, 1X polynucleotide kinase buffer (New England Biolabs, Beverly, Massachusetts), and 20 units polynucleotide kinase for 30 minutes at 37°C. PCRs were performed in 20 μ l volumes containing 2 pmole of both forward and reverse end-labeled primers in an otherwise standard PCR reaction. Radiolabeled PCR products were electrophoresed on a 43 cm non-denaturing 6% polyacrylamide gel, dried, and exposed to film.

Markers considered to show statistically significant linkage to phenotypes of interest (see below) on the genome scanning experiment were then used to genotype all obese progeny determine the correlation between genotype and all aspects of phenotype and any locus interactions.

Statistical analysis

Selected genotypes across the genome were analyzed by chi-square analysis of deviation of the observed results from the expected 1:2:1 ratio for any autosomal locus unlinked to the obesity mutation. Markers with a chi square greater than 6.0 ($p < 0.05$) were tested further as described below.

MapMaker QTL (version 1.1, 1993) was used to analyze chromosomal segments for which three or more markers had been typed on a majority of the obese progeny. All phenotypic parameters were tested under all modes of inheritance (free, additive, dominant, recessive).

Analysis of variance (ANOVA) between groups defined by genotypic class and sex for the dependent variables BMI, fasting plasma [glucose], and fasting plasma [insulin], HbA1c, pancreatic grade, pancreatic [insulin], pancreatic [glucagon], pancreatic [insulin]/[glucagon], number of islets, number of hyperplastic islets, average islet size, and islet area as a percentage of total pancreatic area was performed using the BMDP (7D,

2V) statistical package with post hoc analysis using the Bonferroni test (Dixon et al. 1988). Interactions among selected loci were also tested by analysis of variance.

Chapter 4

Identification of diabetes susceptibility regions

in

F2 B6DBA and DBAB6 segregating for *ob*

Selective genotyping selection criterion

Forty-six F1 B6xDBA (*ob/+*) dams were mated to produce a total of 358 obese offspring (195 females and 163 males) which were sacrificed at the age of approximately 110-120 days. Some obese mice died prematurely (<5%) and were extremely diabetic males (5+ urine dipstick tests for glycosuria). Similarly, 12 F1 DBAxB6 (*ob/+*) dams were mated to produce a total of 46 obese progeny (28 females and 18 males).

Twelve *ob/ob* mice were initially classified as insulin-resistant/non-diabetic (fasting plasma [glucose] < mean [glucose] of the lean animals [317 mg/dl]; fasting plasma [insulin] > one standard deviation above the obese mean [378 μ U/ml], and non-glycosuric) and twelve other mice as diabetic (fasting plasma [glucose] > one standard deviation above the obese mean [899 mg/dl]; fasting plasma [insulin] < 40 μ U/ml; maximal glycosuria; prolonged and or severe disease indicated by HbA1c > 14%; and pancreatic [insulin]/[glucagon] < 0.1) (Table 3). Both groups were selectively genotyped with a panel of 91 polymorphic markers located throughout the genome (Table 4). Subsequently, twelve mice (some of which had also been designated earlier as insulin-resistant/non-diabetic) were designated as having pancreatic morphology and hormone content which was protective against diabetes, protective pancreas (pancreatic grade < 2 and pancreatic [insulin] >1000 mU/mg protein) and were genotyped for a total of 133 SSR's throughout the genome (Table 5).

ID	Age (days)	sex	Bodyweight (g)	BMI (g/cm ²)	urine	Hba1c (%)	glucose (mg/dl)	insulin (uU/ml)	Pancreatic grade	Pancr [Insulin] (uU/mg protein)	Pancr [glucagon] (uU/mg protein)	Pancr [Insulin]/[glucagon]
Protected pancreases												
103	132	F	58.2	0.528	0.0	2.8	409	925.5	1.0	123053	93381	1.32
121	109	F	55.7	0.557	ND	2.5	406	616.7	1.0	127804	84245	1.52
202	96	F	68.3	0.620	0.1	4.8	318	301.6	1.0	117844	71654	1.64
246	103	F	52.4	0.557	0.5	3.7	232	316.0	1.0	132348	75974	1.74
259	115	F	67.2	0.633	0.0	4.3	373	612.6	1.0	109653	84744	1.28
323	114	F	66.3	0.613	ND	4.8	363	646.0	1.3	118203	77246	1.53
501	112	F	62.1	0.583	ND	4.6	366	151.6	1.5	105571	44359	2.38
540	115	F	56.8	0.591	0.1	3.7	264	162.8	1.5	146192	44281	3.30
116	125	F	70.1	0.636	ND	2.2	273	438.8	1.0	213657	70996	3.01
148	120	F	66.3	0.590	0.0	4.0	282	614.5	1.0	357278	115216	3.10
175	123	F	79.3	0.719	0.0	4.2	257	538.4	1.0	181051	128063	1.41
176	101	F	76.0	0.617	0.0	4.1	226	542.1	1.0	104526	66478	1.57
Mean	114		64.9	0.602	0.1	3.8	316	489.1	1.1	152848	79718	1.98
Insulin-resistant/non-diabetic												
118	125	F	70.1	0.636	ND	2.2	273	438.8	1.0	213657	70996	3.01
136	122	F	59.6	0.541	0.0	4.8	284	442.7	1.0	77888	66278	1.18
148	120	F	66.3	0.590	0.0	4.0	282	614.5	1.0	357278	115216	3.10
175	123	F	79.3	0.719	0.0	4.2	257	538.4	1.0	181051	128063	1.41
217	94	F	60.5	0.605	1.0	5.3	217	552.2	1.3	79405	105632	0.75
255	101	F	76.0	0.617	0.0	4.1	226	542.1	1.0	104526	66478	1.57
284	115	M	59.6	0.584	0.0	6.3	279	634.5	1.5	16743	25163	0.67
291	115	M	71.9	0.652	0.0	5.1	268	891.4	1.5	65088	54594	1.19
382	97	F	52.3	0.580	0.1	3.9	266	530.0	1.8	36344	83513	0.44
504	107	F	66.8	0.606	ND	1.5	224	364.3	1.3	66519	49386	1.35
535	122	F	64.3	0.594	0.0	5.2	224	540.5	1.0	45955	27561	1.67
Mean	113		66.1	0.611	0.1	4.2	255	555.4	1.2	113132	72060	1.48
Diabetic												
112	132	M	59.1	0.591	5.0	14.2	962	25.2	4.0	7878	99101	0.08
122	133	F	57.3	0.573	5.0	15.1	900	21.8	4.0	3983	173139	0.02
124	125	M	53.7	0.595	5.0	17.0	944	11.0	4.0	20008	275571	0.07
173	115	F	51.7	0.538	5.0	14.9	988	35.5	5.0	1006	100335	0.01
183	114	M	53.7	0.537	5.0	14.1	1022	30.8	4.0	3559	77196	0.05
197	103	M	21.5	0.271	5.0	17.9	1016	26.5	5.0	1932	51065	0.04
211	100	F	33.5	0.414	5.0	16.0	950	33.3	4.3	2924	83803	0.03
285	115	M	52.3	0.523	5.0	14.9	1111	25.7	4.3	3569	38900	0.09
290	114	M	50.1	0.522	5.0	19.5	949	30.3	4.0	2495	35986	0.07
299	107	M	42.0	0.496	5.0	15.8	943	32.3	4.0	1724	36638	0.05
312	110	F	48.4	0.484	5.0	17.0	1051	16.4	4.0	1039	44581	0.02
Mean	115		47.6	0.504	5.0	16.0	985	26.3	4.2	4556	82392	0.05

Table 3. Phenotypic characteristics of obese animals used in each genome scanning experiment of the F2 B6DBA and DBAB6 segregating for *ob*. The average for the group for each phenotypic parameter.

Table 4. Genome scanning results for the eleven *ob/ob* F2 B6DBA or DBAB6 mice with extreme insulin resistance and eleven *ob/ob* mice with severe diabetes. B6/B6 indicates homozygosity for B6 alleles, B6/DBA for heterozygosity and DBA/DBA homozygosity for DBA alleles at a particular locus. Total indicates the total number of animals genotyped for a particular marker. All chi square >6 are boxed and are significant at $p<0.05$. All markers are listed in order along the chromosome from centromere to telomere.

MARKER	OBESE, INSULIN RESISTANT					OBESE, DIABETIC				
	B6/B6	B6/DBA	DBA/DBA	TOTAL	CHI SQUARE	B6/B6	B6/DBA	DBA/DBA	TOTAL	CHI SQUARE
D1Mit70	2	9	0	11	5.18	2	6	3	11	0.27
D1Mit79	4	6	1	11	1.73	1	4	6	11	5.36
D1Mit76	8	2	1	11	13.36	4	2	4	10	3.60
D1Mit24	5	5	1	11	3.00	1	4	6	11	5.36
D1Mit80	5	5	1	11	3.00	1	5	5	11	3.00
D1Mit84	5	5	1	11	3.00	1	5	4	10	1.80
D1Mit90	5	5	1	11	3.00	1	4	6	11	5.36
D1Mit94	5	5	1	11	3.00	1	4	6	11	5.36
D1Mit108	5	6	0	11	4.64	2	4	5	11	2.45
D1Mit110	8	3	0	11	13.91	2	5	4	11	0.82
D2Mit80	2	5	4	11	0.82	4	4	3	11	1.00
D2Mit61	1	6	4	11	1.73	3	6	2	11	0.27
D2Mit12	0	5	6	11	6.64	3	6	2	11	0.27
D2Mit42	0	4	6	10	7.60	2	7	2	11	0.82
D2Mit43	0	5	6	11	6.64	3	6	2	11	0.27
D2Mit58	0	5	6	11	6.64	2	7	2	11	0.82
D2Mit17	0	6	5	11	4.64	2	7	2	11	0.82
D3Mit62	3	5	2	10	0.20	1	7	3	11	1.55
D3Mit65	3	7	1	11	1.55	1	8	2	11	2.45
D3Mit51	2	7	2	11	0.82	1	7	3	11	1.55
D3Mit12	3	6	2	11	0.27	1	7	3	11	1.55
D3Mit17	2	6	3	11	0.27	2	5	4	11	0.82
D4Mit89	5	2	4	11	4.64	3	3	5	11	3.00
D4Mit76	3	7	1	11	1.55	2	4	5	11	2.45
D4Mit54	3	6	2	11	0.27	2	5	4	11	0.82
D4Mit42	4	7	0	11	3.73	2	5	4	11	0.82
D5Mit79	2	7	2	11	0.82	1	6	4	11	1.73
D5Mit65	0	8	2	10	4.40	2	7	2	11	0.82
D5Mit99	2	8	1	11	2.45	3	7	1	11	1.55
D6Mit49	7	0	0	7	21.00	11	0	0	11	33.00
D6Mit9	11	0	0	11	33.00	7	4	0	11	9.73
D7Mit57	3	6	2	11	0.27	2	8	1	11	2.45
D7Mit84	1	5	3	9	1.00	3	5	2	10	0.20
D7Mit100	3	6	2	11	0.27	3	6	2	11	0.27
D7Mit12	3	6	2	11	0.27	1	8	2	11	2.45
D8Mit3	2	3	6	11	5.18	2	8	1	11	2.45
D8Mit4	2	3	6	11	5.18	5	5	1	11	3.00
D8Mit69	3	2	6	11	6.09	5	6	0	11	4.64
D8Mit25	3	3	5	11	3.00	5	6	0	11	4.64
D8Mit31	3	4	4	11	1.00	5	5	0	10	5.00
D8Mit45	3	5	1	9	1.00	6	5	0	11	6.64
D8Mit86	4	4	0	8	4.00	4	2	0	6	6.00
D9Mit64	3	8	0	11	3.91	3	5	3	11	0.09
D9Mit71	2	9	0	11	5.18	1	7	3	11	1.55
D9Mit10	0	9	2	11	5.18	1	7	3	11	1.55
D9Mit18	1	8	2	11	2.45	1	7	3	11	1.55
D10Mit51	2	7	2	11	0.82	3	6	2	11	0.27
D10Mit42	1	7	1	9	2.78	4	5	2	11	0.82
D10Mit70	1	7	3	11	1.55	5	4	1	10	3.60

MARKER	OBESE, INSULIN RESISTANT					OBESE, DIABETIC				
	B6/B6	B6/DBA	DBA/DBA	TOTAL	CHI SQUARE	B6/B6	B6/DBA	DBA/DBA	TOTAL	CHI SQUARE
D11Mit78	3	4	4	11	1.00	1	5	5	11	3.00
D11Nds9	2	6	2	11	0.45	3	6	1	11	1.18
D11Mit39	2	5	4	11	0.82	3	6	2	11	0.27
D11Mit41	1	5	3	9	1.00	2	5	0	7	2.43
D11Mit99	2	7	2	11	0.82	4	5	1	10	1.80
D11Mit103	1	5	1	7	1.29	5	2	2	9	4.78
D11Mit104	1	3	3	7	1.29	6	3	2	11	5.18
D12Mit12	3	5	3	11	0.09	1	8	2	11	2.45
D12Mit62	4	3	4	11	2.27	1	7	3	11	1.55
D12Mit17	3	6	2	11	0.27	0	7	4	11	3.73
D13Mit17	1	5	5	11	3.00	4	4	3	11	1.00
D13Mit11	1	2	7	10	10.80	4	6	0	10	3.60
D13Mit9	2	1	8	11	13.91	5	6	0	11	4.64
D13Mit69	2	1	8	11	13.91	3	8	0	11	3.91
D13Mit77	2	2	7	11	9.00	2	9	0	11	5.18
D13Mit76	2	2	7	11	9.00	3	8	0	11	3.91
D13Mit35	2	2	7	11	9.00	3	6	1	10	1.20
D14Mit54	2	6	3	11	0.27	3	7	1	11	1.55
D14Mit30	1	6	1	8	2.00	2	7	2	11	0.82
D14Mit28	1	5	5	11	3.00	2	6	3	11	0.27
D14Mit39	1	5	5	11	3.00	2	6	3	11	0.27
D14Mit7	2	3	6	11	5.18	2	5	4	11	0.82
D14Mit35	2	3	6	11	5.18	2	5	4	11	0.82
D14Mit75	2	3	6	11	5.18	0	8	3	11	3.91
D15MIT12	4	6	1	11	1.73	2	5	4	11	0.82
D15Mit5	3	7	1	11	1.55	1	6	4	11	1.73
D15Mit34	3	6	1	10	1.20	1	7	3	11	1.55
D16Mit12	2	7	2	11	0.82	6	4	1	11	5.36
D16Mit5	3	5	3	11	0.09	6	4	1	11	5.36
D16Mit50	4	3	1	8	2.75	6	4	1	11	5.36
D17Mit46	3	4	1	8	1.00	3	3	1	7	1.29
D17Mit39	3	5	3	11	0.09	3	6	2	11	0.27
D18Mit17	3	5	3	11	0.09	1	7	3	11	1.55
D18Mit50	3	4	4	11	1.00	2	4	5	11	2.45
D18Mit48	4	2	5	11	4.64	2	4	5	11	2.45
D19Mit28	0	6	5	11	4.64	2	5	4	11	0.82
D19Mit13	0	8	3	11	3.91	2	4	5	11	2.45
D19Mit19	1	8	2	11	2.45	2	5	4	11	0.82
D19Mit10	1	7	3	11	1.55	1	7	3	11	1.55
D19Mit36	3	5	2	10	0.20	1	8	1	10	3.60
D19Mit1	4	5	2	11	0.82	1	8	2	11	2.45
D19Mit34	4	5	2	11	0.82	1	8	2	11	2.45
DXMit89	4	6	1	11	1.06	4	1	5	10	1.55
DXMit105	4	6	1	11	1.06	3	2	6	11	2.92
DXMit37	4	5	2	11	0.11	4	1	6	11	2.69
DXMit182	4	5	2	11	0.11	4	1	6	11	2.69

Table 5. Genome scanning results for the twelve *ob/ob* F2 B6DBA or DBAB6 mice with pancreata mostly likely to protect against diabetes. B6/B6 indicates homozygosity for B6 alleles, B6/DBA for heterozygosity and DBA/DBA homozygosity for DBA alleles at a particular locus. Total indicates the total number of animals genotyped for a particular marker. All chi square >6 are boxed and are significant at $p < 0.05$. All markers are listed in order along the chromosome from centromere to telomere.

MARKER	Protected Pancreas				CHI SQUARE
	B6/B6	B6/DBA	DBA/DBA	TOTAL	
D1Mit70	2	8	2	12	1.33
D1Mit300	6	4	2	12	4.00
D1Mit24	3	8	1	12	2.00
D1Mit84	3	8	1	12	2.00
D1Mit90	2	8	2	12	1.33
D1Mit110	6	6	0	12	6.00
D1Mit56	4	3	1	8	2.75
D2Mit80	6	2	4	12	6.00
D2Mit61	1	4	7	12	7.33
D2Mit42	0	4	8	12	12.00
D2Mit17	0	4	8	12	12.00
D2Mit423	0	4	4	8	4.00
D2Mit229	2	4	6	12	4.00
D3Mit62	3	8	1	12	2.00
D3Mit65	2	8	2	12	1.33
D3Mit51	2	7	3	12	0.50
D3Mit12	3	5	3	11	0.09
D3Mit257	1	4	1	6	0.67
D3Mit17	2	7	3	12	0.50
D4Mit149	0	5	3	8	2.75
D4Mit227	0	4	3	7	2.71
D4Mit192	0	3	8	11	13.91
D4Mit237	1	1	9	11	19.00
D4Mit286	1	0	9	10	22.80
D4Mit89	2	1	9	12	16.50
D4Mit111	2	1	8	11	13.91
D4Mit288	2	1	7	10	11.40
D4Mit81	1	1	7	9	13.44
D4Mit110	1	3	5	9	4.56
D4Mit76	3	4	6	13	3.31
D4Mit54	4	5	3	12	0.50
D4Mit42	4	5	3	12	0.50
D5Mit193	2	9	1	12	3.17
D5Mit79	1	5	2	8	0.75
D5Mit93	2	3	3	8	0.75
D5Mit65	2	7	3	12	0.50
D5Mit99	1	10	0	11	7.55
D6Mit55	5	2	1	8	6.00
D6Mit159	8	0	0	8	24.00
D6Mit9	10	1	1	12	21.83
D6Mit17	7	1	0	8	16.75
D6Mit14	7	1	0	8	16.75
D7Mit178	1	5	2	8	0.75
D7Mit57	6	5	1	12	4.50
D7Mit84	2	9	1	12	3.17
D7Mit62	0	2	2	4	2.00
D7Mit100	2	6	4	12	0.67
D7Mit12	5	6	1	12	2.67
D7Mit291	4	3	1	8	2.75
D8Mit3	4	2	6	12	6.00
D8Mit4	5	1	6	12	8.50
D8Mit69	4	2	6	12	6.00
D8Mit45	4	6	1	11	1.73
D8Mit207	4	4	2	10	1.20
D8Mit113	2	7	2	11	0.82
D8Mit272	0	6	2	8	3.00
D9Mit64	1	8	3	12	2.00
D9Mit71	1	3	0	4	1.50
D9Mit10	1	7	4	12	1.83
D9Mit243	1	5	2	8	0.75
D9Mit18	0	11	1	12	8.50
D10Mit51	4	6	2	12	0.67
D10Mit44	1	5	2	8	0.75
D10Mit194	2	5	1	8	0.75
D10Mit94	2	5	1	8	0.75
D10Mit61	2	5	1	8	0.75
D10Mit42	4	6	2	12	0.67
D10Mit70	5	6	2	13	1.46

MARKER	Protected Pancreas				CHI SQUARE
	B6/B6	B6/DBA	DBA/DBA	TOTAL	
D11Mit148	0	5	5	10	5.00
D11Mit78	1	4	7	12	7.33
D11Mit185	0	4	6	10	7.60
D11Mit174	1	2	4	7	3.86
D11Mit217	1	2	5	8	6.00
D11Mit260	2	4	2	8	0.00
D11Mit157	2	4	2	8	0.00
D11Mit39	2	7	2	11	0.82
D11Mit99	2	8	2	12	1.33
D11Mit103	3	6	2	11	0.27
D12Mit105	2	6	0	8	3.00
D12Mit12	4	8	0	12	4.00
D12Mit62	3	7	2	12	0.50
D12Mit34	2	6	0	8	3.00
D12Mit239	2	6	0	8	3.00
D12Mit17	3	7	2	12	0.50
D12Mit141	3	5	0	8	2.75
D12Mit133	3	5	0	8	2.75
D13Mit17	3	7	2	12	0.50
D13Mit198	1	5	0	6	3.00
D13Mit88	1	1	2	4	1.50
D13Mit9	3	5	4	12	0.50
D13Mit77	3	6	3	12	0.00
D13Mit76	3	5	4	12	0.50
D14Mit1	2	5	1	8	0.75
D14Mit54	1	7	4	12	1.83
D14Mit28	2	3	7	12	7.17
D14Mit39	0	1	5	6	11.00
D14Mit193	1	2	5	12	7.17
D14Mit35	2	3	7	12	1.83
D14Mit75	2	5	5	13	1.77
D15Mit12	2	5	5	12	1.83
D15Mit5	2	9	1	12	3.17
D15Mit144	1	5	0	6	3.00
D15Mit95	4	8	0	12	4.00
D15Mit34	5	3	0	8	6.75
D15Mit217	3	3	0	6	3.00
D16Mit181	2	5	1	8	0.75
D16Mit165	2	5	1	8	0.75
D16Mit103	1	6	1	8	2.00
D16Mit12	2	8	2	12	1.33
D16Mit5	2	7	3	12	0.50
D16Mit50	2	5	4	11	0.82
D17Mit46	5	5	2	12	1.83
D17Mit22	3	2	3	8	2.00
D17Mit66	3	2	3	8	2.00
D17Mit54	4	0	4	8	8.00
D17Mit39	4	4	4	12	1.33
D18Mit132	1	4	1	6	0.67
D18Mit17	1	8	3	12	2.00
D18Mit50	2	8	2	12	1.33
D18Mit48	3	4	1	8	1.00
D18Mit49	3	4	1	8	1.00
D19Mit69	1	3	4	8	2.75
D19Mit28	2	4	6	12	4.00
D19Mit16	1	1	1	3	0.33
D19Mit40	3	2	5	10	4.40
D19Mit13	3	3	6	12	4.50
D19Mit19	2	5	5	12	1.83
D19Mit10	2	6	3	11	0.27
D19Mit34	2	7	3	12	0.50
DXMit89	4	7	1	12	0.37
DXMit105	4	7	1	12	0.37
DXMit37	4	6	2	12	1.20
DXMit182	5	5	2	12	1.17

<u>Class designation</u>	<u>Criterion for selection</u>
Insulin resistant	Plasma [glucose] < 317 mg/dl Plasma [insulin] > 378 μ U/ml
Diabetic	Plasma [glucose] > 899 mg/dl Plasma [insulin] < 40 μ U/ml HbA1c > 14%
Protected pancreas	Pancreatic grade < 2 Pancreatic [insulin] > 1000mU/mg protein

Results

Comparison of lean and obese animals

As a group, the genetically obese animals were readily distinguishable from the lean animals on the basis of weight which was 70% higher in the obese animals and BMI which was 56% greater than the lean controls (Table 6). Although there was variability in all phenotypic parameters within the lean class of mice, none of the lean mice were diabetic, as indicated by the glycosylated hemoglobin fractions which were consistently less than 5%. All phenotypic parameters were distinguishable between the two groups with the exception of fasting plasma [insulin] which tended to overlap between the two groups. On the whole, the lean animals weighed less, were euglycemic, and had a higher pancreatic insulin/glucagon ratio.

Phenotype	Lean	Obese
Age (days)	113 (11)	112 (16)
Weight (g)	35.2 (6.9)	59.7 (11.5)
BMI (g/cm ²)	.382 (.051)	.597 (.087)
HbA1c (%)	3.9 (1.4)	10.7 (4.7)
Fasting plasma [glucose] (mg/dL)	317 (86)	697 (222)
Fasting plasma [insulin] (uU/ml)	135.9 (207)	168.1 (209.7)
Normalized pancreatic insulin content (uU/mg protein)	43050 (35010)	25410 (48900)
Normalized pancreatic glucagon content (pg/mg protein)	26790 (19570)	74130 (40840)
Pancreatic [insulin]/[glucagon]	2.12 (2.0)	0.364 (.623)

Table 6. Phenotypic characteristics of F2 B6DBA and DBAB6 segregating for *ob*. Means for the lean and obese groups are indicated with standard deviations in parentheses.

Comparison of reciprocal crosses

Although the lean animals from the two reciprocal crosses were indistinguishable, the obese animals demonstrated significant variations in some phenotypic parameters. The majority of animals (358) were derived from the F2 B6DBA reciprocal cross in which the grandmother of the F2 progeny was B6. Only 46 of the obese progeny were derived from the cross in which the grandmother was DBA (F2 DBAB6). Despite the small numbers of obese progeny from the second cross, differences in phenotypes of the animals were observed (Table 7). Both crosses were equally obese; however, progeny from the cross in which the grandmother was DBA (F2 DBAB6) were significantly less diabetic as demonstrated by lower HbA1c (9.2 vs 11.6 %), plasma [glucose] (574 vs 713 mg/dl), plasma [insulin] (187.3 vs 201.1 μ U/ml), and pancreatic grade (1.5 vs 3.1).

Phenotype	B6DBA		DBAB6		P
	Mean	Variance	Mean	Variance	
Body Weight (g)	59.3	133.7	62.4	110.3	NS
BMI (g/cm ²)	0.596	0.007	0.604	0.009	NS
HbA1c (%)	11.6	17.4	9.2	30.0	0.01
Fasting plasma [glucose] (mg/dl)	713	47771	574	45434	0.0001
Fasting plasma [insulin] (uU/ml)	201.1	58451	187.3	40619	NS
Pancreatic grade	3.1	1.4	2.5	1.5	0.006

Table 7. Phenotypic comparison of obese (*ob/ob*) F2 B6DBA and F2 DBAB6 animals. The mean, variance, and number of animals studied (N) is indicated for each phenotype under BxD (F2 B6DBA) or DxB (F2 DBAB6). NS indicates that the comparison was not statistically significant.

When the obese progeny from the two reciprocal crosses were then analyzed by separating the sexes, the results were less statistically significant since the numbers of animals were smaller, but the same trends were observed (Table 8). Both males and females from the F2 DBAB6 cross had lower fasting plasma [glucose], and higher pancreatic [insulin]/[glucagon] than F2 B6DBA. In addition, this analysis showed the expected sexual dimorphism in the progeny of both crosses. The females in the aggregate tended to weigh slightly more and to have a higher BMI. The sexual dimorphism was also apparent in the diabetes phenotypes. The females were consistently less diabetic whether the phenotypes included biochemical profiles (HbA1c, plasma [glucose] or plasma [insulin]) or pancreatic profiles (hormone content or histopathology).

Biochemical characterization

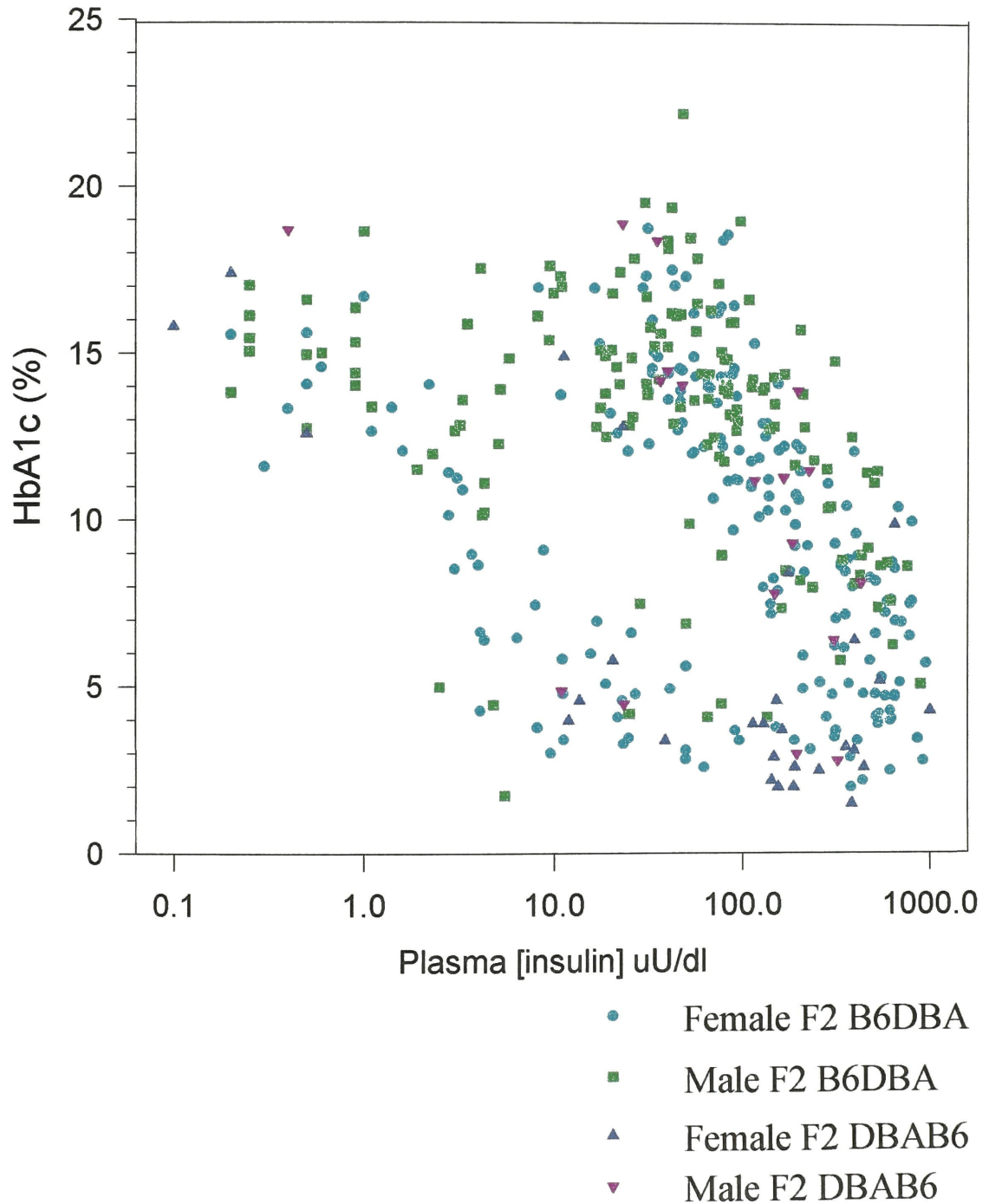
In addition to the variation noted both between the two reciprocal crosses and between the two sexes, there was extensive variation in phenotype within either cross or sex. The biochemical profile in Figure 4 demonstrates the evolution of the diabetes in animals of the same age but at different stages of their disease. A small group of animals, mainly females, was relatively non-diabetic based on their HbA1c < 7% and was not hyperinsulinemic based on their fasting plasma [insulin] < 100 μ U/ml. The second group of animals in the lower right quadrant was hyperinsulinemic and insulin-resistant (plasma [insulin] as high as 1000 μ U/ml). This group was also relatively non-diabetic on the basis of their HbA1cs. This group of insulin-resistant animals was largely, although not exclusively, composed of females. There was no clear demarcation between the insulin-

Phenotype	B6DBA, FEMALES			DBAB6, FEMALES			P			B6DBA, MALES			DBAB6, MALES			P	
	Mean	Variance	N	Mean	Variance	N	Mean	Variance	N	Mean	Variance	N	Mean	Variance	N		
Body Weight (g)	60.9	109.7	195	64.6	94.5	28	57.4	156.3	163	58.9	120.1	18	58.9	120.1	18	NS	NS
BMI (g/cm ²)	0.615	0.006	195	0.633	0.007	28	0.572	0.008	163	0.561	0.007	18	0.561	0.007	18	NS	NS
HbA1c (%)	10.2	17.0	178	6.9	26.3	20	13.3	12.8	149	12.2	20.0	15	12.2	20.0	15	NS	NS
Fasting plasma [glucose] (mg/dl)	681	51824	195	527	56811	28	751	40474	163	647	20884	18	647	20884	18	0.01	0.01
Fasting plasma [insulin] (uU/ml)	244.6	61810	189	218.1	55338	28	150.0	45641	161	139.4	15641	18	139.4	15641	18	NS	NS
Pancreatic grade	2.8	1.6	195	2.4	1.6	27	3.4	1.0	155	2.7	1.3	18	2.7	1.3	18	0.03	0.03
Normalized pancreatic insulin content (uU/mg protein)	34100	3.9E+07	168	44550	2.7E+07	28	10570	1311000	120	14940	818900	18	14940	818900	18	NS	NS
Number of islets	16.7	120.1	186	15.8	67.7	14	11.7	61.9	154	12.5	44.1	14	12.5	44.1	14	NS	NS
Number of hypertrophied islets	2.2	10.7	186	1.9	5.0	14	1.0	3.1	154	1.1	1.8	14	1.1	1.8	14	NS	NS
Average islet area (mm ²)	0.021	0.00001	186	0.018	0.00003	39	0.023	0.00015	154	0.02	0.00006	14	0.02	0.00006	14	NS	NS
Total islet area (mm ²)	0.41	0.18	186	0.36	0.06	14	0.24	0.05	154	0.27	0.05	14	0.27	0.05	14	NS	NS
Islet area as a percentage of total pancreatic area (%)	1.60	2.00	186	1.69	1.14	13	0.9	0.8	154	0.97	0.32	14	0.97	0.32	14	NS	NS

Table 8. Phenotypic comparison obese (*ob/ob*) F2 B6DBA and F2 DBAB6 animals divided by sex. The mean, variance, and number of animals studied (N) is indicated for each phenotype under BxD (F2 B6DBA) or DxB (F2 DBAB6). NS indicates that the comparison was not statistically significant.

Figure 4. Fasting plasma [insulin] versus HbA1c in obese (*ob/ob*) male and female F2 B6DBA and F2 DBAB6. Each point represents a single animal.

Biochemical profile



resistant and the progressively diabetic animals. As the animals' fasting plasma [insulin] declined, there was an inverse and proportionate increase in fractional HbA1c and severity of diabetes. Eventually, the HbA1c plateaued at approximately 15% while fasting plasma [insulin] continued to decline. This last group of hypoinsulinemic, extremely diabetic animals was comprised predominantly of males.

If severity of diabetes was measured with fasting plasma [glucose] rather than HbA1c, a similar although less obvious clinical picture was apparent (Figure 5). In this case, fasting plasma [glucose] < 500 mg/dl (although not as low as the lean controls), was approximately the equivalent of the 7% HbA1c used to define a relatively non-diabetic group.

Figures 4 and 5 are slightly discrepant because of the imperfect correlation between fasting plasma [glucose] and HbA1c (Figure 6). Three explanations for the discordance between the two measures are: inadequate lengths of fasting (3 hours) which could result in persistent post-prandial hyperglycemia, differential effects of the stress of terminal asphyxiation in different animals, and the temporal difference between the two parameters, instantaneous versus the average of the previous 60 days with HbA1c.

Figure 5. Fasting plasma [glucose] versus fasting plasma [insulin] in obese (*ob/ob*) male and female F2 B6DBA and F2 DBAB6. Each point represents a single animal.

F2 *ob/ob* B6DBA and DBAB6

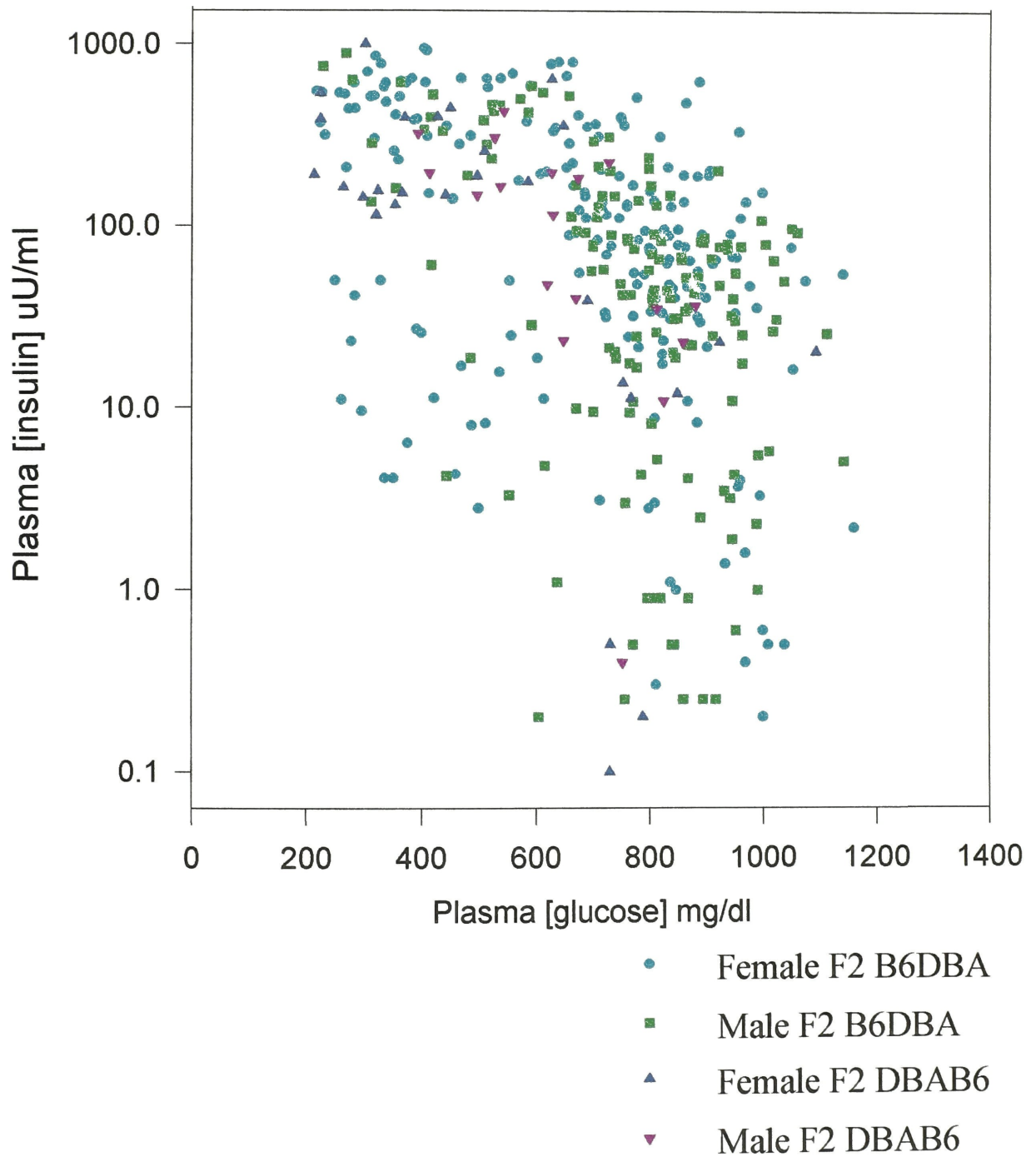
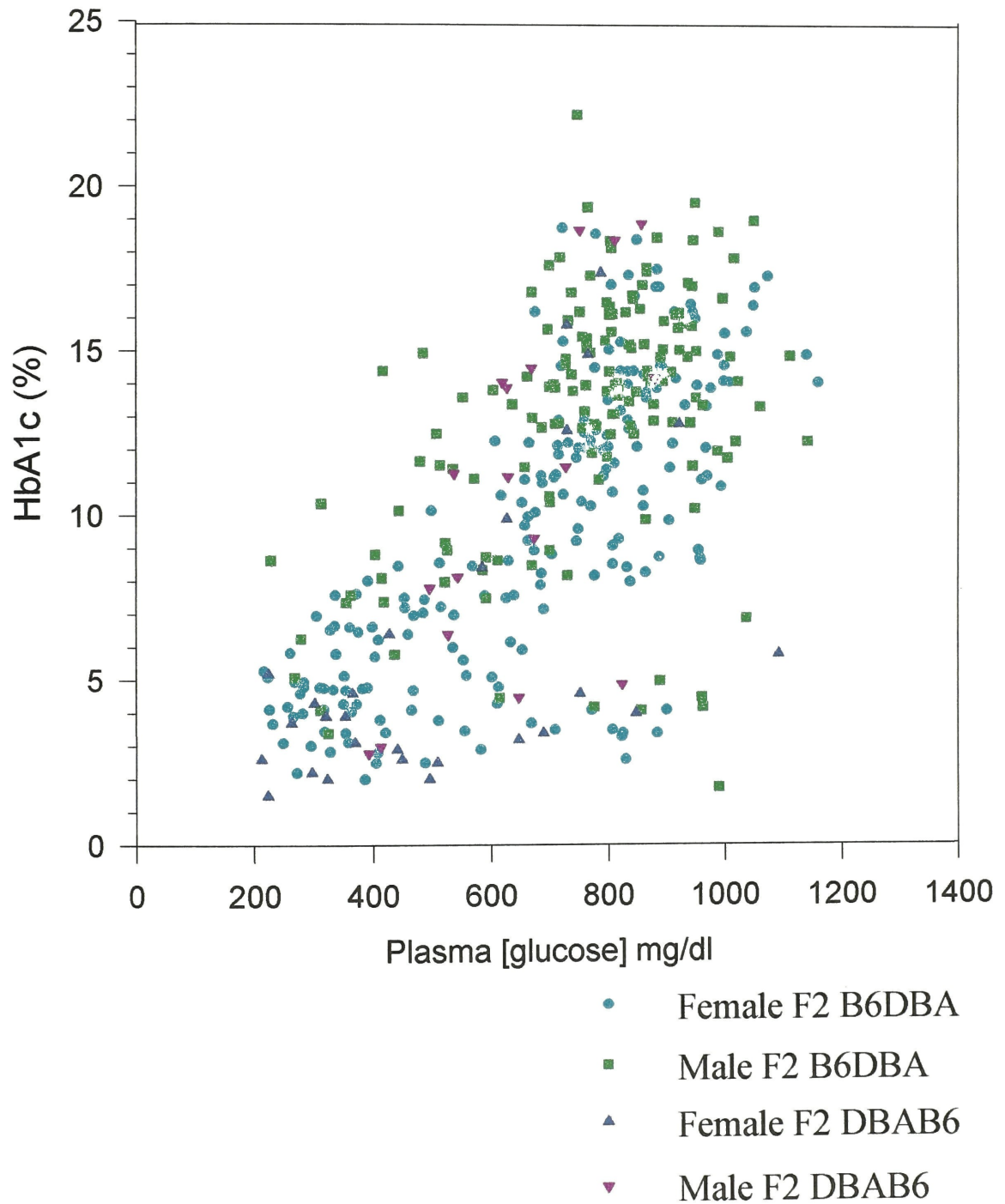


Figure 6. Fasting plasma [glucose] versus HbA1c in obese (*ob/ob*) male and female F2 B6DBA and F2 DBAB6. Each point represents a single animal.

F2 *oblob* B6DBA and DBAB6



Pancreatic phenotypes

Pancreatic grade was judged qualitatively using three criteria: size, and number of islets, and islet integrity. These same parameters were measured using an image analysis system to quantify average islet area, number of islets, and number of hypertrophic islets. Quantitation of pancreatic hormone content protected the above phenotypic parameters from measurements based on only two sections and the possibility that any particular section was not representative of the entire pancreas.

Qualitative pancreatic grade (1=best, 5=worst) was inversely related to average islet area determined quantitatively, as would have been predicted (Figure 7). Similarly, pancreatic grade was inversely related to pancreatic [insulin]/[glucagon] (Figure 8). However, for the majority of the animals, pancreatic [insulin]/[glucagon] appeared to be unrelated to average islet size (Figure 9), probably because each of these parameters was itself a composite of phenotypes. Pancreatic [insulin]/[glucagon] would be elevated if beta cells were hyperplastic and hypertrophic but could be “normal” either if such hyperplasia and hypertrophy did not occur or if the beta cells which were once abundant had subsequently died or lost the ability to produce insulin. Similarly, average islet size could not distinguish between islets which never grew and those which had grown only to subsequently atrophy.

Figure 7. Pancreatic grade versus average islet area in obese (*ob/ob*) male and female F2 B6DBA and F2 DBAB6. Each point represents a single animal.

F2 *obl/obl* B6DBA and DBAB6

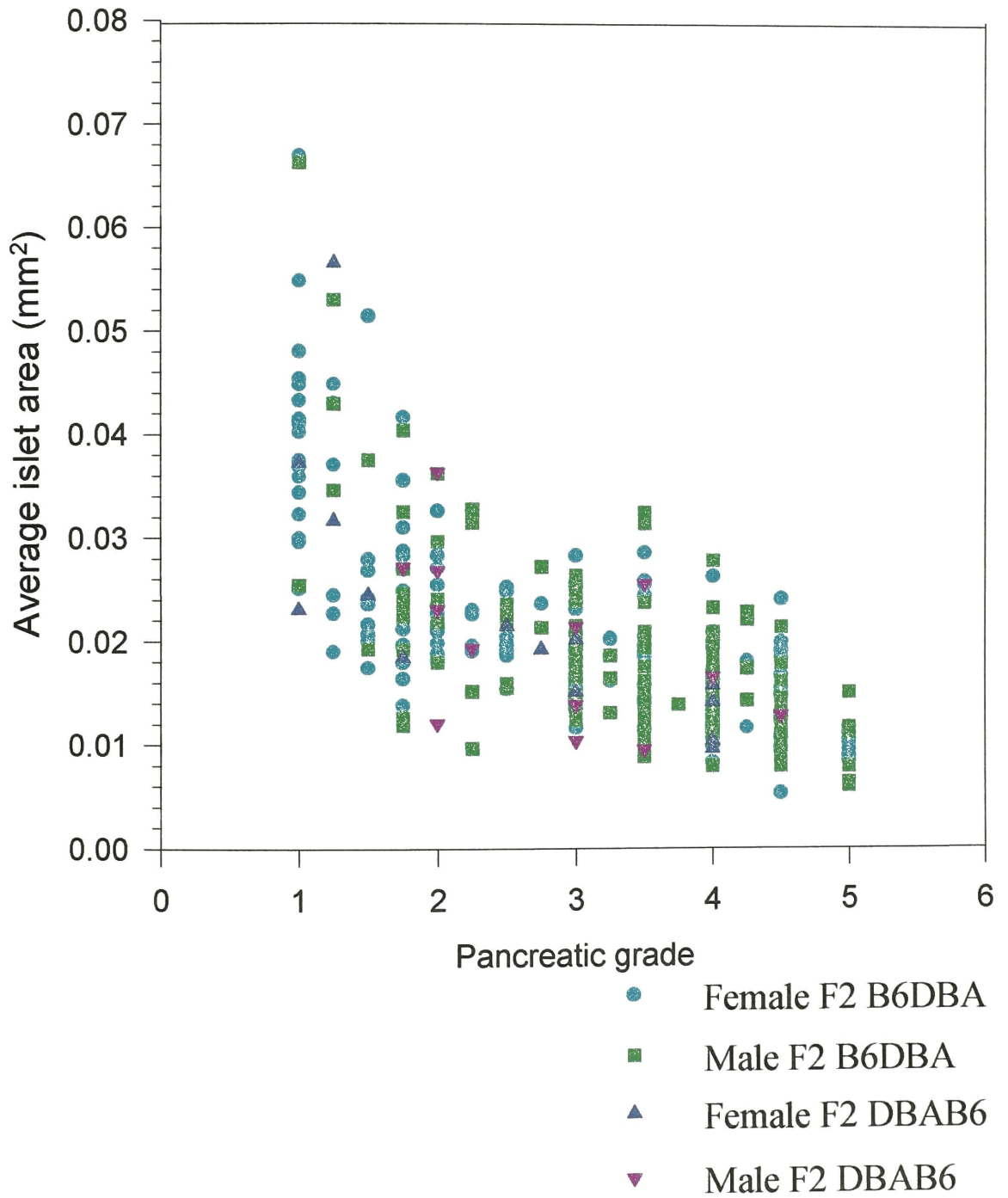


Figure 8. Pancreatic grade versus pancreatic [insulin]/[glucagon] in (*ob/ob*) obese male and female F2 B6DBA and F2 DBAB6. Each point represents a single animal.

F2 *ob/ob* B6DBA and DBAB6

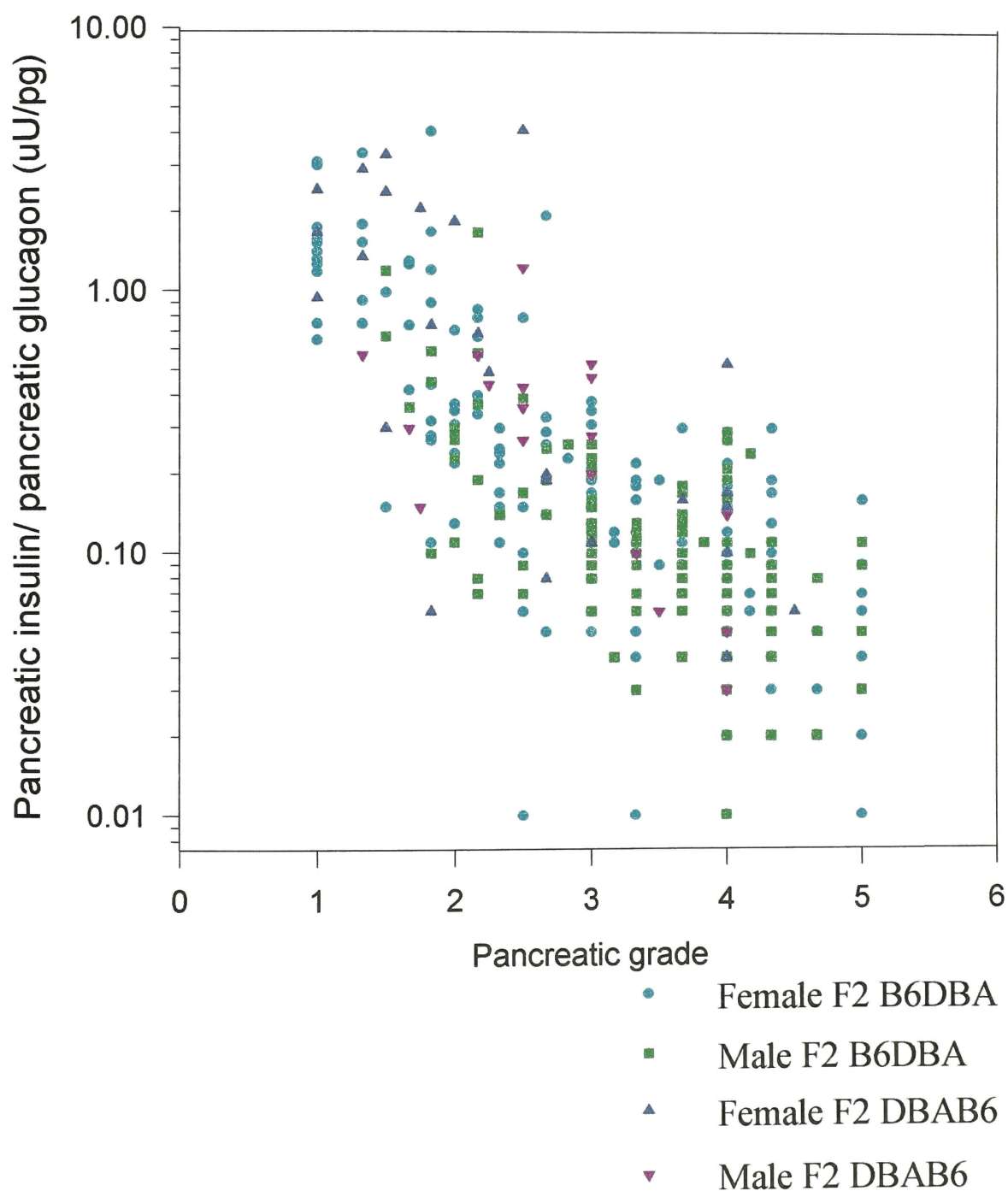
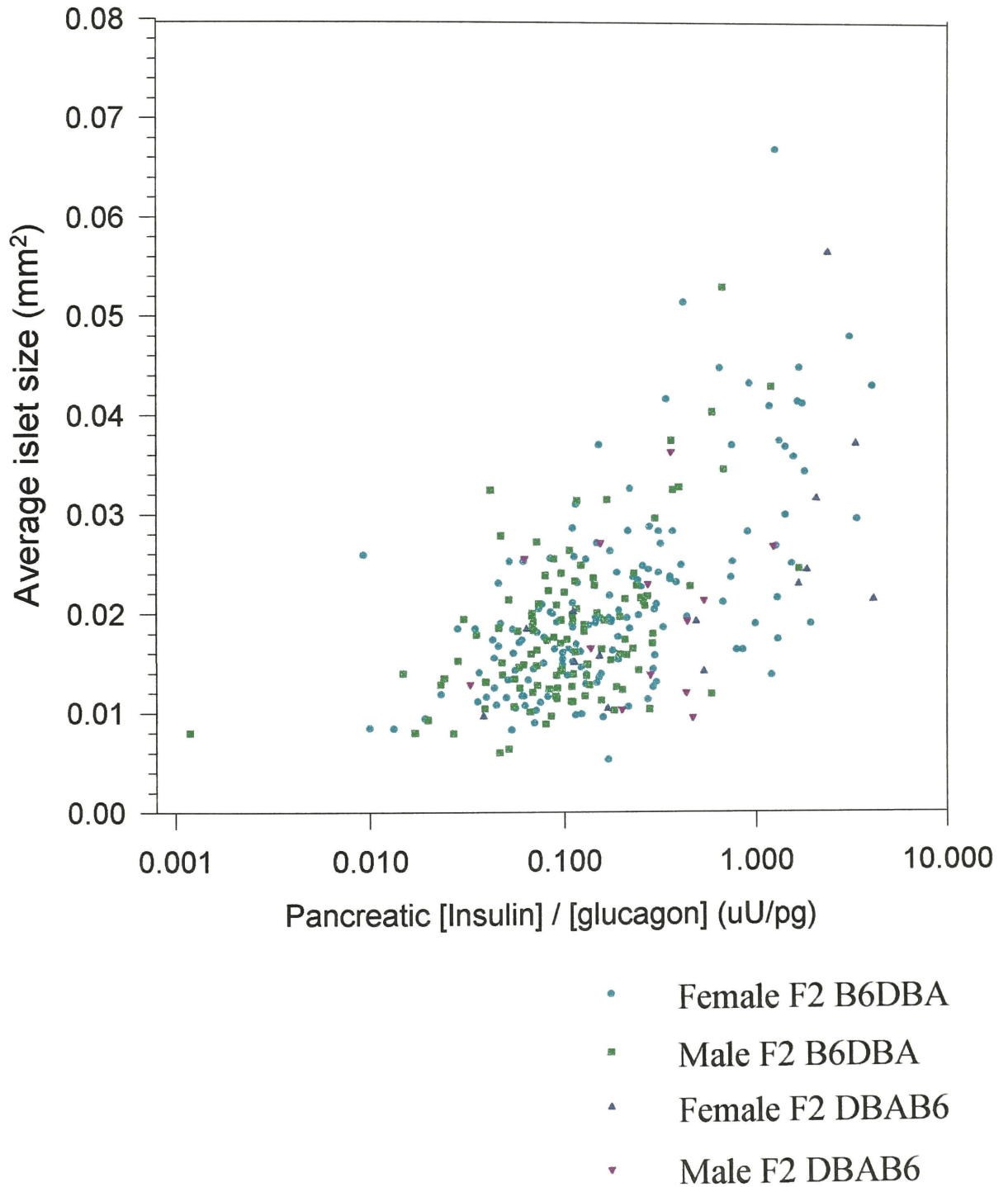


Figure 9. Pancreatic [insulin]/[glucagon] versus average islet size in obese (*ob/ob*) male and female F2 B6DBA and F2 DBAB6. Each point represents a single animal.

F2 *ob/ob* B6DBA and DBAB6



Genome scanning results

The results of the genome scanning experiments for the subgroups demonstrating the most extreme phenotypes of protected pancreata, insulin resistance, and diabetes are shown in Tables 4 and 5 respectively.

Chromosome 1 contained a locus or loci with powerful effects on diabetes phenotypes since consistent, significant linkage disequilibrium of the parental alleles was demonstrated for each of the three phenotypic classes. Both D1Mit76 and D1Mit110 demonstrated enrichment of B6 alleles within the insulin resistant class ($p < 0.01$). Similarly there was also an enrichment of B6 alleles at D1Mit110 within the protected pancreas class ($p = 0.05$). Conversely, there was enrichment of DBA alleles within the diabetic class for D1Mit24, D1Mit90, and D1Mit94 ($p < 0.05$). The number of loci contributing to the phenotype or their precise location(s) could not be determined on the basis of these three experiments. The correlation of B6 alleles with the non-diabetic classes, and DBA alleles with the diabetic class was consistent with the phenotypes of *ob* on the parental strains (Chua et al. Unpublished material).

Conversely, a locus on proximal chromosome 2 demonstrated enrichment of DBA alleles within the protected pancreas and insulin resistant groups for D2Mit61/D2Mit42/D2Mit17 and D2Mit12, D2Mit42/D2Mit43/D2Mit58 respectively ($p < 0.05$). The association of DBA alleles with the non-diabetic classes contradicted the

prediction based on the phenotypes of the parental strains and is an example of transgression (chapter 1).

The locus on chromosome 4 was unique because the linkage disequilibrium was only observed in one of the three genome scanning experiments (protected pancreas). However, within this class defined by a very specific phenotype- i.e. extreme hypertrophy and hyperplasia of the beta cells and complete integrity of the islets- the majority of the twelve animals were homozygous for DBA alleles for D4Mit237, D4Mit286, D4Mit 89, D4Mit111, D4Mit288, and D4Mit81. Based on this initial result, the chromosome 4 locus would be predicted to have its primary effect on the beta cell.

The precise location of the gene on chromosome 8 was difficult to determine on the basis of the genome scanning experiments. Although statistically there was a deviation from the expected Mendelian ratio for the most centromeric markers within the protected pancreas class, the significance was produced by virtue of a paucity of heterozygotes. Although it is possible that the protein products of such a gene might form multimers which would not work well as “homomers”, given the distribution of alleles for the same markers in the other two classes, such an explanation is unlikely. Within the insulin resistant class, there is a relative enrichment of DBA alleles for D8Mit3, D8Mit4, D8Mit69; and within the diabetic class, conversely enrichment of B6 alleles for D8Mit69, D8Mit25, D8Mit31, and D8Mit45.

D9Mit18, the most telomeric marker typed on chromosome 9, derived its statistical significance from the preponderance of heterozygotes within the protected pancreas class. A model similar to that proposed above in which the protein product of this locus produces a multimer in which the two alleles instead act in a manner to reduce diabetes susceptibility is a possible explanation for the genotypic distribution observed. However, because the genotypic deviation was only observed in a single phenotypic class, the locus was not further characterized.

Within the protected pancreas class, there was enrichment of DBA alleles for D11Mit78, D11Mit185, D11Mit174, and D11Mit217. Markers in the same region in the other classes failed to produce significant results.

The majority of markers tested on chromosome 13- D13Mit11, D13Mit9, D13Mit69, D13Mit77, D13Mit76, and D13Mit35- demonstrated biasing of DBA alleles within the insulin resistant class. Additionally, there were few if any DBA homozygotes for the same markers within the diabetic class although none of the markers demonstrated statistically significant deviation from expected within this class.

D14Mit28, D14Mit39, and D14Mit193 demonstrated enrichment of DBA alleles within the class of protected pancreas. A similar tendency toward enrichment of DBA alleles within the insulin resistant class was observed for markers telomeric of D14Mit193.

Single loci (D15Mit34 and D17Mit54) demonstrated statistically significant results within the class of the protected pancreas; but because these results were not replicated with adjacent markers, they were likely to be spurious and were not pursued further.

Interestingly, for none of the classes was any effect of markers on the X chromosome observed.

On the basis of the genome scanning experiments, approximate locations for each QTL were determined; and markers within this interval were used to genotype all the obese progeny from both reciprocal crosses to determine which of the regions indicated by the genome scanning experiments were likely to be biologically relevant. Additionally, because the number of contributing genes on chromosome 1 could not be determined on the basis of the original experiments, and because precise localization of the QTL would be relevant to subsequent experiments, a total of 16 polymorphic markers spanning the majority of chromosome 1 were used to genotype the obese progeny. Two markers on chromosome 10 were tested although nothing of significance was noted on the genome scanning experiments because evidence from a previous cross also segregating *ob* on B6DBA had indicated the presence of a QTL related to overnight fasting plasma [insulin] in the vicinity of D10Mit3 (chapter 8). In total, 37 markers from ten different chromosomes were tested: D1Mit245, D1Mit300, D1Mit24, D1Mit83, D1Mit84, D1Mit338, D1Mit90, D1Mit286, D1Mit103, D1Mit101, D1Mit55, D1Mit110,

D1Mit355, D1Mit150, D1Mit56, D1Mit408, D2Mit367, D2Mit7, D2Mit61, D2Mit42, D2Mit17, D4Mit192, D4Mit286, D4Mit288, D6Mit74, D6Mit54, D8Mit69, D10Mit3, D10Mit51, D11Mit104, D11Mit78, D13Mit9, D13Mit11, D14Mit28, D14Mit35, D14Mit75, and D16Mit15.

Quantitative Trait Locus Analysis and Analysis of Variance (ANOVA)

Genes in the interval from D1Mit84 to D1Mit56 on chromosome 1 exerted effects on almost every phenotype examined (Tables 9 and 10). On the basis of the LOD scores for body weight (Figure 10), there were two separate QTL's, one centered around D1Mit90 and another located telomerically and centered around D1Mit110-D1Mit150 (Figure 11 and 12). For both regions, B6 alleles were associated with increased weight in both sexes although the effect of heterozygosity at either locus differed between the two sexes, with female heterozygotes more closely resembling B6 homozygotes and male heterozygotes more closely resembling DBA homozygotes. The locus centered at D1Mit90 appeared to act more co-dominantly if BMI was considered rather than weight. This more centromeric of the two QTL's exerted a greater and more specific influence on body weight and accounted for 4.7% of the variance for this trait compared to the 3.2% of the variance accounted for by the telomeric QTL.

Table 9. Quantitative trait locus analysis for diabetes-susceptibility loci in obese (*ob/ob*) F2 B6DBA and DBAB6 mice. Data is summarized by trait/phenotype, the marker or interval which demonstrated the peak LOD score, the confidence interval as defined by interval on either side of the peak 1 LOD unit less than the maximum peak using a free genetic model, the maximum LOD score observed under a free genetic model, and the genetic models which most likely fit the data. Genetic models which demonstrated a LOD score < (peak LOD score under a free genetic model -1) were determined to be unlikely genetic models. In cases in which an alternative genetic model was not strictly rejected based on the above criteria but demonstrated a peak LOD score < (peak LOD score under a free genetic model -0.75), a “>” sign indicates that such a model is possible but unlikely.

Phenotype	Marker with peak LOD score	Confidence Interval	LOD score	% variance for which locus can account	Genetic model
Weight	D1MI100	D1MI83/D1MI101	3.8	4.7	Free, additive
	D1MI110		3.2	3.2	
	D2MI7-D2MI61	D2MI367/D2MI42	8.9	11.7	Free, additive
	D6MI74		2.4	10.3	
BMI	D2MI7-D2MI61	D2MI367/D2MI42	7.1	9.2	Free, additive
HbA1c	D1MI150-D1MI56		6.5	11.4	Free, additive
Fasting plasma [glucose]	D1MI110-D1MI355	D1MI55/D1MI56	8.5	10.5	Free, additive
	D4MI286-D4MI288		2.6	3.6	Free, recessive > additive
	D6MI74-D6MI54		2.4	3.5	Free, dominant, additive
	D13MI11		2.2	3.2	Free, additive
	D14MI75		4.0	14.4	Free
Fasting plasma [Insulin]	D1MI355-D1MI150	D1MI55/D1MI56	2.8	3.4	Free, dominant
	D4MI286-D4MI288		2.6	3.5	Free, recessive
	D6MI74-D6MI54		3.5	10	Free, dominant, additive
	D13MI9-D13MI11		3.4	5.7	Free, additive
Pancreatic grade	D1MI150-D1MI56	D1MI355/D1MI56	9.0	13.7	Free, dominant, additive
	D13MI9-D13MI11		2.8	3.9	Free, additive
Pancreatic [Insulin]	D1MI150-D1MI56		5.7	11.6	Free, dominant > additive
Pancreatic [Insulin]/[glucagon]	D1MI150-D1MI56	D1MI150/D1MI56	5.1	13.9	Free, dominant > additive
	D4MI286-D4MI288		2.5	4.2	Free, recessive
Number of Islets	D1MI150-D1MI56	D1MI55/D1MI56	2.4	3.3	Free, additive
	D2MI61-D2MI42		2.7	4.9	Free, additive
Number of hypertrophic Islets	D1MI150-D1MI56	D1MI55/D1MI56	4.0	7.9	Free, dominant, additive
	D4MI286-D4MI288		5.8	9.2	Free, recessive
	D6MI74		2.8	13.6	Free, dominant, additive
	D14MI75		3.3	18.1	Free, recessive, additive
Average Islet size	D1MI150-D1MI56	D1MI55/D1MI56	5.6	12	Free, dominant > additive
	D4MI286-D4MI288		4.4	7.2	Free, recessive
	D13MI9-D13MI11		3.3	6.8	Free, recessive
	D14MI75		2.1	11.8	Free, recessive
Beta cell area	D1MI150-D1MI56	D1MI55/D1MI56	3.8	7.4	Free, dominant, additive
	D4MI286-D4MI288		3.9	6.3	Free, recessive
	D14MI75		3.1	6.5	Free, recessive
Beta cell area/total pancreatic area	D1MI150-D1MI56	D1MI55/D1MI56	4.8	10.2	Free, dominant, additive
	D4MI286-D4MI288		3.4	5.6	Free, recessive
	D13MI9-D13MI11		2.3	5.1	Free, recessive
	D14MI75		2.0	6	Free, recessive

Table 10. Analysis of variance by sex and genotype at the marker designated for *ob/ob* F2 B6DBA and DBAB6. All traits for all markers demonstrating statical significance are summarized. Number of animals genotyped and phenotyped for each a given marker and trait is indicated. Mean values for each trait are listed first by sex and genotypic class.

Marker	Phenotype	Number	B6/B6	Female B6/DBA	DBA/DBA	B6/B6	Male B6/DBA	DBA/DBA	P
D1Mit300	Weight (g)	356	64.4	61.8	59.6	58.2	57.8	52.5	0.001
	[Glucose] (mg/dl)		606	661	681	721	769	790	0.04
	Pancreatic Grade		2.3	2.8	3.0	3.2	3.4	3.3	0.01
	Average islet size (mm2)		0.0229	0.0212	0.0209	0.0212	0.0167	0.0181	0.04
D1Mit24	Weight (g)	330	65.1	61.0	59.0	58.0	57.3	54.9	0.01
	HbA1c (%)		8.2	10.9	10.6	13.1	13.7	13.1	0.01
	[Glucose] (mg/dl)		596	658	721	700	744	781	0.002
	Pancreatic Grade		2.4	2.9	3.3	3.1	3.4	3.4	0.0008
	Pancreatic [Insulin] (uU/mg protein)		45335	39693	19803	12574	11119	8724	0.02
	Pancreatic [Insulin]/[Glucagon]		0.63	0.54	0.3	0.19	0.2	0.13	0.02
	Number of hyperplastic islets		3.0	1.9	1.5	1.4	0.7	0.5	0.003
	Average islet size (mm2)		0.0234	0.0207	0.0189	0.0219	0.0169	0.0171	0.0007
	Islet area/total area (%)		1.864	1.524	1.18	1.03	0.809	0.83	0.04
D1Mit84	Weight (g)	312	64.6	61.7	59.4	59.1	56.0	54.4	0.005
	HbA1c (%)		7.9	10.6	10.9	12.9	13.8	13.2	0.003
	[Glucose] (mg/dl)		581	664	734	704	748	771	0.001
	[Insulin] (uU/ml)		335.1	234.8	212.2	173.6	116.5	165.7	0.04
	Pancreatic Grade		2.4	2.8	3.2	3.1	3.4	3.5	0.001
	Pancreatic [Insulin] (uU/mg protein)		42428	33677	16220	12295	10840	8335	0.03
	Pancreatic [Insulin]/[Glucagon]		0.57	0.51	0.24	0.18	0.22	0.14	0.02
	Number of hyperplastic islets		3.2	2.2	1.4	1.5	0.9	0.5	0.01
	Average islet size (mm2)		0.0227	0.0213	0.0186	0.0204	0.0181	0.0165	0.03
	Islet area (mm2)		0.52	0.44	0.28	0.27	0.21	0.21	0.03
D1Mit338	Weight (g)	270	67.7	61.3	60.0	59.9	56.6	53.9	0.0001
	BMI (g/cm2)		0.65	0.61	0.63	0.59	0.56	0.55	0.02
	HbA1c (%)		7.8	10.5	11.5	12.6	13.6	13.6	0.0002
	[Glucose] (mg/dl)		562	679	720	695	764	783	0.0004
	[Insulin] (uU/ml)		341.71	238.77	216.76	180.11	97.26	157.34	0.02
	Pancreatic Grade		2.3	2.9	3.5	3.0	3.5	3.5	0.00001
	Pancreatic [Insulin] (uU/mg protein)		47823	34847	14076	13735	10592	8828	0.01
	Pancreatic [Insulin]/[Glucagon]		0.61	0.48	0.17	0.2	0.21	0.13	0.001
	Number of hyperplastic islets		3.3	2.1	1.1	1.2	0.6	0.6	0.003
	Average islet size (mm2)		0.0236	0.0207	0.0184	0.0213	0.0169	0.0165	0.003
	Islet area (mm2)		0.52	0.4	0.24	0.22	0.19	0.21	0.01
	Islet area/total area (%)		1.895	1.544	0.98	1.012	0.763	0.81	0.02
D1Mit90	Weight (g)	324	64.0	63.1	57.5	61.9	55.5	54.7	0.000001
	BMI (g/cm2)		0.63	0.63	0.61	0.59	0.55	0.55	0.02
	HbA1c (%)		8.1	10.1	11.4	11.5	14.0	13.4	0.00001
	[Glucose] (mg/dl)		586	644	730	658	758	771	0.0001
	[Insulin] (uU/ml)		267.8	255.5	214.3	220.5	95.2	152.5	0.06
	Pancreatic Grade		2.4	2.8	3.3	2.7	3.5	3.5	0.0000001
	Pancreatic [Insulin] (uU/mg protein)		55487	38288	17251	15417	10375	8931	0.005
	Pancreatic [Insulin]/[Glucagon]		0.66	0.57	0.25	0.22	0.2	0.15	0.004
	Number of islets		19.1	16.6	13.5	13.6	9.6	11.2	0.01
	Number of hyperplastic islets		3.0	2.2	1.5	1.7	0.6	0.5	0.001
	Average islet size (mm2)		0.0249	0.0207	0.0191	0.0225	0.0173	0.0165	0.004
	Islet area (mm2)		0.51	0.41	0.3	0.31	0.17	0.2	0.001
	Islet area/total area (%)		2.028	1.57	1.066	1.304	0.724	0.765	0.0006
D1Mit286	Weight (g)	281	65.0	63.2	58.1	58.4	56.7	56.7	0.03
	HbA1c (%)		8.2	10.2	12.6	12.5	14.3	13.7	0.00001
	[Glucose] (mg/dl)		574	660	753	695	784	766	0.0002
	Pancreatic Grade		2.4	2.9	3.5	3.0	3.6	3.5	0.000001
	Pancreatic [Insulin] (uU/mg protein)		50322	38369	11143	14617	9184	9435	0.006
	Pancreatic [Insulin]/[Glucagon]		0.63	0.51	0.15	0.21	0.19	0.15	0.001
	Number of hyperplastic islets		2.9	2.1	1.4	1.4	0.5	0.5	0.01
	Average islet size (mm2)		0.0221	0.0204	0.0193	0.0215	0.0167	0.0164	0.01
	Islet area (mm2)		0.48	0.4	0.28	0.27	0.15	0.21	0.03
	Islet area/total area (%)		1.726	1.531	1.1	1.09	0.628	0.756	0.04
D1Mit103	Weight (g)	313	63.8	63.6	57.3	59.0	56.5	55.3	0.006
	HbA1c (%)		7.7	10.1	11.8	12.5	13.8	13.4	0.0001
	[Glucose] (mg/dl)		558	652	741	689	785	752	0.0007
	Pancreatic Grade		2.2	2.8	3.4	2.9	3.5	3.5	0.000001
	Pancreatic [Insulin] (uU/mg protein)		60793	35332	12971	14266	8986	7147	0.003
	Pancreatic [Insulin]/[Glucagon]		0.65	0.49	0.18	0.21	0.17	0.14	0.003
	Number of islets		21.2	16.8	12.2	12.3	10.5	12.7	0.01
	Number of hyperplastic islets		3.4	2.0	1.3	1.5	0.9	0.6	0.0007
	Average islet size (mm2)		0.025	0.0204	0.0187	0.0219	0.0179	0.0164	0.0002
	Islet area (mm2)		0.58	0.4	0.26	0.28	0.21	0.22	0.0008
	Islet area/total area (%)		2.069	1.524	1.035	1.165	0.838	0.835	0.001
D1Mit101	Weight (g)	288	65.3	62.8	57.0	59.4	56.5	52.7	0.0001
	HbA1c (%)		9.0	9.8	12.2	12.4	13.7	14.1	0.0008
	[Glucose] (mg/dl)		578	634	750	698	805	773	0.0004
	Pancreatic Grade		2.5	2.7	3.4	2.8	3.5	3.6	0.000001
	Pancreatic [Insulin] (uU/mg protein)		41976	39280	14406	15363	8385	8482	0.03
	Pancreatic [Insulin]/[Glucagon]		0.46	0.53	0.17	0.22	0.15	0.11	0.006
	Number of islets		19.8	16.9	12.4	11.6	11.3	11.6	0.05
	Number of hyperplastic islets		3.3	2.0	1.1	1.7	0.8	0.6	0.0004
	Average islet size (mm2)		0.0245	0.0205	0.0184	0.0226	0.0173	0.0157	0.0001
	Islet area (mm2)		0.55	0.41	0.25	0.28	0.22	0.2	0.003
	Islet area/total area (%)		1.933	1.628	1.037	1.188	0.828	0.752	0.005

Marker	Phenotype	Number	B6/B6	Female B6/DBA	DBA/DBA	B6/B6	Male B6/DBA	DBA/DBA	p
D1Mit55	Weight (g)	272	62.0	64.2	59.8	59.4	56.9	53.8	0.02
	HbA1c (%)		9.5	10.1	11.5	12.1	13.8	13.6	0.01
	[Glucose] (mg/dl)		603	649	744	660	782	743	0.001
	Pancreatic Grade		2.5	2.7	3.5	2.8	3.5	3.5	0.00001
	Pancreatic [insulin] (uU/mg protein)		44199	39087	16921	15893	9657	9653	0.03
	Pancreatic [insulin]/[glucagon]		0.6	0.6	0.21	0.24	0.19	0.15	0.004
	Number of hyperplastic islets		2.7	1.9	1.4	1.6	0.5	0.6	0.01
	Average islet size (mm2)		0.0226	0.0205	0.0174	0.022	0.0166	0.0169	0.001
	Islet area (mm2)		0.46	0.39	0.27	0.29	0.16	0.22	0.04
D1Mit110	Weight (g)	330	61.1	63.5	57.5	60.3	56.4	54.0	0.001
	HbA1c (%)		8.7	9.6	12.2	11.6	14.1	14.3	0.00001
	[Glucose] (mg/dl)		585	645	773	646	770	784	0.00001
	[Insulin] (uU/ml)		258.3	272.2	178.5	235.1	83.1	148.1	0.01
	Pancreatic Grade		2.4	2.7	3.7	2.7	3.5	3.6	0.00001
	Pancreatic [insulin] (uU/mg protein)		64674	35963	7696	15890	9445	8384	0.00001
	Pancreatic [insulin]/[glucagon]		0.8	0.54	0.12	0.25	0.17	0.13	0.00001
	Number of islets		18.0	17.6	11.5	13.6	9.8	10.9	0.005
	Number of hyperplastic islets		3.0	2.3	0.9	1.7	0.5	0.6	0.0001
	Average islet size (mm2)		0.0248	0.0208	0.0171	0.022	0.0166	0.0171	0.00001
D1Mit355	Weight (g)	172	6.9	9.6	10.6	11.8	12.9	13.0	0.02
	[Glucose] (mg/dl)		501	695	751	598	778	749	0.0001
	[Insulin] (uU/ml)		422.5	284.9	224.5	283.8	98.9	273.2	0.03
	Pancreatic Grade		2.2	2.6	3.3	2.8	3.5	3.2	0.01
	Pancreatic [insulin] (uU/mg protein)		40659	27218	10699	21551	7746	7254	0.02
	Pancreatic [insulin]/[glucagon]		0.63	0.41	0.18	0.41	0.15	0.16	0.02
	Number of islets		23.2	18.7	11.9	12.8	11.9	12.4	0.02
	Number of hyperplastic islets		4.5	2.4	1.0	1.8	0.5	1.6	0.04
	Average islet size (mm2)		0.027	0.0219	0.0179	0.023	0.0159	0.0217	0.05
D1Mit150	Weight (g)	296	63.5	63.4	58.6	59.0	57.0	54.2	0.008
	HbA1c (%)		7.9	10.6	12.0	12.0	14.2	13.8	0.00001
	[Glucose] (mg/dl)		525	691	780	669	786	794	0.00001
	[Insulin] (uU/ml)		272.6	289.7	175.7	224.32	98.2	138.5	0.04
	Pancreatic Grade		2.2	2.9	3.7	2.8	3.7	3.6	0.00001
	Pancreatic [insulin] (uU/mg protein)		66284	33798	7965	15877	8071	8446	0.0001
	Pancreatic [insulin]/[glucagon]		0.77	0.41	0.11	0.24	.12	0.12	0.000001
	Number of islets		20.2	16.8	11.7	12.5	10.9	11.2	0.01
	Number of hyperplastic islets		3.5	2.2	1.0	1.7	0.7	0.6	0.000001
	Average islet size (mm2)		0.0258	0.0209	0.0175	0.0224	0.0167	0.0167	0.00001
D1Mit58	Weight (g)	301	6.9	9.6	10.6	11.8	12.9	13.0	0.02
	HbA1c (%)		7.9	10.6	12.0	12.0	14.2	13.8	0.00001
	[Glucose] (mg/dl)		525	691	780	669	786	794	0.00001
	[Insulin] (uU/ml)		272.6	289.7	175.7	224.32	98.2	138.5	0.04
	Pancreatic Grade		2.2	2.9	3.7	2.8	3.7	3.6	0.00001
	Pancreatic [insulin] (uU/mg protein)		66284	33798	7965	15877	8071	8446	0.0001
	Pancreatic [insulin]/[glucagon]		0.77	0.41	0.11	0.24	.12	0.12	0.000001
	Number of islets		20.2	16.8	11.7	12.5	10.9	11.2	0.01
	Number of hyperplastic islets		3.5	2.2	1.0	1.7	0.7	0.6	0.000001
	Average islet size (mm2)		0.0258	0.0209	0.0175	0.0224	0.0167	0.0167	0.00001
D1Mit406	Weight (g)	334	6.9	9.6	10.6	11.8	12.9	13.0	0.02
	HbA1c (%)		7.9	10.6	12.0	12.0	14.2	13.8	0.00001
	[Glucose] (mg/dl)		525	691	780	669	786	794	0.00001
	[Insulin] (uU/ml)		272.6	289.7	175.7	224.32	98.2	138.5	0.04
	Pancreatic Grade		2.2	2.9	3.7	2.8	3.7	3.6	0.00001
	Pancreatic [insulin] (uU/mg protein)		66284	33798	7965	15877	8071	8446	0.0001
	Pancreatic [insulin]/[glucagon]		0.77	0.41	0.11	0.24	.12	0.12	0.000001
	Number of islets		20.2	16.8	11.7	12.5	10.9	11.2	0.01
	Number of hyperplastic islets		3.5	2.2	1.0	1.7	0.7	0.6	0.000001
	Average islet size (mm2)		0.0258	0.0209	0.0175	0.0224	0.0167	0.0167	0.00001
D2Mit367	Weight (g)	358	58.3	61.7	65.6	53.3	56.4	61.7	0.000001
	BMI (g/cm2)		0.59	0.62	0.65	0.55	0.56	0.6	0.00001
	Number of islets		14.5	15.3	19.1	9.7	12.1	12.0	0.04
	Number of hyperplastic islets		1.3	1.7	3.5	0.8	0.9	0.8	0.01
	Islet area (mm2)		0.32	0.35	0.54	0.19	0.24	0.21	0.03
	Islet area/total area (%)		1.277	1.237	2.135	0.857	0.93	0.83	0.04
D2Mit7	Weight (g)	281	57.7	62.6	66.6	52.7	55.7	62.3	0.00001
	BMI (g/cm2)		0.58	0.62	0.65	0.54	0.55	0.6	0.00001
	Number of islets		14.3	16.7	20.4	8.0	11.7	12.0	0.003
	Number of hyperplastic islets		1.4	2.1	3.5	0.9	0.8	0.9	0.04
	Islet area (mm2)		0.31	0.4	0.54	0.17	0.23	0.22	0.03
D2Mit61	Weight (g)	368	57.4	62.4	66.2	52.3	57.5	60.3	0.00001
	BMI (g/cm2)		0.59	0.62	0.65	0.55	0.57	0.59	0.0001
	Number of islets		14.7	15.6	20.2	10.0	11.9	12.4	0.01
	Number of hyperplastic islets		1.3	1.8	3.4	1.1	0.9	0.6	0.03
	Islet area (mm2)		0.32	0.37	0.54	0.22	0.23	0.21	0.03
D2Mit42	Weight (g)	392	57.3	61.5	65.8	52.9	57.0	59.5	0.00001
	BMI (g/cm2)		0.58	0.62	0.65	0.56	0.56	0.58	0.001
	[Insulin] (uU/ml)		154.17	266.9	320.32	137.67	129.33	131.82	0.01
	Number of islets		11.9	17.0	19.3	9.8	11.5	13.0	0.0005
	Number of hyperplastic islets		0.8	2.3	3.0	1.0	1.0	0.8	0.04
	Islet area (mm2)		0.24	0.43	0.49	0.23	0.23	0.24	0.02
	Islet area/total area (%)		0.927	1.617	1.907	0.939	0.892	0.878	0.04

Marker	Phenotype	Number	Female			Male			P
			B&B6	B6/DBA	DBA/DBA	B&B6	B6/DBA	DBA/DBA	
D2Mit17	Weight (g)	373	57.8	61.7	66.1	53.4	57.1	58.6	0.00001
	BMI (g/cm ²)		0.6	0.61	0.66	0.56	0.56	0.57	0.009
	[Insulin] (uU/ml)		155.73	282.11	303.89	127.89	124.42	121.18	0.02
	Number of islets		12.0	17.2	20.6	10.4	10.8	13.2	0.0003
	Number of hyperplastic islets		0.9	2.4	3.5	0.8	0.8	0.7	0.002
	Islet area (mm ²)		0.25	0.45	0.54	0.21	0.21	0.23	0.002
	Islet area/total area (%)		0.978	1.65	2.087	0.869	0.806	0.905	0.006
D4Mit192	Number of islets	360	16.3	15.4	20.9	8.9	12.4	12.6	0.03
	Number of hyperplastic islets		1.9	1.7	4.0	0.5	1.1	0.9	0.008
	Average islet size (mm ²)		0.0209	0.0197	0.0266	0.0166	0.0187	0.019	0.009
	Islet area (mm ²)		0.39	0.35	0.64	0.16	0.25	0.25	0.003
	Islet area/total area (%)		1.552	1.318	2.284	0.624	1.029	0.87	0.02
D4Mit288	HbA1c (%)	332	10.01	10.94	8.39	14.79	13.38	12.58	0.003
	[Glucose] (mg/dl)		677	702	564	832	850	730	0.0006
	[Insulin] (uU/ml)		240.8	215.8	362.9	78.0	132.2	145.4	0.006
	Pancreatic Grade		2.9	3.1	2.2	3.6	3.3	3.4	0.02
	Pancreatic [Insulin]/[glucagon]		0.39	0.25	0.84	0.11	0.19	0.17	0.003
	Number of islets		16.0	14.9	21.2	10.3	11.8	12.6	0.02
	Number of hyperplastic islets		1.8	1.4	4.6	0.5	1.0	1.1	0.0001
	Average islet size (mm ²)		0.0206	0.0194	0.0278	0.0156	0.0186	0.0189	0.0003
	Islet area (mm ²)		0.37	0.33	0.68	0.17	0.24	0.25	0.0007
	Islet area/total area (%)		1.515	1.249	2.415	0.623	0.978	0.967	0.002
D4Mit288	BMI (g/cm ²)	331	0.63	0.62	0.61	0.58	0.57	0.55	0.04
	[Glucose] (mg/dl)		667	708	623	826	769	710	0.02
	[Insulin] (uU/ml)		277.0	213.7	316.7	90.5	118.1	185.3	0.02
	Number of hyperplastic islets		2.0	1.5	3.2	0.5	0.7	1.6	0.002
	Average islet size (mm ²)		0.0202	0.02	0.0242	0.0169	0.0175	0.0213	0.004
	Islet area (mm ²)		0.41	0.32	0.5	0.21	0.19	0.3	0.01
	Islet area/total area (%)		1.539	1.259	1.963	0.756	0.793	1.188	0.01
D6Mit74	[Glucose] (mg/dl)	328	632	798	780	745	754	752	0.0008
	[Insulin] (uU/ml)		268.7	101.6	83.9	143.3	88.2	41.9	0.00001
	Pancreatic [Insulin] (uU/mg protein)		39807	12453	2087	10530	11083	5323	0.00001
	Number of islets		17.1	10.8	8.0	11.5	9.2	4.0	0.02
	Islet area (mm ²)		0.43	0.22	0.16	0.22	0.16	0.09	0.00001
	Islet area/total area (%)		1.665	0.868	0.42	0.903	0.57	0.509	0.02
D6Mit54	[Glucose] (mg/dl)	336	622	679	735	728	726	803	0.006
	[Insulin] (uU/ml)		295.0	216.5	117.2	156.4	148.1	69.6	0.00001
	Number of islets		18.0	15.0	12.2	12.0	11.8	6.6	0.0001
	Number of hyperplastic islets		2.4	2.0	1.0	1.2	0.9	0.3	0.003
	Average islet size (mm ²)		0.0216	0.0209	0.0178	0.0194	0.0187	0.0153	0.009
	Islet area (mm ²)		0.46	0.36	0.24	0.25	0.23	0.11	0.0002
	Islet area/total area (%)		1.693	1.494	0.868	0.977	0.944	0.405	0.00001
D8Mit89	[Glucose] (mg/dl)	332	643	717	561	729	746	735	0.02
	[Insulin] (uU/ml)		217.6	203.1	348.0	146.8	138.5	150.4	0.02
	Pancreatic grade		2.7	3.2	2.4	3.2	3.4	3.3	0.01
	Number of hyperplastic islets		1.8	1.6	3.6	0.9	0.9	0.9	0.02
	Islet area (mm ²)		0.37	0.32	0.56	0.2	0.24	0.22	0.04
D13Mit9	[Insulin] (uU/ml)	317	169.71	217.99	332.89	101.28	170.52	144.73	0.001
	Pancreatic grade		3.2	2.9	2.6	3.6	3.3	3.1	0.01
	Average islet size (mm ²)		0.02	0.0193	0.0232	0.0164	0.0185	0.0209	0.02
	Islet area/total area (%)		1.3	1.22	2.031	0.763	0.925	1.035	0.02
D13Mit11	[Glucose] (mg/dl)	297	726	669	594	736	759	698	0.02
	[Insulin] (uU/ml)		168.9	229.2	322.7	99.7	155.7	180.0	0.0009
	Pancreatic Grade		3.2	2.9	2.7	3.6	3.4	2.8	0.001
	Pancreatic [Insulin] (uU/mg protein)		18701	33252	49951	11846	8365	17420	0.01
	Pancreatic [Insulin]/[glucagon]		0.31	0.37	0.6	0.18	0.14	0.26	0.03
	Number of hyperplastic islets		1.7	1.8	2.9	0.6	0.8	1.5	0.01
	Average islet size (mm ²)		0.0194	0.0199	0.0241	0.0166	0.0178	0.0216	0.003
	Islet area (mm ²)		0.32	0.36	0.51	0.19	0.21	0.28	0.04
	Islet area/total area (%)		1.198	1.438	1.956	0.792	0.805	1.137	0.04
D14Mit28	[Glucose] (mg/dl)	368	678	688	560	768	756	724	0.005
	Number of islets		15.2	15.5	21.6	11.6	11.3	11.6	0.05
	Number of hyperplastic islets		1.4	1.9	4.2	0.5	1.1	1.0	0.0008
	Islet area (mm ²)		0.34	0.38	0.61	0.2	0.24	0.24	0.008
D14Mit35	[Glucose] (mg/dl)	331	694	688	570	734	746	722	0.02
	Number of islets		14.6	14.7	20.1	12.5	10.2	11.5	0.05
	Number of hyperplastic islets		1.6	1.6	3.9	0.8	0.8	1.1	0.0006
	Average islet size (mm ²)		0.0184	0.0201	0.0239	0.0179	0.0176	0.0198	0.02
	Islet area (mm ²)		0.31	0.34	0.57	0.23	0.19	0.25	0.006
	Islet area/total area (%)		1.221	1.371	2.082	0.917	0.76	1.036	0.01

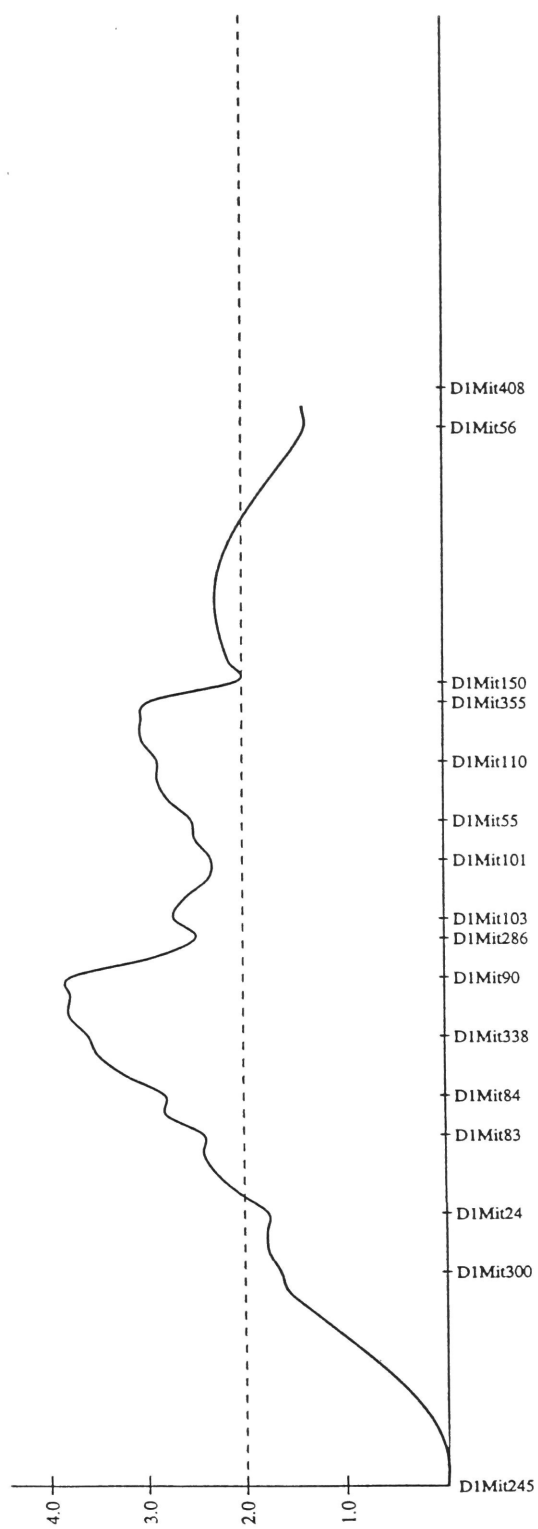
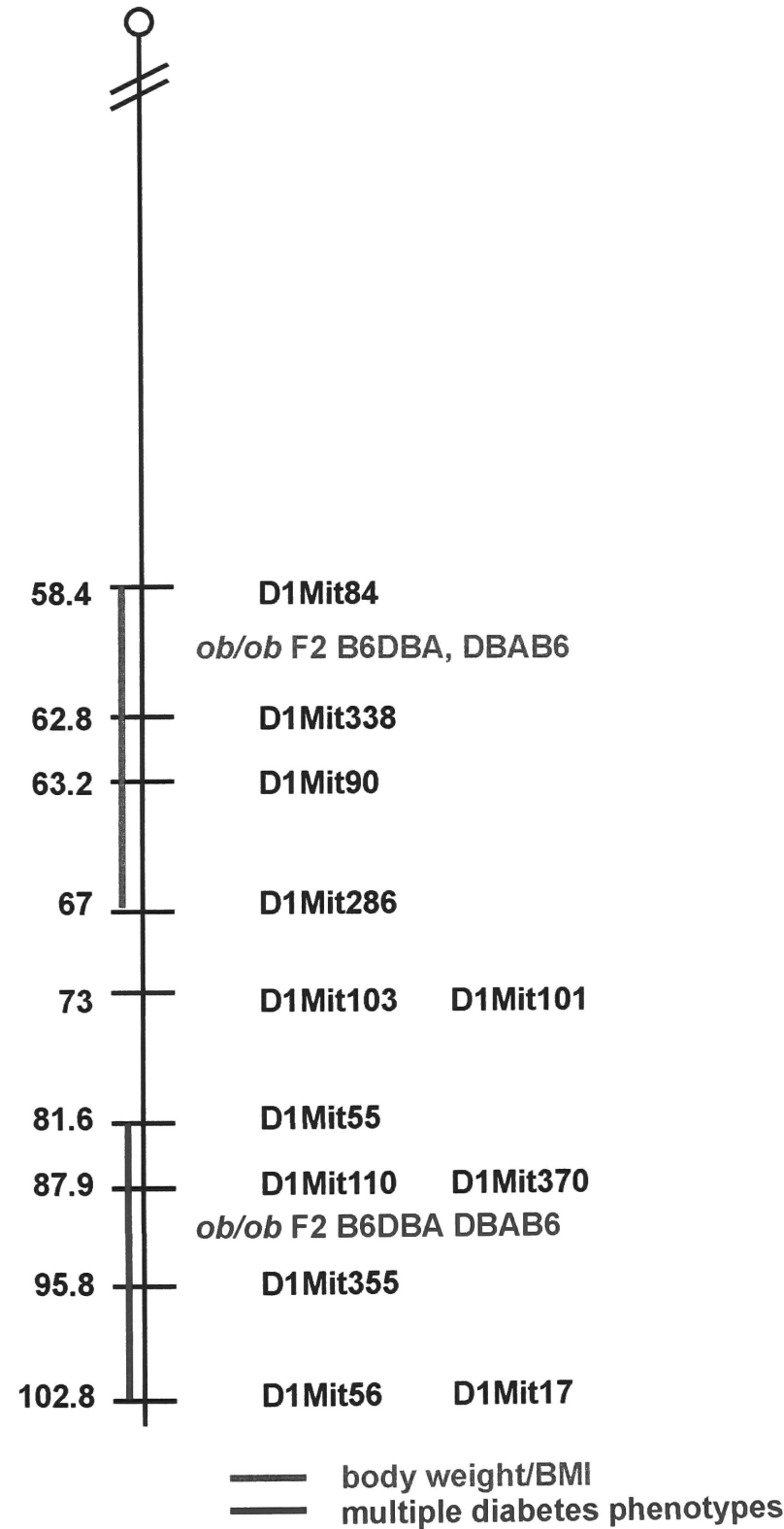


Figure 10. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for weight in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

Figure 11. Summary of the QTLs for obesity/diabetes on mouse chromosome 1. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 1



F2 *ob/ob* B6DBA, DBAB6

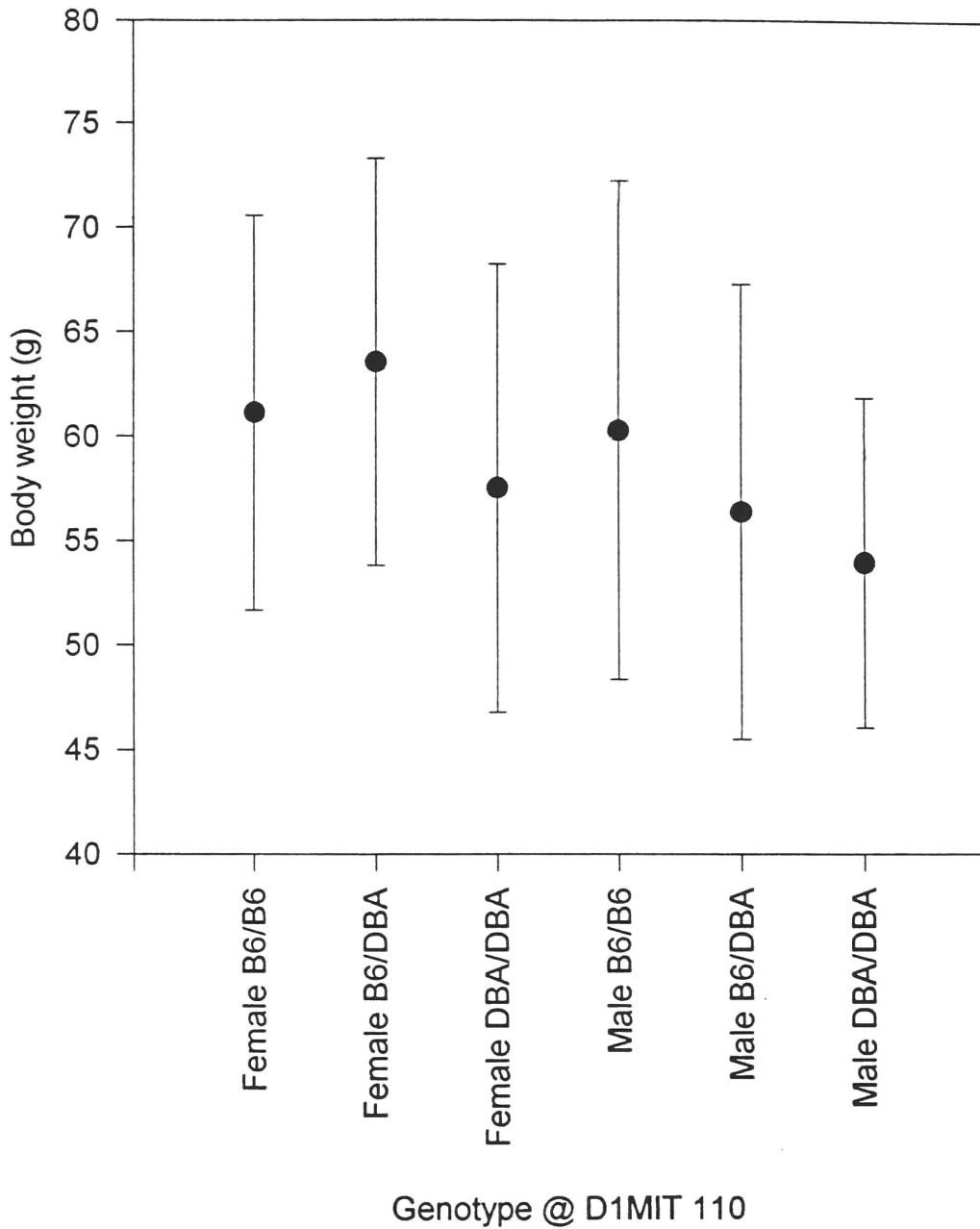


Figure 12. Relationship of genotype at D1Mit110 on body weight by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

Conversely, for other traits more directly related to diabetes, the telomeric chromosome 1 QTL exerted the greater effect of the two regions, and the influence of the proximal QTL could not be distinguished from that of the telomeric diabetes QTL (Figures 13-22). Of the three plasma/blood biochemical traits used to characterize diabetes severity, HbA1c and plasma [glucose] accounted for a greater fraction of the variance (11.4 and 10.5%, respectively) than did [insulin] (3.4%). As with the traits measuring degree of obesity, B6 alleles were associated with relative protection from diabetes (Table 10). In females, the influence of DBA alleles was co-dominant or additive; however, in males the DBA allele acted dominantly to increase diabetes susceptibility for all diabetes phenotypes (Figures 23-30). It is possible that a phenotypic ceiling had been reached for males with a single copy of the DBA allele at this locus since the males were relatively more diabetic than the females in which an effect of gene dosage could still be observed. For the phenotypes related to the pancreas, pancreatic grade demonstrated the most significant results, with a peak LOD of 9.0 accounting for 13.7% of the variance. Pancreatic grade was a qualitative judgement which assessed the likelihood that a given pancreas was able to function to meet the demands imposed by insulin resistance and hyperglycemia. The traits pancreatic [insulin] and pancreatic [insulin]/[glucagon] demonstrated comparable results to each other, and genotype in the D1Mit150 to D1Mit56 interval accounted for approximately the same variance (11.6 and 13.9% respectively) as pancreatic grade and provided a quantitative measure of the functional beta cell reserve in a given pancreas. For both males and females, B6/B6 animals demonstrated pancreatic [insulin] two to eight fold higher than those of the DBA/DBA class for markers in the

D1Mit110/D1Mit150 interval. Of the traits quantifying pancreatic morphology, genotype at the chromosome 1 QTL accounted for a greater fraction of the variance in the number of hypertrophic islets and average islet area (an indirect measure of extent of islet hypertrophy)(7.9% and 12.0%) than did the number of islets (3.3%) which was a more direct measure of islet hyperplasia. Genes on chromosome 1 accounted for 10.2% of the variance of normalized total islet area, a similar result to that observed with the direct measure of pancreatic insulin content.

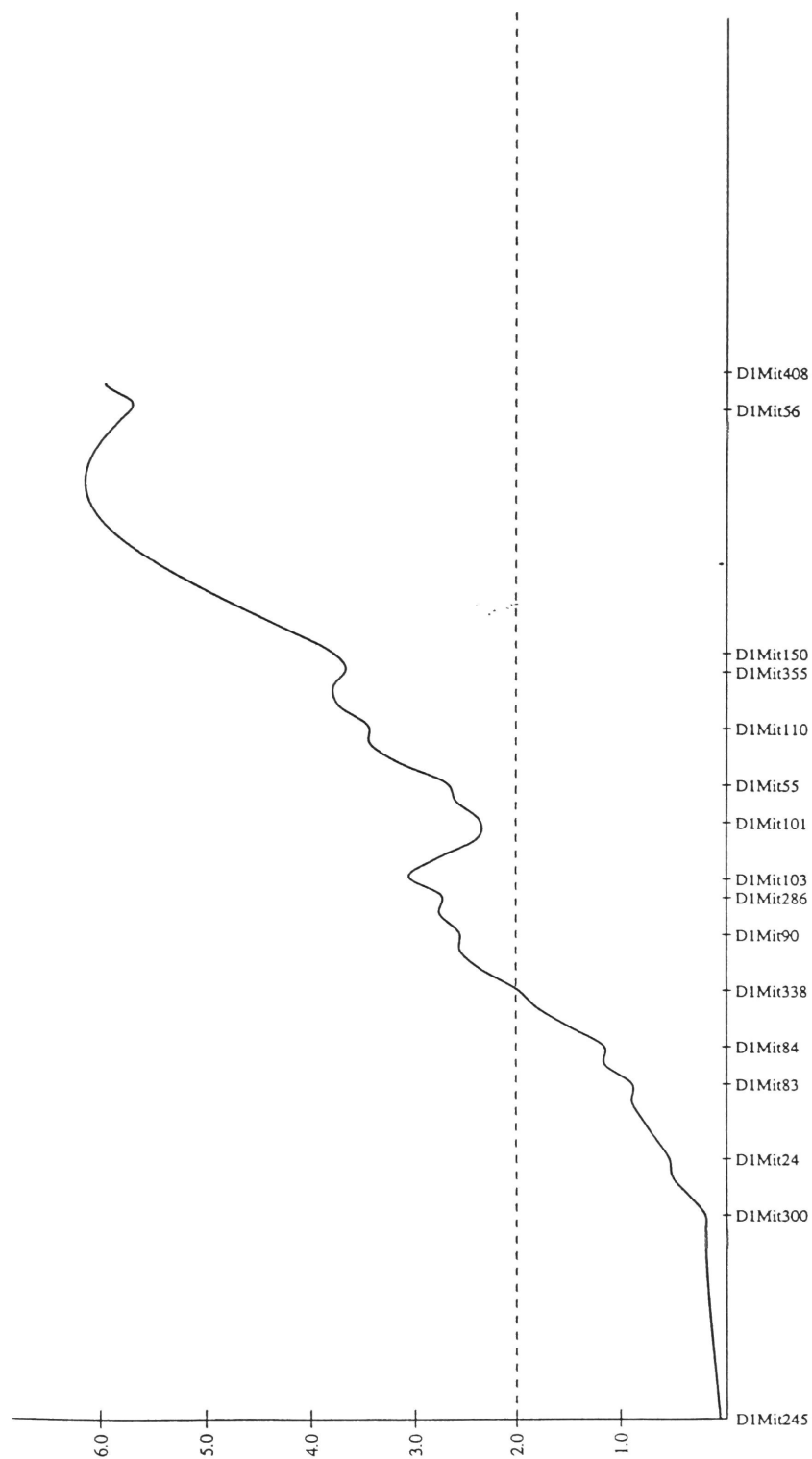


Figure 13. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for HbA1c in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

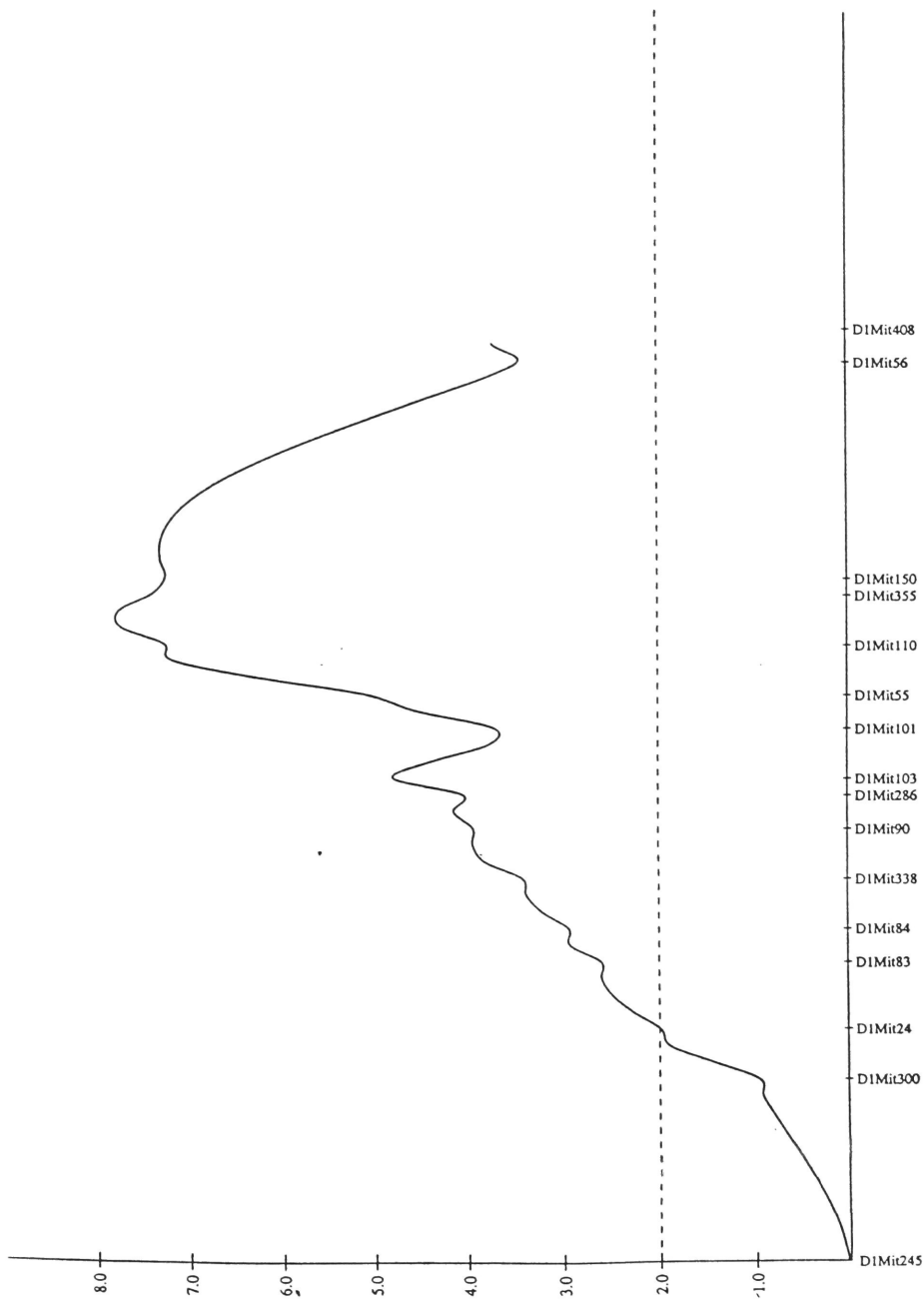


Figure 14. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for fasting plasma [glucose] in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

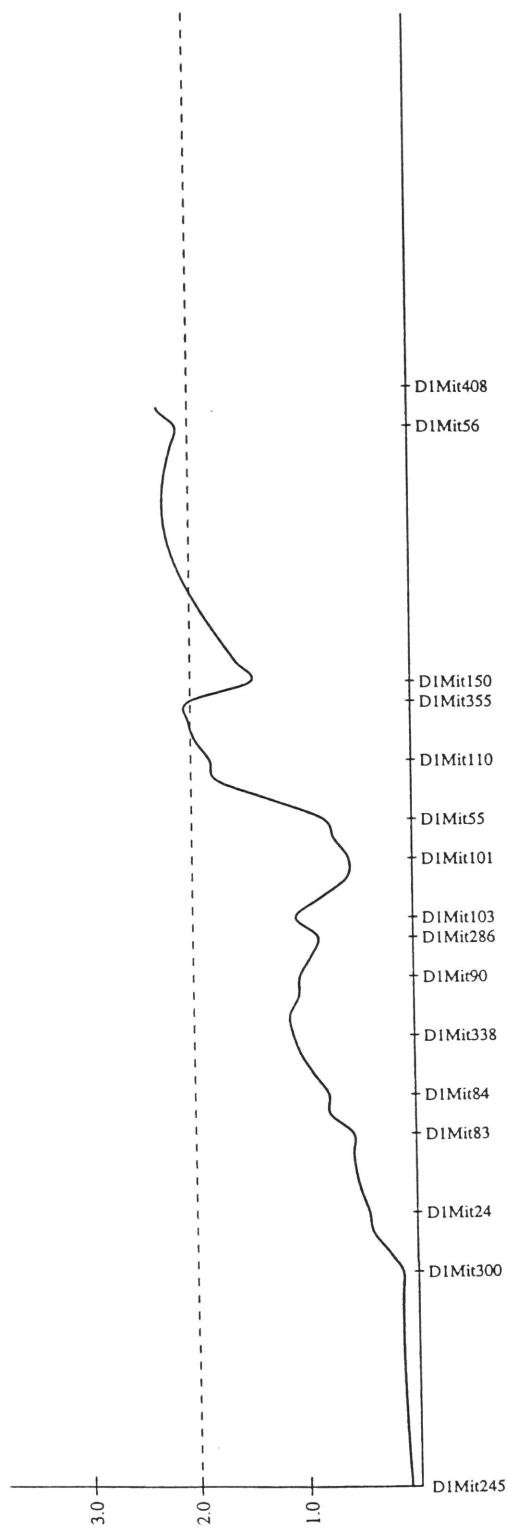


Figure 15. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for fasting plasma [insulin] in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

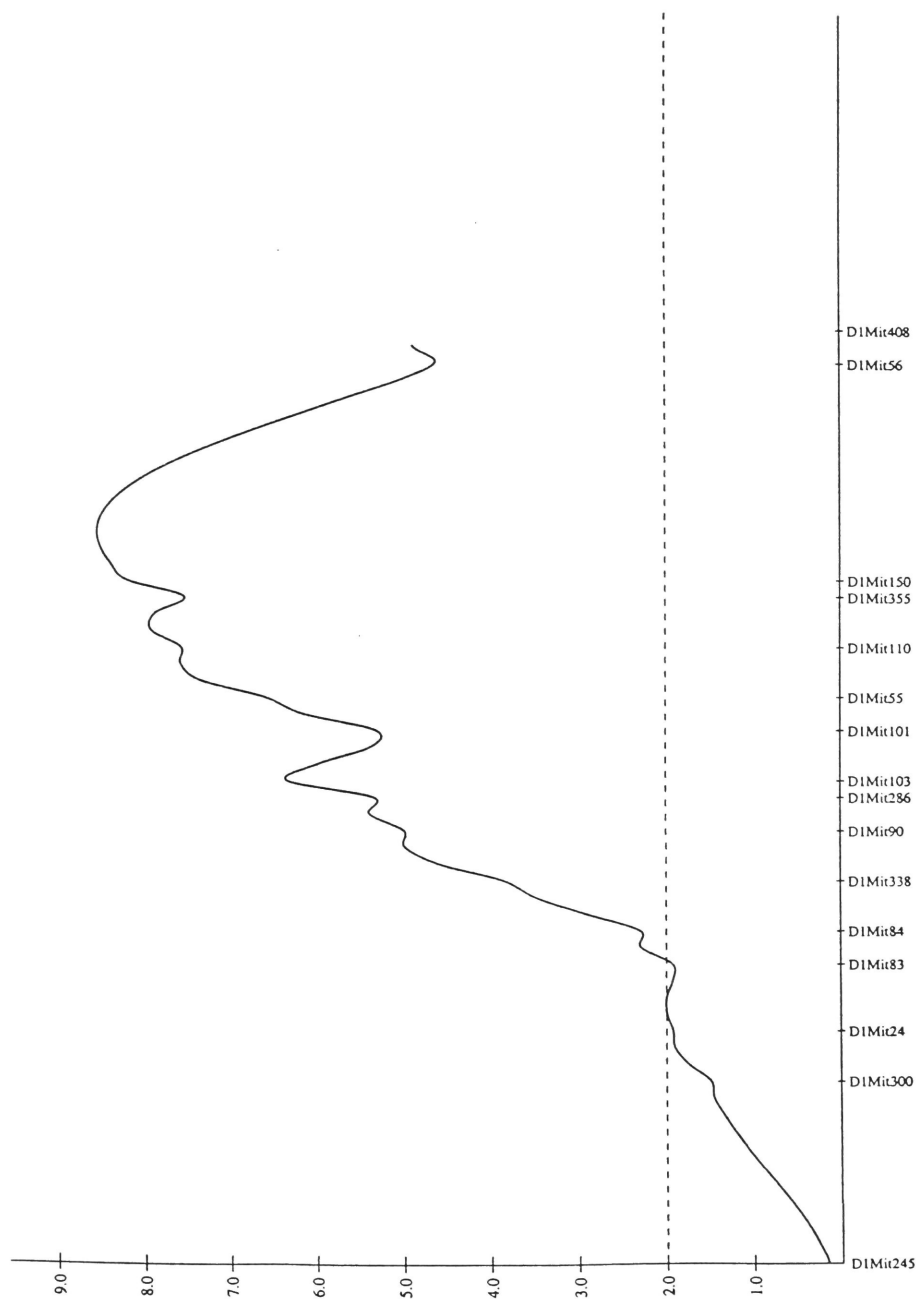


Figure 16. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for pancreatic grade in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

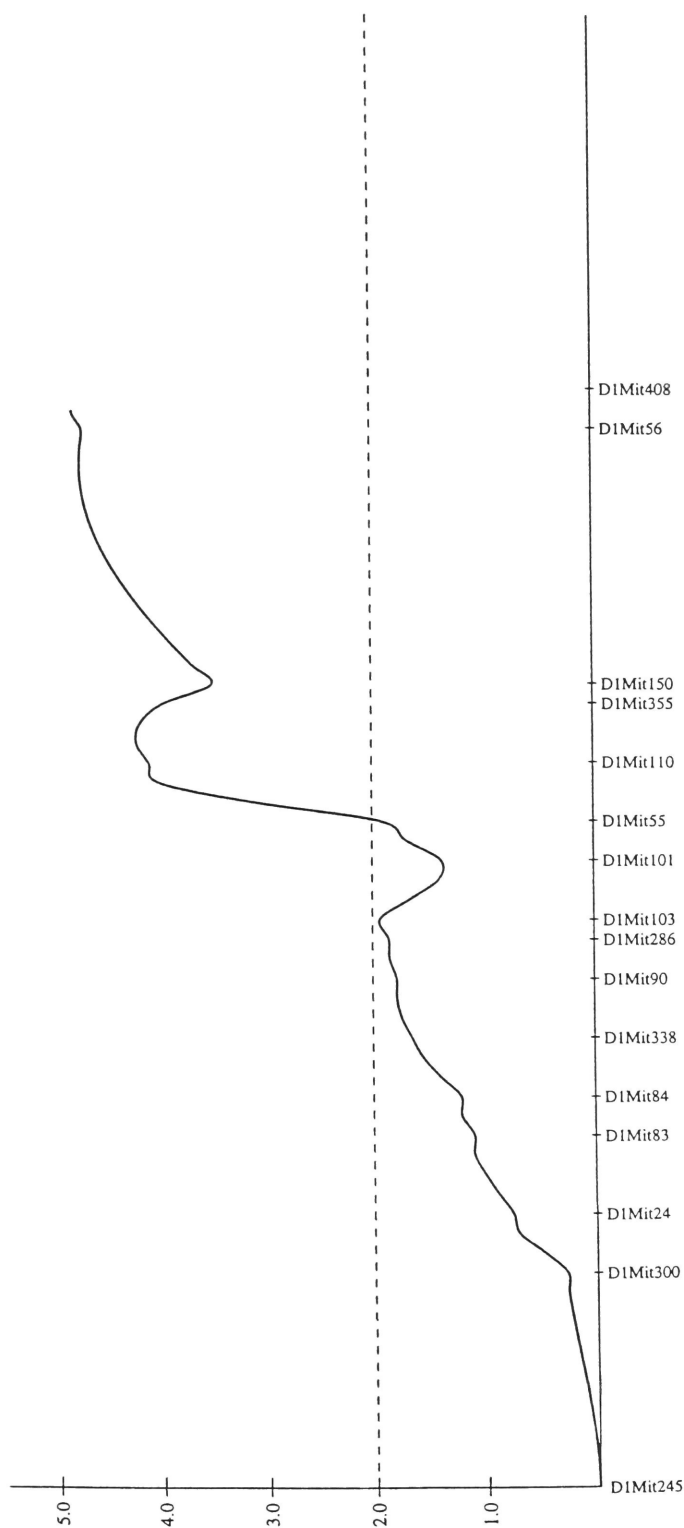


Figure 17. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for pancreatic insulin content normalized to pancreatic protein in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

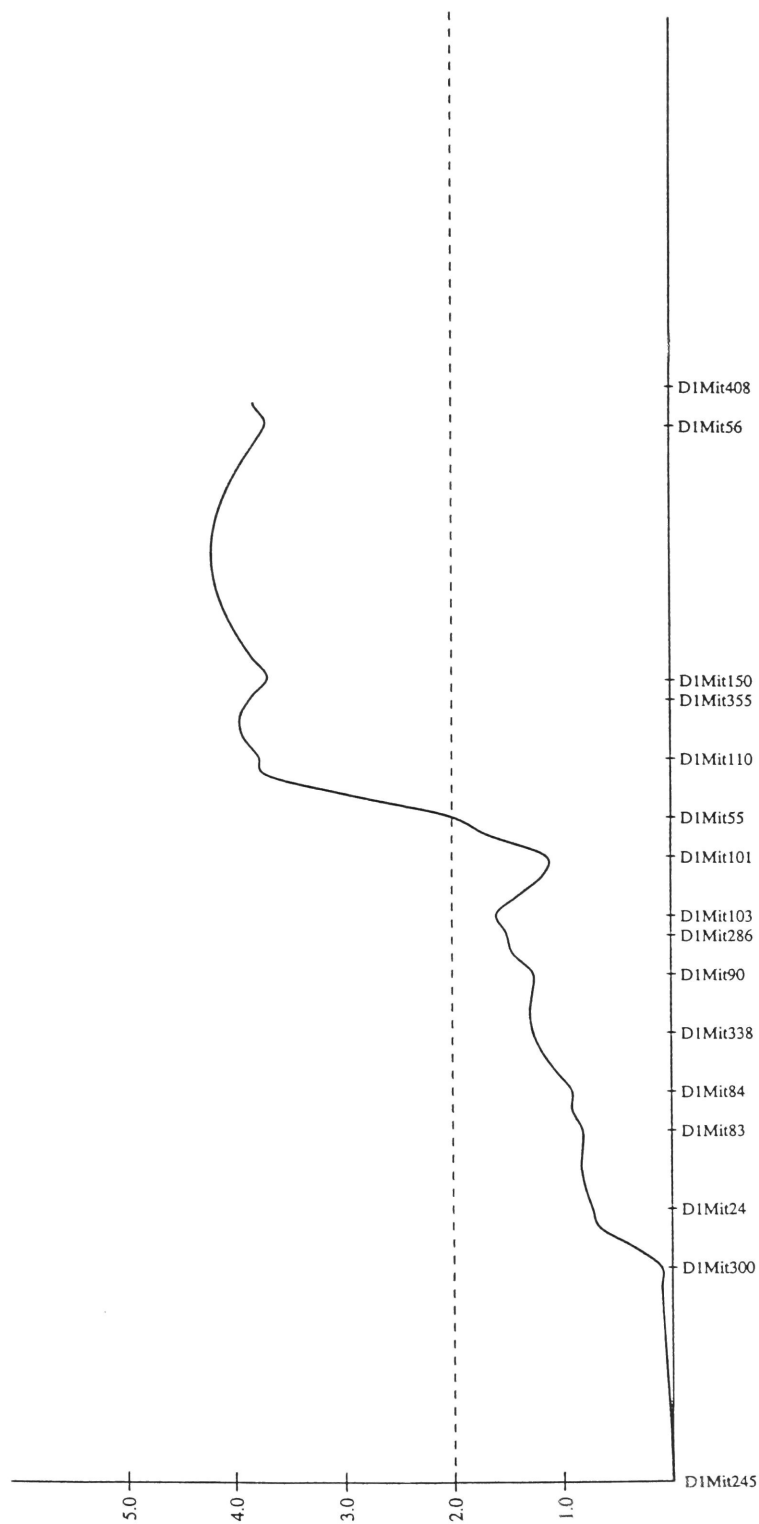


Figure 18. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) ratio of pancreatic [insulin]/[glucagon] in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

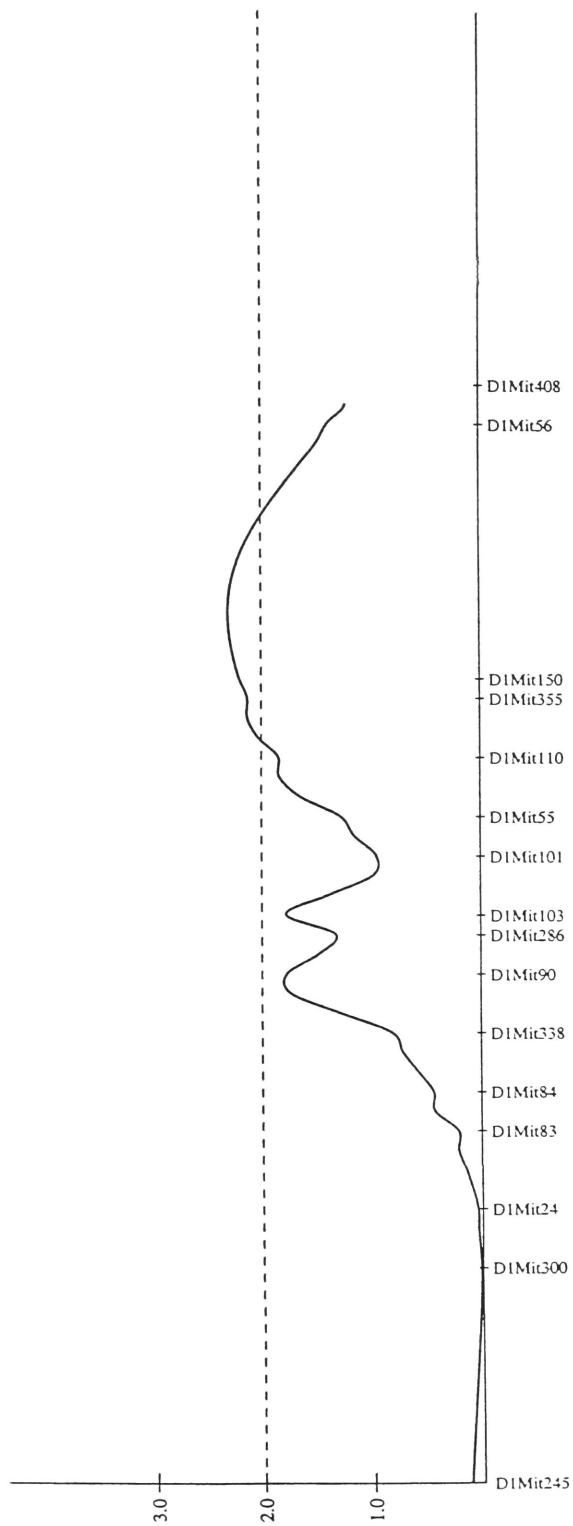


Figure 19. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for total number of islets in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

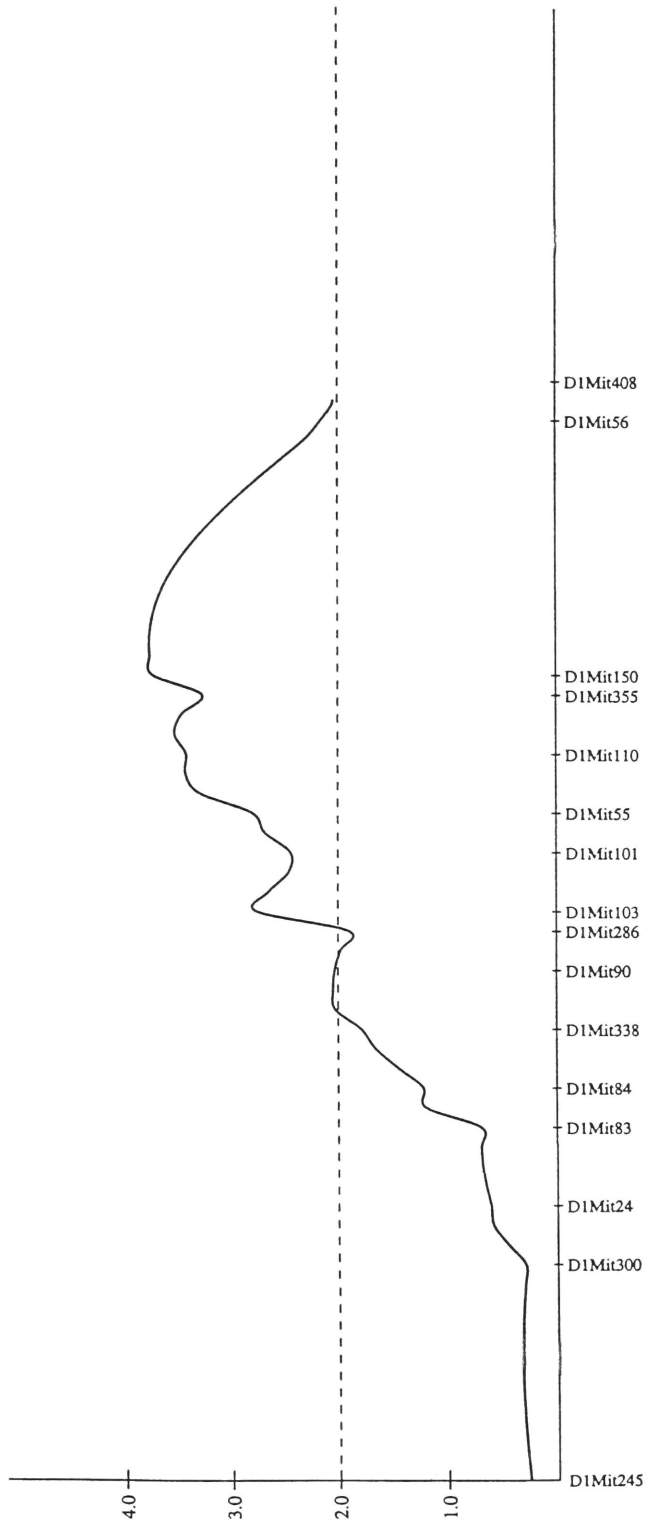


Figure 20. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for number of hypertrophic islets in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

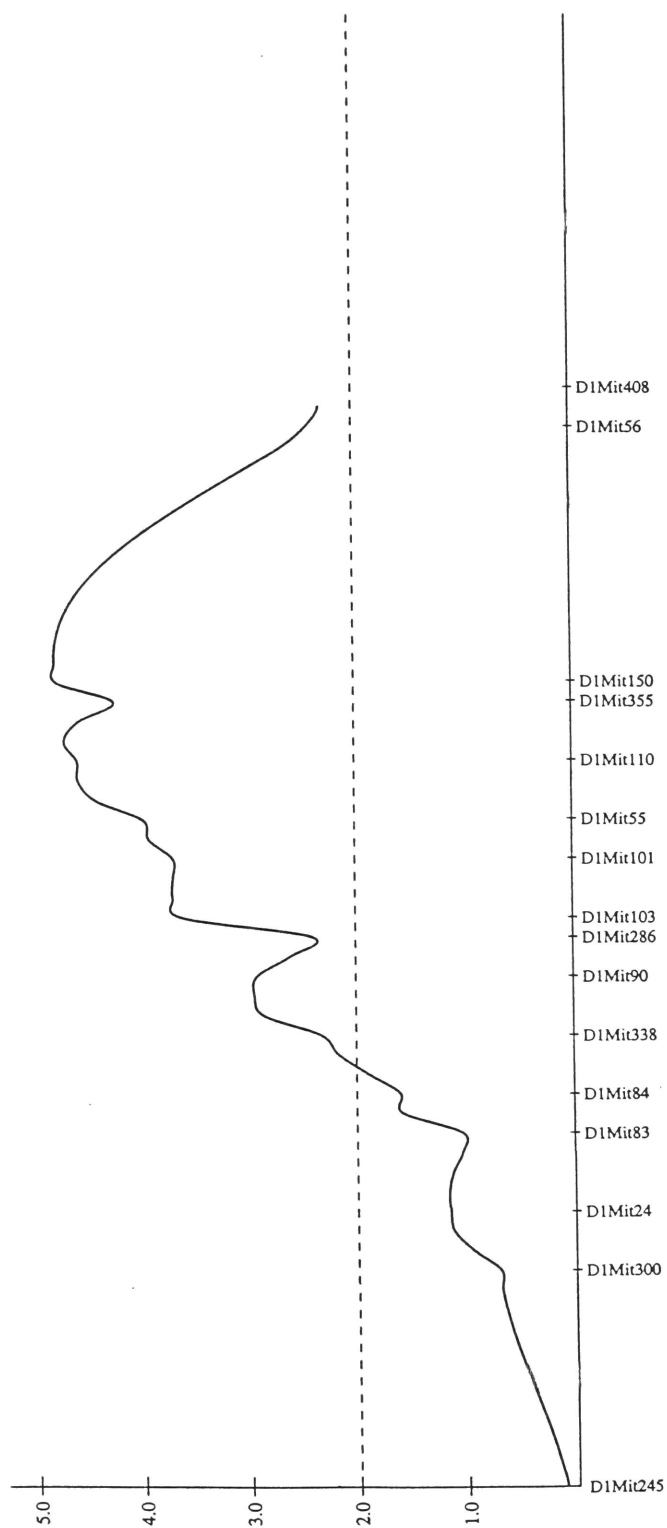


Figure 21. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for average islet area in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

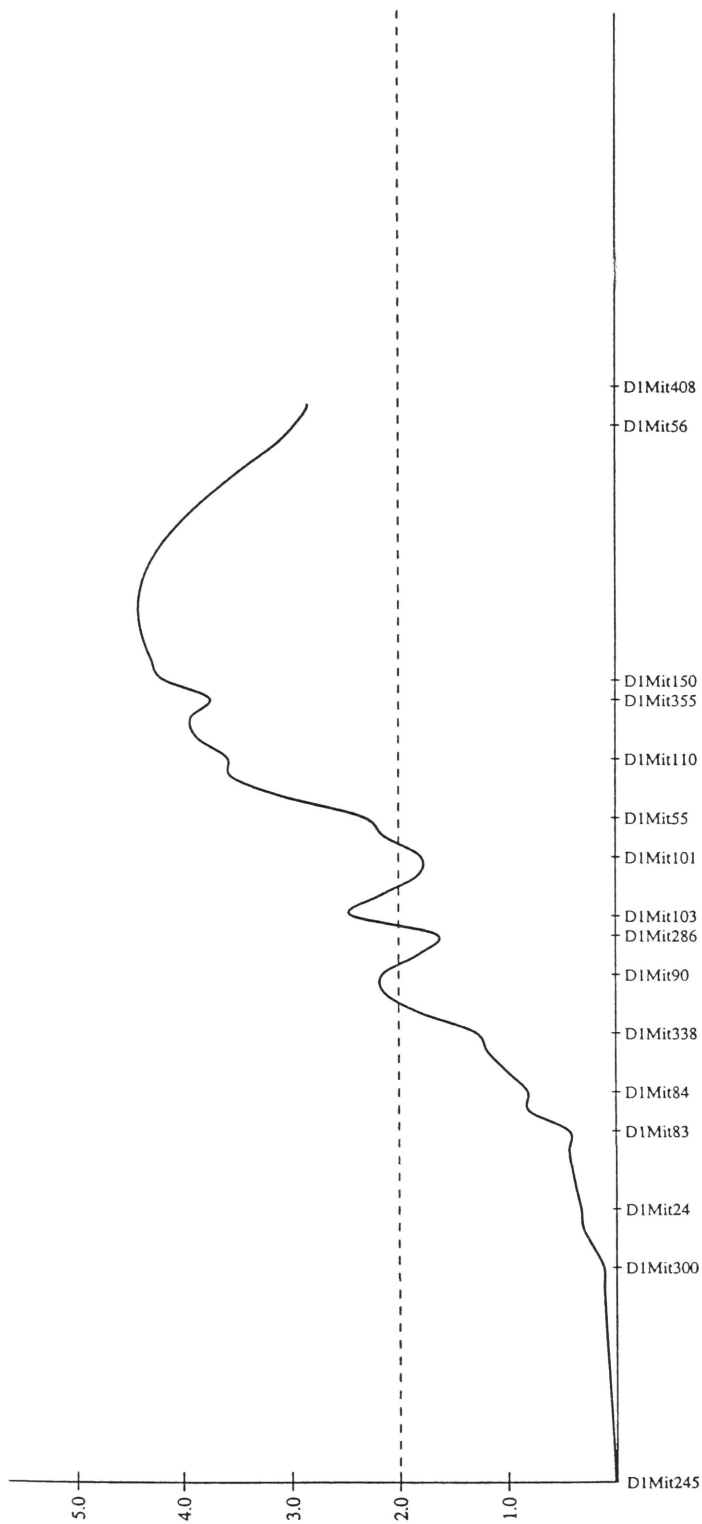


Figure 22. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for total islet area normalized to pancreatic area in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

F2 *ob/ob* B6DBA, DBAD6

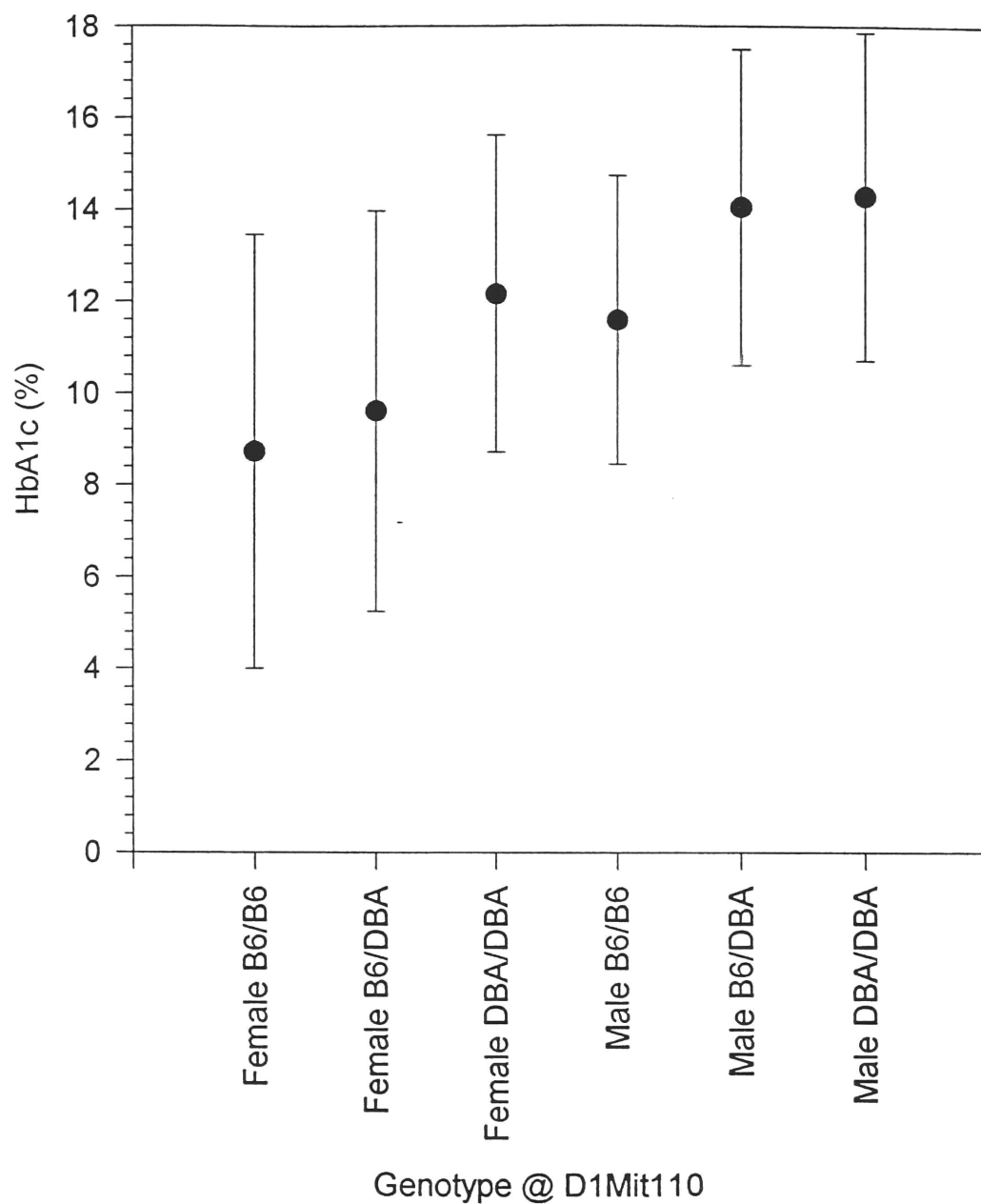


Figure 23. Relationship of genotype at D1Mit110 on HbA1c by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

F2 *ob/ob* B6DBA, DBAB6

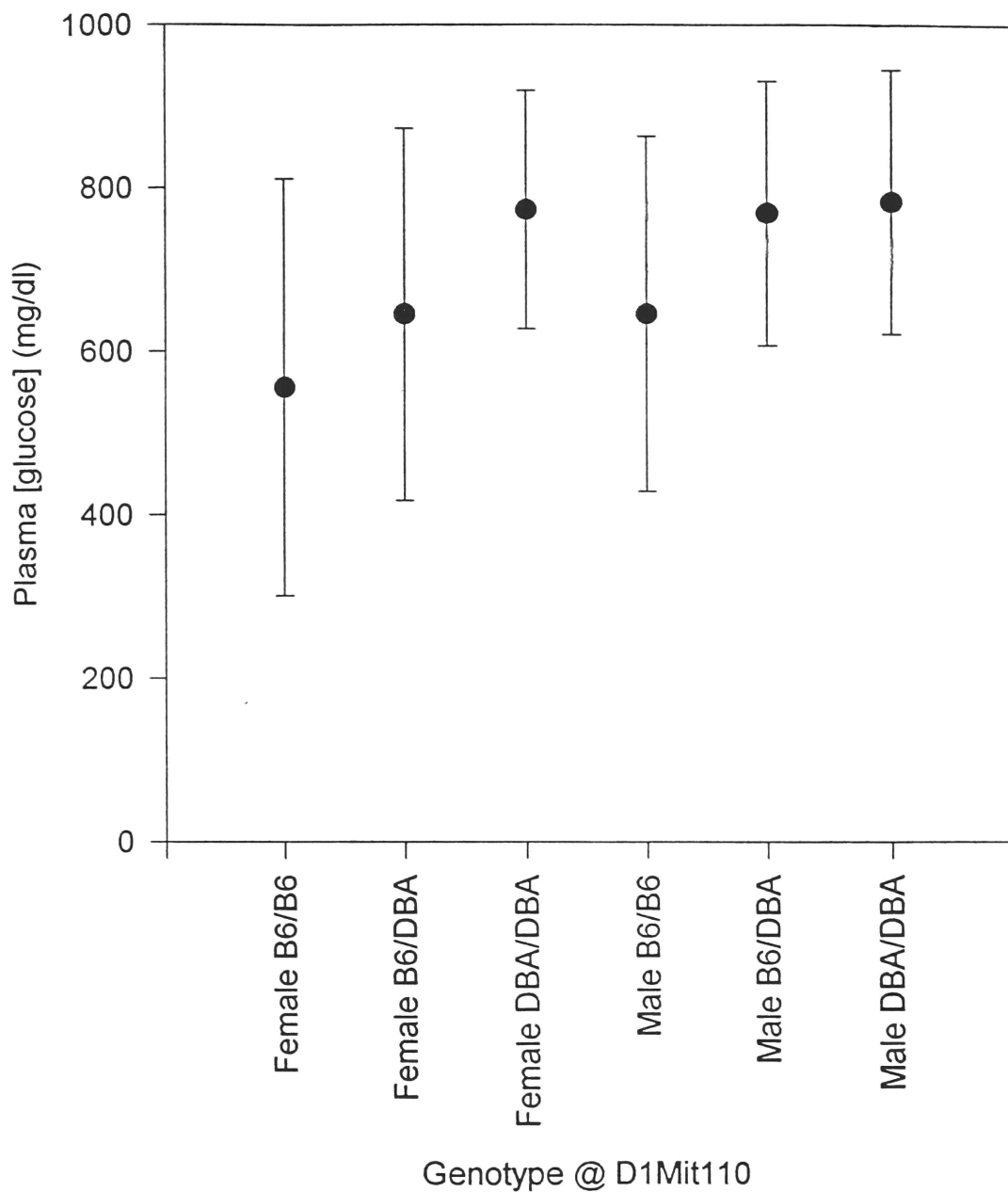


Figure 24. Relationship of genotype at D1Mit110 on plas

F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

F2 *ob/ob* B6DBA, DBAB6

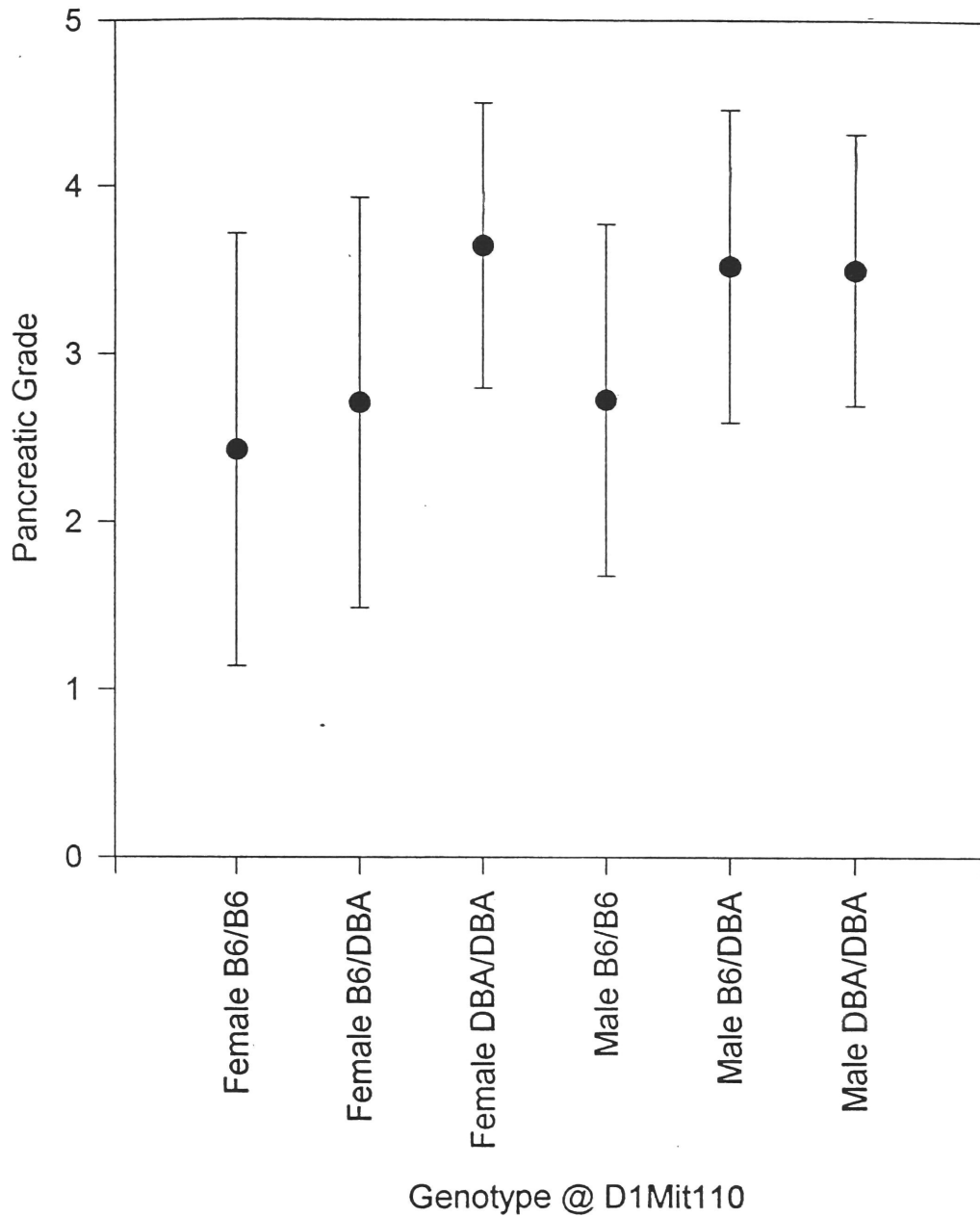


Figure 25. Relationship of genotype at D1Mit110 on pancreatic grade by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

F2 *ob/ob* B6DBA, DBAB6

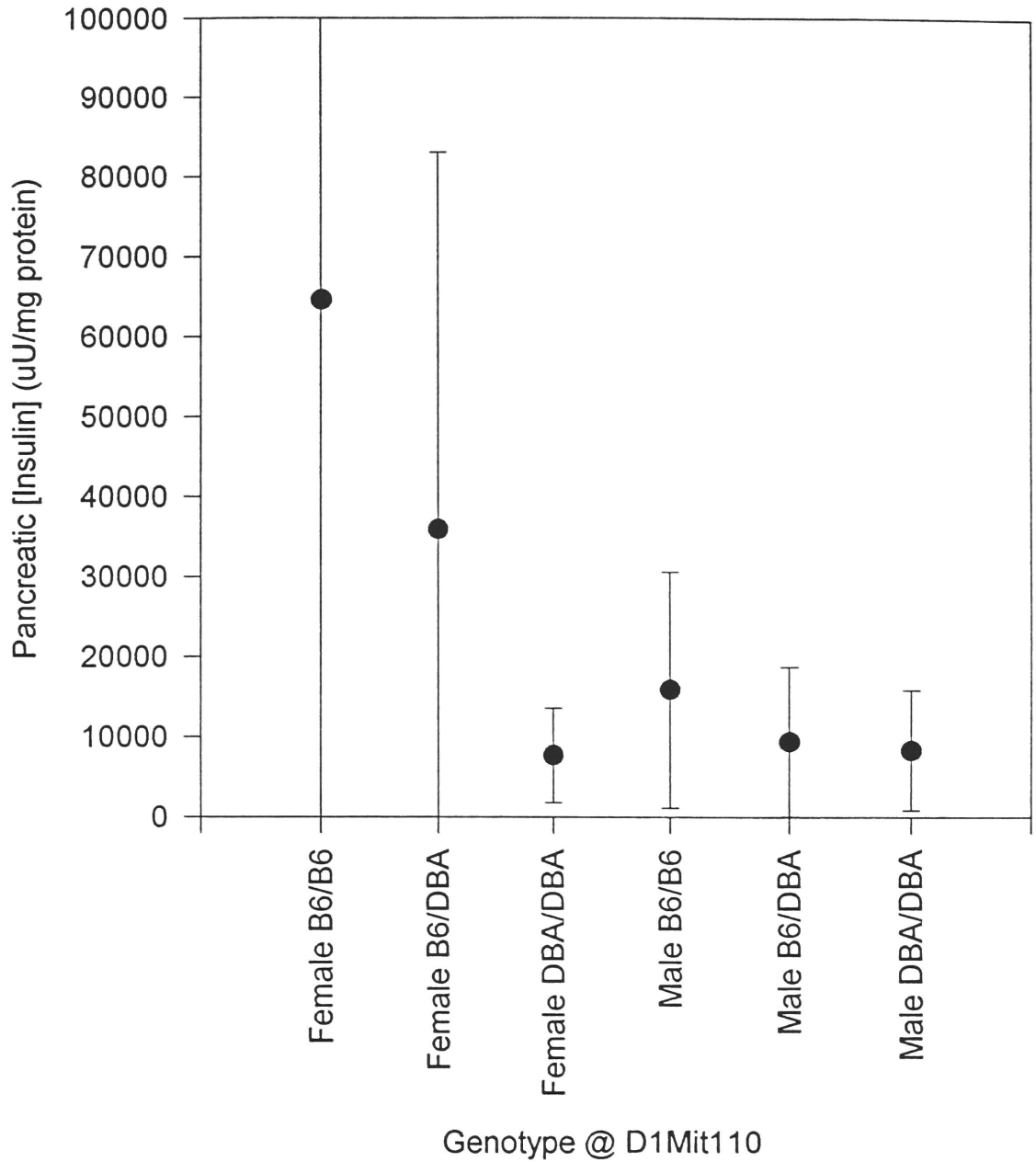


Figure 26. Relationship of genotype at D1Mit110 on pancreatic [insulin] by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

F2 *ob/ob* B6DBA, DBAB6

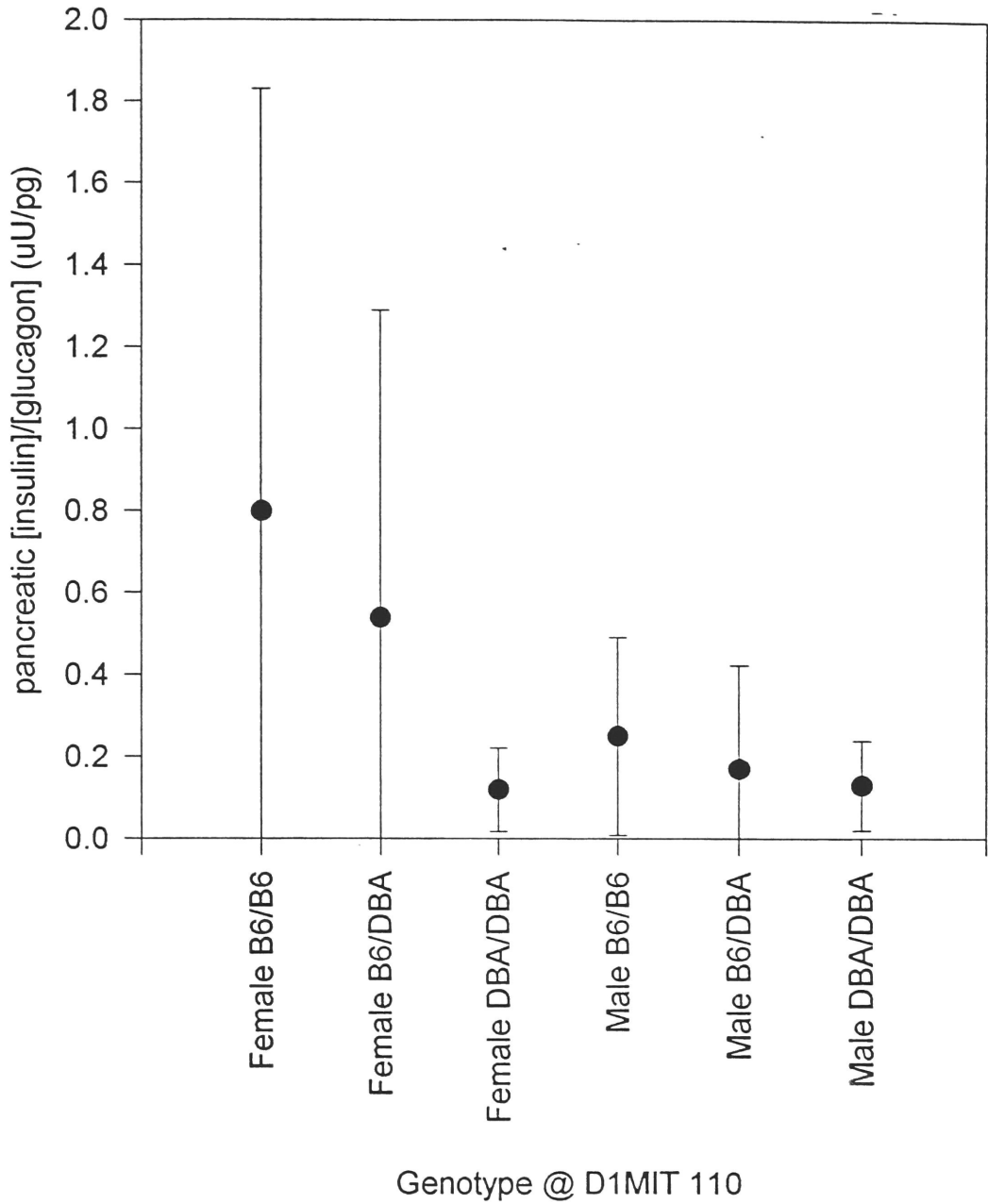


Figure 27. Relationship of genotype at D1Mit110 on pancreatic [insulin]/[glucagon] by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

F2 *ob/ob* B6DBA, DBAB6

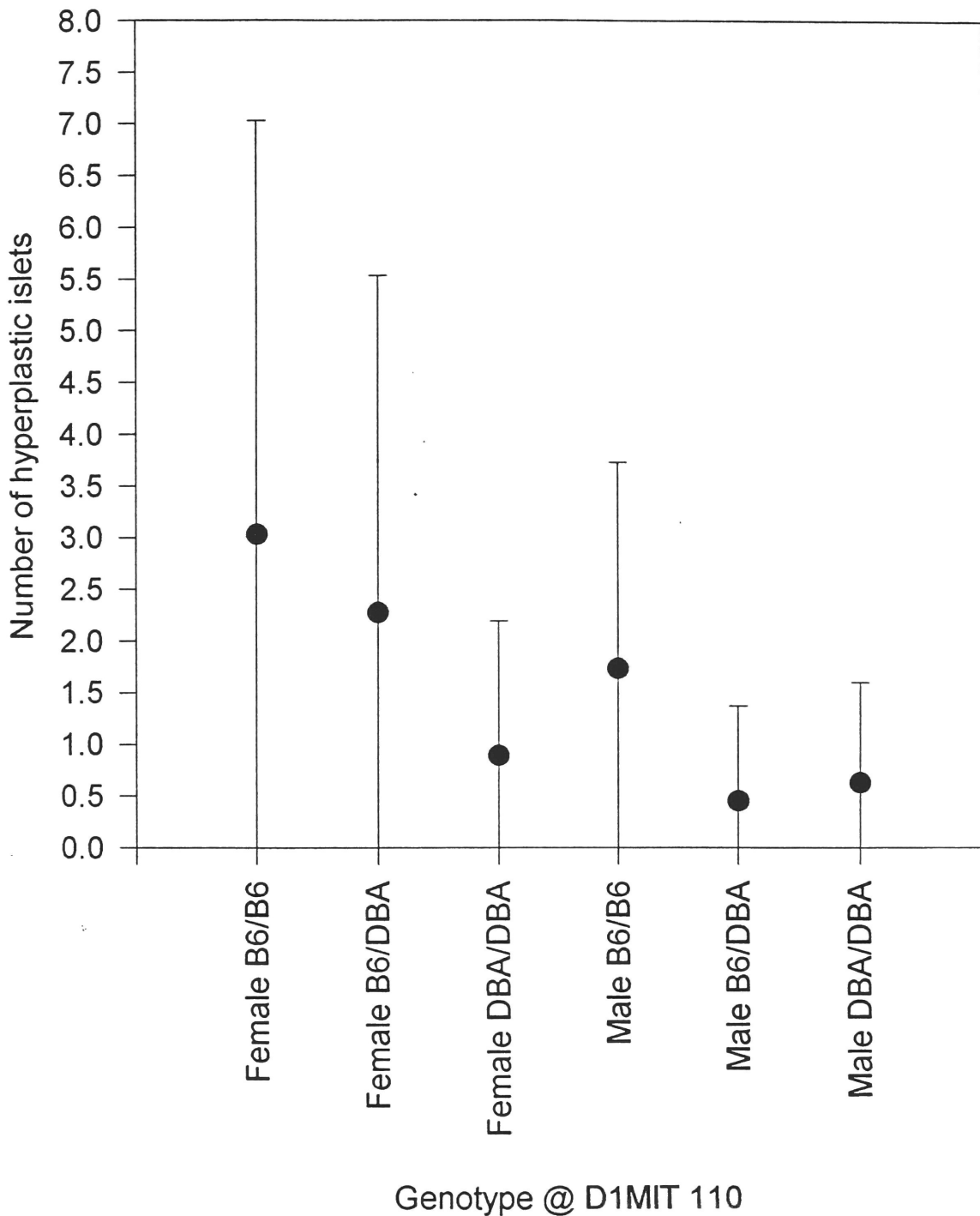


Figure 28. Relationship of genotype at D1Mit110 on number of hyperplastic islets by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

F2 *ob/ob* B6DBA, DBAB6

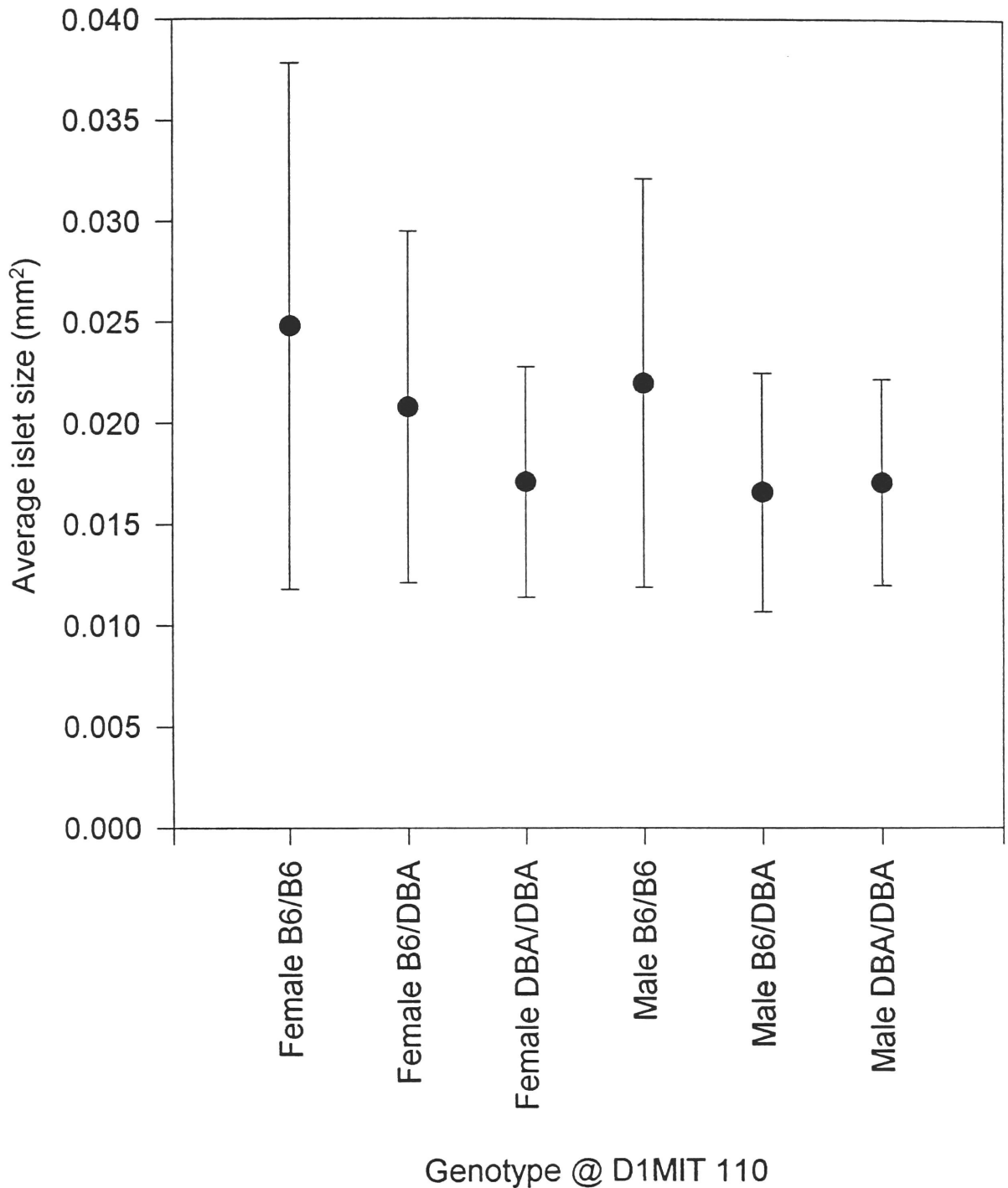


Figure 29. Relationship of genotype at D1Mit110 on average islet size by sex in *ob/ob*

F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

F2 *ob/ob* B6DBA, DBAB6

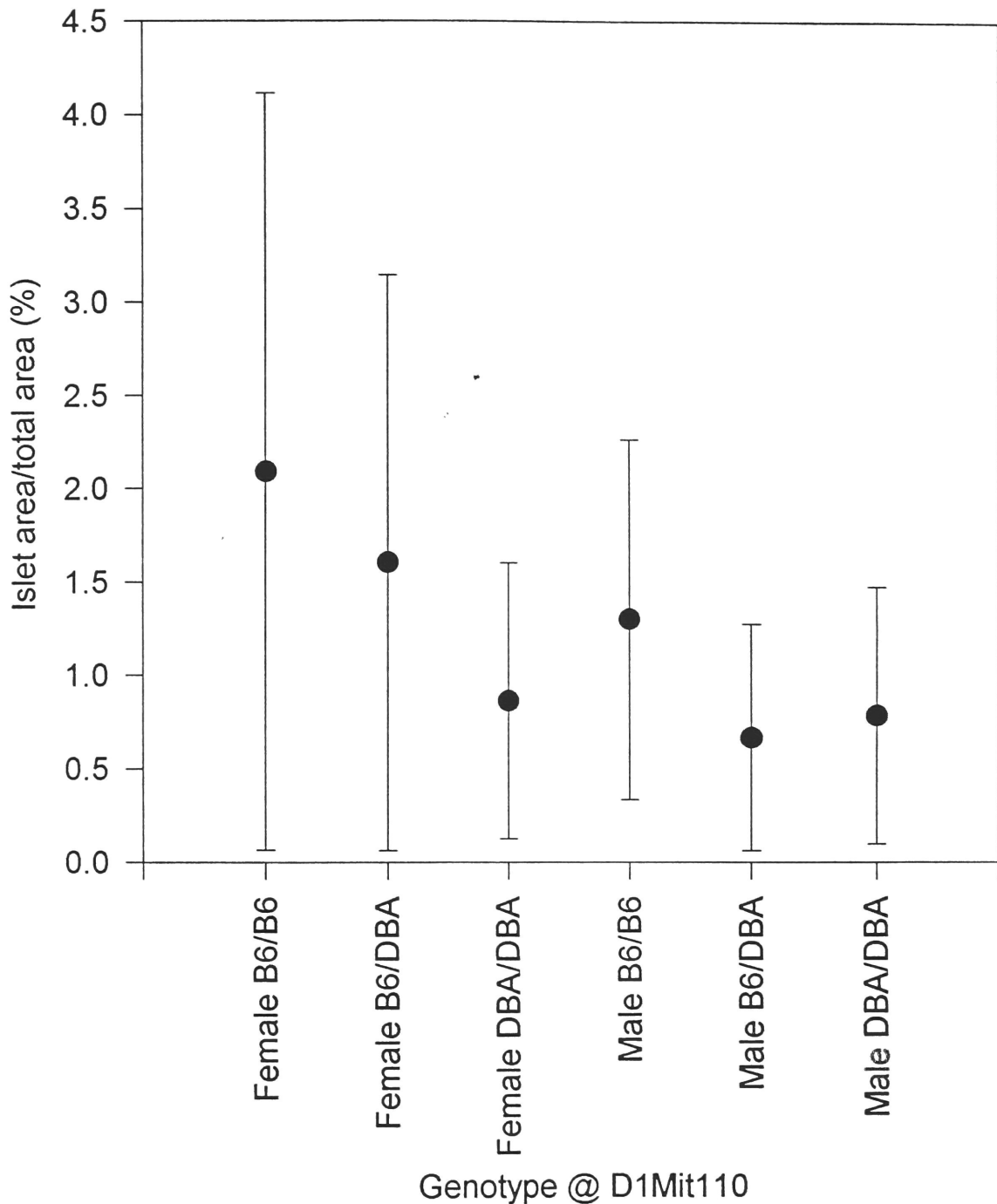


Figure 30. Relationship of genotype at D1Mit110 on normalized islet area by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

The chromosome 2 locus exerted its primary effect on weight and BMI with small, likely secondary, effects on the number of islets, number of hyperplastic islets and total islet area (Figures 31, 32, 33, Table 9 and 10). The LOD scores for weight (8.9) and BMI (7.1) were the same order of magnitude as those observed for several of the phenotypes with the telomeric chromosome 1 locus. The chromosome 2 QTL accounted for 11.7 and 9.2% of the variance of weight and BMI, respectively. The chromosome 2 locus behaved co-dominantly in both sexes (Figure 34, 35). Interestingly, weight and BMI were not explicitly used as selection criteria for the animals selected for the genome scanning experiments. However, the animals comprising the protected pancreata and insulin resistant classes were among the heaviest, presumably as a consequence of lack weight loss due to diabetes.

The chromosome 4 locus exerted its primary effect on phenotypic parameters related to islet hypertrophy: number of hypertrophic islets, average islet area, and total islet area, with secondary effects on HbA1c, plasma [glucose] and plasma [insulin] (Figures 36, 37, 38; Tables 9 and 10). In females, DBA homozygotes at D4Mit286 had twice as many hyperplastic islets and twice as much total islet area as B6 homozygotes. Similar to the QTL on chromosome 2, the effects of the QTL on chromosome 4 were specific and in this case limited to the ability of the pancreas to produce and sustain large islets.

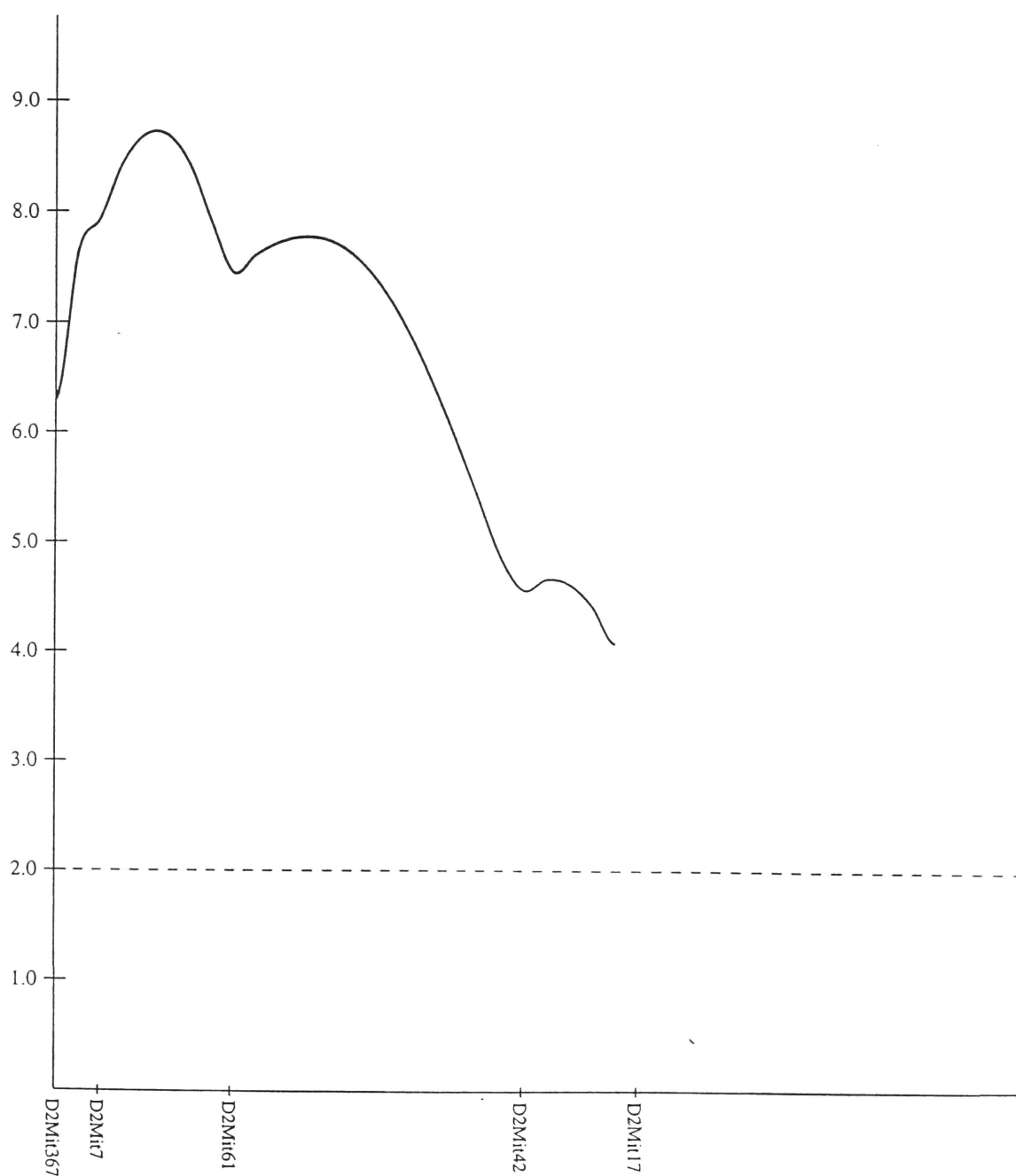


Figure 31. LOD scores for markers along chromosome 2 from centromere (left) to telomere (right) for weight in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

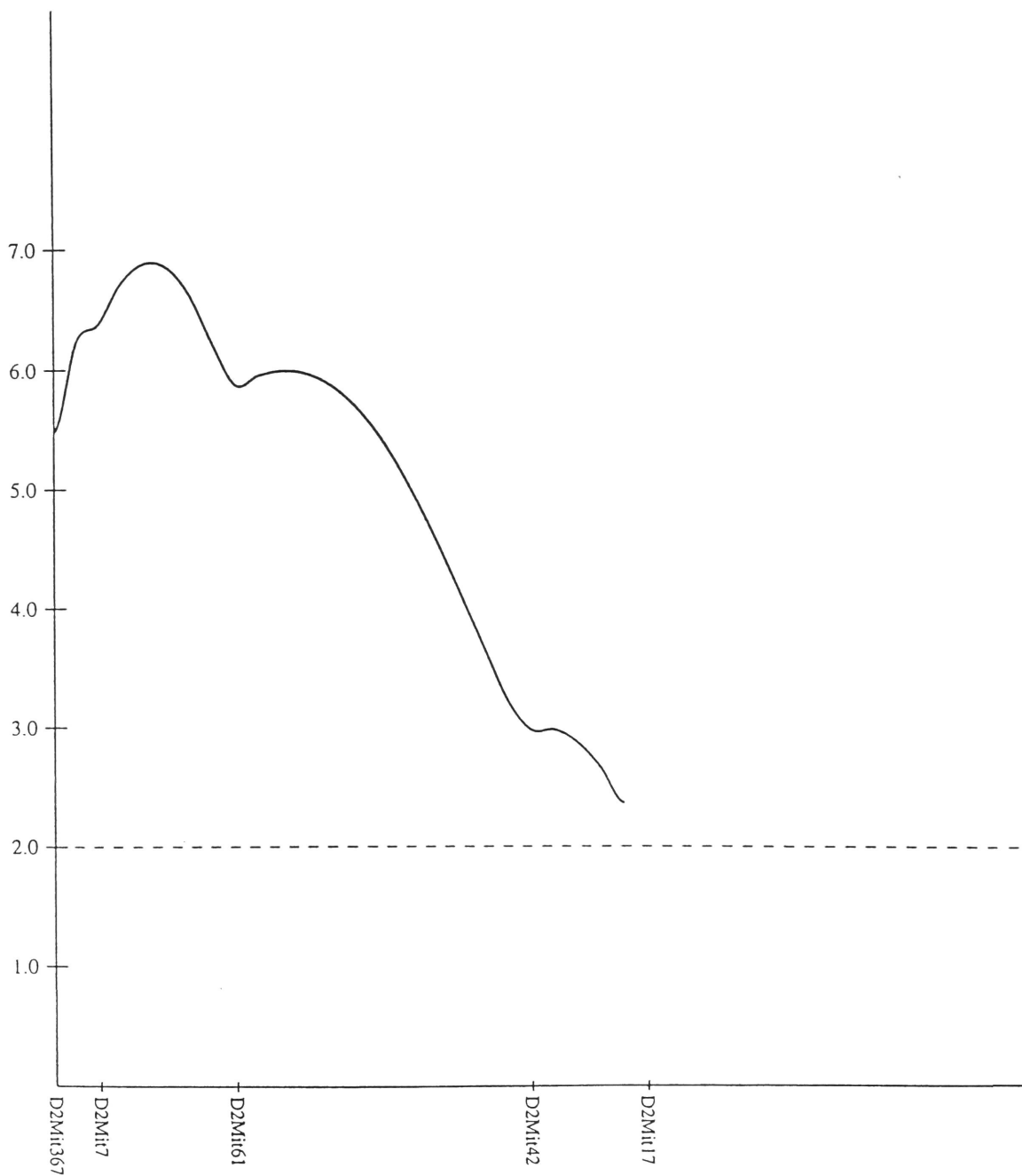


Figure 32. LOD scores for markers along chromosome 2 from centromere (left) to telomere (right) for BMI in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

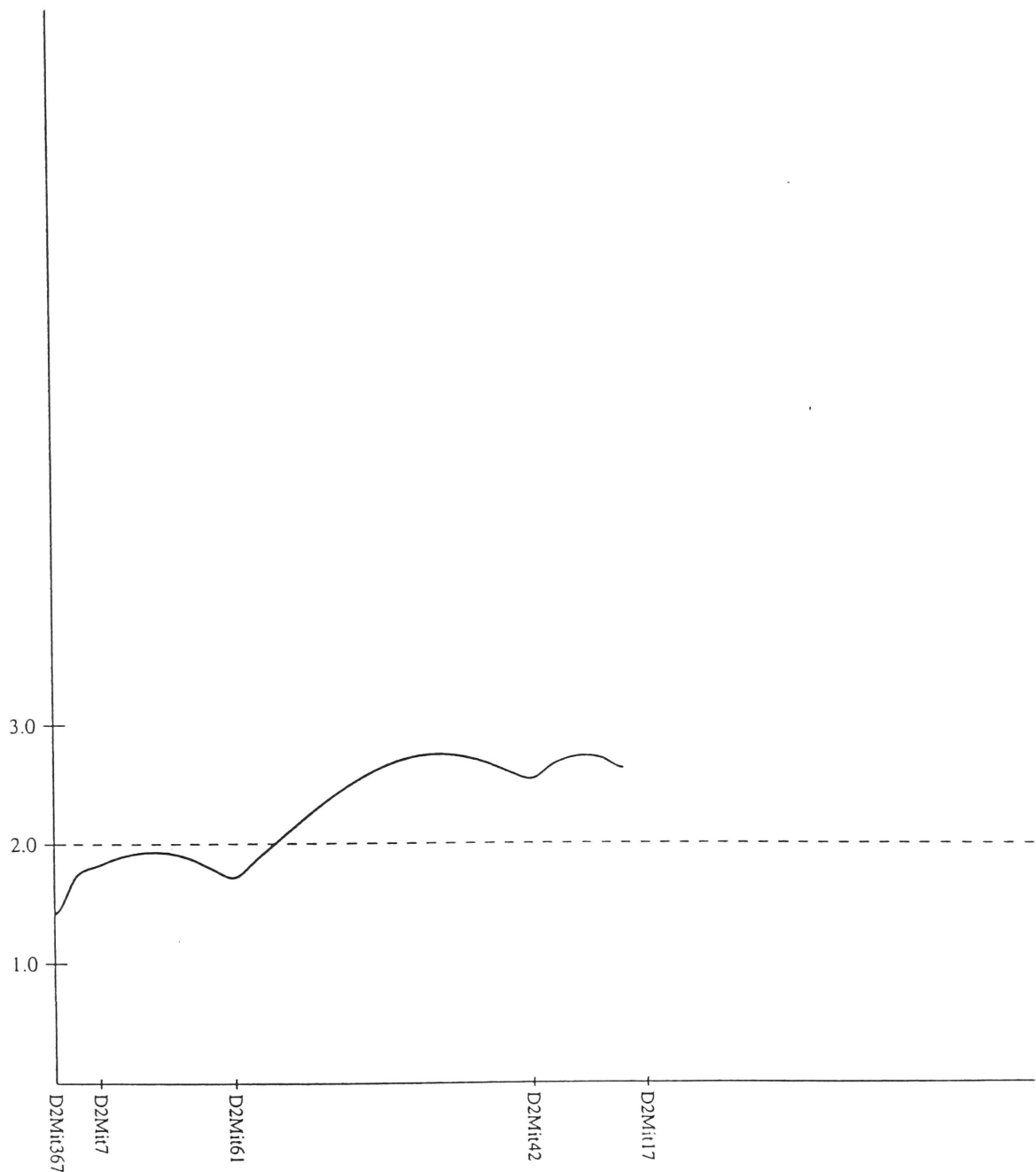


Figure 33. LOD scores for markers along chromosome 2 from centromere (left) to telomere (right) for number of islets in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

ob/ob F2 B6DBA, DBAB6

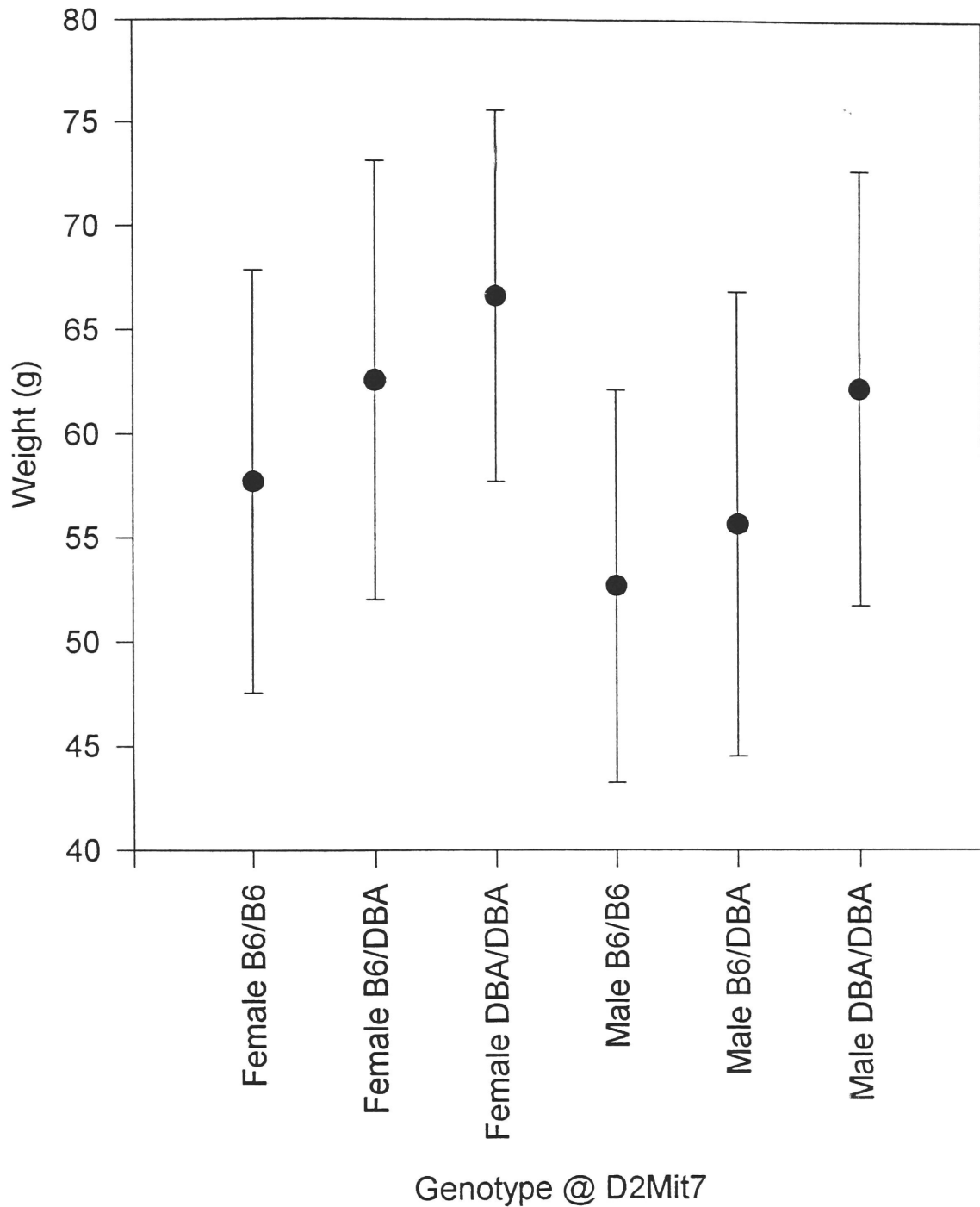


Figure 34. Relationship of genotype at D2Mit7 on weight by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

F2 *ob/ob* B6DBA, DBAB6

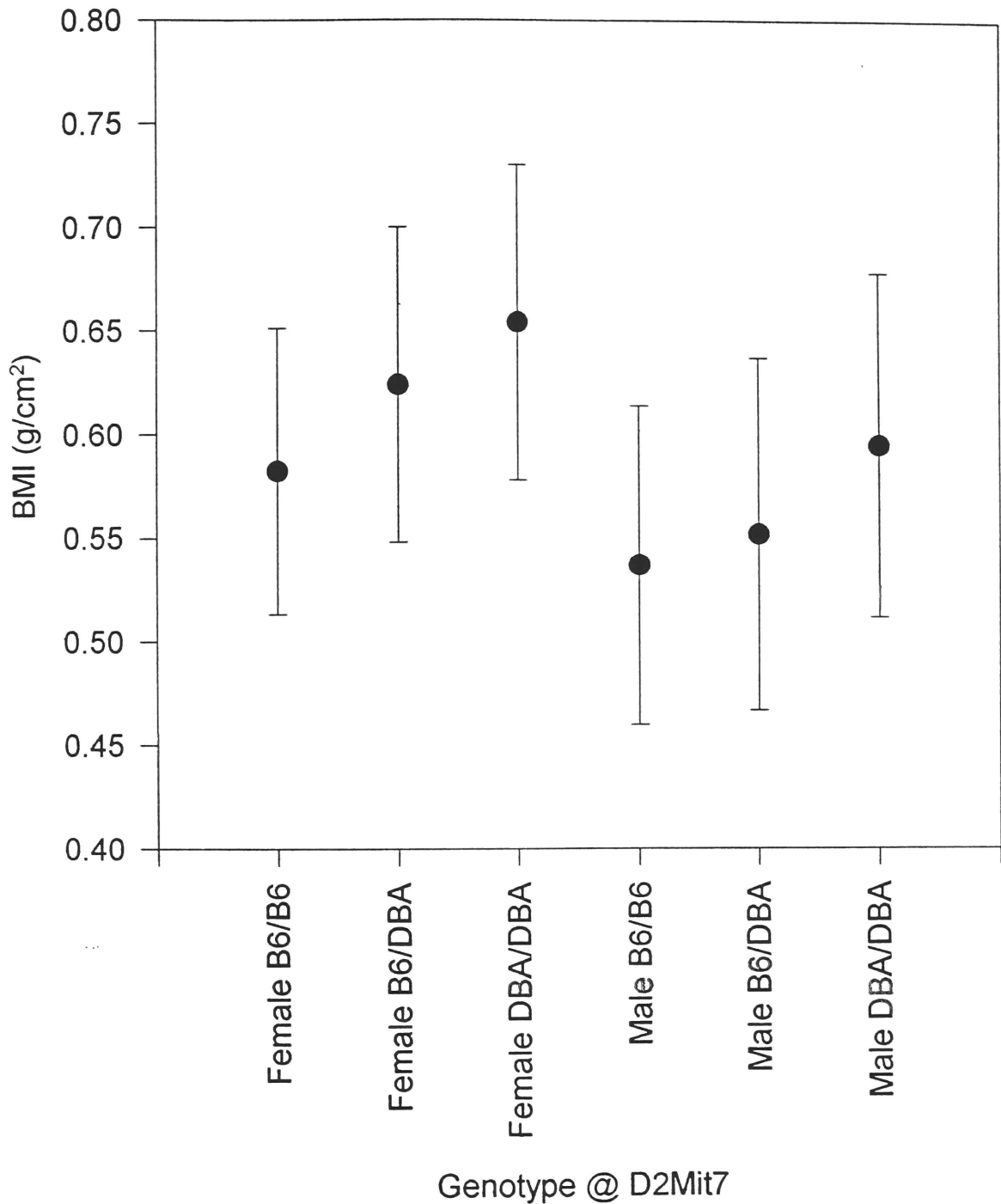


Figure 35. Relationship of genotype at D2Mit7 on BMI by sex in *ob/ob* F2 B6DBA and DBAB6. Circles represent the mean of each class with error bars of standard deviations.

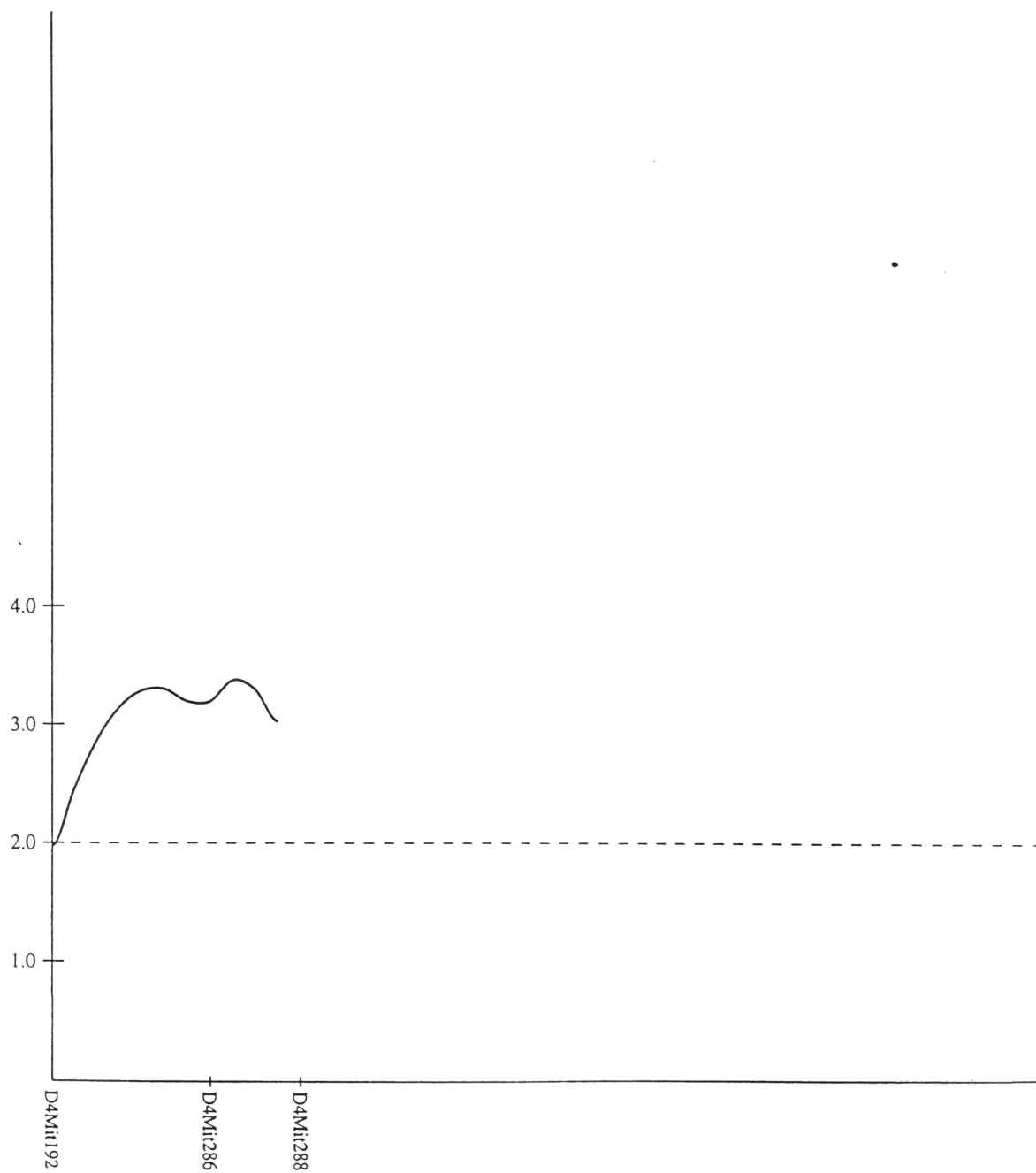


Figure 36. LOD scores for markers along chromosome 4 from centromere (left) to telomere (right) for number of hypertrophic islets in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

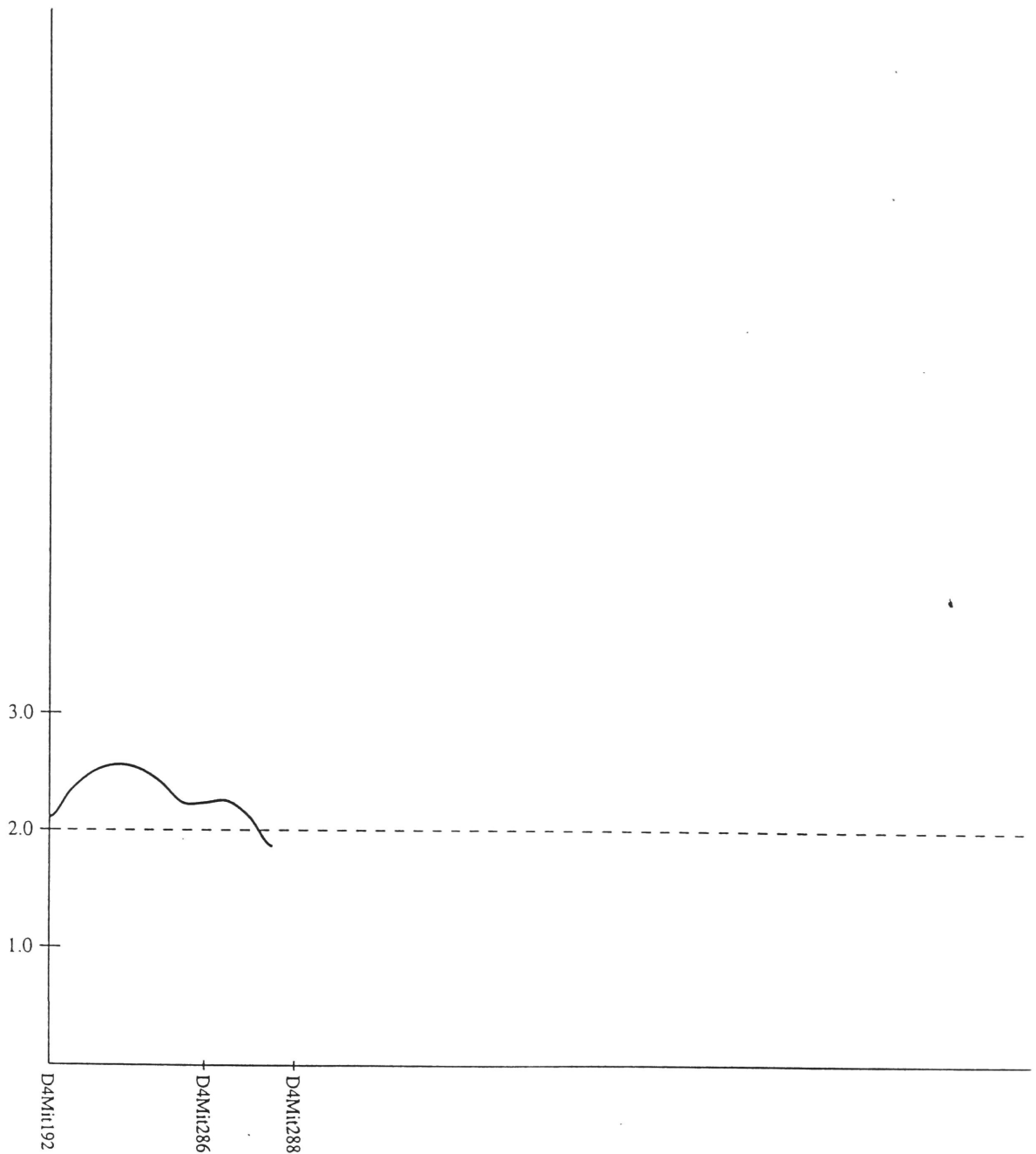


Figure 37. LOD scores for markers along chromosome 4 from centromere (left) to telomere (right) for average islet area in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

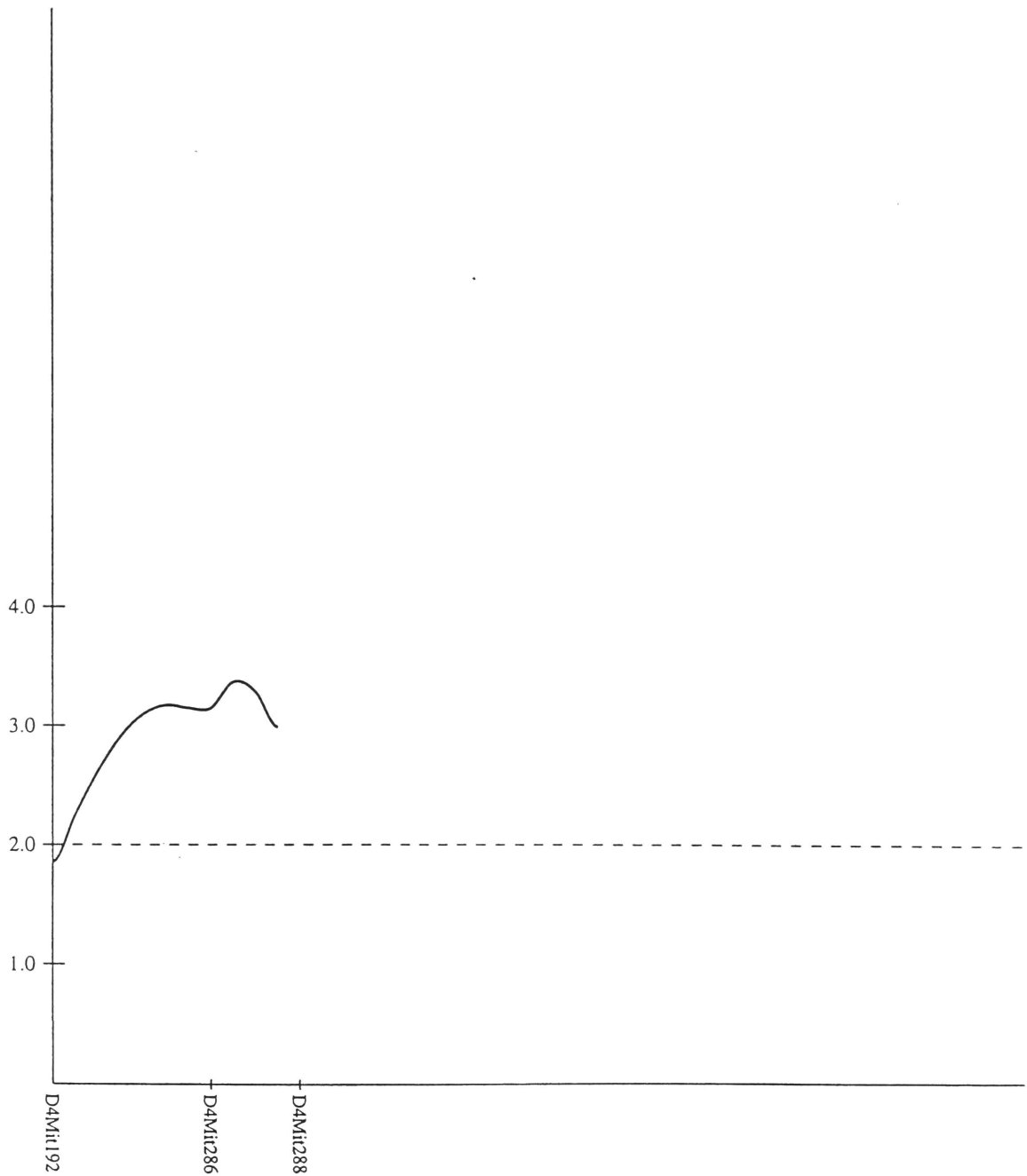


Figure 38. LOD scores for markers along chromosome 4 from centromere (left) to telomere (right) for total islet area in *ob/ob* F2 B6DBA and DBAB6. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

The chromosome 6 locus centered around D6Mit38 and D6Mit54 was not identified on the original genome scanning experiments because these crosses segregated the *ob* mutation on proximal chromosome 6 which produced linkage disequilibrium of markers within 50 cM of *ob* for the obese animals. This chromosome 6 locus had been previously identified in a cross segregating *db* (F2 B6CAST-chapter 9) and was tested in the current crosses segregating *ob* to test for its general relevance to diabetes genetics. The main influence of this locus was upon fasting plasma [glucose] and [insulin] and pancreatic [insulin] (Table 10). B6 alleles were protective against diabetes, only the second locus (in addition to that of chromosome 1) which demonstrated this protective effect in these crosses. The locus behaved co-dominantly. In both sexes, B6/B6 had plasma [insulin] three fold higher than DBA/DBA; and female B6/B6 had pancreatic [insulin] which was 19 fold increased relative to DBA/DBA.

The locus centered at D8Mit69 demonstrated marginal effects on plasma [glucose] and [insulin], pancreatic grade, number of hyperplastic islets, and islet area (Table 10). The diabetes-susceptibility related to this QTL for both sexes resulted from heterozygosity at the locus, recapitulating the paucity of heterozygotes within the protected pancreas class of the genome scanning experiments. The genotype at D8Mit69 exerted weak effects on plasma [glucose] and [insulin], pancreatic grade, number of hypertrophic islets, and islet area (Table 10).

D11Mit104 and D11Mit78 demonstrated no statistically significant association with any trait.

The locus near D13Mit11 was correlated with a multiplicity of diabetes phenotypes including fasting plasma [glucose] and [insulin], pancreatic grade, and average islet area (Tables 9 and 10). Although the variety of phenotypes affected was similar to that of the chromosome 1 locus, the magnitude of the effect was not nearly as pronounced. The precise location of the QTL was difficult to define on the basis of only two markers used to type the animals; the QTL may lie telomeric of D13Mit11.

Statistically, the most significant phenotypic effects of the chromosome 14 locus were number of hypertrophic islets and correlated with that, total islet area. D14Mit75 accounted for 18.1% of the variance in number of hypertrophic islets (LOD of 3.3), the greatest percentage of variance accounted for by any QTL in this cross (Table 9). DBA/DBA animals of both sexes demonstrated, on average, at least twice as many hypertrophic islets as B6/B6 animals (Table 10). Secondary effects of the locus were observed on plasma [glucose] and [insulin].

ANOVA with interactions between loci

In several instances there was evidence of interactions between QTL loci (Table 11). The most significant of these interactions was between D4Mit286 and D13Mit11. The primary action of the chromosome 4 locus was likely to be on islet hypertrophy and

survival. There was a pronounced effect on number of hypertrophic islets of genotypes either at D1Mit150 or D13Mit11 within the class of animals which were DBA/DBA at D4Mit286. Within the class of animals DBA/DBA at D4Mit286, animals homozygous B6 at D1Mit150 had 2.5 times as many hypertrophic islets as did DBA homozygotes. Similar differences were observed between the genotypes for either D1Mit150 or D4Mit286 alone. However, within the class of animals DBA/DBA at D4Mit286, animals DBA/DBA at D13Mit11 had 13.7 times as many hypertrophic islets as the B6/B6 class. The effect of genotype at D4Mit286 resulted in a two to three fold difference in number of hypertrophic islets, and genotype at D13Mit11 accounted for a two-fold maximal difference in the same phenotype. Therefore, these two QTL appeared to act synergistically to affect number of hypertrophic islets. Several other diabetes phenotypes were probably secondarily affected by these loci in a similar direction although smaller magnitude, including plasma [glucose] and [insulin], pancreatic [insulin], and islet area. Classical genetics has repeatedly emphasized the importance of epistasis, and such interactions among QTLs is supported by evidence from *Drosophila* (Spassky 1965), rice (Kinoshita and Shinbashi 1982), and tomato (Tanksley and Hewitt 1988).

First locus	Second locus	Phenotype	B6/B6	B6/DBA	DBA/DBA	B6/B6	B6/DBA	DBA/DBA	P	Genotype demonstrating synergism			
D1Mit160 D1Mit160	D2Mit7 D4Mit286	pancreatic glucagon (uU/mg protein)	54684	66321	89859	70469	70013	67708	74340	74976	61937	0.03	
		plasma [insulin] uU/ml	130.6	217.2	363.8	150.6	214.4	251.8	125.0	175.0	118.9	0.05	
D4Mit286 D4Mit286	D6Mit84 D13Mit11	number of hyperplastic islets	1.9	1.5	5.1	1.2	1.3	2.1	0.6	0.9	0.5	0.008	B6/B6 @ D1Mit150/ DBA/DBA @ D4Mit286
		pancreatic islet area (mm2)	0.35	0.36	0.71	0.27	0.31	0.39	0.22	0.22	0.16	0.04	B6/B6 @ D1Mit150/ DBA/DBA @ D4Mit286
		pancreatic [insulin]/[glucose]	0.4	0.14	0.22	0.25	0.21	0.21	0.78	0.47	0.07	0.02	DBA/DBA @ D4Mit286/ B6/B6 @ D8Mit54
		plasma [glucose] mg/dl	758.3	736.2	757.1	689.5	779.1	640.4	750.3	632.1	521.6	0.03	B6/DBA and DBA/DBA @ D4Mit286
D4Mit286 D14Mit76 D6Mit84	D13Mit11	pancreatic insulin (uU/mg protein)	17708	13250	43562	17983	18643	19841	14276	29492	76541	0.03	DBA/DBA @ D4Mit286/ DBA/DBA @ D13Mit11
		pancreatic [insulin]/[glucose]	0.20	0.22	0.44	0.29	0.18	0.26	0.31	0.34	0.94	0.05	DBA/DBA @ D4Mit286/ DBA/DBA @ D13Mit11
		number of hyperplastic islets	1.4	1.3	1.1	0.8	0.9	1.8	0.4	2.2	5.9	0.004	DBA/DBA @ D4Mit286/ DBA/DBA @ D13Mit11
		pancreatic islet area (mm2)	0.28	0.31	0.27	0.22	0.24	0.37	0.28	0.38	0.83	0.01	DBA/DBA @ D4Mit286/ DBA/DBA @ D13Mit11
D4Mit286 D14Mit76 D6Mit84	D13Mit11	number of islets	10.8	13.0	18.5	7.9	12.3	13.3	22.4	12.3	8.3	0.03	
		pancreatic [insulin]/[glucose]	0.09	0.21	1.20	0.10	0.44	0.19	0.14	0.43	0.12	0.02	B6/B6 @ D14Mit75/ DBA/DBA @ D13Mit11
		weight (g)	58.1	61.7	60.9	61.1	57.5	62.8	51.9	54.1	64.9	0.01	

Table 11. ANOVA for interactions of loci on diabetes phenotype in obese (*ob/ob*) male and female F2 B6DBA and F2 DBAB6.

Means for the traits are listed by genotypes at the first and second loci. Specific combinations of genotypes at the two loci which result in a synergistic effect are indicated.

Discussion of individual loci

The telomeric chromosome 1 locus exerts a profound effect on all aspects of diabetes phenotypes examined. The most powerful effect is that on pancreatic grade (Table 12). Although the responsible gene(s) need not necessarily act within the pancreas, on the basis of quantitative and qualitative measures for pancreatic morphology, the gene(s) appears to influence islet hypertrophy and sustainable function. B6 alleles are associated with the ability to produce additional beta cells and/or to maintain them in the face of the additional demands imposed by obesity-mediated insulin resistance. The more normal HbA1c associated with B6 alleles indicates that the pancreatic morphology has some long-term functional consequences for glucose homeostasis. The chromosome 1 locus affects measures of glucose homeostasis-plasma [glucose] and [insulin] following a short (3 hour) fast. This locus' minimal effect on plasma [insulin] and profound effect on plasma [glucose] may indicate that this gene plays a minor role in insulin resistance but a larger role in hepatic glucose production.

The chromosome 2 locus appears to primarily influence weight and BMI, although the time course of this effect is not yet known since longitudinal weight data are not available. On the basis of the results from this cross, the locus does not appear to have effects on diabetes phenotypes. The locus was, however, identified by preselecting animals with specific diabetes phenotypes, and it is possible that the time course of weight gain can itself have an effect on the diabetes parameters studied. For instance, if weight gain were too rapid, it is possible that the sudden increase in insulin resistance could not be

compensated for by the islets. Conversely, if the weight increase occurred after a critical developmental period for beta cells (Finegood et al. 1995), the beta cells would be incapable of hyperplasia in response to the additional metabolic stress.

The chromosome 4 and 14 loci appear to primarily affect the ability of the pancreas to produce and sustain hypertrophic islets; in both cases, DBA alleles are associated with the greater number of hypertrophic islets. In humans, obesity without NIDDM is associated with a doubling of beta cell volume. In equally obese individuals with NIDDM there is a 50% reduction in beta cell volume (probably by selective beta cell death) (Kloppel et al. 1985, Porte 1991). Bonner-Weir has demonstrated that beta cell mass can be increased by replication of preexisting islet cells, and by proliferation and differentiation of precursor cells in the ductal epithelium in adult rats (Bonner-Weir et al. 1993). She has also suggested that apoptosis counterbalances the 3% replication rate of beta cells (Finegood and Scaglia 1995). Based on mass balance equations, a dynamic reorganization of the islets occurs around the time of weaning with a “wave of beta cell death” and increased rates of replication between 15-30 days of age (Finegood and Scaglia 1995). The proliferation rate of islet cells is two fold greater in B6 than BKS mice. The chromosome 4 or 14 QTLs may account, in part, for that difference (Svenne and Andersson 1984). By careful cross sectional examination of the pancreata of obese mice between 10-30 days of age, it may be possible to correlate dynamic changes in beta cell mass with genotypes at the chromosome 4 and 14 QTLs.

The primary effect of the chromosome 13 locus is difficult to discern. Its effect could be on insulin resistance in peripheral tissues with secondary induction of beta cell in the pancreas (increased islet size and total islet area). Islet stress due to insulin resistance imposed by B6 alleles at this locus could accelerate pancreatic decompensation, resulting in decreased plasma [insulin] and increased plasma [glucose]. However, it is also possible that the significance of the locus is conveyed primarily through its effects on the ability of the pancreas to respond to insulin resistance. Based on the endpoint data available, it is not possible to distinguish between these two alternative possibilities.

Although a QTL was identified on chromosome 11 based on the genome scanning experiments ($p < 0.05$), when the full set of obese F2 progeny was tested, no diabetes-related trait was linked to this region. This result was not unexpected since multiple independent tests were performed in the genome scanning experiments, thereby increasing the likelihood of false positive results. The additional advantage of using a selected group of animals for the initial genome scanning experiments and then testing the loci on all of the obese progeny is that regions which then produce statistical significance are unlikely to be falsely positive since a limited number of independent tests are performed.

Definition of the specific roles of these five or six loci will require additional subphenotypic characterization, final determination of the number of responsible genes on chromosome 1, removal of confounding interacting loci by a series of introgressions to

fix these other regions to homozygosity (discussed in chapter 18), and cross sectional or longitudinal studies of the animals to determine which metabolic/morphological phenotypes are primary and which are secondary (chapter 17). Once these loci have been characterized individually, it will be possible to study the specific interactions of the loci. Until these experiments are done, attempting to define their physiological roles based on statistical arguments is largely speculative.

The significance of these loci will be further discussed in following chapters in which they were tested in additional crosses.

Locus	Primary phenotype	Secondary phenotype	Diabetes susceptible allele	Putative mechanism
D1Mit90	body weight/BMI		DBA	hyperphagia, energy expenditure, caloric partitioning
D1Mit110-D1Mit150	pancreatic morphology	HbA1c, fasting plasma [glucose]	DBA	beta cell hyperplasia/ acute insulin release
D2Mit367-D2Mit61	body weight/BMI		B6	hyperphagia, energy expenditure, caloric partitioning
D4Mit286-D4Mit288	islet hypertrophy	fasting plasma [glucose]	B6	islet hypertrophy
D13Mit11	plasma [insulin]	pancreatic grade	B6	unknown
D14Mit28-D14Mit35	islet hypertrophy		B6	islet hypertrophy

Table 12. Summary of major QTLs in *ob/ob* F2 B6DBA and F2 DBAB6. The interval likely to contain the QTL or the markers representing the peak of the QTL is indicated under the column locus. Primary and secondary phenotypes were determined on the basis of the statistical significance and extent of effect of the genotype at the locus and diabetes phenotypes.

Discussion

The set of F2 progeny of the B6DBA and DBAB6 crosses segregating *ob* provide interesting data without the necessity of any genotypic information. For the effect of genotype on any particular locus discussed above, the two reciprocal crosses behave identically although the statistical significance for the F2 DBAB6 was usually small since the number of obese progeny from the cross only totalled 46 animals. However, when characterizing the animals in the aggregate either analyzed by sex or as a single group, the progeny of the reciprocal crosses were not phenotypically identical. The F2 DBAB6 progeny, although the numbers were small, were not as hyperglycemic as the F2 B6DBA.

Genetically, the difference between these two crosses is limited. The X chromosome appears not to play a role based on the genome scanning results. The Y chromosome could have a role and has not yet been specifically tested, but this would not explain the observed effects on the female mice. Imprinting is an attractive hypothesis, but the traditional model of imprinting assumes that imprinting effects are erased with each generation. If this were the case, then the F1 animals from each cross would be identical with the exception of the sex chromosomes, and should produce the same set of imprinted genes. Only if two generations of information could be stored via the imprinting mechanism could such a theory explain the observed results. The only genetic system which has already been shown to “remember the parent” from which it came over multiple generations is that of the mitochondrial genome. All of the mitochondria in the F2 progeny would have been derived from the grandmaternal strain and could differ

between B6 and DBA. This hypothesis has not yet been specifically tested, but this would not be the first case in which sequence variation in a mitochondrial gene had caused diabetes (Katagiri et al. 1994). Any mutation which compromises mitochondrial integrity could also compromise the beta cell and predispose to diabetes.

Based on the phenotypes of *ob/ob* on B6 being insulin resistant but non-diabetic, and *ob/ob* on DBA being extremely diabetic with wasting due to glycosuria and pre-mature death due to total beta cell failure, it was assumed that most of the QTLs would have B6 alleles associated with diabetes-resistance and DBA alleles with diabetes-susceptibility. This did prove to be the case for the loci on chromosomes 1 and 6, but not for loci on chromosomes 2, 4, 13, and 14. The locus or loci on chromosome 1 statistically were quite significant and biologically may be the most powerful loci because B6 alleles on that single chromosome appear to be largely responsible for the diabetes resistance of *ob/ob* F2 B6DBA and DBAB6 animals.

Some comments on the strategy and design of the experiment can be made. The pancreatic phenotype was well characterized in these mice, including a genome scan specifically to identify the genes which were responsible for islet hypertrophy and sustenance. In fact, as a result of the care which was taken to identify these loci, two such QTLs on chromosomes 4 and 14 were identified which probably primarily affect these specific parameters of islet hypertrophy and maintenance of function. On the other hand, no assay was performed to specifically characterize insulin resistance either by

glucose disposal by skeletal muscle, glucose tolerance, or hepatic glucose production. These assays were not performed because large numbers of animals must be characterized for these genomic mapping experiments, and such extensive physiologic phenotyping was not feasible at the time. However, because these phenotypes were not directly characterized, it is not possible with the available data to determine if the chromosome 1, 6 or 13 loci may primarily affect one of these phenotypes, or if other loci exist which have profound effects on these narrowly defined sub-phenotypes but which may not have had large, discernible effects on the mechanistically indiscriminate endpoint phenotypes: HbA1c or plasma [glucose] and [insulin]. As the specific effects of each locus are defined, fine phenotypic assays will be performed to clearly delineate the relevant tissues affected and the likely physiological function of the locus.

The genome scanning experiments are obviously more efficient if a group of animals is identified which have a phenotype so unique that mechanistically there can be only a single pathway which leads to that phenotype. In the case of the obese progeny from these crosses, the majority of the mice, and especially the majority of the males, were diabetic. Therefore, the rare phenotype was that of the disease-free state or large numbers of large, intact islets. Mechanistically, there was probably a limited number of ways of producing such diabetes-resistant animals because particular genotypes at several loci were required to produce a non-diabetic animal in this cross. Thus, all of the significant QTL were identified from the genome scanning results of either the protected pancreas or insulin resistant classes. The diabetic class was useful to confirm positive

findings in the other two classes but could not be used independently to determine the locations of QTLs. This result is not surprising since the diagnosis of diabetes in this cross is likely a threshold phenomenon. The individual loci mainly act additively to bring the obese animal closer to the diabetes threshold in the face of profound insulin resistance. If there is a hypothetical total of six loci, a single major and minor locus may be enough to move an individual across the threshold to diabetes. Thus, even the animals which were chosen as the most severely affected were unlikely to be genetically homogeneous. The situation is likely to be the same in human NIDDM.

Chapter 5:

Confirmation of diabetes susceptibility regions

in

F3 B6DBA segregating for *ob*

Strategy

In an attempt to quickly assess the reproducibility and strength of the association of the chromosome 1 locus with diabetes phenotypes, lean F2 B6DBA progeny were tail clipped and genotyped with markers for markers flanking *ob* (Cftr and D6Mit74) to identify *ob*/+ animals. Heterozygous animals were subsequently genotyped for D10Mit51 and D10Mit38, markers 13 cM apart flanking a locus on chromosome 10 previously associated with diabetes-susceptibility (chapter 8), and for D1Mit90 and D1Mit110, markers 18 cM apart, close to but not necessarily flanking the diabetes susceptibility QTL on chromosome 1 (chapter 4). Although it would have been more conclusive to use markers which definitively flanked the chromosome 1 locus, this set of markers would have been spaced approximately 50 cM apart, and it would not have been feasible to obtain animals genetically homogeneous across the interval. Animals heterozygous for *ob* and concordant for genotype at either D10Mit51 and D10Mit38, or D1Mit90 and D1Mit110, were mated to genotypically similar mice of the opposite sex. No attempt was made to simultaneously fix specific combinations of genotypes for the two QTL's. Twenty eight such breeding pairs were established which produced a total of 87 male and 105 obese females which were sacrificed at a mean age of 114.5 and 120.3 days, respectively (Table 13). The mice were phenotypically characterized and analyzed as previously described. All of the obese progeny were initially typed for seven markers along a 94 cM interval on chromosome 1 (D1Mit245, D1Mit300, D1Mit103, D1Mit101, D1Mit110, D1Mit150, and D1Mit56) and two markers on chromosome 10 (D10Mit38 and D10Mit51). Subsequently, on the basis of the results obtained with the F2 progeny,

five markers on chromosome 2 (D2Mit367, D2Mit7, D2Mit61, D2Mit42, and D2Mit17) spanning a total of 31 cM, three markers on chromosome 4 (D4Mit192, D4Mit286, and D4Mit288) spanning a total of 15.5 cM, and D14Mit28 were also typed on all the obese progeny. Although the crosses were not specifically designed to test for the phenotypic effects of these additional loci, the F3 progeny provided an opportunity to test their replicability.

Results

Phenotypically, the obese F3 progeny were most readily distinguishable from the lean on the basis of body weight and BMI (Table 13). None of the lean mice were diabetic, as indicated by the glycosylated hemoglobin fractions which were consistently less than 5%. All phenotypic parameters were distinguishable between the two groups.

Phenotype	Lean female	male	Obese female	male
N	22	17	105	87
Age (days)	132 (39)	123 (38)	120 (32)	115 (37)
Weight (g)	31.7 (5.6)	33.0 (5.0)	65.5 (14.5)	55.9 (10.8)
BMI (g/cm ²)	0.381 (0.052)	0.402 (0.061)	0.644 (0.093)	0.572 (0.081)
HbA1c (%)	3.8 (0.5)	3.9 (0.9)	9.6 (3.8)	12.5 (3.2)
Fasting plasma [glucose] (mg/dL)	278 (43)	273 (47)	667 (228)	756 (210)
Fasting plasma [insulin] (uU/ml)	87.2 (45.9)	110.2 (64.5)	230.6 (301.9)	191.9 (312.4)
Normalized pancreatic insulin content (uU/mg protein)	29874 (13231)	24894 (11921)	19546 (28428)	23964 (104615)
Normalized pancreatic glucagon content (pg/mg protein)	ND	ND	20396 (7061)	15290 (5024)
Pancreatic [insulin]/[glucagon]	ND	ND	0.84 (1.15)	1.56 (5.49)

Table 13. Phenotypic characteristics of F3 B6DBA progeny segregating for *ob*. Means for the lean and obese groups by sex are indicated with standard deviations in parentheses. ND indicates that values were not determined.

As in the case of the F2 progeny, obese F3 females tended to be heavier than males: weights (65.5 g vs 55.9 g) and BMIs (0.64 g/cm² vs 0.57 g/cm²). The obese females also tended to be less diabetic: HbA1c's (9.6% vs 12.5%) and plasma [glucose] (667 mg/dl vs 756 mg/dl). Additionally, the F3 progeny were phenotypically comparable to, and statistically indistinguishable from, the obese F2 progeny.

The biochemical and pancreatic profiles of the obese F3 progeny were comparable to those of the F2 progeny and displayed similar variations and correlations (Figures 39, 40).

Figure 39. Fasting plasma [insulin] versus HbA1c in obese (*ob/ob*) male and female F3 B6DBA. Each point represents a single animal.

F3 B6DBA

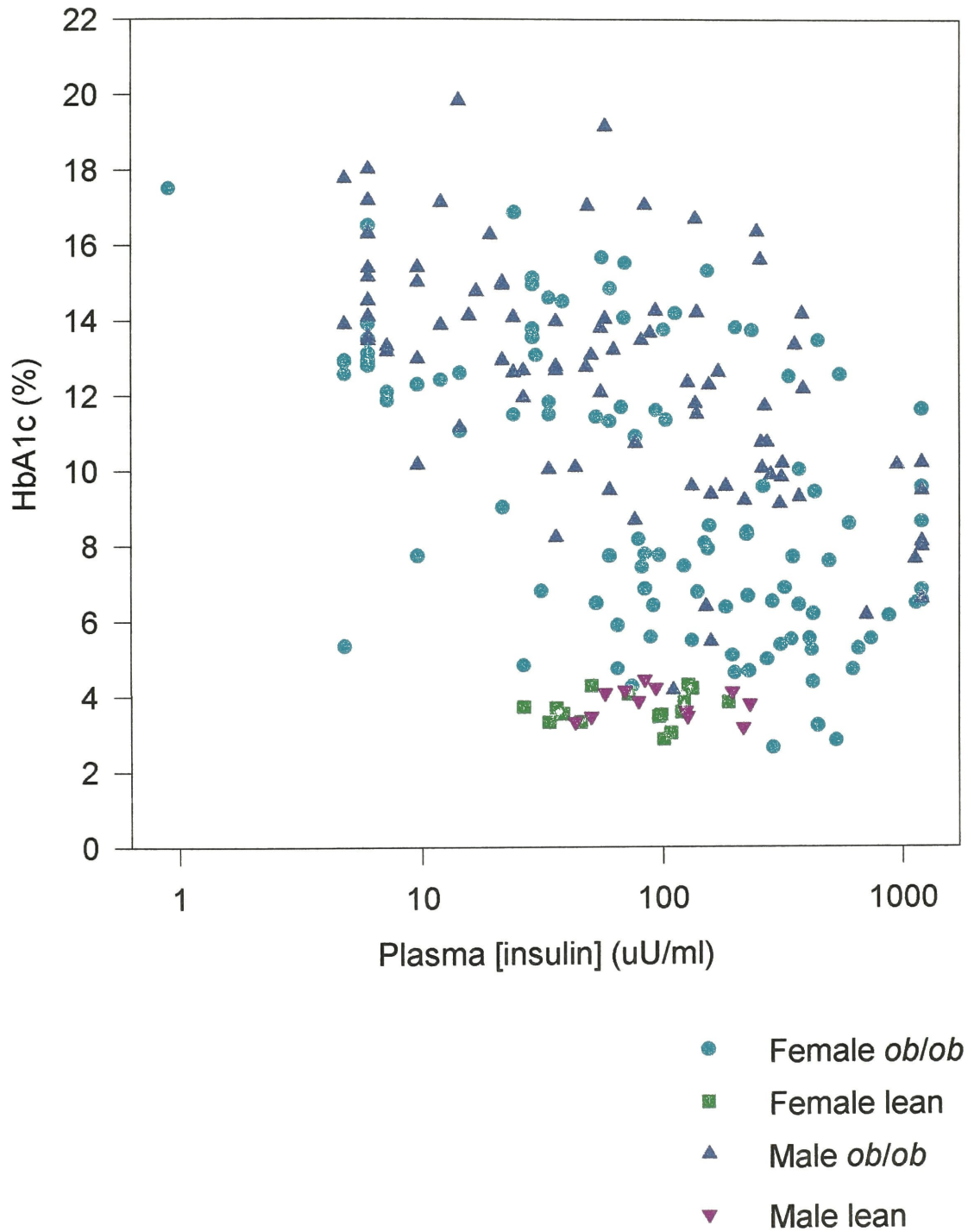
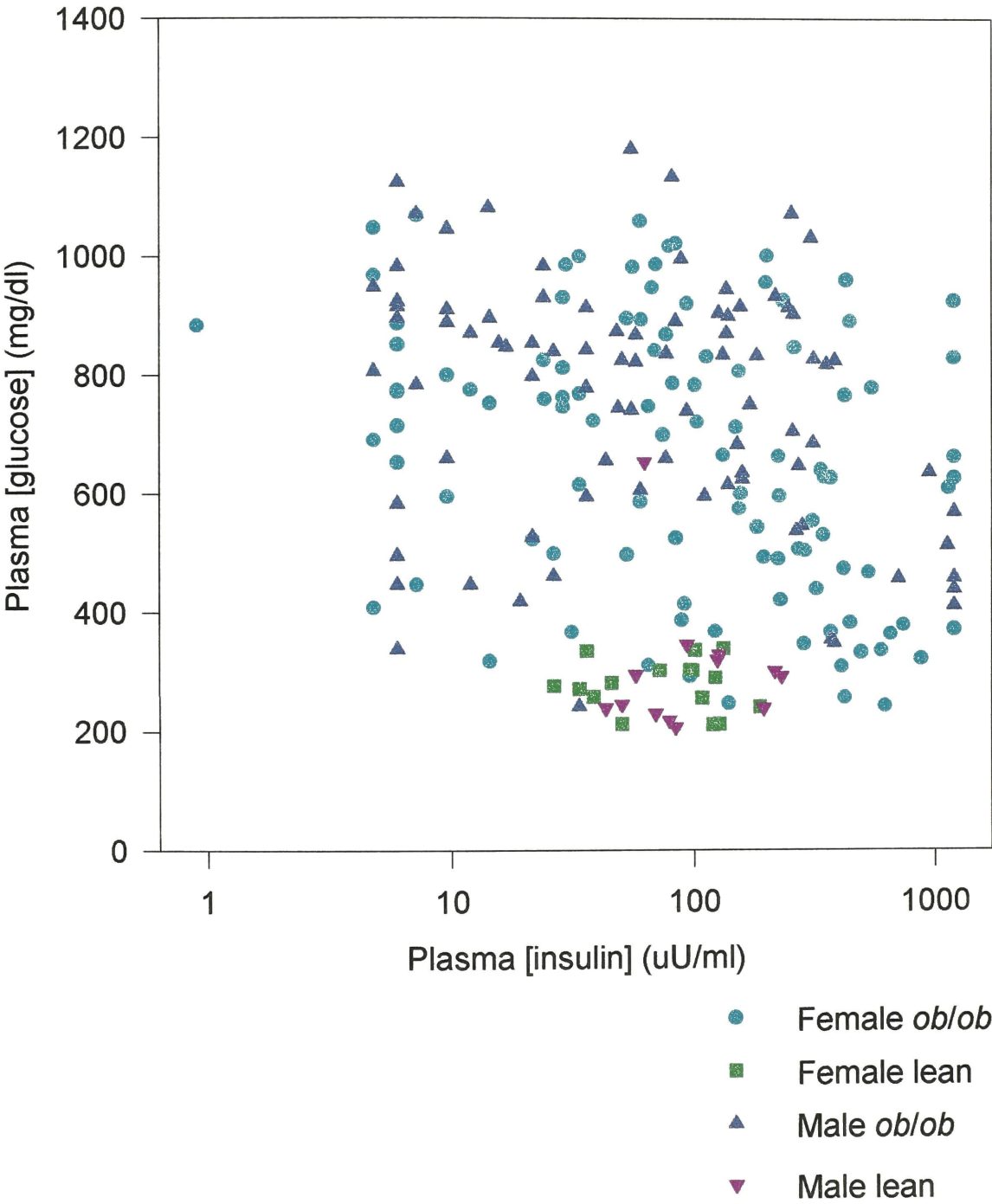


Figure 40. Fasting plasma [glucose] versus fasting plasma [insulin] in obese (*ob/ob*) male and female F2 B6DBA. Each point represents a single animal.

F3 B6DBA



QTL and ANOVA results

Only one of the two QTLs fixed in the F2 breeding pairs and analyzed in the F3 progeny replicated the results previously demonstrated. The chromosome 10 locus which had previously been associated with variation in plasma [insulin] was not replicated in the *ob/ob* F3 B6DBA progeny, but this result was not entirely unexpected since linkage of this region to plasma [insulin] had not been replicated in the more recent *ob/ob* F2 B6DBA progeny discussed in the previous chapter. However, the chromosome 1 locus which had demonstrated powerful effects on all aspects of diabetes phenotype showed similar effects in the F3 progeny. Again, B6 alleles were associated with diabetes resistance. As in the F2 progeny, there was the possibility of two separate peaks for weight, BMI, HbA1c, and pancreatic grade but more clearly a single peak between D1Mit103 and D1Mit110 for the other parameters related to pancreatic phenotype (Table 14, Figures 41-47). Although the number of obese F3 progeny was approximately half that of the F2 progeny, the LOD scores for weight were comparable in the two crosses. Females which were B6/B6 at D1Mit103 weighed 35% more than DBA/DBA females, and males weighed 24% more. Additionally, the chromosome 1 interval between D1Mit300 and D1Mit101 accounted for 26.3 % of the variance of BMI, a much larger proportion of the variance than observed in the F2 progeny (Table 14). Segments of chromosome 1 between D1Mit300 and D1Mit101 accounted for 34.7% of the variance in HbA1c, 30.7% of the variance in pancreatic grade, and 27.2% of the variance in normalized beta cell area and regions between D1Mit103 and D1Mit101 for 25% of the variance in fasting plasma [glucose]. Single loci accounting for so large a proportion of the

variance were not encountered in the F2 progeny. Unlike the F2 progeny, in the F3 progeny the chromosome 1 QTL tended to behave co-dominantly for the majority of traits in both sexes, possibly because the males were not as severely diabetic in this cross and had not yet reached the “ceiling” of phenotype related to diabetes. Statistically, the traits which were most closely related to genotype for the QTL were HbA1c and pancreatic grade (Figure 48, 49). Whether or not this locus acted primarily in the pancreas was still difficult to determine without further studies, but this possibility was not eliminated by the additional F3 data.

Table 14. Analysis of variance by sex and genotype at the marker designated for *ob/ob* F3 B6DBA. All traits for all markers demonstrating statical significance are summarized. Number of animals genotyped and phenotyped for each a given marker and trait is indicated. Mean values for each trait are listed first by sex then genotypic class.

Marker	Phenotype	Number	B6/B6	Female		B6/B6	Male		P
				B6/DBA	DBA/DBA		B6/DBA	DBA/DBA	
D1Mit245	BMI (g/cm2)	160	0.66	0.66	0.61	0.58	0.57	0.53	0.01
	Pancreatic Grade	145	2.3	2.6	2.8	3.0	3.0	3.8	0.02
	Number of hyperplastic Islets	151	5.3	2.7	1.8	1.3	1.1	38.0	0.02
	Average Islet size (mm2)	151	0.027	0.024	0.022	0.020	0.018	0.014	0.04
	Islet area/total area (%)	151	2.29	1.76	1.43	1.07	1.02	0.62	0.05
D1Mit300	Weight (g)	188	67.6	69.3	61.7	56.9	61.2	49.5	0.0002
	BMI (g/cm2)	166	0.65	0.67	0.62	0.58	0.60	0.51	0.0001
	HbA1c (%)	166	8.7	9.0	10.3	11.0	13.0	13.0	0.03
	Pancreatic Grade	151	2.5	2.2	3.1	2.9	2.9	3.7	0.0004
	Number of hyperplastic Islets	157	4.1	3.5	1.8	1.3	1.3	0.3	0.05
	Average Islet size (mm2)	157	2.39	2.58	1.98	1.85	1.85	1.48	0.02
	Islet area/total area (%)	157	1.9	2.07	1.36	1.04	1.08	0.63	0.04
D1Mit103	Weight (g)	88	80.7	67.2	59.6	61.7	58.5	49.8	0.0002
	BMI (g/cm2)	88	0.71	0.66	0.63	0.61	0.57	0.52	0.008
	HbA1c (%)	88	7.7	10.1	11.0	11.3	13.1	13.3	0.007
	[Glucose] (mg/dl)	88	528	633	837	747	766	763	0.02
	[Insulin] (uU/ml)	88	345.8	213.4	87.1	302.6	89.0	43.7	0.001
	Pancreatic Grade	86	2.0	2.5	3.5	2.8	2.9	3.8	0.0001
	Number of Islets	86	27.4	20.3	12.3	16.7	18.2	10.7	0.0008
	Number of hyperplastic Islets	86	5.5	3.0	0.6	1.2	1.4	0.1	0.0004
	Average islet size (mm2)	86	0.0289	0.0246	0.0164	0.0185	0.0186	0.0145	0.0009
	Islet area (mm2)	86	0.907	0.525	0.209	0.326	0.348	0.159	0.0003
	Islet area/total area (%)	86	2.56	1.76	0.69	1.10	1.14	0.59	0.0001
D1Mit101	Weight (g)	127	75.2	66.3	60.6	59.8	60.0	50.0	0.0002
	BMI (g/cm2)	127	0.69	0.65	0.61	0.6	0.58	0.54	0.004
	HbA1c (%)	127	8.1	9.5	10.7	10.0	12.3	14.0	0.0001
	[Insulin] (uU/ml)	125	309.4	181.4	109.0	283.9	197.8	42.0	0.0006
	Pancreatic Grade	117	2.1	2.3	3.0	2.7	2.7	3.6	0.0002
	Number of Islets	121	23.7	20.9	16.3	20.0	16.5	9.8	0.0007
	Number of hyperplastic Islets	121	4.2	3.5	1.3	1.3	1.6	0.3	0.008
	Average Islet size (mm2)	121	2.78	2.48	1.74	1.81	2.02	1.6	0.002
	Islet area (mm2)	121	0.743	0.57	0.315	0.361	0.349	0.155	0.0007
	Islet area/total area (%)	121	2.4	1.86	1.01	1.23	1.07	0.57	0.0001
D1Mit110	Weight (g)	154	70.1	69.3	58.7	60.5	57.9	50.5	0.0001
	BMI (g/cm2)	154	0.67	0.67	0.6	0.64	0.56	0.55	0.004
	HbA1c (%)	154	7.6	9.3	11.1	10.8	12.5	13.8	0.00001
	[Glucose] (mg/dl)	154	546	687	682	698	773	736	0.03
	[Insulin] (uU/ml)	152	243.4	231.4	75.8	236.4	160.1	39.1	0.0003
	Pancreatic Grade	140	1.8	2.6	3.1	2.7	2.8	3.6	0.000001
	Number of Islets	145	20.9	22.4	17.1	17.6	16.3	10.5	0.02
	Number of hyperplastic Islets	145	3.3	3.9	1.9	1.2	1.5	0.2	0.02
	Average Islet size (mm2)	145	0.0278	0.0246	0.0179	0.019	0.0187	0.0158	0.0009
D1Mit150	Weight (g)	150	70.9	70.2	58.4	62.0	57.4	52.7	0.0001
	BMI (g/cm2)	150	0.68	0.67	0.6	0.61	0.56	0.55	0.0006
	HbA1c (%)	150	8.3	9.0	11.0	10.6	12.9	13.2	0.0006
	[Glucose] (mg/dl)	150	553	686	690	641	845	760	0.0003
	[Insulin] (uU/ml)	149	244.8	211.2	83.28	300.96	142.8	66.72	0.001
	Pancreatic Grade	137	1.8	2.6	3.0	2.6	3.1	3.5	0.00001
	Number of Islets	141	18.5	23.0	16.5	18.9	17.1	10.7	0.007
	Number of hyperplastic Islets	141	3.4	3.9	1.5	1.4	1.1	0.4	0.01
	Average Islet size (mm2)	141	0.0287	0.0251	0.0177	0.019	0.0164	0.0172	0.004
D1Mit56	Islet area (mm2)	141	0.586	0.669	0.329	0.374	0.301	0.183	0.001
	Islet area/total area (%)	141	2.24	2.09	1.07	1.29	0.92	0.66	0.0006
	HbA1c (%)	94	11.0	8.5	12.5	10.2	12.9	13.2	0.03

Marker	Phenotype	Number	B6/B6	Female		B6/B6	Male		P
				B6/DBA	DBA/DBA		B6/DBA	DBA/DBA	
D2Mit367	Weight (g)	102	56.1	70.6	74.0	55.7	55.8	62.7	0.02
	BMI (g/cm2)	102	0.58	0.66	0.7	0.56	0.56	0.6	0.04
D2Mit7	Weight (g)	113	61.4	68.7	67.9	50.8	62.4	53.9	0.03
	BMI (g/cm2)	113	0.61	0.66	0.67	0.51	0.61	0.54	0.02
	Pancreatic Grade	111	3.0	2.5	2.4	4.0	2.9	3.1	0.01
	Average Islet size (mm2)	109	0.0194	0.025	0.0277	0.0137	0.0188	0.0176	0.009
D2Mit61	Number of islets	140	17.0	23.2	20.7	12.5	17.5	18.2	0.02
D2Mit42	Weight (g)	102	70.6	72.9	58.6	61.5	58.1	54.3	0.03
	BMI (g/cm2)	102	0.69	0.67	0.61	0.59	0.58	0.55	0.05
D2Mit17	Pancreatic Grade	149	2.5	2.5	2.8	3.2	2.9	3.9	0.05
D4Mit192	Weight (g)	156	72.5	65.9	60.3	60.9	55.9	48.9	0.00001
	BMI (g/cm2)	156	0.68	0.65	0.61	0.6	0.56	0.51	0.00001
D4Mit286	Weight (g)	158	68.6	69.5	61.1	58.5	56.3	53.4	0.03
	BMI (g/cm2)	158	0.66	0.67	0.61	0.58	0.57	0.54	0.009
D4Mit288	Weight (g)	138	67.8	68.6	61.0	59.5	58.1	51.7	0.009
	BMI (g/cm2)	138	0.67	0.66	0.61	0.59	0.58	0.53	0.003
D14Mit28	Weight (g)	147	61.4	66.3	77.7	57.8	55.1	60.6	0.01
	HbA1c (%)	147	10.4	9.3	7.9	12.4	13.1	9.9	0.006
	[Insulin] (uU/ml)	146	211.7	122.2	313.0	170.9	139.0	277.7	0.02
	Number of hyperplastic islets	139	1.5	2.7	6.6	0.9	1.0	1.8	0.009
	Average islet size (mm2)	139	0.0171	0.025	0.0301	0.0162	0.0186	0.0196	0.004
	Islet area (mm2)	139	0.375	0.514	0.958	0.284	0.281	0.379	0.02
	Islet area/total area (%)	139	1.28	1.76	2.53	0.87	0.93	1.3	0.03

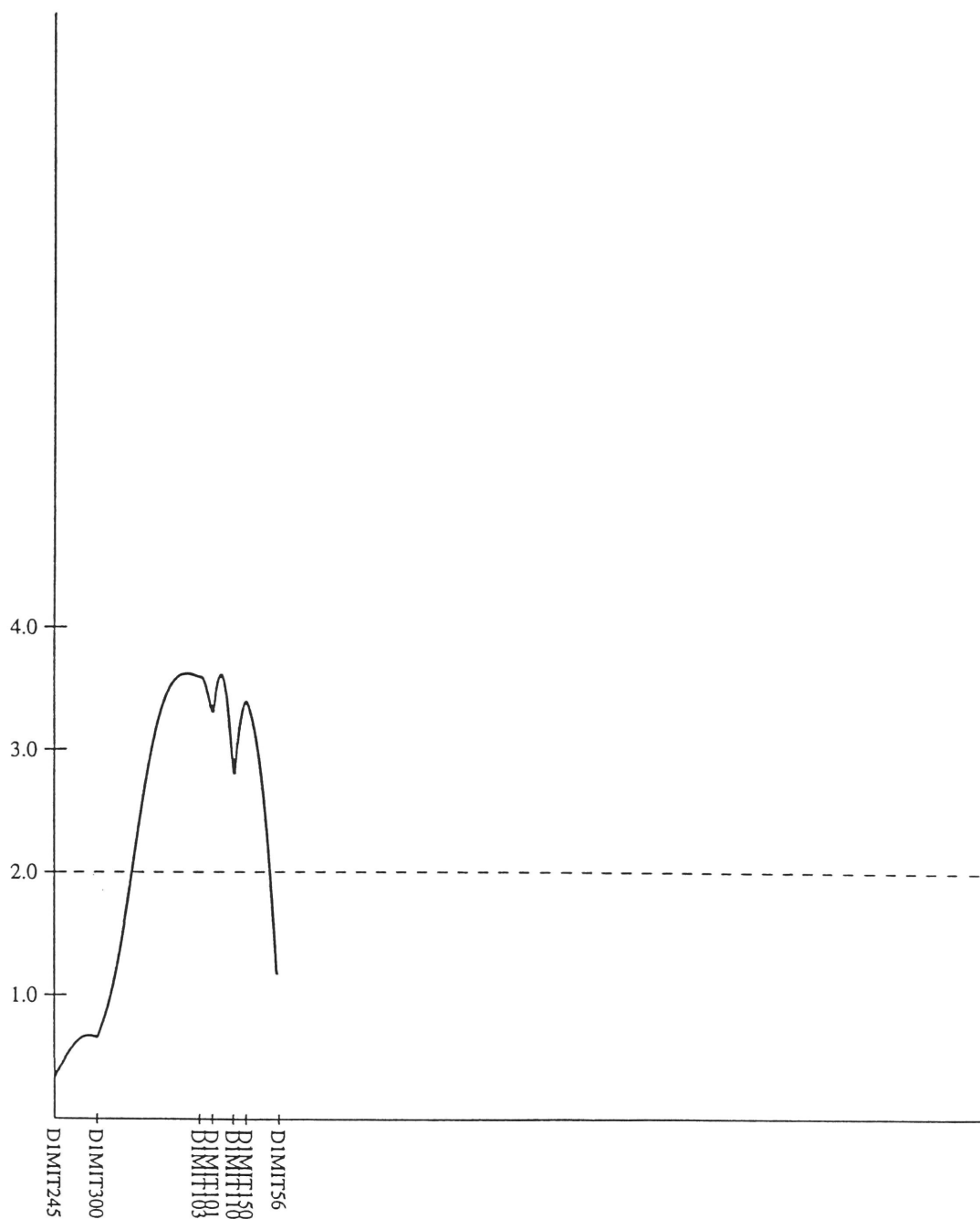


Figure 41. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for weight in *ob/ob* F3 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

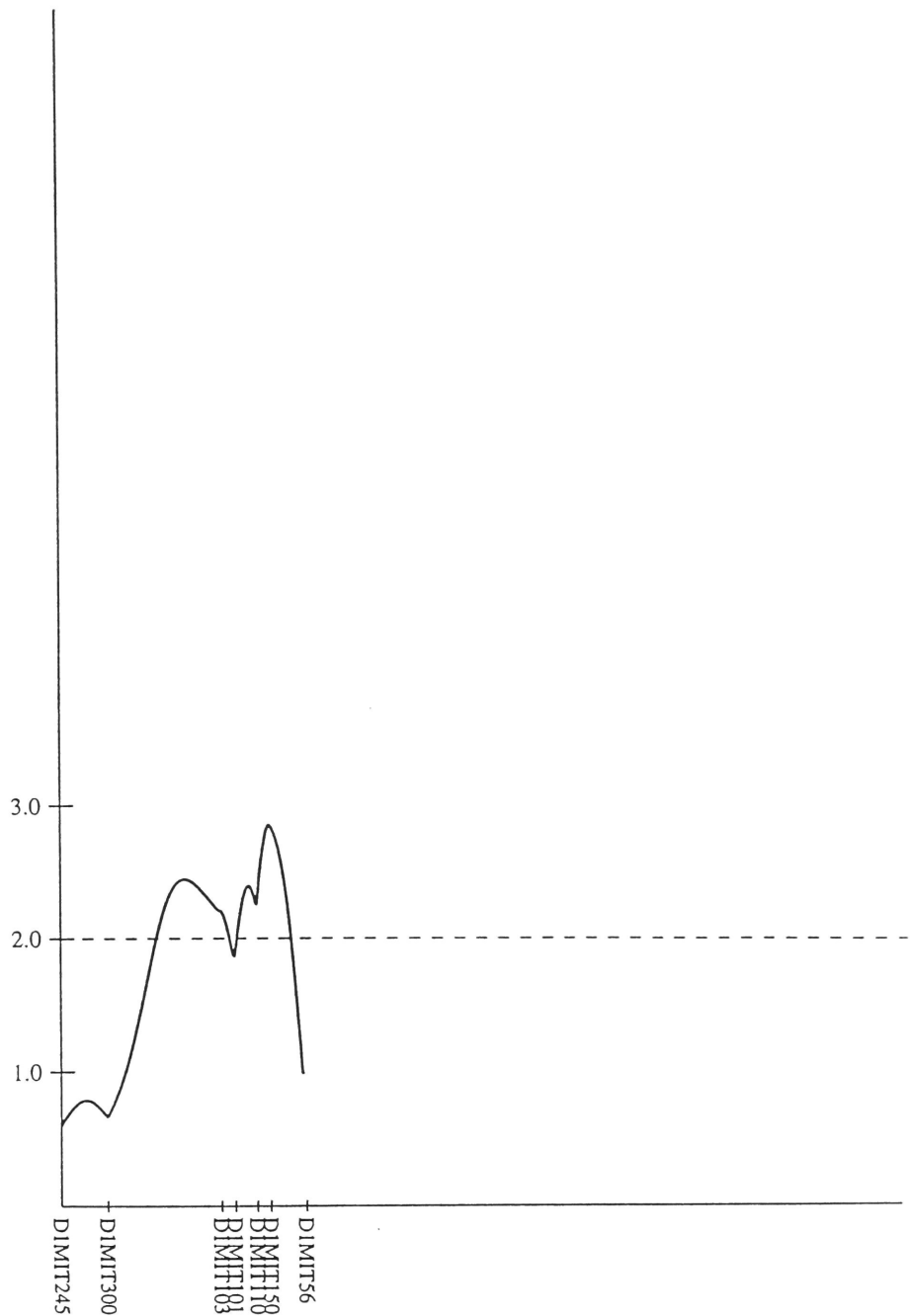


Figure 42. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for BMI in *ob/ob* F3 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

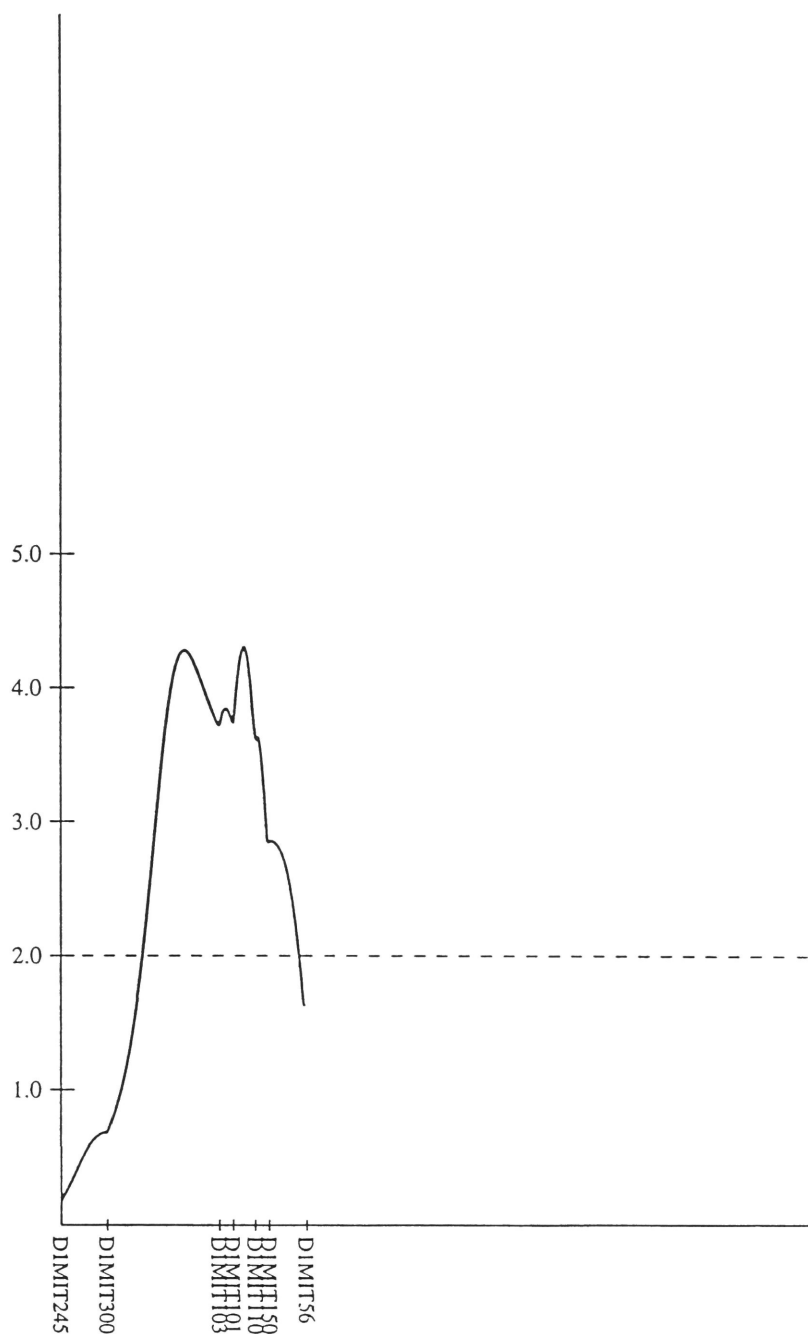


Figure 43. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for HbA1c in *ob/ob* F3 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

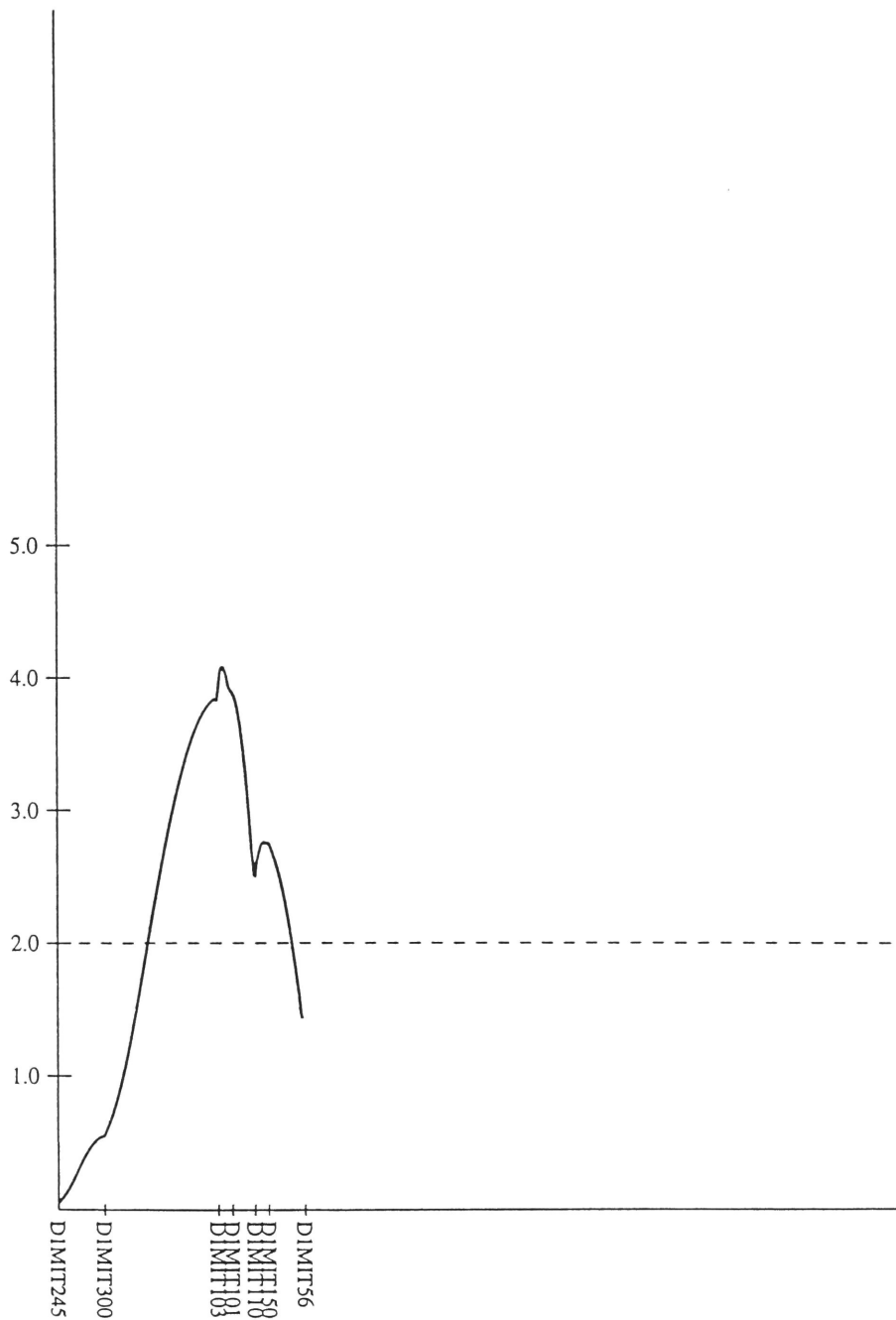


Figure 44. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for fasting plasma [insulin] in *ob/ob* F3 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

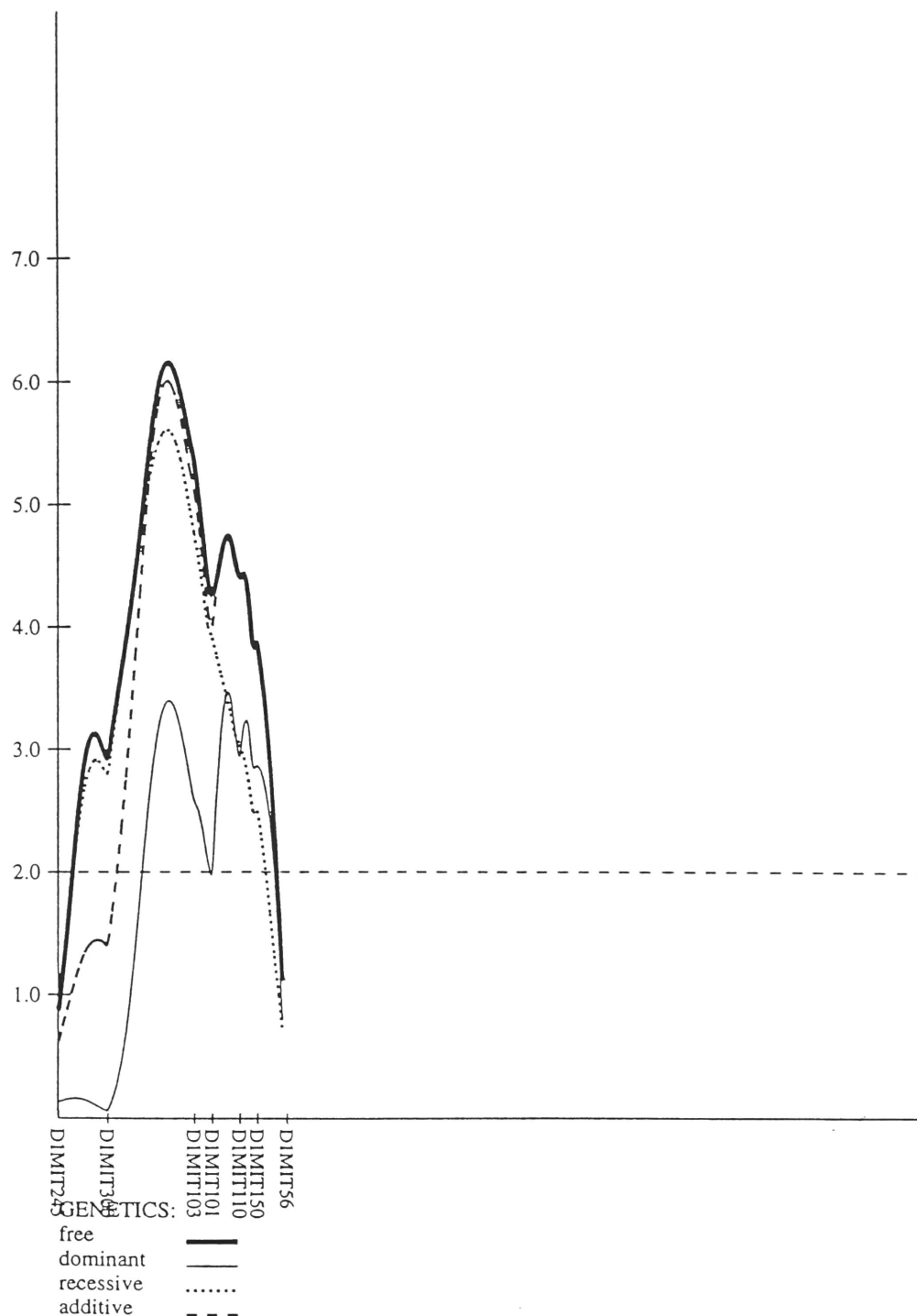


Figure 45. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for pancreatic grade in *ob/ob* F3 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown. LOD scores for four different genetic models are shown.

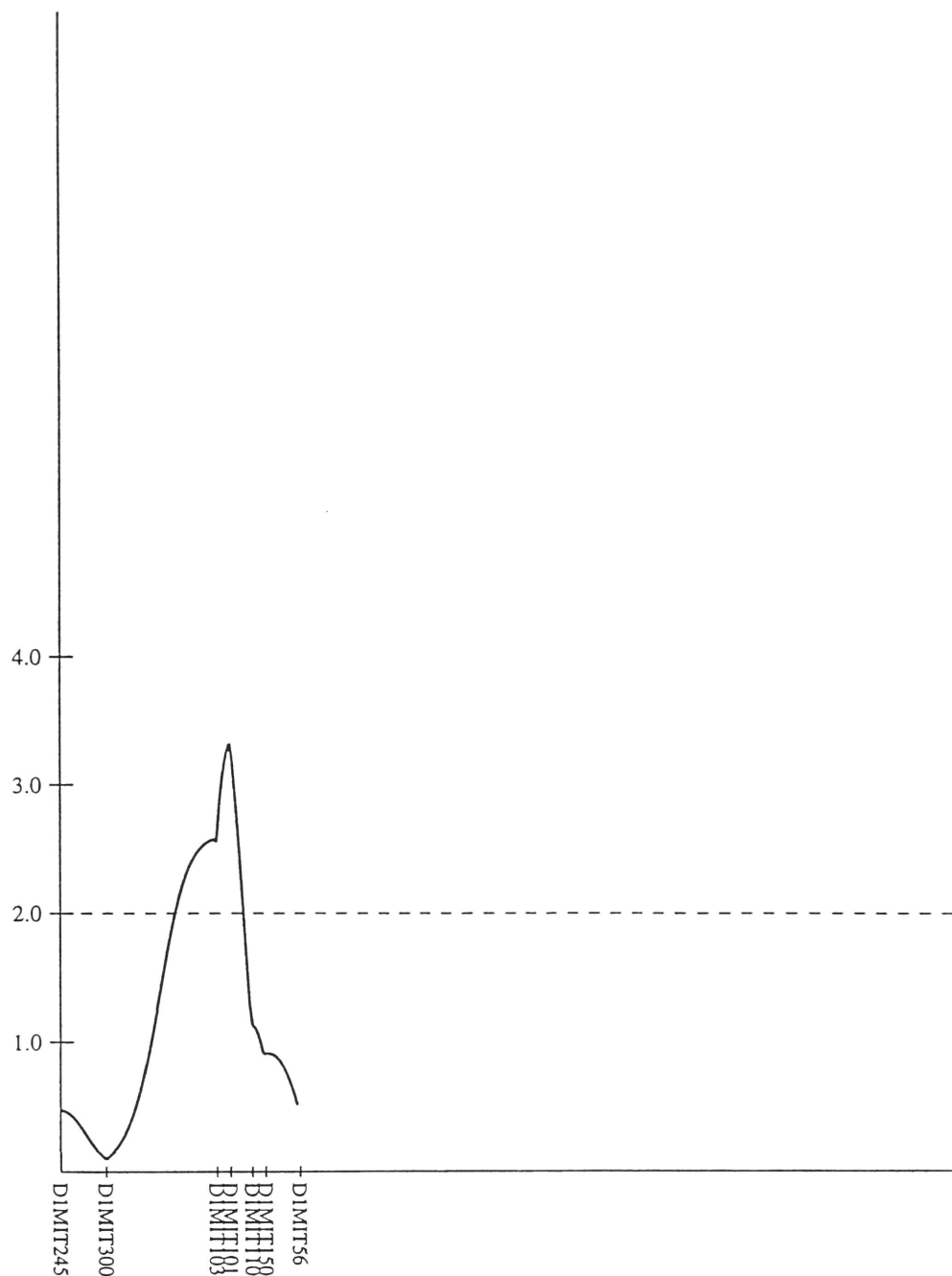


Figure 46. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for number of islets in *ob/ob* F3 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

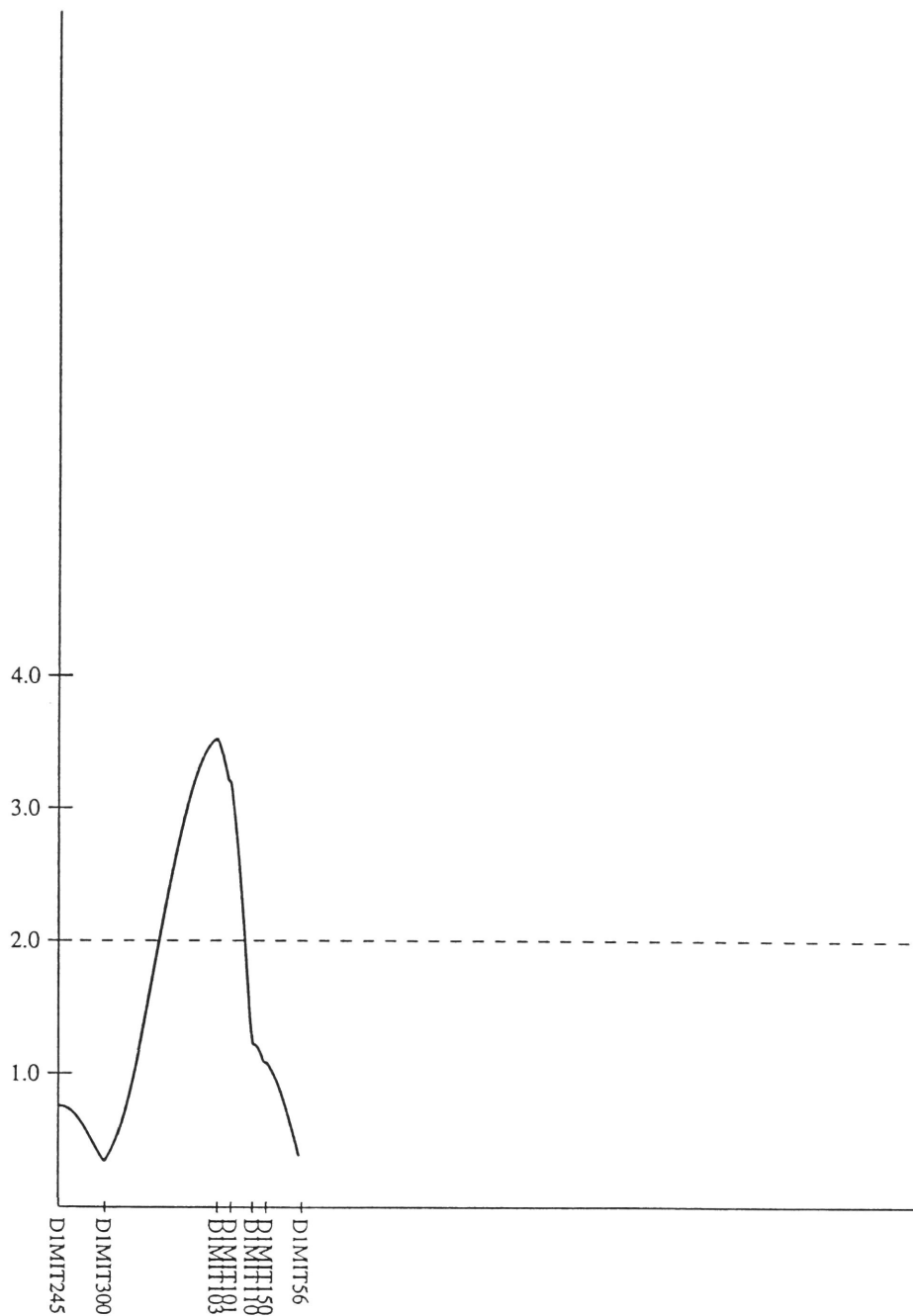


Figure 47. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for total islet area in *ob/ob* F3 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

F3 *ob/ob* B6DBA

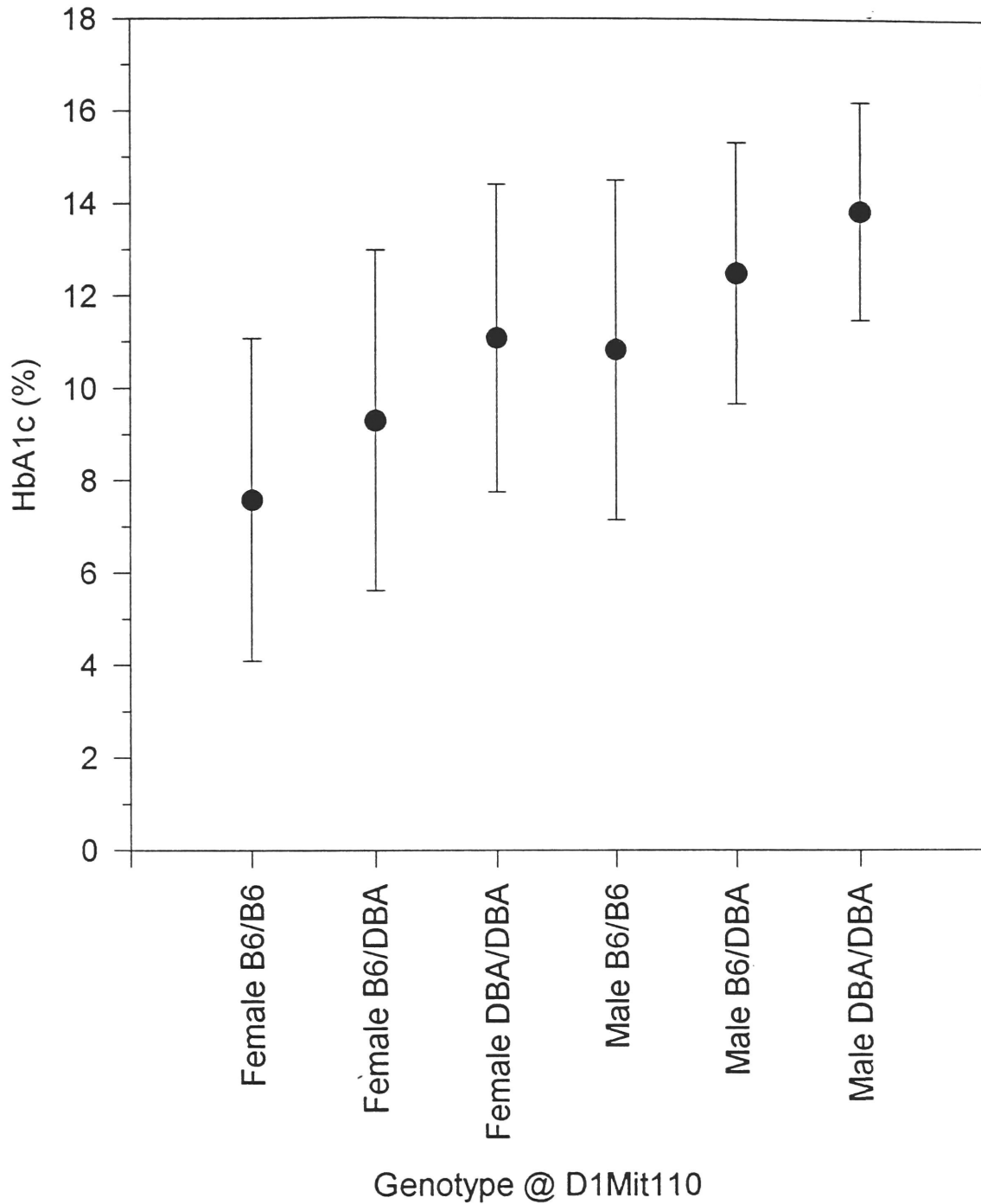


Figure 48. Effect of genotype at D1Mit110 on HbA1c by sex in *ob/ob* F3 B6DBA.

Circles represent the mean of each class with error bars of standard deviations.

F3 *ob/ob* B6DBA

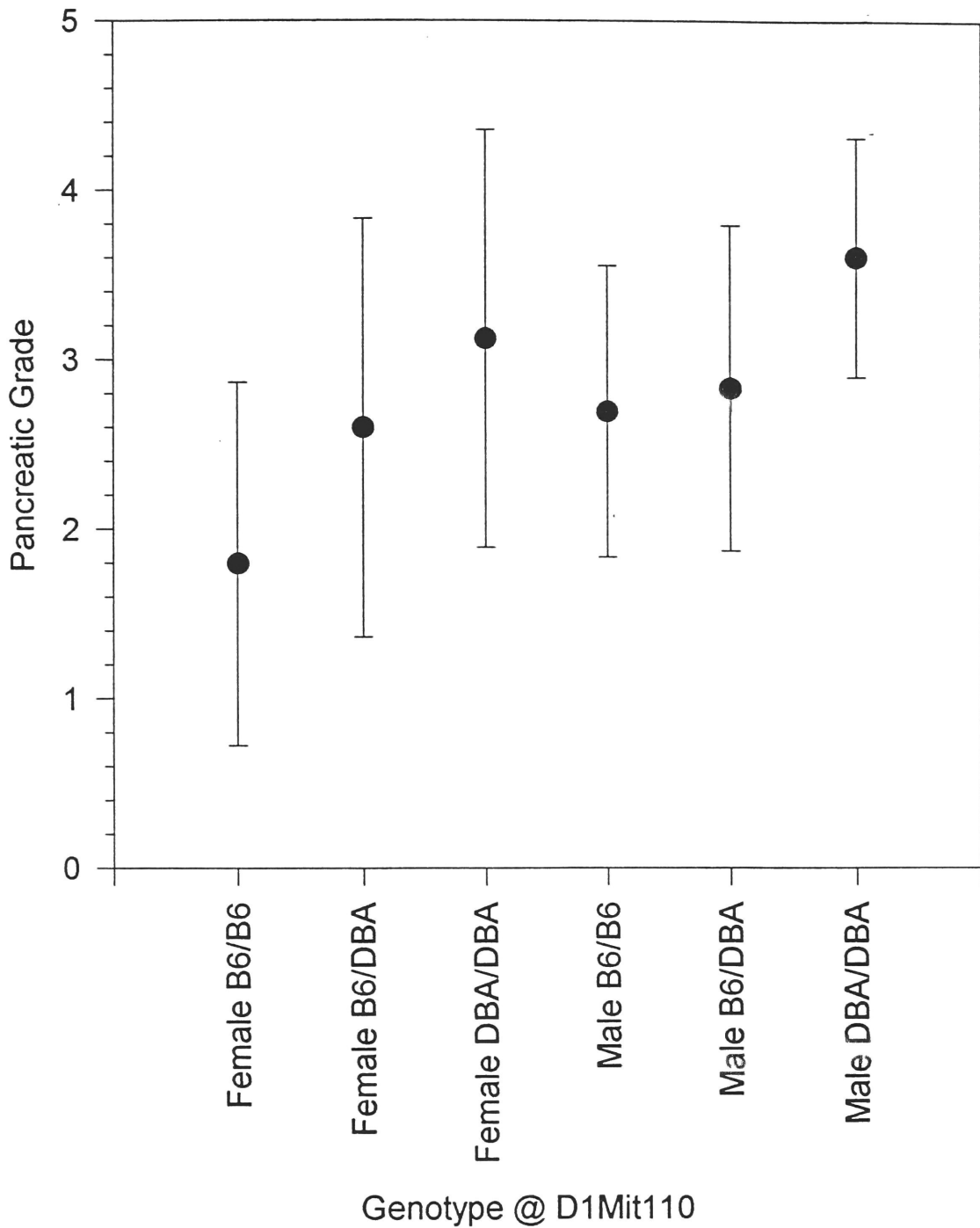


Figure 49. Effect of genotype at D1Mit110 on pancreatic grade by sex in *ob/ob* F3 B6DBA. Circles represent the mean of each class with error bars of standard deviations.

The second locus which demonstrated effects similar to those observed in the F2 progeny was that on chromosome 2 centered around D2Mit367 for which DBA alleles were associated with increased body weight and BMI (Table 14, Figure 50). In this case, the LOD scores were much lower than those observed with the F2 progeny (2-3 vs 7-8), yet the variance attributable to the genotype at this locus was 44.3% for weight and 43% for BMI (Table 15). Homozygosity for DBA alleles at D2Mit367 was associated with a 32% greater body weight in females and 12.5% greater body weight in males compared to the B6/B6 animals. Other traits were also weakly correlated with other loci in the region, with DBA/DBA animals associated with lower pancreatic grade, larger average islet size, and greater islet numbers.

Table 15. Quantitative trait locus (QTL) analysis for diabetes-susceptibility loci in obese (*ob/ob*) F3 B6DBA mice. Data are summarized by trait/phenotype, the marker or interval which demonstrated the peak LOD score, the confidence interval as defined by interval on either side of the peak 1 LOD unit less than the maximum peak using a free genetic model, the maximum LOD score observed under a free genetic model, and the genetic models which most likely fit the data. Genetic models which demonstrated a LOD score < (peak LOD score under a free genetic model -1) were determined to be unlikely genetic models.

Phenotype	Marker with peak LOD score	LOD score	% variance for which locus can account	Genetic model
Weight	D1Mit101	4.2	10.7	free, recessive
	D2Mit367	2.7	44.3	free, recessive
	D4Mit192	3.3	8.4	free, dominant, additive
BMI	D1Mit300/D1Mit101	3.7	26.3	free, recessive, additive
	D1Mit150	3.1	7.9	free, recessive, additive
	D2Mit367	2.2	43	free
	D4Mit192	2.8	7.2	free, dominant, additive
HbA1c	D1Mit300/D1Mit101	5.1	34.7	free, dominant
	D1Mit150	4.6	15.3	free, dominant, additive
Fasting plasma [glucose]	D1Mit110/D1Mit150	2.9	9	free, dominant
Fasting plasma [insulin]	D1Mit103/D1Mit101	5.1	25	free, dominant
Pancreatic grade	D1Mit300/D1Mit101	6.2	30.7	free, recessive, additive
Number of islets	D1Mit103/D1Mit101	3.3	9.9	free, recessive, additive
Number of hyperplastic islets	D1Mit103	2.8	9.9	free, additive
Average islet area	D1Mit103	3.5	11.6	free, additive
Total islet area	D1Mit103	3.5	11.9	free, additive
Beta cell area/total pancreatic area	D1Mit300/D1Mit103	4.4	27.2	free, dominant, additive

F3 *ob/ob* B6DBA

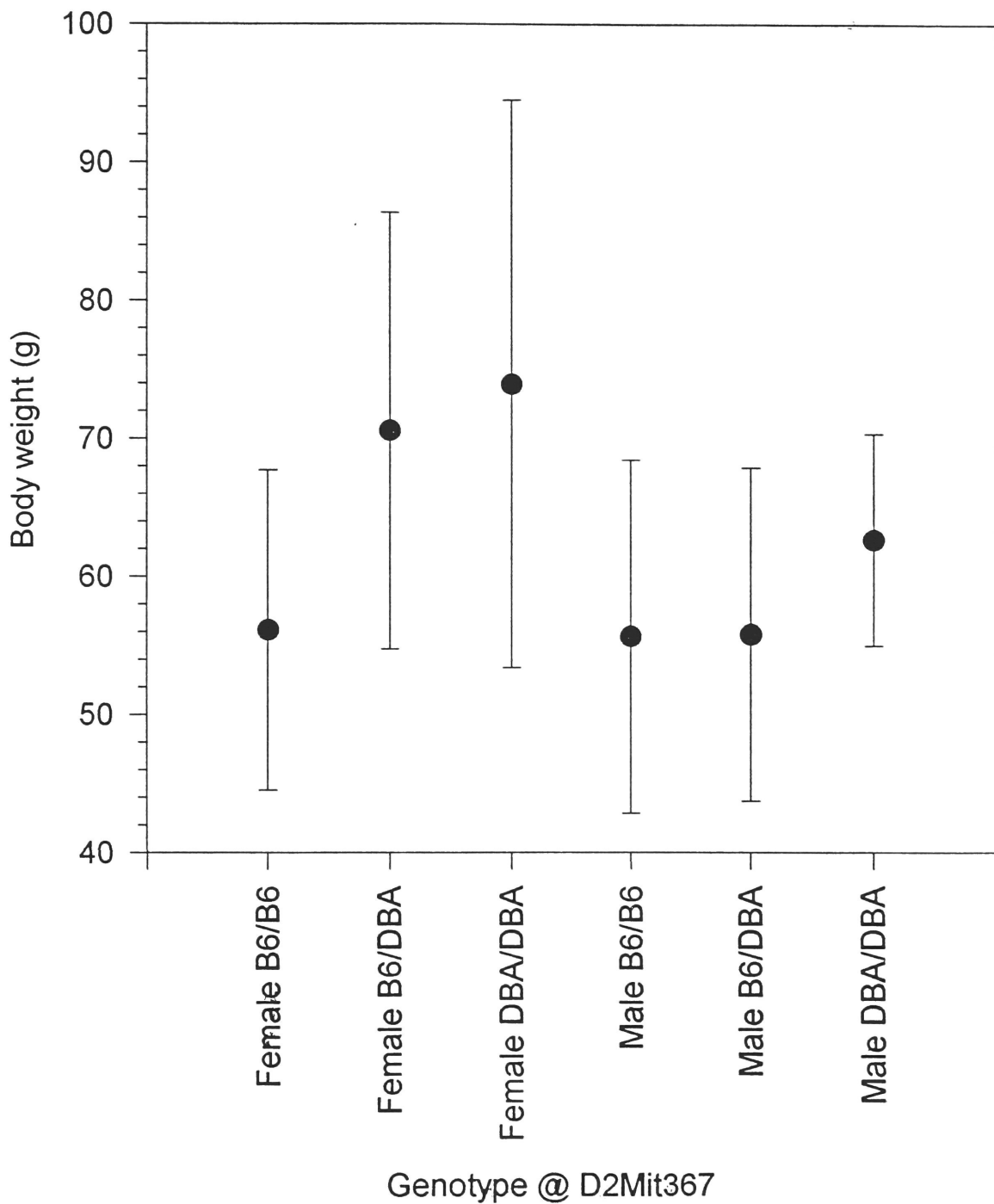


Figure 50. Effect of genotype at D2Mit367 on weight by sex in *ob/ob* F3 B6DBA.

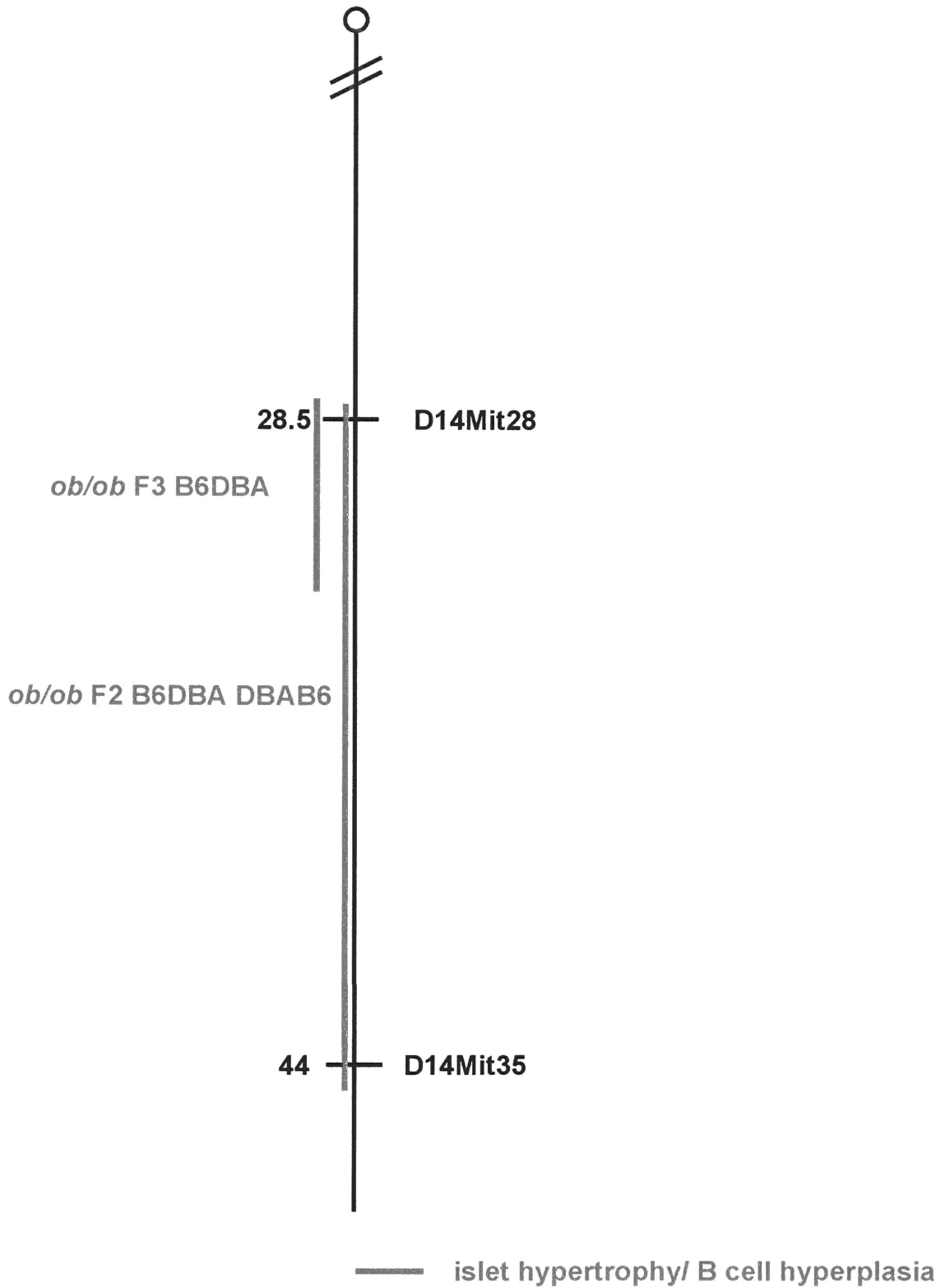
Circles represent the mean of each class with error bars of standard deviations.

In contrast to the results observed with the F2 progeny, with F3 progeny the chromosome 4 locus at D4Mit192 was associated with effects on weight and BMI and demonstrated no effect on islet hypertrophy. This locus accounted for 8.4% of the variance in weight and 7.2% of the variance in BMI (Table 15). Homozygosity for B6 at D4Mit192 was associated with a 20% greater body weight in females and 24% greater body weight in males than homozygotes for DBA (Table 14). Recall that in the F2 progeny, DBA alleles were associated with diabetes protection, so that if the effect of the chromosome 4 locus observed in the F3 progeny had been secondary to a primary effect on a diabetes phenotype, the DBA homozygotes would have been the heaviest. Therefore, it is unlikely that the effects observed were the same as those described for the chromosome 4 F2 QTL. Rather, the effects on body weight and BMI may be the result of the chromosome 4 locus observed in the *ob/ob* F2 B6CAST progeny affecting body weight and BMI (chapter 8).

A single marker, D14Mit28, was used to test the locus on chromosome 14; therefore, no QTL analysis was performed. The number of hyperplastic islets was greatly increased in DBA/DBA animals compared to B6/B6 (6.57 vs 1.45 in the females and 1.75 vs 0.93 in the males) (Figure 51). Average islet size was 76% greater in the females and 21% greater in the male DBA/DBA relative to sex-matched B6/B6. Other traits were similarly affected including weight, HbA1c, and measures of total islet area. The results observed for the F3 animals were comparable to those of the F2 progeny.

Figure 51. Summary of the QTLs for obesity/diabetes on mouse chromosome 14 in *ob/ob* F3 B6DBA. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 14



Discussion

The data from the F3 progeny are primarily important to validate the associations observed in the F2 progeny. Whenever multiple independent comparisons are performed, there is a probability proportionate to the number of analyses performed that the results observed are by chance alone and not biologically meaningful. This problem was addressed in the design of the genome scanning experiments by having a two-tiered approach to evaluate any QTL. No increases were made in the thresholds of statistical significance required with either the QTL analysis or ANOVA to account for the number of independent tests performed. Rather, we independently replicated the results in a cross segregating the same obesity mutation between the same parental strains. In addition to providing confirmation of the F2 results, the inbreeding of F2 *ob/+* animals to produce F3 progeny, results in a 19.8 % loss of genetic variability. If a locus is acting independently, this loss of genetic heterogeneity should act to decrease the phenotypic “noise” produced by the additional loci segregating independently in the background which make small contributions to a particular phenotype. Therefore, the primary locus influencing a phenotype would account for increasingly larger percentages of the variance as the minor loci become genetically fixed. It has been previously demonstrated in sorghum and rice that if genes explaining a large proportion of the phenotypic variance are rendered homozygous, the other genes contributing to the phenotypic variance will account for a larger proportion of the variance and the threshold for QTL detection will be decreased (Paterson et al. 1990). Therefore, it is not surprising that in the F3 the loci on chromosomes 1 and 2 account for more than twice the genetic variance observed with the

F2 progeny. If the loci on chromosomes 1, 2, and 4 act independently, 63.4% of the variance for weight and 76.5% of the variance for BMI can be accounted for even if only the more centromeric of the two loci on chromosome 1 is assumed to influence BMI.

The additional implication of data derived from the F3 progeny is that the loci on chromosomes 1 and 2 are likely to breed true after they are introgressed to produce congenics (see chapter 18). Genetic heterogeneity is randomly and inefficiently lost in producing recombinant inbred lines (the first step of which was taken in producing the F3 progeny). Through a series of backcrosses the genetic heterogeneity is systematically and efficiently eliminated in the production of congenic mice (especially if “speed congenic” techniques are used). Therefore, if the effects of the chromosomes 1 and 2 loci are enhanced by minor reductions of genetic heterogeneity in the F3 progeny, it is likely that (assuming that the chromosome 1 and 2 loci act independently) their effects will be dramatically enhanced if all other regions of the genome producing minor effects on the phenotype are eliminated.

The negative results are as useful as the positive ones for designing future experiments. Because the chromosome 10 locus could not be confirmed in either the most recent F2 or F3 B6DBA crosses segregating *ob*, it will not be fruitful to continue pursuing either the precise location or function of this locus using our current methods of phenotypic characterization. The inability to replicate the original results may be due to a difference in the manner in which the animals were prepared for sacrifice, (an overnight fast was

used in the first cross in contrast to a three hour fast in the most recent crosses) (chapter 8), or changes in subtle features of the environment which may have changed with time such as the constituents of the rodent chow. The correlation that was originally observed in the F2 B6DBA-1988 and F2 B6CAST crosses segregating *ob* was elevated overnight fasting plasma [insulin] in animals homozygous for DBA or CAST at D10Mit3. The plasma [insulin] that has been measured in all the subsequent crosses is following only a three hour fast. Elevations in plasma [insulin] after an overnight fast should not be correlated with previous meal ingestion but rather may be a manifestation of the pancreas' attempt to control fasting hyperglycemia due to increased hepatic glucose production. Therefore, if the location and physiology of the chromosome 10 locus were pursued, different fasting regimes would be required.

The chromosome 4 locus affecting weight and BMI originally observed in the *ob/ob* F2 B6CAST progeny may have been replicated in the *ob/ob* F3 B6DBA and could be further studied; however, better localization of the QTL would be required. The chromosome 4 locus apparently controlling islet hypertrophy however was not successfully replicated possibly due to: 1). A false positive result in the F2 progeny 2). Inaccurate map localization within the F2 progeny, and therefore still present but not yet detected in the F3 progeny 3). Dependence upon some environmental factor which has changed between the twelve months when the F2 and F3 progeny were raised or 4). Requirements for interactions with other loci (for which evidence was presented in the previous chapter) which may have been randomly fixed in enough animals to mask the effect the locus was

having in a subset of the F3 progeny. Although the data were analyzed for interactions between the loci by ANOVA, the numbers of animals with particular genotypes or combinations of genotypes was not large enough to detect any statistical differences. Further study of this locus will require precise genetic localization and investigation of its interactions with other loci, possibly only after multiple congenics have been made.

The phenotypic effects of the chromosome 14 locus were replicated in the F3 progeny, with characteristics similar to those observed with the F2 progeny. Although the effects of this locus on the phenotypes studied were not as extreme as the chromosome 1 and 2 loci, the locus may be more genetically tractable once a larger panel of more specific phenotypes is used to characterize the animals. That is, a sub-phenotype may correlate with genotype of the locus better than the current phenotypic measures in use.

Chapter 6

Identification of diabetes susceptibility regions in F2 B6DBA and segregating for *db*

Selective genotyping selection criterion

All of the mice were typed with D4Mit76 and D4Mit12 which lie centromeric of *db*, and with Bam7 and c-Jun which lie telomeric to *db* (Chua et al. 1996) to confirm gene dosage at *db* in phenotypically obese animals. Concordance between obesity genotype and phenotype was 100%.

Three groups of animals with extreme phenotypes were chosen based on biochemical determinants of progression through three stages of disease: disease free, insulin resistant, and diabetic. Five obese mice were initially classified as disease free (HbA1c < 7%; fasting plasma [glucose] < 1 standard deviation below the mean [glucose] of the obese animals [436 mg/dl]; fasting plasma [insulin] < the obese mean [331 μ U/ml]; and non-glycosuric); 10 mice as insulin resistant (HbA1c < 7%; fasting plasma [glucose] < 500 mg/dl; fasting plasma [insulin] > one standard deviation above the obese mean [643 μ U/ml]; and non-glycosuric); and 12 other mice as diabetic (fasting plasma [glucose] > 900 mg/dl; fasting plasma [insulin] < 40 μ U/ml; maximal glycosuria and prolonged disease indicated by HbA1c > 14%) (Table 16). Also, 17 mice (some of which had also been designated insulin-resistant/non-diabetic) were chosen solely on the basis of pancreatic morphology protective against diabetes (pancreatic grade < 1.5) and were designated protective pancreas. Each group was typed for 130-140 SSR's spaced throughout the genome (Table 17).

ID	Age (days)	Sex	Weight (g)	BMI (g/cm ²)	Islets/pan (μm ²)	Insulin (μU/ml)	pancreatic grade	pancreatic insulin (μU/mg protein)	pancreatic glucagon (pg/mg protein)	pancreatic (Insulin/glucagon) (μU/ps)	number of islets per section	number of hyperatrophic islets	average islet size (mm ²)	islet area (mm ²)	islet area (%)
Non-diabetic															
103	107	F	44.7	0.664	4.1	320	234.4	33394	17932	1.96	19	2	0.022	0.416	0.843
216	111	F	37.7	0.622	3.9	340	77.1	4766	22429	0.21	16	2	0.020	0.293	1.070
243	100	F	20.6	0.446	3.0	296	242.8	1289	32467	0.04	7	0	0.013	0.091	0.889
268	106	M	31.7	0.480	9.3	140	113.6	12623	17786	0.71	22	0	0.012	0.287	0.799
268	106	F	41.2	0.926	6.1	412	263.0	6978	20639	0.43	22	4	0.036	0.630	2.422
Average	106		39.4	0.645	4.5	284	184.2	12216.26	22269.95	0.81	17.06	1.06	0.02	0.36	1.16
Insulin resistant															
122	103	F	63.8	0.686	4.6	364	828.4	41096	20991	1.97	39	8	0.024	0.519	3.654
123	102	F	53.3	0.616	7.1	392	209.6	21387	26638	0.93	23	6	0.031	0.702	3.208
199	106	F	66.9	0.669	8.9	632	889.4	4053	17764	0.20	37	12	0.038	1.461	0.994
207	122	F	66.8	0.696	8.2	698	1034.4	9432	13476	0.70	29	10	0.048	1.317	7.396
208	133	F	412	0.897	3.8	412	366.6	27920	21944	1.27	27	9	0.034	0.906	3.781
209	133	F	69.8	0.810	4.8	444	161.7	22146	13472	1.84	22	7	0.029	0.894	2.191
248	98	F	50.3	0.821	6.2	340	661.7	16276	16246	1.00	11	1	0.04	0.34	0.844
248	98	F	53.3	0.816	6.1	392	889.4	4053	17764	0.20	37	12	0.038	1.461	0.994
253	102	M	63.8	0.686	4.6	364	828.4	41096	20991	1.97	39	8	0.024	0.519	3.654
253	102	M	63.8	0.686	4.6	364	828.4	41096	20991	1.97	39	8	0.024	0.519	3.654
104	109	M	48.6	0.697	6.0	244	876.1	13206	18282	0.89	13	2	0.031	0.404	1.917
107	109	M	49.2	0.634	6.8	412	1156.0	21068	19242	1.09	13	6	0.044	0.867	3.223
142	109	F	52.3	0.623	3.8	492	1167.6	33739	24283	1.39	43	6	0.026	1.127	3.026
170	113	F	47.2	0.503	3.9	308	662.6	26090	16639	1.87	20	4	0.031	0.837	2.096
174	112	F	47.3	0.503	3.9	308	662.6	26090	16639	1.87	20	4	0.031	0.837	2.096
224	99	F	43.4	0.461	6.6	384	874.6	21062	24906	0.93	28	2	0.016	0.560	2.176
263	87	F	44.3	0.501	6.0	360	863.2	18600	24906	0.85	20	0	0.016	0.560	2.176
271	120	F	61.4	0.590	6.6	404	1142.3	21062	24906	0.93	31	10	0.053	1.569	4.126
Mean	111		52.7	0.576	5.4	404	726.1	21818.58	26317.48	1.08	25.63	8.06	0.05	0.87	3.96
Insulin resistant															
104	109	F	48.6	0.697	6.0	244	876.1	13206	18282	0.89	13	2	0.031	0.404	1.917
107	109	M	48.2	0.634	6.8	412	1156.0	21068	19242	1.09	13	6	0.044	0.867	3.223
135	102	F	66.6	0.665	9.2	472	1357.4	15603	10287	1.02	20	2	0.017	0.336	1.116
142	106	F	52.3	0.523	3.8	492	1157.6	33739	24283	1.39	43	6	0.026	1.127	3.026
160	87	F	66.1	0.696	6.3	496	1761.1	34096	18633	0.76	19	1	0.016	0.649	2.008
170	113	F	47.2	0.523	3.8	302	664.4	26090	16639	1.87	20	4	0.031	0.837	2.096
174	112	F	47.3	0.523	3.8	308	662.6	26090	16639	1.87	20	4	0.031	0.837	2.096
224	99	F	43.4	0.461	6.6	384	874.6	21062	24906	0.93	28	2	0.016	0.560	2.176
263	87	F	44.3	0.501	6.0	360	863.2	18600	24906	0.85	20	0	0.016	0.560	2.176
271	120	F	61.4	0.590	6.6	404	1142.3	21062	24906	0.93	31	10	0.053	1.569	4.126
Mean	105		54.6	0.540	5.5	378	871.3	21635.44	26126.95	0.88	23.68	4.68	0.03	0.74	2.24
Diabetic															
117	103	M	36.8	0.442	16.7	962	39.9	9354	24062	0.39	ND	ND	ND	ND	ND
119	103	M	34.2	0.473	17.4	1024	29.3	11964	17734	0.68	ND	ND	ND	ND	ND
144	106	M	47.6	0.606	16.4	904	0.0	13360	26469	0.47	1	0	0.013	0.013	0.047
166	113	M	38.0	0.431	17.1	928	0.0	7817	18652	0.40	ND	ND	ND	ND	ND
167	113	M	38.0	0.431	17.1	928	0.0	7817	18652	0.40	ND	ND	ND	ND	ND
182	112	M	66.2	0.822	15.6	1060	26.3	5343	21862	0.24	9	0	0.008	0.098	0.031
182	112	M	66.2	0.822	15.6	1060	26.3	5343	21862	0.24	9	0	0.008	0.098	0.031
182	112	M	66.2	0.822	15.6	1060	26.3	5343	21862	0.24	9	0	0.008	0.098	0.031
182	112	M	66.2	0.822	15.6	1060	26.3	5343	21862	0.24	9	0	0.008	0.098	0.031
204	124	F	47.7	0.576	16.9	960	30.7	842	23266	0.04	2	0	0.008	0.017	0.087
204	124	F	47.7	0.576	16.9	960	30.7	842	23266	0.04	2	0	0.008	0.017	0.087
211	88	M	37.1	0.448	20.2	1066	0.0	6966	16248	0.39	2	0	0.012	0.026	0.076
211	88	M	37.1	0.448	20.2	1066	0.0	6966	16248	0.39	2	0	0.012	0.026	0.076
216	114	F	40.2	0.608	18.9	1148	24.0	11633	17463	0.16	14	0	0.011	0.161	0.673
216	114	F	40.2	0.608	18.9	1148	24.0	11633	17463	0.16	14	0	0.011	0.161	0.673
228	100	F	41.1	0.468	16.3	928	37.9	6611	17687	0.31	6	0	0.017	0.163	0.621
240	93	M	37.0	0.410	16.6	824	24.0	6611	17687	0.31	6	0	0.017	0.163	0.621
Mean	108		41.1	0.485	16.7	1028	26.7	7354.67	26443.96	0.36	6.67	0.66	0.02	0.66	0.22

Table 16. Phenotypic characteristics of obese animals used in each genome scanning experiment of the F2 B6DBA segregating for *db*.

The average for the group for each phenotypic parameter is indicated as the last row of the group in bold.

Table 17. Genome scanning results for the five *db/db* F2 B6DBA non-diabetic, 17 protected pancreas, ten insulin resistant, and twelve diabetic mice. B indicates homozygosity for B6 alleles, H heterozygosity or B6/DBA and D homozygosity for DBA alleles at a particular locus. Total indicates the total number of animals genotyped for a particular marker. All chi square >6 are boxed and are significant at $p < 0.05$. All markers are listed in order along the chromosome from centromere to telomere.

Disease free MARKER	B	Genotype H	D	Total Animals Scorec	CHI SQUARE
D1MIT70	1	1	3	5	3.4
D1MIT300	1	1	3	5	3.4
D1Mit76	1	1	3	5	3.4
D1MIT24	1	2	2	5	0.6
D1MIT84	1	2	2	5	0.6
D1MIT90	1	1	2	4	1.5
D1MIT110	2	2	1	5	0.6
D1MIT56	0	4	0	4	4
D2MIT80	2	3	0	5	1.8
D2MIT61	2	3	0	5	1.8
D2MIT42	1	4	0	5	2.2
D2MIT17	1	4	0	5	2.2
D2MIT423	1	4	0	5	2.2
D2MIT229	2	3	0	5	1.8
D3MIT62	2	3	0	5	1.8
D3MIT65	3	2	0	5	3.8
D3MIT51	3	1	1	5	3.4
D3MIT12	3	1	1	5	3.4
D3MIT254	2	1	1	4	1.5
D3MIT257	2	2	1	5	0.6
D3MIT17	1	3	1	5	0.2
D3MIT86	1	4	0	5	2.2
D3MIT200	1	2	0	3	1
D3MIT129	1	2	0	3	1
D4MIT149	2	1	2	5	1.8
D4MIT227	2	0	2	4	4
D4MIT89	3	2	0	5	3.8
D4MIT76	4	1	0	5	8.2
D4MIT54	3	2	0	5	3.8
D4MIT42	2	3	0	5	1.8
D5MIT193	2	1	2	5	1.8
D5MIT79	2	3	0	5	1.8
D5MIT93	2	3	0	5	1.8
D5MIT65	2	3	0	5	1.8
D5MIT99	1	3	1	5	0.2
D6MIT55	0	4	1	5	2.2
D6MIT159	0	3	2	5	1.8
D6MIT9	0	5	0	5	5
D6MIT17	0	5	0	5	5
D6MIT14	0	4	1	5	2.2
D7MIT178	0	3	2	5	1.8
D7MIT57	2	2	1	5	0.6
D7MIT229	2	2	1	5	0.6
D7MIT84	0	2	1	3	1
D7MIT62	0	0	5	5	15
D7MIT100	0	1	4	5	8.2
D7MIT12	0	3	2	5	1.8
D7MIT291	0	3	2	5	1.8
D8MIT3	1	3	1	5	0.2
D8MIT4	1	3	1	5	0.2
D8MIT132	2	2	0	4	2
D8MIT236	2	2	1	5	0.6
D8MIT69	2	2	1	5	0.6
D8MIT45	2	3	0	5	1.8
D8MIT207	2	3	0	5	1.8
D8MIT242	3	2	0	5	3.8
D8MIT113	3	2	0	5	3.8
D8MIT272	1	4	0	5	2.2
D9MIT64	3	2	0	5	3.8
D9MIT10	3	2	0	5	3.8
D9MIT243	2	2	0	4	2
D9MIT18	1	4	0	5	2.2
D10MIT51	0	4	1	5	2.2
D10MIT44	0	2	3	5	3.8
D10MIT194	0	4	1	5	2.2
D10MIT94	0	4	1	5	2.2
D10MIT61	3	1	1	5	3.4
D10MIT42	2	2	1	5	0.6
D10MIT70	1	3	1	5	0.2

Disease free MARKER	B	Genotype H	D	Total Animals Scorec	CHI SQUARE
D11MIT78	1	3	1	5	0.2
D11MIT174	1	2	2	5	0.6
D11MIT217	1	3	1	5	0.2
D11MIT260	0	4	0	4	4
D11MIT39	0	5	0	5	5
D11MIT99	0	5	0	5	5
D11MIT103	0	5	0	5	5
D12MIT105	3	2	0	5	3.8
D12MIT12	3	2	0	5	3.8
D12MIT171	3	2	0	5	3.8
D12MIT60	3	1	1	5	3.4
D12MIT62	1	3	1	5	0.2
D12MIT34	2	2	1	5	0.6
D12MIT239	2	2	1	5	0.6
D12MIT17	1	1	3	5	3.4
D12MIT141	1	1	3	5	3.4
D12MIT133	1	1	3	5	3.4
D13MIT17	1	1	3	5	3.4
D13MIT198	1	1	3	5	3.4
D13MIT66	0	1	2	3	3
D13MIT77	2	2	1	5	0.6
D13MIT76	0	4	1	5	2.2
D14MIT1	2	3	0	5	1.8
D14MIT54	2	3	0	5	1.8
D14MIT28	1	4	0	5	2.2
D14MIT39	0	1	1	2	1
D14MIT193	1	3	1	5	0.2
D14MIT35	1	3	1	5	0.2
D14MIT75	3	2	0	5	3.8
D15MIT12	3	1	1	5	3.4
D15MIT5	1	3	1	5	0.2
D15MIT91	0	2	0	2	2
D15MIT95	1	4	0	5	2.2
D15MIT34	1	4	0	5	2.2
D15MIT217	1	2	0	3	1
D16MIT181	3	1	1	5	3.4
D16MIT165	3	1	1	5	3.4
D16MIT103	2	3	0	5	1.8
D16MIT12	1	4	0	5	2.2
D16MIT5	1	3	1	5	0.2
D16MIT50	2	2	1	5	0.6
D17MIT46	2	2	1	5	0.6
D17MIT22	2	2	1	5	0.6
D17MIT66	1	3	1	5	0.2
D17MIT54	1	1	2	4	1.5
D17MIT39	2	1	2	5	1.8
D18MIT66	0	2	2	4	2
D18MIT132	0	3	2	5	1.8
D18MIT87	3	1	3	7	3.57
D18MIT17	0	1	4	5	8.2
D18MIT123	0	0	3	3	9
D18MIT50	0	2	3	5	3.8
D18MIT9	0	2	3	5	3.8
D18MIT186	1	1	3	5	3.4
D18MIT49	2	0	3	5	5.4
D19MIT28	0	1	4	5	8.2
D19MIT13	1	1	3	5	3.4
D19MIT19	2	1	2	5	1.8
D19MIT10	2	2	1	5	0.6
D19MIT34	2	2	1	5	0.6
DXMIT89	4	1	0	5	1.9
DXMIT105	3	1	1	5	1.1
DXMIT182	3	2	0	5	0.6

Protected pancreas MARKER	B	Genotype H	D	Total Animals Scored	CHI SQUARE
D1MIT70	5	7	5	17	0.53
D1MIT76	3	12	2	17	3.00
D1MIT24	4	11	2	17	1.94
D1MIT84	5	10	2	17	1.59
D1MIT90	5	8	2	15	1.27
D1MIT110	7	7	2	16	3.38
D1MIT56	3	6	4	13	0.23
D2MIT80	3	8	6	17	1.12
D2MIT61	3	9	5	17	0.53
D2MIT42	2	12	2	16	4.00
D2MIT17	2	10	4	16	1.50
D2MIT423	3	8	6	17	1.12
D2MIT285	2	8	5	15	1.27
D2MIT310	0	8	7	15	6.60
D2MIT311	1	7	7	15	4.87
D2MIT229	1	8	8	17	5.82
D2MIT147	3	6	7	16	3.00
D2MIT266	2	9	6	17	1.94
D3MIT62	3	12	1	16	4.50
D3MIT65	1	10	5	16	3.00
D3MIT51	1	10	5	16	3.00
D3MIT12	4	8	4	16	0.00
D3MIT257	6	6	4	16	1.50
D3MIT17	8	4	5	17	5.82
D3MIT86	4	2	4	10	3.60
D3MIT200	4	3	4	11	2.27
D3MIT129	6	4	5	15	3.40
D4MIT149	7	4	3	14	4.86
D4MIT227	10	4	3	17	10.53
D4MIT89	9	6	2	17	7.24
D4MIT76	11	4	0	15	19.40
D4MIT54	9	8	0	17	9.59
D4MIT42	7	9	1	17	4.29
D5MIT193	5	8	4	17	0.18
D5MIT79	4	12	1	17	3.94
D5MIT93	4	9	4	17	0.06
D5MIT65	3	8	6	17	1.12
D5MIT99	0	9	8	17	7.59
D5MIT222	0	10	7	17	6.29
D5MIT223	0	10	7	17	6.29
D6MIT55	3	9	5	17	0.53
D6MIT159	1	13	3	17	5.24
D6MIT9	2	12	3	17	3.00
D6MIT17	1	12	4	17	3.94
D6MIT14	4	11	2	17	1.94
D7MIT178	4	7	4	15	0.07
D7MIT57	4	7	6	17	1.00
D7MIT229	5	5	4	14	1.29
D7MIT84	4	9	4	17	0.06
D7MIT62	6	4	2	12	4.00
D7MIT100	6	7	4	17	1.00
D7MIT12	6	10	1	17	3.47
D7MIT291	5	8	1	14	2.57
D8MIT3	4	11	2	17	1.94
D8MIT4	4	9	3	16	0.38
D8MIT69	5	10	2	17	1.59
D8MIT45	4	9	4	17	0.06
D8MIT207	4	9	3	16	0.38
D8MIT113	4	7	6	17	1.00
D8MIT272	5	5	7	17	3.35
D9MIT64	2	11	4	17	1.94
D9MIT10	3	10	4	17	0.65
D9MIT243	2	9	4	15	1.13
D9MIT18	7	7	3	17	2.41
D10MIT51	2	10	5	17	1.59
D10MIT44	3	9	5	17	0.53
D10MIT194	3	7	5	15	0.60
D10MIT94	3	7	7	17	2.41
D10MIT61	3	8	6	17	1.12
D10MIT42	4	8	5	17	0.18
D10MIT70	2	4	2	8	0.00

Protected pancreas MARKER	B	Genotype H	D	Total Animals Scored	CHI SQUARE
D11MIT78	5	8	4	17	0.18
D11MIT174	4	9	4	17	0.06
D11MIT217	5	9	2	16	1.38
D11MIT260	3	9	4	16	0.38
D11MIT157	3	9	4	16	0.38
D11MIT39	3	11	3	17	1.47
D11MIT99	4	10	3	17	0.65
D11MIT103	5	7	4	16	0.38
D12MIT105	7	8	0	15	6.60
D12MIT12	7	7	0	14	7.00
D12MIT171	9	8	0	17	9.59
D12MIT60	6	7	0	13	5.62
D12MIT62	6	9	2	17	1.94
D12MIT34	5	12	0	17	5.82
D12MIT239	4	12	1	17	3.94
D12MIT17	1	6	1	8	2.00
D12MIT141	2	11	4	17	1.94
D12MIT133	0	11	2	13	6.85
D13MIT17	4	11	2	17	1.94
D13MIT198	3	11	1	15	3.80
D13MIT66(9)	0	7	2	9	3.67
D13MIT77	4	9	4	17	0.06
D13MIT76	3	9	5	17	0.53
D14MIT1	3	12	2	17	3.00
D14MIT54	5	9	3	17	0.53
D14MIT28	1	9	7	17	4.29
D14MIT39	1	7	5	13	2.54
D14MIT193	1	9	5	15	2.73
D14MIT35	0	9	6	15	5.40
D14MIT75	2	7	7	16	3.38
D15MIT12	6	8	3	17	1.12
D15MIT5	4	9	4	17	0.06
D15MIT91	4	7	3	14	0.14
D15MIT95	5	9	3	17	0.53
D15MIT34	5	8	1	14	2.57
D15MIT217	7	5	3	15	3.80
D16MIT181	3	9	4	16	0.38
D16MIT165	3	10	4	17	0.65
D16MIT103	2	8	6	16	2.00
D16MIT12	2	9	6	17	1.94
D16MIT5	2	8	6	16	2.00
D16MIT50	2	10	5	17	1.59
D17MIT46	3	11	3	17	1.47
D17MIT22	3	9	3	15	0.60
D17MIT66	5	10	2	17	1.59
D17MIT54	6	8	0	14	5.43
D17MIT39	5	8	4	17	0.18
D18MIT66	6	3	6	15	5.40
D18MIT132	6	5	6	17	2.88
D18MIT87	6	5	6	17	2.88
D18MIT17	5	6	6	17	1.59
D18MIT123	4	7	4	15	0.07
D18MIT50	7	8	1	16	4.50
D18MIT9	5	9	3	17	0.53
D18MIT186	6	6	4	16	1.50
D18MIT49	4	9	2	15	1.13
D19MIT28	4	10	2	16	1.50
D19MIT46	3	10	3	16	1.00
D19MIT13	3	12	2	17	3.00
D19MIT19	3	12	2	17	3.00
D19MIT10	3	13	1	17	5.24
D19MIT34	3	11	2	16	2.38
DXMIT89	6	10	0	16	2.57
DXMIT105	11	4	1	16	2.37
DXMIT182	9	6	1	16	0.33

Insulin resistant MARKER	B	Genotype H	D	Total Animals Scored	CHI SQUARE
D1MIT70	4	5	1	10	1.80
D1MIT76	2	8	0	10	4.40
D1MIT24	1	8	0	9	5.67
D1MIT84	2	7	1	10	1.80
D1MIT90	2	6	1	9	1.22
D1MIT110	2	7	0	9	3.67
D1MIT56	1	5	3	9	1.00
D2MIT80	1	7	2	10	1.80
D2MIT61	1	8	1	10	3.60
D2MIT42	2	5	2	9	0.11
D2MIT17	2	4	3	9	0.33
D2MIT423	3	4	3	10	0.40
D2MIT229	2	4	4	10	1.20
D3MIT62	0	8	2	10	4.40
D3MIT65	0	7	3	10	3.40
D3MIT51	0	8	2	10	4.40
D3MIT12	3	4	2	9	0.33
D3MIT257	3	5	1	9	1.00
D3MIT17	6	3	1	10	6.60
D4MIT149	6	1	1	8	10.75
D4MIT227	8	1	1	10	16.20
D4MIT89	8	2	0	10	16.40
D4MIT76	7	3	0	10	11.40
D4MIT54	3	7	0	10	3.40
D4MIT42	3	6	1	10	1.20
D5MIT193	5	4	1	10	3.60
D5MIT79	3	6	1	10	1.20
D5MIT93	4	4	2	10	1.20
D5MIT65	4	2	4	10	3.60
D5MIT99	1	5	4	10	1.80
D6MIT55	3	4	3	10	0.40
D6MIT159	1	7	2	10	1.80
D6MIT9	2	6	2	10	0.40
D6MIT17	1	3	3	7	1.29
D6MIT14	4	3	3	10	1.80
D7MIT178	3	3	3	9	1.00
D7MIT57	5	2	3	10	4.40
D7MIT117	5	3	2	10	3.40
D7MIT229	2	3	2	7	0.14
D7MIT84	3	4	3	10	0.40
D7MIT62	5	1	3	9	6.33
D7MIT100	4	3	3	10	1.80
D7MIT12	5	5	0	10	5.00
D7MIT291	4	4	0	8	4.00
D8MIT3	3	6	1	10	1.20
D8MIT4	4	4	1	9	2.11
D8MIT69	5	4	1	10	3.60
D8MIT45	5	3	2	10	3.40
D8MIT207	5	2	2	9	4.78
D8MIT113	4	1	5	10	6.60
D8MIT272	2	4	4	10	1.20
D9MIT64	1	6	3	10	1.20
D9MIT10	1	6	3	10	1.20
D9MIT243	0	5	4	9	3.67
D9MIT18	3	3	4	10	1.80
D10MIT51	1	6	3	10	1.20
D10MIT44	4	5	1	10	1.80
D10MIT194	2	3	3	8	0.75
D10MIT94	2	3	5	10	3.40
D10MIT61	2	4	4	10	1.20
D10MIT42	1	5	3	9	1.00

Insulin resistant MARKER	B	Genotype H	D	Total Animals Scored	CHI SQUARE
D11MIT78	1	5	4	10	1.80
D11MIT174	1	6	3	10	1.20
D11MIT217	3	4	2	9	0.33
D11MIT260	1	5	4	10	1.80
D11MIT157	1	5	4	10	1.80
D11MIT39	1	7	2	10	1.80
D11MIT99	2	7	1	10	1.80
D11MIT103	2	5	2	9	0.11
D12MIT105	2	5	1	8	0.75
D12MIT12	3	4	0	7	2.71
D12MIT171	5	4	1	10	3.60
D12MIT60	3	4	1	8	1.00
D12MIT62	4	5	1	10	1.80
D12MIT34	1	9	0	10	6.60
D12MIT239	2	8	0	10	4.40
D12MIT141	1	5	4	10	1.80
D12MIT133	1	6	2	9	1.22
D13MIT17	3	6	1	10	1.20
D13MIT198	2	7	0	9	3.67
D13MIT66	0	4	1	5	2.20
D13MIT77	1	5	4	10	1.80
D13MIT76	1	4	5	10	3.60
D14MIT1	3	6	1	10	1.20
D14MIT54	5	5	0	10	5.00
D14MIT28	1	7	2	10	1.80
D14MIT39	1	7	1	9	2.78
D14MIT193	1	7	1	9	2.78
D14MIT35	0	5	3	8	2.75
D14MIT75	2	4	3	9	0.33
D15MIT12	3	6	1	10	1.20
D15MIT5	2	6	2	10	0.40
D15MIT91	2	6	2	10	0.40
D15MIT95	3	5	2	10	0.20
D15MIT34	2	5	0	7	2.43
D15MIT217	3	5	2	10	0.20
D16MIT181	2	6	1	9	1.22
D16MIT165	3	6	1	10	1.20
D16MIT103	2	6	1	9	1.22
D16MIT12	2	7	1	10	1.80
D16MIT5	2	6	1	9	1.22
D16MIT50	3	6	1	10	1.20
D17MIT46	1	9	0	10	6.60
D17MIT22	1	7	0	8	4.75
D17MIT66	1	9	0	10	6.60
D17MIT54	3	5	0	8	2.75
D17MIT39	2	5	3	10	0.20
D18MIT66	3	5	2	10	0.20
D18MIT132	3	5	2	10	0.20
D18MIT87	3	5	2	10	0.20
D18MIT17	2	6	2	10	0.40
D18MIT123	1	4	1	6	0.67
D18MIT50	4	5	0	9	3.67
D18MIT9	1	2	3	6	2.00
D18MIT186	2	4	3	9	0.33
D18MIT48	3	1	2	6	3.00
D18MIT49	3	5	2	10	0.20
D19MIT28	1	6	2	9	1.22
D19MIT13	1	8	1	10	3.60
D19MIT19	1	8	1	10	3.60
D19MIT10	2	7	1	10	1.80
D19MIT34	3	5	1	9	1.00
DXMIT89	4	6	0	10	1.20
DXMIT105	6	3	1	10	1.20
DXMIT182	6	3	1	10	1.20

Diabetic MARKER	Genotype			Total Animals Scored	CHI SQUARE
	B	H	D		
D1MIT70	1	7	4	12	1.83
D1MIT76	3	5	4	12	0.50
D1MIT24	2	6	4	12	0.67
D1MIT84	2	5	5	12	1.83
D1MIT90	2	5	5	12	1.83
D1MIT110	2	5	5	12	1.83
D1MIT66	0	2	5	7	8.43
D2MIT80	2	6	4	12	0.67
D2MIT61	2	4	2	8	0.00
D2MIT42	4	3	5	12	3.17
D2MIT17	4	3	5	12	3.17
D2MIT423	3	4	5	12	2.00
D2MIT229	2	4	5	11	2.45
D3MIT62	2	3	6	11	5.18
D3MIT66	2	5	4	11	0.82
D3MIT61	2	3	1	6	0.33
D3MIT12	4	6	2	12	0.67
D3MIT267	2	8	1	11	2.45
D3MIT17	0	10	2	12	6.00
D4MIT149	8	4	0	12	12.00
D4MIT227	7	5	0	12	8.50
D4MIT89	11	1	0	12	28.50
D4MIT76	10	2	0	12	22.00
D4MIT64	7	5	0	12	8.50
D4MIT42	6	5	0	11	6.64
D6MIT193	1	9	2	12	3.17
D6MIT79	6	3	2	11	5.18
D6MIT93	4	6	2	12	0.67
D6MIT66	7	2	2	11	9.00
D6MIT99	4	6	1	11	1.73
D6MIT66	2	5	5	12	1.83
D6MIT169	5	5	1	11	3.00
D6MIT9	4	4	4	12	1.33
D6MIT17	4	4	4	12	1.33
D6MIT14	2	7	3	12	0.50
D7MIT178	6	6	0	12	6.00
D7MIT67	6	6	0	12	6.00
D7MIT229	2	5	1	8	0.75
D7MIT84	6	6	0	12	6.00
D7MIT62	1	3	0	4	1.50
D7MIT100	4	7	1	12	1.83
D7MIT12	3	5	4	12	0.50
D7MIT291	3	3	4	10	1.80
D8MIT3	2	7	3	12	0.50
D8MIT4	1	8	3	12	2.00
D8MIT69	1	8	2	11	2.45
D8MIT46	1	6	5	12	2.67
D8MIT207	0	5	6	11	6.64
D8MIT113	0	7	3	10	3.40
D8MIT272	0	9	3	12	4.50
D9MIT64	2	4	6	12	4.00
D9MIT10	2	4	6	12	4.00
D9MIT243	3	2	7	12	8.00
D9MIT18	2	3	6	11	5.18
D10MIT61	3	8	1	12	2.00
D10MIT44	2	6	4	12	0.67
D10MIT194	5	5	1	11	3.00
D10MIT94	5	5	2	12	1.83
D10MIT61	5	6	1	12	2.67
D10MIT42	3	7	2	12	0.50

Diabetic MARKER	B	Genotype H	D	Total Animals Scored	CHI SQUARE
D11MIT78	5	6	1	12	2.67
D11MIT174	4	7	1	12	1.83
D11MIT217	2	9	1	12	3.17
D11MIT260	2	8	1	11	2.45
D11MIT167	2	8	1	11	2.45
D11MIT39	3	6	2	11	0.27
D11MIT99	3	7	2	12	0.50
D11MIT103	4	8	2	11	2.45
D12MIT106	2	4	5	11	2.45
D12MIT12	4	5	0	9	3.67
D12MIT171	2	5	5	12	1.83
D12MIT60	2	3	3	8	0.75
D12MIT62	1	8	3	12	2.00
D12MIT34	1	8	2	11	2.45
D12MIT239	1	9	2	12	3.17
D12MIT141	1	5	5	11	3.00
D12MIT133	1	7	4	12	1.83
D13MIT17	1	7	4	12	1.83
D13MIT198	2	8	1	11	2.45
D13MIT66	2	0	1	3	3.67
D13MIT77	3	6	1	10	1.20
D13MIT76	2	9	1	12	3.17
D14MIT1	4	7	1	12	1.83
D14MIT64	5	7	0	12	4.50
D14MIT28	5	3	2	10	3.40
D14MIT39	2	2	2	6	0.67
D14MIT193	5	4	2	11	2.45
D14MIT36	6	4	2	12	4.00
D14MIT76	2	3	3	8	0.75
D16MIT12	3	6	2	11	0.27
D16MIT6	2	7	3	12	0.50
D16MIT91	3	5	4	12	0.50
D16MIT96	0	6	4	10	3.60
D16MIT34	2	1	0	3	3.00
D16MIT217	1	4	2	7	0.43
D16MIT181	2	6	3	11	0.27
D16MIT166	2	7	3	12	0.50
D16MIT103	3	6	3	12	0.00
D16MIT12	3	7	2	12	0.50
D16MIT6	3	6	2	11	0.27
D16MIT60	3	6	0	9	3.00
D17MIT46	3	4	4	11	1.00
D17MIT28	3	4	5	12	2.00
D17MIT22	2	2	6	10	6.80
D17MIT49	3	4	5	12	2.00
D17MIT177	3	4	5	12	2.00
D17MIT66	3	4	5	12	2.00
D17MIT64	4	3	2	9	1.89
D17MIT39	4	4	4	12	1.33
D18MIT66	4	3	1	8	2.75
D18MIT132	4	7	1	12	1.83
D18MIT87	1	3	1	5	0.20
D18MIT17	4	7	1	12	1.83
D18MIT123	4	4	1	9	2.11
D18MIT60	3	6	2	11	0.27
D18MIT9	3	6	2	11	0.27
D18MIT186	2	7	2	11	0.82
D18MIT48	1	6	0	7	3.86
D18MIT49	2	7	3	12	0.50
D19MIT28	3	5	3	11	0.09
D19MIT13	2	7	2	11	0.82
D19MIT19	1	9	2	12	3.17
D19MIT10	1	7	3	11	1.55
D19MIT34	2	6	3	11	0.27
DXMIT89	8	0	4	12	2.22
DXMIT106	7	0	4	11	1.72
DXMIT182	3	1	6	10	2.17

<u>Class designation</u>	<u>Criterion for selection</u>
Disease free	HbA1c < 7% Plasma [glucose] < 436 mg/dl Plasma [insulin] < 331 μ U/ml
Insulin resistant	HbA1c < 7% Plasma [glucose] < 500 mg/dl Plasma [insulin] > 643 μ U/ml
Diabetic	HbA1c > 14% Plasma [glucose] > 900 mg/dl Plasma [insulin] < 40 μ U/ml
Protected pancreas	Pancreatic grade < 1.5

Results

Comparison of lean and obese animals

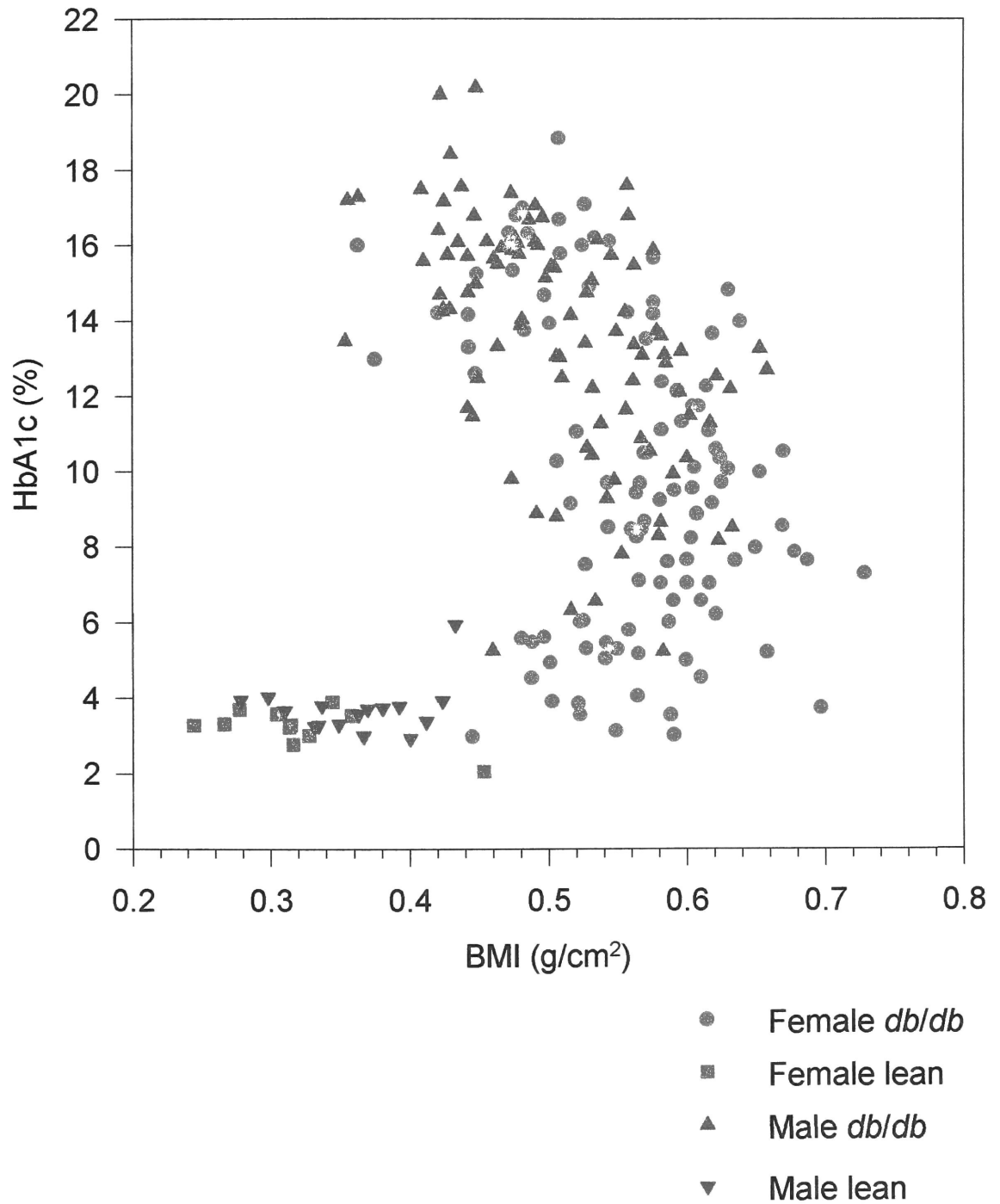
As a group, the genetically obese animals were readily distinguishable from the lean animals on the basis of weight and BMI which were 64% and 51% higher respectively, in the obese animals than the lean controls (Table 18). Although there was the expected variability in all phenotypic parameters within the lean class of mice, none of the lean mice were diabetic, as demonstrated most convincingly by the glycosylated hemoglobin fractions which were consistently less than 6%. By analysis of BMI and HbA1c, the groups of lean and obese progeny were readily distinguishable (Figure 52), although there was a small degree of overlap between the heaviest lean animal and the lightest obese animal. In general, the females tended to have higher BMI's and lower HbA1c's than the males.

Phenotype	Lean	Obese
Age (days)	108(8)	104 (15)
Weight (g)	29 (7)	48 (10)
BMI (g/cm ²)	0.351 (0.052)	0.532 (0.081)
HbA1c (%)	3.7 (0.8)	11.6 (4.2)
Fasting plasma [glucose] (mg/dL)	282 (59)	685 (249)
Fasting plasma [insulin] (uU/ml)	86.6 (73.4)	331.2 (312.0)
Pancreatic grade		3.3 (1.2)
Normalized pancreatic insulin content (uU/mg protein)		11986 (11298)
Normalized pancreatic glucagon content (pg/mg protein)		22804 (14302)
Pancreatic [insulin]/[glucagon]		0.524 (0.527)

Table 18. Phenotypic characteristics of F2 B6DBA segregating for *db*. Means for the lean and obese groups are indicated with standard deviations in parentheses.

Figure 52. BMI versus HbA1c in lean and obese male and female F2 B6DBA segregating for *db*. Each point represents a single animal.

F2 B6DBA



Biochemical characterization

In addition to sex-related differences, there was large phenotypic variation within either sex. The biochemical profiles in Figures 53 and 54 demonstrate the evolution of the diabetes and the rationale for identification of the disease free, insulin resistant, and diabetic classes. HbA1c demonstrated less variance than [glucose] at sacrifice since it is an integrated measure of glycemia over time. A small group of animals, mainly females, was relatively euglycemic based on their HbA1c < 7% and based on their fasting plasma [insulin] < 331 $\mu\text{U/ml}$ was not hyperinsulinemic. The second group of animals, mainly females, shown in the lower right quadrant of the plot was hyperinsulinemic and insulin-resistant (plasma [insulin] as high as 1000 $\mu\text{U/ml}$) but was again relatively non-diabetic based on their HbA1c. There was no clear demarcation in the plot between the insulin-resistant and the progressively diabetic animals. As the animals' fasting plasma [insulin] declined, there was an increase in HbA1c and severity of diabetes. Based on Figure 53, it can also be seen that as HbA1c increased, BMI decreased. This last group of extremely diabetic animals was composed predominantly of males.

Figure 53. Fasting plasma [insulin] versus HbA1c in obese and lean male and female F2 B6DBA segregating for *db*. Each point represents a single animal.

F2 B6DBA

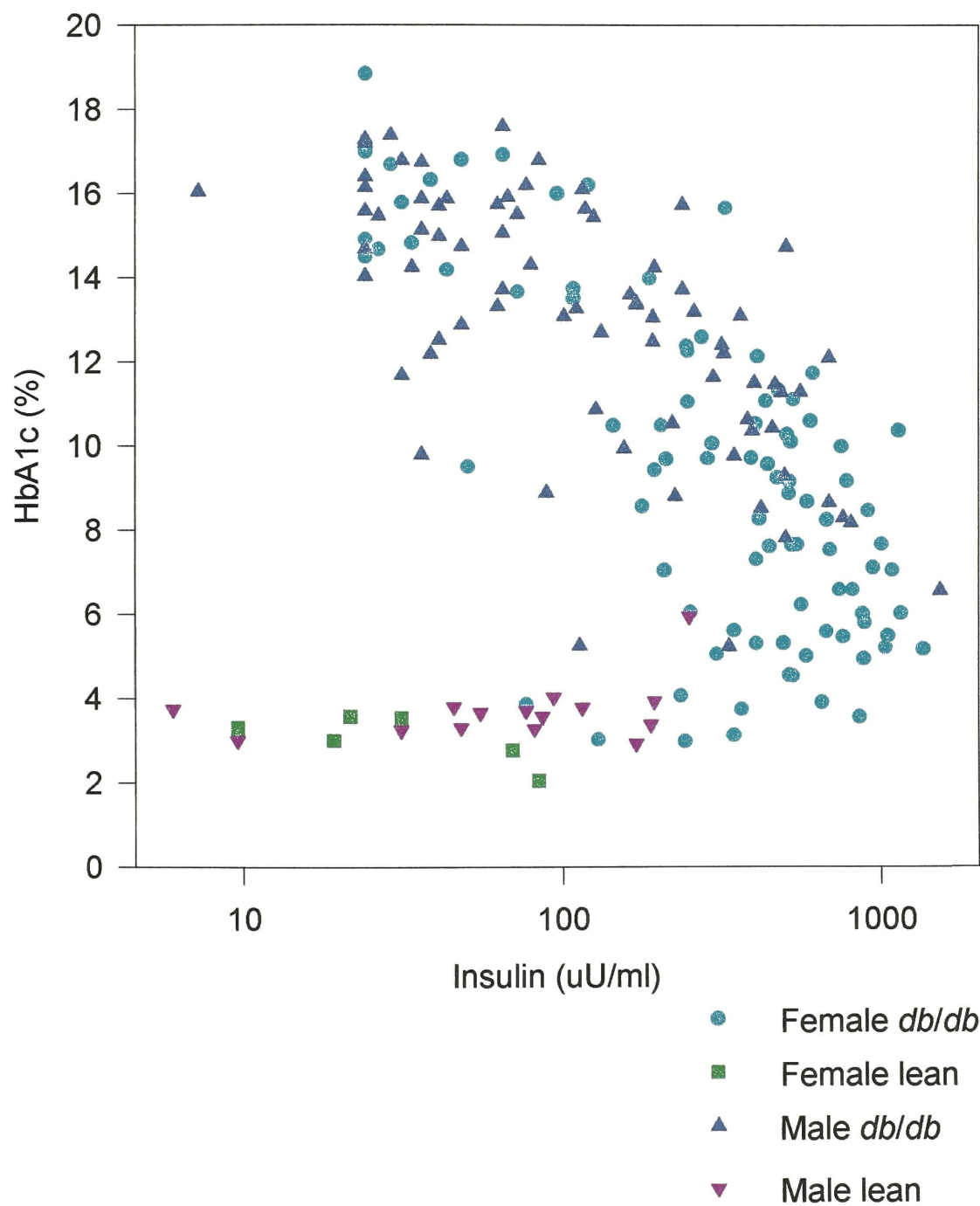
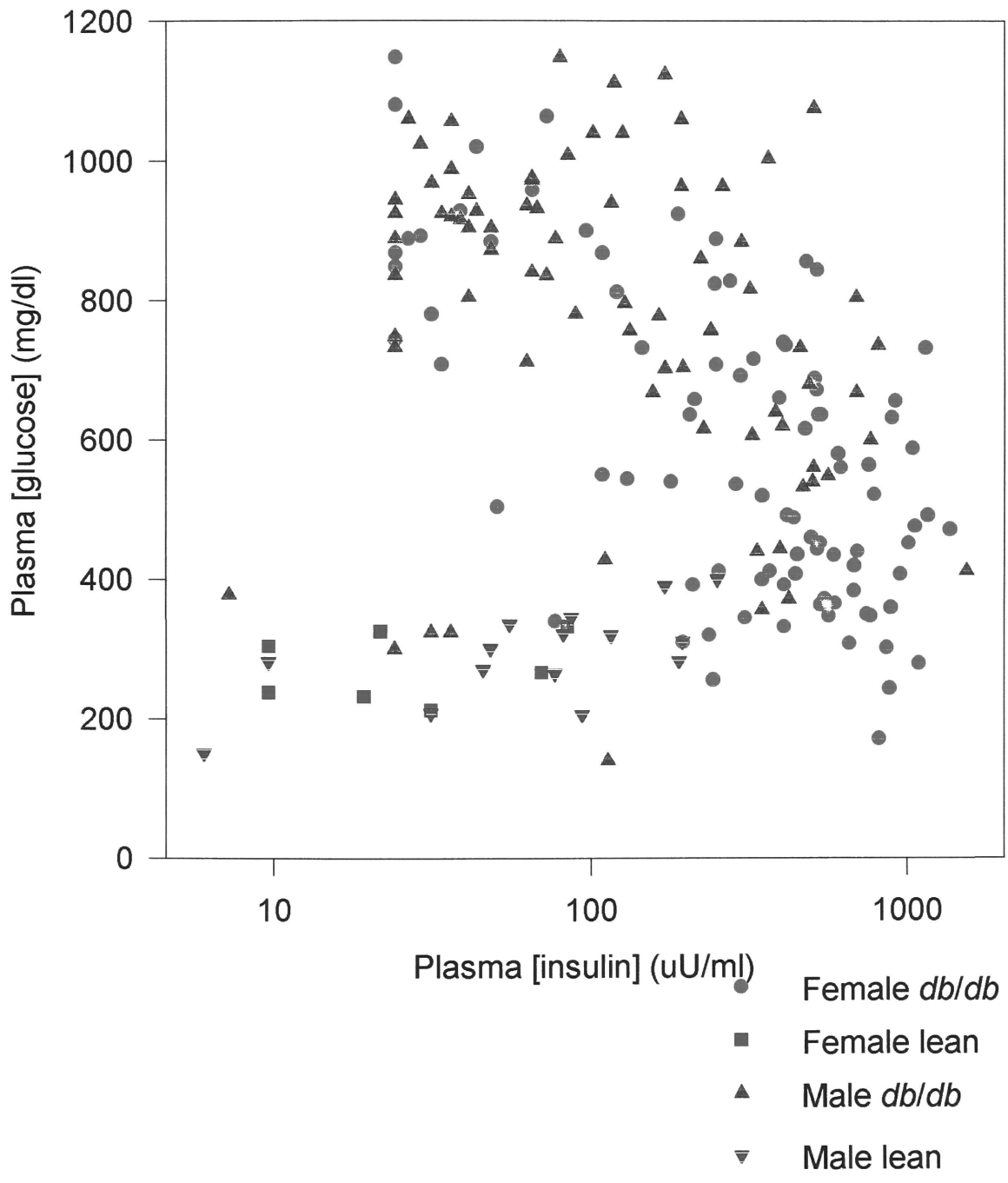


Figure 54. Fasting plasma [insulin] versus fasting plasma [glucose] in obese and lean male and female F2 B6DBA segregating for *db*. Each point represents a single animal.

F2 B6DBA



Genome scanning results

The results of the genome scanning experiments for the groups demonstrating the most extreme phenotypes: disease free, protected pancreas, insulin resistant, and diabetic mice are shown in Table 17.

On chromosome 1 telomerically, in approximately the same region as the QTL for all diabetes phenotypes in the *ob/ob* F2 B6DBA and DBAB6, there was a statistically significant enrichment of DBA alleles within the diabetic class for D1Mit56, and a relative enrichment of B6 alleles within the protected pancreas class for D1Mit110.

On chromosome 2 at D2Mit310 there was an enrichment of DBA alleles within the protected pancreas class with no B6/B6 animals. DBA alleles acted as diabetes-protective at this locus.

Telomerically on chromosome 3 at D3Mit17, there was a predominance of B6/B6 animals (6/10) within the insulin resistant, non-diabetic class and only 1/10 DBA/DBA animals in this phenotypic class. This marker demonstrated statistically significant linkage disequilibrium only within this one class of animals.

Because *db* is located on chromosome four, other markers along this chromosome which were linked to *db* necessarily were more likely carry B6 alleles since this was the strain

carrying *db*. Therefore, none of the scanning results for chromosome 4 were taken as indications for the presence or absence of QTLs.

Telomerically on chromosome 5 there was enrichment of DBA alleles for D5Mit99, D5Mit222, and D5Mit223 within the protected pancreas class with no B6/B6 at any of the markers. Conversely, there was enrichment for B6 alleles within the diabetic class at D5Mit65, slightly proximal of the previous three chromosome 5 markers (7 B6/B6, 2 B6/DBA, 2 DBA/DBA)

Chromosome 7 markers demonstrated significant deviations from Mendelian ratios in multiple phenotypic classes, but at slightly different genetic locations in the various classes. Within the non-diabetic class, all five animals were DBA/DBA at D7Mit62, implying that DBA alleles were protective against insulin resistance. Within the insulin resistant class this same marker was statistically significant due to a paucity of heterozygotes. Possibly more relevant were the genotypes at D7Mit12 (more telomerically) at which there were no DBA homozygotes and five B6/B6 and five B6/DBA. The genotypes at D7Mit12 would indicate that B6 alleles were associated with insulin resistance. Finally, at markers proximal to D7Mit62 and D7Mit12 (i.e. D7Mit178, D7Mit57, and D7Mit84) there was enrichment for B6 alleles within the diabetic class. Again, B6 alleles at this locus could have been associated with increased insulin resistance in these animals and the pancreas unable to compensate leading to overt diabetes.

D8Mit207 demonstrated increased numbers of DBA alleles within the diabetic class (5 B6/DBA and 6 DBA/DBA). For the same marker, the insulin resistant class demonstrated an increased number of B6 alleles (5 B6/B6, 2 B6/DBA, and 2 DBA/DBA) although this degree of distortion was not statistically significant.

Within the diabetic class, D9Mit243 demonstrated increased number of DBA alleles (3 B6/B6, 2 B6/DBA, and 7 DBA/DBA).

Unique to the protected pancreas class was an association between B6 alleles of centromeric markers on chromosome 12 (D12Mit105, D12Mit12, D12Mit171, and D12Mit60). Of the four markers, D12Mit171 demonstrated the most significant deviation with 9 B6/B6, 8 B6/DBA, and 0 DBA/DBA.

Although not statistically significant, for D14Mit35 there were relatively more DBA alleles within the protected pancreas class (0 B6/B6, 9 B6/DBA, and 6 DBA/DBA) and relatively more B6 alleles within the diabetic class (6 B6/B6, 4 B6/DBA, 2 DBA/DBA). These results were concordant with those observed in the *ob/ob* mice of the same strains. However, this result has not yet been confirmed in all the obese progeny from the *db/db* F2 B6DBA cross.

Both the insulin resistant and diabetic classes demonstrated statistically significant results with markers on chromosome 17. For centromeric markers D17Mit46 and D17Mit66 there was an excessive number of heterozygotes within the insulin-resistant class (9/10). Within the diabetic class, the deviation at D17Mit22 was the result of an excessive number of DBA/DBA (6/10). Hybrid vigor (heterosis) might explain the apparent protection of the heterozygous class from overt diabetes.

D18Mit17 and D19Mit28 each demonstrated 4/5 DBA/DBA within the non-diabetic class. These results were not replicated at adjacent markers or within other classes. Because the number of animals in this class was small, it is likely that these results are not biologically significant.

On the basis of the genome scanning experiments, approximate map locations for each QTL were determined. Markers within this interval were then used to genotype all of the obese progeny to determine which of the regions indicated by the genome scanning experiments were likely to contain biologically relevant genes. Twenty-seven markers from twelve different chromosomes were tested: D1Mit90, D1Mit110, D1Mit370, D1Mit56, D1Mit408, D2Mit229, D3Mit62, D3Mit17, D5Mit79, D5Mit65, D5Mit99, D5Mit222, D6Mit54, D7Mit57, D7Mit84, D7Mit62, D7Mit100, D8Mit207, D9Mit18, D9Mit243, D12Mit218, D12Mit12, D12Mit171, D17Mit22, D17Mit53, D18Mit17, and D19Mit28.

Quantitative Trait Locus Analysis and ANOVA

QTL analysis can only be performed when multiple markers along a chromosome have been typed. Therefore, only loci on chromosomes 1, 3, 5, 7, 9, 12 and 17 were tested with this analysis.

The locus on chromosome 1 behaved similarly in the *db/db* F2 B6DBA cross as it had in the *ob/ob* F2 animals resulting from the same interstrain cross (Figure 55). There was a major peak more proximally between D1Mit90 and D1Mit110 for weight and BMI (Tables 19 and 20, Figure 56). There was a single peak in the *db/db* animals for traits related directly to degree of obesity. However, for traits relating to phenotypes of diabetes (HbA1c, plasma [insulin], and pancreatic morphology) there were two peaks, a centromeric peak coincident with that for body weight and a telomeric peak between D1Mit370 and D1Mit56 (Figures 57, 58). The levels of statistical significance for the chromosome 1 loci in the *db/db* was smaller than that observed in the *ob/ob*, but the number of animals was also smaller (190 vs 450). Of all the traits affected, weight had the highest LOD score (5.3) and accounted for the greatest proportion of the variance (15.6%). For both loci, B6 alleles were associated with increased weight (as much as 28% higher in B6/B6 compared to DBA/DBA males, Figure 59), BMI, or protection from diabetes. Of the diabetes phenotypes, pancreatic grade (Figure 60) and average islet size demonstrated the most significant results (LOD 2.7 and 2.9 respectively) and accounted for 9.5 and 9.4% of the variance of these phenotypes.

Figure 55. Summary of the QTLs for obesity/diabetes on mouse chromosome 1. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 1

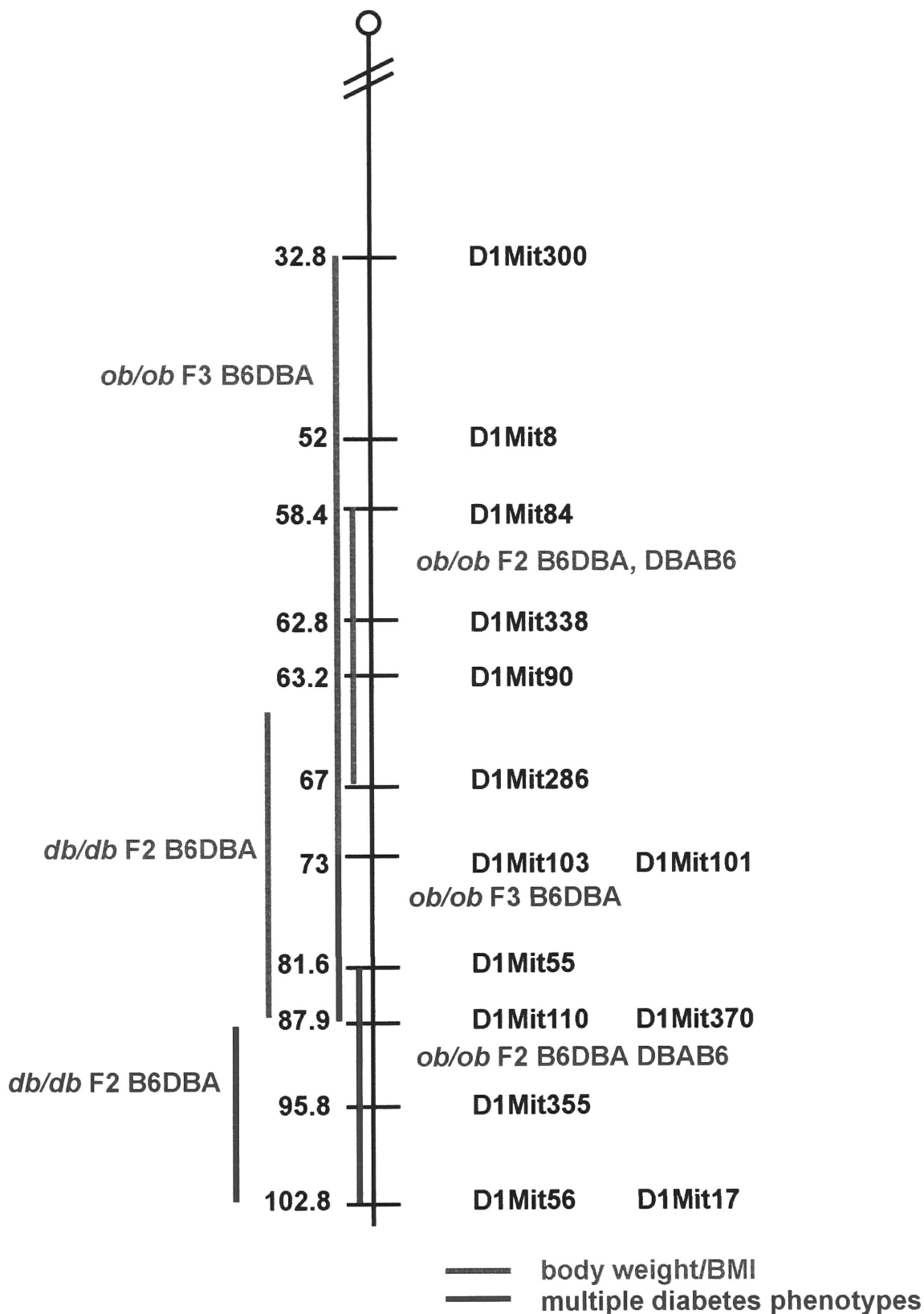


Table 19. Quantitative trait locus analysis for diabetes-susceptibility loci in *db/db* F2 B6DBA. Data are summarized by trait/phenotype, the marker or interval which demonstrated the peak LOD score, the confidence interval as defined by interval on either side of the peak 1 LOD unit less than the maximum peak using a free genetic model, the maximum LOD score observed under a free genetic model, and the genetic models which most likely fit the data. Genetic models which demonstrated a LOD score < (peak LOD score under a free genetic model -1) were determined to be unlikely genetic models.

Phenotype	Marker with peak LOD score	Confidence Interval	LOD score	% phenotypic variance for which locus can account	Genetic model
Weight	D1Mit110	D1Mit90/D1Mit370	5.33	15.6	free, additive
BMI	D1Mit110	D1Mit90/D1Mit56	2.25	5.6	free, recessive, additive
HbA1c	D1Mit110 D9Mit18 D12Mit12/D12Mit17		2.15 2.23 3.66	7.4 7.2 9.2	free, additive free, additive free, additive
Fasting plasma [glucose]	D12Mit12/D12Mit17		2.13	6.2	free, recessive, additive
Fasting plasma [insulin]	D1Mit110 D12Mit12/D12Mit17	D1Mit90/D1Mit56	2.44 2.29	8.6 8.5	free, recessive free, recessive
Pancreatic grade	D1Mit370 D9Mit18 D12Mit21/D12Mit12		2.7 2.95 4.32	9.5 8.5 11.2	free, dominant, additive free, dominant, additive free, dominant, additive
Number of islets	D1Mit370 D9Mit18 D17Mit22	D1Mit370/D1Mit56	2.24 3.53 2.67	11 11.7 12.2	free, dominant, additive free, additive free, recessive, additive
Number of hyperplastic islets	D1Mit110	D1Mit90/D1Mit56	2.1	5.4	free, dominant, additive
Degree of hypertrophy/hyperplasia	D1Mit110 D9Mit18		2.93 2.03	9.4 6.8	free, dominant, additive free, additive
Average islet size	D1Mit90/D1Mit110		2.93	9.4	free, dominant, additive
Beta cell area	D1Mit110 D9Mit18	D1Mit90/D1Mit370	2.3 2.03	6 6.8	free, dominant, additive free, additive
Beta cell area/total pancreatic area					

Table 20. Analysis of variance by sex and genotype at the marker designated for *db/db* F2 B6DBA. All traits for all markers demonstrating statistical significance are summarized. Number of animals genotyped and phenotyped for each a given marker and trait is indicated. Mean values for each trait are listed first by sex and genotypic class. P value shown is for the effect of genotype on phenotype.

Marker	Phenotype	Number	B6/B6	Female		B6/B6	Male		P
				B6/DBA	DBA/DBA		B6/DBA	DBA/DBA	
D1Mit90	Weight (g)	181	52.4	51.6	47.8	52.4	45.6	41.0	0.00001
	[Insulin] (uU/ml)	162	468.7	469.0	247.9	215.8	255.8	121.7	0.001
	Pancreatic Grade	181	2.6	2.9	3.5	3.6	3.7	4.0	0.007
	Number of Islets	169	16.1	16.4	11.2	11.0	11.2	7.1	0.007
	Number of hypertrophic Islets	169	2.7	2.2	1.1	1.2	0.8	0.0	0.03
	Average Islet size (mm2)	169	0.0274	0.0202	0.0171	0.0191	0.0167	0.0121	0.0007
	Islet area (mm2)	169	4.79	3.94	2.37	2.38	2.02	0.93	0.006
	Islet area/total area (%)	169	1.7	1.54	0.89	0.76	0.75	0.36	0.006
D1Mit110	Weight (g)	183	53.0	52.1	47.2	51.1	47.1	41.6	0.0001
	BMI (g/cm2)	183	0.580	0.570	0.540	0.530	0.510	0.490	0.02
	HbA1c (%)	183	7.1	10.3	11.3	13.4	12.7	14.9	0.0005
	[Glucose] (mg/dl)	183	472	624	684	815	723	844	0.02
	[Insulin] (uU/ml)	165	460.6	464.9	270.0	157.7	261.1	154.8	0.01
	Pancreatic Grade	183	2.0	3.1	3.5	3.7	3.6	4.1	0.0001
	Number of Islets	171	20.4	14.7	13.4	11.0	11.2	6.7	0.005
	Number of hypertrophic Islets	171	3.8	2.0	0.8	0.8	0.9	0.1	0.0003
	Average Islet size (mm2)	171	0.0299	0.0201	0.0162	0.0158	0.0169	0.014	0.0004
	Islet area (mm2)	171	6.15	3.62	2.47	2.08	2.07	1.03	0.0004
	Islet area/total area (%)	171	2.3	1.3	0.96	0.67	0.69	0.44	0.0007
D1Mit370	Weight (g)	178	54.9	51.1	46.8	51.1	45.2	41.3	0.00001
	BMI (g/cm2)	178	0.600	0.560	0.530	0.530	0.500	0.480	0.002
	HbA1c (%)	178	7.1	10.3	9.9	13.2	12.9	14.7	0.01
	[Insulin] (uU/ml)	144	469.4	481.4	337.9	172.8	323.8	151.7	0.02
	Pancreatic Grade	178	2.1	3.2	3.0	3.6	3.7	3.9	0.009
	Number of Islets	168	21.7	14.2	14.9	12.1	10.6	8.5	0.006
	Number of hypertrophic Islets	168	3.5	1.9	1.5	1.1	0.7	0.6	0.02
	Islet area (mm2)	168	5.93	3.56	3.15	2.44	1.83	1.84	0.01
	Islet area/total area (%)	168	2.2	1.26	1.22	0.78	0.66	0.67	0.01
D1Mit408	HbA1c (%)	145	8.0	9.6	11.4	11.9	13.9	13.9	0.01
	[Glucose] (mg/dl)	125	467	598	659	712	787	827	0.05
	Pancreatic Grade	145	2.3	3.1	3.4	3.4	3.8	4.0	0.007
	Pancreatic [Insulin] (uU/mg protein)	121	23556	11243	11285	9920	8035	6751	0.01
	Number of Islets	139	20.7	14.2	12.4	11.4	10.3	10.3	0.02
	Pancreatic [Insulin]/[glucagon]	144	0.856	0.538	0.530	0.510	0.297	0.306	0.04
D2Mit229	HbA1c (%)	171	12.0	10.7	7.4	14.8	12.9	12.5	0.00001
	[Glucose] (mg/dl)	152	660	632	505	777	819	707	0.03
	[Insulin] (uU/ml)	137	282.7	379.9	593.5	140.6	244.8	267.4	0.002
	Pancreatic Grade	171	3.5	3.3	2.4	4.1	3.5	3.5	0.002
	Pancreatic [Insulin] (uU/mg protein)	149	9715	14273	19833	7989	9959	10599	0.02
	Number of Islets	160	11.0	13.8	19.8	7.8	10.9	13.5	0.0004
D3Mit62	Pancreatic [Insulin] (uU/mg protein)	148	20752	11466	11736	9805	8752	9703	0.04
D3Mit17	[Glucose] (mg/dl)	134	454	637	613	677	827	741	0.02
D5Mit99	[Insulin] (uU/ml)	125	280.8	420.5	524.9	162.2	234.7	221.3	0.04
	Pancreatic [glucagon] (uU/mg protein)	138	21250	20252	23727	21802	20297	34011	0.02
D6Mit222	Weight (g)	176	44.5	52.3	49.9	45.1	43.9	49.6	0.04
	HbA1c (%)	176	10.8	9.7	8.3	13.7	14.0	12.5	0.02
	[Insulin] (uU/ml)	143	324.5	424.6	565.4	173.0	217.2	250.1	0.03
	Pancreatic Grade	176	3.3	3.1	2.2	3.9	3.8	3.6	0.003
	Average Islet size (mm2)	166	0.0188	0.0196	0.0252	0.0143	0.0161	0.0188	0.04
	Islet area/total area (%)	166	1.18	1.30	1.90	0.47	0.62	0.91	0.03
D6Mit54	Weight (g)	182	51.2	51.5	50.4	49.5	47.2	41.2	0.02
	HbA1c (%)	182	11.1	8.6	10.3	12.3	13.1	15.4	0.01
	[Glucose] (mg/dl)	182	677	547	618	710	763	885	0.05
	[Insulin] (uU/ml)	164	418.6	487.2	333.6	321.8	205.0	102.5	0.03
	Islet area (mm2)	170	2.28	5.10	3.34	2.76	1.82	1.03	0.03
D7Mit57	Weight (g)	177	46.4	52.2	50.5	44.6	48.8	45.8	0.04
	BMI (g/cm2)	177	0.520	0.570	0.560	0.500	0.530	0.500	0.02
	[Glucose] (mg/dl)	160	684	557	576	808	817	678	0.05
D8Mit207	Number of Islets	165	13.47	16.49	10.55	10.93	11.44	9.22	0.05

Marker	Phenotype	Number	B6/B6	Female		B6/B6	Male		P
				B6/DBA	DBA/DBA		B6/DBA	DBA/DBA	
D9Mit18	HbA1c (%)	142	8.3	8.9	11.2	12.5	13.5	15.1	0.001
	Pancreatic Grade	142	2.4	2.9	3.5	3.6	3.6	4.2	0.002
	Number of Islets	134	18.2	16.8	10.7	13.2	10.0	7.0	0.001
	Islet area (mm2)	134	4.73	3.72	2.21	2.03	2.18	1.03	0.005
	Islet area/total area (%)	134	1.89	1.32	0.91	0.64	0.75	0.36	0.02
D9Mit243	Number of Islets	166	15.5	15.6	11.6	14.2	9.3	7.6	0.008
D12Mit21	HbA1c (%)	154	8.3	10.2	11.5	12.4	13.1	14.3	0.005
	Pancreatic Grade	154	2.3	3.3	3.6	3.3	3.8	3.8	0.002
	Number of hypertrophic Islets	147	2.1	2.4	1.1	1.8	0.7	0.3	0.04
	Pancreatic [Insulin]/[glucagon]	151	0.71	0.53	0.35	0.54	0.3	0.38	0.02
D12Mit17	HbA1c (%)	177	8.8	9.6	10.7	12.5	12.6	15.4	0.003
	[Glucose] (mg/dl)	158	541	566	654	706	753	858	0.03
	Pancreatic Grade	177	2.5	3.0	3.3	3.4	3.6	4.0	0.004
	Pancreatic [Insulin] (uU/mg protein)	154	18341	14541	11144	10554	8971	6029	0.03
	Number of hypertrophic Islets	167	2.3	2.4	1.2	1.1	1.3	0.3	0.04
	Pancreatic [Insulin]/[glucagon]	175	0.74	0.69	0.48	0.5	0.4	0.29	0.04
D17Mit22	Number of Islets	156	12.1	14.6	17.7	7.6	11.0	14.5	0.003
	Average Islet size (mm2)	156	1.89	2.16	2.01	1.24	1.99	1.66	0.02
	Islet area (mm2)	156	3	3.84	4.35	1.05	2.58	2.58	0.05

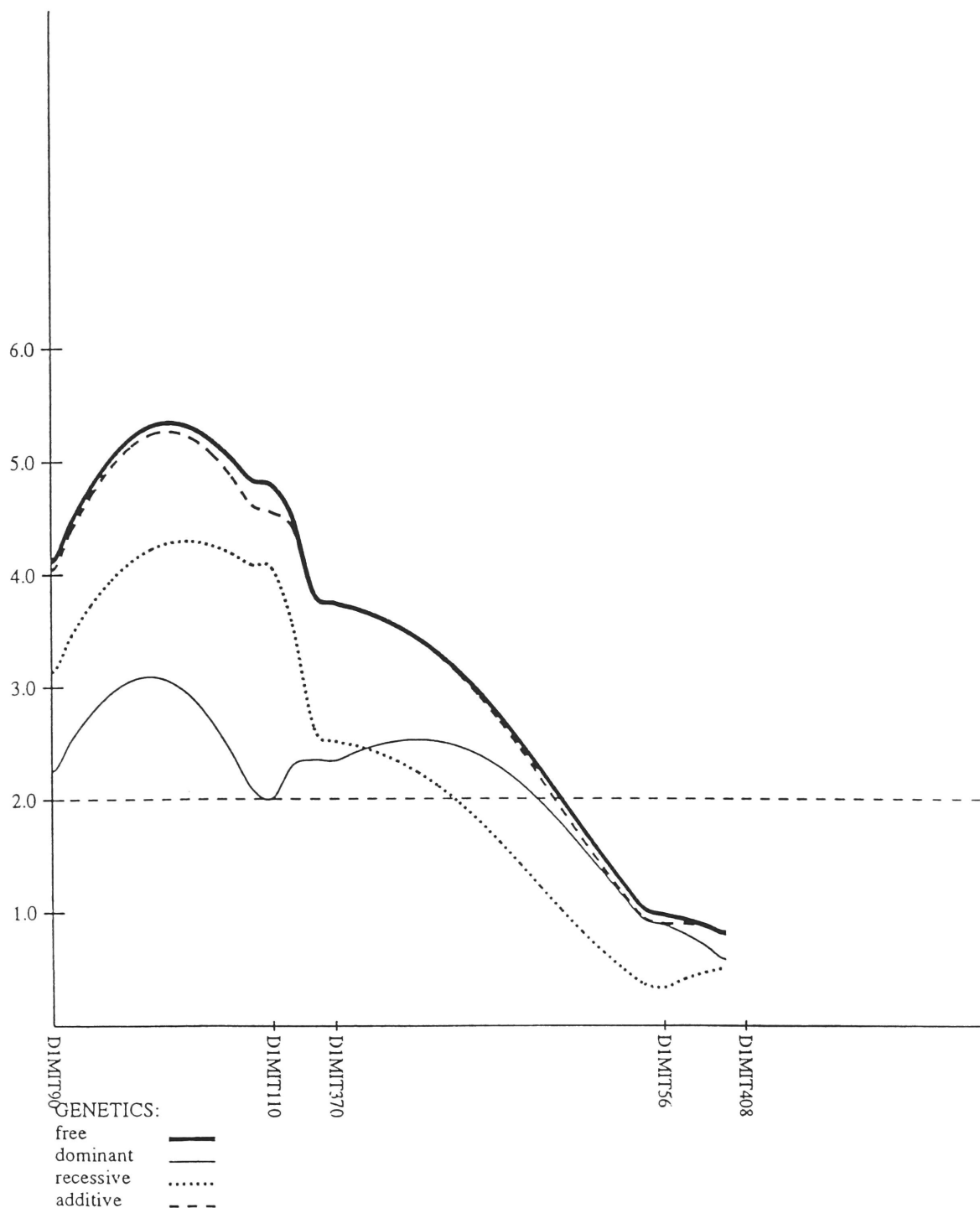


Figure 56. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for weight for *db/db* F2 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

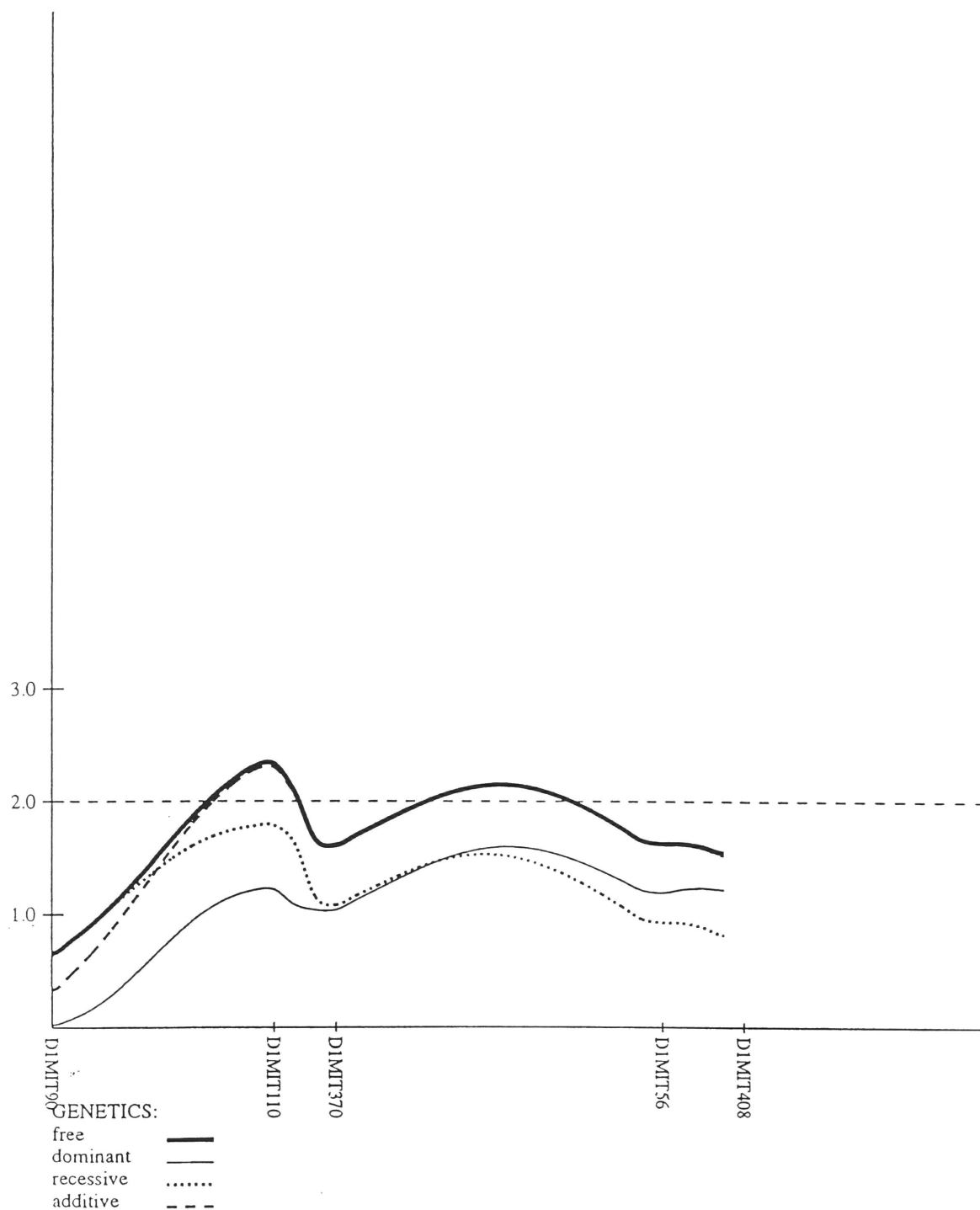


Figure 57. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for HbA1c for *db/db* F2 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

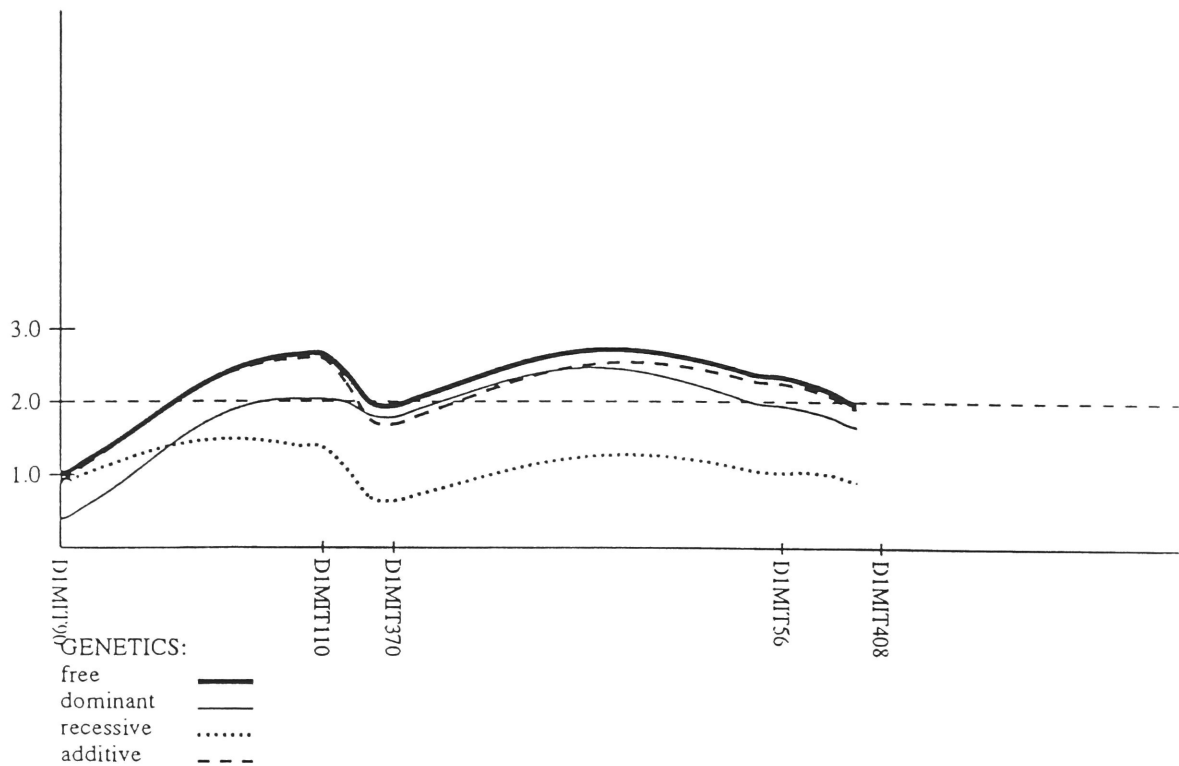


Figure 58. LOD scores for markers along chromosome 1 from centromere (left) to telomere (right) for pancreatic grade for *db/db* F2 B6DBA. Markers are spaced proportionately to their genetic distance. The results for an additive genetic model are shown.

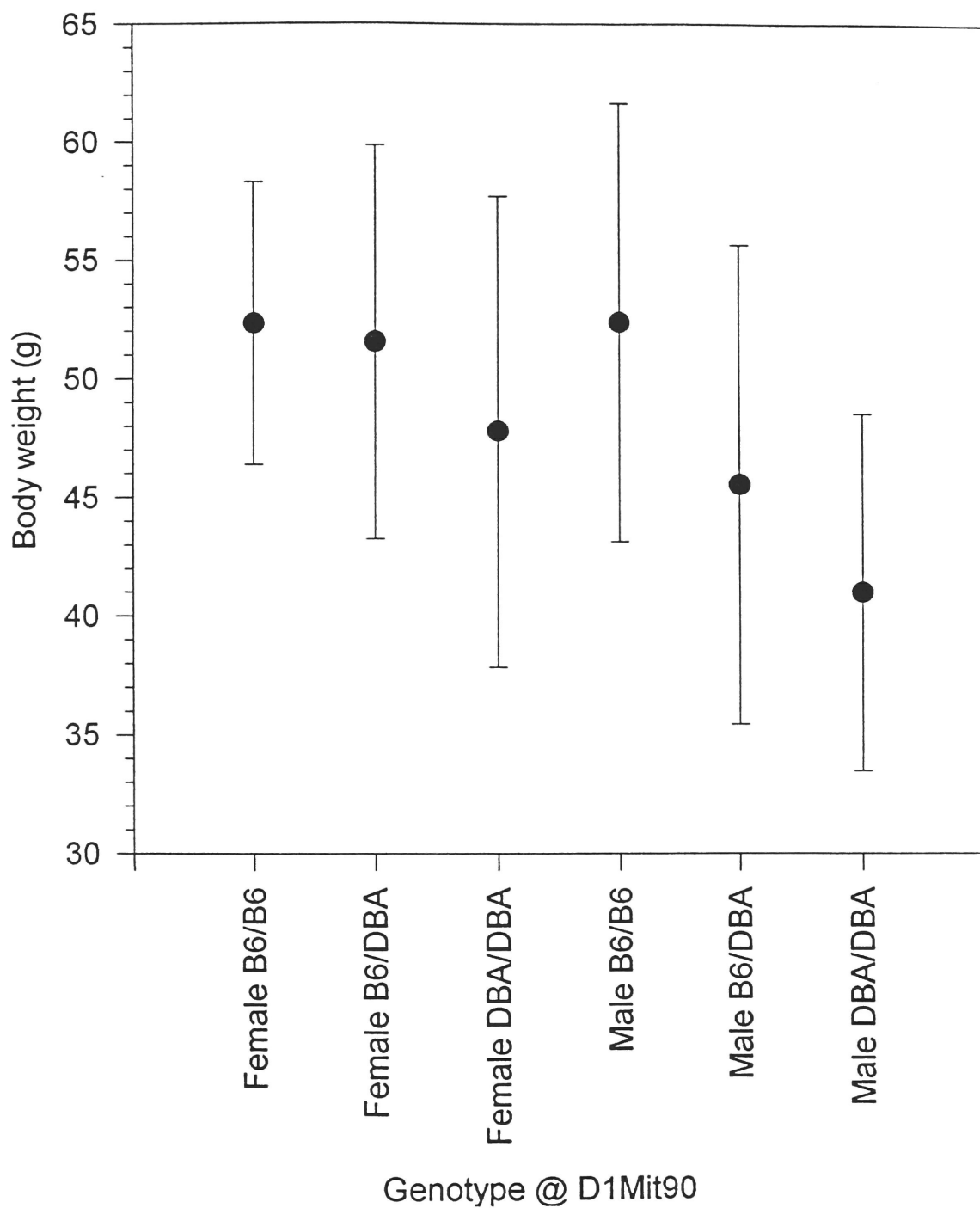


Figure 59. Relationship of genotype at D1Mit90 on weight-by-sex in *db/db* F2 B6DBA.

Circles represent the mean of each class with error bars of standard deviations.

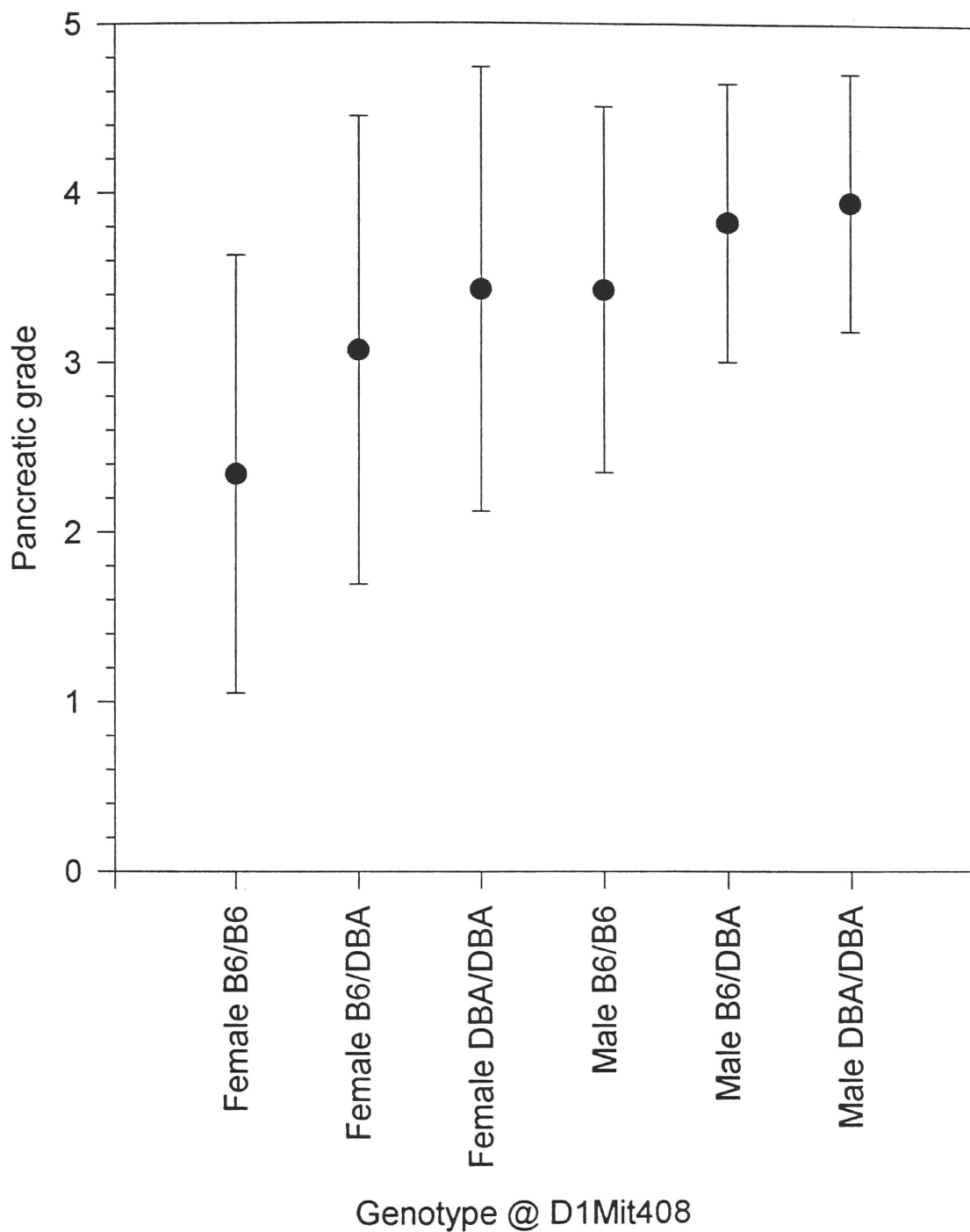
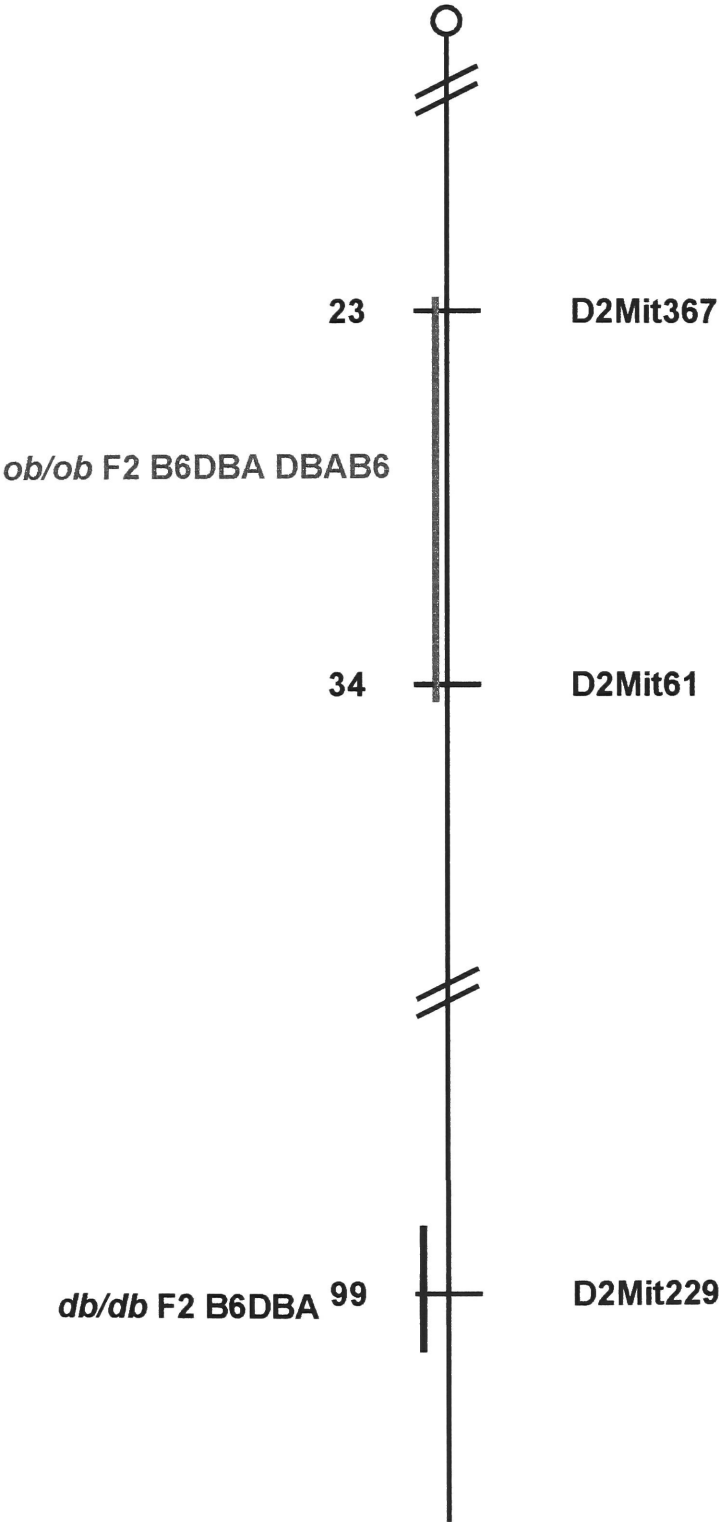


Figure 60. Relationship of genotype at D1Mit408 on pancreatic grade by sex in *db/db* F2 B6DBA. Circles represent the mean of each class with error bars of standard deviations.

Figure 61. Summary of the QTLs for obesity/diabetes on mouse chromosome 2. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 2



body weight/BMI
plasma [glucose]

The telomeric chromosome 2 locus in the *db/db* demonstrated no statistically significant association with weight or BMI, unlike the *ob/ob*; however, it did significantly affect HbA1c, plasma [glucose], plasma [insulin], pancreatic grade, pancreatic [insulin], and number of islets (Table 20). D2Mit229 is approximately 45 cM telomeric of the center of the QTL described in the *ob/ob* F2 B6DBA and DBAB6, and is likely to be an independent locus from the one previously reported to influence weight and BMI (Figure 61). DBA alleles were protective against diabetes and were associated with HbA1c's 40% lower in the DBA/DBA vs B6/B6 females (Figure 62). More markers in this region will be required to further define the interval containing the relevant gene(s).

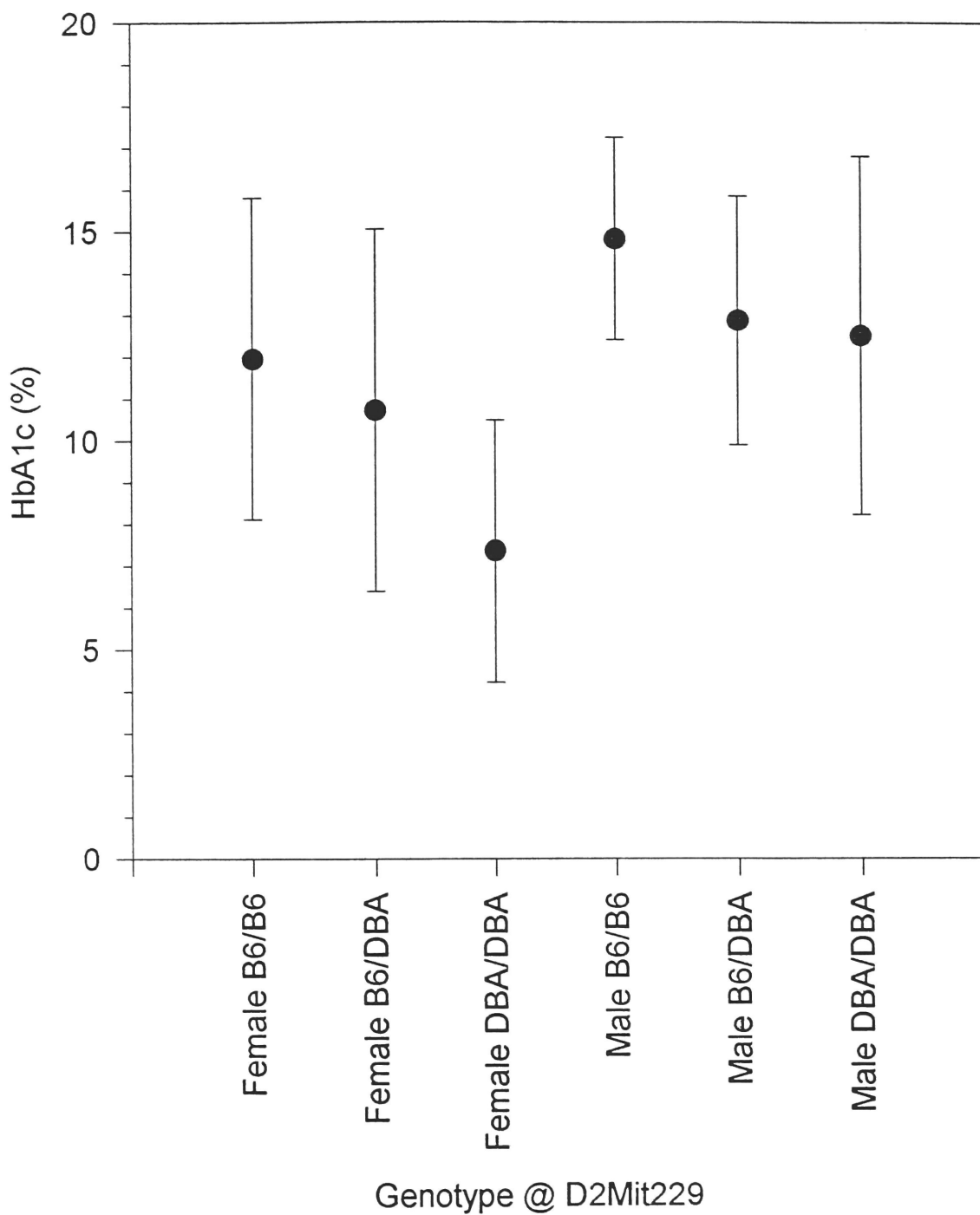


Figure 62. Relationship of genotype at D2Mit229 on HbA1c by sex in *db/db* F2 B6DBA.

Circles represent the mean of each class with error bars of standard deviations.

The chromosome 3 markers D3Mit62 and D3Mit17 demonstrated weak effects on pancreatic [insulin] and plasma [glucose], respectively. Within the females, B6 alleles at the locus were potent in increasing pancreatic [insulin] (20752 μ U/mg protein vs 11736 μ U/mg protein in B6/B6 vs DBA/DBA) and decreasing hyperglycemia (454 mg/dl and 613 mg/dl in B6/B6 and DBA/DBA); however, no clear effect of genotype at this locus was observed in the males.

The locus on chromosome 5 appeared to be mostly closely linked to D5Mit222, although D5Mit99 also demonstrated a statistically significant effect on plasma [insulin] (Table 20). Weight, HbA1c, plasma [insulin], pancreatic grade, average islet size, and normalized islet area were approximately equally affected by this locus, but of the six phenotypes affected, pancreatic grade demonstrated slightly more significant results. For all phenotypes except weight, both males and females were affected similarly, with DBA alleles providing protection from diabetes by co-dominant effects. Interestingly, although there was an effect of the locus on weight, of the three genotypes, heterozygotes were heaviest among the females and lightest among the males. This could indicate either that the QTL has not been sufficiently localized or that it interacts with sex.

The marker D6Mit54 was chosen because it was linked to multiple diabetes phenotypes in numerous crosses segregating both *ob* and *db* (*db/db* F2 B6CAST, *ob/ob* F2 B6CAST, *ob/ob* B6DBA and *ob/ob* F1N2 B6CAST). The chromosome 6 locus displayed similar

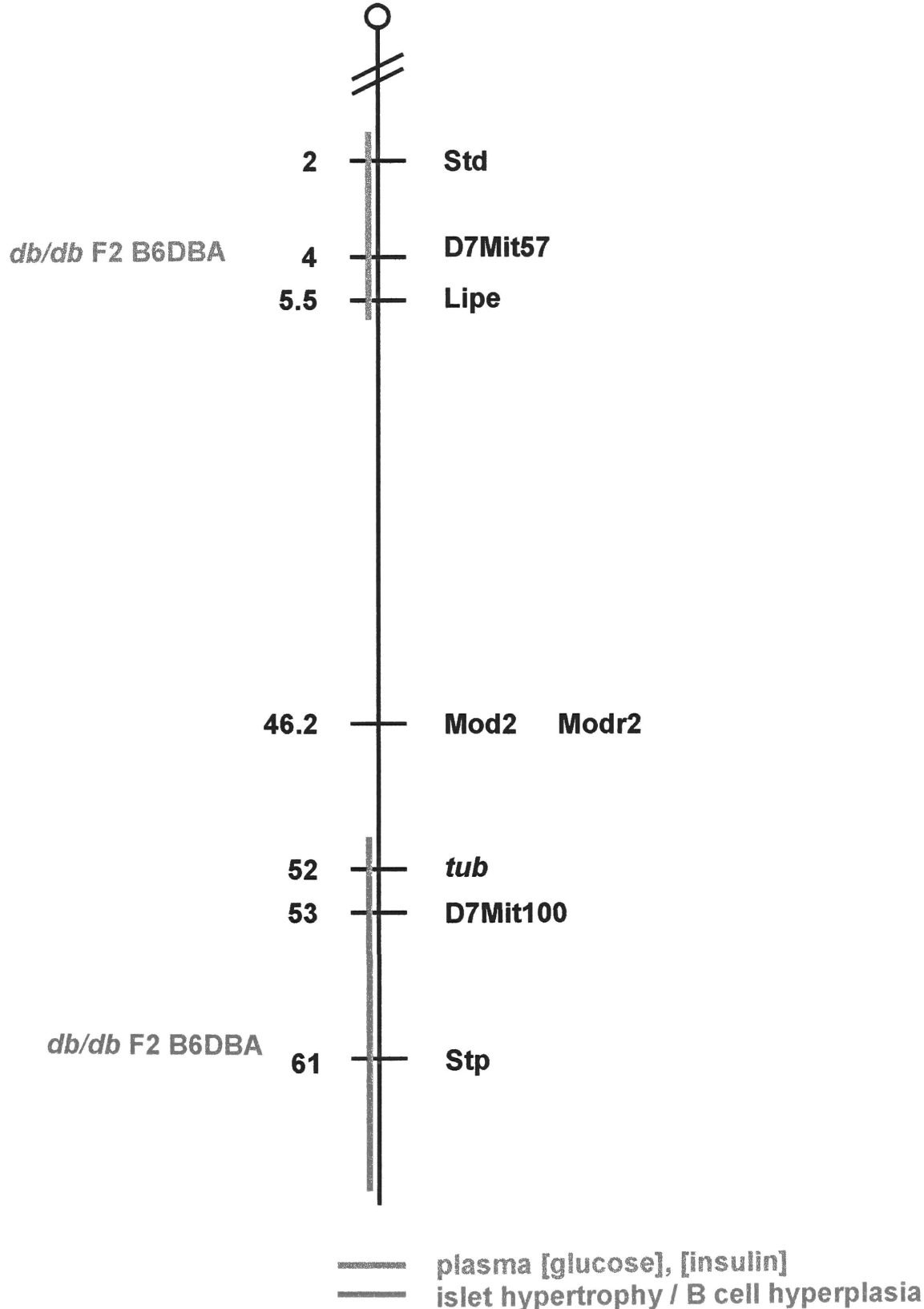
effects in the *db/db* F2 B6DBA males but not the females. In the males, B6 homozygotes were relatively less diabetic, and had higher BMI's, lower HbA1c's, lower [glucose], higher [insulin], and greater islet area (Table 20). For the females, the heterozygotes were protected from diabetes for the same phenotypic parameters for which B6 homozygous males had been protected. Similar interactions between this locus and sex had not been observed in other crosses.

Two different regions of chromosome 7 were tested because identification of a single interval based on the scanning experiments proved difficult because different classes mapped to different parts of the chromosome. D7Mit57 represented the more centromeric location, and was the same marker used previously in crosses segregating *ob* and *db* B6CAST (Figure 63). For this marker, heterozygosity (B6CAST) was associated with diabetes protection, higher weight and BMI and lower [glucose] in females (Table 20). D7Mit62 and D7Mit100 were separated by 12 cM from each other and were 27 cM telomeric of D7Mit57. These telomeric markers indicated that the responsible gene(s) was located more centromerically closer to D7Mit57.

D8Mit207 demonstrated a marginal effect on a single diabetes phenotype, number of islets. Heterozygotes displayed the greatest number of islets vs either homozygous class. Although this relationship could be biologically relevant, only a single phenotype was marginally affected and that phenotype was not used as a specific criteria for selecting the insulin resistant or diabetic animals used for the scans.

Figure 63. Summary of the QTLs for obesity/diabetes on mouse chromosome 7. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 7



D9Mit243 and D9Mit18 were 9.6 cM apart on telomeric chromosome 9. Of the two markers, D9Mit18 appeared more tightly linked with the diabetes phenotypes: HbA1c, pancreatic grade, number of islets and measures of islet area (Tables 19 and 20). For each phenotype in both sexes, DBA alleles were associated with more severe diabetes as had been observed in the original scanning results (Figure 64). D9Mit18 accounted for 7.2% of the variance in HbA1c, 8.5% of the variance in pancreatic grade, 11.7% in the number of islets, and 6.8% of the islet area. This QTL was one of the five loci influencing diabetes related phenotypes in the *db/db* F2 B6DBA.

Two of the three chromosome 12 markers, D12Mit21 and D12Mit17 demonstrated the association of B6 alleles with less severe disease as had been found on the initial genome scan. HbA1c, plasma [glucose], pancreatic grade, pancreatic [insulin], number of hypertrophic islets, and pancreatic [insulin]/[glucagon] were affected by genotype at one or both loci (Tables 19 and 20 and Figure 65). 9.2% of the variance of HbA1c, 8.5 % of the variance of pancreatic grade, 11.7 % of the variance in number of islets, 6.8% of the variance in number of hypertrophic islets, and 6.8% of the variance in islet area was accounted for by the chromosome 12 QTL. This QTL was the last of the five major loci influencing diabetes-related phenotype in the *db/db* F2 B6DBA.

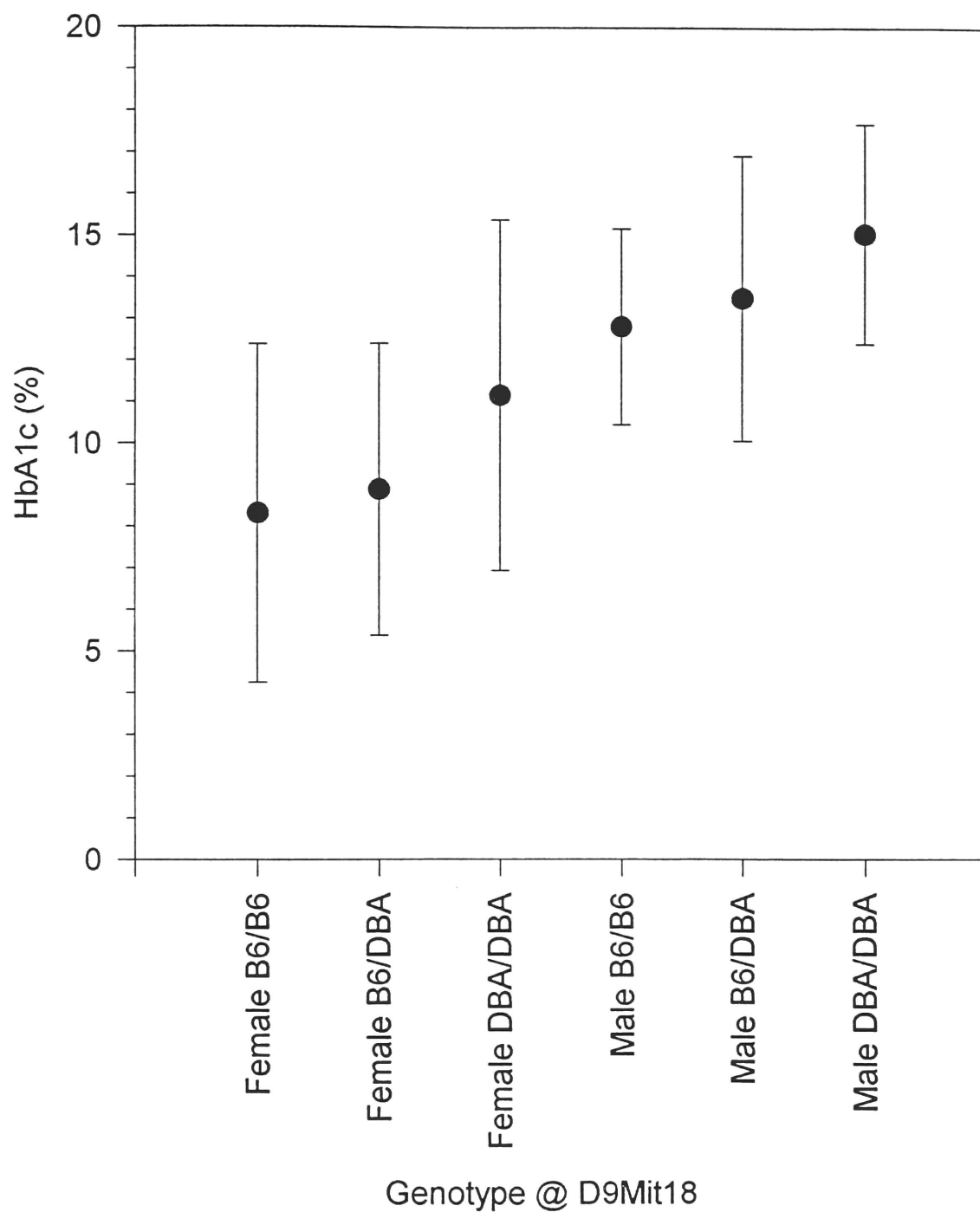


Figure 64. Relationship of genotype at D9Mit18 on HbA1c by sex in *db/db* F2 B6DBA.

Circles represent the mean of each class with error bars of standard deviations.

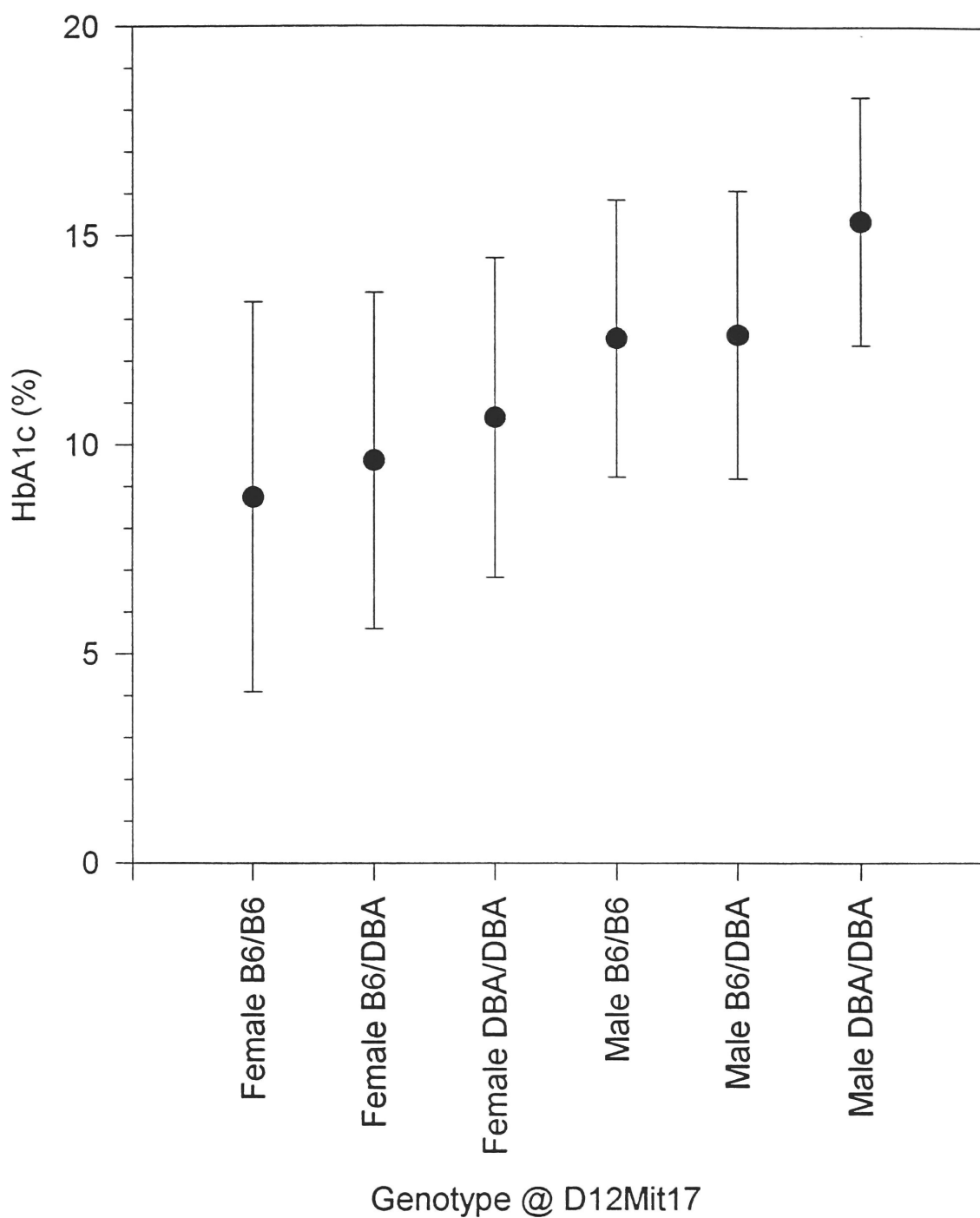


Figure 65. Relationship of genotype at D12Mit17 on HbA1c by sex in *db/db* F2 B6DBA.

Circles represent the mean of each class with error bars of standard deviations.

The marker D17Mit22 demonstrated a weak effect on the diabetes parameters related to the numbers, sizes and total areas of islets (Table 20). However, the effects were not consistent with those predicted on the basis of the original scan data which would have predicted that DBA alleles at this locus would have been associated with a lower number of smaller islets. Therefore, it is unlikely that a biologically significant locus resides near D17Mit22 in this cross.

Genotypes at D18Mit17 and D19Mit28 were not significantly associated with any of the phenotypes examined.

ANOVA with interactions between loci

Several interactions between loci were statistically significant; however, because the number of animals within each of the nine possible genotypic classes was small, caution must be exercised in generalizing from these data. Only loci which demonstrated $p < 0.01$ will be described (Table 21). Based upon the independent results of each individual locus, animals B6/B6 for the telomeric loci on chromosome 1 and DBA/DBA for the telomeric locus on chromosome 2 would have been expected to be the healthiest animals. In fact, the animals with the largest average islet area were those which were B6/B6 at both loci, suggesting that genotype at the telomeric chromosome 1 locus influenced expression of phenotype based on genotype at the chromosome 2 telomeric locus.

First locus	Second locus	Phenotype	B6/B6	B6/DBA	DBA/DBA	B6/B6	B6/DBA	DBA/DBA	B6/B6	B6/DBA	DBA/DBA	P	Genotype demonstrating synergism
D1Mit110	D2Mit229	average islet area (mm ²) normalized islet area (%)	0.0356 2.42	0.021 1.50	0.0222 1.55	0.017 0.51	0.0185 0.83	0.0227 1.74	0.0114 0.21	0.0181 0.79	0.0187 0.99	0.007 0.05	B6/B6 ② D1Mit100/ B6/B6 ② D2Mit229 B6/B6 ② D1Mit100/ B6/B6 ② D2Mit229
D1Mit110	D6Mit222	BMI (g/cm ²) HbA1c (%)	0.520 7.8	0.560 11.2	0.570 10.1	0.500 13.2	0.560 10.8	0.540 11.0	0.580 12.9	0.490 15.3	0.510 9.3	0.002 0.003	
D1Mit110	D6Mit54	number of hyperplastic islets	1.8	3.4	2.0	1.5	1.8	0.9	0.7	0.2	0.0	0.03	B6/B6 ② D1Mit100/ B6/DBA ② D6Mit54
D1Mit110	D7Mit57	pancreatic [insulin]/[glucose]	1.1	0.5	0.75	0.5	0.59	0.48	0.45	0.7	0.3	0.03	
D2Mit229	D6Mit222	pancreatic insulin (uU/mg protein)	7008	8872	11003	8598	15101	9809	21870	10325	15549	0.05	
D2Mit229	D12Mit17	pancreatic [insulin]/[glucose]	0.8	0.42	0.3	0.83	0.51	0.41	0.51	0.91	0.44	0.03	
D6Mit222	D6Mit54	number of islets pancreatic [insulin]/[glucose] pancreatic islet area (mm ²)	7.1 0.38 1.05	22.0 0.89 5.65	7.9 0.23 1.66	11.9 0.58 2.18	12.4 0.54 2.98	12.3 0.58 2.44	21.0 0.58 4.47	14.7 0.48 3.9	10.7 0.55 2.72	0.0003 0.05 0.05	B6/B6 ② D5Mit222/ B6/DBA ② D6Mit54 B6/B6 ② D5Mit222/ B6/DBA ② D6Mit54
D6Mit54	D7Mit57	HbA1c (%) pancreatic grade pancreatic insulin (uU/mg protein) pancreatic [insulin]/[glucose]	11.6 3.4 12438 0.60	11.3 3.3 9327 0.44	14.6 4.2 14732 0.81	11.2 3.4 18095 0.68	11.8 3.4 10587 0.51	9.9 2.9 12082 0.55	15.2 4.1 6204 0.30	11.9 3.0 16480 0.77	11.5 3.1 7889 0.38	0.01 0.01 0.03 0.03	DBA/DBA ② D6Mit54/ B6/B6 ② D7Mit57
D6Mit54	D12Mit17	weight (g) BMI (g/cm ²) HbA1c (%) plasma [glucose] mg/dl pancreatic insulin (uU/mg protein) pancreatic glucose (uU/mg protein) pancreatic [insulin]/[glucose] number of islets	52.1 0.560 9.1 488 11178 19684 0.63 19.6	49.8 0.550 12.5 717 11023 22290 0.48 10.0	57.5 0.600 9.8 618 13055 24719 0.58 15.0	47.0 0.530 10.4 827 12278 21000 0.59 15.7	52.5 0.560 8.9 570 14681 22536 0.7 18.8	47.0 0.500 13.8 785 7938 20565 0.39 9.8	48.1 0.520 9.8 711 24548 24541 1.09 13.3	42.7 0.510 13.2 748 8512 21023 0.41 8.2	47.9 0.530 13.7 794 7163 20864 0.34 13.3	0.02 0.03 0.01 0.04 0.008 0.01 0.03 0.05	B6/B6 ② D6Mit54/ DBA/DBA ② D12Mit17 B6/B6 ② D6Mit54/ DBA/DBA ② D12Mit17 B6/B6 ② D6Mit54/ B6/DBA ② D12Mit17 B6/B6 ② D6Mit54/ B6/DBA ② D12Mit17 DBA/DBA ② D6Mit54/ B6/B6 ② D12Mit17

Table 21. ANOVA for interactions of loci on diabetes phenotype in *db/db* F2 B6DBA. Means for the traits are listed by genotypes at the first and second loci. Specific combinations of genotypes at the two loci which result in a non-additive effect are indicated.

DBA alleles at D5Mit222 were protective from diabetes. Again, B6 alleles at D1Mit110 locus primarily influenced BMI and HbA1c, but the expression of phenotype for the chromosome 5 locus was dependent upon genotype for the telomeric chromosome 1 locus. Based on HbA1c, the healthiest class of animals were B6/B6 at both loci rather than being DBA/DBA at telomeric chromosome 2.

Genotype at D5Mit222 also interacted with D6Mit54. Based on the phenotypic effects of the individual loci, the DBA/DBA class at D5Mit222 and B6/B6 class at D6Mit54 would be expected to have additive effects on phenotypes such as number of islets, islet area, and pancreatic [insulin]/[glucagon]. In addition to observing this effect, animals B6/B6 at D5Mit222 and B6/DBA at D6Mit54 were similarly protected from diabetes in a non-additive manner. Neither one of these genotypes alone protected the animals from diabetes, but the combination produced animals with the largest number of islets and total islet areas.

The interaction between the loci on chromosomes 6 and 12 was particularly interesting. B6 alleles were associated with diabetes resistance when the loci were analyzed independently. However, the combination of DBA/DBA alleles at D6Mit54 and B6/B6 at D12Mit17 produced concentrations of pancreatic insulin which were at least two fold increased over any other combination of genotypes. Many of the other phenotypes were also affected, but the combinations of genotypes which demonstrated non-additive effects differed for the phenotype (heterozygosity at both loci for [glucose] and B6/B6 genotype

at chromosome 6 and DBA/DBA genotype at chromosome 12 for weight). These results could have been the result of imprecise localization of the two loci producing marginally significant and apparently contradictory results.

The biology of the interactions will be more accessible once the relevant congenics have been produced.

Summary of individual loci (Table 22)

Cross	Marker	Primary Phenotype(s)	Secondary Phenotype(s)	Pathogenesis
db/db F2 B6DBA	D1Mit90	body weight		obesity
	D1Mit110	HbA1c, pancreatic morphology	[insulin]	non-specific
	D2Mit229	HbA1c	number of islets	hepatic glucose production
	D5Mit222	pancreatic grade		beta cell proliferation/survival
	D6Mit54	[insulin]	[glucose]	insulin resistance
	D7Mit57	[glucose]	BMI	hepatic glucose production
	D9Mit18	number of islets	HbA1c	islet proliferation
	D12Mit17	HbA1c	[glucose], pancreatic grade	non-specific
	D17Mit22	number of islets		islet proliferation

Table 22. Summary of QTLs for obesity/diabetes in *db/db* F2 B6DBA with primary and secondary phenotypes associated with the QTL and potential pathogenic mechanisms.

The most important piece of information gained from this genome scanning experiment in the *db/db* F2 B6DBAs is the independent replication of the two QTL's on chromosome 1 between D1Mit90 and D1Mit110 and between D1Mit370 and D1Mit56. These regions were identified in the genome scan and validated after typing all of the animals with four markers located on chromosome 1. Although the density of the map is not nearly as great as determined for the *ob/ob* cross with the same strains, the locations of the proximal and distal QTLs is concordant with the locations previously described in the *ob/ob* animals. Therefore, this locus interacts in the same manner with *ob* and *db* with the strains B6 and DBA. This result is not surprising given that *ob* and *db* are loss of function alleles for a ligand and receptor system controlling body weight and adiposity. Like the *ob/ob* progeny, the *db/db* animals demonstrated diabetes resistance in association with B6 alleles at the chromosome 1 loci. Also as in the *ob/ob*, weight and BMI were the predominant phenotypes affected by the centromeric QTL, and all diabetes phenotypes were affected by the more telomeric QTL. Again, proximal chromosome 1 locus accounted for a similar percentage of the variance in the F2 progeny as previously observed. The replication of the phenotypic impact of the chromosome 1 QTLs in F2 and F3 *ob/ob* B6DBA and F2 *db/db* B6DBA attests to the importance of this locus in mediating diabetes-related phenotypes.

The *db/db* experiment provided new insight into the chromosome 2 locus. What was previously thought to be a single locus on proximal 2 affecting predominantly weight and BMI is more likely two independent loci at opposite ends of the chromosome. Telomeric

markers were never specifically tested in the *ob/ob* F2 B6DBA and DBAB6, and the apparently secondary effects of weight and BMI on blood biochemistries and pancreatic phenotype may actually have been mediated by the telomeric locus for which DBA alleles were also associated with protection from diabetes. This hypothesis can and will be simply tested by genotyping the *ob/ob* animals for D2Mit229. The telomeric chromosome 2 locus affects HbA1c most significantly; however, this parameter is the best indicator of extent of disease but also the most remote from primary pathogenic mechanisms. Therefore, although this region is obviously relevant to diabetes, its mechanism of action is not immediately obvious.

If the locations of the QTL's were accurately approximated with the markers chosen for each region, the strongest QTLs reside on chromosomes 1 and 2 based upon replication and statistical strength. Three other loci not observed in the *ob/ob* B6DBA were characterized in this cross. Both because they were not previously detected and because the relative statistical significance associated with these loci is not as great, they are likely to play smaller although possibly interesting interactive roles in diabetes. The chromosome 5 locus most specifically affects pancreatic grade; chromosome 9 pancreatic grade in addition to other quantitative measures of pancreatic morphology, and chromosome 12 both biochemical and pancreatic phenotypes. Two of these three new loci (chromosomes 9 and 12) demonstrated association of B6 alleles with protection from diabetes. The previous *ob/ob* cross had been significant for its lack of QTLs for which B6 provided protection from diabetes. Thus, these two minor loci which could have been

missed in the *ob/ob* and may explain the preponderance of QTLs previously described for which DBA alleles confer resistance to diabetes. Alternatively, it is possible that the chromosome 1 loci are so powerful, that they are largely responsible for the phenotype of *ob/ob* or *db/db* B6 animals. The additional loci may not have been observed in the *ob/ob* F2 B6DBA because the genome scanning method utilizing only the extremely affected animals initially may be more specific than sensitive and may uncover the most powerful loci all the time but only randomly uncover the less powerful loci.

Discussion

Some insight into the design of the genome scanning experiments can be gained by comparison of the *db/db* and *ob/ob* F2 B6DBA experiments. The number of progeny in the *db/db* cross was less than half of that in the *ob/ob* cross (190 vs 450). The smaller number of animals affects statistical power but more importantly affects the number of animals available at the extremes of phenotype for use in the genome scanning part of the experiment. Ten or more animals of a particular class are required to perform the scan. When fewer animals (5 in the case of the non-diabetic class) are used, only extreme deviations (4 or 5 animals homozygous for a genotype) result in statistically significant deviation from expected Mendelian ratios. In this case, because the class of non-diabetic/non-insulin resistant animals was so biologically interesting, the reduced power was tolerated, but largely without success. Only a single weak locus was identified with the genome scanning experiments using this class. If at least ten animals are then required for the genome scan, the smaller the total number of progeny in the cross, the less extreme

the phenotype demonstrated by the ten animals. Thus, small numbers of progeny increase the likelihood of genetic heterogeneity in animals at the extremes of phenotype if an absolute minimum number of animals is required. Therefore, unless extremely well defined subphenotypes are measured which are immediately proximal to disease development, at least 400 obese progeny should be generated from which the extreme 5% at each tail of the distribution can be selected.

The impact on statistical analysis of the reduced number of F2 progeny is also recognized when attempting to localize the QTL without analysis of a locus in entire obese F2 progeny. If sufficient numbers of animals are used in the scan, the QTL can be accurately localized to within 30 cM by typing the subset of extremely affected animals. If the number of extremely affected progeny is not great enough, a small number of animals is unlikely to have enough sufficiently informative recombination events to allow precise localization, necessitating extensive genotyping of all obese progeny to localize the QTL. For most of the regions, extensive genotyping (6-8 markers per region) has not yet been performed. Thus, some current localizations may be sufficiently inaccurate to impair detection of statistical significance and/or to generate apparently biologically inconsistent results between sexes or interacting loci.

Of the four phenotypic classes used for the scanning experiments, the protected pancreas and extremely diabetic classes identified the greatest number of loci with an apparent effect on phenotype. The protected pancreas class derived its power from two factors:

1) The relatively large number of animals analyzed (17) and 2) The use of a specific subphenotype which is less likely to display genetic heterogeneity. The diabetic class may have been informative because it was the most extreme physiological endpoint.

Again, the most robust phenotypes were HbA1c and pancreatic grade. The assay for HbA1c is precise, and, in contrast to plasma [glucose] and [insulin], the values are unaffected by the stress of fasting and/or euthanasia. Similarly, pancreatic grade is precisely determined and not susceptible to short term perturbations; variance of scoring has been decreased by the use of two independent readers. Additional phenotypes which are more precise and proximal to disease etiology will be necessary for future experiments.

If *ob* and *db* are ligand and receptor respectively for a pathway determining body weight via food intake and energy expenditure; since both alleles are loss of function, exactly the same phenotypes and experimental results should have been observed with the *ob/ob* and *db/db* F2 B6DBA progeny. In fact, this was not the case (Table 23). Because the *ob/ob* animals were slightly older, all phenotypes were adjusted for age before comparison. Despite this adjustment, the *ob/ob* were 23.5% heavier and had BMIs which were 12.5% higher than the *db/db* progeny ($p < 0.00001$). The *ob/ob* animals were also less diabetic with slightly lower pancreatic grades (3.14 vs 3.3, $p < 0.0005$) and pancreatic insulin content twice as high as the *db/db* progeny.

Phenotype	F2 <i>ob/ob</i> B6/DBA	F2 <i>db/db</i> B6/DBA	P
Age (days)	112 (16)	104 (15)	
Weight (g)	59.3 (11.5)	48.0 (10.1)	0.00001
BMI (g/cm ²)	.596 (.087)	0.531 (0.082)	0.00001
HbA1c (%)	11.0 (4.5)	11.6 (4.2)	
Fasting plasma [glucose] (mg/dL)	713 (219)	685 (249)	
Fasting plasma [insulin] (uU/ml)	165.5 (210.9)	331.2 (312.0)	
Pancreatic grade	3.1 (1.1)	3.3 (1.2)	0.0005
Normalized pancreatic insulin content (uU/mg protein)	24208 (49722)	11986 (11298)	0.0002
Number of islets	14.4 (10.0)	12.9 (9.2)	
Number of hypertrophic islets	1.6 (2.8)	1.4 (2.6)	
Average islet area (mm ²)	0.02 (0.009)	0.02 (0.01)	
Total islet area (mm ²)	0.33 (0.36)	0.29 (0.33)	
Normalized islet area (%)	1.26 (1.33)	1.06 (1.19)	

Table 23. Comparison of *ob/ob* and *db/db* F2 B6/DBA progeny by ANOVA. Means for each trait corrected for age are indicated with standard deviations in parentheses.

There are at least two possible explanations for these results: 1) *ob* and *db* have as yet undetermined roles in addition to binding one to the other. Loss of these additional functions then results in similar although distinguishable phenotypes. Although this explanation is possible, previous descriptions of the similarity of phenotypes of these mutations on the same genetic backgrounds would suggest otherwise (Leiter, Coleman 1981). 2) Alternatively (the more likely explanation), the crosses are not perfectly genetically controlled for all loci besides *ob* and *db*, and these additional loci influence

measures of body weight and pancreatic insulin content. *Ob* originally arose on stock V at the Jackson Laboratory and was transferred to C57BL/6J by a series of backcrosses. Animals which are now obtained from Jackson are F56 and genetically homogeneous for B6 alleles. *Db* is co-isogenic on C57BL/KsJ and was transferred to C57BL/6J also by a series of five backcrosses. On average after 5 backcrosses, 98.4375% of the genome is B6. However, loci linked to *db* on chromosome 4 are more likely be BKS the closer they lie to *db*. Therefore, although only a small fraction of the alleles segregating may be BKS in the *db/db* F2 B6DBA's, they may account for the phenotypic differences in the two crosses. The BKS itself is likely to be a result of contamination of B6 with DBA alleles. For reasons given above, the most likely location for the loci is on chromosome 4, but they could be located anywhere in the genome. Systematic searches for the markers which are polymorphic between B6 and BKS in the parental B6 (*db*) animals would define the markers which could be tested for effect on phenotype. However, most markers are not polymorphic between B6 and BKS and the length of the regions which are still BKS is likely to be small, hence they could easily be missed. Therefore, a systematic search for such a locus would only be fruitful if limited to chromosome 4. In fact, a locus on chromosome 4 for body weight/BMI centromeric of *db* was described in the *ob/ob* animals and could be tested in the *db/db* progeny.

Chapter 7

Confirmation of diabetes susceptibility regions

in

F3 B6DBA segregating for *db*

Strategy

In an attempt to determine the reproducibility and strength of the association of the chromosome 1 locus with diabetes phenotypes, lean F2 B6DBA progeny were tail clipped and genotyped with Bam7, a marker 0.1 cM telomeric of *db* to identify *db*/+ animals. Heterozygous animals were subsequently genotyped for D1Mit90 and D1Mit110, markers 18 cM apart, in the region of the diabetes susceptibility QTL on chromosome 1. The selection of these markers was based on the more precise localization of the QTL in the *ob* crosses under the assumption that the same QTL was detected in the *db/db* F2 B6DBA. Animals heterozygous for *db* and concordant for genotype at D1Mit90 and D1Mit110 were mated to a genotypically similar mouse of the opposite sex. Seventeen such breeding pairs were established which produced a total of 119 progeny. The mice were phenotypically characterized and analyzed as previously described. All of the obese progeny were initially typed for three markers along a 22 cM interval on chromosome 1 (D1Mit370, D1Mit56, and D1Mit408), two markers at opposite ends of chromosome 3 (D3Mit17 and D3Mit62), four markers on chromosome 5 spanning 57 cM (D5Mit79, D5Mit65, D5Mit99, and D5Mit222), four markers on chromosome 7 spanning 39 cM (D7Mit57, D7Mit84, D7Mit62, and D7Mit100), two markers on chromosome 12 separated by 4.3 cM (D12Mit218 and D12Mit171) and two markers on chromosome 17 separated by 22 cM (D17Mit53 and D17Mit22). In addition, single markers were run on several chromosomes: D2Mit229, D8Mit207, D18Mit17, and D19Mit28. Gene dosage at *db* in the obese progeny was determined by genotyping the animals for c-jun, Bam7 and D4Mit76.

Results

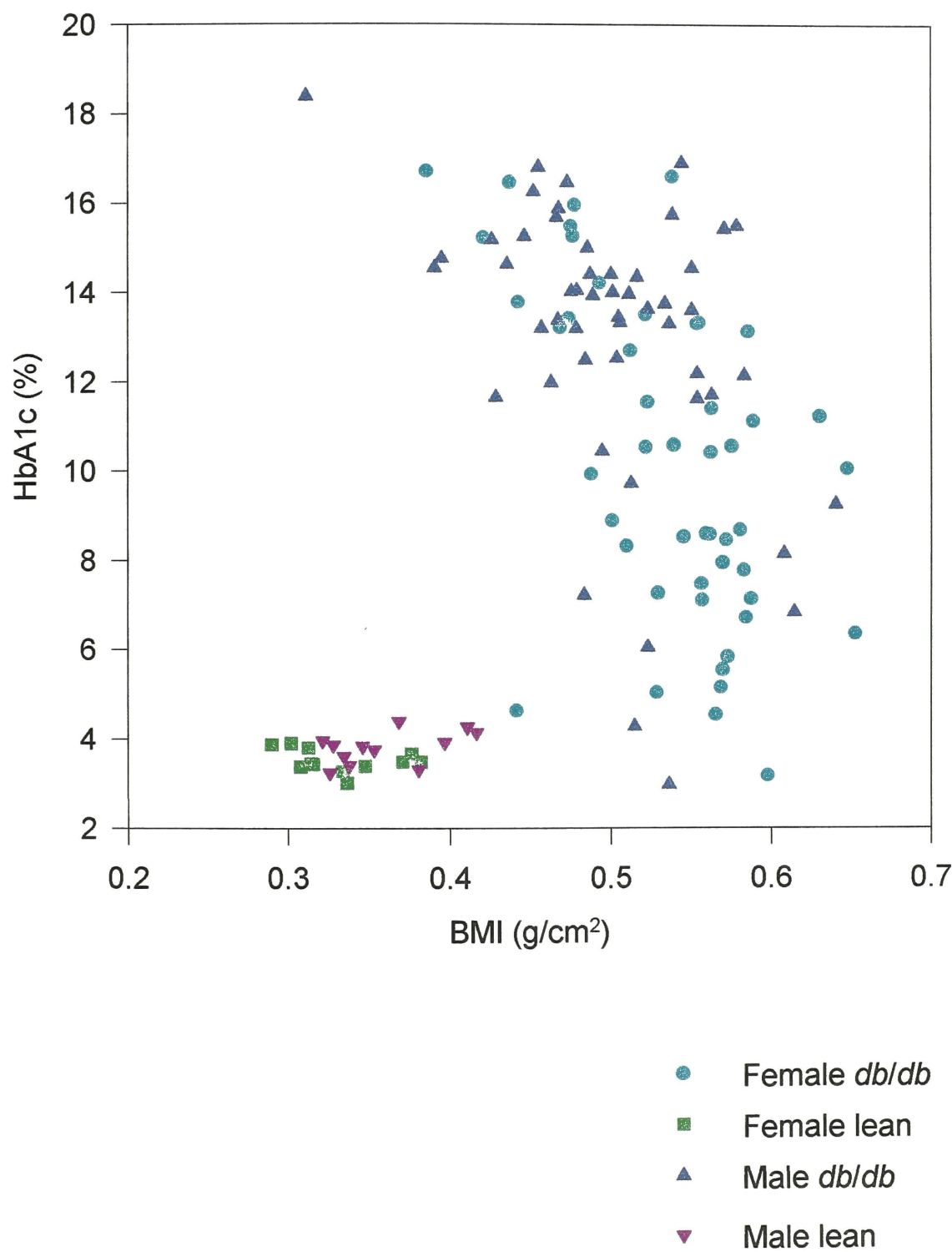
Phenotypically, the obese (*db/db*) progeny were most readily distinguishable from the lean (*db/+* or *+/+*) on the basis of body weight and BMI (Table 24 and Figure 66). None of the lean mice were diabetic, as demonstrated by the glycosylated hemoglobin fractions which were consistently less than 5%. All phenotypic parameters were distinguishable between the two groups.

Phenotype	Lean	Obese
Age (days)	99 (9)	102 (12)
Weight (g)	28.2 (4.4)	45.4 (7.6)
BMI (g/cm ²)	0.346 (0.28)	0.521 (0.066)
HbA1c (%)	3.7 (0.3)	11.7 (3.7)
Fasting plasma [glucose] (mg/dL)	321 (44)	654 (208)
Fasting plasma [insulin] (uU/ml)	100.8 (85.1)	361.3 (369.0)
Pancreatic grade	ND	3.3 (1.2)
Number of islets	ND	15.36 (8.89)
Number of hyperplastic islets	ND	1.41 (2.36)
Average islet area (mm ²)	ND	0.0184 (0.008)
Total islet area (mm ²)	ND	0.318 (0.289)
Normalized islet area (%)	ND	0.974 (0.859)

Table 24. Phenotypic characteristics of F3 B6DBA progeny segregating for *db*. Means for the lean and obese groups by sex are indicated with standard deviations in parentheses. “ND” indicates that values were not determined.

Figure 66. BMI versus HbA1c in *db/db* and lean male and female F3 B6DBA. Each point represents a single animal.

F3 B6DBA



In Figure 67 the usual inverse relationship between plasma [insulin] and HbA1c in the obese was observed. All of the animals were at least insulin resistant and some demonstrated varying degrees of decompensation as manifested by decreasing plasma [insulin] despite increases in HbA1c. Of note is that two animals were confirmed to be genotypically as well as phenotypically lean yet demonstrated elevated plasma [insulin] > 400 μ U/ml. These two lean males were euglycemic, suggesting that, although rare, it is possible for a non-obese animal to become insulin resistant. Phenotypically equivalent (hyperinsulinemic) lean animals were not observed in the F2 progeny. Figure 68 recapitulated the relationship between fasting plasma [insulin] and hyperglycemia observed in figure 67 although the relationship with glucose was less clear owing to the variability when collected post-mortem after asphyxiation. A positive linear relationship between pancreatic grade and HbA1c was observed (Figure 69) with animals (predominantly females) in the lower left quadrant representing relatively healthy animals and those animals (predominantly males) in the upper right quadrant representing diabetic animals.

The biochemical and pancreatic profiles of the obese progeny were comparable to those of the F2 progeny and displayed similar variation (Table 25).

Figure 67. Plasma [insulin] versus HbA1c in *db/db* and lean male and female F3 B6DBA.

Each point represents a single animal.

F3 B6DBA

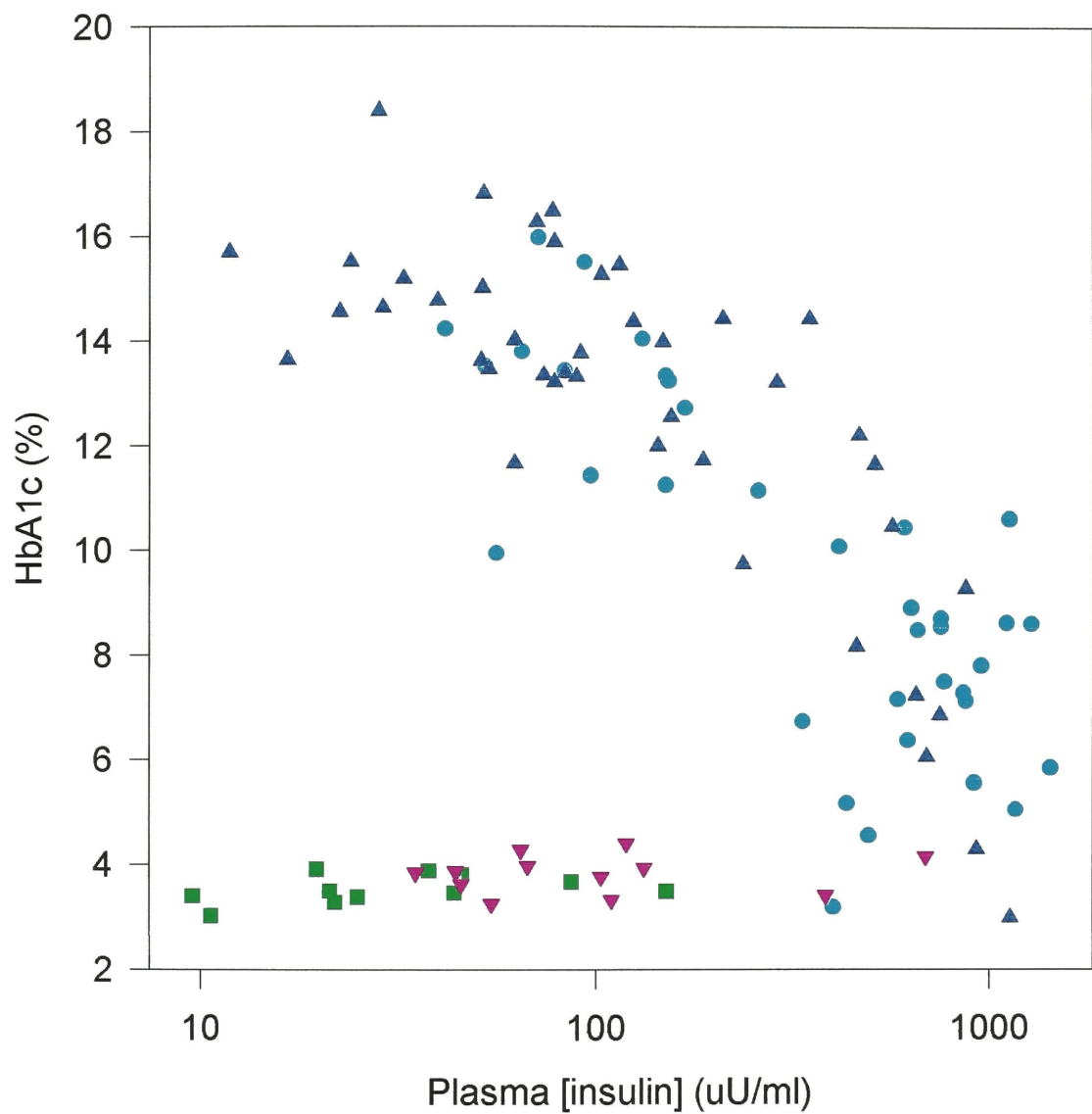
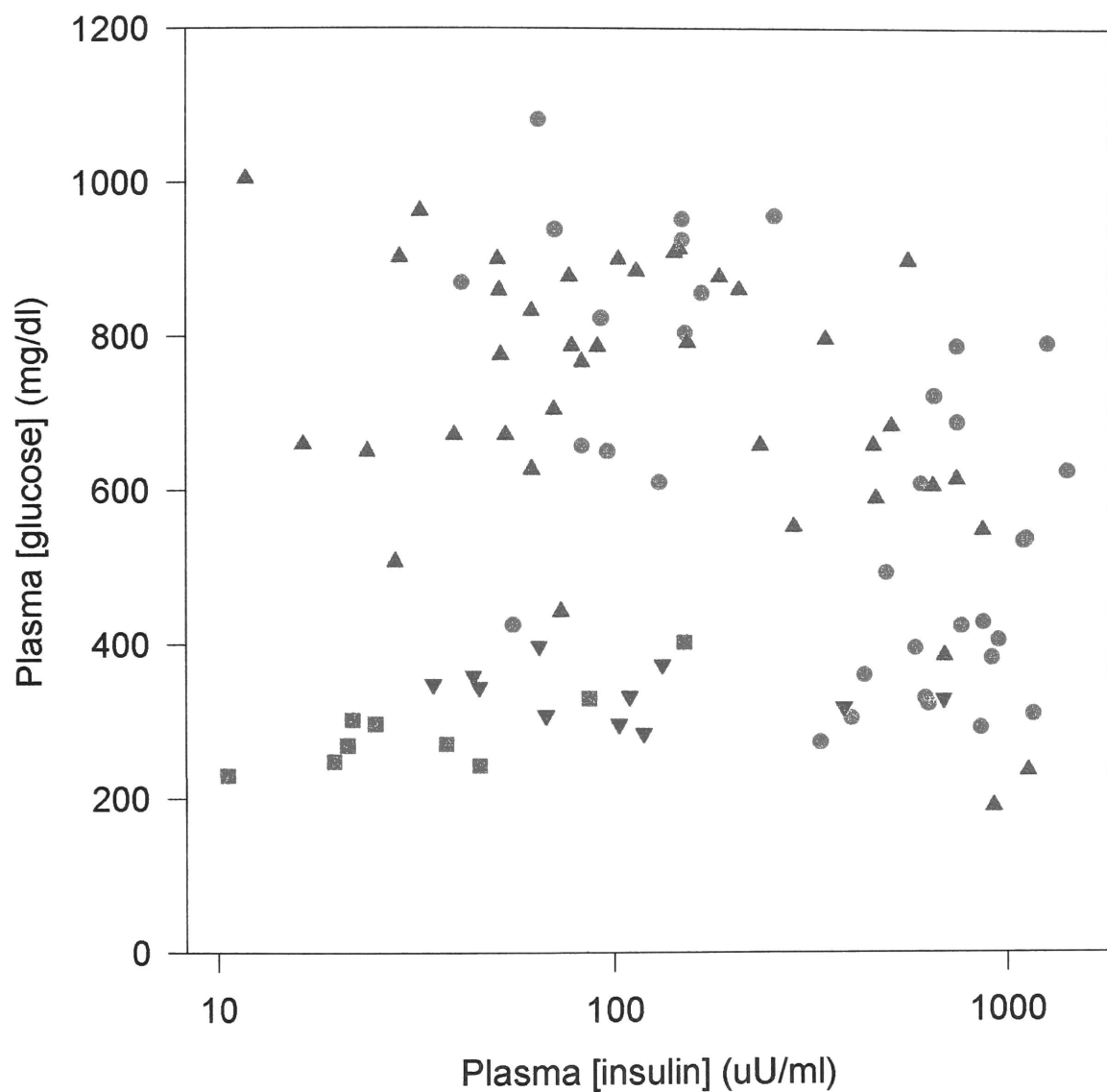


Figure 68. Plasma [insulin] versus plasma [glucose] in *db/db* and lean male and female F3 B6DBA. Each point represents a single animal.

F3 B6DBA

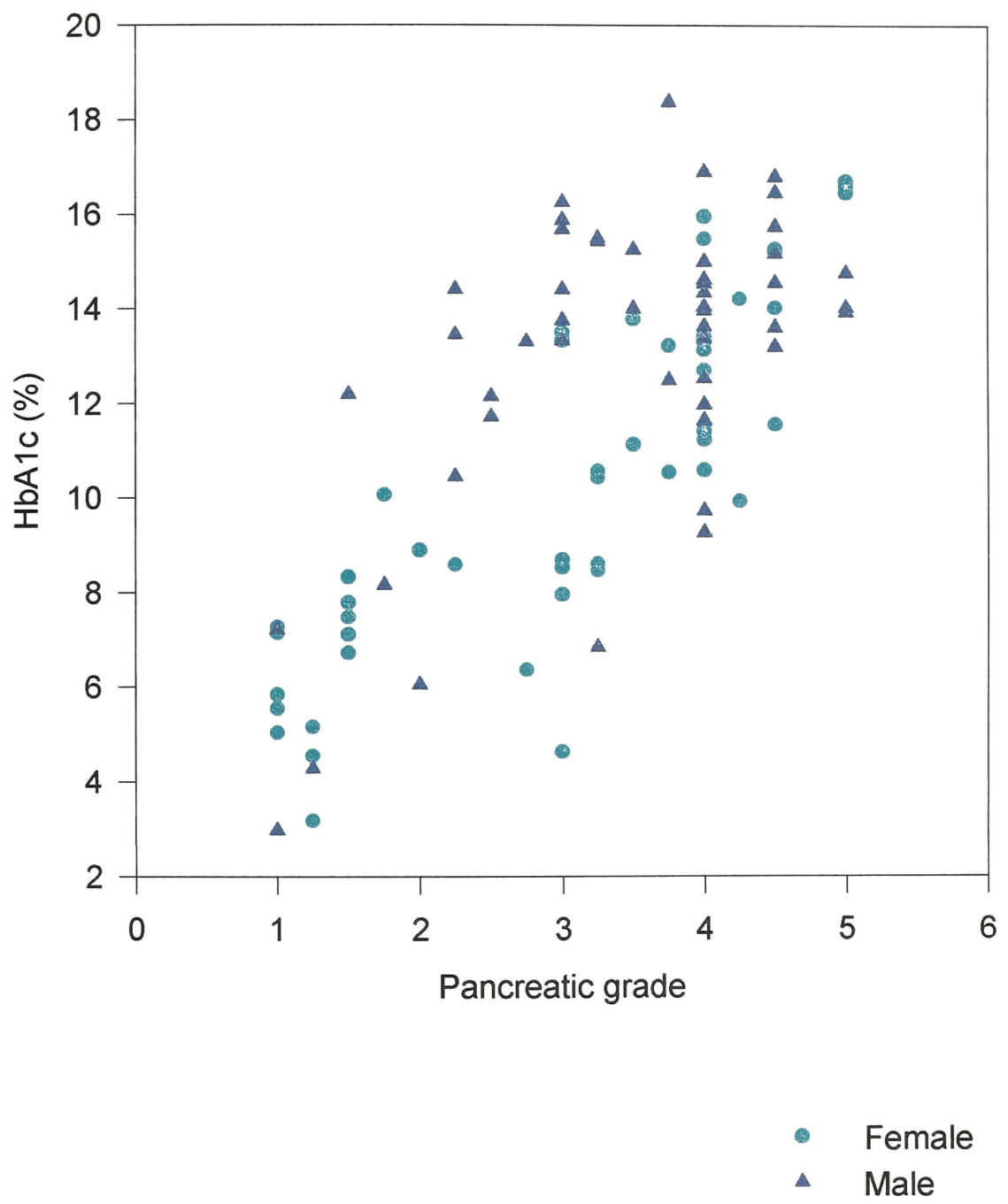


- Female *db/db*
- Female lean
- ▲ Male *db/db*
- ▼ Male lean

Figure 69. Pancreatic grade versus HbA1c in *db/db* and lean male and female F3 B6DBA.

Each point represents a single animal.

F3 *db/db* B6DBA



Phenotype	F2 db/db B6DBA	F3 db/db B6DBA
Age (days)	104 (15)	102 (12)
Weight (g)	48.0 (10.1)	45.4 (7.6)
BMI (g/cm ²)	0.532 (0.081)	0.521 (0.066)
HbA1c (%)	11.6 (4.2)	11.7 (3.7)
Fasting plasma [glucose] (mg/dL)	685 (249)	654 (208)
Fasting plasma [insulin] (uU/ml)	331.2 (312.0)	361.3 (369.0)
Pancreatic grade	3.3 (1.2)	3.2 (1.2)
Number of islets	12.94 (9.16)	15.36 (8.89)
Number of hyperplastic islets	1.41 (2.55)	1.41 (2.36)
Average islet area (mm ²)	0.0188 (0.01)	0.0184 (0.008)
Total islet area (mm ²)	0.290 (0.329)	0.318 (0.289)
Normalized islet area (%)	1.055 (1.19)	0.974 (0.859)

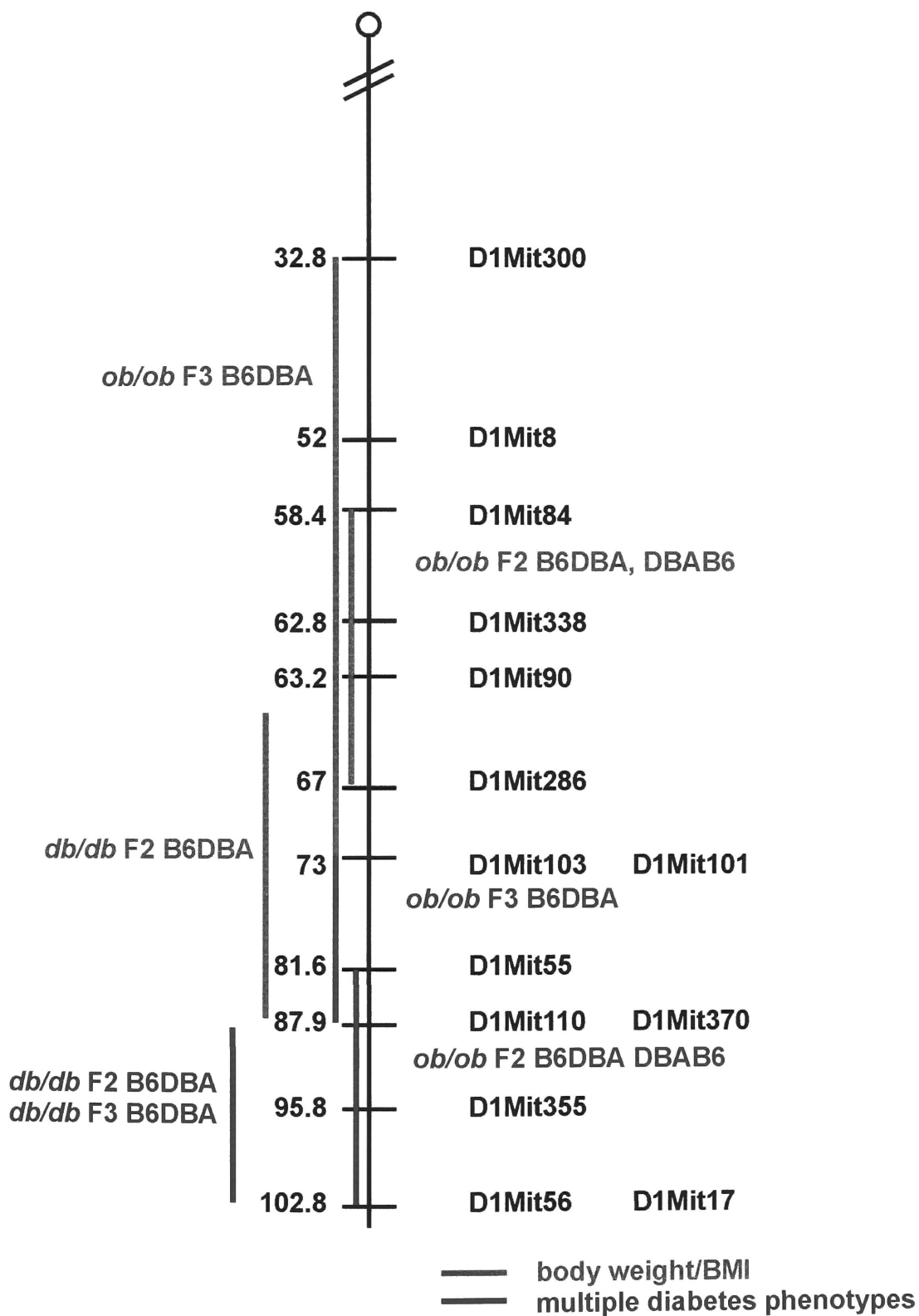
Table 25. Phenotypic comparison of *db/db* F2 and F3 B6DBA progeny. Means are indicated with standard deviations in parentheses. None of the traits were statistically distinguishable in the two crosses.

QTL and ANOVA results

F2 *db/+* breeders which carried the same genotypes at D1Mit90 and D1Mit110 (13 cM apart) were mated to genetically equivalent animals for these two markers. As had been observed in the *ob/ob* F3 B6DBA progeny, the *db/db* F3 B6DBA progeny replicated and independently confirmed the results observed in the *db/db* F2 progeny (Figure 70, Table 26). More specifically, locus D1Mit370, the most centromeric of the three markers typed on chromosome 1, accounted for 16% of the variance in pancreatic grade with a LOD score of 2.1 (Table 27). All three of the markers typed -D1Mit370, D1Mit56, and D1Mit408- demonstrated significant effects of genotype on pancreatic grade, plasma [insulin], HbA1c, and average islet size (Table 26). In addition, D1Mit370 also demonstrated an effect of genotype on weight. Most likely the apparent effect of D1Mit370 on weight is due to influence of the more centromeric QTL previously described, acting over a distance. As has been observed with all previous crosses segregating B6 or DBA at this locus, B6 alleles were protective for diabetes in both sexes. The effect of the chromosome 1 telomeric QTL on all diabetes phenotype has now been replicated in F2 progeny twice, suggesting that the trait will continue to breed true with introgression.

Figure 70. Summary of the QTLs for obesity/diabetes on mouse chromosome 1. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 1



Marker	Phenotype	Number	B6/B6	Female		B6/B6	Male		P
				B6/DBA	DBA/DBA		B6/DBA	DBA/DBA	
D1Mit370	Weight (g)	88	51.8	47.3	46.6	45.1	41.4	41.7	0.04
	Pancreatic Grade	88	2.4	3.3	3.2	3.1	3.6	4.3	0.003
	Islet area/total area (%)	87	1.45	0.85	1.21	1.05	0.71	0.38	0.05
D1Mit56	HbA1c (%)	87	8.7	11.0	10.8	11.9	13.0	14.5	0.03
	[Insulin] (uU/ml)	71	656.8	473.8	321.8	333.7	240.9	103.8	0.01
	Pancreatic Grade	87	2.3	3.3	3.3	3.1	3.5	4.1	0.003
	Average Islet size (mm2)	85	0.023	0.017	0.021	0.017	0.016	0.024	0.04
D1Mit408	HbA1c (%)	91	8.4	11.1	11.4	12.0	13.0	14.7	0.007
	[Insulin] (uU/ml)	71	656.8	589.1	258.5	300.6	244.5	58.2	0.002
	Pancreatic Grade	92	2.4	3.3	3.4	3.3	3.6	4.0	0.01
D2Mit229	Islet area/total area (%)	90	0.85	1.29	1.18	0.51	1.02	1.05	0.05
D5Mit65	Weight (g)	81	54.0	48.3	47.3	40.5	45.0	37.5	0.05
	BMI (g/cm2)	81	0.641	0.532	0.531	0.482	0.513	0.472	0.01
D7Mit100	HbA1c (%)	87	13.0	9.2	11.7	13.7	12.5	13.4	0.006
	Pancreatic Grade	88	3.9	2.6	3.7	3.7	3.3	3.6	0.009
	Number of hyperplastic Islets	85	0.7	2.9	0.7	0.3	1.5	0.5	0.002
	Average Islet size (mm2)	85	0.016	0.022	0.017	0.016	0.02	0.015	0.03
	Islet area (mm2)	85	0.262	0.494	0.26	0.154	0.319	0.196	0.006
	Islet area/total area (%)	85	0.78	1.33	0.79	0.52	1.06	0.60	0.01
D12Mit17	HbA1c (%)	94	14.2	9.4	10.5	12.5	12.5	13.7	0.04
	Pancreatic Grade	95	4.3	2.7	3.1	3.7	3.2	3.6	0.004
	Number of Islets	92	11.7	20.6	15.7	15.0	14.6	10.0	0.04
	Islet area (mm2)	92	1.74	4.95	3	3.43	2.82	1.44	0.02
	Islet area/total area (%)	92	0.67	1.41	0.76	1.33	0.91	0.45	0.03
D17Mit53	Weight (g)	84	51.5	42.0	46.6	44.8	43.7	43.9	0.04
	BMI (g/cm2)	84	0.569	0.500	0.529	0.521	0.512	0.499	0.04

Table 26. Analysis of variance by sex and genotype at the marker designated for *db/db* F3 B6DBA. All traits for all markers demonstrating statistical significance are summarized. Number of animals genotyped and phenotyped for each a given marker and trait is indicated. Mean values for each trait are listed first by sex then genotypic class.

Phenotype	Marker with peak LOD score	LOD score	% variance for which locus can account	Genetic model
Weight	D17Mit22	2.17	11	free, dominant
Pancreatic grade	D1Mit370	2.14	16	free, dominant
Number of hyperplastic islets	D7Mit62-D7Mit100	2.15	10.4	free
Total islet area	D7Mit62-D7Mit100	2.15	10.9	free

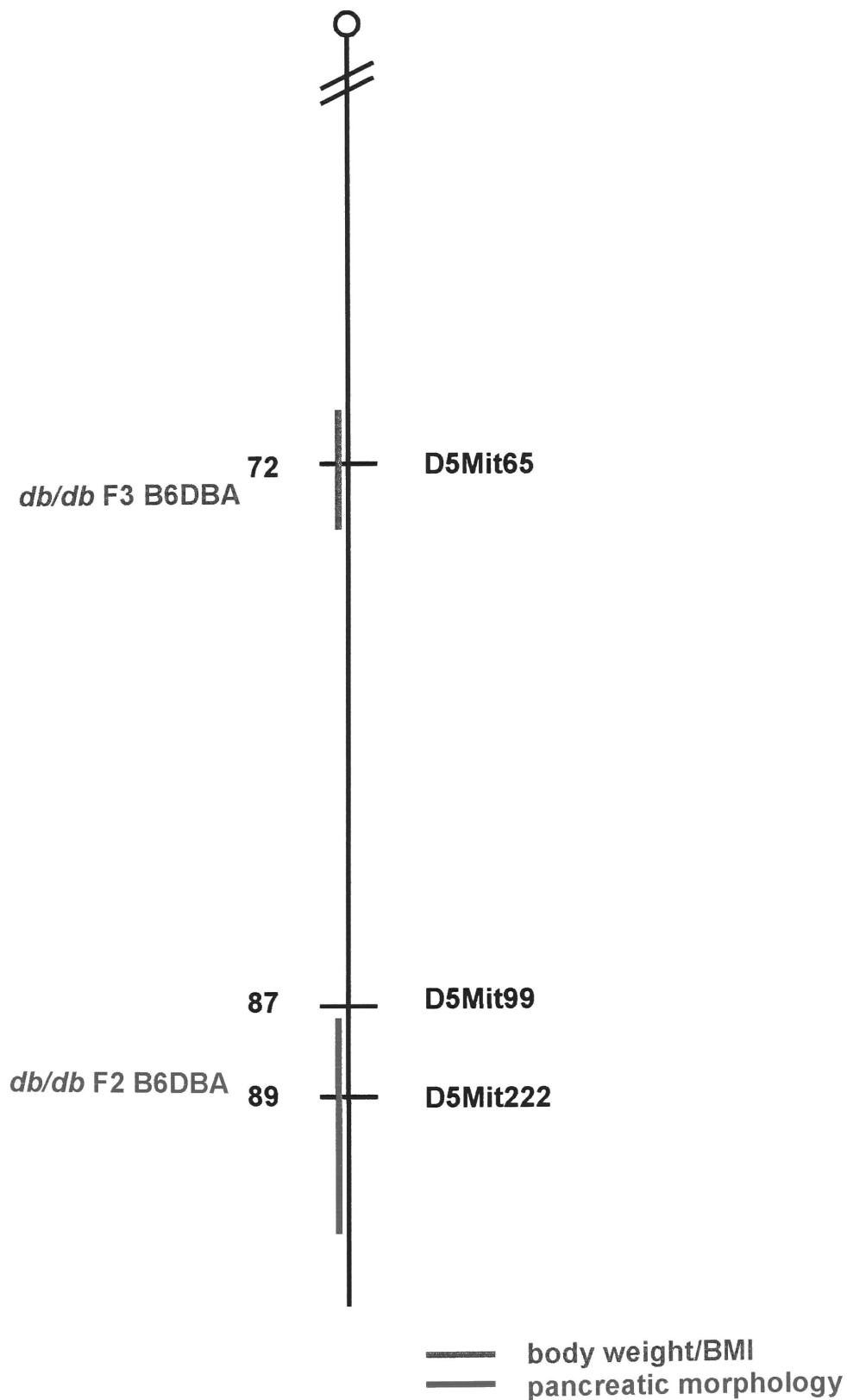
Table 27. Quantitative trait locus analysis for diabetes-susceptibility loci in *db/db* F3 B6DBA mice. Data are summarized by trait/phenotype, the marker or interval which demonstrated the peak LOD score, the maximum LOD score observed under a free genetic model, and the genetic models which most likely fit the data.

The other markers tested on chromosomes 2, 3, 5, 7, 8, 17, 18, and 19 were not controlled for within the breeders. The 119 progeny were produced from only 17 breeder pairs. Because the sample size was small and because many combinations of loci were randomly fixed within the breeders, it may not be valid to test for the effects of these other loci in a limited sample size.

The most centrometic of the chromosome 5 regions, between D5Mit79 and D5Mit65, was associated with a significant effect on plasma [glucose] and accounted for 12 % of the variance of this phenotype. B6 alleles at D5Mit65 were also associated with increased weight and BMI, and differed in gene dosage required between the sexes (two B6 alleles required in females but only one in males). These results in the F3 progeny are unexpected because the QTL had been localized more telomerically on chromosome 5 in the *db/db* F2 B6DBA (Figure 71). More importantly, DBA had been associated with increased weight and diabetes protection in the F2 progeny rather than the B6 protection observed in the F3 progeny. Therefore, the biological significance of the results obtained for this locus with the F3 progeny are questionable. It is possible that two different loci exist on chromosome 5, and that B6 alleles produce diabetes resistance at this one locus and DBA alleles are diabetes resistant at the other. However, more likely, because the results of the F3 progeny are only marginally significant, the possibility that other loci which were simultaneously fixed by chance in some of the F2 progeny are producing some of the same proportion of the effect observed.

Figure 71. Summary of the QTLs for obesity/diabetes on mouse chromosome 5. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 5



The QTL between D7Mit62 and D7Mit100 accounted for a maximum of 10.4 % of the variance in number of hypertrophic islets and 10.9% of the variance in total islet area (Table 27). In the F3 progeny, this locus influenced HbA1c, pancreatic grade, number of hypertrophic islets, average islet size, and islet area. As had been observed with the F2 progeny, heterozygosity at this locus produced protection from diabetes. This hybrid vigor (heterosis) was observed in both sexes for all phenotypes and was most striking with regard to the number of hypertrophic islets and normalized islet area which were twice as great in heterozygotes as either homozygote in both sexes. This hybrid vigor could be the result of gene products with activities which vary under different conditions. Genetic access to a side repertoire of responses could be protective against disease if each allele were favorable under different conditions.

The locus on chromosome 12 represented by D12Mit17 produced significant effects on HbA1c, pancreatic grade, number of islets and islet area (Table 26). The protective effect of homozygosity for B6 observed in the F2 progeny was only replicated in the males for a subset of phenotypes (number of islets and islet area). For the other phenotypes, and for the females for all phenotypes, heterozygosity was most protective. This result could have been a consequence of inaccurate localization of the QTL or interactions of other loci which were not controlled for.

B6 homozygosity at D17Mit53 was associated with increases in body weight and BMI, the effect being greater in females than males (Table 26). The effect of genotype at this

marker on phenotypes was marginal, and the phenotypes affected in the F3 (weight and BMI) differed from the F2, (measures of islet number and size). Because measures of body fatness and islet insulin production capacity are likely to be inter-related, such results are consistent.

There was insufficient power to analyze the interactions of loci since there were not enough animals in each of nine possible combinations of genotypes.

Discussion

Data from the *db/db* F3 B6DBA indicate that the diabetes-related effects of the locus on telomeric chromosome 1 (qualitative and quantitative islet morphology) are replicable in an independent population. The results obtained for the other regions which had been indicated on the basis of the *db/db* F2 B6DBA progeny were not as dramatic and were probably influenced by other unidentified loci which were not controlled for and which may have been genetically fixed in a non-random manner within particular F2 breeding pairs. In general, a greater number of F3 progeny are necessary to replicate the genotypic effects observed in similar F2 progeny.

Chapter 8

Identification of diabetes susceptibility regions

in

F2 B6CAST segregating for *ob*

Selective genotyping selection criterion

The F2 B6CAST progeny segregating for *ob* were produced, maintained, and characterized as previously described (Bahary et al. 1993). A total of 339 *ob/ob* F2 progeny, 173 males and 166 females were produced. Genotype for the *ob* locus was determined as previously described (Bahary et al. 1990). Because these animals were generated for the purpose of cloning *ob* rather than studying the diabetes phenotype, the animals were less extensively characterized phenotypically (BMI, fasting plasma [insulin] and [glucose]). The animals were fasted overnight before sacrifice rather than for 3 hours as with the more recent crosses.

Animals were assigned to the insulin-resistant or diabetic groups solely on the basis of overnight fasting plasma [insulin] because variability in the plasma [glucose] was thought to be affected by method of sacrifice to such a degree that it would not be informative in distinguishing the animals phenotypically. It is now known that CO₂ asphyxiation with cardiac puncture does raise the plasma [glucose]; but despite this elevation, the exact concentrations observed do indicate degree of diabetes. Eleven insulin resistant animals were selected with plasma [insulin] > two standard deviations above the mean of all the obese progeny (966.4 μU/ml) (Table 28). Nine animals with the plasma [insulin] < 70 μU/ml were selected as the diabetic class. The group of insulin resistant animals was composed largely of females (9/12) and the diabetic class largely of males (7/9).

Cross designation**Criterion for Selection**

Insulin resistant

Plasma [insulin] > 966.4 μ U/ml

Diabetic

Plasma [insulin] < 70 μ U/ml

ID	Sex	Age (days)	Weight (g)	BMI (g/cm ²)	Plasma [glucose] mg/dl	Plasma [insulin] uU/ml
Insulin resistant						
107	M	93	37.8	0.447	704	1378.8
114	F	77	36.3	0.469	451	1948.4
160	F	64	48.6	0.527	322	1173.4
193	F	93	60.2	0.667	570	1487.2
202	F	75	43.6	0.504	542	1182.4
213	F	68	40.2	0.531	598	1327.8
295	F	71	39.3	0.464	500	1139.8
308	M	70	35.7	0.472	616	1862.0
337	M	71	38.1	0.441	826	1237.8
338	F	77	37.6	0.408	261	1773.2
392	F	61	30.7	0.435	320	1251.1
Average		75	40.7	0.488	519	1432.9
Diabetic						
126	M	76	28.8	0.408	648	20.0
174	M	62	38.9	0.491	868	20.2
184	M	77	22.7	0.355	682	26.8
211	F	61	23.4	0.375	640	21.4
233	M	75	28.2	0.364	734	19.8
250	M	73	39.8	0.460	936	7.4
283	M	75	29.6	0.463	896	60.2
287	M	65	29.7	0.402	866	54.2
356	F	59	22.8	0.365	694	29.0
Average		69	29.3	0.409	774	28.8

Table 28. Phenotypic characteristics of *ob/ob* F2 B6CAST used in each genome scanning experiment. The average for the group for each phenotypic parameter is indicated below the individual animals.

Results

Phenotypic characterization

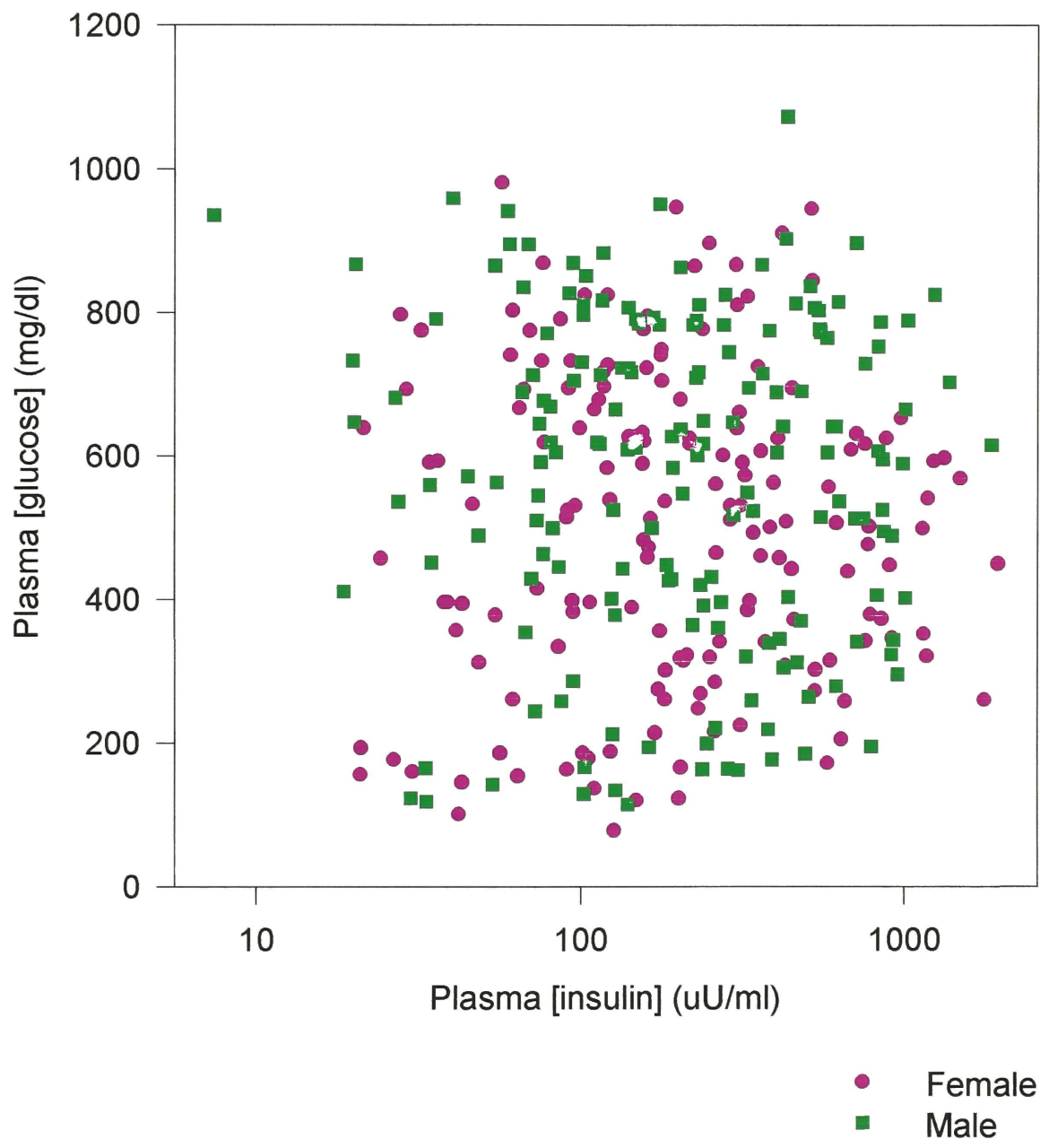
The *ob/ob* F2 B6CAST were not followed as long as the more recent crosses since the purpose of studying these animals was only to determine whether or not the animals were obese, and obesity could clearly be discerned by the age of approximately 70 days. The *ob/ob* F2 B6CAST were shorter animals that weighed less than those from other crosses employing strains other than CAST; however, the *ob/ob* were equally obese (based on BMI) to obese progeny from other crosses (Table 29). Unlike many of the other crosses, there was little sexual dimorphism in the *ob/ob* F2 B6CAST, although in general the most diabetic animals were males and the most insulin-resistant animals were females (Figure 72). Extensive variability in degree of diabetes (degrees of glycemia) existed within the cross (Figure 72) in animals of both sexes.

	Female <i>ob/ob</i>	Male <i>ob/ob</i>
Age (days)	71 (12)	70 (12)
Weight (g)	34.5 (6.8)	35.7 (6.1)
BMI (g/cm ²)	0.471 (0.061)	0.472 (0.051)
Plasma [glucose] mg/dl	503 (215)	573 (309)
Plasma [insulin] uU/ml	313.7 (342.6)	316.6 (308.6)

Table 29. Phenotypic characteristics of *ob/ob* F2 B6CAST separated by sex. Means for groups are indicated with standard deviations in parentheses.

Figure 72. Overnight fasting plasma [insulin] vs [glucose] of *ob/ob* F2 B6CAST. Each point represents a single animal.

F2 *ob/ob* B6CAST



Genome scanning results

Between 95 and 100 markers were used to genotype the animals at the extremes of phenotype for the genome scanning experiment (Table 30).

D1Mit17 at the telomeric end of chromosome 1 demonstrated enrichment of CAST alleles in the insulin resistant class (3 B6/B6, 2 B6/CAST, and 6 CAST/CAST) and enrichment of B6 alleles in the diabetic class (5 B6/B6, 4 B6/CAST, 0 CAST/CAST). D1Mit17 was located 1 cM telomeric of D1Mit408 and could have represented the same region of chromosome 1 harboring a QTL in the B6 DBA crosses.

There was extreme distortion of genotype distribution in both the diabetic and insulin resistant classes for markers in the middle of chromosome 2. In the diabetic class, there were 8 B6/B6, 1 B6/CAST and 0 CAST/CAST for D2Mit43. Conversely, in the insulin resistant class, there was enrichment of CAST/CAST with 3 B6/B6, 2 B6/CAST, and 6 CAST/CAST. Based on the genome scanning results, this locus would be predicted to be relatively powerful. In addition, because it potentially represented a transgression, other regions should have been identifiable for which B6 alleles were protective against diabetes.

Table 30. Genome scanning results for the twelve *ob/ob* F2 B6CAST insulin resistant mice with elevated fasting plasma [insulin] and nine diabetic *ob/ob* F2 B6CAST. B6/B6 indicates homozygosity for B6 alleles, B6/CAST for heterozygosity and CAST/CAST homozygosity for CAST alleles at a particular locus All chi square >6 are boxed in bold and are significant at $p < 0.05$. Results with marginal significance ($p < 0.01$) are boxed with a dashed line. All markers are listed in order along the chromosome from centromere to telomere.

Diabetic Marker	B6/B6	B6/CAST	CAST/CAST	Total	Chi Squared
D1Mit24	3	4	1	8	1.00
D1Mit8	4	4	1	9	2.11
D1Mit30	5	4	0	9	5.67
D1Mit33	4	4	0	8	4.00
D1Mit17	5	4	0	9	5.67
D2Mit61	3	2	2	7	1.57
D2Mit43	8	1	0	9	19.67
D2Mit58	3	5	1	9	1.00
D2Mit59	3	2	0	5	3.80
D3Mit21	4	3	2	9	1.89
D3Mit12	4	2	2	8	3.00
D3Mit14	4	2	2	8	3.00
D3Mit19	2	5	2	9	0.11
D4Mit5	0	4	1	5	2.20
D4Mit27	1	4	3	8	1.00
D4Mit31	1	5	3	9	1.00
D4Mit12	4	1	4	9	5.44
D4Mit40	2	3	4	9	1.89
D4Mit16	3	1	5	9	6.33
D4Mit72	1	3	5	9	4.56
D4Mit71	1	3	5	9	4.56
D4Mit70	1	3	5	9	4.56
D4Mit69	1	4	4	9	2.11
D4Mit68	1	4	4	9	2.11
Tnfr1	1	4	4	9	2.11
D4Mit33	1	4	3	8	1.00
D5Mit1	2	4	1	7	0.43
D5Mit41	4	4	1	9	2.11
D5Mit24	2	6	0	8	3.00
D6Mit46	7	0	0	7	21.00
D6Mit29	5	3	0	8	6.75
D6Mit39	4	2	2	8	3.00
D6Mit14	4	2	3	9	3.00
D7Mit57	5	4	0	9	5.67
D7Mit31	2	5	1	8	0.75
D7Mit43	2	6	1	9	1.22
D7Mit12	2	4	2	8	0.00
D8Mit1	2	2	4	8	3.00
D8Mit24	2	3	3	8	0.75
D8Mit8	1	4	3	8	1.00
D8Mit11	3	5	0	8	2.75
D8Mit35	2	5	1	8	0.75
D9Mit4	1	4	2	7	0.43
D9Mit31	2	5	1	8	0.75
D9Mit35	2	3	4	9	1.89
D9Mit18	2	4	2	8	0.00

Diabetic Marker	B6/B6	B6/CAST	CAST/CAST	Total	Chi Squared
D10Mit28	4	2	1	7	3.86
D10Mit3	6	2	1	9	8.33
D10Mit5	4	2	1	7	3.86
D10Mit21	3	2	4	9	3.00
D10Mit10	2	2	3	7	1.57
D10Mit14	1	3	5	9	4.56
D10Mit35	1	2	6	9	8.33
D11Mit1	0	7	2	9	3.67
D11Mit20	1	4	3	8	1.00
D11Mit5	1	3	5	9	4.56
D11Mit15	1	2	6	9	8.33
D11Mit33	2	1	6	9	9.00
D11Mit36	2	1	6	9	9.00
D11Mit38	3	1	5	9	6.33
D11Mit41	2	1	6	9	9.00
D11Mit54	0	5	4	9	3.67
D11Mit14	0	4	5	9	5.67
D12Mit9	1	6	2	9	1.22
D12Mit11	1	6	2	9	1.22
D12Mit46	1	3	5	9	4.56
D12Mit5	2	2	5	9	4.78
D12Mit7	2	3	4	9	1.89
D13Mit17	1	4	3	8	1.00
D13Mit9	1	3	4	8	2.75
D13Mit30	0	4	3	7	2.71
D14Mit10	2	3	4	9	1.89
D14Mit39	2	2	5	9	4.78
D14Mit35	1	4	4	9	2.11
D15Mit13	2	6	1	9	1.22
D15Mit29	3	4	2	9	0.33
D15Mit33	2	2	2	6	0.67
D15Mit42	1	5	3	9	1.00
D15Mit15	0	6	3	9	3.00
D16Mit29	0	5	4	9	3.67
D16Mit12	0	5	2	7	2.43
D16Mit6	2	3	3	8	0.75
D17Mit46	3	5	1	9	1.00
D17Mit21	4	4	1	9	2.11
D17Mit39	2	3	2	7	0.14
D17Mit41	2	3	2	7	0.14
D18Mit17	2	3	2	7	0.14
D18Mit7	1	3	2	6	0.33
D18Mit4	3	3	3	9	1.00
D19Mit1	1	3	5	9	4.56
D19Mit4	1	2	6	9	8.33
D19Mit10	3	0	6	9	11.00
D19Mit11	2	1	6	9	9.00
D19Mit14	2	3	4	9	1.89
D19Mit16	2	3	4	9	1.89

Insulin resistant+A36 Marker	B6/B6	B6/CAST	CAST/CAST	Total	Chi Squared
D1Mit8	3	6	2	11	0.27
D1Mit30	4	1	4	9	5.44
D1Mit33	3	3	5	11	3.00
D1Mit37	3	2	5	10	4.40
D1Mit115	1	3	5	9	4.56
D1Mit116	1	3	5	9	4.56
D1Mit17	3	2	6	11	6.09
D2Mit76	3	3	3	9	1.00
D2Mit79	2	3	4	9	1.89
D2Mit64	2	3	4	9	1.89
D2Mit72	2	2	5	9	4.78
D2Mit61	3	2	6	11	6.09
D2Mit92	1	4	4	9	2.11
D2Mit94	2	3	4	9	1.89
D2Mit58	2	4	5	11	2.45
D2Nds3	2	3	5	10	3.40
D2Mit59	2	3	5	10	3.40
D3Mit21	3	7	1	11	1.55
D3Mit12	2	5	3	10	0.20
D3Mit14	2	3	6	11	5.18
D3Mit19	1	8	2	11	2.45
D4Mit5	1	8	2	11	2.45
D4Mit31	1	7	3	11	1.55
D4Mit12	1	7	2	10	1.80
Tnfr1	3	7	0	10	3.40
D4Mit33	2	6	2	10	0.40
D5Mit1	1	9	1	11	4.45
D5Mit22	5	4	1	10	3.60
D5Mit41	6	4	1	11	5.36
D5Mit24	5	3	1	9	4.56
D6Mit48	9	0	0	9	27.00
D6Mit33	8	1	0	9	19.67
D6Mit29	6	1	0	7	13.86
D7Mit21	1	4	3	8	1.00
D7Mit57	1	7	2	10	1.80
D7Mit55	1	4	3	8	1.00
D7Mit31	1	3	2	6	0.33
D7Mit43	4	3	3	10	1.80
D7Mit12	3	3	2	8	0.75
D8Mit1	1	6	4	11	1.73
D8Mit24	0	7	2	9	3.67
D8Mit11	0	3	5	8	6.75
D8Mit35	3	3	4	10	1.80
D9Mit23	4	4	1	9	2.11
D9Mit4	6	3	1	10	6.60
D9Mit46	5	3	1	9	4.56
D9Mit21	6	4	1	11	5.36
D9Mit72	5	2	2	9	4.78
D9Mit31	6	2	3	11	6.09
D9Mit32	5	2	2	9	4.78
D9Mit73	5	2	2	9	4.78
D9Nds2	5	2	2	9	4.78
D9Mit9	6	2	2	10	6.80
D9Mit10	5	2	2	9	4.78
D9Mit35	7	1	2	10	11.40
D9Mit36	6	2	1	9	8.33
D9Mit18	6	3	2	11	5.18

Insulin resistant+A36					
Marker	B6/B6	B6/CAST	CAST/CAST	Total	Chi Squared
D10Mit28	3	2	5	10	4.40
D10Mit5	0	4	6	10	7.60
D10Mit21	0	8	1	9	5.67
D10Mit11	0	3	0	3	3.00
D10Mit14	2	8	1	11	2.45
D11Mit1	3	4	3	10	0.40
D11Mit20	2	4	2	8	0.00
D11Mit26	1	5	3	9	1.00
D11Mit5	0	1	7	8	16.75
D11Mit30	1	6	2	9	1.22
D11Nds1	1	5	1	7	1.29
D11Mit33	0	5	2	7	2.43
D11Mit36	1	4	2	7	0.43
D11Mit14	2	5	3	10	0.20
D12Mit9	4	3	2	9	1.89
D12Mit11	5	6	0	11	4.64
D12Mit46	5	4	2	11	2.45
D12Mit5	3	5	1	9	1.00
D12Mit7	4	7	0	11	3.73
D13Mit17	2	6	3	11	0.27
D13Mit9	3	2	4	9	3.00
D13Mit30	2	6	3	11	0.27
D14Mit10	1	6	4	11	1.73
D14Mit39	2	5	2	9	0.11
D14Mit36	3	5	3	11	0.09
D15Mit13	3	7	1	11	1.55
D15Mit49	3	6	0	9	3.00
D15Nds1	3	5	1	9	1.00
D15Mit29	4	4	1	9	2.11
D15Mit33	4	3	2	9	1.89
D15Mit42	4	3	4	11	2.27
D16Mit29	1	6	1	8	2.00
D16Mit12	5	5	1	11	3.00
D16Mit48	4	4	3	11	1.00
D16Mit6	3	5	1	9	1.00
D17Mit46	3	5	3	11	0.09
D17Mit21	1	5	3	9	1.00
D17Mit39	2	7	2	11	0.82
D17Mit41	2	7	1	10	1.80
D18Mit17	2	5	4	11	0.82
D18Mit7	2	7	2	11	0.82
D19Mit1	1	9	1	11	4.45
D19Mit10	2	6	0	8	3.00

D4Mit16, approximately 12 cM telomeric of *db*, demonstrated a paucity of heterozygotes with 3 B6/B6, 1 B6/CAST, and 5 CAST/CAST within the diabetic class. No distortion of genotypes was observed in the insulin resistant class. The three markers telomeric of D4Mit16 - D4Mit72, D4Mit71, and D4Mit70 - demonstrated enrichment of CAST alleles within the diabetic class with 1 B6/B6, 3 B6/CAST, and 5 CAST/CAST and may have been closer to the true location of the QTL although the relationship of these markers to phenotypic class was not statistically significant ($p>0.05$).

D8Mit11, approximately 10 cM telomeric of *fat* and a QTL for plasma [insulin] in the *ob/ob* F2 B6DBA, demonstrated enrichment of CAST homozygotes in the insulin resistant class with 0 B6/B6, 3 B6/CAST, and 5 CAST/CAST. No linkage disequilibrium was observed in the diabetic class.

Several markers along the length of chromosome 9 demonstrated enrichment of B6 alleles within the insulin resistant class (6 B6/B6, 2 B6/CAST, 1 CAST/CAST for D9Mit36). The variability in the statistical significance for the markers was largely a result of incomplete genotypic information. Most of the markers for which only nine animals were typed failed to demonstrate statistical significance at $p<0.05$. Therefore, a 28 cM interval from D9Mit4 to D9Mit36 was potentially implicated for a QTL.

Two centromeric markers on chromosome 10, D10Mit3 and D10Mit5, demonstrated enrichment of opposite alleles within the diabetic and insulin resistant classes. At

D10Mit3 there were 6 B6/B6, 2 B6/CAST, and 1 CAST/CAST within the diabetic class. Conversely, within the insulin resistant class, there were 0 B6/B6, 4 B6/CAST, and 6 CAST/CAST at D10Mit5. This was the second example in this cross of a potentially powerful locus demonstrating transgression.

Markers over a 14 cM interval on mid chromosome 11 demonstrated enrichment of CAST alleles within both the insulin resistant and diabetic classes. For D11Mit5, there were 0 B6/B6, 1 B6/CAST, and 7 CAST/CAST in the insulin resistant class. For D11Mit15, less than 1 cM from D11Mit5, there were 1 B6/B6, 2 B6/CAST, and 6 CAST/CAST within the diabetic class. Because the same alleles were enriched in BOTH classes, it was unlikely that this region of chromosome 11 was involved in disease susceptibility.

Three markers on the mid to distal part of chromosome 19 demonstrated deviation from expected Mendelian ration in the diabetic class. For D19Mit4 there were 1 B6/B6, 2 B6/CAST, and 6 CAST/CAST in the diabetic class, but no significant genotypic deviations in the insulin resistant class.

ANOVA

To ensure that there was no linkage disequilibrium within the obese animals as a whole for any of the regions of interest (as might have been the case for chromosome 11 where both the diabetic and insulin resistant animals were enriched for CAST alleles), markers located

near the peak of the QTL based on the initial scanning experiments were used to type a panel of 50 randomly selected lean animals from the cross. D1Mit17, D4Mit16, D7Mit57, D8Mit11, D9Mit4, D9Mit31, D9Mit35, D10Mit3, D11Mit15, D11Mit33, D11Mit38, D19Mit4, D19Mit10, and D19Mit11 demonstrated no linkage disequilibrium within the 50 randomly selected lean progeny.

At the time this cross was originally analyzed, genotyping was considerably more labor intensive. Therefore, as a screening device to determine which loci were likely to be most relevant, the first 50 *ob/ob* animals from the cross were typed with D1Mit17, D2Mit61, D4Mit58, D4Mit12, D4Mit16, Tnfr1 (chromosome 4), D6Mit54, D7Mit57, D7Mit12, D8Mit11, D9Mit35, D10Mit3, D11Mit15, and D19Mit10. The selection of the majority of the markers was based upon the genome scanning experiments; however, D6Mit54 was selected on the basis of its effect on plasma [insulin] and plasma [glucose] in the *db/db* F2 B6CAST cross (see chapter 9). Because *ob* was located on chromosome 6, all *ob/ob* animals necessarily demonstrated a distortion of Mendelian ratios for any regions linked to *ob* on chromosome 6. Because *db* was located on chromosome 4, crosses segregating *db* were used to localize QTL regions on chromosome 6 and, conversely, *ob* crosses were used to localize comparable regions on chromosome 4.

For all markers except D1Mit17 and D8Mit11 there was a genotype effect on one of the three phenotypes (BMI, plasma [glucose] or plasma [insulin]). The results based on only

50 animals were not statistically significant, so the rest of the progeny were genotyped for the remaining twelve markers.

Of the eight different chromosomal regions tested, four represented by D7Mit57, D9Mit4, D9Mit31, D9Mit35, D9Mit4, D11Mit15, D11Mit33, D11Mit38, D19Mit10, and D19Mit11 demonstrated no statistically significant effect of genotype on BMI, or overnight fasting plasma [insulin] or [glucose].

Three of the four regions which did demonstrate an effect of genotype on phenotype each affected a different phenotype primarily.

Progeny who were CAST/CAST at D2Mit61 demonstrated approximately 20% decreases in overnight fasting plasma [glucose] compared to animals of either sex which were B6/B6 at this locus ($p < 0.0001$) (Table 31 and Figure 73). CAST alleles acted recessively to protect the animals from diabetes. Although the correlation between genotype and BMI was not as good, CAST/CAST females were lighter than either the heterozygotes or B6/B6 ($p < 0.05$). Unlike many of the other loci described, the most diabetic class was also the heaviest class, possibly a reflection of the age at which the animals were studied. Animals in this cross were on average only 70 days old at the time of sacrifice, compared to 110-120 days for the more recent studies. Therefore, the disease progression may have been such that the animals which were initially the heaviest were the ones that went on to become diabetic and subsequently would have been the lightest if

they had been followed longer. D2Mit61 was located in the same region as the centromeric chromosome 2 locus previously defined in the *ob/ob* F2 B6DBA animals (Figure 74). In the *ob/ob* F2 B6DBA,

Locus	Phenotype	Female		Male		p
		B6/B6	B6/CAST	B6/B6	B6/CAST	
D2Mit61	BMI (g/cm2)	0.536	0.508	0.507	0.511	0.05
	Plasma [glucose] (mg/dl)	706	676	745	729	0.00007
D4Mit58	BMI (g/cm2)	0.488	0.472	0.483	0.469	0.0027
D4Mit12	BMI (g/cm2)	0.486	0.473	0.471	0.473	0.00001
D4Mit16	BMI (g/cm2)	0.501	0.485	0.496	0.471	0.0012
	Plasma [insulin] (uU/ml)	242.8	212.5	376.5	212.9	0.0446
TNFR1	BMI (g/cm2)	0.483	0.468	0.481	0.467	0.0002
D6Mit54	BMI (g/cm2)	0.481	0.469	0.471	0.475	0.022
	Plasma [insulin] (uU/ml)	611.3	194.4	286.7	258.1	0.037
D10Mit3	Plasma [insulin] (uU/ml)	236.6	310.4	241.8	249.1	0.0022

Table 31. Analysis of variance by sex and genotype at the marker designated in *ob/ob* F2B6CAST. All traits for all markers demonstrating statistical significance are summarized. Mean values for each trait are listed by genotypic class.

F2 *ob/ob* B6CAST

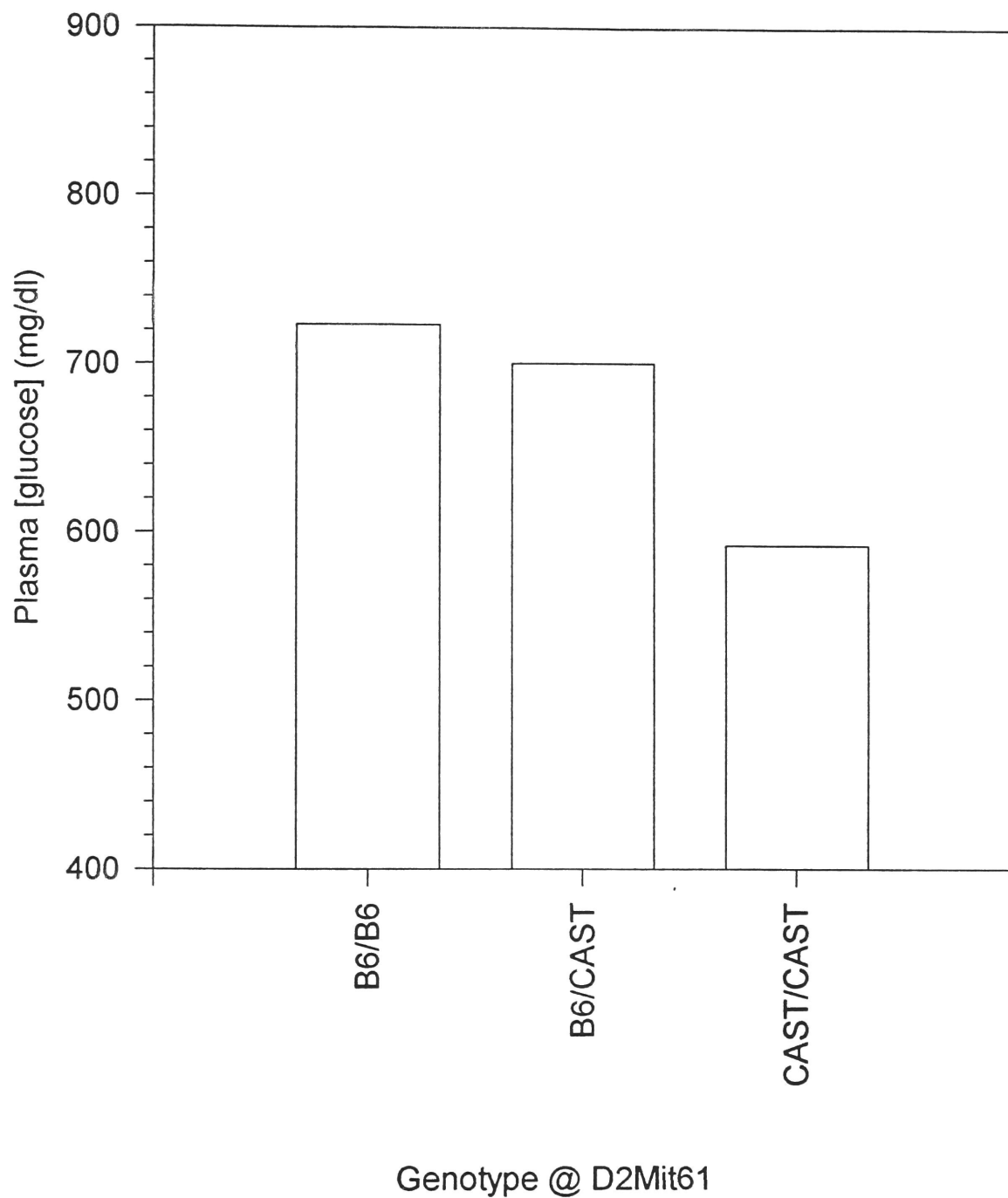
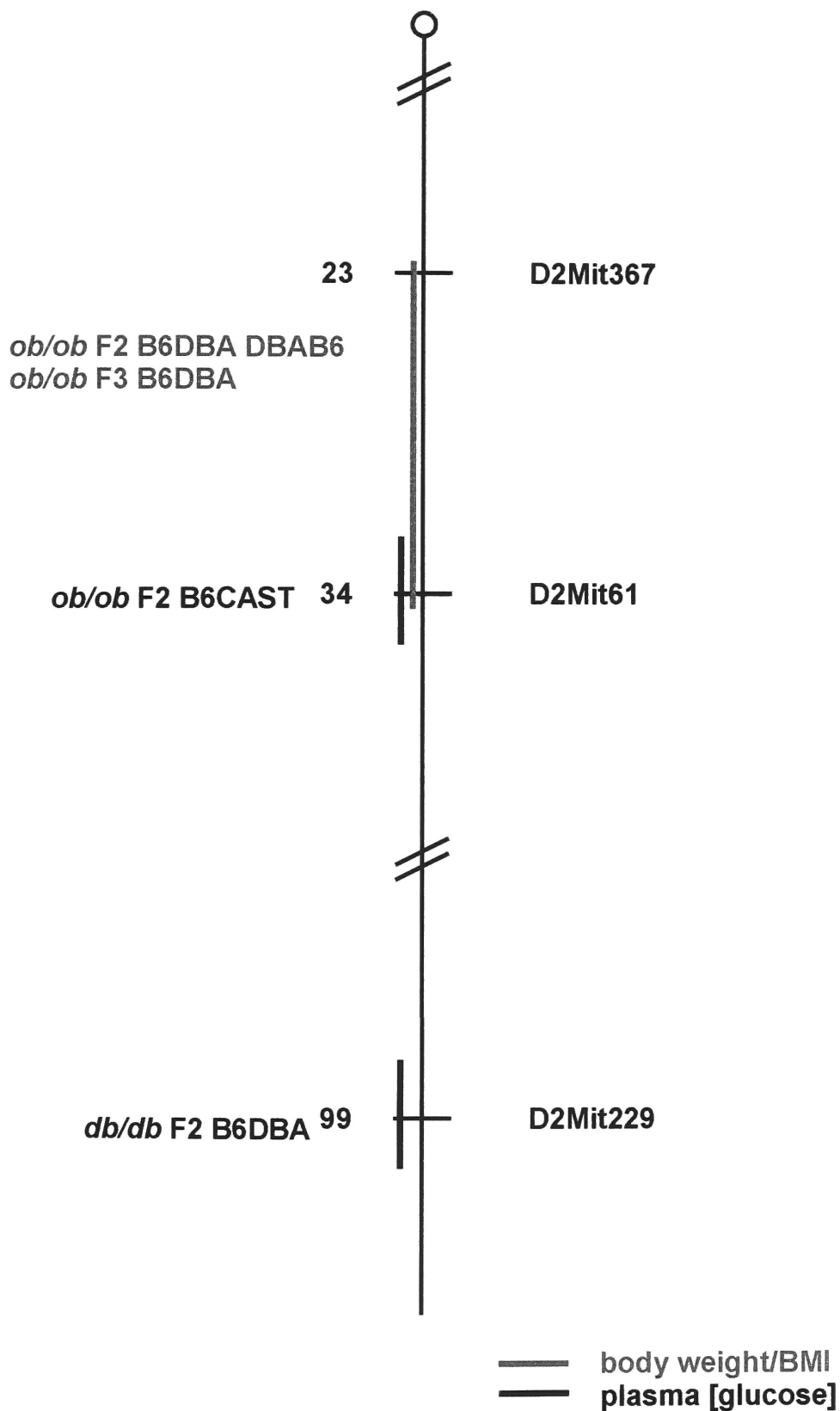


Figure 73. Effect of genotype at D2Mit61 on overnight fasting plasma [glucose] in *ob/ob* F2 B6CAST. Means for each genotypic group are indicated by heights of the bars.

Figure 74. Summary of the QTLs for obesity/diabetes on mouse chromosome 2. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 2

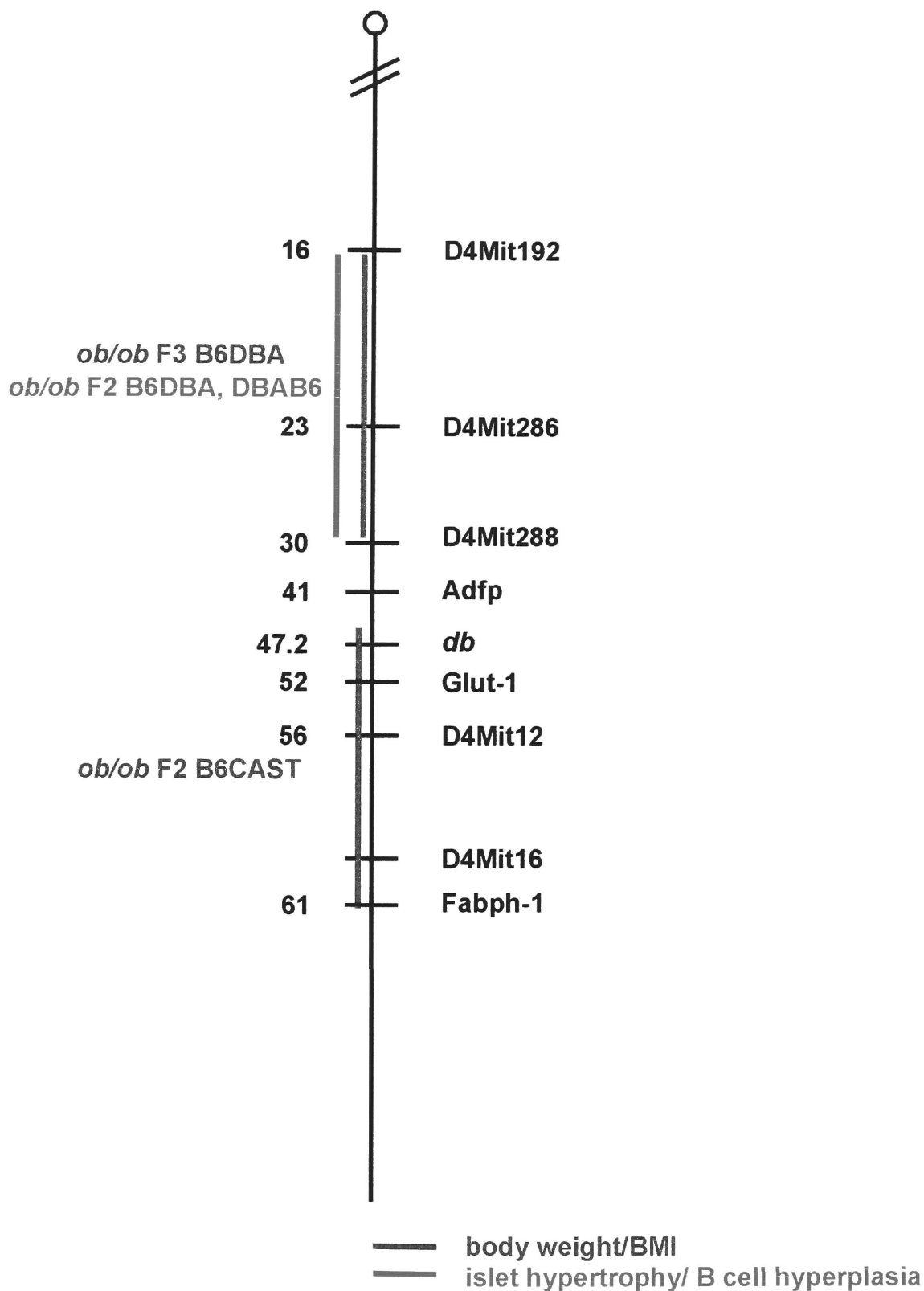


DBA alleles were associated with increased BMI which may have been equivalent to the decreased plasma [glucose] associated with CAST/CAST in the *ob/ob* F2 B6CAST.

Of the four markers on chromosome 4 which demonstrated a relationship of genotype to BMI, D4Mit12 appeared to be the closest to the responsible gene ($p < 0.00001$). In both sexes, CAST/CAST had BMIs 8.5% less than B6/B6. The location of the chromosome 4 QTL in the *ob/ob* F2 B6CAST did not coincide with the QTL from the *ob/ob* F2 B6DBA which primarily influenced pancreatic hypertrophy and was located more centromerically on chromosome 4 (Figure 75). Therefore, there are likely to be two separate QTLs located on chromosome 4, as has been shown for chromosomes 1 and 2. The candidate gene tumor necrosis factor receptor 1 (*Tnfr1*) was scored in this cross by sequencing the 3' untranslated region (UTR) of this gene in B6 and CAST mice. A G=>T substitution in the CAST allele which destroyed a *Sau 96* (GGNCC) restriction site was used to score the respective alleles. Another A=>G substitution was detected in the CAST progenitor, but was not directly used in scoring the animals.

Figure 75. Summary of the QTLs for obesity/diabetes on mouse chromosome 4. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 4



Tnfr1 is a good candidate gene based on reports that circulating concentration of TNF- α were elevated in *ob/ob* and *db/db* mice and *fa/fa* rats, and associated with insulin resistance compared to lean littermates (Hotamisligil et al. 1993). This effect may be paracrine due to adipocytes in skeletal muscle. Administration of solubilized TNF receptor immunoglobulin chimeric protein reduced insulin resistance as demonstrated by increased glucose disposal in insulin clamp experiments. Because the elevation in plasma concentrations of TNF- α could have been due to diminished receptor activity, it was useful to test the location of Tnfr1 in the *ob/ob* F2 B6CAST which demonstrated a QTL on chromosome 4. Based on the lower statistical association of the Tnfr1 polymorphism with BMI compared to D4Mit12, it is unlikely that Tnfr1 was the gene responsible for the effect seen on BMI (Figure 76). Additionally, if the elevations of circulatory TNF- α observed by Hotamisligil were associated with insulin resistance and Tnfr1 was the gene responsible for the elevations in TNF- α , some effect of the genotype should have been observed on plasma [glucose] or [insulin].

F2 *ob/ob* B6CAST

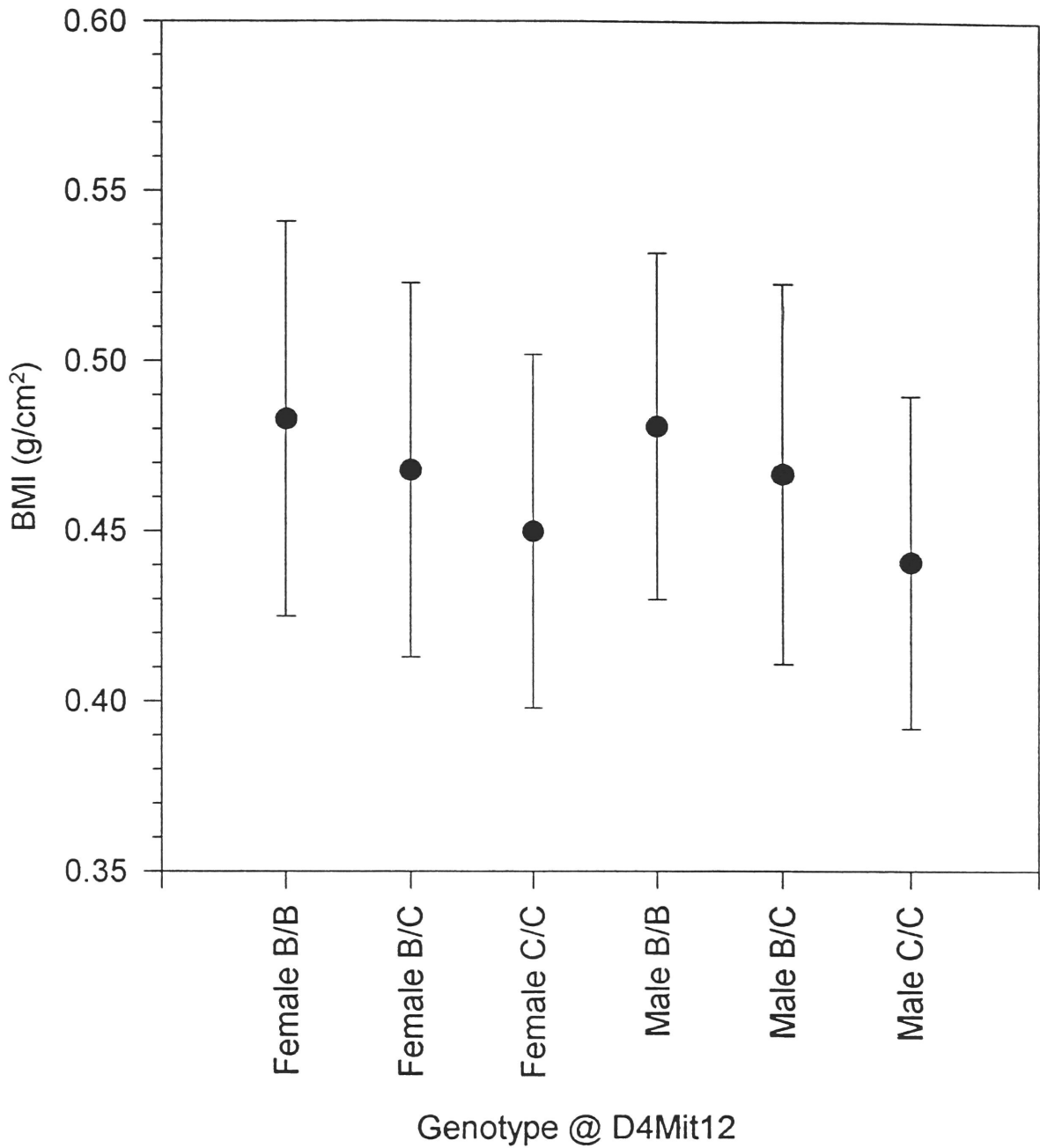


Figure 76. Effect of genotype at D4Mit12 on BMI in *ob/ob* F2 B6CAST separated by sex. Means indicated by the dot and standard deviations by the error bars.

The locus on chromosome 6 was associated with both BMI and plasma [insulin] to approximately the same extent. CAST/CAST alleles were associated with 11% lower BMI, and 45% lower plasma [insulin] in both sexes (Figure 77). This was similar to the effect of this locus observed in the *db/db* F2 B6CAST which will be reported in the following chapter.

B6/B6 animals at D10Mit3 demonstrated decreases in overnight fasting plasma [insulin] of 35-45% compared to CAST/CAST of the same sex (Table 31 and Figure 78). Although many of the B6/B6 animals clustered in the upper left quadrant with lowest plasma [insulin] and highest plasma [glucose], there were clearly animals of the same genotype in all four quadrants, emphasizing the fact that other genes must be involved in diabetes progression in this cross.

Figure 77. Summary of the QTLs for obesity/diabetes on mouse chromosome 6. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 6

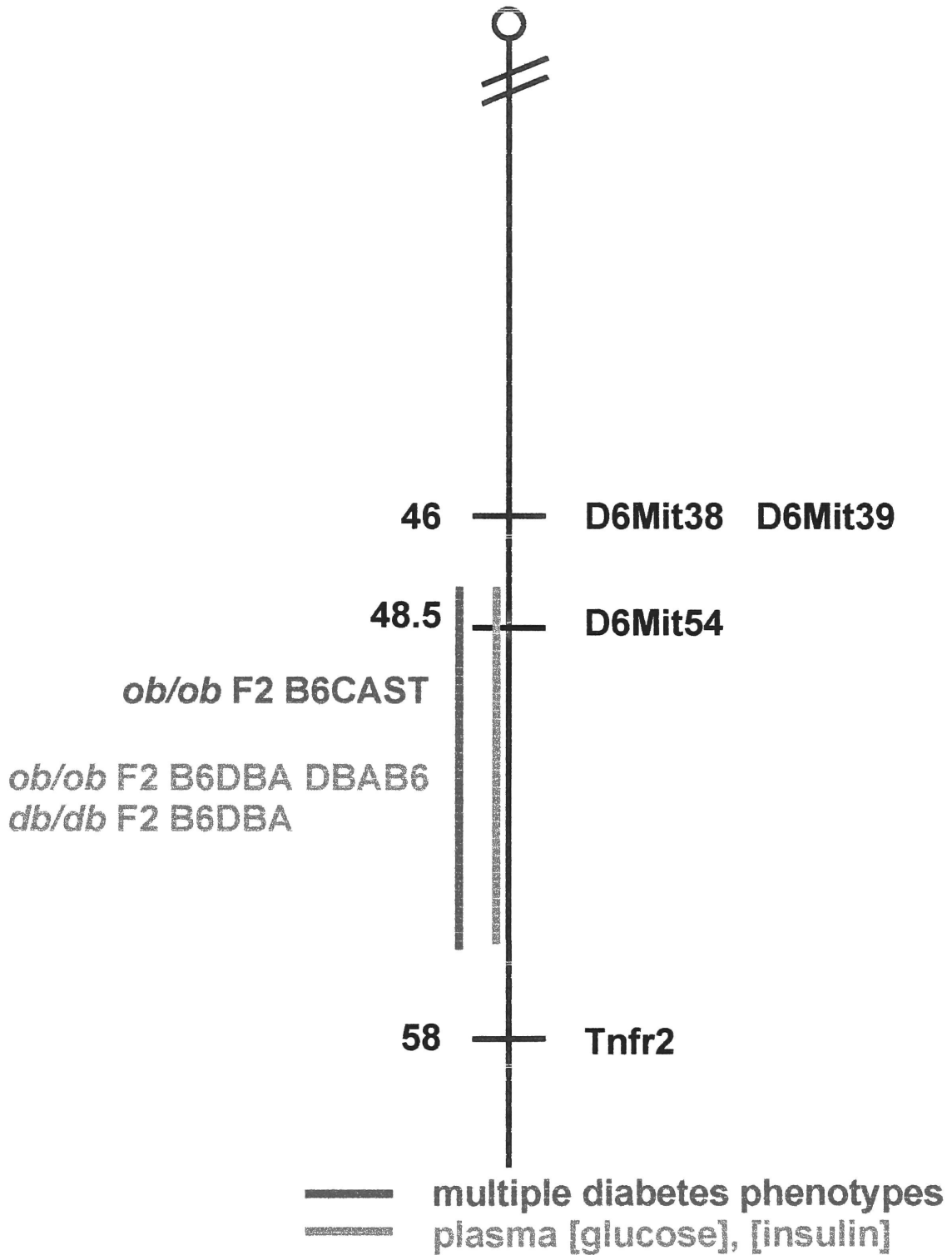
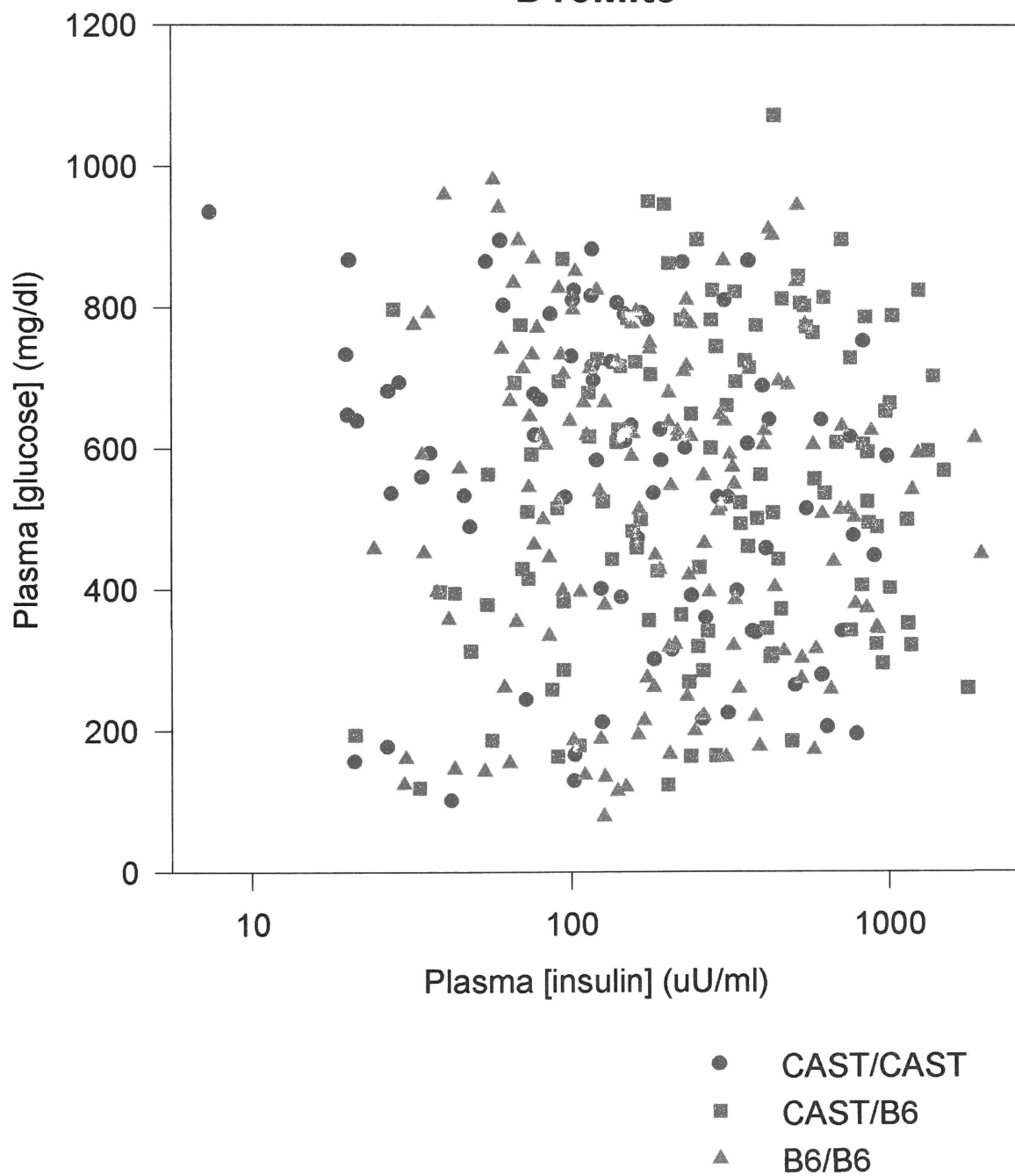


Figure 78. Effect of genotype at D10Mit3 on overnight fasting plasma [glucose] and [insulin] in *ob/ob* F2 B6CAST. Each symbol represents a single animal.

F2 *ob/ob* B6CAST

D10Mit3



Discussion

In the *ob/ob* F2 B6CAST additional regions on chromosomes 4 relating to BMI and on chromosome 10 correlated with overnight fasting plasma [insulin] were identified. One locus on chromosome 2 affecting overnight fasting plasma [glucose] was mapped to the same region implicated for this phenotype in the *ob/ob* F2 B6DBA and represents independent confirmation of this locus with a different (CAST) counterstrain. Finally, the locus on chromosome 6 identified in the *db/db* F2 B6CAST (and discussed in the following chapter) was confirmed although the number of CAST/CAST animals at this locus was small due to the proximity of D6Mit54 to *ob*.

The *ob/ob* F2 B6CAST cross demonstrates some important differences from the more recent phenotypic and genotypic characterizations. Phenotypically, because the animals were not originally studied for diabetes phenotype, overnight fasting concentrations of insulin and glucose were determined. Because the animals were fasted for 16-18 hours, plasma insulin concentrations should have been (and were) negligible in non-obese animals. Within the obese animals, elevated concentrations of insulin and glucose after such a relatively prolonged fast likely represent the effects of increased hepatic gluconeogenesis due to the metabolic stress of fasting in context of insulin resistance. In contrast, the more recently studied mice and rats have only been fasted for 3-4 hours, and plasma insulin and glucose concentrations probably reflect both the effects of delayed post-prandial nutrient clearance as well as hepatic glucose production.

The second phenotypic difference between the *ob/ob* F2 B6CAST cross and the more recent crosses is the age of the animals at sacrifice. Progeny in the earlier crosses were terminated at considerably younger ages, and may represent an opportunity to identify genes which control the earlier stages of the disease. Ultimately, both ages as well as intervening ages should be examined by longitudinal studies of the same animals. However, destructive experiments such as the pancreatic morphology are only possible as terminal events. Therefore, the age of sacrifice should be optimized according to the stage at which the genes of greatest interest are likely to produce the most profound phenotypic differences. Although it may be difficult to determine this a priori, there may be substantial advantages (aside from cost) to studying at least a subset of a cross of animals from 40-70 days of age, during the early stages of disease progression.

The animals selected for the two genome scanning experiments were chosen based upon the sole phenotypic criterion of overnight fasting plasma [insulin]. This single criterion was probably not sufficiently accurate to produce robust results in the scanning experiment. Whereas, 75-90% of the region identified on the initial genome scans usually represent QTLs after complete genotypic work up, only half of the regions identified in this cross were statistically significant after typing all of the obese progeny.

Additionally, the regions on chromosome 1 and 8 which were typed in only 50 obese progeny now deserve reconsideration, especially in light of the identification of the

telomeric chromosome 1 QTL which was independently detected in both *ob/ob* F2 B6DBA and *db/db* F2 B6DBA and confirmed in the corresponding F3 progeny.

Chapter 9

Identification of diabetes susceptibility regions

in

F2 B6CAST segregating for *db*

Selective genotyping selection criterion

The F2 B6CAST progeny segregating for *db* were produced by using animals with ovarian transplants from *db/db* B6 N5 females mated to CAST males to produce *db/+* F1 progeny which were then intercrossed . A total of 577 *db/db* F2 progeny, 278 males and 299 females were produced. Genotype for the *db* locus was determined as previously described (Lee et al. 1996). Because these animals were generated for the purpose of cloning *db* rather than studying the diabetes phenotype, the animals were less extensively characterized phenotypically (BMI, fasting plasma [insulin] and [glucose]). The animals were fasted overnight rather than for 3 hours as with the more recent crosses.

Animals were selected for the insulin-resistant or diabetic groups on the basis of overnight fasting plasma [insulin] and [glucose]. Twelve insulin resistant animals were selected with plasma [insulin] > 1.5 standard deviations above the mean of all the obese progeny (966.7 μ U/ml) and plasma [glucose] < mean (655.1 mg/dl) (Table 32). Seventeen animals with plasma [glucose] > one standard deviation above the mean (866 mg/dl) and plasma [insulin] < 200 μ U/ml were selected as the diabetic class. The group of insulin resistant animals was comprised largely of females (8/12) but the diabetic class contained equal numbers of males (9) and females (8).

Table 32. Phenotypic characteristics of *db/db* F2 B6CAST used in each genome scanning experiment. The average for the group for each phenotypic parameter is indicated below the individual animals.

ID	Age (days)	Sex	Body weight (g)	BMI (g/cm2)	Plasma [insulin] uU/ml	Plasma [glucose] mg/dl
Insulin resistant						
1310	70	F	41.5	0.536	1067	530
1317	72	F	38.9	0.538	1077.6	596
1347	66	M	39.0	0.481	1089	357
1348	66	F	35.4	0.490	1236.2	484
1385	74	M	48.4	0.598	1433.4	540
1389	70	F	44.4	0.548	1150.4	341
1395	58	M	40.1	0.495	1103.8	462
1403	68	F	40.2	0.556	1609.8	564
1479	66	F	37.1	0.580	1076	321
2002	74	F	36.3	0.448	1423.2	256
2010	68	F	34.8	0.544	1279.8	374
2037	70	M	32.9	0.406	1168.8	332
Average	69		39.1	0.518	1226.3	430
Diabetic						
1001	94	F	45.1	0.624	139.9	914
1318	58	F	44.8	0.553	197.8	970
1322	62	M	34.0	0.471	92	1064
1337	65	M	38.2	0.529	153	972
1345	57	M	32.2	0.398	51	1096
1351	65	M	26.1	0.464	105.4	1000
1366	74	M	32.3	0.574	152.8	1004
1398	76	M	38.8	0.537	163.8	906
1424	64	F	28.3	0.578	48	906
1426	78	F	36.2	0.447	139.8	922
1429	64	F	36.1	0.500	192.4	902
1434	69	F	31.2	0.555	64.4	934
1448	58	F	32.4	0.506	82	928
1471	56	M	29.0	0.401	113.2	914
1494	66	M	37.8	0.447	98.6	918
2041	81	F	28.4	0.444	74.4	900
2049	71	F	41.5	0.512	142.4	908
Average	68		34.8	0.502	118.3	950

Phenotypic extreme Parameters for selection

Insulin resistant Plasma [insulin] > 966.7 µU/ml Plasma [glucose] <655.1 mg/dl

Diabetic Plasma [insulin] < 200 µU/ml Plasma [glucose] >866 mg/dl

Results

Phenotypic characterization

The *db/db* F2 B6CAST were shorter, lighter than crosses to DBA or Ckc, similar to the *ob/ob* F2 B6CAST and were equally obese (based on BMI) to obese progeny from other crosses (Table 33). The obese animals were readily distinguishable from the lean for all three phenotypic parameters (Table 33). As for the *ob/ob* F2 B6CAST, there was little sexual dimorphism in the *db/db* F2 B6CAST (Figures 79 and 80). Extensive variability in phenotype existed in the cross among both the obese and lean progeny (Figures 79 and 80).

Phenotype	Lean Female	Male	Obese Female	Male
Age	65 (10)	64 (9)	65 (10)	66 (11)
Body weight (g)	18.4 (2.9)	23.0 (4.0)	35.6 (6.1)	37.3 (6.8)
BMI (g/cm ²)	0.262 (0.033)	0.298 (0.039)	0.499 (0.069)	0.500 (0.073)
Fasting plasma [insulin] uU/ml	40.3 (56.1)	88.7 (115.0)	422.5 (354.1)	413.0 (373.4)
Fasting plasma [glucose] mg/dl	213 (76)	244 (100)	646 (198)	664 (223)

Table 33. Phenotypic characteristics of lean and obese F2 B6CAST segregating for *db*, separated by sex. Means for groups are indicated with standard deviations in parentheses.

Figure 79. BMI vs fasting plasma [insulin] of lean and obese F2 B6CAST segregating for *db*. Each point represents a single animal.

F2 B6CAST

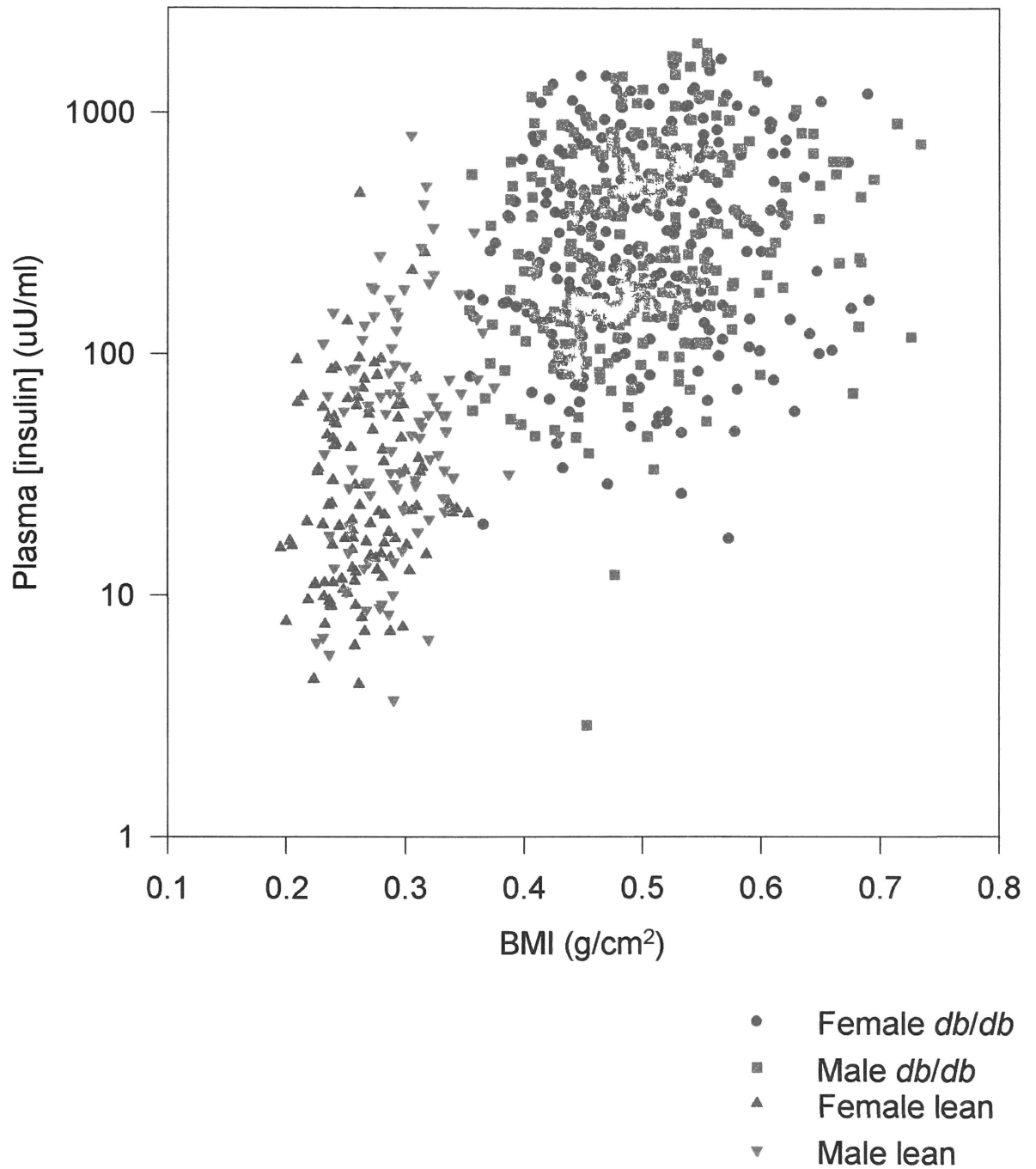
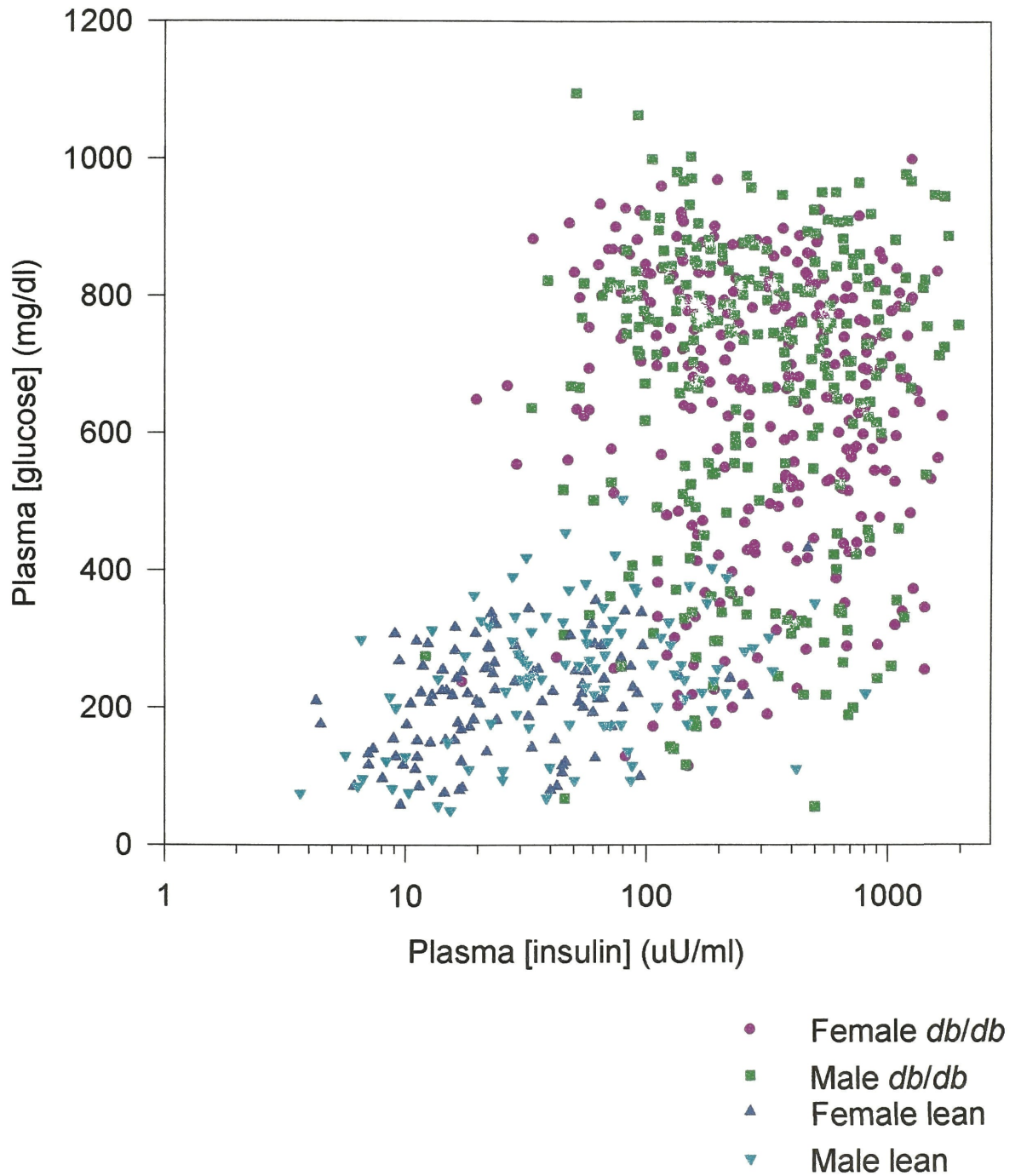


Figure 80. Fasting plasma [glucose] vs fasting plasma [insulin] of lean and obese F2 B6CAST segregating for *db*. Each point represents a single animal.

F2 B6CAST



Genome scanning results

Between 96 and 109 markers were used to genotype the animals at the extremes of phenotype for the genome scanning experiment (Table 34).

For the majority of markers on chromosome 6 the insulin resistant group demonstrated enrichment of B6 alleles and conversely, the diabetic group enrichment of CAST alleles. Both groups were significant at $p < 0.01$, making it extremely likely that a QTL influencing NIDDM was present on this chromosome. On the basis of the insulin resistant animals, the QTL was located between D6Mit16 centromerically and D6Mit54 telomerically. On the basis of the diabetic animals, the QTL localized between D6Mit38 centromerically and D6Mit54 telomerically approximately 41 cM telomeric of *ob*.

Markers on proximal chromosome 7 demonstrated enrichment of B6 alleles within the diabetic class at $p < 0.01$. Based on the scanning animals, the boundaries of the region containing the QTL extended from D7Mit76 centromerically to D7Mit55 telomerically. For a more telomeric marker on chromosome 7 (D7Mit43), there was enrichment of B6 alleles within the insulin resistant class ($p < 0.05$). However, it is unlikely that both regions are part of the same QTL since B6 alleles should then have been predominant in both the insulin resistant animals as well as the extremely diabetic animals.

Table 34. Genome scanning results for the twelve *db/db* F2 B6CAST insulin resistant and eighteen diabetic *db/db* F2 B6CAST. B6/B6 indicates homozygosity for B6 alleles, B6/CAST for heterozygosity and CAST/CAST homozygosity for CAST alleles at a particular locus. All chi square >6 are boxed in bold and are significant at $p < 0.05$. All markers are listed in order along the chromosome from centromere to telomere.

Insulin resistant Marker	B6/B6	B6/CAST	CAST/CAST	Total	Chi-squared
D1Mit8	2	4	5	11	2.45
D1Mit33	4	5	2	11	0.82
D1Mit17	3	5	3	11	0.09
D2Mit61	3	8	1	12	2.00
D2Mit43	2	4	5	11	2.45
D2Nds3	2	6	3	11	0.27
D2Mit59	1	5	3	9	1.00
D3Mit21	4	4	2	10	1.20
D3Mit12	2	5	1	8	0.75
D3Mit14	1	7	2	10	1.80
D3Mit19	1	6	4	11	1.73
D4Mit5	5	6	1	12	2.67
D4Mit31	12	0	0	12	36.00
D4Mit33	7	4	0	11	9.73
D5Mit1	2	6	2	10	0.40
D5Mit22	3	5	0	8	2.75
D5Mit41	5	5	1	11	3.00
D5Mit24	4	6	1	11	1.73
D6Mit50	7	2	3	12	8.00
D6Mit46	7	1	3	11	10.27
D6Mit16	8	4	0	12	12.00
D6Mit33	8	3	1	12	11.17
D6Mit21	8	4	0	12	12.00
D6Mit29	6	2	0	8	11.00
D6Mit40	8	4	0	12	12.00
D6Mit65	8	4	0	12	12.00
D6Mit38	7	4	1	12	7.33
D6Nds5	5	3	0	8	6.75
D6Mit11	8	3	1	12	11.17
D6Mit54	8	4	0	12	12.00
Tnfr2	8	3	1	12	11.17
D6Mit55	8	3	1	12	11.17
D6Mit44	5	3	1	9	4.56
D6Mit24	8	3	1	12	11.17
D6Mit14	4	6	1	11	1.73
D7Mit21	3	6	3	12	0.00
D7Mit57	1	6	5	12	2.67
D7Mit55	3	3	3	9	1.00
D7Mit31	1	6	2	9	1.22
D7Mit43	6	6	0	12	6.00
D7Mit12	3	5	0	8	2.75
D8Mit1	3	7	2	12	0.50
D8Mit24	0	8	4	12	4.00
D8Mit40	1	5	4	10	1.80
D8Mit83	1	7	3	11	1.55
D8Mit11	2	7	1	10	1.80
D8Mit33	3	7	2	12	0.50
D8Mit86	2	7	2	11	0.82
D8Mit35	2	9	1	12	3.17
D9Mit23	7	2	2	11	9.00
D9Mit4	6	3	2	11	5.18
D9Mit46	8	3	1	12	11.17
D9Mit21	7	3	2	12	7.17
D9Mit72	7	4	1	12	7.33
D9Mit31	8	3	1	12	11.17
D9Mit32	6	4	1	11	5.36
D9Mit73	6	5	1	12	4.50
D9Nds2	6	5	1	12	4.50

Insulin resistant Marker	B6/B6	B6/CAST	CAST/CAST	Total	Chi-squared
D9Nds2	7	4	1	12	7.33
D9Mit9	7	4	1	12	7.33
D9Mit10	6	5	1	12	4.50
D9Mit35	3	6	2	11	0.27
D9Mit36	7	4	1	12	7.33
D9Mit18	5	6	1	12	2.67
D10Mit28	3	5	1	9	1.00
D10Mit5	2	4	5	11	2.45
D10Mit21	3	6	2	11	0.27
D10Mit11	3	6	3	12	0.00
D10Mit14	3	8	1	12	2.00
D11Mit1	3	4	4	11	1.00
D11Mit20	3	4	4	11	1.00
D11Mit26	3	4	4	11	1.00
D11Mit5	3	1	6	10	8.20
D11Mit30	3	5	4	12	0.50
D11Nds1	2	6	4	12	0.67
D11Mit33	1	7	4	12	1.83
D11Mit36	1	5	4	10	1.80
D11Mit14	1	5	4	10	1.80
D12Mit9	3	6	3	12	0.00
D12Mit11	4	7	1	12	1.83
D12Mit46	3	6	2	11	0.27
D12Mit5	4	7	1	12	1.83
D12Mit7	4	7	1	12	1.83
D13Mit17	1	8	3	12	2.00
D13Mit9	3	3	6	12	4.50
D13Mit30	3	3	5	11	3.00
D14Mit10	5	4	2	11	2.45
D14Mit39	4	1	2	7	4.71
D14Mit35	5	5	1	11	3.00
D15Mit13	2	10	0	12	6.00
D15Mit49	0	4	2	6	2.00
D15Mit46	0	7	5	12	4.50
D15Nds1	1	5	5	11	3.00
D15Mit29	2	4	4	10	1.20
D15Mit33	2	5	2	9	0.11
D15Mit42	4	3	3	10	1.80
D16Mit29	3	3	2	8	0.75
D16Mit12	3	4	4	11	1.00
D16Mit6	1	3	4	8	2.75
D17Mit48	5	5	2	12	1.83
D17Mit46	3	7	2	12	0.50
D17Mit21	1	9	1	11	4.45
D17Mit39	2	7	2	11	0.82
D17Mit41	4	4	3	11	1.00
D18Mit17	0	8	3	11	3.91
D18Mit7	0	8	2	10	4.40
D19Mit1	4	3	4	11	2.27
D19Mit10	3	5	3	11	0.09
DXMit19	4	4	4	12	1.33

Diabetic Marker	B6/B6	B6/CAST	CAST/CAST	Total	Chi Squared
D1Mit8	4	8	4	16	0.00
D1Mit33	5	9	3	17	0.53
D1Mit17	5	10	2	17	1.59
D2Mit61	6	10	1	17	3.47
D2Mit58	6	7	3	16	1.38
D2Nds3	7	8	2	17	3.00
D2Mit59	3	11	1	15	3.80
D3Mit21	3	9	3	15	0.60
D3Mit12	2	9	6	17	1.94
D3Mit14	4	7	6	17	1.00
D3Mit19	4	9	4	17	0.06
D4Mit5	8	9	0	17	7.59
D4Mit31	17	0	0	17	51.00
D4Mit33	11	3	1	15	18.73
D5Mit1	2	10	4	16	1.50
D5Mit22	3	5	6	14	2.43
D5Mit41	6	5	6	17	2.88
D5Mit24	4	6	6	16	1.50
D6Mit50	0	11	5	16	5.38
D6Mit46	0	11	6	17	5.71
D6Mit33	0	12	4	16	6.00
D6Mit16	1	9	6	16	3.38
D6Mit21	0	8	9	17	9.59
D6Mit29	1	6	6	13	3.92
D6Mit40	0	8	9	17	9.59
D6Mit65	1	6	10	17	11.00
D6Mit38	0	7	10	17	12.29
D6Nds5	0	5	8	13	10.54
D6Mit11	0	7	10	17	12.29
D6Mit54	0	7	10	17	12.29
Tnfr2	0	8	9	17	9.59
D6Mit55	0	8	9	17	9.59
D6Mit44	0	8	9	17	9.59
D6Mit24	0	8	9	17	9.59
D6Mit14	3	6	8	17	4.41
D7Mit21	7	9	0	16	6.38
D7Mit56	9	5	1	15	10.20
D7Mit76	11	4	1	16	16.50
D7Mit57	11	5	1	17	14.65
D7Mit77	11	4	1	16	16.50
D7Mit72	10	5	2	17	10.41
D7Mit55	11	4	1	16	16.50
D7Mit52	9	5	1	15	10.20
D7Mit31	2	6	6	14	2.57
D7Mit43	2	9	5	16	1.38
D7Mit12	1	8	7	16	4.50
D8Mit1	4	11	2	17	1.94
D8Mit24	4	11	2	17	1.94
D8Mit11	5	8	3	16	0.50
D8Mit35	7	4	6	17	4.88

Diabetic Marker	B6/B6	B6/CAST	CAST/CAST	Total	Chi Squared
D9Mit4	2	13	2	17	4.76
D9Mit31	3	11	3	17	1.47
D9Mit35	4	7	6	17	1.00
D9Mit18	3	10	4	17	0.65
D10Mit28	5	6	6	17	1.59
D10Mit5	2	9	5	16	1.38
D10Mit21	4	7	4	15	0.07
D10Mit11	2	8	4	14	0.86
D10Mit14	3	10	4	17	0.65
D11Mit1	4	7	6	17	1.00
D11Mit20	3	9	3	15	0.60
D11Mit15	3	8	4	15	0.20
D11Mit36	4	8	5	17	0.18
D11Mit14	2	12	3	17	3.00
D12Mit9	3	10	3	16	1.00
D12Mit46	4	9	4	17	0.06
D12Mit5	2	9	5	16	1.38
D13Mit17	5	10	2	17	1.59
D13Mit9	6	10	1	17	3.47
D13Mit30	6	9	2	17	1.94
D14Mit10	5	9	2	16	1.38
D14Mit39	5	5	5	15	1.67
D14Mit35	3	6	7	16	3.00
D15Mit13	5	9	3	17	0.53
D15Mit49	5	7	2	14	1.29
D15Mit46	7	8	1	16	4.50
D15Nds1	8	8	1	17	5.82
D15Mit29	7	8	2	17	3.00
D15Mit28	7	8	2	17	3.00
D15Mit70	5	9	3	17	0.53
D15Mit71	6	9	2	17	1.94
D15Mit2	6	8	3	17	1.12
D15Mit33	4	9	3	16	0.38
D15Mit42	5	8	4	17	0.18
D16Mit29	3	10	3	16	1.00
D16Mit12	6	5	6	17	2.88
D16Mit6	2	5	2	9	0.11
D17Mit46	5	8	4	17	0.18
D17Mit48	4	7	4	15	0.07
D17Mit21	5	10	2	17	1.59
D17Mit39	5	10	2	17	1.59
D17Mit41	7	7	3	17	2.41
D18Mit17	4	6	1	11	1.73
D18Mit7	2	11	4	17	1.94
D19Mit1	2	8	7	17	3.00
D19Mit10	5	7	5	17	0.53

Eight markers on chromosome 9 demonstrated enrichment of B6 alleles within the insulin resistant class; no linkage disequilibrium was observed in the diabetic class for chromosome 9. The markers demonstrating statistically significant results in the insulin resistant class spanned a total distance of 32 cM in the middle of chromosome 9.

A single marker in the middle of chromosome 11, D11Mit5 demonstrated linkage disequilibrium within the insulin resistant class with a paucity of heterozygotes (3 B6/B6, 1 B6/CAST, and 6 CAST/CAST).

A single marker on chromosome 15, D15Mi13, demonstrated enrichment of heterozygotes and a paucity of either homozygous class within the insulin resistant class (2 B6/B6, 10 B6/CAST, and 0 CAST/CAST).

ANOVA

Based on the results of the genome scanning experiments and on the results from the *ob/ob* F2 B6CAST, nine markers from seven different chromosomes were selected with which to genotype all of the *db/db* F2 B6CAST. These markers: D6Mit38, D6Mit54, D7Mit57, D7Mit12, D9Mit35, D11Mit15, and D15Nds1 were selected from the regions demonstrating the greatest linkage disequilibrium in the genome scanning experiments. D2Mit61 and D10Mit3 were chosen on the basis of significant correlation between genotype at one locus and either plasma [glucose] or plasma [insulin] in the *ob/ob* F2 B6CAST.

Analogous to the results in the *ob/ob* F2 B6CAST, D2Mit61 demonstrated an association of B6 alleles with increased overnight fasting plasma [glucose] ($p < 0.0001$) (Figure 81) but not plasma [insulin] (Table 35). The means of plasma [glucose] for the three genotypic classes was virtually identical between the two crosses (shown below in mg/dl):

Cross	B6/B6	B6/CAST	CAST/CAST
<i>ob/ob</i> F2 B6CAST	723	700	592
<i>db/db</i> F2 B6CAST	744	729	600

Locus	Phenotype	Female			Male			<i>p</i>
		B6/B6	B6/CAST	CAST/CAST	B6/B6	B6/CAST	CAST/CAST	
D2Mit61	BMI (g/cm ²)	0.536	0.508	0.499	0.507	0.511	0.496	0.06
	Plasma [glucose] (mg/dl)	706	676	582	744	729	601	0.0003
D6Mit54	BMI (g/cm ²)	0.512	0.505	0.497	0.5151	0.512	0.474	0.0087
	Plasma [glucose] (mg/dl)	579	646	676	660	680	751	0.001
	Plasma [insulin] (uU/ml)	587.5	470.3	329.1	476.3	427.0	319.2	0.0016
D7Mit57	Plasma [insulin] (uU/ml)	343.7	466.3	472.8	325.0	440.0	484.0	0.025
D9Mit35	BMI (g/cm ²)	0.501	0.505	0.503	0.475	0.501	0.515	0.03
	Plasma [insulin] (uU/ml)	455.1	446.9	374.9	529.8	461.3	290.7	0.001
D11Mit15	BMI (g/cm ²)	0.499	0.519	0.517	0.486	0.511	0.518	0.0152

Table 35. Analysis of variance by sex and genotype at the marker designated in *db/db* F2B6CAST. All traits for all markers demonstrating statistical significance are summarized. Mean values for each trait are listed by genotypic class.

F2 *db/db* B6CAST

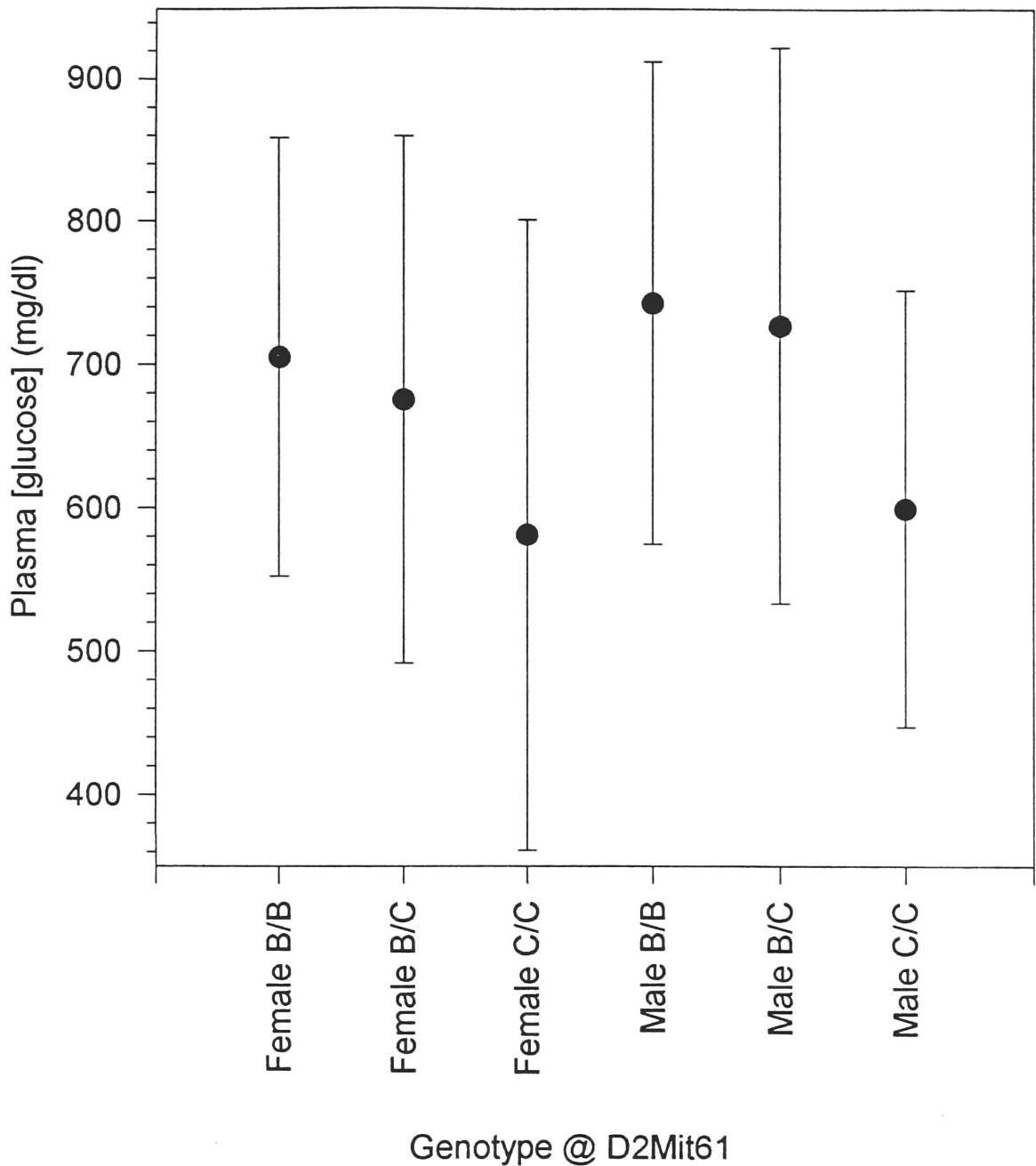
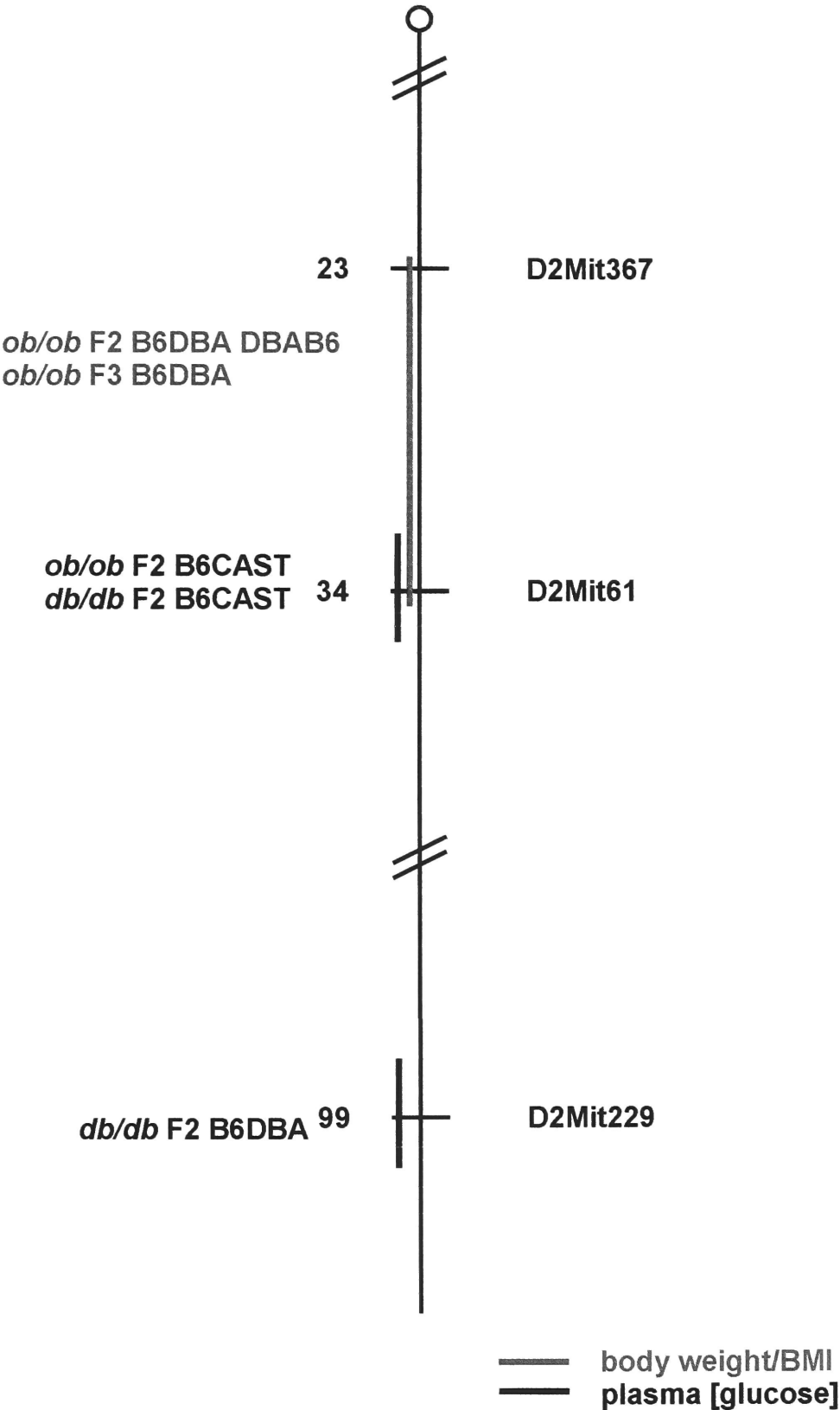


Figure 81. Relationship of genotype at D2Mit61 on overnight fasting plasma [glucose] in *db/db* F2 B6CAST. Means for each genotypic group are indicated by dot and standard deviations by the error bars. “B/B” stands for B6/B6; “B/C” for B6/CAST; and “C/C” for CAST/CAST.

D2Mit61 was within the confidence interval defining the centromeric chromosome 2 locus related to body weight and BMI which was discovered in the *ob/ob* F2 B6DBA (Figure 82). Therefore, it was possible that the locus described in the *ob/ob* F2 B6CAST and *db/db* F2 B6CAST was the same as the centromeric chromosome 2 locus previously reported to be correlated with plasma [glucose] in *ob/ob* and *db/db* F2 B6 DBA. In the *db/db* F2 B6CAST, BMI was probably only secondarily affected by degree of hyperglycemia since the biological correlation between genotype at D2Mit61 and BMI was not strong, especially in the males.

Figure 82. Summary of the QTLs for obesity/diabetes on mouse chromosome 2. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

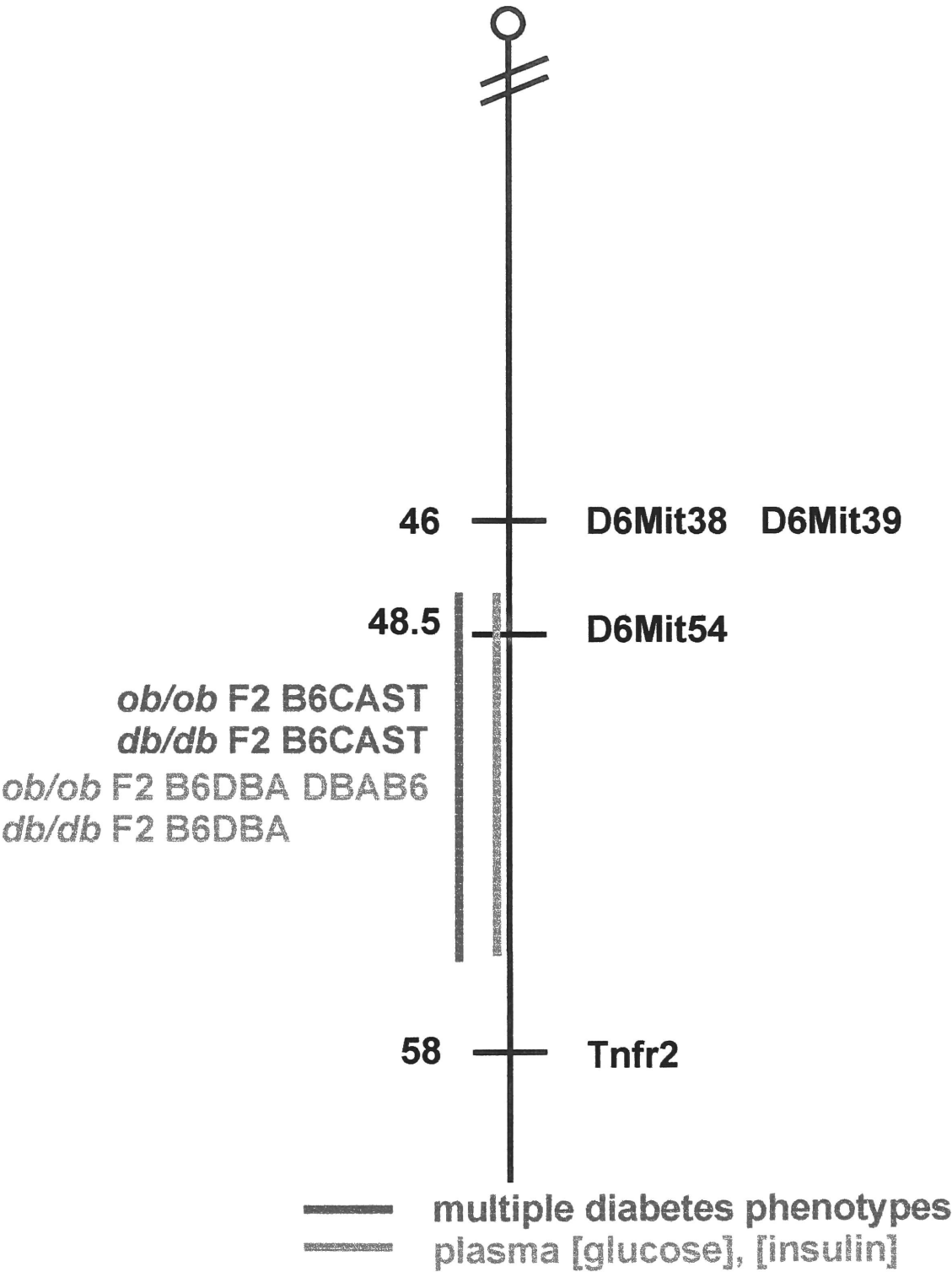
Chromosome 2



Based on the genome scanning experiments, the locus on chromosome 6 was predicted to be the most powerful locus in the *db/db* F2 B6CAST with B6 alleles protective from diabetes. Genotype at D6Mit54 accounted for 16% of the phenotypic variance in plasma [insulin]. Plasma [insulin] was increased 78% in the B6/B6 class relative to the CAST/CAST in the females and 49% in the males (Table 35). Plasma [glucose] was similarly affected with the CAST/CAST class, approximately 100 mg/dl higher than the B6/B6 class of either sex. BMI was greatest in the B6/B6 class which was also the least diabetic. The statistical significance of the results of the associations of genotype at D6Mit54 and various phenotypes are likely greater in the *db/db* F2 B6CAST compared to the *ob/ob* F2 B6CAST because *db* is unlinked to D6Mit54 and therefore free to segregate independently within the *db/db* animals (Figure 83). The candidate gene tumor necrosis factor receptor 2 located on mid-chromosome 6 approximately 9.5 cM centromeric of D6Mit54 was also considered because of its relevance as a candidate gene based on the work alluded to in the previous chapter. The statistical significance of the results obtained with D6Mit38 were similar to those with D6Mit54 (Figures 84 and 85) and support the potential role for one of the TNF receptors rather than the TNF molecule as mediators of insulin peripheral sensitivity.

Figure 83. Summary of the QTLs for obesity/diabetes on mouse chromosome 6. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 6



F2 *db/db* B6CAST

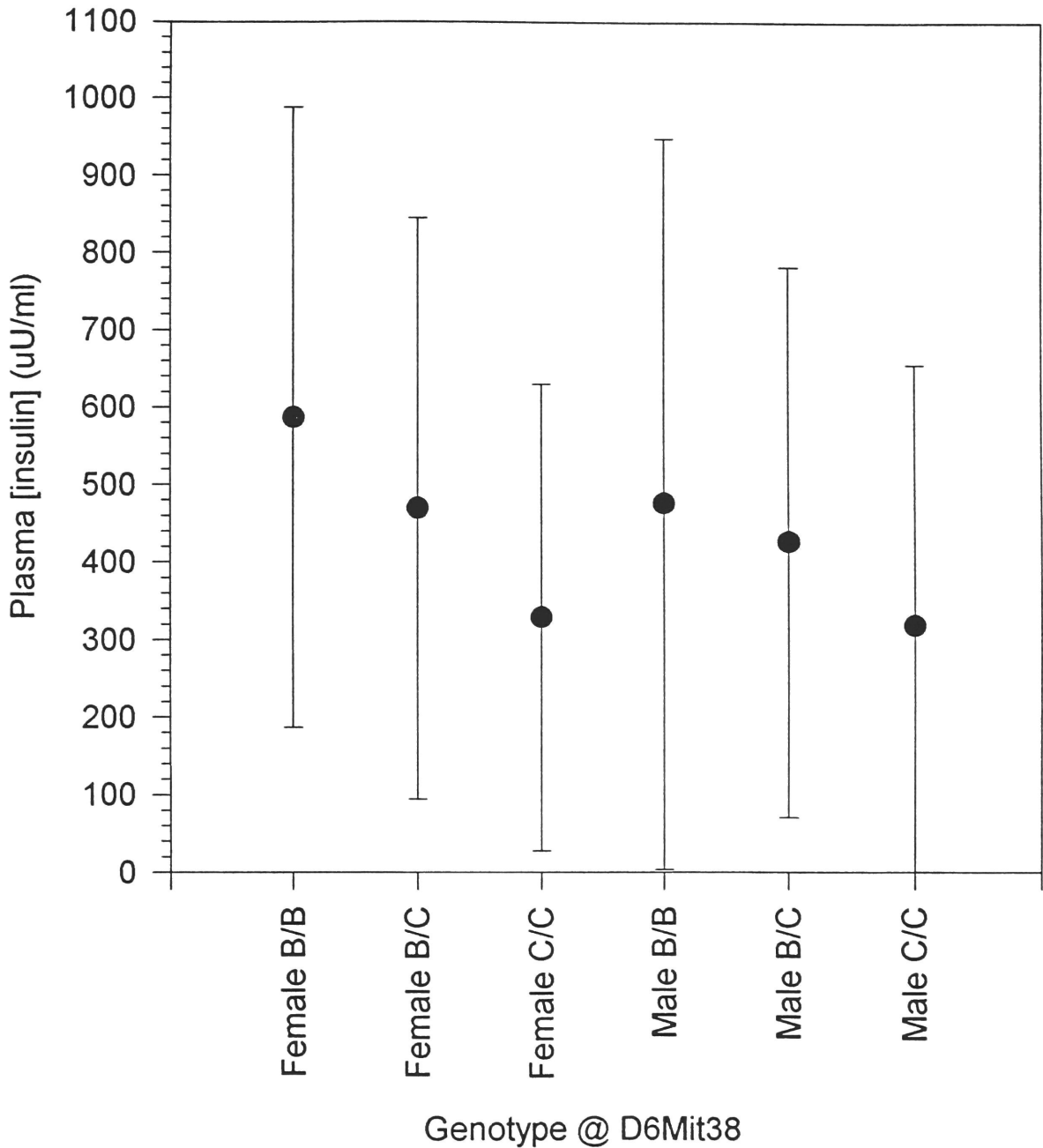


Figure 84. Relationship of genotype at D6Mit38 on fasting plasma [insulin] in *db/db* F2 B6CAST separated by sex. Means indicated by the dot and standard deviations by the error bars. “B/B” stands for B6/B6; “B/C” for B6/CAST; and “C/C” for CAST/CAST.

F2 *db/db* B6CAST

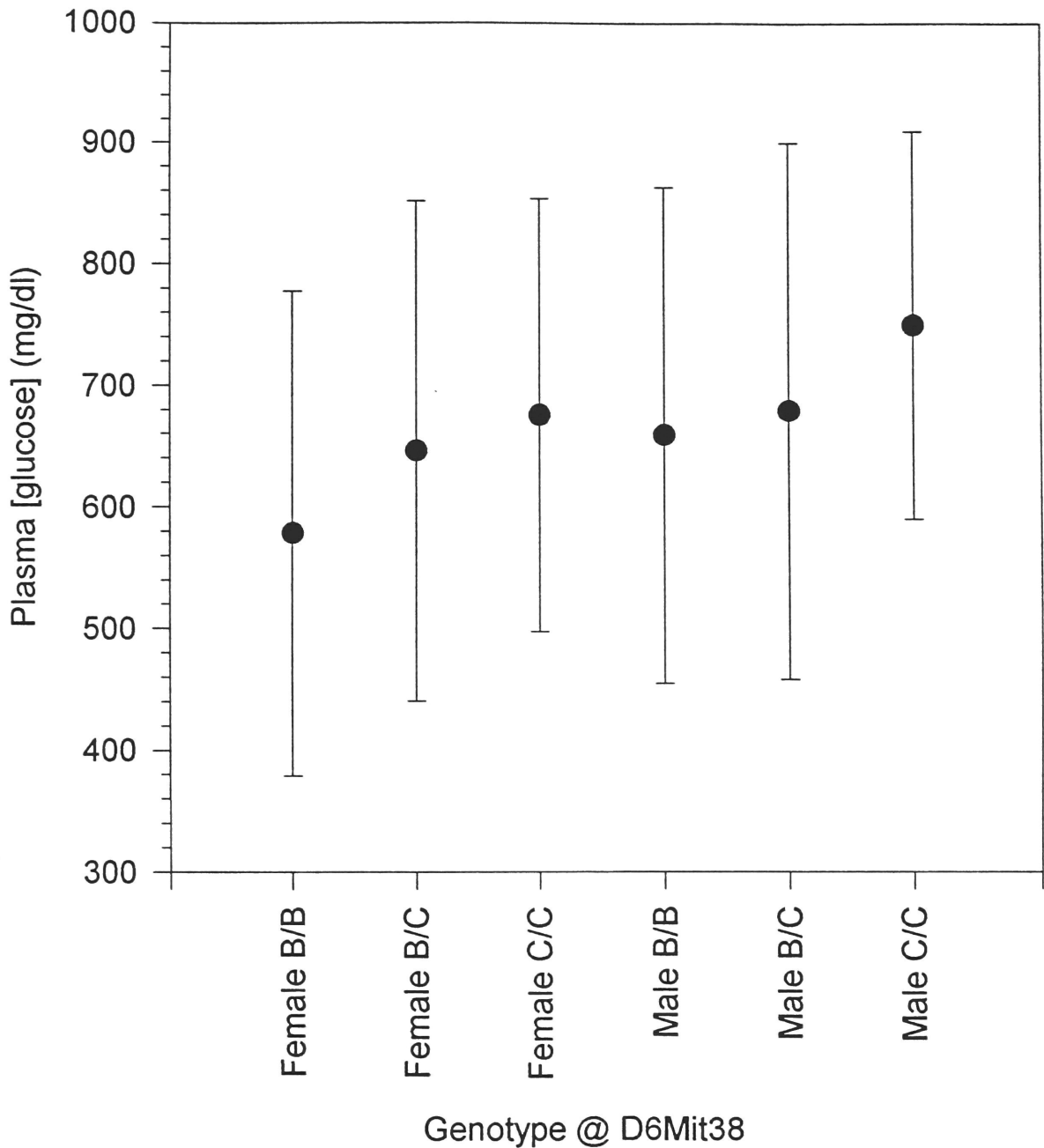


Figure 85. Relationship of genotype at D6Mit38 on fasting plasma [glucose] in *db/db* F2 B6CAST separated by sex. Means indicated by the dot and standard deviations by the error bars. “B/B” stands for B6/B6; “B/C” for B6/CAST; and “C/C” for CAST/CAST.

Of the two markers typed on chromosome 7, only D7Mit57 demonstrated a statistically significant effect of genotype on any of the three phenotypic parameters (Figure 86). As had been observed with the scanning results, B6 alleles were associated with more diabetic animals with plasma [insulin] approximately 30% lower in the B6/B6 compared to the CAST/CAST of either sex (Table 35). Neither BMI nor plasma [glucose] was correlated with genotype at this chromosome 7 locus.

B6 alleles at D9Mit35 had been associated with the insulin resistance group in the genome scanning experiments and were associated with a 22% increase in plasma [insulin] in the females and a 82% increase in the males compared to the CAST/CAST class (Figure 87). The effect of this locus on BMI was equal in relative magnitude to its effect on plasma [insulin]. In the females, for which the effect of the locus on plasma [insulin] was marginal, there was no effect of the locus on BMI. However, in the males, which had demonstrated a greater effect of the locus on plasma [insulin], B6/B6 males had BMIs which were 8% less than CAST/CAST. Interestingly, the class with the heaviest males was the same class with the lowest plasma [insulin], the inverse of the correlation usually observed between the two phenotypes for most QTLs. The interpretation of these data depends greatly on the time point in the progression of the disease being studied. These animals were -as noted - much earlier in the development of their diabetes than animals generated in subsequent crosses. If the animals had not yet begun to lose weight due to diabetes, the heaviest animals may have imposed the greatest stress on the beta cells which had started to become dysfunctional resulting in decreased plasma [insulin].

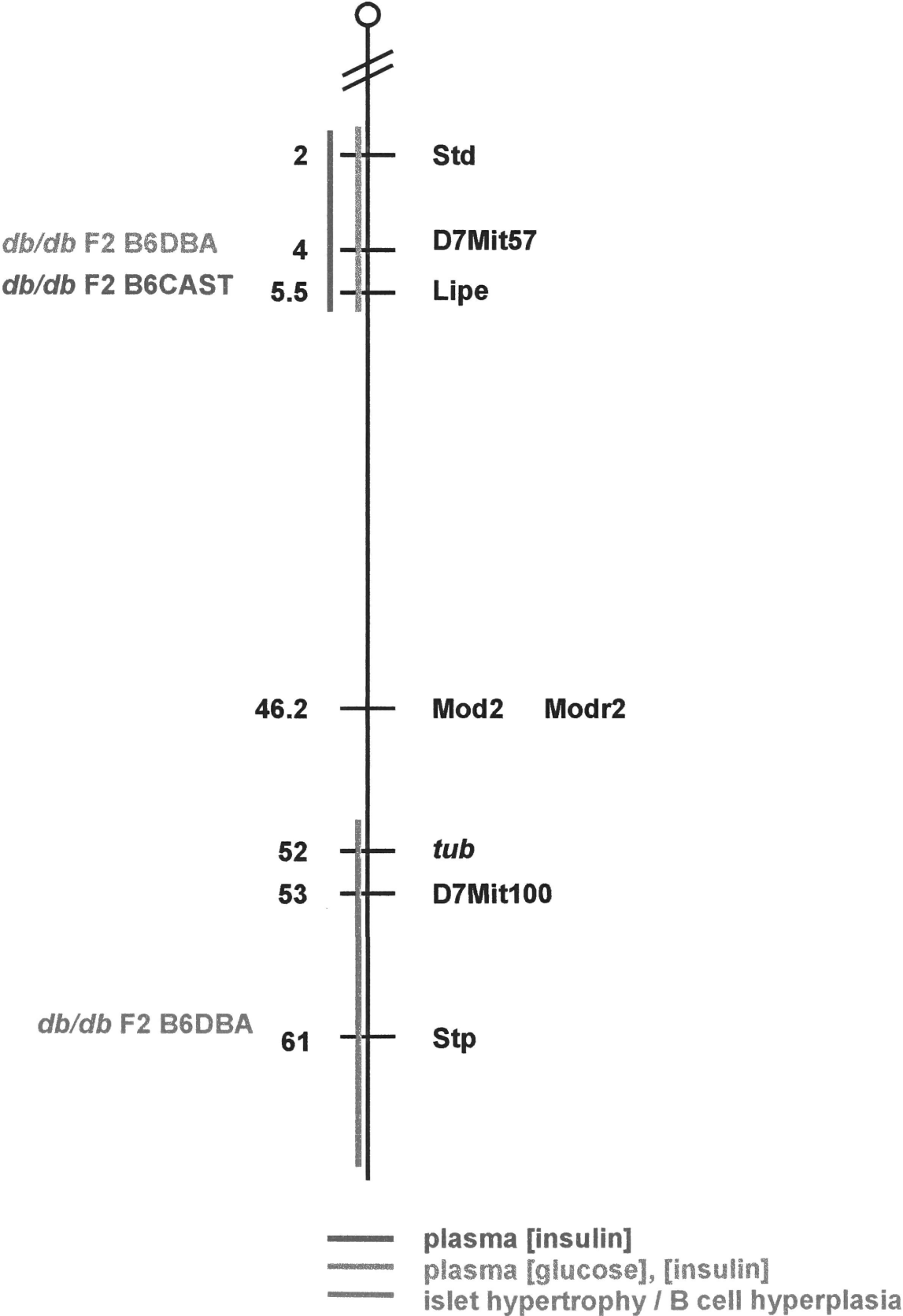
D9Mit35 is approximately 32 cM centromeric of D9Mit18, the marker associated with the QTL for number of islets in the *db/db* F2 B6DBA and probably represents a second locus for plasma [insulin] on chromosome 9 (Figure 88).

B6/B6 animals at D11Mit15 demonstrated a 5% decrease in BMI in both sexes relative to CAST/CAST animals. This result would not have been predicted on the basis of the genome scanning experiments in which a paucity of heterozygotes in the insulin resistant class underlay the statistical significance of D11Mit15.

D10Mit3 and D15Nds1 failed to demonstrate any effect of genotype on phenotypes.

Figure 86. Summary of the QTLs for obesity/diabetes on mouse chromosome 7. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 7



F2 *db/db* B6CAST

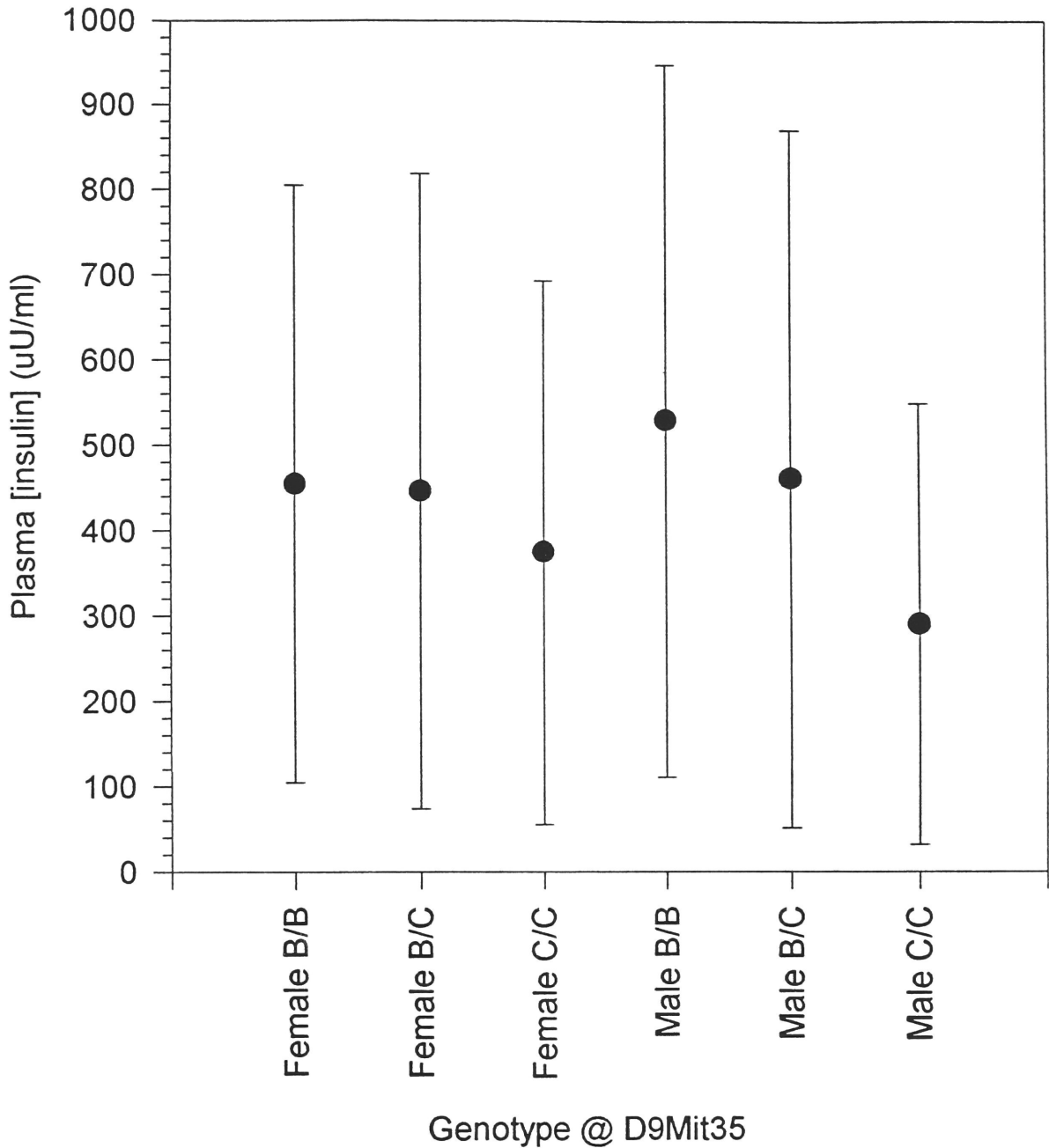
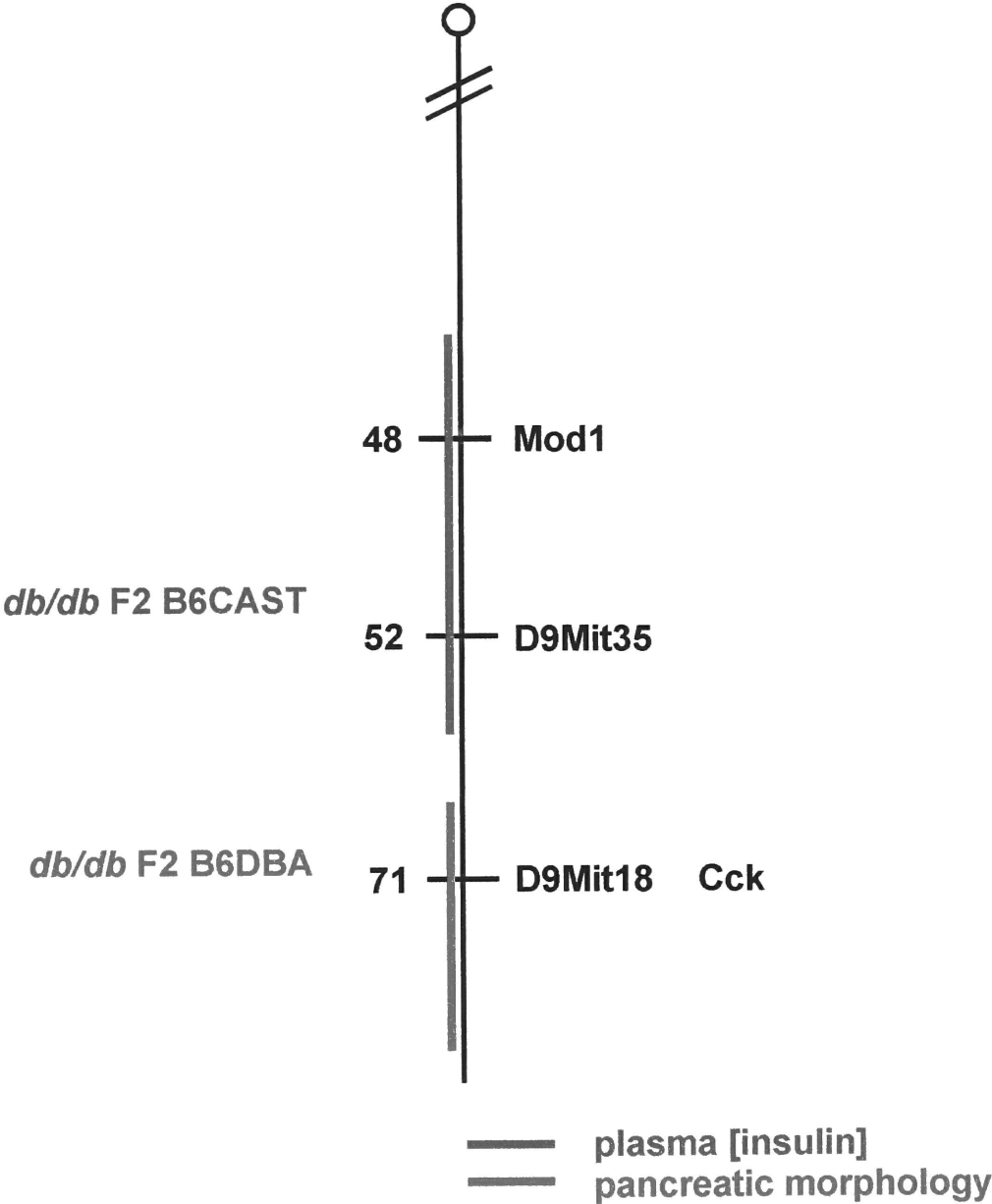


Figure 87. Relationship of genotype at *D9Mit35* on fasting plasma [insulin] in *db/db* F2 B6CAST separated by sex. Means indicated by the dot and standard deviations by the error bars. “B/B” stands for B6/B6; “B/C” for B6/CAST; and “C/C” for CAST/CAST.

Figure 88. Summary of the QTLs for obesity/diabetes on mouse chromosome 9. The most likely location for the QTL is indicated by the vertical bar. Coloring of the vertical bar indicates the primary phenotype(s) associated with the locus.

Chromosome 9



Discussion

Although *Tnfr1* was excluded as a candidate gene for the chromosome 4 QTL influencing body weight and BMI in the *ob/ob* F2 B6CAST, *Tnfr2* on chromosome 6 has not yet been excluded as a candidate for the chromosome 6 QTL influencing fasting plasma [insulin] and [glucose] in the *db/db* F2 B6CAST. TNF- α may mediate many of its different effects from cachexia and wasting to insulin resistance through a combination of dose, receptor specificity and hormonal milieu. Immunologically, it has been argued that the two receptors have distinct and non-redundant functions (Tartaglia and Goeddel 1992), and *Tnfr2* is thought to be the biologically more relevant receptor at the low TNF- α concentrations observed by Homatigil (Hotamisligil, Shargill 1993). The role of the two receptors and their function outside the immunological system has not been well studied, although knockouts of both of these receptors in mice are now available. Preliminary evidence suggests that *Tnfr2* but not *Tnfr1* expression is elevated in fat tissues of genetically obese mice in parallel with increases in Tnf α in adipose tissue (Hofmann et al. 1994). In humans, TNFR2 mRNA expression levels in adipose tissue were strongly correlated with the degree of relative hyperinsulinemia (Hotamisligil). Additionally, TNF α has been hypothesized to play a role in autoimmune diabetes which could have commonalities with NIDDM in processes affecting beta cell survival. For example, non obese diabetic (NOD) mice are low producers of endogenous Tnf (Satoh et al. 1989), and administration of recombinant human TNF α suppressed the development of diabetes in this mouse model of IDDM. It is, therefore, possible that TNF α plays a

role in peripheral insulin sensitivity and/or beta cell longevity. Differential mediation of the TNF α signal through genetically variant receptors is a plausible mechanism to explain the observed hyperinsulinemia effects of the mid-chromosome 6 locus. Direct sequencing of *Tnfr2* in B6 and CAST mice will determine the plausibility of this candidate gene.

The parallel *ob/ob* F2 B6CAST and *db/db* F2 B6CAST crosses also allow comparison of the two obesity mutations on approximately the same genetic backgrounds. When transferred to the same genetic backgrounds, *ob* and *db* are phenotypically indistinguishable (Coleman 1978). Additionally, *ob* has been identified as a null allele of the adipose derived hormone leptin (Zhang, Proenca 1994), and *db* is the result of mutations in the receptor for *ob* (*Obr*) which result in complete loss of (Chua, Chung 1996, Lee, Proenca 1996, Tartaglia and Goeddel 1992). Therefore, if *ob* and *db* function as monogamous ligands and receptor, respectively and have no additional functions, one would expect the same phenotype in the two mutations on the same genetic backgrounds. As a group, the *ob/ob* F2B6CAST mice were less obese ($p<0.00001$), and more diabetic with higher fasting plasma [glucose] ($p<0.00001$) and lower plasma [insulin] ($p<0.01$) than the *db/db* F2B6CAST (Table 36). This result is in direct contrast to comparison of the *ob/ob* F2 B6DBA and *db/db* F2 B6DBA for which the *db/db* were the less obese and more diabetic (see chapter 6). Again, the question arises whether the difference in the animals is attributable to the obesity mutations themselves or to BKS alleles which persist in the genomes of the *db* B6 parentals at the N5 generation. Whichever

explanation is correct, it must be assumed that either the obesity mutation or the BKS gene interacts differently with other genes in the genome (e.g. DBA or CAST) since the phenotypes of the mice relative to each other differ depending on the counterstrain.

Phenotype (age adjusted)	F2 <i>ob/ob</i> B6/CAST	F2 <i>db/db</i> B6/CAST	P
BMI (g/cm ²)	0.469 (0.061)	0.513 (0.081)	0.00001
Fasting plasma [glucose] (mg/dL)	624 (221)	539 (224)	0.00001
Fasting plasma [insulin] (uU/ml)	317.5 (325.6)	377 (357)	0.01

Table 36. Comparison of *ob/ob* and *db/db* F2 B6CAST animals. Phenotypes adjusted for age.

If *ob* and *db* interact only with each other why should QTLs behave differently in the two crosses? The QTL's on chromosome 2 and 6 produce the same qualitative and quantitative effects in the two crosses. The locus on chromosome 4 could not be adequately studied in the *db/db* due to its proximity to *db*. However, the loci on chromosome 7, 9, 10, and 11 were tested in both sets of progeny with significant linkages to phenotypes in one cross but not the other. Do these data indicate that the correlation between genotype at these loci and phenotypes were spurious or that the small proportion of the BKS alleles contaminating the B6 genome of the *db* mice exert a large effect on the phenotypes. Alternatively, these data may indicate that either *ob* and/or *db*

have additional physiologic roles which are yet undefined, and which do not depend on leptin-Obr interactions.

Chapter 10

**Confirmation of diabetes susceptibility regions
in
N2F1 B6CAST segregating for *ob***

Strategy

In an attempt to determine the reproducibility and strength of the association of the chromosome 10 locus with increased fasting plasma [insulin], lean *ob/+* F1 B6CAST progeny were backcrossed to CAST males to generate N2 progeny which were on average 75% CAST and heterozygous or homozygous at every locus for CAST alleles. N2 B6CAST progeny were then tail clipped and genotyped with markers flanking *ob* (Cftr and D6Mit74), to identify *ob/+* animals. Heterozygous animals were subsequently genotyped for markers 13 cM apart flanking the QTL on chromosome 10 (D10Mit51 and D10Mit38). Animals heterozygous for *ob* and concordant for genotype at either D10Mit51 and D10Mit38 were mated to a genotypically identical mouse of the opposite sex. Twenty six such breeding pairs were established, yielding a total of 102 male and 114 *ob/ob* females which were sacrificed at approximately 90 days of age (Table 37). The mice were phenotypically characterized and analyzed as previously described. All *ob/ob* progeny were typed for D10Mit3 to test the original hypothesis that a QTL near this marker would influence fasting plasma [insulin]. Additionally, once the chromosome 6 locus near *Tnfr2* influencing fasting plasma [insulin] had been identified, this locus was tested in the *ob/ob* N2F1 B6CAST by genotyping the animals with D6Mit38 (near *Tnfr2*). For any locus randomly segregating in the cross (i.e. not on chromosome 10 linked to the QTL or on chromosome 6 linked to *ob*), the Mendelian ratio of alleles expected would be 1/16 B6/B6: 6/16 B6/CAST: 9/16 CAST/CAST. However, because the mice were all carrying B6 alleles at *ob*, a large percentage of the *ob/ob* N2F1 B6CAST progeny were carrying B6 alleles for markers on chromosome 6 allowing greater statistical

power to analyze regions of chromosome 6 relative to other chromosomes. Although the power to detect linkage between obesity and diabetes phenotypes was low due to the paucity of B6 homozygotes in the N2F1 progeny, the markers D2Mit236 and D2Mit7 were used in an attempt to confirm the previously identified QTL modifying diabetes/obesity on centromeric chromosome 2.

Phenotype	Obese, females	Obese, males	Lean females	Lean males
Age (days)	94 (21)	87 (21)	94 (20)	86 (22)
Weight (g)	34.1 (6.5)	34.4 (7.2)	18.0 (2.9)	21.4 (3.3)
BMI (g/cm ²)	0.500 (0.076)	0.515 (0.071)	0.274 (0.03)	0.310 (0.03)
HbA1c (%)	13.6 (3.6)	13.6 (2.8)	4.1 (0.5)	4.2 (0.7)
Fasting plasma [glucose] (mg/dL)	639 (183)	648 (204)	117 (114)	156 (130)
Fasting plasma [insulin] (uU/ml)	445.8 (331)	399.2 (258.5)	138.9 (123.1)	125.6 (106.8)
Normalized pancreatic insulin content (uU/mg protein)	4863 (3443)	4463 (4173)	10657 (7471)	11826 (10170)
Normalized pancreatic glucagon content (uU/mg protein)	22743 (50130)	19722 (17546)	5045 (3890)	7299 (10137)
Pancreatic [insulin]/[glucagon]	0.91 (1.7)	0.93 (2.66)	5.05 (7.96)	7.07 (12.91)
Number of islets	12.5 (7.0)	11.8 (8)	ND	ND
Number of hypertrophic islets	1.22 (1.77)	0.81 (1.51)	ND	ND
Average islet area (mm ²)	0.019 (0.019)	0.018 (0.007)	ND	ND
Total islet area (mm ²)	0.25 (0.2)	0.23 (0.21)	ND	ND
Normalized islet area (%)	1.81 (1.4)	1.57 (1.2)	ND	ND

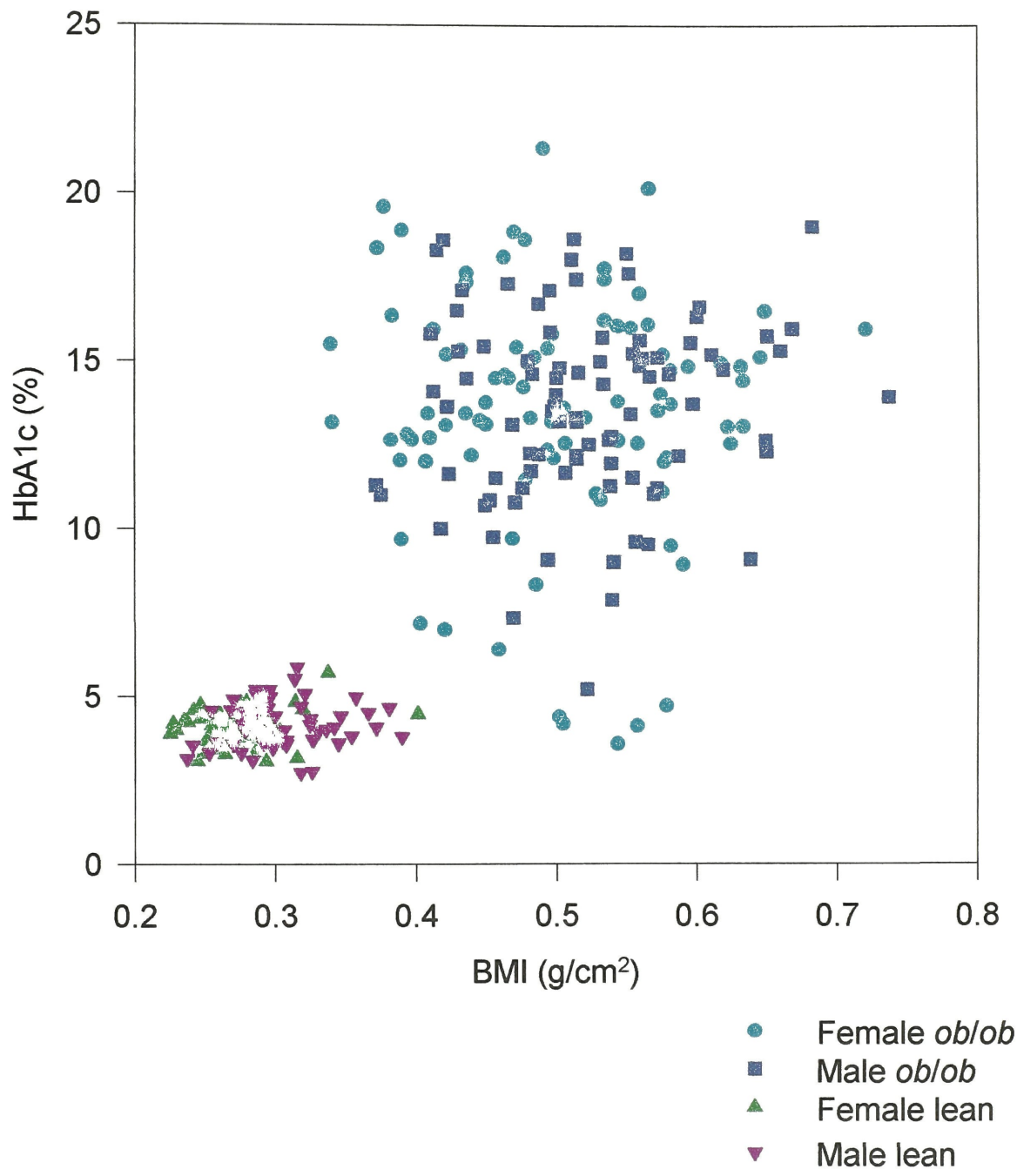
Table 37. Phenotypic characteristics of N2F1 B6CAST progeny segregating for *ob*. Means for the lean and obese groups by sex are indicated with standard deviations in parentheses. “ND” indicates that the values have not been determined.

Results

Phenotypically, the obese progeny were distinguishable from the lean for all parameters (Table 37). As was true of all the previous *ob* and *db* crosses with CAST, there was little sexual dimorphism. There was variability in extent of obesity and diabetes (Figure 89), but the majority of the animals (90%) were diabetic, with HbA1c greater than 8%. The *ob/ob* N2F1 B6CAST were more diabetic than the *ob/ob* F2 B6CAST, with fasting plasma [glucose] 27% higher in the females (639 vs 503 mg/dl) and 13% higher in the males (648 vs 573 mg/dl). These results were predicted since 75% of the genome of the animals carried CAST alleles in the *ob/ob* N2F1 B6CAST compared to 50% in the *ob/ob* F2 B6CAST.

Figure 89. BMI versus HbA1c N2F1 B6CAST progeny segregating for *ob*. Each point represents a single animal.

N2F1 B6CAST



ANOVA

Genotype at D10Mit3 was not correlated with any phenotype relating either to obesity or diabetes.

Genotype at D6Mit38 was most significantly correlated with fasting plasma [insulin] ($p < 0.0001$) and pancreatic grade ($p < 0.00001$), B6 alleles conveyed relative protection from diabetes, as had been observed in the *ob/ob* F2 B6CAST and *db/db* F2 B6CAST. The fasting plasma [insulin] was 2.5 times greater in B6/B6 females relative to CAST/CAST, and 1.6 times greater in males (Table 38). The extent of the association between genotypes at D6Mit38 and plasma [insulin] is apparent in Figure 90 in which the majority of B6/B6 animals demonstrated fasting plasma [insulin] $> 600 \mu\text{U/ml}$. Similarly, pancreatic grade was one full class lower (“healthier”) in B6/B6 animals of both sexes relative to CAST/CAST. The chromosome 6 QTL accounted for 27% of the phenotypic variance in plasma [insulin]. Additional phenotypes including body weight, pancreatic [insulin], number of islet and total islet area were similarly affected. Fasting plasma [glucose] and HbA1c were notably not correlated with genotype at the chromosome 6 locus. Although the majority of B6/B6 animals demonstrated pancreatic grades less than 2, they also demonstrated a wide variation in HbA1c (Figure 91). The majority of the B6/B6 animals had both fasting plasma [insulin] $> 600 \mu\text{U/ml}$ and pancreatic grades < 2 (Figure 92). A QTL located in the vicinity of D6Mit38 and D6Mit54 which affects fasting plasma [insulin] as well as other diabetes phenotypes has

now been shown to segregate in a total of five different crosses: *db/db* F2 B6CAST, *ob/ob* F2 B6CAST, *ob/ob* N2F1 B6CAST, *db/db* F2 B6DBA, and *ob/ob* F2 B6DBA.

Although the *ob/ob* N2F1 B6CAST were not the optimal animals to test the effects of the chromosome 2 locus previously demonstrated to affect BMI and plasma [glucose] due to the small numbers of B6/B6 progeny at this locus, a strong correlation between genotype at D2Mit236 and a weaker correlation at D2Mit7 was observed for both BMI and plasma [glucose]. As had been previously demonstrated in the *db/db* F2 B6CAST and *ob/ob* F2 B6CAST, B6 homozygotes had the highest plasma [glucose], 16% higher than the CAST/CAST in the females and 24% higher in the males. However, in contrast to the *db/db* F2 B6CAST and *ob/ob* F2 B6CAST, B6 homozygotes had the highest BMI of the three genotypic classes despite their hyperglycemia. In the *ob/ob* N2F1 B6CAST, the centromeric chromosome 2 locus may primarily affect BMI with the most obese animals becoming secondarily hyperglycemic resulting in subsequent weight loss with disease progression.

Marker	Phenotype	Number	Female		Male		P
			B6/B6	CAST/CAST	B6/CAST	CAST/CAST	
D1Mit110	[glucose] (mg/dl)	202	874	643	644	641	0.05
D2Mit236	BMI (g/cm2)	204	0.501	0.492	0.522	0.502	0.002
	[glucose] (mg/dl)	199	724	626	660	635	0.0007
D2Mit7	BMI (g/cm2)	184	0.501	0.494	0.512	0.502	0.05
D6Mit38	Weight (g)	208	36.9	30.8	34.6	32.3	0.0001
	[Insulin] (uU/ml)	201	651.2	258.4	295.6	341.6	0.00001
	Pancreatic Grade	159	2.0	3.1	2.6	2.7	0.00001
	Pancreatic [Insulin] (uU/mg protein)	203	5905	4188	3406	3967	0.001
	Number of islets	151	15.1	9.4	11.9	9.9	0.007
	Islet area/total area (%)	150	2.4	1.4	1.4	1.3	0.001

Table 38. Analysis of variance by sex and genotype at D6Mit38, D2Mit236, and D2Mit7 for *ob/ob* N2F1 B6CAST. All traits for all markers demonstrating statistical significance are summarized. Number of animals genotyped and phenotyped for each a given marker and trait is indicated. Mean values for each trait are listed first by sex then genotypic class.

Figure 90. Fasting plasma [insulin] vs [glucose] separated by genotype at D6Mit38 in *ob/ob* N2F1 B6CAST. Each symbol represents a single animal.

***ob/ob* N2F1 B6CAST
D6Mit38**

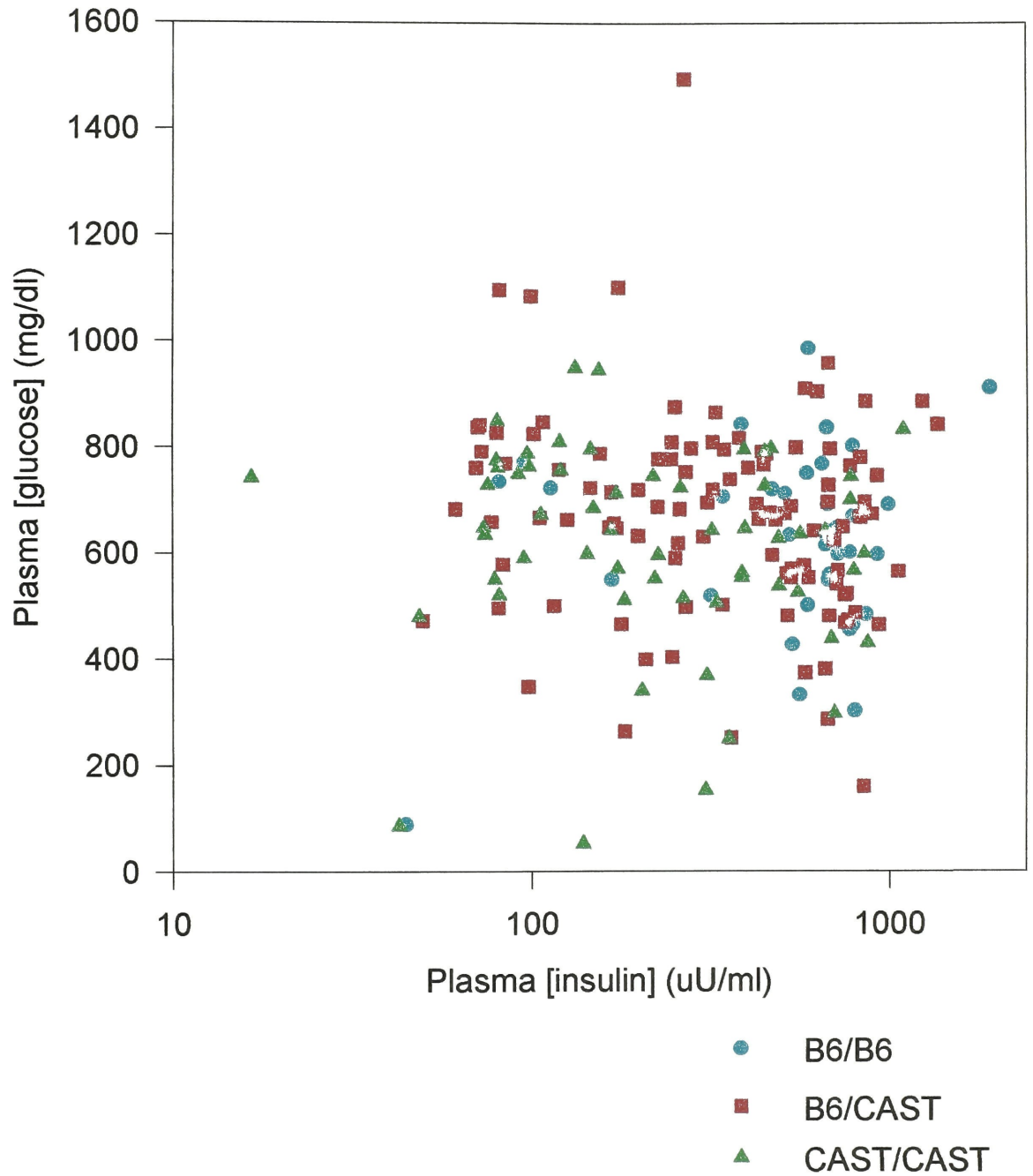


Figure 91. Pancreatic grade vs HbA1c separated by genotype at D6Mit38 in *ob/ob* N2F1 B6CAST. Each symbol represents a single animal.

ob/ob N2F1 B6CAST
D6Mit38

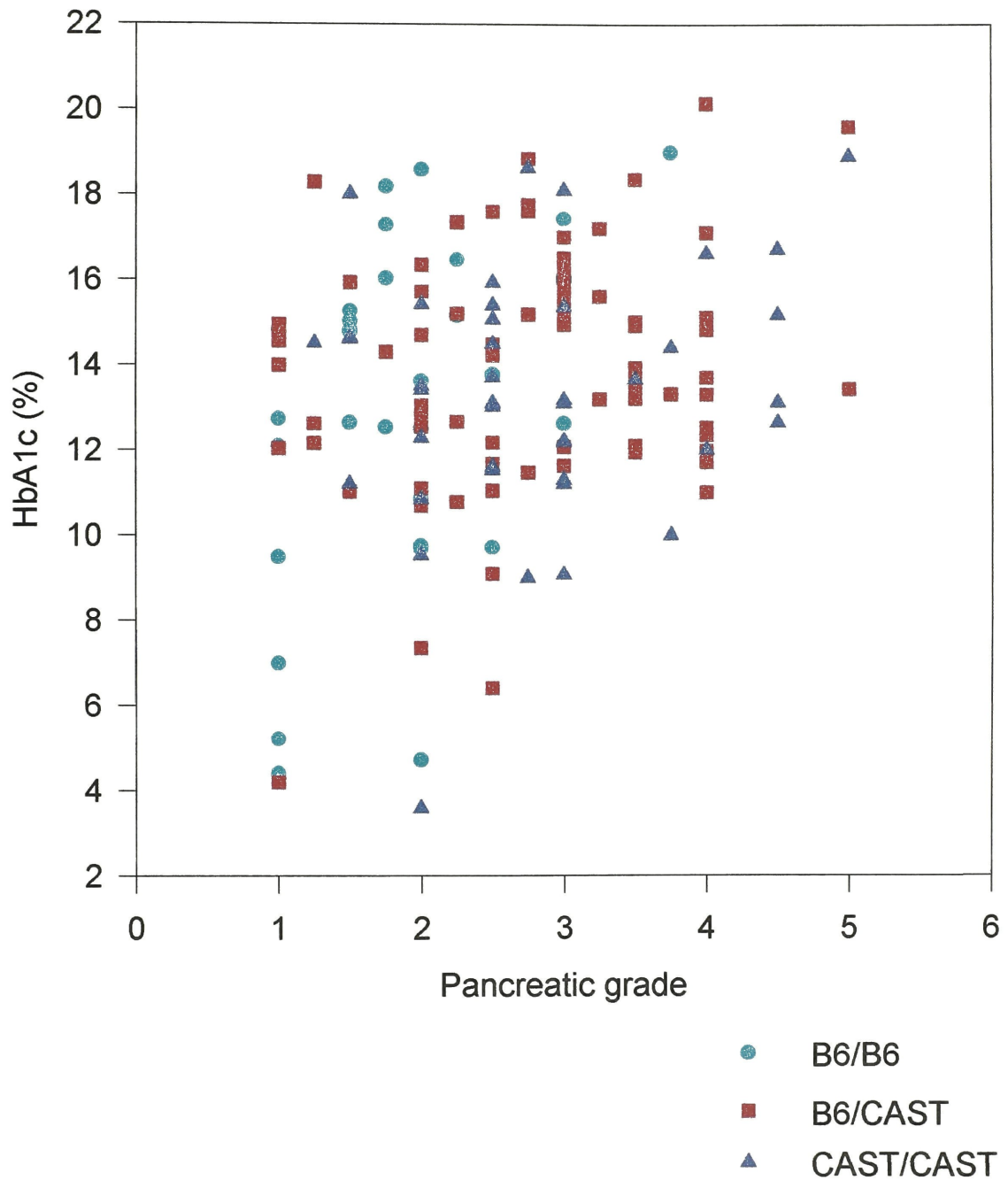
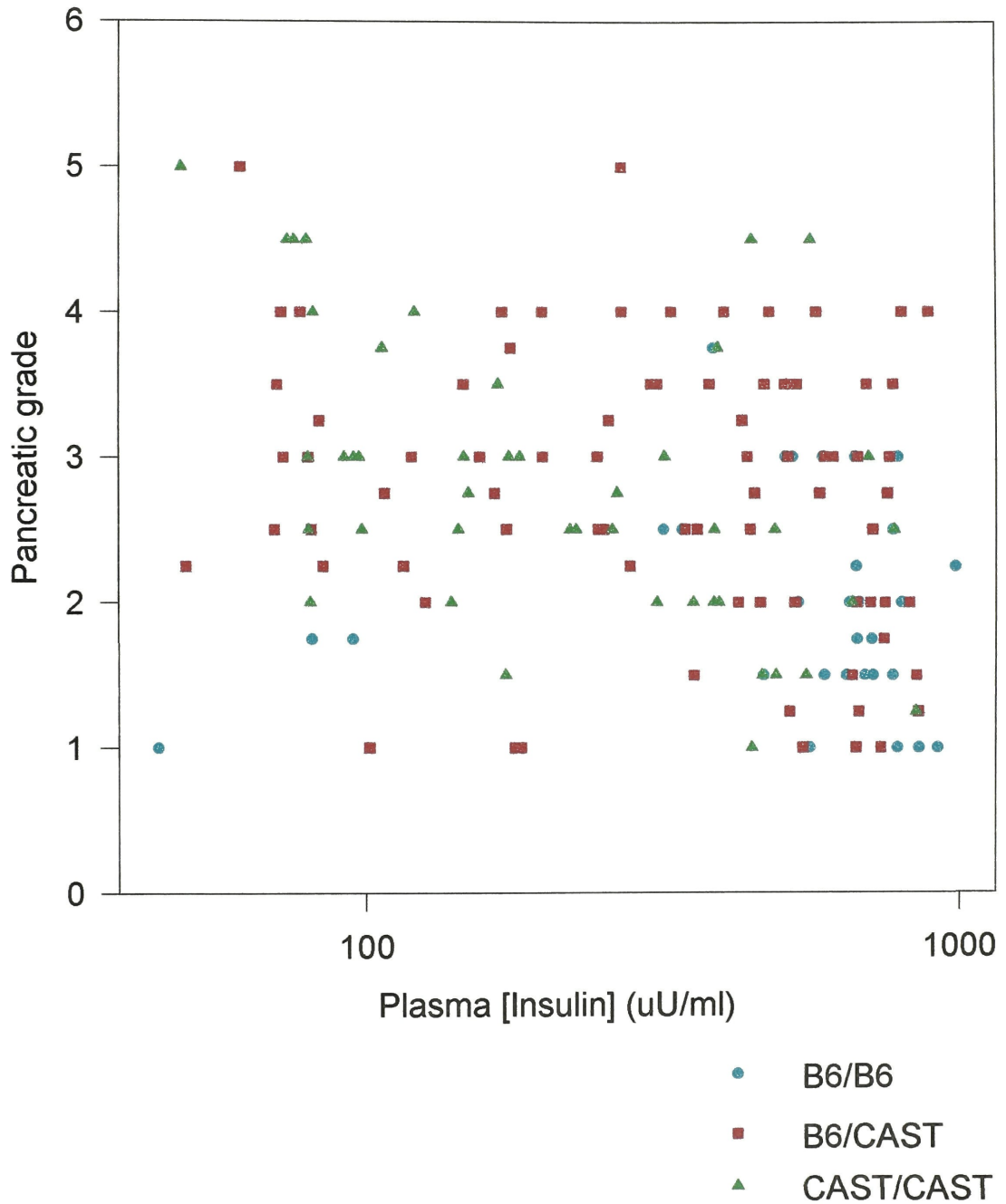


Figure 92. Fasting plasma [insulin] vs pancreatic grade separated by genotype at D6Mit38. Each symbol represents a single *ob/ob* N2F1 B6CAST animal.

ob/ob N2F1 B6CAST
D6Mit38



Discussion

Some of the negative results in the *ob/ob* N2F1 B6CAST are very informative. Failure to replicate the locus on proximal chromosome 10 which was correlated with overnight fasting plasma [insulin] in the *ob/ob* F2 B6CAST suggests that either the original result was not biologically relevant or that the effect of this locus is critically dependent upon the duration of food restriction before sacrifice. The original animals in which this locus was discovered were fasted overnight rather than for three hours as in the *ob/ob* N2F1 B6CAST. If the chromosome 10 locus is biologically relevant, its primary effect may be on the pancreatic response to fasting hyperglycemia or on hepatic sensitivity to effects of insulin on gluconeogenesis during a fast. To test this hypothesis, it would be necessary to challenge the N2F1 animals with an overnight fast and then measure plasma [glucose] and [insulin].

The mid-chromosome 6 locus did exert a major affect on the same phenotypes for which correlations were demonstrated in the F2 progeny (i.e. plasma [insulin] and pancreatic grade). The interval around D6Mit54 and D6Mit38 appears primarily to effect beta cell physiology. A series of repeated backcrosses to CAST until the N5 generation was attempted in an effort to generate congenic mice which were heterozygous across the interval containing the chromosome 6 locus. However, each successive generation was less fecund. By N5, the mice were no longer able to reproduce. It should prove more productive to use B6 as the recurrent strain for backcrossing in the future because of the increased fertility of B6 compared to CAST.

Tnfr2 remains a good candidate gene for the chromosome 6 locus. Based on the correlation of genotype near Tnfr2 with several phenotypes including body weight, plasma [insulin], pancreatic grade and number of islets, allelic variation at Tnfr2 might exert effects on beta cell performance as well as mediate effects on peripheral insulin sensitivity. Tnfr2 is expressed in all tissues, and its message levels have been positively correlated with Tnf α and degree of insulin resistance. Tnfr2 expression in beta cells in non-immunologically based diabetes has never been studied but certainly warrants further investigation.

The centromeric chromosome 2 locus was confirmed for its effects associated both with obesity (BMI) and diabetes (plasma [glucose]). Additional longitudinal studies will be necessary to determine whether the obesity susceptibility of this locus is the primary or secondary effect.

Chapter 11

Confirmation of diabetes susceptibility regions

in

F2 B6DBA segregating for *ob* or *db*

and

testing of diabetes susceptibility regions in two other

genetic models of obesity using F2 BKSCAST

segregating for *fat* and F2 B6CAST segregating for *tub*

Strategy

Four crosses segregating for *ob*, *db*, *fat* and *tub* were originally established to map and clone these rodent obesity genes. Progeny of these crosses were used to test the reproducibility of certain diabetes-susceptibility QTLs identified in other crosses, and to examine the applicability of these QTL's in the context of less severe forms of genetic obesity seen in the *fat* and *tubby* mice. The *ob/ob* F2 B6DBA-1988 and *db/db* F2 B6DBA-1988 animals were produced in 1988, independently of the more recently produced animals segregating the same obesity mutations on the same parental strains which were described in the previous chapters. The methods of phenotypic and genotypic characterization of the *ob/ob* F2 B6DBA-1988 (Bahary, Leibel 1990), *db/db* F2 B6DBA-1988 (Bahary, Siegel 1993), *fat/fat* F2 BKSCAST (Chung et al. 1996), and *tub/tub* F2 B6CAST (Chung et al. 1996) have been previously described. Animals from all four crosses were sacrificed from 110-130 days, i.e. at ages similar to those of the more recent crosses. Body weight, BMI, and overnight fasting plasma [glucose] and [insulin] were determined. All the obese animals from the respective crosses were genotyped for the markers listed below:

ob/ob F2 B6DBA-1988: D4Mit12, D6Mit54, D7Mit12, D7Mit57, D9Mit35, D10Mit3, and D11Mit15.

db/db F2 B6DBA-1988: D6Mit54, D7Mit21, D9Mit35, D10Mit3, and D11Mit15

fat/fat F2 BKSCAST: Tnfr1, D6Mit38, D7Mit77, D9Mit35, and D10Mit3

tub/tub F2 B6CAST: D6Mit38, D6Mit54, D9Mit35, D10Mit3, and D11Mit15

Results

Cross	Sex	Age	BMI	Plasma [insulin]	Plasma [glucose]
ob/ob F2 B6DBA	Female	141 (31)	0.603 (0.082)	1131.4 (761.1)	352 (141)
	Male	134 (19)	0.600 (0.063)	992.2 (662.0)	485 (168)
db/db F2 B6DBA	Female	123 (23)	0.583 (0.062)	170.2 (219.3)	414 (178)
	Male	126 (28)	0.549 (0.081)	131.8 (151.4)	546 (179)
fat/fat F2BKsCAST	Female	104 (28)	0.451 (0.063)	561.1 (370.5)	358 (178)
	Male	116 (32)	0.428 (0.053)	564.5 (476.7)	352 (135)
tub/tub F2 B6CAST	Female	125 (50)	0.440 (0.053)	238.4 (358.2)	237 (97)
	Male	135 (34)	0.429 (0.070)	486.7 (638.8)	260 (114)

Table 39. Phenotypic characteristics of *ob/ob* F2 B6DBA-1988, *db/db* F2 B6DBA-1988, *fat/fat* F2 BKSCAST, and *tub/tub* F2 B6CAST progeny divided by sex. Mean of each group is indicated with the standard deviation in parentheses.

The *ob/ob* F2 B6DBA-1988 were as obese as the more recent *ob/ob* F2 B6DBA (mean BMI = 0.602 g/cm² vs 0.597 g/cm²) but were more hyperinsulinemic (1051 μ U/ml vs 168.1 μ U/ml) and less hyperglycemic (426 mg/dl vs 697 mg/dl) (Table 39). The *db/db* F2 B6DBA-1988 were approximately as obese as the more recent *db/db* F2 B6DBA (mean BMI = 0.562 g/cm² vs 0.530 g/cm²) and were less hyperinsulinemic (140.2 μ U/ml vs 331.2 μ U/ml) and less hyperglycemic (501 mg/dl vs 685 mg/dl). The *fat/fat* F2 BKSCAST and *tub/tub* F2 B6CAST were less obese than any of the crosses segregating for *ob* or *db*, with an average BMI of 0.435 g/cm² and 0.433 g/cm², respectively. The obesity observed in the *fat/fat* and *tub/tub* animals was of later onset and more uniform

anatomic distribution. The apparent plasma [insulin] of the *fat/fat* F2 BKSCAST was elevated (562.2 μ U/ml) relative to other obesity mutations outcrossed to CAST; however, the radioimmunoassay for insulin did not distinguish between pro-insulin and insulin, and it is now known that *fat* is a mutation in Cpe, one of the enzymes that processes pro-insulin to insulin. The *fat/fat* F2 BKSCAST mice were not as hyperglycemic as the *ob/ob* and *db/db* mice, and might have been expected to be the most severely diabetic since two diabetes-susceptible strains, BKS and CAST, were the parental strains used for this cross. The *tub/tub* F2 B6CAST animals demonstrated unique sexual dimorphism in which the males rather than the females were relatively hyperinsulinemic. Animals of both sexes were euglycemic (plasma [glucose] = 247 mg/dl).

***ob/ob* F2 B6DBA-1988**

Of the seven markers tested in the *ob/ob* F2 B6DBA-1988, two demonstrated significant associations between marker genotypes and one of the three phenotypes (BMI, plasma [glucose] or plasma [insulin]). As had been demonstrated in the *ob/ob* F2 B6CAST, B6 alleles at D10Mit3 were associated with lower concentrations of overnight fasting plasma [insulin] which were 29% lower in the females and 38% lower in the males B6/B6 at D10Mit3 relative to the CAST/CAST class (Table 40). Genotype at D10Mit3 accounted for 5% of the phenotypic variance in plasma [insulin] in the *ob/ob* F2 B6DBA-1988. Homozygosity for DBA alleles at D4Mit12 was associated with decreased BMI as had been observed with the *ob/ob* F2 B6CAST which were raised at approximately the same time. Animals which were DBA/DBA at D4Mit12 had BMIs approximately 8% less

than B6/B6 animals (Figure 93) and had plasma [glucose] which were 25% greater than B6/B6 animals.

Locus	Phenotype	B6/B6	Female B6/DBA	DBA/DBA	B6/B6	Male B6/DBA	DBA/DBA	p
D4Mit12	BMI (g/cm ²)	0.611	0.621	0.554	0.633	0.621	0.586	0.002
	Glucose (mg/dl)	321	354	385	413	479	531	0.05
D10Mit3	Insulin (uU/ml)	875.3	1060.1	1239.8	696.8	1193.7	1133.5	0.03

Table 40. ANOVA results of *ob/ob* F2 B6DBA-1988. Only markers which demonstrated significant effects on one of the three phenotypic parameters (BMI, fasting plasma [insulin] or [glucose] are shown. Mean of each genotypic group divided by sex is listed.

F2 *ob/ob* B6DBA

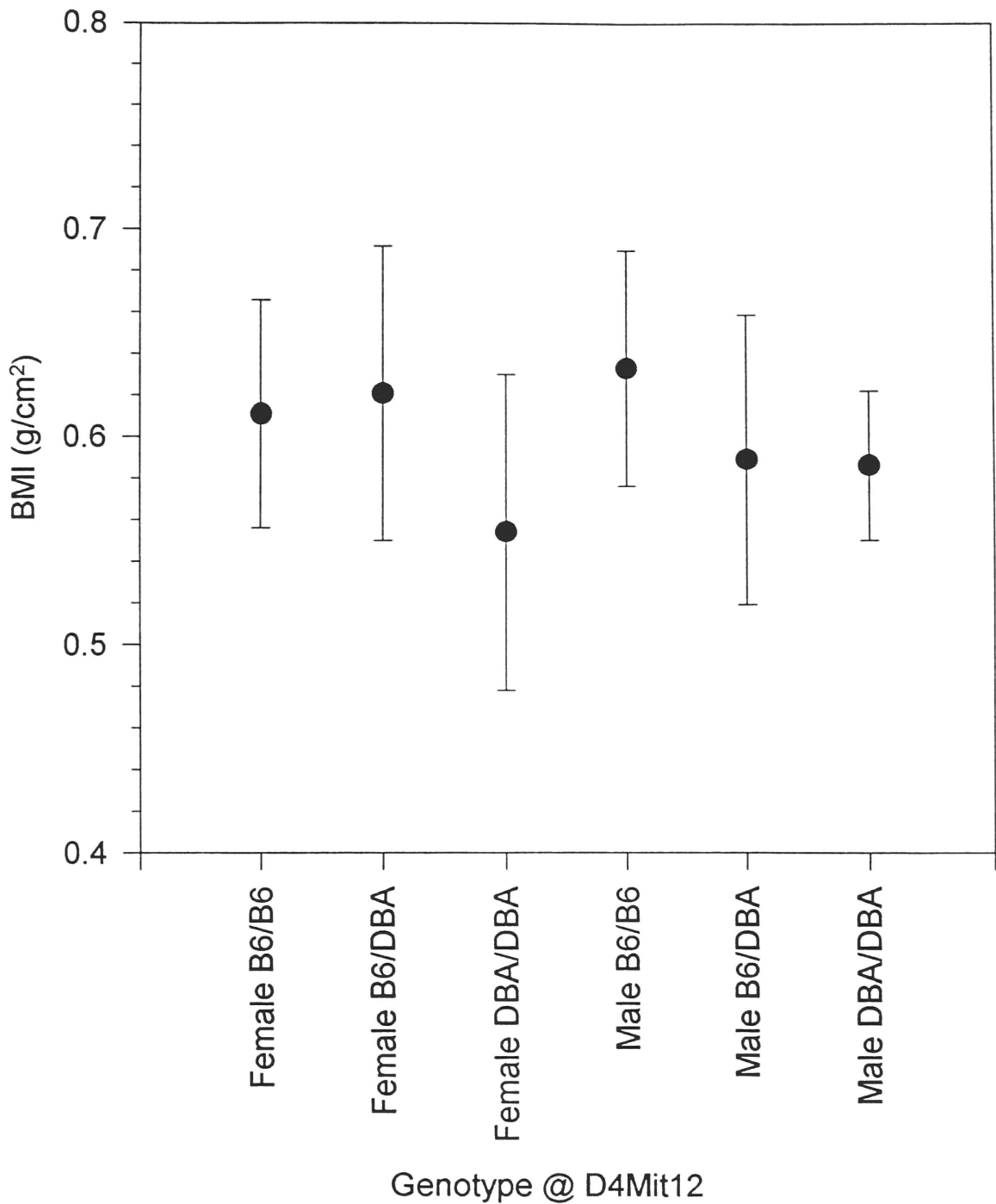


Figure 93. Relationship between genotype at D4Mit12 and BMI in *ob/ob* F2 B6DBA-1988. Mean indicated by the dot and standard deviations by error bars.

db/db F2 B6DBA-1988

Of the five markers tested in the *db/db* F2 B6DBA-1988, two demonstrated significant correlation with a specific phenotype on phenotype. B6 alleles at D6Mit54 were associated with increased fasting plasma insulin concentrations in both sexes as had been observed in five previous crosses (*ob/ob* F2 B6DBA, *db/db* F2 B6DBA, *ob/ob* F2 B6CAST, *db/db* F2 B6CAST, and *ob/ob* N2F1 B6CAST) (Table 41). The fasting plasma [insulin] was 70% higher in the B6/B6 females relative to the CAST/CAST and three times higher in the males of the same genotypic classes (Figure 94). Unlike the *db/db* F2 B6CAST for which B6 alleles had been associated with higher fasting plasma [insulin], DBA alleles at D9Mit35 were associated with lower BMIs (Table 41). B6/B6 females had BMIs which were 5.5% lower than DBA/DBA and males were 9.5% lower in their respective classes (Figure 95).

Locus	Phenotype	B6/B6	Female B6/DBA	DBA/DBA	B6/B6	Male B6/DBA	DBA/DBA	p
D6Mit54	Plasma [insulin] (uU/ml)	170.8	213.6	100.6	169.9	128.9	52.9	0.002
D9Mit35	BMI (g/cm ²)	0.566	0.584	0.600	0.522	0.546	0.577	0.005

Table 41. ANOVA results of *db/db* F2 B6DBA-1988. Only markers which demonstrated significant effects on one of the three phenotypic parameters (BMI, fasting plasma [insulin] or [glucose] are shown. Mean of each genotypic group divided by sex is listed.

F2 *db/db* B6DBA

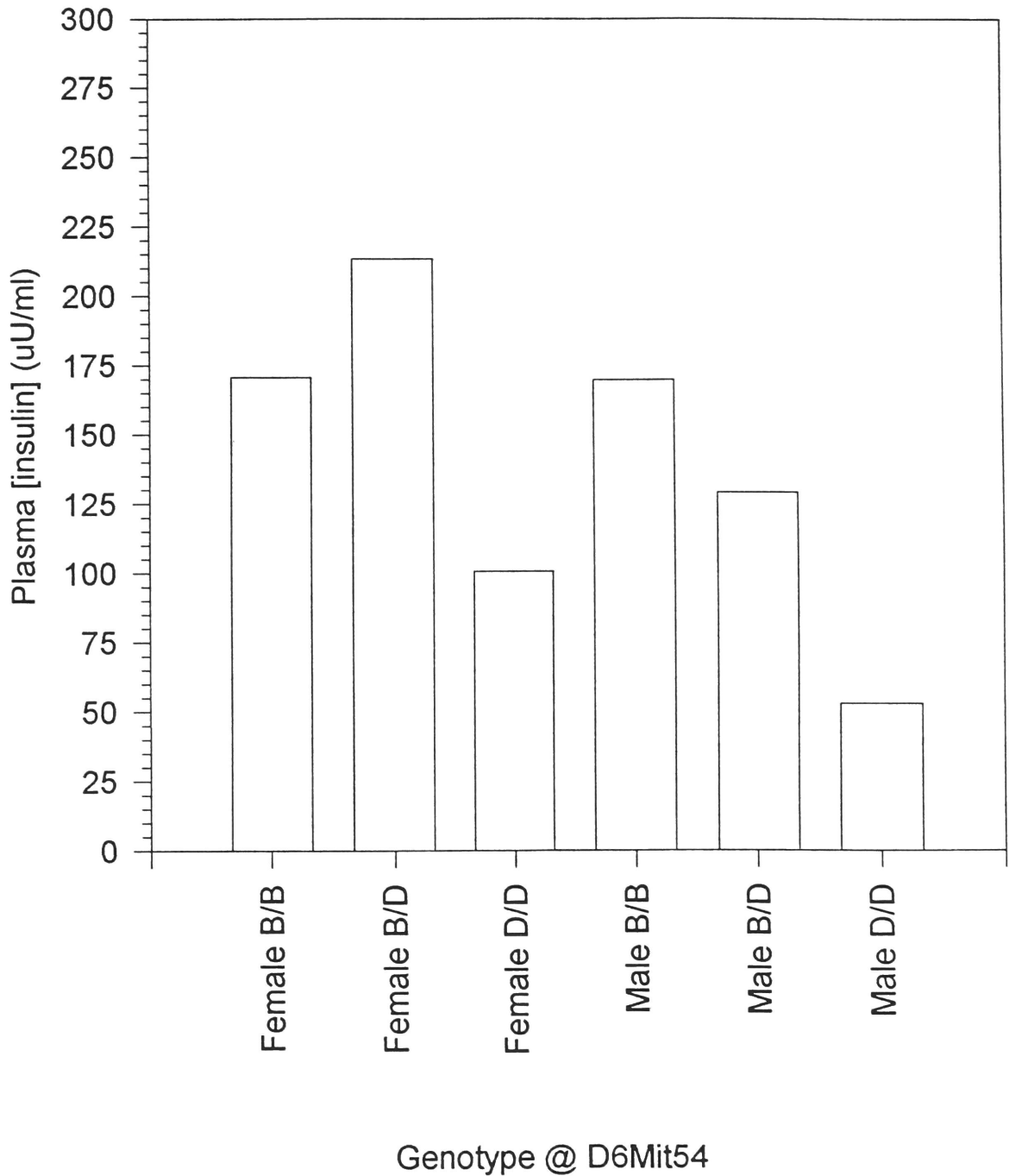


Figure 94. Relationship between genotype at D6Mit54 and plasma [insulin] in *db/db* F2 B6DBA-1988. Means indicated by the heights of the bars.

F2 *db/db* B6DBA

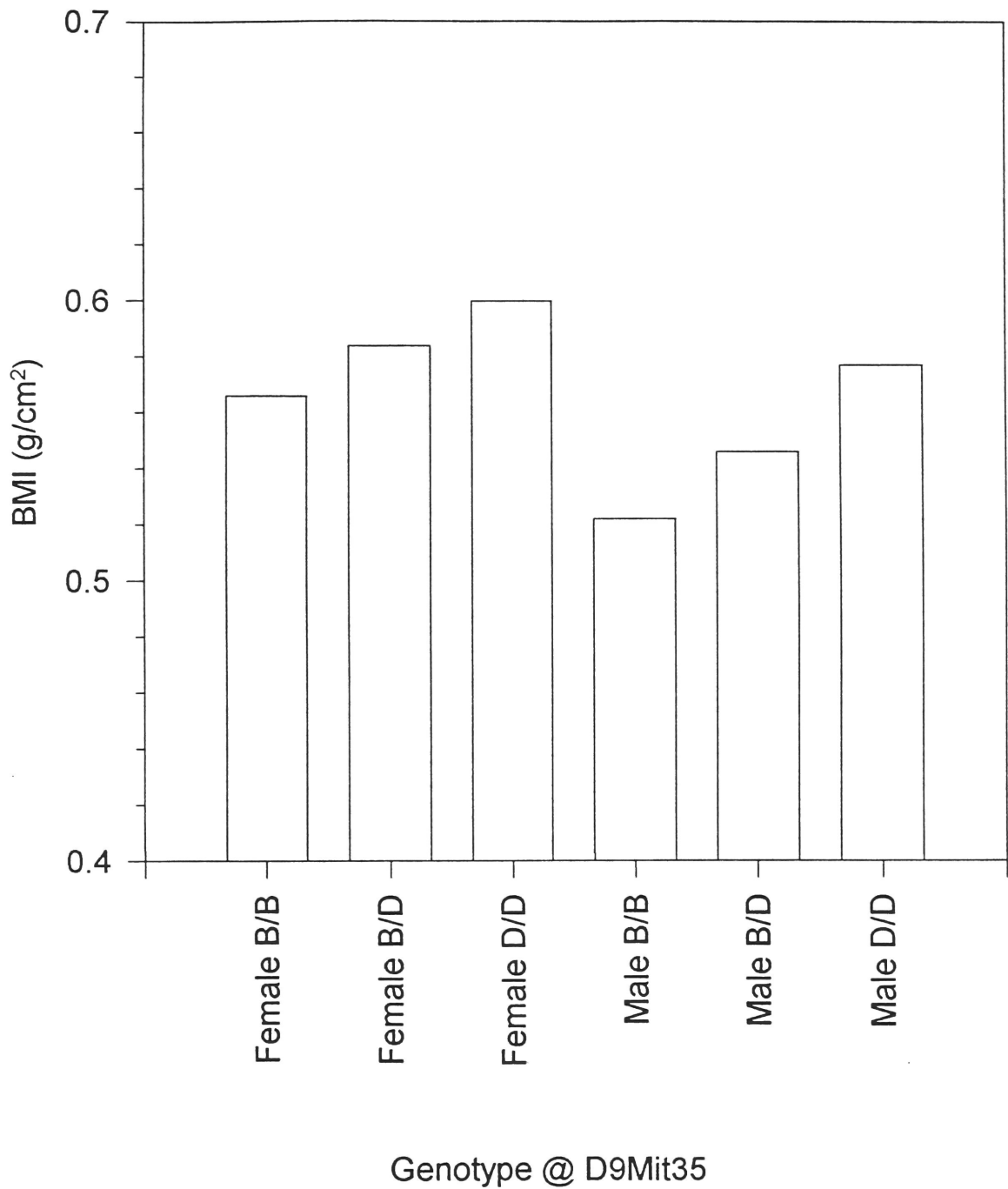


Figure 95. Relationship between genotype at D9Mit35 and BMI in *db/db* F2 B6DBA-1988. Means indicated by the heights of the bars.

fat/fat F2 BKSCAST

Of the five markers tested in the *fat/fat* F2 BKSCAST, only one demonstrated an apparent effect of genotype at the marker on any one of the three phenotypes. BKS alleles at D9Mit35 were associated with decreased BMI and decreased plasma [insulin], similar to the effect of B6 alleles at this locus observed in the *db/db* F2 B6DBA-1988 (Table 42). BKS/BKS females at D9Mit35 had 11.4% lower BMIs and 2.8 times higher fasting plasma [insulin] than CAST/CAST (Figure 96 and 97). Similarly, male BKS/BKS at D9Mit35 had 15.6% lower BMIs and 1.6 times higher fasting plasma [insulin] than CAST/CAST.

Locus	Phenotype	BKS/BKS	Female		BKS/BKS	Male		p
			BKS/CAST	CAST/CAST		BKS/CAST	CAST/CAST	
D9Mit35	BMI (g/cm ²)	0.397	0.447	0.448	0.394	0.440	0.467	0.007
	Plasma [insulin] (uU/ml)	281	584.5	777.7	441.2	662.4	725.6	0.02

Table 42. ANOVA results of *fat/fat* F2 BKSCAST. Only markers which demonstrated significant effects on one of the three phenotypic parameters (BMI, fasting plasma [insulin] or [glucose] are shown. Mean of each genotypic group divided by sex is listed.

F2 *fat/fat* BKsCAST

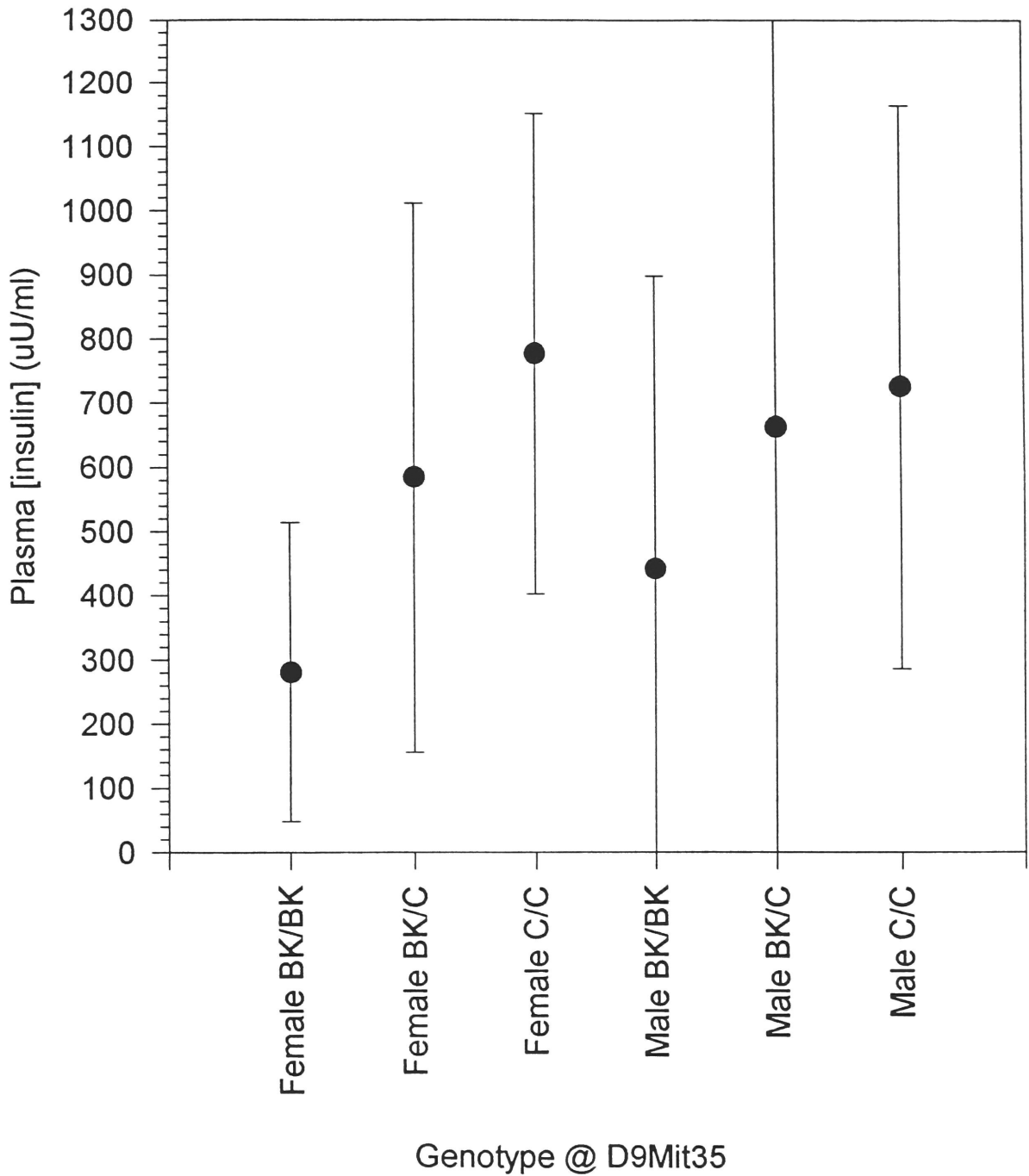


Figure 96. Relationship between genotype at D9Mit35 and BMI in *fat/fat* F2 BKSCAST.

Mean indicated by the dot and standard deviations by error bars.

F2 *fat/fat* BKsCAST

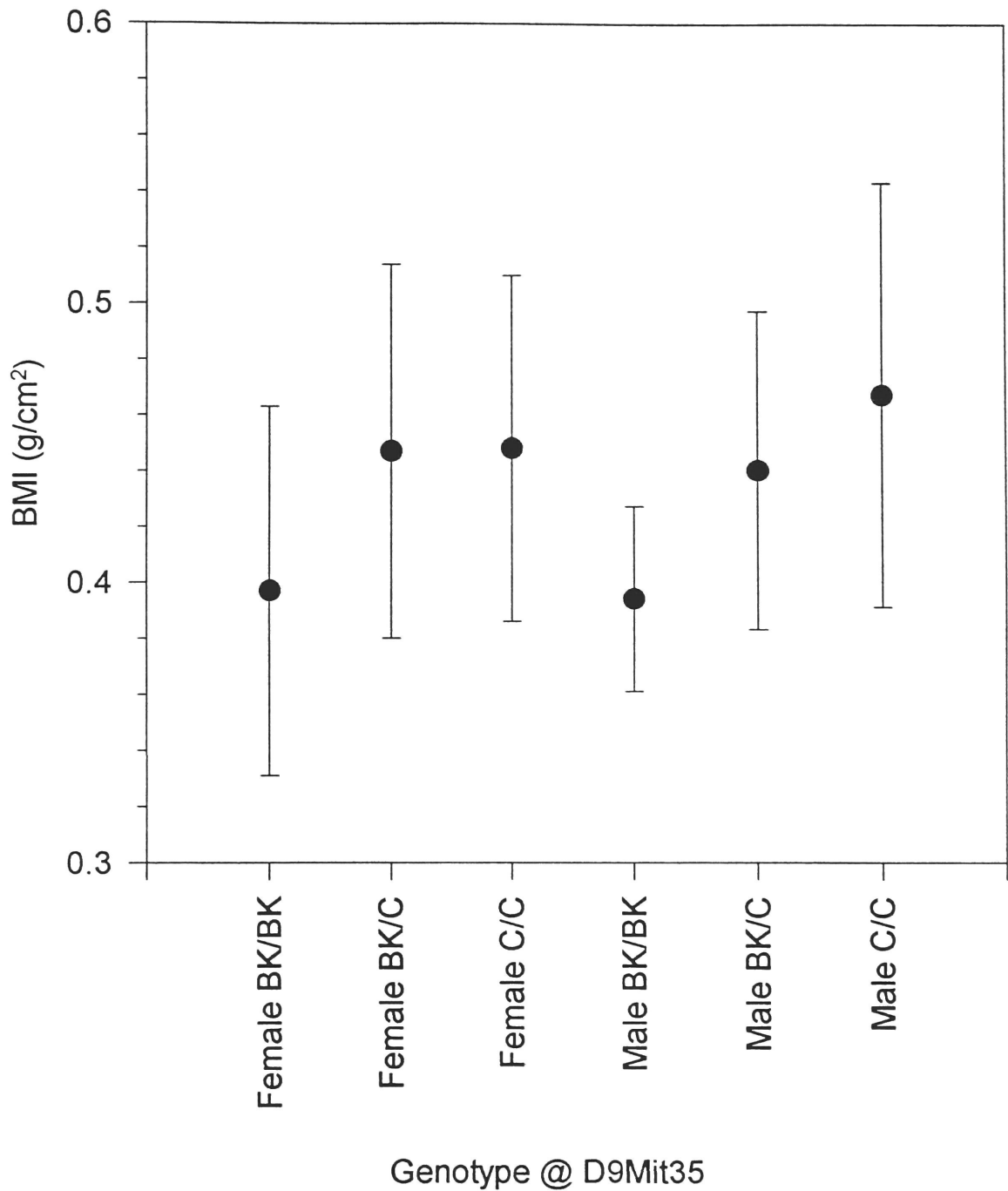


Figure 97. Relationship between genotype at D9Mit35 and plasma [insulin] in *fat/fat* F2 BKsCAST. Mean indicated by the dot and standard deviations by error bars.

tub/tub F2 B6CAST

Of the five markers tested in the *tub/tub* F2 B6CAST, two markers demonstrated an effect of genotype on obesity- or diabetes-related phenotypes. B6 alleles at *Tnfr1* on chromosome 4 were associated with decreased concentrations of plasma insulin (Table 43). Animals of both sexes which were CAST/CAST at *Tnfr1* had plasma [insulin] which were three times greater than B6/B6 animals (Figure 98). The *tub/tub* animals were the only mutation for which the apparent primary effect of this locus was on plasma [insulin]. The predominant phenotypic parameter affected by the chromosome 4 locus was body weight or BMI in the *ob/ob* F2 B6DBA and *ob/ob* F2 B6CAST. Finer QTL maps of the interval will allow evaluation of *Tnfr1*'s candidacy for a causative role in the hyperinsulinemia of the *tubby* progeny. The effect of genotype at D11Mit15 on BMI in the *tub/tub* F2 B6CAST was exactly the opposite of the effect observed in the *db/db* F2 B6CAST since B6 alleles were associated with increased BMIs in the *tub/tub* and decreased BMIs in the *db/db*.

Locus	Phenotype	B6/B6	Female		CAST/CAST	B6/B6	Male		p
			B6/CAST				B6/CAST	CAST/CAST	
Tnfr1	Plasma [insulin] (uU/ml)	184.0	196.9		535.3	346.8	427.4	1171.7	0.003
D11Mit15	BMI (g/cm ²)	0.455	0.431		0.403	0.454	0.417	0.423	0.04

Table 43. ANOVA results of *tub/tub* F2 B6CAST. Only markers which demonstrated significant effects on one of the three phenotypic parameters (BMI, fasting plasma [insulin] or [glucose] are shown. Mean of each genotypic group divided by sex is listed.

F2 *tub/tub* B6CAST

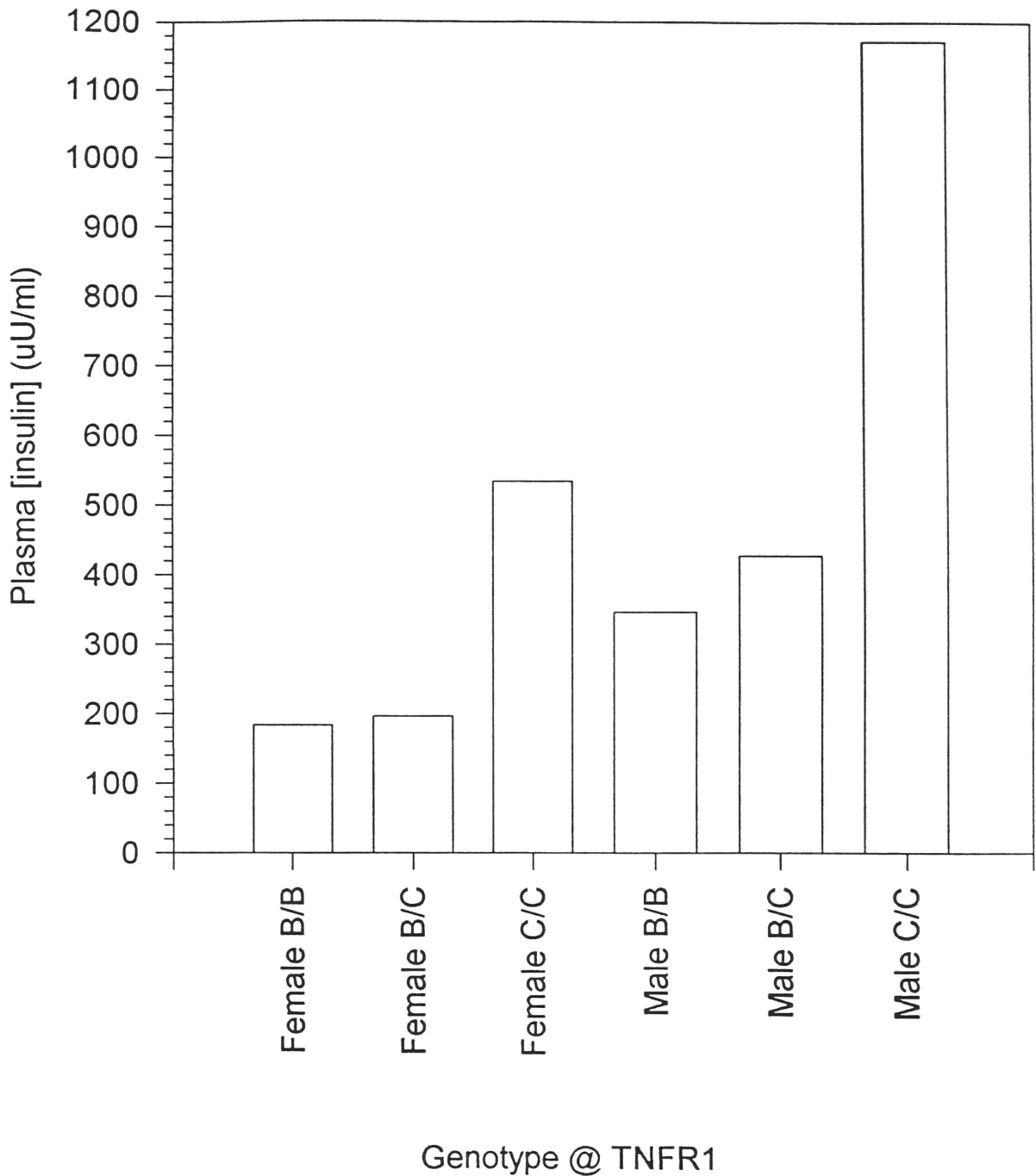


Figure 98. Relationship between genotype at Tnfr1 and plasma [insulin] in *tub/tub* F2 B6CAST. Means indicated by the heights of the bars.

Discussion

The difference in fasting plasma [insulin] and [glucose] between the *ob/ob* F2 B6DBA from 1988 and 1994 is dramatic. The ages of the animals and degree of obesity were approximately the same in the two crosses. The *ob/ob* F2 B6DBA-1988 were fasted overnight rather than for three hours, but the prolonged fast should have decreased plasma [insulin] and [glucose] as was observed in the *db/db* F2 B6DBA-1988. The *ob/ob* F2 B6DBA-1988 and *db/db* F2 B6DBA-1988 were maintained in the same facility at the same time yet demonstrated the most dramatic differences in plasma [insulin] observed between any two crosses segregating *ob* and *db* between the same strains. The most likely explanation for the high plasma [insulin] observed in the *ob/ob* F2 B6DBA-1988 is a technical problem with the radioimmunoassay for which standards to compare interassay variability were not available.

The *fat/fat* F2 BKSCAST clearly demonstrate that either the degree of obesity, onset and/or the specific genetic mechanism by which the animals become obese is relevant to the degree of diabetes susceptibility conferred by the parental strains. Both BKS and CAST are diabetogenic strains, yet *fat/fat* the mice were not as hyperglycemic as the majority of outcrossed *ob/ob* or *db/db*.

The *tub/tub* F2 B6CAST mice were not diabetic but did demonstrate varying degrees of insulin resistance based on the large variance in plasma [insulin], especially in the males. Once the *tubby* mutation has been published, it may be possible to account for the

hyperinsulinemia in the males. Both the *fat* and *tubby* mice provide an opportunity to test the relevance of the diabetes QTLs in settings of later onset, more moderate obesity which may be more relevant to human NIDDM.

Of the six QTL regions tested, only the locus on chromosome 7 failed replication in at least one of the four crosses. Two of the regions, located on chromosomes 4 and 9, were replicated in two crosses, one of which was either segregating *fat* or *tub*, the models of moderate, later onset obesity. The phenotypes correlated with the QTL's in the four crosses were often but not always the same phenotype related to the genotype at the locus in previous crosses. The new phenotypes correlated with the QTLs may represent different expression of the genes under different fasting conditions, effects of different obesity mutations, and/or effects of parental strains.

End