MAPPING OF BEHAVIOURAL QUANTITATIVE TRAIT LOCI

Maria Grazia Turri

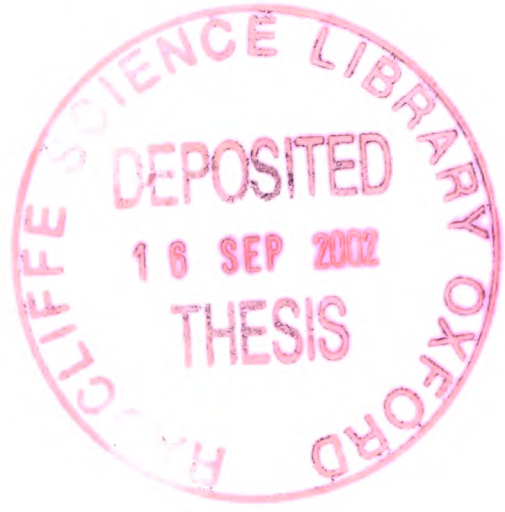
Wadham College

University of Oxford

A thesis submitted for the degree of

Doctor of Philosophy

Trinity 2002



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ABSTRACT

Anxiety is a common disorder which affects about 25% of the population and whose pathophysiology is still poorly understood. Animal models of disease have been widely used to investigate the molecular basis of human disorders, including psychiatric illnesses. This thesis is about the study of the genetic basis of a mouse model of anxiety.

I have carried out a QTL mapping study of behavioural measures thought to model anxiety. I report results from 1,636 mice, assessed for a large number of phenotypes in five ethological tests. Mice belonged to two F2 intercrosses originated by four lines generated in a replicate selection experiment. By comparing mapping results between the two crosses, I have demonstrated that selection operated on the same relatively small number of loci in the four selected lines.

Analysis of genetic effect of QTL across phenotypes has allowed me to identify loci with specific roles on different dimensions of anxious behaviour, therefore enhancing our understanding of the anxiety phenotype in mice. For some of these QTL I have also accomplished fine mapping experiments: a locus on chromosome 15 is now contained in an interval of only 3 centimorgans.

This work is the basis for further molecular dissection of the genetic loci that underlie anxiety and provides a starting point for the discovery of genes involved in a common psychiatric condition.

Dicono che la felicità dell'uomo non può consistere fuorché nella verità. Così parrebbe, perché qual felicità in una cosa che sia falsa? E come, se il mondo è diretto alla felicità, il vero non deve render felice? Eppure io dico che la felicità consiste nell'ignoranza del vero.

Giacomo Leopardi, Zibaldone

It is said that human happiness cannot consist of anything other than truth. So it seems, and indeed which happiness could be found in something false? And if the world is heading for happiness, how could the truth not make one happy? Yet I say that happiness consists of being ignorant of the truth.

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Original Papers

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Contents

Abbreviations

Abbreviations used in the text are listed alphabetically and are defined when the first appear.

°C	degrees Celsius
5-HT	5-hydroxytryptamine
ANOVA	analysis of variance
BAC	bacterial artificial chromosome
bp	base pairs (of deoxyribonucleic acid)
C1, C2	DeFries control strains
Chr	Chromosome
cm	centimetre(s)
cM	centimorgan(s)
DNA	deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
EDTA	ethylene diaminotetra-acetic acid
EMO	Emotionality
G0	Original H1XL1 F2 intercross (Flint, Corley et al. 1995)
G1	Group 1 (H1XL1 F2 intercross)
G2	Group 2 (H2XL2 F2 intercross)
GABA	gamma-amino butyric acid

GAD	Generalized Anxiety Disorder
EPM	Elevated Plus Maze
H1, H2	DeFries High OFA selected strains
ICD	International Classification of Diseases
L1, L2	DeFries Low OFA selected strains
LD	Light Dark Box
LOD	logarithm to the base 10 of the odds ratio of linkage against no linkage
LR	likelihood ratio
M	molar
mg	milligram(s)
µg	microgram(s)
min	minute(s)
MIT	Massachusetts Institute of Technology
µl	microlitre(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MR	Mirror Chamber
ng	nanogram(s)
No.	Number

OF	Open Field
OFA	Open field activity
OFD	Open field defecation
QTL	quantitative trait locus/ quantitative trait loci
PCR	polymerase chain reaction
RI	Recombinant Inbred
RIST	Recombinant Inbred Segregation Test
rpm	revolutions per minute
sec	second(s)
SQ	Elevated Square Maze
TE	Tris-EDTA
Var	variance

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CHAPTER 1

Introduction

Anxiety is a very common condition whose aetiology is poorly understood and, when it manifests as a psychiatric disorder, one for which we have few effective long term therapies. Anxiety disorders have a high morbidity: the life-time prevalence for any anxiety disorder is about 25%, which means that 1 in 4 people will experience anxiety at a disturbing level (Kessler, McGonagle et al. 1994). They represent a heavy burden for society and have been estimated to cost the US economy about $42.3 billion dollars in 1990 (Greenberg, Sisitsky et al. 1999).

The benzodiazepines, the standard anti-anxiety drugs, are among the most commonly prescribed drugs in the Western countries: prescriptions for benzodiazepines in Great Britain were estimated to be 26 million in 1987 (Taylor 1987). Although there is much psychopharmacological research on anti-anxiety drugs, and although already a few different classes of drugs are available (Argyropoulos, Sandford et al. 2000), current therapeutic regimes are effective in the short term, but are far from providing a cure.

One reason why we do not have an effective cure for anxiety disorders is that we have such a poor understanding of their pathophysiology. This thesis concerns one approach to advancing our understanding of the causes of anxiety, by investigating the genetic basis of an animal model of the condition.

Genetic analysis of anxiety has the potential to identify the molecular components of the trait, therefore advancing our understanding of its pathophysiology and eventually resulting in the development of novel therapies. The reason for studying an animal model is that it is expected to overcome the limitations of the genetic approaches currently being attempted with humans. However, using an animal model of a human behaviour has the drawback that the results may not be applicable to the human phenotype. One of the main concerns of my thesis is to evaluate the mouse phenotype for anxious behaviour.

In this chapter I will first describe the key features of human anxiety, and briefly discuss the results of the genetic approach in research on anxiety. I will then discuss the validation of rodent models of anxiety and give a general overview and a detailed description of five of such models.

1.1 Definition of anxiety in humans

1.1.1 Anxiety as a normal emotion

Anxiety is an emotion that every human being will have experienced at some point of her life, at least in a mild and fleeting form. Descriptions of normal anxiety provide quantitative measures and address the question of how can we distinguish anxiety from other emotions. The common view of anxiety is that it consists of an unpleasant, diffuse and vague sense of apprehension, lack of energy, restlessness and muscular tension, accompanied by a variable number of peripheral manifestations such as trembling, shortness of breath, palpitations, tightness on the chest, mild stomach discomfort, and others (Kaplan, Sadock et al. 1994).

In the last century a number of psychological explanations of anxiety have been proposed. Darwin (Darwin 1998) was the first to suggest the adaptive role of anxiety and other emotions and their importance for evolutionary development. His arguments stimulated enquires into the manner in which emotional reaction may promote survival, leading to the evolutionary psychology view that anxiety is the process that arouses and organises the biological activities required to render the individual capable of dealing with the threats of life.

Another psychological formulation is the proposed distinction between fear and anxiety, on the basis of the nature of the threat and its relationship to the perceptions of the subject. There have been differing theories about the relationship between fear and anxiety. On one hand, the Blanchards (Blanchard and Blanchard 1990; Blanchard, Yudko et al. 1993) classify a mouse's reaction to a cat as fear when the mouse is faced with an 'actually present' cat, and as anxiety when the mouse is put in a situation where the cat could be present, but is not, for instance in a cage where the mouse had been previously faced with a cat. On the other hand, Gray (Gray and McNaughton 2000b) suggests that the distinction between fear and anxiety is not so much based on the nature (actual or potential) of the threat, but the way in which the animal responds to the threat: it is fear when the mouse's behaviour is aimed at avoiding a danger, like a cat actually or potentially present; it is anxiety when the behaviour functions to facilitate the approach to a danger (again, were it actual or perceived as potential).

However, although these distinctions have been of help in the investigations into the nature of anxiety, our present understanding does not allow for a clear-cut distinction between fear and anxiety.

State and trait anxiety

The accounts of anxiety that I have briefly discussed are based on the definition of anxiety as an emotional state. The work of Cattell and Scheier (Cattell and Scheier 1963; Cattell 1966) and the application of multivariate analysis to defining and measuring anxiety, introduced the concept of trait-anxiety, as somewhat independent for that of state-anxiety.

If state-anxiety is defined as the transitory emotional reaction that manifests when the imminence of threat is experienced, trait-anxiety refers instead to a relatively stable characteristic of individual persons in their tendency to perceive stressful situations as threatening, and to react to such situations with elevations in the intensity of state-anxiety.

Cattell and Scheier's multivariate approach indicated that while physiological variables, such as shortness of breath or palpitations, loaded strongly on the state-anxiety factor, as one would expect for symptoms that fluctuate over time and express the arousal of the autonomic nervous system during the emotional experience, variables indicating some stable characteristics of personality, like ego-weakness, guilt-proneness, or tendency to embarrassment, loaded on a second, relatively independent axis, that was hence defined as trait-anxiety (Cattell and Scheier 1963; Cattell 1966).

The concept of trait-anxiety sets the ground for what we can call 'susceptibility' to anxiety, i.e. the individual differences in the frequencies and the modes in which people (and indeed animals) make access to their state-anxiety repertoire. Measuring trait anxiety is a matter of some dispute, but there is now convincing evidence that increased

susceptibility to anxiety (and depression) can be attributable to genetic variation. The data in support of this assertion come from studies of variation in personality (Kendler, Neale et al. 1993b).

1.1.2 Anxiety as pathology

Psychological descriptions of pathological anxiety and attempts to differentiate it from normal anxiety date back to the end of the nineteenth century when Freud (Freud 2001) coined the term 'anxiety neuroses' and proposed that pathological anxiety was the experience of the conscious self faced with the appearance of unconscious processes attempting to escape repression and pressing for conscious representation and discharge.

Psychodynamic explanations of pathological anxiety have now largely been replaced by purely descriptive accounts, formulated in the two classification systems in use today: the International Classification of Diseases (ICD)-10 (World Health Organisation 1992), and the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV (American Psychiatric Association 1994). The general mark for pathological anxiety in these classification systems requires that its psychological or physical symptoms are sufficiently severe to lead the sufferer to seek a medical help and/or to cause impairment in social or occupational functioning.

Table 1.1 reports the sub-categories identified by the DSM-IV for classifying anxiety disorders (American Psychiatric Association 1994).

Table 1.1

Classification of Anxiety Disorders according to DSM-lV (American Psychiatric Association 1994).

Panic Disorder Without Agoraphobia
Panic Disorder With Agoraphobia
Agoraphobia Without History of Panic Disorder
Specific Phobia
Social Phobia
Obsessive-Compulsive Disorder
Posttraumatic Stress Disorder
Acute Stress Disorder
Generalized Anxiety Disorder
Anxiety Disorder Due to a General Medical Condition
Substance-Induced Anxiety Disorder
Anxiety Disorder Not Otherwise Specified

These are (American Psychiatric Association 1994; Kaplan, Sadock et al. 1994):

- Panic disorder, characterised by the overwhelming and precipitous feeling of anxiety, so intense that the autonomic and cognitive signs are exaggerated into attacks of palpitations, tachypnoea, choking, dizziness, and fainting, with intense fear of death, insanity, or uncontrollability (so-called panic attacks);
- Phobias, defined as the irrational experience of fear in the presence a situation (agoraphobia and social phobia) or object (simple phobia), resulting in severe distress in the presence or anticipation of the phobic entity;
- Obsessive-compulsive disorder, characterised by one or more obsessions, in the form of recurrent and intrusive thoughts, ideas, images and impulses, and compulsive and standardised rituals such as hand washing, checking, or counting, that are time-consuming activities, which are performed by the affected person in an attempt to reduce the anxiety level associated with the obsession(s);
- Posttraumatic and acute stress disorders, consisting in a state of hyper-arousal as a consequence of a previously lived trauma (like combat experience, natural catastrophes, assault, or rape), which is re-experienced with great distress through dreams and waking thoughts;
- Generalized anxiety disorder (GAD), which is a non-specific anxiety syndrome, defined by excessive and pervasive worry, with no obvious precipitant, and accompanied by a variety of somatic symptoms;
- Anxiety disorder due to an organic cause, such as a general medical condition, or drug intoxication or withdrawal.

Categories in DSM-IV are established by collecting a vast amount of clinical data, and rely solely on description of disease symptoms, while no space is given to etio-

pathogenetical theories (with the exception of the cases where an organic cause such as a disease, or the use of a substance, is used as a tool for differential diagnosis).

Table 1.2 reports lifetime and 12-month prevalence for anxiety disorders as reported by the National Comorbidity Survey, one of the largest epidemiological study carried out to date on a sample in the US. The table reports recently revised 12-month prevalence for the same disorders (Kessler, McGonagle et al. 1994; Narrow, Rae et al. 2002).

The advantages of such a classification are immediately evident: it allows a standardisation of diagnosis so that epidemiological data can be collected and compared across the world and any finding of clinical or scientific trials can be generalised (Kramer 1969; Zubin 1969).

There are though several limitations to the categorical classification. A major difficulty is that it does not produce a clear distinction between disorders. Not only do we find that the symptom profiles of the disorders overlap, so that it is often difficult to unequivocally make a diagnosis, but also there is a high level of comorbidity between different categories of anxiety disorders and also between anxiety disorders and depression.

Data from the large national comorbidity study carried out in the US (Kessler, McGonagle et al. 1994) show that comorbidity between mental disorders is a very common phenomenon: as reported in Table 1.3, out of the great proportion of people (about 48% of a national probability sample of the US population) that will ever experience a mental disorder, more than half (27% of the sample) has been affected by 2 or more mental disorders.

Table 1.2

Lifetime and 12-month prevalence for anxiety disorders as reported by the National Comorbidity Survey on a national probability sample in the US (Kessler, McGonagle et al. 1994) and recently revised 12-month prevalence for the same disorders (Narrow, Rae et al. 2002).

Disorder	lifetime	12-month	12-month revised
Any anxiety disorder	24.9%	17.2%	12.1%
Panic disorder	3.5%	2.3%	1.7%
Agoraphobia	5.3%	2.8%	2.2%
Simple (specific) Phobia	11.3%	8.8%	4.4%
Social Phobia	13.3%	7.9%	3.7%
Generalized Anxiety Disorder	5.1%	3.1%	2.8%

Table 1.3

Comorbidity data of any mental disorder from (Kessler, McGonagle et al. 1994).

Number of life-time disorders	Proportion of sample
0	52%
1	21%
2	13%
≥3	14%

In order to give an idea of the occurrence of this phenomenon in anxiety disorders, Table 1.4 reports results of comorbidity between generalized anxiety disorder (GAD) and other disease from a study on 109 patients with GAD (Brawman-Mintzer, Lydiard et al. 1993), showing that as high as 74% of the patients have suffered from at least one additional mental disorder. Table 1.5 reports the most common comorbid diagnosis in the same sample: it is noticeable that 42% of the GAD patients have suffered in the past of at least one major depressive episode, and that 55% of them have been diagnosed another anxiety disorder a part from GAD.

The loose boundaries between disorders not only exist within the anxiety disorder group, but also between anxiety disorders and depression, as shown not only by the high comorbid data between GAD and major depression, but also from the fact that typical antidepressant drugs as tryciclic antidepressants are effective also across the whole range of anxiety disorders.

1.2 Genetics of anxiety disorders

Family and twin studies support the familial nature of anxiety disorders such as panic disorder (Crowe, Noyes et al. 1983; Weissman 1993; Kendler, Neale et al. 1993a), agoraphobia (Harris, Noyes et al. 1983), phobias (Kendler, Neale et al. 1992a; Fyer, Mannuzza et al. 1995; Kendler, Myers et al. 2001), obsessive compulsive disorder (Lenane, Swedo et al. 1990), and GAD (Noyes, Clarkson et al. 1987; Hettema, Prescott et al. 2001), and have also reported a familial relationship between anxiety disorders and depression (Kendler, Neale et al. 1992b; Roy, Neale et al. 1995).

Table 1.4

Prevalence of comorbid mental disorders in 109 patients with GAD (Brawman-Mintzer, Lydiard et al. 1993).

Any additional mental lifetime disorder	74%
One additional disorder	37%
Two additional disorders	20%
Three or more additional disorders	17%

Table 1.5

Type of comorbid mental disorders in 109 patients with GAD (Brawman-Mintzer, Lydiard et al. 1993).

Social phobia	23%
Simple phobia	21%
Panic disorder	11%
Past major depressive episode	42%
Past substance abuse	24%

While family studies can only demonstrate that anxiety disorders run in families, twin studies can also discern between the influence of genetic factors and of a common environment on their transmission. For example, two large studies of phobias in male and female twins respectively (Kendler, Neale et al. 1992a; Kendler, Myers et al. 2001) addressed the question of how genetic and environmental effects operate in these syndromes.

1.2.1 Molecular Genetic approaches to anxiety disorders

A genetic approach to anxiety is justified by the evidence that anxiety disorders and more generally anxiety as a trait are dependent on genetic factors: it should therefore be possible to establish which genes contribute to susceptibility to anxiety.

By delivering the molecular variants that are responsible for susceptibility to anxiety, molecular genetic techniques may provide new insights into the causes of the condition. This in turn could lead to the development of novel therapies and provide insights into the biological relationships hidden in our classificatory systems.

Two methods have been used to try to find the molecular determinants of anxiety in humans: linkage and association studies. Linkage studies use pedigrees to test whether a particular allele (marker) is co-inherited with the expression of the disorder of interest. Linkage studies of anxiety are rare, because large numbers of pedigrees are needed in order to detect the small genetic effects that are likely to be responsible for the genetic variation contributing to anxiety disorders. Association studies compare the frequency of a particular marker between a sample of affected patients and a sample of non-affected individuals (controls). Association studies have analysed the role of many putative genes

in the genetic predisposition of anxiety disorders (like genes involved in the gamma-amino butyric acid (GABA) and 5-hydroxytryptamin (5-HT) transmission pathways). Neither association nor linkage study has produced conclusive evidence for the detection of a genetic factor involved in anxiety disorders, as reviewed in (Lesch 2001).

1.3 Validating animal models of anxiety

An operational definition of rodent anxiety would require the animal to show a consistent pattern of responses in a series of behavioural tasks, so that for example, a fearful animal would be slow to emerge into a threatening environment but quick to avoid a predator. The difficulty here is to prove that these tests are measuring the animals' fearful behaviour in a way that is related to human anxiety.

According to one of the first papers that discussed criteria to be used to validate attempts at modelling psychopathology (McKinney and Bunney 1969), an animal model should faithfully represent symptoms of the human disorder, in a way that produces observable behavioural changes that can be objectively evaluated by independent observers. Moreover, symptoms and behavioural changes should be reverted by treatments that are effective in the corresponding human disorder. Since then researchers have discussed validation of animal tests from three distinct points of view: predictive, face, and construct validity (Willner 1990).

1.3.1 Predictive validity

Predictive validity maintains that performance in the test predicts performance in the corresponding human condition, and implies a similarity of effect on the model by factors known to influence the disorder (Willner 1990).

In practise, in animal models of anxiety this has been determined through pharmacological studies, using drugs known to have an effect on anxiety in humans and asking if these drugs affect the model in a predictable way. Most of the evidence that animal models of anxiety represent anxious behaviour in a predictive way, comes from pharmacological studies investigating the effect of anxiolytic drugs, in particular the benzodiazepines, together with anxiogenic compounds, on the performance of animals in the tests (Green and Hodges 1991).

When testing anxiolytic agents, the value of the variables thought to represent high anxiety levels should decrease, while the value of the variables thought to represent low anxiety levels should increase. On the other hand, anxiogenic compounds should affect the same variables in the opposite direction. Finally, drugs with no effect on human anxiety should have no effect in the model. Generally speaking, then, a good test for anxiety should be sensitive to anxiolytic and anxiogenic drugs in an opposed and predictable way, while other drugs should have no effect on it.

Although these results have been used to claim validation for many animal models of anxiety, conclusive evidence has been difficult to obtain. One major problem has been that the action of benzodiazepines in the tests can be attributed to their effects on locomotor activity rather than to their anxiolytic effects (Dawson, Crawford et al. 1995).

A further problem concerns the spectrum of action of anxiolytic drugs in humans and in the models. Benzodiazepines are not effective in all human anxiety disorders, while other drugs known to act as anxiolytics in humans (for instance buspirone), have inconsistent effects on the tests. Moreover, some anxiety disorders, like panic disorders, respond to antidepressant drugs, which are rarely used to test for predictive validity of the models (Willner 1990).

1.3.2 Face validity

Face validity refers to the acknowledgement of similarities between the model and the correspondent disorder (Willner 1990), i.e. the expression of fearful behaviour in the model should represent symptoms and manifestations of human anxiety (Green and Hodges 1991). It has been shown in rodents that fear elicits responses that have human equivalents such as: autonomic changes, like tachycardia, hypertension and increased defecation, analgesia, and increased release of several hormones (Fendt and Fanselow 1999). But although some of these aspects have been used to test anxiety in rodents, most of them require too laborious and invasive procedures. Moreover, anxiety disorders in humans range from the more definite manifestations of phobias or panic attacks to the vague symptoms of generalized anxiety disorders and include cognitive changes and self-reported subjective experiences that are not testable in rodents.

However, as I have previously discussed, anxiety not only exists as a pathological condition. It is also an emotional reaction that is experienced by every human being in relation to threat, and it is this latter aspect that is amenable to modelling in rodents,

regardless of the discrepancies between the manifestations of the response to threat between animals and humans.

Studies (Blanchard and Blanchard 1989; Blanchard, Griebel et al. 2001) investigating defensive behaviour in rats and mice exposed to a cat in an ethological test called the visible burrow system, found that immediate flight to burrows was followed by freezing or immobility, which could last several hours, and long-latency re-entry on the surface after the cat had been removed was characterised by inhibition of non-defensive behaviours throughout, until normal activities were resumed. This result only confirms the widely accepted concept that fearfulness in rodents manifests itself as competition to innate activities such as exploratory drive (Lister 1990). In fact, the most widely used animal models of anxiety are based on the idea that a fear-inducing situation inhibits spontaneous behaviour, and therefore measure the degree of such disruption, for instance in terms of decrease in locomotor exploration, as in many unconditioned paradigms, or in terms of suppression of escape behaviour, as in some conditioned tests such as conditioned freezing or the shuttle box.

1.3.3 Construct validity

As I have just discussed, attempt to validate rodent models of anxiety through any direct comparisons between manifestations in humans and animals, is jeopardised by an important limitation: the assumption that the same condition presents similar manifestations in different species. Although some similarities between fearful behaviours across species indeed exist (for instance immobility or freezing can be considered a reaction to fear which is well conserved), obvious examples showing that

this is not always the case can be directly drawn by the experience with animal models of anxiety. For instance, while rearing on the hind legs in rodents has been shown to represent a stress-induced stereotyped behaviour, this is clearly not true for human beings. In order to claim that rearing in rodents is a symptom of anxious behaviour as, for instance, scratching (a very different manifestation) in humans, we would need an account of a common biological mechanism underlying the two conditions in the two species (Willner 1991). Here construct validity comes into play.

Construct validity is based on the construction of theories accounting for the physiological and biochemical mechanisms that underlie brain circuits thought to be implied both in the model and in the correspondent disorder.

The most comprehensive neuropsychological account of anxiety (Gray and McNaughton 2000) argues that anxiety corresponds to activation of the so-called Behavioural Inhibition System (BIS). In brief, when the BIS, which is thought to be anatomically placed in the septo-hippocampal brain region, detects signals associated with potential threats like novelty, punishment, and non-reward, it suppresses ongoing behaviour so that attention and arousal are increased (Gray and McNaughton 2000a). The theory has been supported by evidence coming both from the effect of anxiolytic drugs across a range of animal models of anxiety, and from experiments involving behavioural study of rodents subjected to lesions of the septo-hippocampal system.

There is enough evidence that the anatomical and physiological brain structures that underlie animal physiological fear and human anxiety are the same to suggest that the same biological mechanisms might underlie the two conditions (Davis 1992; Lang, Bradley et al. 1998; LeDoux 1998; Gorman, Kent et al. 2000; Lang, Davis et al. 2000).

On this basis, tests for stress-induced adaptive behaviour in rodents should have some value for studying human pathological anxiety (File 1992).

1.4 Distinction between conditioned and unconditioned tests

Broadly speaking, rodent models of fearful behaviour involve exposure of the animal to a stress-provoking situation presenting one or more aversive stimuli, and they may be distinguished in two general categories involving either conditioned or unconditioned responses. Approaches using learned fear examine conditioned behaviours provoked by stimuli that have become associated with something aversive (more commonly an electric foot-shock), while unconditioned tests rely on stimuli that mimic a natural environmental threat and therefore are innately recognised by the animal without need of any prior learning experience. This distinction is important because conditioned tests are amenable to experimental manipulation to a degree that is impossible with unconditioned tests and offer the possibility of a strong control over the experiment and its potential confounds (Willner 1990). From an evolutionary perspective, conditioned fear, by activating protective behaviours, is thought to provide a tool for survival in the face of threat and has been widely investigated though neurophysiological studies (Fendt and Fanselow 1999). Consequently with conditioned tests we know more about the reasons for the behaviour we are testing (Gray 1982). However, conditioning requires a long training of subjects, and the outcome of this training depends on cognitive and memory abilities, which may act as confound factors in the subsequent testing of anxious behaviour.

The study of unconditioned responses typically involves assessing the animal's behaviour in a mildly stressful environment, where anxiety is elicited by stimuli that naturally

provoke fear, like exposure to a predator or to a novel place where a predator can potentially be in ambush. Fearfulness is measured either by response inhibition, in the form of suppression of other natural drives, typically exploration, or by response emission, for example in the form of risk assessment behaviours, like grooming or stretching. As unconditioned tests take advantage of the natural response of rodents when faced with ecologically relevant environmental challenges, they are also defined ethological, and are thought of providing a high degree of ecological validity for the research (Rodgers and Cole 1994).

1.5 Tests of anxiety in rodents

Table 1.6 lists the conditioned and unconditioned behavioural tests used to measure fearful behaviour in rodents. Although a great number of tests have been developed, and most of them have been used both in rat and mouse, only a small fraction of these are in common use in the mouse.

Table 1.7 lists the number of references currently (19 March 2002) available in PubMed (http://www.ncbi.nlm.nih.gov/entrez/) for studies involving use of each test in the mouse. Search words are given in the table.

Perusal of the table shows that the ethological tests (Elevated plus maze, Open field) are preferred and this is for two reasons. First, most of the tests are designed to validate drugs as potential anxiolytic agents, for which purpose a high throughput, easy to administer test is required.

Table 1.6

Animal behavioural models of anxiety in rodents

(Rodgers 1997; Rodgers and Dalvi 1997).

Conditioned responses	**Unconditioned responses**
Foot-shock induced freezing (punished locomotion)	Elevated plus maze
Active/passive avoidance (shuttle box)	Elevated zero maze
Fear potentiated startle	Open field
Geller-Seifter conflict	Light-dark box
Vogel water-lick conflict	Mirror chamber
Four-plate test	Free exploration
Conditioned emotional response	Hole-board
Conditioned taste aversion	Staircase test
Conditioned suppression of drinking	Social interaction
Conditioned defensive burying	Social competition
Learned helplessness	Separation-induced ultrasonic vocalisations
	Anxiety/defence test battery
	Fear/defence test battery
	Mouse defence test battery
	Hyponeophagia
	Pain-induced ultrasonic vocalisations
	Startle response (baseline)

Table 1.7

Number of references currently (19 March 2002) available in PubMed (http://www.ncbi.nlm.nih.gov/entrez/) for behavioural models of anxiety in the mouse. Search words are given in the table.

TEST	Search Words	Number of References
Conditioned responses		
Foot-shock induced freezing (punished locomotion)	Foot-shock freezing mice Punished locomotion mice	5 7
Active/passive avoidance (shuttle box)	Shuttle box mice	131
Fear potentiated startle	Fear potentiated startle mice	6
Geller-Seifter conflict	Geller Seifter mice	13
Vogel water-lick conflict	Water-lick conflict mice	10
Four-plate test	Four-plate mice	19
Conditioned emotional response	Conditioned emotional response mice	25
Conditioned taste aversion	Conditioned taste aversion mice	103
Conditioned suppression of drinking	Conditioned suppression drinking mice	12
Conditioned defensive burying	Conditioned defensive burying mice	0
Learned helplessness	Learned helplessness mice	49

Continues next page

Continued from previous page

TEST	Search Words	Number of References
Unconditioned responses		
Elevated plus maze	Elevated plus maze mice	407
Elevated zero maze	Elevated zero maze mice	7
Open field	Open field mice	891
Light-dark box	Light-dark box mice	72
Mirror chamber	Mirror chamber mice	4
Free exploratory paradigm	Free exploratory paradigm mice	17
Hole-board	Hole-board mice	76
Staircase test	Staircase test mice	45
Social interaction	Social interaction mice	132
Social competition	Social competition mice	14
Separation-induced ultrasonic vocalisations	Ultrasonic vocalisation mice	1
Anxiety/defence test battery	Anxiety/defense battery mice	18
Mouse defence test battery	Mouse defense battery	28
Hyponeophagia	Hyponeophagia mice	2
Pain-induced ultrasonic vocalisations	Pain vocalisation mice	3
Baseline startle response	Baseline startle response mice startle response mice	6 149

Second, most of the conditioned tests were developed in the rat, where they have certainly played a very important role in advancing our understanding of the neurobiology of fear and anxiety, but equivalent tests have yet to be developed in the mouse.

The genetic analysis that I will be discussing in the succeeding chapters has been performed in mice using five ethological tests of anxiety. This is for two main reasons. First, genetic analysis is mainly carried out in mice rather than rats, because the genetic information available for mice is more complete to carry out whole genome scans. Second, the genetic analysis of behaviour requires the use of large numbers of animals, which have to be phenotyped individually in the test/tests employed to measure fear-related behaviour, therefore high throughput assays are best employed. While most ethological tests respond to this requirement, this is not the case for conditioned paradigms, which require training of animals.

I will now describe the five ethological tests of anxiety used in this study: the open field, the elevated plus maze, the elevated zero maze, the light-dark box, and the mirror chamber. For two of them, the open field and the elevated plus maze, a wide range of data has been collected by studies aimed at validating these tests as models of anxiety. I will report, when available, data from pharmacological studies, often aimed at predicting the validating the model, and data from multivariate analysis, generally used to provide a framework for construct validation of these tests.

1.5.1 Open Field

History

The open field was first devised by Calvin Hall in the '30s (Hall 1934; Hall 1936b), who implied it to be mildly stressful for the rats because it represented a novel environment. Rats were assessed for the presence of anxiety through defecation scores, a measure thought to represent an autonomic reaction to fear. Hall observed that high defecating (high emotional) rats were also moving around less than low emotional ones, establishing a negative correlation between these two behaviours (Hall 1936b). From these early observations, frightened animals were described as those that urinate and defecate more, and ambulate less than less emotional animals. Consistent with this view, increased defecation and urination and reduced activity are taken as indices of fearfulness (Green and Hodges 1991; Ramos and Mormède 1998). Since then, the open field test has been one of the most popular tests in behavioural research.

Description

The most common variant of the apparatus (Figure 1.1) consists of a large arena, usually circular in shape (although square open fields have also been used), which is brightly lit and surrounded by a wall, so that the animal is unable to escape. The animal is introduced at the periphery of the open field, left to explore, and its behaviour recorded for 5 minutes. Due to its wide employment, there is a great degree of variability in the characteristics of the test, the testing conditions, procedures and protocols used by different studies, as reviewed in (Walsh and Cummins 1976).

Figure 1.1

The Open Field

The open field is constructed to be a fear-inducing environment for rodents. This figure shows a mouse in a circular open field with white floor and walls and brightly lit.

Rationale

Rodents' exposure to the open field in the form of a strongly illuminated, novel, open space, is thought to result in a general state of arousal. Aubry and colleagues (Aubry, Bartanusz et al. 1995) have shown that plasma corticosterone B levels were increased by 4 to 6 fold in rats after exposure to the open field for 10 minutes: as acute release of corticosterone into the blood stream is one of the effects of the activation of the hypothalamo-pituitary-adrenal axis during acute stress, this study has provided evidence that exposure to the open field represents a stressful stimulus for rodents.

From a behavioural point of view, the most popular indices of anxiety in this test are defecation and ambulation scores, as stress arousal is known to manifest thorough the activation of the autonomic nervous system (measured by an increase in defecation) and the inhibition of instinctive exploratory drive (thought to result in decreased ambulation) (Lister 1990).

Another important anxiety-related behaviour in the open field is thigmotaxis, or wall-seeking behaviour, which is the tendency of rodents to stay in close contacts with the walls of the open field in order to avoid the open central area, and has been widely introduced in the analysis of rodents fearful behaviour in the open field (Archer 1973; Ivinskis 1976; Ramos and Mormède 1998). The concept that thigmotactic behaviour is felt as protective by rodents has evolved into the view that the open field, although physically determined by one single space, can be considered as virtually separable in two compartments: the more fearful centre, in which illumination is stronger and exposure to an open space at its utmost, and the more protected periphery. Hence, evaluation of anxiety is based on measures of avoidance of the central area, like

percentage time spent in the periphery, and latency to enter the centre after the starting of test.

Some studies have put an emphasis on the development of ethological paradigms, i.e. the analysis of naturally occurring behaviours, like sniffing or grooming. Ethological analysis has attempted to assign specific meaning to such behaviours as representative of different aspects of fear and anxiety. For instance, rearing and sniffing are thought to represent exploratory behaviour and therefore are expected to decrease in response to anxiogenic stimuli; instead stretch attend postures (stretching of the body typically from a more protected area, like the periphery of the open field, towards a more exposed area, like the centre) are meant to represent risk assessment, therefore to increase with fear (Green and Hodges 1991; Choleris, Thomas et al. 2001).

Due to its wide use (891 references in PubMed on 19th March 2002) a vast range of additional measures of anxious behaviour in the open field have been introduced, as review by (Walsh and Cummins 1976).

Pharmacological study

Early pharmacological analysis in rats of the classical behaviours in the open field (Fukuda and Iwahara 1974) has shown that chlordiazepoxide, a benzodiazepine, increases ambulation in the first few minutes of test and decreases defecation and urination, compatible with detection of the anxiolytic action of these drug. However, the validity of defecation and urination as appropriate measures of anxiety has been questioned (Archer 1973) and chlordiazepoxide has been shown to decrease defecation and urination also in the home cage (Oishi, Iwahara et al. 1972), an effect more compatible with a direct action

on autonomic system rather than on fear. However, the effect on ambulation is suggestive of an anxiolytic effect, and more recent analysis (Choleris, Thomas et al. 2001) has shown that both chlordiazepoxide and diazepam decrease ethologically significant anxious behaviour in the open field: they reduce thigmotactic behaviour and risk assessment in the form of stretch attend postures.

Studies employing centrally administered atypical anxiolytics have reported an anxiolytic effect (measured as an increase in entries and time spent in the centre of the open field) for buspirone and other 5-HT1a receptor agonists, and for 5-HT3 receptor antagonists. This effect was shown to be dependent on the specific site of injection of the drugs (Stefanski, Palejko et al. 1993a; Stefanski, Palejko et al. 1993b).

Multivariate analysis

Since Hall's early observations (Hall 1936b), studies of open field behaviour have looked for a negative correlation between ambulation and defecation as a definition of anxious behaviour. However, data on this point have been far from consistent (Pare 1964). Moreover, ambulation itself has been shown to be an ambiguous measure, because it can represent both exploratory activity (a dimension which should be influenced by fear) and general locomotion (a dimension which should be independent of fear) (Whimbey and Denenberg 1967).

Using factor analytical studies of open field behaviours, Royce (Royce 1977) has attempted to account for the construct validity of the test by reporting the identification of three invariant first-order factors: motor discharge (indicated primarily by activity, penetration to the centre and latency), autonomic balance (indicated primarily by

defecation), and territorial marking (indicated primarily by urination). Other multivariate analyses have dissected open field behaviour in at least two components: exploration and fear (Whimbey and Denenberg 1967; Crusio 2001), however the introduction of ethological measures such as grooming or sniffing implies a third factor (Crusio 2001; Aguilar, Gil et al. 2002).

1.5.2 Elevated Plus Maze

History

In the 1950s Montgomery (Montgomery 1955; Montgomery and Monkman 1955) performed a series of experiments to demonstrate that stimulation by exposure to a novel environment evoked both exploratory drive and fear, thus generating what he called an 'approach-avoidance' conflict. This concept brought to the development of one of the most common test for fearful behaviour in rodents: the Elevated Plus Maze (EPM).

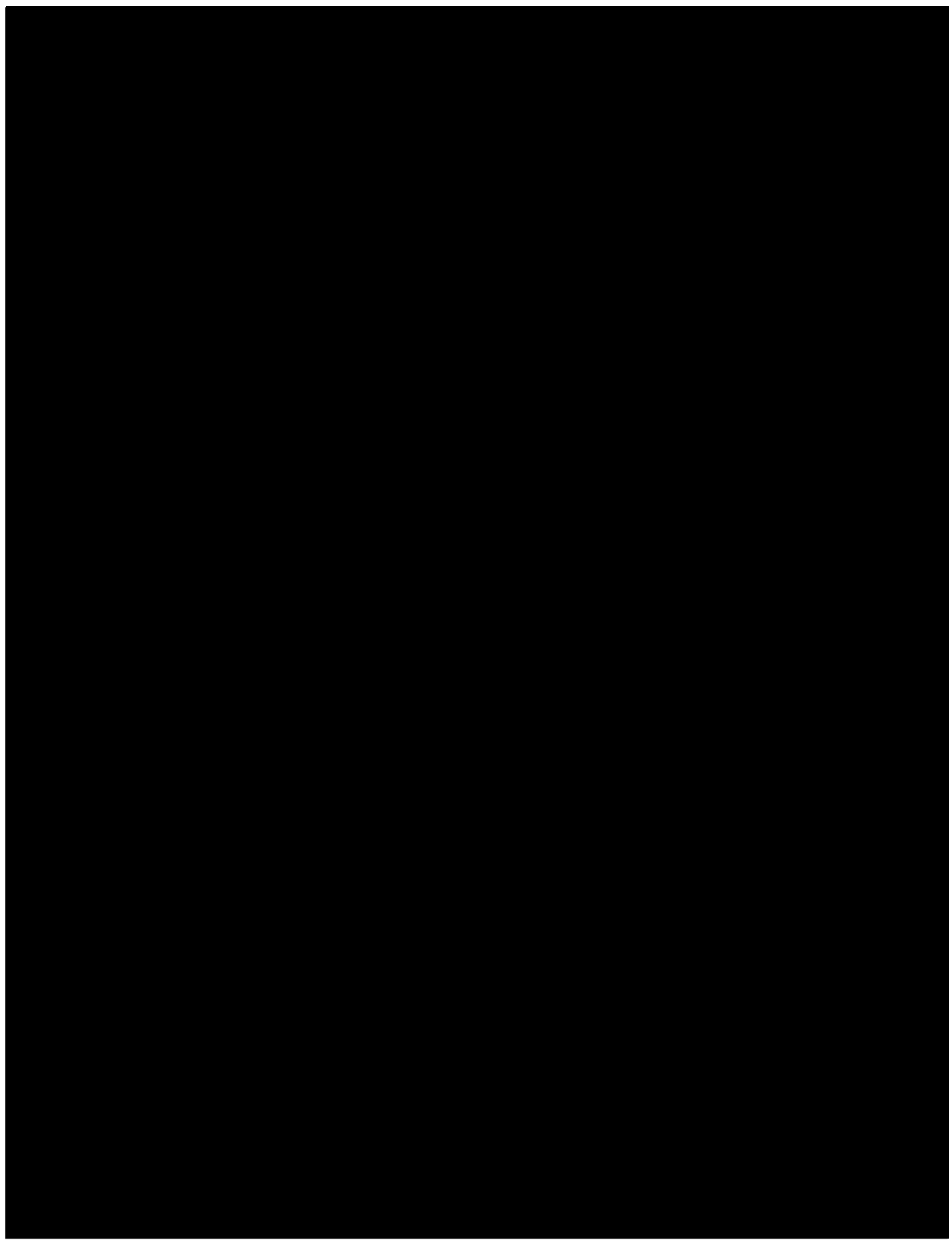
Description

The EPM (Figure 1.2) consists of four arms, elevated from the floor about 50 centimetres (cm), and positioned like a cross, two of which are enclosed by walls 40 cm high and two open and exposed. The animal is placed in the central platform and allowed free exploration, while its behaviour is recorded (usually for 5 minutes) (Ramos and Mormède 1998).

Figure 1.2

The Elevated Plus Maze

The elevated plus maze, one of the most popular tests for measuring fearful behaviour in rodents, consists of four arms, elevated from the floor. Two arms are enclosed by walls, two are open. In this picture a mouse is shown on an open arm.



Rationale

The EPM is a conflict test in which exposure to a novel environment excites both an approach drive (exploration or curiosity) and an avoidance drive (fear) (Handley and McBlane 1993). It is based on the concept that rodents' preference for the closed arms is due to the greater aversive potential of the open arms. Conflict between spontaneous exploration and fear is realised through sudden introduction of the animal in the central platform, a choice point from where the four alleys depart, two of which are enclosed by walls, while the other two are open in the space (Pellow, Chopin et al. 1985).

Pellow and colleagues (Pellow, Chopin et al. 1985) have shown that plasma corticosterone concentrations were significantly elevated in rats confined to the open arms of the EPM for 20 minutes, compared to concentrations in home cage controls, and also compared to animals confined for 20 minutes to the closed arms. This strongly suggests that the open arms are a stressful stimulus for the rat. As plasma corticosterone concentrations were also elevated in rats confined to the closed arms, albeit less than in those confined to the open arms, it should be maintained that the closed arms are also mildly stressful.

It has been long debated what stimuli would generate the avoidance of the open alleys. The apparatus is elevated from the floor about 50 cm, thus the open alleys present at least three possible sources of fear: the novelty, the height and the openness, while of these only novelty is also displayed by the closed arms. Although novelty and height have been reasonably proposed to act as fearful stimuli, one study (Treit, Menard et al. 1993) has provided strong evidence that it is the aversion of rodents for open spaces that mainly accounts for rodents' emotional behaviour in the EPM.

Because of the partition of the test in two compartments, the exposed open arms and the protected closed arms, typical measures of fearfulness are represented by relative activity performed or time spent in open versus closed arms, i.e. percentage of open arm entries on the total number of entries in the four arms, and percentage of time spent in the open arms on total test time (Lister 1990). More recently, it has been suggested that the analysis of a wider range of measures would provide a more detailed account of different facets of rodent anxiety as displayed in the EPM. In fact most animals, placed in the central platform at the beginning of test, spend quite some time there and engage in high levels of risk assessment before venturing into one of the four arms (Rodgers 1997). Sniffing, grooming and stretched attend postures are considered indices of risk assessment, while percentage time spent in the central platform may represent decision-making. Finally, rearing and head-dipping have been proposed as indices of exploration (Rodgers and Johnson 1995; Rodgers and Dalvi 1997).

Pharmacological study

Earlier pharmacological work in the EPM showed that benzodiazepines increase the percentage of entries into, and the time spent on, the open arms, while anxiogenic compounds have mixed effects but they tend to reduce exploration of the open arms; stimulants such as amphetamine or caffeine, neuroleptics, and antidepressant do not increase entries or time in the open arms, therefore suggesting that these are quite specific measures of anxious behaviour (Pellow, Chopin et al. 1985; Pellow and File 1986; Lister 1987). From this evidence it was maintained that the EPM has a particularly good predictive validity in the sense that it is bi-directionally sensitive to anxiogenic as well as to anxiolytic effects (Green and Hodges 1991), and it became one of the most popular ethological tests for anxious behaviour in rodents. However, more recent studies using

atypical anxiolytics, like buspirone, have reported remarkable discrepant results (Rodgers and Cole 1994), and concern about the validity of the test has arisen.

Dawson and colleagues have suggested that the effect of chlordiazepoxide, a benzodiazepine, on the EPM may be due to an increase in locomotor activity, rather than to its anxiolytic properties (Dawson, Crawford et al. 1995). Although a drastic rejection of the predictive validity of the EPM is not justified (Rodgers 1997), the test has proved to be very sensitive to variables affecting pre-test manipulation of animals or test conditions, all of which can affect baseline behaviour as well as response to drugs (Hogg 1996). In conclusion, a greater understanding of EPM behavioural patterns and of their interaction with other variables is needed in order to provide a framework for the interpretation of pharmacological studies.

Multivariate analysis

As with other tests employing ambulation-related measure as an index of anxiety, researchers have been concerned with dissecting locomotor activity from anxiety. Factor analysis of EPM behaviour has shown that the standard spatio-temporal measures of relative activity and time in open versus closed arms can be accommodated by 2 factors, one representing anxiety (with loadings from percentage of open arms entries and percentage of open arms time), and the other representing general locomotor activity (with loadings from total arm entries) (Lister 1987; Rodgers and Johnson 1995). In further support to the concept that total arm entries represent locomotor activity, a distinct dimension from anxiety, comes from evidence that this measure consistently correlates with locomotor activity in the hole-board (Lister 1987). However, it has also been demonstrated that total arm entries do not load exclusively on the activity factor, but also,

albeit less strongly, on the anxiety factor. Therefore the number of enclosed arm entries has been more recently proposed as a purer index of activity, as it loads highly and solely on the activity factor (File 1992).

As I have discussed previously, recent studies have suggested the opportunity to analyse a wider range of behaviours in the test. In this case, when factor analysis is performed measures can be accommodated by six factors: besides the two 'classic' ones for anxiety and locomotor activity, four additional ones are found and have been proposed to represent decision-making, risk assessment, vertical activity and exploration (Rodgers and Johnson 1995).

1.5.3 Elevated Zero Maze

The elevated zero maze has been recently developed (Shepherd, Grewal et al. 1994a; Shepherd, Grewal et al. 1994b) as a modified version of the more famous EPM. In this test the four arms are placed in a 'zero' shape, instead of a 'plus', resulting in the removal of the central platform and the abolishment of any measure related to it. This provides a direct and inverse relationship between time spent in the open arms and time spent in closed arms. Besides, the arms continue one into the other, promoting uninterrupted exploration.

The apparatus consists of a circular raised platform in which open and closed arms are alternated, so that the two open arms are forming two opposite quadrants, and the same is true for the closed arms.

The rationale of the elevated zero maze is analogous to that of the EPM, i.e. an exploration/fear conflict paradigm in which the open arms are thought to be more aversive than the closed arms.

The animal is placed on a closed quadrant at the beginning of test and left to explore the maze for 5 minutes. A great number of measures, developed from the experience with the EPM, are scored: relative time spent in either open and closed arms, number of transitions between open and closed arms, head dips over the edge of the open arms, stretched attend postured from closed to open arms, defecation, rearing and grooming in both open and closed arms (Shepherd, Grewal et al. 1994a; Shepherd, Grewal et al. 1994b; Tarantino, Gould et al. 2000). Activity in the closed arms and latency to enter an open arms for the first time, have also been measured (Cook, Williams et al. 2001).

A pharmacological study in the rat has shown that benzodiazepines increase open arm entries, while consistently increasing head dips and decreasing stretched attend postures, and that anxiogenic compounds (5-HT receptor ligands) have an opposite effect (Shepherd, Grewal et al. 1994a; Shepherd, Grewal et al. 1994b); results for atypical anxiolytics acting on 5-HT receptors were more ambiguous.

The test has been recently used to assess the anxiety profile of mutant mice (Heisler, Chu et al. 1998; Kash, Tecott et al. 1999).

1.5.4 Light Dark Box

History

The light-dark box was devised by Crawley (Crawley and Goodwin 1980; Crawley 1981) in an attempt to provide a rapid and simple model for the anxiolytic effect of benzodiazepines.

Description

It consists of two compartments, one dark and the other brightly lit, communicating through an opening cut into the partition that separates the two sides. The light side is illuminated by a fluorescent light placed above the apparatus (Crawley and Goodwin 1980). In the most common version, the dark side is smaller than the lit one, being about one third of the apparatus; however a variant with both sides of the same dimensions has been devised and used in some studies (Belzung, Misslin et al. 1987; Misslin, Belzung et al. 1989; Belzung, Misslin et al. 1990).

Rationale

The model is based on conflict between the natural tendency of exploration in rodents and their aversion for a novel brightly lit space (Lister 1990).

Light has been demonstrated to be the aversive stimulus in this test (Costall, Jones et al. 1989; Misslin, Belzung et al. 1989), because when bright illumination is removed, no preference is shown for either of the two compartments.

Typically the animal is placed in the light compartment at the beginning of tests and let free to explore the apparatus for 5 or 10 minutes. Rodents display a natural tendency to

escape into the dark side and show a clear preference for it, but they also exhibit some incursions into the lit side (Misslin, Belzung et al. 1989). The original measure proposed to reflect anxious behaviour is the number of transitions between the light and dark compartments (Crawley and Goodwin 1980; Crawley 1981), but more recently other indexes have been proposed: percentage time spent in the light side, ambulation and rearing performed in the light side, and latency to enter the dark side (Costall, Kelly et al. 1988; Costall, Jones et al. 1989).

Although in the classical procedure the animal is first put in the light side of the box, some studies have also employed a variant (sometimes called dark-light box) in which the animal is instead put in the dark side at the beginning of test (Costall, Jones et al. 1989; Misslin, Belzung et al. 1989; Chaouloff, Durand et al. 1997). Here a new measure, latency to emerge from the dark into the light side, can be taken (Costall, Jones et al. 1989). Interestingly, in the only factor analysis available for the dark/light version of this test, latency to enter the dark side loads on the same factor as the classic 'anxiety' measures, i.e. transitions and percentage time spent in the light side, while this is not true for latency to enter the dark side when the light/dark version is employed (Chaouloff, Durand et al. 1997).

Pharmacological study

Benzodiazepines have been shown to increase light/dark crossings and their effect acts selectively on exploratory locomotion rather than general motor activity (Crawley and Goodwin 1980; Crawley 1981; Crawley, Skolnick et al. 1984). This effect is blocked by benzodiazepines antagonists and inverse agonists (Crawley 1981; Crawley, Skolnick et al. 1984).

The test has also been shown to be sensitive to atypical anxiolytics like buspirone (Costall, Kelly et al. 1988) and the 5-HT3 antagonists (Jones, Costall et al. 1988), and to the anxiogenic properties of benzodiazepines inverse agonists (Belzung, Misslin et al. 1987; Belzung, Misslin et al. 1990).

Increased light/dark transitions are thought to represent release of exploratory behaviour after fear suppression. However, stimulants also increase light/dark crossings, and it is unclear whether the effect of benzodiazepines on crossings might be due to a direct stimulation of locomotion as they also increase locomotion in the dark box, although this might also be a result of increased exploratory behaviour (Costall, Jones et al. 1989). Anxiolytics give a consistent increase in time, ambulation and rearing in the light side (Costall, Kelly et al. 1988; Costall, Jones et al. 1989) and latency to enter the light side when the animal is first placed in the dark compartment is decreased (Costall, Jones et al. 1989).

1.5.5 Mirror Chamber

The mirror chamber was introduced by Toubas and colleagues (Toubas, Abla et al. 1990) as a test for detecting anxiolytic agents. The test is based on the observation that many species show approach/avoidance conflict behaviour in front of a mirror.

The apparatus (Figure 1.3) consists of a cube with the internal surface completely covered by a mirror surface, placed into a square plexiglas box. The cube is open on one side, and a sixth mirrored wall is placed to face the mirrored chamber on the side of the opening. Placement of the mirror cube into the centre of the plexiglas box forms a 5 cm corridor surrounding the mirror chamber, which communicates with the corridor via its

Figure 1.3

The Mirror Chamber

The mirror chamber consists of a cube with the internal walls covered by a mirror surface and dark external walls, placed into a square box with dark walls. The mirrored cube is open on one side. The figure shows a photo of the apparatus and a schematic that illustrates the position of the mirror (shown in blue).

opened side. The corridor walls, consisting in the external walls of the mirror cube and the internal walls of the plexiglas box, are opaque and black.

The animal is first put in a corner of the corridor and allowed to explore the apparatus for 30 minutes. Latency to enter the mirror chamber is taken as an index of fearfulness. The benzodiazepine diazepam has been shown to shorten the latency taken by the mice to enter the mirror chamber in a dose-dependant way, and this was imputed to be due to its anxiolytic effect rather than to motor activity stimulation (Toubas, Abla et al. 1990).

However, Lamberty (Lamberty 1998) has pointed out that the validity of the concept that the mirror chamber acts as an aversive stimulus is not proven. His study demonstrated that the fearfulness of the mirror chamber could not be unequivocally attributed to the aversive effect of the mirror stimulus. In fact, increased latency to enter the chamber was also obtained when the walls of the cube where painted in white or grey instead of mirrored. Thus, the central position of the chamber, or the brightness of the walls could be responsible for its fearful properties, rather than the mirror effect. No further data are available on this test.

CHAPTER 2

Genetic analysis of anxious behaviour in rodents

Until the advent of molecular maps in the 1980's, the study of any genetic character depended on an analysis of phenotype in relation to its pattern of inheritance. Two approaches have proved productive for the study of behaviour in rodents: the analysis of the pattern of expression of behavioural characteristics displayed by different inbred strains, and the application of selective breeding for the development of lines that differed in behaviour. With the advent of complete genetic maps for mice (Van Etten, Steen et al. 1999) and rats (Watanabe, Bihoreau et al. 1999; McCarthy, Bihoreau et al. 2000) it has become possible to extend the analysis of inbred and selected strains and to identify the genetic loci underlying the heritable differences in behaviour.

In the present chapter I start by describing the pre-molecular approaches: analysis of inbred strains and selective breeding. I particularly focus on selective breeding and provide a detailed description of this technique, reporting in detail the selection experiment that was used to establish the murine inbred lines from which the mice analysed in this thesis have been generated. I then describe the molecular methods, focusing especially on genetic mapping and reporting results of its application to the genetic analysis of anxious behaviour in rodents.

2.1 Analysis of inbred strains

Inbred strains of mice have been developed since the beginning of the 20th century for the study of inheritance in mice (Beck, Lloyd et al. 2000). As variation in behaviour is thought to be determined partly by genetic factors and partly by environment, inbred strains represent a unique resource for psychogenetics. If a number of animals from several strains, reared in the same standardised laboratory conditions, are tested in behavioural paradigms, variation within each strain represents pure environmental variation, as each strain is constituted by genetically identical animals, while variation between animals of different strains represents genetic plus environmental factors. Since it is possible to establish the contribution of environmental effect within each strain, it will then be possible to estimate the genetic effect by comparing the degree of variation between strains with the degree of variation within strains.

Since the 1950s inbred strains have been analysed for their anxious behaviour in the open field (Thompson 1953; Mathis, Paul et al. 1994; Clément, Martin et al. 1995; Crawley, Belknap et al. 1997), the EPM (Trullas and Skolnick 1993; Ågmo, Belzung et al. 1999; Griebel, Belzung et al. 2000), the light-dark box (Misslin, Belzung et al. 1989; Mathis, Paul et al. 1994; Griebel, Belzung et al. 2000), and other tests for fearful behaviour (Crawley, Belknap et al. 1997). Although these data have provided important demonstration of the existence of a genetic basis of behaviour, they have not always produced consistent results. For instance, the inbred strain BALB/cJ is found to be respectively more (Ågmo, Belzung et al. 1999) or less anxious (Trullas and Skolnick 1993) than the inbred strain C57BL/6J, when both are tested in the EPM.

In a recent paper (Crabbe, Wahlsten et al. 1999) the issue of replication of behavioural measurements is addressed in a systematic way. Six inbred strains of mice were tested for a cluster of behaviours, including measures in the open field and the EPM, in three different laboratories. Interestingly, despite rigorous equation of testing devices, testing protocols, animal husbandry and housing between the three laboratories, significant effects of site were found for nearly all variables, including measures of ambulation and rearing in the open field, and measures of total arm entries and time spent in the open arms of the EPM. Again, discrepancies between results obtained for the same strains in different laboratories were evident. For instance, when mice were tested for anxious behaviour by means of time spent in the open arms of the EPM, the inbred strain C57BL/6J was found to be respectively more anxious than the inbred strain BALB/cByJ by two laboratories, and less anxious by the third laboratory. However it should also be noted that the paper confirmed a highly significant effect of genotype on behaviour, and analysis of inbred strains remains a powerful tool for providing firm evidence that behaviour is partly dependent on genetic factors.

Comparisons of inbred strains can only give a very crude measure of how genes are operating. A more powerful approach, which provided some of the most compelling evidence that genes influence behaviour, is selective breeding. I will now discuss such approach in detail and present a review of selection experiments for anxious behaviour in rodents.

2.2 Selective breeding

2.2.1 Theoretical considerations

Definition and phenotypic meaning

When variation in a phenotype is due, even in part, to genetic variation, then the value of the phenotype in a population is subject to selection, either by natural or artificial means. Consequently it is possible to determine whether a trait has a genetic contribution by establishing a programme of artificial selection. In each generation parents are picked for the expression of the phenotype at the maximum or minimum level, until, through generations, the expression of the character is fixed at one extreme or the other of the spectrum.

The usual method in psychogenetics is to select in more than one direction at once, generating and establishing two breeding lines derived from the same original strains or population, typically defined as 'high' and 'low'. For fear-related behaviour, these lines would ideally have the maximum difference in anxiety-related behaviour and the minimum difference in other behaviours and physiological parameters not primarily related to anxiety.

In almost all cases at the end of a selection experiment the lines are inbred, thus providing with inbred lines, which are a very useful tool in genetic analysis for the reasons discussed above and also for their application to molecular methods, as I will discuss below.

Genetic meaning

From a genetic point of view, a selection experiment increases the frequency of alleles influencing the trait, until, if selection is applied long enough, such alleles become fixed in the population. In other words, one outcome of the selection experiment is that all animals become homozygous at the selected loci. Besides, selection should act not only on loci directly influencing the selected trait, but also on loci determining phenotypes that are biologically correlated to that trait. Therefore artificial selection experiments tell us not only whether a trait has a genetic component but also whether there are other traits that are genetically correlated, providing information about biological mechanisms influencing the selected trait.

A good example is defecation and activity in the open field. As I have discussed in chapter 1, these two phenotypes have been used to measure fearful behaviour in rodents. Studies of artificial selection have first suggested that these traits might be related to a common biological process that underlies anxious behaviour in rodents. Broadhurst performed a genetic selection experiment in the open field in which he selected rats with high and low rates of defecation, eventually producing two strains that consistently differed in their defecation in the open field: the Maudsley Reactive (MR) strain with high defecation and the Maudsley Non-Reactive (MNR) strain with low defecation (Broadhurst 1960). In both lines during and after selection there was a negative correlated change in open field activity: selection for high defecation resulted in low activity animals, selection for low defecation resulted in high activity animals. DeFries carried out a similar experiment in mice, this time selecting the animals for activity rather than defecation in the open field (DeFries, Wilson et al. 1970; DeFries, Gervais et al. 1978). Again, selection for activity was correlated to a change in the opposite direction for defecation scores.

Problems in the interpretation of selection experiments

Although the analysis of selected strains can provide interesting insights in the underlying biology of behaviour, results of a selection experiment need to be interpreted with caution and it should not be assumed that the genetic architecture, inferred from the analysis of a selection, is representative.

First, in traits where the underlying genetic effects are multiple and small, not all of them may be driven to fixation during selection; chance will play a part (depending on the size of the breeding population during selection, the length of time selection is applied, as well as the genetic effect size) so that some of them may be lost. On the other hand, some loci not involved in the selected phenotype may go to fixation due to random drift.

Second, the results of selection will depend on the starting material. When selection is carried out in the offspring of a cross between two inbred strains, the selection experiment will be limited to act on the genetic material already present in the two given strains. Therefore, homologous selection experiments conducted on different strains may result in very different genetic architectures.

Finally, inferences about genetic correlations are also subject to the same limitations. Correlated changes may also arise by chance due to genetic drift, or they may be limited to a particular population design (strains of origin). Moreover, a further limitation undermines the inference about correlated strains. When selection operates on a given locus and brings it to fixation, it automatically acts also on the genetic material immediately adjacent to that locus (i.e. material that it is genetically linked). Therefore, based on a pure phenotypic analysis it is impossible to exclude the occurrence that two

traits are correlated because they are determined by loci that lie very close on the genome and not because they are part of the same biological process.

It is possible to deal with some of the problems of interpreting the results of artificial selection experiments by good experimental design. There are four strategies that can help.

I. Bi-directional selection

A main advantage of bi-directional selection is that it helps with the detection of true correlations between traits, lowering the possibility of mistaking chance correlations for true ones. This occurs because the experimenter can consider as true correlations only those that occur in both 'high' and 'low' lines, and ignore those that occur only in one line. So, for open field defecation to be considered as biologically related to activity, it would have to correlate with activity both in the high and in the low activity strains. Moreover, the direction of the correlation would have to be consistent in the two strains: if defecation is consistently decreasing in the high activity line, we should expect that it were consistently increasing in the low activity lines. In the DeFries strains, in fact, defecation is negatively correlates to activity both in the high and low lines in a consistent way.

II. Use of a large population and avoiding inbreeding

The use of a large effective population size (the number of breeding pairs) limits the effects of random genetic drift. Nevertheless, it is not feasible to abolish such a phenomenon, since allele frequencies will vary by chance in a population of any size. Large population sizes however increase the number of generations before an allele goes to fixation by chance.

III. Replication

The replication of a selection experiment may help in several ways. First, it will show if selection operates on the trait in a consistent way across replicas. If this is the case, heritabilities of the trait and genetic correlations between traits should be similar in replicated experiments. Replicated selected lines become an even more valuable resource when molecular techniques are applied to their analysis. As I will describe in chapter 3, the comparison between genetic loci involved in anxiety traits in replicated lines allows us to see how robustly selection acts on these traits. For the genetic basis of anxious behaviour to be analysed through scientific methods, we should expect that it is amenable to replicable scientific manipulation, i.e. the genetic basis of two replicated selection experiments should be fairly consistent.

IV. Use of a control line

The use of a control group, maintained from the same parental stock as the selected lines, but differing from them only by the absence of selective pressure, is one possible tool that has been advocated for at least measuring, if not controlling, environmental variance. The evaluation of the phenotypic variation of the selected behaviour in the control line should provide a measure of the influence of environmental pressures on the phenotype, and should alert when these pressures are cause of big fluctuations of the trait of interest.

2.2.2 Application of selection experiments to the genetic analysis of anxious behaviour in rodents

Selection experiments have shown that bi-directional artificial selection can produce strains of animals with striking behavioural differences. Such results have been

particularly important both for the investigation of the genetic mechanisms underlying behavioural traits, as well as for providing stable biological material for further psychological and pharmacological investigations. This has been especially true when the extreme differences produced by selection have been maintained for long periods after withdrawal of the selective pressure.

Selective breeding studies in psychogenetics have involved a wide range of cognitive (Tryon 1932; Heron 1935) and behavioural aspects, which comprise alcohol addiction (Eriksson 1968), depressive-like behaviour (Scott, Cierpial et al. 1996), and preponderantly emotional behaviour. Table 2.1 gives a summary of the selection experiments performed for anxiety-like behaviour in rodents. Of twelve experiments, seven have selected the animals for ethological parameters, while five have employed conditioned paradigms. Rats have been the species of choice, while only two selection experiments for fearful behaviour have employed mice, one of which is the DeFries selection experiment, which has generated the mice strains whose genetic background I have analysed.

2.2.3 The DeFries selection experiment

Genetic analysis of the DeFries strains is the main concern of this thesis. Here I describe in detail the selection experiment that resulted in the creation of the two high open field activity (OFA) strains used in my work and referred to as H1 and H2, the two low OFA strains L1 and L2, and the two control strains C1 and C2. The careful experimental design of the selection experiment provides an opportunity to investigate thoroughly the genetic architecture of the response to behavioural selection.

Table 2.1

Selection experiments for anxiety-related behaviours in rodents.

Selected strains	Key features of selection experiment	References
Mausdley Reactive (MR) and Non reactive (MNR) rats	Bi-directional selection for emotional defecation (and urination) in the open field. Parental population: outbred Wistar rats.	(Broadhurst 1960)
Hall's emotional and non-emotional rats	Bi-directional selection for emotional defecation and urination in the open field. Parental strain not designated.	(Hall 1940)
Wistar-Kyoto hyperactive (WKHA) rats	Uni-directional selection for spontaneous hyperactivity in a novel environment. Progenitor strains: outbred Wistar-Kyoto and inbred Wistar-Kyoto spontaneously hypertensive (SHR) strains.	(Hendley, Wessel et al. 1986; Hendley and Ohlsson 1991)
Naples high (NHE) and low (NLE) excitability rats	Bi-directional selection for locomotor 'forced' exploratory activity. Parental population: Sprague-Dawley rats.	(Sadile, Cerbone et al. 1983; Sadile, Cerbone et al. 1984)
Tsukuba high (THE) and low emotional rats (TLE)	Bi-directional selection for activity in the runway tests. Parental population: outbred Wistar rats.	(Kitaoka and Fujita 1991)
DeFries Low and High activity mice	Bi-directional selection for open field activity. Parental inbred strains: C57BL/6J and BALB/cJ.	(DeFries, Wilson et al. 1970; DeFries, Gervais et al. 1978)
High (HAB) and low anxiety-related behaviour (LAB) rats	Bi-directional selection for anxiety-related behaviour in the elevated plus maze. Parental population: outbred Wistar rats.	(Liebsch, Montkowski et al. 1998)
Roman high (RHA) and low avoidance (RLA) rats	Bi-directional selection for avoidance learning in a shuttle-box. Parental population: outbred Wistar rats.	(Bignami 1965)

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Selected strains	**Key features of selection experiment**	**References**
Syracuse high (SHA) and low avoidance (SLA) rats	Bi-directional selection for avoidance learning in a shuttle-box. Parental population: Long-Evans rats.	(Brush, Froehlich et al. 1979)
High (HBP) and low (LBP) behavioural performance rats	Bi-directional selection for behavioural performance in a footshock-motivated brightness discrimination test (avoidance learning). Parental population: outbred Wistar rats.	(Hess, Lesser et al. 1992)
Koltushi high (KHA) and low (KLA) avoidance rats	Bi-directional selection for avoidance learning. Parental population: Krushinsky-Molodkina rat strain.	(Ryzhova, Kulagin et al. 1983)
High and low avoidance mice	Bi-directional selection for avoidance learning in a shuttle-box. Parental population: the 12th generation of a laboratory-maintained wild trapped *Mus musculus* population.	(Smith 1978)

The foundation for the selection experiment consisted of an F3 generation derived from a cross between two inbred lines, BALB/cJ and C57BL/6J. Ten F3 litters that contained at least two males and two females were chosen at random. The most active male and female within each litter (highest OFA score) were selected to become the progenitors of one selected line, termed H1. The least active male and least active female (lowest OFA score) from each of these same 10 litters were selected to serve as progenitors of a low-activity line, L1. Exactly the same selection procedure was applied to members of a second randomly chosen group of ten F3 litters, thereby providing ten pairs of parents for replicate selected lines H2 and L2. From a third set of F3 litters a male and female were chosen at random to serve as the progenitors of a control line, C1. In the same manner, parents for a replicate control line (C2) were chosen from a fourth set of ten F3 litters. Within each of these six lines, animals chosen for breeding were mated at random after selection. The resulting progeny represented the first selected generation. In subsequent generations, one male and female were chosen when possible from each litter. Within the two high lines the most active male and female were selected from each litter, whereas the least active male and female were selected from each litter in the two low lines. Within the control lines, one male and one female were chosen entirely at random from each litter. After selection the chosen males and females were mated at random within line. The number of mice tested in each of the six lines varied between 44 and 108. Thirty generations of selection were completed and in generation 30 open field activity scores of the high lines were 30 times greater than those of the low lines. Following the suspension of selection the six lines were randomly mated within line for 18 generations and then inbred using brother sister mating (DeFries and Hegman 1970; DeFries, Wilson et al. 1970; DeFries, Gervais et al. 1978).

The selection experiment provides conclusive evidence that genetic variation is in part responsible for variation in the phenotype. I have previously described how some of the animal tests that have been subject to genetic investigation correlate with fearfulness and, possibly, with anxiety in humans. I have also reported that the current genetic methodologies used to find genes in studies of human psychiatric disease, including anxiety, have not been successful. Therefore we may ask if molecular genetic investigation of the animal models is more likely to be successful. Available data indicate that genetic mapping in animals is in fact a very powerful strategy, and I will now explain why this is so and then outline the relevant animal studies.

2.3 QTL mapping

Molecular genetic approaches applied to the study of anxious behaviour comprise a number of techniques that have been developed for the wider purpose of analysing the genetic basis of all quantitative traits. A quantitative trait is one that, in contrast with qualitative traits, which are expressed in the form of distinct phenotypes, exhibits a continuous variation of its expression in the population. Paradigmatic examples of quantitative characters are height and weight.

Quantitative variation is determined by a combination of multiple environmental and genetic effects. From a genetic point of view, quantitative traits result from the segregation of multiple genes of small effect, which are transmitted according to the Mendelian laws, and which contribute in a cumulative way to the phenotype. Mapping the loci that contribute to the genetic variation in a quantitative trait is termed quantitative

trait locus (QTL) mapping. I will now describe in detail the technique of QTL mapping, as this has been the first molecular tool applied to the study of anxious behaviour in rodents, has proved quite popular and is the method used in this thesis.

In one of the most influential publications in molecular genetics Eric Lander and David Botstein suggested how a map of genetic markers could be used to pinpoint genetic effects anywhere in the genome in humans (Lander and Botstein 1986). They went on to develop a robust methodology for doing the same in inbred crosses in mice (Lander and Botstein 1989). In the 1980s, Eric Lander's group at the Whitehead Institute at the Massachusetts Institute of Technology (MIT) (http://www-genome.wi.mit.edu/) published the sequence of deoxyribonucleic acid (DNA) polymorphisms in the mouse suitable for genotyping using the versatile and relatively easy molecular technique known as polymerase chain reaction (PCR) (Mullis, Faloona et al. 1986). The availability of the MIT markers as they became known revolutionized mouse genetics. For the first time a complete genetic map of the mouse genome was available and could be utilized by any laboratory in the world.

The power of mapping genetic effects in inbred strains is due to the simplicity of the genetics, as I will illustrate. QTL explaining as little as 5% of the phenotypic variance can be reliably detected with sample sizes of a few hundred animals - for reviews see (Falconer and Mackay 1996; Lynch and Walsh 1998). Two methods for QTL detection are popular, using either recombinant inbred strains or a cross between two inbred strains. Recombinant inbred (RI) strains (Taylor 1989) are established starting with an F2 intercross between two inbred strains and then using brother-sister mating for about 20 generations to obtain inbreeding. Each resulting strain will have a different combination

of the maternal and paternal genomes, which can be determined by genotyping with a set of polymorphic markers. Once the position of recombinants across the genome is known, a QTL can be mapped by comparing the phenotypes of each recombinant inbred strain and determining what genetic material they have in common.

The second popular method for mapping QTL in mice consists of constructing crosses between two inbred strains and searching for segregation of the genetic effect. Figure 2.1 shows an intercross between two inbred strains giving rise to an F2 generation, at which point recombination has produced genetically unique individuals so that genetic and phenotypic variation can be correlated (note that the figure shows a single chromosome pair). Only two alleles segregate at each locus (for example: *a* and *b*), whose identity can be determined by a PCR based assay. Mapping consists of comparing the phenotypes of the three possible genotypes (*aa*, *ab* and *bb*) at each locus. If there is no genetic effect at, or near to a marker locus, the difference between the three genotypes will not exceed chance expectation. If there is a genetic effect, then not only will the means of the three groups differ, but also the pattern of differences can tell us about genetic action. Figure 2.2 shows the distribution of a given phenotype for the three genotypes, in which an additive effect is suggested, because the distribution of the *ab* genotype is intermediate in respect to that of *aa* and *bb*. If instead the distributions of *aa* and *ab* genotypes are equal and greater than that of *bb*, the effect of the *a* allele is probably dominant to *b*. The statistical significance of the genetic effect on the phenotype is determined by carrying out a test for association, for example an analysis of variance (ANOVA).

Figure 2.1

Genetic design of an F2 intercross between two inbred strains (a single chromosome pair is shown). Because the parental strains are inbred there will be just two alleles segregating at each locus in the F2, one from each parental strain, and consequently the genotype at any locus in the F2 must be one of *aa*, *bb* (homozygous for either parent) or *ab* (heterozygous).

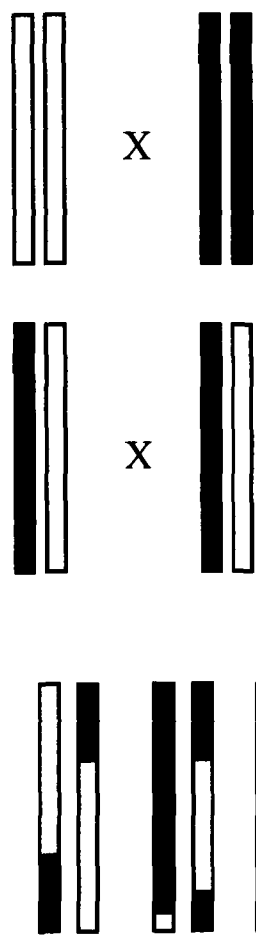
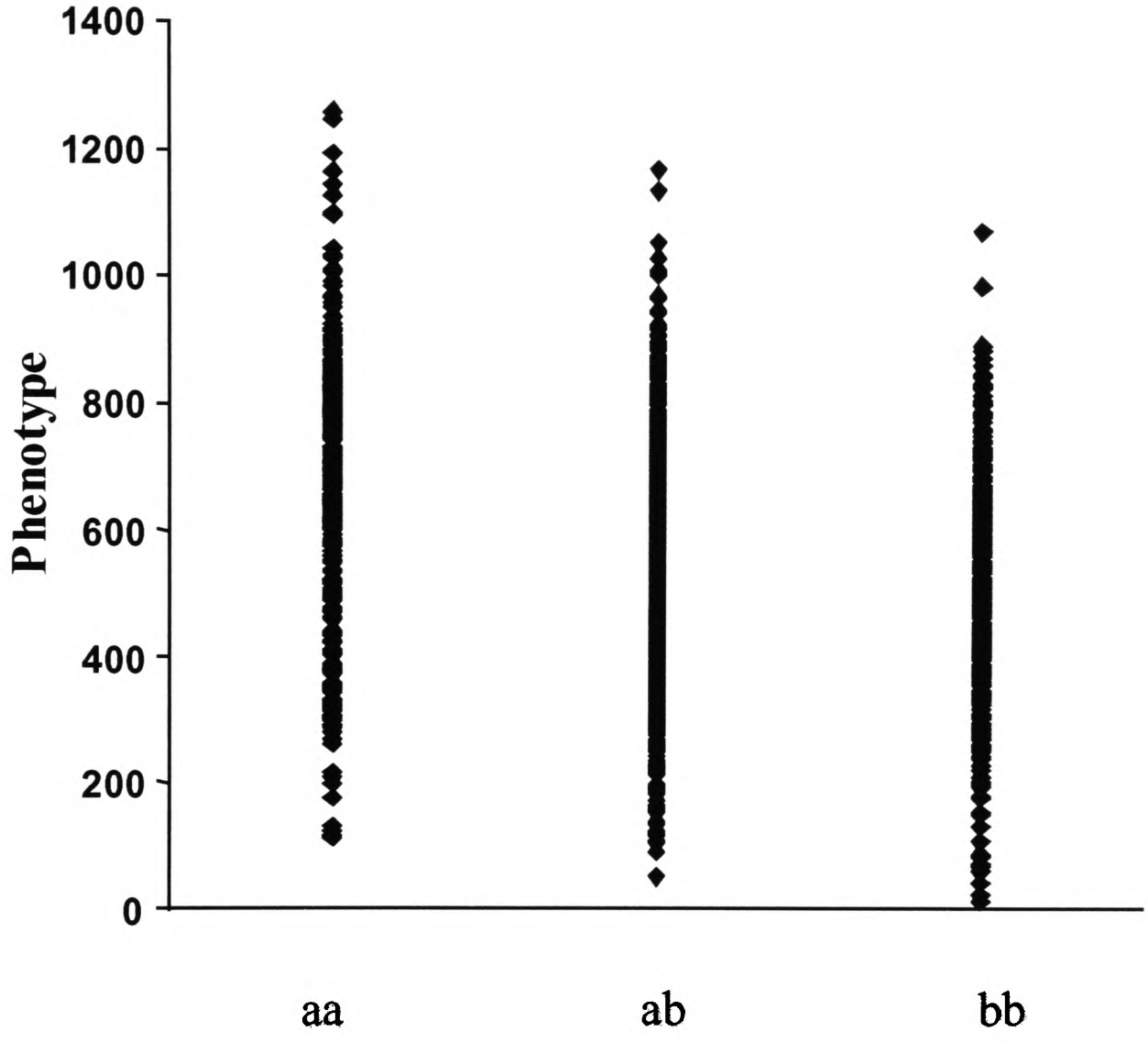


Figure 2.2

Phenotypic distribution of the three genotypes in an F2 intercross at a locus that influences a trait.



The problem with QTL mapping in both methods is that it cannot distinguish between a small genetic effect close to the marker from a large genetic effect a long way from the marker. In order to deal with this problem Lander (Lander and Botstein 1989) introduced interval mapping, in which an algorithm is used to work out the most likely recombinations between two markers. Using this approach it is possible to distinguish the position of a QTL from its effect. Since the introduction of interval mapping, there have been a number of statistical innovations to improve the detection of small effect loci, increase mapping resolution and more recently to detect interactions between loci and to map multiple traits. While of substantial interest for QTL analysis, these methodological advances are not of direct relevance to this thesis and will not be reviewed here.

2.3.1 Application of QTL mapping to the genetic analysis of anxious behaviour in rodents

The first QTL mapping study for behavioural phenotypes in rodents (Flint, Corley et al. 1995) was carried out using the DeFries strains, mice inbred lines established through selection for high and low anxious-related behaviour. The mapping experiment consisted of a wide genome scan in an F2 intercross population derived from H1 and L1 DeFries strains.

879 mice F2 animals were tested for open field activity (OFA), open field defecation (OFD), entries into the open and closed arms of an EPM, and activity in a Y-maze. Of these, OFA, OFD, entries into the open arms of an EPM, and activity in the Y-maze, are thought to represent fearful behaviour, while entries into the closed arms of the EPM is meant to test general locomotor activity. If selection in these mice has operated on loci

determining anxious behaviour, and if the interpretation of the phenotypes is correct, it is expected that QTL for OFA, OFD, entries into the open arms of the EPM, and activity in the Y-maze, are found on the same chromosomal regions, while no overlapping QTL for entries into the closed arms of the EPM should be found.

A genome scan was performed on 192 F2 animals that had the highest (96 mice) and lowest (96 mice) OFA scores. Significant association was found for six markers on chromosomes 1, 4, 12, 15, 17 and 18, indicating that six QTL influenced OFA. These were then tested for association with the other measures in a total of 384 animals, including the highest and lowest 10% of all measures. Out of six, three QTL (on chromosomes 1, 12 and 15) were found to contribute to most of the genetic variance of fearful behaviour as measured in the open field, EPM and Y-maze, while no QTL for entries into the closed arms of the elevated plus maze was found in these regions. This result was consistent with the view that anxious behaviour in mice has a relatively simple architecture, with three major QTL representing the main genetic variance of OFA and correlated traits. Table 2.2 lists the results of subsequent experiments that have mapped QTL that influence anxious-related behaviour in crosses between inbred strains of mice and rats.

The first lesson to be learnt from mapping fearful behaviour in rodents is that the genetic basis need not be complex. In fact most studies agree that in inbred strains individual differences in the phenotype can be produced by a small number of genes. Three loci (on chromosomes 1, 12 and 15) have been identified as contributing to almost all the genetic variance of emotionality (defined as open field activity and open field defecation) in the DeFries high and low activity mice (Flint, Corley et al. 1995). Open field activity was

Table 2.2

QTL mapping experiments for anxiety-related behaviours in rodents.

Species	Phenotype[1]	Strains[2]	Method[3]	No.[4]	Chr[5]	References
Mouse	OF Activity (first trial, 5 minutes)	A/J C57BL/6J	F2	518	1 10 15 19	(Gershenfeld, Neumann et al. 1997)
		A/J C57BL/6J	RI	28 strains	9	(Mathis, Neumann et al. 1995)
	OF Activity habituated (second trial)	A/J C57BL/6J	F2	518	1 3 10 19	(Gershenfeld, Neumann et al. 1997)
	OF Rearing (first trial, 5 minutes)	A/J C57BL/6J	F2	518	1 10 19	(Gershenfeld, Neumann et al. 1997)
		A/J C57BL/6J	RI	28 strains	3 9 11	(Mathis, Neumann et al. 1995)
	OF Rearing habituated (second trial)	A/J C57BL/6J	F2	518	1 8 10 11 19	(Gershenfeld, Neumann et al. 1997)
	OF Centre Time (first trial, 5 minutes)	A/J C57BL/6J	F2	518	1 19	(Gershenfeld and Paul 1997)
	EPM % open arms entries	A/J CBA/J	F2	219	5	(Cohen, Kang et al. 2001)
	EPM closed arms entries	A/J CBA/J	F2	219	7	(Cohen, Kang et al. 2001)
	LD transitions (first trial)	A/J C57BL/6J	F2	518	10 19	(Gershenfeld and Paul 1997)
		A/J C57BL/6J	RI	28 strains	9 11	(Mathis, Neumann et al. 1995)
	LD transitions (second trial)	A/J C57BL/6J	F2	518	10 19	(Gershenfeld and Paul 1997)
	LD transitions (third trial)	A/J C57BL/6J	F2	518	10 19 X	(Gershenfeld and Paul 1997)

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Species	Phenotype[1]	Strains[2]	Method[3]	No.[4]	Chr[5]	Reference
Mouse	LD transitions average (over three trials)	A/J C57BL/6J	F2	518	1 6 10 15 19	(Gershenfeld and Paul 1997)
	FC: contextual	C3H/HeJ C57BL/6J	BC	473	1 3 8 9	(Caldarone, Saavedra et al. 1997)
		C57BL/6J DBA/2J	F2	479	1 2 3 10 16	(Wehner, Radcliffe et al. 1997)
		C57BL/6J BALB/cJ	F2	199	3 8 9	(Valentinuzzi, Kolker et al. 1998)
		C57BL/6J DBA/2J	RI	25 strains	1 9 11 17 19	(Owen, Christensen et al. 1997)
	FC: altered context	C3H/HeJ C57BL/6J	BC	473	1 8	(Caldarone, Saavedra et al. 1997)
		C57BL/6J DBA/2J	F2	479	2 3	(Wehner, Radcliffe et al. 1997)
		C57BL/6J DBA/2J	RI	25 strains	3 12 13 19	(Owen, Christensen et al. 1997)
	FC: cued	C3H/HeJ C57BL/6J	BC	473	1 9	(Caldarone, Saavedra et al. 1997)
		C57BL/6J DBA/2J	F2	479	1 10 16	(Wehner, Radcliffe et al. 1997)
		C57BL/6J DBA/2J	RI	25 strains	1	(Owen, Christensen et al. 1997)

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Species	Phenotype[1]	Strains[2]	Method[3]	No.[4]	Chr[5]	Reference
Rat	OF Activity in periphery	RHA RLA	F2	908	3 5	(Fernández-Teruel, Escorihuela et al. 2002)
		LEW SHR	F2	192	5	(Ramos, Moisan et al. 1999)
	OF Activity in centre	RHA RLA	F2	908	15	(Fernández-Teruel, Escorihuela et al. 2002)
		LEW SHR	F2	192	4	(Ramos, Moisan et al. 1999)
	OF Defecation	RHA RLA	F2	908	3 6 19 X	(Fernández-Teruel, Escorihuela et al. 2002)
	EPM % open arms entries	RHA RLA	F2	908	5	(Fernández-Teruel, Escorihuela et al. 2002)
		LEW SHR	F2	192	6	(Ramos, Moisan et al. 1999)
	EPM closed arms entries	RHA RLA	F2	908	15	(Fernández-Teruel, Escorihuela et al. 2002)
		LEW SHR	F2	192	7	(Ramos, Moisan et al. 1999)
	FC: contextual	RHA RLA	F2	908	5 10	(Fernández-Teruel, Escorihuela et al. 2002)
	FC: cued	RHA RLA	F2	908	5	(Fernández-Teruel, Escorihuela et al. 2002)
	Two way active avoidance (shuttle box)	RHA RLA	F2	908	5 10	(Fernández-Teruel, Escorihuela et al. 2002)

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1

OF = open field
EPM = elevated plus maze
LD = light-dark box
FC = fear conditioning

2

RHA = Roman High Avoidance
RLA = Roman Low Avoidance
LEW = Lewis
SHR = Spontaneously Hypertensive Rats

3

F2 = F2 intercross
BC = backcross
RI = recombinant inbred strains

4

No. = number of animals employed in study

5

Chr = chromosome

also mapped in a cross between A/J and C57BL/6J mice and loci on chromosomes 1, 10, 15 and 19 were found to contribute to about a quarter of the phenotypic variance (hence again almost all the genetic variance) (Gershenfeld, Neumann et al. 1997).

The second lesson has been that most loci are pleiotropic. The DeFries mice were scored for open field activity and defecation and elevated plus maze behaviours. Of six loci that were significantly associated with activity, three were also significantly associated with defecation and entry into the open arms of the elevated plus maze. A QTL on chromosome 1 for OF measures was also found by two other studies (Gershenfeld, Neumann et al. 1997; Gershenfeld and Paul 1997), though its position was more centromeric than that of the previously identified locus for OFA.

Genetic loci contributing to variation in fear conditioning have also been mapped, providing an opportunity to test the hypothesis that fear conditioning and unconditioned anxiety-related behaviours have a common genetic basis. Two F2 intercrosses and one study of recombinant inbred strains identified a locus on chromosome 1 that lies within the region identified by the work on open field behaviours (Caldarone, Saavedra et al. 1997; Owen, Christensen et al. 1997; Wehner, Radcliffe et al. 1997). Therefore it appears very likely that a locus on chromosome 1 determines variability in a genetic system mediating fear.

However, all these results have been obtained using crosses between inbred animal strains. The strategy has great power (it can detect genes that contribute to as little as 5% of the phenotypic variance of the trait) but it also samples only a fraction of the total variability of a population. Each time a cross is set up, we can only look at two alleles at

any locus and many loci may not be polymorphic between the strain pair that we pick. This explains why results of mapping experiments differ: for example a locus on chromosome 10 that influences open field activity was found in a cross between A/J and C57BL/6J (Gershenfeld and Paul 1997) but not in cross between BALB/cJ and C57BL/6J (Flint, Corley et al. 1995).

CHAPTER 3

QTL mapping in laboratory mice derived from a replicated selection experiment for open-field activity

3.1 Introduction

This chapter describes the results of a QTL analysis of the DeFries selected strains. The DeFries (DeFries, Gervais et al. 1978) selection experiment and the first QTL mapping study on the DeFries strains (Flint, Corley et al. 1995) were described in chapter 2. Here I report QTL analyses of two crosses (H1 X L1 and H2 X L2) and comparison with previous results.

The QTL experiment I describe here has several important features that distinguish it from other QTL mapping studies of behaviour. First, a much higher number of animals have been employed in the phenotypic and genotypic analysis: 1636 mice were phenotyped and genotyped. Such large numbers are unique and should allow detection of small effect QTL. Second, the mice in this experiment derive from two distinct crosses, one originated from the H1 and L1 strains (like the original experiment on the DeFries lines), the other from the H2 and L2 DeFries strains. This allows comparison of the genetic effect of strains derived from two replicate but distinct selection experiments. Third, in this project a much higher number of phenotypes for anxious behaviour were

analysed: 64 (for a complete list of phenotypes and their description see Table A.1 in Appendix A). This has allowed an in depth and refined analysis of different dimensions of anxious behaviour in mice, and results for the complete set of phenotypes will be given in chapter 4.

3.2 Population design and genotyping

Two F2 populations were derived from the DeFries inbred strains, as described in Appendix A, from two intercrosses between the DeFries inbred strains: Group 1 (G1) mice (815) were the progeny of the H1XL1 intercross and Group 2 (G2) mice (821) were the progeny of the H2XL2 intercross, for a total of 1636 animals. Results from G1 and G2 will be compared in this chapter with data from the original DeFries QTL mapping experiment (384 mice) (Flint, Corley et al. 1995), which for practical purposes will be referred to as G0.

All mice in G1 and G2 were subject to a genome scan. 80 and 77 MIT markers were used respectively in G1 and G2, in order to have a genome coverage of a marker every 20 centimorgan (cM) on average. List of markers used in the two Groups and their MIT cM position are given in Appendix B. Genotyping protocols are given in Appendix A.

3.3 Phenotypes

Table A.1 in Appendix A reports the complete list of the 64 phenotypes analysed in G1 and G2, with a brief description.

In this chapter I will limit the discussion to a subset of well-validated measures taken in the open field (OF) (Walsh and Cummins 1976), the elevated plus maze (EPM) (Pellow, Chopin et al. 1985), and the light-dark box (LD) (Costall, Jones et al. 1989):

- two measures in the OF: total activity (OFA) and defecation (OFD),
- three measures in the EPM: entries into the open arms (EPM-open entries), entries into the closed arms (EPM-closed entries), and time spent in the open arms (EPM-open time),
- three measures in the LD: latency to enter the light side for the first time (LD-latency), transitions between the dark and the light side (LD-transitions), and time spent in the light side (LD-light time).

For a description of these measures and how they were taken in the present experiment, see Appendix A. For a description of the phenotyping in the original experiment (G0), see (Flint, Corley et al. 1995).

3.4 QTL mapping results

Table 3.1 reports results for mapping OFA and OFD in G0, G1 and G2. Table 3.2 reports mapping results for LD and EPM measures in G1 and G2. G0 data are not available for these measures. In both tables, mapping results for the combined data set of G1+G2 are also given. The tables give LOD (logarithm to the base 10 of the odds ratio of linkage against no linkage) scores, position on the chromosome and percent of the phenotypic variance explained for QTL that exceed the 5% significance threshold (LOD equal to or greater than 3.2) for at least one intercross. The numbers of animals in the two replicates are nearly equivalent, so we can directly compare the LOD scores. However the first

Table 3.1

QTL with LOD scores exceeding a 5% significance threshold for OFA and OFD in at least one of three F2 intercrosses: G0 (Flint, Corley et al. 1995), G1 and G2. The results of a combined data set of G1+G2 are also shown.

The table shows the chromosome (Chr), the position (Pos.) in cM of the LOD score (LOD) and the percentage of the phenotypic variance (%Var) explained. All values, including map distances, are derived from MAPMAKER and MAPMAKER-QTL (Lincoln, Daly et al. 1992).

Significant LOD are highlighted in blue.

		G0			G1			G2			G1+G2		
Phenotype	**Chr**	**LOD**	**Pos.**	**%Var**	**LOD**	**Pos.**	**%Var**	**LOD**	**Pos.**	**%Var**	**LOD**	**Pos.**	**%Var**
OFA	1	13.4	74	10.7	12.8	72	10.9	14.8	74	10.0	27.3	74	10.0
	4	6.2	34	4.2	2.5	36	1.7	1.6	62	1.2	3.5	36	1.1
	7	1.3	46	1.2	7.6	66	6.5	7.0	50	6.0	13.1	52	5.8
	12	4.3	2	3.5	2.9	16	1.8	1.4	50	1.0	3.6	40	1.2
	15	11.0	12	8.3	5.6	12	3.5	7.3	26	4.8	12.4	20	4.0
	17	3.4	12	2.5	1.0	14	0.6	1.4	0	0.8	0.4	14	0.2
	18	4.0	10	4.4	2.2	34	1.4	3.4	32	2.2	5.3	32	1.7
	X	1.9	32	0.8	2.1	14	1.5	3.3	12	1.9	5.2	14	1.9
OFD	1	4.8	80	4.1	7.6	80	5.8	7.4	72	5.3	14.4	74	5.5
	7	2.8	32	9.6	1.2	82	0.9	4.0	42	3.1	3.3	44	1.4
	12	4.4	6	4.6	1.5	16	0.9	0.4	44	0.3	1.7	38	0.6
	14	2.5	0	5.9	4.0	18	2.5	5.9	22	4.5	9.4	20	3.3
	X	1.2	38	1.3	2.1	2	1.6	3.9	52	3.9	5.3	50	2.7

Table 3.2

Light Dark Box and Elevated Plus Maze: LOD scores exceeding a 5% significance threshold for G1, G2 or for the combined data set G1+G2. The table shows the chromosome (Chr), the position (Pos.) in cM of the LOD score (LOD) and the percentage of the phenotypic variance (%Var) explained. All values are derived from MAPMAKER-QTL (Lincoln, Daly et al. 1992).

Significant LOD are highlighted in blue.

		G1			G2			G1+G2		
Phenotype	**Chr**	**LOD**	**Pos.**	**%Var**	**LOD**	**Pos.**	**%Var**	**LOD**	**Pos.**	**%Var**
EPM-open entries	1	4.0	82	3.3	13.8	80	8.6	15.4	80	5.3
	4	3.7	42	2.5	1.1	0	0.7	3.2	46	1.1
	15	4.8	14	3.1	8.9	24	5.6	12.9	22	3.8
	18	4.9	26	3.5	3.5	20	2.7	8.0	22	2.9
	X	1.3	14	1.1	2.2	16	2.0	3.2	16	1.4
EPM-closed entries	1	3.9	80	2.7	5.5	80	3.7	9.4	80	3.1
	4	4.7	32	3.0	1.5	60	1.1	5.5	34	1.8
	7	11.6	50	10.1	6.0	52	4.8	16.5	50	6.9
	8	4.5	62	3.6	2.0	76	1.9	5.9	68	2.6
	18	3.3	26	2.3	3.8	24	2.5	7.1	26	2.3
EPM-open time	1	2.5	86	1.6	1.4	80	0.8	3.4	82	1.1
	5	2.5	34	4.5	3.1	0	3.6	3.6	40	2.1
	15	2.0	18	1.7	3.0	24	1.9	4.4	22	1.3
	X	2.0	4	1.6	4.1	0	2.6	5.2	0	1.9
LD-latency	1	2.9	76	2.2	2.5	80	1.7	4.9	80	1.7
	2	3.4	56	2.5	0.3	0	0.3	2.1	60	1.0
	11	2.8	20	1.8	2.0	16	1.7	4.8	20	1.5
	14	4.6	18	3.1	7.0	18	4.5	11.4	18	3.6
	15	7.3	14	5.1	12.7	26	8.0	19.6	24	6.4
	18	1.8	34	1.1	2.5	34	1.6	3.4	34	1.0

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		G1			G2			G1+G2		
Phenotype	**Chr**	**LOD**	**Pos.**	**%Var**	**LOD**	**Pos.**	**%Var**	**LOD**	**Pos.**	**%Var**
LD-transitions	1	6.7	78	5.1	10.7	74	7.8	16.7	76	6.2
	4	2.4	42	1.4	1.6	56	1.8	3.9	42	1.3
	11	3.6	18	2.3	3.6	16	3.0	7.0	20	2.2
	12	2.2	16	1.5	2.0	40	1.9	4.3	38	1.4
	14	3.4	18	2.3	3.9	12	2.6	7.0	16	2.3
	15	5.1	14	3.7	8.9	26	5.5	13.5	24	4.4
	18	2.8	34	1.8	3.7	34	2.3	5.7	34	1.8
	X	0.6	40	0.4	3.2	24	2.9	2.9	22	1.3
LD-light time	1	4.2	78	3.2	5.9	82	3.9	9.9	80	3.3
	4	0.7	42	0.4	3.7	56	3.1	3.2	40	1.1
	11	2.5	20	1.6	3.2	18	4.2	5.7	20	1.7
	14	4.5	14	3.2	3.1	12	2.1	7.6	12	2.6
	15	5.4	18	4.4	10.3	24	6.7	14.7	24	5.0
	16	3.1	50	2.9	1.6	16	1.6	4.3	50	1.9
	18	2.3	34	1.4	4.3	34	2.7	5.7	32	1.9

experiment genotyped only 384 animals, including those with the most extreme OFA scores, so direct comparison of the LOD scores is not practicable.

3.4.1 Comparing G0 with G1

Significant QTL are found on 7 chromosomes for OFA and on 3 chromosomes for OFD in either cross. Of these, consistent results for both crosses are found only for a QTL on chromosome 1 for both OFA and OFD and a QTL on chromosome 15 for OFA. Additional significant LOD scores in G0 are found on chromosomes 4, 17 and 18 for OFA and on chromosome 12 for OFA and OFD. In G1 instead QTL are detected on chromosome 7 for OFA and on chromosome 14 for OFD. Out of a total of 10 chromosomes with significant LOD scores for two measures in the OF, only 3 are found to be replicable in two mapping experiments that share the same design.

3.4.2 Comparing G1 with G2

Results for the two crosses are reported in Tables 3.1 and 3.2. For OFA, out of the 5 chromosomes harbouring a significant QTL in either G1 or G2, consistent results are found for chromosomes 1, 7 and 15. For OFD, out of 4 chromosomes with significant LOD scores, consistent results are found on chromosomes 1 and 14. Additional significant QTL are found in G2 on chromosomes 18 and X for OFA and on chromosomes 7 and X for OFD. In G1 no additional QTL is detected. In summary, out of a total of 9 chromosomes with significant LOD scores for OFA and OFD, 5 results are replicated in the two crosses.

Results in the light-dark box and the elevated plus maze show that out of 25 significant LOD scores found in either G1 or G2 for six phenotypes, 14 consistent results are found in both crosses, while 5 additional QTL are found specifically in G1, and 6 in G2. More than half QTL for LD and EPM behaviours are replicated in the two crosses, albeit there is less consistency in the LOD scores than seen for the open-field results, with inter-group differences of well over 5 LOD for the light-dark box on chromosome 15, and entries into the open-arms of the elevated plus maze on chromosome 1. Nevertheless, when we consider loci explaining more than 3% of the phenotypic variance, the data do indicate that the same loci are contributing to the phenotype in each cross.

To analyse the consistency of mapping data between G1 and G2, in Table 3.3 I report direction of the allele effect and dominance component of significant QTL in either G1 or G2 on the eight phenotypes analysed. In both crosses the direction of allelic effects were the same and the estimated dominance components were similar for most of the phenotypes. Not all QTL are additive: some QTL have a substantial dominance component like the one on chromosome 1, other QTL act recessively, like the one on chromosome 7.

3.4.3 Identifying QTL of small effect

Results for QTL influencing eight behaviours in two F2 intercrosses in the DeFries strains indicate that the same QTL are segregating in the two populations. However the results do not rule out the possibility that many additional smaller QTL could be present that differentiate the crosses. Using a power calculation given by Darvasi (Darvasi 1998), it can be shown that each F2 does not have the power to detect reliably QTL contributing

Table 3.3

Additive and dominance components of QTL for behaviours in the OF, the LD and the EPM. Data are given for the two F2 intercrosses that derive from the replicate selection experiment. Additive and dominance components were estimated with MAPMAKER-QTL (Lincoln, Daly et al. 1992). Alleles were categorized as either BALB/cJ or C57BL/6J (progenitors of the selection experiment) from the size of the microsatellite alleles (see Appendix B for a list of microsatellites used in this project and their allelic characterisation in respect to BALB/cJ and C57BL/6J alleles). The direction of allelic effects is given with respect to the BALB/cJ allele, which in each case decreases activity and increases defecation in the open field.

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		G1		G2	
Phenotype	**Chr**	**Additive**	**Dominant**	**Additive**	**Dominant**
OFA	1	-0.39	-0.36	-0.43	-0.27
	7	-0.17	0.39	-0.27	0.31
	15	-0.25	0.11	-0.30	0.08
	18	-0.16	0.00	-0.21	0.06
	X	0.11	-0.14	0.15	-0.14
OFD	1	0.31	0.17	0.27	0.26
	7	0.12	-0.09	0.18	-0.22
	14	-0.21	0.12	-0.25	0.18
	X	-0.01	-0.28	-0.08	-0.31
EPM-open entries	1	-1.03	-0.54	-1.65	-0.84
	4	-0.98	-0.14	0.36	-0.43
	15	-1.08	0.33	-1.25	0.54
	18	-1.16	-0.19	-0.72	-0.77
EPM-closed entries	1	-1.19	-0.91	-1.49	-0.90
	4	-1.28	0.93	-0.91	-0.22
	7	1.98	-2.59	1.77	-1.06
	8	1.60	-0.11	1.11	-0.05
	18	-1.24	0.02	-1.35	0.47
EPM-open time	X	0.02	-0.27	-0.09	-0.31

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		G1		G2	
Phenotype	**Chr**	**Additive**	**Dominant**	**Additive**	**Dominant**
LD-latency	2	-0.12	0.01	-0.02	0.00
	14	-0.14	0.01	-0.16	0.01
	15	0.18	-0.05	0.22	-0.05
LD-transitions	1	-1.57	-0.81	-1.85	-1.56
	11	-1.20	-0.05	-1.09	0.46
	14	1.18	-0.15	1.20	-0.12
	15	-1.40	0.56	-1.74	0.42
	18	-1.01	-0.53	-1.10	0.49
	X	-0.21	-0.71	0.83	-1.15
LD-light time	1	-0.23	-0.11	-0.24	-0.21
	4	-0.09	-0.04	-0.18	-0.22
	11	-0.18	0.01	-0.20	0.02
	14	0.25	0.02	0.20	0.04
	15	-0.26	0.20	-0.33	0.11
	18	-0.17	-0.07	-0.21	0.12

less than 2% to the phenotypic variance. However, combining the two data sets does confer power to detect such small effects.

Tables 3.1 and 3.2 show LOD scores in the combined data set G1+G2 for OF measures, and LD and EPM measures respectively. Where there is evidence that QTL segregate in both crosses, the LOD scores in the combined data set are approximately the sum of the two LOD in G1 and G2. For example, a LOD of 27.3 for OFA is found on chromosome 1 in the combined data set, whereas corresponding LOD are 12.8 in G1 and 14.8 in G2. Overall results for the combined data set for measures in the OF, LD and EPM, indicate that much more often than not LOD in the combined data set are the approximate sum of the LOD found in G1 and G2.

Significant LOD scores in the combined set are sometimes derived from the sum of two non-significant LOD scores in G1 and G2, indicating that the large number of animals in the combined data set allows detection of small effect QTL that would be missed in analysing G1 or G2 alone. An example is given by LOD for OFA on chromosome 4.

A QTL for OFA on this chromosome was originally found in G0, but was not replicated in either G1 or G2. However, the combined G1+G2 data set is indeed finding a significant LOD score of 3.5, which is approximately the sum of G1 LOD of 2.5 and G2 LOD of 1.6. However it is notable that this occurrence is relatively rare; there are remarkably few changes to the QTL identified in the individual crosses: regarding the loci in each behavioural test as independent, 32 QTL found in the combined data are also found in both or either of the two crosses, while only 12 are found exclusively in the combined data set.

Only 2 exceptions exist when the combined data set does not replicate a QTL present either in G1 or G2, and the LOD score does not appear to be the sum of G1 and G2 LOD. This happens for a locus on chromosome 2 detected in G1 as having an effect on the latency to enter the light side of the LD (LOD 3.4), which is not replicated either in G2 or in the combined set; and for a locus on chromosome X found in G2 to affect transitions between the dark and the light side of the LD (LOD 3.2), neither replicated in G1 and the combined set.

3.5 Discussion

By reporting mapping results of genetic analysis of anxious behaviour in three F2 intercrosses (G0, G1 and G2), which are derived from the DeFries inbred strains, I set out to establish whether mapping results for behaviour are reliable and reproducible, and whether a similar simple architecture is responsible for the genetic determination of behaviour in replicate selection experiments.

G0 and G1, which are exact replicas as they are both derived from an H1XL1 intercross, would be expected to present overlapping mapping data. However, when results are compared, only two loci (on chromosomes 1 and 15) occur in both experiments at significance thresholds of less than 5%. Procedural differences between the two crosses might explain the discrepancies: the first intercross employed a circular open field and testing occurred during the light cycle with uniform illumination of the apparatus. Testing in the open field was carried out with ambient noise. By contrast, phenotyping in this project was performed in the first half of the dark cycle (when mice are active), used a square open field (allowing the animals access to relatively safe corners) that was

soundproofed and had graded illumination (the centre being brighter and hence more threatening than the periphery).

There is greater agreement between the analyses between G1 and G2. These two crosses were bred and phenotyped contemporaneously using the same phenotypic assessments, even though they are not genetically identical, as they were derived from two F2 intercrosses between distinct pairs of the DeFries inbred strains (H1XL1 and H2XL2 respectively), which on their part were generated from a replicate selection experiment. Of five loci for OFA that exceed the 5% significance threshold, three are found in both crosses and the difference between LOD is much smaller than the differences observed between the non-contemporaneous H1XL1 replicates. Analyses of the other phenotypes (in the elevated plus maze and the light-dark box) and high consistency of direction of effect and dominance component between loci detected on same chromosomes in the two crosses are consistent with the conclusions reached from the open field data, namely that the same small numbers of loci influence the phenotypes in both crosses.

Overall, these data indicate that selection acted on the same relatively small group of genes in the two replicated DeFries experiments.

High consistency of data from G1 and G2 has allowed me to perform genetic analysis of the combined data set G1+G2. Overall results indicate that much more often than not LOD in the combined data set are the approximate sum of the LOD found in G1 and G2, therefore confirming that most loci are replicated in the two crosses. Moreover, use of the combined data set allows detection of small effect QTL that would be missed in analysing G1 or G2 alone.

Interestingly, by comparing data from the combined data set with results from the original G0 experiment, I find that out of 6 loci found for OFA in G0, 5 are now replicated, with the only exception of the locus on chromosome 17 not reaching significance, and the additional detection of two loci on chromosomes 7 and X.

These results show that it is possible to replicate QTL analysis for behaviour, but that confirmation at significance levels of 5% or lower requires large numbers of animals.

CHAPTER 4

Interpreting QTL action

4.1 Introduction

It is generally assumed that different ethological tests of anxiety in mice measure the same psychological construct (Treit 1985; Lister 1990). Consequently, consistent behaviour across tests is interpreted as evidence that an animal is anxious. However it is not clear that the ethological tests really do measure the same phenomenon, or indeed whether anxiety in rodents is a unitary phenomenon (Ramos and Mormède 1998). For instance, lack of function of receptor-2 for the corticotrophin releasing hormone (Crhr2) has been found to affect anxious behaviour in the OF, EPM and LD by one study (Kishimoto, Radulovic et al. 2000), to affect anxious behaviour in the OF and the EPM but not in the LD by a second study (Bale, Contarino et al. 2000), and to not affect anxious behaviour as measured in the OF and EPM by a third study (Coste, Kesterson et al. 2000).

I argue here that the difficulties of interpreting behavioural test results can be dealt with genetically. If we apply QTL mapping to animals whose behaviour has been assessed in multiple tests, we can expect QTL underlying the inferred psychological process (for example anxiety) to influence more than one measure in a way consistent with our predictions. We would also expect such QTL to have no influence on measures unrelated

to the phenotype. In short we need to be able to identify pleiotropic QTL with a specific pattern of influence.

In this chapter I will first summarise mapping data for 64 phenotypes in the combined data set G1+G2. In order to address the issue of how the QTL operate, I will then present analysis of a subset of the data laid out as follows:

- an analysis of the direction of allelic effect of QTL on measures across the five ethological tests;
- an analysis of the effect of QTL on repeated measures, i.e. re-exposure of the mice to an anxious test for a second trial;
- an analysis of specificity of QTL genetic action on anxious behaviour as opposed to a control measure for general locomotor activity (homecage activity) and a control measure for an aversive situation unrelated to anxiety (tail suspension test).

4.2 Genotyping and phenotyping data

The genotyping data analysed in this section are restricted to the combined data set G1+G2. As shown in chapter 3, QTL mapping across the two groups is fairly consistent. Moreover, the analysis of the combined data set, consisting of 1636 mice, allows detection of QTL of small effect, and is therefore more powerful.

The phenotyping data presented in this section comprise a large set of the measures in the five ethological tests - open field (OF), elevated plus maze (EPM), light-dark box (LD), elevated square maze (SQ), mirror chamber (MR) - plus two additional phenotypes:

homecage activity and tail suspension. The complete list is reported in Table A.1 in Appendix A.

4.3 Reporting results for 62 phenotypes in five ethological tests of anxiety

Appendix C reports significant LOD scores (>3.2) for QTL influencing 62 phenotypes measured in five tests of anxious behaviour in mice, in G1, G2, and the combined data set G1+G2. Results are given also for non-significant LOD scores for chromosomes where a significant LOD has been found at least in one of the two F2 crosses or in the combined data set.

Table 4.1 summarizes the data. For each phenotype and each chromosome the table reports if a significant LOD has been found in G1 (indicated as 1 in the corresponding cell), G2 (indicated as 2), combined set G1+G2 (c, for 'combined'), and finally reports if the LOD score in the combined data set results from the summing of LOD in G1 and G2 (s, for 'sum'). When LOD scores sum up, the corresponding cell has also been coloured in blue. A total of 289 significant LOD are found if we consider single combinations of phenotype and chromosome disrespectfully of the fact that the LOD is found in G1, G2, the combined data set or a combination of those.

Table 4.1

Summary of mapping data for 62 anxious-related phenotypes. For each phenotype and each chromosome the table reports if a significant LOD has been found in G1 (indicated as 1 in the corresponding cell), G2 (indicated as 2), combined set G1+G2 (c, for 'combined'), and reports if the LOD score in the combined data set results from the summing of LOD in G1 and G2 (s, for 'sum'). When LOD scores sum up, the corresponding cell has also been coloured in blue.

No.	Name of variable	chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chrX
1	OFD	1,2,c,s						2,c							1,2,c,s						2,c,s
2	OFA	1,2,c,s			c,s			1,2,c,s					c,s			1,2,c,s			2,c,s		2,c,s
3	OFA-centre	1,2,c,s						1,2,c,s					1,c			1,2,c,s			2,c,s		
4	OF-centre time	c,s						1,2,c,s					1,c			1,c,s			2,c,s		
5	OF-latency							c,s								1,2,c,s					
6	OF-rears				1,2,c,s			1,2,c,s				2,c,s	1,c			1,2,c,s			2,c,s		2,c,s
7	OF-rearing time				c,s			1,2,c,s								1,2,c,s			2,c		
8	OFA-2	1,2,c,s	c,s		1,2,c,s			1,2,c,s	1,c,s					1,c,s		1,2,c,s		1	1,2,c,s		2,c,s
9	OFA-centre-2	1,2,c,s			1,2,c,s			1,2,c,s					1			2,c,s		1	c,s		c,s
10	OF-centre time-2				2,c,s			1,2,c,s								2,c,s					
11	OF-latency-2							1,2,c,s								1,2,c,s					
12	EPM-boli	1,c,s							c,s						1,2,c,s						1,2,c,s
13	EPM-shifts	1,2,c,s			1,c,s	1		1,c						c,s		1,2,c,s			1,2,c,s		
14	EPM-closed entries	1,2,c,s			1,c,s			1,2,c,s	1,c,s										1,2,c,s		
15	EPM-closed ambulation	2,c,s			1,c,s			1,2,c,s	1,c,s										1,2,c,s		
16	EPM-closed time	1,c,s					1,c,s														

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No.	Name of variable	chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chrX
17	EPM-open entries	1,2,c,s			1											1,2,c,s			1,2,c,s		c,s
18	EPM-open ambulation	1,2,c,s			1,c											1,2,c,s			1,c,s		c,s
19	EPM-open time	c,s				c,s										c,s					2,c,s
20	EPM-open latency															1,2,c,s					
21	EPM-end entries	2,c,s			1,c			2,c							2,c,s	1,2,c,s			1,c,s		
22	EPM-end ambulation	1,2,c,s			1,c											1,2,c,s			1,c,s		c,s
23	EPM-end time							2,c,s							c,s						
24	EPM-closed rears				1,2,c,s											1,2,c,s			1,2,c,s		
25	EPM-closed rearing time				2,c,s									2		1,2,c,s			1,2,c,s		
26	EPM-open rears					c,s										c,s		2			
27	EPM-open rearing time					1															
28	EPM-open scans	1,2,c,s					2,c,s									1,2,c,s			1,c		
29	EPM-end scans			1,c,s	1,c,s	2,c		1,2,c,s								1,2,c,s		2	1,c	1	
30	EPM-end scanning time			1,c	1,c,s	2,c		1,2,c,s								2,c,s		2	1		c,s
31	EPM-centre entries	1,2,c,s			1,c,s			1,2,c,s	1,c,s					c,s					1,2,c,s		
32	EPM-centre time							1,2,c,s	c,s						c,s	2,c,s					
33	SQ-boli	1,c,s							2,c						2,c						1,2,c,s
34	SQ-shifts	1,2,c,s			1,c,s				c,s						2	2,c,s					1,2,c,s
35	SQ-closed entries	1,2,c,s											c,s			1,2,c,s			1,c,s		
36	SQ-closed ambulation	1,2,c,s			1,c			1,c	2,c,s										1,2,c,s		c,s
37	SQ-open entries	1,2,c,s			c,s											1,2,c,s			c,s		
38	SQ-open ambulation	1,2,c,s			1,c,s			2,c								1,2,c,s			1,2,c,s		2,c,s
39	SQ-open latency															2,c,s					

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No.	Name of variable	chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chrX
40	SQ-closed rears				c,s											1,2,c,s			1,c,s		
41	SQ-closed rearing time															1,2,c,s			c,s		
42	SQ-open rears				1,c		c,s									1,2,c,s					
43	SQ-open rearing time															2,c,s					
44	SQ-open scans	1,2,c,s			c,s											1,2,c,s			1,c,s		
45	SQ-open scanning time							c,s								2,c,s					
46	SQ-corner entries	1,2,c,s			1,c			1,c						2,c					1,2,c,s		
47	SQ-corner time							2,c,s								1,2,c,s			1,c,s		2,c,s
48	LD-boli	1,c,s		2	c,s										1,c						c,s
49	LD-dark ambulation	2,c,s	c,s		1,c,s	1												1,c,s			c,s
50	LD-transitions	1,2,c,s			c,s							1,2,c,s	c,s		1,2,c,s	1,2,c,s			2,c,s		2
51	LD-light ambulation	1,2,c,s										2,c,s			1,c,s	1,2,c,s			2,c,s		
52	LD-light time	1,2,c,s			2,c							2,c,s			1,c,s	1,2,c,s	c,s		2,c,s		
53	LD-latency	c,s	1									c,s			1,2,c,s	1,2,c,s	c,s		c,s		
54	LD-transitions-2	1,2,c,s			1,2,c,s								c,s						1,2,c,s		2,c
55	LD-light ambulation-2	1,2,c,s			1,2,c,s			1,c,s											1,c,s		2,c,s
56	LD-light time-2	1,c,s			1,2,c,s																2,c,s
57	LD-latency-2	2,c,s										1,2,c,s			2,c,s	1,2,c,s			2,c		
58	MR-boli	c,s			1							2				c,s			c,s		2,c,s
59	MR-entries							1,2,c,s				2			2,c,s	1,2,c,s			c,s		
60	MR-latency					c,s							c,s			1,2,c,s					
61	MR-rears	1,2,c,s			c,s		1	1,c					1,c,s			1,2,c,s			1,c		c,s
62	MR-rearing time	1,c,s					1	1,c,s					1,c,s			1,2,c,s			1,c		1,2,c,s

The table shows that many phenotypes report significant LOD scores on the same chromosomes in both G1 and G2 (104 cases out of 289). Moreover, if we also consider cases when a significant LOD is found either in G1 or G2 and the combined data set or only in the combined data set, LOD score in G1+G2 derives from the summing of the individual intercrosses LOD in most of the cases (236 out of 289, i.e. 81.7%). These data confirm on a much larger scale what has been already discussed in chapter 3, namely that mapping results from the two intercrosses were highly consistent, and justify the decision to combine genetic data from G1 and G2 to maximize power and mapping resolution.

Table 4.2 reports a further summary of the overall data. For each of the five tests, and overall across the tests, the table shows how many phenotypes present a significant LOD score on each chromosome. For example, the number of phenotypes that present a significant LOD on chromosome 1 are: 6 out of 11 in the OF, 12 out of 21 in the EPM, 8 out of 15 in the SQ, 10 out of 10 in the LD, 3 out of 5 in the MR, for a total of 39 out of 62, which corresponds to 63%.

On six chromosomes (1, 4, 7, 15, 18 and X) LOD scores are found for almost half or more of the 62 tested phenotypes. In five further chromosomes (5, 8, 11, 12 and 14) at least 10% or more (usually around 15-20%) of the measures have a significant LOD score. In the remaining 9 chromosomes, significant LOD scores are found only for one or two measures, up to a maximum of 6 out of 62. Overall, out of 289 QTL, 214 (74%) are found on 6 chromosomes, namely chromosomes 1, 4, 7, 15, 18 and X.

Table 4.2

Summary of mapping data for 62 anxious-related phenotypes. For each chromosome the table reports: the number of QTL mapped for each of the five ethological tests, the overall number of phenotypes mapped across the five tests (tot, for total) and what percentage they represent in relation to the total number of 62 phenotypes analysed in this project. Percentages above 40% have been highlighted in yellow, percentages between 10% and 40% have been highlighted in blue.

	OF	EPM	SQ	LD	MR	tot	%
No. phenotypes	11	21	15	10	5	62	
chr1	6	12	8	10	3	39	63.0
chr2	1			2		3	4.8
chr3		2		1		3	4.8
chr4	6	12	8	7	2	35	56.5
chr5		6		1	1	8	12.9
chr6		2	1		2	5	8.1
chr7	11	9	5	1	3	29	46.8
chr8	1	5	3			9	14.5
chr9						0	0.0
chr10						0	0.0
chr11	1			5	2	8	12.9
chr12	5		1	2	3	11	17.7
chr13	1	3	1			5	8.1
chr14	1	4	2	6	1	14	22.6
chr15	10	14	12	5	5	46	74.2
chr16				2		2	3.2
chr17	2	3		1		6	9.7
chr18	7	13	9	7	4	40	64.5
chr19		1				1	1.6
chrX	5	6	5	6	3	25	40.3

These results attest that a very large number of phenotypes for anxious behaviour map to a few chromosomes, suggesting that a small number of loci are acting pleiotropically on many different measures of fearful behaviour in five distinct tests. Unfortunately determining pleiotropic action is not easy, primarily because of the poor resolution of the F2 analyses: we cannot be certain that two QTL whose positions coincide, do not contain different genes that happen, by chance, to influence the same phenotype. However, I suggest that it is possible to address the issue of pleiotropy by exploiting the predicted direction of allelic effects.

4.4 Analysis of direction of allelic effects

Using the information contained in the direction of allelic effects has a number of advantages. First, unlike the effect size, once a QTL is detected, the direction is robust, so we do not need to worry whether we estimated it correctly or not. Second, inconsistent direction of effects unambiguously excludes a QTL from consideration as influencing the psychological process we believe the test measures. I should stress here that consistent allelic effects do not prove that a QTL acts on the phenotype, since the joint action may be due to two linked QTL rather than a single pleiotropic locus, but it makes joint action less likely. In fact, alleles in different genes that are physically linked in an inbred strain do not necessary have allelic effects that operate in the same direction (except for alleles that specifically act on measures for which the lines have been selected, i.e. OFA for the DeFries strains).

To understand the value of the direction of an allele's effect, it is important to understand the principles of the tests for anxiety and I will briefly recapitulate the critical points from my earlier description in chapter 1. In each test employed in this study, the animal is presented with a choice between threatening and non-threatening environments. In the EPM and in the elevated square maze, mice have a choice between two relatively anxiogenic regions (the open arms) and two relatively safe regions (the closed arms) (Pellow, Chopin et al. 1985; Hogg 1996; Rodgers and Dalvi 1997). The light-dark box and the mirror chamber also permit the animal to explore a novel situation or remain in a relatively non-threatening dark enclosure. The difference lies in the nature of the novel environment: either a well lit exposed area (Crawley and Goodwin 1980) or a mirrored chamber (Lamberty 1998). Even within the open field, there are distinctions in the level of threat the exposed area provides: the periphery is safer than the centre (Ramos and Mormède 1998; Choleris, Thomas et al. 2001) and latency to reach the centre of the maze is thought to measure the same behaviour as latency to emerge from the LD or enter the mirrored chamber (Lamberty 1998).

Here I set out to establish if I can identify QTL allelic effects that act across the five tests in a way predictable with their action on anxiety. Because one of the main concerns with ethological tests of anxiety is that they use ambulation-related measures, and these could be measuring general locomotor activity rather than anxiety (Whimbey and Denenberg 1967; Dawson, Crawford et al. 1995), I also wanted to test if I could distinguish between the profile of an allele influencing anxiety and one influencing activity.

An allele increasing anxiety is expected to decrease activity measures taken in anxiogenic regions of the apparatus, while an allele decreasing locomotor activity will affect activity measures in anxiogenic as much as in non-anxiogenic regions. Moreover, an allele

increasing anxiety will increase time spent in the non-exposed part of the test as opposed to time spent in the exposed side, and increase latency to move from the non-exposed to the exposed side. Time indexes that measure the choice of the animal between safe and exposed sides are particularly appealing because they should be devoid of any influence by alleles acting on locomotor activity. Finally, an allele increasing anxiety should also act on defecation but with an opposite direction of effect in respect of that on activity, i.e. increasing it, while an allele decreasing locomotor activity should have no effect on defecation.

Table 4.3 reports the expected direction of effect in measures in five ethological tests of anxiety for a QTL allele increasing anxious behaviour (first column, 'anxious'), or a QTL allele decreasing locomotor activity (second column, 'inactive'). The table also reports significant LOD scores and direction of effect for the QTL mapped in this project on chromosomes 1, 4, 7, 8, 11, 12, 14, 15, 18, X. The reported directions of effect refer to the allele from the less active strain (L1 and L2) and are shown in the table as a plus (+) or minus (-) according to whether the QTL allele respectively increases or decreases the trait.

To facilitate an easier visualisation of results, in Table 4.4 I have used colours to highlight direction of effects reported in table 4.3, according to their action across tests. Red indicates results that are compatible with an anxiogenic effect, while blue indicates the opposite situation where the direction of effect is compatible with a QTL allele that decreases anxiety ('anxiolytic QTL allele'). Yellow indicates a QTL allele effect decreasing locomotor activity, and finally green shows a QTL allele effect increasing locomotor activity.

Table 4.3

LOD scores and direction of allelic effect (L strains-derived) of QTL influencing measures in five ethological tests of anxiety

Phenotype	Anxious	Inactive	Chr 1	Chr 4	Chr 7	Chr 8	Chr 11	Chr 12	Chr 14	Chr 15	Chr 18	Chr X
OFA	-	-	27.3 -	3.5 -	13.1 -			3.6 -		12.4 -	5.3 -	5.2 +
OFA-centre	-	-	14.8 -		13.5 -			3.2 -		5.1 -	6.2 -	
OF-centre time	-		3.9 -		7.3 -			3.7 -		5.2 -	6.0 -	
OF-latency	+				4.3 +					9.0 +		
OFD	+		14.4 +		3.3 +				9.4 -			5.3 -
EPM-closed entries		-	9.4 -	5.5 -	16.5 +	5.9 +					7.1 -	
EPM-closed ambulation		-	6.7 -	6.5 -	15.8 +	6.2 +					6.4 -	
EPM-open entries	-	-	15.4 -	3.2 -						12.9 -	8.0 -	3.2 +
EPM-open ambulation	-	-	12.1 -	3.5 -						11.8 -	7.7 -	4.1 +
EPM-open time	-		3.4 -							4.4 -		5.2 -
EPM-open latency	+									10.5 +		
SQ-closed entries		-										
SQ-closed ambulation		-	21.3 -	3.2 -	9.7 +	5.1 +					10.4 -	3.5 +
SQ-open entries	-	-	9.2 -	3.6 -						9.3 -	4.6 -	
SQ-open ambulation	-	-	11.3 -	4.9 -	4.4 -					15.4 -	6.9 -	5.4 +
SQ-open latency	+									5.7 +		
LD-dark ambulation		-	7.4 -	6.2 -								3.7 +
LD-transitions	-	-	16.7 -	3.9 -			7.0 -	4.3 -	7.0 +	13.5 -	5.7 -	
LD-light ambulation	-	-	24.8 -				6.0 -		5.6 +	11.4 -	4.1 -	
LD-light time	-		9.9 -	3.2 -			5.7 -		7.6 +	14.7 -	5.7 -	
LD-latency	+		4.9 +				4.8 +		11.4 -	19.6 +	3.4 +	
MR-entries	-	-			12.8 +				3.6 +	13.2 -	4.8 -	
MR-latency	+							3.2 +		17.7 +		

Table 4.4

Direction of allelic effect (L strains-derived) of QTL influencing measures in five ethological tests of anxiety. A coloured square indicates that a QTL has been detected on the chromosome, the colour referring to the QTL type of action on the tests (red and blue = anxiety increaser and decreaser; green and yellow = activity increaser and decreaser).

Phenotype	Anxious	Inactive	Chr 1	Chr 4	Chr 7	Chr 8	Chr 11	Chr 12	Chr 14	Chr 15	Chr 18	Chr X
OFA	-	-	-	-	-			-		-	-	+
OFA-centre	-	-	-		-			-		-	-	
OF-centre time	-		-		-			-		-	-	
OF-latency	+				+					+		
OFD	+		+		+				-			-
EPM-closed entries		-	-	-	+	+					-	
EPM-closed ambulation		-	-	-	+	+					-	
EPM-open entries	-	-	-	-						-	-	+
EPM-open ambulation	-	-	-	-						-	-	+
EPM-open time	-		-							-		-
EPM-open latency	+									+		
SQ-closed entries		-										
SQ-closed ambulation		-	-	-	+	+					-	+
SQ-open entries	-	-	-	-						-	-	
SQ-open ambulation	-	-	-	-	-					-	-	+
SQ-open latency	+									+		
LD-dark ambulation		-	-	-								+
LD-transitions	-	-	-	-			-	-	+	-	-	
LD-light ambulation	-		-				-		+	-	-	
LD-light time	-		-	-			-		+	-	-	
LD-latency	+		+				+		-	+	+	
MR-entries	-	-			+				+	-	-	
MR-latency	+							+		+		

Ideally, a QTL allele that increases anxiety should show red, with no yellow, green or blue squares, while a QTL allele decreasing activity should show only yellow. Empty boxes indicate that no significant LOD score was detected for that measure, and they are non-informative.

By categorizing QTL allelic effect in this way, we can define how a QTL operates.

The locus on chromosome 15 emerges with a profile fully consistent with a role in anxiety. On the other hand, the QTL on chromosome 4 is consistent with a role restricted to locomotor activity, except for a single anxiogenic effect in the LD, for which however the LOD is at the bottom of the significance level (3.2).

The loci on chromosomes 1 and 18 influence a broader range of measures, in both anxiogenic and non-anxiogenic compartments: data suggest that they act as 'anxious' QTL but their effect is less specific to more threatening areas.

Other QTL operate in a way that is not so easy to explain, in that they appear to be test specific or the mode of action does not fit easily with our expectations. The loci on chromosomes 7 and 12 are compatible with an 'anxious' QTL for measures in the OF. The chromosome 12 QTL influences a couple of additional anxious measures, namely it decreased LD transitions and increases latency to enter the mirror chamber. The chromosome 7 QTL, instead, also acts as a locomotor activity-increaser in the EPM, elevated square maze and mirror chamber. The locus on chromosome 11 is specific for the LD and is compatible with an 'anxious' QTL. The locus on chromosome 14 is compatible with the pattern of an anxiolytic QTL, although it affects only a subset of measures. Its profile in the LD is exactly opposite to the chromosome 11 locus. Also the

QTL on chromosome X has an anxiolytic profile, and also increases activity in the SQ and LD. Finally, the locus on chromosome 8 specifically acts by increasing activity in the EPM and elevated square maze only in protected areas.

4.5 Analysis of QTL effect on repeated measures

The five ethological tests of anxiety employed in this study are based on exposure to a novel environment as a source of mild stress in mice. Novelty is maintained to represent a fearful stimulus for rodents, and to evoke a conflict between their instinctive drive to explore and the inhibition of such drive due to fear. Re-testing in the same apparatus reduces novelty, and hence the anxiogenic stimulus. It has already been shown that animals habituate to the OF so that activity decreases across sessions (Hegmann and DeFries 1968; Walsh and Cummins 1976).

A detailed ethological analysis of the OF (Choleris, Thomas et al. 2001) has found that mice exposed to this test for 30 minutes tended to display more fear-related behaviours, such as thigmotaxis and stretch attend postures, in the first part of the session (5-10 minutes) while increasingly engaging in investigative and locomotor activity towards the end of the session. Also, mice tended initially to spend more time in the peripheral part of the OF, while, as time was passing, gradually shifting more towards the centre. This pattern was claimed to be consistent with a time dependent reduction in anxiety due to habituation.

On this premise, I set out to establish if QTL influencing anxiety have a limited temporal action and if their effect is attenuated on repeated exposure to the tests.

Mice in this project have been re-tested on a separate day in two of the five tests: the OF and the LD. Table 4.5 reports LOD scores on chromosomes 1, 4, 7, 8, 11, 12, 14, 15, 18, X for repeated measures taken in the OF and the LD.

The first remark to be made is that most QTL are still detected on repeated measures, with the exception of the QTL on chromosome 12 for OF measures, which disappear on day 2. However, some changes can be detected in their effect size.

For instance, in the LD on day 2 there is a dramatic fall in the importance of the chromosome 15 locus: for the latency measures it drops from a LOD score of 19.6 to 12.3, while the LOD scores for transitions and time spent in the light box fall from 13.5 and 14.7 on day 1 to a non-significant value on day 2 (while the LOD for ambulation in the light box remains stable). A similar but less marked drop in day 2 LOD scores can be seen with respect to OFA. In contrast, on chromosome 4, Day 2 LOD scores increase for both LD and OF measures.

On chromosomes 1 and 18, day 2 LOD are more similar to day 1 LOD. However, interestingly, the tendency is for activity-related measures (like OFA, ambulation in the centre of the OF, LD transitions and ambulation in the light side of the LD) to keep or increase their LOD on day 2, while for time and latency measures the contrary is true, i.e. there is a tendency of the LOD on day 2 to fall.

Interestingly, the QTL on chromosome X, which was found to have an anxiolytic action through analysis of the direction of the allelic effect, is found to have an anxiolytic action in the LD on day 2, as compared to no action on day 1.

Table 4.5

LOD scores and direction of allelic effect (L strains-derived) of QTL influencing measures in the OF and LD on an initial trial (day 1) and a subsequent re-exposure to the tests (day 2).

Test	Phenotype	Chr 1	Chr 4	Chr 7	Chr 8	Chr 11	Chr 12	Chr 14	Chr 15	Chr 18	Chr X
OF	OFA	27.3 -	3.5 -	13.1 -			3.6 -		12.4 -	5.3 -	5.2 +
day 1	OFA-centre	14.8 -	6.0 -	13.5 -			3.2 -		5.1 -	6.2 -	
	OF-centre time	3.9 -		7.3 -			3.7 -		5.2 -	6.0 -	
	OF-latency			4.3 +					9.0 +		
OF	OFA-2	24.3 -	10.2 -	9.9 -	4.7 +				5.4 -	9.2 -	5.1 +
day 2	OFA-centre-2	10.2 -	10.1 -	14.6 -					4.8 -	5.4 -	3.2 +
	OF-centre time-2		6.8 -	20.8 -					7.2 -		
	OF-latency-2			17.5 +					9.8 +		
LD	LD-transitions	16.7 -	3.9 -			7.0 -	4.3 -	7.0 +	13.5 -	5.7 -	
day 1	LD-light ambulation	24.8 -				6.0 -		5.6 +	11.4 -	4.1 -	
	LD-light time	9.9 -	3.2 -			5.7 -		7.6 +	14.7 -	5.7 -	
	LD-latency	4.9 +				4.8 +		11.4 -	19.6 +	3.4 +	
LD	LD-transitions-2	23.8 -	10.4 -				4.2 -			7.2 -	5.0 +
day 2	LD-light ambulation-2	24.8 -				6.0 -		5.6 +	11.4 -	4.1 -	3.8 +
	LD-light time-2	5.1 -	6.7 -								4.1 +
	LD-latency-2	5.1 +				4.9 +		5.2 -	12.3 +	4.0 +	

For the other chromosomes, the pattern is not easy to interpret. The locus on chromosome 7, which acts only in the OF, shows an increased LOD for time and latency measures on day 2. The loci on chromosomes 11 and 14, which act only in the LD, on day 2 present a parallel fall in significance of the LOD for transitions and time spent in the light box. Finally, the activity-increaser QTL on chromosome 8 shows a day 2 specific effect of increasing OFA.

4.6 Analysis of specificity of QTL action

The data I have presented above concern the action of QTL on anxiety-related phenotypes as measured in five ethological tests: consistency of direction of effect across tests and on repeated measures strongly suggest pleiotropic action of identified loci on the construct of anxiety.

Here I describe two experiments that seek to determine whether identified QTL action is specific to anxiety-related traits, by analysing two measures unrelated to anxiety: activity in the homecage as a measure of general activity, and tail suspension test, as a measure of anxiety-unrelated stress.

4.6.1 QTL influencing homecage activity

In this project we included a control measure for general activity in the form of homecage activity. The homecage is a safe environment and should be deprived of any element of novelty or fearfulness as presented in anxiety tests. Therefore any ambulatory and vertical activity inside it should represent a measure of 'pure' general activity, devoid of any anxiety-related component.

I have performed QTL mapping of homecage activity: LOD scores and direction of effect (L strains-derived) are reported in Table 4.6.

QTL for home cage activity are found on chromosomes 4, 8, 11, 14, and X. As I have reported above, these loci are also found to harbour QTL for anxious behaviour. Interestingly, the analysis of the direction of effect has suggested that the QTL on chromosomes 4, 8 and X have all an activity-related component, and moreover the chromosomes 4 and 8 QTL work solely as activity QTL. Only the result on chromosome 14 is inconsistent with its above-reported anxiolytic action. Finally, it is reassuring that QTL for homecage activity are not found on chromosomes 1, 7 and 15 and 18. A large proportion of anxiety measures are found to map on these chromosomes, suggesting that their QTL are rather specific to measures from the ethological tests.

4.6.2 QTL influencing tail suspension test

Although clinical data in humans have shown that comorbidity between anxiety and depression is a common phenomenon (Kessler, McGonagle et al. 1994), it is not yet clear if these two conditions represent different manifestations of a same underlying trait, or if they are biologically distinct albeit with common features.

In animal models, specific tests for screening antidepressants have been developed, of which the most commonly used are the forced swim test (Porsolt, Le Pichon et al. 1977) and the tail suspension test (Steru, Chermat et al. 1985). In both tests, when mice are confronted with an inescapable stressful situation, like being forced to swim in a water-filled cylinder or being suspended by the tail, they will adopt a characteristic immobile posture, which is maintained to represent a state of

Table 4.6

LOD scores and direction of allelic effect (L strains-derived) of QTL influencing homecage activity and tail suspension.

Phenotype	Chr 3	Chr 4	Chr 5	Chr 8	Chr 11	Chr 14	Chr 19	Chr X
Homecage activity		4.3 -		3.3 +	4.1 +	4.2 -		3.2 +
Tail suspension	6.7 +		10.9 -		6.9 -		6.2 -	

behavioural despair. Pharmacological studies have shown that antidepressants reduce immobility in both tests.

In this project, mice have been subjected to a tail suspension test (see Appendix A for a description). As this test is meant to measure 'despair' as a response to a stressful situation, I set out to establish if the genetic effect responsible for such 'despair' was distinct from the genetic basis of anxious behaviour. Previous study on inbred strains suggested that genetic determinants for tail suspension immobility were distinct from those for open field activity as a measure of anxiety (Trullas, Jackson et al. 1989).

Table 4.6 reports LOD scores and direction of effect for QTL influencing tail suspension. Significant QTL are found on chromosomes 3, 5, 11 and 19. Only the locus on chromosome 11 coincides with QTL already identified for anxious behaviour.

4.7 Discussion

In summarizing mapping data for 62 phenotypes in five ethological tests for anxious behaviour, I have shown that QTL in two distinct intercrosses derived from the DeFries selected strains are highly consistent: often a QTL found in one population is replicated in the other, and nearly always, QTL identified in the combined data set present a LOD that is approximately the sum of the LOD in the two individual groups (236 cases out of 289, i.e. 81.7%).

Moreover, only six chromosomes are found to harbour the majority of QTL: out of 289 QTL detected for the 62 phenotypes, 214, i.e. 74%, are found on chromosomes 1,

4, 7, 15, 18 and X. This strongly suggests that QTL act pleiotropically across phenotypes as measured in five distinct tests.

However, due to the poor resolution of the F2 analyses, it remains possible that, within the large regions identified by the mapping data, different molecular variants may be segregating in the two crosses. I have addressed the issue of pleiotropy by analysing the direction of allelic effects. Moreover, I have also analysed change in genetic effect on repeated measures to test for attenuation of effect in response to habituation. Finally, I have reported mapping data for homecage activity and tail suspension, in order to test for specificity of genetic effect on anxiety-related traits.

First, I have found that most detected QTL have a profile of action across phenotypes, which substantiates the view that they act pleiotropically. This is especially evident for QTL on chromosomes 1, 4, 15 and 18. Although the other QTL detected act on a smaller number of phenotypes, usually limited to a subset of tests, still consistency of results suggest pleiotropy, at least for some of the measures they influence.

Second, the QTL do not influence behaviour equally. In my analysis of direction of allelic effects, I exploited the fact that the tests employed allow to extract information about different components of behaviour.

According to my analysis, the profile of the QTL on chromosome 4 is compatible with an effect decreasing locomotor activity rather than increasing anxiety. Interestingly, a locus on this chromosome is also found to decrease activity in the homecage, a measure that is maintained to be anxiety-unrelated.

I have found that QTL on chromosomes 1, 15 and 18 influence anxiety-related phenotypes with a consistent pattern across the tests, although only the QTL on chromosome 15 has a very specific profile with effect only on more threatening areas of the tests, while loci on chromosome 1 and 18 also decrease activity measures in less exposed areas. However, no QTL for homecage activity or tail suspension are found on these chromosomes, showing that their effect is specific for measures of anxiety-related behaviours.

More detailed examination of the phenotypes upon which these three chromosomes act suggest that the effects of chromosomes 1 and 18 are more similar and they can be dissociated from that of chromosome 15 QTL in three ways.

First, the locus on chromosome 15 has reduced or no influence on behaviour in non-threatening regions (such as the enclosed arms of the EPM and dark compartment of the LD box) while the loci on chromosomes 1 and 18 have also an effect in non-threatening regions. This is not merely that the chromosome 15 QTL effects slip below the level at which they can be detected: the LOD score for activity in the open arms of the EPM is over 11, but only 1.2 for activity in the closed arms (data non shown). Second, the QTL on chromosome 15 is the only locus with consistent and large effects on latency to enter aversive regions of the test apparatus. Third, analysis of effect of QTL on repeated measures shows that prior exposure to the apparatus diminishes the chromosome 15 QTL effect, especially in the LD. The QTL has no detectable influence on transitions in the light-dark box and time spent in the light side in the second day of testing, while the LOD score for the first day are around 14 for both measures. Interestingly, on day 2 LOD on chromosomes 1 and 18 tend to increase for activity-related measures and to fall for time and latency measures. Also, the LOD score on chromosome 4 increases on day 2 for both LD and OF phenotypes.

From these data I suggest that the QTL on chromosome 15 acts primarily to promote avoidance behaviour, while the QTL on chromosomes 1 and 18 influence exploratory behaviour. We can interpret the findings of the re-test experiment in the light-dark box as a decrease in avoidance behaviour following prior-exposure to the tests on day 1 with a consequent increase in exploration and locomotor activity, shown by increased genetic effects on chromosomes 1, 4 and 18, as if the chromosome 15 QTL is releasing its effect.

Interpretation of the effect of other QTL is more complex. The QTL on chromosome 7 and 12 have a consistent profile of an anxiety QTL only in reference to measures taken in the OF, to which their action is mainly although not exclusively, restricted. Instead the QTL on chromosome 11 has an anxiogenic profile restricted to the LD. The QTL on chromosome 14 and X have a consistent profile of an anxiolytic QTL. Finally, the locus on chromosome 8 acts as an activity-increaser with a specific effect in the EPM and elevated square maze.

CHAPTER 5

High resolution mapping of QTL

In the previous chapters I have reported mapping data for a large number of anxious-related phenotypes in two F2 intercrosses derived by the DeFries inbred strains. My analysis of the results has shown that the genetic architecture of the DeFries selected strains is similar and is relatively simple. Mapping results from two crosses, G1 and G2, are in agreement: in both crosses the same chromosomes are found to harbour QTL accounting for more than 3% of the phenotypic variance. Moreover, 6 chromosomes (1, 4, 7, 15, 18 and X) are found to harbour the vast majority of QTL for 62 anxiety-related phenotypes in five tests of anxiety; analysis of the genetic effects, as discussed in chapter 4, supports the idea that the same QTL act pleiotropically across many phenotypes.

The identification of such genetic loci indicates that one or more genes located on those chromosomes are involved in the susceptibility to anxious behaviour in rodents. Cloning the putative genes would allow to identifying the biochemical pathways and the specific molecules that affect rodents' anxiety, and would allow the application of such knowledge to the advancement of our understanding and therapy of human anxiety.

However, from QTL mapping to gene cloning the route is very long and laborious. Mapping resolution of QTL in F2 intercrosses is far too poor: for QTL contributing 5% to

the phenotypic variance, mapped with 800 animals, the 95% confidence intervals of the QTL location still encompass about 40 cM, or about half a mouse chromosome (Darvasi and Soller 1997). Fine resolution of QTL is a necessary step towards gene cloning.

Here I report results from two strategies that I have employed to increase the mapping resolution of some of the identified QTL: haplotype analysis and a Recombinant Inbred Segregation Test (RIST).

5.1 Haplotype analysis

As I have previously discussed, the genetic structure of F2 mice derived from an intercross between inbred strains allows the segregation of only two alleles at each locus (see Figure 2.1 in chapter 2). So, for example, at each locus animals in G1 will either be homozygous for the H1 allele, homozygous for the L1 allele or heterozygous.

The DeFries inbred strains from which the F2 mice are generated, are on their part derived from an intercross between C57BL/6J and BALB/cJ, therefore at each locus they can have only one of two possible conformations: homozygosity for the C57BL/6J-derived allele or homozygosity for the BALB/cJ-derived allele.

On the basis of the results of QTL mapping and analysis of direction of allelic effect in the F2 intercrosses I make some predictions about the haplotype structure of the region containing the QTL in the parental strains.

First, in the F2 mice, QTL cannot be segregating in regions where the two progenitors have the same allele conformation. For example QTL found in G1 can only be in regions where either H1 is C57BL/6J-like and L1 BALB/cJ-like or vice versa.

Second, the consistency of direction of allelic effect in the two intercrosses suggests that a region containing a QTL in the H1 and H2 strains should derive from the same progenitor, and consequently the corresponding region in L1 and L2 would have to be derived from the other progenitor (for example H1 and H2 both C56BL/6-derived and L1 and L2 both BALB/cJ-derived, or vice versa).

Third, we know from the original F2 intercross analysis that QTL increasing OFA were carried by the C57BL/6J strain (Flint, Corley et al. 1995). Since the selection experiment was carried out four times (twice for high OFA and twice for low OFA) and the mice subsequently inbred, the QTL is likely to be in a region where H1 and H2 DeFries strains (selected for high activity) are C57BL/6J and where L1 and L2 DeFries strains (selected for low activity) are BALB/cJ.

I therefore set out to analyse the chromosomal structure around the QTL on chromosomes 1, 4, 7 and 15 and see if I could find the expected conformation. DNA from the four selected strains (H1, L1, H2 and L2) as well as DNA from the progenitor animals C57BL/6J and BALB/cJ was amplified with markers spaced every 5 cM across the regions on chromosomes 1, 4, 7 and 15 found to harbour the QTL. Primers used, their cM position and working conditions are given in Appendix B. For these chromosomes, composite interval mapping (Zeng 1994) for mapping multiple traits was also used, and results are plotted over the haplotype pictures, as shown in Figure 5.1. The chromosomes

of the selected strains are coloured according to their progenitor origin: black indicates C57BL/6J and white BALB/cJ.

The combination of composite interval mapping for multiple traits and haplotype analysis decreases the intervals containing QTL and goes some way to demonstrating pleiotropic action. Under each peak of the composite interval mapping, there lies a chromosomal region of the expected haplotype structure.

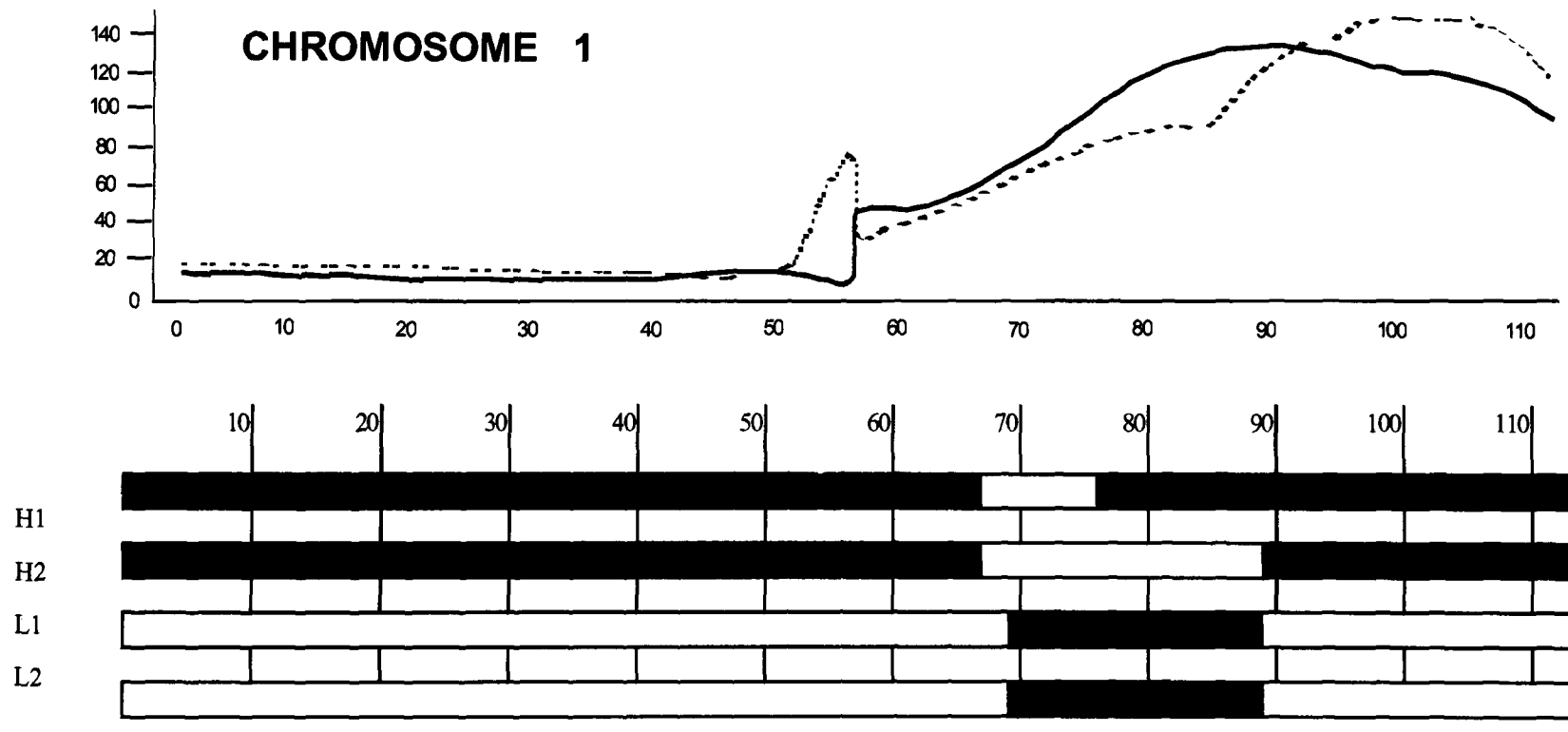
On chromosome 1, composite interval mapping defines the position of the QTL somewhere from 70 cM to the end (an interval of about 45 cM). However, between 70 and 78 cM, both H1 and H2 are BALB/cJ in origin while L1 and L2 are C57BL/6J, suggesting that this region does not contain the QTL, while the region from 90 to 114 cM has the converse pattern and is therefore more likely to harbour the QTL.

Also on chromosomes 4, 7 and 15 there is a region with the expected haplotype in the four parental strains under each peak of significance given by composite interval mapping.

Although the regions on chromosome 1 and 7 are still large (about 15 cM for chromosome 1 and 25 cM for chromosome 7), haplotype analysis identifies smaller intervals of less than 10 cM on chromosomes 4 and 15. On chromosome 15 in particular, in the H strains there is a small C57BL/6 derived region at about 30 cM (coloured black in figure 5.1) under the likelihood ratio peak, of less than 3 cM.

Figure 5.1

Haplotypes and composite interval mapping of QTL on four chromosomes (1, 4, 7 and 15). The haplotypes of the four inbred selected strains (H1, H2, L1 L2) are shown as composites of either BALB/cJ (white bar) or C57BL/6J (black bar). The likelihood ratio (LR) test statistic (derived from Jzmapqtl (Basten, Zeng et al. 1996)) is plotted above the picture of the haplotype structure. A black continuous line is used to plot data from the G1 intercross and a black dotted line the data from the G2 intercross. The vertical axis is the LR test statistic and the horizontal axis the distance in centimorgans along the chromosome. All map distances are those calculated in MAPMAKER. Composite interval mapping did not include genotypes for the terminal 5 cM of chromosome 15, so the haplotype data for this chromosome extend beyond the mapping data.



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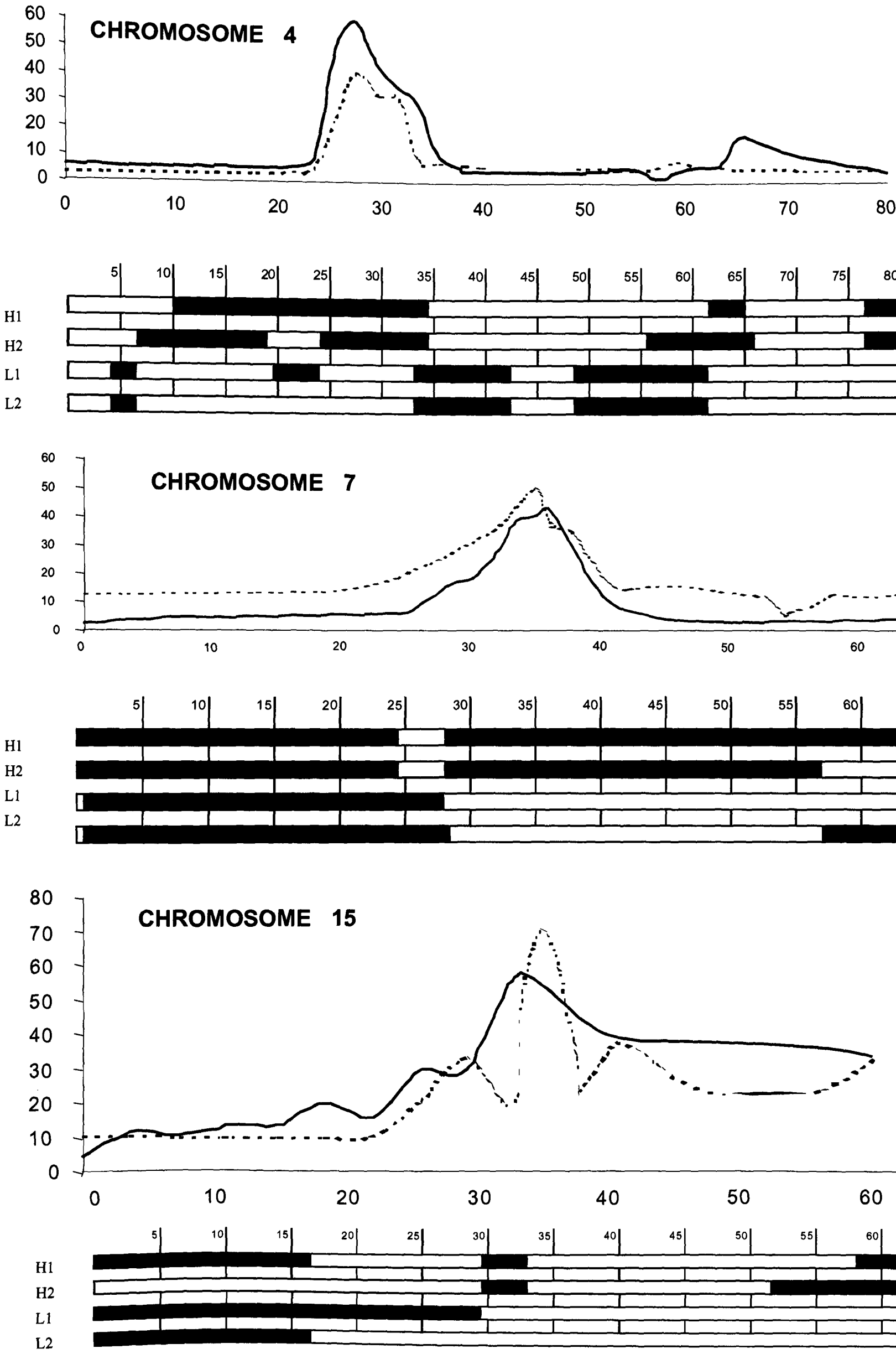


5.2 Recombinant Inbred Segregation Test (RIST)

In order to confirm the results of the haplotype analysis and to attempt further refined mapping of QTL I carried out a variant of the recombinant inbred segregation test (Darvasi 1998). The experiment described in this project diverges from a classical RIST, in that we did not use standard recombinant inbred (RI) strains. We instead established an F2 intercross between two of DeFries selected inbred strains, namely H2 and C1, which on their part are genetically equivalent to RI strains derived from C57BL/6J and BALB/cJ, as their genomes derive from C57BL/6J and BALB/cJ inbred strains.

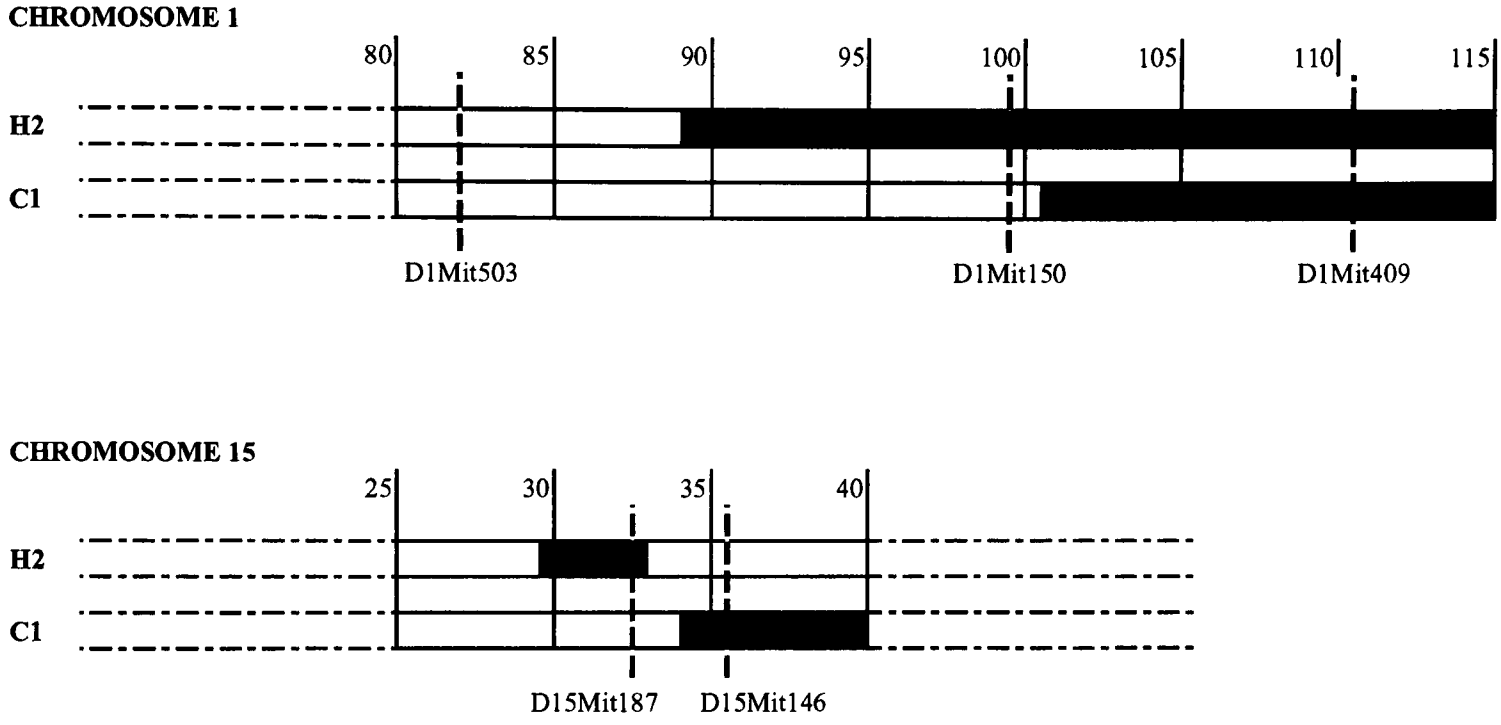
5.2.1 Experiment design

RIST analysis was limited to chromosomes 1 and 15 QTL. Figure 5.2 shows haplotypes of H2 and C1 across the region between 80 cM and the end on chromosome 1, and between 25 and 40 cM on chromosome 15, and some marker positions. By comparing this picture with the genetic interval identified to harbour the QTL by haplotype analysis (Figure 5.1), it is possible to identify which markers should be used to design an informative RIST experiment.

For chromosome 15, the region identified by haplotype analysis to harbour the QTL is C57BL/6J-derived in H2 and BALB/cJ-derived in C1. By searching for association between marker D15Mit187 and the F2 progeny we can establish if the QTL is contained in that region (positive association, segregation of QTL) or not (no association, no segregation of QTL). No further high-resolution mapping is possible, as C1 does not harbour any recombination breakpoints in that region.

Figure 5.2

Haplotypes of H2 and C1 strains across the segments of chromosomes 1 and 15 identified to harbour the QTL by haplotype analysis. White bar represents BALB/cJ alleles, black bar C57BL/6J alleles. Dashed lines indicate mapping position of the corresponding MIT markers. Scale at top is in cM.



For chromosome 1, the region identified by haplotype analysis to harbour the QTL is C57BL/6J-derived in H2 while in C1 there is a recombination breakpoint so that its allelic conformation is BALB/cJ-derived between about 90 and 100 cM and C57BL/6J-derived from about 100 cM and the end. By searching for association between marker D1Mit150 and the F2 progeny we can refine the mapping of the QTL, and I will now explain how.

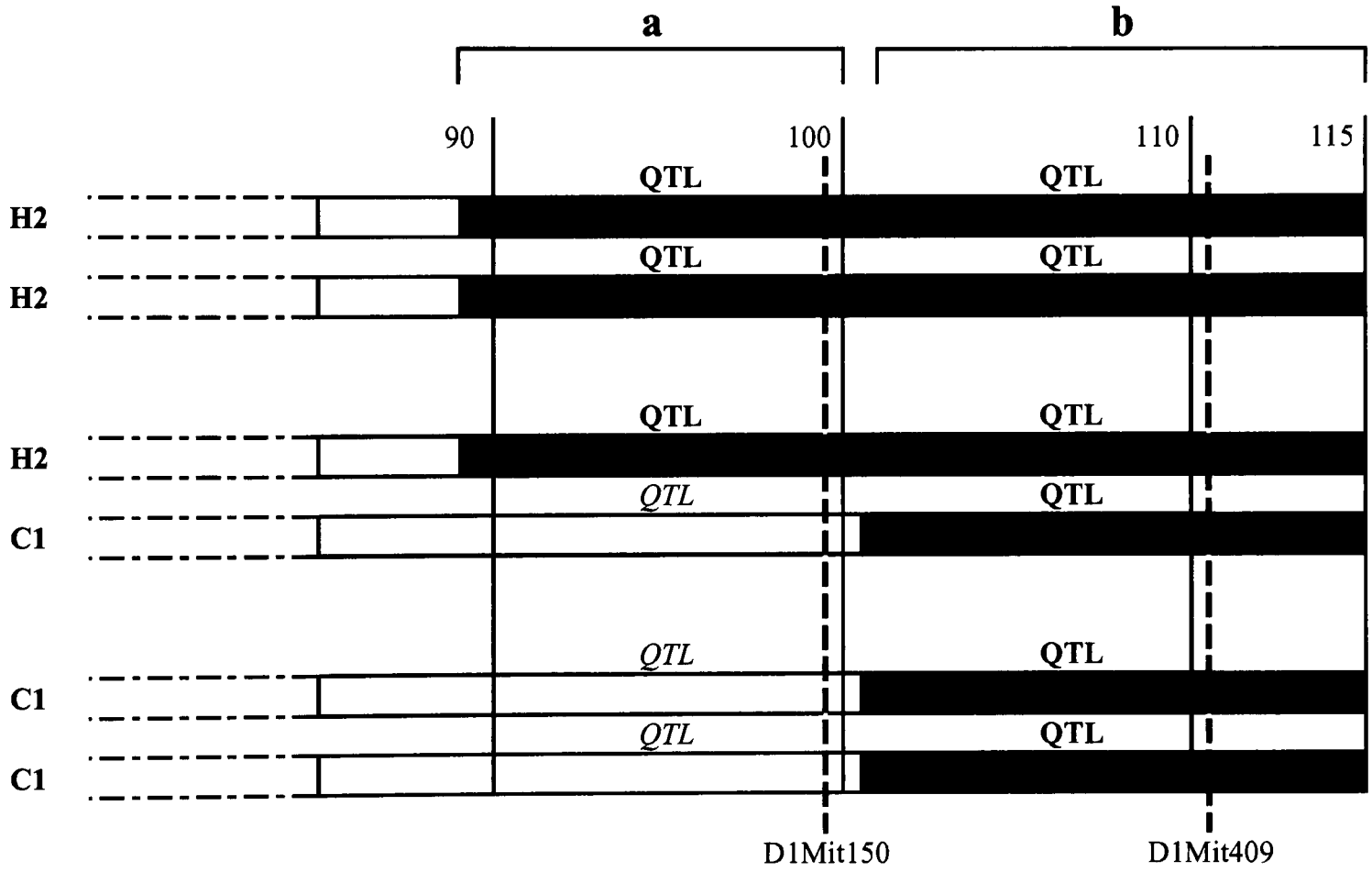
Figure 5.3 shows the expected results of a cross between H2 and C1 for the two possible QTL locations. If the QTL is contained in the 90-100 cM interval (marked as 'a' in the picture), we expect a positive association of the phenotype with marker D1Mit150, as there is a segregation of the QTL in the F2 mice. If instead the QTL is contained in the terminal 15 cM region (marked as 'b'), no association should be found between marker and phenotype, because there will be no phenotypic differences segregating. However, in the case that no association is found, we cannot rule out an alternative hypothesis, i.e. that the QTL is placed outside the region, more proximally than 90 cM, where H2 and C1 allelic conformations are both BALB/cJ-derived.

5.2.2 Population design, genotyping, phenotyping and statistical analysis

An F2 was generated from a cross between C1 and H2 and 71 animals were tested in an open-field arena for OFA and OFD, as described in Appendix A. A derived phenotype, EMO (emotionality) (Talbot, Nicod et al. 1999), was computed as follows: OFA and OFD scores were standardised to a mean of 0 and variance of 1, the sign of OFA was reversed (to take into account the known negative genetic correlation of the measures)

Figure 5.3

Outcome of an F2 cross between the H2 and C1 strains, which determines whether the QTL lies in region 'a' or 'b'. The figure shows non-recombinant chromosomes over the terminal 30 cM of chromosome 1. The OFA-increasing QTL allele (associated with C57BL/6J) is shown in bold, the OFA-decreasing QTL allele (associated with BALB/cJ) is shown in italics. If the QTL lies in region 'b', there will be no phenotypic differences segregating with D1Mit150; in contrast if the QTL lies in region 'a' D1Mit150, will detect an effect.



and the mean of the derived scores defined EMO.

In order to test for segregation of the QTL I used marker D1Mit150 on chromosome 1 and marker D15Mit187 on chromosome 15.

I tested for a difference between genotypes by an analysis of variance (ANOVA) in which the phenotypic mean and variance associated with each genotype is calculated and a variance ratio used to test for the equality of the variances.

5.2.3 Results

Table 5.1 reports the means of OFA, OFD and EMO associated with each genotype at two markers: D1Mit150 for chromosome 1 and D15Mit187 for chromosome 15. The ANOVA *P*-value for EMO, shown in the table, was significant for both markers.

On chromosome 1, the marker D1Mit150 gave an ANOVA P-value of 0.0006, indicating that the QTL must be located in the interval between 90 and 100 cM (marked as 'a' in figure 5.3).

On chromosome 15, the marker D15Mit187 gave an ANOVA P-value of 0.0038, confirming that a QTL is segregating in this F2 at that location, therefore suggesting that the QTL is contained in the region between 30 and 33 cM.

Table 5.1

Animals from an F2 intercross between H2 and C1 DeFries strains have been classified by genotype at two loci (D1Mit150 and D15Mit187). Alleles are given the name of the inbred strain from which they derive (either BALB/cJ or C57BL/6J). The means of three phenotypes (OFA, OFD and EMO) associated with each genotype are shown. ANOVA *P*-values are given for the EMO phenotype only (at both loci the F statistic degrees of freedom are 2, 71).

LOCUS	GENOTYPE		ANOVA	EMO	OFA	OFD
			P-Value	Mean	Mean	Mean
D1Mit150	BALB/cJ	BALB/cJ	0.0006	0.76	185.94	7.76
	C57BL/6J	BALB/cJ		0.22	210.24	5.64
	C57BL/6J	C57BL/6J		-0.12	235.22	4.72
D15Mit187	BALB/cJ	BALB/cJ	0.0038	0.31	190.45	5.41
	C57BL/6J	BALB/cJ		0.08	225.69	5.5
	C57BL/6J	C57BL/6J		-0.44	264.74	4.03

5.3 Discussion

In order to refine the mapping position of some of the major QTL for anxious behaviour in rodents, found in this project, I applied two strategies: haplotype analysis and a variant of the Recombinant Inbred Segregation Test.

These strategies have proved valuable in increasing resolution. From 95% confidence intervals of the QTL location which is around 40 cM for a classical F2 intercross with our number of animals (about 800 in each cross) (Darvasi and Soller 1997), haplotype analysis defines regions of 25 cM for chromosome 1 and 7, 10 cm for chromosomes 4, and only 3 cM for chromosome 15.

Furthermore, I was able to fine map the chromosome 1 locus with a RIST experiment that defined the QTL mapping in a region of about 10 cM. This experiment also confirmed the presence of the QTL on the 3 cM region on chromosome 15.

Because in the QTL mapping experiment I had found that QTL were consistent between the two crosses G1 and G2, in the haplotype analysis I hypothesised that the QTL should be contained in regions where both High strains were C57BL/6J and both Low strains were BALB/cJ. The finding of such haplotype conformation under each peak of LOD score identified by composite interval mapping support the idea, previously analysed, that selection has operated on the same QTL in the two crosses. The interval on chromosome 15 is now small enough to consider physical mapping towards cloning of the gene. The other QTL are still too large and will require alternative approaches to reduce the interval further.

CHAPTER 6

Discussion

6.1 The action of selection on genes and replicability of QTL mapping

My project is the largest genome scan of animal behaviour so far reported, comprising 1636 animals tested with 62 anxiety-related phenotypes in five ethological tests of anxiety. Moreover, it is the first QTL analysis of lines derived from a replicated behavioural selection experiment.

By analysing four lines derived from a replicated selection experiment, I have been able to investigate the effects of selection on behaviour. Highly consistent QTL mapping data between the two intercrosses indicate that selection has operated on the same relatively small number of loci in the four selected strains.

Further genetic analysis of the combined sample of the two crosses increases the power to detect small effect loci. Very few additional QTL are found in respect to the individual populations, indicating that the genetic architecture of the two selected strains is relatively simple, with six chromosomes (1, 4, 7, 15, 18 and X) contributing to most of the genetic variance of 62 anxiety related phenotypes.

The evidence suggests that the QTL are acting pleiotropically, as their effects operate in a consistent direction across phenotypes. For example the QTL on chromosome 15 influences measures of anxious behaviour across the five tests, in a highly consistent and predictable way, like decreasing ambulation scores and time spent in threatening areas of the tests while increasing latency to reach these areas.

The enhanced power of QTL detection in the combined data sample allows the detection of QTL for OFA that replicate results of a previous mapping experiment of anxious behaviour in the DeFries strains (Flint, Corley et al. 1995). The effect sizes of the QTL were in general small. The highest effect is obtained for the effect of the locus on chromosome 1 on OFA, which accounts for 10% of the total phenotypic variance of this measure. Most of the QTL detected in the individual crosses account for 3-6% of the phenotypic variances, while the combined data set detects QTL which account for as low as 1% of the phenotypic variance (see Tables 3.1 and 3.2 of chapter 3).

The issue of replicability of mapping experiments is well known in human genetics, where inconsistent results for complex traits, including psychiatric disorders and personality traits, are the rule. The relatively low number of QTL mapping studies of anxious behaviour in rodents (reviewed in Table 2.2 in chapter 2) has not yet posed the question for animal genetic studies of anxiety. However, a recent paper (Crabbe, Wahlsten et al. 1999) which studied inbred strains genetic differences in behaviour across three different laboratories and found several inconsistent results, has brought forward the issue of how robustly behavioural measures can detect genetic effect, particularly when searching for small size effects. Although the concerns raised by the paper were mainly intended at studies of transgenic mice, they can easily be transposed to QTL mapping.

Replicability of QTL has been shown for alcohol preference drinking in mice through a meta-analysis of eight independent studies (Belknap and Atkins 2001). The present project also demonstrates that genetic mapping of behaviour is robust and replicable, although it generally requires large number of animals as demonstrated by the fact that mapping results for OFA in the original experiment on the DeFries strains (Flint, Corley et al. 1995) could be highly replicated only when the full set of 1636 mice were analysed and consistency was much lower for analysis of a single intercross (815 mice) although this population was an exact replica of the one in the original study (both were H1XL1 intercrosses).

6.2 Dissecting different components of anxious behaviour

One of the first operational definitions of anxious behaviour in rodents was based on the concept that fearful animals would ambulate less and defecate more when placed in a novel, potentially stressful, environment. However, the validity of this construct has been questioned. A negative correlation between defecation and activity in the open field has been found by selection experiments for anxious behaviour in both rats and mice. The two paradigmatic examples are: the Maudsley reactive (MR) and non-reactive (MNR) strains of rats (Broadhurst 1960), in which selection for OFD acted indirectly on OFA scores, so that for example the MR strain was selected for high OFD and turned out to have low OFA; the DeFries strains (DeFries, Gervais et al. 1978), described in chapter 2, in which selection for low OFA resulted in high OFD and vice versa. However, a number of studies have not replicated these findings (Pare 1964) and there are discrepancies in the correlations reported between defecation and other measures of anxiety (Archer 1973), suggesting a complex relationship.

In the mapping experiments reported in my thesis, results for mapping OFD also point in the direction of a complex relationship between defecation and anxiety. OFD is increased by two QTL, on chromosomes 1 and 7, in a consistent way with the role of these QTL in anxiety (although limited to OF measures for the chromosome 7 locus). QTL on chromosomes 14 and X decrease OFD in a way compatible with their anxiolytic profile. However, no QTL for defecation are found on chromosome 15 and 18, indicating that defecation and anxiety are partly genetically independent.

Another important concept at the basis of ethological tests of anxiety in rodents is that mild stress in the form of exposure to an unfamiliar environment results in inhibition of spontaneous exploratory behaviour, which can be quantified in terms of ambulation scores in the test. However, multivariate analysis in both the OF and the EPM has pointed out that ambulation is an ambiguous measure, representing both exploratory activity (which should be fear-related) and general locomotor activity (which on the contrary should be fear-unrelated) (Whimbey and Denenberg 1967; Lister 1987; Rodgers and Johnson 1995). Pharmacological studies in the EPM (Dawson, Crawford et al. 1995) and the LD (Costall, Jones et al. 1989) have shown that measures of ambulation in these tests (like entries into the arms of the EPM and transitions between the light and dark side of the LD) may not be considered pure indexes of anxiety, as they are contaminated by locomotor activity.

A recent detailed study of the open field (Choleris, Thomas et al. 2001) has suggested that it is possible to dissect exploratory ambulation from general locomotor ambulation, by simple observation of the modality in which ambulation is performed: exploratory ambulation is slow, non-straight and accompanied by investigative sniffing of the air and the floor of the chamber; locomotory ambulation is faster and straight, and does not

involve sniffing. Unfortunately, in the vast literature on the open field, such sophisticated ethological analysis of ambulation is virtually non-existent, as most studies rely on the simple measurement of OFA.

Again, my genetic mapping data can be used to advance understanding of the relationship between ambulation and anxiety. I have found that a QTL on chromosome 15 influences anxiety measures in a very consistent way, and its effects on ambulation scores are restricted to decreasing ambulation in threatening areas of the tests. On the other hand, a locus on chromosome 4 decreases activity in a way restricted to the non-threatening areas and also decreases activity in the homecage (a measure that should be anxiety-independent) therefore indicating a role of this QTL as an activity-decreaser. These findings support the concept already suggested by factor analysis that ambulation scores in threatening areas, like the open arms of the EPM, are an index of anxiety, while ambulation scores in non-exposed areas, like the closed arms of the EPM, are an index of locomotor activity.

However I also found two QTL, on chromosome 1 and 18, which have a profile consistent with their effect on anxiety, but they also decrease activity in non-threatening areas, indicating that some genetic effect is common to these two dimensions.

One possible argument here is that non-exposed areas of tests, albeit being less stressful, still maintain some threatening potential. This has been demonstrated at least for the EPM, where a study of plasma corticosterone concentrations in rats exposed either to the closed arms or the open arms has shown that, although exposure to the open arms resulted in significant higher concentrations than exposure to the closed arms or controls

left in the home cage, exposure to the closed arms also provoked enhanced concentrations compared to the home cage controls (Pellow, Chopin et al. 1985). This stands for an interpretation of the closed arms as a stress-inducing situation of lower potential than the open arms, rather than a completely safe environment.

Another explanation for the profile of the loci on chromosomes 1 and 18 comes from further analysis of allelic effect on repeated measures. It has been shown that animals exposed to the OF show an increase in OFA scores upon re-exposure, compatible with a decrease in the anxiogenic potential of the test due to habituation (Hegmann and DeFries 1968; Walsh and Cummins 1976). In line with these findings, a detailed ethological analysis of the OF (Choleris, Thomas et al. 2001) has found that mice exposed to this test for 30 minutes tended to display more fear-related behaviours, such as thigmotaxis and stretch attend postures, in the first part of the session (5-10 minutes) while increasingly engaging in investigative and locomotor activity towards the end of the session.

The analysis of allelic effect of loci on chromosomes 1, 15 and 18 in this project show an interesting pattern. The chromosome 15 QTL decreases activity only in threatening areas and its effect tends to fall with repeated exposure, compatible with its role in avoidance behaviour. Also, latency measures map largely and consistently only on this chromosome. The QTL on chromosome 1 and 18 decrease activity measure in a less specific way, affecting also ambulation in non-threatening areas, and their pattern on habituation is more complex, but there is a tendency for effect on time and latency measures to fall, and for effect on activity measures to increase. These data suggest a role of these loci on exploration, as their effect is released when avoidance drops due to

habituation. So, ambulation scores in these tests seem to reflect at least three dimensions: general locomotor activity, avoidance and exploration.

Interestingly, QTL that I have mapped for tail suspension, a test devised for screening antidepressants (Steru, Chermat et al. 1985) and thought to measure 'behavioural despair', do not overlap with QTL for anxiety. Analysis of differences in tail suspension and OF behaviour between inbred strains had already suggested that genetic effect for anxiety and behavioural despair is different (Trullas, Jackson et al. 1989). This study confirms this finding, although out of four QTL found to influence tail suspension, one is found on chromosome 11, where also QTL for anxiety limited to the LD are found.

The QTL for tail suspension on chromosome 11 has been replicated in a recent mapping study (Yoshikawa, Watanabe et al. 2002), where additional loci for tail suspension were found on chromosomes 4, 8 and 14. A simple explanation for the inconsistencies between Yoshikawa's and my mapping results is that in Yoshikawa's experiment, analysis was carried out on an F2 intercross between C57BL/6 and C3H/He, while here the inbred strains of origin are C57BL/6J and BALB/cJ.

In conclusion, this study of QTL action establishes the relative independence of the genetic effects for anxiety-related and depression-related animal models, identifies at least three dimensions of anxiety behaviour: general locomotor activity, avoidance and exploration, and suggests that some time and latency indexes represent relatively purer indexes of anxiety as compared to ambulation scores. However, other constructs, such as decision-making, make contributions to what we term anxiety and it will be necessary to test for their presence.

Although my results may only apply to the inbred strains studied in this project, they indicate the power of genetic mapping to inform behavioural analysis.

6.3 Fine resolution mapping of QTL for anxious behaviour

The detection of loci influencing anxious behaviour provides the starting tool to identify the molecular variants responsible for this effect. However, gene cloning is a very laborious procedure, and it is impossible unless genetic mapping refines a locus to a very small interval.

F2 intercrosses are a simple genetic system, with only two alleles segregating at each locus at only one round of meiotic recombination (as shown in Figure 2.1 in chapter 2). This is what makes them an ideal system for mapping experiment, but also renders refined mapping impossible. A series of strategies for fine mapping that can be applied to results from F2 intercrosses have been proposed (Darvasi 1998), and more recently the use of heterogeneous stocks of mice has been proposed and recently applied to the analysis of mice anxious behaviour (Talbot, Nicod et al. 1999; Mott, Talbot et al. 2000). These latter studies replicated the finding of QTL on chromosome 1 and 15 for open field anxious behaviour.

In this project I have applied haplotype analysis and a recombinant inbred segregation test to increase the mapping resolution of some of the QTL identified to influence anxiety. Of these, the QTL on chromosome 7 still encompasses a large region of 25 cM, the QTL on chromosomes 1 and 4 have been mapped to an interval of about 10 cM, and the QTL on chromosome 15 has been fine mapped to a 3 cM region.

Although the chromosome 7 locus is still mapped to a very large region, a series of data are consistent with it representing the albino c locus. First, it acts recessively. Second, it has already been shown that albinism affects open field behaviour (Fuller 1967; DeFries 1969), in a consistent way with the profile of the QTL found in this project (decreasing OFA and increasing OFD). Although its effect on open field behaviour has been attributed to the fact that albino mice are more photophobic than pigmented mice and therefore the bright illumination of the open field constitutes a more aversive stimulus, albinism has also been associated with illumination-independent effects, like reduced cognitive abilities (Lassalle and Le Pape 1981) and fear of heights (van Abeelen and Kroes 1968). Consistent with these findings, in this project the chromosome 7 locus is found to have an effect in the elevated plus maze and elevated square maze, and in particular to increase entries and activity in the closed arms of these tests. Moreover, this locus has no effect in the light-dark box, reinforcing the idea that photophobia is not enough to explain its action.

6.4 Future work

6.4.1 Multivariate analysis and mapping of principal components

Multivariate analysis has been widely used to analyse anxious behaviour in rodents. In chapter 1 I have presented data of multivariate analysis for the 5 ethological tests used in this project. Multivariate analysis has also been used across two or more tests in the attempt of dissecting different dimensions of anxious behaviour (Lister 1987; Maier, Vandenhoff et al. 1988; Trullas and Skolnick 1993; Belzung and Le Pape 1994; Ramos, Berton et al. 1997). Factor analysis across measures in the five ethological tests, followed

by genetic mapping analysis of the factors identified, would represent an important approach for the analysis of the multidimensionality of anxious behaviour, as recently emphasised by (Ramos and Mormède 1998), which is complementary to the univariate mapping experiment presented here.

I have done some preliminary multivariate analysis on these data and found that identified factors tend to be test specific, rather than across tests, and they tend to map to multiple chromosomes without differentiating specific genetic effect. However, more detailed and refined analysis is required to draw some conclusions.

6.4.2 Analysis of sex differences in the genetic basis of anxiety

Gender differences have been reported in many tests of anxious behaviour in rodents, as reviewed in (Palanza 2001). Recently, the importance of analysing sex differences in mapping of behavioural traits has been suggested by findings of sex-specific QTL for various behaviours, among which ethanol preference in mice (Melo, Shendure et al. 1996; Peirce, Derr et al. 1998) and OFA in rats (Ramos, Moisan et al. 1999). In this project, a QTL influencing anxiety and operating with an anxiolytic profile has been identified on chromosome X. Moreover, our collaborator Prof. N. Henderson has set out to analyse QTL mapping in male and female mice separately, and his results will show if sex-specific QTL can be identified on autosomal chromosomes in our population.

6.4.3 Genome-wide epistatic interaction analysis

It is known that genetic variance of complex traits is not only determined by additive effects of multiple loci across the genome, but also by gene-gene interactions, a

phenomenon known as epistasis (Frankel and Schork 1996). Standard QTL mapping procedures are not designed to search for epistatic interactions. Studies of complex traits in mice have searched for epistatic interactions intervening between loci already identified as affecting the trait (Fijneman, de Vries et al. 1996; van Wezel, Stassen et al. 1996), however, only very recently computer programs have been developed to use in wide-genome analysis of epistasis. This makes it possible not only to detect interactions between identified QTL but also to discover loci that act only as epistatic modifiers and would therefore have been missed with standard QTL mapping analyses. Studies have demonstrated the importance of epistasis in contributing to genetic effect for complex phenotypes in mice, like circadian behaviour (Shimomura, Low-Zeddies et al. 2001), pentobarbital withdrawal convulsions (Hood, Belknap et al. 2001), and hypertension (Sen and Churchill 2001). A wide-genome analysis of epistasis is in progress for some of the phenotypes analysed in this project.

6.4.4 Fine mapping and gene cloning

Fine mapping through haplotype analysis and the RIST experiment should be extended to QTL not analysed in this project. Moreover, other resources like the heterogeneous stock of mice, are available to confirm the QTL and perform high resolution mapping, as has already been achieved for chromosomes 1 and 15 QTL (Talbot, Nicod et al. 1999; Mott, Talbot et al. 2000).

Through this project, I have already achieved a high resolution mapping of the chromosome 15 locus. Moreover, in recent work, I have constructed a bacterial artificial chromosome (BAC) contig of 40 BACs that encompasses the whole 3-cM region

identified by haplotype analysis (about 3 megabases). This constitutes a valuable resort for sequencing and candidate gene analysis that will hopefully lead to the cloning of the molecular variant(s) responsible of the QTL effect in this locus.

APPENDIX A

Methods

All mice were bred and phenotyped in the specific pathogen free colony of the Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado, under the supervision of Professor J. DeFries and Professor N.D. Henderson.

A.1 Mice breeding

For the QTL mapping experiment, the DeFries H1 and L1 lines were crossed and the progeny intercrossed to produce 815 F2 animals, here called Group 1 (G1). The F1 cross was reciprocal, consisting of equal numbers of male H1 X female L1 and vice versa. Similarly 821 F2 animals were derived from the DeFries H2 and L2 animals, here called Group 2 (G2). In this way four different F2 combinations occur for each replicate, taking into account strain and sex of the progenitor inbred strains. Animals were weaned at 25 days and housed with like-sex littermates (no more than five per cage) until day 40 when they were individually housed prior to testing. Testing took place between day 40 and day 75.

For the RIST experiment, an F2 of 71 animals was generated from a cross between the DeFries H2 and C1 lines.

A.2 Mice phenotyping

The 71 mice used in the RIST experiment were tested for 5 minutes (min) under white light in a 60 centimetres (cm) circular open field made of white plastic, which is divided into 16 sections by photo-beams spaced 15 cm apart. OFA is detected automatically when a photo-beam is broken. OFD is measured by the number of faecal boli produced in the open field during the 5 min session. Testing occurred during the light cycle.

For the G1 and G2 mice, all testing was done during the first four hours of the dark cycle. A complete list of phenotypes recorded is given in table A.1 and testing apparati are described below.

All measures were standardised to a mean of 0 and a variance of 1. Only measures with a normal distribution or one that could be transformed, by 'taking the square root of the natural logarithm' of the value, to an approximately normal distribution were used in this study.

A.2.1 Open Field

The open field is a 61 cm white plastic lined square box, 38 cm deep. The apparatus was covered and a 100-Watt compact florescent bulb placed over the centre of the ceiling, providing a graded illumination, approximately 600 Lux at the centre of the arena. The field was divided into an 8 X 8 grid of 76 millimetres (mm) squares using infrared emitters and photo detectors used to monitor horizontal movement. In addition, four light beams at each corner were placed 6 cm from the floor to detect rearing on and near the

Table A.1

List of phenotypes measured in this project.

Test apparatus	No.	Abbreviation	Name of variable
Open Field Arena (OF)	1	OFD	defecation
	2	OFA	total activity
	3	OFA-centre	activity in the centre
	4	OF-centre time	time spent in the centre
	5	OF-latency	latency to move from perimeter to centre
	6	OF-rears	rears
	7	OF-rearing time	time spent rearing
	8	OFA-2	total activity on second trial
	9	OFA-centre-2	activity in the centre on second trial
	10	OF-centre time-2	time spent in the centre on second trial
	11	OF-latency-2	latency to move from perimeter to centre on second trial
Elevated Plus Maze (EPM)	12	EPM-boli	defecation
	13	EPM-shifts	shifts between open and closed arms
	14	EPM-closed entries	entries into the closed arms
	15	EPM-closed ambulation	ambulation in the closed arms
	16	EPM-closed time	time spent in the closed arms
	17	EPM-open entries	entries into the open arms
	18	EPM-open ambulation	ambulation in the open arms
	19	EPM-open time	time spent in the open arms
	20	EPM-open latency	latency to enter either of the open arms
	21	EPM-end entries	entries into the ends of the open arms
	22	EPM-end ambulation	ambulation in the ends of the open arms
	23	EPM-end time	time spent in the end of the open arms
	24	EPM-closed rears	rears in the closed arms
	25	EPM-closed rearing time	time spent rearing in the closed arms
	26	EPM-open rears	rears in the open arms
	27	EPM-open rearing time	time spent rearing in the open arms
	28	EPM-open scans	scans over the open arms
	29	EPM-end scans	scans over the ends of the open arms
	30	EPM-end scanning time	time spent scanning over the ends of the open arms
	31	EPM-centre entries	entries into the central platform
	32	EPM-centre time	time spent in the central platform

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Test apparatus	No.	Abbreviation	Name of variable
Elevated Square Maze (SQ)	33	SQ-boli	defecation
	34	SQ-shifts	shifts between open and closed arms
	35	SQ-closed entries	entries into the closed arms
	36	SQ-closed ambulation	ambulation in the closed arms
	37	SQ-open entries	entries into the open arms
	38	SQ-open ambulation	ambulation in the open arms
	39	SQ-open latency	latency to enter either of the open arms
	40	SQ-closed rears	rears in the closed arms
	41	SQ-closed rearing time	time spent rearing in the closed arms
	42	SQ-open rears	rears in the open arms
	43	SQ-open rearing time	time spent rearing in the open arms
	44	SQ-open scans	scans over the open arms
	45	SQ-open scanning time	time spent scanning over the open arms
	46	SQ-corner entries	entries into corners
	47	SQ-corner time	time spent in corners
Light Dark Box (LD)	48	LD-boli	defecation
	49	LD-dark ambulation	ambulation in the dark side
	50	LD-transitions	transitions between the dark and the light side
	51	LD-light ambulation	ambulation in the light side
	52	LD-light time	time spent in the light side
	53	LD-latency	latency to enter the light side
	54	LD-transitions-2	transitions between dark and light side on second trial
	55	LD-light ambulation-2	ambulation in the light side on second trial
	56	LD-light time-2	time spent in the light side on second trial
	57	LD-latency-2	latency to enter the light side on second trial
Mirror Chamber (MR)	58	MR-boli	defecation
	59	MR-entries	entries into new alleys
	60	MR-latency	latency to enter the mirror chamber
	61	MR-rears	rears
	62	MR-rearing time	time spent rearing
Homecage	63	homecage activity	
Tail suspension test	64	tail suspension	freezing time while suspended by tail

walls of the open field. The Digiscan optical system allowed detailed assessment of horizontal locomotor activity, including total ambulation and ambulation in centre, vertical locomotor activity, i.e. rearing, plus time measures like time spent in centre, latency to reach the centre and time spent rearing.

Animals were placed in a plexiglas cylinder in one corner of the apparatus, the cylinder removed and activity monitored for a 5 min interval. At the end of the session the number of faecal boli deposited were recorded and the floor was cleaned with 70% ethanol solution.

A.2.2 Elevated Plus Maze

The elevated plus maze consisted of four runways (5 cm X 30 cm) arranged in a cross and elevated 37.5 cm above the ground. Two of the arms on opposite sides were enclosed with transparent acrylic plastic walls 21 cm high. The open arms contained a slight raised, 0.25 cm, edge to provide additional grip for the animal. A low output compact fluorescent bulb was located over the centre of the maze, which provided approximately 20 Lux illumination on the floor of the maze. Movement was detected by infrared emitter-detector pairs located around the perimeter of the apparatus. Beams were positioned to detect both horizontal activity and vertical rearing in each runway and scanning over the edges and ends of open runways. Animals were placed in a rectangular bottomless start-box in the centre of the maze. The start-box was lifted and mice tested for a 5 min period. Activity and time spent in each arm was recorded as well as transitions into different arms. A complete list of phenotypes is given in table A.1. At the

end of the session the number of faecal boli deposited were recorded and the floor was cleaned with 70% ethanol solution.

A.2.3 Elevated Square Maze

The square maze differs from the elevated plus maze in providing a continuous circuit for the animal to traverse, with alternating enclosed and open regions. It is a variant of the elevated zero maze, with the difference that here the four arms are placed in a square instead of a zero, therefore presenting four corners at the intersection of the arms.

The maze was a square 30 cm X 30 cm large, with 5 cm-wide runways, two of which, on opposing sides, were enclosed by 20 cm black walls. The open runways contained 0.25 cm edges, similar to those on the plus maze. Illumination level and infrared activity monitoring methods were the same as those described for the elevated plus maze. Animals were placed in a bottomless start-box in a corner of one closed arm. The start-box was lifted and mice tested for 5 min. A complete list of phenotypes is given in table A.1. At the end of the session the number of faecal boli deposited were recorded and the floor was cleaned with 70% ethanol solution.

A.2.4 Light Dark Box

The light dark box consisted of two chambers. The black-walled, enclosed, compartment was 27 cm by 15 cm and had an exit, 8 cm wide and 9 cm high, in the middle of the wall adjoining the white-walled light compartment (31 cm by 27 cm). A 60-Watt bulb was positioned 30 cm above the floor, over the centre of the light compartment, providing a

light level of approximately 400 Lux. Infrared emitters and detectors recorded horizontal activity in both light and dark compartments. Animals were placed in the dark chamber and movement recorded over a 5 min period. Measures of latency to emerge from the dark compartment, time spent in the light side, ambulation in both compartments and light-dark transitions were recorded. At the end of the session the number of faecal boli deposited were recorded and the floor was cleaned with 70% ethanol solution.

A.2.5 Mirror Chamber

The test chamber used in this project consisted of an outer box 40 cm X 40 cm large and 30.5 cm high. Located within this box was a 30 cm X 30 cm X 30 cm cube, open on one end. The three inner walls, ceiling and floor of the cube were mirrored. The illumination within the mirrored chamber was approximately 10 Lux. The space between the cube and outer box provided the animal with a 4.6 cm dark-walled dim (1-2 Lux) alley surrounding the cube. The mouse was placed in the narrow alley at the farthest point from the opening to the mirrored chamber. Using infrared emitters and detectors, alley activity and rearing were recorded as well as latency to enter the mirrored chamber. At the end of the session the number of faecal boli deposited were recorded and the floor was cleaned with 70% ethanol solution.

A.2.6 Homecage Activity

All mice were caged singly several days prior to homecage activity monitoring and subsequent testing. Activity in homecages was measured during the first two hours of the dark cycle on two consecutive days. Two different activity-monitoring systems were used

simultaneously. The first consisted of infrared photo emitter-detector pairs, located outside the clear plastic homecage that divided the cage onto three equal areas. This system recorded activity in a manner similar to that used in the test apparatus, recording gross locomotor activity on the floor of the homecage as the animal broke the photo beams. The beam-break activity score consisted of the square root of number of beam breaks for each detector summed across the two test days.

The second homecage activity monitoring system employed an infrared motion detector located above the top of the homecage. This system covered the whole homecage and converted all motion sensed within the homecage into arbitrary units, based on the magnitude of activity detected. For example, rapid gross motor activity, such as climbing on wire cage lids, and cage floor locomotion generated more score units per second (sec) than grooming or nest activity. Unlike the beam-break system, which only monitored floor locomotion, mice showing high levels of activity restricted to one area of the homecage could obtain relatively high activity scores with motion detection monitoring.

A.2.7 Tail suspension

Approximately 2 cm of the animal's tail (beginning 1 cm from the tip) was taped to the vertical surface of a narrow horizontal board. The mouse was observed for 3 min, with the total number of seconds the mouse remains immobile recorded for each minute. The dependent variable was total seconds of immobility during the final two minutes of the test session.

A.3 Mice genotyping

All commonly used stock solutions and buffers were prepared as outlined in (Sambrook, Fritsch et al. 1989). Unless otherwise stated, all the chemicals were supplied by Sigma.

A.3.1 Extraction of DNA from spleen tissue

Genomic DNA was prepared from spleen tissue using the following procedure.

I. Lysis

Approximately one fifth of the spleen of the mouse was chopped finely using a razor blade and put in a Falcon polypropylene tube.

The tissue was put to lysate in 2 millilitres (ml) lysis buffer, consisting of Reagent B (50 millimolar (mM) Tris pH8, 10 mM ethylene diaminotetra-acetic acid (EDTA), 100 mM sodium chloride (NaCl,), 1% sodium dodecyl sulphate) plus 50 microlitres (µl) of 100 milligrams (mg) per ml of proteinase K added just prior to use. The sample was incubated at 50 degrees Celsius (°C) overnight. The next day 500 µl of 5 molar (M) sodium perchlorate were added and the sample was incubated at 65°C for 25 min.

II. Extraction

The sample was then extracted with phenol/chloroform as follows. An equal volume (2.5 ml) of phenol/chloroform/isoamylalcohol (25:24:1) was added to the sample, mixed by vortexing and centrifuged for 15 min at 3,200 revolutions per minute (rpm) in a Sorvall RT7-Plus centrifuge (Rotor RTH-250). The aqueous layer (upper phase) was carefully removed to a clean Falcon polypropylene tube with a disposable Pasteur pipette. The

upper phase was extracted once again with phenol/chloroform/isoamylalcohol, and then with an equal volume of chloroform. The upper phase was removed to a clean Falcon polypropylene tube.

III. Precipitation

DNA was precipitated as follows: 2 volumes (approximately 5 ml) of cold absolute ethanol were added to the sample, mixed thoroughly inverting the tube several times, and finally centrifuged for 15 min at 3,200 rpm. The DNA pellet, which was left in the tube after removing the supernatant, was washed in 70% ethanol as follows: 2 ml of cold 70% ethanol were added, the sample was centrifuged for 5 min at 3,200 rpm and the supernatant removed. The pellet was left to air-dry and finally resuspended in 100 µl Tris-EDTA (TE) 10:0.1 (10 mM Tris, 0.1 mM EDTA).

IV. Dilution to working concentration

The final DNA concentration was estimated by measuring the absorption at 260 nanometres on a DV-640 spectrophotometer (Beckman). An optical density of 1 unit approximates to 50 mg/ml double-stranded DNA. Genomic DNA was stored at 4°C. DNA was diluted to a working concentration of 10 nanograms (ng) per µl for use in PCR reactions.

A.3.2 Amplification of DNA by polymerase chain reaction (PCR)

All PCRs were carried out in 96 well plates (Costar) using AmpliTaq Gold polymerase (Applied Biosystems) and 50 ng of genomic DNA, in a total volume of 10 µl.

Constituents of PCR reactions and their concentrations were as follows:

Reagent[1]	**Working concentration**	**Final concentration**	**Volume added**
Genomic DNA	10 ng/µl	50 ng	5.0 µl
AmpliTaq Gold Buffer (Applied Biosystems)	10X	1X	1.0 µl
$MgCl_2$ (Applied Biosystems)	25 mM	1.5 mM	0.6 µl
dNTP mix (dATP, dCTP, dGTP, dTTP)	8 mM (2 mM for each dNTP)	0.8 mM	1.0 µl
Primer forward (Sigma Genosys)	10 picomoles/µl	1 picomoles/µl	1.0 µl
Primer reverse (Sigma Genosys)	10 picomoles/µl	1 picomoles/µl	1.0 µl
AmpliTaq Gold polymerase (Applied Biosystems)	5 units/µl	0.05 units	0.1 µl
H_2O	-	-	0.3 µl

1

$MgCl_2$ = Magnesium Chloride
dNTP = deoxynucleotide triphosphate
dATP = 2'-deoxyadenosine 5'-tryphosphate
dCTP = 2'-deoxycytidine 5'-tryphosphate
dGTP = 2'-deoxyguanosine 5'-tryphosphate
dTTP = 2'-deoxythymidine 5'-tryphosphate

Mineral oil was added to each sample to prevent evaporation during PCR amplification.

Amplifications were performed on Tetrad PCR machines (MJ Research).

The general PCR protocol was:

Step	Temperature	Time	
Pre-cycling denaturation	95°C	12 min	
Denaturation	95°C	30 sec	30 cycles
Annealing	50/55/60°C	30 sec	
Extension	72°C	30 sec	

The annealing temperature for each primer pair was determined by a test reaction to assay which temperature gave a specific product. Annealing temperatures for each primer pair are reported in Appendix B.

Primers

In genotyping of G1 mice I employed MIT markers previously used in the initial H1XL1 cross (Flint, Corley et al. 1995). I also screened these markers on the parental inbred strains H2 and L2 looking for strain differences and where I found that a marker was not polymorphic in this cross, I screened the parental strains with nearby markers. When available, I chose markers with a difference between the two alleles of at least 6 base pairs (bp), so that all products could be analysed on 4% agarose gels and alleles scored manually. In cases where only markers with smaller differences in size between the two parental strains were available, I used fluorescently labelled primers, ordered with one

ABI dye (TET, 6-FAM or HEX) incorporated at the 5' end. Fluorescent PCR products were analysed on an ABI 377 sequencers (Applied Biosystems).

In the haplotype analysis additional markers, spaced every 5 cM across the regions on chromosomes 1, 4, 7 and 15 found to harbour the QTL, were used.

Appendix B reports the full list of primers used in this project, their cM position, annealing temperature and dye name only for fluorescently labelled primers. For each primer pair, I report the bp length of the corresponding microsatellite in C57BL/6J and BALB/cJ strains, and characterisation of H1, H2, L1 and L2 for their origin strain (either C57BL/6J, indicated as C, or BALB/cJ, indicated as B).

A.3.3 Nucleic acid electrophoresis

After amplification with PCR, DNA fragments were separated for analytical purposes by horizontal agarose gel electrophoresis if the difference between the two alleles was of at least 6 bp, otherwise by vertical acrylamide gel electrophoresis on ABI 377 (fluorescently labelled primers were used in these cases).

Agarose gel electrophoresis of PCR products

4% agarose gels were made by melting agarose in 1X TAE buffer (0.04 M Tris-acetate, 1 mM EDTA) in a microwave. The agarose was allowed to cool with stirring until hand-hot, hence ethidium bromide was added (0.2 micrograms (μg) per ml). Gels were cast in large gel tanks (Flowgen) taking 600 ml of gel and fitted with six 50-wells combs, so that

three 96 well plates could be loaded on each gel. Gels were allowed to cool for about 30 min before loading.

For loading, 5 µl of each PCR sample were taken and added to 5 µl of Orange G loading buffer (Orange G dye in 70% Glycerol) and loaded on the gel. In each gel lane the first well was loaded with 1 Kb ladder (Gibco BRI) to serve as a size marker.

Gels were electrophoresed submerged in tanks containing 1X TAE buffer. Electrophoresis was carried out at a constant voltage between 2.5 and 5.0 Volts/cm. Ethidium bromide stained gels were visualised using a long-range UV transilluminator at 254 nanometers. The image was then electronically captured (MultiImage™, Alpha Innotech Corporation) and then recorded onto thermo-sensitive paper (Mitsubishi) via a digital graphic printer (Sony).

ABI 377 gel electrophoresis of fluorescent PCR products

6% polyacrylamide gels were prepared as follows: 12 cm glass plates were washed with Alconox detergent and rinsed thoroughly with deionised water. The plates were allowed to air-dry and then were assembled with a set of 0.2 mm spacers and 64-well square-tooth comb. The gel mix was composed of 30 ml of Acrylamyde (Severn Biotech), 72 µl of 10% Ammonium Persulfate and 36 µl TEMED (N,N,N',N'-Tetramethylethylene-diamine). The gel was allowed to set for two hours and then loaded on to the ABI 377. The gel was checked for dust and fluorescent contamination with module "Plate Check". The top and bottom buffer chambers were assembled and filled with 1X TBE (0.09 M Tris-borate, 2 mM EDTA). The gel was pre-run with module "PR GS 36C-2400" for 20 min. In the meantime, samples were prepared for loading.

2 µl of each PCR sample were taken and diluted 1:3 with water. 0.6 µl of the dilution were added to 0.25 µl of TAMRA/ROX 350 standard (Applied Biosystems) and 2.5 µl of ABI Loading buffer. Samples were denatured at 94°C for 2 min before loading.

The apparatus was electrophoresed using module "GS 36C-2400 " for three hours. Data collection was managed by a Power Macintosh 7100/80 running ABI 377 Data Collection Software (Applied Biosystems). Data were analysed using Genescan Analysis and Genotyper Softwares (Applied Biosystems).

A.4 QTL mapping procedure

Each marker was genotyped on the C57BL/6J and BALB/cJ inbred strains, unless these data were available from the MIT web site (http://www-genome.wi.mit.edu/), and on the DeFries parental strains H1, L1, H2, L2. This allowed me to classify H and L alleles as either C57BL/6J-derived or BALB/cJ-derived; and to classify F2 mice at each locus as either homozygous for the L parent allele, homozygous for the H parent allele, or heterozygous.

The order of all markers was checked using the MAPMAKER software package (Lincoln, Daly et al. 1992) and using the map distances derived from MAPMAKER, I analysed all data by interval mapping (Lander and Botstein 1989) in QTL-MAPMAKER (Lincoln, Daly et al. 1992). Data on chromosomes 1, 4, 7 and 15 were also analysed by composite interval mapping (Zeng 1994) in QTL-CARTOGRAPHER (Basten, Zeng et al. 1996).

In order to obtain an appropriate threshold value for detecting a QTL effect, permutation test was employed (Churchill and Doerge 1994; Doerge and Churchill 1996). For a single phenotype the values obtained were 3.2 and 3.9 for the 5% and 1% genome wide significance levels respectively. Phenotypes within each test are significantly correlated. There were no significant correlations with measures in other tests. Therefore I should have divided the *P*-values obtained for one phenotype by the number of tests to obtain significance levels appropriate to my experiment. However, I have given all LOD scores that equal or exceed the 5% threshold for a single phenotype (LOD 3.2).

In the RIST experiment, I tested for a difference between genotypes by an analysis of variance (ANOVA) in which the phenotypic mean and variance associated with each genotype is calculated and a variance ratio used to test for the equality of the variances.

APPENDIX B

Primers

Full list of primers used in this project. Primers highlighted in orange were used in the mapping experiments. All primers were used in haplotype reconstruction. Primers D1Mit150 and D15Mit187 were also used in the RIST.

For each primer the table reports:

- cM position (cM) taken from the MIT website (http://www-genome.wi.mit.edu/)
- if it was used in G1 and/or G2 mapping experiment (only for primers highlighted in orange) (tick in G1 or G2 corresponding cell)
- annealing temperature (T)
- dye name, for fluorescently labelled primers only (label)
- bp length of the corresponding microsatellite in C57BL/6J (C57) and BALB/cJ (BALB) strains
- characterisation of H1, H2, L1 and L2 for their origin strain (either C57BL/6J, indicated as C, or BALB/cJ, indicated as B).

Primer	cM	G1	G2	T	label	C57	BALB	H1	H2	L1	L2
D1Mit70	15.3	✓	✓	55		176	190	C	C	B	B
D1Mit132	43.7	✓	✓	60		148	162	C	C	B	B
D1Mit26	64.5	✓	✓	60		194	216	C	C	B	B
D1Mit443	66.7			50		103	93	C	C	B	B
D1Mit421	67.8			60	6-FAM	171	169	B	B	B	B
D1Mit218	69.9	✓	✓	55		147	162	B	B	C	C
D1Mit202	82		✓	55		143	96	C	B	C	C
D1Mit503	82			50		103	109	C	B	C	C
D1Mit452	84.2			55		98	92	C	B	C	C
D1Mit 113	91.8			55		206	224	C	C	B	B
D1Mit 149	92.9			50		96	146	C	C	B	B
D1Mit 206	94			53	HEX	123	118	C	C	B	B
D1Mit 355	95.1			50		112	96	C	C	B	B
D1Mit150	99.5	✓	✓	60		138	126	C	C	B	B
D1Mit 361	101.6			55		176	194	C	C	B	B
D1Mit223	110.4	✓	✓	55		149	169	C	C	B	B
D1Mit 409	110.4			50	6-FAM	99	101	C	C	B	B
D1Mit 293	113.7			60		154	116	C	C	B	B
D1Mit 155	115.8			55		252	216	C	C	B	C
D2Mit365	21.9	✓	✓	60	6-FAM	102	106	C	C	B	B
D2Mit43	50.3	✓	✓	55		210	244	B	B	C	C
D2Mit525	61.2	✓	✓	55		310	240	C	C	B	B
D2Mit226	80.9	✓	✓	60		102	124	B	B	C	C
D3Mit241	24	✓	✓	60		116	130	B	B	C	C
D3Mit11	37.2	✓	✓	60		145	163	B	B	C	C
D3Mit216	41.5	✓	✓	60		122	142	B	B	C	C
D3Mit199	54.6	✓		55		141	114	B	C	C	C
D3Mit116	64.5	✓	✓	55		263	275	B	B	C	C
D4Mit149	0			55		114	130	B	B	B	B
D4Mit235	3.3			55		116	90	B	B	B	B
D4Mit181	4.4			55		133	143	B	B	C	C
D4Mit101	5.5	✓	✓	60		102	96	B	B	C	C
D4Mit18	8.7			55		228	216	B	C	B	B
D4Mit2	10.9			55		187	172	C	C	B	B
D4Mit108	16.4	✓	✓	60		133	157	C	C	B	B

Primer	cM	G1	G2	T	label	C57	BALB	H1	H2	L1	L2
D4Mit196	16.4			55		272	306	C	C	B	B
D4Mit214	21.9			55		126	160	C	B	C	B
D4Mit89	23			55		132	118	C	B	C	B
D4Mit111	25.1			55		126	140	C	C	B	B
D4Mit178	30.6	✓		60		201	165	C	C	B	B
D4Mit81	31.7		✓	60		160	204	C	C	B	B
D4Mit152	33.9			55		143	161	C	C	C	C
D4Mit27	35			55		150	118	B	B	C	C
D4Mit15	39.3	✓	✓	55		279	329	B	B	C	C
D4Mit153	40.4			55		122	112	B	B	C	C
D4Mit58	45.9			55		192	178	B	B	B	B
D4Mit175	47			55		110	126	B	B	B	B
D4Mit31	50.3			55		122	112	B	B	C	C
D4Mit12	54.6			55		198	168	B	B	C	C
D4Mit16	56.8			55		217	235	B	C	C	C
D4Mit203	60.1	✓		55		118	144	B	C	C	C
D4Mit339	62.3		✓	60		122	133	C	C	B	B
D4Mit69	63.4			55		128	106	C	C	B	B
D4Mit54	68.9			55		150	190	B	B	B	B
D4Mit32	71			55		148	184	B	B	B	B
D4Mit127	72.1			55		164	176	B	B	B	B
D4Mit254	80.9			55		135	117	C	C	B	B
D5Mit145	0	✓	✓	60		149	121	C	C	B	B
D5Mit304	27.3	✓	✓	60		123	140	B	B	C	C
D5Mit24	45.9	✓	✓	60		174	198	B	B	C	C
D5Mit101	74.3	✓	✓	60		130	118	C	C	B	B
D6Mit166	1.1	✓		55		100	114	C	B	B	B
D6Mit93	12		✓	60		140	152	B	C	B	B
D6Mit4	24	✓		55		102	90	B	C	C	C
D6Mit36	40.4	✓	✓	60		196	178	B	B	C	C
D6Mit15	66.7	✓	✓	60		260	195	C	C	B	B
D7Mit178	0	✓	✓	60		201	165	C	C	B	B
D7Mit340	1.1			55		112	140	C	C	C	C
D7Mit341	2.2			55		124	114	C	C	C	C
D7Mit117	10.9			55		151	171	C	C	C	C

Primer	cM	G1	G2	T	label	C57	BALB	H1	H2	L1	L2
D7Mit55	12			55		150	204	C	C	C	C
D7Mit309	13.1			55		168	128	C	C	C	C
D7Mit310	15.3			55		164	118	C	C	C	C
D7Mit231	24			55		116	92	C	C	C	C
D7Mit86	25.1			55		194	206	B	B	C	C
D7Mit84	26.2	✓	✓	60		170	184	B	B	C	C
D7Mit276	26.2			55		114	137	B	B	C	C
D7Mit91	27.3			55		144	132	B	B	C	C
D7Mit90	28.4			55		260	280	C	C	B	C
D7Mit212	28.4			60		150	160	C	C	B	B
D7Mit301	33.9			55		115	131	C	C	B	B
D7Mit96	37.2			55		78	108	C	C	B	B
D7Mit222	38.3	✓	✓	55		147	123	C	C	B	B
D7Mit281	38.3			55		113	203	C	C	B	B
D7Mit237	41.5			55		122	110	C	C	B	B
D7Mit238	42.6			55		156	116	C	C	B	B
D7Mit105	49.2	✓	✓	55		260	242	C	C	B	B
D7Mit46	64.5	✓	✓	55		184	196	C	B	B	C
D7Mit174	64.5			55		109	123	C	B	B	C
D7Mit362	67.8			55		84	108	C	B	B	B
D8Mit58	0	✓	✓	55		164	152	C	C	B	B
D8Mit94	10.9	✓	✓	55		156	130	B	B	C	C
D8Mit339	25.1	✓	✓	55		122	106	C	C	B	B
D8Mit207	41.5	✓	✓	60		138	154	B	B	C	C
D8Mit121	62	✓	✓	60		250	224	B	B	C	C
D9Mit67	13.1	✓		60		124	136	B	B	C	B
D9Mit130	21.9	✓	✓	60		121	141	B	B	C	C
D9Mit353	33.9	✓	✓	55		131	151	C	C	B	B
D9Mit182	53.6	✓	✓	60		99	117	C	C	B	B
D10Mit170	20.8	✓	✓	60		135	147	B	B	C	C
D10Mit230	47	✓		55		116	138	C	C	B	C
D10Mit134	57.9		✓	55		101	119	B	B	B	C
D10Mit233	63.4		✓	55		130	110	B	B	B	C
D10Mit270	75.4	✓		55	6-FAM	108	106	C	B	B	B

Primer	cM	G1	G2	T	label	C57	BALB	H1	H2	L1	L2
D11Mit71	0	✓	✓	60		214	228	B	B	C	C
D11Mit242	31.7	✓	✓	60		122	104	C	C	B	B
D11Mit212	48.1	✓	✓	60		148	164	C	C	B	B
D11Mit360	64.5		✓	60		124	102	C	B	C	C
D11Mit181	69.9	✓		60		125	113	C	B	B	B
D12Mit37	1.1		✓	55		140	118	B	B	C	C
D12Mit147	13.1	✓		60		151	181	C	B	B	B
D12Mit285	19.7		✓	50		127	139	C	C	B	B
D12Mit114	23	✓		60		146	126	C	B	B	B
D12Mit5	31.7	✓	✓	60		176	160	C	C	B	B
D12Mit132	47	✓	✓	60		131	112	B	B	C	C
D13Mit248	19.7		✓	55		114	94	C	B	B	C
D13Mit142	24	✓		60		145	161	B	B	C	B
D13Mit159	32.8	✓	✓	60		142	160	C	C	B	B
D13Mit78	59	✓	✓	60		226	210	B	B	C	C
D14Mit50	9.8	✓	✓	60		190	148	B	B	C	C
D14Mit101	26.2	✓	✓	60		133	117	B	B	C	C
D14Mit92	52.5	✓	✓	60		90	128	C	C	B	B
D15Mit265	7.7			55		200	160	C	B	C	C
D15Mit220	16.4		✓	55	6-FAM	113	111	C	B	C	C
D15Mit151	16.4			55		142	148	B	B	C	B
D15Mit100	18.6	✓		60		111	127	B	B	C	B
D15Mit121	24			55		148	163	B	B	C	B
D15Mit167	25.1			55	HEX	124	122	B	B	C	B
D15Mit209	26.2			60		127	106	B	B	C	B
D15Mit208	26.2			55		147	139	B	B	C	B
D15Mit122	28.4			55		150	144	B	B	C	B
D15Mit234	28.4			50	HEX	123	127	B	B	C	B
D15Mit233	28.4			50		108	114	B	B	C	B
D15Mit65	28.4			50		152	140	B	B	C	B
D15Mit63	28.4	✓		55		146	124	B	B	C	B
D15Mit47	29.5			55	TET	138	140	B	B	C	B
D15Mit123	29.5			60		141	129	B	B	C	B
D15Mit156	29.5			55		145	125	B	B	C	B

Primer	cM	G1	G2	T	label	C57	BALB	H1	H2	L1	L2
D15Mit91	29.5			55	HEX	195	197	B	B	C	B
D15Mit212	29.5			55	6-FAM	88	86	B	B	B	B
D15Mit66	29.5			55		152	146	B	B	B	B
D15Mit3	29.5			50	HEX	137	135	B	B	B	B
D15Mit67	29.5			55	TET	190	186	B	B	B	B
D15Mit185	29.5			55	6-FAM	122	120	B	B	B	B
D15Mit186	29.5			55	TET	122	120	B	B	B	B
D15Mit257	29.5			55	HEX	124	122	B	B	B	B
D15Mit90	29.5			50	HEX	113	109	B	B	B	B
D15Mit105	30			55	6-FAM	125	131	C	C	B	B
D15Mit28	32.8	✓	✓	55		164	148	C	C	B	B
D15Mit187	32.8			55		194	202	C	C	B	B
D15Mit93	32.8			55	HEX	148	154	C	C	B	B
D15Mit68	32.8			55		112	120	B	B	B	B
D15Mit146	32.8			50	HEX	124	126	B	B	B	B
D15Mit2	35			55		93	84	B	B	B	B
D15Mit1	35			55	TET	184	188	B	B	B	B
D15Mit214	35			55		126	134	B	B	B	B
D15Mit31	35			55		190	184	B	B	B	B
D15Mit70	35			55		152	144	B	B	B	B
D15Mit71	35			55		118	132	B	B	B	B
D15Mit118	35			50		122	116	B	B	B	B
D15Mit158	35			50		152	170	B	B	B	B
D15Mit239	36.1			55		112	100	B	B	B	B
D15Mit134	33.9			50		148	154	B	B	B	B
D15Mit189	40.4			55		128	120	B	B	B	B
D15Mit107	41.5			55		151	145	B	B	B	B
D15Mit241	45.9			55		102	90	B	B	B	B
D15Mit262	45.9			50		122	114	B	B	B	B
D15Mit159	49.2			50		146	118	B	B	B	B
D15Mit96	49.2			50		140	134	B	B	B	B
D15Mit171	52.5			55		133	139	B	C	B	B
D15Mit242	53.6		✓	60		104	90	B	C	B	B
D15Mit34	55.7			55		148	192	B	C	B	B
D15Mit14	61.2			60	TET	188	192	C	C	B	B
D15Mit77	61.2			55	TET	168	164	C	C	B	B
D15Mit79	63.4	✓		50		276	282	C	B	B	B

Primer	cM	G1	G2	T	label	C57	BALB	H1	H2	L1	L2
D16Mit130	3.3		✓	50		146	126	B	B	C	C
D16Mit34	12	✓		60		138	110	C	C	B	C
D16Mit173	33.9	✓	✓	55		121	141	C	C	B	B
D16Mit106	51.4	✓	✓	60		148	140	C	C	B	B
D17Mit57	1.1	✓	✓	55		300	320	C	C	B	B
D17Mit24	10.9	✓	✓	50		142	126	C	C	B	B
D17Mit66	19.7	✓	✓	55		132	108	C	C	B	B
D17Mit88	29.5	✓	✓	60		242	186	B	B	C	C
D17Mit142	41.5	✓	✓	55		147	123	C	C	B	B
D18Mit67	2.2	✓	✓	55		128	140	B	B	C	C
D18Mit53	16.4	✓	✓	55		144	130	C	C	B	B
D18Mit9	45.4	✓	✓	55		170	160	C	C	B	B
D19Mit68	3.3		✓	55		136	122	B	B	C	C
D19Mit61	9.8	✓		55		131	149	C	B	B	B
D19Mit19	24	✓		60		142	116	C	B	B	B
D19Mit46	24		✓	55		115	129	B	B	C	C
D19Mit34	44.8	✓	✓	60		156	198	B	B	C	C
DXMit166	18.6	✓	✓	60		114	122	C	C	B	B
DXMit16	29.5	✓	✓	60		118	86	B	B	C	C
DXMit153	50.3	✓		60		145	158	B	C	C	C
DXMit189	51.4		✓	50		120	108	B	B	C	C

APPENDIX C

LOD scores

List of significant LOD scores (>3.2) for QTL influencing 62 phenotypes measured in five tests of anxious behaviour in mice, in G1, G2, and the combined data set G1+G2. Results are given also for non-significant LOD scores for chromosomes (Chr) where a significant LOD has been found at least in one of the two F2 crosses or in the combined data set.

No.	Name of variable	Chr	G1	G2	G1+G2
1	OFD	1	7.6	7.4	14.4
		7	1.2	4.0	3.3
		14	4.0	5.9	9.4
		X	2.1	3.9	5.3
2	OFA	1	12.8	14.8	27.3
		4	2.5	1.6	3.5
		7	7.6	7.0	13.1
		12	2.9	1.4	3.6
		15	5.6	7.3	12.4
		18	2.2	3.4	5.3
		X	2.1	3.3	5.2
3	OFA-centre	1	8.4	5.9	14.8
		7	5.1	4.0	13.5
		12	3.8	1.3	3.2
		15	3.3	3.9	5.1
		18	2.7	3.8	6.2

No.	Name of variable	Chr	G1	G2	G1+G2
4	OF-centre time	1	3.0	2.0	3.9
		7	5.5	3.7	7.3
		12	3.9	1.2	3.7
		15	4.0	2.5	5.2
		18	2.4	4.0	6.0
5	OF-latency	7	2.9	1.6	4.3
		15	5.5	5.0	9.0
6	OF-rears	4	3.7	6.9	9.6
		7	12.6	14.3	25.0
		11	1.9	3.5	5.2
		12	3.6	0.9	3.2
		15	11.8	16.7	28.3
		18	2.3	4.1	5.5
		X	2.4	3.8	5.2
7	OF-rearing time	4	1.4	3.0	3.8
		7	7.1	8.2	14.4
		15	8.9	9.8	19.3
		18	1.2	4.0	3.7
8	OFA-2	1	10.8	14.5	24.3
		2	2.9	1.8	3.5
		4	7.7	3.3	10.2
		7	7.1	5.4	9.9
		8	3.7	1.8	4.7
		13	4.5	2.0	4.7
		15	3.2	4.3	5.4
		17	3.3	0.4	1.2
		18	3.8	5.3	9.2
		X	2.4	3.7	5.1
9	OFA-centre-2	1	7.1	4.7	10.2
		4	5.7	4.5	10.1
		7	9.8	7.0	14.6
		12	3.4	0.3	2.2
		15	2.6	3.9	4.8
		17	3.7	1.4	1.7
		18	3.0	2.4	5.4
		X	2.1	1.5	3.2
10	OF-centre time-2	4	2.8	4.1	6.8
		7	9.7	11.8	20.8
		15	2.5	5.4	7.2

No.	Name of variable	Chr	G1	G2	G1+G2
11	OF-latency-2	7	6.1	12.5	17.5
		15	3.8	6.9	9.8
12	EPM-boli	1	5.7	2.6	7.4
		8	1.6	2.0	3.6
		14	4.1	3.3	7.1
		X	4.0	4.7	8.9
13	EPM-shifts	1	3.8	3.4	7.2
		4	6.2	2.4	8.0
		5	3.3	0.7	1.3
		7	6.3	0.2	3.3
		13	2.4	2.4	3.7
		15	4.3	4.6	8.4
		18	4.5	3.6	8.2
14	EPM-closed entries	1	3.9	5.5	9.4
		4	4.7	1.5	5.5
		7	11.6	6.0	16.5
		8	4.5	2.0	5.9
		18	3.3	3.8	7.1
15	EPM-closed ambulation	1	2.4	4.8	6.7
		4	4.9	2.1	6.5
		7	12.5	5.0	15.8
		8	4.5	2.1	6.2
		18	3.4	3.2	6.4
16	EPM-closed time	1	4.0	1.2	4.3
		6	3.2	1.5	4.4
17	EPM-open entries	1	4.0	13.8	15.4
		4	3.7	1.1	3.2
		15	4.8	8.9	12.9
		18	4.9	3.5	8.0
		X	1.3	2.2	3.2
18	EPM-open ambulation	1	4.0	10.1	12.1
		4	3.6	1.0	3.5
		15	4.5	8.4	11.8
		18	5.9	2.6	7.7
		X	2.0	2.8	4.1
19	EPM-open time	1	2.5	1.4	3.4
		5	2.5	3.1	3.6
		15	2.0	3.0	4.4
		X	2.0	4.1	5.2

No.	Name of variable	Chr	G1	G2	G1+G2
20	EPM-open latency	15	5.2	6.9	10.5
21	EPM-end entries	1	3.0	5.4	8.2
		4	4.6	0.7	4.1
		7	1.0	3.8	3.6
		14	1.3	3.6	3.8
		15	4.5	10.4	13.2
		18	4.3	1.3	4.8
22	EPM-end ambulation	1	4.0	10.0	12.1
		4	3.6	1.0	3.5
		15	4.5	8.4	11.8
		18	5.9	2.6	7.6
		X	2.0	2.8	4.1
23	EPM-end time	7	2.5	3.8	5.7
		14	0.9	3.1	3.6
24	EPM-closed rears	4	3.3	5.5	8.2
		15	15.0	15.7	32.6
		18	3.7	3.7	5.7
25	EPM-closed rearing time	4	2.3	4.5	6.3
		15	13.5	13.5	29.1
		18	4.2	4.1	6.9
26	EPM-open rears	5	1.8	2.3	3.8
		15	2.0	2.6	4.2
		17	0.8	3.5	2.2
27	EPM-open rearing time	5	3.3	1.0	2.9
28	EPM-open scans	1	4.4	7.3	10.5
		6	2.2	3.7	4.1
		15	4.0	6.5	10.2
		18	4.7	0.6	4.1
29	EPM-end scans	3	4.2	1.7	4.3
		4	4.1	0.9	4.3
		5	1.1	4.8	3.8
		7	3.2	4.6	7.5
		15	3.4	6.3	8.3
		17	1.1	3.5	2.2
		18	4.4	0.3	3.3
		19	3.6	0.2	1.5

No.	Name of variable	Chr	G1	G2	G1+G2
30	EPM-end scanning time	3	3.8	1.5	3.5
		4	4.0	1.0	4.2
		5	1.5	5.4	4.5
		7	3.8	5.4	8.5
		15	2.9	5.1	6.7
		17	1.1	3.6	2.2
		18	3.4	0.7	2.3
		X	2.0	1.7	3.4
31	EPM-centre entries	1	6.3	10.0	15.6
		4	4.4	1.8	5.0
		7	13.5	8.0	20.7
		8	5.4	2.7	7.3
		13	1.3	2.7	3.4
		18	3.5	4.8	8.0
32	EPM-centre time	7	4.1	11.9	14.5
		8	1.8	2.3	4.0
		14	2.7	1.7	4.2
		15	2.6	9.9	10.1
33	SQ-boli	1	3.4	1.8	5.0
		8	1.1	3.5	3.4
		14	1.1	3.6	3.3
		X	4.5	4.0	8.0
34	SQ-shifts	1	6.6	11.3	17.2
		4	3.3	2.1	3.8
		8	2.1	3.0	4.1
		14	0.1	3.3	2.1
		15	3.0	6.4	8.2
		18	5.1	3.3	8.0
35	SQ-closed entries	1	4.0	6.1	9.8
		12	2.8	1.3	3.9
		15	4.2	3.2	6.6
		18	5.0	2.5	7.1
36	SQ-closed ambulation	1	6.2	16.6	21.3
		4	4.6	2.6	3.2
		7	10.7	1.9	9.7
		8	2.6	3.8	5.1
		18	4.5	6.0	10.4
		X	2.0	2.2	3.5

No.	Name of variable	Chr	G1	G2	G1+G2
37	SQ-open entries	1	5.1	4.3	9.2
		4	2.2	1.6	3.6
		15	4.8	5.3	9.3
		18	2.5	2.0	4.6
38	SQ-open ambulation	1	3.7	8.5	11.3
		4	4.5	1.1	4.9
		7	1.3	4.7	4.4
		15	6.4	10.6	15.4
		18	3.9	3.2	6.9
		X	2.2	3.7	5.4
39	SQ-open latency	15	0.9	4.1	5.7
40	SQ-closed rears	4	3.0	3.0	4.8
		15	11.6	9.8	22.3
		18	4.0	1.0	4.2
41	SQ-closed.rearing.time	15	5.3	6.3	16.1
		18	1.0	1.4	3.4
42	SQ-open.rears	4	4.0	0.8	3.9
		6	1.9	2.0	3.4
		15	6.7	7.8	14.6
43	SQ-open.rearing.time	15	2.2	3.7	5.5
44	SQ-open.scans	1	4.2	3.9	7.4
		4	2.6	1.5	4.0
		15	7.7	9.5	16.9
		18	3.6	1.3	4.5
45	SQ-open.scanning.time	7	1.9	2.1	3.6
		15	2.8	4.7	7.2
46	SQ-corner.entries	1	9.3	14.9	23.4
		4	3.6	1.9	3.4
		7	11.6	1.0	7.7
		13	1.5	4.6	4.0
		18	3.6	4.3	8.0
47	SQ-corner.time	7	2.4	6.7	7.6
		15	7.4	4.3	12.4
		18	3.5	2.1	4.5
		X	2.6	3.6	4.0

No.	Name of variable	Chr	G1	G2	G1+G2
48	LD-boli	1	4.0	2.1	5.6
		3	1.3	4.1	2.2
		4	1.8	2.2	3.3
		14	4.2	1.0	4.1
		X	2.4	2.1	3.7
49	LD-dark ambulation	1	2.0	7.1	7.4
		2	2.1	2.2	4.2
		4	5.2	1.8	6.2
		5	3.3	0.5	2.9
		17	3.5	2.0	4.5
		X	2.3	3.0	3.7
50	LD-transitions	1	6.7	10.7	16.7
		4	2.4	1.6	3.9
		11	3.6	3.6	7.0
		12	2.2	2.0	4.3
		14	3.4	3.9	7.0
		15	5.1	8.9	13.5
		18	2.8	3.7	5.7
		X	0.6	3.2	2.9
51	LD-light ambulation	1	10.3	15.5	24.8
		11	2.9	3.3	6.0
		14	3.5	2.4	5.6
		15	4.4	7.3	11.4
		18	1.5	3.6	4.1
52	LD-light time	1	4.2	5.9	9.9
		4	0.7	3.7	3.2
		11	2.5	3.2	5.7
		14	4.5	3.1	7.6
		15	5.4	10.3	14.7
		16	3.1	1.6	4.3
		18	2.3	4.3	5.7
53	LD-latency	1	2.9	2.5	4.9
		2	3.4	0.3	2.1
		11	2.8	2.0	4.8
		14	4.6	7.0	11.4
		15	7.3	12.7	19.6
		16	2.3	1.7	3.4
		18	1.8	2.5	3.4

No.	Name of variable	Chr	G1	G2	G1+G2
54	LD-transitions-2	1	12.3	11.9	23.8
		4	7.0	6.2	10.4
		12	2.0	2.5	4.2
		18	3.7	3.9	7.2
		X	1.1	5.3	5.0
55	LD-light ambulation-2	1	11.4	8.1	18.9
		4	6.5	7.4	10.8
		7	3.5	2.7	5.6
		18	3.5	3.0	5.3
		X	0.8	3.6	3.8
56	LD-light time-2	1	4.2	1.6	5.1
		4	4.8	5.1	6.7
		X	1.0	3.6	4.1
57	LD-latency-2	1	2.4	3.4	5.1
		11	4.3	3.4	4.9
		14	2.1	3.7	5.2
		15	6.8	5.1	12.3
		18	1.6	4.8	4.0
58	MR-boli	1	1.4	1.9	3.3
		4	3.3	0.3	2.0
		11	1.8	3.5	2.6
		15	1.8	1.7	3.4
		18	1.9	2.6	3.3
		X	1.7	4.4	5.6
59	MR-entries	7	5.0	9.1	12.8
		11	1.2	4.2	2.5
		14	1.7	3.5	3.6
		15	4.8	8.7	13.2
		18	3.0	2.4	4.8
60	MR-latency	5	1.5	2.6	3.5
		12	0.6	3.1	3.2
		15	8.7	9.4	17.7

No.	Name of variable	Chr	G1	G2	G1+G2
61	MR-rears	1	3.4	3.5	6.5
		4	3.1	1.1	3.4
		6	3.5	0.4	1.6
		7	4.6	0.8	4.3
		12	3.9	1.1	4.0
		15	15.1	20.3	36.7
		18	7.8	0.2	4.8
		X	1.8	2.0	3.6
62	MR-rearing time	1	3.3	2.4	5.1
		6	3.4	0.6	1.5
		7	4.7	1.2	5.2
		12	3.4	1.1	3.5
		15	21.1	17.7	40.5
		18	8.0	0.7	5.6
		X	3.3	3.8	5.5

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