A Molecular and Genetic Approach to an Investigation of Pyrimethamine Resistance In Malaria Parasites

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I hereby declare that I alone have composed this thesis, and that, except where stated, the work is my own

Richard Culleton

January 2005

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* Heights are based purely on guesswork, and not on any actual measurements. They are dependent entirely on how large I picture the people involved to be in my mind's eye. This order may not accurately reflect the true standings, physical or otherwise, of the people involved.

$^{\Psi}$While neither is particularly tall, they come first by virtue of combined height

STATEMENT OF CONTRIBUTIONS

The following people contributed to the work presented in this thesis:

Drug Selection Experiment B (Chapter III) was designed and performed in collaboration with Dr. Jaap de Roode

The production of the genetic crosses described in Chapter V were performed with Professor Richard Carter

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Some of the proportional sequencing of *dhfr*, *dhfs*, *dhps* and *6-pthps* described in Chapter IV was performed by Mr Richard Fawcett

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ABBREVIATIONS

6-PTHPS	6-pyruvoyl tetrahydropterin synthase
AFLP	Amplified Fragment Length Polymorphism
AMA-1	Apical Membrane Antigen -1
ATP	Adenosine Tri-Phosphate
BCE	Before Common Era
CE	Common era
cM	centiMorgan
CQ	Chloroquine
CSP	Circumpsorozoite protein
DDT	Dichloro-diphenyl-trichloroethane
DHFR	Dihydrofolate Reductase
DHFS	Dihydrofolate synthase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
dTMP	Thymidylate
EDTA	Ethyleninediamine tetraacetic acid
GDP	Gross Domestic Product
HIV	Human Immunodeficiency Virus
Kb	Kilobase
LGS	Linkage Group Selection
LGS	Linkage Group Selection
Mg/kg	Milligrams (of drug) / Kilogram (of body weight)
MSP-1	Merozoite Surface Protein-1
PABA	Paraminobenzoic Acid
PCR	Polymerase Chain Reaction
PYR	Pyrimethamine
TAE	Tris-acetate/EDTA buffer
TBE	Tris-borate/EDTA buffer
TE	Tris/EDTA buffer

TRAP	Thrombospondin related adhesive protein
v/v	volume for volume
w/v	weight for volume
WHO	World Health Organisation

Amino Acids

C	cysteine
I	isoleucine
L	leucine
N	asparagine
R	arginine
S	serine

ABSTRACT

The identification of parasite genes controlling phenotypes such as drug resistance, virulence, immunogenicity, and transmission is vital to malaria research. Classical genetic methods have achieved these goals only rarely and with difficulty. The work presented in this thesis describes the development of, and an investigation into a novel genetic method, Linkage Group Selection (LGS), which achieves rapid *de novo* location of genes encoding selectable phenotypes of malaria parasites. A phenotype-specific selection pressure is applied to the uncloned progeny of a genetic cross between two malaria parasites that differ in the phenotype under investigation. Selected and unselected progeny are analyzed using genome-wide quantitative genetic markers. Markers of the "sensitive" parent, which are reduced after selection, are sequenced and located in genomic databases. They are expected to be closely linked to gene(s) determining the phenotype under selection. The validation of LGS presented in this work was carried out with the rodent malaria parasite *Plasmodium chabaudi chabaudi* using a phenotype, pyrimethamine resistance, whose controlling gene, that encoding dihydrofolate reductase (*dhfr*), is known. The results show that molecular markers closely linked to *dhfr*, and only those linked to this gene, were reduced or removed by pyrimethamine treatment in accordance with the expectations of LGS.

In addition, experiments investigating the "fitness cost" of pyrimethamine resistance are described. The recombinant progeny of a cross between drug-sensitive and drug-resistant parasites were grown in infections in mice, and the proportions of the sensitive and resistant alleles of *dhfr* were measured throughout the infection. It was found that parasites carrying the pyrimethamine resistance mutation at *dhfr* grew more slowly than those carrying the sensitive allele, indicating this specific mutation for drug resistance carries a fitness cost to the parasite. This result may have important implications for the design of effective drug policies in malaria endemic areas.

CHAPTER I
INTRODUCTION

The aim of the work presented in this thesis is to explore and validate a novel method (Linkage Group Selection (LGS)) for the identification of genes controlling important phenotypes in malaria parasites. The results presented herein relate to the accomplishment of these aims through investigating LGS using pyrimethamine resistance in the rodent malaria parasite, *Plasmodium chabaudi*. The genetic basis underlying the ability of resistant parasites to proliferate under drug pressure is analysed, along with the related subjects of fitness costs associated with drug resistance, competition between sensitive and resistant parasites in mixed infections, and the genetic basis of differing growth rates.

In this introduction, the historical aspects of malaria are discussed (including an overview of the current status of the disease and its socio-economic causes and consequences), followed by the current understanding of the parasite's life cycle and essential genetics. There follows a discussion on the emergence and genetics of drug resistance, focussing specifically on pyrimethamine. The subject of fitness costs associated with mutations conferring drug resistance is then addressed, succeeded by a brief discussion of virulence and growth rate differences between strains of parasites. Finally, there is an introduction to the principles behind linkage group selection including the rationale behind the work presented in this thesis.

1.1 Ancient history

Every nasty bug has schemed to plague me with disease,
The twin Gods of Illness bore holes in my chest and diaphragm
("He's old – why not kill him off?"),
Send vermin of darkness to release their poisonous venom.

The suffocating heat of my body has steamed my vitality away,
I quake as though struck by thunder;
Heaven and earth have become a gigantic two-part steamer
And all the gravy pours out of me.
But suddenly comes a chill to chase away the heat,
And I shiver as though drowning under ice,
Pile up more blankets of coverlets and quilts,
No end to the number I can take.

Why, in such a brief span of time,
Do Yin and Yang alternate so violently?
I cough and sneeze,
Tears running mixed with phlegm,
Toss and turn but can't find peace
No matter how I rearrange pillow and mattress;
I have trouble whether I'm up or down,
For the slightest movement someone must support me,
So dizzy I confuse square and round,
Fall over all the time go black and pass out.

All my life I've eaten a vegetarian diet,
And it's always suited my taste just fine:
So the five whole mullet they've set before me now
Taste about as appetizing as frozen quinine bark.
In the short while I've been confined to this hammock,
My scrawny body has turned to Jerky;
People passing by look in
Wondering if I'm not dead and just pretending to be alive.

All day groans issue from my mouth –
Whatever comes brings anger or terror.
Returning to consciousness, I compose myself:
A visitor from far across the seas,
No one knows my mind
Or has pity for a strange accent

-Chugan Engetsu (1300-1375 CE)

Writing in China in the 14th Century, the Japanese monk Chugan Engetsu describes the symptoms of a disease we can now identify as malaria. The disease had been known in China since at least 2700 BCE when it was described in the *Nei Ching* – the Canon of Medicine (edited by the Emperor Huang Ti). In the second century BCE, the first mention is made in Chinese medicine of a "cure" for the disease – the Quinhao plant (Sweet Wormwood – *Artemisia annua*), which is described in the medical treatise *52 remedies.* It is from this ancient herbal remedy that the current antimalarial drug artemisinin and its derivatives have recently been developed, which offer one of the most promising new treatments for the disease. It is possible to speculate that Engetsu himself may have been treated with Quinhao, as it is known that he made a full recovery from his illness before returning to Japan. His intriguing reference to another ancient herbal cure for malaria – "quinine bark", is, unfortunately, an elaboration by the poem's translator who was attempting to introduce some revisionist continuity on the theme of malaria (David Pollack *pers. comm.*).

The disease has also been described outside of China since ancient times. Fevers associated with splenomegaly (two of the most characteristic symptoms of the disease) were described in the Egyptian Ebers papyrus - reputedly found between the legs of a mummy in the Assassif district of the Theben necropolis and dating from about 1550 BCE. It is also from ancient Egypt that the first mention is made of "bed-nets" in human history, with Herodotus (c. 490 - 431 BCE) describing how Egyptian fishermen would arrange their nets around them as they slept to protect themselves from mosquitoes (Schmidt and Roberts, 2004).

Hippocrates (c. 460-377 BCE) was the first person to differentiate between different types of malarial fever when he categorised the disease into "quotidian" (daily), "tertian" (alternate days) and "quartan" (three days apart) depending upon the fever periodicity. He also noted that the disease was common among those who lived near swamps (but attributed this to the drinking of stagnant water). Indeed, this association of the disease with swampy habitats has given us the name "malaria", a contraction of the Italian "*mala*

aria" which means "bad or evil air", in reference to the foul vapours associated with swamps.

1.2 Recent History

Although the symptoms of malaria were known since ancient times, the discovery of the *cause* of the disease had to wait until the end of the nineteenth century. A French military doctor - Alphonse Laveran - was the first person to recognise a parasite as the causative agent of malaria. Medicine in Europe had been revolutionised by Robert Koch and Louis Pasteur's work in the 1860s and '70s that showed that most infectious diseases are caused by microbial agents (the "germ theory" of disease). With the wide-spread acceptance of this idea, researchers (the first modern malariologists) were soon looking for the microbial agent that caused malaria, and many studies were conducted on various algae, protozoa, fungi and bacteria that were associated with malarious areas.

It appeared that the problem was solved when, in 1879, Corrado Tommasi-Crudeli and Edwin Klebs (already famous for his isolation of the diphtheria bacillus) identified a bacterium in the soil of the swamps of the Roman Campagna, which, they claimed, could induce a malaria-like disease when injected into rabbits. It was duly named "*Bacillus malariae*", and despite some unsuccessful attempts to reproduce the rabbit-infection experiment, such was Klebs' reputation at the time that this bacterium was widely accepted as the causative agent of malaria.

In fact, this theory actually ignored evidence to the contrary that had been known for over 150 years before the discovery of *Bacillus malariae*. It had long been observed that the spleen and brain of malaria victims contained a dark pigmented material which had first been described by Giovanni Maria Lancisi in 1717 (Lancisi was a remarkable character in the history of malaria research who not only appears to be the first person to have described evidence of malaria parasites, but who also suggested a link between the disease and the mosquitoes associated with malarious areas). Laveran's breakthrough was

to realise that this dark pigmented material was associated with the *cause*, and not the result, of malaria.

In 1878 he began working with malarious French soldiers in Constantine, Algeria, and his autopsies of the victims of the disease constantly revealed the same pigmented bodies in the spleen and brain that had first been described by Lancisi. He was unable, however, to collate any evidence from tissue samples that these pigmented bodies were anything other than a pathological symptom of the disease, and he switched his investigations to the study of fresh blood from malaria patients. This was to prove to be the decisive step in the discovery of the malaria parasite, when on November 6th 1880, Lavern observed the vigorous movement of one of the pigmented bodies in fresh blood. He instantly realised that the pigmented malarious bodies that he and other researchers had long observed in the tissue and blood of malaria patients were living parasites, and were the causal agents of the disease (see Figure 1.1). Laveran communicated his discovery to the Academy of Medicine in Paris, and named the parasite "*Oscillaria malariae*" (Lehrer, 1979; National Center for Infectious Diseases, 2004; Schmidt and Roberts, 2004).

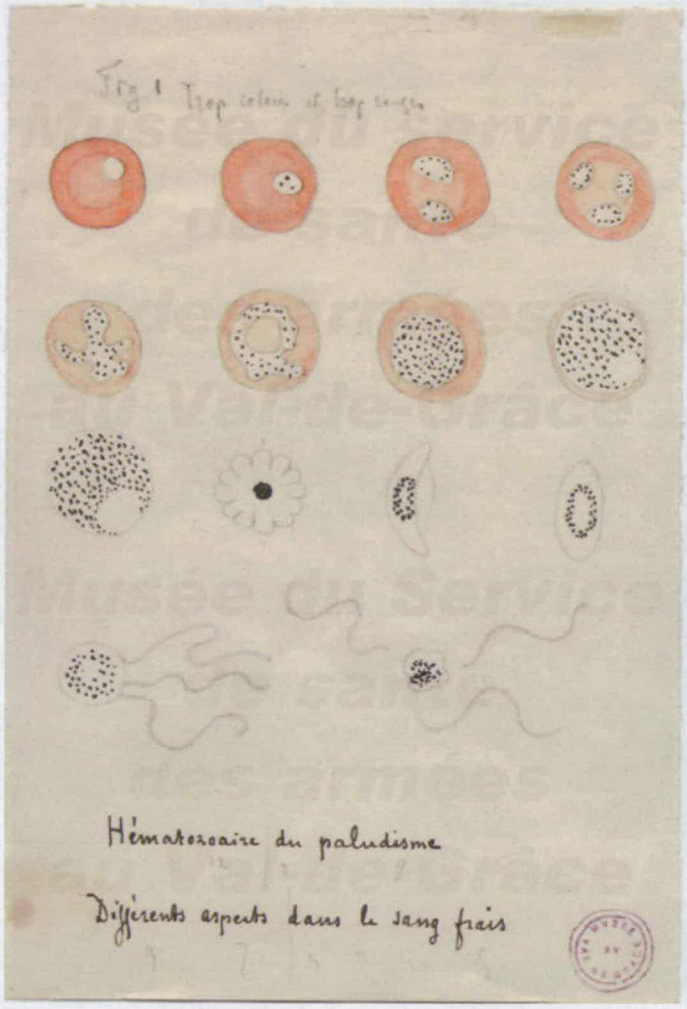


Figure 1.1 One of Laveran's original drawings of the parasites he observed in the blood of a malaria patient. Source: National Center for Infectious Diseases

There followed a period of some years in which the scientific community were slow to accept Laveran's discovery, such was the enthusiasm for *Bacillus malariae*, but by the early 1890s, it was generally accepted that Laveran's parasites were, indeed, responsible for malaria. This acceptance was bolstered by the new blood staining techniques developed by Romanovsky in Russia that allowed the parasites to be visualised in great detail in blood smears. In 1884, Ettore Marchiafava (a long time advocate of *Bacillus malariae*) and Angello Celli verified Laveran's claims, and named the parasite they'd observed "*Plasmodium malariae*". Camillo Golgi was the first to realise that there were different species of the parasite, each with a characteristic morphology and pathology,

and by 1897 three distinct human parasite species had been described; *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium falciparum*. A fourth, *Plasmodium ovale,* was described in 1922.

1.2.1 Discovery of the mosquito transmission of parasites

It was now known that malaria was caused by a *parasite* rather than by vapours, bacteria, or other such airborne agents. The question arising from this discovery, then, was how does the parasite get from one person to the next? Again, Giovanni Maria Lancisi had a theory. "Mosquitoes", he proposed, "always inject their salivary juices into the small wounds on the surface of the body." And "...no controversy can arise concerning the harmful effects which [they], by mixing their injurious juices with the saliva...inflict upon us" (quoted in Lehrer, 1979). His idea, presented in 1717, was not significantly elaborated upon until the wonderfully named Albert Freeman Africanus King proposed a similar theory in a lecture to the Philosophical Society of Washington in 1882 (Cook and Webb, 2000). King went further than Lancisi, stating that the mosquito was the *source* of malaria, and that eradication of the disease could be achieved by preventing mosquitoes from biting people. He elaborated on this idea by suggesting that Washington city could be covered by a giant mosquito net as tall as the Washington Monument in order to rid the city of the disease. This last comment proved too much for his scientific audience, and somewhat undermined his fundamental ideas, which were, with the benefit of hindsight, extremely prescient.

It was a Scotsman, Patrick Manson, who became the most vigorous exponent of the mosquito vector theory of malaria transmission, when in the 1880s, inspired by Laveran's publication of the parasitic nature of the disease, he began to wonder if it could be transmitted by a mosquito in the same way that he had recently shown for the filarial nematodes (*Wuchereria bancrofti)* that cause elephantiasis. He was further inspired by the work of Smith and Kilbourne, who in 1893, proved that Texas Cattle Fever (bovine babesiosis) was transmitted by the tick *Boophilus bovis,* and of David Bruce who proved the following year that the tsetse fly, *Glossina morsitans*, was the vector of nagana

(Gubler, 2001). In the light of these findings Manson postulated that the flagellated forms of malaria parasites were taken up by mosquitoes during feeding, and these would then migrate into the tissues where they would become forms that were infectious to another human. He went on to theorise, however, that as mosquitoes were, at the time, thought to die straight after laying their eggs, the parasites escaped from the dead body of the flies into the marshy waters and infected people who drank them, thus returning to the ideas of Hippocrates.

Manson clearly saw the urgency of experimental work to prove this theory, and initially applied for funding to travel to malarious areas to undertake such endeavours himself. He was refused, however, but managed to convince Surgeon-Major Ronald Ross to investigate his theories while serving in the Indian Medical Service. Ross quickly applied his considerable enthusiasm and dedication to the attempt to find evidence of malaria parasites in mosquitoes. After much laborious dissection of countless mosquitoes of various different genera, Ross eventually found what he was looking for – on August 20th 1897 he saw the pigmented parasites first described by Laveran in the mid-gut of an *Anopheles* mosquito known to have fed on a malarious patient. He immediately reported this discovery to Manson, and published a description of his work in the British Medical Journal. He had planned to prove that infected mosquitoes were able to transmit the parasites to an uninfected host, but his was interrupted when he was posted to a non-malarious area of India. Manson, however, arranged for him to continue his work the following year when he embarked upon a study of the avian malaria parasite *Plasmodium relictum*, with which he demonstrated the presence of sporozoites (which he named "germinal rods") in the salivary glands of a mosquito that had fed on an infected lark.

Manson presented the results of Ross' work at the Edinburgh meeting of the British Medical Association on the 28th of July 1898; the transmission of malaria by mosquitoes had been proven (Cook, 1997). It remained to be shown, however, that *human* malaria could be induced in man by the bite of an infected mosquito. This was finally proven by Amigo Bignami and Giovanni Grassi, who successfully infected Abele Sola (a volunteer

with no history of malaria) with malaria from an infected mosquito in October 1898 (Ascenzi A, 1999).

1.3 Malaria Today

Today, over 100 years after the work of the pioneering malariologists who uncovered the causes of the disease, malaria is responsible for 300-500 million cases worldwide every year, of which 0.5 – 2.5 million are fatal (White et al., 1999b). The majority of these deaths are of children under five years old, and it is estimated that every 30 seconds a child dies of malaria (WHO, 2004). Most of these deaths occur in sub-Saharan Africa where the most virulent species of human malaria, *Plasmodium falciparum*, is endemic. The disease is also widespread in South Asia, Central and South America, and the Western Pacific. The mortality from malaria is less in these areas than in Africa, but morbidity is high leading to considerable social and economic costs (Trigg and Kondrachine, 1998). These enormous socio-economic costs are made even more significant by the fact that the areas affected are amongst the poorest in the world (Figure 1.3). In 1995 alone, the direct and indirect cost attributed to the malaria problem world-wide was estimated at US$1800 million (The Wellcome Trust, 1998), despite concerted efforts by organisations such as the World Health Organisation (WHO) to control the disease. In fact, the situation is getting worse (WHO, 1998)

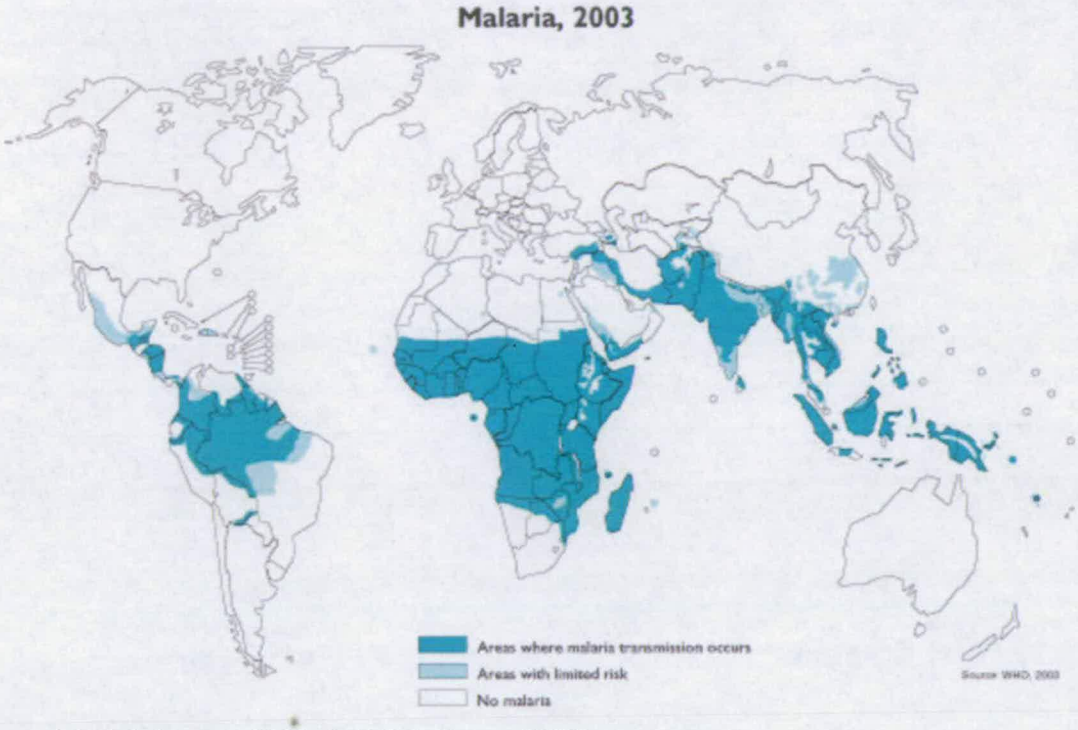


Figure 1.2 Global status of Malaria (2003). Source: WHO

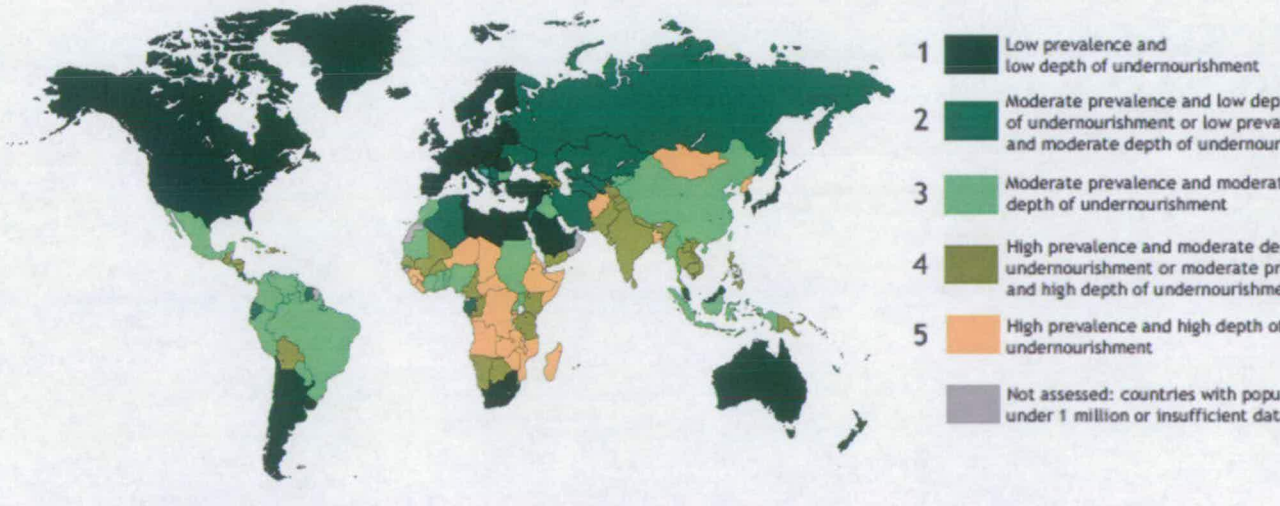


Figure 1.3 Global prevalence of undernourishment (1998) The prevalence of chronic under-nutrition is a relevant and valid measure of endemic poverty and is a better indicator than estimates of per capita income (Food and Agriculture Organisation of the United Nations, 2004). Source: (UN, 2004)

Comparing Figures 1.2 and 1.3, which show the distribution of malaria and the prevalence of global undernourishment respectively, it is easy to observe the correlation between the disease and poverty. In 1995 the gross domestic product (GDP) of malarious countries was more than 5 times less than that of non-malarious areas (Gallup and Sachs, 2001). This correlation between malaria and poverty can be interpreted in a number of ways. It is possible that the link between poverty and malaria is purely co-incidental, with tropical countries being impoverished for reasons of geography, climate, political history and other tropical diseases, independent of malaria. Gallup and Sachs (2001) analysed the income per capita of malarious countries and included these other factors in their analysis. They concluded that the "association of malaria with poverty seems to be more than just a mask for other plausible causes of low income".

Is, then, malaria a cause or an effect of poverty? It is true that the richer countries of the world have been able to eradicate malaria in the last 50 years. In 1946, for example, large areas of the USA, Southern Europe and northern Australia were afflicted with the disease, but managed to eradicate it by the mid 1960s (Sachs and Malaney, 2002). Certainly this was due, in part, to the economic power of the nations involved, which were able to organise efficient and effective strategies of mosquito control. The control attempts were probably aided by the affluence of the people living in these countries, particularly in relation to good quality housing and reliable access to medical services – both features of Western affluence that are sadly lacking in poorer nations. It is easy, then, to postulate that rich countries can *afford* to be malaria-free, while poorer nations are burdened with the disease due to the poor infrastructure associated with poverty. This explanation is overly simplistic, however, for though there is undoubtedly some truth in it, it ignores the very real biological differences in malaria parasite transmission between the rich and poor countries. It is often overlooked that it is far easier to eliminate malaria in regions where transmission is low, (an effect of human population density, mosquito population density and, crucially, climate), than where transmission is high and endemic. Countries that have successfully eradicated malaria are predominantly found in temperate climate zones, which are associated with less efficient vectors, lower night-time temperatures and marked seasonality in parasite transmission (Gallup and Sachs, 2001). Control of malaria

in tropical zones, then, is far more difficult. This argument against a purely economic *cause* of malaria is supported by rich countries in tropical locations that have not been able to eradicate the disease. Oman and Saudi Arabia, for example, whilst both enjoying high GDPs due to oil revenues, still suffer from the presence of malaria.

The incidence of malaria, then, cannot be directly attributed to the poverty of the country in which it is found. What is attributable to poverty, to a certain degree, is *death* from the disease. The startling statistic mentioned above, that "a child dies of malaria every 30 seconds" is often quoted in the introduction to scientific papers concerned with malariology, so much so that its impact is somewhat reduced on those working in the field. The fact is that this is a sickening statement to make in the 21st Century. Malaria is completely curable today thanks to highly effective anti-malarial drugs. Yes, there are huge problems of drug resistance in large areas of the world, but the effectiveness of combination therapy with artemisinin has yet to be undermined by this problem. The simple fact is that any child that contracts malaria need not die if treated with effective drugs such as Coartem® (artemisinin-derivative in combination with lumefantrine), and yet 2000 children are dying every day, because, fundamentally, their parents simply cannot get access to a medicine that costs US$2 for an effective dose.

There can be no doubt that increases in the economic development of malarious countries will decrease the terrible death rates presently associated with the disease. A case study conducted by Médecins Sans Frontières involving 5 African countries suffering huge malaria burdens (Burundi, Kenya, Rwanda, Tanzania and Uganda) estimated that for the 25.3 million cases of malaria within these 5 countries to be treated with artemisinin combination therapy would cost an extra US$19.1 million per year (Kindermans et al., 2002). Extrapolating this estimate to include the whole of Africa (in which there are approximately 200-450 million cases of the disease annually), the figure rises to approximately US$300 million extra a year. To put this figure into context, the combined yearly military expenditure of the 5 African countries listed above totals US$414.4 million (SIPRI, 2004).

If the prevalence of malaria is a not direct result of poverty, then the problem is certainly exacerbated by it, but what of the effects of the disease on the prevalence of poverty? There can be little doubt that countries in which malaria is endemic bear a great economic burden which can be directly attributed to the disease. Gallup and Sachs (2001) showed that countries that suffered severe malaria in 1965 had "dramatically lower economic growth in the next 25 years" than malaria-free countries (even after controlling for other factors such as geographic location, initial poverty, political economic policies, etc.). This reduction in the economic viability of malarious countries can be attributed to a number of factors. There is the direct personal economic cost borne by the people living in malarious areas who are forced to pay for preventative and curative measures against the disease. There is also the proportion of government spending on programmes designed to control the disease. Then there is the loss of revenue from foreign tourism and investment, which suffers due to reluctance on the part of would-be tourists and investors to travel to countries with a serious malaria problem. When malaria was eradicated from Portugal, Spain, and Greece in the mid 1950s, for example, there was huge economic development in these countries, which can be directly attributed to large increases in tourism and foreign investment (Sachs and Malaney, 2002) .

Less quantifiable, but certainly as important, is the reduction in economic growth associated with having a large proportion of the population unable to work due to sickness. High morbidity reduces the effective productivity of the population directly, in terms of the numbers of people unable to work, and indirectly, with the loss of earnings of the many people who have to look after affected family members (Nur, 1993). The fact that malaria is mostly prevalent among children has huge implications for education in the poorest regions of the world, sick children being unable to attend school. A study by Brooker *et al.* (2000), attributed 13-50% of medically related school absences in Kenya to malaria (Brooker et al., 2000). Malaria has been shown to have an adverse effect on the school performance of infected children in Sri Lanka (Fernando et al., 2003). There is also evidence to suggest that people who suffer from the disease in infancy experience impairment of cognitive development and learning capabilities (Holding and Snow, 2001). Related to this is the fact that mothers are particularly prone to malaria during

pregnancy. Malaria during pregnancy is directly associated with low birth-weights, which is also a risk factor for diminished cognitive development (Mccormick et al., 1992).

Another malaria-related factor that affects economic development is that the disease limits the movement of people within malarious areas. When people move away from a malarious area for prolonged periods, there is an associated loss of immunity against the disease. This prevents those people who have gone to non-malarious areas to work or study returning to the areas they left, and so contributes to the lack of skills and education in endemic areas. Similarly, as immunity to malaria is known to be strain-specific (so that immunity induced against a local population of parasites is not effective against a genetically different population of parasites), there is a risk involved in workers migrating from one endemic area to another, thus preventing the movement of labour to regions where it is most productive (Sachs and Malaney, 2002). All these factors combine to present the depressing picture of a disease strongly associated with poverty, both as a cause and as an effect. There can be little doubt that improving the economic development of the poorest countries will greatly aid the fight against malaria, while, at the same time, effectively reducing the disease burden will increase the economic development of the countries affected.

1.4 The parasite and its life-cycle

Malaria is caused by parasitic protozoa of the genus *Plasmodium*, with four species (*Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax* and *Plasmodium malariae*) infectious to man. The life cycle of these parasites is complex (see Figure 1.4)

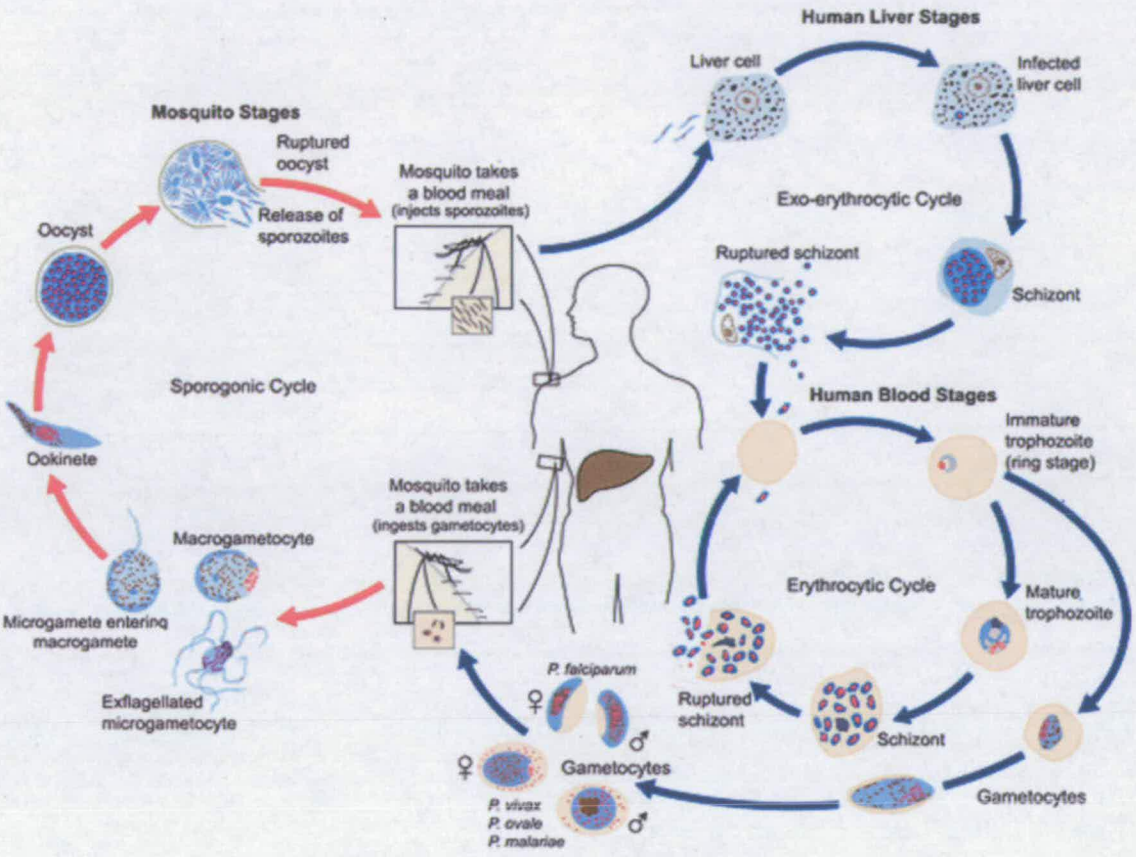


Figure 1.4 Life-cycle of human *Plasmodia*. (Adapted from www.dpd.cdc.gov)

Mammalian malaria parasites are parasites of erythrocytes that require a vector host for transmission and sexual reproduction. The life cycle in the vertebrate begins with the bite of a mosquito. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host. Despite the fact that the salivary glands of an infected mosquito are likely to contain many thousands of sporozoites (11,000 in *Plasmodium berghei* infections of *Anopheles stephensi*, for example (Sinden, 1997)), less than 100 of these are believed to be transmitted in any one bite (Rosenberg et al., 1990). These sporozoites (typically around 11μm in length, and 1 μm in diameter for *P. falciparum*) then enter the bloodstream and within 30-45 minutes migrate to the liver where they invade hepatocytes. This is achieved by the binding of the thrombospondin domains of the circumsporozoite and thrombospondin-related adhesive proteins (CSP and TRAP, respectively), to heparin sulphate proteoglycans on hepatocytes (Frevert et al., 1993). Here they undergo asexual reproduction (schizogony), lasting for 5-15 days, the

end product of which is around 10,000 – 30,000 merozoites. In *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading erythrocytes weeks, or even years later (Mangoni et al., 2003). Merozoites are released into the bloodstream following rupture of the hepatocyte, where they invade red blood cells. Merozoites are approximately 1.5 μm in length, and 1μm in width, and contain 3 membrane bound organelles at the apical end (dense granules, rhopteries and micronemes), characteristic of all Apicomplexan parasites (see Figure 1.5).

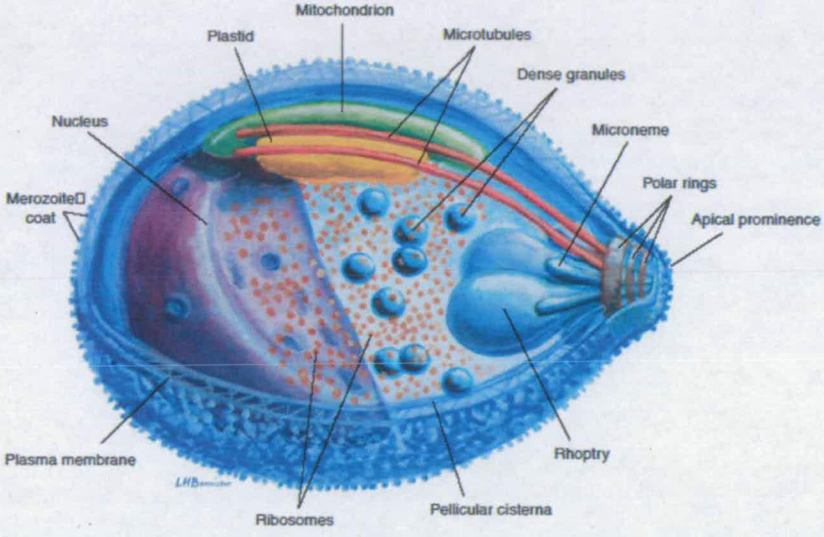


Figure 1.5 The ultra-structure of the *P falciparum* merozoite (Bannister et al., 2000)

Invasion of erythrocytes by merozoites involves an initial low affinity interaction between proteins on the surface coat of the merozoite (possibly merozoite surface protein-1 (MSP-1)) and the surface of the erythrocyte. The merozoite then orientates itself so that the apical prominence is in contact with the surface of the red blood cell, and there follows the release of the contents of the rhoptries, dense granules and micronemes. These assist with the penetration of the merozoite into the host cell (Chitnis and Blackman, 2000). Inside the erythrocytes the merozoites commence feeding, becoming trophozoites. These in turn become schizonts after undergoing another asexual reproductive stage (erythrocytic schizogony), and release more merozoites into the bloodstream, causing lysis of the host cell (see Figure 1.6 for a detailed explanation of the erythrocytic cycle).

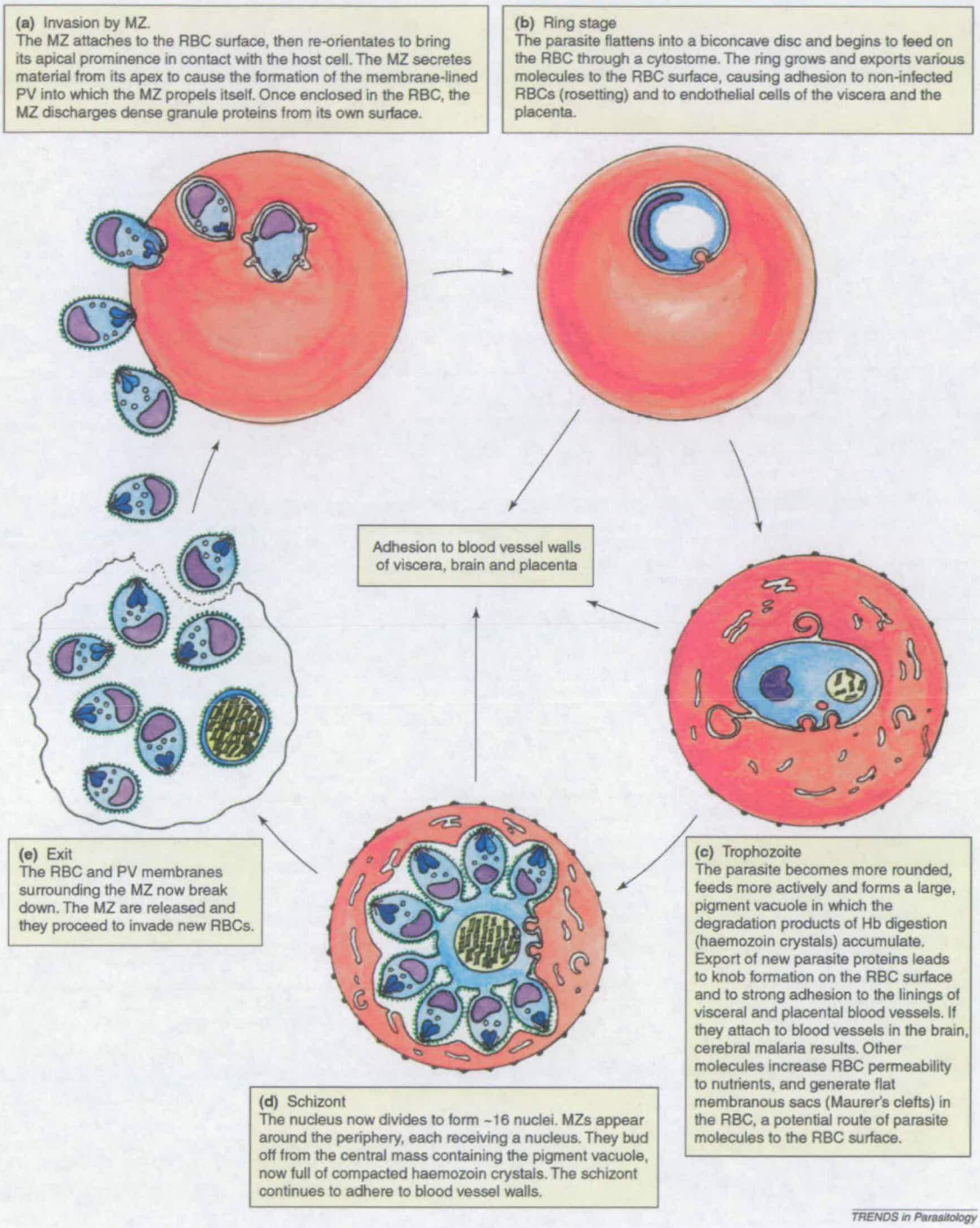


Figure 1.6 The erythrocytic cycle of *P. falciparum* (Bannister and Mitchell, 2003)

As well as releasing merozoites (that are able to invade more red blood cells), some parasites undergo gametocytogenesis within the erythrocyte, producing micro- and macrogametocytes, male and female respectively, that remain in the blood circulation

where they are available for ingestion by a feeding mosquito. Inside the insect mid-gut the gametocytes undergo gametogenesis, in which the female macrogametocyte escapes from the erythrocyte membrane, and the male microgametocyte undergoes the process of exflagellation which produces 8 motile microgametes. The micro- and macro-gametes fuse to form a zygote that in turn becomes an ookinete (the only diploid stages of the parasite) that crosses the gut wall and encysts. This oocyst then undergoes several rounds of division, producing thousands of haploid sporozoites that are subsequently released into the haemocoel of the mosquito where they migrate to the salivary glands (Barnwell and Galinski, 1998; Beier and Vanderberg, 1998; Frevert and Crisanti A., 1998; Sinden, 1998).

1.5 Clinical manifestations of the disease

The physical symptoms of infection with malaria parasites vary with a number of factors, the two most important being the species of parasite and the immune status of the host. As the parasites proliferate during the erythrocytic cycle, and the percentage of infected erythrocytes increases, patients typically experience headaches, chills, joint pains and, characteristically, fever. The chills and fever (known as a paroxysm) are typically periodic, recurring at intervals characteristic of the species of parasite. A paroxysm lasts for 4-12 hours, after which time there is typically a cessation of symptoms for a period, before another wave of paroxysm. These paroxysms are extremely stressful for patients, which begin with an intense feeling of cold, and which can last for up to an hour. After this, the body temperature begins to rise and can reach to between 39 and 41°C. Associated with this fever are feelings of nausea and sometimes convulsions. The paroxysms are associated with the rupture of infected erythrocytes and the release of merozoites into the blood stream, and the length of the inter-paroxysm period is defined by the parasite species, with *P. vivax, P. falciparum* and *P. ovale* cycling every 48 hours, and *P. malariae* every 60 hours.

The most severe manifestations of the disease, such as cerebral malaria, severe anaemia, splenomegaly, hepatomegaly and nephritic syndromes are usually associated only with *P.*

falciparum. Cerebral malaria is, at least in part, caused by sequestration of the parasites in the capillary vasculature in the brain. This is thought to occur due to parasitized erythrocytes binding to each other (rosetting) (Pongponratn et al., 1991), and to the endothelium of the capillaries themselves (Macpherson et al., 1985) resulting in hypoxia. Another factor contributing to the severity of cerebral malaria is the release by the host of large amounts of toxic cytokines (Clark and Schofield, 2000). This combination of hypoxia and elevated cytokine levels has been shown to increase levels of nitric oxide synthase which in turn generates large amounts of pathogenic nitric oxide (Melillo et al., 1995). Severe anaemia is caused by the destruction of erythrocytes infected by the parasite, but there is also evidence that the presence of the parasite reduces the host's capacity to produce more erythrocytes by suppressing the response of bone marrow to erythropoietin (Kurtzhals et al., 1997).

1.6 Natural history of the malaria parasites

The four human malaria parasites are members of a genus that includes some 180 species (Ash and Orihel, 1990). The genus *Plasmodium* is thought to have evolved from a coccidian ancestor that was parasitic on an insect host (Garnham, 1966). The *Plasmodium* genus is a member of the phylum Apicomplexa, the extant members of which are obligate intra-cellular animal parasites. The Apicomplexa are themselves members of the Alveolata superphylum which includes ciliates, dinoflagellates, perkinsida and marine alveolates. The phylum consists of 5000 described species, but there may be as many as 60,000 still to be described (Escalante and Ayala, 1995). All members of the Apicomplexa contain a vestigial plastid called an apicoplast - a discrete organelle bound by three or four membranes (Kohler et al., 1997) (see Figure 1.5). The origin of this organelle is likely to have been in a "secondary endosymbiotic event" in which the ancestor of all Apicomplexans engulfed a photosynthetic unicellular alga and retained the algal plastid (which was itself the product of a previous endosymbiotic event) (Kohler et al., 1997; Palmer and Delwiche, 1996; Wilson et al., 1996). Supporting this theory is the evidence that present day Apicomplexa such as *Plasmodium* and *Toxoplasma* have highly

organized cytoplasmic compartments which are associated with organisms of a phagotrophic nature (Robibaro et al., 2001).

The Apicomplexa phylum is thought to be very ancient, and may have originated earlier than the multicellular kingdoms of plants, fungi and animals. The *Plasmodium* lineage may have diverged several hundred million years ago (before the appearance of vertebrates), with the radiation that gave rise to human parasites beginning around 130 million years ago (Escalante and Ayala, 1995). Digenetic parasitism (requiring an alternation of hosts), a feature of *Plasmodium* and other Apicomplexa, may have evolved independently several times within the phylum (Barta, 1989; Escalante and Ayala, 1995). The phylogenetic relationship of the extant human parasites is somewhat contentious, with debate ongoing about the origin and age of *P. falciparum* in particular (see Tanabe et al. (2004) for the most recent addition to the literature). An extensive phylogeny of malaria parasites is presented in Figure 1.7 (Perkins and Schall, 2002).

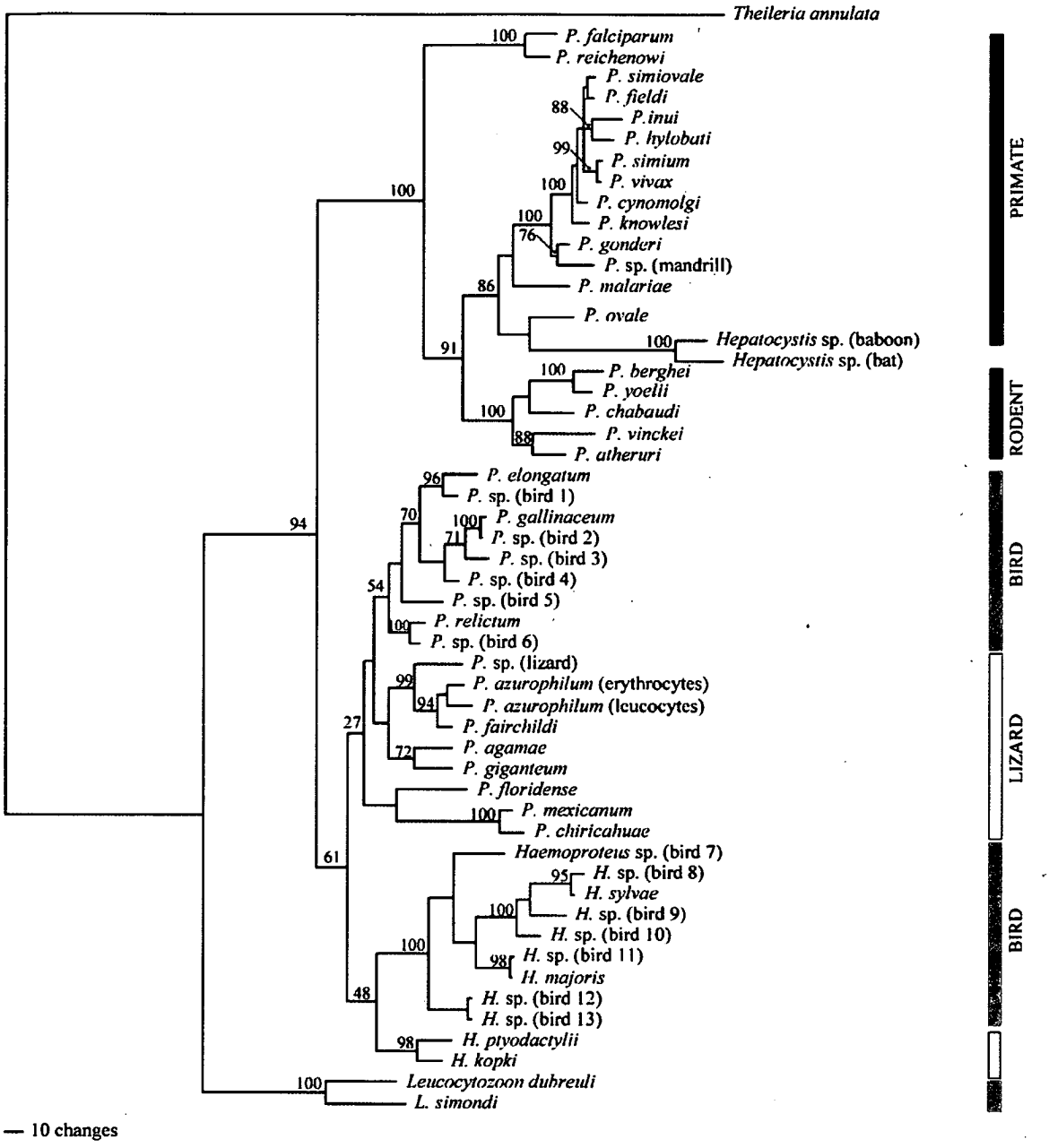


Figure 1.7 Phylogeny of malaria parasites using mitochondrial cytochrome *b* gene. Vertebrate class exploited as the parasite's host is indicated with bars. This phylogeny is rooted with *Theileria annulata* (a related Apicomplexan parasite). Branch lengths are proportionate to number of nucleotide changes (Perkins and Schall, 2002)

1.7 The genetics of malaria parasites

Malaria parasites are, like all members of the Apicomplexa, haploid for almost their entire life cycle. An exception is the zygote stage, which is diploid prior to the meiotic division that results in the production of sporozoites. The parasite possesses three individual genomes; an extra-chromosomal mitochondrial genome, known as the 6kb element, a 35kb circular genome associated with the apicoplast, and a large nuclear genome. The mitochondrial genome encodes two truncated ribosomal RNAs and three components of respiration (Cytochrome *c* oxidase subunits I and II, and cytochrome *b* (Funes et al., 2004). The apicoplast genome encodes 30 proteins which are primarily involved in gene expression (Funes et al., 2004; Gardner et al., 2002). It has been shown, however, that about 10% of the genes encoded by the nuclear genome are targeted to the apicoplast, supplementing its proteome via post-translational targeting into the organelle (Gardner et al., 2002; Roos et al., 1999b; Zuegge et al., 2001). The exact role of the apicoplast remains unclear, but it is known to be involved in the anabolic synthesis of fatty acids, isoprenoids and haem. (Gardner *et al.*, 2002).

The nuclear genome of *P. falciparum* consists of 14 chromosomes and encodes about 5,300 genes with a total genome size of 22.8 megabases. It is extremely (A+T)-rich, with an overall (A+T) content of 80.6%, rising to 90% in intronic and intergenic regions (Gardner *et al.*, 2002). There is considerable chromosomal size polymorphism between strains of parasites (Corcoran and Kemp, 1986), which could be due to unequal crossing-over of homologous chromosomes during meiosis, or non-meiotic chromosome breaking and healing events (Babiker et al., 1994; Gardner et al., 2002; HernandezRivas et al., 1996; Scherf et al., 1992).

Malaria parasites exhibit extensive genetic polymorphism between species and between strains within species. Some of these genetic polymorphisms manifest themselves as phenotypic differences between strains for quantifiable traits such as virulence and susceptibility to drugs. This genetic diversity is subject to natural selection, which is most intuitively apparent in the way that drug-resistance appears and spreads under

strong drug pressure. This diversity arises both by spontaneous genetic mutations, and by novel genotypes arising through recombination. The meiotic division and recombination which the parasites undergo in the mosquito can result in independent assortment of genes on different chromosomes, crossing-over events between linked genes on homologous chromosomes (both producing novel genotypes), and intragenic recombination (resulting in novel alleles of genes) (Walliker et al., 1998).

Meiosis occurs after the fusing of the micro- and macrogametocytes to form a zygote. If gametocytes from two genetically distinct parasites are taken up by the mosquito, then crossing resulting in heterozygous zygotes can occur. Each heterozygote will, through the process of meiosis, produce 4 genetically distinct haploid daughter cells (recombinant progeny). If there are equal numbers of male and female gametes present from each parental strain in the mosquito, then selfing will occur 50% of the time, resulting in 25% of the zygotes being genetically identical to one parental strain, and 25% identical to the other. The remaining 50% will be hybrid between the two parentals (see Figure 1.8).

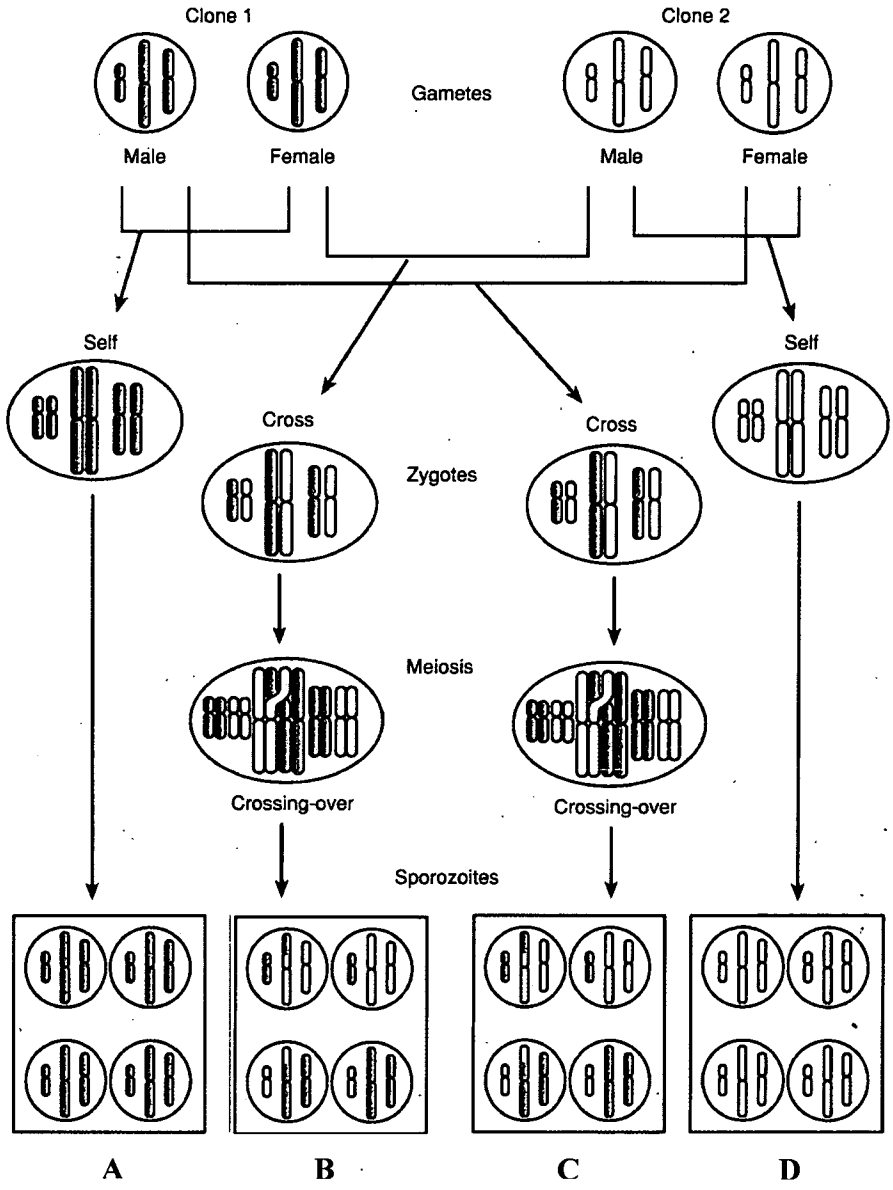


Figure 1.8 Crossing and chromosomal events in Plasmodium. A and D show the results of selfing, while B and C show the products of recombination (Walliker, 2000).

Recombination between malaria parasites was first formally proven with genetic crossing experiments conducted with the rodent parasites *Plasmodium yoelii* (Walliker et al., 1971), and *Plasmodium chabaudi* (Walliker et al., 1975a). In the *P. chabaudi* experiments, two cloned parasites which differed in their response to the anti-malarial drug pyrimethamine and in the electrophoretic patterns of two enzymes (6-phosphogluconate dehydrogenase (6-PGD) and lactate dehydrogenase (LDH)) were

mixed in mosquitoes and the resulting progeny were cloned and characterised for their enzyme type and their phenotypic response to pyrimethamine. It was found that not only had the two enzyme isoforms recombined, but that pyrimethamine susceptibility segregated independently (Walliker et al., 1975a). This showed that crossing between gametes, and recombination between the parental characters had occurred.

The production of heterozygotes (in the oocyst or zygote stage in the mosquito) between two heterologous malaria parasites has been shown to occur experimentally with *P. falciparum* (Ranford-Cartwright et al., 1993). This study involved the typing of alleles of *msp-1* and *msp-2* from individual oocysts dissected from mosquitoes that had fed on a mixed blood infection of clones 3D7 and HB3. It was found that some oocysts contained alleles exclusively from HB3, some contained alleles only from 3D7, and the remainder of the oocysts contained alleles from both parents, and were therefore hybrids - the products of fertilisation between the two different parental strains. The proportion of the homozygous and heterozygous forms was consistent with random fertilisation between parents (Ranford-Cartwright *et al.*, 1993).

Evidence for intragenic recombination resulting in novel alleles of genes was demonstrated by the production of a novel *msp-1* gene resulting from a crossing over event in a conserved region of the gene (Kerr et al., 1994). The possibility of intragenic recombination during mitosis has also been proposed, based on studies of the gene encoding circumsporozoite protein (*csp*) (Rich et al., 1997).

The inheritance of the 6 kb element appears to follow the same pattern as other mitochondrial genomes in eukaryotes – it is inherited from the female parent only (1994; Creasey et al., 1993).

The complete genome of *Plasmodium falciparum* (clone 3D7) was published in 2002, and heralded the "post-genomic" age of malariology (Gardner *et al.*, 2002). The genome presents researchers with a powerful new resource with which to work toward new drug and vaccine development.

1.8 Control strategies

Current methods used for the control of malaria invariably rely on prevention through minimizing exposure to mosquitoes, and drug treatment of clinical disease. The fact that the parasite has to rely on an insect vector for its transmission immediately highlights a weakness that may be currently under-exploited. Malaria can be stopped by attacking the vector host effectively, as was proven by the WHO's (ultimately abandoned) eradication campaign in the early 1950s. By residual spraying of effective insecticides such as DDT, it is possible to reduce mosquito numbers to levels which do not permit the transmission of the parasite.

Large scale public anti-mosquito measures such as the introduction of larvicides to breeding sites, non-selective residual insecticide spraying and effective drainage of potential breeding sites have all achieved the successful reduction of malaria in the past. Before the wide-scale use of DDT in India, for example, there were 75 million malaria cases and 0.8 million deaths from the disease yearly. This fell to 100,000 cases per year during the 1960s when around 18,000 tonnes of DDT were used annually. By the year 2000, when DDT use had been considerably reduced due to the development of resistance in mosquitoes and environmental concerns, there were over 3 million incidences of the disease (Sharma GK, 1987).

Personal anti-mosquito measures such as insecticide treated bed-nets have also been shown to greatly reduce the prevalence of disease in areas where they are used correctly. Admittedly, anti-mosquito control strategies work best in areas of low transmission, as shown by the effective elimination of the disease from many temperate countries in the latter half of the 20^{th} century. There are powerful arguments, however, for the introduction of free insecticide treated bed-nets to areas of Africa where the disease is endemic in order to attempt to reduce the terrible burden inflicted by the disease on children.

There are, however, many problems associated with effective anti-mosquito measures. The indiscriminate use of large amounts of insecticides may have environmental repercussions, and outcry by powerful environmental lobbyists, had, rightly or wrongly, greatly reduced the (very effective) use of residual DDT spraying until the United Nations Environmental Program sanctioned its use for malaria control (but not for agriculture) in 2000 (Taverne J, 2001)*. Mosquitoes have evolved resistance to widely used insecticides, both biologically and behaviorally. For example, the rationale behind spraying the inner walls of houses with insecticide was that mosquitoes settle there after taking a blood-meal. Extensive implementation of this technique was shown to select for those mosquitoes that flew straight out of the window after feeding. Other problems include the need to educate local populations effectively about the correct way to implement personal control methods, especially the need to maintain effective bed-nets.

1.8.1 Anti-malarial drugs

In areas of high transmission, where large numbers of malaria cases are presently inevitable, there is a reliance on anti-malarial drugs to treat the disease. The drugs used vary depending on the local conditions (prevalence and specificity of drug resistance, economic status, etc). Table 1.1 lists the main groups of anti-malarial drugs used today.

* For a balanced review of the arguments for and against the use of DDT see Curtis and Lines (2000)

Main groups of antimalarial drug in use	
Generic Chemical Group	**Example**
antifolic drugs	pyrimethamine, proguanil
sulpha drugs	a) sulphones e.g. dapsone b) sulphonamides e.g. sulphadoxine
cinchona alkaloids	Quinine
4-aminoquinolines	chloroquine, amodiaquine
8-aminoquinolines	Primaquine
4-quinoline methanols	Mefloquine
9-phenanthrene methanols	Halofantrine
sesquiterpene lactones	a) artemisinin b) artemisinin derivatives e.g. artemether
antibiotics	tetracycline, chloramphenicol
drug combinations	pyrimethamine + sulphadoxine ('Fansidar') pyrimethamine + sulphadoxine + mefloquine ('Fansimef') atovaquone + proguanil ('Malarone')

Table 1.1 The main groups of anti-malarial drugs in use today (adapted from http://www.ncbi.nlm.nih.gov/projects/Malaria/antimalarialdrug.html)

The following section discusses the problem of anti-malarial drug resistance, and focuses on our current understanding of the genetics behind pyrimethamine resistance.

1.9 Drug Resistance

The emergence and spread of parasite resistance to anti-malarial drugs has presented one of the largest obstacles hindering the effective treatment and control of malaria. The WHO's official definition of malaria parasite resistance dates from 1973; "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject" (World Health Organisation, 1973). Since then several authors,

including Basco & Ringwald (2000), have attempted to update this definition in the light of more advanced techniques available in the fields of genetics, pharmacology, and molecular biology that are able to elucidate factors such as the presence of genetic mutations that are linked to drug resistance *in vitro*, high IC50 values and varying plasma concentrations of drugs. The WHO (1973)'s definition of the *level* of parasite drug resistance remains in use;

> **Sensitive (S)**: The asexual parasite count reduces to 25% of the pre-treatment level in 48 hours after starting the treatment, and complete clearance after 7 days, without subsequent recrudescence - Complete Recovery.
>
> **RI Delayed Recrudescence**: The asexual parasitaemia reduces to < 25% of pre-treatment level in 48 hours, but reappears between 2-4 weeks.
>
> **RI Early Recrudescence**: The asexual parasitaemia reduces to < 25% of pre-treatment level in 48 hours, but reappears within 2 weeks.
>
> **RII Resistance**: Marked reduction in asexual parasitaemia (decrease >25% but <75%) in 48 hours, without complete clearance in 7 days.
>
> **RIII Resistance**: Minimal reduction in asexual parasitaemia, (decrease <25%) or an increase in parasitaemia after 48 hours.

A number of factors influence the likelihood of resistance occurring and the speed with which it spreads. The mechanism by which the drug works against the parasite is important; simple modes of action such as enzyme inhibition are likely to lead to rapid evolution of resistance, as the number of genetic mutations required to alter enzyme structure is low. This is the case with pyrimethamine resistance, which evolved very quickly after the introduction of the drug, in contrast to the pattern seen with the emergence of chloroquine resistance, which took much longer to evolve.

The pharmacokinetic dynamics of drugs are also important in determining the selection pressure for drug resistance. Watkins and Mosobo (1993), for example, showed that the long half-life of sulfadoxine-pyrimethamine was a considerable factor in the selection pressure for resistant mutants, as the drug was present in patients at sub-therapeutic levels for long periods of time. Drugs with high efficiencies of parasite killing, rapid

achievement of levels above the minimal inhibitory concentrations and short half-lives, will be the most effective at minimizing the selection pressure for resistant mutants (Winstanley et al., 2002).

Resistance has been recorded to every anti-malarial drug currently in use, with the exception (to date) of artemisinin and its derivatives. Quinine, the first drug used specifically to treat malaria was "discovered" in the 17th century by Jesuit priests in Peru, who identified the anti-fever properties of the bark of the Cinchona plant. The active alkaloid, quinine, was purified in Paris in 1820 by Pelletier and Caventou, and was used extensively as a treatment for malaria thereafter throughout the world. The first reports of resistance to the drug occurred at the beginning of the 20th century, when Couto (1908) and Nocht and Werner (1910) reported the treatment of patients who did not respond to quinine treatment (cited in Peters, (1987). Despite the early appearance of quinine resistance, the drug remains remarkably useful today, especially as a first line drug for treating complicated cerebral malaria. In fact, quinine resistance is surprisingly uncommon, and in the few instances it has emerged, it is often associated with parasites that are already resistant to other drugs such as chloroquine and mefloquine (Looareesuwan et al., 1990; Peters, 1987; Pukrittayakamee et al., 1994). Quite why quinine remains so effective to the present day is not clearly understood (Meshnick, 1997). Its use has certainly declined since the introduction of less unpleasant (in terms of their side-effects) anti-malarials, but even so, resistance to the drug seems very reluctant to emerge.

Chloroquine, itself based on the structure of quinine, was developed in Germany in the early 1940s, and was first used extensively shortly after the Second World War. Resistance to chloroquine was far more forthcoming than with quinine, and the first reports of parasites failing to respond to the drug emerged independently from South America and South East Asia in the late 1950s (Harinasuta et al., 1965; Moore and Lanier, 1961; Young and Moore, 1961). The spread of resistance from these pioneer areas was

relatively rapid (see Figure 1.9), and chloroquine resistance is now a major problem throughout the malaria affected areas of the world.

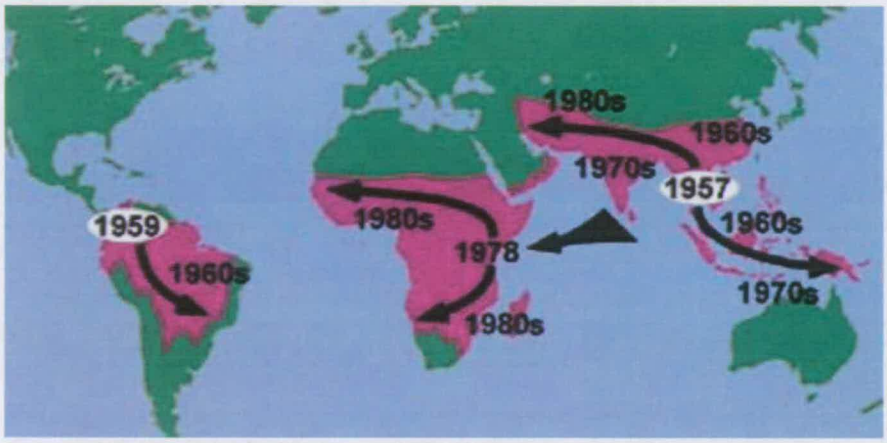


Figure 1.9 The emergence and distribution of chloroquine resistant malaria parasites. Source: http://www.rnw.nl/health/html/drug_resistance.html

One of the proposed mechanisms for the emergence of drug resistance is through the presence of a drug at sub-therapeutic levels within a population (see below). There can be no doubt that the emergence of chloroquine resistance in South America was facilitated by the policy of distributing chloroquinated salt to the area as part of a well-intentioned control problem. This resulted in a large proportion of the population being exposed to the drug at sub-curative doses, thus considerably enhancing the chances of selection of chloroquine resistant parasites (Payne, 1988). At a recent British Society of Parasitology meeting, a researcher was describing the difficulties of selecting artemisinin resistant parasites in rodents. One member of the audience suggested a method he was sure would work; "add the drug to the rodent's salt" he advised.

The proliferation of chloroquine resistance prompted the US Army to search for a more effective alternative drug. A drug research programme was established specifically for this goal in the early 1960s, and resulted in the development of mefloquine, a drug that was effective against chloroquine resistant parasites. Initial indications that mefloquine resistance was likely to emerge came, however, in 1977, when resistance was experimentally induced in a rodent malaria parasite (Peters et al., 1977). Efforts to reduce the possibility of the emergence of resistant parasites in the field by using mefloquine in

combination with other drugs (especially pyrimethamine) met with failure, however, and reports of mefloquine resistant parasites emerged throughout the 1980s (reviewed in Peters (1998)). Introduced as a first line treatment to Thailand in 1984, substantial resistance had developed within 6 years (Price et al., 2004). Mefloquine resistance is now widespread in South East Asia, and there is a particularly high incidence in the Thailand/Cambodia/Mayanmar borders (Wernsdorfer, 1994).

1.9.1 Resistance to pyrimethamine

Pyrimethamine was one of two highly effective antifolate malarial drugs first introduced in 1952 (the other being proguanil). Hopes were high for this new weapon against malaria, as it was cheap to produce, and had been shown to be highly effective against *Plasmodium* spp. in relatively low concentrations. There was little toxicity to humans using the drug, and side-effects were negligible. Soon after its introduction, however, reports of pyrimethamine resistant *P. falciparum* emerged, when in 1952 treatment at established drug-concentrations failed to cure a baby in the Gambia (McGregor and Smith, 1952), reviewed in Peters (1987)). Soon after this case, several more reports of drug-tolerant malaria appeared from different areas around the world. Resistance to pyrimethamine is, at present, widespread in all malaria endemic areas (Talisuna et al., 2004). One interesting aspect of the emergence of pyrimethamine resistance is the fact that it appears to have arisen independently in several locations around the world very shortly after its introduction.

Pyrimethamine belongs to a group of drugs known as the "antifolates" as they target the folate biosynthesis pathway of the parasite (see Figure 1.10). Other drugs in this category include proguanil (and its active form, cycloguanil, which is metabolised from proguanil *in vivo*), the sulfonamides including sulfadoxine and sulfalene, and the sulfone, dapsone. Pyrimethamine and proguanil resistance arose in response to the extensive use of the drugs on their own. In order to minimize the development of drug resistance, and also to exploit the synergistic properties of antifolate and sulfonamide drugs (Chulay et al.,

1984), pyrimethmine is now commonly used in combination with sulfadoxine (Fansidar®).

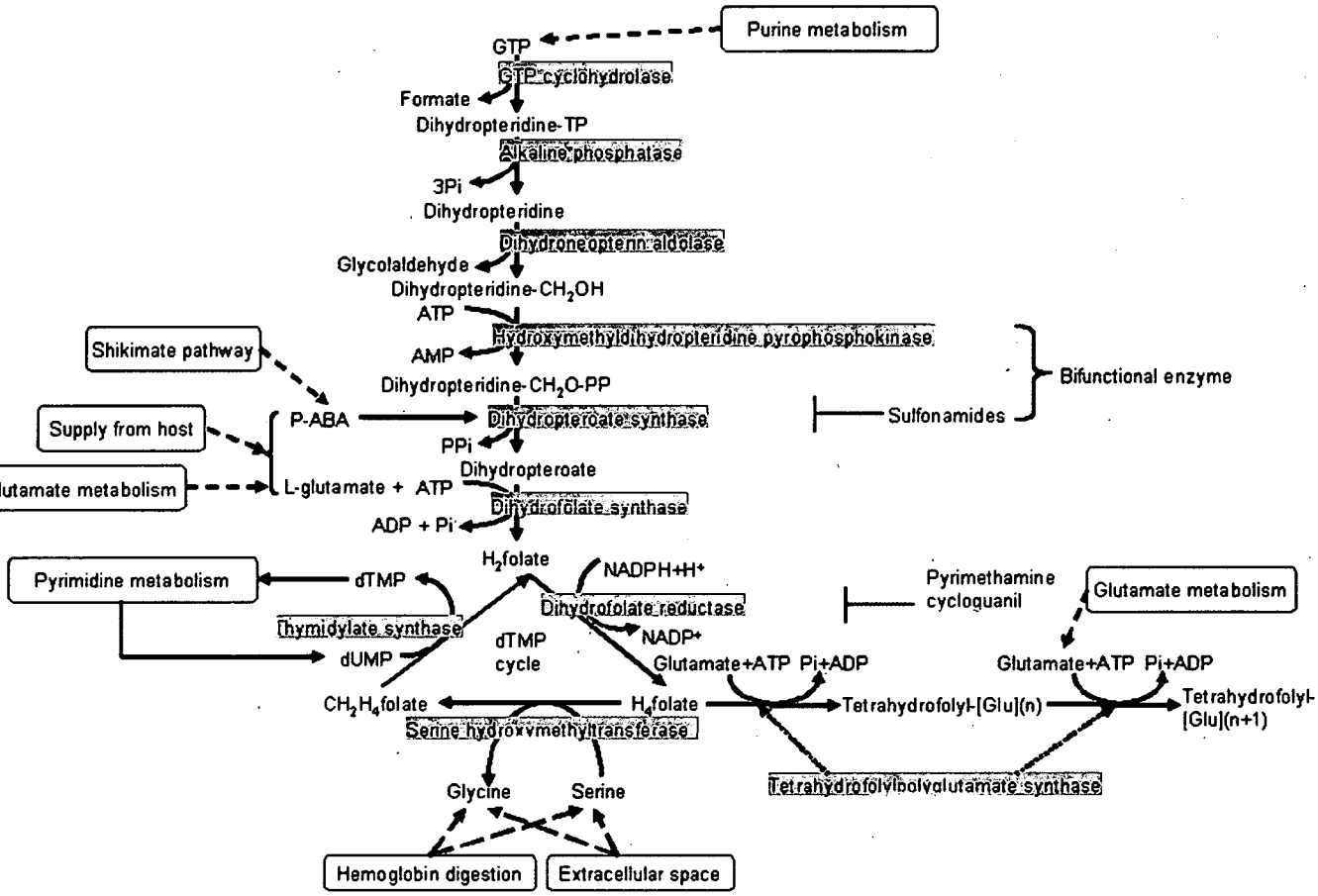


Figure 1.10 The folate biosynthesis pathway of *P. falciparum*, showing the stages at which inhibition by pyrimethamine, cycloguanil and the sulfonamides occurs (adapted from http://sites.huji.ac.il/malaria/maps/folatebiopath.html).

Malaria parasites are able to scavenge purines, but not pyrimidines from their hosts (Le Bras and Durand, 2003). They must, therefore, synthesise the latter themselves, a process that involves the folate biosynthesis pathway. Pyrimethamine works by competitively inhibiting the dihydrofolate-reductase enzyme (DHFR) which is used by the parasite to synthesise thymidylate (dTMP) from dihydrofolate (Ferone, 1977). Specifically, DHFR catalyses the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-

tetrahydrofolate (Sirawaraporn et al., 1997a). Pyrimethamine works by binding to the active enzyme site of DHFR, preventing the binding of the natural substrate of DHFR (dihydrofolic acid). The chemical structure of pyrimethamine and dihydrofolic acid are shown in Figure 1.11.

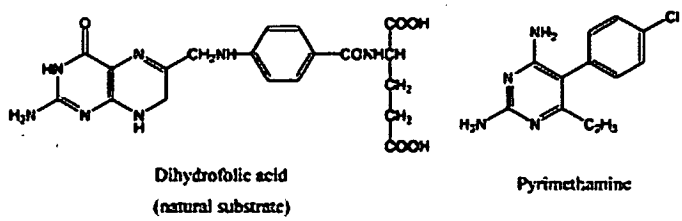


Figure 1.11 The chemical structures of the natural substrate of DHFR (dihydrofolic acid) and of pyrimethamine. From Yuthavong (2002).

The inhibition of DHFR gives rise to thymidylate depletion with ensuing disruption of DNA synthesis and resultant parasite death. DHFR is required for folate metabolism in both the host and the parasite, but pyrimethamine has a much higher affinity for binding to the parasites DHFR (several hundred times), and so disrupts the parasite without adversely affecting the host (Olliaro and Yuthavong, 1999).

The rapid way in which pyrimethamine resistance arose in the field suggested that a single point mutation might be responsible. This was proven correct by many different researchers working on a variety of malaria parasites including *in vitro* culture of *P. falciparum*, and murine models *P. chabaudi*, *P.berghei* and *P. yoelii* (Bzik et al., 1987; Cowman et al., 1988; Diggens et al., 1970; Foote et al., 1990; Morgan, 1972; Peterson et al., 1990; Rosario et al., 1976). Conclusive proof that a mutant allele of the DHFR gene confered pyrimethamine resistance to *P. falciparum* was obtained through transfection studies in 1996 (Wu et al.).

Resistance to the drug has been to shown to be conferred by cumulative single nucleotide mutations in the *dhfr* gene (Cowman et al., 1988; Foote et al., 1990; Peterson et al., 1990; Sirawaraporn et al., 1997a). Changes in the amino acid sequence of this gene reduce the ability of pyrimethamine to bind to the DHFR enzyme. There are 4 single nucleotide

mutations causing amino acid replacements associated with pyrimethamine resistance in the wild; a change from serine to asparagine at amino acid position 108 (S108N), from asparagine to isoleucine at position 51 (N51I), from cysteine to arginine at position 59 (C59R), and from isoleucine to leucine at position 164 (I164L). It is thought that these mutations arise sequentially, starting with S108N, as this mutation confers a 10-fold increase in the K_i value for pyrimethamine (the dissociation constant of the enzyme-inhibitor complex) , while maintaining similar kinetic parameters to the wild type enzyme (Sirawaraporn et al., 1997a). This is the only single mutation seen in the field. Each sequential mutation adds further increases in K_i values, with the quadruple mutant exhibiting the highest value (Figure 1.12)

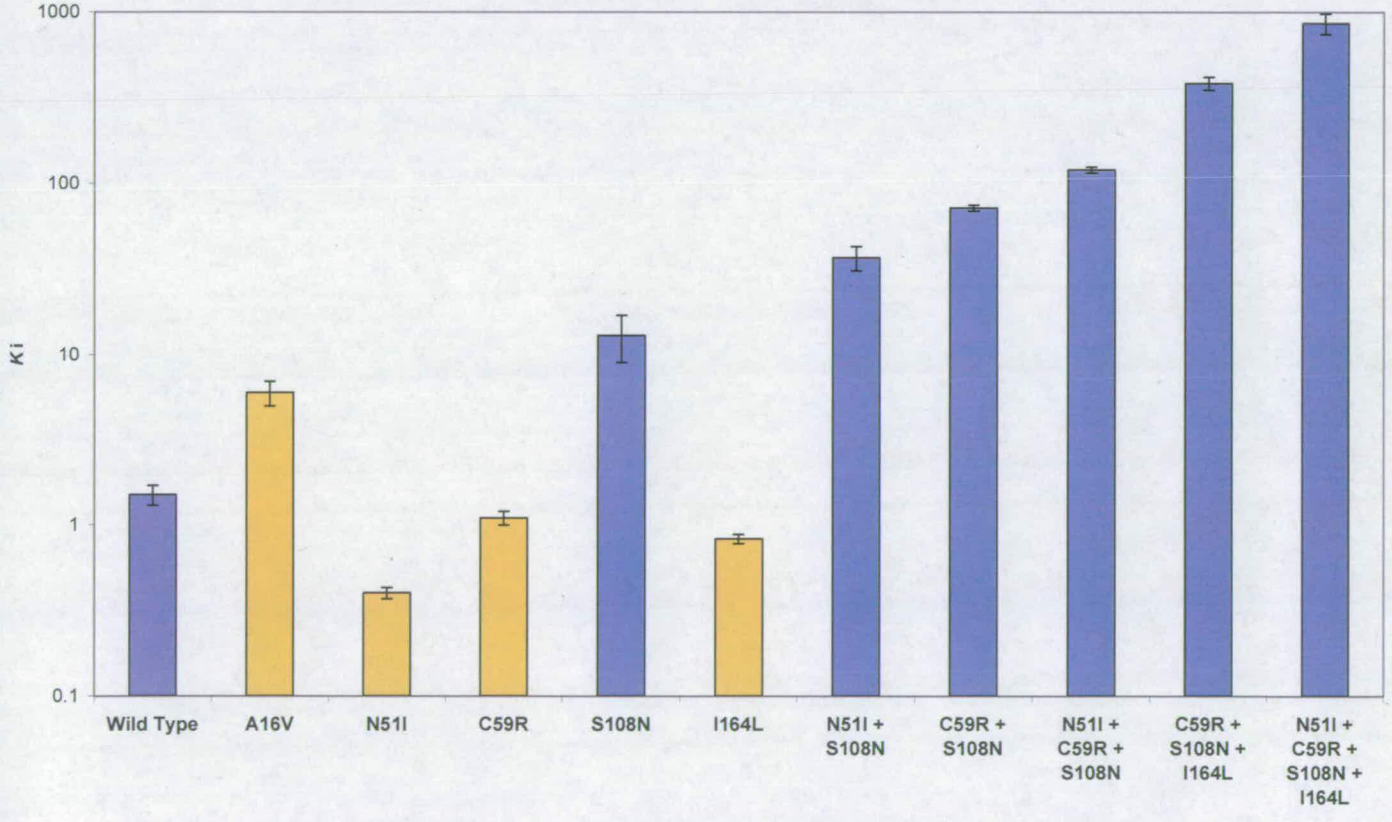


Figure 1.12 Inhibition by pyrimethamine, *K*i (nM), of enzymes encoded by the various mutant alleles of *dhfr* that arise to confer drug resistance. Orange bars show mutants which do not appear in the field. Data for this figure was obtained from Sirawaraporn (1997a).

It is likely that the evolution of increased resistance to pyrimethamine involves the initial S108N mutation arising in a single event, followed by subsequent mutations at the other positions in sequential fashion (Figure 1.13). Each further mutation in the gene acts synergistically with the preceding mutations to increase the overall resistance of the parasite. Indeed, it has been shown that each mutation in a double, triple or quadruple mutant allele contributes more to the overall resistance of the enzyme to pyrimethamine than the sum of the component mutations (Sirawaraporn et al., 1997a).

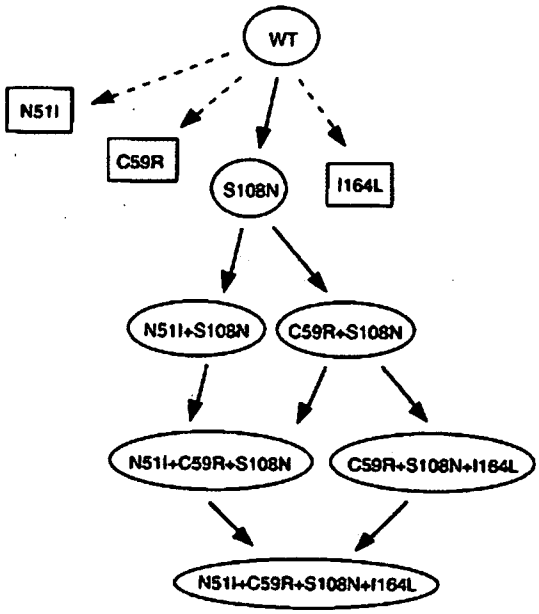


Figure 1.13 Proposed sequence of sequential evolution of *dhfr* mutants, beginning with the wild type (WT) and progressing to the quadruple mutant. Broken arrows shows single amino acid polymorphisms that do not occur in the wild. Adapted from Sirawaraporn et al (1997a).

There is evidence to suggest that there are other mechanisms, apart from the mutations described above, which may contribute to pyrimethamine resistance. Chromosomal rearrangement and gene amplification were found to have occurred in *in vitro* cultures which were subjected to increased levels of pyrimethamine over long periods (Thaithong et al., 2001). More recently it has been shown that pyrimethamine sensitive *P. falciparum* parasites are able to up-regulate DHFR enzyme translation upon challenge with pyrimethamine (Nirmalan et al., 2004).

As previously mentioned, the speed with which parasites developed resistance to pyrimethamine in the field led to the policy of combining the drug with sulfadoxine (Fansidar®). This drug also targets the folate biosynthesis pathway, competitively inhibiting dihydropteroate synthase (DHPS) (see Figure 1.10). Thus, the pyrimethamine-sulfadoxine combination acts synergistically, and any loss of efficiency in either component results in the reduction of the effectiveness of the combination as whole. For this reason, it has been suggested that pyrimethamine-sulfadoxine should not be considered a real "combination" therapy in the sense that the two components do not act independently with different mechanisms of action (Sibley et al., 2001).

1.9.2 Fitness cost of resistance

The development of resistance to drugs such as pyrimethamine involves the mutation of genes in a "wild type" parasite into alleles that encode modified protein products. It is possible that such mutations may render the parasite less fit than the wild type parasite in the absence of drug selection (Walliker et al., 2005). The emergence and survival of drug resistant mutants depends on a number of factors including; recombination between resistant loci (if resistance is controlled by more than one locus), the geographical transmission dynamics of the parasites, the strength of the selection pressure imposed by the drug and *the effect of natural selection on the mutant* (assumed to be unfavourable, indicating a fitness cost) (Mackinnon, 1997). Various theoretical models of the spread of drug resistance have identified natural selection against resistant mutants as an important factor determining the rate of emergence of drug resistance (Hastings, 1997; Hastings and D'Alessandro, 2000; Hastings and Mackinnon, 1998). In a study by Nair et al (2003), it was found that, contrary to expectations, pyrimethamine resistance had spread to a large geographical area of Southeast Asia from a single point of origin rather than by many independent mutations in different regions. This was unexpected, as the mutations that lead to pyrimethamine resistance are few, and so would be expected to arise frequently and independently within the parasite population. The explanation favoured by Nair et al, and supported by Hastings (2004), is that most of these mutations would have been eliminated by natural selection except for the extremely rare case in which such a

mutation was accompanied by a "compensatory" mutation, which increases the mutant's intrinsic fitness to levels comparable with the wild type.

Drug resistance mutations that carry a fitness cost might be expected to lead to the removal of drug resistant parasites through natural selection in the absence of drug pressure. It has been suggested that the cessation of a drug to which resistance has emerged would lead to the competitive exclusion of resistant parasites, returning the parasite population to sensitivity (Hayward et al., 2005). It has been proposed, therefore, that a time-fixed cycle of different anti-malarial drugs could diminish the impact of drug-resistant parasites on human health. Such a conclusion is not, however, supported by the findings of Clyde et al (1967) who reported the presence of pyrimethamine parasites 10 years after the withdrawal of the drug in Tanzania.

Fitness costs associated with drug resistance have been documented for numerous organisms. Studies on *Escherichia coli* have shown that streptomycin resistant parasites bear a substantial fitness cost associated with the ribosomal mutations that confer resistance (Bilgin et al., 1992; Zengel et al., 1977). Rifampin resistance carries a physiological cost to *Mycobacterium tuberculosis* (Billington et al., 1999), the replication rate of the HIV virus with multiple drug resistance-associated mutations is retarded (Devereux et al., 2001), and mosquitoes (*Culex pipiens*) resistant to organophosphates have been shown to be less fit than their wild type contemporaries (Wang et al., 1998). The evidence for fitness costs associated with drug resistance is not confined to parasites and pathogens, however, as Baucom and Maurico (2004) show that weedy Morning Glory (*Ipomoea purpurea*) can develop resistance to the herbicide glyphosate, but that resistant plants produce fewer seeds than susceptible varieties.

Evidence for fitness costs associated with drug resistance in malaria parasites is surprisingly sparse, given the large volume of research dedicated to the parasite and to drug resistance in particular. Circumstantial evidence from the field suggests there may be a cost levied by resistance to chloroquine among *P falciparum* parasites; studies from Hainan island in China (Liu et al., 1995) and Vietnam (Thanh et al., 2001) both show the

re-emergence of chloroquine sensitive parasites in areas with previously high rates of resistance (the former reporting a prevalence of 84% resistant parasites in 1981, dropping to 40% in 1991 after the cessation of chloroquine usage in 1979). The same pattern has been reported from Malawi which switched its first line drug treatment from chloroquine to sulfadoxine-pyrimethamine in 1993 due to an 80% failure rate of chloroquine treatment. It was shown that 7 years later the prevelance of the K76T *pfcrt* mutation (a marker for chloroquine resistance) was substantially lower (9%) than in other countries where chloroquine use had continued (41-80%) (Mita et al., 2003). A further survey from a different area of Malawi found a reduction in the proportion of parasites carrying the same allele from 85% in 1992 to 13% in 2000 (Kublin et al., 2003a). Two possible mechanisms for the re-emergence of sensitivity were proposed: Firstly, back mutation of resistant parasites, and secondly, the resistant parasites were out-competed by fitter chloroquine-sensitive parasites after the removal of the drug pressure. Extensive sequence analysis by Mita et al (2004) favoured the latter explanation, estimating the relative fitness of the resistant compared to the sensitive parasites to be 0.76 in the absence of chloroquine.

Direct experimental evidence for a fitness cost associated with atovaquone resistance was presented by Peters et al (2002). Atovaquone resistance is conferred by mutations in the cytochrome *b* genes of *P falciparum* (Korsinczky et al., 2000), and in much the same way as for pyrimethamine resistance, there is a cumulative effect of single amino acid polymorphisms leading to increased levels of resistance. Peters et al. (2002) considered 2 mutants; one in which there is a single mutation in the gene (a change from methionine to isoleucine at amino acid position 133 (M133I)), and a double mutant which includes the M133I mutation and a further mutation at position 280 from glycine to aspartic acid (G280D). The single mutant had previously been shown to have a 25-fold increase in resistance to atovaquone, and the double mutant an 800-fold increase compared to the wild-type (Korsinczky *et al.*, 2000). Direct competition through mixed infections in culture between wild-type parasites and the two resistant mutants revealed a 5-9% reduction in growth rate between the double mutant and the wild-type, but no difference in the growth rates of the single mutant and the wild-type.

It was suggested that the way in which the mutations altered the structure of the cytochrome *b* enzyme was responsible for the difference in fitness between the mutants. Both mutations confer drug resistance by reducing the binding affinity of cytochrome *b* to atovaquone. Mutation M133I, however, achieves this reduction in atovaquone affinity without altering the enzyme's affinity for ubiquinone (its natural substrate), whereas mutation G280D, whilst conferring higher levels of drug resistance also lowered the ability of cytochrome *b* to bind to ubiquinone.

There are few data available on the potential fitness cost associated with pyrimethamine resistance in malaria parasites. Sirawaraporn et al (1997a) conducted an extensive experiment in which kinetic analyses were performed on each of the DHFR isoforms encoded by the various *dhfr* mutants (described in Figure 1.11). They showed that for the majority of the naturally occurring resistant mutants, the k_{cat}/K_m values (the maximal catalytic rate of the enzyme when substrate is saturating) of the enzyme isoforms decreased by 2- 50-fold (Figure 1.14)

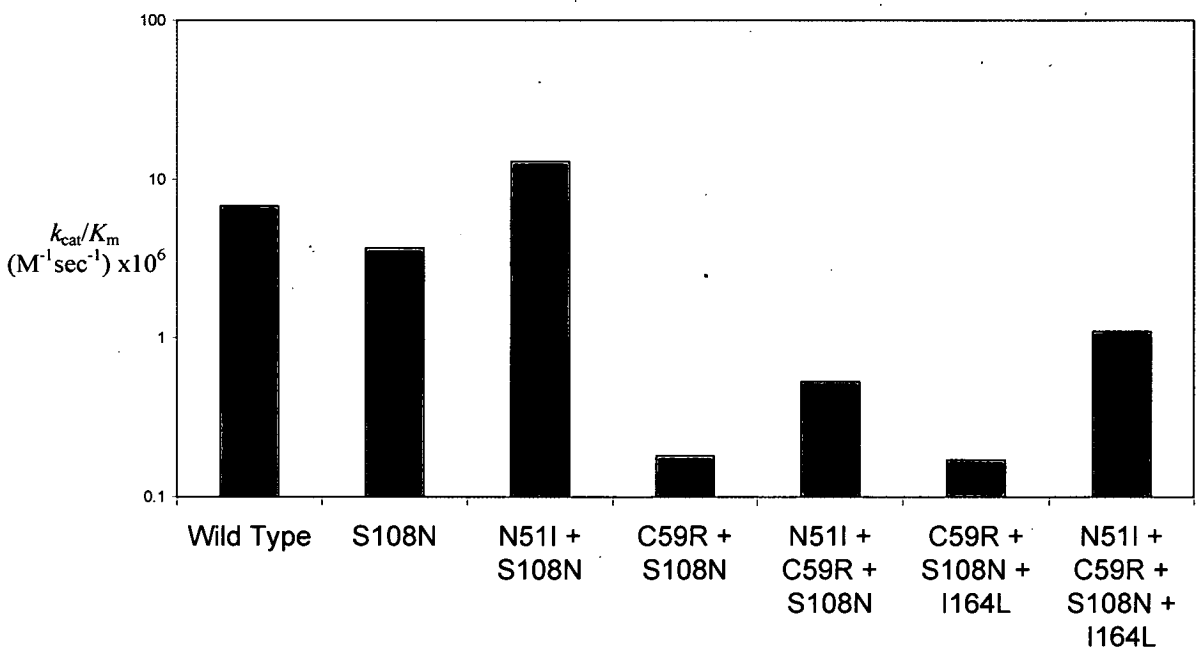


Figure 1.14 k_{cat}/K_m values of the enzyme isoforms associated with the various mutations in the *dhfr* gene, calculated from K_m values for dihydrofolate. Data for this figure was obtained from Sirawaraporn (1997a)

There is very little difference between the k_{cat}/K_m values for the wild type, the S108N and the N51I + S108N mutation carrying parasites, but the other naturally occurring mutants show large reductions in their catalytic rates. This reduction in the efficiency of resistant enzymes suggests that there may be fitness costs associated with these resistant mutations, although this cannot be proven without competing the mutants against the wild type parasites in a mixed infection.

In a study on the rodent malaria parasite *P. berghei*, pyrimethamine resistant parasites displayed no growth rate reduction compared to sensitive parasites during the erythrocytic cycle in mice, but did proceed through the sporogonic development at a reduced rate compared to sensitive parasites (Shinondo et al., 1994). Earlier work with chloroquine and pyrimethamine resistant *P. chabaudi* parasites by Rosario et al (1978), in which sensitive parasites were competed with isogenic resistant clones in mixed infections in mice, showed that whereas pyrimethamine resistant parasites were out-competed by sensitive clones in the absence of drug treatment, chloroquine resistant parasites appeared to posses a selection advantage over the sensitive parasites.

In perhaps the most extensive investigation into fitness costs associated with pyrimethamine resistance to date, Fohl & Roos (2003) describe experiments with *Toxoplasma gondii* parasites in which pyrimethamine resistant mutants were grown in competition, both *in vitro* and in mice, with sensitive parasites. *T. gondii* is an Apicomplexan parasite related to *Plasmodium*, which, although an important human and veterinary pathogen in its own right, has been used as a model for *P. falciparum* (Roos, 1996; Roos et al., 1999a). Fohl & Roos (2003) engineered allelic replacements in *T. gondii* parasites at the *dhfr* locus that were analogous to the pyrimethamine resistant mutations observed in *P. falciparum*, and competed the mutants (S108N + C59R) against wild type parasites both *in vivo* (in mice) and through *in vitro* culture. They found that the pyrimethamine resistant mutants had a significant growth rate disadvantage compared to the wild type parasites (Figure 1.15)

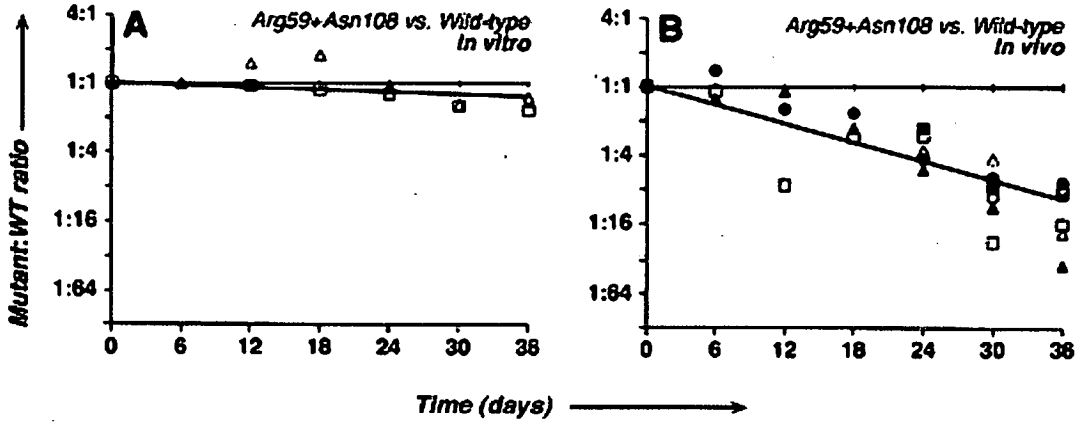


Figure 1.15 Competition between *T. gondii* parasites transformed to include mutations homologous to the S108N + C59R pyrimethamine resistant mutation in *P. falciparum*, and wild-type parasites, *in vitro* (A) and *in vivo* (B). The ratio of mutant/wild type parasites is plotted for several replicate experiments, indicated by the circle, square and triangle symbols. Each best-fit line represents the average of all data points on the graph up until fixation. Adapted from Fohl & Roos (2003).

From these results, the authors calculated that there was a fitness defect of 0.8% per generation for the 133 generations under observation (1 generation = 6.5h) *in vitro* and 1.8% *in vivo*. These results correlate well with the large difference in k_{cat}/K_m values observed by Sirawaraporn et al (1997), (Figure 1.14), between the S108 + C59R mutant DHFR and the wild type. How well transfected *T. gondii* parasites model natural populations of *P. falciparum* in the field is open to some debate.

1.10 Growth Rates

It has long been established that different strains of the same species of *Plasmodia* differ in the rate of their replication through the erythrocytic cycle. For example, several authors have shown that differences exist in the growth rates (or replication rates) of different strains of *P. falciparum* (Ariey et al., 2001; Hayward et al., 1999; Robert et al., 1996). Field data show an over-representation of certain clones in severe malaria cases (Engelbrecht et al., 1995; Kun et al., 1998) suggesting that these clones are more virulent

than others (growth rate is an important factor in parasite virulence). Replication rate has been shown to be positively related to parasite virulence (Mackinnon and Read, 1999b), where the term virulence is defined as "harm to the host following infection, where harm is disease severity (morbidity) and/or mortality".

When *P. falciparum* malaria was used as a cure for neuro-syphilis in the first half of the last century, it was possible to collect data on the infection dynamics of malaria parasites in experimentally infected humans. Two such investigations by James et al. (1936), and Covell & Nicol (1951) demonstrated differences in virulence between two strains of *P. falciparum.* Evidence for genetic differences in growth rates between strains of the rodent malaria parasite *P. chabaudi* has also been reported (Mackinnon and Read, 1999b; Taylor et al., 1997; 1998; Timms et al., 2001). Figure 1.16 shows the average parasitaemias (percentage of erythrocytes infected by parasites) of mice infected with three different strains of *P. chabaudi.* It can be seen that the three strains differ greatly in the peak parasitaemia, and in the length of time it takes to reach this peak.

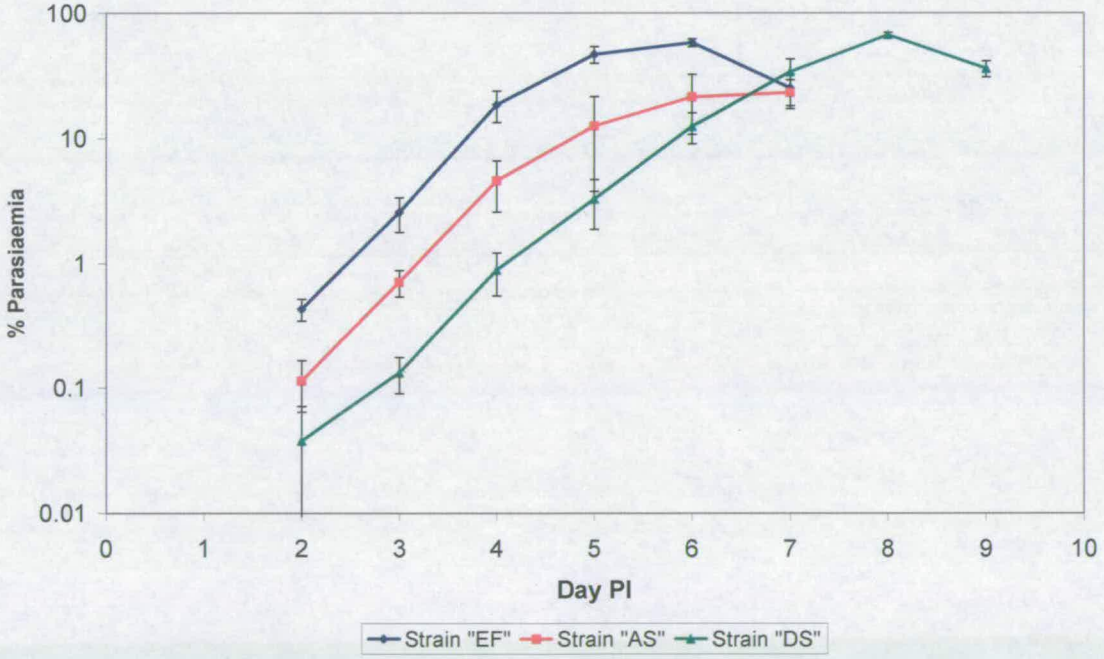


Figure 1.16 Parasitaemia through time of three different strains of *P. chabaudi*. Strains "AS" and "EF" are sub-species *Plasmodium chabaudi chabaudi*, and "DS" *Plasmodium chabaudi adami*. Each data point represents the mean of 5 mice (7 for strain "EF"), and error bars represent 95% confidence intervals of the means. Data collected during this project.

The mechanisms that enable one strain to grow faster than another are little understood. Possible phenotypic determinants of parasite growth rate include; erythrocyte selectivity (Simpson et al., 1999); antigenic variation (some parasites may elicit a stronger immune response in some hosts than others); intra-erythrocytic replication rate (simply the numbers of merozoites produced by each schizont, and the rate at which they are produced); the ability of the parasites to cause uninfected erythrocytes to bind to infected cells (rosette-formation) (Carlson 1990, and Rowe 1997), or to adhere to the walls of blood vessels (sequestration). These last two cytoadherence factors may be considered more determinants of the *virulence* of parasites rather than growth rate *per se*, but some authors have suggested that the ability of parasites to form rosettes may increase the numbers of invadable erythrocytes in the vicinity of infected cells (Wahlgren et al., 1989). Sequestration could be advantageous to the parasite, and so increase growth rate, because it prevents their free circulation in the blood system, thus avoiding clearance by the spleen.

Understanding the genetic mechanisms of parasite virulence is important for predicting parasite response to any malaria vaccine that may be introduced. It has been hypothesized that the wide-scale introduction of vaccines that are less than 100% efficient could lead to the evolution of more virulent parasite strains (Gandon et al., 2001). There are conflicting theories on this subject, however, with some evidence suggesting that the right sort of vaccine could lead to the proliferation of more avirulent strains (Ewald, 1996) (see also (Bergstrom et al., 1999; Bull, 1994; Ebert, 1998; Ewald, 1994). A clearer understanding of the genetic determinants of virulence would lead to a clearer picture of how parasites are likely to respond when faced with large-scale vaccination.

The fact that there are so many factors involved in replication rates (see above) suggests that the genetic basis for differences in growth rates between parasite strains is likely to be complex, with many genes possibly involved.

1.11 *Plasmodium chabaudi*

The experiments described within this PhD thesis were carried out using the rodent malaria parasite, *Plasmodium chabaudi*. *P. chabaudi* belongs to a group of four *Plasmodium* species that infect murine rodents from Central Africa the other species being *Plasmodium vinckei*, *Plasmodium yoelii* and *Plasmodium berghei*. A map of the locations from which the various rodent parasites were isolated is shown in Figure 1.17. The first of the rodent parasites to be discovered was *P. berghei*, which was found in the blood of a thicket rat, *Grammomys surdaster*, by Vincke and Lips in 1948. The discovery of *P. vinckei* followed in 1952, *P. chabaudi* in 1965, and *P. yoelii* was added to the taxonomy in 1974 (Killick-Kendrick and Peters, 1978).

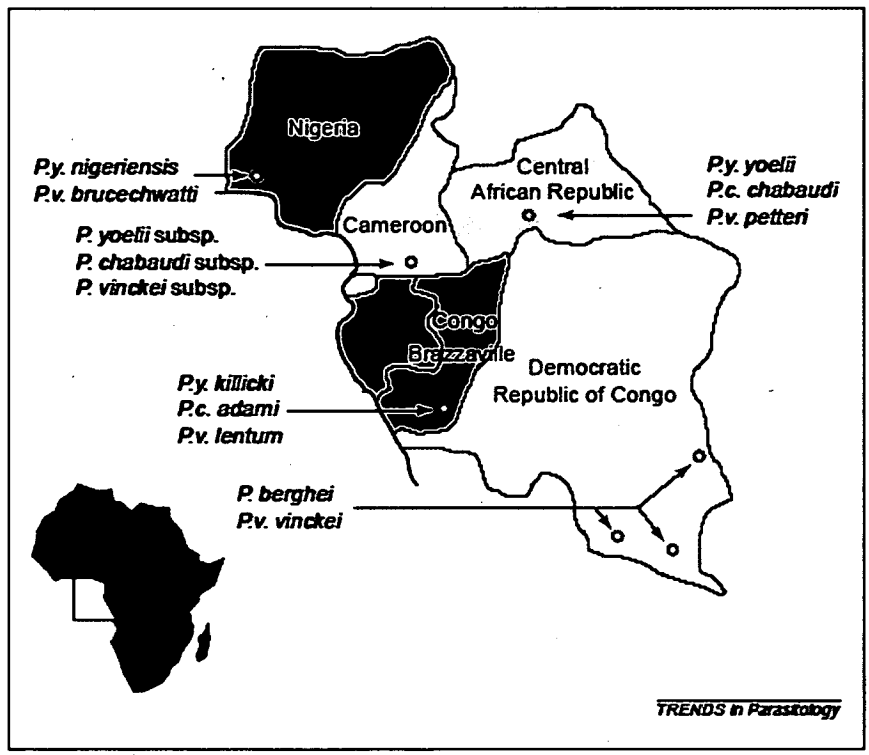


Figure 1.17 The origins of the rodent malaria parasites (From Carlton et al (2001))

There are many reasons for using rodent malaria parasites as models for human malaria. The most obvious of these is the ease with which the whole life cycle (including the sexual stages in mosquitoes) can be achieved in the laboratory. This is a crucial factor in

studying malaria parasites, and one which is impossible to achieve with the human parasites with current methods and technology. Apart from the similarities in basic biology between the rodent and human malaria parasites, they share conserved genetics and genome organization, conserved housekeeping genes and biochemical processes, and there is considerable evidence for conservation of the molecular basis of drug-sensitivity and resistance (Janse and Waters, 2004). Table 1.2 shows some similarities and differences between *P. chabaudi* and the human malaria parasites.

	P. chabaudi	***P. falciparum***
Merozoites per schizont	6-8	8-24
Reticulocyte preference	No	No
Synchronous blood infection	Yes	Yes
Optimum temperature range mosquito transmission (sporogony)	24-26	>26
Oocyst size (μm) at optimum temperature	50	50-60
Sporozoites in glands at optimum temp. (days after infection)	11-13	10-12
Mean diameter of mature pre-erythrocytic schizonts (μm)	38-45	45-60
Duration of pre-erythrocytic development	50-58 hours	5.5 - 6 days
Duration of the asexual blood stage cycle (hours)	24	48
Developmental time of gametocytes	36 hours	8-11 days
Developmental time ookinete (hours)	18-24	12-24
Sporozoite (size in μm)	10-15	10-14

Table 1.2 Comparisons of the features of *P. chabaudi* and *P. falciparum* parasites (adapted from (Janse and Waters, 2004) .

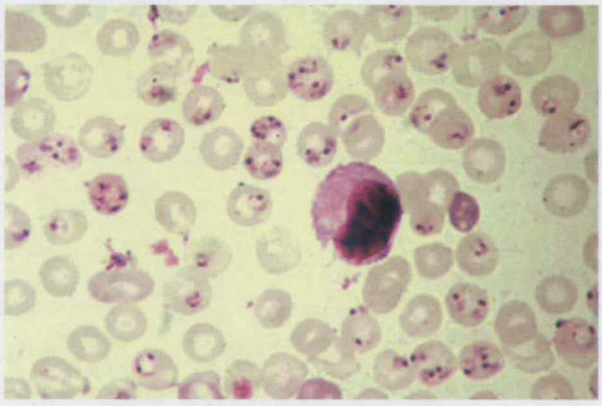


Figure 1.18 Plasmodium chabaudi parasites (early trophozoite stage) in mouse blood. Picture from http://www.ncbi.nlm.nih.gov/projects/Malaria/Rodent/csmear_mid.html (NCBI 2004)

1.12 Linkage Group Selection

The identification of genes that control important parasite phenotypes such as drug resistance, growth rate (as a virulence factor) and strain-specific immunity, is of immense importance in the fight against malaria. Knowledge of the gene(s) controlling resistance to a specific anti-malarial drug, for example, enables the monitoring of the spread of resistance, as well as potentially increasing the effectiveness of decisions concerning drug policy. Knowing which genes are involved in resistance to a drug can also help us to understand the molecular basis of drug resistance and aid in the design of new versions of drugs which are unaffected by the mutations causing parasite drug resistance (Yuvaniyama et al. 2003). Similarly, the identification of genes involved in the parasite's immunogenicity is crucial to the development of vaccines. Other parasite traits for which the genetic basis is unknown include virulence (*i.e.* what causes some parasites to be more harmful to a host than others) and transmissibility through particular vectors. Both would be better understood if the genes underlying these traits were known.

Existing methods in the genetic study of malaria parasites that attempt to locate genes controlling such traits, such as linkage analysis of individual cross progeny clones, are labour-intensive and expensive. Without the generation of an extremely large number of recombinant clones (>1,000), they have poor resolution which makes the actual

identification of the underlying genes extremely difficult unless strong candidates are already suspected. There is an inverse relationship between the size of the locus within which possible target genes may be located and the number of recombinant clones that must be generated (Su et al., 1997; Wellems, 1991).

Linkage Group Selection (LGS), whose principles are investigated in this project, was devised for application to malaria parasites in order to locate genes that control selectable phenotypes such as drug sensitivity, growth rate and strain-specific immunity without the disadvantages of classical linkage analysis. LGS (Figure 1.19) uses a genetic cross between two unrelated (genetically distinct) parasites of the same species, one of which is sensitive and the other resistant to the relevant selection pressure (e.g. drug treatment). Following cross-fertilisation between gametes of each parasite and zygote formation in the mosquito, there is recombination between the parental genomes during meiosis, producing haploid recombinant progeny. Each individual recombinant parasite will have inherited a random assortment of parental alleles; 50% of the parasites will have inherited the "resistant" allele(s) of the gene(s) for the phenotype under investigation, and 50% the "sensitive" allele(s). The uncloned population of haploid recombinant parasites is then exposed to the relevant selection pressure, as well as being passaged in the absence of the selection pressure. After such selection, alleles from both parents should still be equally represented except at those loci linked to the gene(s) that determine(s) the parasites' response to this selection. At these loci, alleles from the "sensitive" parent should have been reduced or eliminated in the 'selected' population, relative to the untreated population.

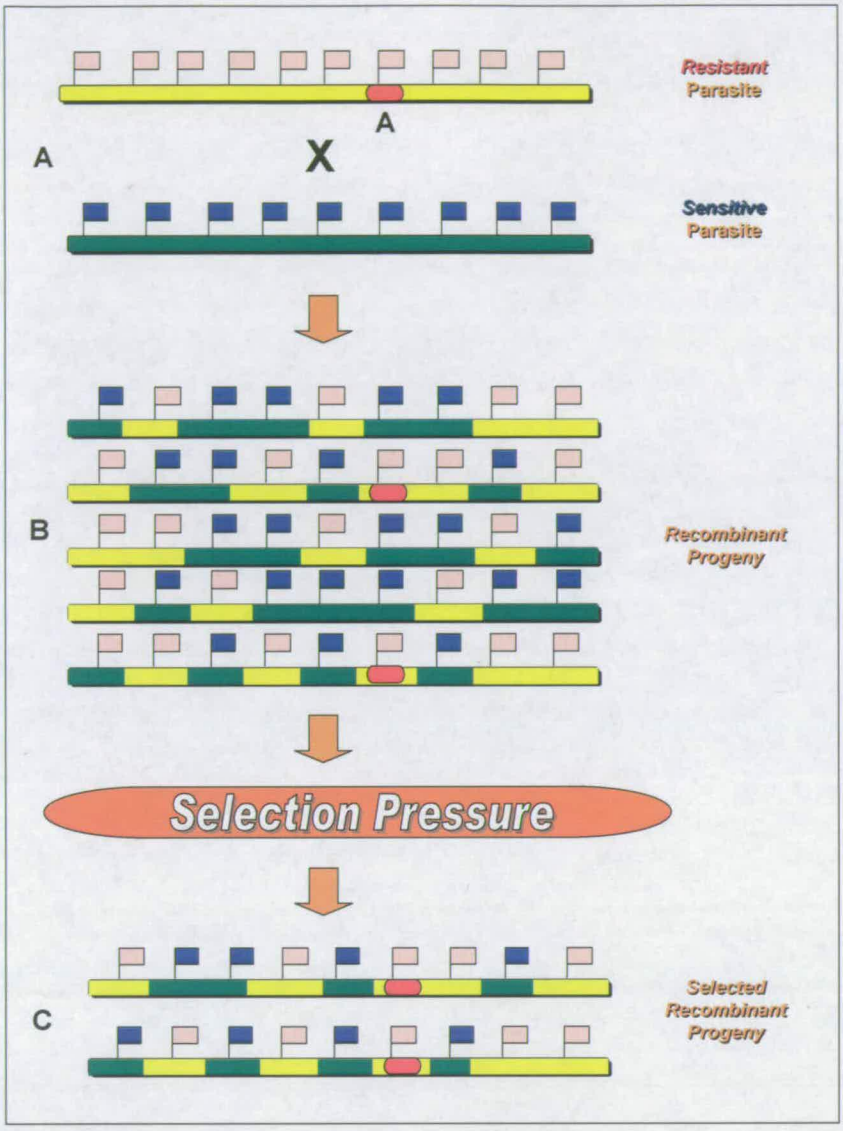


Figure 1.19 A schematic representation of the genetic principles of LinkageGroup Selection

a) Two cloned haploid parasites which differ in their sensitivity to a particular selection pressure are genetically crossed. In this example, resistance to the selection pressure is conferred by a gene at locus "A". The genome is represented by the coloured bars, while the flags represent dense, genome-wide markers between the two strains.

b) The resulting recombinant progeny population will consist of thousands of parasites, each bearing a random assortment of parental alleles, and also a random assortment of parental markers.

c) This uncloned population is then subjected to the selection pressure under investigation, and the resulting selected population will consist solely of parasites that possess the resistant allele of the gene that controls the phenotype of interest, and, therefore, any markers linked to this locus will be mainly from the resistant parent, their proportion increasing as the distance from the locus under selection decreases. We look for markers of the sensitive parent that are removed or reduced by the selection pressure; the greater the degree of reduction of a sensitive marker, the closer it is linked to the allele removed by the selection pressure.

By typing both the selected and the unselected recombinant populations with large numbers of quantitative, genome-wide, markers, it is possible to identify those markers from the sensitive parent that are removed or reduced by the selection pressure. These markers are likely to be linked to the loci involved in controlling the response of the parasite to the selection pressure. The closer markers are genetically linked to the target locus, the greater the reduction in their representation in the selected population. The markers can then be sequenced, and their positions located in the parasite genome (using the *Plasmodium* genome databases in conjunction with bioinformatics techniques) in order to identify the genes to which they are linked.

1.12.2 Amplified Fragment Length Polymorphism (AFLP)

LGS requires that the two parental clones are distinguished by a large enough number of genetic markers to ensure that some will be linked to the genes of interest. To obtain such markers, Amplified Fragment Length Polymorphism (AFLP) has been used, a PCR-based method for amplifying DNA fragments from genetically distinct cloned lines of parasites. AFLP is a technique in which large numbers of markers are generated across a genome (Masiga et al., 2000) (Figure 1.20). The high density of these markers in the genome means that there is a high probability that some of them will be linked to the gene(s) of interest. It has been previously shown that AFLP meets the requirements of LGS both as regards numbers of markers generated in different strains of *P. c. chabaudi* (Grech et al., 2002b) and their quantitation in a mixture of the strains (Martinelli et al., 2004).

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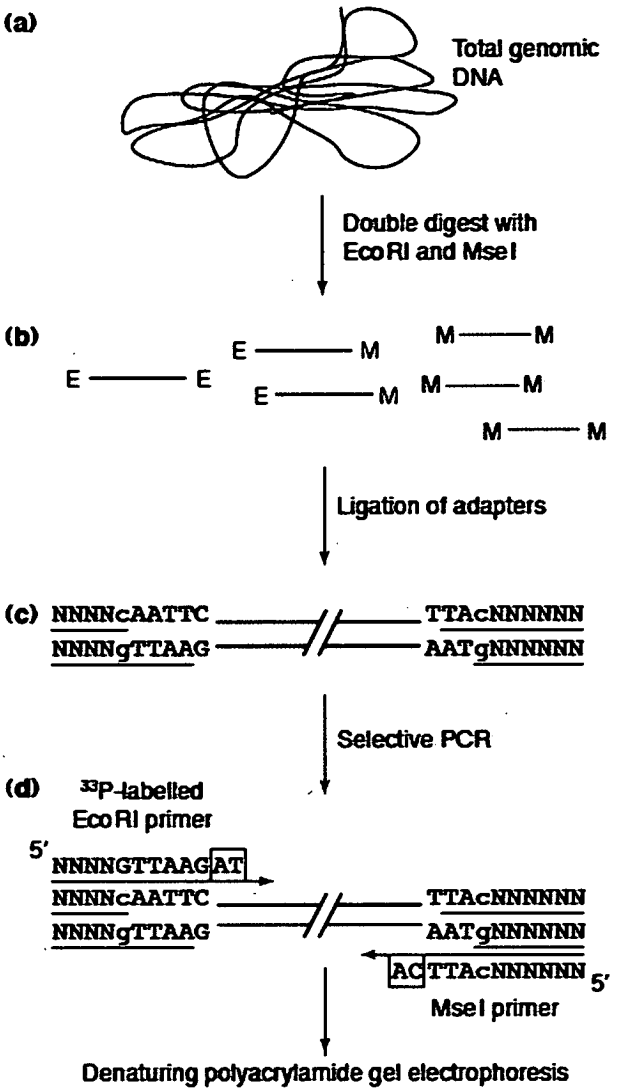


Figure 1.20 An outline of the AFLP process. (a) Genomic DNA is digested with 2 restriction endonucleases (usually *Eco*R1 (E) and *Mse*1 (M)). (b) 3 types of restriction fragments are produced, with various combinations of the two primers at either ends. (c) Adapters which create binding sites for PCR primers are ligated to the ends of the fragments. (d) PCR amplification of the fragments is selective, using primers with 3' nucleotide extensions (boxed). These selective extensions define a subset of restriction fragments in order to reduce the numbers of fragments that are amplified to a manageable number. One of the primers is labelled with radioactive ^{33}P to allow visualisation on denaturing poly-acrylamide gels. From Masiga et al (2000).

The AFLP technique allows the visualisation of restriction fragments of DNA. This enables the detection of the variation in DNA between two strains without prior knowledge of the nucleotide sequence. Depending upon the polymorphism between the two strains, large numbers of genetic markers can be produced in a relatively short time

(Vos et al., 1995). AFLP involves cutting genomic DNA into a large number of fragments with two different restriction enzymes (a "frequent" and a "rare" cutter). The resulting DNA fragments are then amplified by PCR, using primers matching the adapters. The frequent cutter produces many small fragments, which are easily amplified and are the optimal size for separation on sequencing gels. The rare cutting enzyme produces fewer (but much longer) fragments. The AFLP procedure also results in the amplification of those restriction fragments which have a rare cutter sequence on one end and a frequent cutter sequence on the other end (Vos *et al.*, 1995), thus generating optimal size fragments for visualisation on poly-acrylamide gels. Radio-labeling of one primer allows visualisation of the products. Genetic differences between strains occurring at a cutting site result in fragments of different sizes which can then be identified on a gel as being present in one strain and absent in the other (Figure 1.21). Polymorphic bands can be produced as a result of polymorphisms between strains at the enzyme recognition sites, polymorphisms between strains at the selective bases used in each PCR, or as a result of insertion/deletion polymorphisms within fragments.

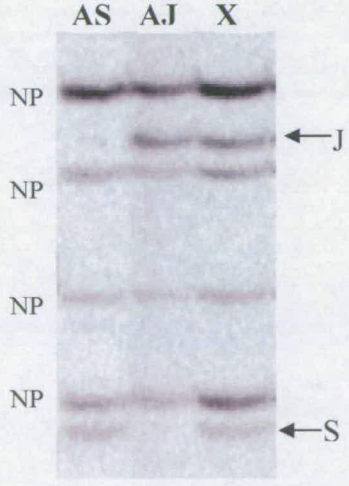


Figure 1.21 Example of a section of an AFLP gel showing polymorphic markers in two strains of *P. chabaudi* parasites (AS and AJ), and their representation in the uncloned recombinant progeny of a cross between the two strains (X). Two markers distinguishing the strains are shown, one unique to AJ (J), and one to AS (S). 4 non-polymorphic bands (NP), present in both strains, are also shown.

1.12 Aims of the project

This project involves a "proof of principle" of the Linkage Group Selection approach to gene identification. Experiments were conducted using a selectable phenotype for which the controlling genetic factors were already understood. Two cloned *P. chabaudi* strains were available that differed in their sensitivity to pyrimethamine. These were ASpyr1 (resistant) and AJ (sensitive). The ASpyr1 clone had been selected for pyrimethamine resistance by passage under drug pressure. In this parasite, pyrimethamine resistance is conferred by a single base mutation in codon 106 of *pcdhfr* (S106N), which is homologous to the S108N mutation in *pfdhfr* that confers pyrimethamine resistance to *P falciparum* (Cheng and Saul, 1994). The results of LGS experiments using these parasites are presented in **Chapter IV**.

Before proceeding with the full LGS investigation, it was necessary to investigate the interaction dynamics of the two parental strains (ASpyr1 and AJ) under pyrimethamine pressure in mixed infections in mice. Experiments were designed in which mixtures of ASpyr1 and AJ were passaged through mice under different levels of drug pressure, and data were gathered on the growth of each individual strain in the mixture throughout the infection. Results from these experiments are described in **Chapter III**. The purpose of these experiments was primarily to decide on a suitable drug regimen for the LGS experiments, and also to gain an understanding of how the two strains respond to drug pressure in the presence of the other strain. Experiments in this chapter also report on the effect of host genotype on competition between drug-sensitive and resistant strains.

Fitness costs associated with pyrimethamine resistance in *P. chabaudi* are the subject of investigation in the experiments described in **Chapter V.**

1.12.1 Summary of main aims

- To investigate the interaction between pyrimethamine resistant and sensitive parasites in mixed infections, in the presence and absence of drug pressure, and within hosts with differing genotypes.
- To investigate the Linkage Group Selection technique as a method for identifying genes that control selectable phenotypes in malaria parasites using pyrimethamine selection in *P. chabaudi*.
- To determine if there is a measurable fitness cost associated with the *dhfr* S106N pyrimethamine resistance mutation in *P. chabaudi*.

CHAPTER II
MATERIALS AND METHODS

All work was carried out in accordance with the Animals (Scientific Procedures) Act 1986 (UK)

2.1 Mice

Inbred female CBA/Ca and C57B1/6J mice (*Mus musculus*) aged 4–6 weeks were used in the experiments described in this work. They were housed in polypropylene cages with sawdust bedding, and were provided with 41B rat and mouse maintenance diet (Harlan-Tekld, England) and water supplemented with 0.05% paraminobenzoic acid (PABA), *ad libitum*. Cages were kept at a constant 25 ± 1°C, on a 12h light/dark cycle (light phase between 07.00 and 19.00, controlled by an automatic time-switch). Mice were supplied by the University of Edinburgh.

2.2 Mosquitoes

Anopheles stephensi mosquitoes were maintained in a temperature and humidity controlled room (25-27°C, 75-85 % humidity), kept on a 12-hour light/dark cycle. Larvae were maintained on Liquifry™ until first instar and thereafter on ground Tetramin™ fish food, and adult flies on 10% glucose and 2% PABA-supplemented water solution. Stock adults also receive bi-weekly rat-blood feeds, which are essential for the production of eggs.

2.3 Parasites

All parasites used in these experiments were *Plasmodium chabaudi chabaudi* (refered to as *P. chabaudi* from here).

2.3.1 Definition of terms

Isolate- refers to a population of parasites that have been collected from a single wild-caught thicket rat, *Thamnomys rutilans*, on a single occasion. An "isolate" does not necessarily represent a homogeneous parasite population and may contain more than one representative of the same species or different species.

Strain- refers to all parasites of a single subspecies present in a single isolate. For example, *P. c. chabaudi* strain AJ is composed of all the parasites belonging to the *P. c. chabaudi* sub-species in blood isolated from the *T. rutilans* designated "AJ".

Line- refers to a population of parasites that have undergone one or more *in vitro* or *in vivo* laboratory passages. Usually, a "line" is produced when the parasites being passaged in the laboratory have been subjected to some kind of induced pressure, such as drug treatment. As above, parasites constituting a line may not be genetically identical.

Clone- refers to parasites all of which have been derived from a single cell by asexual reproduction. Therefore, any particular clone should represent a population of genetically identical parasites, assuming that no spontaneous mutations had taken place during parasite growth.

Stabilate - refers to a population of parasites that have been preserved in a viable condition in liquid nitrogen on a unique occasion.

The clones used in these experiments were:-

- *P. chabaudi* ASpyr1 - Pyrimethamine Resistant
- *P. chabaudi* AJ - Pyrimethamine Sensitive
- *P. chabaudi* CB - Pyrimethamine Sensitive
- *P. chabaudi* AS(sens) - Pyrimethamine Sensitive

These strains were derived from parasites initially isolated from thicket rats, *Thamnomys rutilans*, in the Central African Republic(Landau, 1965; Landau and Chabaud, 1965). *P. chabaudi* AS(sens), CB and AJ were then cloned by limiting dilution of blood forms (Walliker et al., 1975a), and AS(sens) was subjected to selection for pyrimethamine resistance to produce clone ASpyr1.

2.4 Routine Maintenance of Parasites

All parasite clones were available as cryopreserved stabilates, deep-frozen in liquid nitrogen. To establish new infections, these stabilates were thawed by immersion of tubes in water and the contents inoculated intra-peritoneally into mice, in which a patent infection normally developed 5-10 days later.

Parasites were maintained in laboratory mice by the collection of parasitised red blood cells from the tail vein of infected mice, and their inoculation into uninfected mice in suspension in citrate saline solution (Appendix 1).

Thin blood smears were taken from the tail veins of infected mice daily. The smears were fixed with 70% ethanol solution and stained with Giemsa's solution for 30 minutes. They were then studied with a light stage microscope (100x objective lens, oil immersion), and percentage parasitaemias were determined. This was done by counting the number of infected red blood cells (iRBCs) in ten microscope fields, and dividing by the total number of blood cells in the same ten fields and multiplying by 100; -

Percentage iRBC = (No. of iRBCs in 10 fields / Total No RBCs in 10 fields) **X 100**

Infected red blood cells were identified as those containing trophozoites or schizonts, which stain purple with Geimsa's solution.

When necessary, infected blood was cryopreserved according to the following procedure: mice were anaesthetised by halothane and bled from the brachial artery. Blood was

collected into a tube containing citrate saline (2-3 times the volume of blood). The red cell suspension was centrifuged at 2000 r.p.m. for 5 minutes after which the red cell pellet was mixed with an equal volume of deep-freeze solution (Appendix 1). This mixture was then aliquoted into glass capillaries and deep-frozen in liquid nitrogen.

2.5 Experiments with mixed infections of ASpyr1 and AJ (Chapter III)

2.5.1 Preparation of inocula for the production of mixed infections containing equal proportions of 2 parasite clones (AJ and ASpyr1)

Infections of both ASpyr1 and AJ clones were induced in mice as described above. Absolute numbers of parasites per volume of blood were worked out at peak parasiteamia for both infections by multiplying the parasitaemia recorded by thin blood smear against the numbers of red blood cells per volume of blood of each mouse (calculated by flow cytometry; Beckman Coulter). A volume of blood containing known parasite numbers was then taken from both ASpyr1 and AJ infected mice by incision of the distal portion of the tail, and collected in glass capillaries. This blood was then mixed to produce a 50/50 mixture of the two clones, and diluted to a concentration of 1 x 10^6 parasites per 0.1 ml with 50% ringers solution (Appendix 1), 45% heat-inactivated calf serum and 5% 200 units/ml heparin solution. This solution was kept on ice, and administered intra-peritoneally to each experimental mouse in 0.1 ml aliquots.

2.5.2 Preparation of Pyrimethamine

Pyrimethamine solution was prepared by dissolving pyrimethamine powder in dimethylsulfoxide (DMSO). This was administered to mice orally in 0.1 ml volumes using a lubricated catheter. Doses of drug varied between experiments, from 5mg/kg to 20 mg/kg for four days. At the time of drugging, mice were individually weighed so that the amount of drug given could be adjusted accurately to each mouse.

2.5.3 Blood collection for Proportional Sequencing analyses

5µl of blood was removed from each infected mouse daily using a glass capillary. This was immediately placed in an eppendorf tube containing 2 drops of citrate saline solution. The samples were then spun in a microcentrifuge at 10,000 rpm for 2 minutes, and the supernatent removed. The resulting pellet was frozen at -70°C.

2.5.4 Extraction of parasite DNA

Frozen blood pellets were thawed at room temperature, and the DNA extracted using the InstaGene™ Matrix (Bio-Rad), following the manufacturers handbook protocol. The final volume of DNA was 49µl.

2.5.5 Determination of the proportions of the strains in the mixed infections by Proportional Sequencing

The blood samples taken from each mouse were analysed using Proportional Sequencing. This technique allows the determination of the percentages of each parasite in a mixture on a given day of the infection. By applying this technique it was possible to determine the proportions of two parasites in mixed infections over the course of an infection.

To directly quantify the proportions of the sensitive (ASpyr1) and resistant (AJ) parasites present in the selected and unselected mixed infections, DNA was amplified by nested PCR at the *pcdhfr* locus using primers common to non-polymorphic sequences from both parental alleles. The resulting PCR products were purified and sequenced. Sequencing results were analyzed using the SeqED v 1.0.3 software (Applied Biosystems, Inc., 1992), which allows the visualization of chromatograms of sequenced DNA and the quantitation of individual fluorescent peaks. The relative heights of peaks at the polymorphic sites in *pcdhfr* can be used as an index of the relative proportions of the AJ and ASpyr1 parasites in each sample. With five replicate samples, and with reference to a calibrated series of mixtures between ASpyr1 and AJ (Cheesman et al. 2003), it is

possible to estimate the percentage of parasites carrying the ASpyr1 or AJ alleles of the *pcdhfr* gene with a standard error of <3% (P. Hunt, R. Fawcett, R. Carter, and D. Walliker, in prep.).

A detailed description of the Proportional Sequencing technique is available elsewhere (P. Hunt, R. Fawcett, R. Carter, and D. Walliker, submitted). Briefly, the technique exploits the fact that during a PCR reaction, DNA is amplified in proportion to the initial template. If DNA containing a mixture of both ASpyr1 and AJ type alleles is amplified in the same reaction, then the proportions of these clones will remain constant throughout the PCR. This means that the amplified PCR product will contain the same proportions of ASpyr1 and AJ DNA as the template DNA that was amplified. When this DNA is sequenced, it is possible to determine the proportions of the clones in a population by measuring the proportions of a single base that differs between them. It is possible to distinguish AJ and ASpyr1 parasites by 8 single nucleotide polymorphisms on the *pcdhfr* gene.One of these polymorphisms, which occurs at position 317 on the *pcdhfr* gene is responsible for conferring pyrimethamine resistance to ASpyr1 (see Figure 2.1).

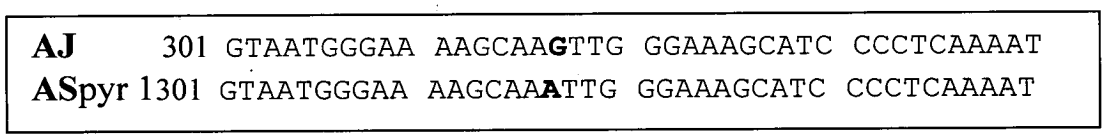


Figure 2.1 The single point mutation on the *pcdhfr* gene that confers pyrimethamine resistance to ASpyr1 parasites. At position 317, AJ has a G, and ASpyr1 has an A.

When both these parasites are present in an infection, both bases will be present at position 317 in the sequence. By measuring the proportions of each base (by comparing the size of the peaks on a sequence chromatograph) it is possible to determine the proportions of each parasite in the mixture. The use of a nested PCR may, however, introduce a bias towards the amplification of one of the alleles in a mixed infection, as the efficiency of amplification of the products may not be equal.

2.5.6 PCR reagents and reaction conditions

Extracted parasite DNA was subjected to a nested PCR, amplifying a portion of the *pcdhfr* gene that contains 8 single nucleotide polymorphisms between ASpyr1 and AJ. The oligonucleotide primers used were as follows:-

Outer PCR

pcdhfr –09 : **GAAGATATCTCTGAAATATTCG**

pcdhfr –14 : **GGTGTGTGTGATGAAAAAAATA**

Inner PCR

pcdhfr –16 : **TTTTAAGGGACTTGGGAATG**

pcdhfr –07 : **CCAGATATCAATGAAGATTTAT**

Individual PCR reactions were carried out in 50µl volumes containing 10pM of both oligonucleotides, 1 X PCR buffer, 1 X dNTP solution, 1 unit *Taq* DNA polymerase (Boehringer Mannheim), and 20µl of extracted DNA solution for the outer PCR (from a total extracted DNA volume of 49 µl) and 1µl of the resulting PCR product for the inner PCR. Negative controls were also prepared, which contained 1µl of sterile distilled water in place of template DNA. Positive controls were prepared using a previously amplified DNA template.

PCR reactions were carried out using a UNO-Thermoblock machine (Biometra), under the following standard conditions;

Denaturing	95°C for 1 minute	x 30 cycles
Annealing	52°C for 1 minute	
Extension	65°C for 1 minute	

PCR conditions were the same for both rounds of the nested reaction.

PCR products were run and visualised on a 1.5% agarose gel in TBE solution. The resulting PCR products were then purified using the QIAGEN QIAquick™ PCR purification kit, following the manufacturer's instructions.

2.5.7 Sequencing and purification of PCR products

Sequencing PCR reactions were set up using the ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit, (PE Applied Biosystems). PCR reactions were prepared to a final volume of 10µl, containing the following reagents: 4µl template DNA (100-250ng), 4µl Terminator Ready Reaction Mix, 1µl primer (3.2pmol), 1µl sterile distilled water

Sequencing PCR conditions were as follows;

Denaturing	95°C for 30 seconds	x 25 cycles
Annealing	50°C for 20 seconds	
Extension	60°C for 4 minutes	

Following the sequencing PCR, the products were purified by precipitation with sodium acetate and 95% ethanol, and washed in 70% ethanol.

For sequencing of purified PCR products, internal primers covering the extreme 5' and 3' ends of the fragments were used. All fragments were sequenced in opposite directions.

2.5.8 Analyses of sequencing results

Sequencing results were analysed using the SeqED V 1.0.3 software (Applied Biosystems Inc., 1992). The programme allows the visualisation of chromatograms of the sequenced DNA. The relative proportions of the two strains in each sample were

determined by measuring the two peaks associated with the single nucleotide polymorphism that confers pyrimethamine resistance. It was possible, therefore, to calculate the percentages of each allele of a gene in each sample.

2.6 Production of cross progeny

The experiments described in Chapters IV and V involve the analysis of uncloned recombinant progeny of crosses between parasite strains ASpyr1 + AJ, ASpyr1 + CB, and AS(sens) + AJ. All three crosses were carried out using the procedure outlined below.

2.6.1 Overview of procedure

To produce a genetic cross between two strains, the two parental clones are inoculated into mice to produce a mixed infection (as described in Section 2.5.1), and the resulting infections followed by microscopy for the presence of gametocytes. When gametocytes are present in the infection, mosquitoes are allowed to feed on the mice, and the sporozoites present in the salivary glands of infected mosquitoes 14 days later are inoculated intra-peritoneally into mice.

During the early stages of this project, work was carried out to determine the optimum day post mouse inoculation to achieve infections in mosquitoes. The results of this work showed that feeding mosquitoes on mice at day 6 post-inoculation produced the greatest number of infected mosquitoes. All mosquito feeds in this experiment, therefore, were carried out at day 6 post mouse-infection.

2.6.2 Mosquito feeds

Six $30cm^3$ mosquito cages were set up containing $\approx$ 200 female *Anopheles stephensi* mosquitoes, 5-7 days old. Mosquitoes were maintained on glucose and water solution, which was removed 24 hours prior to infective mouse feeds. Blood smears were taken from all infected mice on day 6 post infection, and the presence of gametocytes was

confirmed. Mice were then anaesthetized with rohypnol solution (Appendix 1), and attached to cork boards placed on top of the mosquito cages. Mosquitoes were allowed to feed for 30 minutes before the mice were removed. Mice were then killed before awakening from the anesthesia. Glucose and water solution was placed back into the cages, and egg bowls were provided 2 days post feed. 10 mosquitoes were dissected from each cage 7 days after the feeds, to check for the presence of oocysts.

2.6.3 Infection of mice from sporozoites

Fourteen days after the infective feed, mosquitoes from each cage were dissected, and the salivary glands examined for the presence of sporozoites. If the presence of sporozoites was confirmed, all mosquitoes from the cage were dissected, and the salivary glands removed. These were placed in a glass tube containing 50% Ringer's solution (Appendix 1), 45% heat-inactivated calf serum and 5% 200 units/ml heparin solution. The glands were then gently crushed using a pestle and mortar in order to release the sporozoites. This solution was kept on ice, and injected intra-peritoneally into mice in 0.1 ml aliquots. Thin blood smears were taken from infected mice daily, and parasitaemias recorded.

2.7 Selection of cross progeny

When the sporozoite-induced infections reached peak parasitaemia (10%–15%), the parasites were harvested, pooled, and inoculated into a group mice (designated the "non-passaged" group, and containing 3 -5 mice depending on the experiment). Each mouse in the group received 1 x 10^6 parasites. This initial experimental group was left untreated, and the parasites were harvested at peak parasitaemia for AFLP analysis to provide a reference point for markers analyzed in the subsequent treatment groups. For the ASpyr1 + AJ and the ASpyr1 + CB crosses, parasites were pooled and sub-inoculated from the "non-passaged" group into two further groups of mice, one of which was treated with pyrimethamine ("PYR" group), and the other left untreated ("untreated" group). These two groups provided the material for the comparison of markers between drug-treated and untreated parasite populations. Pyrimethamine was administered orally at a dose of

10 mg/kg of mouse body weight daily at 24-h intervals for 4 days, starting 3h after parasite challenge. Both the pyrimethamine-treated and the untreated blood-stage cross progeny were allowed to grow to peak parasitaemia (30%–40% for untreated, and 15%–20% for treated), at which point the blood was harvested. Two samples of parasite DNA were prepared for AFLP and other molecular analyses by pooling separately the blood from the treated and untreated mice. For the AS(sens) + AJ cross, blood from the sporozoite induced infections was pooled and inoculated into groups of 5 mice serially, for 4 passages. Blood was collected for analysis from each of these groups.

2.8 Amplified Fragment Length Polymorphism (AFLP) analysis

2.8.1 Preparation of Parasite DNA from experimental groups

When infections reached peak parasitaemia, blood was extracted from all mice in each group by severance of the brachial artery, pooled, and prepared for DNA extraction. Blood was filtered in order to remove any mouse lymphocytes or other nucleated cells, by running through a 5 ml column of powdered cellulose (Sigma) washed with citrate saline. This was repeated twice. Blood was then filtered through Plasmodipur™ filters (Euro-Diagnostica) twice. The filtrate was centrifuged for 5 mins at 3000 rpm and the supernatant removed, leaving a pellet of packed cells. Twice the pellet-volume of 0.15% saponin in Phosphate Buffered Saline (PBS) was added to lyse cells. As soon as lysis of erythrocytes was judged to have occurred (associated with a change in colour of the solution from bright red to burgundy colour), PBS was added in excess to prevent parasite lysis. This solution was then centrifuged again at 4000 rpm for 5 minutes and washed twice in PBS. Supernatant was discarded and pellets stored at -70°C. Three thick blood smears were taken prior to filtration, after cellulose filtration and after Plasmodipur™ filtration in order to determine the efficiency of host cell removal at each stage.

The frozen pellet was re-suspended in 0.4 ml buffer A (Appendix 1), and 10 µl of 10% SDS and 50 µg Proteinase K (Sigma) were added. The pellet was left at 37°C overnight. The following day, an equal volume of 1:1 phenol/chloroform mixture was added, mixed for 3 min, and centrifuged at 5,000 g for 1-2 minutes. The upper aqueous layer was transferred to a fresh tube. The step was repeated 2-3 times. Then an equal volume of chloroform was added, and the tube was centrifuged as before for 1-2 minutes. The upper aqueous layer was removed to a fresh tube. This procedure was repeated once before an equal volume of ether was added, the solution centrifuged as before for 1-2 minutes and the upper layer removed. The remaining ether was left to dry in the air. Three volumes of absolute ethanol (0°C) and $1/10^{th}$ volume of 3M sodium acetate (pH 5.2) were added to the dried pellet, and the tube was mixed and placed on ice for 15-45 minutes to precipitate the DNA. The tube was then centrifuged at 10,000 g in a Speed Vac (Savant) for 10 minutes and the ethanol mixture removed. The tube was again centrifuged at 10,000 g for 5-10 minutes to remove final traces of ethanol and the pellet was resuspended in 100-200 µl of TE buffer (pH 8.0) (see Appendix 1) and left at 37°C for 10 minutes before storage at -20°C. Final DNA concentration was measured by spectophotometry (using a BioPhotometer spectrophotometer (Eppendorf)).

2.8.2 AFLP analysis

0.5µg of parasite genomic DNA were cut with two enzymes. Firstly, 10 U of *Eco*RI (MBI Fermentas, recognition sequence: G↓AATTC) were added and DNA incubated at 37°C for 1 h, then 5 U of *TruI*/*Mse*I (MBI Fermentas, recognition sequence: T↓TAA) were added and DNA incubated for further 3 h at 65°C. The digestion stage was performed in a 40 µl solution containing 2X Y^+/ Tango buffer (Promega). The fragments were then ligated with adapters matching the cut ends produced by the enzymes. All primers used were provided by MWG-Biotech UK Ltd. Adapters disrupted the cutting site recognised by the enzymes in order to prevent cutting of the adapters from the DNA fragment. These adapters also provided a recognition site for primers.

-*Mse*I adapters: MeI.a1 and MeI.a2

-MeI.a1: 5'-GACGATGAGTCCTGA**G**-3'

-MeI.a2: 3'-TACTCAGGACT**C**AT-5'

-*Eco*RI adapters: EoI.a1 and EoI.a2

-EoI.a1: 5'-CTCGTAGACTGCGTAC**C**-3'

-EoI.a2: 3'-CATCTGACGCATG**G**TTAA-5'

Bold letters indicate base substitution to disrupt enzyme cutting site.

Adapters were prepared by adding equimolar amounts of both strands and then performing the following procedure:

Heat adapters at 94°C for 5 mins, then cool to 21°C for 5 mins

Heat adapters at 72°C for 60 s., then cool to 21°C for 5 mins

Heat adapters at 65°C for 60 s., then cool to 21°C for 5 mins.

Adapters were then diluted to 50 pmol/µl. 10 µl of ligation mixture (Appendix 1) were then added to 40 µl of digested DNA and this incubated at 37°C for 3h, then overnight at 15°C. Ligated material was diluted 1:10 in TE buffer (pH 8.0) (Appendix 1) and stored at -20°C.

A preliminary PCR amplification was performed involving the use of "non-selective" primers (i.e. primers with no extra nucleotides added at their 3' ends extending beyond the adapters sequence) matching the adapters:

Non-selective *Eco*RI primer: 5'-GACTGCGTACCAATTC-3'

Non-selective *MseI* primer: 5'-GATGAGTCCTGAGTAA-3'

A 20µl PCR solution containing 0.32 µM of each of the non-selective primers, 1 µl template DNA, 0.4 U *Taq* polymerase (Promega), 1X Mg-free PCR Buffer (Promega),

1.5 mM $MgCl_2$, and 0.2mM of all 4 dNTPs was set up. The following cycles were performed:

- 94°C for 60 s., then
- 94°C for 30 s.
- 56°C for 60 s.
- 65°C for 60 s.
- repeat the three steps for 20 cycles

The resulting PCR material was diluted 50-fold in TE buffer (pH 8.0) and stored at -20°C.

Selective amplification was performed using radiolabelled primer (*Eco*RI-primer) and selective primers (i.e. primers with a selective extension at the 3'-end, in order to reduce the number of fragments amplified). For radiolabeling, 2.5X Kinase buffer (Promega), 20U T4 polynucleotide Kinase (Promega), 100 μCi [γ-^{33}P] ATP / [γ-^{32}P] ATP (ICN) and 500 ng of oligonucleotide primer were incubated in a 20 μl solution at 37°C for 60 min. The reaction was stopped by adding 1 μl of 0.1 M EDTA pH 8.0 and heating at 70°C for 10 min. The mixture was then made up to a volume of 50 μl by adding sterile, distilled water.

Primer purification was performed using TE Micro Select-D, G-25 microcentrifuge spin columns produced by Eppendorf-5 Prime Inc.

Hot PCR with the radiolabelled primer was performed as follows: 0.32 μM of the selective *MseI* primer, 0.05 μM of the labelled selective *Eco*RI primer, 1 μl template DNA, 0.4 U *Taq* polymerase (Promega), 1X Mg-free PCR Buffer (Promega), 2.5 mM $MgCl_2$, and 0.2mM of all 4 dNTP's were added to a 20μl total volume PCR solution.

PCR conditions for this stage were:

- 94°C for 60 s., then
- 94°C for 30 s.
- 65°C for 60 s., annealing temperature is reduced at each cycle by 0.7°C for the next 12 cycles, then remained at 56°C for the remaining 23 cycles
- 65°C for 60 s.
- repeat the preceding three steps for 35 cycles

The PCR products were mixed with 20 µl of loading dye specific for poly-acrylamide sequencing gels (Anachem), then heated at 99°C for 3 min and immediately cooled on ice. 5µl of each sample was loaded onto a 5% denaturing polyacrylamide gel (5% acrylamide, 0.25% methylene bisacryl, 7.5 M urea in 50mM Tris/50mM Boric acid/1mM EDTA). 500 µl of a 10% Ammonium Persulfate solution (APS) and 100µl of TEMED (Sigma) were added to 100 ml of gel solution and the gel cast using a SequiGen 38x50 cm gel apparatus (BioRad). Electrophoresis was performed at 110 W for 2 h in 1x TBE buffer. Gels were then dried in a vacuum gel drier (model 583, Bio Rad) and exposed overnight in phosphorimager screens (Fuji) at -70°C. Results were visualised on an autoradiography film (Kodak XAR-5). They were developed in an automatic autoradiographer developer (Exograph).

The entire AFLP procedure is summarised in Figure 1.18

2.8.3 Measurement and comparison of the intensity of AFLP markers

Markers between ASpyr1 and AJ were identified as bands that were present in one clone, but not the other. Marker band intensities were measured with both PhosphorImager (Molecular Dynamics) and IMAGEQUANT software (Molecular Dynamics), and the values were converted to relative intensity indices (RIIs). The RII of an AFLP marker in a genetically mixed population of parasites is defined as the intensity of the AFLP marker

band in the mixture divided by the intensity of a designated non-polymorphic band in the same mixed parasite sample on the same AFLP gel (its intensity index), divided by the equivalent ratio (intensity index) of the same two bands (AFLP and non-polymorphic band) when measured in a sample of the pure parental strain (Martinelli et al., 2004). For the purpose of determining an intensity index, non-polymorphic reference bands were chosen close to the polymorphic AFLP bands to minimize error due to any variation in gel or image quality (Grech et al., 2002a).

Comparative Intensity (CI) is defined as the RII of an AFLP marker in the cross progeny selected by pyrimethamine treatment of mice (RIIp), divided by the RII of the marker of the cross progeny grown in a parallel non-pyrimethamine treated group of mice (RIInp), and expressed as a percentage, i.e., CI = (RIIp/RIInp) x 100. An example of the calculation of the CI of a marker under selection is given below.

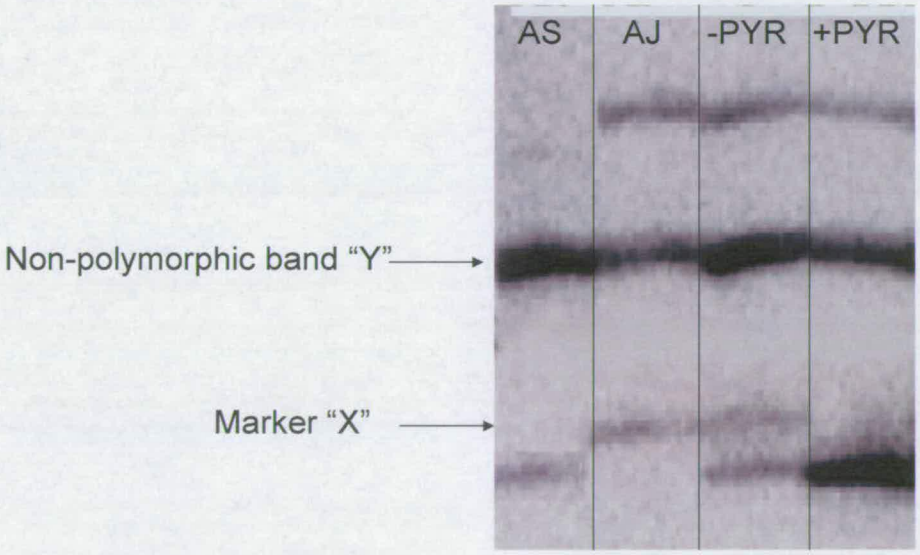


Figure 2.2 A section of an AFLP polyacrylimide gel showing a marker of the AJ parent which is reduced under pyrimethamine selection.

In order to determine the Comparative Intensity of marker "X" (Figure 2.2, above), the following procedure was followed:

1) The intensity of marker "X" was measured in each treatment group, resulting in the following values;

Treatment Group	AS	AJ	-PYR	+PYR
Intensity	20	300	150	26

2) These intensity readings were then corrected for lane bias by dividing the values by the intensities of a nearby non-polymorphic band (marked "Y" in Figure 2.2);

Treatment Group	AS	AJ	-PYR	+PYR
Intensity "X"	20	300	150	26
Intensity "Y	400	400	400	400
X/Y	0.05	0.75	0.375	0.065

3) "Relative Intensities" were then calculated by dividing the corrected intensities by the value for the AJ parent (0.75), giving;

Treatment Group	AS	AJ	-PYR	+PYR
Relative Intensity	0.06	1	0.50	0.08

4) The degree by which the marker under PYR treatment was removed compared to the same marker in the non-treated group is given by the Comparitive Intensity – in which the Relative Intensity of the marker under PYR treatment is divided by the Relative Intensity of the same marker in the absence of drug treatment and the result expressed as a percentage;

$$C.I = \left(\frac{0.08}{0.5}\right) X\ 100 = 16\%$$

2.8.4 Assignment of AFLP markers to locations in a *P. chabaudi* genetic linkage map.

Markers were ordered on a genetic linkage map of *P. c. chabaudi* (Martinelli, 2003) obtained from previously generated crosses between AS-derived clones and AJ strains of *P. c. chabaudi* (Walliker et al. 1975; Rosario 1976; Carlton et al. 1998). A total of 674 AFLP markers were typed for each of 28 cross-progeny clones, and were subsequently

assigned to linkage groups using the Map Manager QTX software (Manly et al. 2001). A total of 44 RFLP markers characterized in a previous study (Carlton et al. 1998) were used as genetic anchors to allow the assignment of the various linkage groups to chromosomes. In total, 11 chromosomes could be identified, while 12 linkage groups of as yet unknown assignment remain, which include the three remaining chromosomes (i.e. chromosomes 2, 4, and 14). A more detailed account of this process is available (Martinelli, A., Hunt, P., Fawcett, R., Cravo, P., Walliker, D. & Carter, R., in prep.).

2.8.5 Sequencing of AFLP markers, and their location on the *Plasmodium falciparum* genome

AFLP bands that appeared to be under selection were excised from acrylamide gels using a sterile scalpel. The gel fragments were then soaked in an Eppendorf tube in 50 µl of autoclaved, distilled water (sdH_2O) overnight. Gel slices were centrifuged at 12000 g prior to removal of the liquid phase containing the DNA. DNA was precipitated using 1/10 volume of 3M sodium acetate (pH 5.2) and 3 volumes of ice-cold absolute ethanol. The solution was placed at -20°C for at least 1 h. Thereafter the tubes were spun for 30 minutes at high speed. The liquid phase was removed and the DNA pellet washed twice in 70% ethanol, before air drying. The pellet was then dissolved in 50 µl TE buffer (8.0 pH). The extracted DNA fragment was amplified by PCR, using the same selective AFLP primers and PCR conditions that produced it. Sequencing reactions were carried out using the protocol described in Section 2.5.7. The sequences of markers were then physically mapped in the *P. falciparum* genome (sequence data for *P. falciparum* were obtained from the Sanger Centre website, which can be accessed at www.sanger.ac.uk_Projects_P_falciparum), using BLAST searches.

2.9 Proportional sequencing of *dhps*, *dhfs*, *6-pthps* and *mdr-1*

The analysis of the cross progeny described in Chapters IV and V involved Proportional Sequencing (Section 2.5.5) of alleles of *dhps*, *dhfs*, *6-pthps* and *mdr-1*. The same PCR

reaction conditions as described for *dhfr* (Section 2.5.6) were used, with the following primer combinations:

***dhps*:**

Outer PCR

pcdhps –11 : **GTACGCAGAATATTTCAAATG**

pcdhps –12 : **CTTTTTATTGGGTATTCAAGG**

Inner PCR

pcdhps –07 : **CTTTTGTTTCTCATAATCCAG**

pcdhps –07 : **GGTTTAGGTTTTGCAAAGAA**

***dhfs*:**

Outer PCR

pcdhfs –03 : **ATTAGGAGGAAGATTGGATG**

pcdhfs –10 : **GTTTTTTACTTGCCGTCAAT**

Inner PCR

pcdhfs –04 : **TTACATCAATTGGTTATGACC**

pcdhfs –09 : **GGCAAGATATATACATTTG**

***6-pthps*:**

Outer PCR

pc11-cg25-02 : **CAGAACTTTTAGTTGAATCA**

pc11-cg25-04 : **GTGAACAAATCTGTGCAGCTA**

Inner PCR

pc6pthps –05 : **AATCACCAGCATTTACATTTC**

pc6pthps –06 : **GGTATATATAAGAGAAATCAGG**

RESULTS

The results of the present work are divided into three chapters:

- **Chapter III** describes the results of experiments designed to determine the optimum drug regimen for the selection of pyrimethamine resistant parasites from a mixture containing both sensitive and resistant parasites. These experiments also involved the use of a quantitative technique (proportional sequencing) for following the fate of single clones in mixed infections. The second round of these experiments also investigated the results of competition between resistant and sensitive clones in two different strains of mice.

- **Chapter IV** is concerned with the results of an LGS experiment conducted with the uncloned recombinant progeny of a cross between a pyrimethamine resistant (ASpyr1) and a sensitive (AJ) cloned strain of *P. c. chabaudi*. This data forms the basis of the "proof of principle" for the validation of the theory of LGS.

- **Chapter V** presents the results of an investigation into the fate of the resistant allele of *dhfr* in a population of parasites containing both the sensitive and resistant alleles in the absence of drug treatment. It also describes the results of experiments conducted on a cross between the pyrimethamine-sensitive precursor of ASpyr1 and AJ parasites. These experiments were designed to investigate the "fitness cost" associated with the *dhfr* PYR resistance mutation.

CHAPTER III

EXPERIMENTS WITH DRUG SELECTION OF MIXED INFECTIONS OF PYRIMETHAMINE RESISTANT AND SENSITIVE STRAINS.

This chapter presents the results of experiments designed to investigate the drug response of mixtures of pyrimethamine resistant and sensitive parasites. The main aim of these experiments was to determine the optimum drug regimen for the selection of pyrimethamine resistant parasites grown in a mixture with a sensitive strain.

3.1 Introduction

The experiments described in this chapter are concerned with the dynamics of malaria infections in which both drug-resistant and drug-sensitive parasites are present. In order to carry out a complete LGS analysis on the progeny of a cross between ASpyr1 and AJ (Chapter IV) it was essential to understand how mixtures of drug-sensitive and drug-resistant parasites would respond to drug pressure. Although drug regimens had previously been optimised which effectively remove sensitive parasites but are tolerated by resistant clones, these had been tested only on pure clonal infections. It is possible that the presence of drug-sensitive parasites in an infection may alter the drug response of resistant parasites and vice versa (Burudi et al., 1994). The first experiment described here attempts to address this problem by following the fate of drug-sensitive and drug-resistant clones in a 50:50 mixture through mice in the presence of two differing drug regimens, as well as in the absence of the drug. This experiment also allowed an evaluation of the Proportional Sequencing technique's ability to quantify the proportions of two different alleles of a gene throughout the course of an infection. The second experiment described in this chapter also addresses the differences in the growth of drug-sensitive and drug resistant parasites in the presence and absence of drug treatment. In addition it includes an analysis of the effect of host genotype on the dynamics of such

infections. Some of the results from these experiments have been published (de Roode et al., 2004a; de Roode et al., 2004b).

3.2 Drug Selection Experiment A

The aim of this experiment was to determine whether it was possible to follow the disappearance of a strain under a selection pressure in a mixed infection of two strains, as well as to provide data on how the two strains respond to different levels of drug treatment in a mixed infection.

Two cloned strains of *P. chabaudi* which differ in their sensitivity to pyrimethamine ASpyr1 (resistant) and AJ (sensitive) were passaged through mice in 1:1 mixtures ($5x10^5$ parasites of each clone) under two different drug regimens (20mg/kg, and 5mg/kg PYR for 4 days), and also through mice left untreated. Figure 3.1 shows a graphical summary of the experimental procedure.

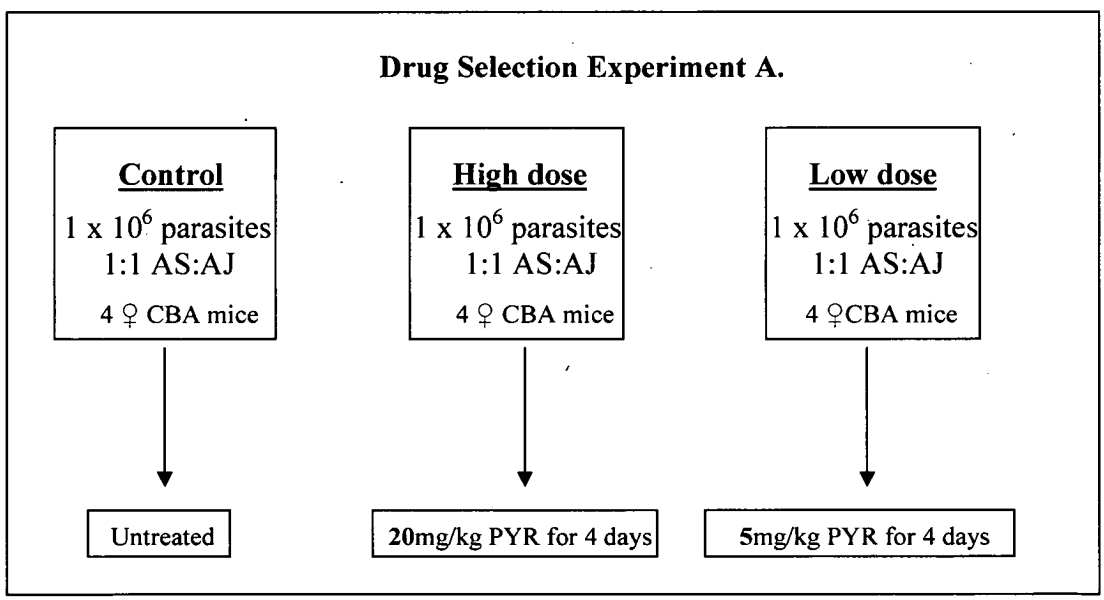


Figure 3.1 The experimental groups used in drug selection experiment A. Infections were followed by thin blood smear on days 4,6,7,8 and 10 post-inoculation (PI), and blood samples were taken for proportional sequencing on days 4, 6, and 8 PI.

3.2.1 Results

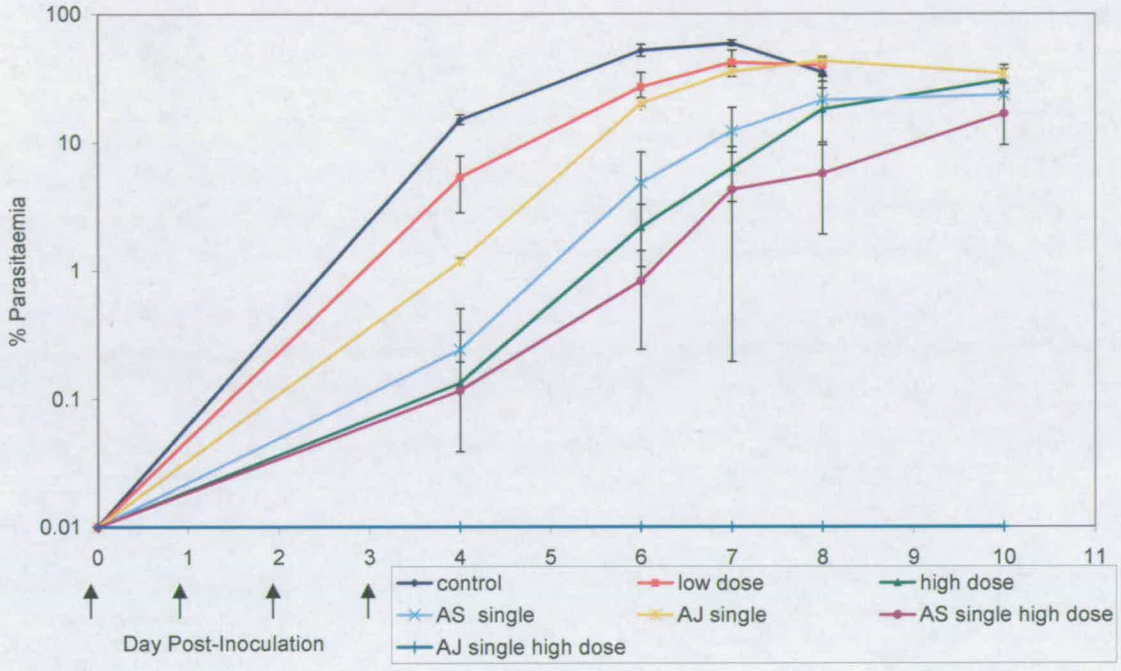


Figure 3.2 The mean parasitaemias of the three treatment groups through time, including single infection data for the two constituent clones. The arrows (↑) indicate the days upon which PYR was administered to the drug-treated group. Each group consists of 4 mice, with the error bars representing 95% confidence intervals of the means. The untreated and low dose groups were terminated at day 8 due to high parasitaemias. Control = Untreated Mice. Low dose = 5mg/kg PYR for 4 days. High dose = 20mg/kg for 4 days.

Figure 3.2 shows the parasitaemias of the mice in all three treatment groups, as well as data for single clone infections in the presence and absence of pyrimethamine. The untreated control group shows the natural course of a 50:50 mixed infection of ASpyr1+AJ, with the mean parasitaemia reaching a peak of 59.3% (n = 4, 95% confidence interval (CI) = 2.86) at day 7 post infection. The low dose group shows lower total numbers of parasites than the control group, with a mean peak parasitaemia of 42.2% (n = 4, CI = 9.89) also at day 7 post infection. The infections in the high drug dose group show considerable retardation of parasite growth, with a mean peak parasitaemia of 30.6% (n = 4, CI = 6.43) attained at day 10 post infection. This is comparable with the single AS(pyr1) infection subjected to pyrimethamine pressure.

Using Proportional Sequencing based on the *pcdhfr* gene (see Chapter 2), it was possible to quantify the proportions of each strain in the infections. Figure 3.3 shows the percentage of the drug resistant strain (ASpyr1), in the above infections.

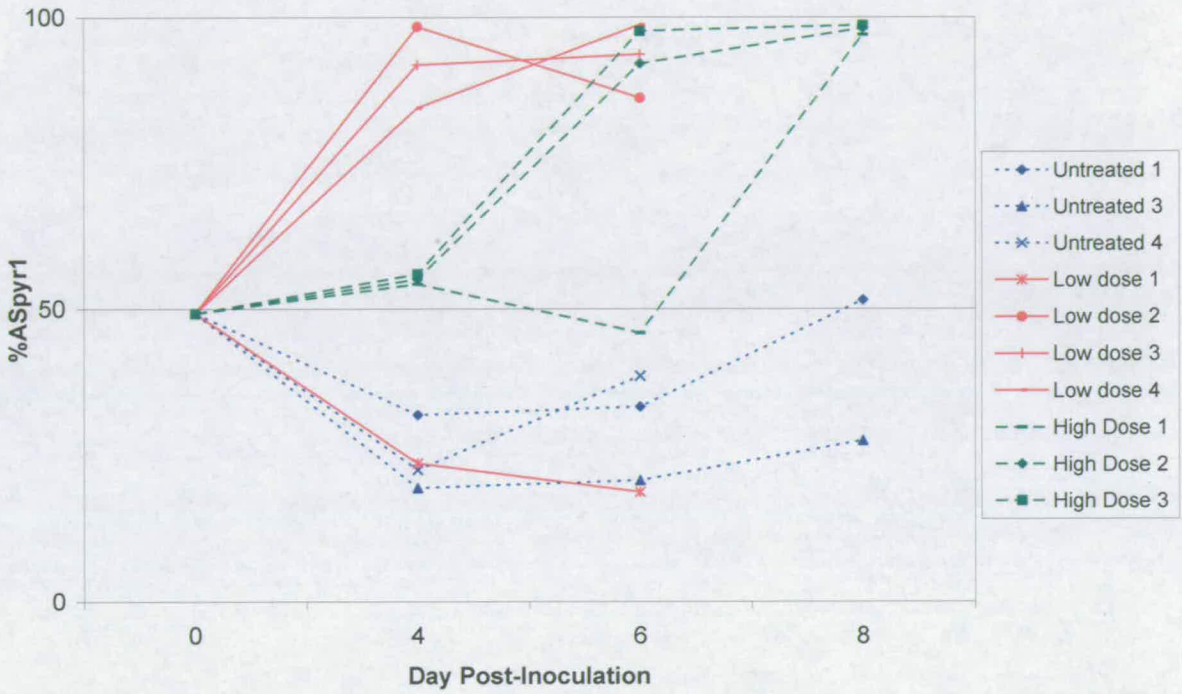


Figure 3.3 The percentage of ASpyr1 parasites in the three treatment groups through time. Each line represents one mouse. Data is available for 3 out of 4 mice in the untreated and high dose groups due to problems during DNA extraction. Control = Untreated Mice. Low dose = 5mg/kg PYR for 4 days. High dose = 20mg/kg for 4 days.

The initial inoculum (i.e. the mixture of AS+AJ administered to all mice) was shown to contain 49.3% ASpyr1. In the control group, the percentage of ASpyr1 dropped to a mean of 24.8% (n = 3, CI = 7.37) by day 4 post infection. By day 8, however, ASpyr1 recovered to 51.6% and 27.7% in the two mice for which data is available. In three out of four mice in the low dose group, the percentage of ASpyr1 in the mixtures rose to a mean of 91.63% (n = 3, CI = 7.92) by day 4 post infection. This remained constant at day 6, the last day of sampling for this group. One infection in this group showed a reduction in the proportion of ASpyr1 present, dropping to 23.8% on day 4, and 18.6% on day 6. In the group receiving the high drug dose, ASpyr1 increased from 49.3% to a mean of 55% (n =

3, CI = 0.96) by day 4. In two infections, ASpyr1 reached a mean of 94.9% by day 6 (n = 2, CI = 5.49), and one infection reached 97.1% AS by day 8.

These results show that AJ outgrows ASpyr1 by day 4 in non drug-treated infections. This domination of AJ in a mixed infection is not total, however, as some ASpyr1 parasites remain to recrudesce later in the infection. The high dose group sees the elimination of pyrimethamine-sensitive AJ parasites by day 6 in 3 mice, and by day 8 in the remaining mice. This high level of pyrimethamine also significantly inhibits the growth of ASpyr1 parasites in the mixture, with little growth seen until day 6 post-infection (the last pyrimethamine dose was administered on day 3 PI). In the low pyrimethamine dose group, ASpyr1 dominates the mixed infection by day 4 PI in 3 out of 4 mice. This dose of pyrimethamine inhibits the growth of AJ in these mice, while allowing ASpyr1 to grow normally. The fourth infection in this group sees AJ dominating the mixture on days 4 and 6 PI. It is possible that in this individual, enough AJ parasites survived the drug treatment to out-compete ASpyr1 due to their faster growth rate.

3.2.2 Discussion

The following discussion of the results of drug selection experiment A is divided into 3 paragraphs, each discussing one of the treatment groups

3.2.2.1 Untreated control group

In this group, the only selection pressure on the strains is direct competition with the other strain in the mixture. The proportion of AJ is seen to increase significantly by day 4 of the infection, due to its faster growth rate compared to ASpyr1 (Figure 3.3). It does not, however, completely dominate the infection, as ASpyr1 is seen to recover slightly by day 8 in two mice. This result can be explained in the context of strain specific immunity, in which the host's immune response to an infection of malaria parasites is specific to the strain that induces the response (Eisen et al., 2002). As AJ is the dominant parasite in the

mixture in the early stage of the infection, it is possible that it elicits an immune response from the host that is specific in targeting only AJ parasites. This could allow ASpyr1 parasites that are not targeted by such a strain-specific immune response to out-compete AJ in the infection by day 8.

3.2.2.2 High drug-dose group

In this group, the proportions of the two strains appear to remain constant up to day 4 PI. This could be due to the high drug dose arresting the growth of both the drug sensitive and drug resistant parasites. When the total parasitaemias of the group are analysed, it can be seen that very little growth occurs in the first 4 days post infection (Figure 3.1). As the growth of both strains has been inhibited during this early period, no change in proportions is seen by day 4. By day 6, however, ASpyr1 has increased to almost 100% in two mice. This shows that some resistant parasites have survived the high drug pressure, and remain viable to start growing when the drug pressure is removed (pyrimethamine is administered up until day 3). Indeed, single clone infections composed of only AS(pyr1) are equally retarded in the presence of pyrimethamine. However, it also indicates the presence of a small proportion of the sensitive AJ parasites. It has been shown that DNA from dead parasites remains detectable by PCR up to 48 hours after parasite death (Jarra and Snounou, 1998). As drug treatment is given over 4 days with the last dose on day 3 PI, any AJ DNA amplified by PCR at day 4 of the infection could be due to residual "non-viable" parasites that have yet to be removed from the circulation.

3.2.2.3 Low drug-dose group

The application of a lower drug dose to this group allows ASpyr1 to outgrow AJ by day 4 PI. This dose of drug inhibits the growth of AJ while allowing the resistant ASpyr1 to grow. Three infections in this group all show the domination of ASpyr1 parasites in the mixture by day 4 PI, and this remains constant at day 6. In one mouse, however, the proportion of ASpyr1 drops by days 4 and 6 PI in much the same way as in the untreated control group. This cannot, however, be due to failure of drug treatment, as the

parasitaemia of this mouse is comparable with the other mice in the group, which show a marked effect of drug treatment inhibiting the growth of the infection. It is possible that in this infection, enough AJ parasites survived the drug pressure to allow the growth rate factor to influence the infection. This drug regimen, then, acts as an "imperfect selection pressure" in which it is possible for growth rate differences to overcome an applied selection pressure. Even though there is a selection pressure (pyrimethamine treatment) against the faster growing parasite, if it is not strong enough to completely arrest the growth of the faster growing strain, then growth rate factors can still determine the outcome of an infection.

The overall results of this experiment show that it is possible to follow the disappearance of a single cloned strain of parasites under an applied selection pressure in infections containing a mixture of two genetically distinct clones. This experiment is the first to follow the growth of two strains in the same host throughout an infection using the Proportional Sequencing technique. It shows that the growth rate differences between ASpyr1 and AJ are extremely important in determining the proportions of parasites in a mixture, especially in the early stages of infection

The results from this experiment led to the design of the following drug selection experiment (Drug Selection Experiment B) involving mixtures of ASpyr1 and AJ. It was seen from the previous experiment (Drug Selection Experiment A) that domination of the mixture by one or other of the strains was usually achieved by day 4 post infection. Drug Selection Experiment B was, therefore, designed to include daily blood sampling from day 1 post infection, in order to understand what is happening to ASpyr1 and AJ in a mixture during the early stages of infection.

3.3 Drug Selection Experiment B

This experiment was carried in order to understand the interactions between ASpyr1 and AJ in mixtures in the early stages of infection. Nine experimental groups were used, with 50:50 mixtures of ASpyr1 and AJ grown in both C57 and CBA mice, along with the

appropriate single infection controls (see Figure 3.4). The drug regimen used was 10 mg/kg for 4 days. Both CBA and C57 mice were used in order to investigate whether there was any difference in parasite growth between these 2 mouse strains. Single strain infections were used to assess any differences in drug response between strains growing in mixed and single clone infections.

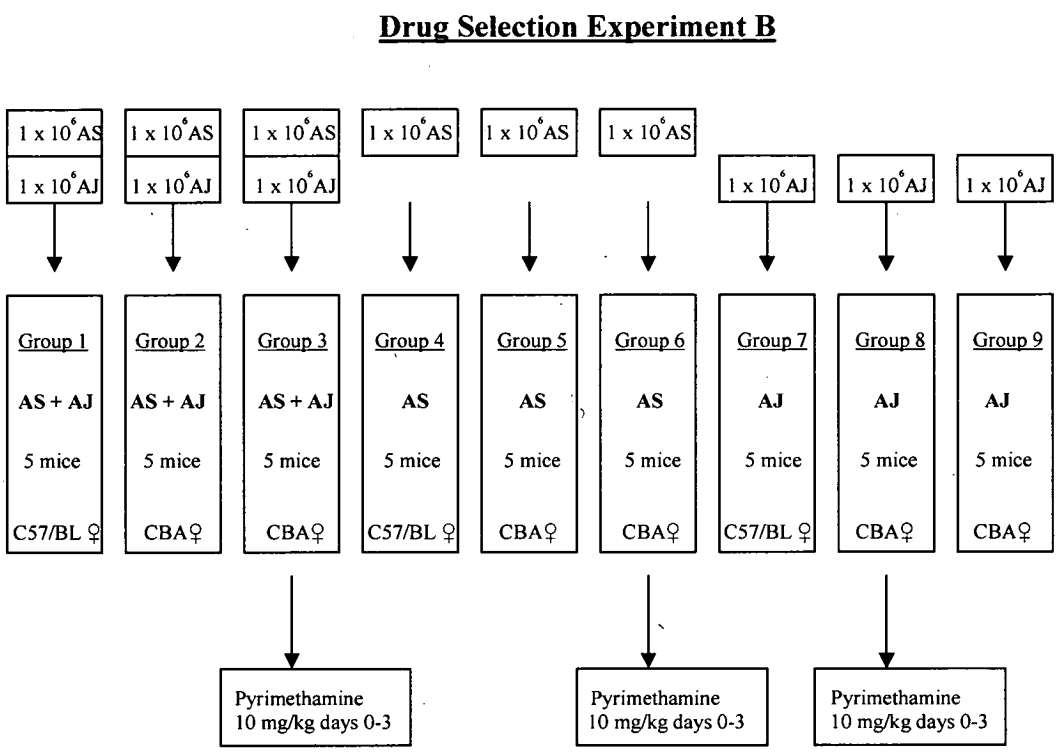


Figure 3.4 The experimental groups used in drug selection experiment B. Infections were followed by thin blood smear daily from day 1 until day 21 PI, and blood samples were taken daily on days 1-7 PI.

3.3.1 Results

The parasitaemias throughout the course of the infections for all treatment groups are given, along with the total areas under the parasitaemia curves, in Figure 3.5

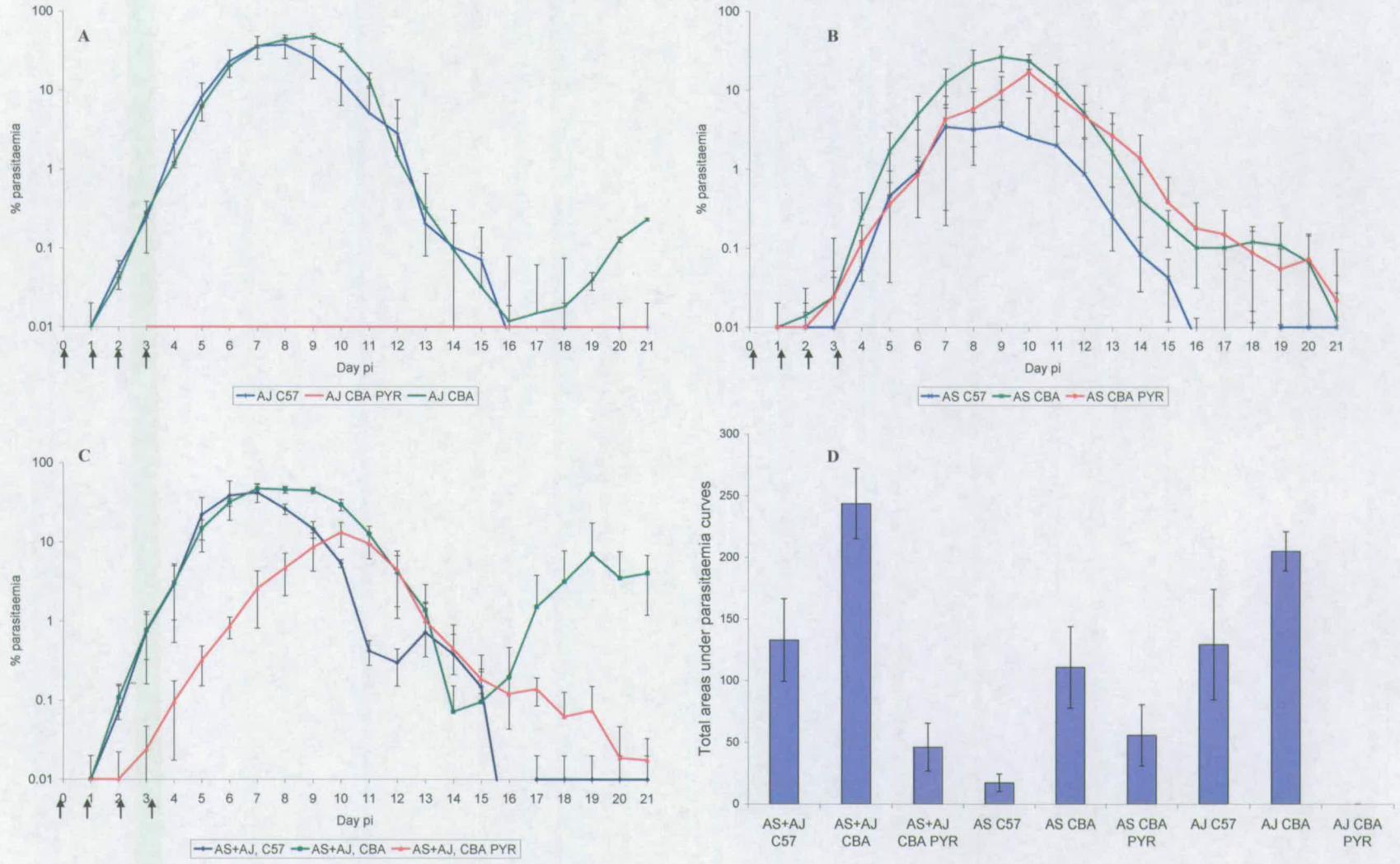


Figure 3.5 The mean parasitaemias of all treatment groups (n=5) up to day 21 PI. The arrows (↑) indicate the days upon which PYR (10mg/kg) was administered to the drug-treated groups. The 9 groups have been split into 3 graphs for clarity; the scales of all graphs are equal. Panel **A** shows AJ single infections, **B** shows ASpyr1 single infections and **C** shows mixed ASpyr1+AJ infections. Panel **D** shows the total areas under the parasitaemia curves for each treatment group. Error bars indicate 95% confidence intervals for the means.

3.3.1.1 Statistical analysis of parasitaemia differences

Data were analysed using a General Linear Model ANOVA in Minitab (v. 13.1, Minitab Inc.). The explanatory variables used were mouse strain, parasite strain and drug treatment. Mouse strain and drug treatment had two factors each (CBA and C57, and PYR-treated and non-treated respectively) and parasite strain had three factors (AS, AJ and AS+AJ). Analysis was performed on the total areas under the parasitaemia graphs for each treatment group, and these data were log transformed to meet the necessary normality and homogeneity-of-variance assumptions. Significant differences between individual treatment groups were identified by t-test analyses.

There was a significant effect of parasite strain ($F_{2,31} = 11.4$, $p < 0.001$), mouse strain ($F_{1,31} = 20.6$, $p < 0.001$) and drug treatment ($F_{1,31} = 17.4$, $p < 0.001$) on the parasitaemias of mice in each of the 9 groups. Differences between individual treatment groups were analysed by Student's t-tests, the results of which are summarized in Table 3.1.

	AS+AJ *C57*	**AS+AJ** *CBA*	**AS+AJ** *CBA* pyr	**AS** *C57*	**AS** *CBA*	**AS** *CBA* pyr	**AJ** *C57*	**AJ** *CBA*	**AJ** *CBA* Pyr
AS+AJ *C57*		0.002**	n/a	<0.001**	n/a	n/a	0.892	n/a	n/a
AS+AJ *CBA*			<0.001**	n/a	0.002**	n/a	n/a	0.043*	n/a
AS+AJ *CBA* pyr				n/a	0.011*	0.563	n/a	n/a	<0.001**
AS *C57*					<0.001**	0.020*	<0.001**	n/a	n/a
AS *CBA*						0.031*	n/a	0.001**	n/a
AS *CBA* pyr							n/a	n/a	<0.001**
AJ *C57*								0.011*	n/a
AJ *CBA*									<0.001**
AJ *CBA* pyr									

Table 3.1 The results of t-tests between individual treatment groups. $^{*}p < 0.05$, $^{**}p < 0.01$, n/a = comparison not applicable

It can be seen that there was a significant growth rate difference between the two parasite strains, with AJ growing faster and reaching a higher parasitaemia than ASpyr1 in single clone infections. This result was independent of mouse strain, with AJ outgrowing ASpyr1 in both C57 and CBA mice. Mouse strain had a significant effect on parasite growth regardless of the strain of parasite and whether the infection was single clone or mixed, with parasite growth in C57 mice being lower than in CBA mice. Mixed infections produced significantly more parasites than single clone infections in CBA mice, but in C57 mice, there was no difference between the total parasitaemias of single clone AJ (the faster growing parasite) infections and mixed infections. Drug treatment completely arrests the development of AJ, with no parasites being visible up to day 21 PI. The growth of ASpyr1 under pyrimethamine treatment was also significantly reduced,

but, interestingly, mouse strain had a more significant effect on the growth of ASpyr1 than drug treatment, with ASpyr1 growing significantly faster in CBA mice than in C57 mice. Drug treatment had a significant effect on the growth of the parasites in the mixtures, comparable with that seen in drug selection experiment A. There was no difference between the growth of parasites in the drug treated mixture and the drug treated ASpyr1 single infection.

3.3.1.2 Effect of parasite strain on host body weight

Previous work with *P. chabaudi* has shown that genetically distinct clones differ in their virulence (measured as anaemia, weight loss and mortality) (Mackinnon and Read, 1999a). In order to investigate differences in virulence between the parasite strains used in this experiment (and to assess if mixed infections were more virulent than single clone infections), the effect of parasite strain on host body weight was measured. A comparison of parasitaemias between parasite strains elucidates the differences in growth rates between parasites, but is not a direct measure of virulence (where virulence is defined as "the ability of the pathogen to produce disease"). As a measure of the virulence of a parasite must include its effect on the host, changes in mouse body weight were recorded daily for the first 21 days of the infection. The results are shown in Figure 3.6

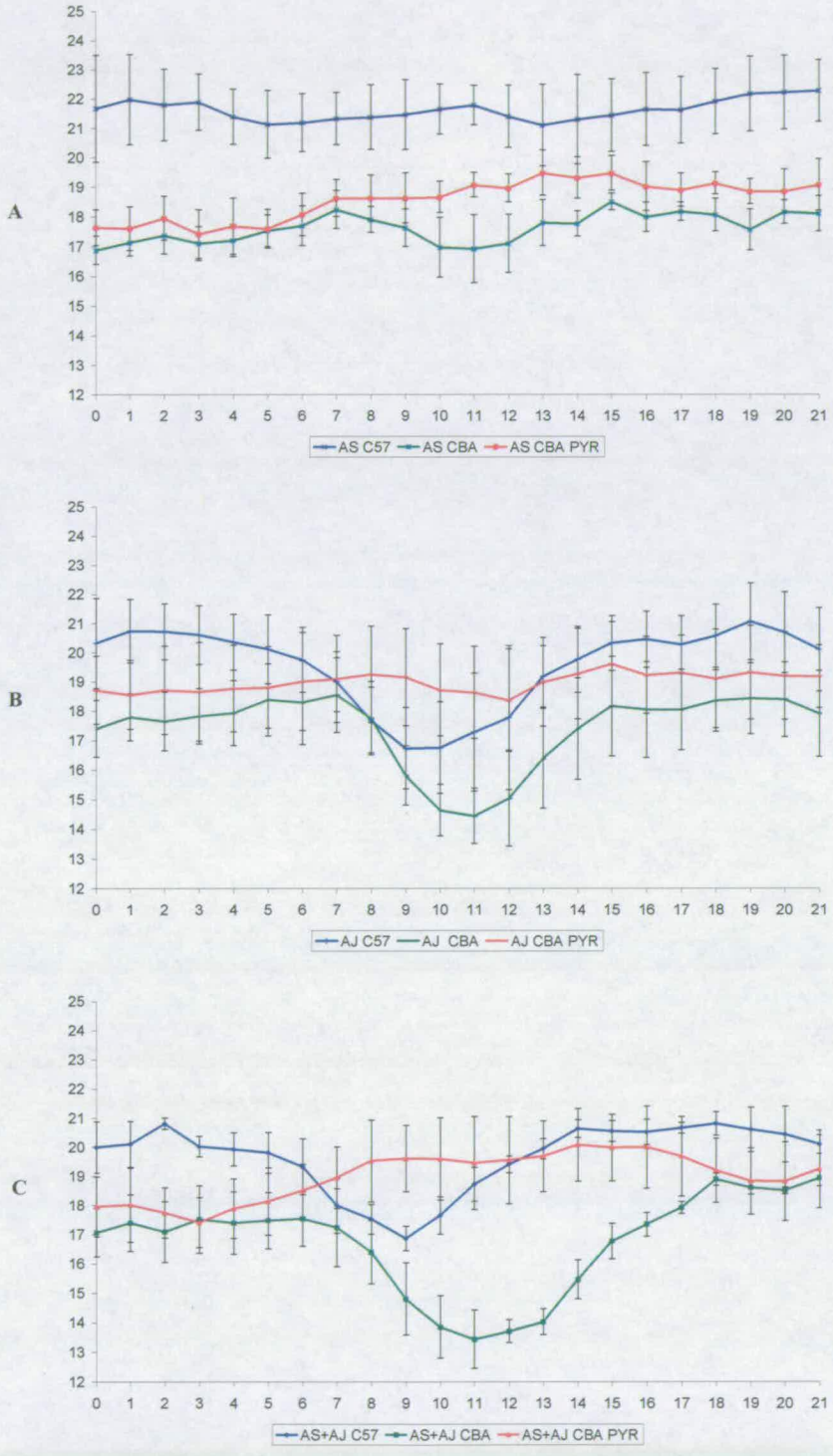


Figure 3.6 The mean weights of mice in all treatment groups (n=5) up to day 21 PI. The 9 groups have been split into 3 graphs for clarity; the scales of all graphs are equal, with the y-axis showing weight in grammes and the x-axis day post-inoculation. Panel **A** shows ASpyr1 single infections; **B** shows AJ single infections and **C** shows mixed AS+AJ infections. Error bars indicate 95% confidence intervals for the means.

ASpyr1 parasites in single clone infections elicit no apparent weight loss in C57 mice, consistent with the low parasitaemia in this group (Figure 3.5). There is some weight loss peaking at day 11 PI in the CBA mice, but this effect is removed with the addition of drug treatment. Infections with AJ parasites cause a marked weight loss in both CBA and C57 mice, with the latter effect being less pronounced. There is no apparent weight loss in the drug treated AJ infections. Mixed clone infections produce similar weight loss effects to single AJ inections, with the effect more pronounced in CBA mice compared to C57 mice. Again, the drug treated group shows no weight-loss effect of infection.

3.3.1.3 Analysis of strain composition of mixed infections

In order to quantify the proportions of each clone in the mixed infections, Proportional Sequencing was carried out as for drug selection experiment A. The data shown in Figure 3.7 represents the Proportional Sequencing results for the mixed infections in CBA mice in drug treated and untreated infections. There are 8 single nucleotide polymorphisms between ASpyr1 and AJ in the *dhfr* gene, and the proportions given in Figure 3.5 represent the mean of the proportion measurements for one of these polymorphic nucleotides.

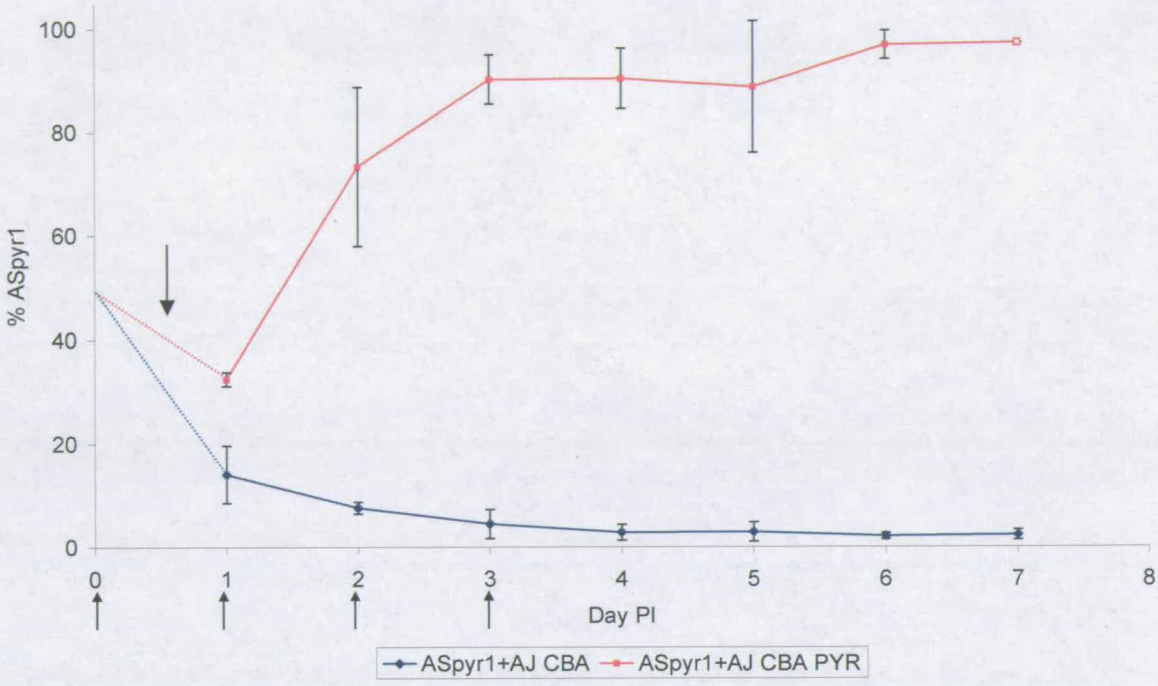


Figure 3.7 The proportion of ASpyr1 in mixed infections of ASpyr1+AJ for the drug treated (PYR, 10mg/kg for 4 days). The arrows (↑) indicate the days upon which PYR was administered to the drug-treated group. The dotted lines between day 0 and day 1 PI, indicated by an arrow (↓), represent an extrapolation from the hypothetical proportions of the alleles at day 0 (50%), which were not measured directly. Error bars indicate 95% confidence intervals for the means. There were five mice in each group, but each data point represents the mean for those mice for which proportional sequencing data was available (between 2 and 5). The open data point at day 7 in the drug-treated group indicates the Proportional Sequencing data for one mouse in the group.

Both groups were infected on day 0 PI with the same initial inoculum comprising of 50:50 ASpyr1: AJ parasites. No molecular data is available on the actual proportions of the parasite clones in this inoculum, but previous experiments have shown that the techniques used to produce the mixed inoculum are accurate, and a variation of greater than 10% in favour of one particular clone is unlikely. It can be seen that by day 1 PI, there was already a significant difference between the two groups, with the untreated infection composed of more than 80% AJ, a reflection of the higher growth rate of this parasite. By day 4 PI, the proportion of the AJ clone had increased to above 95%, at which level it remained constant until day 7, the last day of sampling. There was not, however, complete exclusion of the ASpyr1 parasite from the infection, and some residual ASpyr1 parasites remained. Indeed, further analysis of this group at days 35 – 45

PI showed relapse parasitaemias consisting of mainly ASpyr1 parasites (de Roode *et al.*, 2004b). In the drug treated group, the AJ parasites are removed by day 6 PI, leaving only the resistant ASpyr1 parasites present in the infection. Subsequent sampling of this infection after day 7 showed no re-emergence of AJ parasites (data not shown).

3.3.2 Discussion

In the pyrimethamine treated mixture, the proportion of ASpyr1 initially drops to 32% at day 1 (from an initial inoculum containing 50% of each strain), before recovering to 88 % by day 5, and 97% by day 7 post infection. This represents an increase of AJ from 50% to 68% by day 1 and can be explained in the context of the drug regimen administered. Pyrimethamine is given over the course of 4 days, and it is likely that the first drug dose is not enough to kill off all AJ parasites. This allows the faster growing AJ parasites to outgrow ASpyr1 after this initial pyrimethamine treatment. Drug treatment on subsequent days, however, further inhibits the growth of AJ parasites, allowing the resistant ASpyr1 strain to dominate the infection. A further explanation is that DNA from "dead" AJ parasites was amplified on days 1, as it has been shown that DNA from dead parasites remains detectable by PCR up to 48 hours after parasite death (Jarra and Snounou, 1998).

In the untreated mixture, AJ is seen to outgrow ASpyr1, reaching a proportion of 96% by day 3 post infection. This shows that growth rate is a very strong selection pressure between these two parasite strains. The possibility that the growth rate differences between these parasites are related to a fitness cost associated with the S106N pyrimethamine-resistance mutation is discussed further in Chapter V. No recrudescence of ASpyr1 was seen up to day 7 post infection, although it was subsequently shown that ASpyr1 was not completely eliminated from the infection, as it re-appeared after the chronic phase (days 35-45) (de Roode et al., 2004c). The rate by which AJ outgrows ASpyr1 in a mixed infection had not been previously determined, and this result has important implications for understanding growth rate effects as a confounding factor in the LGS approach to gene identification.

The results from this experiment confirm the findings of drug selection experiment A, that AJ out-competes ASpyr1 in mixed infections, and that it is possible to follow the proportions of genetically distinct parasites through mixed infections using proportional sequencing. This experiment gives an indication of the time scale over which the drug-resistant strain outgrows the sensitive strain under drug pressure, and the rate at which the sensitive parasites dominate the infection in the absence of such pressure. This is important to know for the optimal design of LGS experiments, in which parasites should be harvested from infections at the point in time at which there is the largest difference in proportions between the "sensitive" and "resistant" parasites for the selection pressure under investigation.

3.3.2.1 Host heterogeneity and the dynamics of co-infections with parasites of differing growth rates and susceptibilities to pyrimethamine

The results from both Drug Selection Experiment A and B show that ASpyr1 is at a competitive disadvantage when grown in mixed infections with AJ. This can be explained by the fact that ASpyr1 has a slower growth rate than AJ (observed by comparisons between single strain infections), and so the proportion of AJ in a mixed infection of the two strains would be expected to increase as the infection proceeds. What controls this difference in growth rate between the two strains is currently unknown, although suggestions for factors influencing growth rate differences between malaria parasites include erythrocyte selectivity, antigenic differences, intra-erythrocytic replication rates and cytoadherence factors (reviewed in Chapter I). The two strains used in these experiments also differ in their response to pyrimethamine, with the ASpyr1 strain possessing a mutation in the *dhfr* gene that confers resistance to the drug. It is possible that this resistance mutation also confers a cost to the parasite that lowers its fitness compared to sensitive parasites in the absence of drug pressure. This possibility is explored in more detail in Chapter V.

One of the more striking results of these experiments was the differences in the growth of parasites observed between mouse strains. Different strains of mice are known to have

varying levels of susceptibility to infection with *P. chabaudi*, and the genetic determinants regulating this effect are complex (Fortin et al., 2002; Stevenson et al., 1982). In the current experiments, both strains of parasite achieved significantly higher levels of parasitaemia in CBA mice than in C57 mice, (this was also true for mixed infections). Most interestingly, the differences in growth rates of parasites observed between mouse strains was more pronounced for ASpyr1 parasites (p <0.001) than for AJ parasites (p =0.01).

One possible explanation for this effect is that ASpyr1 parasites may provoke a stronger immune response from the host than AJ parasites (differences in immunogenicity), or they may be more *susceptible* to the immune response than AJ parasites. Such differences would not be detectable in single strain infections through one host genotype, as they would manifest simply as growth rate differences between strains. However, the fact that the growth of ASpyr1 is suppressed to a greater degree in C57 mice (which have greater intrinsic immunity against the parasite than CBA mice (Stevenson et al, 1982)), than AJ suggests that host immune status is a greater restraint on the growth rate of ASpyr1 than it is for AJ. In experiments conducted as part of an investigation into strain specific immunity in *P. chabaudi*, it was found that immunity induced against ASpyr1 conferred a greater level of protection against challenge with other, genetically diverse strains, than did immunity induced against the other strains tested (R. Carter, *pers. com.*). This supports the hypothesis that the relatively slow growth rate associated with ASpyr1 infections is a function of the strong immunity which this strain induces against itself. Of course, such host-immunity mediated strain-specific growth rate differences are unlikely to be manifest in the early phase of an infection (i.e days 0-5), as strain-specific host immunity will not have developed during this period.

3.3.2.2 Implications of these results for the design of an LGS investigation into pyrimethamine resistance

The results from these two drug selection experiments provide important information on the interactions between ASpyr1 and AJ parasites in a mixture. They provide information

on the time scale over which growth rate factors influence the proportions of parasites in mixed infection, and how the parasites respond to drug treatment. One of the main purposes of the experiments was to assess different levels of pyrimethamine dose, in order to carry out the LGS "proof of principle" experiment on the progeny of a cross between ASpyr1 and AJ under pyrimethamine pressure (Chapter IV). Three levels of pyrimethamine dose were been investigated in these experiments. The lowest dose (5 mg/kg) appears to provide the closest approximation of an imperfect selection pressure with some sensitive AJ parasites surviving the drug treatment. The highest dose used (20mg/kg) not only arrests the growth of drug sensitive parasites but also significantly affects the growth of the resistant strain. The intermediate drug dose (10mg/kg) was therefore chosen as the most suitable to use in the LGS experiment with the progeny of a cross between ASpyr1 and AJ.

CHAPTER IV

EXPERIMENTS TO PROVE THE PRINCIPLE OF LINKAGE GROUP SELECTION USING PYRIMETHAMINE SELECTION.

This chapter describes the application of LGS to a study of pyrimethamine resistance in *P. c. chabaudi*. The results of experiments aimed at testing the principle of LGS using a selectable phenotype for which the controlling genetic locus has been previously identified are presented.

4.1 Introduction

The main aim of these experiments was to test the principle of LGS by using a system in which the gene controlling the selectable phenotype is already known. A complete LGS experiment was carried out using the uncloned recombinant progeny of a cross between *P. chabaudi* clones AJ and ASpyr1. These are genetically distinct cloned isolates of *P. chabaudi* that differ in their response to pyrimethamine, with ASpyr1 being resistant to the drug, while AJ is sensitive. As discussed in Chapter I, resistance to pyrimethamine in *P. chabaudi* is conferred by a single base mutation in codon 106 (S106N) of *pcdhfr* (the *P. chabaudi* dihydrofolate reductase gene locus) which is homologous to the *pfdhfr* S108N mutation associated with pyrimethamine resistance in *Plasmodium falciparum* (Cheng and Saul, 1994). By applying pyrimethamine drug pressure to the uncloned recombinant progeny of a cross between these two parasites, it was hoped to identify AFLP markers of the sensitive (AJ) parent that were selected against by the drug treatment, while all other unlinked markers remained unselected by the drug pressure. By showing that markers linked to the AJ allele of *dhfr* were removed or reduced in proportion to their physical proximity to the allele by the selection pressure, the principles underlying LGS would be validated.

4.2 LGS experiment on previously generated crosses

The first of these experiments involved applying pyrimethamine pressure (following the drug regimen optimised by the experiments described in Chapter III) to the uncloned recombinant progeny of 3 previously generated crosses which were available as deep frozen stabilates in this laboratory (described in Table 4.1).

Cross Name	Year Published	Number of Recombinant Clones analysed	Reference
Walliker *et al*	1975	10	(Walliker et al., 1975b)
Rosario	1976	38	(Rosario, 1976)
Carlton *et al*	1998	20	(Carlton et al., 1998)

Table 4.1 Information relating to the history of the three crosses used in this experiment

The decision to use these crosses was taken in order to avoid the potentially time-consuming task of generating a new cross between these parasites. One of the conditions of LGS is that large numbers of recombinant progeny clones are present in the selected population. As these crosses had previously yielded reasonable numbers of cloned recombinants (68 independent recombinant clones in total) it was hoped that the uncloned progeny would contain adequate numbers of recombinants for an LGS analysis. Concerns were raised, however, about the viability of these recombinants after long term storage in liquid nitrogen, and the possible selection of faster-growing parasites through previous serial passage, so it was decided to pool all three crosses in order to maximise the numbers of recombinants present. The three crosses were removed from the deep freeze, pooled and inoculated into 4 experimental CBA mice, and the resulting infections monitored as described in Chapter II. When these infections reached their peak parasitaemias, blood was collected from all mice by incision of the distal portion of the tail, pooled and sub-inoculated into two further groups of 3 mice each according to the protocols previously described. One group was left untreated, so that a normal course of parasitaemia was observed, while the other group was treated with pyrimethamine

according to the regimen previously determined. Parasites from all three experimental groups were collected at peak parasitaemia, and prepared for AFLP as previously described (Chapter II). See Figure 4.1 for a schematic representation of this experiment. The relative intensities of AFLP markers of each of the 3 experimental groups were compared for evidence of the effects of pyrimethamine selection.

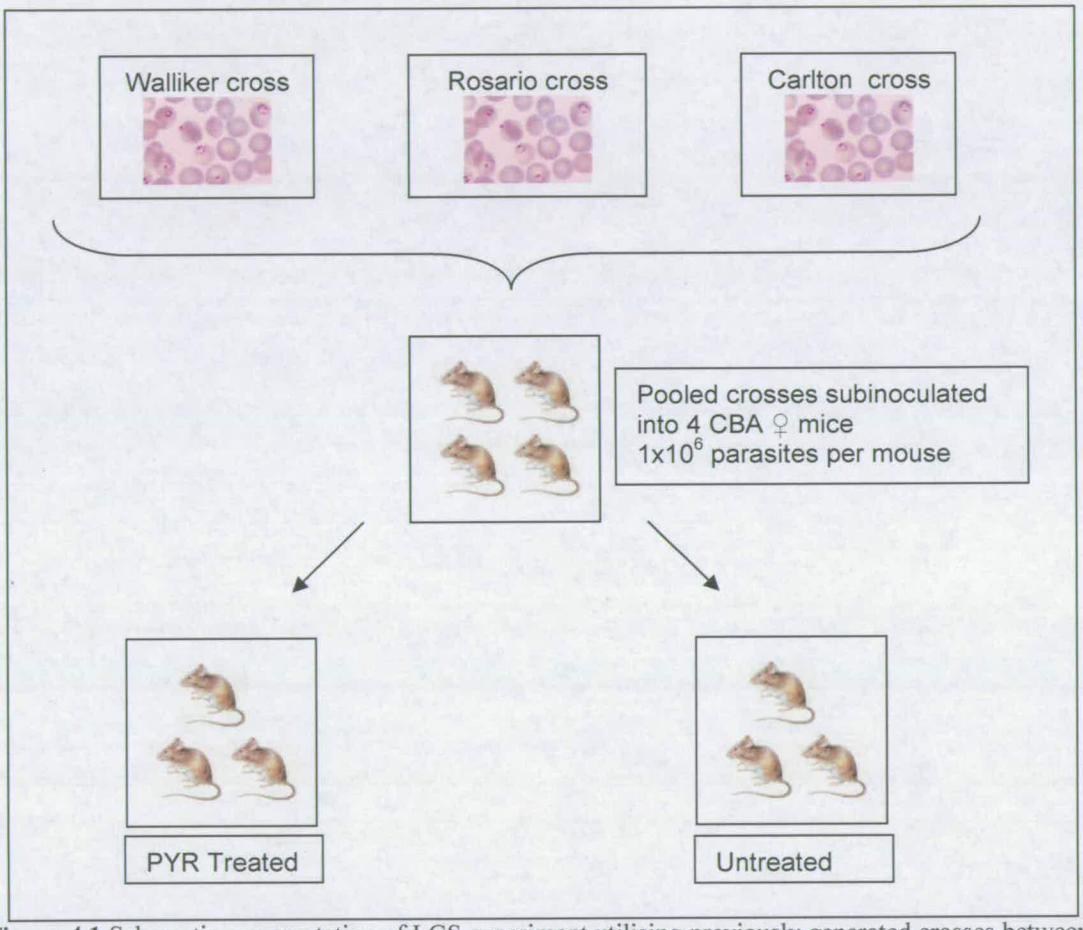


Figure 4.1 Schematic representation of LGS experiment utilising previously generated crosses between AJ and ASpyr1

4.2.1 Results

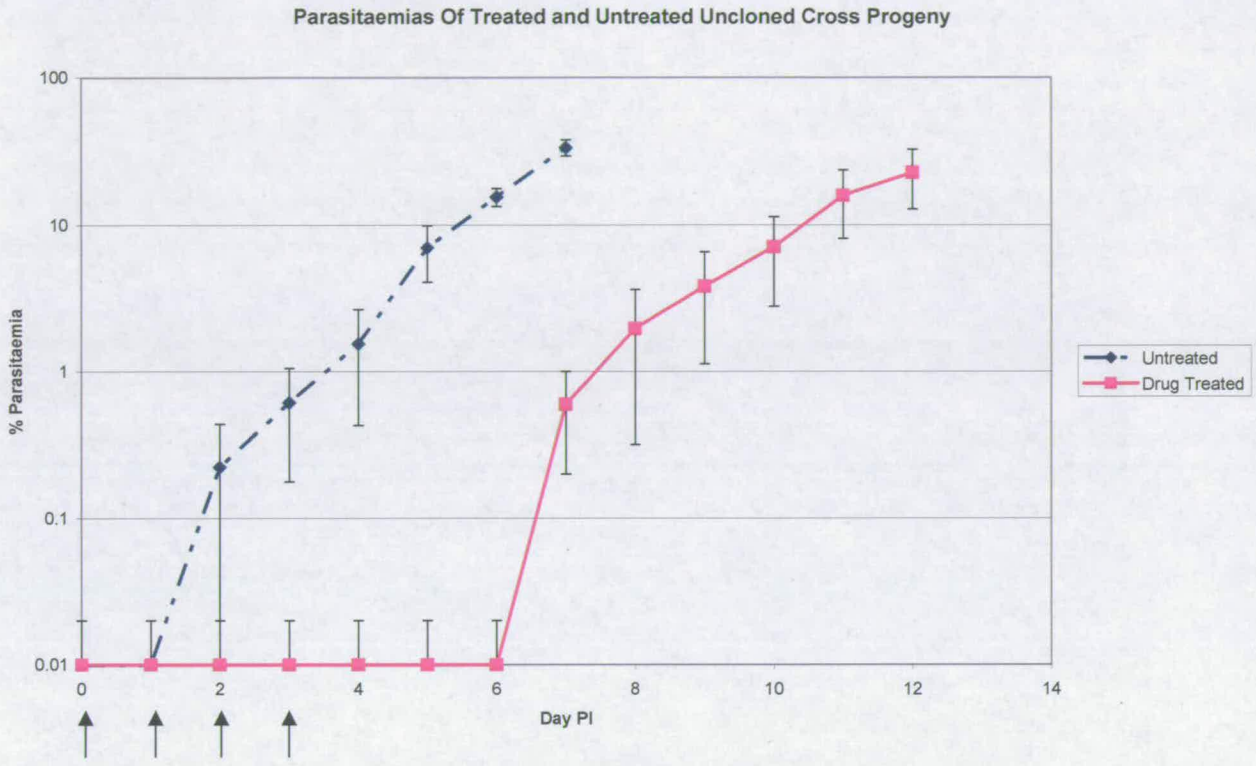


Figure 4.2 Parasitaemia curves for the Untreated and Pyrimethamine Treated uncloned cross progeny of 3 independent crosses of AJ and ASpyr1. The arrows (↑) indicate the days upon which PYR (10mg/kg) was administered to the drug-treated group. Mice were killed and bled when parasitaemias exceeded 20%. Each group contains 3 mice, with the error bars indicating the standard deviation of the means.

Figure 4.2 shows the mean parasitaemias of the mice in the two experimental groups. It can be seen that PYR treatment suppresses the growth of the parasites until day 7, when the resistant parasites recover and follow a normal growth curve. Mice were killed and bled when parasitaemias exceeded 20%, and parasite DNA was extracted as described in Chapter II. This DNA was then prepared for AFLP analysis, and AJ marker intensities were compared between the untreated and PYR-treated groups. A total of 4 AFLP gels were run, comprising of 20 *EcoR1/Mse1* combinations, from which it was possible to analyse 63 AJ markers. An example of two of the *EcoR1/Mse1* combinations for this experiment is shown in Figure 4.3.

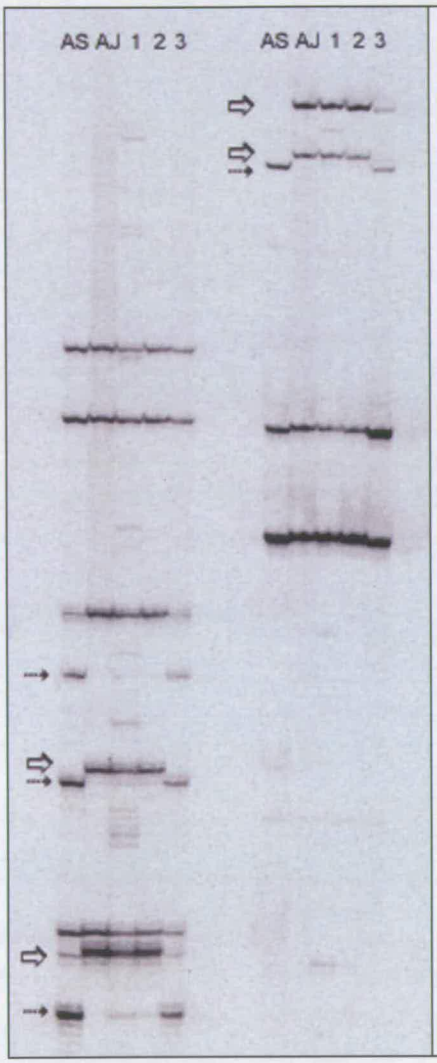


Figure 4.3 Example of an AFLP gel showing markers generated from the experiment described above. This gel shows the results of bands generated with *Mse1* (CA selective bases) + *EcoR1* (AG selective bases) in the left hand set of lanes, and *Mse1* (GG selective bases) + *EcoR1* (AG selective bases) in the right hand set of lanes.

The lanes are labelled as follows;

AS: DNA from single ASpyr1 clone infection
AJ: DNA from single AJ clone infection
1 : DNA from NON-PASSAGED pooled crosses
2 : DNA from UNTREATED group
3 : DNA from PYR TREATED group

Each lane represents the pooled DNA of all mice in the groups.
ASpyr1 specific markers are marked with a broken arrow (⇢), and AJ specific markers with an open arrow (⇨)

It can be seen from this gel that all AJ markers are present only in the non-passaged and untreated groups (lanes 1 and 2), and are completely absent from the PYR selected group (lane 3). This was true for all 63 AJ markers analysed, suggesting that there were very few recombinants in the cross material used. If the cross population contained only parental type parasites, then it would be expected that all the markers associated with the sensitive parent (AJ) would be removed under PYR selection, leaving only the resistant (ASpyr1) parental markers. Moreover, it was also observed that AS markers were absent from the pre-passaged and untreated groups, which is consistent with the theory that there

were very few recombinant parasites in the cross material, and that the AJ parent competitively excludes ASpyr1 when grown in competition (de Roode et al., 2004c).

4.2.2 Discussion

These results show that there were very few, if any, recombinant parasites in the cross material used in this experiment. Although recombinant parasites were undoubtedly generated from these crosses (as demonstrated by the successful cloning of recombinants from the crosses shown in previous studies) it seems likely that multiple freezing and thawing, and subsequent selection of fast-growing parasites through serial passage before they were used in this experiment greatly depleted the numbers of recombinants in this material. Consequently, it was decided to generate a new cross between ASpyr1 and AJ with which to perform an LGS analysis, utilising the cross-generating techniques outlined in Chapter II. This fresh cross material would be subjected to LGS analysis without being frozen down and with the minimum number of passages in order to maximise the survival of recombinant parasites.

4.3 LGS analysis of a new cross between ASpyr1 and AJ

A cross was generated between ASPyr1 and AJ following the protocols described in Chapter II. Briefly, single clone infections of ASpyr1 and AJ were grown individually in donor mice. Blood from these mice was then extracted and mixed together to produce an inoculum containing equal proportions of the two clones. This inoculum was administered intra-peritoneally to 3 mice, so that each mouse received a total of 2×10^6 parasites (1×10^6 of each clone). Six days post-inoculation, the presence of gametocytes was confirmed in the mice by thin blood-smear microscopy. The mice were then anaesthetised and each was placed on top of a mosquito cage containing ~200 female mosquitoes 5-7 days post emergence from pupae. The mosquitoes were allowed to feed on the mice for 30 minutes. Ten mosquitoes from each cage were dissected 7 days post-feed, and the mid-guts were checked for the presence of oocysts. Oocyst numbers are presented in Table 4.2.

Mosquito Cage Number[1]	Gametocytaemia on day of feed	Numbers of Oocysts per mosquito dissected[2]	Mean number of Oocysts per Mosquito	Number of mosquitoes Dissected for sporozoites	Estimated number of recombinants[3]
Parental AJ	0.025	2,0,0,0,0 1,0,1,5,0	0.9	-	-
Parental ASpyr1	0.100	4,29,11,3,6 8,0,21,9,4	9.5	-	-
Cross 1	0.030	8,20,28,0,37 28,0,16,9,11	15.7	143	4490
Cross 2	0.075	2,5,20,17,15 0,15,8,27,9	11.8	120	2832
Cross 3	0.030	0,0,0,3,0 0,0,1,0,1	0.5	98	98

Table 4.2 Numbers of oocysts present in mosquitoes 8 days post-infection.
[1]200 ♀ mosquitoes per cage. Numbers in brackets indicate the individual mouse upon which the cage was fed
[2]10 mosquitoes were dissected from each cage, on day 8 post infective blood-meal
[3]Assumes 50% of oocysts are hybrid, with each producing 4 recombinants (see Figure 1.6)

Mosquito salivary glands were dissected at day 14 post-infective blood meal, pooled from all three cages and sporozoites liberated by gentle crushing in a pestle and mortar containing 50:50 Ringer's solution : foetal calf serum. This inoculum was injected into 2 mice and the resulting infections followed by thin blood film microscopy.

When the sporozoite-induced infections reached peak parasitaemia (10-15%), the parasites were harvested, pooled, and inoculated into a group of 3 mice. Each mouse in the group received 1 x 10^6 parasites. This initial experimental group was left untreated, and the parasites were harvested at peak parasitaemia for AFLP analysis, to provide a reference point for markers analysed in the subsequent treatment groups. Parasites were pooled and sub-inoculated from this group into two further groups of 3 mice one of which was treated with pyrimethamine and the other left untreated.

Pyrimethamine was administered orally at a dose of 10 mg per kg of mouse body weight daily at 24-hour intervals for four days, starting three hours after parasite challenge.

Both the pyrimethamine treated and the untreated blood-stage cross progeny were allowed to grow to peak parasitaemia (30-40 % for untreated and 15-20% for treated), at which point the blood was harvested. Two samples of parasite DNA were prepared for AFLP and other molecular analyses, by pooling separately the blood from the treated and untreated mice. A summary of this experimental procedure is depicted in Figure 4.4.

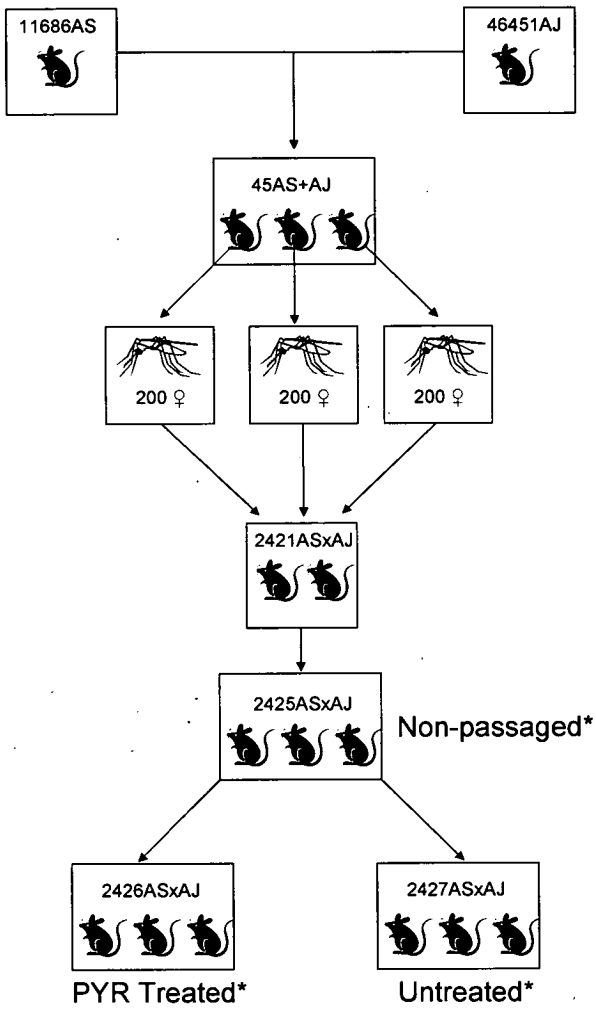


Figure 4.4 A schematic representation of the procedure for the experiment to test LGS with a newly generated cross *Experimental groups are referred to in the text by these names.

4.3.1 Results

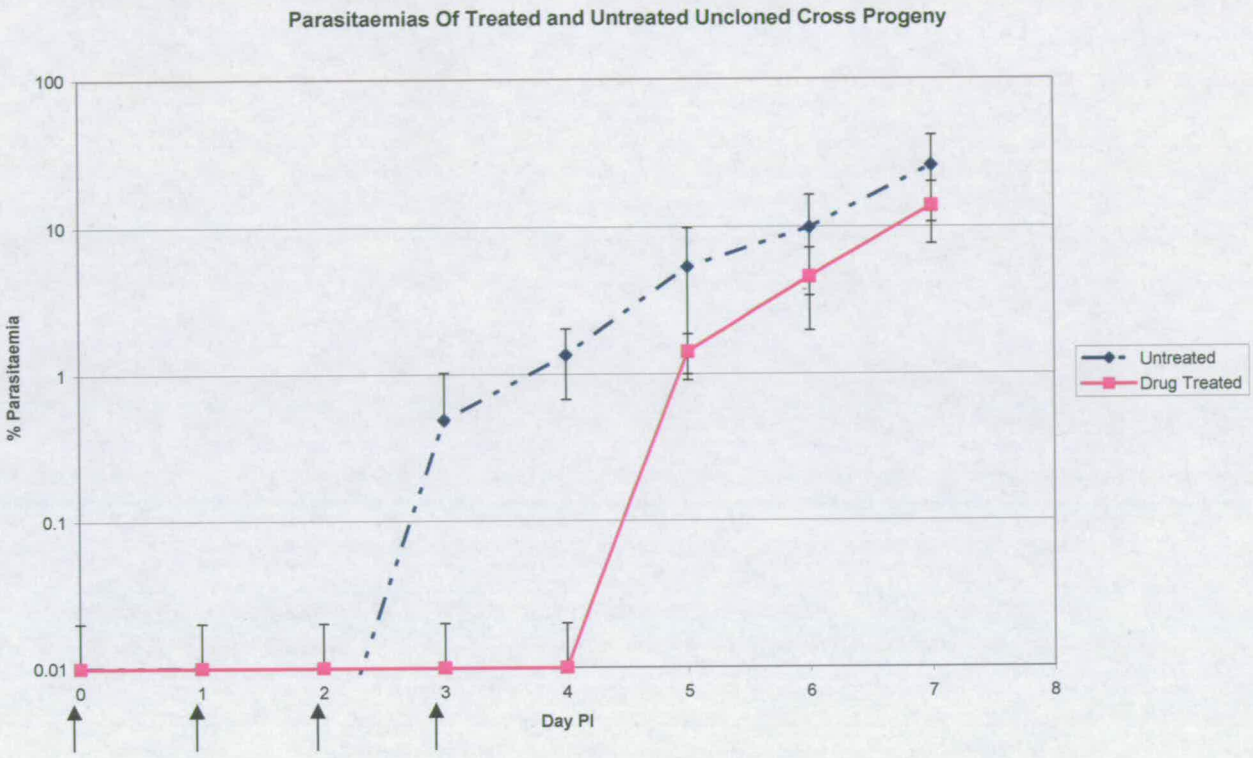


Figure 4.5 Parasitaemia curves for the Untreated and Pyrimethamine Treated uncloned cross progeny of a new AJ and ASpyr1 cross. The arrows (↑) indicate the days upon which PYR (10mg/kg) was administered to the drug-treated group. Mice were killed and bled when parasitaemias exceeded 10%. Each group consists of 3 mice, with the error bars representing the standard deviation of the means.

Parasitaemia curves for both drug treated and untreated groups are shown in Figure 4.5. In contrast to the previous experiment, the drug treated group does not show such a marked inhibition of parasite growth when compared to the untreated group. This is an indication that there are many parasites in the cross material carrying the resistant dhfr allele. A comparison of the areas under these parasitaemia curves shows that there is no significant difference between the two groups (Student's t-test, $p = 0.22$).

The relative intensities of 325 AFLP markers distinguishing the parental clones were compared between the untreated, passaged and drug-treated groups. 206 of these were markers unique to the sensitive parent (AJ) and 119 of the resistant parent (ASpyr1). A Table showing marker relative intensity index comparisons between all treatment groups

is included in Appendix 2 and a statistical analysis of the effects of treatment on the Comparative Intensities of markers in each treatment group is presented in Appendix 3.

It was possible to assign 203 of the AJ parental AFLP markers to a genetic linkage map of *P. c. chabaudi* generated from previous crosses between AS and AJ (see Chapter II for a description of how markers were assigned to this map).

When the PYR treated group was compared to the untreated group, 10 of the 206 AJ markers showed Comparative Intensities of less than 0.5 (i.e. reductions of greater than 50 %). These are shown in Table 4.3, along with their derived positions as located on the linkage map. It can be seen that the markers that show the greatest reductions in intensity are located on chromosome 7. There is also some reduction in 3 linked markers on chromosome 13, and in one marker that was not assigned to a chromosomal location on the linkage map.

Marker Name	Comparative Intensity	Chromosomal Location in genetic linkage map
AJAA05CA	0.01	7
AJAG02CA	0.13	7
AJTG01AG	0.08	7
AJTG01GA	0.06	7
AJAC02TT	0.42	7
AJAT02AT	0.44	13
AJGT01AG	0.41	13
AJAA01AG	0.48	13
AJTT03CT	0.17	Group "E" [1]
AJAT03AT	0.38	Unallocated

Table 4.3 Comparative Intensities of AJ AFLP markers under selection in the PYR treated group, compared to the untreated group, and their chromosomal locations according to the AS/AJ linage map. [1]Group E is a linkage group that could not be assigned to a particular chromosome.

Figure 4.6 shows an example of an AFLP gel for the *Mse1* (CA selective bases) and *EcoR1* (AG selective bases) combinations. Marker AJAG02CA is highlighted, one of the markers that displays almost complete removal in the selected material, while all other markers on the gel remain present.

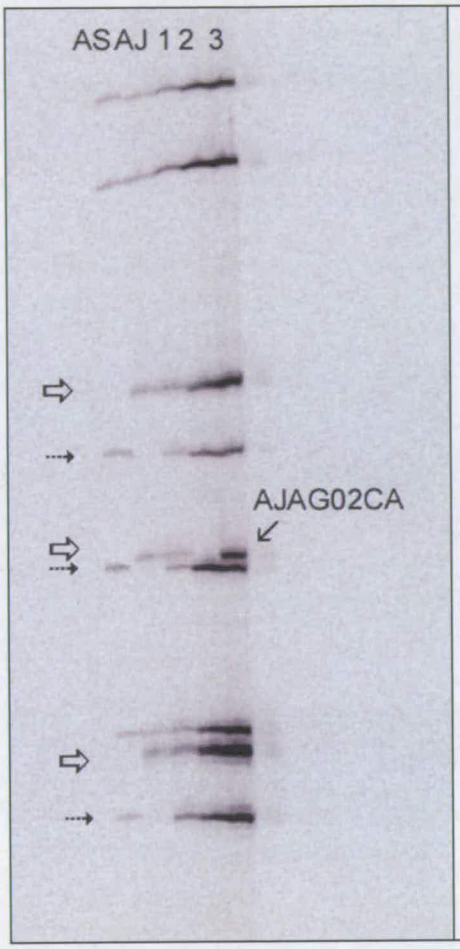


Figure 4.6 AFLP gel showing bands generated from the experiment described above. This gel shows the results of bands generated with *Mse1* (CA selective bases) + *EcoR1* (AG selective bases). The five lanes are labelled as follows;

AS: DNA from single ASpyr1 clone infection
AJ: DNA from single AJ clone infection
1 : DNA from Non-Passaged pooled crosses
2 : DNA from PYR TREATED group
3 : DNA from UNTREATED group

Each lane represents the pooled DNA of all mice in the groups.

ASpyr1 specific markers are marked with a broken arrow (⇢), and AJ specific markers with an open arrow (⇨)

One marker (AJAG02CA) is almost completely removed (Comparative Intensity = 0.13) in the PYR treated group.

By assigning AFLP markers to the AS/AJ linkage map, it is possible to generate graphs showing the change in marker intensities between treatment groups as they appear across the *P.c.chabaudi* genome. Figure 4.7 shows the Comparative Intensities of AJ (sensitive parent) markers between the PYR treated and the Untreated groups. It was found that all of the AJ markers which were most reduced under pyrimethamine pressure lie on chromosome 7 of *P. c. chabaudi*. Moreover, when these markers are put into order according to the genetic map, they form a "selection valley" around the position of the marker (AJAA05CA) which had been eliminated by pyrimethamine treatment. Figure 4.8 shows the same graphs for the AS (resistant) parental markers. No markers show significant reductions in Relative Intensity Indices between the PYR treated and Untreated groups. Figure 4.9 was created by superimposing Figure 4.7(A) on to Figure 4.7(B). This allows a direct comparison between the Comparative Intensities of both sets of parental markers.

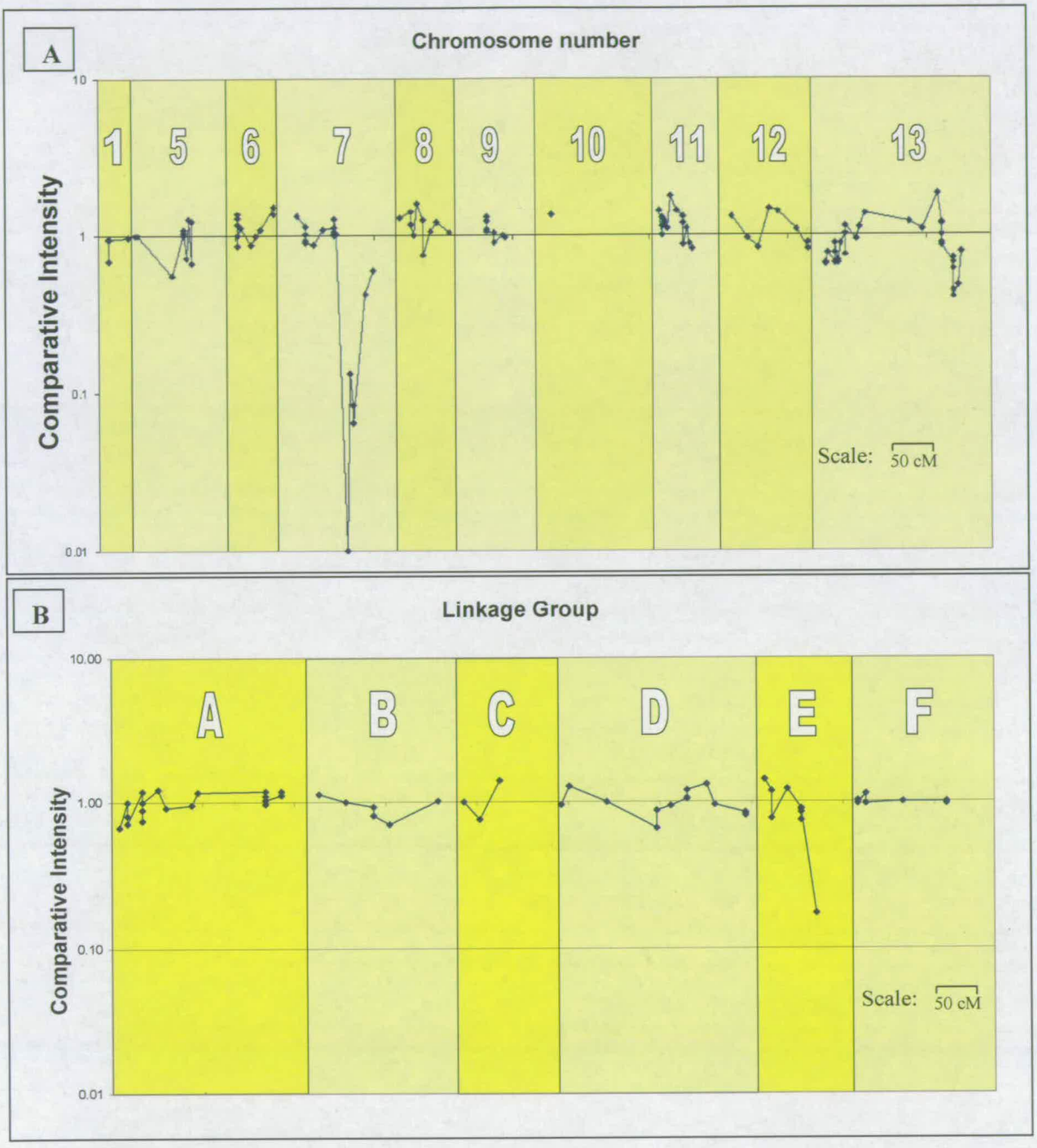


Figure 4.7 Comparative Intensities of AJ (sensitive parent) markers in the PYR treated group compared to the untreated group, for markers assigned to *P.c.chabaudi* chromosomes (A) and non-chromosomally allocated linkage groups (B). Markers are positioned on the X axis according to their positions in cM along the chromosomes/linkage groups, and the size of the chromosomes/linkage groups in cM according to the map are indicated by the width of the coloured columns.

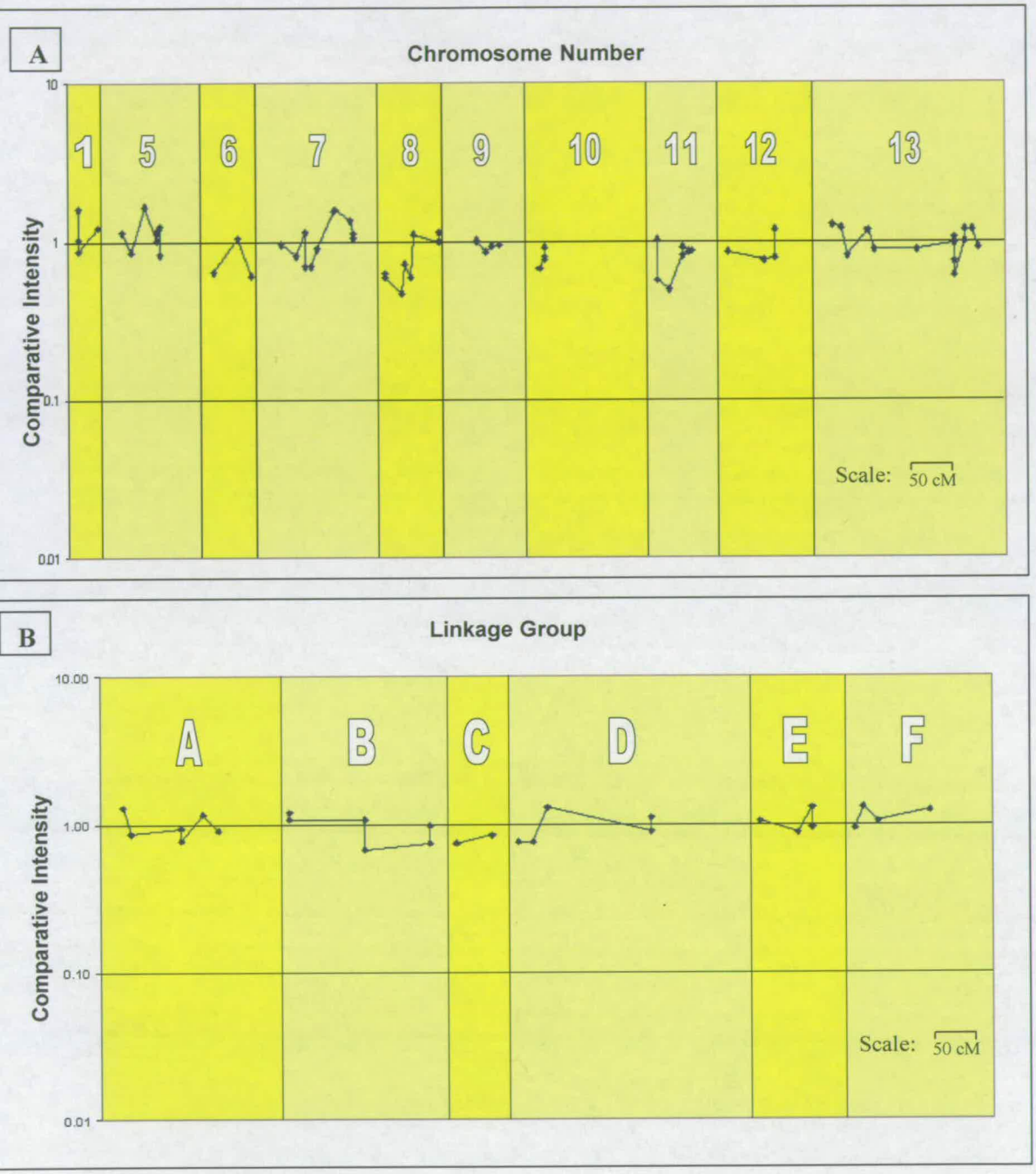


Figure 4.8 Comparative Intensities of ASpyr1 (resistant parent) markers in the PYR treated group compared to the untreated group, for markers assigned to *P. c .chabaudi* chromosomes (A) and non-chromosomally allocated linkage groups (B). Markers are positioned on the X axis according to their positions in cM along the chromosomes/linkage groups, and the size of the chromosomes/linkage groups in cM according to the map are indicated by the width of the coloured columns.

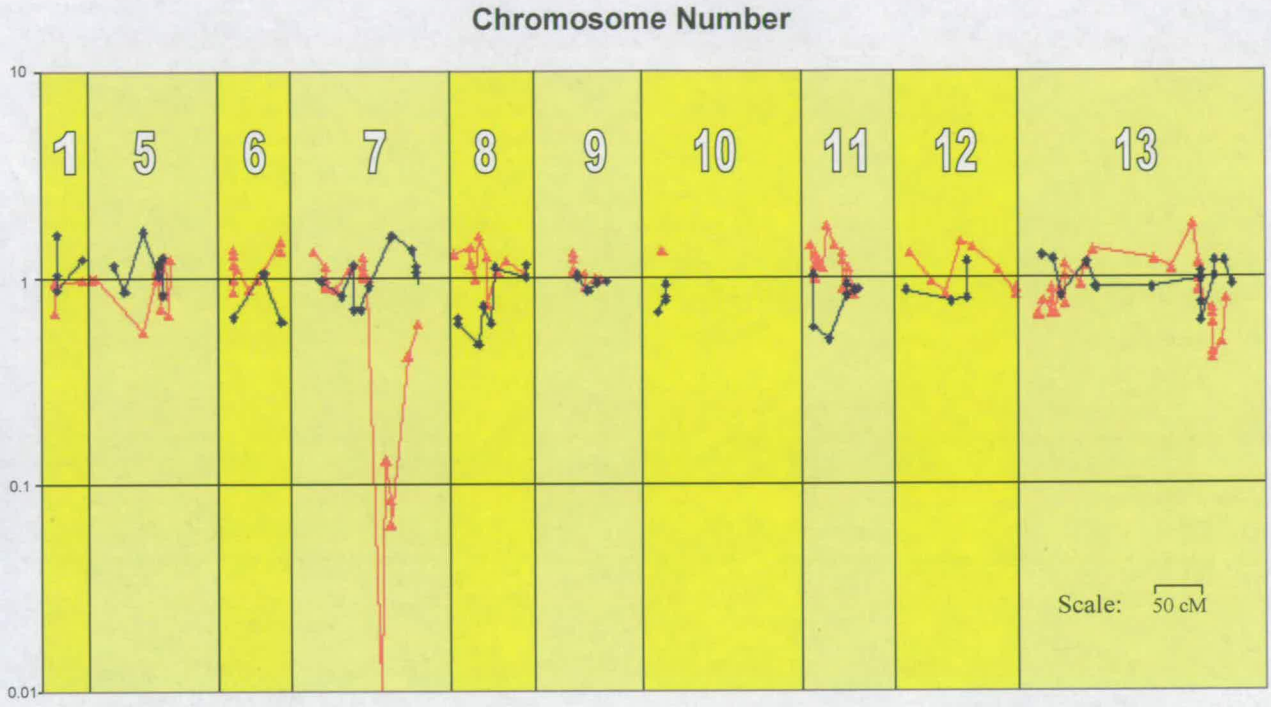
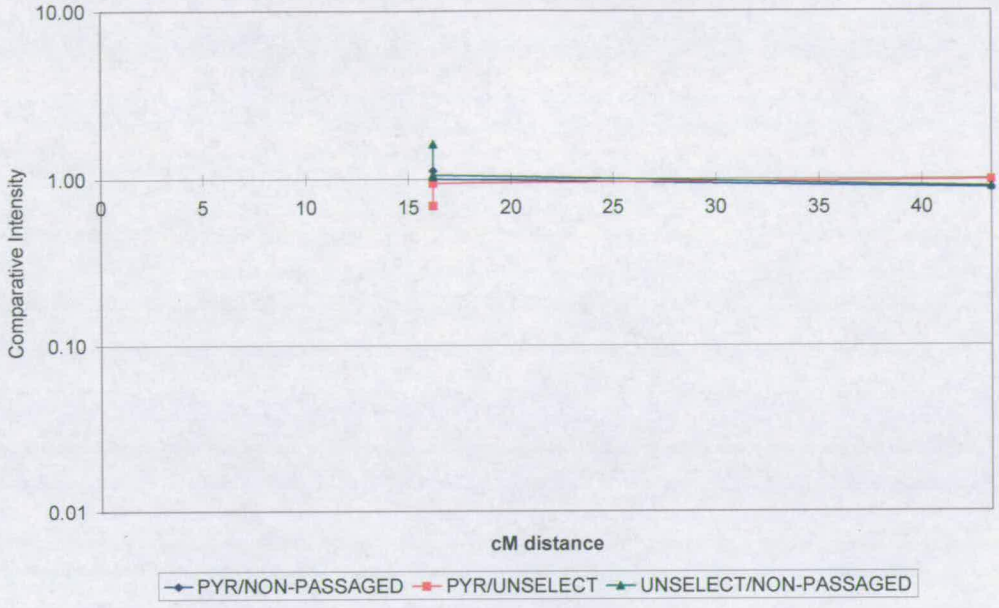


Figure 4.9 The Comparative Intensities of AJ markers (red line), and ASpyr1 markers (blue line) in the PYR treated group compared to the untreated group (data combined from Figures 4.7 and 4.8).

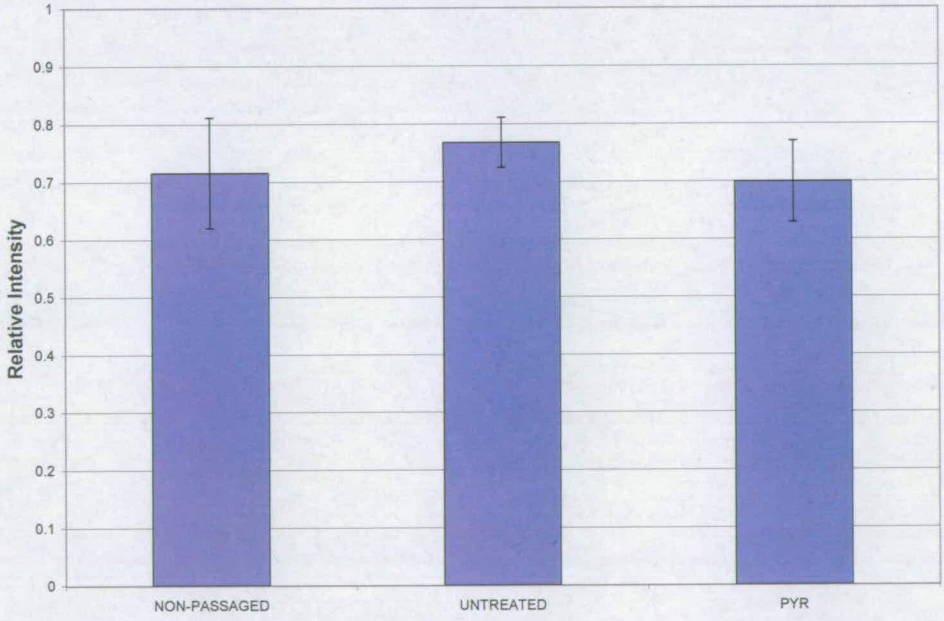
4.3.1.1 Analysis of individual linkage groups and data summaries

In this section, the relative intensities of AJ markers are compared between each treatment group for each chromosome/unassigned linkage group individually, in order to highlight the differences in marker relative intensity between treatment groups in detail. General Linear Model (GLM) analyses of variance were carried out on each chromosome / linkage group in order to determine if there were statistically significant differences in marker intensities between treatment groups. The groups are referred to throughout by the names designated in Figure 4.4 (i.e. "non-passaged", "untreated" and "PYR"). The analysis of each chromosome / linkage group consists of a graph showing the comparative intensities of markers between the three groups (A), in order to demonstrate the changes in relative intensities between treatment groups for individual markers, and a bar chart showing the mean relative intensities of markers in each treatment group with error bars indicating the standard error of the means (B). Each figure also contains a Table showing the results of individual GLMs (C). The section ends with a summary for all markers analysed.

A



B

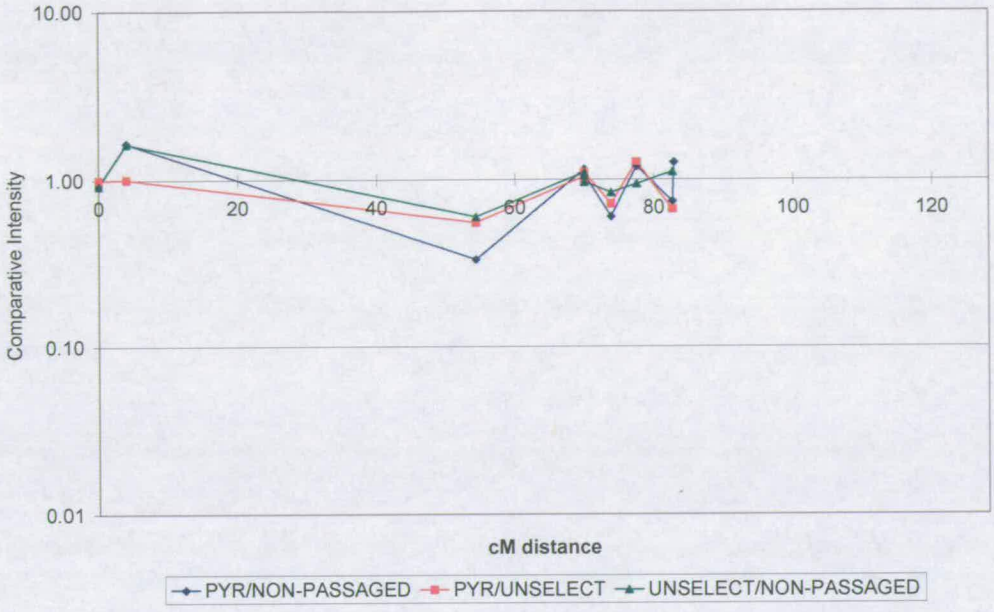


C

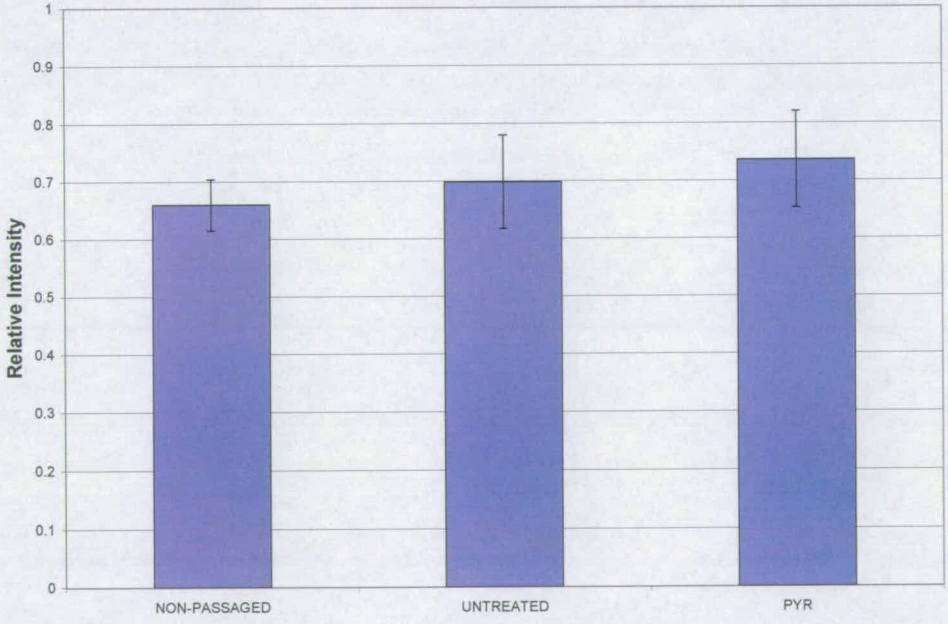
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	5	0.31	0.73	No
Pairwise Comparisons				
PYR / Non-Passaged			0.99	No
PYR / Untreated			0.76	No
Non-Passage / Untreated			0.78	No

Figure 4.10 AJ Markers on **Chromosome 1**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

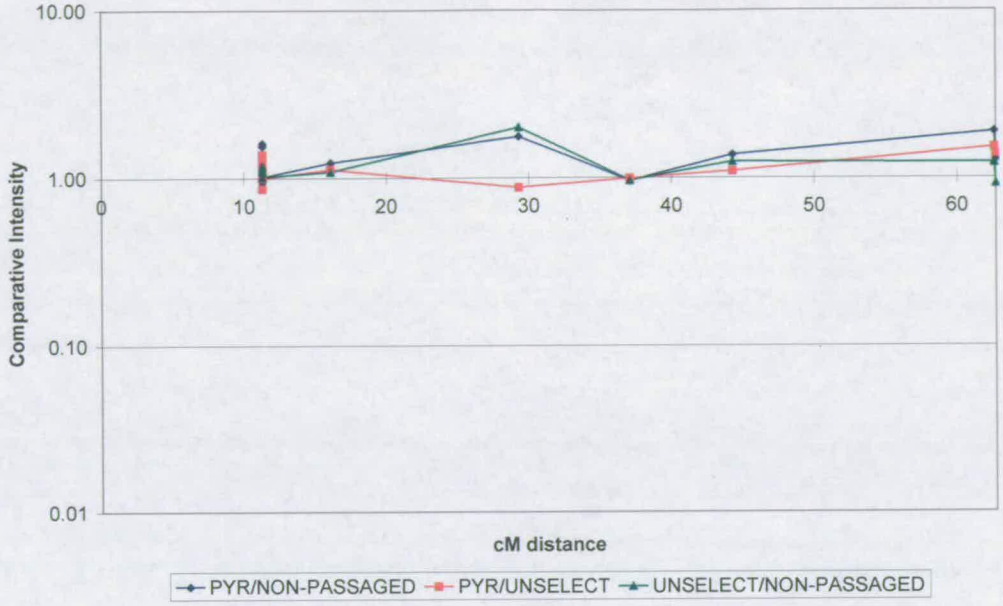


C

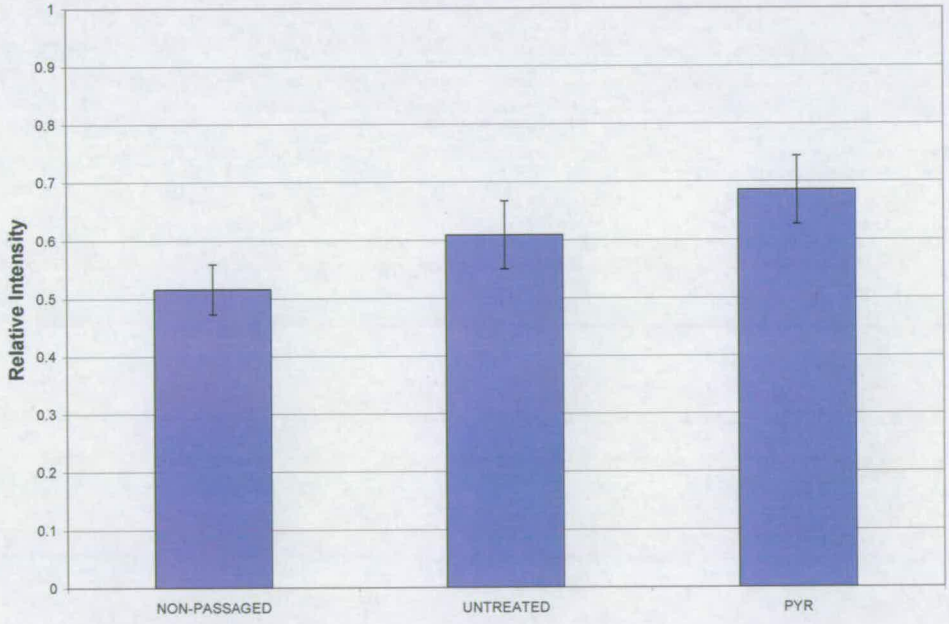
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	9	0.09	0.91	No
Pairwise Comparisons				
PYR / Non-Passaged			0.90	No
PYR / Untreated			0.96	No
Non-Passage / Untreated			0.98	No

Figure 4.11 AJ Markers on **Chromosome 5**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

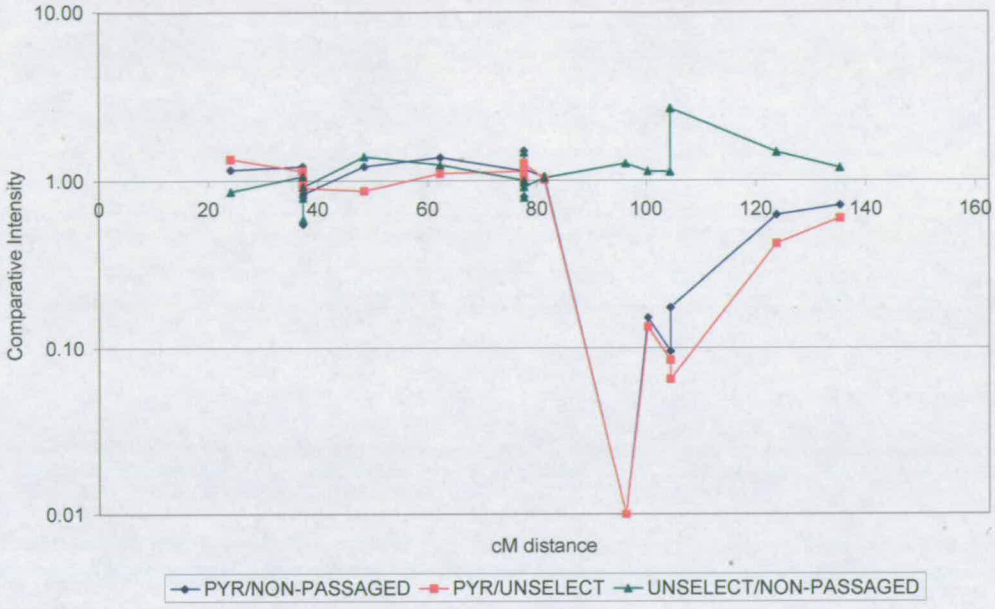


C

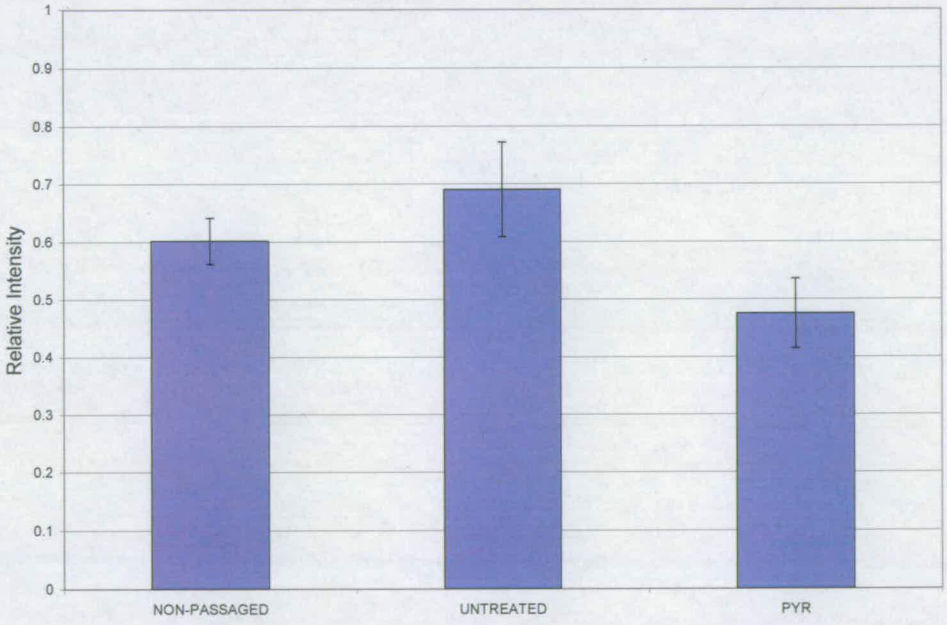
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	11	2.45	0.10	No
Pairwise Comparisons				
PYR / Non-Passaged			0.08	No
PYR / Untreated			0.58	No
Non-Passage / Untreated			0.45	No

Figure 4.12 AJ Markers on **Chromosome 6. A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

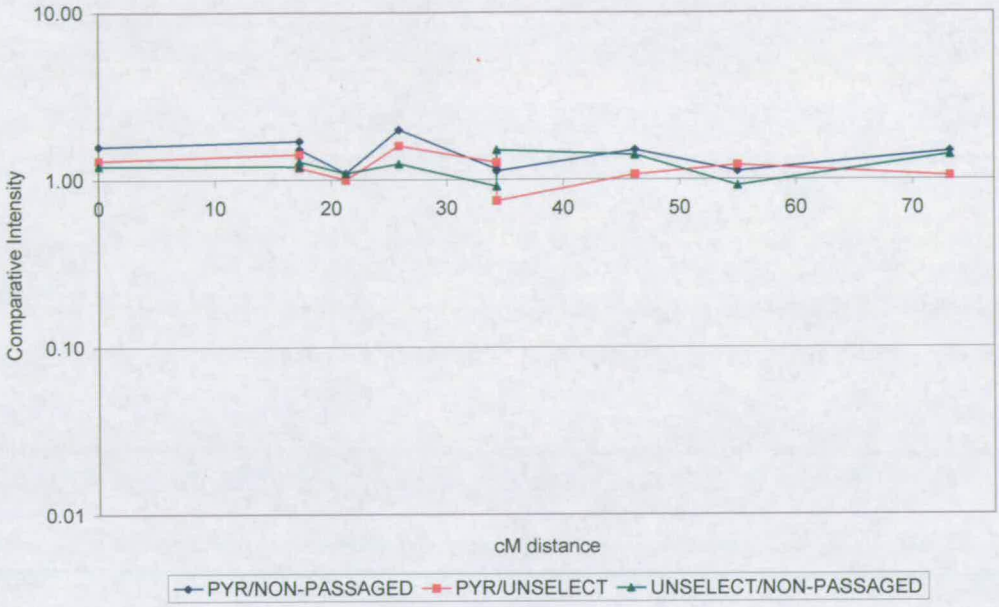


C

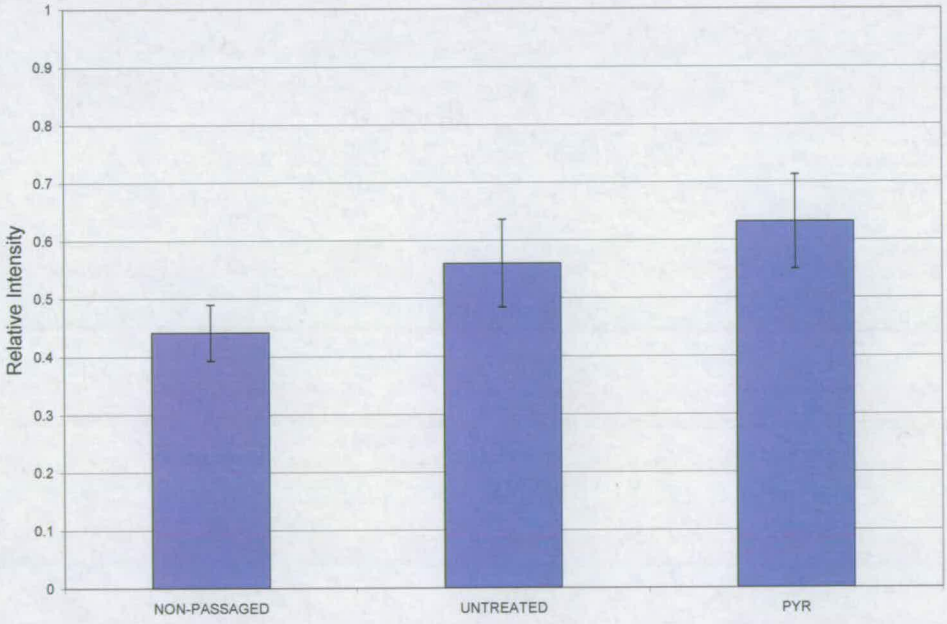
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	19	3.54	0.03	Yes
Pairwise Comparisons				
PYR / Non-Passaged			0.06	No
PYR / Untreated			0.06	No
Non-Passage / Untreated			0.99	No

Figure 4.13 AJ Markers on **Chromosome 7**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

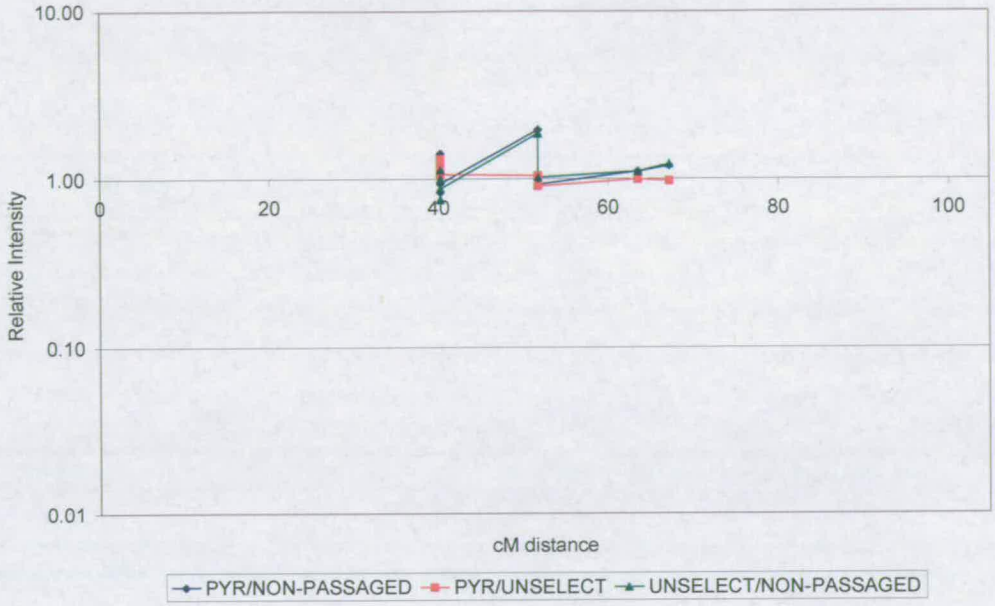


C

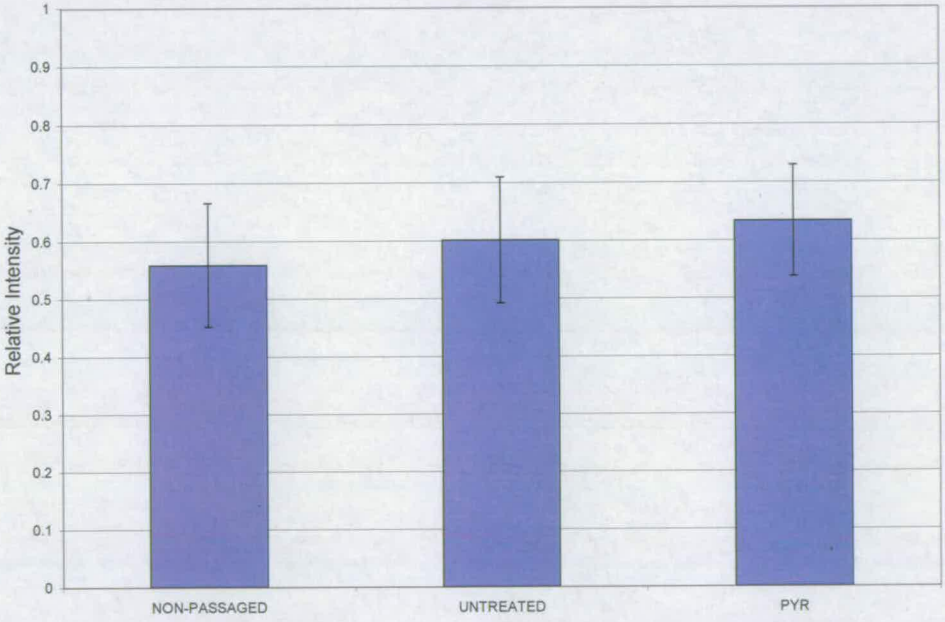
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	10	1.87	0.18	No
Pairwise Comparisons				
PYR / Non-Passaged			0.08	No
PYR / Untreated			0.58	No
Non-Passage / Untreated			0.45	No

Figure 4.14 AJ Markers on **Chromosome 8**. **A; t**he Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

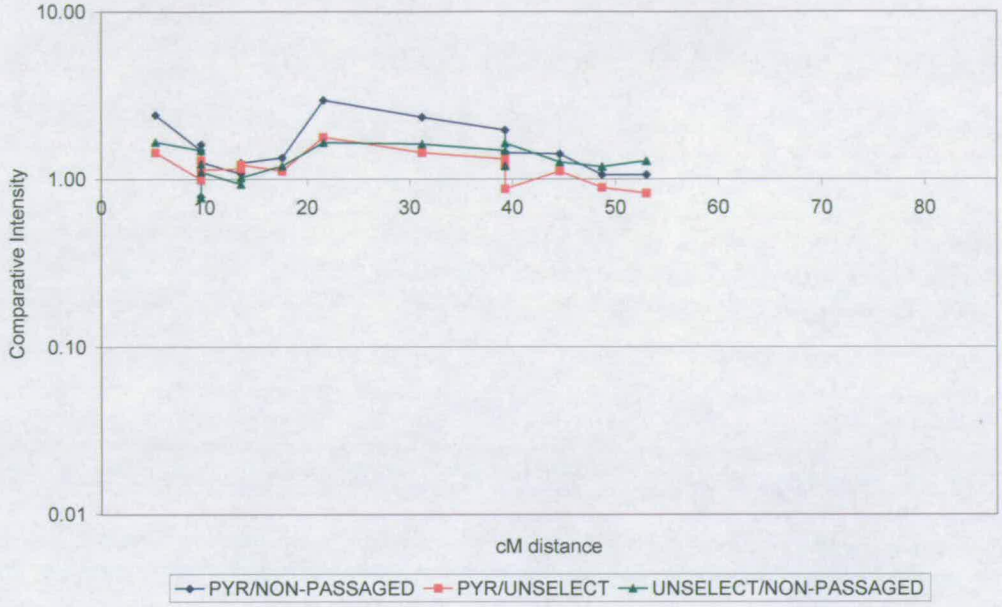


C

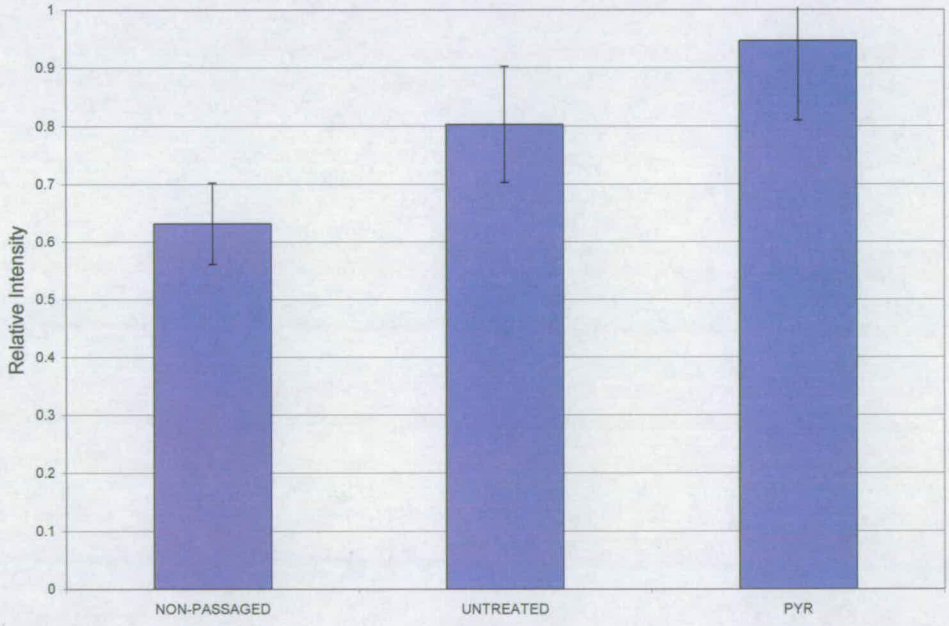
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	8	0.13	0.87	No
Pairwise Comparisons				
PYR / Non-Passaged			0.86	No
PYR / Untreated			0.97	No
Non-Passage / Untreated			0.95	No

Figure 4.15 AJ Markers on **Chromosome 9**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B



C

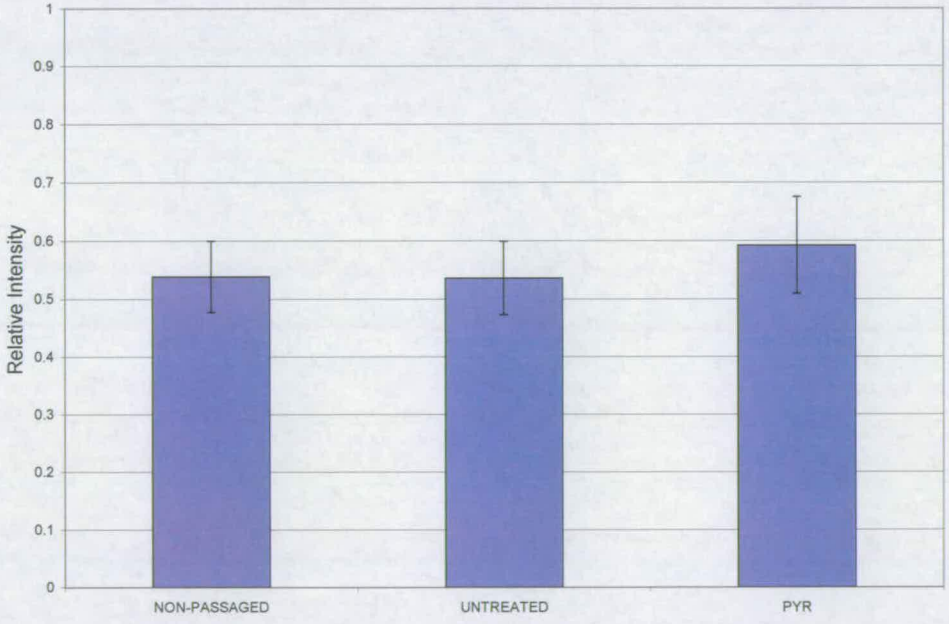
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	16	3.88	0.03	Yes
Pairwise Comparisons				
PYR / Non-Passaged			0.02	Yes
PYR / Untreated			0.54	No
Non-Passage / Untreated			0.21	No

Figure 4.16 AJ Markers on **Chromosome 11**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B



C

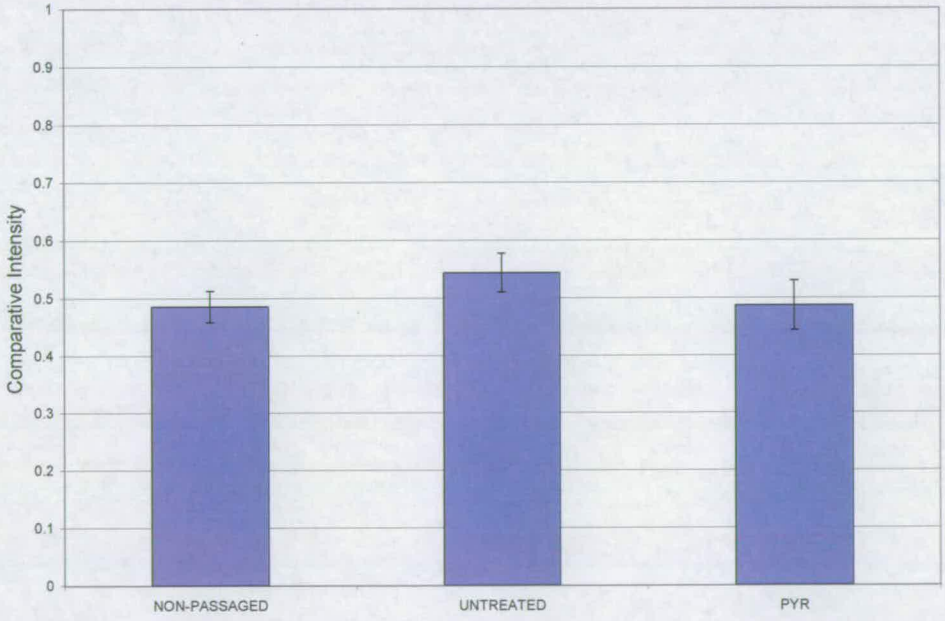
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	8	0.21	0.81	No
Pairwise Comparisons				
PYR / Non-Passaged			0.85	No
PYR / Untreated			0.83	No
Non-Passage / Untreated			0.99	No

Figure 4.17 AJ Markers on **Chromosome 12**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

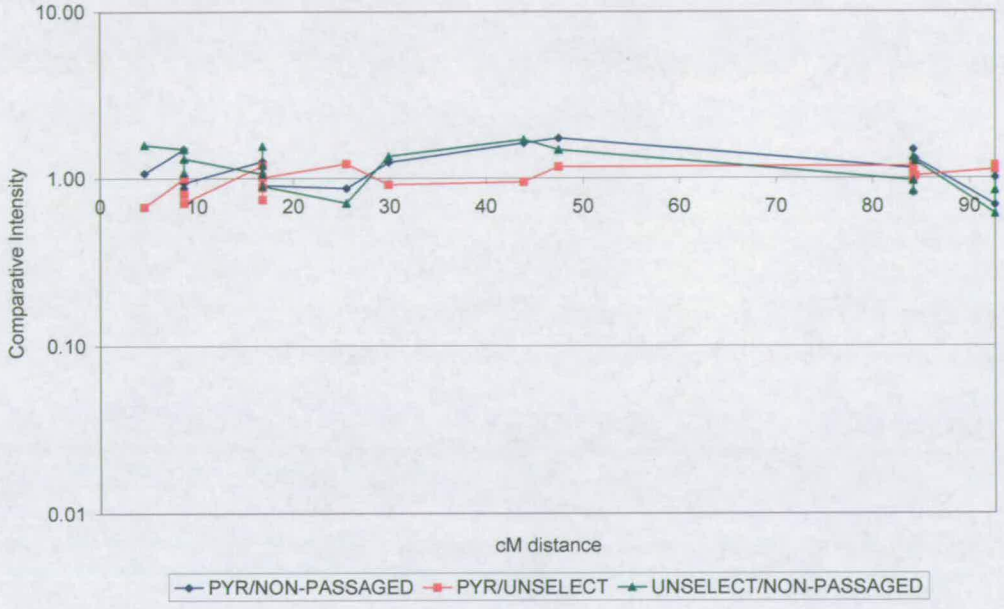


C

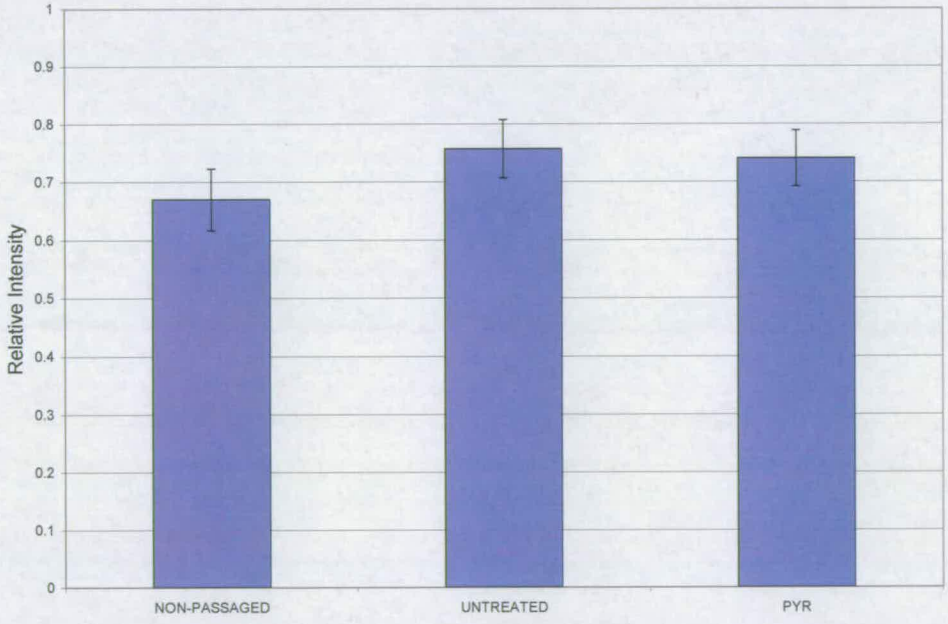
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	32	0.89	0.41	No
Pairwise Comparisons				
PYR / Non-Passaged			0.99	No
PYR / Untreated			0.48	No
Non-Passage / Untreated			0.47	No

Figure 4.18 AJ Markers on **Chromosome 13**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

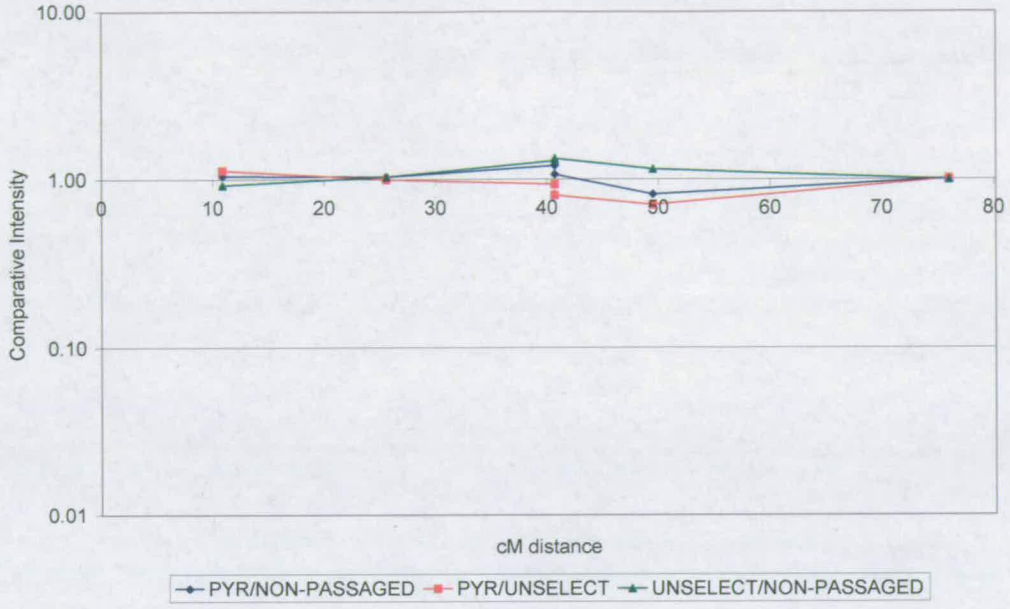


C

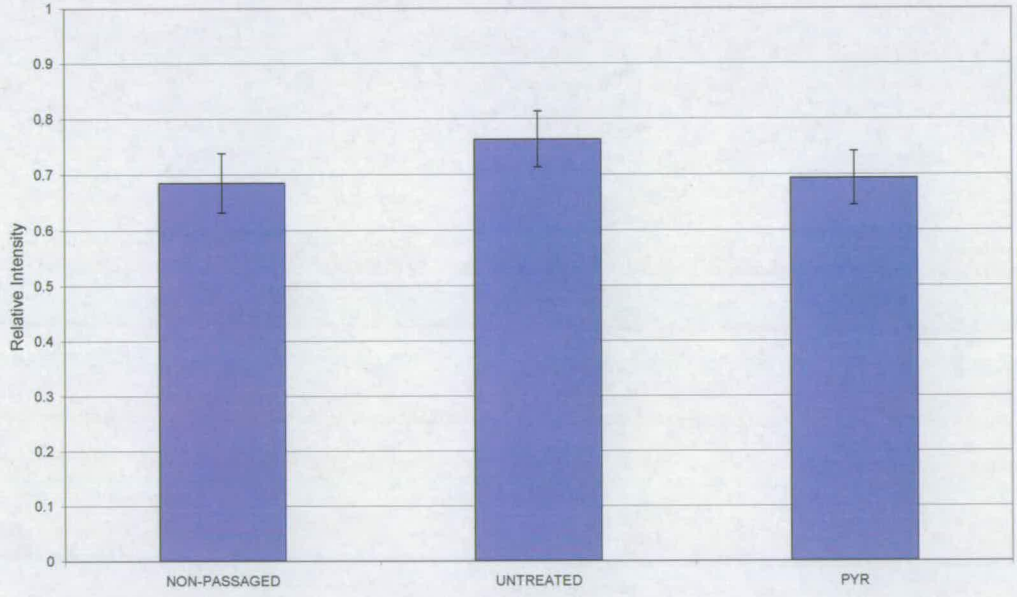
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	18	0.84	0.44	No
Pairwise Comparisons				
PYR / Non-Passaged			0.58	No
PYR / Untreated			0.97	No
Non-Passage / Untreated			0.44	No

Figure 4.19 AJ Markers in **Linkage group A**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

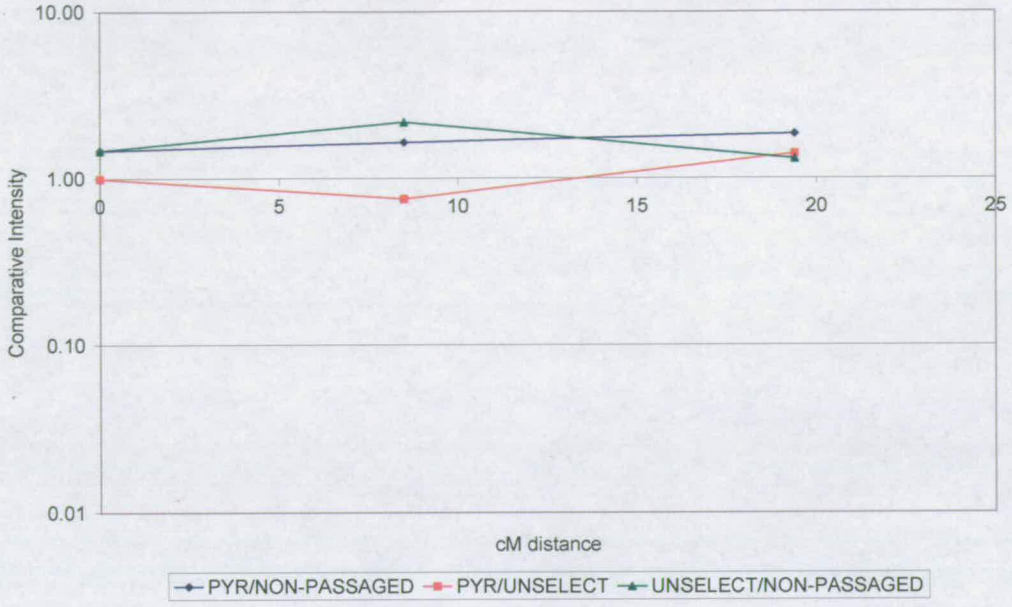


C

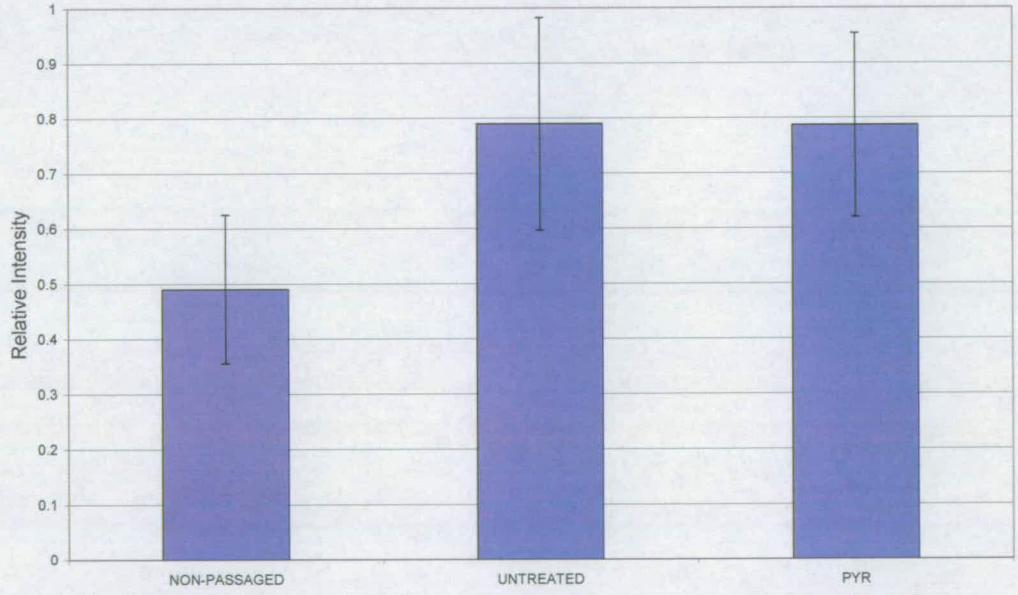
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	6	0.33	0.71	No
Pairwise Comparisons				
PYR / Non-Passaged			0.99	No
PYR / Untreated			0.78	No
Non-Passage / Untreated			0.71	No

Figure 4.20 AJ Markers in **Linkage group B**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

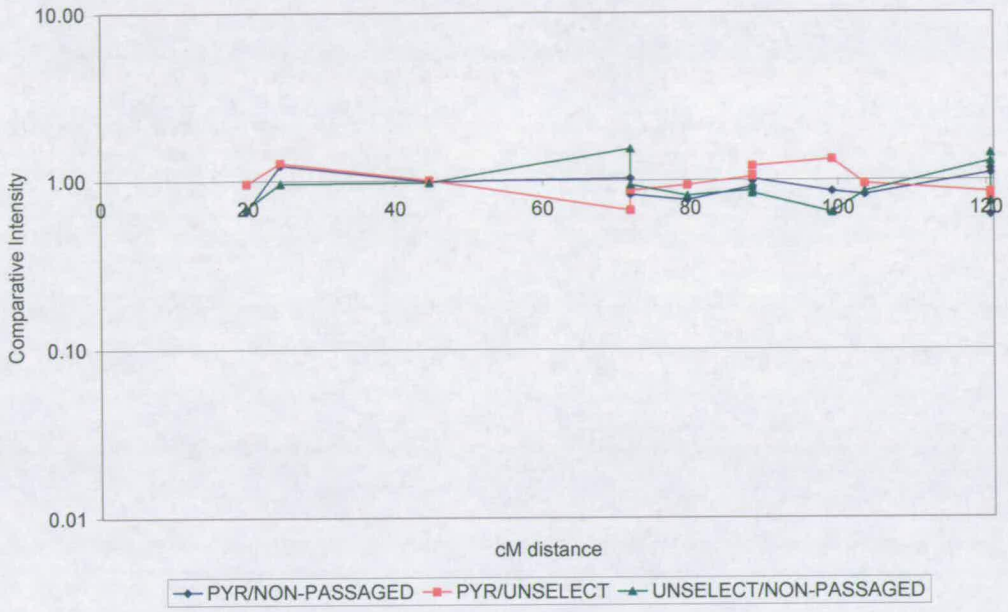


C

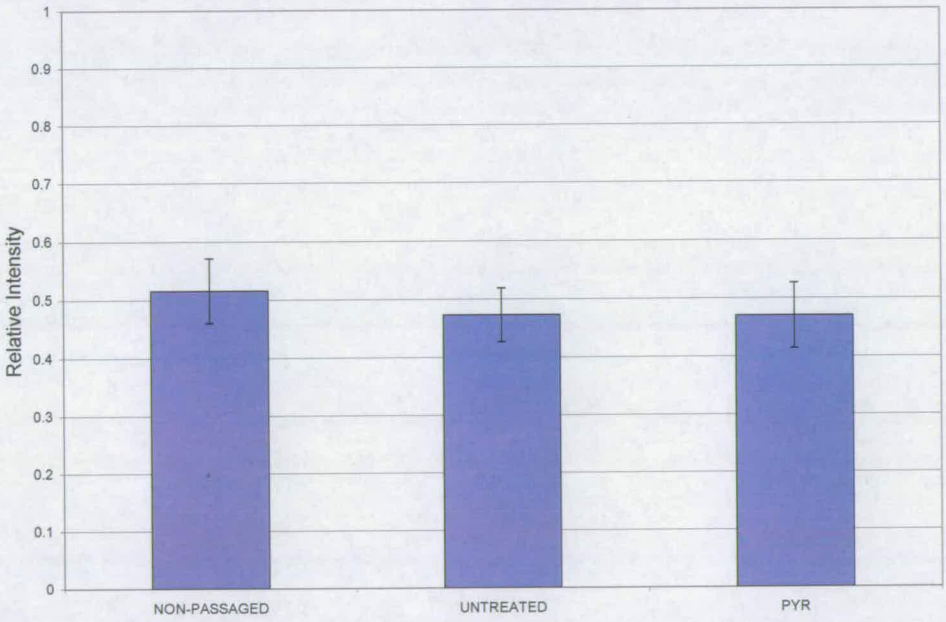
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	3	0.3	0.75	No
Pairwise Comparisons				
PYR / Non-Passaged			0.83	No
PYR / Untreated			0.99	No
Non-Passage / Untreated			0.77	No

Figure 4.21 AJ Markers in **Linkage group C. A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

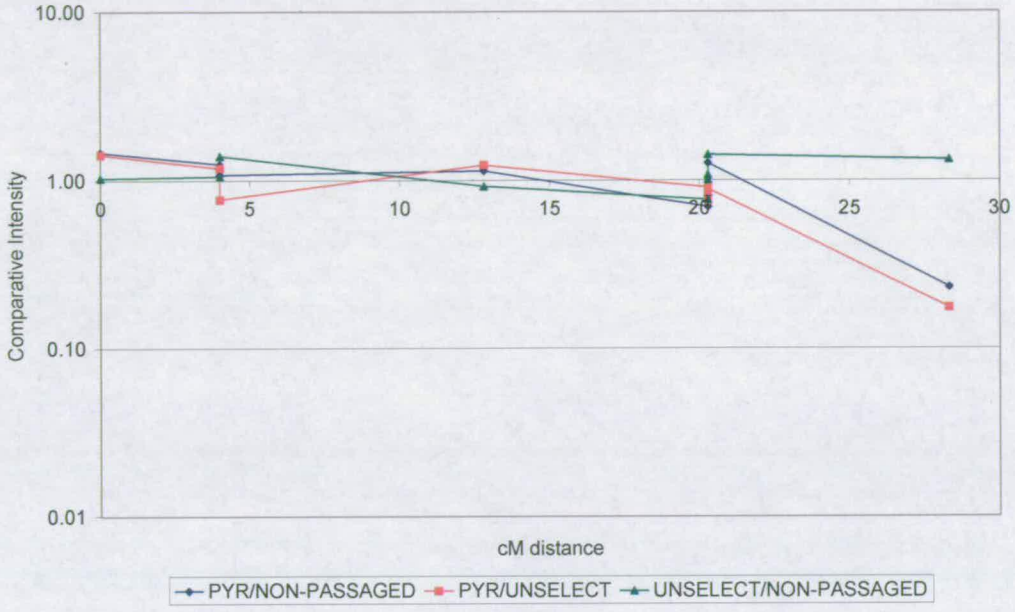


C

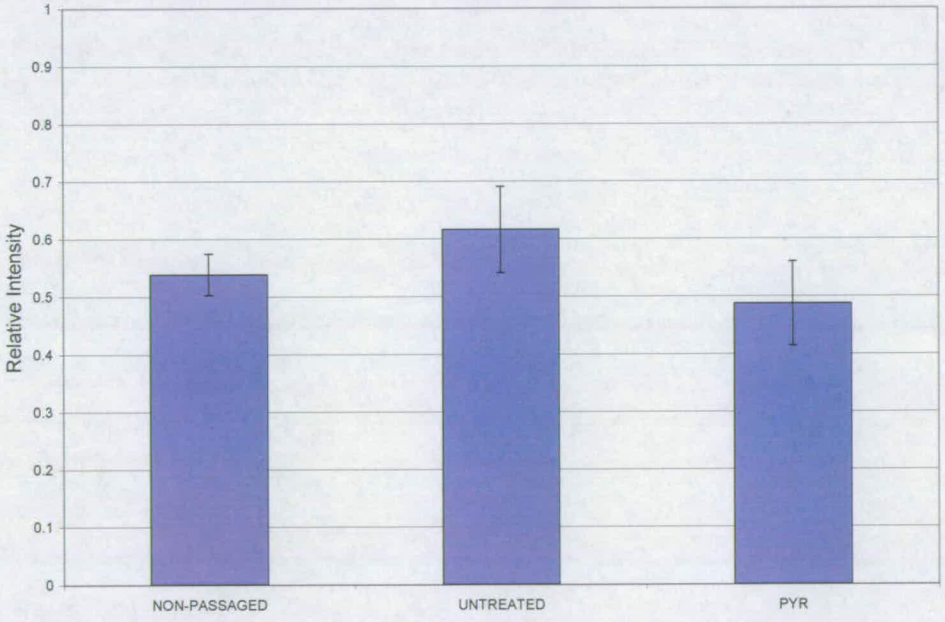
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	14	0.24	0.78	No
Pairwise Comparisons				
PYR / Non-Passaged			0.77	No
PYR / Untreated			0.97	No
Non-Passage / Untreated			0.88	No

Figure 4.22 AJ Markers in **Linkage group D**. **A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



B

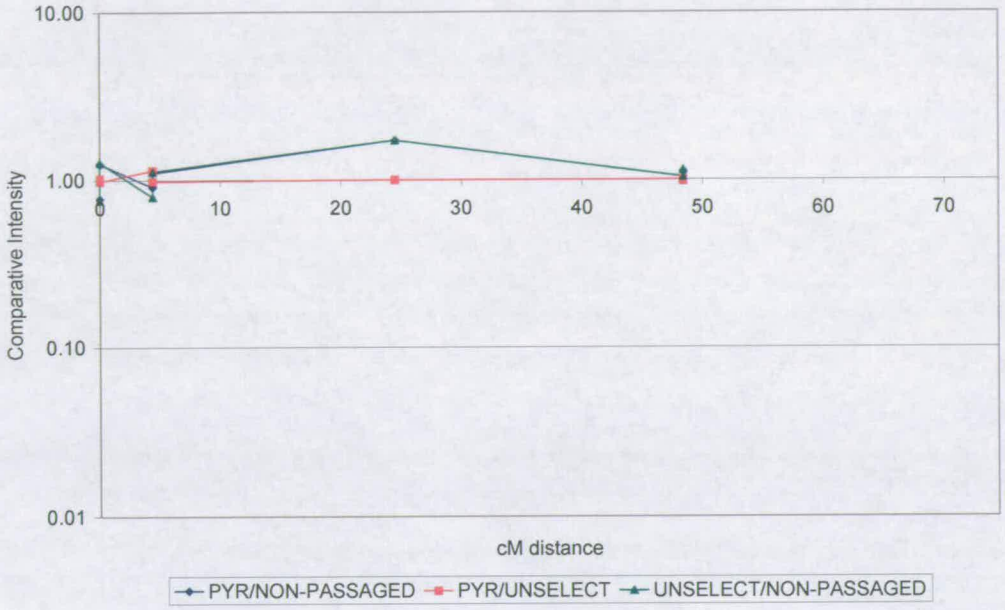


C

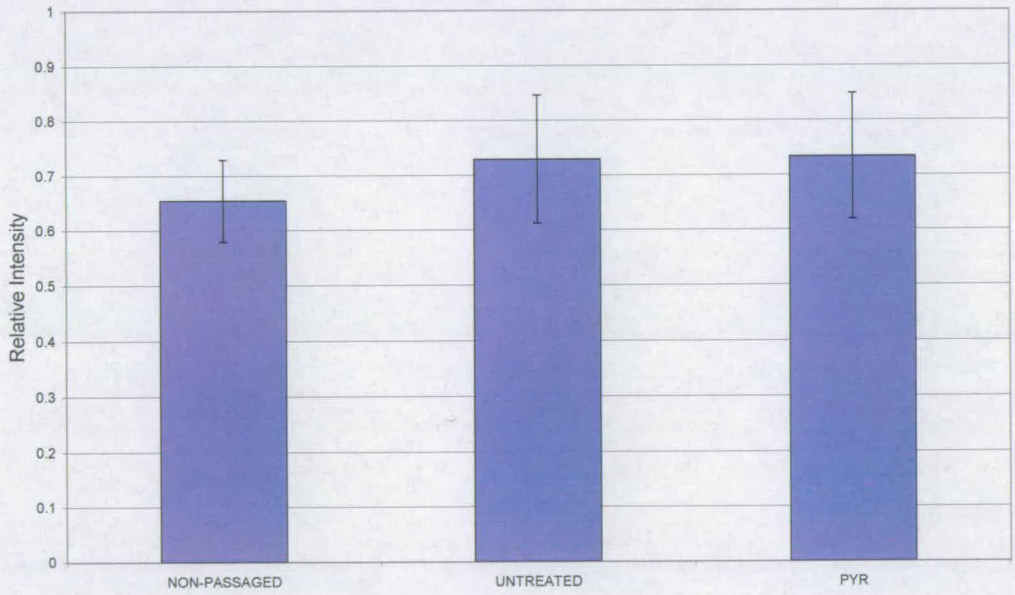
GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	9	1.03	0.37	No
Pairwise Comparisons				
PYR / Non-Passaged			0.83	No
PYR / Untreated			0.34	No
Non-Passage / Untreated			0.67	No

Figure 4.23 AJ Markers in **Linkage group E. A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

A



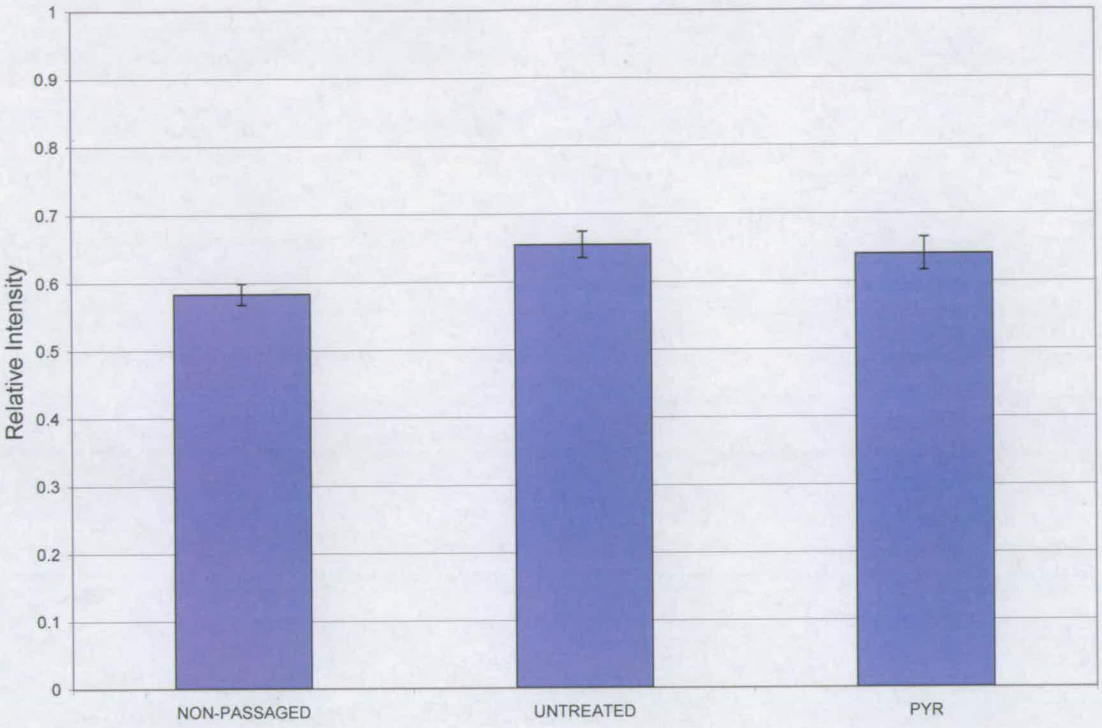
B



C

GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	7	0.17	0.84	No
Pairwise Comparisons				
PYR / Non-Passaged			0.85	No
PYR / Untreated			0.99	No
Non-Passage / Untreated			0.88	No

Figure 4.24 AJ Markers in **Linkage group F. A;** the Relative Intensities of markers positioned along the chromosome according to the genetic linkage map are compared between all three treatment groups. **B;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **C;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.



GLM Results	Number of Markers	F-Value	P-value	Significant
Overall effect	204	2.99	0.05	No
Pairwise Comparisons				
PYR / Non-Passaged			0.53	No
PYR / Untreated			0.35	No
Non-Passage / Untreated			0.03	Yes

Figure 4.25 AJ Markers in **All chromosomes / linkage groups. A;** the average Relative Intensities of markers in the three treatment groups (error bars indicate the standard error of the means), **B;** the results of a General Linear Model analysis of variance performed on the Relative Intensities with "treatment" as an explanatory variable.

Taken overall, the AJ markers in the three treatment groups show no significant effects of treatment. There is, however, a significant difference between the non-passaged and the untreated group, possibly reflecting the AJ parasite's faster growth rate. This effect is most pronounced with markers on chromosome 11, and Linkage Group C (see above). This effect is unlikely to be caused by the parental form of AJ overgrowing parental ASpyr1 and recombinants, however, as the same trend (although not statistically significant) can be seen in the comparison of the non-passaged and PYR treated groups.

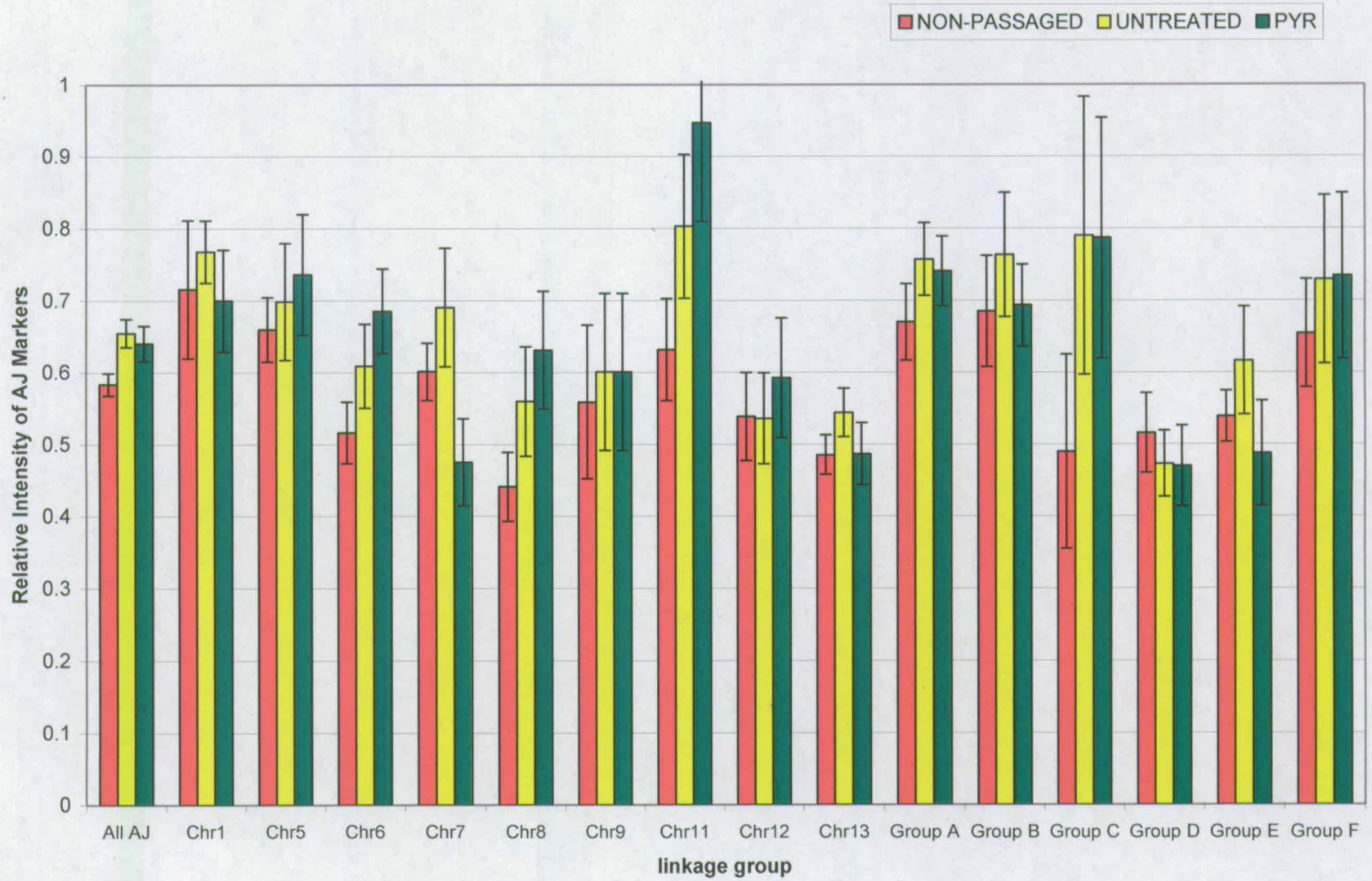


Figure 4.26 Mean Relative Intensity values of AJ markers for all chromosomes / linkage groups. Error bars represent the standard error of the means.

4.3.1.2 Data Summary

The following results (Figures 4.27 – 4.29) describe the distribution of markers on the genome according to their positions on the genetic linkage map, and the results of repeated measurements on the same markers to assess the degree of reproducibility of marker relative intensities.

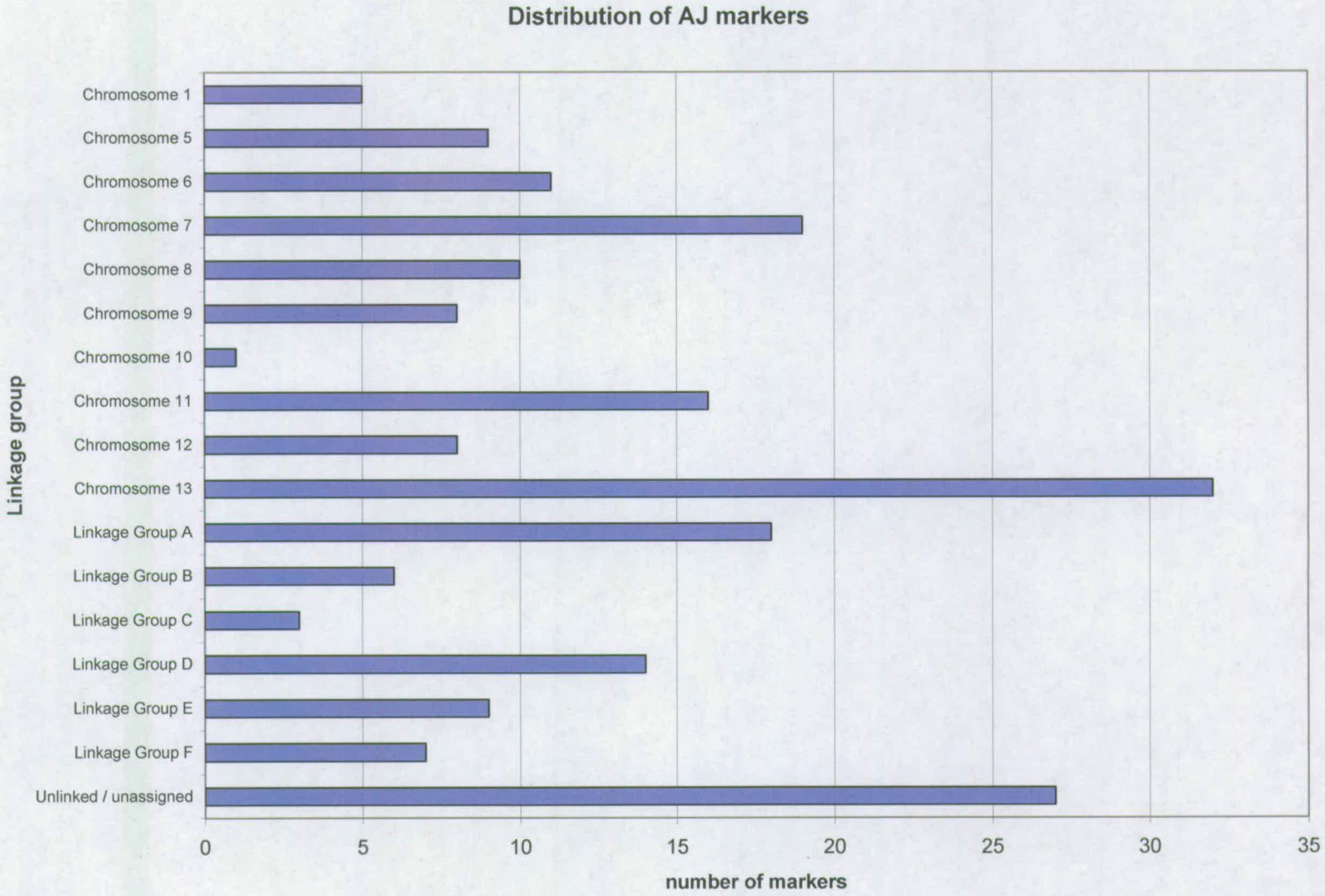


Figure 4.27 The distribution of numbers of AJ markers to chromosomes and linkage groups on the genetic linkage map between ASpyr1 and AJ. The group "unassigned / unlinked" contains markers that could not be assigned to linkage groups on the map.

Figure 4.28 shows the numbers of markers present on each chromosome / linkage group as they were assigned to the linkage map. The uneven distribution of markers reflects the sizes of the chromosomes / linkage groups, with more markers present on the largest chromosomes. 27 markers could not be assigned to either a chromosome or a linkage group and were designated "unassigned / unlinked". Chromosomes 2, 3, 4 and 14 are missing from the linkage map, and markers from these chromosomes may map to linkage groups A-F.



Figure 4.28 The relative intensity indices of repeated intensity measurements of selected specific markers. Each pair of columns represents the results for the same marker in the PYR treatment group. The measurments were repeated from the DNA stage (i.e. each marker of a pair was independently subjected to the AFLP process from the digested and ligated DNA stage, and run on different polyacrylamide gels at different times)

Figure 4.28 shows the results of repeated measurements of relative intensity of selected markers. Each individual reading of a pair of measurements on the same marker comes from a separate AFLP PCR and was run on a different gel. The largest discrepancy between marker relative intensities was 0.3 (AJTT03AA), while the smallest variation was 0.03 (AJTT01AA).The mean difference was 0.11 (standard error of the mean = 0.02) Figure 4.29 shows the differences between these same pairs of repeated measurements of markers for the *comparative* intensity between the PYR treated and the unselected groups. Differences between measurements is crucial, as a high variation between repeats could result in an inaccurate estimation of the number of markers under selection in an LGS analysis. The mean discrepancy between the repeated measurements was 0.12 (standard error of the mean = 0.03).

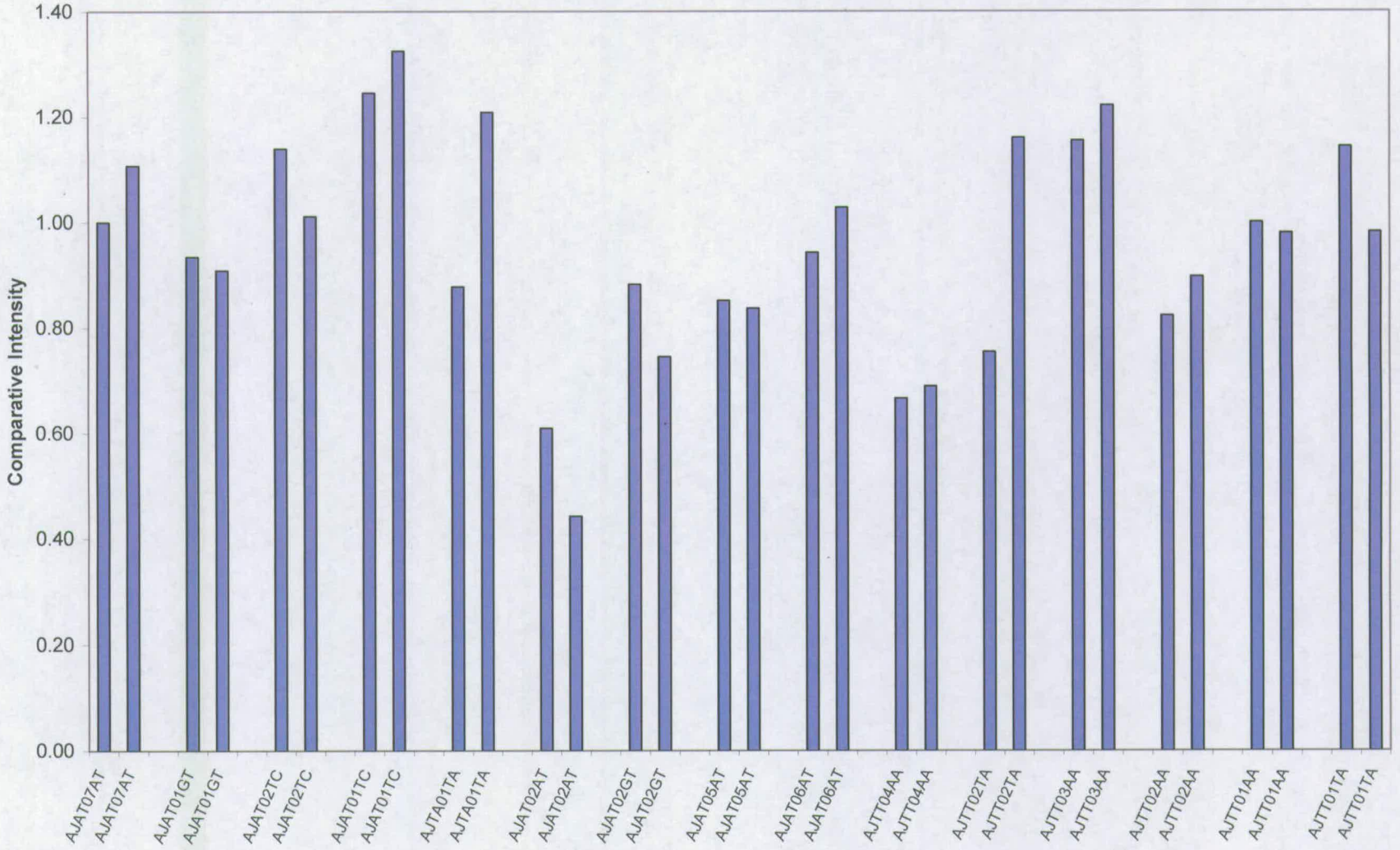


Figure 4.29 The comparative intensities of repeated intensity measurements of selected specific markers between the PYR and unselected groups. Each pair of columns represents the results for the same marker. The measurements of intensity were repeated from the DNA stage (i.e. each marker of a pair was independently subjected to the AFLP process from the digested and ligated DNA stage, and run on different polyacrylamide gels at different times).

4.3.2 Further investigation of markers under selection on chromosome 7

The genetic linkage map allows the positioning of markers upon the *P. chabaudi* genome according to genetic linkage data based on the characterization of 28 clones of a cross between AS and AJ (Martinelli, A., Hunt, P., Fawcett, R., Cravo P., Walliker, D. & Carter, R., submitted). It is impossible to be confident, however, that the genetic linkage observed reflects actual physical linkage on the genome. Markers that appeared to be greatly reduced in the PYR treated group compared to the non-selected group, were assumed to be physically linked to genes that control the PYR response of sensitive parasites. In order to confirm this physical linkage, it was necessary to obtain the genetic sequences of such markers, and map their physical location on the genome. Ideally, markers would be located on the *P. chabaudi* genome, but unfortunately, the genome sequence of this species remains incomplete. It was possible, however, to utilize the strong synteny between *P falciparum* and the rodent malarias (Carlton et al., 1998; Hunt et al., 2004), in order to physically locate the markers in relation to closely linked genes. The markers that are strongly selected against on *P chabaudi* chromosome 7 according to the linkage map, are genetically linked to *dhfr* in this species. Indeed, the marker that shows the greatest reduction in intensity under PYR treatment (AJAA05CA) lies only 5.3 cM from *dhfr* according to the *P. chabaudi* linkage map (see Table 4.4), while the comparative intensities of the other markers under strong selection decrease with increasing proximity to this locus.

In order to determine the physical linkage with *dhfr* of the 6 AFLP markers which showed the greatest comparative intensity reductions in the selected population (Table 4.4), they were sequenced and their homologues located in the *P.falciparum* genome wherever possible. It was possible to identify *P. falciparum* homologues for 3 of these markers, all of which lay within 120 Kb of the *P. falciparum dhfr* locus (*pfdhfr*) (Figure 4.30). The homologue of AJAA05CA which was virtually eliminated following pyrimethamine selection lay 10 Kb from the location of *pfdhfr*.

Marker Name	Comparative Intensity of Marker	Genetic Distance (cM) from *dhfr* (*P. chabaudi*)
AJAA05CA	0.00	5.3
AJAG02CA	0.13	9.5
AJTG01AG	0.06	13.4
AJTG01GA	0.08	13.4
AJAC02TT	0.42	33.0
AJAA03AT	0.60	44.5
AJAA01GA[a]	1.02	53.5

Table 4.4. The Comparative Intensities of markers under pyrimethamine selection, and their positions on the *P. chabaudi* linkage map. The Comparative Intensities of the markers are seen to decrease with increasing proximity to the gene under selection. Markers were assigned to a linkage map between AS and AJ as described in the methods.

[a]This marker is not under pyrimethamine selection, and is included as an example of a flanking marker to give an indication of the size of the selection valley.

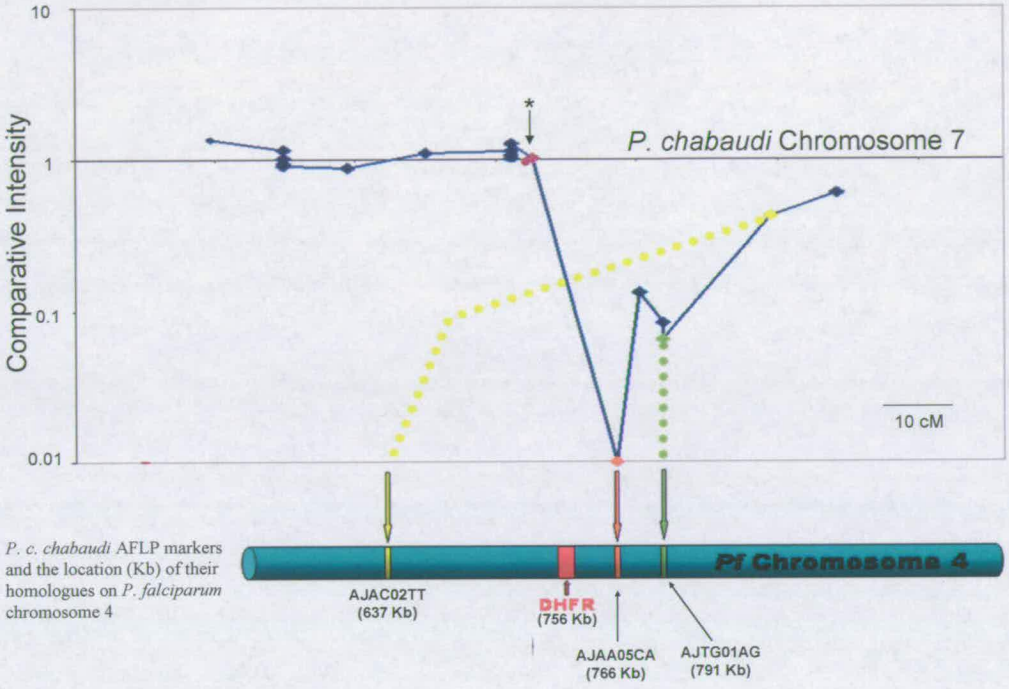


Figure 4.30 The Comparative Intensities of AJ AFLP markers after selection of the cross progeny, and their positions on *P. chabaudi* chromosome 7. Homologues of markers that were reduced in intensity under pyrimethamine pressure were located on *P. falciparum* chromosome 4. Marker intensity decreased with increasing proximity to *pfdhfr*. AJAA05CA, whose intensity was most reduced, was located only 10 Kb from *pfdhfr*. Numbers in brackets below marker names give the distances in kilobases of the markers along the *P. falciparum* chromosome 4. Two discrepancies were observed between the *P. chabaudi* linkage map and the *P. falciparum* genome database: Marker AJAC02TT was located on opposite sides of *dhfr* by the two resources, and the homologue of the marker indicated with an asterisk (*) was found to lie on *P. falciparum* chromosome 6, and was not linked to *pfdhfr*. It is thought that these differences are due to errors in the production of the *P. chabaudi* linkage map.

4.3.3 Proportional Sequencing of the *dhfr* locus

The presence of a selection valley around *dhfr* is strong evidence that this gene was involved in the response to PYR treatment of the cross-progeny population. It is possible, however, that PYR selection may be acting on a locus closely linked to *dhfr*, and not on the gene itself. Therefore, in order to confirm that *dhfr* was, indeed, the target of drug selection, the proportion of the sensitive to the resistant allele was directly measured using Proportional Sequencing in all three treatment groups. Additionally, three other polymorphic genes (dihdropteroate synthase (*dhps*), dihydrofolate synthetase (*dhfs*), and

6-pyruvoyl tetrahydropterin synthase (*6-pthps*)) were measured in the same way, as controls. These additional loci were chosen because they are unlinked to *pcdhfr*, yet all encode enzymes that are associated with the folate biosynthesis pathway, but are not known to be involved in PYR resistance.

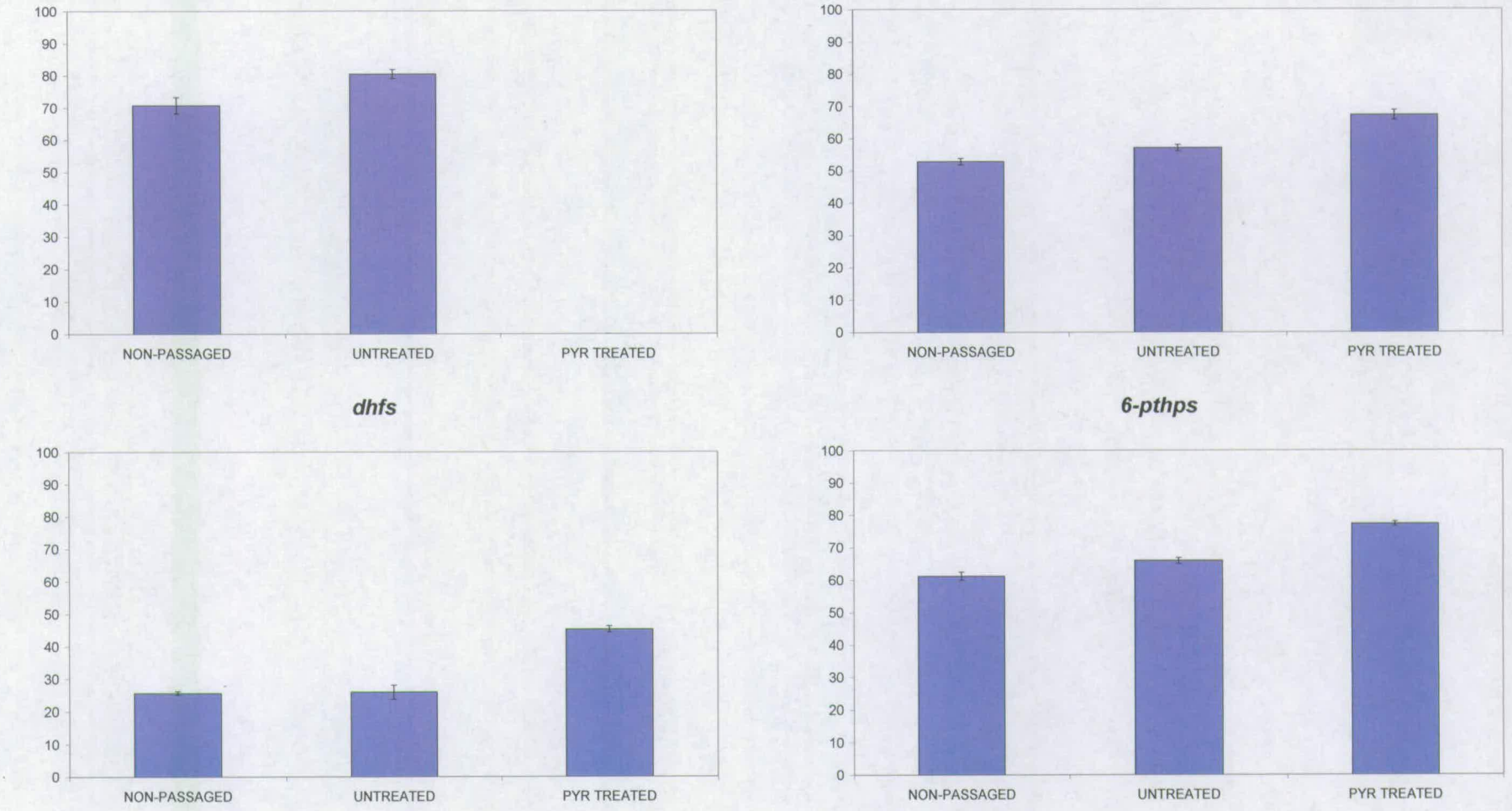


Figure 4.31 The proportions of AJ alleles of 4 genes associated with the *Plasmodium* folate biosynthesis pathway, as determined by Proportional Sequencing. Bars show the percentage of the AJ allele of four genes in 3 different treatment groups. Each individual measurement was repeated 5 times from the PCR stage, and error bars give the standard errors of the means.

Figure 4.31 shows the proportions of AJ alleles of 4 genes, *dhfr* (the gene responsible for PYR resistance) and three other genes involved in the folate biosynthesis pathway. Individual GLM analyses were performed on the proportion of each allele's representation in the three treatment progeny populations to test for significant differences between treatment groups.

For each of the four genes analysed, there was a significant difference in allele proportions between all groups (Non-passaged versus Untreated, Non-passaged versus PYR Treated, and PYR Treated versus Untreated) ($p<0.001$), except for the Non-passaged versus Untreated for *dhfs* ($p=0.9558$). The AJ allele of *dhfr* was reduced to 0% in the PYR treated population, whereas the AJ alleles of the three other genes were significantly increased in the PYR group compared to the untreated group. This result shows that the AJ allele of *dhfr* is completely removed in the drug treated population, confirming the indication from the LGS analysis that it lies at the bottom of the selection valley observed on chromosome 7 of *P. chabaudi* (Figure 4.10).

4.3.4 Evidence of a selection valley on chromosome 13

In addition to the selection valley observed around *dhfr*, a less prominent valley was identified on chromosome 13 (see Table 4.3). Markers that appeared under selection at this locus were also sequenced, and their homologues located in the *P. falciparum* genome database wherever possible (Figure 4.32)

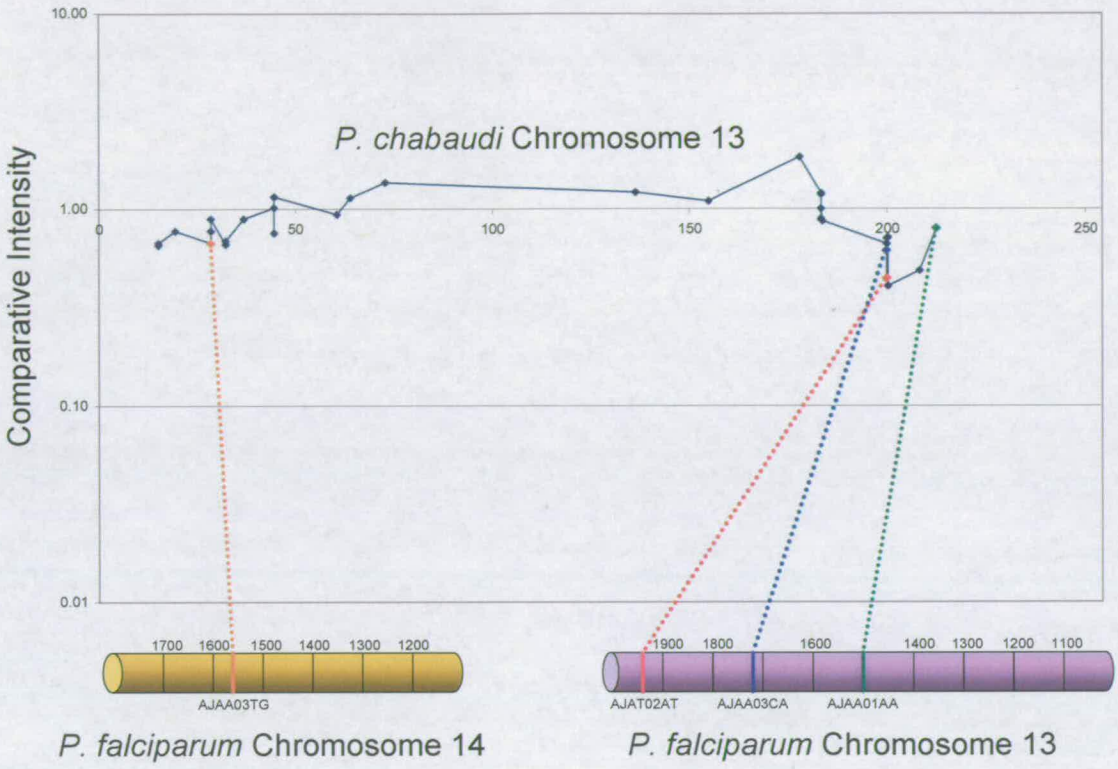


Figure 4.32 The Comparative Intensities of AJ AFLP markers after selection of the cross progeny, and their positions on *P. c. chabaudi* chromosome 13. Homologues of markers that were reduced in intensity under pyrimethamine pressure were located on the *P. falciparum* genome database. Dotted lines link the markers on *P. c. chabaudi* chromosome 13 to their homologues on *P. falciparum* chromosomes 13 and 14. Numbers above the *P. falciparum* chromosomes indicate distances in kilobases. Marker names are given below the chromosomes.

4.3.5 Estimate of size of a centiMorgan in the *dhfr* selection valley

For practical purposes a selection valley will comprise the genomic region on either side of a locus under selection, in which markers linked to the selected locus can be expected to show measurable reductions (or increases) in frequency (Comparative Intensity) among recombinant progeny following selection. In reality, this is going to lie within a region of about 50 centiMorgans (cM) on either side of the target locus. The physical size of a cM is likely to vary within a genome. It is necessary, therefore, to calculate the size of a cM in the region of the genome under investigation, in order to determine the physical size of a selection valley in this region.

An estimate of the physical width (in Kb) of selection valleys is important for several reasons. It allows the estimation of the number of markers across the genome required in order to be certain of locating a selection valley. The size of a selection valley (in Kb) will also determine how close the results of an LGS analysis will locate markers under selection to the selected locus itself. The "narrower" a selection valley is, the closer its apex will be to the locus under selection.

One centiMorgan (cM), the unit of measurement for recombination frequency between two loci, is equal to a 1% chance that a marker at one genetic locus will have recombined away from a marker at the second locus as the result of a crossing over event in a single generation. The number of kilobases per centiMorgan varies between genomes. In humans, for example, one cM is equivalent to around 1000 Kb (Human genome Project, 2004). For *P. falciparum*, one cM has been estimated to be 17 Kb in size (Su *et al.*, 1999).

The data generated in the experiments described above allow an estimation of the size of one cM in *P. chabaudi*, or, more accurately, the size of 1cM in the locality of the selection valley associated with *dhfr* (cM distances are likely to vary across the genome, there may, for example, be "recombination hotspots"- areas of the genome where recombination is particularly frequent (Lichten and Goldman, 1995)). A 1% difference in representation of a marker in a population translates to a change in Comparative Intensity of 0.01. By plotting a graph of the Comparative Intensities of AFLP markers in the selection valley against their known physical distances (Kb) from *dhfr* in the *P. falciparum* genome, a line of best fit can be drawn through the points (Figure 4.33).

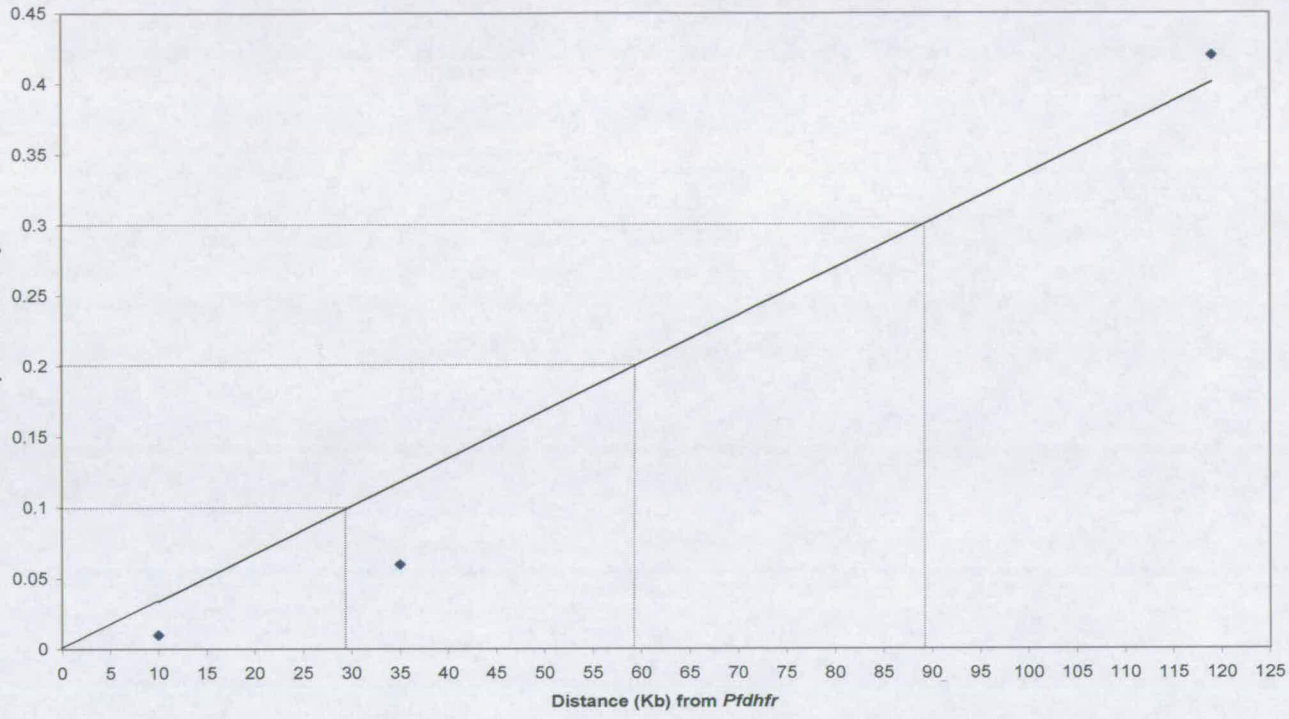


Figure 4.33 The Comparative Intensities of AJ AFLP markers in the PYR versus the Unselected treatment groups. The x axis shows Kb distances from *dhfr* as determined from marker homologue location on the *P. falciparum* genome. Dotted lines link Comparative Intensities of 0.1, 0.2 and 0.3 (10, 20, and 30 cM respectively) to their extrapolated distances from *dhfr*.

From this graph it is possible to estimate the distances in Kb of markers that show Comparative Intensities of any given value. By extrapolating the distance in Kb of markers showing a given Comparative Intensity, it is possible to calculate the size of a centiMorgan in the region of the genome on which these markers lie. In this case, a Comparative Intensity of 0.1 (10cM) translates to approximately 30 Kb, giving one cM a size of 3 Kb at this locus. This is much smaller than that estimated for *P. falciparum*. This value can only be taken as a rough estimate, however, as it relies on the results of only three markers, and assumes that the comparative intensities are accurate. If 1cM = 3kb, then the width of the selection valley around *dhfr* is about 150 Kb (from the target locus to the edges of the valley).

4.3.5 Summary of results

These results show that there was significant reduction in the intensities of 10 out of 206 AJ (drug-sensitive) AFLP markers in a population of recombinant progeny of a cross between a drug-resistant (ASpyr1) and a drug-sensitive (AJ) *P. chabaudi* parasite under PYR pressure, compared to a non-drug treated population of the same recombinant progeny. Five of these AJ markers were genetically linked to *dhfr* in *P. chabaudi*, the gene that controls the drug sensitivity of the parental parasites from which the cross progeny were generated. This linkage to *dhfr* was confirmed by physically mapping the individual AFLP markers that appeared under selection to the genome of a related malaria parasite, *P. falciparum*. It was observed that the degree of reduction in intensity of the markers was directly correlated to their physical distance from *dhfr* in *P. falciparum,* so that the closer the markers were linked to the gene, the greater their reduction in intensity under drug treatment, forming a "selection valley" around the *dhfr* locus. In order to confirm that the AJ allele of *dhfr* was the target of the drug treatment, the proportions of the sensitive and resistant alleles were measured directly, using Proportional Sequencing at this locus. It was found that the sensitive allele of the gene was not detectable after PYR treatment, and was therefore likely to be the target locus of PYR selection.

In addition to the selection valley observed on *P. chabaudi* chromosome 7, a lesser effect of PYR pressure on AJ AFLP markers was seen on chromosome 13, where three genetically linked markers were reduced in intensity under PYR pressure by more than 50% compared to the untreated population. Physical location of these markers placed them on chromosome 13 of *P. falciparum*.

4.4 Discussion

These results have demonstrated the validity of LGS as a method to locate the genes controlling a selectable phenotype such as drug resistance in malaria parasites. Using PYR selection on the progeny of a cross generated between a sensitive and resistant line

of *P. chabaudi*, and by measuring the intensity of numerous, genome-wide AFLP markers in populations of progeny after growth in the presence or absence of the drug, it was possible to visualize a selection valley around *dhfr*, the gene known to control PYR sensitivity in the parental parasites. These results have exciting consequences for the future investigations of the genetic determinants of drug resistance, and, indeed, of other selectable phenotypes such as transmissibility, parasite growth rate and strain-specific immunity. LGS offers a relatively quick and cost-effective laboratory-controlled experimental procedure for gene identification in comparison to previous methods of genetic investigation.

The selection valley produced around *dhfr* on *P. chabaudi* chromosome 7 consists of 5 consecutive linked markers that have a Comparative Intensity of less than 0.5 (and one closely linked marker with a Comparative Intensity of 0.67). Nowhere else on the genome was such a pronounced reduction in marker Comparative Intensity observed in a group of linked markers. Furthermore, the Comparative Intensities of these markers decreased with increasing genetic distance from *dhfr*. This effect, when visualized on a graph showing all AJ markers distributed along the entire genome, produces a dramatic selection valley that points to *dhfr* (Figure 4.7).

By sequencing the markers in this selection valley and locating their homologues in the *P. falciparum* database, their physical linkage to *dhfr* was confirmed, as was the fact that the Comparative Intensity of the markers was proportional to their proximity to *pfdhfr* in *P. falciparum* (Figure 4.30). As well as strengthening the evidence for the selection valley around *dhfr*, these results also reflect the high degree of synteny between *P. falciparum* and *P. chabaudi*.

Discrepancies were, however, discovered between the *P. chabaudi* linkage map and the *P. falciparum* genome database. One marker (AJAC02TT) was physically located on the opposite side of *pfdhfr* in the *P. falciparum* genome from that predicted by the *P. chabaudi* linkage map. This disparity can best be explained by errors in the linkage map resulting from the relatively low number of progeny clones, 28, analysed for the

generation of the map. Nevertherless, this marker was linked to *pfdhfr* and at a distance in accordance with that shown on the linkage map.

Another marker (AJTT03TG) presented a problem when it was first located on the linkage map. The map indicates that this marker was genetically linked to *pcdhfr* at a distance of 9.6 cM. A marker so closely linked to *pcdhfr* would be expected to be significantly reduced under PYR pressure, yet it did not appear to be under any selection. When the homologue of this marker was located on the *P. falciparum* genome, however, it was found to lie on chromosome 6, and was, therefore, completely unlinked to *pfdhfr*. It seems likely, therefore, that this marker has been incorrectly assigned to the genetic linkage map, and is not closely linked to *pcdhfr*.

The occasional inaccuracy of the *P. chabaudi* genetic linkage map in locating markers on the *P. chabaudi* genome is not a limitation for LGS itself. A successful LGS analysis could have been carried out without the use of a linkage map at all, simply by sequencing markers that appeared under selection and finding their homologues in the published parasite genomes. For the present work, it was necessary to rely on the high degree of synteny between *P. falciparum* and the rodent malarias, but as the sequencing project for *P. chabaudi* continues, it will eventually be possible to locate markers directly onto the species specific genome, and so by-pass any difficulties encountered due to breakdowns in synteny.

Figure 4.9 shows the Comparative Intensities of AJ markers in the PYR selected cross progeny on the same graph as those of ASpyr1. Small movements in Comparative Intensity seen in AJ makers across the genome are, for the most part, mirrored by movements in the opposite direction of closely linked ASpyr1 markers. This suggests that such small differences in marker intensities represent real effects in the regions of these markers and are not the result of general inaccuracies in the measurement of marker intensity.

4.4.1 Evidence for PYR selection of ASpyr1 markers on *P. chabaudi* Chromosome 13

In addition to the selection valley around *dhfr* on chromosome 7, another group of linked markers was shown to be under selection on chromosome 13 (Figures 4.7 and 4.32). The markers in this valley were not under as strong a selection as those linked to *dhfr* (the lowest comparative index of the three markers under strong selection was 0.41, compared with 0.01 in the *dhfr* selection valley). This was the only other area on the genome that appeared under significant selection and which had more than one marker showing a significant reduction at the same locus. When these markers were sequenced and their homologues located on the *P. falciparum* genome, they were found to lie on chromosome 13 (known to contain loci syntenic with parts of chromosome 13 in *P. chabaudi*) and formed a linkage group that spanned a 430 Kb stretch of the chromosome (Figure 4.32).

The presence of selection for ASpyr1 markers other than those linked to *dhfr* was unexpected, as control of pyrimethamine resistance in ASpyr1 is known to be caused by a single point mutation in *dhfr*, and no other genes are thought to be involved. However, the possibility of mutations in other genes caused by selection with PYR *after* the *dhfr* mutation has occurred cannot be ruled out. Sequential mutations of resistance genes are well documented, in which a first mutation leads to a level of drug resistance, and further sequential mutations in the same gene increase the level of resistance to the drug (Sirawaraporn et al., 1997a). This is known to occur in PYR resistance in *P. falciparum*, where an initial mutation from a serine to an asparagine at amino acid position 108 in DHFR appears to be essential to confer PYR resistance, and further mutations at positions 51 (asparagine to isoleucine), and 59 (cysteine to arginine) increase this resistance (Sirawaraporn et al., 1997a) (see also chapter I). It is conceivable, then, that amino acid sequence differences on a gene on chromosome 13 in *P. chabaudi* ASpyr1 compared to AJ confers an additional level of resistance when combined with the mutation at *dhfr*. Such a difference could be the result of a mutation in that gene selected by drug pressure during the generation of ASpyr1 from AS(sens), or could be the result of intrinsic differences between AS and AJ.

The fact that complete selection of ASpyr1 AFLP markers is not seen at this locus, in contrast to the *dhfr* selection valley, suggests that the ASpyr1 allele to which the markers are linked is not essential to confer PYR resistance. It does seem, however, to convey a significant advantage to those parasites possessing the ASpyr1 rather than the AJ allele, but not enough to out-compete the AJ parasites in the 7 days for which they were grown. It is possible, however, that further passage of this population under PYR pressure would have produced a population monomorphic for the ASpyr1 allele at this locus. By physically locating the homologues of markers in this selection valley, it was discovered that the marker under the greatest selection (AJAT02AT) was 39 kb away from a gene (cytidine and deoxycytidylate deaminase family, putative) whose product is involved in the deoxypyrimidine nucleotide/side metabolism pathway, which also involves the product of *dhfr* (Yeh et al., 2004) (Figure 4.34).

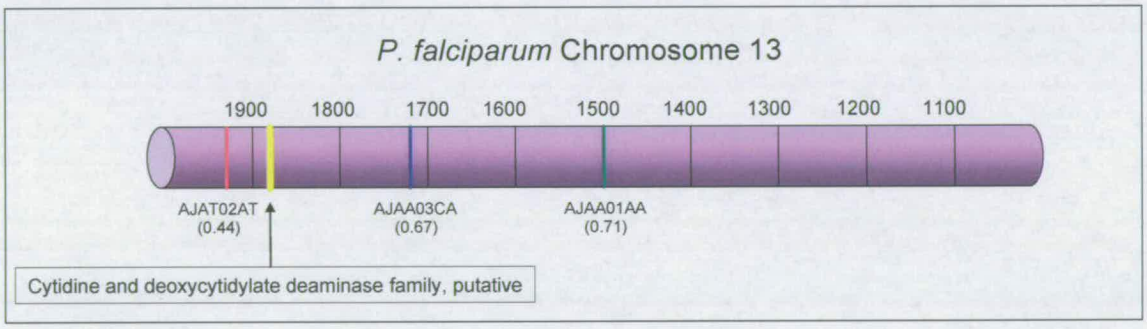


Figure 4.34 Positions of homologues of AJ markers within the chromosome 13 selection valley. The marker displaying the lowest comparative intensity (AJAT02AT) is 39 Kb from the "cytidine and deoxycytidylate deaminase family" gene which is involved, along with DHFR / Thymidylate synthase in the deoxypyrimidine nucleotide/side metabolism pathway. Numbers in brackets below the marker names show comparative intensities on the PYR treated population versus the untreated population.

Deoxycytidylate deaminase and thymidylate synthase (a product of the *dhfr* gene) are functionally associated with one another, since they catalyze sequential reactions (McGaughey et al., 1996). This association can be seen in Figure 4.35.

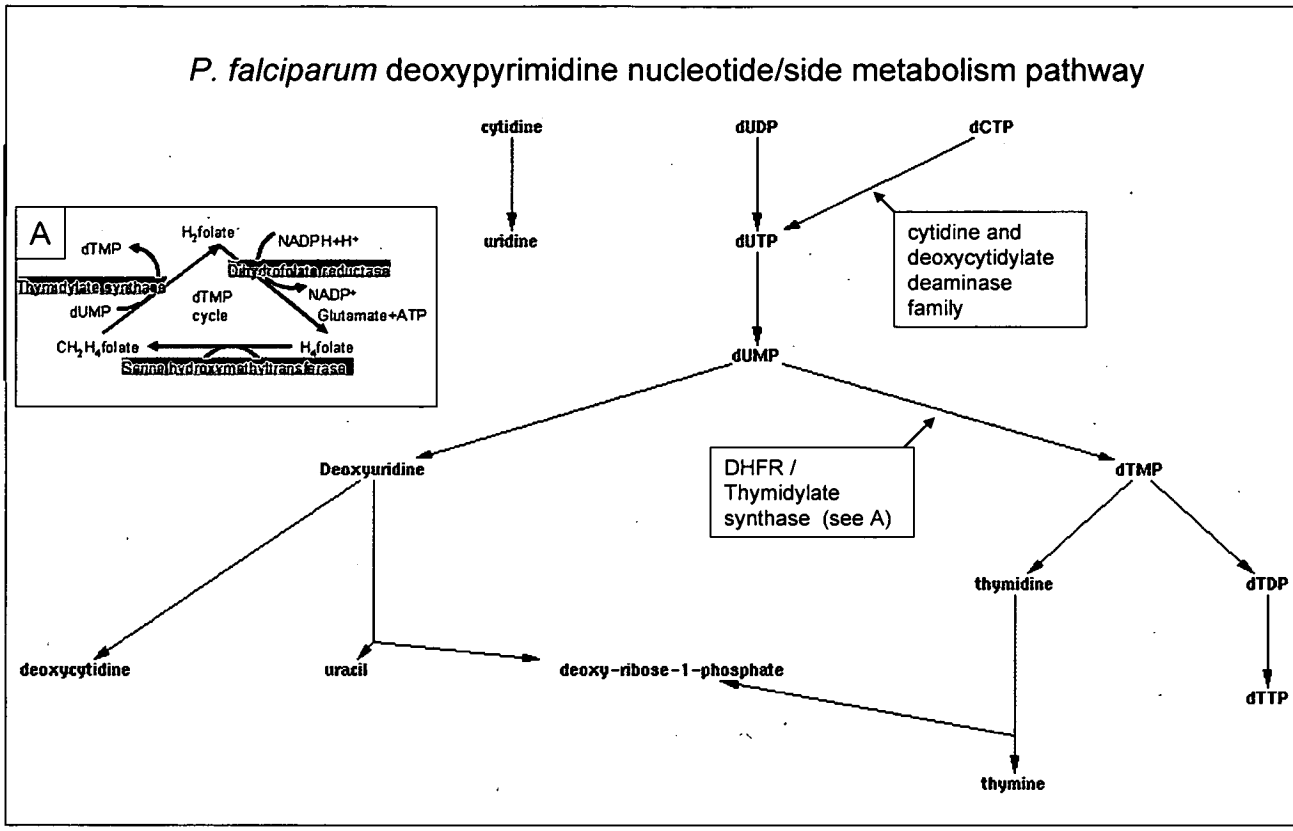


Figure 4.35 The deoxypyrimidine nucleotide/side metabolism pathway of *P falciparum* showing the actions of cytidine and deoxycytidylate deaminase and DHFR / thymidylate synthase. Box A shows the interaction between DHFR and thymidylate synthase. Adapted from the PlasmoCyc website; http://biocyc.org/PFA

From the deoxypyrimidine nucleotide/side metabolism pathway it is possible to develop a hypothesis to explain why a mutation at the cytidine and deoxycytidylate deaminase gene may lead to increased parasite growth under PYR pressure. PYR is known to interfere with the binding of DHFR to its substrate, dihydrofolate. This leads to the rate of the reaction catalysed by DHFR being reduced in the presence of PYR (even in the resistant parasites, it is likely that inhibition of DHFR by PYR still occurs, only to a much lesser extent than in sensitive parasites).

One way of increasing the rate of an enzyme-catalysed reaction is to increase the amount of substrate available to the enzyme. From Figure 4.35, it can be seen that an increase in the activity of cytidine and deoxycytidylate deaminase, upstream of the DHFR / thymidilate synthase catalysed reaction, will increase the amount of substrate in the

DHFR / thymidilate synthase catalysed part of the pathway. Therefore, in a resistant parasite under PYR pressure, any mutation in cytidine and deoxycytidylate deaminase that leads to an increased efficiency of the enzyme is likely to be positively selected. Such an up-regulation of the activity of cytidine and deoxycytidylate deaminase could also be beneficial to resistant parasites in the absence of PYR pressure.

ASpyr1 parasites have a slower growth rate than the AS clone from which they are derived. As these parasites are thought only to differ genetically at the single point mutation in *dhfr*, then one the explanation for their differing growth rates is that the mutated resistant form of *dhfr* is "less fit" than the wild-type allele. If this is the case, then resistant parasites growing in the absence of PYR pressure would benefit from an increase in the efficiency of another enzyme in the same pathway as DHFR, such as cytidine and deoxycytidylate deaminase. Such a mutation, arising from the inhibition of a metabolic pathway due to a change in a constituent enzyme caused by selection for a drug resistant mutation, can be thought of as a "compensatory mutation" which increases the overall efficiency of the pathway involved. A further discussion of possible "fitness costs" associated with drug resistance mutations is given in Chapter V.

4.4.2 Selection for an AJ locus on Chromosome 11

There appears to be some selection for AJ markers on chromosome 11 under pyrimethamine pressure. These markers show a "selection peak" on this chromosome (Figure 4.9), which is mirrored by a slight selection valley of ASpyr1 markers. Markers on the peak, either side of the marker showing the greatest increase, map to the end of chromosome 6 in *P. falciparum*, indicating that the target locus is between 998 and 1334 Kb along the chromosome. The marker in the corresponding ASpyr1 selection valley that is most greatly reduced in intensity maps to a position 1310 Kb along the same chromosome. Intriguingly, a gene called *MAL6P1.121* which codes for "FolD bifunctional protein" is found at 1329kb along chromosome 6 (see Figure 4.36), closely linked to those markers that appear under selection.

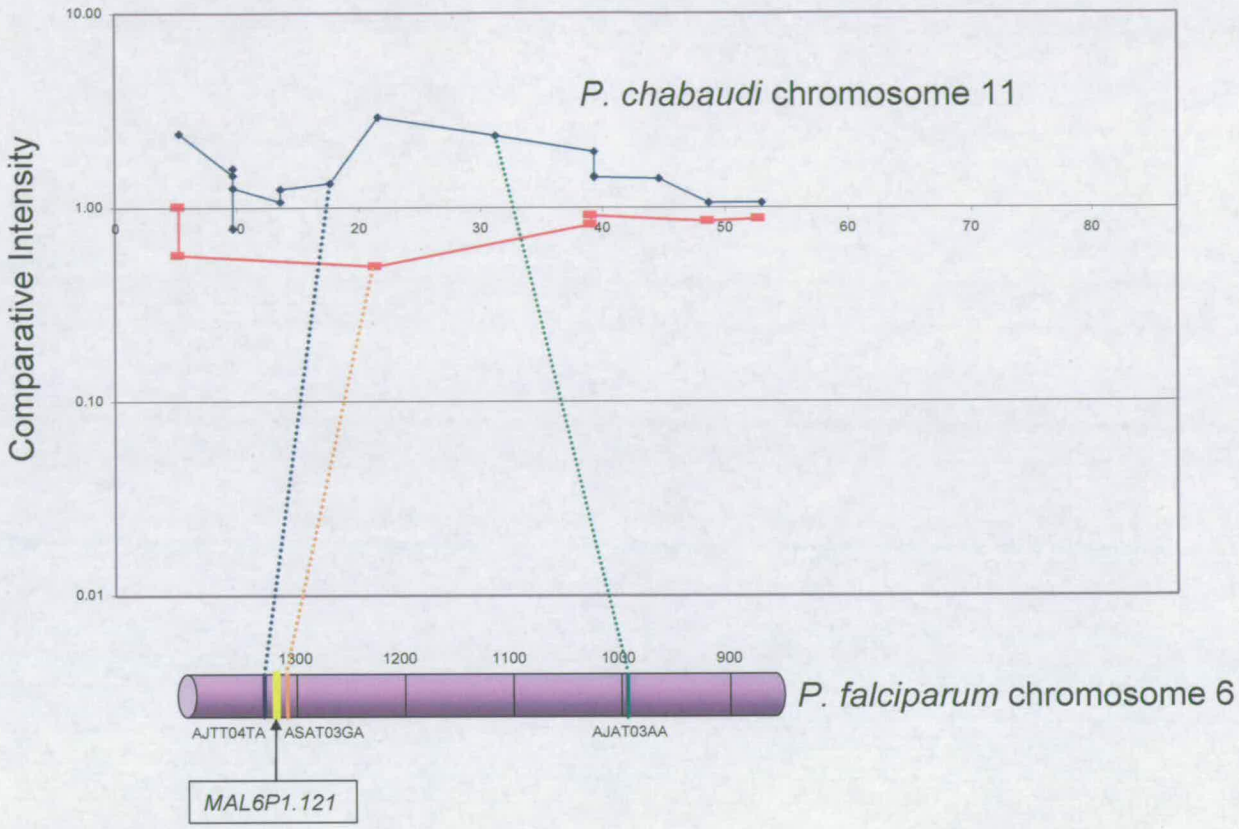


Figure 4.36 The comparative intensities of AJ (blue line) and ASpyr1 (red line) AFLP markers after selection of the cross progeny, and their positions on *P. c. chabaudi* chromosome 11. Homologues of markers that appear to be selected for under pyrimethamine pressure were located on the *P. falciparum* genome database. Dotted lines link the markers on *P. c. chabaudi* chromosome 11 to their homologues on *P. falciparum* chromosome 6. Numbers above the *P. falciparum* chromosomes indicate distances in Kilobases. Marker names are given below the chromosomes.

The two components of this protein are methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate cyclohydrolase, two enzymes that are closely associated with DHFR / thymidilate synthase in both the folic acid biosynthesis and formyl THF biosynthesis pathways (see Figures 4.37 and 4.38).

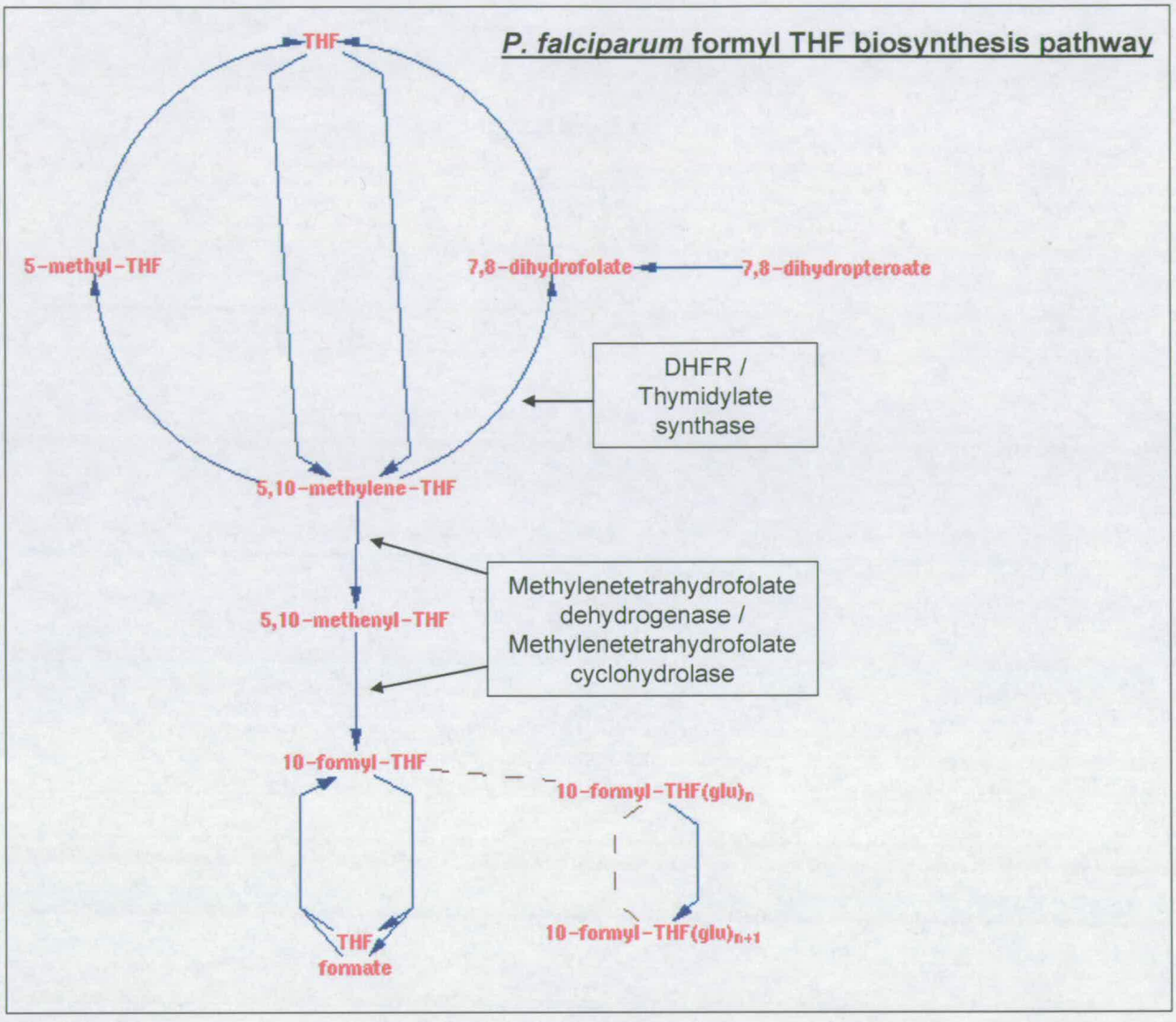


Figure 4.37 The formyl THF biosynthesis pathway of *P. falciparum* showing the actions of methylenetetrahydrofolate dehydrogenase / methylenetetrahydrofolate cyclohydrolase and DHFR / thymidylate synthase. Adapted from the PlasmoCyc website; http://biocyc.org/PFA

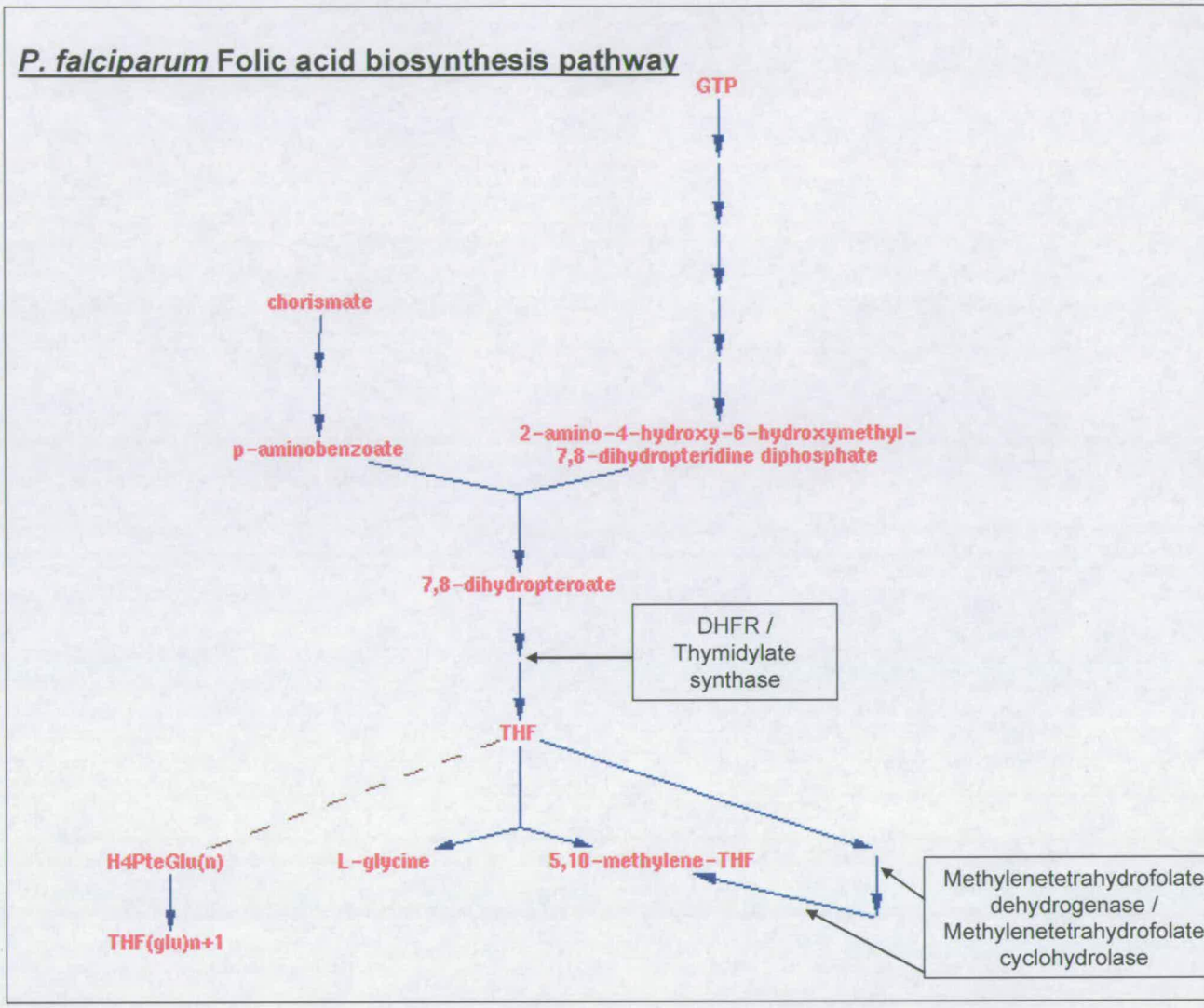


Figure 4.38 The folic acid biosynthesis pathway of *P. falciparum* showing the actions of methylenetetrahydrofolate dehydrogenase / methylenetetrahydrofolate cyclohydrolase and DHFR / thymidylate synthase. Adapted from the PlasmoCyc website; http://biocyc.org/PFA

Following the arguments presented for the selection of more efficient alleles of genes encoding enzymes in the same pathway(s) as the target enzyme of a drug under drug-pressure (Section 4.4.1), it is conceivable that selection may occur for the AJ allele of *MAL6P1.121* if its enzyme product is more efficient than that of the ASpyr1 allele and leads to greater productivity in the pathway(s) overall. This effect must be due, however, to intrinsic differences between ASpyr1 and AJ parasites, and cannot be the result of a compensatory mutation, as it involves the selection of an allele from the AJ parent which has not been subject to selection for PYR resistance.

4.4.3 Growth rate advantage of AJ alleles

Analysis of the change in AJ marker intensities between treatment groups (Non-passaged, Untreated and PYR treated) shows that there is a significant increase in the average AJ marker intensity between the Non-passaged and the Untreated progeny. This effect is seen in 14 out of the 16 chromosome / linkage groups with the only exceptions being chromosome 12 and linkage group D. This effect could be explained by parental type AJ parasites out-competing ASpyr1 parasites due to their faster growth rate (as seen in Chapter III), leading to an increase in AJ marker intensity overall. This explanation cannot, however, account for the observation that there is also an increase in AJ marker intensity from the Non-passaged to the PYR treated group overall (although not significant), and in 11 out of the 16 chromosome / linkage groups, as the parental AJ in this population was completely removed by the drug treatment (Figure 4.31 – *dhfr*).

This effect is supported by the proportional sequencing data (Figure 4.31), which shows a significant increase in the proportion of the AJ allele in all genes except *dhfr*. One possible explanation for this effect is that AJ is "fitter" than ASpyr1 at many different loci across the genome, which would mean that AJ type alleles would out-compete their ASpyr1 equivalents at all these loci. This would explain the growth rate differences between the two parental parasites in terms of genome-wide differences in fitness, rather than there being a small number of "growth-rate genes".

CHAPTER V
EXPERIMENTS INVESTIGATING THE FITNESS COST OF PYRIMETHAMINE RESISTANCE

This chapter contains further analysis of the results of the experiments described in chapter IV. The analysis in this chapter concerns growth rate differences and the apparent fitness cost of the mutation at *dhfr* which results in PYR resistance. In addition, further experiments are described which attempt to establish whether parasites carrying the PYR resistance mutation are at a selection disadvantage when in competition with parasites carrying PYR sensitive alleles of *dhfr*.

5.1 Introduction

Evidence for fitness costs associated with drug resistance in malaria parasites is relatively scarce. There is circumstantial evidence to suggest that chloroquine resistance may carry a selective disadvantage to parasites in competition with sensitive strains when there is no chloroquine pressure applied (Kublin et al., 2003a; Liu et al., 1995; Mita et al., 2003; Mita et al., 2004; Thanh et al., 2001), and experimental evidence has shown that there is a cost associated with atovaqoune resistance (Peters et al., 2002). For pyrimethamine resistance the evidence for a related fitness cost is inconclusive, with some studies apparently revealing a cost for some mutations (Fohl and Roos, 2003; Sirawaraporn et al., 1997a; 1997b), while others find no resistance-associated fitness disadvantage (Rosario et al., 1978). These studies were discussed in more detail in Chapter I.

The results described in Chapter III show that ASpyr1 parasites are out-competed by AJ parasites when grown in mixed infections. As the two parasite clones are genetically divergent (there is, on average one single nucleotide polymorphism per 150 base pairs, (Hunt, *pers. com.*)), it is impossible to attribute this growth rate difference to any particular genetic determinant, such as the mutation that confers PYR resistance. In order to ascertain whether the S106N mutant allele of *pcdhfr* confers a reduction in growth rate, it is necessary to investigate the results of competition between parasites that differ *only*

at this mutation in mixed infections. This was, in effect, the approach taken by Rosario et al. (1978) when ASpyr1 was grown in mixed infections with AS(sens), the parasite line from which ASpyr1 was derived. It is supposed that these parasites differ genetically only at the SNP in *pcdhfr* responsible for the S106N mutation, but confirmation of this is impossible without extensive sequencing at other loci. The results of these experiments were inconclusive, with both sensitive and resistant parasites present in 3 independent infections at day 10 post-inoculation, and sensitive parasites appearing to competitively exclude the resistant strain by between days 26 and 36 in two out of the 3 infections.

The production of large numbers of recombinant progeny of a cross between a pyrimethamine sensitive and a resistant parasite effectively introduces the resistant allele into a wide range of diverse genetic backgrounds. The growth of this recombinant progeny population in mice without the presence of pyrimethamine, and the direct measurement of the proportions of parasites carrying the resistant and sensitive alleles of *dhfr* allows a direct measurement of the relative fitness of both alleles which is independent of genetic differences at other loci. This approach solves the inherent problems associated with using the isogenic lines described above, namely the inability to be completely confident that the lines differ only at the *dhfr* locus, and the fact that the different number of passages each line was subjected to may have selected for differences in growth-rates between them. The results of such experiments involving the progeny of crosses between ASpyr1 (resistant) and AJ (sensitive), and ASpyr1 and CB (sensitive) are described here.

A further experiment was conducted on the progeny of a cross between AS(sens) and AJ in order to control for the possibility that single nucleotide polymorphisms in the *dhfr* gene between AS(sens) and AJ are responsible for the apparent difference in fitness between ASpyr1 and AJ

5.2 Further analysis of the progeny of a cross between ASpyr1 and AJ described in Chapter IV (section 4.3)

The uncloned progeny of the cross between ASpyr1 and AJ described in Chapter IV (Section 4.3) were grown in a group of 3 mice for 6 days until a parasitaemia of 30-40% was reached. At this point all mice were bled, the blood was mixed and 0.3 ml was used to inoculate a further 3 mice, the remainder being used for the extraction of parasite DNA for AFLP analysis. The resulting infection in the second group of mice was allowed to proceed, without the administration of pyrimethamine, until peak parasitaemia was achieved (6 days post-inoculation) at which point blood was collected, pooled and parasite DNA extracted. AFLP analysis was then performed on samples from both groups as previously described, and the relative intensities of markers of both parental parasites were compared between the two groups (First Passage and Second Passage, respectively, see Figure 5.1). This allowed the comparison of the intensities of AFLP markers before and after six days of erythrocytic growth in the absence of pyrimethamine treatment.

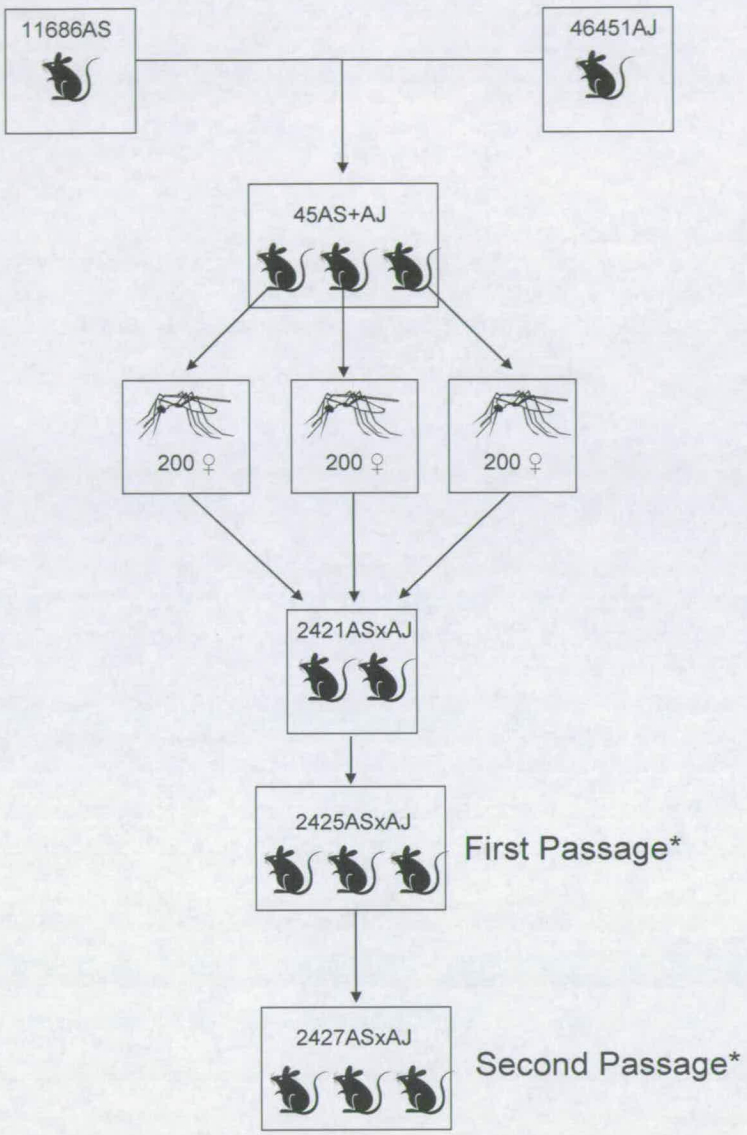


Figure 5.1 A schematic representation of the procedure for the generation of a cross between ASpyr1 and AJ. Numbers in the boxes refer to the cage numbers of the mice used in the experiment. *Experimental groups are referred to in the text by these names

5.2.1 Results

Figure 5.2 shows the Comparative Intensities of ASpyr1 makers in the Second Passage progeny of the cross compared to the First Passage progeny. This graph therefore shows the effect on marker representation of 6 days of erythrocytic cycle growth in a population of genetically diverse parasites without the influence of drug selection. The same

comparison of marker intensities is shown for AJ markers in Figure 5.3, superimposed upon the data from Figure 5.2 in order to facilitate comparisons between the markers from the two parents.

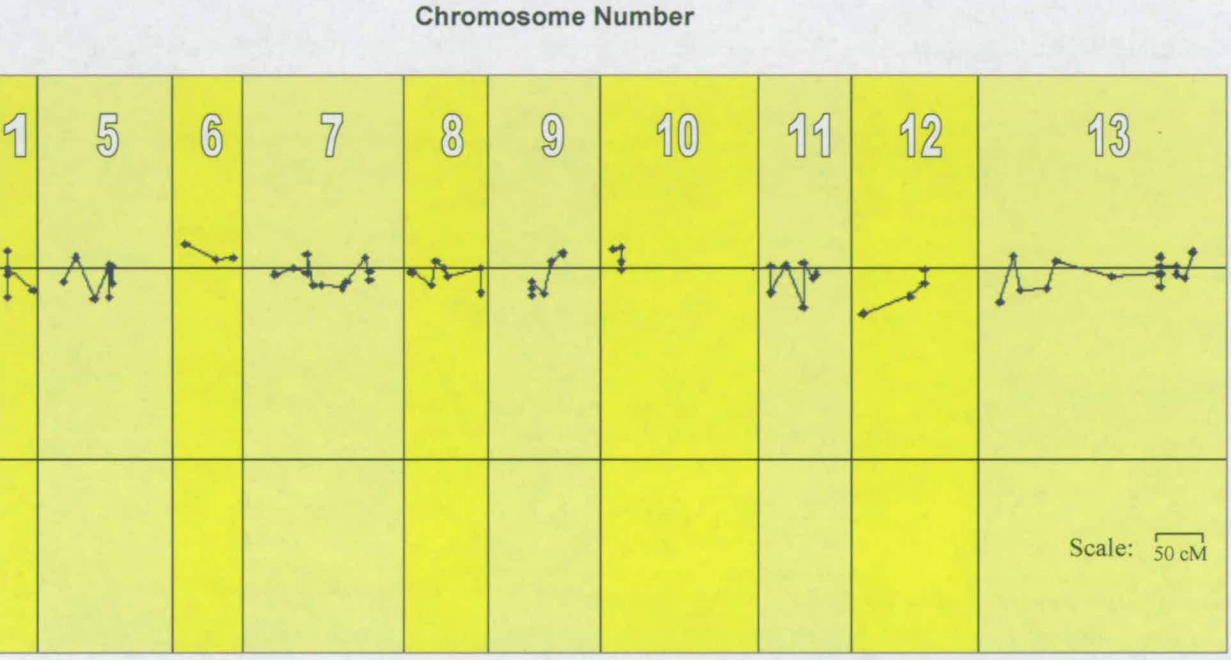


Figure 5.2 Comparative Intensities of ASpyr1 (resistant parent) markers in the Second Passage group compared to the First Passage group, for markers assigned to *P. chabaudi* chromosomes. Markers are positioned on the X axis according to their positions in cM along the chromosomes, and the sizes of the chromosomes in cM according to the map are indicated by the width of the columns.

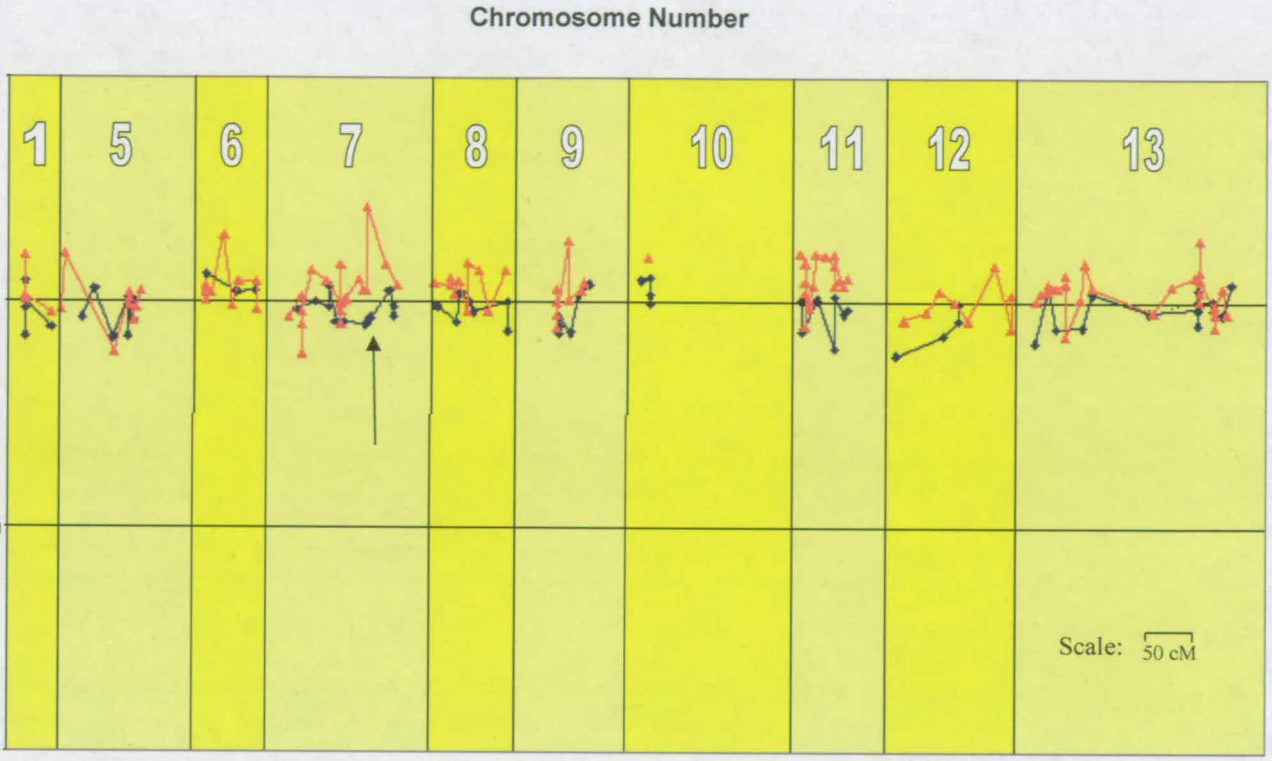


Figure 5.3 Comparative Intensities of **AJ** (sensitive parent) (red line) and **ASpyr1** (resistant parent) (blue line) markers in the Second Passage group compared to the First Passage group, for markers assigned to *P. chabaudi* chromosomes. Markers are positioned on the X axis according to their positions in cM along the chromosomes, and the sizes of the chromosomes in cM according to the map are indicated by the width of the columns. The arrow points to the position of *pcdhfr*.

From these results there appears to be little selection against linked groups of ASpyr1 markers. There is, however, a slight selection valley present on chromosome 7 around the position of *dhfr* (marked with an arrow), which is associated with a large "selection peak" of an AJ marker in the same region. In order to investigate this effect in more detail, the proportions of ASpyr1 and AJ alleles of *dhfr* in both the First Passage and Second Passage groups were measured by Proportional Sequencing. The proportions of ASpyr1 and AJ at 3 other loci (*dhps*, *dhfs*, and *6-pthps)* were likewise measured as controls. The measured proportions of these alleles are presented in Figure 5.4.

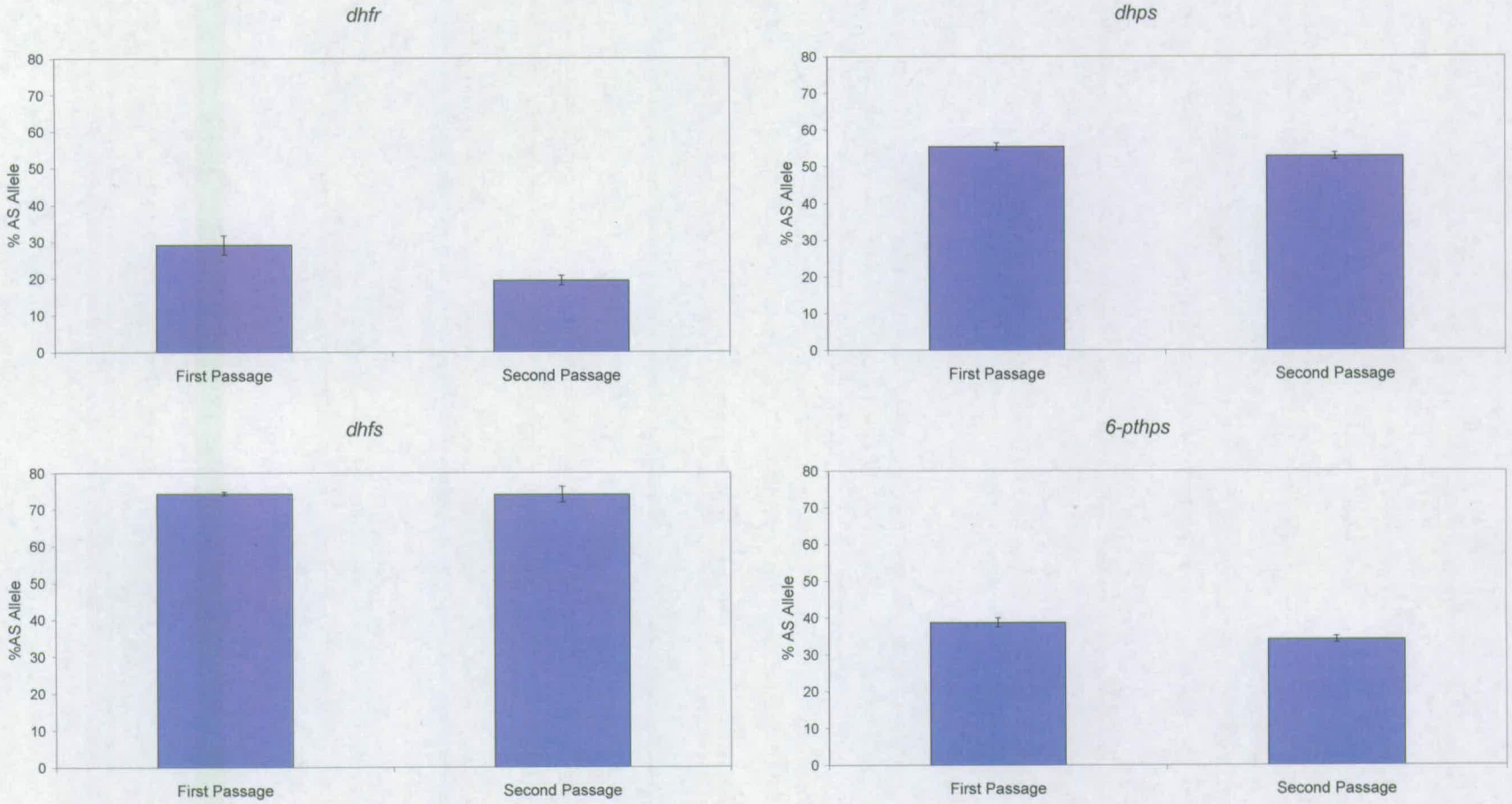


Figure 5.4 Proportions of ASpyr1 alleles of *dhfr, dhps, dhfs* and *6-pthp* in the First Passage and Second Passage progeny of the cross between ASpyr1 and AJ, measured by Proportional Sequencing. Each individual proportional sequencing measurement was repeated 5 times on the same DNA (so that each measurement represents an independent nested PCR and measurement of sequencing peaks from the same DNA sample – each DNA sample represents about $6x10^9$ parasites). Error bars give the standard errors of the means.

The proportion of the ASpyr1 allele of *dhfr* is 29.2% in the First Passage progeny, compared to 55.5%, 74.3% and 38.8% for *dhps*, *dhfs*, and *6-pthps* respectively. This suggests that there has been some selection against parasites carrying the ASpyr1 allele of *dhfr* during the growth of the progeny of the cross in the 4 mice in which the progeny were initially grown from sporozoites prior to inoculation into the First Passage group (assuming a 50% representation of alleles from both parents in the sporozoites produced during the cross). Furthermore, the proportion of the ASpyr1 allele of *dhfr* decreases by an average of 9.72% between the First Passage group and the Second Passage group, a much greater decrease than those observed for the other 3 alleles investigated (*dhps* = 2.73, *dhfs* = 0.26, and *6-pthps* = 4.66). This suggests that, in the absence of drug pressure, the ASpyr1 allele of *dhfr* confers a growth rate disadvantage upon the parasites carrying it compared with those carrying the AJ allele. This difference is measurable over the course of 6 days growth in mice (the difference between the First Passage and Second Passage groups of mice). It is possible that further passage without drug treatment may produce a larger effect of selection against the ASpyr1 allele *dhfr*.

5.2.2 Estimate of the relative fitness of parasites carrying the ASpyr1 allele of *dhfr*

From this data it is possible to calculate the relative fitness "*s*" of those parasites carrying the pyrimethamine resistance mutation at the *dhfr* allele, using the formula:

$$Srt = \ln\left[\frac{a_{t2}}{b_{t2}}\right] - \ln\left[\frac{a_{t1}}{b_{t1}}\right]$$

where a = the relative proportion of those parasites carrying the ASpyr1 allele of *dhfr* (resistant)

b = the relative proportion of those parasites carrying the AJ allele of *dhfr* (wild-type)

r = the replication rate

t = time (or number of generations - in this case "6" (for 6 days of growth in the Second Passage group)

Using the values a_{t1} = 29.20, a_{t2} = 19.48, b_{t1} = 70.08, and b_{t2} = 80.52, obtained from the Proportional Sequencing data described above, and r = 5 (given by the slope of the parasitaemia

curve from day 0 to day 6 in the Second Passage group), the selection co-efficient (S) of parasites carrying the ASpyr1 allele of *dhfr* is **-0.018,** and hence their relative fitness (1+S),compared to those carrying the AJ allele in the absence of pyrimethamine pressure is **0.98.**

5.3 Analysis of a cross between ASpyr1 and CB (pyrimethamine sensitive)

It is possible that the growth rate disadvantage conveyed by the ASpyr1 allele of *dhfr* measured in the experiment above is only apparent when parasites carrying this allele are in competition with parasites carrying the AJ *dhfr* allele. It is conceivable, therefore, that the growth rate differences observed above are due simply to inherent differences in the relative fitness of the ASpyr1 *dhfr* allele compared to the AJ allele (independent of the specific mutation that confers PYR resistance to ASpyr1), and so it is impossible to conclude that the ASpyr1 allele carries a fitness cost due to the PYR-resistance mutation itself.

In order to address the question of whether the fitness disadvantage conveyed by the ASpyr1 allele of *dhfr* is only apparent when in competition with the AJ *dhfr* allele, a further genetic cross was generated between ASpyr1 and another sensitive parasite clone, CB. The cross (Figure 5.5) was generated as previously described for ASpyr1 x AJ, and in an analogous fashion, First Passage and Second Passage progeny were analysed.

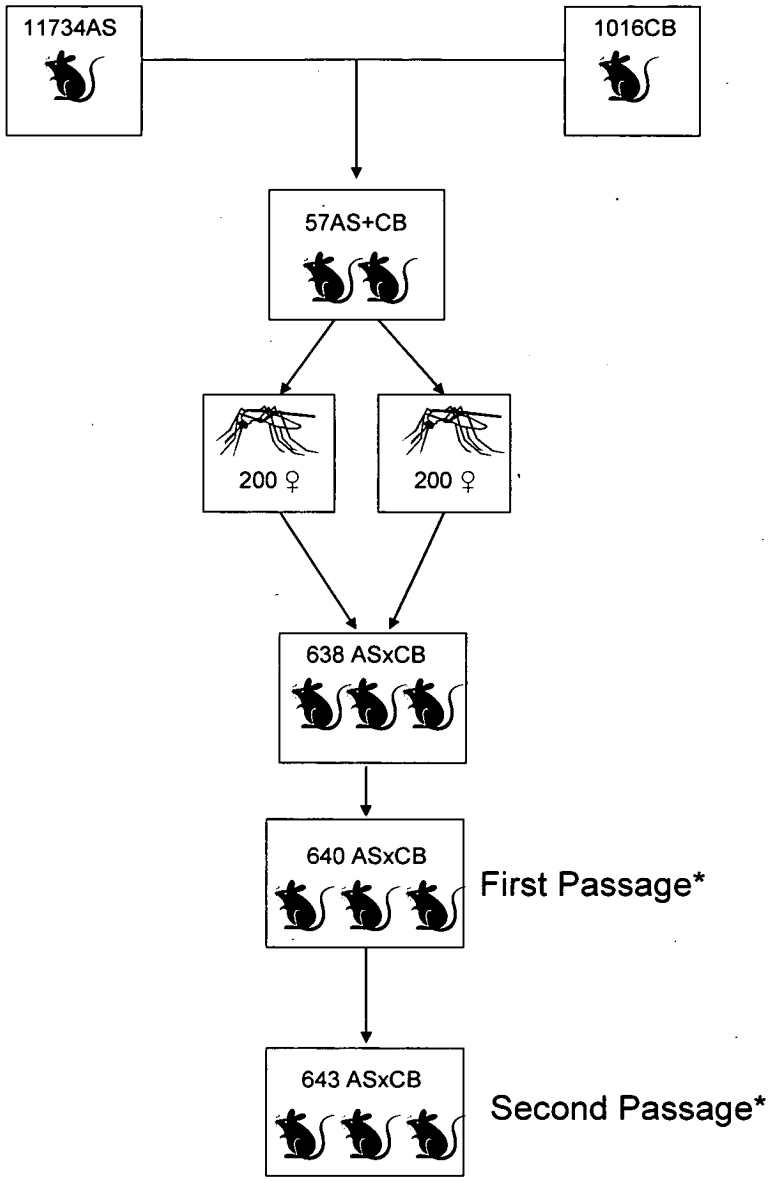


Figure 5.5 A schematic representation of the procedure for the generation of a cross between ASpyr1 and CB. Numbers in the boxes refer to the cage numbers of the mice used in the experiment. *Experimental groups are referred to in the text by these names.

Mosquito Cage Number[1]	% Gametocytaemia on day of feed	Number of oocysts per mosquito dissected[2]	Mean number of oocysts per Mosquito	Number of mosquitoes Dissected for sporozoites	Estimated number of recombinants[3]
Parental CB	0.13	0,0,5,1,1, 11,9,1,16,1	4.5	-	-
Parental ASpyr1	0.07	3,5,6,6,4, 2,9,1,63,37	12.36	-	-
Cross 1	0.11	0,5,14,0,68	17.4	122	4245
Cross 2	0.04	36,0,18,27,0	16.2	153	4957

Table 5.2 Numbers of oocysts present in mosquitoes 8 days post-infection.

[1]200 ♀ mosquitoes per cage. Numbers in brackets indicate the individual mouse upon which the cage was fed.

[2]10 mosquitoes were dissected from each parental cage, and 5 from each cross cage, on day 8 post infective blood-meal

[3]Assumes 50% of oocysts are hybrid, and each produces 4 recombinants (see Figure 1.6).

5.3.1 Results

Figure 5.6 shows the proportions of ASpyr1 alleles of *dhfr*, *dhps* and *6-pthps* in the First Passage and Second Passage groups as measured by Proportional Sequencing.

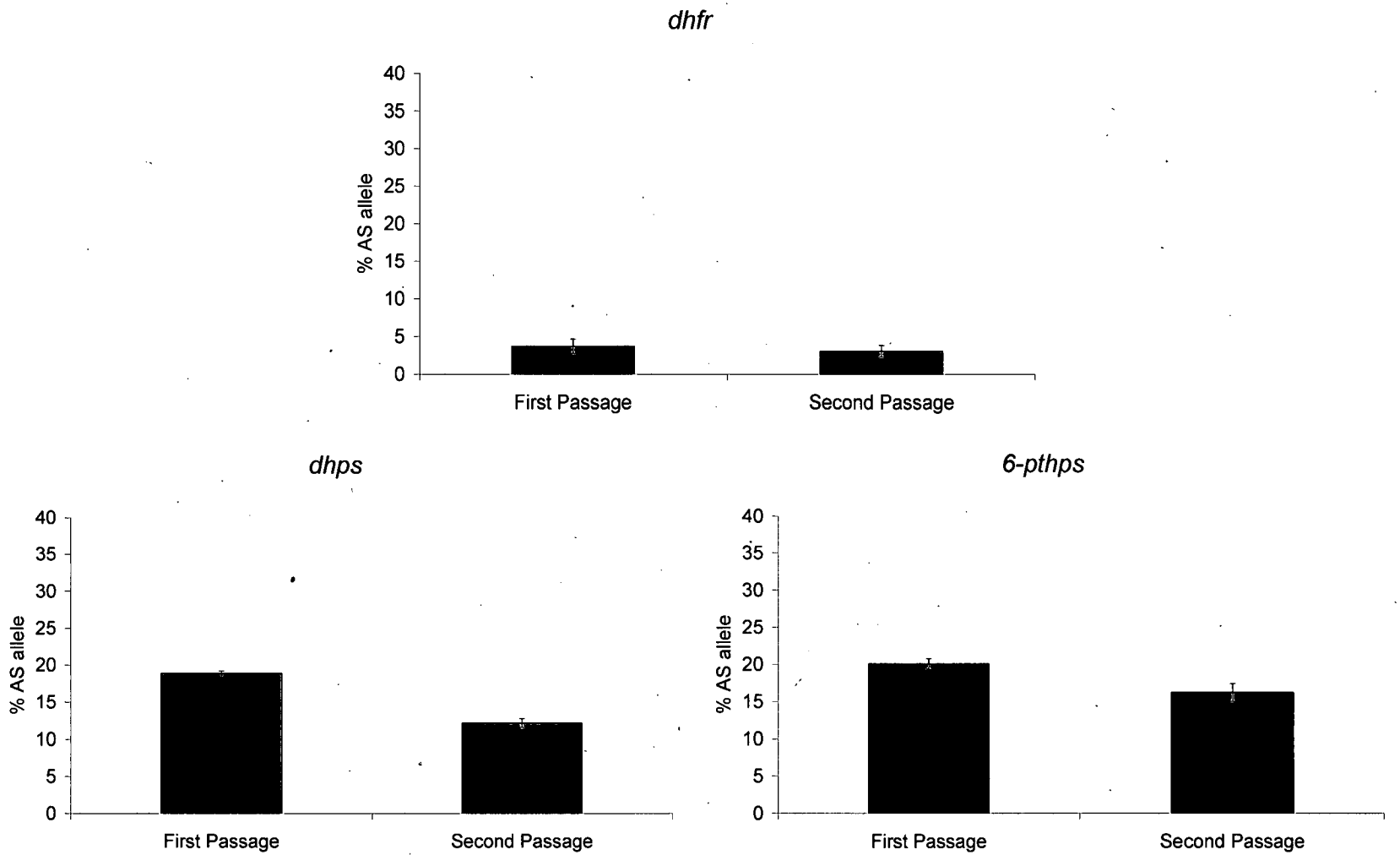


Figure 5.6 Proportions of ASpyr1 alleles of *dhfr, dhps,* and *6-pthps* in the First Passage and Second Passage progeny of the cross between ASpyr1 and CB, measured by Proportional Sequencing. Each individual proportional sequencing measurement was repeated 5 times on the same DNA, and error bars give the standard errors of the means.

These results show that, in the cross between ASpyr1 and CB, the proportion of the ASpyr1 allele of *dhfr* is greatly reduced in the First Passage cross progeny compared to the proportions of the ASpyr1 alleles of *dhps* and *6-pthps*. This suggests that selection against the ASpyr1 allele of *dhfr* had already occurred in the sporozoite-induced infections of the cross progeny population prior to inoculation into the First Passage experimental group. In contrast to the results from the ASpyr1 x AJ cross, however, there was little further decrease in proportion of ASpyr1 *dhfr* the First Passage and Second Passage progeny (indeed, there appears to be a greater decrease between the representation of ASpyr1 alleles for *dhps* and *6-pthps* than for *dhfr*).

5.4 Analysis of a cross between AS(sens) and AJ (both pyrimethamine sensitive)

The results from the two experiments described above suggest that there is a growth rate cost conferred to parasites carrying the ASpyr1 allele of *dhfr* when grown in competition with parasites carrying a pyrimethamine sensitive allele of the same gene.

In the experiment involving the progeny of a cross between ASpyr1 and AJ, the ASpyr1 allele of *dhfr* appears to be selected against in the absence of drug pressure in a mixed infection containing the uncloned recombinant progeny of a cross between ASpyr1 and AJ. It is conceivable that this is evidence of a fitness cost associated with the mutation in *dhfr* which confers resistance to pyrimethamine. It is known that the mutation responsible for pyrimethamine resistance in the *dhfr* gene is a single nucleotide polymorphism which results in a change in amino acid at position 106 from a serine to an asparagine (Cheng and Saul, 1994). However, ASpyr1 and AJ differ not only at this SNP, but at 7 other bases within the gene (one of which is non-synonymous). It is possible, therefore, that the fitness cost observed for the ASpyr1 *dhfr* allele when in competition with the AJ allele, may be due to these other polymorphisms, and may not be associated with pyrimethamine resistance.

In order to investigate this possibility, an experiment was conducted in which the pyrimethamine sensitive progenitor of ASpyr1, AS(sens), was crossed with AJ and the resulting uncloned recombinant progeny were passaged through a series of groups of mice

(Figure 5.7) in the absence of any selection pressure other than growth competition between parasites. Blood samples were taken from all groups on day 4 post-inoculation and the proportions of AS(sens) and AJ alleles of *dhfr* were measured in order to determine if there was selection against AS(sens) *dhfr*. The relative proportions of alleles of another, unrelated, polymorphic gene, multi-drug resistance -1 (*mdr-1*) were also measured as a control.

In the experiments conducted with the uncloned progeny of crosses between ASpyr1 and AJ, and ASpyr1 and CB (see above), there had appeared to be selection against the *dhfr* allele of ASpyr1 already in the sporozoite induced infections in which the cross progeny population was grown before inoculation into the First Passage groups. In order to measure any possible selection at this stage, samples were taken daily from the sporozoite induced infections in which the progeny of the cross between AS(sens) and AJ were grown, and were analysed for the proportions of AS(sens) and AJ alleles of *dhfr* and *mdr-1*.

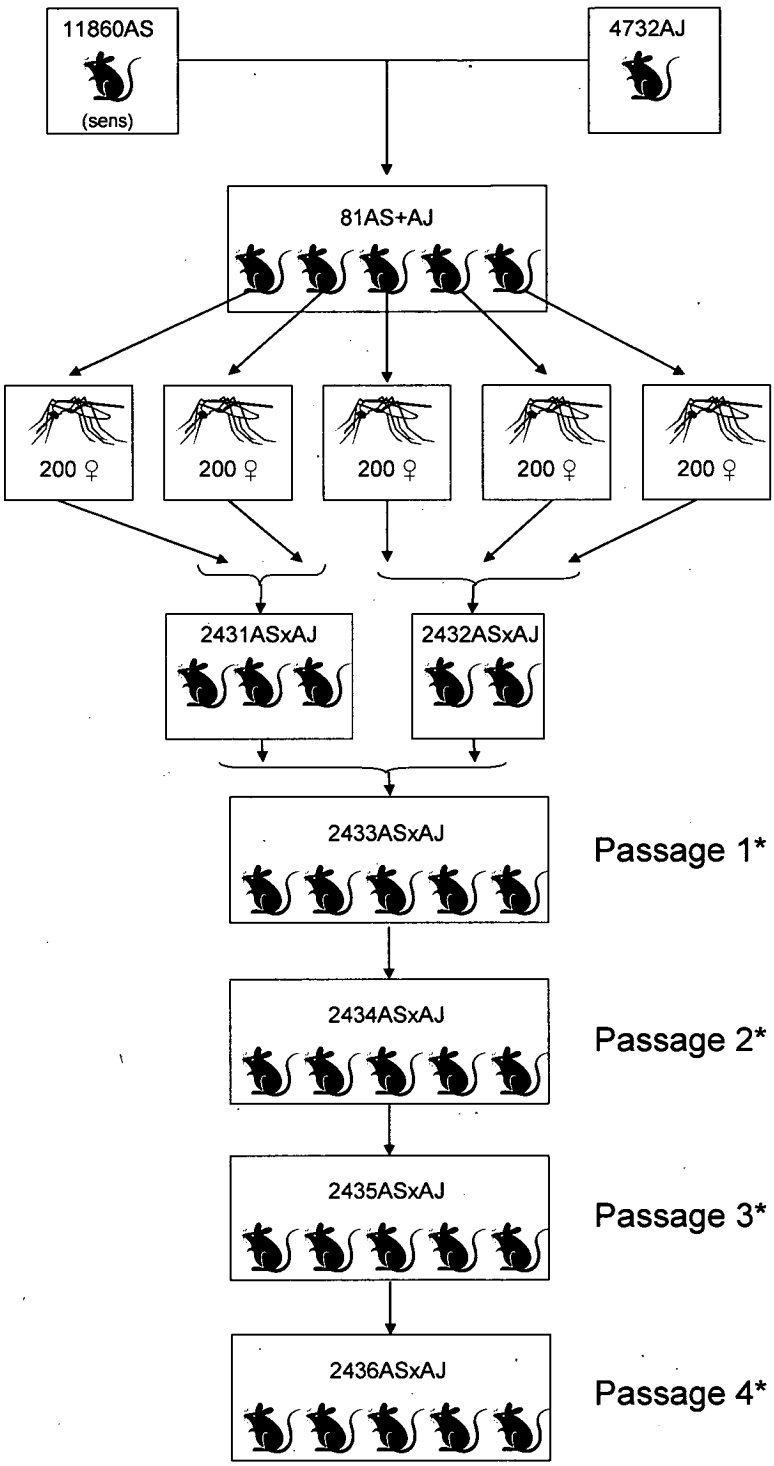


Figure 5.7 A schematic representation of the procedure for the generation of a cross between AS(sens) and AJ, and the subsequent passage of the uncloned recombinant progeny through a series of groups of mice. Numbers in the boxes refer to the cage numbers of the mice used in the experiment. *Experimental groups are referred to in the text by these names.

5.4.1 Results

Parasitaemias of all mice infected with sporozoites from the cross between AS(sens) and AJ are shown in Figure 5.8. The course of an infection induced by sporozoites differs from that seen when blood stage parasites are directly inoculated into hosts. The fact that sporozoites must enter the liver where they undergo pre-erythrocitic development (which takes around 48 hours before release of merozoites), means there is a period of time post-inoculation in which there are no blood-form parasites present in the circulation. In such sporozoite induced infections, peak parasitaemia is only approached by day 10 post-inoculation; whereas infected blood induced infections do so by day 5 or 6 post-inoculation.

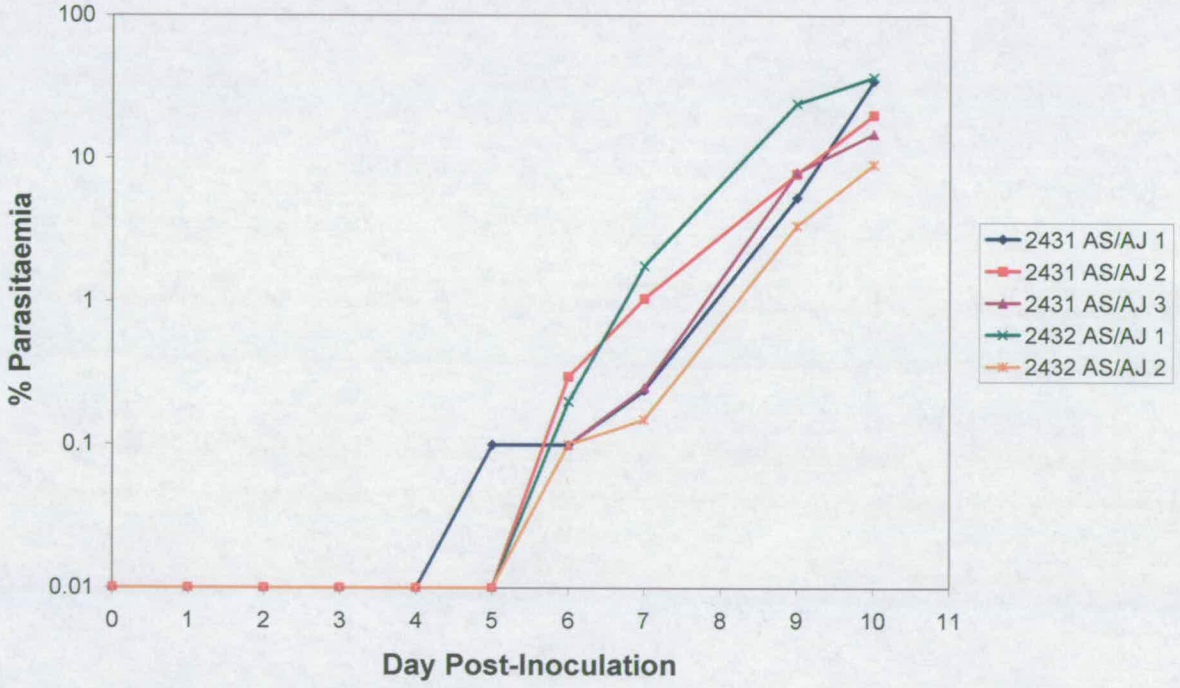


Figure 5.8 The parasitaemias (on a log scale) of individual mice inoculated with sporozoites from a cross between AS(sens) and AJ.
The key refers to each mouse by the cage numbers given in Figure 5.7

All mice from the sporozoite induced infections were blood-sampled daily, and the infections monitored by Proportional Sequencing in order to determine the proportions of AS(sens) and AJ alleles of *dhfr* and of the control gene (*mdr-1*). Of the two groups of mice (2431AS/AJ and

2432AS/AJ) inoculated with sporozoites from the cross, both mice in group 2432AS/AJ were found to contain both AS(sens) and AJ alleles of *dhfr* and *mdr-1* genes as assayed by Proportional Sequencing. Although a patent parasitaemia was observed in the mice in 2331AS/AJ (Figure 5.8), only alleles from AJ were present in the infection, suggesting that only the AJ parent was present, with zero (or very few) recombinants represented in the population. As both these groups were inoculated with sporozoites from different cages of mosquitoes (Figure 5.7), differences in the compositions of the resulting infections are not unexpected.

The proportions of the AS(sens) alleles of *dhfr* and *mdr-1* in the infections from the progeny in mouse group 2432AS/AJ as determined by Proportional Sequencing assays are presented in Figure 5.9.

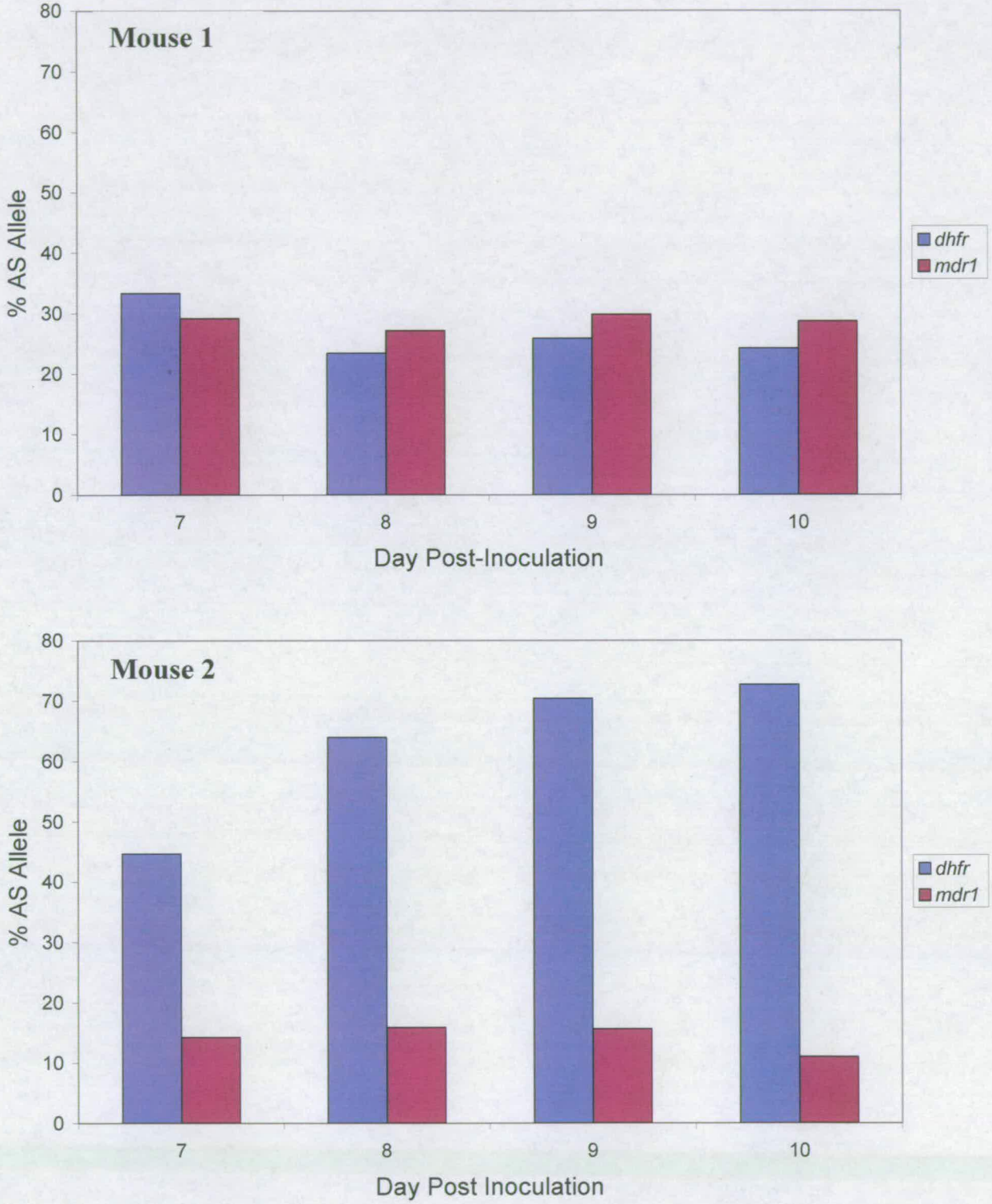


Figure 5.9 The proportions of AS(sens) and AJ alleles of *dhfr* and *mdr-1* in two individual mice (from group 2432AS/AJ) inoculated with sporozoites from a cross between the two parasites, as determined by Proportional Sequencing.

The Proportional Sequencing results for the sporozoite induced infections vary between the two mice analysed. In mouse 1, the proportions of both alleles are approximately the same, and continue at similar levels throughout the infection. In mouse 2, however, the proportion of the AS(sens) allele of *dhfr* was much greater than that of *mdr-1*. The discrepancies between the mice can be explained by the fact that each mouse received a separate inoculation of cross progeny sporozoites, resulting in each infection being composed of a different subset of the total progeny population. In neither infection, however, was selection against the AS(sens) *dhfr* allele observed over the course of the infection.

Blood from all sporozoite inoculated mice, including those subsequently found to contain only AJ alleles of *dhfr* and *mdr-1*, was pooled and passaged in 4 serial experimental infections in mice as shown in Figure 5.7. The parasitaemias of these infections are given in Figure 5.10, and the proportions of the AS(sens) alleles of *dhfr* and *mdr-1* from parasites present in these infections (at day 4 post-inoculation for each group), are shown in Figure 5.11.

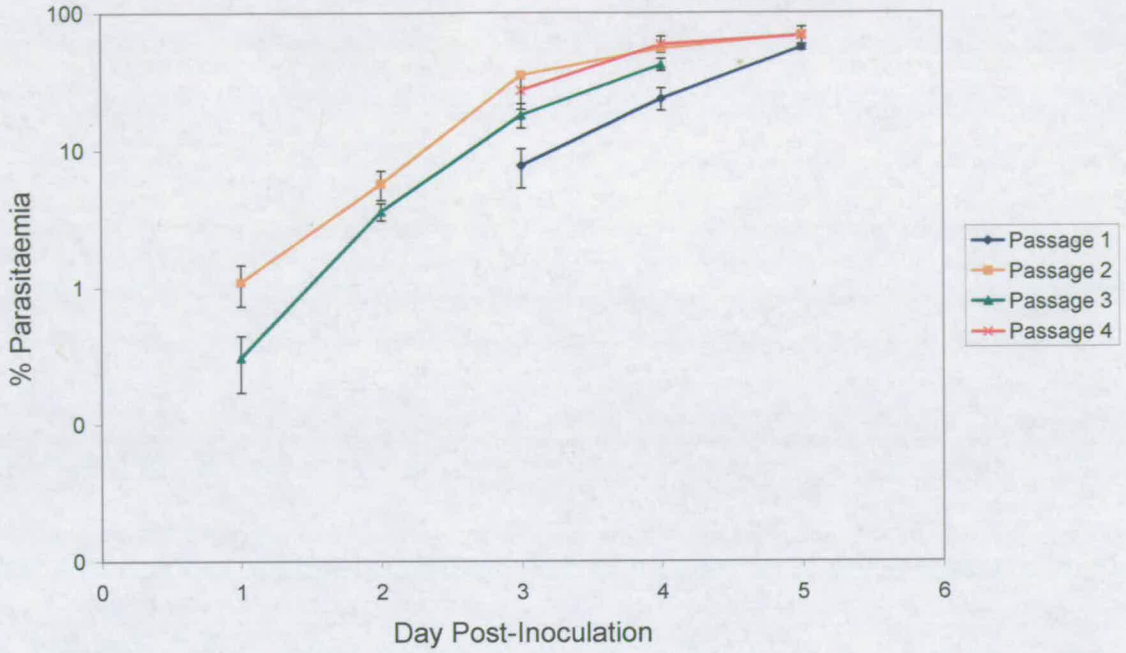


Figure 5.10 Mean parasitaemias (n=5) of infections of uncloned AS(sens) x AJ cross progeny in 4 serial passage infections in mice. Error bars indicate the 95% CI for the mean parasitaemia of the 5 mice in each group. The key refers to the groups as described in Figure 5.7

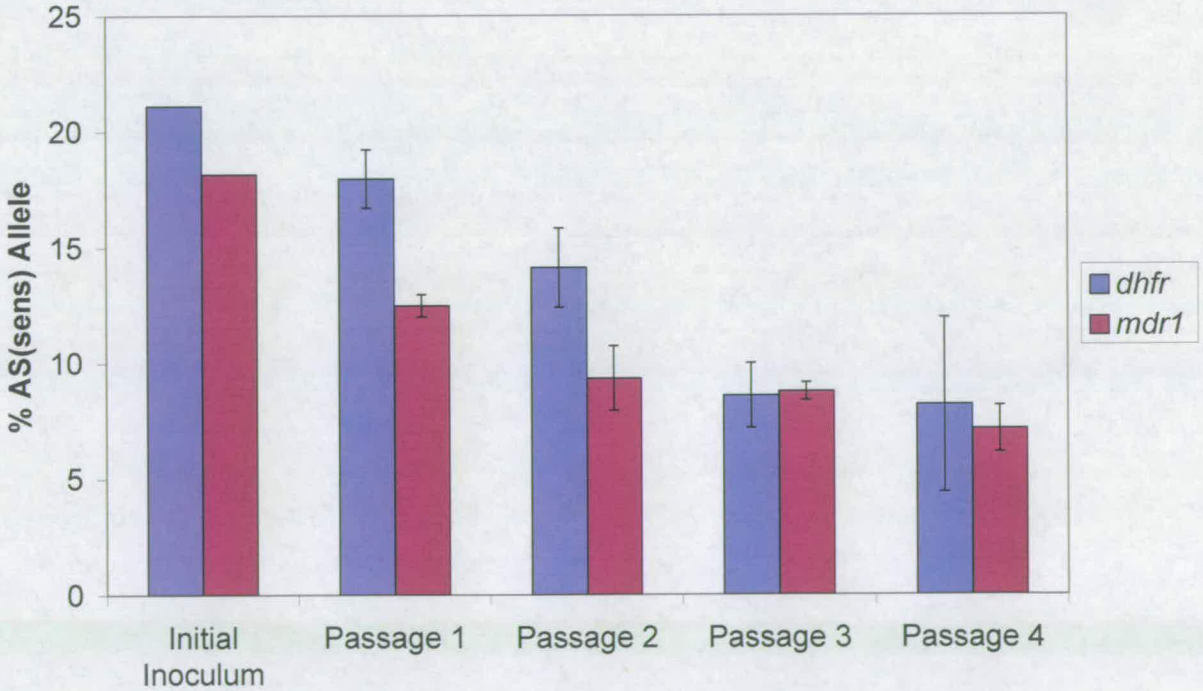


Figure 5.11 The proportions of AS(sens) alleles of *dhfr* and *mdr-1* in infections of uncloned progeny of a cross between AS(sens) and AJ in serial passage through 4 groups of 5 mice, as determined by Proportional Sequencing. Errors bars indicate the 95% CI for the mean of the 5 mice in each group. Samples were taken on day 4 post-inoculation. Groups are referred to as described in Figure 5.7.

It can be seen that the proportions of AS(sens) alleles of both *dhfr* and *mdr-1* decrease within 4 serial passages, starting from around 20% in the initial inoculum and decreasing to around 8% in the 4th passaged group. There is, however, no significant difference in the rate of decrease between *dhfr* and *mdr-1*, suggesting that there is no preferential selection against the ASpyr1 *dhfr* allele. The fact that there is a decrease in AS(sens) alleles of both genes throughout the passages is probably due to the AJ parental type parasites out-growing AS(sens) parental types, and most other recombinant types, as was seen in the experiments described in Chapter III.

5.5 Discussion

The results from the experiments in which a clone of ASpyr1 (pyrimethamine resistant) was crossed with a clone of either AJ or CB (both pyrimethamine sensitive), and the resulting uncloned progeny of the crosses were grown in mice in the absence of drug treatment, indicated that parasites carrying the ASpyr1 allele of *dhfr* were at a growth rate disadvantage compared to those carrying the sensitive allele (whether AJ or CB) of the same gene.

In the ASpyr1 x AJ cross, this growth rate disadvantage manifested itself both as an overall reduction in the proportion of the ASpyr1 *dhfr* allele compared to other ASpyr1 alleles in the First Passage group (possibly explained by selection against ASpyr1 *dhfr* in the sporozoite induced infection from which the First Passage progeny were obtained), and as a significant reduction in the proportion of the allele from progeny in the First Passage group to the Second Passage group, when compared to reductions of other alleles between progeny in the two groups.

In experiments involving the uncloned recombinant progeny of a cross between AS(sens) and AJ, the possibility that the growth rate disadvantage associate with the ASpyr1 allele of *dhfr* was due to polymorphisms in the gene between ASpyr1 and AJ other than that which confers pyrimethamine resistance, was investigated. It was shown that the AS(sens) allele of *dhfr*, conferred no significant growth rate disadvantage to parasites when compared to another, unlinked, gene. It can be concluded, therefore, that the growth rate differences attributed to the ASpyr1 *dhfr* allele in the cross between ASpyr1 and AJ, are due to the S106N mutation that confers pyrimethamine resistance, and not to any other polymorphisms within this gene between the two parasite clones.

5.5.1 Summary

The results from these experiments show that ASpyr1 alleles of *dhfr* confer a selection disadvantage on parasites when grown in the presence of parasites containing AJ and CB alleles of the gene. This effect was subsequently shown to be due to the S106N mutation in the *dhfr* gene that confers resistance to pyrimethamine and is independent of other SNP polymorphisms between AS and AJ.

This is the first time a direct fitness cost of pyrimethamine resistance directly attributable to the S106N mutation has been measured and demonstrated. Previous work involving measuring the kinetic parameters of pyrimethamine-resistant enzymes of DHFR (Sirawaraporn et al., 1997a) and studies in which pyrimethamine resistant alleles of *dhfr* were transfected into *T. gondii* parasites (Fohl and Roos, 2003), fail to address the natural situation in which parasites possessing resistant alleles are competed against parasites with sensitive alleles of *dhfr* in genetically diverse backgrounds.

In an investigation into the enzyme kinetics of pyrimethamine-resistant DHFR enzymes, Sirawaraporn et al (1997a) found that the isoform of the enzyme encoded by the S108N *P.*

falciparum allele of *dhfr* (homologous to *P. chabaudi* S106N) did not exhibit a significantly lower k_{cat}/K_m value than the wild-type allele (Figure 1.12). Such a result may be interpreted to suggest that there would be no fitness cost associated with this single amino acid mutation. The results presented here, however, imply that there is a fitness cost associated with this mutation. It would be interesting to perform these experiments using the other mutations in *dhfr* that are know to influence resistance to pyrimethamine (N51I, C59R and I164L in *P. falciparum*), but there are no homologous mutants of *P. chabaudi* as yet available. It would be also be useful to subject the progeny of the ASpyr1 x AJ and ASpyr1 x CB crosses to further passages without selection, as performed with the AS(sens) x AJ cross.

As previously discussed (Chapter I) the repercussions of this result for drug policy in endemic areas are significant. If resistant parasites are selected against in the absence of drug pressure, then it is possible that resistance will disappear from a population when the drug pressure is removed. This opens the possibility that a carefully controlled drug programme in which two independently acting drugs were provided as first line treatments in alternate succession, could alleviate the problems currently associated with the spread of drug resistance.

CHAPTER VI
DISCUSSION

6.1 Summary

The main aim of the work presented within this thesis was the validation of the Linkage Group Selection approach to the identification of genes controlling selectable phenotypes in malaria parasites. The approach taken to achieve this aim involved the application of LGS to an investigation of the genetic determinants of pyrimethamine resistance in *Plasmodium chabaudi*. By showing that LGS was capable of identifying *pcdhfr* as the gene responsible for controlling pyrimethamine resistance in this parasite, the technique was shown to be reliable. During the course of this work, the dynamics of the interactions between pyrimethamine resistant and sensitive parasites grown in mixed infections were investigated, and data were produced that indicated there was a fitness cost associated with pyrimethamine resistance in *P. chabaudi*.

6.2 Experiments described in Chapter III

In Chapter III, experiments were described in which ASpyr1 (pyrimethamine resistant) and AJ (pyrimethamine sensitive) cloned strains of *P. chabaudi* were grown in mixed infections under varying conditions, including different mouse strains and varying levels of pyrimethamine treatment. Throughout these experiments, ASpyr1 was shown to have a reduced growth rate (in terms of the total numbers of parasites produced during the acute phase of the infection) compared to AJ in the absence of drug pressure, irrespective of host genotype. This difference in growth rates between the two clones was reflected in differences in *virulence* between them (as measure by host weight loss during Drug Selection Experiment B), as the faster growing AJ clone caused a greater degree of weight loss in infected mice than did ASpyr1. Whether this difference in growth rate reflects a difference in *fitness* between the clones (where fitness is defined as "the reproductive success of individuals of a particular genotype relative to the most fit genotype"), is unknown, as the comparative ability of the clones to transmit through mosquitoes was not measured.

Fitness differences between the clones are likely to manifest themselves in mixed infections, however, especially as one clone was seen to dominate mixed infections both in the absence of pyrimethamine (clone AJ), and in its presence (clone ASpyr1). In order to quantify fitness differences between the clones, however, further experiments would need to be designed in which the gametocyte production of each clone in a mixed infection was measured, and the infectivity of these gametocytes to mosquitoes determined. This would be an interesting direction for further work on this subject.

6.3 Linkage Group Selection (Chapter IV)

Linkage Group Selection is "the application of a specific selection pressure (such as drug treatment) to the entire recombinant progeny of a genetic cross between two members of the same species that differ phenotypically in their response to the selection pressure, and evaluation of the effects of the selection upon the progeny at loci across the entire genome using quantitative genetic markers" (Culleton et al., 2005). This approach ultimately allows the identification of specific genes that control the phenotype under investigation. The LGS approach is ideally suited to organisms such as the malaria parasites whose genetics are conventionally Mendelian but which are in a haploid state for most of their life cycle when phenotype-specific selection pressures can be applied. These circumstances apply to all the protozoan parasites of the phylum Apicomplexa which include not only the malaria parasites but also their relatives *Theileria*, *Babesia*, *Toxoplasma* and *Coccidia,* all of which are important pathogens of humans and/or animals.

Using the rodent malaria parasite *P. chabaudi*, and applying the LGS method, the work presented in this thesis has demonstrated that a group of linked AFLP markers were reduced in intensity when the uncloned progeny of a genetic cross between a pyrimethamine resistant (ASpyr1) and a pyrimethamine sensitive clone (AJ) were subjected to pyrimethamine treatment. These markers were linked on a *P. chabaudi* genetic linkage map to *pcdhfr,* the gene already known to determine pyrimethamine resistance in the resistant parental line used in the cross. The homologues of these markers were also found to be physically linked to *pfdhfr,* the

homologous gene in *P. falciparum*. The data demonstrate the existence, under pyrimethamine treatment, of a "selection valley" with the *dhfr* locus at its apex and have, therefore, validated LGS as a method for locating a gene controlling a selectable phenotype such as drug resistance.

A full discussion of the potential uses of LGS as a method to identify genes controlling selectable phenotypes has recently been published (Culleton et al., 2005), and includes a discussion of the potential application of the technique to diploid organisms, and its ability to identify multiple genes controlling certain phenotypes. Additionaly, LGS has recently been applied to an investigation of the genes controlling strain-specific immunity in *P. chabaudi* (Martinelli, 2003).

6.4 Growth-rate cost associated with Pyrimethamine Resistance

The results presented in Chapter IV indicate that there is a growth-rate cost associated with the mutation at S106N in *P. chabaudi* that confers pyrimethamine resistance. This is the first time that such a cost has been shown for pyrimethamine resistance in malaria parasites in which growth-rate differences between sensitive and resistant parasites were measured directly in mixed infections.

Whether the impaired growth rate of a parasite carrying the pyrimethamine resistant mutant allele of *dhfr* would translate to *fitness* cost that would manifest in a reduced ability to transmit through mosquitoes was not determined. This is because the experimental design was confined to analysis of the blood stages of the parasite life-cycle. Further experiments in which the proportion of the resistant allele of *dhfr* in the uncloned recombinant progeny of a cross between a pyrimethamine resistant and a sensitive clone was measured throughout the parasite life cycle (including transmission through mosquitoes) would show whether there is a fitness cost associated with the resistant allele. Such an experiment provides an exciting potential direction for future work on this subject.

As previously discussed (Chapter I), some evidence form the field suggests that resistant mutants are at a selection disadvantage compared to sensitive parasites in the absence of drug pressure. Studies on the prevalence of chloroquine resistant mutants show a decline in the prevalence of resistant parasites after periods of cessation of chloroquine usage in several geographic locations (Kublin et al., 2003b; Liu et al., 1995; Mita et al., 2003; Mita et al., 2004; Thanh et al., 2001). This evidence is supported by a recent *in vitro* study in which chloroquine resistant mutants were competed in culture with sensitive parasites, in which the proportion of resistant parasites declined through time (Hayward et al., 2005). This finding, combined with the aforementioned field observations, prompted the authors to suggest that chloroquine may be effectively re-introduced to areas from which it was previously withdrawn due to parasite resistance after a suitable time period.

This idea has been expanded to include the possibility of "cycling" two drugs with different modes of action for fixed time periods, allowing parasites resistant to the first drug to be removed by natural selection while the second drug was in use, so that it could be used effectively at the end of the second drug's usage period, and so on (Beck, HP *pers. com*).

Such suggestions should be treated with caution, however, as the declining rate at which resistant strains disappear from the population must be taken into account. For example, in a study reported from Tanzania in 1967, Clyde et al (1967) report the presence of pyrimethamine resistant parasites 10 years after the cessation of the use of the drug in this country. It should also be noted that in all the field reports described above which show a drop in the prevalence of chloroquine resistant parasites in the absence of chloroquine pressure, resistant parasites do still persist in the population. The re-introduction of drugs to parasite populations where even a small proportion of resistant mutants persist is likely to lead to the rapid selection of resistance once more.

It is probable, however, that the most effective approach to anti-malaria chemotherapy is the use of combinations of drugs with different modes of action. This type of approach was first developed for antituberculous chemotherapy, and has also been adopted for the treatment of AIDS and early HIV-1 (Barnes and White, 2005; White et al., 1999). The chance of selecting a

mutant that is simultaneously resistant to two drugs in a combination is given by the product of the individual mutation rate per parasite for each drug, multiplied by the number of parasites exposed to the drugs (White et al., 1999), and their use will, therefore, greatly diminish the selection of resistant parasites compared with single drug regimens. It is for this reason that the future of malaria chemotherapy is likely to be based on this type of drug therapy, especially using drug combinations that include artemisinin and its derivatives, to which there is currently no known parasite resistance.

APPENDIX 1

SOLUTIONS AND BUFFERS

1:1 FCS : Ringer's Solution

50% (v/v) heat inactivated foetal calf serum

50% (v/v) mammalian Ringer's solution

20 units heparin/ml mouse blood

Buffer A

150 mM NaCl

25 mM EDTA

Citrate saline

0.9% (w/v) NaCl

1.5% (w/v) Sodium citrate

pH 7.2

Deep-freeze solution

28% (v/v) glycerol

3.0% (v/v) sorbitol

0.65% (v/v) NaCl- sterilise by filtration.

Mammalian ringer solution

27mM KCl

27mM $CaCl_2$

0.15M NaCl

TBE buffer (sterilised by filtration)

100nM Tris

100 mM Boric Acid

2 mM EDTA (Ethylenediaminetetraacetic Acid)

TE buffer (Tris/EDTA)

10mM Tris-HCl

1mM EDTA

APPENDIX 2

THE RELATIVE INTENSITY INDICES AND COMPARATIVE INTENSITIES OF ALL AFLP MARKERS ANALYSED IN THE LGS EXPERIMENT DESCRIBED IN CHAPTER IV

Marker Name	CM distance along Chromosome / Linkage Group	Relative Intensity			Comparative Intensity		
AJ Markers		Non-Passaged	PYR treated	Untreated	PYR / NON-PASSAGED	PYR / UNTREATED	UNTREATED /NON-PASSAGED
Chromosome 1							
AJAC03TA	16.20	0.63	0.63	0.67	1.00	0.94	1.06
AJTA03AT	16.20	0.42	0.47	0.68	1.13	0.69	1.63
AJAG01GT	16.20	0.80	0.79	0.82	0.99	0.96	1.03
AJAG02TC	16.20	0.73	0.73	0.77	1.00	0.94	1.06
AJTG01AA	43.40	1.00	0.88	0.90	0.88	0.98	0.90
Chromosome 5							
AJAT02AG	0.00	0.38	0.35	0.35	0.92	1.00	0.92
AJTA05AT	4.00	0.62	1.03	1.02	1.66	1.01	1.64
AJGT06AA	54.40	0.73	0.24	0.44	0.33	0.56	0.60
AJAA04AC	70.00	0.71	0.82	0.79	1.15	1.05	1.10
AJAT07AT	70.00	0.80	0.83	0.83	1.05	1.00	1.05
AJAT07AT	70.00	0.74	0.79	0.72	1.07	1.11	0.97
AJAT01AT	73.90	0.67	0.40	0.56	0.60	0.72	0.84
AJTA01AC	77.50	0.66	0.79	0.62	1.20	1.27	0.94
AJAG01TT	82.70	0.42	0.31	0.46	0.74	0.67	1.11
AJAA03TA	82.90	0.70	0.88		1.26		0.00
Chromosome 6							
AJAG02AG	11.30	0.33	0.54	0.39	1.61	1.39	1.16
AJAT02TA	11.30	0.51	0.49	0.56	0.95	0.87	1.10
AJGT01TA	11.30	0.44	0.59	0.50	1.33	1.18	1.13
AJTG02AA	11.30	0.66	1.03	0.79	1.56	1.31	1.19
AJTT05AT	11.30	0.73	0.75	0.75	1.02	1.00	1.02
AJAG01CT	16.10	0.32	0.39	0.35	1.24	1.13	1.10
AJAT03TG	29.30	0.46	0.81	0.92	1.76	0.88	2.00
AJTA02GA	37.10	0.61	0.58	0.59	0.96	1.00	0.97
AJTT01CA	44.30	0.69	0.95	0.87	1.37	1.10	1.25
AJGT03AT	62.60	0.40	0.74	0.49	1.88	1.52	1.24
AJTA02AC	62.70	0.53	0.67	0.49	1.28	1.38	0.93
Chromosome 7							
AJAA02TG	24.10	0.50	0.58	0.43	1.16	1.35	0.86
AJAG01AG	37.20	0.54	0.66	0.57	1.22	1.15	1.06
AJAA01GA	37.30	0.68	0.54	0.54	0.80	1.02	0.79
AJAT01GT	37.30	0.92	0.51	0.54	0.55	0.93	0.59
AJAT01GT	37.30	0.40	0.33	0.37	0.83	0.91	0.92
AJTT06AT	48.50	0.41	0.50	0.57	1.22	0.88	1.39

AJTA01CA	62.30	0.45	0.61	0.55	1.38	1.11	1.24
AJAT02TC	77.70	0.36	0.41	0.36	1.14	1.14	1.00
AJTG02AT	77.70	0.76	0.79	0.74	1.04	1.07	0.97
AJAT02AC	77.70	0.71	1.06	1.04	1.50	1.02	1.47
AJAT02TC	77.70	0.79	0.63	0.62	0.80	1.01	0.79
AJTA02TA	77.70	0.44	0.52	0.41	1.17	1.27	0.92
AJTT03TG	81.40	0.65	0.68	0.67	1.05	1.02	1.04
AJAA05CA	96.30	0.33	0.00	0.41	0.01	0.01	1.26
AJAG02CA	100.30	0.86	0.13	0.97	0.15	0.13	1.14
AJTG01AG	104.40	0.58	0.06	0.65	0.10	0.08	1.13
AJTG01GA	104.40	0.71	0.12	1.91	0.17	0.06	2.69
AJAC02TT	123.80	0.64	0.40	0.94	0.63	0.42	1.48
AJAA03AT	135.30	0.70	0.50	0.83	0.71	0.60	1.19
Chromosome 8							
AJAG03AG	0.00	0.46	0.73	0.56	1.58	1.30	1.21
AJTA03TA	17.30	0.31	0.53	0.38	1.70	1.42	1.20
AJTT02AT	17.30	0.61	0.93	0.79	1.52	1.18	1.28
AJAT01TA	21.30	0.46	0.51	0.51	1.09	1.00	1.09
AJTT07AT	25.90	0.45	0.89	0.55	1.99	1.60	1.24
AJGT02TA	34.30	0.38	0.44	0.35	1.15	1.27	0.91
AJTA04AT	34.30	0.53	0.60	0.80	1.13	0.75	1.50
AJAC02AT	46.20	0.21	0.31	0.29	1.50	1.07	1.40
AJTG03AA	55.00	0.38	0.43	0.35	1.12	1.22	0.92
AJAC03AT	73.20	0.58	0.85	0.81	1.47	1.05	1.40
Chromosome 9							
AJAT01TC	40.20	0.57	0.81	0.65	1.42	1.25	1.14
AJAT01TC	40.20	0.44	0.58	0.44	1.34	1.33	1.01
AJAT05TG	40.20	0.31	0.26	0.23	0.84	1.11	0.76
AJTA01GT	40.20	0.66	0.62	0.58	0.95	1.07	0.88
AJAC01AA	51.70	0.22	0.43	0.41	1.95	1.05	1.86
AJAC03AA	51.70	1.18	1.10	1.21	0.93	0.91	1.03
AJAG01TC	63.50	0.39	0.43	0.43	1.11	1.00	1.11
AJAT04TA	67.20	0.70	0.84	0.86	1.20	0.98	1.23
Chromosome 10							
AJAC02AA	18.40	0.23	0.49	0.36	2.13	1.36	1.57
Chromosome 11							
AJAT03CT	5.30	0.63	1.53	1.05	2.42	1.45	1.67
AJTT02GA	9.70	0.60	0.89	0.89	1.49	1.00	1.49
AJTA01TT	9.70	1.00	0.79	0.78	0.79	1.01	0.78
AJTT03AT	9.70	0.68	1.10	0.84	1.61	1.30	1.24
AJTT03CA	9.70	0.64	0.81	0.71	1.26	1.14	1.11
AJTT03AA	13.60	0.73	0.79	0.68	1.08	1.15	0.93
AJTT03AA	13.60	0.41	0.51	0.42	1.25	1.22	1.02
AJAT03AA	17.60	0.55	0.74	0.66	1.34	1.12	1.20
AJAT01AA	21.60	0.29	0.86	0.48	2.96	1.79	1.65
AJTT04TA	31.20	0.32	0.76	0.53	2.34	1.45	1.62
AJTG05AT	39.30	1.44	2.81	2.13	1.96	1.32	1.48
AJTA01TA	39.30	0.50	0.72	0.59	1.45	1.21	1.20

AJTA01TA	39.30	0.45	0.65	0.74	1.44	0.88	1.64
AJAT06CT	44.60	0.52	0.74	0.66	1.42	1.12	1.26
AJAG01TA	48.70	0.50	0.54	0.60	1.06	0.89	1.19
AJAC01TA	53.00	0.85	0.91	1.09	1.06	0.83	1.28
Chromosome 12							
AJTT04AT	13.30	0.92	1.02	0.77	1.11	1.32	0.84
AJAA02TC	35.90	0.45	0.40	0.42	0.88	0.96	0.92
AJAT01AG	50.90	0.66	0.62	0.74	0.94	0.84	1.12
AJGT02AA	66.40	0.40	0.59	0.40	1.50	1.50	1.00
AJAT04CT	79.90	0.49	0.59	0.41	1.19	1.42	0.84
AJAGO1TG	106.80	0.50	0.81	0.74	1.60	1.09	1.47
AJTT02AA	123.50	0.47	0.30	0.36	0.64	0.82	0.77
AJTT02AA	123.50	0.42	0.41	0.45	0.97	0.90	1.08
Chromosome 13							
AJTT01AG	15.20	0.74	0.49	0.75	0.66	0.66	1.01
AJGT02AG	15.20	0.94	0.65	0.96	0.68	0.67	1.02
AJAA06AA	19.30	0.69	0.59	0.76	0.85	0.77	1.10
AJAA03TG	28.50	0.42	0.34	0.51	0.81	0.67	1.20
AJAC04AT	28.50	0.34	0.31	0.40	0.90	0.78	1.16
AJTA01GA	28.50	0.61	0.61	0.69	1.00	0.89	1.12
AJTT04AA	32.40	0.45	0.34	0.52	0.77	0.67	1.15
AJTT04AA	32.40	0.32	0.26	0.37	0.80	0.69	1.16
AJTT02AC	36.70	0.63	0.65	0.73	1.03	0.89	1.15
AJAA01AT	44.50	0.65	0.82	0.80	1.26	1.02	1.23
AJTT02TA	44.50	0.50	0.51	0.67	1.01	0.76	1.34
AJTT02TA	44.50	0.55	0.45	0.39	0.83	1.16	0.71
AJGT01TG	60.40	0.32	0.31	0.33	0.99	0.94	1.05
AJAT02TG	64.00	0.30	0.52	0.46	1.72	1.14	1.51
AJTA01AG	72.90	0.66	1.07	0.78	1.62	1.37	1.18
AJAA03AC	136.10	0.50	0.56	0.46	1.13	1.23	0.92
AJAA01CT	154.80	0.56	0.74	0.67	1.32	1.10	1.20
AJAG04AG	177.90	0.39	0.93	0.51	2.40	1.84	1.30
AJTG02AG	183.40	0.66	0.81	0.68	1.23	1.20	1.02
AJTG02GA	183.40	0.51	0.86	0.97	1.70	0.89	1.91
AJTT02CA	183.40	0.54	0.71	0.60	1.32	1.19	1.11
AJTT02TG	183.40	0.44	0.54	0.61	1.21	0.88	1.37
AJTT02TT	183.40	0.23	0.32	0.32	1.40	1.00	1.40
AJGT01AT	183.50	0.30	0.33	0.38	1.11	0.87	1.28
AJAA03CA	200.00	0.36	0.23	0.34	0.64	0.67	0.95
AJAC03TT	200.00	0.53	0.35	0.49	0.67	0.71	0.94
AJAT02AT	200.00	0.45	0.27	0.44	0.60	0.61	0.98
AJAT02AT	200.00	0.46	0.18	0.41	0.40	0.44	0.90
AJGT05AA	200.10	0.41	0.23	0.32	0.56	0.71	0.78
AJGT01AG	200.10	0.35	0.14	0.34	0.39	0.41	0.97
AJAA01AG	208.20	0.36	0.20	0.42	0.56	0.48	1.16
AJAA01AA	212.40	0.37	0.26	0.33	0.71	0.79	0.89

Linkage Group A							
AJAT03TA	4.60	0.70	0.75	1.10	1.07	0.68	1.59
AJAA01GT	8.70	0.42	0.64	0.64	1.50	1.00	1.50
AJAT02AA	8.70	0.39	0.34	0.43	0.88	0.81	1.08
AJAT01AC	8.70	0.72	0.67	0.94	0.94	0.72	1.31
AJAA05AC	16.80	0.64	0.81	0.68	1.26	1.19	1.06
AJAT02GT	16.80	0.69	0.73	0.83	1.06	0.88	1.20
AJAT02GT	16.80	0.65	0.76	1.02	1.16	0.75	1.56
AJTT03TA	16.80	1.01	0.91	0.90	0.91	1.01	0.90
AJAT04TG	25.50	0.39	0.34	0.27	0.87	1.22	0.71
AJAT02TT	29.90	0.42	0.53	0.57	1.25	0.92	1.36
AJAT01TG	43.90	0.57	0.92	0.97	1.63	0.95	1.71
AJAT04AA	47.50	0.43	0.75	0.63	1.74	1.18	1.48
AJAT01CT	84.20	0.98	1.12	0.94	1.15	1.18	0.97
AJTG01CT	84.20	0.60	0.89	0.81	1.48	1.11	1.34
AJGT03AG	84.20	0.75	0.61	0.62	0.82	0.98	0.83
AJTT06AA	84.40	0.61	0.79	0.76	1.30	1.03	1.26
AJGT02AT	92.70	1.08	0.75	0.66	0.69	1.13	0.61
AJAG03AT	92.70	1.01	1.02	0.86	1.00	1.19	0.84
Linkage Group B							
AJTA01AT	10.90	0.67	0.70	0.62	1.05	1.13	0.93
AJTA02AT	25.60	0.49	0.51	0.51	1.04	1.00	1.04
AJAC01AT	40.70	0.48	0.58	0.62	1.21	0.94	1.29
AJTT07AA	40.70	0.66	0.71	0.88	1.07	0.80	1.33
AJTT01AC	49.50	0.92	0.75	1.06	0.82	0.71	1.15
AJAA02AA	76.00	0.89	0.91	0.89	1.01	1.01	1.00
Linkage Group C							
AJTT01TG	0.00	0.76	1.12	1.12	1.47	1.00	1.47
AJAA02AT	8.50	0.37	0.61	0.80	1.65	0.76	2.18
AJAG01AT	19.40	0.34	0.63	0.45	1.85	1.41	1.31
Linkage Group D							
AJAG02TT	19.80	0.86	0.57	0.59	0.66	0.96	0.69
AJAC05AT	24.40	0.70	0.86	0.67	1.24	1.28	0.96
AJTT01TC	44.60	0.79	0.79	0.78	0.99	1.01	0.98
AJTT01GT	71.90	0.34	0.35	0.53	1.03	0.67	1.54
AJGT03TA	71.90	0.43	0.35	0.40	0.82	0.88	0.94
AJTA03AC	79.60	0.43	0.33	0.35	0.76	0.94	0.81
AJAC01TT	88.40	0.31	0.28	0.27	0.91	1.05	0.87
AJGT01AA	88.40	0.84	0.83	0.77	0.98	1.08	0.91
AJAG02AT	88.40	0.44	0.44	0.37	1.02	1.22	0.84
AJGT04AA	99.20	0.45	0.38	0.29	0.86	1.33	0.64
AJTG04AA	103.60	0.61	0.50	0.52	0.82	0.96	0.85
AJAT05AT	120.60	0.28	0.30	0.36	1.10	0.85	1.29
AJAT05AT	120.60	0.48	0.30	0.35	0.62	0.84	0.74
AJTA04TA	120.60	0.26	0.30	0.37	1.16	0.81	1.44
Linkage Group E							
AJAG02TA	0.00	0.60	0.88	0.62	1.47	1.42	1.03
AJAC04TA	4.00	0.36	0.45	0.38	1.25	1.18	1.06

AJAT02GA	4.00	0.63	0.68	0.88	1.08	0.77	1.39
AJTG03AT	12.80	0.56	0.64	0.52	1.14	1.23	0.92
AJAC04AA	20.30	0.42	0.29	0.32	0.69	0.91	0.76
AJAT04AT	20.30	0.63	0.54	0.63	0.85	0.85	1.00
AJAT04AT	20.30	0.51	0.41	0.55	0.80	0.75	1.07
AJAT03TT	20.30	0.59	0.75	0.84	1.26	0.89	1.42
AJTT03CT	28.30	0.61	0.14	0.81	0.23	0.17	1.32
Linkage Group F							
AJAT03AC	0.00	1.04	0.80	0.78	0.77	1.02	0.75
AJTG01TT	0.00	0.54	0.68	0.69	1.25	0.98	1.28
AJTT01TA	4.40	0.56	0.51	0.45	0.91	1.14	0.79
AJTT01TA	4.40	0.58	0.64	0.65	1.10	0.98	1.12
AJTG04AT	24.50	0.81	1.39	1.39	1.72	1.00	1.72
AJTT01AA	48.40	0.56	0.58	0.58	1.04	1.00	1.04
AJTT01AA	48.40	0.49	0.54	0.56	1.11	0.98	1.14
Unlinked Markers							
AJAT01CA		0.55	0.50	0.59	10.17	0.84	-6.78
AJGT01TC		0.62	0.81	0.54	-30.91	1.50	12.73
AJAT01TT		0.92	0.78	1.03	14.81	0.75	-12.96
AJTT01TT		0.22	0.28	0.29	-23.08	0.94	-30.77
AJTT02CT		0.60	0.59	0.62	2.27	0.96	-2.27
AJTT01AT		0.81	0.90	0.92	-11.25	0.98	-13.75
AJTA02AG		0.75	0.87	0.85	-16.49	1.03	-13.40
AJAA01AC		0.63	0.55		12.20		
AJAA02AC		0.45	0.45	0.45	0.00	1.00	0.00
AJAA01CA		0.51	0.67	0.85	-30.00	0.78	-66.67
AJAT06AT		0.66	0.81	0.85	-22.13	0.94	-29.51
AJAT03GT		1.01	1.28	1.22	-26.39	1.05	-20.14
AJTG01AT		0.69	0.84	0.50	-20.62	1.67	27.84
AJAT06AT		0.73	0.71	0.69	2.70	1.03	5.41
AJAT02CA		0.57	0.53	0.55	5.88	0.97	2.94
AJTA01CT		0.66	0.77	0.89	-17.54	0.87	-35.09
AJTA02GT		2.00	2.75	1.67	-37.50	1.65	16.67
AJTT03GA		0.87	0.96	1.52	-10.00	0.63	-75.00
AJAG01AA		0.56	0.75	0.65	-33.70	1.15	-16.30
AJAA01TC		0.69	0.71	0.71	-4.17	1.00	-4.17
AJAT03AT		0.59	0.21	0.54	65.22	0.38	8.70
AJAT01GA		0.46	0.32	0.41	30.00	0.78	10.00
AJAT03GT		0.80	1.17	0.97	-46.24	1.21	-20.97
AJTT03AC		0.50	0.66	0.69	-32.26	0.95	-38.71
AJTT02AG		0.62	0.65	0.63	-4.58	1.04	-0.76
AJTT01GA		0.60	0.64	0.57	-7.25	1.12	4.35
AJTT02GT		0.62	0.73	0.73	-17.95	1.00	-17.95

AS MARKERS

Chromosome 1							
ASTA02GT	16.20	0.75	0.85	0.53	1.13	1.62	0.70
ASTA01TT	16.20	0.75	0.80	0.92	1.08	0.87	1.24
ASAA02CA	16.20	0.45	0.43	0.41	0.96	1.04	0.92
ASAA01TC	16.20	0.32	0.28	0.32	0.88	0.88	1.00
ASTG01AA	43.40	0.51	0.48	0.39	0.94	1.23	0.76
Chromosome 5							
ASAT03TC	23.00	1.02	1.00	0.86	0.98	1.16	0.85
ASAT02AG	34.50	0.29	0.29	0.33	0.98	0.86	1.14
ASAA02GA	54.40	0.92	1.07	0.64	1.16	1.67	0.70
ASAC03AT	69.90	0.62	0.65	0.64	1.06	1.02	1.04
ASTA05TA	69.90	1.13	0.90	0.80	0.79	1.13	0.70
ASAA04AC	69.90	0.83	0.94	0.81	1.13	1.17	0.97
ASTA01AC	74.60	0.80	0.84	0.67	1.04	1.25	0.83
ASTA01AC	74.60	0.99	0.83	1.00	0.84	0.83	1.01
Chromosome 6							
ASAC02AT	11.30	0.48	0.40	0.63	0.84	0.64	1.32
ASTT01CA	44.20	1.45	1.70	1.61	1.17	1.06	1.11
ASAG01TG	62.60	0.78	0.53	0.88	0.68	0.60	1.13
Chromosome 7							
ASAT04AG	27.90	1.64	1.45	1.52	0.89	0.96	0.93
ASAA01CA	48.40	0.68	0.56	0.68	0.82	0.82	1.00
ASTT03CA	62.20	0.94	1.01	0.88	1.07	1.15	0.94
ASAC02TT	62.20	0.86	0.70	1.01	0.82	0.70	1.18
ASAT02AT	69.70	0.66	0.38	0.54	0.57	0.70	0.81
ASAT02TC	77.50	0.75	0.55	0.61	0.74	0.90	0.82
ASAG02CA	100.30	0.63	0.78	0.50	1.24	1.56	0.79
ASAA06AT	104.10	0.81	1.10	0.69	1.35	1.59	0.85
ASTT01AG	123.60	0.75	1.14	0.84	1.52	1.36	1.12
ASAC01TA	127.60	0.56	0.61	0.54	1.09	1.13	0.96
ASAT05AT	127.60	0.81	0.73	0.69	0.90	1.06	0.86
Chromosome 8							
ASAG01AG	0.00	0.46	0.27	0.43	0.59	0.63	0.94
ASAA07AC	0.00	0.38	0.22	0.36	0.57	0.59	0.96
ASAT03TA	21.30	0.51	0.19	0.42	0.38	0.47	0.81
ASTG02AA	25.90	0.67	0.53	0.73	0.80	0.73	1.10
ASTA01AT	34.30	0.36	0.21	0.36	0.60	0.60	1.00
ASAG01CA	38.20	0.80	0.80	0.72	1.00	1.11	0.90
ASAC01AT	73.20	0.68	0.68	0.68	1.00	1.00	1.00
ASAT03AC	73.20	1.19	1.03	0.89	0.86	1.16	0.75
Chromosome 9							
ASAT04TC	40.20	0.86	0.62	0.62	0.72	1.00	0.72
ASTA01GT	40.20	0.62	1.14	0.49	1.83	2.33	0.78
ASAA04CA	40.20	0.56	0.48	0.48	0.87	1.02	0.85
ASTT05CA	51.70	0.63	0.40	0.47	0.63	0.86	0.74
ASAT04AA	59.20	0.68	0.68	0.73	1.00	0.93	1.08

ASTT02TT	71.10	0.47	0.54	0.57	1.15	0.95	1.20
Chromosome 10							
ASTT01TT	10.90	0.58	0.49	0.73	0.84	0.67	1.25
ASTA02AG	18.40	0.41	0.42	0.53	1.04	0.81	1.29
ASAT05AA	18.40	0.55	0.50	0.54	0.90	0.92	0.98
ASAT05AG	18.40	0.30	0.25	0.32	0.83	0.77	1.08
Chromosome 11							
ASAG03CA	5.30	0.43	0.45	0.44	1.04	1.02	1.02
ASAT03AA	5.30	0.95	0.41	0.71	0.43	0.57	0.75
ASAT03GA	21.50	0.71	0.37	0.74	0.51	0.50	1.03
ASAT02TA	39.20	0.90	0.46	0.56	0.51	0.82	0.62
ASTA01TA	39.20	0.59	0.56	0.62	0.96	0.91	1.05
ASAG01TA	48.70	0.93	0.70	0.82	0.76	0.86	0.88
ASAC02TA	52.90	0.70	0.57	0.65	0.81	0.88	0.92
Chromosome 12							
ASAC03TT	5.60	0.65	0.32	0.38	0.50	0.86	0.58
ASAA01AT	54.60	0.63	0.34	0.45	0.54	0.76	0.71
ASAT03AG	70.20	0.57	0.37	0.47	0.66	0.79	0.83
ASAT01CT	70.20	1.04	1.22	1.03	1.17	1.19	0.99
Chromosome 13							
ASTA01AA	15.20	0.78	0.65	0.52	0.83	1.27	0.66
ASTT01GA	28.50	0.72	1.01	0.84	1.41	1.21	1.17
ASTA02CA	36.10	0.98	0.61	0.75	0.62	0.81	0.77
ASAT03TG	64.10	1.22	1.11	0.95	0.91	1.17	0.78
ASTA01AG	73.00	0.90	0.87	0.98	0.97	0.88	1.09
ASTT02TC	132.50	0.32	0.25	0.29	0.80	0.89	0.90
ASTG03AA	183.50	0.63	0.58	0.59	0.92	0.97	0.95
ASTA03TA	183.50	0.69	0.68	0.65	0.99	1.06	0.93
ASTT02CA	183.50	0.79	0.64	0.64	0.80	1.00	0.80
ASAT01AG	183.50	1.07	0.93	1.23	0.86	0.75	1.15
ASAT01GA	183.50	0.71	0.44	0.72	0.62	0.61	1.02
ASTA01CT	200.10	1.14	1.15	1.15	1.01	1.00	1.01
ASAT03AT	200.10	0.81	0.90	0.76	1.10	1.18	0.93
ASAT01AT	209.20	0.97	1.02	0.86	1.06	1.19	0.89
ASAG03AT	217.80	0.45	0.50	0.54	1.11	0.92	1.20
Linkage Group A							
ASAG01GA	12.30	0.58	0.38	0.42	0.65	0.91	0.72
ASAA01GA	12.30	0.38	0.52	0.40	1.36	1.30	1.05
ASAA06AC	16.80	0.65	0.46	0.54	0.71	0.87	0.82
ASAA01AG	43.70	0.68	0.54	0.57	0.79	0.95	0.84
ASAT02TG	43.90	0.62	0.62	0.80	1.00	0.78	1.29
ASAA03TC	55.60	0.39	0.40	0.34	1.03	1.17	0.88
Linkage Group B							
ASTA01CA	0.00	0.94	0.89	0.74	0.95	1.21	0.78
ASTA03GT	0.00	0.85	0.76	0.70	0.90	1.08	0.83
ASAG02AT	40.70	0.73	0.70	0.65	0.97	1.08	0.90
ASAT11AA	40.70	0.96	0.60	0.88	0.63	0.68	0.92

ASAT02CT	76.00	0.39	0.43	0.57	1.10	0.75	1.48
ASAA03AG	76.00	0.89	0.68	0.69	0.76	0.98	0.78
Linkage Group C							
ASAC01TT	0.00	0.73	0.38	0.51	0.52	0.75	0.70
ASAG01AT	19.40	0.59	0.44	0.51	0.74	0.85	0.87
Linkage Group D							
ASAA02AT	0.00	0.49	0.50	0.65	1.02	0.76	1.34
ASAG01TT	7.80	0.76	0.60	0.78	0.79	0.76	1.03
ASAT02TT	15.80	0.46	0.62	0.48	1.37	1.30	1.05
AST 01AT	71.90	1.00	1.03	1.16	1.03	0.90	1.16
AST 04CA	71.90	0.60	0.54	0.49	0.90	1.11	0.81
Linkage Group E							
ASAT02GA	0.00	0.81	0.47	0.45	0.58	1.05	0.55
ASAA01GT	20.30	0.56	0.46	0.52	0.82	0.88	0.93
ASTA02TA	28.10	0.76	1.01	0.78	1.33	1.30	1.03
ASTA02TA'	28.10	2.20	2.00	2.10	0.91	0.95	0.95
Linkage Group F							
ASAT04AC	0.00	0.79	0.50	0.53	0.63	0.94	0.67
ASTT01CT	4.40	0.47	0.49	0.37	1.03	1.32	0.78
ASAG01CT	12.40	0.57	0.45	0.43	0.80	1.05	0.76
ASTT01AC	40.30	0.49	0.46	0.37	0.95	1.25	0.76
Unlinked							
ASAT01TC		0.35	0.23	0.35	0.67	0.67	1.00
ASAT03TT		0.57	0.97	0.52	1.69	1.84	0.91
ASAT04TT		0.86	0.51	0.61	0.59	0.83	0.71
ASAG01AA		0.82	1.50	0.90	1.82	1.66	1.10
ASAG02AG		0.79	0.98	0.75	1.25	1.31	0.95
ASAT07AA		1.00	0.59	0.66	0.59	0.90	0.66
ASAT01CA		0.49	0.61	0.73	1.24	0.84	1.48
ASAT03CA		0.70	0.22	0.22	0.32	1.00	0.32
ASAA05AC		0.67	0.81	0.72	1.22	1.12	1.08
ASAA04AT		0.67	0.52	0.58	0.78	0.89	0.87
ASAA01CT		1.22	1.35	1.20	1.11	1.13	0.98
ASAT01TG		0.56	0.38	0.63	0.67	0.59	1.13
ASTA02TT		0.65	0.50	0.62	0.77	0.81	0.96
ASAA03CA		0.67	0.60	0.68	0.89	0.87	1.01
ASAA02CT		1.07	0.83	0.78	0.78	1.06	0.73
ASAA02TC		0.89	0.37	0.57	0.42	0.65	0.64
ASAA01AC		0.51	0.56	0.51	1.09	1.10	0.99
ASAT02CA		0.57	0.72	0.73	1.26	0.99	1.28
ASAT02AC		0.97	1.18	0.92	1.22	1.28	0.95
ASAT04GA		0.61	0.53	0.76	0.87	0.70	1.25
ASAT01TT		0.90	0.53	0.77	0.59	0.69	0.85
ASTA02AA		0.71	0.50	0.59	0.71	0.85	0.83
ASTA03AA		0.92	0.79	0.69	0.86	1.14	0.75

APPENDIX 3

PUBLICATIONS AND PRESENTATIONS

Publications

A Genetic Approach to the de novo Identification of Targets of Strain-Specific Immunity in Malaria Parasites
Axel Martinelli, Sandra Cheesman, Paul Hunt, Richard Culleton, Ahmed Raza, ***Margaret Mackinnon & Richard Carter***
PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA Vol. 102 No. 3 (2005)

Linkage Group Selection: Rapid Gene Discovery in Malaria Parasites
Richard Culleton, Axel Martinelli, Paul Hunt, and Richard Carter
GENOME RESEARCH Vol 15 No. 1 (2005)

Competitive release of drug resistance following drug treatment of mixed malaria infections
Jacobus C. de Roode, Richard Culleton, Andrew S. Bell, and Andrew F. Read
MALARIA JOURNAL 2004, 3:33 (2004)

Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections
de Roode JC, Culleton R, Cheesman SJ, Carter R, Read AF
PROCEEDINGS OF THE ROYAL SOCIETY OF LONDON SERIES B-BIOLOGICAL SCIENCES 271 (1543): 1073-1080 (2004)

Presentations

Talks:

"The Application of Linkage Group Selection To Drug Resistance"
British Society of Parasitology Malaria & Spring Meeting, University College Chester
April 2004

"The Application of Linkage Group Selection To Drug Resistance and Strain-Specific Immunity"
Scottish Universities Molecular Parasitology Meeting, Kindrogan, Scotland
May 2004

Posters:

"Linkage Group Selection and Drug Resistance in Malaria"
Richard Culleton, Axel Martinelli Paul Hunt & Richard Carter
University of Edinburgh PhD Student Presentations
October 2003

"Host Genotype plays an Important Role in Within-Host Competition between Rodent Malaria Parasites"
Jaap de Roode, Richard Culleton, Sandie Cheeseman, Richard Carter & Andrew Read
University of Edinburgh PhD Student Presentations
October 2003

"Linkage Group Selection: A Proof of Principle Using Drug Resistance in Malaria"
Richard Culleton, Axel Martinelli, Paul Hunt & Richard Carter
Molecular Approaches to Malaria, Lorne, Australia
February 2004

"Linkage Group Selection locates genes involved in selectable phenotypes in malaria parasites"
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