THE DEVELOPMENT AND INHERITANCE OF RESISTANCE TO CAMBENDAZOLE IN *NEMATOSPIROIDES DUBIUS* (NEMATODA)

by

Elizabeth Ann Washington

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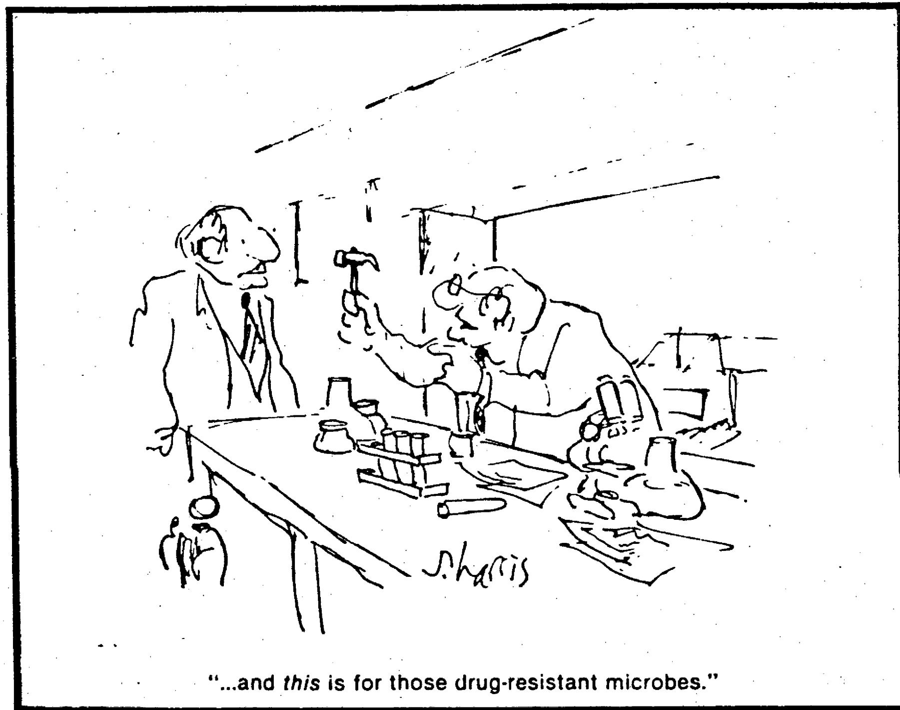
This thesis is my own work; the experimental work is mine, except where specifically acknowledged.

Elizabeth Ann Washington



"...and *this* is for those drug-resistant microbes."

S. Harris, 1981

ABSTRACT

The development and inheritance of benzimidazole resistance was examined in a laboratory model using *Nematospiroides dubius* infections in mice. It was shown that adult *N. dubius* could be selected *in vivo* for resistance to cambendazole. The importance of selection pressure on the development of resistance was demonstrated. Cambendazole resistance was found to be autosomally inherited and co-dominant with susceptibility, in two *N. dubius* isolates. A maternal effect was involved, and the trait appears to be polygenic in nature. The usefulness of this model for augmenting studies of benzimidazole resistance in the nematodes of sheep, is discussed.

No relationship was apparent between the responses to cambendazole of eggs, and adult parasites. Resistance did not develop in the eggs of adult-selected populations. It was demonstrated that resistance in eggs could develop in populations where the eggs themselves were exposed to cambendazole selection in faecal culture. In these populations, adult worms remained susceptible to the anthelmintic. It is suggested that the relationship between benzimidazole resistance in the eggs and adults of sheep nematodes may be more complex than previously assumed, and the implications of this are discussed. An experiment designed to test this hypothesis is proposed.

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CHAPTER I

INTRODUCTION

1.1 *THE PROBLEM*

Helminth parasites cause considerable wastage and serious economic losses in sheep-meat and wool production around the world. Therefore, farmers need to control sheep-helminth numbers. At present, the anthelmintic pesticides provide the only feasible means for control, yet resistance to these compounds is becoming more and more prevalent. In particular, the effectiveness of the benzimidazole family of anthelmintics is being severely undermined, and this thesis presents work on some aspects of the problem.

Resistance to toxicants is encountered in many areas of agriculture and medicine. The World Health Organization Expert Committee on Insecticides (1957) has defined resistance as:

> "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species ....."
>
> (*In* Brown and Pal 1971)

This definition is however applicable to other areas. There is, in fact, a remarkable degree of similarity in the nature and developmental characteristics of pesticide

resistance among such diverse taxa as bacteria, fungi and arthropods. Since resistance in helminth parasites appears to conform with the general pattern, it may be useful to consider anthelmintic resistance in this context. Before doing so, the benzimidazole anthelmintics and their biological properties must be considered.

1.2 *THE BENZIMIDAZOLES*

Benzimidazoles are used in the treatment of a diverse range of pests, including animal parasites, plant-parasitic nematodes, fungi and weeds. Thiophanates are also considered to belong in this chemical group, as they are readily metabolized to benzimidazoles (Selling, Vonk and Sijpesteijn 1970).

These compounds and their metabolites may cause genetic damage, including the induction of point mutations in prokaryotes, and chromasomal abnormalities following the disruption of mitosis and meiosis in eukaryotes. Benzimidazole action on parasitic helminths includes interference with respiratory and possibly other enzymes, plus the breakdown of the internal structure of some cells.

1.2.2 *Chemical nature*

The general structure of the benzimidazole group is similar to that of the purines, guanine and adenine (Fig. 1.1). Benzimidazole is the closest, but lacks the side groups involved in base-pairing with pyrimidines.

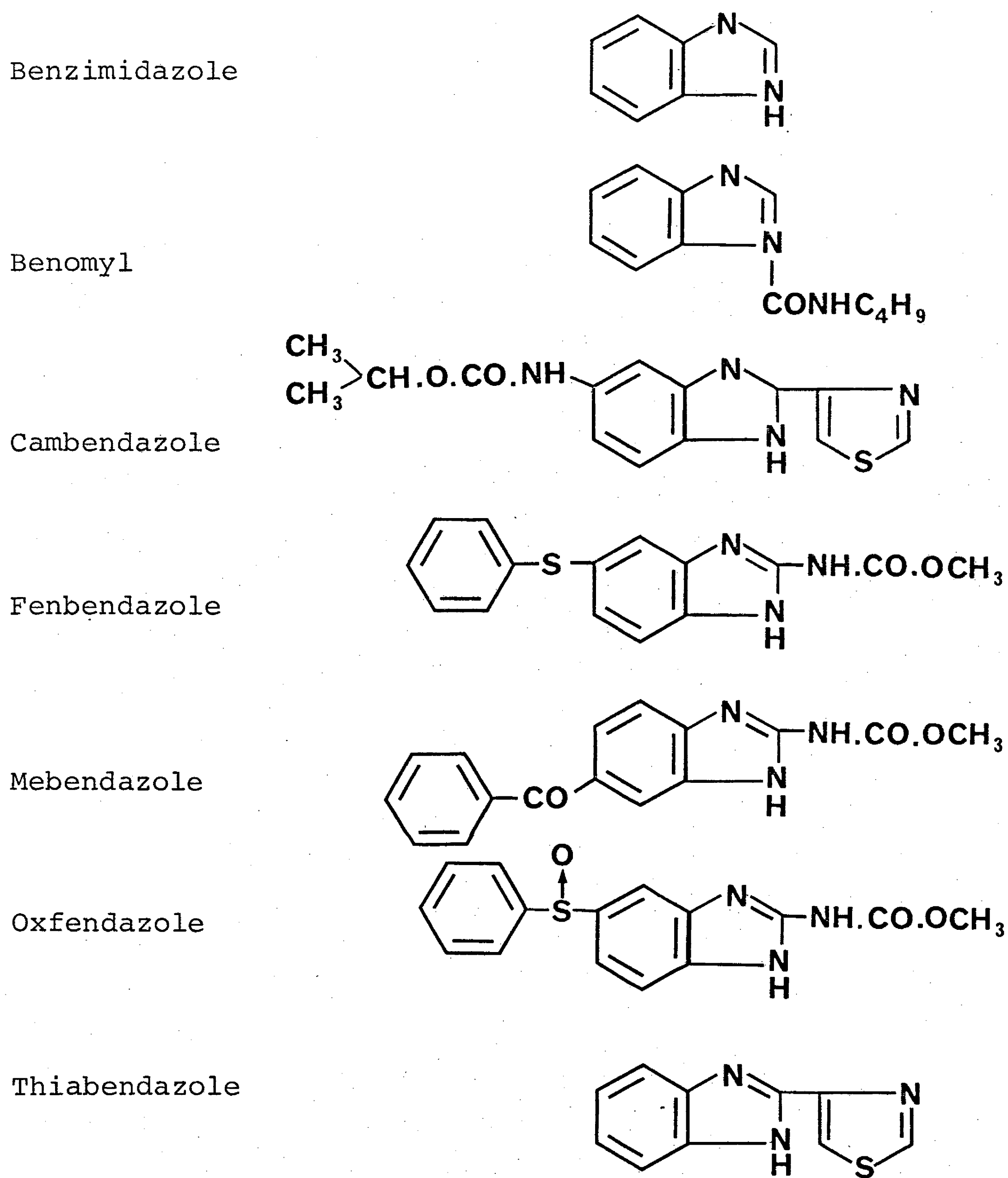


Figure 1.1 Structure of some Benzimidazole molecules

(from Prichard 1978; Seiler 1975)

Seiler (1975) gives a detailed account of the general synthetic and degradative pathways of the benzimidazoles, and Prichard (1978) briefly covers the metabolic fate of those used as anthelmintics.

Most of the derivatives of benzimidazoles used as toxicants have one or more side-chains, which affect their physical and chemical properties, and the significance of this will be referred to later. All of these have a substitution at the 2-postion, mainly a methylcarbamazine or a thiazole group, and the anthelmintics, other than thiabendazole, have side groups at the 5- or 6-positions. Blocking the 5-position, in particular, decreases the rate that the drugs are metabolized and excreted by mammalian systems (Prichard 1978).

Generally, the benzimidazoles have low solubilities at a neutral pH, but greater solubility in the acidic environment of the stomach, enables absorption into the blood (Seiler 1976b). Once in the blood, they are distributed relatively evenly throughout the organs, including the gonads (Noguchi, Ohkuma and Kosaka 1971). The anthelmintics undergo little degradation in the mammalian system, but benomyl and the thiophanates are metabolized, mainly in the liver, by the endoplasmic reticular (microsomal) enzymes (Seiler 1975). The major products of these are carbendazim or 5-hydroxy carbendazim (Seiler 1975).

1.2.3 *Genetic effects in prokaryotes*

The mutagenicity of benzimidazoles has been tested in a number of bacterial test systems (Seiler 1975). Benzimidazole itself induces mutations in *Escherichia coli* (Novick 1956) and *Salmonella typhimurium* (Ames 1971). Many of its derivatives are also mutagenic with the exception of those with large aromatic side-chains or electron accepting groups (Seiler 1975; Seiler and Limacher 1973).

The mechanisms by which benzimidazoles cause these mutations is suggested by their structual similarity to purines. It appears that they do, in fact, act as nucleotide analogues, producing point mutations by base-substitution. Benzimidazole reverts base-substitution mutations in *S. typhimurium*, but not frameshift mutations (Seiler 1972). In *E. coli*, it is incorporated directly into the DNA (Seiler 1973) and the RNA (Seiler 1976a). For the experiments with *E. coli*, the bacteria were grown with radioactively labelled benzimidazole nucleosides, and the incorporation of the latter was monitored by electrophoresis or by gas chromatography. The level of incorporation into DNA was 20% of the total adenosine content, while a value of 16% was obtained for RNA.

Enhanced mutagenicity associated with benzimidazoles having side groups at the 2- and 4-positions, may be due to their closer similarity to, and ability to substitute for, purines (Seiler, 1972). However, the possibility of greater

uptake into cells must also be considered. Seiler (1975) suggests that benzimidazole substitutes for guanine, but is read as adenine during translation, producing a guanine-adenine transition. The actual mechanism of base-substitution involves misrepair of gaps in newly synthesized DNA, rather than misincorporation during replication (Kappas, Green, Bridges, Rogers and Muriel 1976). Once the benzimidazole group is incorporated, the process can be reversed by an excision-repair mechanism (Kappas *et al.* 1976).

1.2.4 *Genetic effects in eukaryotes*

The main effect of benzimidazole compounds on the genetic systems of eukaryotes appears to involve the spindle apparatus, producing chromosome rather than point mutations (Seiler 1975). Dassency and Meyer (1973) did report benomyl-induced point mutations in the haploid conidia of the fungus *Fusarium oxysporum*, but neither benomyl nor carbendazim could be detected in the DNA (Kumari, Decallonne, Meyer and Talpaert 1977). Benomyl may be mutagenic to *Neurospora crassa* (Borck 1973), but in other fungi which have been examined, there is no evidence for benzimidazole-induced point mutations (Hastie 1970; Kappas 1978; Kappas, Georgopoulos and Hastie 1974; Siebert, Zimmermann and Lemperle 1970; Welker and Williams 1980). In mammalian systems, tests for point mutations caused by benzimidazoles have proved negative, and Seiler (1975) discusses this area in detail. By and large, though, the

experiments with mammalian systems have been hampered by the low solubilities of the drugs.

These experiments are limited, but suggest benzimidazoles rarely, if at all, induce point mutations in eukaryotes. Possibly eukaryotic excision repair mechanisms are just very effective in removing incorporated benzimidazoles (Davidse 1977). It would, however, be interesting to know whether the molecules are able to cross the nuclear membrane, and so come into contact with the replicating DNA.

A number of benzimidazole compounds, or their metabolic products, do interfere with mitosis and meiosis in eukaryotic cells. The evidence comes mainly from studies involving fungal cells or cell extracts, but similar effects are also found in nematode, cestode and mammalian cells. Davidse (1977) discusses this mode of action in detail.

Several benzimidazole derivatives disrupt mitosis in fungi (Davidse 1973; Hammerschlag and Sisler 1973; Richmond and Phillips 1975; Welker and Williams 1980; Williams 1980). They also interfere with the fine structure of hyphae and cause nuclear instability, with haploidization or chromosome doubling (Bignami, Aulicino, Velcich, Carere and Morpurgo 1977; Borck 1973; Hammerschlag and Sisler 1973; Hastie 1970; Howard and Aist 1976; Kappas *et al.* 1974;

Kumari *et al.* 1977). Kappas (1978) suggests that chromosome non-junction, resulting from the disruption of mitosis, produces the haploid and diploid recombinants through the formation of unstable aneuploides.

In cell-free extracts, carbendazim binds to fungal tubulin (Davidse and Flach 1977), preventing its polymerization into microtubules (Davidse 1977; Davidse and Flach 1978; Hoebke, Van Nijen and de Brabander 1976). This can be blocked by other tubulin-binding compounds such as the plant alkaloid, colchicine (Davidse and Flach 1977). The toxicity of carbendazim varies between fungal groups (Bollen and Fuchs 1970) and appears to reflect the binding affinities of their tubulin (Davidse and Flach 1977). In strains of *Aspergillus nidulans*, selected for carbendazim resistance, the tubulin also shows reduced binding affinity and this trait is inherited monofactorially (Van Tuyl, Davidse and Dekker 1974; Van Tuyl 1977). The mutation produces electrophoretically abnormal B-tubulin, and appears to be in a structural gene rather than one coding for post-translational modifications (Sheir-Neiss, Lai and Morris 1978).

Benomyl and carbendazim disrupt mitosis in plant cells, but high concentrations are necessary to obtain this result (Boyle 1973; Richmond and Phillips 1975). Mitosis is also disrupted in mammalian cells, both *in vivo* (Styles and Garner 1974; Seiler 1976b) and *in vitro* (Styles and

Garner 1974; de Brabander, Van de Veire, Aerts, Geuens and Hoebeke 1976b). Though here again, a high concentration is necessary to interfere with polymerization (Hoebeke and Van Nijen 1975). Davidse (1977) suggests that plant and mammalian tubulin may have low binding affinities for carbendazim, but the situation is more complicated in mammalian cells because carbendazim is rapidly detoxified (Gardiner, Kirkland, Klöpping and Sherman 1974).

Benomyl, its close relative 2-benzimidazolurea, and carbendazim produce chromosome non-disjunction in mouse and Chinese hamster bone marrow cells (Seiler 1976b). The methylcarbamate, or a similar side chain, seems to be necessary because benzimidazole is inactive against mouse cells, but the chain must be attached to the BZ molecule to be effective. It is interesting to note that benzimidazole is also inactive against *A. nidulans* (Hastie 1970). Seiler (1976b) found no evidence of chromosome breakages, but Styles and Garner (1974) observed a low incidence of breakages using *in vitro* cultures of Chang cells and in rat bone marrow cells *in vivo*. Non-disjunction appears to be the main effect in mammalian cells, as it is in fungal cells, though Davidse and Flach (1977) were unable to show binding to mammalian tubulin.

Oncodazole, an anti-tumor agent (Atassi, Schauss and Tagnon 1975; Atassi and Tagnon 1975) destroys the microtubules of malignant mammalian cells *in vitro*,

affecting both those in mitosis and those in interphase (de Brabander, Van de Veirs, Aerts, Geuens, Borgers, Desplenter and de Crée 1975; de Brander, Van de Veirs, Aerts, Borgers and Janssen 1976a). An interesting point is that oncodazole does not destroy the microtubules of non-malignant cells, only of those undergoing mitosis. The biochemical stability of the microtubules to polymerization by oncodazole may explain this difference (Davidse 1977). Oncodazole has a high affinity for mammalian tubulin and, as is the case in fungi, appears to prevent the *in vitro* polymerization of tubulin into microtubules (Hoebeke *et al.* 1976).

The anthelmintic mebendazole, administered *in vivo*, damages the absorptive surface cells of a number of helminths, including the nematodes *Ascaris suum* and *Syngamus traches*, and also the cestodes *Hymenolepis nana* and *Taenia taeniaeformis* (Borgers and de Nollin 1975; Borgers, de Nollin, de Brabander and Thienpont 1975; Borgers, de Nollin, Verheyen, de Brabander and Thienpont 1975; Borgers, de Nollin, Verheyen, Vanparijs and Thienpont 1975; Verheyen, Borgers, Vanparijs and Thienpont 1976). In all these helminths the cytoplasmic microtubules disappear, preventing transport of secretory granules which then accumulate in the cytoplasm. Cellular autolysis which follows may be due to inadequate nourishment in the absence of granular products or by the products themselves (Verheyen *et al.* 1976).

Mebendazole retards the development of *Hymenolepis diminuta* and *H. nana* in their intermediate host, the beetle *Tribolium confusum* (Evans and Novak 1976; Novak and Evans 1978; Evans, Gray and Novak 1979). Novak and Evans (1978) suggest that interference with spindle formation might inhibit larval growth. All larvae resume development if treatment is discontinued, though in the presence of the drug, some individuals are capable of very slow development (Evans and Novak 1980). An electrophoretic comparison of tubulin from inhibited and partially inhibited larvae might prove interesting. Further studies of the effects of benzimidazoles on helminth tubulin would certainly be worthwhile. Friedman, Platzer and Carroll (1979) are undertaking such a study using *Ascaris suum* embryos and report that the tubulin has colchicine-binding properties similar to those shown by the tubulin of other animals.

1.2.5 *Biochemical effects in helminths*

The benzimidazoles appear to interfere with the mitochondrial energy metabolism of helminth parasites. Several target enzyme systems have already been identified and more may be involved. The biochemical effects of the benzimidazoles are reviewed by Van den Bossche (1976); Behm and Bryant (1979) and only a brief outline of the subject will be given here.

Mebendazole causes an inhibition of glucose uptake and the depletion of glycogen stores in a number of helminths, including *A. suum* (Van den Bossche 1972), *Trichinella spiralis* (de Nollin and Van den Bossche 1973), *Moniezia expansa* (Rahman and Bryant 1977; Rahman, Cornish, Chevis and Bryant 1977). Mebendazole does not inhibit glucose uptake nor cause glycogen depletion in *Fasciola hepatica in vitro*, though glycogen stores are reduced *in vivo* (Cornish and Bryant 1976). Cornish and Bryant (1976) suggest that *Fasciola* differs from the other helminths tested because its glucose uptake is passive, and not energy dependent.

Thiabendazole and mebendazole act as uncouplers of oxidative phosphorylation in *Ascaris* mitochondrial preparations (Van den Bossche 1972; Saz 1972), inhibiting the malate-dependent pathway. The fumarate reductase system of this pathway, which catalyses the conversion of fumarate to succinate, producing ATP, appears to be a target for the drugs. Prichard (1978) in fact regards it as the major target. Thiabendazole inhibits the fumarate reductase system of susceptible *Haemonchus contortus*, but is less effective against that of resistant worms (Bennett 1981; Prichard 1970, 1973; Romanowski, Rhoads, Colglazier and Kates 1975).

The fumarate reductase system of *H. contortus* is also inhibited by cambendzole, oxfendazole and fenbendazole (Malkin and Camacho 1972; Prichard 1970, 1973; Prichard,

Hennessy and Steel 1978a; Romanowski *et al.* 1975). Concentrations which are effective *in vitro* are similar to those found in worms treated *in vivo* (Prichard, Kelly and Thompson 1978b). Mebendazole does not inhibit the fumarate reductase system of *H. contortus* and appears to have a different mode of action (Bennett 1981). In *T. spiralis*, mebendazole inhibits both the fumarate reductase system and succinate dehydrogenase activity (Boczon 1976), while in *M. expasa*, mebendazole and cambendazole inhibit fumarate reductase and phosphoenolpyruvate carboxykinase activities *in vitro* (Rahman and Bryant 1977).

The proportions of ATP, ADP and the total nucleotide levels are altered following mebendazole treatment *in vitro* and *in vivo* of *M. expansa* (Rahman and Bryant 1977; Rahman *et al.* 1977) and *F. hepatica* (Cornish and Bryant 1976; Rahman *et al.* 1977). The relative proportions of respiratory end products are also altered in both species, though the actual products affected differ (Behm and Bryant 1979). These changes may reduce the synthesis of ATP, but they do not appear to fully account for the decline in the total adenine nucleotide levels (Bryant, Cornish and Rahman 1976). Behm and Bryant (1979) suggest that studies of AMP levels may clarify the problem. They suggest that the purine-like structure of the benzimidazoles may lead to interference in any reactions involving purine nucleotides.

It seems that benzimidazoles may interfere with helminth energy metabolism at a variety of sites, the relative importance of which may differ between species. Bryant's group maintains that the final effect of the drugs' actions is reflected in the adenine nucleotide levels. The possibility that interference with pathways other than those involved with energy metabolism, contributes to the elimination of helminth parasites, should not be overlooked.

1.3 *PESTICIDE RESISTANCE*

As emphasized earlier (1.1), resistance is a problem in many areas of pest control, in addition to the well publicized examples from bacteria and arthropods, and has also been found in fungi; comparable problems arise with drug resistance in cancer. Regardless of the taxa, resistance shows a number of common characteristics.

In cases where the mechanisms of resistance have been identified, they appear to be of four main types. These are as follows:

(a) Increased rate of pesticide detoxication or decreased activation, where this is a necessary precursor for its toxic effect.

(b) Decreased sensitivity of the target site.

(c) Possession of an alternative metabolic pathway or reaction, to circumvent the target site.

(d) Reduced absorption or uptake of the toxicant either preventing or decreasing its access to the target site.

The relative importance of these mechanisms is likely to vary according to the toxicant (Sawicki 1979) and may even differ between taxa. All of those listed above have been identified in arthropods and bacteria, where one or more can contribute to decreased sensitivity to toxicants. In methotrexate resistant cancer cells, three of these mechanisms have been identified; namely increased detoxication, changes in the target site, and reduced uptake of the drug (Schimke 1980). All four mechanisms have been reported in fungi (Dekker 1977; Erwin 1973; van Tuyl *et al.* 1974; van Tuyl 1977).

Before a pesticide is applied, alleles conferring protection are probably rare in the target population. It is generally thought that pesticide resistance develops in a population through selection acting to increase the frequency of these alleles. Why some individuals are resistant initially is an intriguing question. Whether the selected variants occur naturally, or are produced by the mutagenic effects of the toxicants themselves, has

stimulated much discussion (Crow 1957). Certainly, some pesticides such as the benzimidazoles do have mutagenic potential (1.2).

One method, other than mutation, involves the multiplication or amplification of genes conferring some protection. Gene amplification itself appears to be a common evolutionary phenomenon, as evidenced by the high incidence of gene clusters and multigene families (Schimke 1980). Also, there are examples of this process occurring during normal physiological development, as requirements for the gene product increase. The multiplication of ribosomal RNA genes in the fruitfly *Drosophila* (Procunier and Tartof 1978), and in plants (Cullis 1977) are good examples. Gene amplification has been demonstrated in the DNA coding for the detoxifying enzyme dihydrofolate reductase produced by methotrexate resistant cancer cells (Alt, Kellems, Bertino and Schimke 1978; Schimke, Kaufman, Alt and Kellems 1978). The process appears to be stepwise, with discrete increases in gene numbers as selection is intensified. A similar mechanism has been reported for penicillin resistance in *Escherichia coli* K_{12} (Normark, Edlund, Grundström, Bergstrom and Wolf-Watz 1977). There is also evidence of a gene amplification resistance mechanism in the aphid *Myzus persicae* (Devonshire 1980; Devonshire and Sawicki 1979). With the extension of recombinant DNA technology to more systems, it will be interesting to discover how widely spread gene amplification is in pesticide resistance.

In bacteria, viruses play an important role in the acquisition of resistance factors (Watanabe 1967). This mechanism may be unique to prokaryotes, but the possibility that DNA sequences conferring protection in eukaryotes are derived from an external source, is an interesting speculation which should not be intirely discounted. DNA can be introduced into mammalian cells using viral vectors (Williamson 1980), and it has recently been suggested that RNA viruses might contribute genetic information to eukaryotic genomes (Green 1980).

At present, the mechanisms by which resistant individuals first appear require much more investigation. There remains the fact that resistance can develop rapidly and, no matter how variants arise, there is ample evidence they are already present in unexposed populations. Selection then increases their frequency. From the practical angle of planning a control programme, this selective process, and the factors influencing it are probably of more immediate importance. Therefore, the development and genetic analysis of resistance in arthropods, which includes the most thoroughly analysed examples from higher organisms, are recounted in detail below. They are strongly suggestive of what may be occurring in the development of resistance to benzimidazoles in nematodes (1.4).

1.4 *INSECTICIDE RESISTANCE*

1.4.1 *Development of resistance*

The rate at which resistance is likely to develop is an important consideration in the design of control programmes, and much attention has been placed on identifying factors which influence the selection process. Insecticides do not appear to be mutagenic and selection of highly inbred insect lines, lacking resistant individuals is ineffective (Crow 1957; Brown and Pal 1971). Even the normal mutation rate over a number of years of selection may not produce a resistant individual (Crow 1957). Therefore, the development of resistance almost certainly depends on the presence of the appropriate alleles in the original population.

The development of resistance is an example of rapid evolution, and the theories of natural selection have been useful in planning and interpreting both experimental studies and recent computer simulation models.

Amongst the factors influencing the spread of resistance in a population are its genetic nature, the type of insecticide and the method of application (Crow 1957; Georghiou 1972; Georghiou and Taylor 1976, 1977a, b). Georghiou and Taylor (1976) suggest the following may all be involved:

(a) Genetic - - Frequency of resistant alleles
Number of resistant alleles
Dominance of resistant alleles
Penetrance; impressivity;
interactions of resistant alleles
Past selection by other chemicals
Extent of integration of resistant
genome with fitness factors

(b) Biological
Biotic - - Generation turn over
Offspring per generation
Monogamy/polygamy; parthenogenesis
Behavioural
- - Isolation; mobility; migration
Monophagy/polyphagy
Fortuitous survival; refugia

(c) Operational
The Chemical
- - Chemical nature of pesticide
Relationship to earlier used
chemicals
Persistence of residues; formulation
The Application
- - Application threshold
Selection threshold
Life stage(s) selected
Mode of application

Space limited selection

Alternating selection

(Reproduced from Georghiou and Taylor 1976).

A major influence on the development of resistance is the intensity of selection (Crow 1957; Brown and Pal 1971; Hoskins and Gordon 1956). In theory, the increase in frequency of an allele conferring resistance is directly proportional to the dose (Georghiou and Taylor 1977b). Intense selection, where few individuals survive, reduces the genetic variation within the population and can retard the development of resistance (Crow 1957; Hoskins and Gordon 1956). In comparison with field populations, development is usually slower in the laboratory, and this may be due to lower genetic variability in the latter populations (Brown and Pal 1971).

Insecticide resistance generally increases very little over the first generations of selection. This lag is followed by a rapid increase until a maximum level is reached. Several theories have been advanced to explain the early lag phase. It may be an artifact caused either by variation in the effectiveness of insecticide treatment at different doses, or of the mathematical analysis (Crow 1957). The general selection model predicts that changes in allelic frequency are a function of the frequency in the preceding generation (Mettler and Gregg 1969). Crow (1957) suggests that as selection increases the frequency of a gene, the

rate of change will also increase. This could account for the initially slow development of resistance, followed by the more rapid change. However, when Georghiou and Taylor (1977a) examined the effect of initial allele frequency, using a computer simulation of monofactorially inherited resistance, they found that values between 10^{-1} and 10^{-8} did not influence the subsequent rate of change.

A further and widely accepted theory is that rare alleles, such as those for resistance, are unlikely to be co-adapted with the background genome common to the population (Wallace 1968) and that the lag phase may represent a period in which favourable combinations are selected (Milani 1960). The relatively more rapid reappearance of resistance, observed following a period without treatment during which the population has reverted to susceptibility, may occur because the remaining resistance alleles are already co-adapted with their background (Milani 1960).

A number of other conditions affect the development of resistance. Reproductive potential and general fitness of resistant genotype are influential, and a number of cases where the resistant genotype is less fertile have been reported (Crow 1957; Brown and Pal 1971). A shorter life cycle, and hence greater generation turnover, is associated with the rapid development of resistance (Georghiou and Taylor 1976). The stage of the life cycle exposed to the insecticide can also affect the development of resistance.

The relative importance of selecting larvae or adults, on the level of resistance attained, varies between species, though it is interesting to note that selection applied to one life stage, affects resistance in others (Brown and Pal 1971).

The nature of resistance and the number of genes involved affect the way in which it develops. Hoskins and Gordon (1956) described three main types of resistance, producing characteristic patterns in dosage mortality responses following selection. These are shown schematically in Fig. 1.2 (redrawn from Brown and Pal 1971), where the percentage mortality is expressed in probability units (probits) and the dosage is given on a logarithmic scale.

Where resistance is specific in nature, the responses are characterized by a decline in the gradient of the log dosage-probit lines, indicating greater genetic heterogeneity. This heterogeneity presumably coincides with the simultaneous selection of a favourable array of alleles at many loci. Eventually, a homogeneous population is achieved in which resistance is co-adapted with its background genome. If the population does not have a component able to respond to selection, the second type of response will occur. Here, the most susceptible individuals are eliminated, giving a steeper line. A third response is characteristic of non-specific polygenic resistance. This so called 'vigour tolerance' produces a series of parallel regression lines

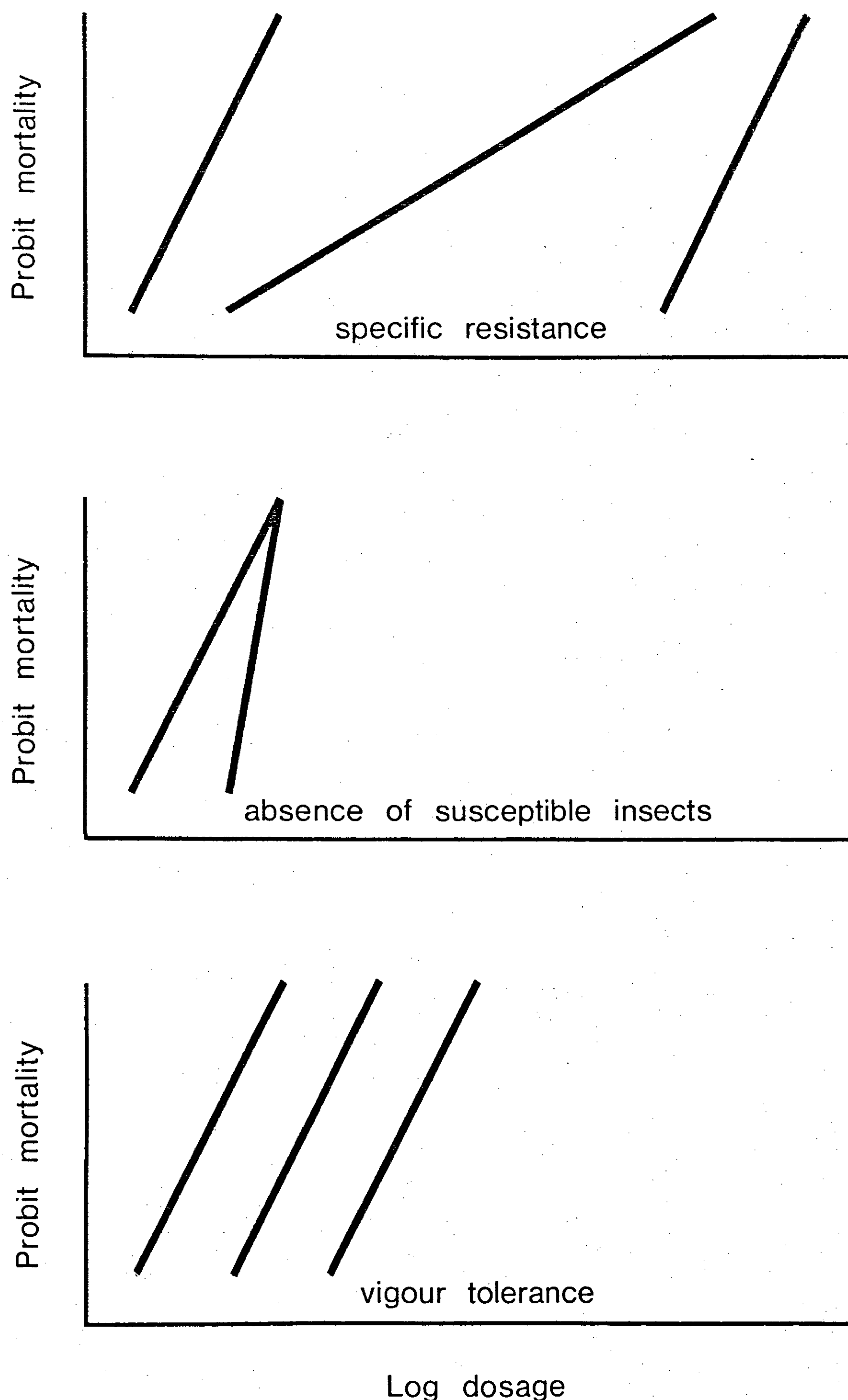


FIGURE 1.2 CHANGES IN DOSAGE MORTALITY LINES AFTER SELECTION FOR INSECTICIDE RESISTANCE
(Adapted from Brown and Pal, 1971)

following selection, and is throught to involve secondary physiological mechanisms.

Assuming that variants carrying alleles for resistance are present, and that the population's variability is not restricted, the rate at which insecticide resistance develops then depends on a complex set of interacting factors. One of these is the mode of inheritance. Insecticide resistance should evolve fastest when the allele, or the major allele of a polygenic system, is fully dominant, and this is supported by field observations (Crow 1957; Georghiou 1972; Georghiou and Taylor 1976). Evolution is rapid because both homozygotes and heterozygotes survive the treatment and the population recovers quickly. Where allele are dominant or partially dominant, the degree of resistance they confer in heterozygous individuals will determine how fast resistance develops (Conway and Comins 1979).

If the rate of change in allele frequency is being considered, then this is fastest when resistance is completely recessive, because only homozygotes survive (Georghiou and Taylor 1977a). The rate at which resistance develops in truly polyfactorial systems, where no single gene makes a major contribution, depends on the amount of genetic recombination possible at reproduction (Conway and Comins 1979).

Two additional factors, which may have significant effects on the rate of development, are refugia and immigration. Refugia, an area (or state) in which members of the population escape treatment, and the immigration of susceptible individuals, both prolong the existence of susceptible alleles in the population (Georghiou and Taylor 1976, 1977a). In computer simulations, they appreciably retard the development of monofactorially inherited resistance (Comins 1977; Georghiou and Taylor 1977a).

The combined effects of these factors and of others not yet studied, are likely to be complex. Nevertheless, their individual manipulation in control programmes may still be possible. Refugia and immigration, which could be preserved by using discontinuous patterns of treatment and restricting insecticide application to limited areas, respectively, may be of practical use in retarding the development of resistance (Comins 1977; Curtis, Cook and Wood 1978; Georghiou and Taylor 1977b).

1.4.2 *Genetical analysis of insecticide resistance*

Analytical approaches used in genetic studies of insecticide resistance generally follow that of classical Mendelian genetics. Georghiou (1969) divides these studies into three main stages. Identification of the basic inheritance patterns forms the first stage. This includes determining the number of loci involved, the dominance relationship between resistant and susceptible alleles, whether resistance is sex-linked and whether ctyoplasmic

factors are also involved. The second stage concentrates on more detailed analysis of the resistance gene or genes, including their location and relationship to other loci. Linkage analysis and mapping, and studies of epistasis depend on the presence of other markers. Complementation tests may also be possible if more than one isolate is available. The final stage is the biochemical identification of the resistance mechanism, or if more than one locus is involved, the mechanisms, and the contribution of each to resistance.

Probably the most important stage for understanding how resistance evolves in a population, is the fundamental study of inheritance. The technique involves reciprocal cross-matings between resistant and susceptible strains. The F_1 hybrids are then inbred to give F_2 hybrids, and also backcrossed to both parental types. Homogeneous parental populations yield the clearest results and it is preferable that both have the same genetic origin (Georghiou 1969). Comparison of the regression lines for each population yields the basic information about the nature of resistance.

The positions of the regression lines for the F_1 hybrids relative to the parental lines, indicate the dominant or recessive nature of the traits. Georghious (1969), lists a number of classifications ranging from complete dominance, through several intermediate stages, to complete recessiveness. The degree of dominance (D) is calculated from Falconer's equation (1964):

$$D = \frac{2.ED_{50}\,F_1 - ED_{50}\,R - ED_{50}\,S}{ED_{50}\,R - ED_{50}\,S}$$

where R, F_1 and S represent the resistant, heterozygotic and susceptible populations (Stone 1968 with modified symbols). Although formulated for monofactorially inherited resistance, this equation is also applied to polygenic systems (Priester and Georghiou 1979). Studies of the dominance heirarchy have shown that resistance to organophosphates and carbamates is dominant, while dieldrin resistance is usually intermediate in nature, and DDT resistance is mainly recessive (Brown and Pal 1971).

Regression lines for the two F_1 populations, from the reciprocal matings, may not be identical. The differences in position may be due to sex-linkage, maternal or paternal inheritance or extrachromosomal factors (Crow 1957). Sex-linkage is identified by finding similar resistance levels in progeny of the homogametic sex (usually females) from reciprocal crosses and differing levels in the heterogametic sex. There is greater difficulty in distinguishing between maternal or paternal, and extrachromosomal inheritance (Sonneborn 1950).

Segregation ratios can be obtained from plateaux in the F_2 and backcross regression lines if one or a few major genes confer resistance. Tsukamoto (1963) discusses the expected shapes of these lines for various combinations of dominance hierarchy and gene number (Fig. 1.3). Interpretation is clearest in cases of monofactorial inheritance,

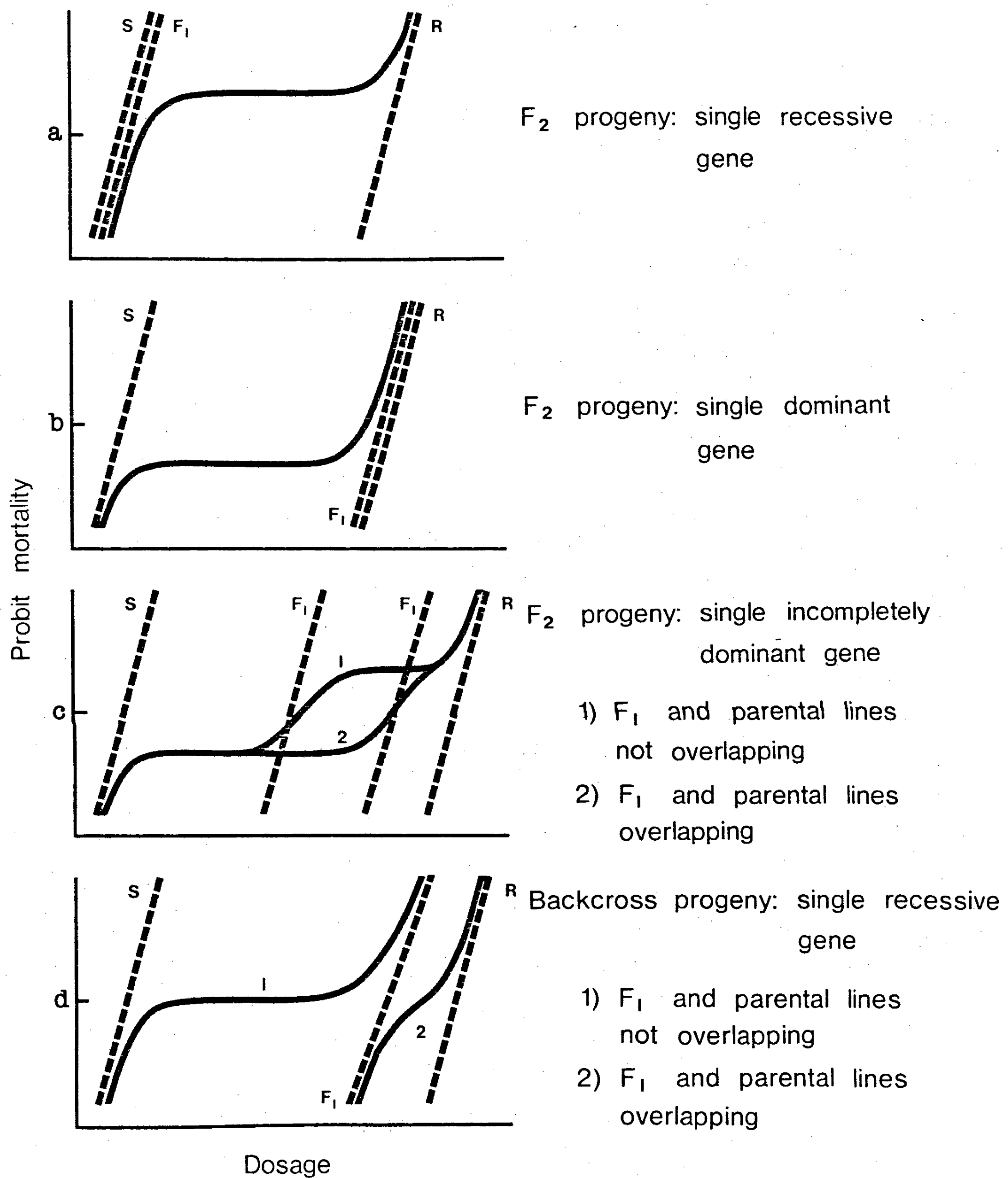


FIGURE 1.3 SCHEMATIC LOG DOSAGE-PROBIT CURVES FOR PROGENY OF A RESISTANT (R) x SUSCEPTIBLE (S) CROSS

(Adapted from Tsukamoto, 1963)

and the following examples illustrate the application of the technique.

If resistance is recessive and monofactorial, three out of four F_2 individuals should have susceptible phenotypes, and a plateau would be expected at the 75% mortality level (Fig. 1.3a). This representa a typical 3: 1 ratio with inflexion points occurring where one genotype is succeeded by the next. Where resistance is co-dominant, there would be two plateaux, one at the 25% and one at the 75% levels giving a ratio of 1 : 2 : 1 (Fig. 1.3c). Finally, in the case of a dominant resistant allele, only one in four individuals would be susceptible, and the plateau should occur at the 25% mortality level indicating a 1 : 3 ratio (Fig. 1.3b). The backcross to a homozygous recessive parental population typically produces a plateau at the 50% level, while the cross to the dominant parental population results in a line without inflexions. For co-dominance, there should be a 1 : 1 segregation in both backcrosses (Fig. 1.3d).

Distinct plateaux are only formed when the regression lines for the parental and F_1 populations do not overlap. If incomplete separation occurs, as between the F_1 and resistant populations in Fig. 1.3, the expected response must be calculated for each dose level using the following equations (Georghiou 1969 with modified symbols). For F_2 progeny, the equation is:

$$Xy = W(S) . 25 + W(F_1) . 50 + W(R) . 25$$

where X is the expected response at dose Y and W is the observed response of the susceptible (S), heterozygotic (F_1) and resistant (R) genotypes at dose Y. These are taken from the respective regression lines. For the back-cross progeny, the equation for the cross to the susceptible population is:

$$Xy = W(F_1) . 50 + W(S) . 50$$

and to the resistant population is:

$$Xy = W(F_1) . 50 + W(R) . 50$$

Where more than one locus is involved, inflexions in the regression lines become more complex and are difficult to identify.

Non-Mendelian segregation patterns may indicate polyfactorial inheritance. This is tested by statistical comparison of the expected regression lines for monofactorial inheritance, calculated using the above equations, and the observed regression lines (Georghiou 1969). A more conclusive demonstration of polyfactorial inheritance, especially when linkage cannot be tested through lack of marker genes, comes from repeated backcrossing to the susceptible population, with selection applied at each generation (Wright 1952). If resistance persists a major gene is probably involved, and if it does not persist, resistance is considered to be polyfactorial.

During the early stages of selection, Crow (1957) advises caution in deciding whether resistance is monofactorial or polyfactorial because polygenic background variability may mask the presence of a major gene. This has occurred a number of times in the study of insecticide resistance. In nearly all those species in which homogeneous parental lines were eventually obtained, resistance appears to be conferred by a single major gene (Brown and Pal 1971).

Further genetic analysis depends on the availability of other markers and most of this work has been confined to studies involving *Drosophila*. In the case of insecticide resistance, linkage determination has special problems because the dose response test gives quantitative rather than qualitative results, and the phenotypes show differential viability. The quantitative nature of insecticide resistance may be regarded as incomplete penetrance. Tsukamoto (1964, 1965) describes techniques for linkage determination which take both this and the problem of differential viability into account. Fisher (1949) and Parsons (1957) advocate balanced backcrossing with coupling and repulsion systems, for non allelic genes. This reduces distortion due to the two factors, but is not applicable in studies of insecticide resistance. Tsukamoto's (1965) approach is to estimate recombination values in three point backcrosses by applying either the coupling or the repulsion test, but not both. In this way the position of the resistance locus in a particular chromosome can be determined using standard mapping procedures.

1.5 *ANTHELMINTIC RESISTANCE*

Resistance to anthelmintics is now a problem world-wide, and involves the helminth parasites of sheep, cattle, horses, goats and, in some instances, man (Kelly and Hall 1979; Prichard, Hall, Kelly, Martin and Donald 1980). The first reports were of phenothiazine resistance in an American strain of *H. contortus* (Drudge, Leland and Wyant 1957). Resistance to this anthelmintic was slow to develop, but for thiabendazole, resistance was recorded within three years of its introduction (Drudge, Szanto, Wyant and Elam 1964). Since this time, there have been numerous reports of anthelmintic resistance. In Australia, it is the increasing incidence of resistance in the nematodes of sheep which currently poses the greatest concern and the following discussion will concentrate on this.

1.5.2 *Incidence in sheep nematodes within Australia*

The full extent of the problem of anthelmintic resistance is not yet known. The three main genera involved are *Haemonchus*, *Trichostrongylus* and *Ostertagia*, and the problem appears to be geographically wide-spread. Benzimidazole resistant strains of *H. contortus* for instance, have been reported from New South Wales, Victoria, Queensland and Western Australia. Anthelmintics other than benzimidazoles are also involved, including levamisole, morantel tartrate and napthalophos. The reported incidences of such resistance in field strains of the three genera are summarized in table 1.1.

Table 1.1 Australian Reports of Anthelmintic Resistance in Field Populations of Nematode Parasites from Sheep.

Nematode species	Anthelmintic	Location	Reference
Haemonchus contortus	thiabendazole	Northern Tablelands, NSW	Smeal, Gough, Jackson and Hotson (1968)
		Southern Queensland	Prichard (1973)
		Northern Tablelands, NSW	Le Jambre, Martin and Webb (1979a)
		Northern Tablelands, NSW	Webb, McCully, Clarke, Greentree and Honey (1979)
		Gippsland, Victoria	Barton (1980)
	thiabendazole with side-resistance to parbendazole	Northern Tablelands, NSW	Webb, Jackson and McCully (1978)
	oxfendazole	Northern Tablelands, NSW	Webb and McCully (1979)
	benzimidazoles	Orange, Goulburn, Dubbo, Newcastle and Sydney districts, NSW	Hall, in Prichard, Hall and Kelly, Martin and Donald (1980)
	thiophanate	Western Australia	de Chaneet, in Prichard *et al.* (1980)
	levamisole	NSW	Kelly, in Kelly and Hall (1979)
	benzimidazoles, levamisole, morantel tartrate and napthalophos	Southern Queensland	Green, Forsyth, Rowan and Payne (1981)

Table 1.1 (continued)

Nematode species	Anthelmintic	Location	Reference
Trichostrongylus spp.	thiabendazole, with side-resistance to other benzimidazoles	Cumberland district, NSW	Hotson, Campbell and Smeal (1970)*; Hogarth-Scott, Kelly, Whitlock, Ng, Thompson, James and Mears, (1976)
	benzimidazoles	Southern Tablelands, South Coast and Central Western districts, NSW	Campbell, Hall and Kelly (1976), in Campbell, Hall, Kelly and Martin (1978)
	thiabendazole, morantel tartrate and oxfendazole	Northern Tablelands, NSW	Le Jambre, Southcott and Dash (1978a); Prichard and Hennessy (1981)
	thiabendazole, morantel tartrate and levamisole	Sydney district, NSW	Sangster, Whitlock, Russ, Gunawan, Griffin and Kelly, (1979); Whitlock, Sangster, Gunawan, Porter and Kelly (1980)
	levamisole	Victoria	Hogarth-Scott in Prichard *et al.* (1980).

* Found in sheep and goats

Table 1.1 (continued)

Nematode species	Anthelmintic	Location	Reference
Ostertagia spp.	thiabendazole	Southern Slopes, NSW	Hall, Campbell and Carroll (1979)
	benzimidazoles	Goulburn and Orange, NSW	Hall in Prichard *et al.* (1980)
	thiabendazole	Canberra, ACT	Donald, Waller, Dobson and Axelsen (1980)
	levamisole and morantel tartrate	Northern Tablelands, NSW	Le Jambre (1979)
	levamisole	Northern Tablelands, NSW	Le Jambre and Martin (1979)
	thiabendazole, morantel tartrate and levamisole	Sydney district, NSW	Sangster *et al.* (1979) Whitlock *et al.* (1980)

A number of TBZ resistant strains show side-resistance to other benzimidazole derivatives to which they have not previously been exposed (Campbell and Hall 1979; Hall, Campbell and Richardson 1978a; Hall, Kelly, Campbell, Whitlock and Martin 1978b; Hogarth-Scott, Kelly, Whitlock, Ng, Thompson, James and Mears 1976; Hotson, Campbell and Smeal 1970; Prichard and Hennessy 1981). Cross-resistance to chemically unrelated anthelmintics not used before, is also evident in some cases (Campbell, Hall, Kelly and Martin 1978; Kelly, Whitlock, Gunawan, Griffin and Porter 1981a; Kelly, Whitlock, Porter, Griffin and Martin 1981b; Le Jambre 1979a; Sangster, Whitlock, Russ, Gunawan, Griffin and Kelly 1979; Whitlock, Sangster, Gunawan, Porter and Kelly 1980).

1.5.3 *Experimental selection for resistance*

The possibility that anthelmintic resistance might develop, was recognized soon after the introduction of phenothiazine in the early 1940's (Drudge 1970). There were several attempts to select nematode parasites for phenothiazine resistance under controlled conditions, but none proved successful (Bennet 1967; Hasche and Todd 1963; Silangwa and Todd 1966; Sinclair 1953). More recently, several groups have succeeded in selecting nematodes for benzimidazole resistance. In the United States of America, Kates' group selected an initially susceptible strain of *H. contortus* for resistance to CBZ. An appreciable level of resistance was detected after four generations, and this was increased with further selection (Colglazier, Kates and Enzie 1974; Kates, Colglazier and Enzie 1973).

Two Australian groups have successfully carried out anthelmintic selection experiments. In both cases, the parasites have been already resistant field isolates. The resistance levels of two TBZ resistant *H. contortus* populations were raised substantially by further selection in the laboratory (Hall, Campbell and Richardson 1978a; Kelly and Hall 1979; Le Jambre, Southcott and Dash 1976). Le Jambre's group also succeeded in selecting their TBZ resistant strain for resistance to levamisole and morantel tartrate. Additional selection of *O. circumcincta* with TBZ, levamisole and morantel tartrate, either singularly or together, proved equally effective, while the rate at which resistance developed was similar in laboratory and field selected isolates (Le Jambre *et al.* 1977, 1978b). Two field isolates of TBZ resistant *Trichostrongylus colubriformis* have not responded to further selection in the laboratory (Hall *et al.* 1978a; Le Jambre *et al.* 1978a). Possibly these populations were already homozygous for resistance (Prichard *et al.* 1980) although the selection pressure applied to one strain was low, producing only a 60% mortality (Le Jambre *et al.* 1978a).

1.5.4 *Biochemical and physiological characteristics of benzimidazole resistant nematodes*

A number of possible target sites in helminths for benzimidazoles have been discussed previously (1.2.5). Any alteration or avoidance of these sites could well reduce the effectiveness of the anthelmintics (1.3). To date, there is evidence of three characteristics which might account for

the ability of benzimidazole resistant worms to survive treatment. These are: the alteration of at least one anthelmintic target site, decreased access to target sites, and a greater ability to use an alternative metabolic pathway to that affected by the toxicant.

In TBZ and CBZ resistant strains of *H. contortus*, the fumarate reductase system is less sensitive to the inhibitory action of benzimidazoles, than that of susceptible strains (Bennett 1981; Prichard 1973; Romanowski, Rhoads, Colglazier and Kates 1975). Uptake of fenbendazole (FBZ), but not of TBZ, is reduced in resistant *H. contortus* and *T. colubriformis* (Prichard, Kelly and Thompson 1978b). Resistance expressed in nematode eggs may also result from reduced uptake of benzimidazoles (Coles 1977). Another mechanism, proposed by Bennett (1981), is the avoidance of the fumarate reductase system through a greater reliance on the aerobic metabolic pathway. Both TBZ and MBZ selected strains of *H. contortus* show this characteristic. It is unlikely that these three represent the only mechanisms of resistance in nematodes. Benzimidazoles appear to have a number of modes of action against helminths, therefore resistance mechanisms should be as numerous.

1.5.5 *Inheritance of resistance to TBZ*

A study of the inheritance to resistance has been conducted using a TBZ-resistant strain of *H. contortus* (Le Jambre, Royal and Martin 1979). The eggs from reciprocal crosses of the resistant strain with a susceptible population,

were examined in the F_1, F_2, F_3 and backcross generations. Adult dose response tests were only performed on the F_2 generation, but adults of the F_1 generation were examined for the presence of sex-linkage.

The results of the study indicated that TBZ-resistance, in this *H. contortus* isolate, is inherited as an autosomal semi-dominant trait, with a maternal effect persisting through to the F_2 generation. There is, however, some doubt whether resistance in the eggs is a direct indication of that in the adults, or is merely a correlated response (Martin, Le Jambre and Claxton 1981). Bearing this in mind, it would be interesting to examine the responses of adults in more of the hybrid generations.

It is clearly evident that the techniques used in studying the inheritance of insecticide resistance can be adopted to similar investigations of anthelmintic resistance.

1.5.6 *Synopsis*

It appears that anthelmintic resistance does conform with the general patterns found in other areas of resistance to toxicants. Benzimidazole resistance develops in response to selection (1.5.3) and the known mechanisms conform to the catagories listed in section 1.3. Recently, refugia was shown to retard the development of TBZ-resistance in a similar manner to that described for insecticide resistance (Martin *et al.* 1981)(1.4.1).

1.6 *THE BIOLOGY OF NEMATOSPIROIDES DUBIUS*

1.6.1 *The parasite*

Nematospiroides dubius is a nematode parasite of rodents, and belongs to the sub-order Trichostrongylina (Cheng 1973). Further classification is somewhat confusing. The parasite may be assigned to the family Trichostrongylidae or else to the closely related family Heligmosomatidae (Chitwood 1974; Skrjabin, Shikhobalova, Schulz, Popova, Boev, and Delyamure 1961). Regardless of the controversy, *N. dubius* is closely related to those trichostrongylid parasites of sheep in which anthelmintic resistance has become a problem.

There is further disagreement over its generic and specific classification. Baylis (1926, 1927) named the parasite *N. dubius* while Schulz (1926, cited in Skrjabin, Shikhobalova and Schulz 1971), apparently a little later, called it *Heligmosomoides skrjabini*. It is also known as *Heligmosomum skrjabini* (Skrjabin *et al.* 1971), and sometimes as *Heligmosomoides polygyrus* (Kerbœuf 1978). *N. dubius* seems to be the most commonly adopted name in non-taxonomic literature and so will be used in this study.

N. dubius is reported from Europe and North America, and its hosts include a number of species of wild mice, jerboa and at least two squirrel species (Baylis 1926, 1927; Bergstrom and Werner 1981). The adults, occupying the lumen of the host's small intestine, are spirally coiled and red

in colour. Males, which are about 6 mm long and 0.11 mm in diameter at their thickest point, are smaller than females which measure about 13 mm in length and 0.12 mm in diameter. The males have a two-lobed, asymmetrical bursa with a small dorsal ray, and females have a single genital tract ending in a posterior vulva (Skrjabin *et al.* 1971).

Each adult female lays about 1200 to 1800 eggs/day (Scott, Cross and Dawson 1959). These eggs measure 75 - 90 by 43 - 58μ and are passed in the host's faeces at the 8 - 16 cell stage. The cleavage and subsequent embryonation conforms with patterns observed in most nematodes (Anya 1976). The life-cycle is direct with two free-living larval stages and an infective third stage larva (L_3).

The development of eggs and free-living larvae is temperature dependent. Eggs will hatch between 5°C and 30°C but development through to the L_3 occurs only between 5°C and 25°C. A high temperature, such as that encountered in the host, will cause abnormal development within the egg (Yasuraoka and Weinstein 1969). The optimum temperature for the larvae in terms of their developmental time and body length appears to be 20°C (Murua 1975). At this temperature eggs hatch after about 36 hours (Bryant 1973a; Murua 1975) but published values for the duration of other stages differ considerably. Bryant (1973a) reported that the time from hatching until the L_3 appeared was between 45 and 49 hours at 20°C while Murua (1975) found it took

about 86 hours. Culture techniques differed though, as did the source of the parsites, with one being a laboratory-maintained strain and the other an isolate from wild mice.

The infectivity of *N. dubius* L_3 depends both on the temperature to which they are exposed and their age (Kerbœuf, 1978). Maximum infectivity is not attained for three weeks after the second moult if the L_3 are kept at 22°C. Infectivity then declines but the rate at which this occurs can be retarded by cooling the L_3 to 4°C. Like the L_3 of most parasitic nematodes, *N. dubius* L_3 obtain their energy from stored lipid (Bryant 1973b) and declining infectivity may be related to the depletion of these reserves.

Under natural conditions, infective larvae are ingested by the host. In the laboratory mouse infected by stomach tube, L_3 exsheath in the host's stomach then penetrate the mucosa within 24 hours (Bryant 1973a). Here they undergo two moults and marked morphogenesis occurs including sexual differentiation and structural changes in the digestive tract (Bryant 1973a; Bonner, Etges and Menefee 1971). Young adults then return to the intestinal lumen around the eighth day after infection and the first eggs appear in the host's faeces two days later (Bryant 1973a). Therefore, when the free-living stages are cultured at 20°C, the life cycle can be completed in 13 to 14 days.

1.6.2 *Physiological and biochemical aspects*

The free-living stages of *N. dubius*, like those of other nematode parasites, are aerobic (Bryant 1973b). Rapid growth and an increasing respiratory rate characterize the first and second larval stages, and extensive lipid stores are laid down during this phase (Bryant 1973b). The nutritional requirements of the larvae are largely unknown (Mauro and Weinstein 1979), but they are bacterial feeders and will die if cultured in germfree faeces (Weinstein, Newton, Sawyer and Sommerville 1969). Infective L_3 do not feed. Instead, they appear to utilize the stored lipid, and with age, their respiratory rate declines (Bryant 1973b).

After being ingested by a mouse, the L_3 exsheath in the host's stomach, most doing so within five miutes (Sommerville and Bailey 1973). From *in vitro* studies, it appears that high concentrations of hydrogen ions and a temperature of about 37°C stimulate exsheathment. While gaseous carbon dioxide enhances this process, it is not an essential component (Sommerville and Bailey 1973).

Parasitic stages exhibit a logarithmic growth phase during the first 14 days of infection, and this is not appreciably interrupted by the two moults occurring within the host (Bryant 1974). Marked changes in the ultrastructure of the worm's intestinal cells are associated with this phase of the life cycle (Bonner, Etges and Menefee 1971). The adults use oxygen *in vitro*, and some oxygen seems to be

essential for their motility and survival (Bryant 1974).

1.6.3 *Non-immunological host-parasite interactions*

In some mouse strains, the host's age and sex can affect both the survival and size of *N. dubius*. Age resistance, in particular, may be caused by increased mucous production in older mice affecting the growth of the parasitic larval stages (Dobson 1966). In both mice and rats, sex hormones influence the size attained by adult worms. Dobson (1966) suggests the hormones could either affect nutrient availability, or act directly on the parasite's metabolic processes.

Infective *N. dubius* larvae are highly active in the presence of bile, and this behaviour may be important in site selection and penetration of the mouse's intestinal sub-mucosa (Sukhdeo and Croll 1981). Certainly, far fewer L_3 become established in the absence of bile.

Newly emerged adults occupy up to 50% of the small intestine, but as the infection ages, they move anteriorly. Lewis and Bryant (1976) suggest higher oxygen tensions close to the stomach may be responsible. The percentage of worms mating does not seem to depend on their position in the small intestine (Beckett and Pike 1980).

The presence of *N. dubius* does not affect the mouse's ability to digest protein, nor does it interfere with absorption of the digestion products (Symons and Jones 1970).

This is despite the fact that the massive infections of 750 L_3 per mouse, caused obvious distress to the hosts.

1.6.4 *Immunity to N. dubius*

Mice are capable of developing immunity to *N. dubius* infections, but as with age and sex resistance, the degree of response varies between strains. In some strains of mice, the primary infection suffers little harm, and the immune responses act mainly on the larval stages before, or during their encystment within the host's intestinal sub-mucosa (Panter 1969a). In others, immunization with repeated infections can lead to the expulsion of already established adult worms (Cypess and van Zandt 1973).

Both humoral and cellular responses have been implicated in the mouse's immunity to *N. dubius* (Bartlett and Ball 1974; Jenkins and Carrington 1981; Panter 1969a, b). Reports differ as to the relative importance of the two types of response, but possibly this is also a characteristic of the particular mouse strains examined by each of the groups investigating the problem. Of interest to such studies is the finding that *N. dubius* can be selected for lower immunogenicity in a particular mouse strain by repeatedly passaging it through that strain.

In the present study, it is important that the mouse strain in which anthelmintic screening tests are conducted, does not show marked immune responses to the adult worms. CBA/H mice, even when immunized by repeated reinfection,

appear not to adversely influence survival of the established adults, or even lower their fecundity (Bartlett and Ball 1974).

1.7 *RESEARCH AIMS*

The aims of the study were to set up a laboratory simulation for nematode parasitism in sheep, then use this model to examine the development and inheritance of resistance to a benzimidazole.

N. dubius was chosen because it is closely related to the sheep nematodes in which anthelmintic resistance is a problem, and has a similar life history. The parasite's ease of maintenance in the laboratory, together with its previous use in anthelmintic screening trials, indicated that *N. dubius* in mice might be an appropriate combination for this study. CBA/H mice were used because this strain has a relatively weak immune response to *N. dubius*.

Of the two benzimidazoles, cambendazole and thiabendazole, which were available, the former proved the more suitable. Some preliminary work was necessary to standardize assays for the responses of *N. dubius* to the anthelmintic. Selection for resistance to cambendazole was applied to adult parasites *in vivo*, or to the eggs and free-living larvae *in vitro*. Having established that resistance could develop in response to either selective regime, its inheritance was then examined in two of the adult-selected isolates.

CHAPTER II

GENERAL METHODS AND MATERIALS

2.1 *THE HOST*

A colony of inbred CBA/H mice was obtained from the Department of Microbiology, John Curtin School of Medical Research, The Australian National University, and maintained by brother-sister matings. Additional males were purchased as required. All mice were fed on Mecon rat and mouse cubes (Fidelity Feeds), and water was freely available.

Both infection and anthelmintic treatment of mice were carried out under light anaesthesia induced by ether (Hoechst Australia), and an overdose of this was used to kill them.

2.2 *THE PARASITE*

The strain of *Nematospiroides dubius* used in the study was obtained from Dr C. Dobson, Department of Parasitology, University of Queensland, and was maintained in female mice. Five mice aged between six and ten weeks, were infected orally with about 200 third stage larvae (L_3). This gave a total population of 1000 or more individuals at each generation. The larvae were counted under a dissecting microscope and administered in about 0.2 ml of tap water, using a blunt 19 gauge needle and a

1 ml tuberculin syringe. An additional 0.2 ml of water was given to flush remaining L_3 from the syringe.

Two to three weeks after infection, mouse faeces containing eggs were collected over a two day period. During this time mice were kept in a wire-bottomed cage suspended over a tray of water. The softened faecal pellets were removed at 24 hour intervals and separated, with forceps, from waste food residue.

The eggs were cultured using the petri dish - filter paper method (Meyer and Olsen 1975). Faeces were divided into 5 ml samples, each containing 55-65 pellets and about 1 ml of water which had been absorbed by the pellets or was held by surface tension. This corresponds to about 2 g of packed freshly passed faeces. Thin slurries were made with an additional 4.5 ml of tap water and spread evenly over double layers of moist filter paper (Whatman, grad 1, 11 cm diameter), in large glass petri dishes (14 cm diameter). The size difference provides an area without filter paper at the perimeter of the dish into which L_3 migrate during the collection procedure. To maintain a high humidity level and minimize evaporation from the surface of the cultures, a double layer of moist filter paper was attached to the inner surface of each petri dish lid.

Cultures were incubated for seven to ten days at 22°C. The dishes were then flooded with tap water and

left for about 24 hours to allow L_3 to migrate away from the filter paper. The L_3 were then transferred to a measuring cylinder to settle overnight before removing the supernatant by suction pump. They were washed once more in this manner then kept at 22°C for a further ten days to ensure infectivity was close to maximum (Kerbœuf 1978a). If L_3 were not used immediately, they were stored at 4°C in plastic cell culture flasks (Costar or Lux, 75 cm^2, 250 ml) containing water to a depth of 1 cm or less.

2.3 *LARVAL YIELD*

The percentage of eggs completing development to the L_3 stage in faecal culture was determined for comparison with yields from other culturing methods which had been reported in the literature. The number of eggs/g fresh faeces was estimated using the McMaster technique. At the same time on three consecutive days, individual faecal samples were collected from five mice. After the samples were weighed and softened in 0.5 ml of tap water, they were macerated and brought to a volume of 5 ml with saturated saline. The suspensions were mixed on a vortex agitator (Scientific Industries, Vortex-genie) for five seconds, then poured into a McMaster slide. Eggs which rose to the top of the slide were counted after ten minutes, and the number per gram of faeces was calculated. Further faecal samples from these mice were then pooled and five cultures were prepared. The L_3 were recovered separately from each culture after 14 days, suspended in a

known volume of tap water, then mixed on a vortex agitator and counted in a McMaster slide.

The arithmetic mean (± standard error) egg count from the five mice over three days was 23,564 (± 1,593) eggs/g fresh faeces, or about 47,128 eggs/culture (on the basis of 2 g fresh faeces/culture, 2.2). Larval recoveries averaged 15,213 (± 1,126) L_3/culture, representing a yield of about 32.3%. This is higher than yields obtained by Cross (1975) for five different techniques using sawdust (24.8%), animal charcoal (24.7%), syracuse dishes (21.6%), wood charcoal (21.5%) or petri dishes without filter paper (3.1%).

2.4 *THE ANTHELMINTICS*

Cambendazole (CBZ) and thiabendazole (TBZ) were supplied as pure micronised powders by Dr R.W. Butler of Merck Sharp and Dohme (Australia) Pty. Ltd. Only CBZ was used in selecting for resistance.

Suspensions of CBZ (4 mg/ml) and TBZ (100 mg/ml) were prepared with distilled water. As the powder forms are difficult to suspend, concentrated pastes were made first and then diluted. All steps were carried out in glassware and the suspensions were stored for no longer than one week before use.

Saturated solutions of CBZ were prepared in 0.1% saline (Le Jambre 1976). Suspensions, containing quantities far in excess of the published solubility value (20 μg/ml water at 20°C, The Merck Index 1976) were kept with regular mixing at 22°C for about one month and then filtered (Whatman filter paper, grade 1).

The exact concentration of CBZ in a saturated solution was determined by high performance liquid chromatography (HPLC). A standard was prepared in specially purified methanol (Unichrom, Ajax Chemicals), and the samples were analysed by Dr C. Bryant, Department of Zoology, ANU, in a Spectra-Physics SP8000B liquid chromatograph. The resulting standard curve is shown in Figure 2.1. Contrary to expectation, the saturated solution of CBZ contained 35 μg/ml, 1.75 times the published value. The disparity may arise from differences in the conditions of preparation, such as a temperature of 22°C instead of 20°C, and the use of micronised CBZ, or from the different methods of analysis.

Originally, the undissolved CBZ residue was re-used in preparing further solutions. However, the concentration fell to only 15 μg CBZ/ml in the 6th batch, so fresh suspensions were used in each subsequent preparation. The problem associated with this change in concentration is discussed in Chapter IV (4.3.2).

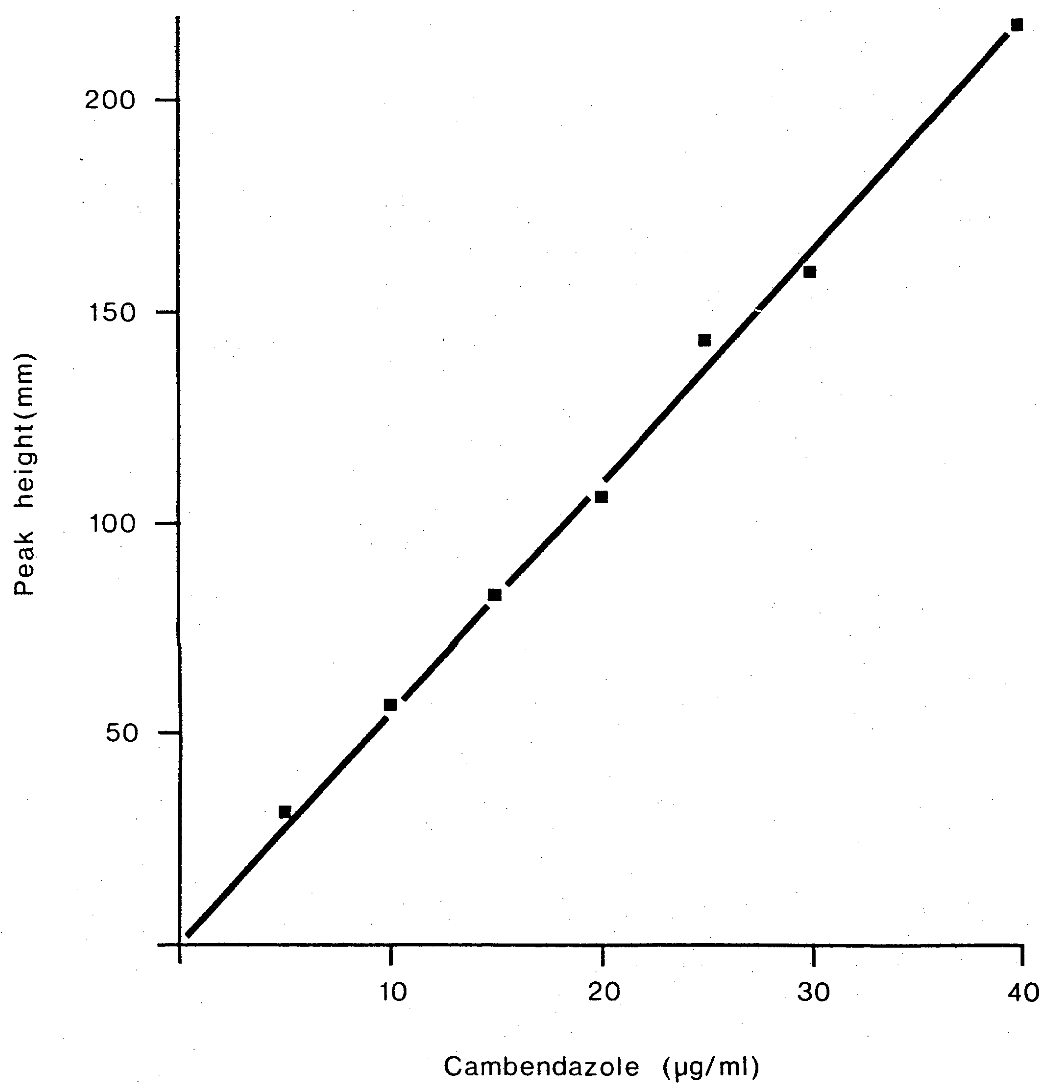


FIGURE 2.1 STANDARD CURVE FOR CAMBENDAZOLE

Determined by high performance liquid chromatography

2.5 *STATISTICAL TREATMENT OF RESULTS*

Dosage-mortality data was examined by probit analysis using two computer programs. The first, written by Davies (1971), was prepared for use on a Sperry Univac 1100/82 system by Dr W.L. Nicholas, Department of Zoology, ANU, and tested with an example provided by Davies. This program fits a log dosage-probit line by iteration involving a maximum likelihood technique, and includes Abbott's formula to correct for response in untreated control groups (Finney 1971). The validity of the model is tested by examination for linearity using the weighted sums of squares for differences between empirical and weighted probits (Finney 1971). This program was used to obtain regression parameters and to examine within group and between group variation in response (2.5.2).

The second program is contained in a package for 'General Linear Interactive Modelling' (GLIM) Release 3, prepared by the Royal Statistical Society, London (1977) and available on the Australian National University's Univac System. It fits the probit model by iteration involving least squares analysis (Finney 1971) and enables the comparison of more than one set of data by analysis of covariance (Baker and Nelder 1978). It does not incorporate a correction for natural response. This package was used to determine whether differences in the slopes of log dosage-probit lines were significant (2.5.4) and for

contingency table analysis (2.5.5).

Data from replicates was pooled by totalling the number of subjects and the number responding at each treatment level, then used to construct log dosage-probit lines (Finney 1971). Responses of each population are summarized by the median effective dose (ED_{50}) for the responses of adults, or median effective concentration (EC_{50}) for those of eggs, and the slope of the regression line. The selected and unselected populations are compared using resistance ratios (or factors) which are the ratios of ED_{50}s or EC_{50}s for the selected isolates to those for the unselected population. Uses of the programs in the statistical analysis of results are described below (2.5.2 - 2.5.5) and examples are included in Appendix 1.

2.5.2 *Within group and between group variation*

Where experiments are replicated, log dosage-probit lines may be fitted using pooled data for each treatment as described above, or by retaining the identity of the within group replicates. Finney (1971) considers either approach valid for determining regression parameters, but if both are used, the difference between the weighted sums of squares from the replicates and from the pooled data gives the within treatment variation. This information was obtained using Davies' program and an example is given in Appendix 1. The detailed analysis of this example is

described in Chapter 3 (Table 3.8b).

2.5.3 *Comparisons of ED_{50}s and EC_{50}s*

Most ED_{50}s and all EC_{50}s were compared by the Student's t-test. The following equations are given in terms of ED_{50}, but apply also to EC_{50}s. The variance of the ED_{50} was calculated from the variance of its logarithm according to the equation given by Finney (1971):

$$\text{Variance } ED_{50} = (ED_{50} \times \log_e 10 \times \sqrt{\text{variance } \log ED_{50}})^2 .$$

Before applying the t-test, ED_{50} variances were checked for equality by an F ratio test (Bliss 1967):

$F = S_1^2/S_2^2$ with (N_1, N_2) degrees of freedom where S_1^2 and S_2^2 are the variances of the two ED_{50}s, S_1^2 is the larger, and N_1 and N_2 are the corresponding numbers of treatments.

In most cases variances were equal and the standard equation for t was used (Bliss 1967):

$$t = \frac{ED_{50}1 - ED_{50}2}{\sqrt{S^2 \ (1/N_1 + 1/N_2)}}$$ with $N_1 + N_2 - 2$ degrees of freedom,

where the common variance of the two populations was:

$$S^2 = \frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{(N_1 - 1) + (N_2 - 1)} .$$

In a few instances where variances were not equal, the following equation was applied (Bliss 1967; Parker 1979):

$$t = \frac{ED_{50}\,1 - ED_{50}\,2}{\sqrt{(S_1^2/N_1 + S_2^2/N_2)}}$$

with reduced degrees of freedom f, where:

$$1/f = \frac{u^2}{(N_1 - 1)} + \frac{(1 - u)^2}{(N_2 - 1)}$$

and,

$$u = \frac{S_1^2/N_1}{(S_1^2/N_1) + (S_2^2/N_2)}$$ with S_1^2 being greater than S_2^2.

The ED_{50} lay outside the tested dose range in some dose-response assays of adults. For these, susceptibility relative to other populations was examined by an analysis of covariance using the GLIM program, and the method is described in the following section (2.5.4).

2.5.4 *Comparison of slopes*

The probit model was fitted to the response data for each pair of populations using the GLIM program. An analysis of covariance, corresponding to a one-way analysis of variance between the two populations, with dose or concentration as the covariate, was performed. Total deviance was determined then partitioned by sequentially removing the effects due to each component from the model (Baker and Nelder 1978). The method used here for determining whether slopes differed significantly is similar

to that described by Finney (1971). In both, the effect of fitting lines with common slopes but separate intercepts is examined, after removing the regression component. This is then compared with the effect of fitting separate slopes and intercepts. The residual deviances are equivalent to weighted sums of squares and have a distribution approximately that of χ^2.

The GLIM program does not incorporate Abbott's formula (2.5), but as little or no difference in natural response was found between the populations being tested, it should not affect the validity of the comparisons. The details of the method are given in Table 2.1 and Appendix 1 contains an example.

2.5.5 *Contingency table analysis*

Comparisons involving other quantal responses, such as the proportion of eggs hatching in the absence of CBZ, were treated as contingency tables (Bliss 1967) and analysed with the GLIM program. A logit transformation (Baker and Nelder 1978) was used and replicates were included in all comparisons so that variation both within and between groups was considered. Results were expressed as the number of subjects and the number responding. Details of the method are contained in Table 2.2 and an example of the analysis is in Appendix 1.

Table 2.1 Details of Analysis of Covariance Using GLIM

(a) Method of partitioning the total deviance

GLIM command	Meaning
1) FIT	calculates the grand mean, giving the total deviance
2) FIT DOSE	fits a single line to the data (removes regression component)
3) FIT DOSE + POPULATION	fits line with common slopes but separate intercepts (removes regression and population components)
4) FIT DOSE * POPULATION	fits lines with separate slopes and intercepts (removes the main effect of regression and population components and their interaction). The fitted deviance indicates departure from linearity.

(b) Analysis of variance table

Source of Deviation	Degrees of Freedom	Deviance
Regression	$df_1 - df_2$	1) - 2)
Population	$df_2 - df_3$	2) - 3)
Parallelism*		
Non-linearity	df_4	4)
Total	df_1	1)

* Deviation from parallel slopes is calculated from the difference between the total deviance and those due to the regression, population and non-linearity components.

Table 2.2 Details of Contingency Table Analysis Using GLIM

(a) Method of partitioning deviances

GLIM command	Meaning
1) FIT POPULATION	removes population component
2) FIT REPLICATES	removes replicate component
3) FIT POPULATION + REPLICATES	removes combined effect of populations and replicates

(b) Analysis of variance table

Source of Deviance	Degrees of Freedom	Deviance
Replicates	$df_1 - df_3$	1) - 3)
Populations	$df_2 - df_3$	2) - 3)

CHAPTER III

AUDLT DOSE RESPONSE ASSAY

3.1 *INTRODUCTION*

Nematospiroides dubius infection in the laboratory mouse is a widely used and documented experimental combination. However, before setting up an assay for monitoring cambendazole (CBZ) resistance, it was necessary to re-examine certain aspects of the infection to determine optimum conditions. Several ways of administering anthelmintics were also investigated to find a reliable technique that was less time-consuming than methods of including them in the mouse's diet for 5-18 days, used in previous screening trials (Brody and Elward 1971; Coles and McNeillie 1977; Theodorides 1976).

The results of the preliminary observations and trials are presented in this chapter. In many cases, these are only of a qualitative nature, because of the large number of variables examined. Results are discussed within each section and the methods adopted are summarized at the end of the chapter.

3.2 *INVESTIGATION OF METHODS*

3.2.1 *Infections and recovery of survivors*

A consistent and accurately determined level of infection is necessary for dose-response tests, so the method of counting third stage larvae (L_3) before infection is important. Indirect and direct approaches were tried. In the former, the concentration of a larval suspension was estimated (2.3), and the volume adjusted to give 200 L_3 in 0.2 ml. In the latter method, 50 or 100 L_3 were counted under a dissecting microscope, using a finely drawn pipette to pick them up. The smaller infections of 50 L_3 were used for this method because of the time involved. All L_3 were administered orally using a blunt 19 gauge needle and a 1 ml tuberculin syringe (2.2).

The entire small intestines, from female mice killed 35 days after infection, were examined. The intestines were slit open with a pointed dissection probe and the worms were teased gently from the tissue into petri dishes of Ringer's solution (BDH Chemicals). Incubation for several hours at 37°C allowed clumped worms to separate, facilitating counting.

The direct method proved to be the more reliable of the two (Table 3.1). Samples of 50 L_3 could be counted quickly and, as the recoveries were both predictable and high, this was adopted as the standard infection size for most experiments.

Table 3.1 Comparison of larval counting techniques and size of infection

Number of L_3	Number of mice	Number of adults recovered Mean (S.E.)	Range	Percent recovery
Indirect counts[1]				
200	10	197.3 (17.5)	85-278	89.7
Direct counts[2]				
50	4	47.5 (1.0)	45-49	95.0
100	4	88.3 (1.0)	86-91	88.3

Tested in female mice killed 35 days after infection.

[1] Numbers of L_3 were estimated by volume after counting a sub-sample

[2] L_3 were counted, under a dissecting microscope, using a finely drawn pipette to pick up each individual.

3.2.2 *Anthelmintic administration*

Three ways of dosing mice were tried to determine which technique was the most suitable. These were inclusion in the diet, intraperitoneal injection and oral gavage. The criteria examined included the accuracy of each method, its suitability for treating large numbers of mice, and the quantities of anthelmintic required.

CBZ and TBZ were fed to infected mice in a dietary supplement. The anthelmintics were added to a dry mixture containing wheatgerm and bran in the ratio of 2:1

by weight. This was made into a dough with about 1 ml of sucrose solution (100 g/l tap-water) per gram of cereal. Mice were housed in pairs and fed the supplement in the evening at a rate of 2.0 g dry weight per mouse. They were accustomed to the new food for three days before adding anthelmintic, and the standard diet of mouse cubes was available at all times. Two regimens were tried, one varying the amount of anthelmintic fed for two days, and the other feeding set doses for one to three days. The levels used were 0.5-2 mg CBZ/day for one to three days, or 10-100 mg TBZ/day for three days.

One test of intraperitoneal administration was made. A single injection of 5 mg CBZ in 0.2 ml distilled water was given with a 23 gauge needle and a 1 ml tuberculin syringe. Care was taken to ensure the suspension was not injected into the internal organs.

The preparation of CBZ suspensions for oral gavage is described in Chapter II (2.4). The suspension was administered using a blunt 19 gauge needle attached to a plastic disposable pipette tip (Oxford, Slim Line) and an adjustable micropipette (Oxford, Adjustable Sampler, 200-1000 µl). A tight seal between needle and plastic tip was achieved by removing about 0.6 mm from the tip. Calibration tests showed that the volumes delivered were 0.5 ml less than the value indicated on the sampler, but the difference was constant throughout the

dose range that was used. This system was adopted because CBZ residue, adhering to the side of a tuberculin syringe, obscured the volume level causing errors of up to 0.2 ml. The doses ranged from 0.6-2.2 mg CBZ in 0.2-0.6 ml water and were administered between 5 pm and 8 pm. Additional water was given where appropriate, so that each mouse received a total volume of 0.6 ml fluid. The dosages, in absolute quantities and in terms of body weight, are tabulated below (Table 3.2).

Table 3.2 Cambendazole Dosage Rates for the Adult Dose Response Assay

Amount of CBZ (mg/mouse)	Dosage rate* (mg/Kg body weight)
0.6	20.0
0.8	26.7
1.0	33.3
1.2	40.0
1.4	46.7
1.6	53.3
1.8	60.0
2.0	66.7
2.2	73.3

* Calculations based on an average weight of 30 g for adult male CBA mice

Of the three techniques, administration by oral gavage was the most suitable, both for accuracy and ease of treating large numbers of mice (Table 3.3). Highly

Table 3.3 Comparison of the Variability Associated with Three Methods of Cambendazole Administration

Method of administration	Size of infection (L_3)	Dosage (mg CBZ)	Number of mice	Number of Surviving Worms		
				mean†	(S.E.)	range
a) Dietary	100	1.0	4	59.5	(18.8)	6-93
	100	1.0	4	75.8	(1.7)	71-79
	100	1.0	8	66.6	(3.5)	50-80
b) Intraperitoneal injection	50	5.0	4	6.8	(4.7)	0-27
c) Oral gavage	50	1.1	10	24.8	(2.3)	16-37

a) Male mice were fed a cumulative dosage of 1.0 mg CBZ (0.5 mg/day for two days) 14 days after infection, and killed five days after the last treatment

b) Female mice were injected with 5.0 mg CBZ in 0.2 ml water 20 days after infection, and killed nine days later

c) Male mice were given 1.1 mg CBZ in suspension 14 days after infection, and killed seven days later.

† Arithmetic mean

variable results were obtained using the dietary technique, samples of which are shown in Table 3.3(a). Differences in the proportion of the supplement eaten by each mouse, and in the amount wasted, were major problems. It should be emphasized that other workers have fed anthelmintics with the complete diet rather than in a supplement. TBZ treatment by this method presented a further problem because the dosage levels required to kill the parasite appeared to make the supplement unpalatable. Low susceptibility to TBZ has been noted by other workers (Brody and Elward 1971; Dobson, personal communication; Coles and McNeillie 1977; Theodorides 1976) and led to the choice of CBZ for use in this study.

Intraperitoneal injection of CBZ was effective, though only at high dosage levels, and was the least relevant to normal methods of administering benzimidazoles.

3.2.3 *Age of infection at treatment*

Most *N. dubius* re-enter the lumen of the intestine by the 8th day of infection (Bryant 1973a). However, maximum body length and respiratory rate are not attained until 14-16 days after infection (Bryant 1974). The effect of administering CBZ on newly emerged and mature adults was investigated to determine which should be used in the adult dose-response assay. Male mice were infected with 100 L_3 and treated with CBZ after 9 or 14 days. A cumulative dose of 1.0 mg was administered in the diet

(0.5 mg/day for 2 days) and the mice were killed 6 days later.

Newly emerged adults were more susceptible to CBZ than were mature adults, and also showed a greater variability in response (Table 3.4).

Table 3.4 The Age of Infection and Its Effect on Response to Cambendazole

Age of infection at treatment (days)	No. of mice	Number of surviving worms Mean†	(S.E.)	Range	Percent mortality
control	4	83.8	(1.7)	80-88	·
9th	4	26.5	(15.3)	7-72[1]	68.4
14th	4	75.8	(1.7)	71-79[2]	9.5

Male mice infected with 100 L_3 were fed 0.5 mg CBZ for two days and killed six days later

† Arithmetic mean

Recoveries compared by contingency table:

[1] Significant variation within group at P = 0.001, others show no significant variation within groups

[1,2] Both treated groups are significantly different from each other and from the control group at $P < 0.05$

Some, but not all of this variation may be due to the method of dosing as discussed previously (3.2.2). So,

14 day old infections were adopted as the standard age for the assay.

3.2.4 *Duration of response*

Trials for determining when to terminate the response were carried out in female mice infected with 50 L_3. CBZ was administered by oral gavage (1.4 mg/mouse) 14 days after infection and the mice were killed at daily intervals after the second day. Worm losses stabilized about 5-6 days after treatment (Table 3.5, Fig. 3.1), and the recoveries of male and female parasites were similar on all but the second day. Subsequent assays were terminated after 7-8 days.

Adult *N. dubius* normally occupy the anterior portion of the mouse's duodenum (Bawden 1969; Dobson 1961; Lewis and Bryant 1976; Panter 1969a), but in the early days following treatment they were found the entire length of the small intestine. These worms were probably damaged and in the process of being eliminated. By the 6th day after dosing the distribution was again normal.

3.2.5 *Effect of the host's age and sex*

Age- and sex-linked resistance to *N. dubius* have been reported in some mouse strains (Bawden 1969; Dobson 1961, 1962, 1966; Hosier and Durning 1975; Newton, Weinstein and Sawyer 1962) but not in others (Behnke and Wakelin 1977; Bryant 1974; Lewis and Bryant 1976;

Table 3.5 Survival of Adult Worms with Time after Cambendazole Treatment

Days after treatment	Number of mice	Number of Surviving Worms: Males mean† (S.E.)	Females mean† (S.E.)	Total mean† (S.E.)	Percent mortality
0	6	21.2 (1.6)	21.0 (0.9)	42.2 (1.6)	-
2	5	11.2 (1.5)[1]	16.6 (2.1)[1]	27.8 (2.2)	34.2
3	5	3.2 (1.4)	4.6 (1.4)	7.8 (1.8)	81.5
4	5	4.2 (1.5)	6.6 (1.4)	10.8 (2.7)	74.4
5	5	2.8 (0.9)	1.6 (0.8)	4.4 (1.5)	89.6
6	5	1.0 (0.3)	2.0 (0.6)	3.0 (0.7)	92.9
7	5	1.5 (0.5)	2.0 (0.8)	3.5 (1.0)	91.7

Female mice were infected with 50 L_3 and treated 14 days later with 1.4 mg CBZ by oral gavage

† Arithmetic means of recoveries

Recoveries compared by contingency table: [1] recoveries of males and females significantly different from each other at P = 0.01 on the 2nd day after treatment. Others show no significant differences at P = 0.05.

Total worm recoveries show no significant decline from the 5th to the 7th days after treatment.

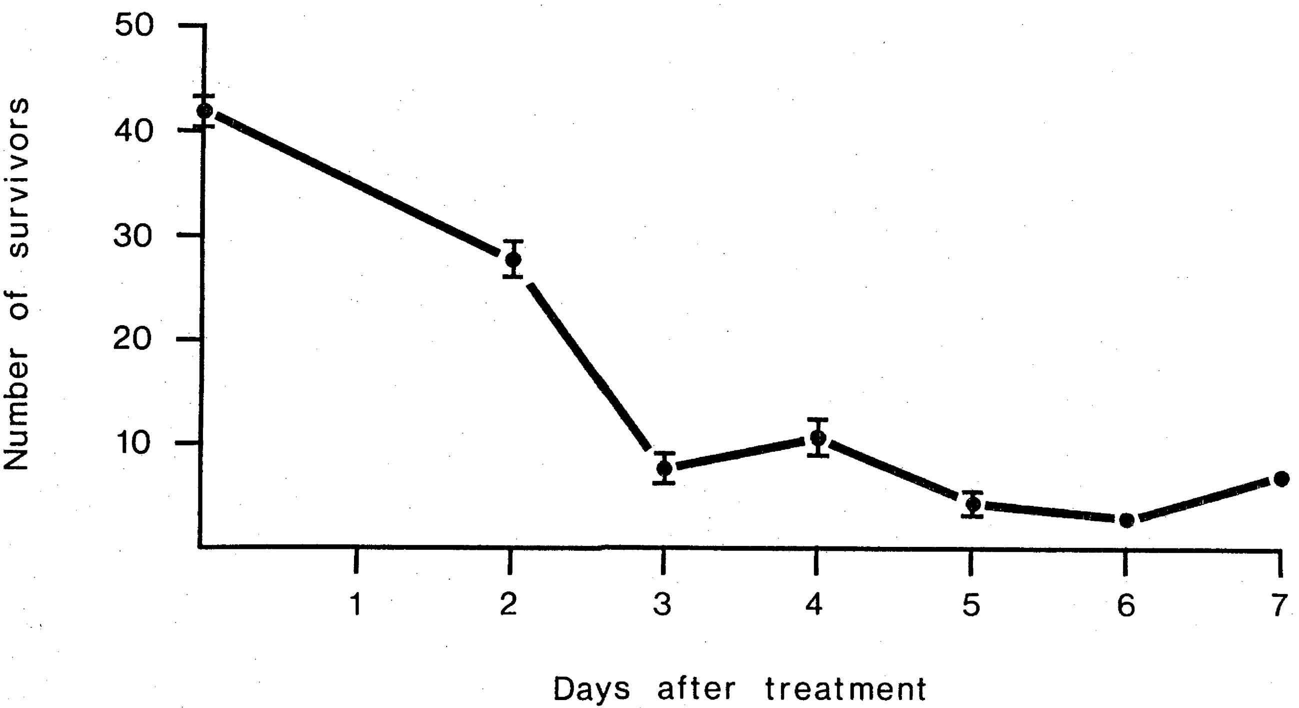


FIGURE 3.1 SURVIVAL OF ADULT WORMS WITH TIME AFTER CAMBENDAZOLE TREATMENT

Mean and standard error for five replicates dosed with 1.4 mg by oral gavage

Panter 1969a; Van Zandt, Cypess and Zidian 1973).

The effect of host age was tested in females 8 or 16 weeks old. These were infected with 50 L_3 and killed after 35 days. Infections in males and females were compared both before and after CBZ treatment. CBZ was administered 14 days after infection and the mice were killed 7 days later.

No age-resistance was observed over this time-span (Table 3.6) and the recoveries from untreated males and females were similar (Table 3.7(a)). However, there was a marked difference in parasite survival in treated mice, with males retaining a greater number of worms (Table 3.7(b)).

Table 3.6 Comparison of Infections in Mice of Different Ages

Age of mice at infection (weeks)	Number of mice	Number of adult worms recovered Mean†	(S.E.)	Range
8	5	42.2	(1.6)	41-46
16	4	47.5	(1.0)	45-49

Female mice were infected with 50 L_3 and killed 35 days later

† Arithmetic mean

Recoveries compared by contingency table: no significant variation within groups but these differ significantly from each other at P = 0.05.

Table 3.7 Comparisons of Infections and Responses to Cambendazole in Male and Female Mice

(a) Recoveries of adult worms from untreated mice

Sex of mice	Number of mice	Number of adults recovered Mean†	(S.E.)
male	5	44.40	(1.90)
female	6	42.21	(1.62)

Mice were infected with 50 L_3 and killed 21 days later
† Arithmetic mean
Recoveries compared by contingency table:
no significant variation within or between groups

(b) Dose responses of adult worms

Sex of mice	Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)
males	4	1.28	(0.01)	3.84	(0.70)
females	4	0.75	(0.01)	4.32	(0.69)

Mice infected with 50 L_3 were treated with CBZ by oral gavage 14 days after infection and killed 7 days later
ED_{50}s compared by Student's t-test:
significantly different from each other at P = 0.001
Slopes compared by analysis of covariance:
not significantly different at P = 0.05

Most subsequent assays were carried out using males aged less than 16 weeks at infection, but some experiments did use females because of difficulties in rearing large numbers of mice.

3.3 *EXAMPLE OF A COMPLETE ADULT DOSE RESPONSE ASSAY*

3.3.1 *Results*

The results of a dose response test on adults from the unselected population are summarized in Table 3.8(a) and displayed in Fig. 3.2. The numbers of worms removed by treatment with CBZ were determined then replicates were examined for variation (2.5.2, Table 3.8(b)) and a log dosage-probit line calculated from the pooled data (2.5). Analysis of sums of squares showed no significant deviation from linearity for pooled data, but marked variation between replicates (Table 3.8(b)). The latter is particularly obvious when depicted graphically (Fig. 3.2).

There was little change in the ED_{50} of the unselected population over the duration of the study, though there was a decline in regression line slopes (Table 3.9).

3.3.2 *Discussion*

The amount of variation between replicates evident in this example was similar for all assays performed on adult worms. In many tests though, there was also significant deviation from linearity using pooled data. Where there is marked variation between replicates, it can contribute to the residual variation between doses and Finney (1971) discusses a number of factors which may influence the overall heterogeneity in a population's response.

Table 3.8 Adult Dose Response for the Unselected Population

(a) Regression parameters

Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)
4	1.24	(0.01)	8.09	(0.54)

(b) Deviation from linearity and variation between replicates

Source of variation	Degrees of freedom	Sum of squares	Mean square
Residual between doses (pooled data)	2	1.2^{ns}	0.6
Between replicates	36	220.6***	6.1
Total residual (retaining identity of replicates)	38	221.8***	

Analyses of sums of squares show no significant deviation from linearity at P = 0.05 (ns) using pooled data, but significant deviation in the total residual at P = 0.001 (***), indicating marked variation between replicates.

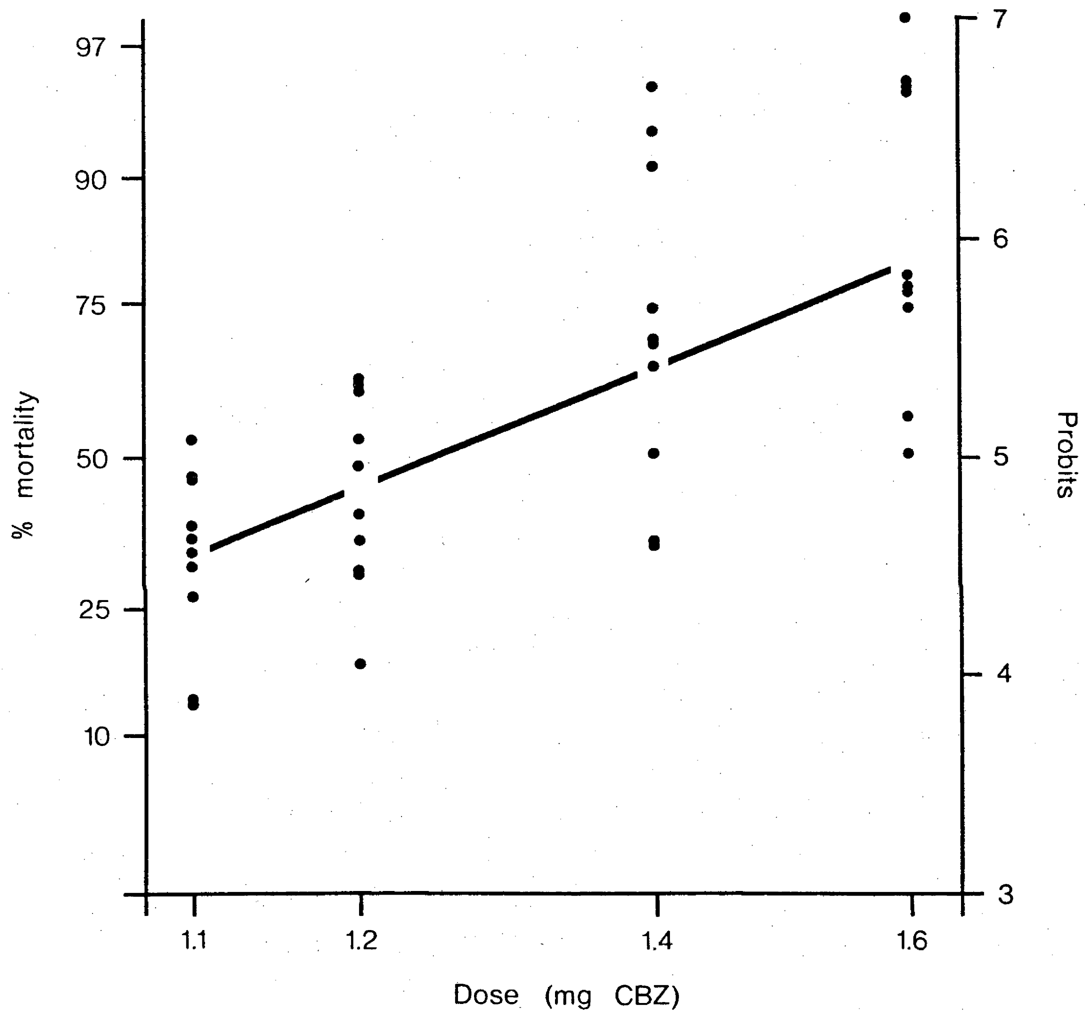


FIGURE 3.2 ADULT DOSE RESPONSE TO CAMBENDAZOLE FOR THE UNSELECTED POPULATION
Tested in male mice

Table 3.9 Dosage-Mortality Responses for the Unselected Population in Male Mice over the Duration of the Study

Generations in CBA/H mice	Number of dosage levels	ED_{50} (mg CBZ) (S.E.)	Slope (S.E.)	Reference Table	Reference Figure
7	4	1.24 (0.01)	8.09 (0.54)[2]	3.8a	3.2
7	4	1.25 (0.13)	7.55 (1.69)[1]	5.3	5.2
8	4	1.17 (0.18)	5.53 (1.45)	5.5	5.4
9	6	1.08 (0.43)	3.45 (1.23)[1,2]	5.3	5.2

ED_{50} compared by Student's t-test: no significant differences at $P = 0.05$

Slopes compared by analysis of covariance: [1] significantly different at $P = 0.05$

[2] significantly different at $P = 0.01$

A failure of individuals to respond independently is one factor which may be important for this parasite. Adults tend to aggregate, sometimes in quite large clumps, and this could interfere with the quantity of anthelmintic that each worm receives. The possibilities that the amount of genetic variation of individuals differs between dosage groups, or that the mice behave differently depending on the dosage given, cannot be eliminated. Nevertheless, the variation observed at each dosage appeared to be similar in most tests suggesting its source was independent of dosage and sampling of the population.

In all assays, the dispersion of pooled responses about the fitted regression lines appeared to be random, so heterogeneity factors were used where necessary when comparing populations (Finney 1971). These were calculated by dividing the weighted sum of squares for differences between empirical and weighted probits with its degrees of freedom, and ranged in value between two and nine. The variances in log ED_{50} were then multiplied by heterogeneity factors to correct for overestimation of weights. Tests for parallelism were also affected and the direct reference to χ^2 tables for this deviance (2.5.4) was replaced by a variance ratio or F-test according to Finney (1978):

$$F = \frac{\text{deviance (parallelism)}}{\text{heterogeneity factor}}$$

with (1, f) degrees of freedom where f is the sum of the degrees of freedom for the two lines.

Variation between replicates would decrease the assay's sensitivity but responses were remarkably constant during the study (Table 3.9). The apparent decline in slopes of the regression lines could indicate an increasing range of susceptibilities in the population, or some variation in assay techniques with time. The latter is more likely though, because similar changes occurred in all of the isolates selected as adults with CBZ (Chapter V). On the whole, this assay proved reliable providing very high sensitivity was not required (Chapter VII).

3.4 *SUMMARY OF METHODS*

Mice were infected orally with 50 L_3 which had been counted under a dissecting microscope. These were given in about 0.4 ml of tap-water using a blunt 19 gauge needle and a 1 ml tuberculin syringe (2.2). Fourteen days later, between the hours of 5 pm and 8 pm, CBZ was administered as a suspension in distilled water (4 mg/ml; 2.4). A modified adjustable micropipette was used and additional water was given where appropriate, so that each mouse received 0.6 ml of fluid. At least four dosage levels were tested, with 7-10 mice in each group.

After seven days, the mice were killed using ether and the entire small intestine was examined for survivors. The number of parasites killed was estimated, and the results were expressed as a log dosage-probit line.

CHAPTER IV

EGG HATCH ASSAY

4.1 *INTRODUCTION*

It is generally accepted that conventional *in vivo* dose response assays are costly, both in time and numbers of host animals. However, an alternative *in vitro* screening test has been devised by Le Jambre (1976) and Coles and Simpkin (1977), and relies on the susceptibility of nematode eggs to benzimidazoles (Egerton 1969
In this assay, the ability of eggs to embryonate and hatch when incubated with anthelmintic is monitored.

Having found that *N. dubius* eggs are susceptible to CBZ in solution, the egg hatch assay was adopted for this study. As with the adult dose response assay, some experimental conditions were examined before choosing a standard method, and the results are discussed within each section. The complete procedure is summarized at the end of the chapter.

4.2 *INVESTIGATION OF METHODS*

4.2.1 *The eggs*

Assays performed on freshly collected *Haemonchus contortus* eggs are more reliable than assays of embryonated

eggs, and Le Jambre (1976) recommends the use of the former. With nematode parasites of sheep, embryonic development is retarded in the period between collecting faeces and setting up the assay by cooling the eggs and all solutions to 4°C (Le Jambre 1976). *N. dubius* also develops very slowly at low temperatures (Mura 1975; and personal observation), so this procedure was adopted.

Faeces were collected from mice over a 15-20 minute interval, then cooled and softened for two to three hours in tap water at 4°C. A slurry was made using a glass stirring rod, and transferred to a 9 cm glass petri dish. After filling the dish to the brim with saturated saline, a thin plastic disc (about 0.2 mm thick), cut to the size of the dish, was placed on the surface for 15 minutes. Eggs which rose to the top were removed on the disc and washed from it with distilled water. The suspension was then diluted to a concentration of about 100-200 eggs/ml. Faecal material can interfere with benzimidazole activity (Coles and Simpkin 1977) and large debris must be removed from sheep faeces by sieving. However, flotation alone was sufficient to recover clean eggs from mouse faeces. No difference in viability was detected between eggs isolated with saturated saline or with sucrose solution, so the former method was retained.

4.2.2 *The assay*

Saturated CBZ solution, prepared as described in Chapter II (2.4), was diluted with 0.1% saline in distilled water (Le Jambre 1976). Originally, the concentration of saturated CBZ was assumed to be 20 μg/ml (The Merck Index 1976) rather than the actual 35 μg/ml (2.4), and dilutions were chosen on the basis of the former (Table 4.1).

Table 4.1 Method of Diluting the Saturated Cambendazole Solution for use in the Egg Hatch Assay.

Volumes of CBZ and Saline Solutions (ml)			Final concentration of CBZ (μg/ml)
CBZ		Saline	
half strength (17.5 μg/ml)	saturated (35 μg/ml)	0.1% in water	
-	-	4.00	0.00
0.75	-	3.25	2.63
-	0.50	3.50	3.50
1.25	-	2.75	4.38
-	0.75	3.25	5.25
1.75	-	2.25	6.13
-	1.00	3.00	7.00
2.25	-	1.75	7.88
-	1.25	2.75	8.75
2.75	-	1.25	9.63
-	1.50	2.50	10.50
-	1.75	2.25	12.25
-	2.00	2.00	14.00
-	2.25	1.75	15.75
-	2.50	1.50	17.50

The volume of each sample was 4 ml, to which 1 ml of egg suspension was added giving a final volume of 5 ml.

All volumes were measured by micropipette (Oxford, P-7000 Sampler, 1000 µl and Adjustable Sampler, 200-1000 µl) into glass test tubes, and five replicates of each concentration were prepared. The samples were mixed on a vortex agitator (Scientific Industries, Vortex-genie), both before and after adding 1 ml of egg suspension, then plated in plastic petri dishes (Falcon 50 x 9 mm with tight lid). Sealable dishes were used to minimize concentration changes caused by evaporation. The depth of liquid was about 3 mm, sufficiently shallow to permit oxygenation (Le Jambre 1976). Day to day differences can be expected in the response of eggs (Le Jambre 1976) so the unselected and selected populations were always tested concurrently to allow direct comparisons.

Incubations of 24 hours (Coles and Simpkin 1977) to 3 days (Le Jambre 1976) at 26-27°C are used for screening sheep nematode eggs. The ability to embryonate is monitored in the shorter assay, while the longer assay tests whether eggs can complete development and hatch. The response of *N. dubius* eggs to incubation with CBZ at 22°C, was investigated to determine the best time to terminate the assay. Samples containing 0, 6.8 or 10.2 µg CBZ/ml were set up in triplicate, and the response was halted after 2, 4.5, 5, 7.5 or 30 days by adding Lugol's iodine (Appendix 2). The numbers of larvae and unhatched eggs in each dish were counted under a dissecting microscope.

In the absence of CBZ, 98% of the eggs hatched after 2 days, and this did not alter throughout the experiment (Table 4.2).

Table 4.2 The Percentage of Eggs Hatching with Duration of Exposure to Cambendazole

Time (days)	% Hatch		
	Control	7.0 µg CBZ/ml	10.5 µg CBZ/ml
2.0	98.0[1]	11.5	1.0
4.5	97.8[1]	37.9[2]	7.2
5.0	97.8[1]	42.6[2]	16.3[4]
7.5	96.8[1]	54.3[3]	18.8[4,5]
30.0	98.6[1]	47.0[3]	23.8[5]

Eggs were incubated for 2-30 days at 22°C with 3 replicates for each time interval.

The proportions of eggs hatching were compared by contingency table: no significant differences were found between replicates at $P = 0.05$

[1-5] No significant difference between values with the same superscript at $P = 0.05$

Untreated and CBZ-treated groups differed significantly in each time interval at $P \leqslant 0.001$

At both CBZ concentrations, the maximum hatch occurred after about 7.5 days, though eggs hatched more slowly at the higher concentration (Fig. 4.1). There appears to be a

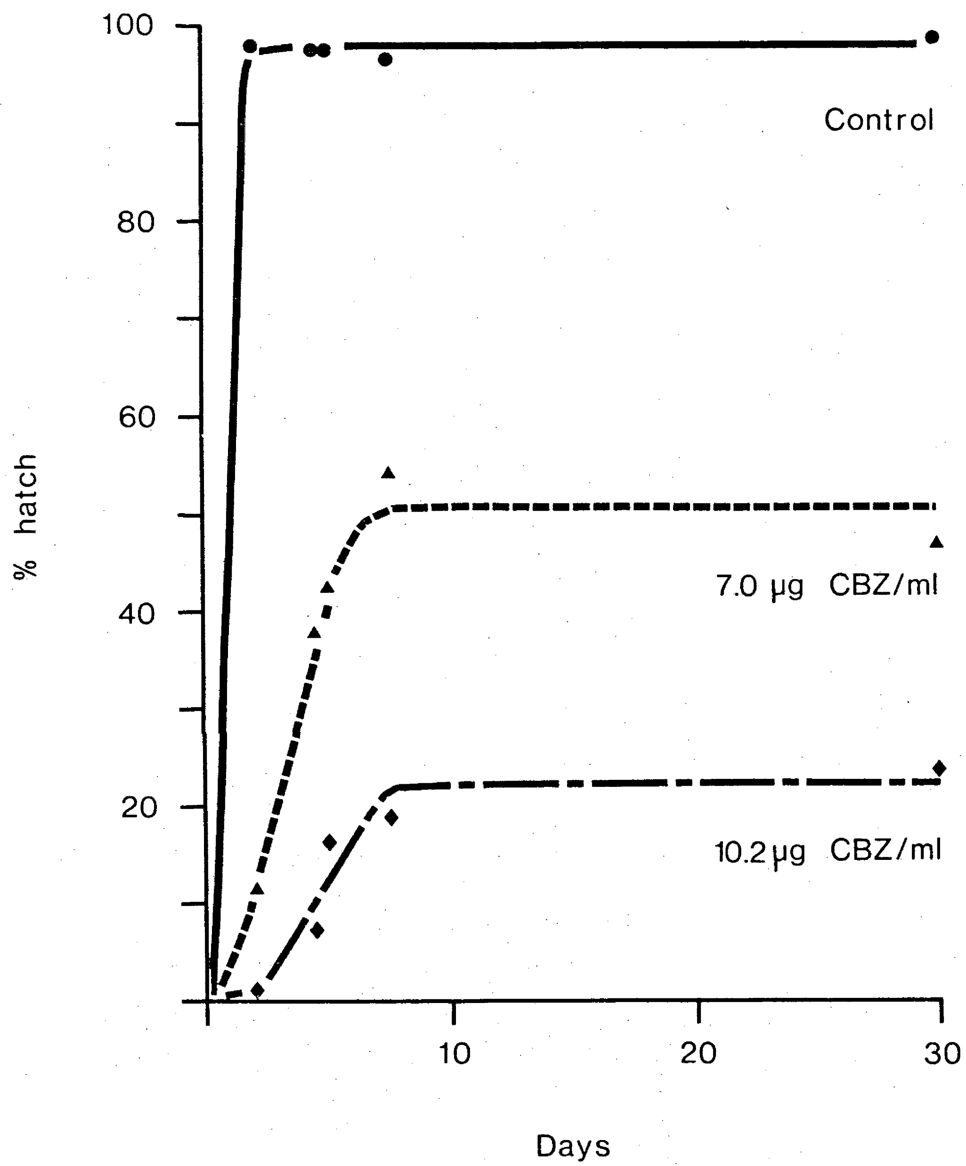


FIGURE 4.1 PERCENTAGE OF EGGS HATCHING WITH DURATION OF EXPOSURE TO CAMBENDAZOLE AT 22 C

continuous range of susceptibilities in non-hatching eggs, from those showing no obvious development to eggs containing vermiform larvae.

Assays terminated before or after the maximum response is achieved should be equally valid screens for anthelmintic resistance, though in the former, any variation in time or temperature between experiments could introduce errors and these would be compounded by the differing rates of development at each concentration (Fig. 4.1). A test of the maximum hatchability was therefore used, and the eggs were incubated for at least 14 days.

4.3 *EXAMPLE OF A COMPLETE EGG HATCH ASSAY*

4.3.1 *Results*

A full assay performed on eggs from the unselected population is included here to demonstrate the treatment and analysis of results (Table 4.3, Fig. 4.2). The number of individuals responding (unhatched eggs) out of the total exposed (eggs plus larvae) was determined. Replicates were tested for variation (2.5.2 and Appendix 1) and a log dosage-probit line calculated from the pooled data (2.5). The tests for differences in the median lethal concentration (EC_{50}) and slope, when comparing populations, are described in Sections 2.5.3 and 2.5.4 respectively.

Table 4.3 Egg Hatch Response for the Unselected Population

(a) Regression parameters

Number of concen- trations	EC_{50} (µg CBZ/ml)	(S.E.)	Slope (S.E.)
5	7.31	(0.07)	7.30 (0.26)

(b) Deviation from linearity and variation between replicates

Source of variation	Degrees of freedom	Sum of squares	P*
Residual between concentrations (pooled data)	3	1.87	ns
Between replicates	20	13.70	ns
Total residual (retaining identity of replicates)	23	15.57	ns

ns not significant at P = 0.05

* Analyses of sums of squares show no deviation from linearity using pooled or separate data, and no significant variation between replicates, indicating that the regression line is an adequate description of the population's response.

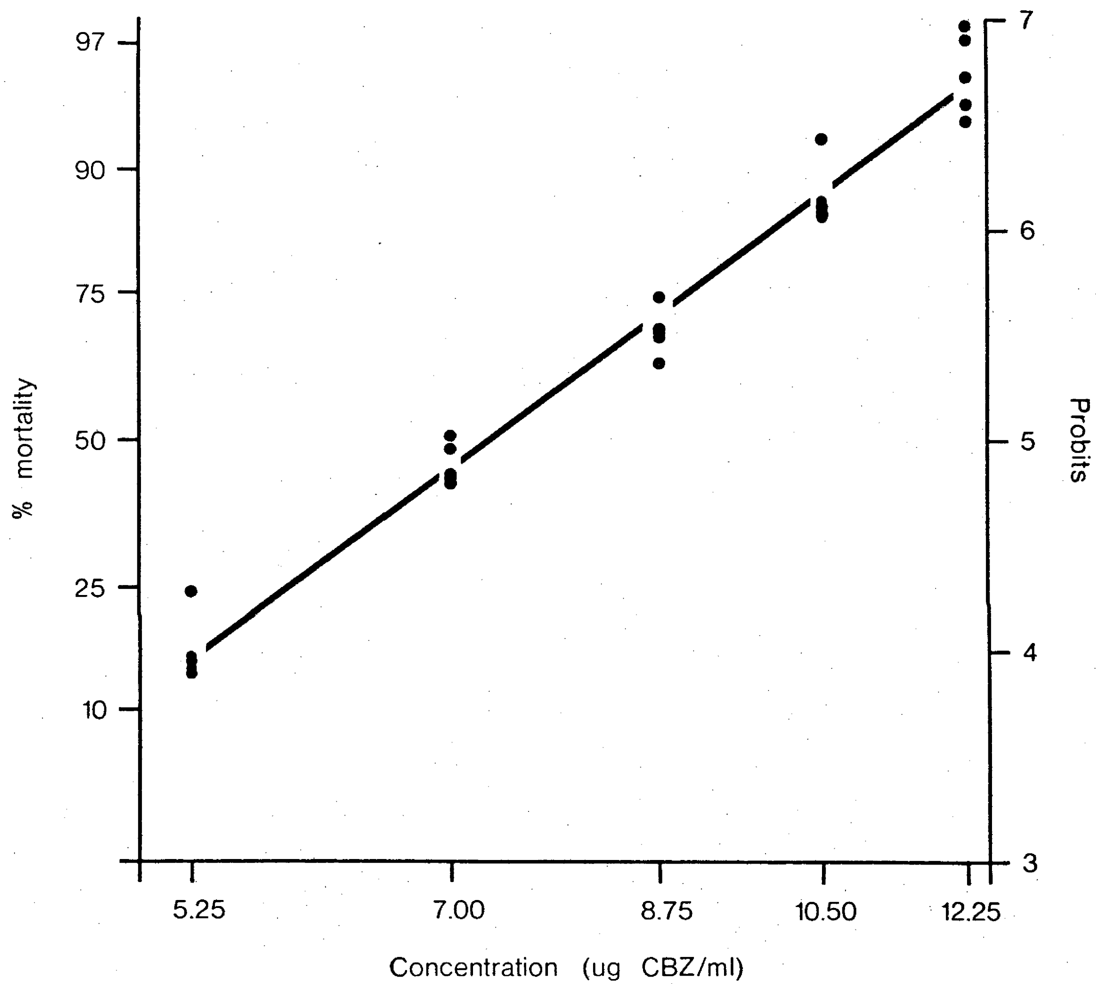


FIGURE 4.2 EGG HATCH RESPONSE TO CAMBENDAZOLE FOR THE UNSELECTED POPULATION

4.3.2 *Discussion*

The egg hatch response of *N. dubius* in the presence of different concentrations of CBZ suggests the assay is applicable to this species. There is little variation between replicates and the results show no significant deviation from linearity (Table 4.3b), indicating that the fitted log dosage-probit line is an adequate description of the population's response (Finney 1971).

One problem encountered early in the study was an apparent decline in the susceptibility of eggs from the unselected population over time (Table 4.4). At this stage, saturated CBZ solution was prepared by re-using the excess residue solids from previous batches (2.4). Once the problem was noticed, the actual concentration in the sixth batch was determined and found to be only half the original strength (2.4). The response of the eggs returned to the original level when a fresh suspension of CBZ was used to prepare the solution (Table 4.4), indicating the phenomenon was in fact due to the anthelmintic solution rather than a change in the nature of the population.

Previous batches of solution could not be analysed, so the numerical values for concentration were not altered for any of the batches. In practice, this would affect both EC_{50}s and slopes of the log dosage-probit lines and absolute values should therefore only be compared between

Table 4.4 The effect of re-using cambendazole solids in preparation of saturated solutions, on egg hatch responses for the unselected population

Batch of CBZ	EC_{50} (μg CBZ/ml)	(S.E.)	Slope	(S.E.)
1st	5.42†	(0.03)	5.87	(0.12)††
2nd	4.17	(0.04)	5.70	(0.16)
3rd	6.62	(0.08)	6.08	(0.28)
4th	7.48	(0.11)	6.33	(0.34)
5th	8.80	(0.07)	6.60	(0.19)††
6th	10.37	(0.24)	5.19	(0.42)
New preparation				
1st	5.35†	(0.23)	6.41	(0.75)

EC_{50} s compared by Student's t-test:

† no significant difference between values with the same superscript

All others differ significantly at P = 0.001

Slopes compared by analysis of covariance:

†† significant difference between slopes with same superscript at P = 0.05

All other slopes show no signficant difference at P = 0.05

populations tested simultaneously. This together with the day to day differences which were observed in the study, support Le Jambre's (1976) recommendation that groups being compared are tested together. For comparisons over time, the EC_{50}s of selected isolates were considered relative to those of their respective controls, using resistance ratios (2.5).

4.4 *SUMMARY OF METHODS*

Faeces were collected from mice over a 15-20 minute interval, and the pellets were softened in cold tap water for two to three hours. The eggs were separated from faecal material by a 15 minute flotation in saturated saline and then resuspended in distilled water, with both steps carried out in 4°C. These eggs were incubated with solutions of CBZ in 0.1% saline using sealed plastic petri dishes. The depth of liquid in the dishes was about 3 mm. After at least 14 days at 22°C, the numbers of larvae and unhatched eggs were counted under a dissecting microscope, and the results were expressed as a log dosage-probit line.

CHAPTER V

SELECTION OF ADULTS FOR RESISTANCE

5.1 *INTRODUCTION*

The usefulness of *Nematospiroides dubius* as a laboratory model for studies of anthelmintic resistance depends upon how it responds to selection. The aims of the work presented in this chapter were to determine whether the population under study was capable of developing resistance, and if so, what conditions would be most effective in promoting this.

Similarities between anthelmintic resistance and the earlier problem of insecticide resistance have been emphasized a number of times (Le Jambre 1978a; Le Jambre, Southcott and Dash 1976; Prichard, Hall, Kelly, Martin and Donald 1980). From the work with insecticides, it is possible to predict how anthelmintic resistance is likely to develop, and in particular, to identify the factors which influence this. It will be recalled from Chapter I that four of the important points affecting the rate of change in the frequency of resistance alleles are:

- (i) the intensity of selection
- (ii) the amount of genetic variability within the population
- (iii) the fitness of the selected individuals
- (iv) the genetic nature of resistance

There have been a number of experimental attempts to produce anthelmintic resistant populations of nematodes. Early selections involved phenothiazine, for a long time the most widely used treatment for nematodes. Members of three genera: *Haemonchus*, *Trichostrongylus* and *Cooperia*; subjected to various selective regimes were tried, but none were successful (Bennett 1968; Hasche and Todd 1963; Silangwa and Todd 1966; Sinclair 1953). It is difficult to judge whether the failures were caused by restrictions in variability common to laboratory populations, or to the limited nature of the experiments themselves.

With benzimidazoles, experimental selection for resistance has proved more successful. There is one report of resistance to cambendazole (CBZ) developing in an initially susceptible strain of *Haemonchus contortus*. In this isolate, resistance appeared after only four generations of selection, and the level was increased with further exposure to CBZ (Colglazier, Kates and Enzie 1974; Kates, Colglazier and Enzie 1973). Other attempts to select for benzimidazole resistance have been confined to increasing the tolerance levels in already resistant field isolates. This has been successful in *Haemonchus* and *Ostertagia*, but field isolates of *Trichostrongylus* have so far failed to respond to further selection under laboratory conditions (Hall, Campbell and Richardson 1978; Kelly and Hall 1979; Le Jambre *et al*. 1976, 1977, 1978a, b).

There have been few previous studies manipulating conditions likely to affect the development of anthelmintic resistance. Le Jambre's group demonstrated that resistance could develop as rapidly to three chemically unrelated anthelmintics as to a single one, if used over the same period of time (Le Jambre *et al.* 1977, 1978a). Recently, Barton (1980) showed the importance of selection frequency on the rate at which resistance developed in *H. contortus*. Weekly or six weekly treatments, repeatedly exposing each generation to selection, were particularly effective. Another factor likely to influence the development of resistance is selection pressure, and the results of experiments investigating its role are reported here.

The retention of viable spermatozoa by female nematodes isolated from males, complicates the design of the selection programme for anthelmintic resistance. Females of *H. contortus* produce fertile eggs for only 48 hours after being separated from males, but those of *Ancylostoma caninum* continue to do so for about three weeks (Beaver, Yoshida and Ash 1964; Le Jambre 1977; Le Jambre and Georgi 1970). If *N. dubius* females stored spermatozoa, then eggs collected from survivors soon after treatment with anthelmintic, could be fertilized by spermatozoa from susceptible males. This possibility was, therefore, considered in designing the selection experiments which are described below.

5.2 *METHOD*

5.2.1 *Selection techniques*

Adult parasites were selected for resistance by administering cambendazole (CBZ) to infected mice, as described in Chapter III. Five mice were each infected with 50 L_3, giving a total population size of about 250 adults exposed to each dosage. Male or female mice were used in alternate generations, and these were dosed 14 - 21 days after infection.

Details of the four selection pressures are given below (Table 5.1). The percentage mortality is that occurring in the first generation.

Table 5.1 Selection Pressures Applied to Adult Parasites

Population	Generations	Dosage mg CBZ per mouse	Dosage mg CBZ/kg body weight	% mortality on first exposure
A	1st - 4th	1.8	60	98^{+}
	5th - 8th	1.6	53	95 - 98
B	1st - 6th	1.6	53	95 - 98
C	1st - 4th	1.6	53	95 - 98
D	1st - 6th	1.4	47	85 - 95
E	1st - 4th	1.2	40	50 - 60

With the exception of one isolate (Population A), the dosages applied were not altered during the course of the study.

5.2.2 *Collection of eggs from survivors*

The length of time that fertile eggs continue to be deposited by inseminated females after removal from the presence of males was tested using the technique of Le Jambre and Royal (1977). Adult worms were recovered from donor mice 14 days after infection, and collected in 0.8% saline. Female worms were separated after allowing them to disentangle from clumps for 15-30 minutes. The females were then transferred orally to male mice, in about 0.5 ml of pasteurized whole milk, using a 1 cm piece of soft plastic tubing (1.14 mm diameter) on the end of a pasteur pipette. Care was taken not to damage the parasites, and all manipulations were carried out at 37°C in a constant temperature room.

Eggs passed in the mouse faeces were isolated by flotation in saturated NaCl, then cultured in 0.1% NaCl for three days at 22°C. After about 14 days, some infertile eggs were found, and between the 18th and 20th day, fertile eggs disappeared. The infertile eggs were misshapen and shrivelled (Plate 5.1), probably because of their exposure to saturated saline. Hypertonic solutions and physical pressure are known to distort the infertile eggs of *H. contortus* and *A. caninum* (Le Jambre 1977, 1979b; Le Jambre and Georgi 1970). To make sure that the saturated saline itself was not responsible for their failure to hatch, some of these eggs were incubated without removal from the faeces, but again hatching was not observed.

The eggs from survivors of CBZ treatment were therefore collected three to four weeks after dosing, to ensure they were the offspring of resistant males. Faecal cultures were set up as described in Chapter II (2.2.2), but for experiments on adult selection, the infective larvae (L_3) were collected after 14 days instead of seven days as routinely used, in case development was slower in resistant populations. There were no other alterations to the methods.

5.2.3 *Assays*

Egg hatch assays were usually performed at each generation. Since the number of eggs collected from surviving adults was generally insufficient for a reliable test, the assays were made on the eggs from their progeny. Resistance levels in the latter could be affected by genetic recombination at reproduction. The responses of eggs from Population A, collected before treatment, were therefore compared with eggs collected after treatment, but no significant differences were found. The details of the assay are described in Chapter IV (4.4).

Full dose response tests of the adults (3.4) were conducted after four generations, and then after the sixth or eighth generation depending on the length of selection.

5.2.4 *General fitness of the selected populations*

The survival of the selected isolates in the absence of CBZ was compared with that in the unselected population

to determine if any reduction in viability had occurred. Four attributes were examined. The proportion of eggs able to hatch in 0.1% NaCl was determined at each generation using the controls from egg hatch assays (4.2.2). Survival of these through to L_3 was measured by their recovery from standard faecal cultures as described in Chapter II (2.2.3). The number of L_3 reaching maturity was then monitored in the control mice through adult dose response assays (3.4), and the egg output of the mature female worms in these mice was examined (2.2.3). The full range of tests was not made in every population.

5.3 *RESULTS*

The exposure of adult *N. dubius* to selection with CBZ proved successful, leading to increases in the median effective dose (ED_{50}) for adults of all isolates. It did not appear to influence the susceptibility of their eggs. Data for egg hatch and adult dose response assays of each isolate are shown in consecutive tables (Tables 5.2 to 5.11). The resistance ratios (2.4.1) for eggs and adults, and the log dosage-probit lines for adults of the final generations of each isolate, are depicted in consecutive figures (Figs 5.1 to 5.10). All tables and figures are placed at the end of this section.

With the exception of Population D, the EC_{50}s of eggs from the selected lines show no consistent deviation from those of the unselected population. Instead, the

resistance ratios tended to fluctuate about the value of 1.0 (Figs 5.1, 5.3, 5.5 and 5.9). The EC_{50}s of eggs from Population D rose slightly and remained higher than those from the unselected population (Fig. 5.7). The regression line slopes for eggs of all isolates were similar to those in the unselected population, though there appeared to be a trend towards steeper log dosage-probit lines in Population A (Table 5.2).

Adult worms developed resistance in response to all selective regimens. The ED_{50} for Population A, when exposed to intense selection with 1.8 mg CBZ (98^{+}% mortality), did not alter after four generations (Table 5.3, Fig. 5.1). However, when the dose level was lowered to 1.6 mg CBZ for a further four generations, a significant increase in ED_{50} resulted.

Two other isolates, Populations B and C, which were also exposed to 1.6 mg CBZ, both showed marked increases in ED_{50} after four generations (Tables 5.5 and 5.7, Figs 5.3 and 5.5). Population B was followed for two more generations after which the resistance ratio had changed only slightly (1.40 to 1.42), and the difference in ED_{50} between this and the control population was not significant.

Lower selection pressure also proved effective. The moderate pressure of 1.4 mg CBZ (85-95% mortality) applied to Population D, led to an increase in ED_{50} after

four generations and a further rise after the sixth (Table 5.9, Fig. 5.4). There was no corresponding change in egg susceptibility even though this remained lower than in the unselected population (Table 5.8, Fig. 5.7). The very low pressure of 1.2 mg CBZ (50-60% mortality) applied to Population E, resulted in a smaller, though still significant increase in ED_{50} (Table 5.11, Fig. 5.9).

During the course of the study, no marked differences were evident between log dosage-probit line slopes for adults from the selected isolates and for those from the unselected population.

As the adult dose response assay showed relatively high repeatability (3.3.2), the results for selected isolates tested at different times have been directly compared in Table 5.12. Nevertheless, because the responses of worms differ in male and female mice (3.2.5), these comparisons were confined to assays conducted in mice of the same sex. Of the generations tested in male mice (Table 5.12a), responses were similar with the exceptions of the fourth generations of Populations A and E, subjected to very high or low selection intensities respectively. Within those generations tested in female mice (Table 5.12b), only the sixth generation of Population B differs significantly from the other isolates. The resistance ratios for all five selected populations are shown in Fig. 5.11.

In general the selected isolates showed no loss of fitness in the attributes examined. Abilities of eggs to hatch in 0.1% saline were generally similar in the selected and unselected lines, except in the last two generations of Population D, and the final generation of Population B, where the proportions hatching were significantly lower ($P = 0.05$) (Table 5.13).

A rigorous test of the number of eggs reaching the L_3 stage was only made on the eighth generation of Population A. In this case, 27.66% of the eggs were recovered as L_3 against 29% for the unselected population. These values were not markedly different ($P = 0.05$) when proportions of eggs and L_3 were compared by contingency table. Less detailed observations of the other isolates did not suggest radical changes either.

The infectivity of the L_3, measured by the percentage recovered as adults from untreated mice, was also similar in selected and unselected lines (Table 5.14). Recoveries of male and female worms remained constant at about 50% of each sex for all isolates and are not tabulated. Egg output by the females was similar in the unselected population and the two isolates tested (Table 5.15).

5.4 *DISCUSSION*

Resistance to CBZ developed rapidly in the adults of *N. dubius*, within four generations in most isolates (Fig. 5.11).

Table 5.2 Population A: Egg Hatch Responses

Generations of selection	Number of concentrations	EC_{50} (µg CBZ/ml)	(S.E.)	Slope	(S.E.)	Resistance ratio
1st	8	5.09	(0.06)***	6.30	(0.20)^{ns}	0.94
unselected	9	5.42	(0.03)	5.87	(0.12)	
2nd	7	4.67	(0.03)***	6.74	(0.15)^{ns}	1.12
unselected	7	4.17	(0.04)	5.70	(0.16)	
3rd	10	6.54	(0.05)***	7.73	(0.22)**	0.87
unselected	9	7.48	(0.11)	6.33	(0.34)	
4th	19	9.08	(0.17)***	5.67	(0.38)*	1.30
unselected	6	6.99	(0.11)	6.32	(0.30)	
5th	7	7.85	(0.10)***	6.23	(0.24)^{ns}	1.09
unselected	7	7.18	(0.06)	5.98	(0.13)	
6th	5	9.46	(0.20)*	6.60	(0.60)^{ns}	0.96
unselected	6	9.89	(0.33)	5.93	(0.74)	
7th	6	10.73	(0.16)*	7.32	(0.46)**	1.03
unselected	6	10.37	(0.24)	5.19	(0.42)	
8th	5	5.52	(0.10)^{ns}	9.01	(0.66) *	1.03
unselected	5	5.35	(0.23)	6.41	(0.75)	

Eggs from adults selected at a 98^{+}% mortality level for four generations then at a 95-98% mortality level
EC_{50}s compared by Student's t-test and slopes compared by analysis of covariance:

^{ns} not significantly different from the unselected population at P = 0.05
* significantly different from the unselected population at P = 0.05
** significantly different from the unselected population at P = 0.01
*** significantly different from the unselected population at P = 0.001

Table 5.3 Population A: Dose Responses of Adults in Male Mice

Generations of selection	Selection pressure (% mortality)	Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)	Resist -ance ratio
4th	98^+%	4	1.26	$(0.12)^{ns}$	7.55	(1.69)	1.01
	unselected	4	1.25	(0.13)	7.55	(1.69)	
8th	95-98%	7	1.89	(0.44)**	4.31	(1.38)	1.75
	unselected	6	1.08	(0.43)	3.45	(1.23)	

ED_{50} s compared by Student's t-test: ns not significantly different from the unselected population
** significantly different from the unselected population at $P = 0.01$

Slopes compared by analysis of covariance: neither significantly different from the unselected population at $P = 0.05$

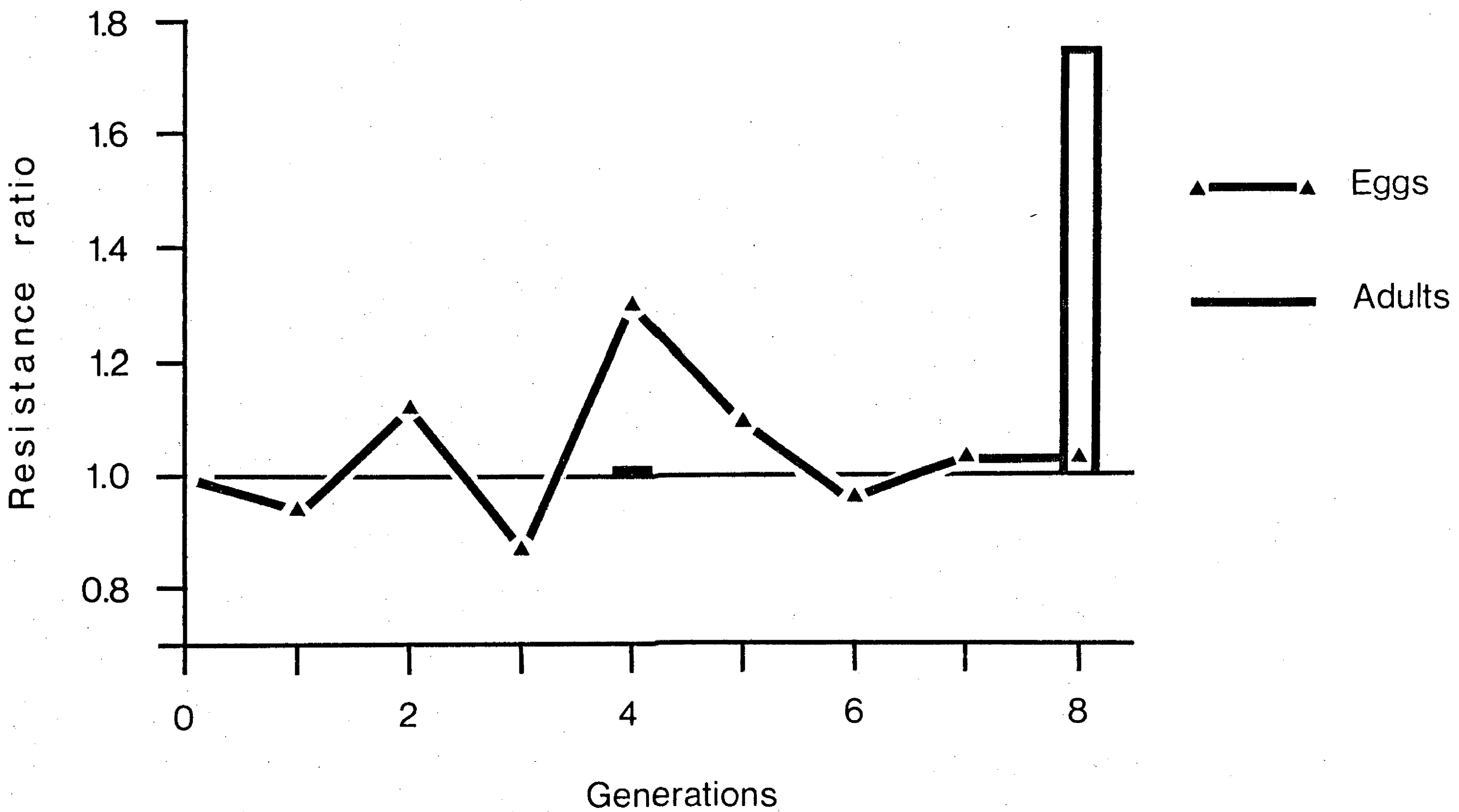


FIGURE 5.1 POPULATION A: COMPARISON OF RESISTANCE RATIOS FOR EGGS AND ADULTS

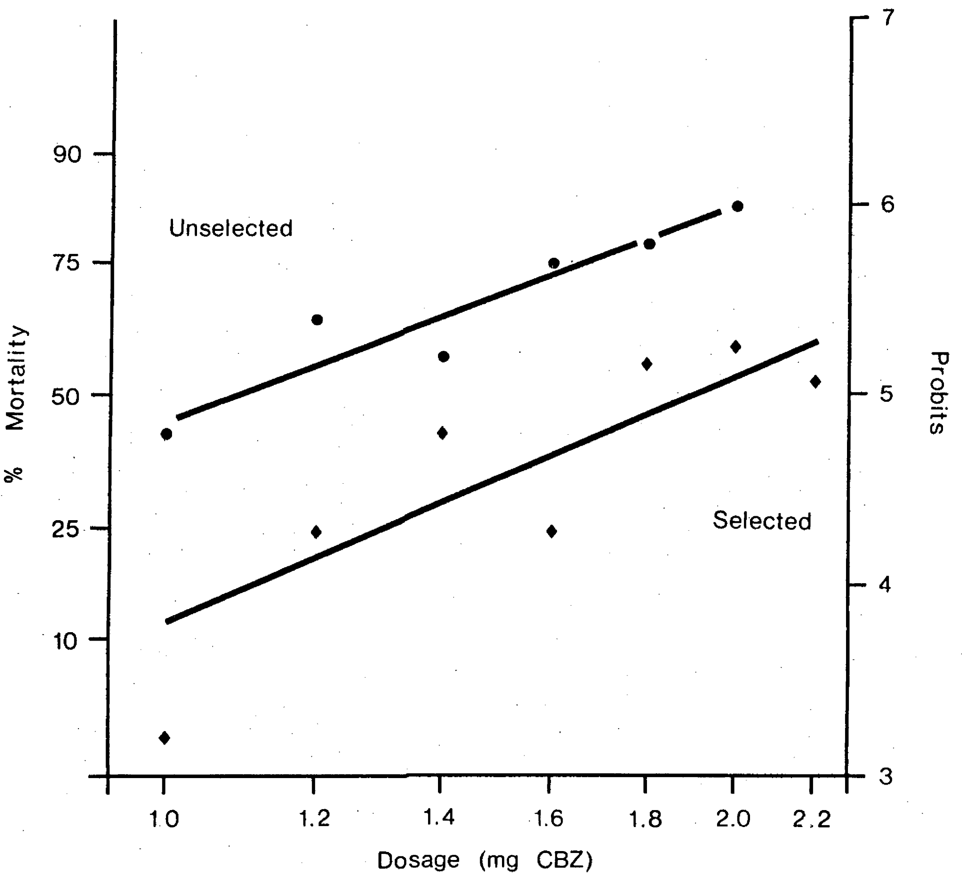


FIGURE 5.2 POPULATION A: DOSAGE MORTALITY LINES FOR ADULTS FROM THE EIGHTH GENERATION AND THE UNSELECTED POPULATION

Tested in male mice

Table 5.4 Population B: Egg Hatch Responses

Generations of selection	Number of concen-tractions	EC_{50} (µg CBZ/ml)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
3rd	7	8.44	(0.12)***	4.77	(0.20)^{ns}	1.07
unselected	7	7.86	(0.15)	4.49	(0.22)	
4th	7	8.73	(0.07)***	7.32	(0.24)***	0.95
unselected	7	9.17	(0.10)	6.04	(0.23)	
5th	6	3.97	(0.06)**	7.50	(0.35)*	0.98
unselected	7	4.06	(0.06)	6.32	(0.22)	
6th	6	5.62	(0.07)***	7.06	(0.25)^{ns}	0.77
unselected	7	7.31	(0.07)	7.36	(0.23)	

Eggs from adults selected at a 95-98% mortality level

EC_{50}s compared by Student's t-test and slopes compared by analysis of covariance:

^{ns} not significantly different from the unselected population at P = 0.05
* significantly different from the unselected population at P = 0.05
** significantly different from the unselected population at P = 0.01
*** significantly different from the unselected population at P = 0.001

Table 5.5 Population B: Dose Responses of Adults

Generations of selection	Selection pressure (% mortality)	Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
4th†	95-98%	4	1.64	(0.34)	4.45	(1.79)	1.40
	unselected	4	1.17	(0.18)	5.53	(1.45)	
6th††	95-98%	5	0.91	(0.43)	5.72	(2.21)	1.42
	unselected	8	0.64	(0.11)	4.04	(0.57)	

† Fourth generation tested in male mice
ED_{50}s compared by Student's t-test: significantly different from the unselected population at $P = 0.05$

†† Sixth generation tested in female mice

Relative susceptibility compared by analysis of covariance: not significantly different from the unselected population at $P = 0.05$

Slopes for both generations compared by analysis of covariance: neither significantly different from the unselected population at $P = 0.05$

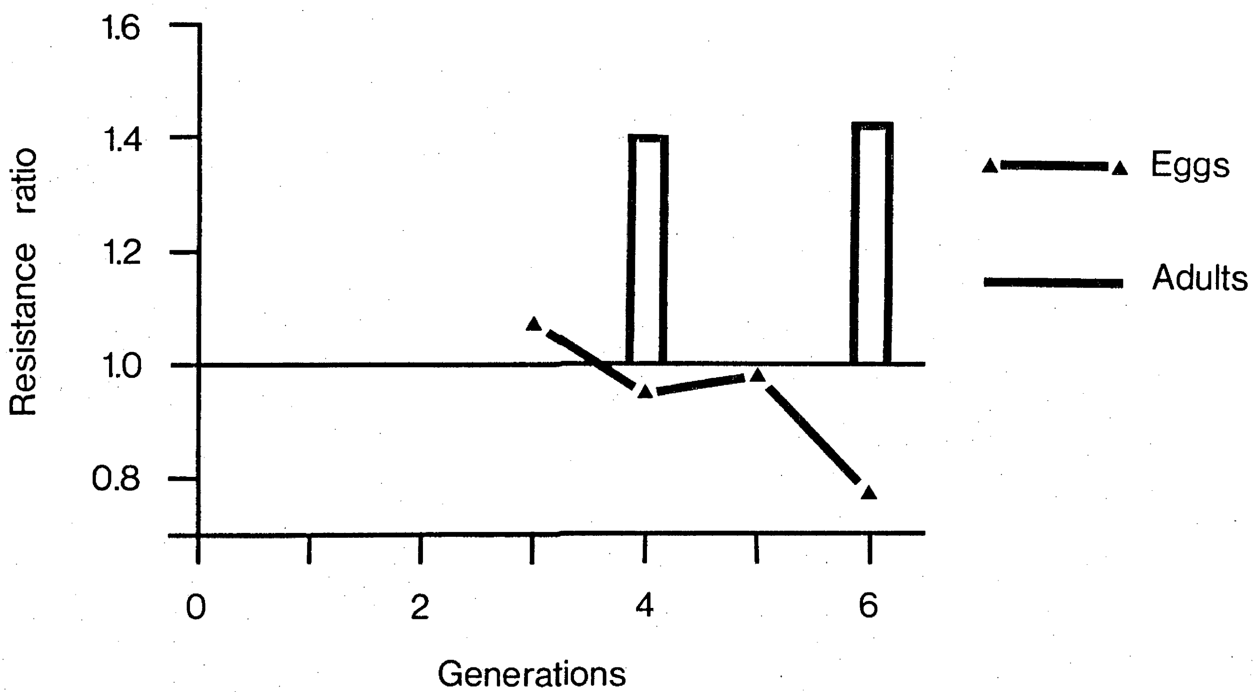


FIGURE 5.3 POPULATION B: COMPARISON OF RESISTANCE RATIOS FOR EGGS AND ADULTS

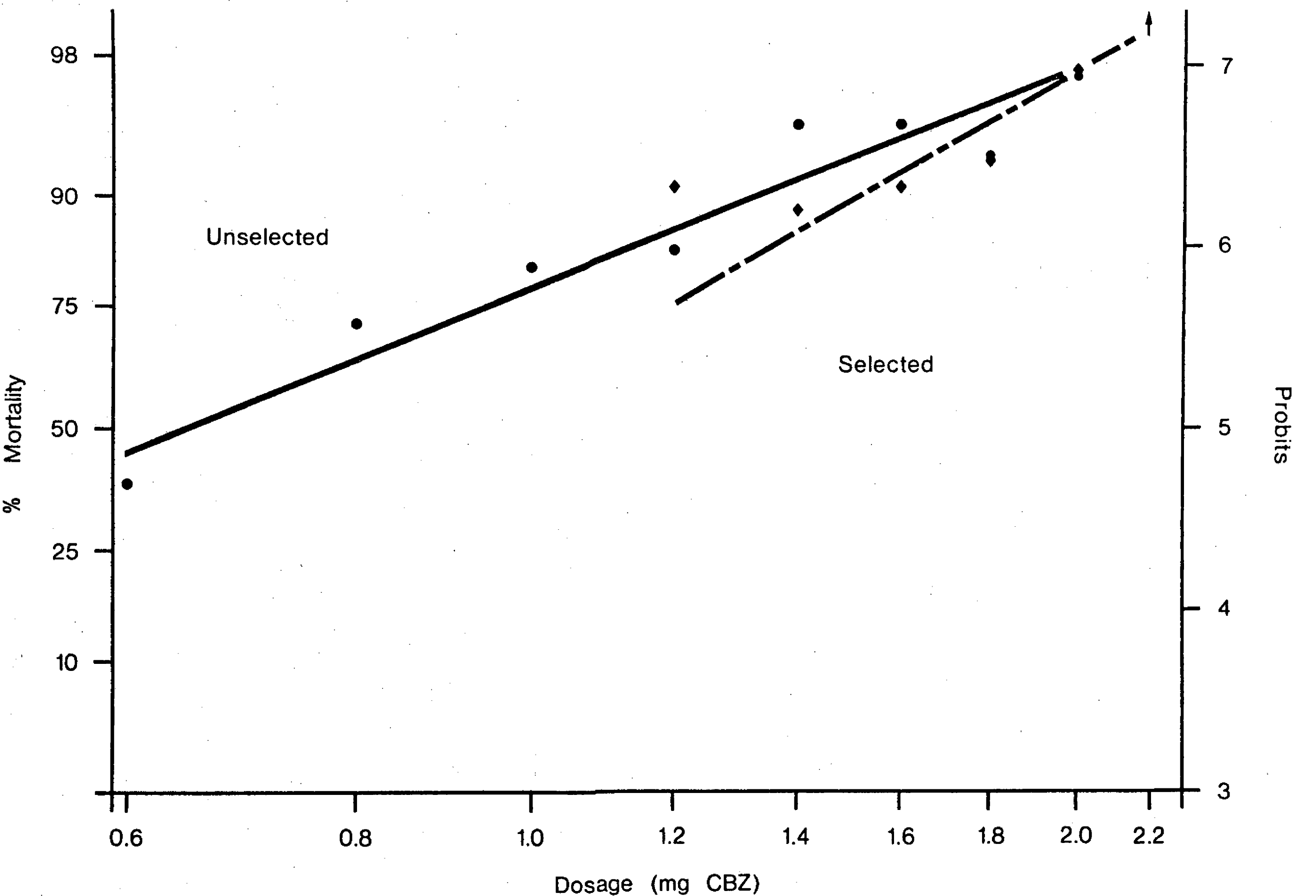


FIGURE 5.4 POPULATION B: DOSAGE MORTALITY LINES FOR ADULTS FROM THE SIXTH GENERATION AND THE UNSELECTED POPULATION

Tested in female mice

Table 5.6 Population C: Egg Hatch Responses

Generations of selection	Number of concen-trations	EC_{50} (µg CBZ/ml)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
1st	8	7.49	(0.21)^{ns}	6.52	(0.51)^{ns}	1.02
unselected	7	7.31	(0.32)	7.10	(0.43)	
2nd	7	11.45	(0.25)***	8.69	(1.23)**	1.16
unselected	6	9.89	(0.33)	5.93	(0.74)	
3rd	7	11.46	(0.15)***	6.93	(0.41)*	1.11
unselected	6	10.37	(0.24)	5.19	(0.42)	
4th	5	5.29	(0.07)*	6.89	(0.27)^{ns}	0.97
unselected	5	5.45	(0.10)	7.91	(0.53)	

Eggs from adults selected at a 95-98% mortality level
EC_{50} s compared by Student's t-test and slopes compared by analysis of covariance:

^{ns} not significantly different from the unselected population at $P = 0.05$
* significantly different from the unselected population at $P = 0.05$
** significantly different from the unselected population at $P = 0.01$
*** significantly different from the unselected population at $P = 0.001$

Table 5.7 Population C: Dose Response of Adults in Female Mice

Generations of selection	Selection pressure (% mortality)	Number of dose levels	ED_{50} (mg CBZ) (S.E.)	Slope (S.E.)	Resist-ance ratio
4th	95-98%	6	1.21 (0.35)	5.24 (1.68)	1.89
	unselected	8	0.64 (0.11)	4.04 (0.57)	

ED_{50} s compared by Student's t-test: significantly different from the unselected population at $P = 0.01$

Slopes compared by analysis of covariance: not significantly different from the unselected population at $P = 0.05$

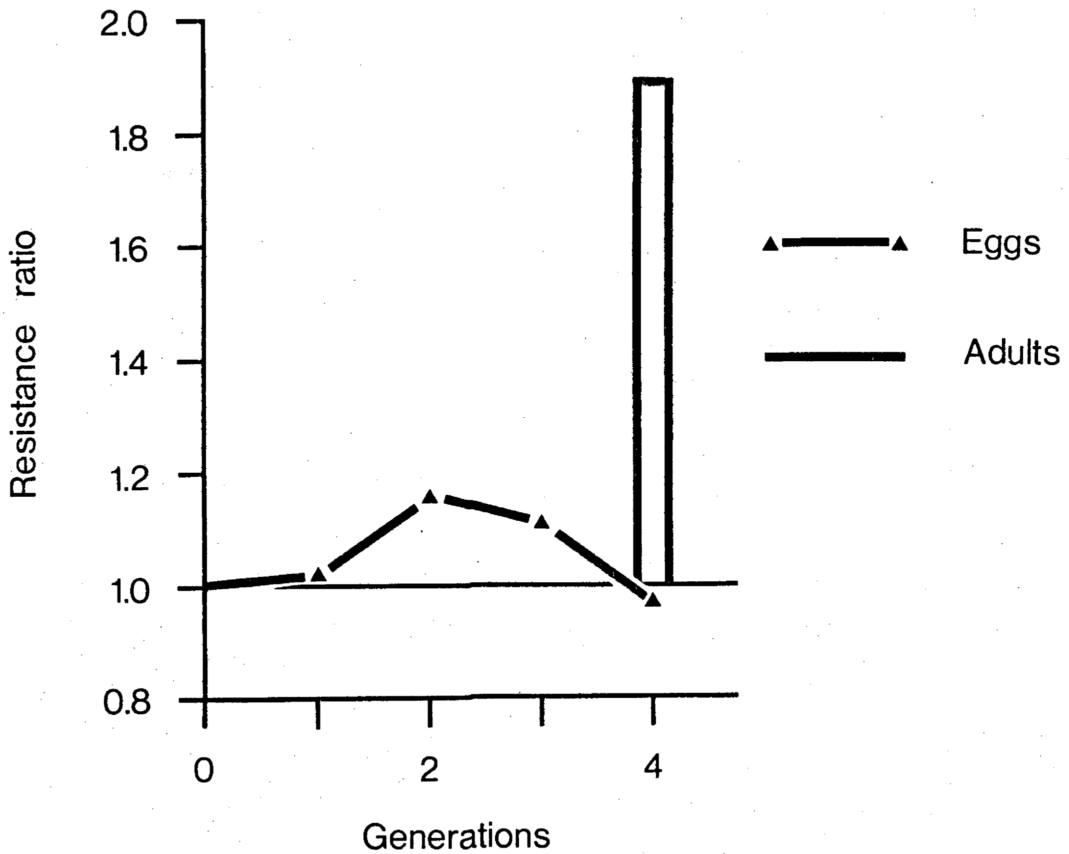


FIGURE 5.5 POPULATION C: COMPARISON OF RESISTANCE RATIOS FOR EGGS AND ADULTS

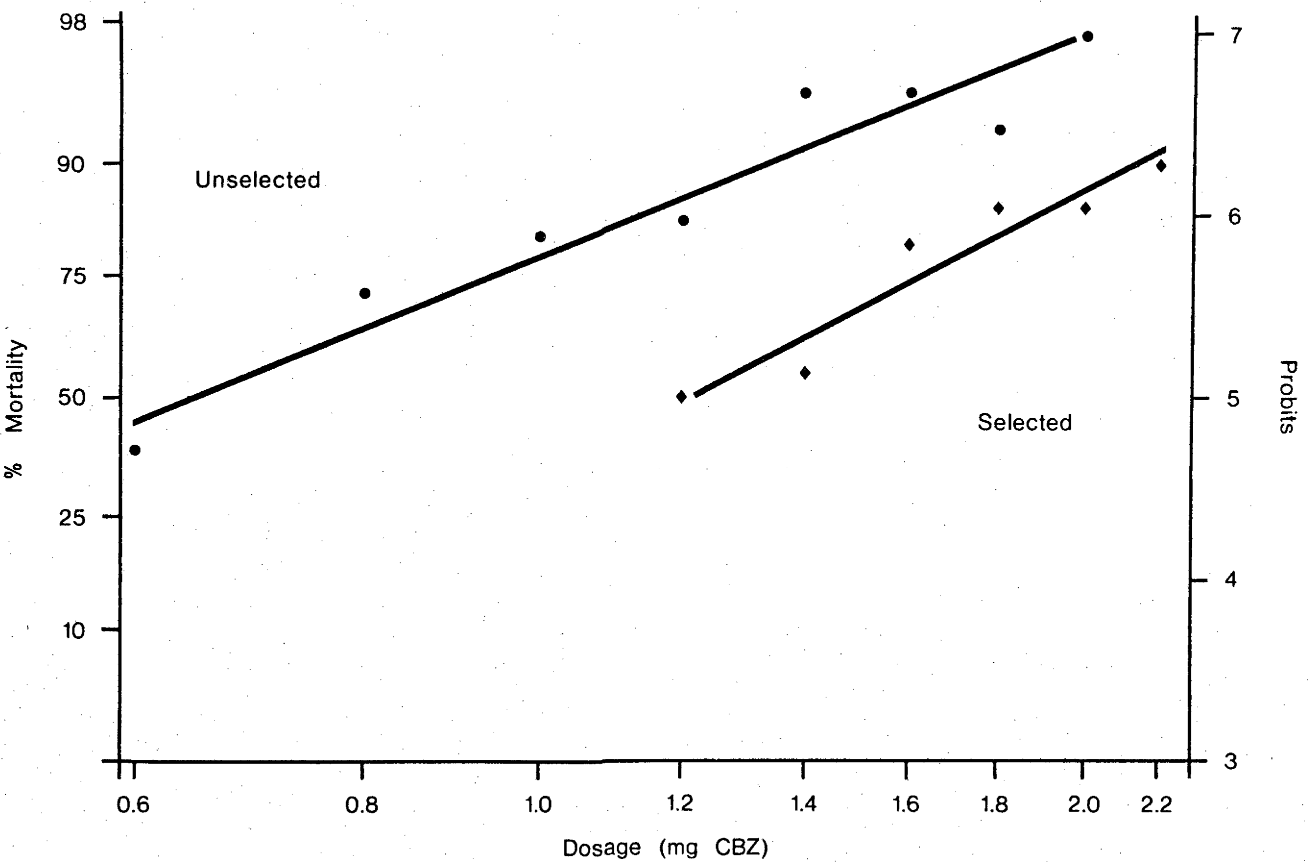


FIGURE 5.6 POPULATION C: DOSAGE MORTALITY LINES FOR ADULTS FROM THE FOURTH GENERATION AND THE UNSELECTED POPULATION

Tested in female mice

Table 5.8 Population D: Egg Hatch Responses

Generations of selection	Number of concen-trations	EC_{50} (µg CBZ/ml)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
1st	6	4.28	(0.08)	7.42	$(0.36)^{ns}$	1.04
unselected	7	4.10	(0.06)	6.41	(0.28)	
3rd	7	9.78	(0.02)	4.44	(0.22)*	1.24
unselected	7	7.86	(0.15)	4.49	(0.22)	
4th	6	11.45	(0.11)	5.83	(0.21)***	1.23
unselected	7	9.17	(0.10)	6.04	(0.23)	
5th	7	4.48	(0.06)	6.15	$(0.19)^{ns}$	1.10
unselected	7	4.06	(0.06)	6.32	(0.22)	
6th	7	8.27	(0.08)	8.09	$(0.25)^{ns}$	1.13
unselected	7	7.31	(0.07)	7.36	(0.23)	

Eggs from adults selected at an 85-95% mortality level
EC_{50}s compared by Student's t-test: all EC_{50}s significantly different from the unselected population at $P = 0.001$

Slopes compared by analysis of covariance:
- ^{ns} not significantly different from the unselected population at $P = 0.05$
- * significantly different from the unselected population at $P = 0.05$
- ** significantly different from the unselected population at $P = 0.001$

Table 5.9 Population D: Dose Responses of Adults

Generations of selection	Selection pressure (% mortality)	Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
4th[†]	85-95%	4	1.81	(0.41)	6.27	(2.52)	1.55
	unselected	4	1.17	(0.18)	5.53	(1.45)	
6th[††]	85-95%	6	1.09	(0.23)	3.15	(0.96)	1.70
	unselected	8	0.64	(0.11)	4.04	(0.57)	

[†] Fourth generation tested in male mice
ED_{50}s compared by Student's t-test: significantly different from the unselected population at $P = 0.05$

[††] Sixth generation tested in female mice
Relative susceptibility compared by analysis of covariance: significantly different from the unselected population at $P = 0.01$

Slopes for both generations compared by analysis of covariance: neither significantly different from the unselected population at $P = 0.05$

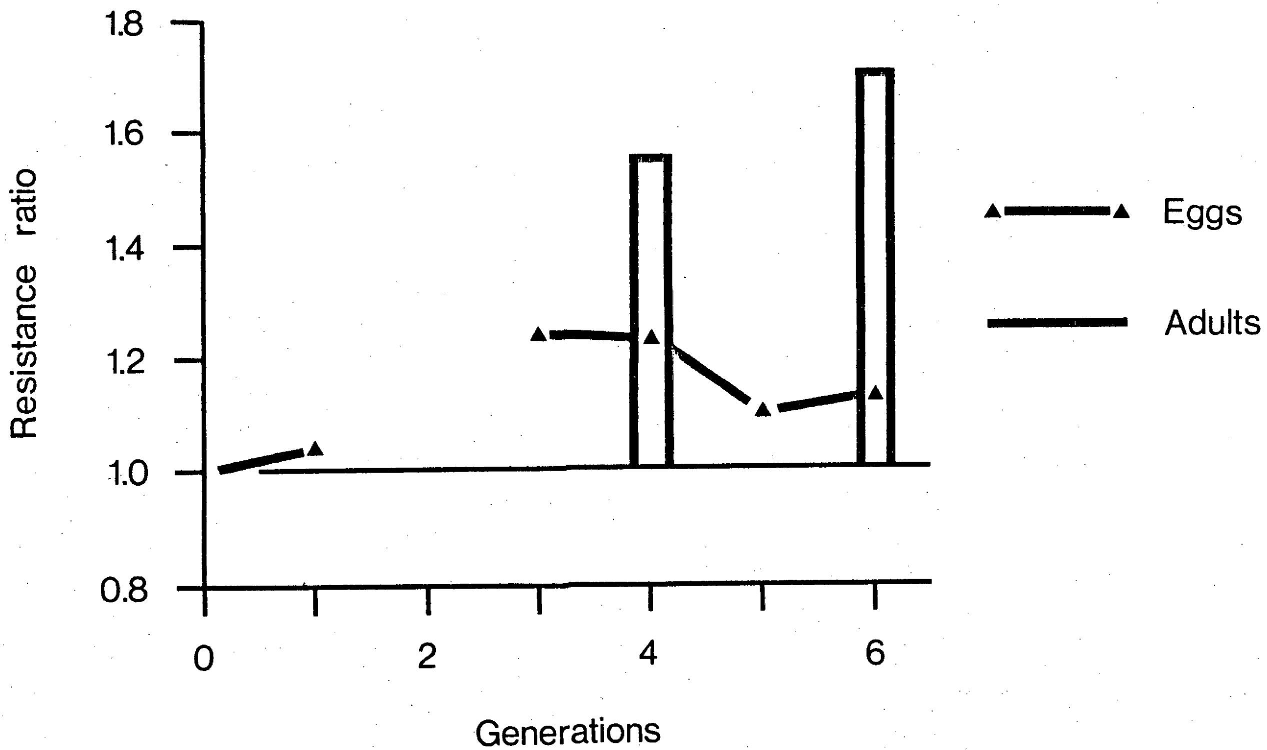


FIGURE 5.7 POPULATION D: COMPARISON OF RESISTANCE RATIOS FOR EGGS AND ADULTS

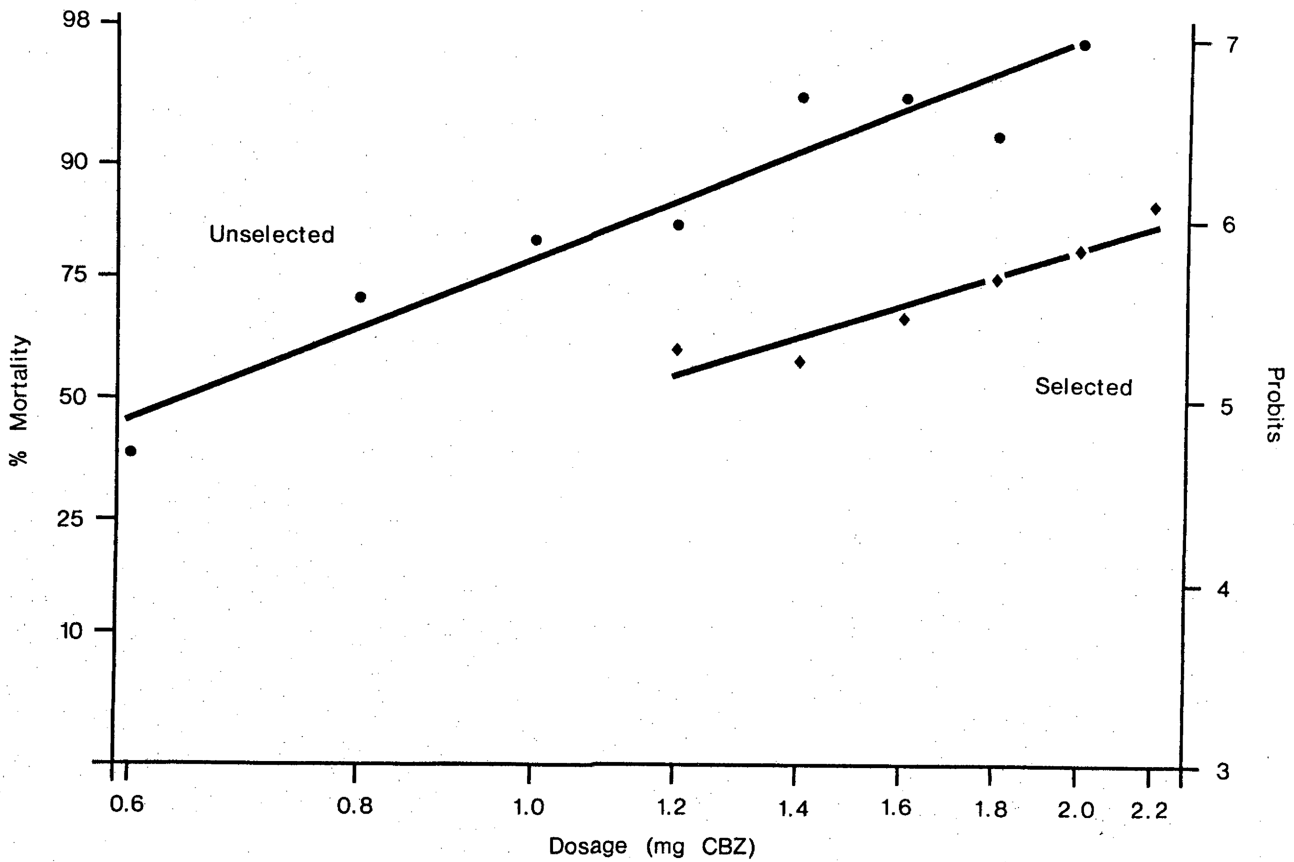


FIGURE 5.8 POPULATION D: DOSAGE MORTALITY LINES FOR ADULTS FROM THE SIXTH GENERATION AND THE UNSELECTED POPULATION

Tested in female mice

Table 5.10 Population E: Egg Hatch Responses

Generations of selection	Number of concen-trations	EC_{50} (µg CBZ/ml)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
1st	7	8.88	(0.11)	6.97	$(0.32)^{ns}$	0.91
unselected	6	9.72	(0.29)	6.01	(0.70)	
2nd	7	9.34	(0.15)	7.14	$(0.44)^{ns}$	0.94
unselected	6	9.89	(0.33)	5.93	(0.74)	
3rd	7	10.84	(0.08)	6.79	$(0.22)^{ns}$	1.05
unselected	6	10.37	(0.24)	5.19	(0.42)	
4th	7	6.14	(0.06)	6.44	(0.16)***	0.84
unselected	7	7.31	(0.07)	7.36	(0.23)	

Eggs from adults selected at a 50-60% mortality level.
EC_{50}s compared by Student's t-test: all significantly different from the unselected population at $P = 0.001$
Slopes compared by analysis of covariance: ns not significantly different from the unselected population at $P = 0.05$
*** significantly different from the unselected population at $P = 0.001$

Table 5.11 Population E: Dose Response of Adults in Male Mice

Generations of selection	Selection pressure (% mortality)	Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)	Resist -ance ratio
4th	50-60%	6	1.39	(0.11)	3.88	(1.11)	1.29
	unselected	6	1.08	(0.43)	3.45	(1.23)	

ED_{50} s compared by Student's t-test: Populations significantly different at $P = 0.05$

Slopes compared by analysis of covariance: not significantly different from unselected population at $P = 0.05$

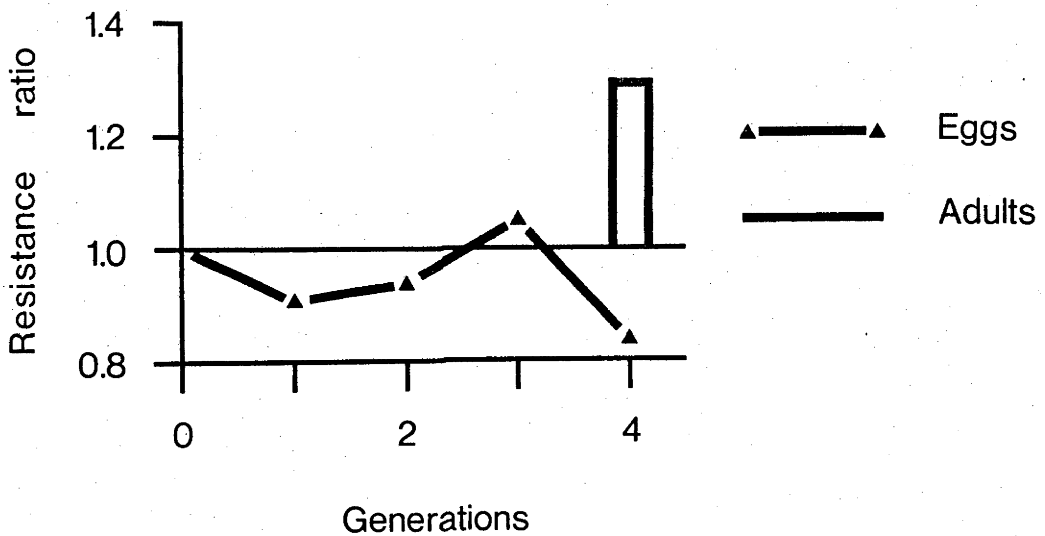


FIGURE 5.9 POPULATION E: COMPARISON OF RESISTANCE RATIOS FOR EGGS AND ADULTS

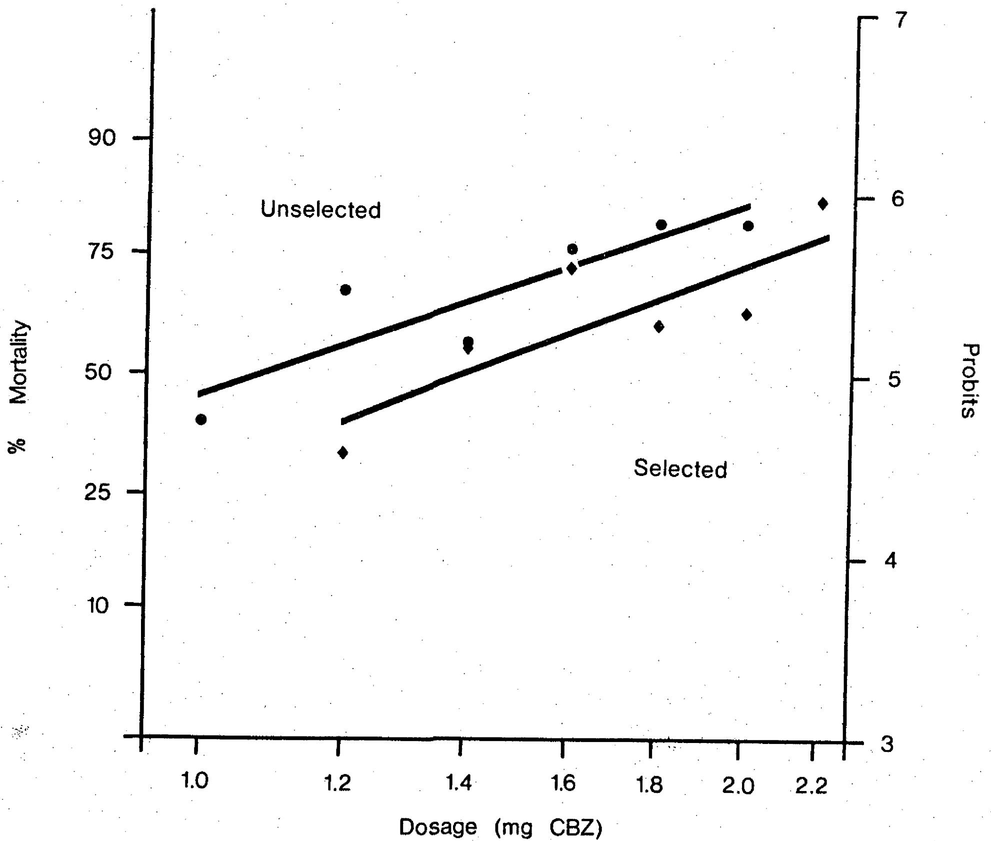


FIGURE 5.10 POPULATION E: DOSAGE MORTALITY LINES FOR ADULTS FROM THE FOURTH GENERATION AND THE UNSELECTED POPULATION Tested in male mice

Table 5.12 Comparison of Dose Responses for Adults from Five Selected Populations

Population	Generations of Selection	Selection pressure (% mortality)	Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
a) Tested in male mice								
A	4th	98†	4	1.26	(0.12)†	7.55	(1.69)††	1.01
	8th	95-98	7	1.89	(0.44)	4.31	(1.38)	1.75
B	4th	95-98	4	1.64	(0.34)	4.45	(1.79)	1.40
D	4th	85-95	4	1.81	(0.41)	6.27	(2.52)	1.55
E	4th	50-60	6	1.39	(0.11)†	3.88	(1.11)††	1.29
b) Tested in female mice								
B	6th	95-98	5	0.91	(0.43)†	5.72	(2.21)	1.42
C	4th	95-98	6	1.21	(0.35)	5.24	(1.68)	1.89
D	6th	85-95	6	1.09	(0.23)	3.15	(0.96)	1.70

ED_{50} s of populations tested in male mice compared by Student's t-test and relative susceptibilities of those tested in female mice compared by analysis of covariance: † significantly different at $P < 0.05$ from all other populations in mice of the same sex

Slopes compared by analysis of covariance: †† significantly different at $P = 0.05$ from other values with the same superscript. No significant differences between other populations in mice of the same sex.

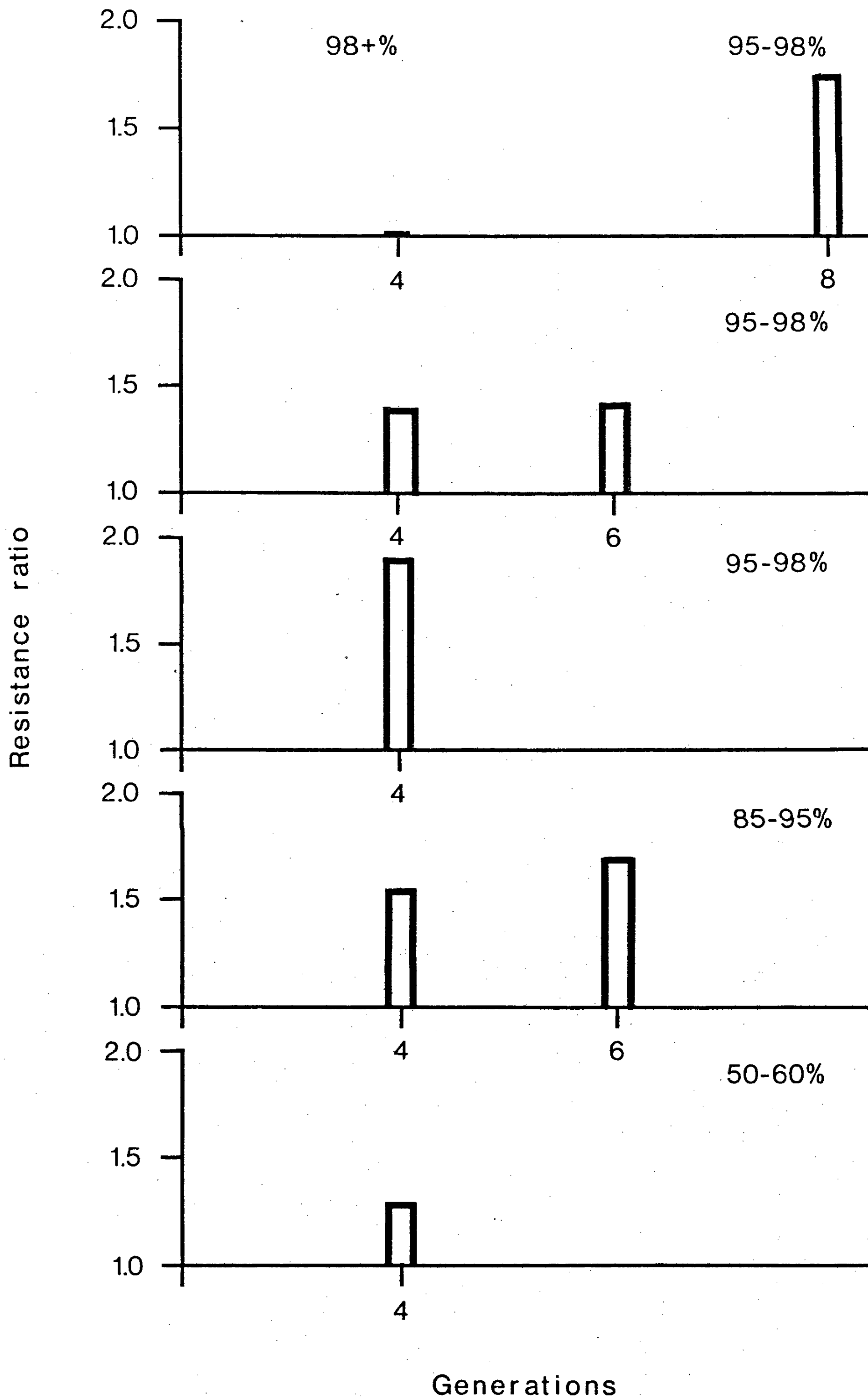


FIGURE 5.11 COMPARISON OF RESISTANCE RATIOS FOR ADULTS OF ALL FIVE ADULT-SELECTED POPULATIONS

Table 5.13 Fitness of Adult-selected Populations: the percentage of eggs able to hatch in 0.1% saline at 22°C

Population	Generations of selection							
	1st	2nd	3rd	4th	5th	6th	7th	8th
A	89.47*	96.37^{ns}	96.95^{ns}	94.21^{ns}	97.26^{ns}	82.42*	96.86^{ns}	92.44^{ns}
unselected	97.19	95.46	98.43	92.06	97.42	94.44	97.66	95.31
B			93.97^{ns}	94.93^{ns}	93.14^{ns}	95.24*		
unselected			94.52	95.03	92.69	97.65		
C	94.92^{ns}	88.57^{ns}	95.02^{ns}	96.27^{ns}				
unselected	95.11	94.44	97.66	97.24				
D	89.73^{ns}		93.35^{ns}	92.70^{ns}	89.24*	91.92**		
unselected	92.69		94.52	95.03	92.69	97.65		
E	95.01^{ns}	94.94^{ns}	96.17^{ns}	97.00^{ns}				
unselected	94.21	94.44	97.66	97.65				

Proportions of eggs able to hatch compared by contingency table:

ns not significantly different from the unselected population at P = 0.05

* significantly different from the unselected population at P = 0.05

** significantly different from the unselected population at P = 0.01

Table 5.14 Fitness of Adult-selected Populations: the percentage of third stage larvae reaching maturity in untreated mice

Population	Generations of selection 4th	6th	8th
A	87.51	-	72.20
unselected	89.40	-	70.00
B	80.67	85.00†	-
unselected	84.80	84.33	-
C	80.00†	-	-
unselected	84.33	-	-
D	84.80	87.11†	-
unselected	84.00	84.33	-
E	71.80	-	-
unselected	70.00	-	-

† Tested in female mice, all others tested in males

Adult worms were recovered from 7-10 mice, 21 days after infection with 50 L_3

Proportions of L_3 recovered as adults compared by contingency table: no significant differences between selected and unselected populations at $P = 0.05$

Table 5.15 Fitness of Adult-selected Populations: the number of eggs/g faeces from mice infected with 50 third stage larvae

Population	Number of mice	Eggs/g fresh faeces mean†	(S.E.)
A (8th generation)	5	38,402	(4,351)
C (4th generation)	5	38,008	(4,753)
unselected	5	39,015	(4,460)

† Arithmetic means for counts over three consecutive days were compared by Student's t-test: no significant difference between populations at $P = 0.05$

Resistance ratios achieved in this time were up to 1.89. This response is only a little lower than that described by Kates' group for *H. contortus* where a resistance ratio of about 3 was obtained (Kates *et al.* 1973). That resistance developed in all five lines indicates a high frequency in the population of the allele or alleles responsible.

The intensity of selection proved important as had been expected from the work on insecticide resistance (Brown and Pal 1971; Crow 1957; Georghiou and Taylor 1977b). Of the pressures tried, those producing between 85 and 98% mortality were the most effective (Table 5.12, Fig. 5.11). A low selection pressure, such as that applied to Population E, would permit the survival of individuals with limited resistance. The interbreeding of these with more resistant individuals would retard the development of a highly resistant population. Intense selection, at greater than 98% mortality had no appreciable effect after four generations, yet the isolate retained the ability to respond when the selective dosage was lowered. Usually, only a few individuals survived, and the severe restriction of genetic variability imposed on the population could, as is the case with insects (Crow 1957), retard the development of resistance.

Before selection, resistance alleles should be relatively rare and so unlikely to be fully co-adapted to the main background genotypes within which they must function

(Wallace 1968). The re-sorting of the genome to accommodate alleles for resistance is thought to account for a lag phase commonly observed in the early stages of selection for insecticide resistance (Brown and Pal 1971; Milani 1960). Resistance levels in all *N. dubius* isolates may have been affected by this process. In the case of Population A, the phenomenon could have been exaggerated by the restriction of variability in alleles both conferring and contributing to resistance, and in those of the remaining genome. In retrospect, it might have been interesting to continue the more severe regimen applied to Population A in parallel with the lower selective dosage to see whether resistance emerged in any case.

Although the sixth generation of Population B had a resistance ratio of 1.42, slightly higher than for the fourth generation, the population's susceptibility did not differ significantly from that of the unselected population (Table 5.5). The standard error for the ED_{50} is disproportionately large for this isolate, and emphasizes the need for a more sensitive assay (3.3.2).

There was no evidence, in the areas examined, of the lethal effects, or losses of fitness, sometimes associated with insecticide resistance (Brown and Pal 1971): nor of any increase in fitness, as reported for one benzimidazole resistant strain of *H. contortus* (Kelly, Whitlock, Thompson, Hall, Martin and Le Jambre 1978). In general, resistant

individuals are expected to be less competitive than their susceptible counterparts. This has in fact been observed for some laboratory populations of benzimidazole resistant *H. contortus* (Cornish and Larkin, personal communication). In this study, fitness was measured by faecal egg counts, hatching rates, survival of free-living stages and infectivity. These criteria conform with classical definitions (Mettler and Gregg 1969). A more complete analysis could include aspects of the interaction with the host, such as those examined by Kelly's group. Probably the most conclusive test of fitness is whether the frequency of the resistant genotype alters once the selection pressure is removed (Mettler and Gregg 1969). After further selection, the possibility of such reversion would be an interesting line to investigate.

The genetic nature of resistance, and whether it differs in separate isolates from the same population, are important considerations in the design of control programmes (Crow 1957; Georghiou and Taylor 1976; Sawicki 1979). During selection, the behaviour of regression slopes can provide some information about these, albeit indirectly (Hoskins and Gordon 1956; Fig. 1.2). Similarities between the slopes of all selected isolates and the unselected population are consistent with the development of polyfactorial rather than monofactorial resistance. This may explain the seemingly complete lack of response in the early generations of Population A, because polygenic systems are particularly

sensitive to restrictions in genetic variability (Le Jambre 1978). Being indirect, this evidence should be treated cautiously.

Selection has spanned very few generations and background variability could mask the presence of a specific mechanism, as it does in the early stages of unselected resistance (Brown and Pal 1971). Even in later stages, analysis of slopes may be misleading. The behaviour of regression lines for adults from a TBZ resistant strain of *H. contortus* suggested that one, or at most a few, major loci were involved (Le Jambre *et al.* 1976). Nevertheless, genetic analysis later indicated resistance was inherited polyfactorially (Le Jambre, Royal and Martin 1979). Obviously the direct analysis is extremely important, and this aspect of the present study is described in Chapter VII.

The absence of any significant variation between isolates selected in the 85-98% mortality range (Table 5.12a, b), could suggest similarities in the resistance mechanism, or possibly even a common mechanism. Some differences in the levels might be expected, even for the latter, due to the influence of polygenic background variability (Crow 1957). More reliable evidence would of course depend on further selection and the complete genetic analysis of all isolates.

Selection of *N. dubius* was applied to the adults alone, so the development of resistance in their eggs should only be expected if the resistance mechanism functions in both stages. That this may not be the case with *N. dubius*, raises an interesting query about the exact relationship between eggs and adults of sheep trichostrongylids. Under normal drenching practices, it is probable that eggs and free-living juveniles are exposed to benzimidazoles on pasture. Theoretically they could therefore undergo simultaneous selection for resistance. At the time of writing, this possibility has not been tested in sheep parasites, although there is evidence suggesting some differences in the genes for TBZ resistance in eggs and adults of *H. contortus* (Martin, Le Jambre and Claxton 1981). The exact relationship between benzimidazole susceptibility in eggs and adults certainly warrants more detailed analysis. Of particular importance is whether eggs will respond to direct selection and this question is examined in the following chapter.

CHAPTER VI

SELECTION OF EGGS FOR RESISTANCE

6.1 *INTRODUCTION*

Selection of adult *Nematospiroides dubius* for cambendazole (CBZ) resistance, described in Chapter V, led to the development of resistance in the adults, but not in their eggs. This is interesting because the egg hatch assay appears to give a good practical indication of benzimidazole resistance in the adults of sheep trichostrongylids (Coles and Simpkin 1977; Le Jambre 1976).

Relationships between DDT resistance in adult and in juvenile insects have been demonstrated for some species of houseflies and mosquitoes (Brown and Pal 1971). Selection of adults or larvae can lead to the development of resistance in both stages, though not necessarily to the same extent. In houseflies, the level of resistance attained by adults was greater when juveniles rather than adults were selected (Decker and Bruce 1952). Although selection of adults or larval *Culex fatigans* also led to resistance in both stages, the level achieved was greatest in the stage to which pressure had been applied (Georghiou, Metcalf and Gidden 1966).

A number of factors are likely to influence the exact resistance relationship between various stages of the life cycle. Presumably, for there to be a relationship, the same resistance mechanism must operate in each stage.

There is no *a priori* reason why the resistance mechanism in trichostrongylid parasites should be the same in eggs and adults. Except for a short period, the two stages occupy separate environments, probably influencing many physiological and biochemical characteristics. Studies of the free-living stages of trichostrongylids have concentrated on the third stage larvae (L_3), which in *Haemonchus contortus*, possess metabolic capabilities similar to those of the adults (Barrett 1977; Bennett 1981; Ward, Schofield and Johnstone 1968). In *Ascaris lumbricoides*, there are however major differences in the metabolism of first stage larvae (which develop in the eggs outside the host) from that of the parasitic adults (Barrett 1977). Since CBZ selection of adult *N. dubius* did not significantly influence the susceptibility of their eggs (Chapter 5.4), the resistance mechanism may not function in the developing eggs, but it might still arise independently in the eggs if these are exposed to selection.

The benzimidazoles, including CBZ, are eliminated from the host's body, in the urine and faeces, either unchanged or as metabolites (Baer, Jacob and Wolf 1977; Düwell 1977; Keystone and Murdoch 1979; Vanden-Heuvel, Wolf, Arison, Buhs, Carlin, Ellsworth, Jacob,

Koniuszy, Smith, Trenner, Walker and Wolf 1978). Some metabolites, such as 5-hydroxythiabendazole from thiabendazole (Prichard, Steel and Hennessy, unpub. obs. in Prichard 1978), may themselves be anthelmintically active. Consequently, the free-living stages are probably exposed to selection on pasture.

Because resistance could arise independently in the eggs, through selection during the free-living phase of the parasites' life cycle, this possibility was examined. A method reproducing natural conditions in which all of these stages were exposed to CBZ in faecal cultures was chosen, and the results are presented in this chapter.

6.2 *METHODS*

6.2.1 *Preparation of cultures*

The method of culturing eggs in the presence of CBZ involved only slight modification to the standard technique described in Chapter II (2.2.2). Faeces were collected from five mice, each infected with 50 L_3 two to three weeks beforehand. Crushed ice was substituted for water in the collection tray to retard the development of the eggs, and the faecal pellets were removed every 12 hours.

Filtered CBZ solution (2.3.2) and distilled water were added to 5 ml of the softened faeces to give final concentrations of 4.2, 3.3 or 2.5 μg CBZ/ml in 7.5 ml of

slurry. Cultures of these slurries, and of controls without anthelmintic, were then prepared in the normal manner (2.2.2). Incubation periods of 14 days rather than seven days were used, because in the eggs, development was slower in the presence of CBZ (4.2.2). After 14 days, the numbers of L_3 recovered from each culture were estimated (2.2.3) to determine the effectiveness of selection. Control cultures contained 12,000 to 18,000 L_3 and the reductions in larval yield at each concentration are tabulated below (Table 6.1). These values refer to the first generation, and are expressed as percentages of the numbers recovered from the control cultures.

Table 6.1 Selection Pressures Applied to Eggs and Free-living Larvae in Faecal Culture

Population	Concentration of CBZ (µg/ml slurry)	% reduction in number of L_3 on first exposure
* ES A	4.2	90^+
ES B	4.2	90^+
ES C	4.2	90^+
ES D	3.3	80 - 90
ES E	2.5	50 - 60

* ES denotes egg-selected

The surviving L_3 were passaged to produce the next generation. Population ES A was selected for seven generations and Population ES B for six, using the same concentration of CBZ. Due to work pressures, the remaining isolates were only examined in the first generation.

6.2.2 *Assays*

An assay of the eggs produced by the survivors after they had reached maturity was performed at each generation (4.4). Adults were tested after the fourth and ninth generation of Population ES A, but because relatively low numbers of larvae were recovered from selective culture, a complete assay was not possible. Instead, only two or three dosage levels were tested. Other methods were not altered (3.4) and responses in selected and unselected populations were compared by contingency table (2.5.5).

6.2.3 *General fitness of the selected populations*

Three areas were examined for signs of reduced survival in the absence of anthelmintic. The viability of eggs was determined for each generation from the proportion which hatched in 0.1% NaCl. These were the controls for egg hatch assays and their preparation is described in Chapter IV (4.2.2). The survival of eggs through to L_3 was checked by the recoveries from standard faecal cultures (2.2.3), and the number of L_3 reaching maturity was monitored in the control mice from adult dose response tests (3.4). Population ES A was the only isolate in which the last two areas were examined.

6.3 *RESULTS*

These experiments showed that *N. dubius* can respond to selection applied to the eggs and free-living juveniles. Eggs from the first generation of all selected isolates, with the exception of Population ES E, were less susceptible than those from the unselected population (Table 6.2). This relationship was maintained in both of the isolates which were exposed to further selection (Tables 6.3 and 6.5; Fig. 6.1) and, while the EC_{50} of the unselected population increased, EC_{50} s of the selected isolates were significantly greater (P = 0.05) at each generation. There was, however, no change in the susceptibility of adults from Population ES A over this period (Table 6.4).

During the early stages of selection, there does not appear to be a steady relationship between slopes in the selected and unselected populations (Tables 6.3 and 6.5). In most generations there were no significant differences (P = 0.05) but from the sixth generation of Population ES A and the fifth of Population ES B, the regression lines for the selected isolates were steeper.

In the absence of CBZ, the survival of eggs was the same in selected and unselected populations for most generations (Table 6.6). The proportions of eggs surviving to the L_3 stage, and of L_3 reaching maturity, were lower in Population ES A than in the unselected line (Table 6.7).

Table 6.2 Comparison of Egg Hatch Responses for the First Generation of Five Selected Populations

Population	Selection pressure *	Number of concen-trations	EC_{50} (μg CBZ/ml)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
	%						
ES A	90†	10	8.16	(0.21)	6.23	$(0.72)^{ns}$	1.21
unselected		10	6.75	(0.13)	6.37	(0.44)	
ES B	90†	16	6.83	(0.07)	6.11	$(0.22)^{ns}$	1.03
unselected		18	6.62	(0.08)	6.08	(0.28)	
ES C	90†	5	6.63	(0.08)	9.45	(1.6) *	1.07
ES D	80-90	5	7.10	(0.04)	6.78	$(1.09)^{ns}$	1.15
ES E	50-60	5	5.98	(0.02)	5.10	(0.63)*	0.97
unselected		5	6.18	(0.01)	7.47	(0.73)	

* Percentage reduction in recovery of L_3.

EC_{50} s compared by Student's t-test: All EC_{50} s significantly different from the unselected population at P = 0.001

Slopes compared by analysis of covariance: ^{ns} not significantly different from the unselected population at P = 0.05
* significantly different from the unselected population at P = 0.05

Table 6.3 Population ES A: Egg Hatch Responses

Generations of selection	Number of concen-trations	EC_{50} (μg CBZ/ml)	(S.E.)	Slope	(S.E.)	Resist-ance ratio
1st	10	8.16	(0.21)	6.23	$(0.72)^{ns}$	1.21
unselected	10	6.75	(0.13)	6.37	(0.44)	
2nd	15	7.63	(0.14)	7.25	$(0.71)^{ns}$	1.15
unselected	18	6.62	(0.08)	6.08	(0.28)	
3rd	10	9.54	(0.19)	6.34	$(0.51)^{ns}$	1.19
unselected	10	8.02	(0.20)	6.22	(0.46)	
4th	7	8.21	(0.26)*	5.66	(0.53)*	1.04
unselected	7	7.86	(0.16)	7.65	(0.49)	
5th	7	8.07	(0.20)	5.85	(0.44)***	1.06
unselected	6	7.58	(0.12)	7.46	(0.39)	
6th	9	9.67	(0.12)	6.99	$(0.37)^{ns}$	1.10
unselected	9	8.80	(0.07)	6.60	(0.19)	
7th	7	10.57	(0.27)	6.13	$(0.66)^{ns}$	1.11
unselected	7	9.54	(0.16)	5.40	(0.32)	

EC_{50}s compared by Student's t-test: * significantly different from the unselected population at P = 0.05; all other EC_{50}s significantly different from the unselected population at P = 0.001

Slopes compared by analysis of covariance:
- ns not significantly different from the unselected population at P = 0.05
- * significantly different from the unselected population at P = 0.05
- *** significantly different from the unselected population at P = 0.001

Table 6.4 Population ES A: Dose responses of adults

Generations of selection	Sex of mice	0 mg CBZ mean† (S.E.)	Number of worms surviving at each dosage: 1.2 mg CBZ mean (S.E.)	1.2 mg CBZ mortality %	1.4 mg CBZ mean (S.E.)	1.4 mg CBZ mortality %
4	males	34.8 (1.46)	24.6 (2.68)	29.3	11.2 (1.36)	68.4
unselected		44.7 (0.82)	30.6 (1.65)	31.6	14.1 (3.15)	68.5
8	females	30.6 (1.72)	4.2 (1.4)	86.9	1.5 (0.1)	95.1
unselected		42.2 (1.60)	7.1 (3.3)	83.2	2.3 (0.8)	94.5

† Arithmetic means of recoveries

Proportions of worms surviving treatment compared by contingency table: selected and unselected populations show no significant differences in response to CBZ at $P = 0.05$.

Table 6.5 Population ES B: Egg Hatch Responses

Generations of selections	Number of concen-trations	EC_{50} (μg CBZ/ml)	(S.E.)	Slope	(S.E.)	Resist-ance ratios
1st	16	6.83	(0.07)	6.11	(0.22)^{ns}	1.03
unselected	18	6.62	(0.08)	6.08	(0.28)	
2nd	10	8.98	(0.21)	5.98	(0.33)^{ns}	1.12
unselected	10	8.02	(0.20)	6.22	(0.46)	
3rd	7	9.14	(0.24)	5.47	(0.48)*	1.16
unselected	7	7.86	(0.16)	7.65	(0.49)	
4th	7	9.23	(0.17)	6.79	(0.50)**	1.22
unselected	6	7.58	(0.12)	7.46	(0.39)	
5th	10	9.45	(0.09)	6.97	(0.26)^{ns}	1.07
unselected	9	8.80	(0.07)	6.60	(0.19)	
6th	7	10.28	(0.19)	6.52	(0.51)^{ns}	1.08
unselected	7	9.54	(0.16)	5.40	(0.32)	

EC_{50}s compared by Student's t-test: all significantly different from the unselected population at $P = 0.001$

Slopes compared by analysis of covariance:
- ns not significantly different from the unselected population at $P = 0.05$
- * significantly different from the unselected population at $P = 0.05$
- ** significantly different from the unselected population at $P = 0.01$

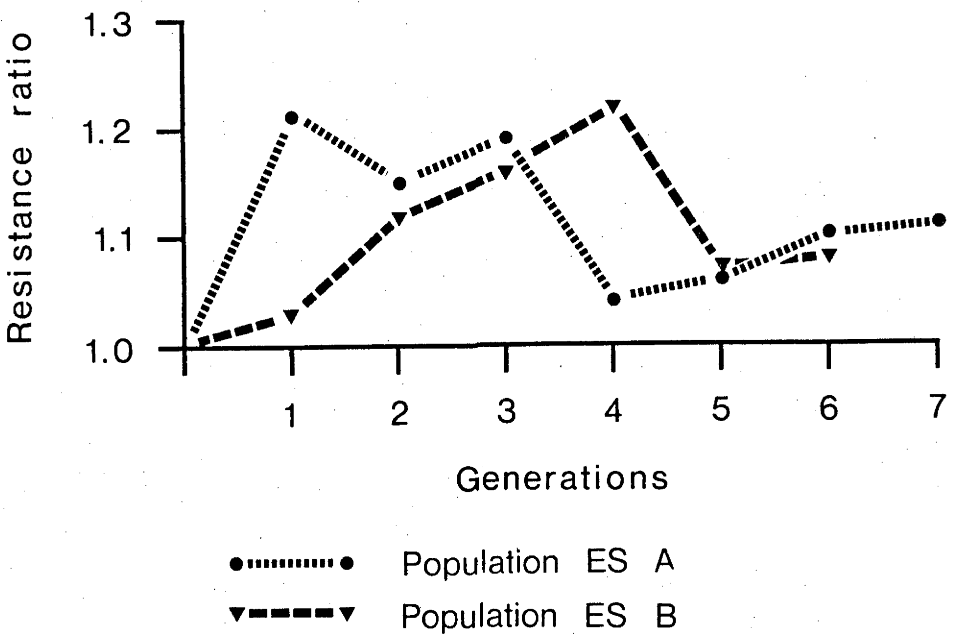


FIGURE 6.1 COMPARISON OF RESISTANCE RATIOS FOR EGGS OF POPULATIONS SELECTED AS EGGS AND FREE-LIVING LARVAE

Table 6.6 Fitness of Egg-selected Populations: the percentage of Eggs able to Hatch in 0.1% saline at 22° C

Population	Generations of Selection						
	1st	2nd	3rd	4th	5th	6th	7th
ES A	92.59	96.30	94.01	92.31	88.89***	93.84	95.00
unselected	96.98	96.83	93.31	97.67	96.72	96.08	94.19
ES B	97.54	93.99	94.19	90.68***	95.99	95.24	
unselected	96.83	93.39	97.67	96.72	96.08	94.19	

Proportions of eggs able to hatch compared by contingency table:

*** significantly different from the unselected population at P = 0.001

all other values not significantly different from the unselected populations at P = 0.05

Table 6.7 Fitness of Population ES A: the percentages of eggs becoming third stage larvae and of the latter reaching maturity in untreated mice.

Population	Generation of selection	% eggs recovered as L_3	% L_3 reaching maturity
ES A	4th	21.0	69.6
unselected		30.8	89.4
ES A	8th	-	61.2
unselected			84.4

L_3 were recovered from 3, 14 day old faecal cultures which originally contained about 40,000 - 45,000 eggs.

Adult worms collected from female mice, 21 days after infection with 50 L_3.

Proportions of eggs recovered as L_3 and of L_3 reaching adulthood were compared by contingency table: selected and unselected populations differ significantly on both accounts at $P = 0.05$.

6.4 *DISCUSSION*

The selection of eggs and free-living larvae led to an increase in the ability of these lines to hatch in cambendazole solutions, although the resistance ratios were much lower than those obtained for roundworms parasitic in sheep and horses. Resistance appeared to develop in the eggs and early larvae independently of the adult's response (Table 6.4). This may support the theory that different mechanisms are involved in the two phases of the life cycle (5.4), though it is also possible that resistance may be expressed in the adults with further selection. Although the levels of resistance attained under fluctuations, the eggs of Populations ES A and ES B remained consistently less susceptible than those of the unselected line (Fig. 6.1). This contrasts markedly with egg hatch results for adult-selected lines (Chapter V).

Changes in the EC_{50} of eggs from the unselected population were caused by a decline in CBZ concentration in the stock solution (2.3.2), as has been fully discussed in Chapter IV (4.3). This means that the absolute values of regression parameters cannot be compared between generations, so resistance ratios must be used.

The selection intensity may influence the level of resistance (Table 6.2), but examination of more than one generation is necessary for verification. Also, differences in EC_{50}, even where statistically significant ($P = 0.05$), should be treated cautiously (Martin, Le Jambre and Claxton 1981) and replicates have therefore been included in this study. The fact that Populations ES A and ES B were consistently less susceptible than the unselected population gives additional credence to the relationship.

It appears that the genotype or genotypes conferring an ability to hatch in the presence of CBZ, are frequent in this strain of *N. dubius* as four out of five isolates responded to selection. Only Population ES E, exposed to a low level of selection, failed to respond (Table 6.2). However, the levels achieved in Populations ES B and ES C were very low in the first generation. With continued selection, the two isolates in the long term study appear to have become more homogeneous for egg resistance, as indicated by their steeper regression lines (Tables 6.3 and 6.5). Further analysis is necessary to confirm this trend.

Selection had no apparent effect on the viability of eggs in the absence of CBZ (Table 6.6), but it may have reduced fitness in some other aspects. In Population ES A, the proportion of eggs surviving through to L_3 and the proportion of L_3 reaching maturity were both reduced (Table 6.7). Although this contrasts with similar tests of the adult selected lines, some loss in fitness is not uncommon in cases of insecticide resistance (Crow 1957; Brown and Pal 1971; Sawicki 1979).

The results of exposing *N. dubius* to CBZ in faecal culture show that resistance can develop in eggs under these conditions. They do not, however, give any indication of where selection is acting to produce the observed response. It is not known if selection of larvae affects the suscepti-bility of eggs, but both are killed by CBZ. Another unknown

factor is whether CBZ kills the bacterial food source of the first and second stage larvae. If these stages were subjected to starvation, reducing the population size and therefore its variability, this could retard the development of resistance.

A number of questions implicit in the hypothesis remain to be answered. The effects of simultaneously selecting adults and juveniles, for instance, might be tested. Furthermore, the study used CBZ solution rather than its metabolites as the selective agent. Faeces from uninfected mice treated with CBZ could have been used, but would not have allowed accurate measurement of the concentration in the cultures.

The lack of a relationship between the development of resistance in eggs and adults of *N. dubius* may not be universal in the trichostrongylids. Whether resistance mechanisms are the same in eggs and adults of other species can only be determined by further testing. For sheep trichostrongylids, the ability of eggs to hatch in the presence of benzimidazoles appears to be related to adult resistance (Coles and Simpkin 1977; Le Jambre 1976). However, the work reported here does question the assumption that both forms of resistance always arises in response to the same form of selection.

Southcott (1980) has raised the point that anthelmintic contamination could reduce pasture productivity. This contamination of the pasture could also select free-living stages for resistance to the anthelmintic. Le Jambre's group has suggested that in *H. contortus*, there may be differences in the genes controlling resistance in eggs and adults (Martin, Le Jambre and Claxton 1981). The strain which they used was originally a resistant field isolate (Le Jambre, Southcott and Dash 1976), so has probably in the past been subjected to selection on pasture as well as in the sheep, and consists of individuals carrying both sets of alleles. If most resistant adults already carried the alleles for egg resistance, there may not have been any extensive loss of these alleles from the population over the 20 or so generations that have been exposed to laboratory selection. As Le Jambre's group points out, variation noted in egg-hatch results may come from a correlated response rather than the selected trait (Martin *et al.* 1981).

The argument could apply equally well to the other two species studied extensively in the laboratory. Both *Ostertaqia circumcincta* and *Trichostrongylus colubriformis* were also exposed to anthelmintics before isolation from the field though both appeared to be susceptible before selection was applied (Le Jambre *et al.* 1977, 1978a, b).

Alternatively, if resistance mechanisms are the same or similar in the eggs and adults of sheep trichostrongylids, then the work reported here has important connotations for the design of control programmes. Selection in this case

could work in both directions, with that on free-living stages contributing to the rate at which anthelmintic resistance develops in the adults. The contribution need not be equal and the intensity of selection applied to adult parasites is likely to be far greater. However, in studies with insecticides, selection of larvae can be highly effective in producing resistant adults (Brown and Pal 1971). Reduction of the extent of refugia, areas where individuals can escape exposure (Comins 1977; Georghiou and Taylor 1976, 1977a), could then be an additional effect of pasture contamination by anthelmintics. The existence of refugia for free-living stages has been suggested as an explanation of why anthelmintic resistance has been slow to develop in the past (Le Jambre 1978a; Martin *et al.* 1981). Any reduction in this refugia could therefore shorten the persistence of susceptible alleles in the population.

In conclusion, it appears that CBZ resistance can develop in *N. dubius* eggs independently of that in adult worms. This supports and extends the theory proposed in the previous chapter (5.4). It also raises some interesting questions about relationships between anthelmintic resistance in the eggs and adults of other nematode parasites.

CHAPTER VII

THE INHERITANCE OF CAMBENDAZOLE RESISTANCE IN ADULTS

7.1 *INTRODUCTION*

The rate at which pesticide resistance develops in a target population depends upon a number of factors, including the biological characteristics of the population and the method of applying the pesticide (Crow 1957; Georghiou 1972; Georghiou and Taylor 1976, 1977 a, b). In particular, a knowledge of the way in which resistance is inherited can aid predictions about its future spread through the population. Hoskins and Gordon (1956) suggested that the genetic nature of resistance could be inferred from changes in the slopes of the log dosage-probit lines following selection. However, direct genetic analysis is preferable.

It has been shown that, following selection, resistance to cambendazole (CBZ) can develop in adult *Nematospiroides dubius* (Chapter V). The slopes of log dosage-probit lines suggested inheritance of this resistance was polyfactorial. The experiments described in this chapter were designed to provide further information about the types of resistance which had developed. The two isolates examined were the eighth generation of Population A and the sixth of Population D, chosen for length of selection and degree of resistance.

Classical genetic analysis, involving cross-mating experiments and the examination of F_1, F_2 and backcross progeny, is widely used for studying the inheritance of insecticide resistance (Crow 1957; Georghiou 1969; Hoskins and Gordon 1956). The major problem is in calculating segregation ratios in these hybrid populations, because the resistance character is itself quantitative, and so genotypes are not usually distinct. Furthermore, the estimation of genotypic ratios must be indirect, because when treatment removes the susceptible individuals, their progeny cannot be analyzed (Tsukamoto 1963).

Behaviour of log dosage-probit lines for the hybrid offspring can provide some answers (Georghious 1969; Hoskins and Gordon 1956; Tsukamoto 1963). Examples of their usage and interpretation in genetic analysis of insecticide resistance are described fully in Chapter 1 (1.4.2). Le Jambre, Royal and Martin (1979) adopted a similar approach in studying the inheritance of thiabendazole resistance in a strain of *Haemonchus contortus*. Their conclusion was that for the population examined, resistance was autosomal, but influenced by a maternal effect, co-dominant with susceptibility, and probably polygenic. All assays, apart from those for sex-linkage and those performed on the F_2 progeny, were conducted on eggs rather than on adults.

Using egg hatch assays does have drawbacks. While the standard errors for regression parameters can be very low, there may be wide variations between results for assays set up on different days, or those using different egg samples. Martin, Le Jambre and Claxton (1981) suggest this casts some doubt on the biological significance of differences between log dosage-probit lines. They also question the assumption that a direct correlation exists between resistance in eggs and in adults (Coles and Simpkin 1977; Le Jambre 1976). Both problems were encountered in the studies of *N. dubius* (4.3.2., 5.4), so in the following series of experiments greater emphasis was placed on the use of adult dose-response assays.

7.2 *METHODS*

7.2.1 *Cross-mating experiments*

The technique for crossing the two selected isolates with the unselected population was similar to that used by Le Jambre's group (Le Jambre and Royal 1977; Le Jambre *et al.* 1979). Donor mice were infected with about 200 third stage larvae (L_3) from the parental lines and the adult worms were recovered 14 days later. All subsequent steps involving handling of adults outside the host were carried out at 37°C in a constant temperature room, and the actual transfer technique is described fully in Chapter V (5.2.2). Two to three male recipient mice were used for every cross, each receiving 50 to 100 worms comprising equal numbers of males and females. Between 10 and 50% of adults survived these transfers.

The problem associated with sperm storage by the transferred females was discussed in 5.1, but applies equally where only the offspring of the imposed matings are wanted. Le Jambre and Georgi (1970) noted that the duration of spermatozoa storage by females of *Ancylostoma caninum* appeared to vary with the worms' age. As other experimental factors could also be influential, controls in which mice received only female *N. dubious* (5.2) were run with every experiment.

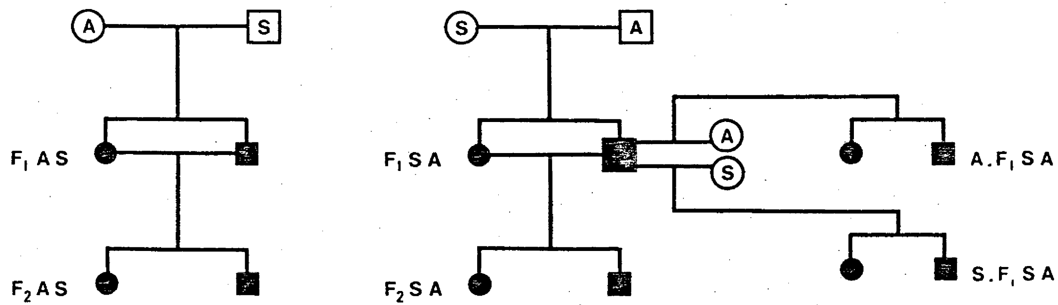
Reciprocal crosses were made for both selected lines, with an additional replicate at a later date for Population A. In the case of Population A, the analysis was extended to include both F_2 generations, and backcrosses for the F_1 progeny derived from resistant males. A hybridization test between males of Population A and females of Population D was also carried out. The terminology for progeny of these crosses conforms with that used in plant and animal breeding, where the female parent is indicated first. This terminology is summarized in Table 7.1 and the pedigrees for all crosses are shown in Fig. 7.1.

7.2.2 *Assays*

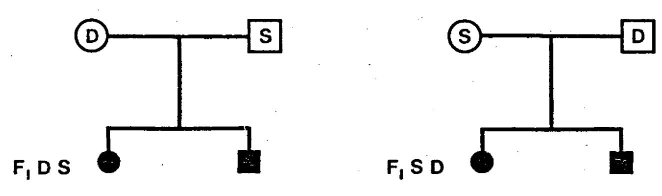
Egg hatch assays (4.4) were performed only on the F_1 generation of Population A. To provide a fully controlled experiment the eggs of both parental lines were also collected from worms which had undergone this transfer technique. Adult dose responses (3.4) were performed on all hybrid progeny, but full simultaneous tests of selected and unselected parents could not be made because too few mice were available. It was

Table 7.1 Summary of Abbreviations for Progeny of Cross-mating Experiments

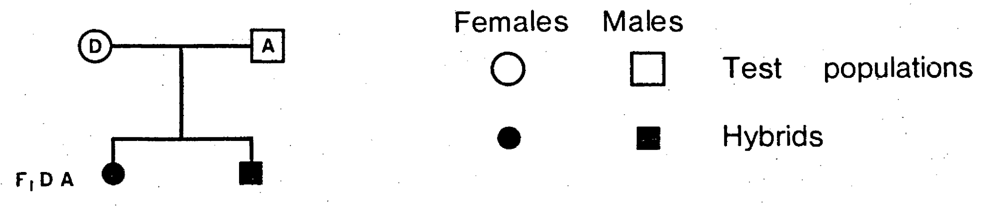
Population	Parents	Progeny
Parental		
Selected populations	Population A : A	
	Population D : D	
Unselected or susceptible population	: S	
F_1	male S x female A	F_1 AS
	male A x female S	F_1 SA
	male S x female D	F_1 DS
	male D x female S	F_1 SD
F_2	F_1 (male S x female A) selfed	F_2 AS
	F_1 (male A x female S) selfed	F_2 SA
Backcross	male F_1 (male A x female S) x female A	A. F_1 SA
	male F_1 (male A x female S) x female S	S. F_1 SA
Hybrids of the selected populations	male A x female D	F_1 DA



Population A x susceptible population



Population D x susceptible population



Population A x Population D

FIGURE 7.1 PEDIGREES FOR CROSS-MATING EXPERIMENTS

therefore necessary to test the progeny derived from Population A in male mice, and those from Population D in females, so that results could be compared with the parental log dosage-probit lines described in Chapter V. As a control for any changes in assay conditions, adults of the unselected population were tested at two or three dosage levels whenever a full assay of hybrids was run. The results for the unselected parents were then compared by contingency table (2.5.5) with those obtained for the same treatment groups in the earlier assay. No significant differences were found in any of these so direct comparisons were presumed to be valid.

7.2.3 *Degree of dominance*

The positions of the F_1 regression lines, relative to those of the two parental populations, are indicative of the dominance relationship between resistance and susceptibility. The degree of dominance (D) for F_1 progeny was calculated according to Falconer's equation:

$$D = \frac{2.ED_{50}\ F_1 - ED_{50}\ R - ED_{50}\ S}{ED_{50}\ R - ED_{50}\ S}$$

where the ED_{50} s are expressed as logarithms, R refers to the resistant population and S to the susceptible one (Stone 1968). A value of about zero indicates co-dominance, while values of -1 or 1 show that resistance is respectively recessive or dominant.

7.2.4 *Tests for sex-linkage*

The karyotype of *N. dubius* was examined to determine whether a simple test for sex-linkage, such as that used by Le Jambre, Royal and Martin (1979) was applicable. The method used was that of Le Jambre (1968), and the preparation of reagents is described in Appendix 2. Gonads, which were usually not intact, were removed from adult worms and fixed in Carnoy's fixative for 15 minutes. They were then stained with lacto-propionic orcein for about five minutes and examined microscopically under oil immersion objective. More success was achieved with the female gonoducts. The karyotype was found to be 2n = 12 in females while males appeared to have one less chromosome. This pattern is similar to that described for *H. contortus* and in fact for most of the Strongylata which have been examined (Bremner 1954, 1955; Le Jambre 1979b; Walton 1959).

As males are heterogametic, receiving their sole sex chromosome from the female parent, male F_1 offspring of resistant males should be more susceptible than their female siblings, if the resistant gene or genes are carried on this chromosome (Le Jambre *et al.* 1979). The numbers of male and female worms from F_1 SA and F_1 SD progeny, surviving dose response tests, were therefore tested for equality using the Student's t-test. Before applying the test, variances in the numbers of each sex were checked for homogeneity by an F ratio test, and when no differences were found, the standard equation for t was used (Bliss 1967). Equations for both F and t are given in Chapter II (2.5.3).

Table 7.2 F_1 Hybrids of Population A: Egg hatch responses

Population	Number of concentrations	EC_{50} (µg CBZ/ml)	(S.E.)	Slope	(S.E.)
Parental					
Selected (A)	5	5.52	(0.08)	9.01	(0.66)
Unselected (S)	5	5.35	(0.10)	6.41	(0.75)*
First generation hybrids					
F_1 A S	4	4.98	(0.09)	8.54	(1.23)
F_1 S A	4	6.04	(0.10)	9.66	(1.13)

EC_{50}s compared by Student's t-test: All differ significantly at P = 0.05

Slopes compared by analysis of covariance: * significantly different from other slopes at P = 0.05
remaining slopes show no significant differences at P = 0.05

7.3 *RESULTS*

7.3.1 *F_1 generation*

Adult F_1 offspring of both selected isolates had responses intermediate in nature compared with those of their parents. The progeny of selected females proved the more resistant of the reciprocal crosses. Neither relationship was evident in the F_1 eggs which were tested.

The susceptibility of eggs from reciprocal crosses of Population A differed significantly, with those derived from resistant males (F_1 SA), having the greater ED_{50} (Table 7.2). This hybrid group was also less susceptible than either parental population, whereas eggs derived from resistant females (F_1 AS) were slightly more susceptible. The regression slopes for hybrid and selected populations were similar but that for the unselected parents was lower.

There was little if any difference between replicates for adults of the crosses involving Population A (Table 7.3; Figs 7.2 and 7.3), and results were similar for the progeny of Population D (Table 7.4; Fig. 7.4). Offspring of females from both selected isolates (F_1 AS and F_1 DS) showed much higher resistance levels than those of the reciprocal crosses (F_1 SA and F_1 SD). In the latter, responses were significantly different from those of either resistant or susceptible populations. The responses of progeny from females of Populations A and D differed only slightly from their respective maternal populations.

Table 7.3 F_1 Hybridgs of Population A: Dose Response of Adults in Male Mice

Population	Number of dose levels	ED_{50} (mg CBZ) (S.E.)	Slope (S.E.)	Degree of dominance
Parental				
selected (A)	7	1.89 $(0.44)^{1}$	4.31 (1.38)	-
unselected (S)	6	1.08 (0.43)	3.45 $(1.23)^{3}$	-
First generation hybrids				
replicate 1				
F_1 A S	7	1.57 $(0.14)^{1}$	4.81 (0.74)	0.35
F_1 S A	7	1.45 $(0.13)^{2}$	3.78 $(0.85)^{4}$	0.06
replicate 2				
F_1 A S	4	1.58 $(0.14)^{1}$	6.44 $(1.31)^{3,4}$	0.35
F_1 S A	4	1.40 $(0.18)^{2}$	4.06 (0.83)	-0.07

ED_{50} s compared by Student's t-test: $^{1\,2}$ no significant difference between populations with the same superscript at P = 0.05; all other ED_{50}s differ significantly at P = 0.05

Slopes compared by analysis of covariance: ^{3,4} populations with the same superscript differ significantly at P = 0.05; others do not differ at P = 0.05.

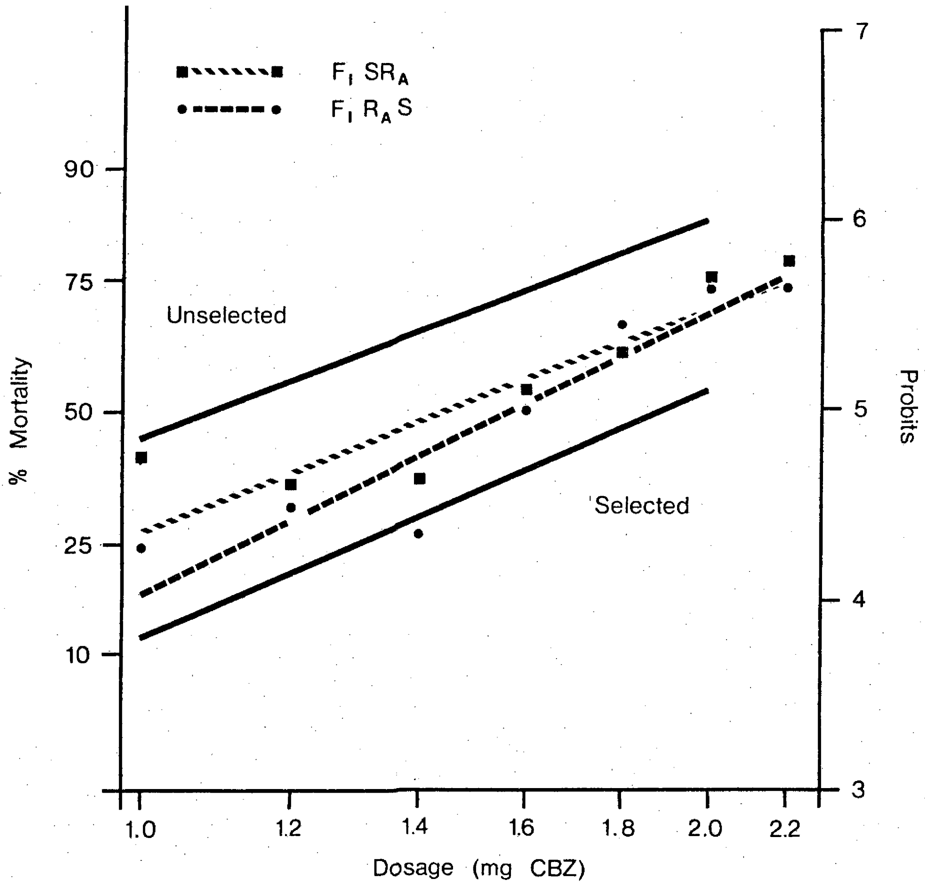


FIGURE 7.2 F_1 GENERATION OF POPULATION A (1): DOSAGE MORTALITY LINES FOR ADULTS FROM HYBRID AND PARENTAL POPULATIONS

Tested in male mice

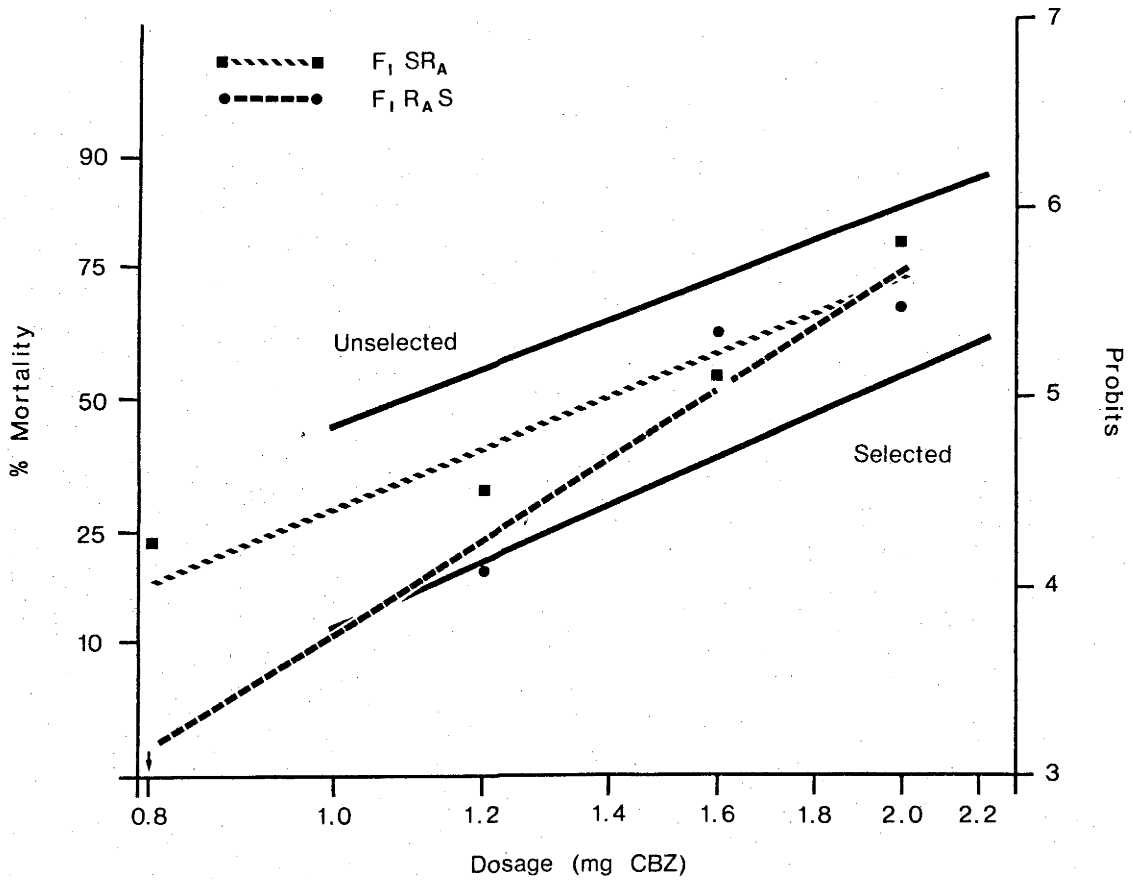


FIGURE 7.3 F_1 GENERATION OF POPULATION A (2) : DOSAGE MORTALITY LINES FOR ADULTS FROM HYBRID AND PARENTAL POPULATIONS
Tested in male mice

Table 7.4 F_1 Hybrids of Population D: Dose Response of Adults in Female Mice

Population	Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)	ED_{60}	Degree of dominance (ED_{60})
Parental							
selected (D)	6	1.09	(0.23)[1]	3.15	(0.96)[3]	1.31	-
unselected (S)	8	0.64	(0.11)	4.04	(0.57)[3]	0.74	-
First generation hybrids							
F_1 D S	8	1.39	(0.09)[1]	2.76	(0.54)[3]	1.72	2.44
F_1 S D	8	0.43	(0.27)	1.00	(0.51)	1.04	0.05

Relative susceptibilities and slopes compared by analysis of covariance:

[1] no significant difference in susceptibility of populations with the same superscript; all others differ significantly at $P = 0.05$

[3] no significant difference between slopes with the same superscript; all other differ significantly at $P = 0.05$

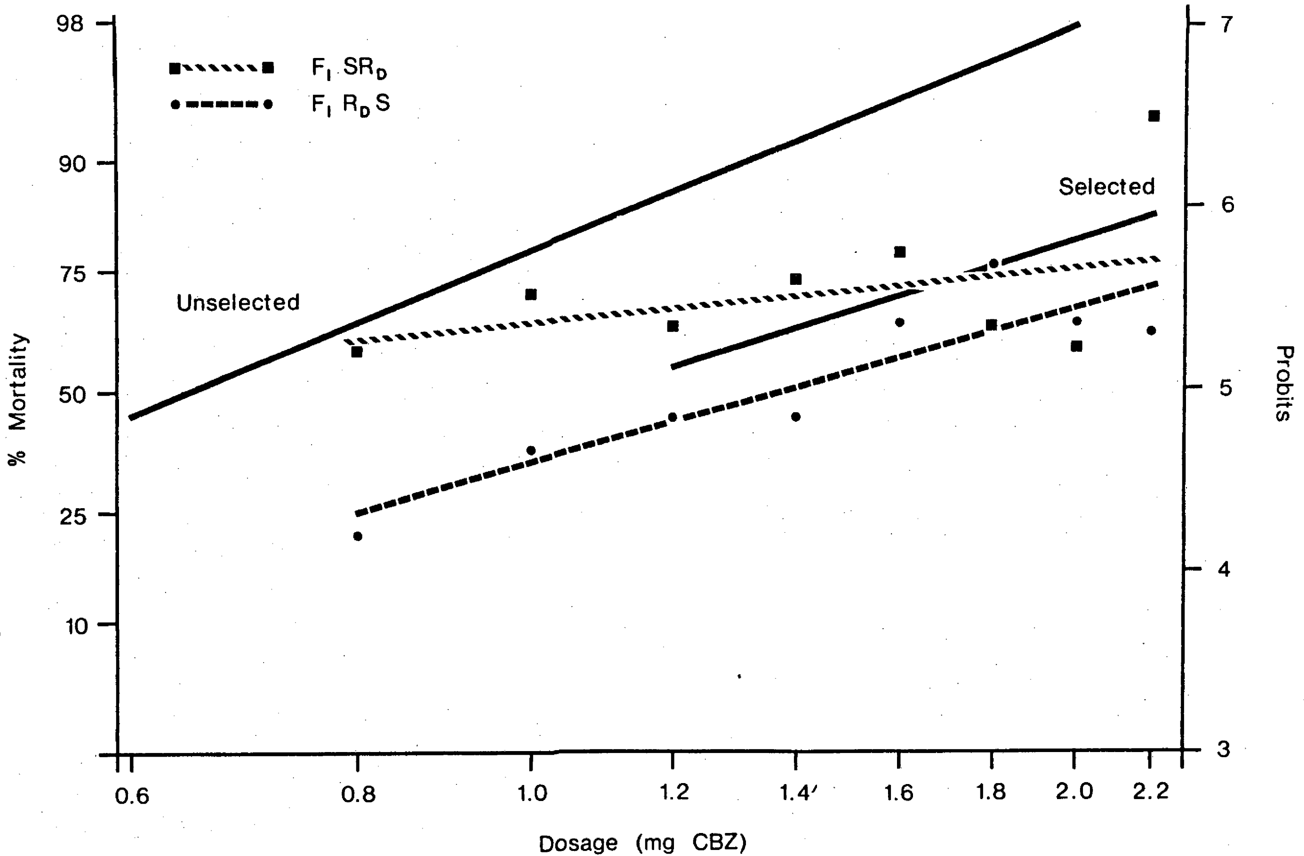


FIGURE 7.4 F_I GENERATION OF POPULATION D: DOSAGE MORTALITY LINES FOR ADULTS FROM HYBRID AND PARENTAL POPULATIONS

Tested in female mice

The most outstanding feature of the regression slopes for hybrid populations was the steepness of the lines for offspring of selected females relative to those for the reciprocal crosses. However, this difference was not marked in the first replicate of the cross involving Population A. Regression slopes for parental and hybrid populations were generally similar, a notable exception being the very low slope obtained for the progeny of males from Population D (F_1 SD).

The relationship between hybrid and parental populations is summarized by the degree of dominance associated with each hybrid (Tables 7.3 and 7.4). In the case of Population D, where the ED_{50} lay outside the tested dose range, ED_{60}s have been used in the calculations. Values of 0.06 and -0.07 for the two F_1 SA replicates and of 0.05 for the F_1 SD hybrids are consistent with co-dominant inheritance of resistance. Degrees of dominance of 0.35 for both F_1 AS replicates and of 2.44 for F_1 DS, indicate the inheritance of an additional component through the female parent.

There was no evidence for sex-linkage of CBZ resistance in either selected isolate. The survival rate for adult male offspring of resistant males (F_1 SA and F_1 SD) was similar to that in their female siblings. In fact, over the complete dosage ranges examined in adult dose response assays, there were no significant differences in the number of male and female worms surviving treatment.

7.3.2 *F_2 and backcross generations of Population A*

Adult dose responses for both F_2 and backcross hybrids were plotted so that any inflections and plateaux would be detectable. Results are, however, less clear than for the F_1 generations. In the F_2 populations, the susceptibilities of adults were similar to those of the selected parents; while for backcross progeny, susceptibility was unexpectedly high.

Visually, there appears to be a plateau at the 25% mortality level for both F_2 populations (Fig. 7.5). In the hybrids derived from selected males there may be another at the 75% mortality level. Neither response, however, deviated from linearity to any greater extent than did those of the selected and unselected populations (2.5, 3.3.2). There was little difference between the levels of resistance measured in either of the two F_2 hybrids or in Population A (Table 7.5). Both hybrids were appreciably more resistant than the unselected population. No marked differences were observed between slopes of the fitted log dosage-probit regression lines for either hybrid or parental populations.

In the progeny of the backcross to the selected population (A. F_1 SA) there may be a plateau in the response curve at the 50% mortality level (Fig. 7.6), but again the deviation from linearity was comparable to that shown by the selected and unselected populations. There was no indication of a plateau in the responses of the other backcross offspring (S. F_1 SA). Progeny of the backcross to the

Table 7.5 F_2 and Backcross Hybrids of Population A: Dose Responses of Adults in Male Mice

Population	Number of dose levels	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)
Parental					
selected (A)	7	1.89	(0.44)[1]	4.31	(1.38)
unselected (S)	6	1.08	(0.43)[2]	3.45	(1.23)
Second generation hybrids					
F_2 A S	7	1.60	(0.35)[1]	3.69	(0.71)
F_2 S A†	7	2.03	(0.83)[1]	3.26	(0.83)
Backcross hybrids					
A x F_1 S A	8	0.84	(0.26)[2,3]	1.92	(0.58)
S x F_1 S A†	8	0.60	(0.35)[3]	3.34	(0.79)

† Relative susceptibilities compared with those of other populations by analysis of covariance; other ED_{50}s compared by Student's t-test: [1,2,3] no significant difference between populations with the same superscript at P = 0.05; all other ED_{50}s differ significantly at P = 0.05

Slopes compared by analysis of covariance: no significant differences between any slopes at P = 0.05

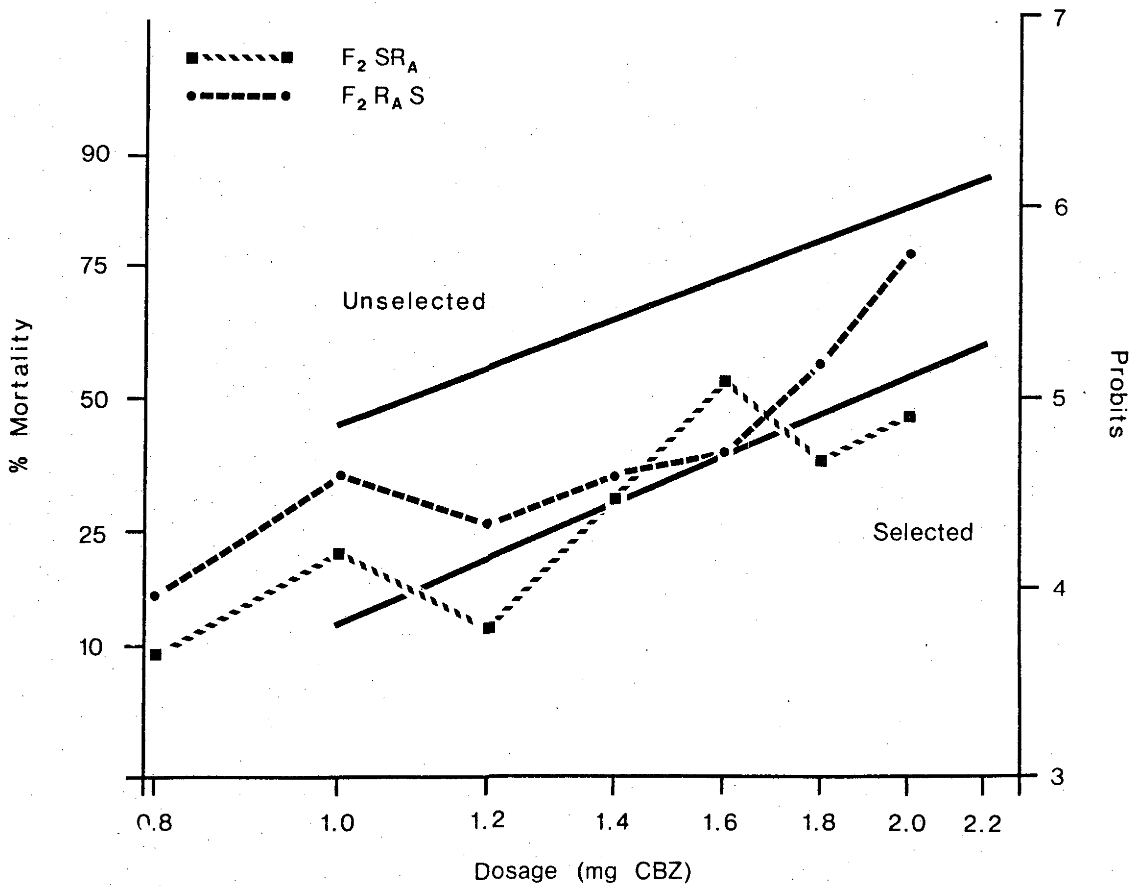


FIGURE 7.5 F_2 GENERATION OF POPULATION A: DOSAGE MORTALITY RESPONSES FOR ADULTS FROM HYBRID, SELECTED AND UNSELECTED POPULATIONS

Tested in male mice

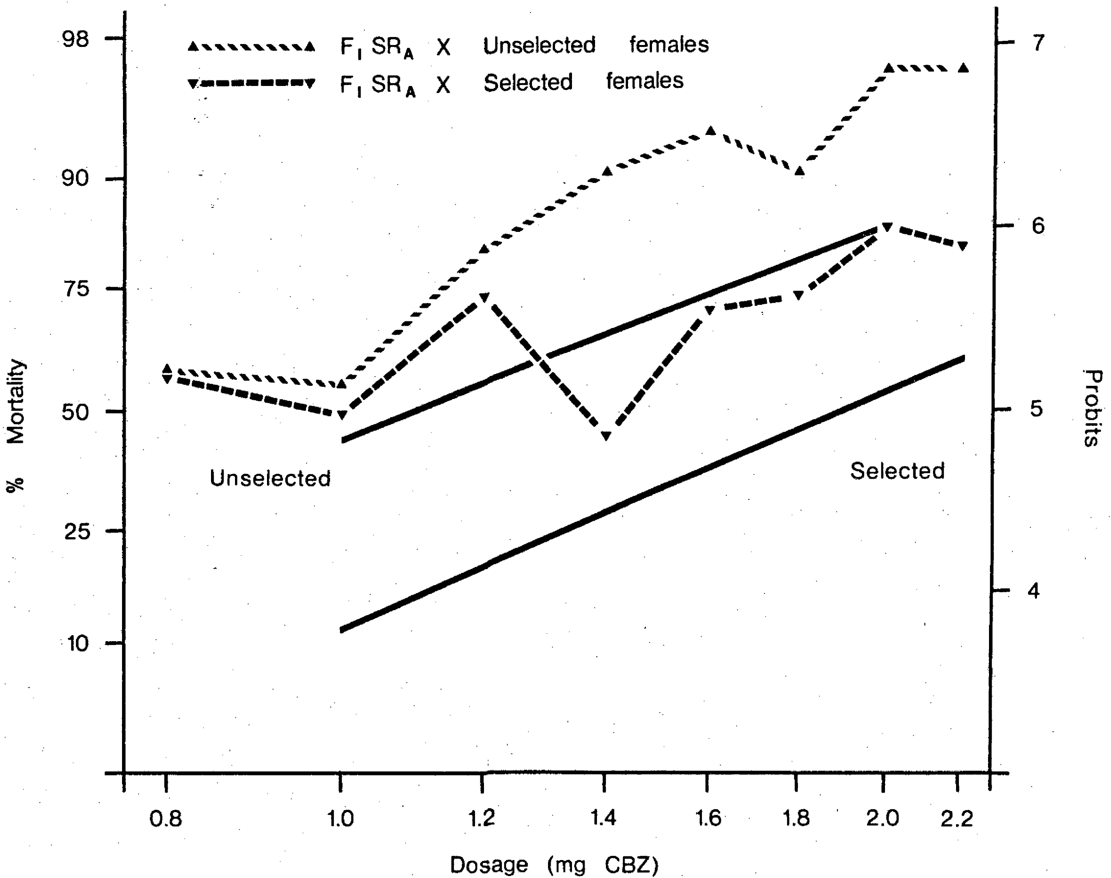


FIGURE 7.6 BACKCROSS GENERATION OF POPULATION A: DOSAGE MORTALITY RESPONSES FOR ADULTS FROM HYBRID, SELECTED AND UNSELECTED POPULATIONS

Tested in male mice

selected isolate were the less vulnerable, although the difference was not significant (Table 7.5). The responses of this particular group and of the unselected population were alike. Offspring of the backcross to the unselected population were appreciably more susceptible than the latter. There were only minor differences in the slopes of the fitted regression lines for hybrid or parental populations.

7.3.3 *Hybrids of Populations A and D*

The offspring of males from Population A and females from Population D exhibited an extremely high level of resistance, with a ratio over double that of either parent (Table 7.6; Fig. 7.7). The hybrid adults were significantly more resistant than those of Population A and their regression slope had a lower gradient. However, because these were both tested in male mice whilst Population D was assayed in females, a direct comparison with the latter was not possible (3.2.5).

7.4 *DISCUSSION*

Cambendazole resistance appears to be co-dominant and autosomal, but influenced by a maternal effect in both of the isolates examined. In the case of Population A, a number of genes may be involved, but the interpretation of segregation ratios should be tentative at such an early stage in the development of resistance (Brown and Pal 1971). The overall impression gained is that the inheritance of CBZ resistance in these *N. dubius* isolates is similar to that described for the TBZ resistant *H. contortus* population studied by Le Jambre's group (Le Jambre *et al.* 1979).

Table 7.6 Adult Dose Response Results for Hybrids of Population A and D

Population	Number of doses	ED_{50} (mg CBZ)	(S.E.)	Slope	(S.E.)	Resistance ratio
Parental						
A	7	1.89	(0.44)	4.31	(1.38)	1.75
D †	6	1.09	(0.23)	3.15	(0.96)	1.70
Hybrid						
F_1 D A	4	3.97	(1.08)	1.75	(1.58)	3.78

† Tested in female mice; all others tested in male mice

Relative susceptibility and slopes of populations A and F_1 D A compared by analysis of covariance:

Populations differ significantly in both respects at P = 0.05

Population D was not included in this comparison because it was tested in mice of a different sex (3.2.5)

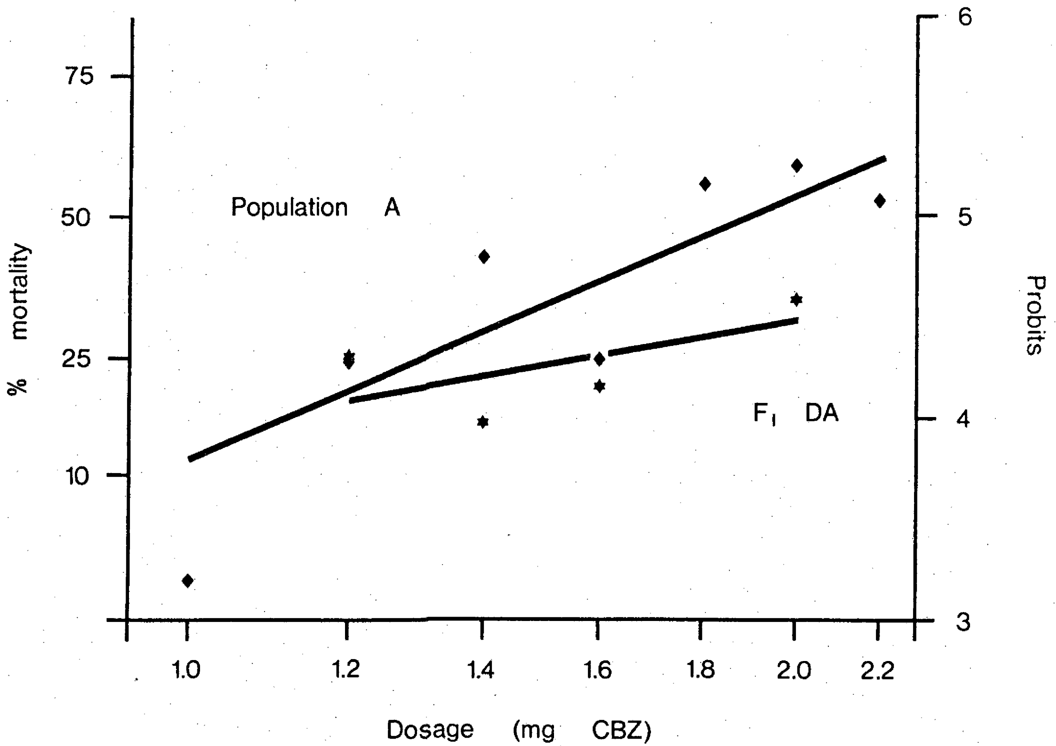


FIGURE 7.7 HYBRID OF POPULATIONS A AND D: DOSAGE MORTALITY LINES FOR HYBRID ADULTS AND THOSE FROM POPULATION A

Tested in male mice

The positions of log dosage-probit lines for F_1 offspring relative to those for the parental populations (Figs 7.2 to 7.4), conform with patterns which in insects are associated with co-dominantly inherited resistance and a maternal influence (Georghiou 1969; Tsukamoto 1963). The degree of dominance is one means of quantifying the phenotypic hierarchy between resistant and susceptible alleles (Falconer 1964; in Stone 1968). Whether ED_{60}s rather than ED_{50}s are used in its calculation should be immaterial provided that all hybrid and parental regression lines are parallel. This is not the case for Population D where the slope for progeny of resistant males (F_1 SD) differs from the slopes, but the use of ED_{60} was necessary to avoid extrapolating beyond the tested dosage range. More emphasis should therefore be placed on trends rather than absolute values for degree of dominance. Nevertheless, even from visual examination of responses, the level of maternal influence does appear to be considerably greater in Population D than in Population A (Figs 7.2 to 7.4).

The higher level of resistance observed in progeny of selected females appears to have a cytoplasmic rather than chromosomal source because no sex-linkage was observed. The autosomally determined inheritance of CBZ resistance in *N. dubius* is consistent with the pattern observed for TBZ resistance in *H. contortus* (Le Jambre *et al.* 1979). Sex-linkage is in fact uncommon in insecticide resistance, though there are many reports of cytoplasmic maternal effects (Brown and Pal 1971).

Maternal effects may be attributed to cytoplasmic factors other than DNA, or to extrachromosomal genes, but it is difficult to differentiate between the two (Sonneborn 1950). The simplest explanation for higher resistance in offspring of selected females, might be the transfer in the egg cytosol of factors such as maternal messenger RNA or proteins. Both have been implicated in maternal effects found in the free-living nematode *Caenorhabditis elegans* (Hirsh 1979). The expression of such factors would have to extend through to adulthood. Extrachromosomal genes, for instance in the mitochondria, could have a more enduring effect. The eggs of *H. contortus* certainly contain large amounts of cytoplasmic DNA, but the function of this has not been investigated (Le Jambre, Crofton and Whitlock 1970). It is interesting to note that the maternal influence although evident in the F_1 adults, was not observed in the F_1 eggs of Population A (Table 7.2). This is consistent with the hypothesis, advanced in Chapter V and developed in Chapter VI, that the mechanism of CBZ resistance differs in eggs and adults of *N. dubius*. By the F_2 generation adults, the effect was no longer apparent (Fig. 7.5). Le Jambre's group did not examine F_1 adults for a maternal effect, but found that F_2 adults descended from resistant females were less susceptible than those descended from resistant males. For the effect to continue so long, they suggest extrachromosomal genes must be involved. The cause of the maternal influence may therefore differ in *H. contortus* and *N. dubius*.

The comparatively steeper regression lines for progeny of selected females indicate a greater homogeneity for their CBZ resistance than in offspring of selected males. The difference in response between reciprocal crosses was most pronounced at low dosages, while at higher ones the regression lines appeared to converge (Figs 7.2 to 7.4). This applied to both resistant isolates, and one explanation may be that the maternal effect is relatively weak, offering additional protection only to the more susceptible individuals. Greater survival in these would tend to mask the population's heterogeneity. The F_1 eggs from reciprocal crosses involving *H. contortus* displayed a similar relationship (Le Jambre *et al.* 1979).

Examinations for segregation patterns in F_2 and backcross generations give no conclusive evidence concerning the number of genes involved. Although deviations from linearity were found in all cases, these were similar to the ones observed in the parental populations. The absence of clear inflexions and plateaux in the log dosage-probit lines of F_2 and backcross generations is open to several interpretations. The one usually advanced is the presence of complicated polyfactorial inheritance (Tsukamoto 1963). The similarity between slopes of selected and unselected populations, which is associated with polygenic inheritance of insecticide resistance (Hoskins and Gordon 1956), gives further credence to this possibility. Gene interactions, even involving relatively few loci, will still produce linear regression curves because their combined effect is synergistic rather than additive (Tsukamoto 1963).

That the slight plateaux observed were real and indicated segregation ratios, should be considered. Plateaux at the 25% and 75% mortality levels in F_2 AS adults could result from a single, co-dominant gene for resistance (Tsukamoto 1963). Alternatively, the sole plateau at the 25% level in F_2 SA adults is suggestive of a single dominant allele, although an additional dosage group is necessary to confirm the absence of a second plateau. The backcross data gave yet another answer. For progeny of the mating back to the resistant isolate (A. F_1 SA), there appeared to be a plateau at the 50% mortality level. This, and a more susceptible response without inflexions for offspring from the mating with the unselected population, are characteristic of a single recessive allele for resistance.

It is probably too early at this stage in the development of resistance, to gain a clear understanding of segregation patterns. If anthelmintic resistance behaves similarly to insecticide resistance in its early stages, then it is most likely to be polygenic (Crow 1957; Brown and Pal 1971). Distinct plateaux should not in any case be expected where log dosage-probit lines overlap to the extent occurring in this study (Tsukamoto 1963). Such analysis also requires an assay sufficiently sensitive to detect all segregants, which the present *in vivo* test may not be (3.3). An *in vitro* assay of adults, such as that developed for *Nippostrongylus brasiliensis* (Jenkins, Armitage and Carrington 1980), appears to be the logical progression for further analysis of this type.

The high susceptibility of both backcross generations, relative to the selected and unselected populations, poses a further query which is difficult to resolve. Selection was not applied to the F_1 generation so reversion to susceptibility may have occurred, yet the F_2 generation with an equivalent background, showed no signs of this. Also, the apparently normal fitness of the selected isolate (Chapter V 5.3) gives no indication of why a sudden reversion should take place. Hoskins and Gordon (1956) suggest there may be 'antagonism' between resistance factors and the remaining genome when two gene pools are combined, especially for polygenically determined characters. This argument seems inconclusive and does not explain what has happened, so the question must presently remain unanswered.

The higher resistance level exhibited by hybrids of the two selected isolates is of interest because it gives further information about the inheritance of the trait. The implications are more readily appreciated by examining some hypothetical genotypes. Two extremes have been considered, one for a single locus and the other involving a number of genes.

If resistance is monogenic for both selected isolates, then three main situations might be envisaged. Only one of the following seems likely to produce an appreciably greater resistance level in the hybrids.

a) Same locus; same alleles:

In this case resistance levels in parental and hybrid populations should be alike.

b) Same locus; different alleles:

As the levels of resistance in Populations A and D were similar, with resistance ratios of 1.75 and 1.70 respectively (Table 7.6), the level in the heterozygotes should be comparable barring some interaction between the different alleles.

c) Different loci:

In this situation, hybrids would be heterozygotic at both loci, and the resistance due to each should resemble that in the F_1 generation. The actual level of resistance due to the combined effect might be additive and therefore similar to that in the parents but could be greater if there was a synergistic interaction.

Polygenic inheritance of resistance in both selected isolates can also be considered in the same manner. Only three major conditions have been described below, but the range of combinations could be continuous.

a) Same loci; different alleles at each:

New combinations of allelesat all loci could confer greater resistance in the heterozygotes,

depending on the dominance and any interactions at each locus.

b) Completely different loci:
In this case, the hybrids are unlikely to be homozygous at any one locus, so resistance levels would depend on the dominance relationship at each. Quite possibly the final effect could exceed that in either parent.

c) Combinations of the same and different loci:
This situation might provide the greatest potential for increased resistance levels. At the common loci, new and more effective combinations could occur in addition to the influence of alleles unique to either parent.

An increased resistance level in the hybrids of Populations A and D, seems to be most likely where new combinations of alleles are possible. This could happen in monogenic situations if different loci are individual for the parental populations, but may be more pronounced where a greater variety of combinations is feasible. Thus, the higher level of resistance demonstrated by hybrids seems to be more in keeping with polyfactored inheritance.

The overall impression gained from these F_2, backcross and hybridization studies is that CBZ resistance in *N. dubius* is polygenic at this stage in selection. It

certainly appears to be co-dominant and autosomal with an additional maternal influence. In all, the resemblence to the TBZ resistant *H. contortus* strains described by Le Jambre and co-workers is noteworthy. With continued selection of these *N. dubius* isolates, the system presents every appearance of being a valuable experimental model for studying the problem.

Further work on the genetic nature of benzimidazole, and indeed any anthelmintic resistance, must be limited until more is known about the parasites' genetic constitution in general. Identification and mapping of marker genes is a necessity. The techniques for producing and selecting such mutants, especially behavioural and physiological ones, are well developed for free-living nematodes (Riddle 1978) and their use with parasitic nematodes should be feasible. The inheritance of morphological characters in *H. contortus* in particular has already been studied by several groups (Daskalov 1971, 1972, 1975; Le Jambre 1977; Le Jambre and Royal 1977 to name a few). So, although a great deal of work is required to describe mutants, there are future prospects for much more detailed analysis of anthelmintic resistance in nematodes.

CHAPTER VIII

CONCLUSIONS

The work described in this thesis demonstrates that *Nematospiroides dubius* may have potential for examining anthelmintic resistance in the laboratory. Both the selection programme for resistance to cambendazole (CBZ), and the rudimentary genetic analysis of the trait in adult selected isolates, were successful. An important question has, however, been raised concerning the relationship between resistance in adult nematodes and resistance in their eggs.

This is the first report of the development of anthelmintic resistance in a laboratory model. It is also only the second report of an anthelmintic resistant population being produced experimentally from a previously unexposed strain of animal parasitic nematodes. The first involved selection of *Haemonchus contortus* with CBZ. All other cases where experimental selection has been successful, have involved field isolates which have either been previously exposed to or already partially resistant to the anthelmintics (1.5.3).

preferable that the resistant strain is derived from the susceptible population to which it is being compared (Georghiou 1969). Such a programme using parasites in

sheep is lengthy and involves a large number of host animals. The development of a laboratory simulation may therefore be an advantage. Important considerations in choosing an appropriate model are that:

(i) the parasite is susceptible to the anthelmintic

(ii) it can be selected for anthelmintic resistance

(iii) the methods used are comparable to those used in studying the nematodes of domestic animals

(iv) the parasite is similar in other respects, such as its life-cycle, to the parasitic nematodes of the domestic animal in question, eg. of sheep.

N. dubius fits these criteria. It is certainly susceptible to CBZ, both in adult and egg stages (Chapters III and IV). As with sheep nematodes, *N. dubius* can be selected for resistance to this anthelmintic (Chapter V), and the response has proved amenable to subsequent genetic analysis (Chapter VII). An oral drenching technique, similar to that used for treating sheep, is effective against adult *N. dubius* in mice, and the *in vitro* egg hatch assay is also applicable. In the case of adult worms, the dosages of benzimidazoles which kill mouse parasites *in vivo*, are relatively high (Coles and McNeillie 1977). The

recommended oral dosage of CBZ for treating sheep is 20 mg/kg body weight (Prichard 1978), whereas about 60 mg/kg body weight is required for a similar effect upon *N. dubius* in mice. Oral drenching, rather than the dietary administration used by other workers for anthelmintic screening tests of mouse parasites, was adopted principally because it is simpler and less time-consuming. It also provides greater analogy with studies in sheep. Finally, the parasite itself shows similarities in other respects to the main economically important trichostrongylids in sheep (Chapter I, 1.7).

The role of selection pressure in the development of resistance has been demonstrated using *N. dubius* (Chapter V). Of the dosages tried, those producing mortalities of 85 - 95% (1.6 mg CBZ/mouse) and 95 - 98% (1.8 mg CBZ/mouse) were the most effective in selecting for resistance. More intense selection resulting in 98% or greater mortality (2.0 mg CBZ/mouse) was the least successful, possibly because the variability of the population was so severely restricted. The smallest pressure of 50 - 60% mortality (1.4 mg CBZ/mouse) led to the development of resistance, but at a lower level. This pattern seems to conform with what might be predicted from work on insecticide resistance (Crow 1957).

N. dubius showed an initial response to cambendazole selection and continued selection may lead to the development of a level of resistance similar to that reported in sheep parasites. If so, *N. dubius* would be a useful model for a number of further studies. Isolates could be examined for side-

resistance to other benzimidazoles, and for cross-resistance to functionally unrelated anthelmintics. Tests for reversion to susceptibility in the absence of anthelmintic pressure would also be of interest, providing more information about the general fitness of selected populations (Chapter V). Another important question is whether the anthelmintic's mode of action against *N. dubius*, and the biochemical basis of resistance in this parasite, is consistent with the presently known situation in sheep nematodes.

Resistance, in the two adult selected isolates examined, appears to be co-dominant with susceptibility, and inherited autosomally (Chapter VII). These characteristics, together with the maternal influence, conform to the pattern of inheritance observed in thiabendazole resistant *H. contortus* (Le Jambre, Royal and Martin 1979). Analysis of segregation in F_2 and backcross generations, suggests CBZ resistance is polyfactorial. Further separation of selected and unselected populations is required before placing too much credance on these results, although other evidence supports this suggestion. For instance: the slopes of log dosage-probit lines following selection, as discussed in Chapter V, and the very high level of resistance expressed in the hybrid progeny of two selected populations (Chapter VII). A more sensitive assay of adult worms is needed for confidence in detecting

segregation of the genes conferring resistance. One possibility is an *in vitro* test, as described by Jenkins, Armitage and Carrington (1980) for *Nippostrongylus brasiliensis*. If such an assay can be adopted, the prospects of investigating more thoroughly the inheritance of anthelmintic resistance will be greatly enlarged.

Resistance to CBZ in the eggs of *N. dubius* appears to be controlled by a different gene, or set of genes, to the ones responsible for resistance in the adults. Selection applied to the adult parasites leads to the development of populations having resistant adults, but has no appreciable effect on the susceptibility of their eggs (Chapter V). Conversely, selection of the eggs and free-living larvae in culture results in populations in which the eggs are less sensitive to CBZ, but which exhibit no change in the susceptibility of the adults (Chapter VI). This lack of correlation between responses in the two stages is contrary to current theories about the expression of resistance in the nematodes of sheep. In the latter, the egg hatch assay is presumed to reflect the degree of resistance in adult parasites (Coles and Simpkin 1977; Le Jambre 1976). It appears to do so in a number of instances where there is information about resistance levels in both eggs and adults (Coles and Simpkin 1977; Donald, Waller, Dobson and Axelsen 1980; Le Jambre, Southcott and Dash, 1977, 1978; Simpkin and Coles 1978). There may therefore be a fundamental difference between benzimidazole resistance in *N. dubius* and in sheep nematodes.

The absence of a direct relationship in the responses of eggs and adults may not be confined to *N. dubius*. There are reports of discrepencies in the degree of resistance expressed in the two stages of sheep nematodes (Hall, Campbell and Richardson 1978). Recently, it was suggested that there could in fact be differences in the genes responsible for TBZ resistance in the eggs and adults of *H. contortus* (Martin, Le Jambre and Claxton 1981). As the eggs and free-living larvae of *N. dubius* can respond to selection with CBZ, it is possible that those of sheep nematodes have the same capacity. Simultaneous selection of parasitic and non-parasitic stages could well be a feature of normal drenching practices, as discussed in Chapter VI. This need not invalidate the egg hatch assay as a screen for resistance in adult worms, because where individuals are selected for both characters, the presence of one can infer that of the other. Alternatively, if the resistance mechanism is common to both stages, selection of eggs and free-living larvae on pasture could influence the rate at which resistance develops in the adults.

This experimental system, involving *N. dubius* in mice, presents many possibilities for further work, and while it should not supplant direct examination of sheep nematodes, it could well augment such studies.

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APPENDIX 1

COMPUTER PRINTOUTS

Probit analysis using Davies' (1971) program:

EGG HATCH ASSAY (UNSELECTED POPULATION) SEPARATE DATA
PROPORTION OF CONTROLS DYING .024

LOG DOSE	NO. EXPOSED	NO. DEAD	OBS. PROBIT	EXPT. PROBIT
.724	88	21	4.18	3.98
.724	102	17	3.95	3.98
.724	101	17	3.95	3.98
.724	103	16	3.89	3.98
.724	110	17	3.89	3.98
.845	121	55	4.85	4.86
.845	92	41	4.83	4.86
.845	105	55	5.03	4.86
.845	102	51	4.97	4.86
.845	92	40	4.80	4.86
.944	100	76	5.69	5.59
.944	96	63	5.38	5.59
.944	110	77	5.50	5.59
.944	115	82	5.54	5.59
.944	99	70	5.52	5.59
1.021	104	90	6.09	6.15
1.021	109	94	6.08	6.15
1.021	82	71	6.09	6.15
1.021	93	81	6.12	6.15
1.021	110	102	6.44	6.15
1.090	123	118	6.73	6.65
1.090	84	82	6.97	6.65
1.090	112	105	6.52	6.65
1.090	106	103	6.90	6.65
1.090	131	124	6.60	6.65

SLOPE .7296+001 VAR SLOPE .6973-001
INTERCEPT -.1302+001 DOF 23
CHI SQUARE .1557+002 LOG ED50 .8637+000
VAR LOG ED50 .1983-004 ED50 .7306+001
HETEROGENEITY .6768+000
NO. CYCLES 2
X AND Y CO-ORDINATES FOR FITTED LINE
.727 4.00
.864 5.00

EGG HATCH ASSAY (UNSELECTED POPULATION) POOLED DATA
PROPORTION OF CONTROLS DYING .024

LOG DOSE	NO. EXPOSED	NO. DEAD	OBS. PROBIT	EXPT. PROBIT
.724	504	88	3.95	3.98
.845	512	242	4.90	4.86
.944	520	368	5.53	5.59
1.021	498	438	6.16	6.15
1.090	556	532	6.70	6.65

SLOPE .7296+001 VAR SLOPE .6976-001
INTERCEPT -.1302+001 DOF 3
CHI SQUARE .1874+001 LOG ED50 .8637+000
VAR LOG ED50 .1984-004 ED50 .7306+001
HETEROGENEITY .6247+000
NO. CYCLES 2
X AND Y CO-ORDINATES FOR FITTED LINE
.727 4.00
.864 5.00

Probit analysis using Davies' (1971) program (cont.):

ADULT DOSE RESPONSE ASSAY (UNSELECTED POPULATION) SEPARATE DATA

PROPORTION OF CONTROLS DYING .106

LOG DOSE	NO. EXPOSED	NO. DEAD	OBS. PROBIT	EXPT. PROBIT
.041	50	20	4.56	4.58
.041	50	22	4.68	4.58
.041	50	26	4.91	4.58
.041	50	11	3.86	4.58
.041	50	21	4.62	4.58
.041	50	29	5.08	4.58
.041	50	17	4.36	4.58
.041	50	11	3.86	4.58
.041	50	26	4.91	4.58
.041	50	19	4.49	4.58
.079	50	13	4.05	4.88
.079	50	34	5.36	4.88
.079	50	29	5.08	4.88
.079	50	21	4.62	4.88
.079	50	19	4.49	4.88
.079	50	19	4.49	4.88
.079	50	34	5.36	4.88
.079	50	33	5.30	4.88
.079	50	27	4.96	4.88
.079	50	23	4.74	4.88
.146	50	48	6.70	5.42
.146	50	47	6.50	5.42
.146	50	46	6.34	5.42
.146	50	28	5.02	5.42
.146	50	37	5.55	5.42
.146	50	37	5.55	5.42
.146	50	21	4.62	5.42
.146	50	35	5.42	5.42
.146	50	39	5.69	5.42
.146	50	21	4.62	5.42
.204	50	48	6.70	5.89
.204	50	49	7.01	5.89
.204	50	48	6.70	5.89
.204	50	48	6.70	5.89
.204	50	28	5.02	5.89
.204	50	41	5.84	5.89
.204	50	40	5.76	5.89
.204	50	31	5.19	5.89
.204	50	40	5.76	5.89
.204	50	39	5.69	5.89

SLOPE .8086+001 VAR SLOPE .2939+000
INTERCEPT .4241+001 DOF 38
CHI SQUARE .2218+003 LOG ED50 .9385-001
VAR LOG ED50 .1867-004 ED50 .1241+001
HETEROGENEITY .5838+001
NO. CYCLES 3
X AND Y CO-ORDINATES FOR FITTED LINE
-.030 4.00
.094 5.00

ADULT DOSE RESPONSE ASSAY (UNSELECTED POPULATIONS) POOLED DATA

PROPORTION OF CONTROLS DYING .106

LOG DOSE	NO. EXPOSED	NO. DEAD	OBS. PROBIT	EXPT. PROBIT
.041	500	202	4.56	4.58
.079	500	252	4.86	4.88
.146	500	359	5.48	5.42
.204	500	412	5.85	5.89

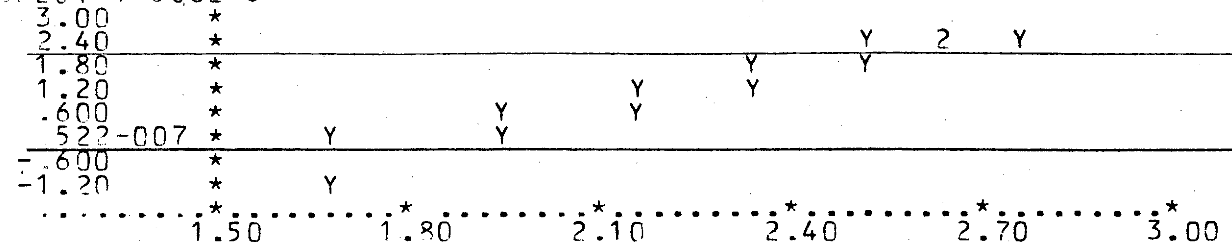
SLOPE .8086+001 VAR SLOPE .2934+000
INTERCEPT .4241+001 DOF 2
CHI SQUARE .1244+001 LOG ED50 .9385-001
VAR LOG ED50 .1866-004 ED50 .1241+001
HETEROGENEITY .6222+000
NO. CYCLES 1
X AND Y CO-ORDINATES FOR FITTED LINE
-.030 4.00
.094 5.00

Analysis of covariance using GLIM: (example of an egg hatch assay)

```
@FTNLIB*GLIM.GLIM
GLIM 3.12 (C)1977 ROYAL STATISTICAL SOCIETY, LONDON
NAG-UNIVAC SL73R1 08/10/81 17:06:39
$UNITS 13 $DATA DOSE TOTAL RESPOND $READ
!CBA 59 11/12/80 (unselected population)
  5.3 504  88
  7.0 512 242
  8.8 520 368
 10.5 498 438
 12.3 556 532
 14.1 490 485
 15.8 478 474
!CBZ S95 6'B 11/12/80 (Population B, 6th generation)
  5.3 738 330
  7.0 775 602
  8.8 777 712
 10.5 793 773
 12.3 764 758
 14.1 782 779
$CALC POP=%GL(2,7)
!ADD GLIM COMMANDS FOR PROBIT ANALYSIS
$LOOK POP DOSE TOTAL RESPOND $
   1      1.000      5.300      504.0      88.00
   2      1.000      7.000      512.0      242.0
   3      1.000      8.800      520.0      368.0
   4      1.000      10 50      498.0      438.0
   5      1.000      12.30      556.0      532.0
   6      1.000      14 10      490.0      485.0
   7      1.000      15 80      478.0      474.0
   8      2.000      5.300      738.0      330.0
   9      2.000      7.000      775.0      602.0
  10      2.000      8.800      777.0      712.0
  11      2.000      10 50      793.0      773.0
  12      2.000      12 30      764.0      758.0
  13      2.000      14 10      782.0      779.0
$FACTOR POP 2
$CALC DOSE=%LOG(DOSE)
$YVAR RESPOND $ERROR BINOMIAL TOTAL $LINK PROBIT
$FIT $
        SCALED
 CYCLE  DEVIANCE      DF
   4     3055.        12

$FIT DOSE $
        SCALED
 CYCLE  DEVIANCE      DF
   4     400.3        11

$FIT DOSE+POP $DISPLAY EC
        SCALED
 CYCLE  DEVIANCE      DF
   3     6.930        10

       ESTIMATE       S.E.       PARAMETER
   1   -6.055        .1515       %GM
   2    3.071        .7033-001   DOSE
   3    .8003        .4159-001   POP(2)
   SCALE PARAMETER TAKEN AS      1.000

CORRELATIONS OF ESTIMATES
  1   1.0000
  2  -.9823   1.0000
  3  -.4065    .2839   1.0000
       1        2        3

$FIT DOSE*POP $
        SCALED
 CYCLE  DEVIANCE      DF
   3     5.994        9

$CALC Y=%ND(RESPOND/TOTAL)
$PLOT Y DOSE $
```

Analysis of covariance (2.5.4, p58)

source of deviation	degrees of freedom	deviance	P
regression	1	2654.7	0.00
population	1	393.37	0.00
parallism	1	0.936	ns
non-linearity	9	5.994	ns
total	12	3055	



Contingency table analysis using GLIM: (example of egg hatchability

```
@FTNLIB*GLIM.GLIM
GLIM 3.12 (C)1977 ROYAL STATISTICAL SOCIETY, LONDON
NAG-UNIVAC SL73R1 08/26/81 14:52:13
$UNITS 10 $DATA POPULATION REPLICATES TOTAL RESPOND $READ
!EGG HATCH CONTROLS (unselected population)
1 1 117  4
1 2  80 10
1 3  87 10
1 4 129  7
1 5 107  7
!EGG HATCH POP D IST GEN (Population D, 1st generation)
2 1  45  6
2 2  37  5
2 3  51  2
2 4  47  5
2 5  44  5
!GLIM COMMANDS FOR CONTINGENCY TABLE ANALYSIS                    TOTAL
$FACTOR POPULATION 2 REPLICATIONS 5 $YVAR RESPOND $ERROR BINOMIAL
$FITS

$FIT POPULATION+REPLICATIONS $
          SCALED
 CYCLE   DEVIANCE        DF
   4       8.211          4

$FIT POPULATION $
          SCALED
 CYCLE   DEVIANCE        DF
   3       12.37          8

$FIT REPLICATIONS $
          SCALED
 CYCLE   DEVIANCE        DF
   4       9.765          5

$DISPLAY ERC
        ESTIMATE       S.E.        PARAMETER
   1    -2.721        .3264        %GM
   2     .8044        .4278        REPL(2)
   3     .3699        .4448        REPL(3)
   4     .1063        .4427        REPL(4)
   5     .2717        .4440        REPL(5)
  SCALE PARAMETER TAKEN AS         1.000

  UNIT   OBSERVED  OUT OF   FITTED      RESIDUAL
    1        4      117     7.222       -1.235
    2       10       80     10.26       -.8575-001
    3       10       87     7.565        .9264
    4        7      129     8.795       -.6272
    5        7      107     8.503       -.5373
    6        6       45     2.778        1.996
    7        5       37     4.744        .1261
    8        2       51     4.435       -1.210
    9        5       47     3.205        1.039
   10        5       44     3.497        .8379

CORRELATIONS OF ESTIMATES

  1   1.0000
  2   -.7630   1.0000
  3   -.7339    .5600   1.0000
  4   -.7374    .5626    .5412   1.0000
  5   -.7353    .5611    .5397    .5422   1.0000
         1        2        3        4        5
```

Analysis of variance (2.5.5, p59)

source of deviation	degrees of freedom	deviance	P
replicates	4	4.159	ns
population	1	1.554	ns

APPENDIX 2

COMPOSITION OF STAINS AND FIXATIVE

Lugol's Iodine (Weigert's modification)

Iodine (pure crystals)	1 g
Potassium iodide	2 g
Distilled water	100 ml

(McGraw-Hill 1960)

Carnoy's Fixative

Glacial acetic acid	1 ml
100% methanol	3 ml

(Le Jambre 1968)

Lacto-propionic Orcein

Natural orcein	2 g
Propionic acid	50 ml
Lactic acid	50 ml

Stock solution filtered and diluted to 45% with distilled water.

(Le Jambre 1968)