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IVERMECTIN SELECTION AND CHARACTERIZATION OF THE LIFE HISTORY TRAITS OF *HELIGMOSOMOIDES POLYGYRUS* (NEMATODA)

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Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfiiment of the requirements for the degree of Master of Science

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ABSTRACT

A stock "parent" (S) strain of the mouse parasite *Heligmosomoides polygyrus* was exposed to increasing levels of ivermectin at the L4 stage for 15 generations. A Passage line was also developed from the parent strain parallel with the ivermectin selected line to control for the effects of rapid passage of the parasite from host to host during drug selection. A dose titration trial indicated 1.5 fold resistance had developed in the ivermectin selected strain at the 8th generation (IVM-8) both at the L4 and adult stage. A higher dose of drug was required to kill the L4 stage compared to the adults at generation 8. Additional selection pressure for 7 generations (IVM-15) did not change the resistance status of adult worms. The Passage strains (P-8 and P-15) remained susceptible to drug. The life history traits of the parent strain (S), the ivermectin selected (IVM-8 and IVM-15) and the Passage (P-8 and P-15) strains were then compared. Eight generations of selection with ivermectin (IVM-8) resulted in an increase in establishment 8 days post-infection (pi) but decreased egg output and worm burden over 4 months compared with strain S. However these effects were not seen after 15 generations of drug selection. The ivermectin selected strain (IVM-15) had similar establishment, egg production and worm burden as the parent strain (S). Establishment in strain P-8 was intermediate and not different from S or IVM-8 however 15 generations of passage (strain P-15) resulted in higher establishment and more rapid development to adult. This was also reflected in the net egg output and worm burden during the first month of infection. There were no differences in per capita fecundity among the five strains. Environmental pressure exerted by passage of *H. polygyrus* from host to host rather than ivermectin selection caused shifts in some life history traits of this nematode.

ABREGE

Une souche-mère du stade larvaire L4 du parasite de la souris, *Heligmosomoides polygyrus*, a été exposée à des niveaux croissants d'ivermectin (IVM) sur 15 générations. Une souche-témoin a aussi été développée à partir de la souche-mère parallèlement à la souche exposée à l'IVM. Cette lignée nous renseignera sur les effets des passages rapides du parasite d'un hôte à un autre pendant la sélection à l'IVM. Un test de dosage effectué sur les adultes et les L4 de la 8ième génération de la souche-IVM (IVM-8) a démontré un niveau de résistance de 1.5. Une dose plus élevée a été cependant nécessaire pour éliminer le stade larvaire L4 en comparaison aux adultes de cette génération. Une exposition additionnelle pendant 7 autres générations (IVM-15) n'a pas changé le niveau de résistance chez les adultes. Les souches-témoins (P-8 et P-15) sont restées sensibles à l'IVM. Les caractéristiques biologiques de la souche-mère (S), de la souche-IVM (IVM-8 et IVM-15) et de la souche-témoin (P-8 et P-15) ont été comparées. En comparaison avec la souche S, 8 générations de sélection à l'IVM (IVM-8) ont résulté en une augmentation de l'implantation au 8ième jour de l'infection, mais ont diminué l'excrétion d'oeufs et le nombre d'adultes sur une période de 4 mois. Toutefois, ces effets n'ont pas été détectés après 15 générations de sélection à l'IVM. La souche-IVM (IVM-15) a démontré une implantation, une production d'oeufs et un nombre d'adultes similaires à la souche S. L'implantation de la souche-témoin, P-8, a été moyenne mais similaire à S ou IVM-8. Toutefois, 15 générations de passage (P-15) ont résulté en une plus grande implantation et un développement plus rapide du stade adulte. Ceci s'est réfleté au niveau de la production nette d'oeufs et du nombre d'adultes durant le premier mois d'infection. Aucune différence chez la fécondité par femelle n'a été décelée parmi les 5 lignées. Les pressions environnementales excercées par le passage d'*Heligmosomoides polygyrus* d'un hôte à un autre, plutôt que la sélection à l'IVM, ont causé des changements au niveau des caractéristiques biologiques de ce nématode.

SUGGESTED SHORT TITLE

The fitness of ivermectin selected strains of *Heligmosomoides polygyrus*

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In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make it perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such thesis serve as an examiner for that thesis."**

STATEMENT BY AUTHOR OF THESIS

With the exception of a particular section in Chapter 2, experiments, analyses and writing of this thesis were performed by the author, under the supervision of Dr. M.E. Scott. In Chapter 2 the ivermectin selection and passage protocol of *Heligmosomoides polygyrus* were carried out by Farzaneh Jalili the research assistant in Dr Scott's lab. She also did Experiment 2 and 4 of the dose response trials for the ivermectin selected (IVM-8), Passage (P-8) and stock (S) strains. Results from the selection protocol and all dose response trials were analyzed by the author.

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This work is dedicated to my parents and my unborn child.

INTRODUCTION

One of the major limitations encountered as a result of increasing productivity, following intensification of grazing livestock, has been the greater range and severity of animal health problems of which diseases caused by nematodes are prominent. The pharmaceutical industry has met this challenge by developing new 'wonder' drugs that include modern broad spectrum drugs, namely the benzimidazoles, imidazothiazoles and avermectins. These drugs have a very high efficacy and this has fostered the view that eradication of the effects of nematode infections can be achieved by frequent treatment. Treatment timing and frequency has often been guided by considerations of convenience rather than being based on epidemiological approaches. Thus, in due course, anthelmintic resistant nematode populations have been selected (Waller and Prichard, 1986). Selection for resistance occurs when resistant individuals or genes in a population increase owing to the effectiveness of an anthelmintic on susceptible individuals or genes causing the resistant survivors to make up a larger percentage of the next generation (Prichard *et al.,* 1980).

Anthelmintic resistance has mainly been reported in gastro-intestinal nematodes of sheep and goats in areas where these animals are raised semi-intensively. The most widespread reports involve benzimidazole resistance. Therefore ivermectin together with other avermectins are currently the drugs of choice for control of gastrointestinal nematodes in sheep, goats and cattle. However ivermectin resistance in sheep and goat nematodes is also spreading very rapidly in the field (Van Wyk and Malan, 1988; Echevarria and Trinidade, 1989; Craig and Miller, 1990; DeVaney *et al.*, 1992; Jackson *et al.*, 1992; Le Jambre, 1993b). It has also been shown that ivermectin resistance can be elicited under laboratory conditions (Egerton *et al.,* 1988; Giordano *et al.,* 1988; Shoop *et al.*, 1990; Echevarria *et al.,* 1993a). These laboratory selected strains can facilitate the study of the evolution, population genetics and mechanisms of ivermectin resistance.

Evolutionary ecologists have proposed that selection of drug resistant nematode strains is accompanied by changes in their fitness characteristics. The characteristics important in multiplication, survival and transmission of nematodes define the fitness of an individual both in the host and in the environment. They determine the contribution of an individual to the next generation. Many features in the biology of an organism determine its overall fitness. The best measure of fitness is the reproductive rate of a parasite. It is defined as the number of eggs produced by a single female parasite that themselves survive to reproduce in the absence of density dependence (Anderson and May, 1982). Under anthelmintic treatment, the frequency of the resistant individuals increases. However the rate of increase will depend on the relative fitness of the resistant heterozygotes and homozygotes compared with the fully susceptible genotype. Once the anthelmintic is introduced, fitness will depend on both general fitness and ability to survive anthelmintic exposure. The latter can be quantified. General fitness of parasitic nematodes in the absence of drugs is difficult to estimate due to lack of genetic markers (Prichard, 1990). Currently the two main measures used to estimate the fitness of susceptible and resistant nematodes are (1) estimating the rate of reversion from resistance to susceptibility and (2) comparing the life history traits of the resistant and susceptible nematode strains in the absence of drug.

A slow rate of reversion to benzimidazole susceptibility in nematodes, after resistance has been established, has been proposed as evidence for an alteration in fitness traits of resistant phenotypes (Le Jambre *et al.,* 1982; Martin *et al.,* 1988b). In fact in some cases, no reversion has been observed in benzimidazole (Hall *et al., 1982)* and ivermectin resistance strains (Egerton *et al.,* 1988). Changes in fitness traits have been investigated mainly in benzimidazole sensitive and resistant strains. *Haemonchus contortus* with high level resistance to benzimidazoles was shown to have a higher establishment (Kelly *et al.,* 1978; Maingi *et al.,* 1990), fecundity, pathogenicity as well as survival in pasture (Kelly *et al.,* 1978) than moderately resistant strains. In

contrast, thiabendazole resistant *Trichostrongylus colubriformis* was reported to have a lower establishment, fecundity and pathogenicity when compared to a naive strain (Prichard *et al.,* 1978; MacLean *et al.*, 1987).

Few comprehensive reports on the characteristics and the properties of ivermectin resistant and sensitive strains are available. The studies so far indicate that the establishment and fecundity of nematode strains resistant or susceptible to ivermectin are similar (Scott and Armour, 1991; DeVaney *et al.*, 1992; Echevarria *et al.,* 1992). Echevarria *et al.* (1993b) however showed that the survival and development of the free living stages of naive and ivermectin resistant strains of *H. contortus* differed at 22C and 27C. The susceptible parasites had the highest development at 27C, while the resistant strain, derived from this strain, had the highest rate of development at 22C. In this trial a strain resistant to ivermectin and other drugs had the lowest developmental rate at both temperatures.

The changes in biological traits of drug resistant and susceptible parasites observed in these and other related studies may have been affected by the source of the parasites. In most cases the resistant and susceptible strains were from different sources and thus may have shown ectopic differences (Maingi *et al.,* 1990; Scott and Armour, 1991; Echevarria *et al.,* 1993b). In addition when the resistant strain has been derived in the lab, the drug selection procedure has involved frequent passage of the parasites from host to host. The effect of the rapid passage on the life history traits of nematodes that are subjected to drug selection pressure is not known.

The question of fitness of strains undergoing selection is an important one since preventive strategies such as those that involve the use of an annual slow rotation of drugs rely upon the possibility of reversion to susceptibility occurring in the intervening years between treatments with a single drug (Jackson, 1993). Indeed studies (Maingi *et al.,* 1990) on the fitness traits of benzimidazole resistant parasites support the view that if reversion is to occur, it is only likely to do so in the heterozygous-resistant phase of development

before selection pressure results in co-adaptation with general fitness characteristics.

The main objectives of this study were therefore to:

1. Develop an ivermectin resistant strain of parasite by subjecting a susceptible parent strain of the mouse parasite, *Heligmosomoides polygyrus*, to increasing doses of ivermectin.

2. Develop a parallel control Passage strain by simultaneous rapid passage of the parent strain with no ivermectin treatment.

3. Determine the efficacy of ivermectin against the parent, the Passage and the ivermectin-selected strains of *H. polygyrus*.

4. Characterize and compare the life history traits of the parent, Passage and the ivermectin selected strains of *H. polygyrus*.

CHAPTER 1
LITERATURE REVIEW

HELIGMOSOMOIDES POLYGYRUS

General Life History (Fig 1:1)

Heligmosomoides polygyrus polygyrus (syn Nematospiroides dubius Baylis according to Durette-Desset *et al.*, 1972).) is a parasite of the duodenum of wild mice (*Mus musculus),* white footed mice (*Peromyscus maniculatus)* and field mice (*Apodemus sylvaticus)* (Spurlock, 1943; Ehrenford, 1954).

Eggs are passed out with the faeces of the infected host. They hatch and develop to infective third stage larvae (L3) in approximately 7 days under moist conditions. Mice acquire the infection by ingesting the L3. The larvae migrate into the serosal musculature of the intestine, moult into L4 and remain there 7-9 days. They then migrate back through the wall of the intestine and live as adult male and female worms in the lumen of the upper small intestine for several months (Byrant, 1973a; Keymer and Hiorns, 1986a).

Females worms are usually more numerous than males (Bawden, 1969; Kerbouef and Jolivet, 1980; Keymer and Hiorns, 1986a). Higher death rates were reported per worm per day during the first 14 days of infection for males than for females (Keymer and Hiorns, 1986b). However the long term survival of males is higher than for females (Keymer and Hiorns, 1986b; Scott, 1991).

Egg Production

There is a high natural variation in egg production *H. polygyrus* within the day (Brindley and Dobson, 1982; Kerboeuf and Jacob, 1983), within a single host, and amongst worms from different hosts (Keymer and Hiorns, 1986a; Richardson and MacKinnon, 1990). In CD1 outbred mice, egg production of female *H. polygyrus* shows a rapid increase as early as 12 days post-infection (pi). The maximum values are observed between day 20 and 40, but between day 40 and 70 there is a steep decrease (Kerbouef, 1982). The decrease may be due to the aging of the worm or to the host's reaction. Some relation has been established between egg production and faecal output (Keymer and Hiorns, 1986a).

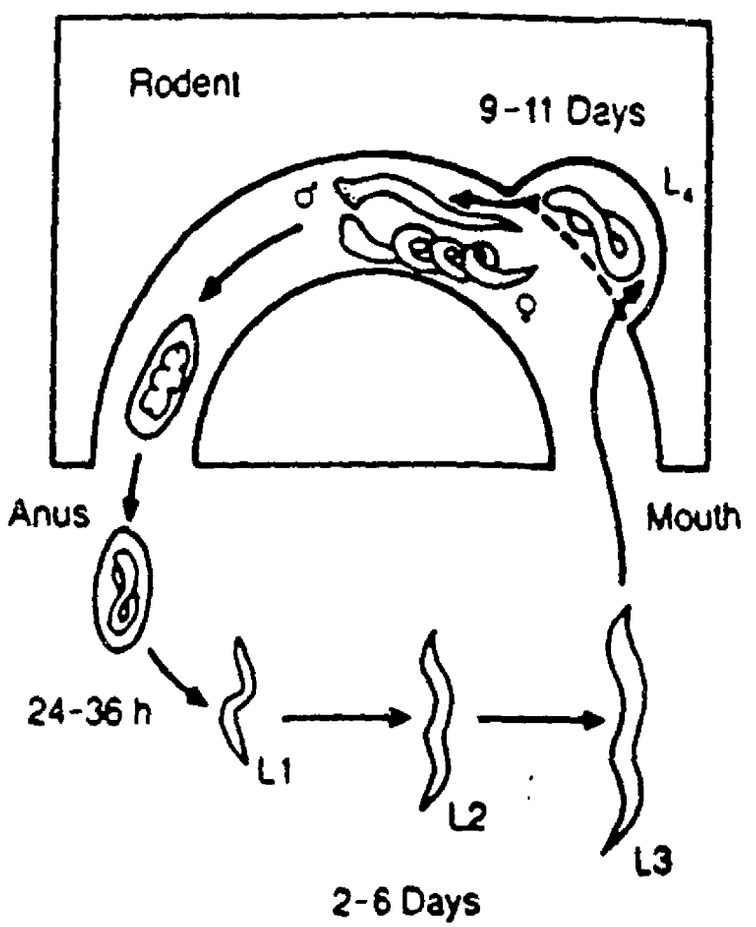

Fig 1.1: The life cycle of *Heligmosomoides polygyrus* (Keymer, 1985)

The high variation in egg production may mask the effect of hormones on the egg output of *H. polygyrus.* A study by Richardson and MacKinnon (1990) showed that cortisone, esteradiol and testosterone significantly decreased egg output by *H. polygyrus* from female mice; cortisone in particular was found to have the greatest effect. Kerbouef (1982) found that the higher the worm burden in the mouse, the lower the fecundity of *H. polygyrus.* High worm burden in a host will cause stress and ultimately an increase in the corticosteroids in the blood stream (Richardson and MacKinnon, 1990). Kerbouef (1982) however, attributed this decrease in fecundity to competition within the nematode population. Kerboeuf (1985) also indicated that variation in per capita egg output depended on the density at which the hosts were maintained. She suggested that variations due to stress-induced hormonal, physiological, nutritional or immunological effects could account for such observations.

The reported effects of mouse strain on per capita fecundity following primary infection are contradictory. Enriquez *et al.* (1988) reported differences in per capita rates of egg production between two strains of laboratory mice (LAF1 and CBA). Scott (1991) however reported no differences in per capita fecundity between Balb/c and C57BL/6 mice in primary infection but the effect of mouse strain on fecundity was noticeable following challenge infection.

Density dependent effects on per capita egg production in *H. polygyrus* have been reported when the worm population was higher than 200 individuals (Kerboeuf, 1978; Kerboeuf, 1982). However Keymer and Hiorns (1986a) failed to show evidence of density dependence in intensities as high as 450 worms/mouse. Re-analysis of these data based on the eggs per female per day revealed that the per capita fecundity decreased with increasing worm burden (Scott and Tanguay, 1994). Density dependent effects have not been observed at densities lower than 200. Thus egg counts can be used to evaluate worm burdens at these intensities (Sitepu and Dobson, 1982; Slater and Keymer,

1986; Scott, 1988).

Infective Stage

Eggs expelled in the faeces hatch after 36 hours to free living first stage larvae (L1). After three days the L1 moult into second stage larvae (L2). Both the L1 and L2 feed on bacteria in the faeces and in the soil. During these two stages the larval body weight increases by 62.44% per day and the stored reserves are used by the non-feeding third stage larva (L3) (Bryant, 1973a, b)

Larval infectivity for *H. polygyrus* has been shown to vary with the age of adults used as the source of larvae (Kerbouef, 1978), with the mouse strain (Scott, 1991) and with season (Sitepu and Dobson, 1982). Kerbouef (1978) also demonstrated that the infectivity of the third stage larvae decreased after exposure to unfavourable conditions. The L3 remained infective for a longer period when maintained at low temperatures (4C) than when exposed to high temperatures (22C). However this loss of infectivity appeared to be partly compensated by an increased egg output.

The infectivity of *H. polygyrus* for outbred Quackenbesh mice has also been shown to increase following serial passage in this host. This change was specific for the outbred mice as the inbred specific pathogen free (SPF) C_3H mice became more refractory to infection and showed greater variation in worm recoveries than Qmice (Dobson and Owen, 1977). During the first 24 hours after infection the L3 can be found migrating in the mucosa and submucosa of the stomach and duodenum (Sukdheo and Mettrick, 1984). The larvae then reach the muscularis externa, usually in the anterior small intestine. The L3 moult into fourth larvae stage (L4) four days (pi) at which time some sexual characteristics may be observed (Bryant, 1973a). The L4 remain in the muscularis externa until they moult into adults. The adults migrate back through the intestinal wall causing haemorrhage (Liu, 1965). Beckett and Pike (1980) observed immature adults in the lumen of the intestine as early as five days pi. After eight days, most of the worms can be found in the intestinal lumen as immature adults.

The first eggs can be detected in the faeces as early as 10 days pi (Bryant, 1973a). The entire life cycle from the egg to egg requires a minimum of approximately 13.5 days (Bryant, 1973a; Spurlock, 1943).

Adult Stage

Adult *H. polygyrus* occur in clumps in the anterior duodenum. This aggregation is thought to be stimulated by specific favourable environmental conditions such as bile secretion (Dobson 1960 cited in Bawden, 1969), supply of dietary nutrients, oxygen tension or pH (Bawden, 1969). Adults feed on tissue in the living host and not on host ingesta or blood (Bansemir and Sukhdeo, 1994). A primary infection with *H. polygyrus* induces a long term chronic infection of 8-10 months (Liu, 1966). However, different mouse strains show varying degrees of resistance to the parasite. For example high responders (SJL and SWR) expel the parasite after about 6 weeks, intermediate responders (NIH, BALB/c, B1-G and DBA) take between 10- 12 weeks, whilst in low responders (CBA, C3H, C57BL/10) it takes up to 35 weeks for expulsion to occur. It is proposed that the time taken for low responder mice to lose the parasite probably reflects the natural life span of the parasite within the hosts rather than an active immune response to it (Behnke *et al.*, 1987).

Pathology

The pathological consequences of infection with *H. polygyrus* include local gastritis, tissue destruction and inflammation of the mucosa and submucosa of the small intestine around the L4 during the first week of infection (Liu, 1965). Mouse mortality at this time is thought to be related to damage and haemorrhage during migration of the larvae back into the lumen approximately one week pi. The longer term infection causes thickening and inflammation of the intestinal wall of the upper duodenum (Mitchell and Prowse, 1979). The remarkable ability to survive in an otherwise immunocompetent host suggests that this parasite regulates the protective immunity it engenders (Monroy and Enriquez, 1992). Dose dependent mortality of mice has been reported in both outbred and inbred strains of mice (Liu,

1966; Forrester, 1971; Mitchell and Prowse, 1979).

A laboratory Model

Heligmosomoides polygrus can be readily maintained in the laboratory because the adult worms are long-lived and therefore frequent passage is not necessary. The infective larvae can be kept in aqueous suspension at 4C for many months and a single culture can provide thousands of infective larvae (Behnke *et al.*, 1991). This parasite also exhibits a life cycle similar to that of directly transmitted nematodes of medical and veterinary importance. All these attributes make it a suitable laboratory model of gastrointestinal trichostrongylid infections (Monroy and Enriquez, 1992). Previous studies have exploited this model to explore host immune responses (Behnke and Wakelin, 1977) and host population dynamics (Slater and Keymer, 1986; Scott, 1990; 1991). In addition this model has been used in the pharmaceutical industry as an anthelminthic screen (Jenkins and Ibarra 1984; Misra *et al.*, 1984; Burg and Stapley, 1989).

IVERMECTIN (IVM)

Pharmacokinetics

Ivermectin (Fig 1:2) belongs to a series of natural and semi-synthetic compounds which include abamectin, moxidectin and doramectin. They are collectively known as avermectins and milbemycines. They share some structural and physiochemical properties and they are potent acaricides, insecticides and anthelmintics (Steel, 1993).

The pharmacokinetic behaviour of ivermectin differs according to the route of adminstration, the formulation and the animal species. Following intravenous (i.v.) adminstration of ivermectin to cattle, elimination half-lives of 2.7 days (Wilkinson *et al.*, 1985) and 3 days (Chiu *et al.*, 1986) have been described. However a slower elimination half life was reported by Prichard *et al.* (1985) in sheep treated with ivermectin (0.2 mg/kg) given i.v. The tissue residue pattern for ivermectin in sheep and cattle has now been established

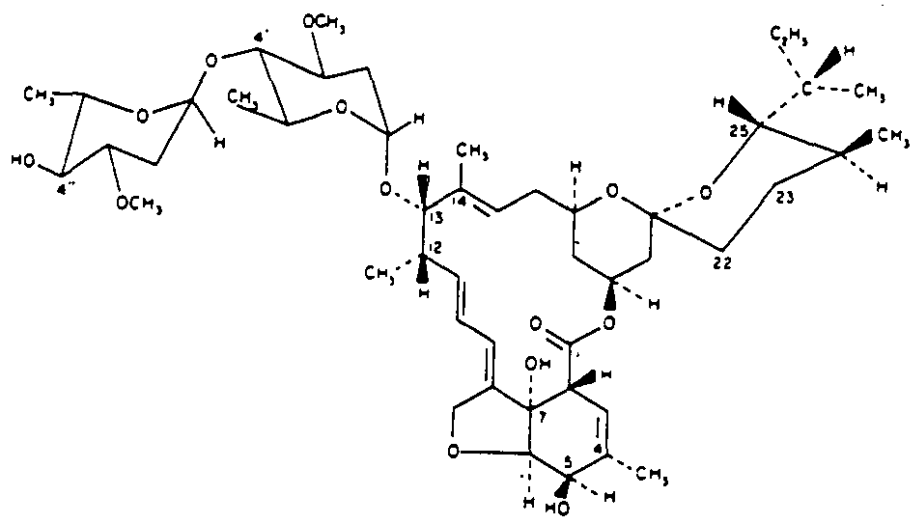

Fig 1.2: Structure of 22,23-dihydroavermectin B_{1a}, a major component of ivermectin. Ivermectin also contains not more than 20% 22, 23-dihydroavermectin B_{1b}, Which is identical except that the substituent in the 25 position is iso propyl instead of sec butyl (Campbell *et al.,* 1983)

using tritium labelled ivermectin (Chiu *et al.,* 1990), which has also been useful in clarifying the metabolism of this compound. After both subcutaneous (s.c.) and intraruminal administration of ivermectin, the highest tissue residues were present in fat and liver, with half lives of 6-8 and 4-5 days respectively. Unchanged parent compound is the major liver residue in cattle, sheep and rats (60%, 48% and 71% of the total radioactive dose, respectively) treated with ivermectin (Chiu *et al.,* 1987). The major liver metabolite in ruminants is 24-hydroxyl-methy-H_2-B_{1a} derivative (Chiu *et al.,* 1987).

Ivermectin has been shown to be excreted in high concentrations in the bile of both sheep and cattle, giving rise to the detection of high concentrations in the duodenal and ileal contents (Bogan and McKellar, 1988). Ivermectin is primarily eliminated in the faeces, with less than 2% of the total administered dose being excreted in the urine (Chiu *et al.,* 1990). However, considerable excretion of ivermectin occurs via the mammary glands in lactating cows (Toutain *et al.,* 1988).

Prichard *et al.* (1985) found that following oral adminstration, ivermectin attains a relatively high concentration in the plasma but the bioavailability of the drug is low compared with that following parenteral adminstration. After intraruminal adminstration in sheep, the plasma concentration was less than one third that reached after intra-abomasal treatment. The plasma peak was not reached until 23.5h and the bioavailability was found to be only 25% following oral treatment. Thus Prichard *et al.* (1985) proposed that metabolism and biodegradation in the rumen may account for the reduced bioavailabilty. After intra-abomasal injection, Prichard *et al.* (1985) reported that the bioavailabilty was close to 100%; the drug was rapidly absorbed and peak plasma concentration was attained within 4h, implying that ruminal by-pass may enhance plasma concentration and consequently potency of orally administered ivermectin. These findings are consistent with ivermectin being degraded by ruminal flora, and would explain the reduced bioavailability of ivermectin following oral and intraruminal adminstration, when compared with parenteral

injection in ruminants. An alternate explanation for the low bioavailability of oral or intraruminary administered ivermectin may be adsorption, or binding to the particulate phase of digesta which has been shown to influence the pharmacokinectics of some drugs such as levamisole (Bogan *et al.*, 1984).

Efficacy

Ivermectin is not ovicidal (Campbell *et al.*, 1983), however it is particularly effective against arrested and fourth larval stages of economically important gastrointestinal nematodes in sheep (McKellar and Marriner, 1987; Echevarria *et al.*, 1992), cattle (Barth and Preston, 1987), and pigs (Murrell, 1981). It has been shown to be effective against both benzimidazole and levamisole/morantel resistant nematodes in sheep (Waller and Donald, 1983).

Ivermectin has also been shown to be effective against the mouse parasite *H. polygrus* (Sayles and Jacobson, 1983; Rajasekariah *et al.*, 1986; Wahid *et al.*, 1989). However, in comparison with the optimal doses required for treating ruminants, dogs and man (0.1- 0.2mg/kg), the doses required to ensure total eradication of *H. polygyrus* from mice are significantly higher. Sayles and Jacobson (1983) reported that a dose of 5 mg/kg was totally effective in removing *H. polygyrus* infection, 6 days following infection. However, Wahid *et al.* (1989) reported that this dose was inappropriate for achieving a complete clearance of the parasite. They reported that the lowest dose required for eradication of worms was 10 mg/kg, but that a dose of 20 mg/kg would guarantee total removal of the worms. However they also found that the efficacy of ivermectin was affected by the mouse strain. Higher doses were required to eradicate worms in NIH (inbred) mice than CFLP (outbred) mice. When 5 mg/kg of ivermectin was administered subcutaneously in CFLP mice 95% of the parasite burden was removed. NIH mice treated at this dose cleared the worms only 82% of the worms. It was apparent that the subcutaneous adminstration was more effective than the oral route (Wahid *et al.*, 1989). In contrast, a dose of 0.3 mg/kg was reported to have 100% efficacy against the adults *H. polygyrus* in MAG outbred mice (Rajasekarial *et*

al., 1986).

Mode of Action (Fig 1.3)

The mode of action of ivermectin in gastrointestinal nematodes is still to be fully elucidated. Recent evidence suggests that ivermectin and related avermectins act by opening a glutamate-dependent chloride ion channel of neuromuscular membranes of nematodes and arthropods (Schaeffer and Haines, 1989; Cully and Paress 1991; Rohrer *et al.*, 1992). The opening of the chloride port leads to permeability to chloride ions which results in worm paralysis and eventually death. The ivermectin receptor has been purified in the free living nematode *Caenorhabditis elegans* (Schaeffer *et al.*, 1993). Cully *et al.* (1993) identified a 1.8-2.0 kd mRNA from *C. elegans* that is sensitive to both ivermectin and glutamate. The *C. elegans* mRNA was expressed in *Xenopus* oocytes and is insensitive to γ-aminobutyric acid (GABA). The effects of ivermectin or glutamate were blocked by picrotoxin, a chloride channel blocker.

ANTHELMINTIC RESISTANCE

Development of resistance by tissue or organisms to drugs is an evolutionary adaptation that puts at risk every tumoricidal, pesticidal and parasiticidal agent. Anthelmintic resistance is defined as a change in the gene frequency of a population that is produced by drug selection whereby more drug is required to exact the same effect than was required prior to selection (Prichard, 1990).

Anthelmintic resistance has been recorded in many countries throughout the world for all the three families of broad spectrum anthelmintics (benzimidazoles, imidazothiazoles and avermectins) which are commonly used in the livestock industry to control nematodes (see reviews by Prichard *et al.*, 1980; Coles, 1986; Waller and Prichard, 1986; Waller, 1987; Prichard, 1990). Resistance has also been recorded in drugs with a narrower spectrum of activity such as salicylanilides (Rolfe *et al.*, 1990).

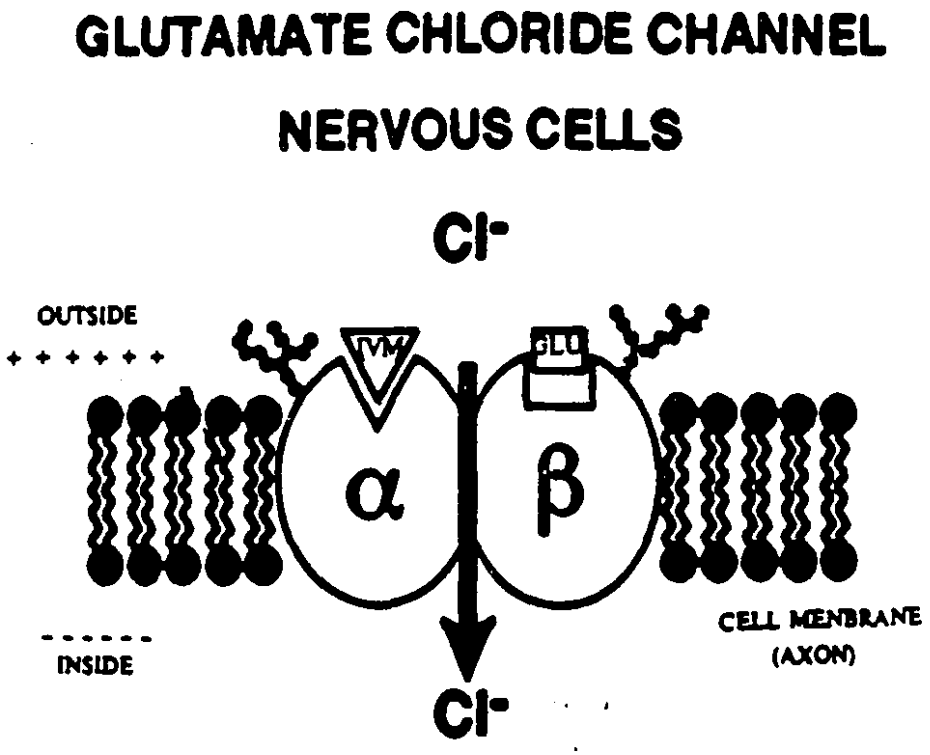

Fig 1.3. The mode of action of avermectins (as adopted from Cully, *et al.*, 1994)

The rate of emergence of anthelmintic resistance appears to vary geographically in accordance with the prevailing climate, parasite species and in particular with the treatment regimes adopted in the region. Sheep and goat producers have tended to adhere to treatment regimes involving frequent treatments and consequently as the prevalence of resistance within one family of drug increased, producers switched to alternate drug families, resulting in the emergence of multiple resistance (Van Wyk and Malan, 1988; Badger and McKenna, 1990; Watson and Hosking, 1990). To date, the majority of problems of drug resistance problems are in sheep and goat nematodes. However there is emerging evidence of anthelmintic resistance in cattle (Anderson, 1977; Anderson and Lord, 1979; Geerts *et al.,* 1987; Borgsteede, 1991; Borgsteede *et al.,* 1992) and pig nematodes (Bjorn *et al.,* 1989).

IVERMECTIN RESISTANCE

Field Reports

The most widespread occurrence of ivermectin resistance involves *H. contortus.* The first field reports of *H. contortus* resistant to ivermectin were confirmed in South Africa (Carmichael *et al.,* 1987), in a mixed nematode infection in sheep, 33 months after the drug was introduced. The recommended dose of ivermectin eliminated *Ostertagia spp, T. colubriformis* and *Nematodirus battus* but its efficacy was less than 50% against *H. contortus*. Other isolates of *H. contortus* resistant to ivermectin in S. Africa have been reported (Van Wyk and Malan, 1988; Van Wyk *et al.,* 1989) and as above, in both cases other nematode species were susceptible to the drug.

Echevarria and Trinidade (1989) detected resistance to ivermectin in a *H. contortus* isolate from sheep in Brazil. An efficacy trial, after 32 treatments given in a 54 month period, showed that 0.2 mg/kg ivermectin produced 59% reduction of adult worms. Subsequent studies of this isolate showed ivermectin resistance occurred at both L4 and adult stages (Echevarria *et al.,* 1992). In

another isolate of *H. contortus* from Brazil ivermectin at use-level removed only 18% of the adults worms in sheep. The history of previous exposure to ivermectin of this strain was not available (Viera *et al.,* 1992).

In Australia, Le Jambre (1993b) also confirmed the presence of an ivermectin resistant strain of *H. contortus.* This strain had contaminated a morphologically marked inbred strain of *H. contortus* in sheep at the research station. The resistant strain was thought to have three possible origins. Firstly it may have arisen when ivermectin was being used at the research farm as a broad spectrum anthelmintic. Secondly it could have arisen through spontaneous mutation during the screening procedure to produce 'worm free sheep'. Thirdly the strain could have originated from elsewhere in the field and passed through the quarantine. Further characterization of this strain (Le Jambre *et al.,* 1995) showed that 0.4 mg/kg of ivermectin reduced the mean worm burden in sheep infected with this by 16%. It was further noted that the female population was unaffected while the male population was substantially reduced (47% reduction), suggesting that ivermectin resistance may be influenced by sex, with males being less resistant than females.

In the USA, Craig and Miller (1990) reported ivermectin resistance in a goat population in Texas infected with *H. contortus.* Ivermectin had been given to goats every three weeks during spring and summer with occasional switching to benzimidazoles for five years. A faecal egg count reduction (FECR) test indicated that treatment at use level produced only a 90% reduction in the parasites. In another report (Miller and Barras, 1994) ivermectin resistance was detected in lambs at the Louisiana research station after 5 years with 4-5 treatments per year.

Other nematode species have also been reported to be resistant to ivermectin. Ivermectin-resistant strains of *Ostertagia circumcinta (=O.trifurcata)* have been reported in goats in New Zealand (Watson and Hosking, 1990; Badger and McKenna, 1990; Pomroy *et al.,* 1992). In the report by Watson and Hosking (1990), kids were given ivermectin 10-12 times

a year and adults dosed 5-9 times a year for 5 years. Necropsy of lambs grazed on the goat pasture and treated with the recommended dose of ivermectin, showed an efficacy of 93% against *O. circumcinta.* This isolate was also multiply resistant to oxfendazole and morantel. It was also noted that treatment with ivermectin eliminated egg production in this strain of *O. circumcinta* despite survival of some worms. Decreased fecundity of worms surviving ivermectin treatment has also been reported in *Cooperia curticei* (Bogan *et al.,* 1988), in *H. contortus* (Scott *et al.,* 1991; Le Jambre *et al.,* 1995) and is well known in filariae (Duke *et al.,* 1990).

In the report by Badger and McKenna (1990), goats were treated exclusively with ivermectin at use level nine times per year for 4 years. At the end of this period, treatment at 0.2 mg/kg reduced *O. circumcinta* burdens by 87%. Pomroy *et al.* (1992) also described an ivermectin resistant strain of *O. circumcinta* in goats using FECR tests. Following treatment with ivermectin at use level FECR was only 27%. This isolate had been treated with ivermectin periodically over a period of eight years and it was also shown to be oxfendazole, fenbendazole and levamisole resistant at their recommended use levels.

Jackson *et al.* (1992) reported a goat strain of *O. ostertagi* resistant to ivermectin from Scotland. Ivermectin was used on 16 occasions over 3 years. This isolate was also fenbendazole resistant but levamisole susceptible. *Oesophagostomum dentatum* and *Oesophagostomum quadrispinulatum,* parasitic in pigs, have also been reported to be resistant to ivermectin. These isolates were also found to be resistant to benzimidazoles and levamisole/morantel (Bjorn *et al.,* 1989).

Apparently there is higher prevalence of ivermectin resistance in goat than in sheep herds. This is probably due to the tendency to treat goats at the same dose rate as sheep. Differences in drug pharmacodymanics (Galtier *et al.,* 1981; Bogan *et al.,* 1987) and the degree of rumen by-pass (Sangster *et al.,* 1991) may result in reduced bioavailability in goats compared with sheep

(Jackson, 1993).

Laboratory Studies

In laboratory studies, ivermectin resistant strains have been selected. Egerton *et al.* (1988) showed that a population of *H. contortus* selected for eight generations at the adult stage with suboptimal doses (0.02mg/kg) of ivermectin produced a four fold resistant population. Passage of this resistant isolate for an additional 11 generations without drug pressure produced no reversion to susceptibility suggesting that resistance to ivermectin was stable. Echevarria *et al.* (1993a) also selected a *H. contortus* isolate for eight generations with one-tenth of the recommended dose (0.02 mg/kg) by treating the adult stage. Analysis of the eggs per gram of faeces during the 14 day period following treatment showed that the egg output from this selected strain, when treated with ivermectin, was not significantly different from that of untreated controls.

An ivermectin resistant isolate of *T. colubriformis* with four fold resistance to this drug was also reported after four generations of ivermectin selection (Giordano *et al.*, 1988). Drug pressure was exerted against immature stages (L4) at each generation. Shoop *et al.* (1990) also showed 20 fold resistance to ivermectin in a thiabendazole resistant strain of *T. colubriformis.* This ivermectin resistant strain was selected with ivermectin for 20 worm generations with a dose that killed 95% of the adult worms at each generation.

POPULATION STRUCTURE AND ANTHELMINTIC RESISTANCE

Drug selection, like all forms of selection, is based on the natural genetic diversity that occurs in a population. Selective removal of susceptible members in a population by drug toxicity leaves resistant members to contribute the greater share of genes to the next generation (Shoop, 1993). Anthelmintic resistance is thought to be a pre-adaptive phenomenon. Therefore, for many species of nematodes, the gene or genes conferring resistance are available within the population prior to exposure to a drug. Anthelmintic resistance thus

arises from selection within the normal phenotypic range. This is supported by selection models described by Martin and McKenzie (1990). Mendelian studies on the inheritance of benzimidazole resistance in *H. contortus* (Le Jambre *et al.*, 1979) and *T. colubriformis* (Martin *et al.*, 1988a) also indicated that the resistance was polygenic. These results have now been confirmed by molecular studies (Le Jambre, 1993a).

Beech *et al.* (1994 and personnal communication) have confirmed the presence of BZ resistance alleles prior to anthelmintic exposure. In comparing the frequency of alleles in two independently derived BZ resistant strains of *H. contortus* and a susceptible strain, the allele frequency in the two resistant strains were found to be similar to each other but they differed significantly from the susceptible strain. The same allele in the resistant strains was favoured by selection with benzimidazoles, suggesting that changes in allele frequency, rather than novel genetic arrangements induced by exposure to drug, are involved in the development of BZ resistance. The molecular genetics of ivermectin resistance remain to be elucidated.

The speed of selection for anthelmintic resistance will be influenced by many factors. Selection pressure will be highest when the proportion of resistant (R) to susceptible (S) genotypes increases most rapidly. If the ratio of R/S is low, selection will be most rapid if all the heterozygous resistant plus homozygous resistant individuals survive (Mani and Wood, 1984). Therefore dose rates should be high enough to kill heterozygous resistant worms, thus rendering resistant genes effectively recessive (Prichard, 1990). It has been shown that when suboptimal treatments are administered, anthelmintic resistance develops rapidly. This is because such low doses tend to give the heterozygotes a selective advantage rather than kill them (Martin, 1989; Egerton *et al.*, 1988). An important factor in the population genetics of controlled populations is that of the refugia where the parasite population is not exposed to drug (Smith, 1990) In most cases, the prominent refugia are those hosts that do not receive treatment and free living stages on pasture.

Refugia have two important but opposite effects that operate on the development of resistance. The presence of refugia may prolong the useful life of the drug. On one hand, if large numbers of parasites are in refugia at the time of treatment, more of the genetic diversity of the parasite population will be maintained, ensuring that the parasite will be able to respond to changing host populations or use of alternate drugs; If treatment occurs when few parasites are in the refugia, the genetic diversity will be severely reduced and the gene pool for future parasite generations will be very limited (founder effect) thus future control efforts will be limited (Medley, 1994).

DRUG RESISTANCE AND PARASITE FITNESS

Parasite fitness is determined by characteristics that are important in their multiplication, survival and transmission both in the host as well as in the environment. These factors determine the contribution by individuals of offspring to the next generation (Anderson and May, 1982). The rate of reversion (return to or towards susceptibility to a drug) is one estimate of the relative fitness of the resistant and the susceptible parasitic phenotypes. This rate may vary and will depend on the re-association of general fitness characteristics with resistance characteristics, genotypic compensations which modify the effect of the resistant genotype and the existence of positive or negative hybrid vigour. Selection pressure and the level of resistance may thus influence the rate of reversion (Prichard, 1990). Martin *et al.* (1988b) showed that benzimidazole resistant strains of *Ostertagia spp* (85% *O. circumcinta* and 15% *O. trifurcata)* showed a reduction in the degree of resistance to benzimidazoles after counter selection with levamisole under field conditions. Other studies have also shown that reversion to benzimidazole susceptibility occurs in sheep nematodes only after being exposed to levamisole for a period of time (Donald *et al.*, 1980; Waller *et al.*, 1983). Thiabendazole treatment has also been observed to decrease levamisole resistance in *T. colubriformis* (Waller *et al.*, 1985).

Reversion towards anthelmintic sensitivity has also been demonstrated by using other approaches. In an elegant study, Van Wyk and Van Schalkwyk (1990) used donor sheep infected with a susceptible strain of *H. contortus* to seed pasture which had been contaminated with a resistant strain of *H. contortus*. The replacement of the resistant by the susceptible strain was attempted at two strategically different times. In two of the five test camps, the susceptible strain was introduced in the autumn after 8-10 weeks of nil grazing; in the remaining three camps the introduction was made in spring (two camps) or summer without having a period of nil grazing. Grazing of worm free sheep on these pastures indicated that reversion to benzimidazole susceptibility had occurred, but in only three of the five camps. These included the two infested with the susceptible strain in the spring and one of the two infested in autumn.

On the other hand, Hall *et al.* (1982) showed that when anthelmintic treatments were deferred, strains of *H. contortus* and *T. colubriformis* showed no loss of benzimidazole resistance after 12 generations. Likewise passage of ivermectin resistant strains of *H. contortus* for 11 generations showed no reversion towards sensitivity to the drug (Egerton *et al.,* 1988). Probably the rate of reversion to anthelmintic susceptibility depends on the reassociation of general fitness characteristics with the resistance characteristics. If, during selection of drug resistance, the general fitness characteristics improve, then reversion will not be expected to occur and if it does it would be after a long time (Prichard, 1990).

The fitness of anthelmintic resistant and susceptible genotypes has also been assessed by comparing their life history traits in the absence of the drug both in the host and the environment. Most of these studies have been based on benzimidazole resistance or susceptible nematode strains. Kelly *et al.* (1978) showed that a benzimidazole-resistant strain of *H. contortus* was significantly more infective (48%) and pathogenic for sheep than a susceptible strain (19%). The rate of egg production, development of eggs and survival of the free-living

stages of the resistant strain was also greater for the resistant worms when compared with the susceptible ones. These authors were amongst the first to suggest that the development of benzimidazole resistance in parasites is accompanied by a developmental and a survival advantage over the susceptible parasites. A strain of *T. colubriformis* exhibiting multi-resistance to thiabendazole, levamisole and morantel was also found to be more infective than a susceptible strain (Kelly *et al.,* 1981). Hall *et al.* (1981) working on *H. contortus* and *Ostertagia spp* in sheep supported the proposal of higher establishment for the resistant strains. They reported an increase in establishment of 3.5 times for *H. contortus* and 1.7 times for *Ostertagia spp.,* over 5 generations of selection with cambendazole, oxfendazole and morantel. Kelly *et al.* (1981) similarly showed higher establishment for BZ-resistant *H. contortus* and *T. colubriformis* but not for BZ-resistant *Ostertagia spp.* Borgesteede and Couwenberg (1987) showed that the eggs per gram of faeces from sheep infected with a fenbendazole resistant strain *H. contortus* remained high during the patency period compared with a susceptible strain. This observation could, however, be due to interhost variation as the observation was only in one sheep per strain.

In direct conflict with these findings, MacLean *et al.* (1987) showed that a susceptible strain of *T. colubriformis* had a higher establishment, fecundity and pathogenicity than the resistant strain. Prichard *et al.* (1978) had earlier demonstrated that susceptible strains of *H. contortus* and *T. colubriformis* caused deaths in one third of the sheep and clinical parasitism in the remainder, whereas the resistant worms cultured similarly and administered at the same dose rate caused no obvious clinical signs.

Maingi *et al.* (1990) further re-examined this issue and found that the selection of a resistant strain of *H. contortus* over four generations from moderate level resistance to higher level of resistance to benzimidazoles resulted in an increase in the rate of establishment in the host which was reflected in higher egg production and pathogenicity. The strain showing

moderate resistance had lower fecundity, establishment and was less pathogenic than both the highly resistant and a susceptible strain from another source. Maingi *et al.* (1990) proposed that the introduction of a drug to a naive nematode population results in selection of a resistant population of nematodes. The moderately resistant individuals (probably heterozygous) show lower biological fitness when compared with the fully susceptible parasites and if the drug is withdrawn at this point reversion may be possible. However if selection continues, resistant alleles reassociate with other fitness characteristics and the highly pressured individuals survive better than moderately resistant worms. Thus the highly resistant individuals may have a level of general fitness approaching that of the fully susceptible parasites.

However while this may be true in cases of benzimidazole resistance and susceptible nematode populations, in situations when the parasite population is resistant to ivermectin or to more than one anthelmintic, very little is known about the fitness of these parasite populations. It has been shown that an ivermectin resistant strain of *H. contortus* had a 5-6 day lag in the onset of egg production, 20-21 days compared with 15-16 days in the susceptible strain (Scott *et al.,* 1990). However, these results may been primarily due to adaptation to sheep, of the resistant parasites which were derived from a population of goats rather than due to drug resistance (Pankavich *et al.,* 1992).

In a study conducted to compare the fitness of a naive strain of *H. contortus* with one that was resistant to ivermectin, benzimidazoles and salicylanilides, Scott and Armour (1991) showed that the two strains had similar rates of establishment and egg production. There were also no significant differences between the two strains for any haematological or biochemical measurements although anaemia and hypoproteinaemia were slower to develop in the animals infected with the resistant strain. The two strains showed differences in development and survival of the free-living stages at a variety of temperatures. The lower percentage of eggs that hatched to larvae indicated that the resistant parasites did not survive as well as the

susceptible parasites. However reduced survival of the resistant strain could have been due to reduced ability to survive in the environment as a result of developing resistance to a variety of anthelmintics . This same phenomena of reduced fitness in a moderately resistant strain was observed by Maingi *et al.* (1990).

Echevarria *et al.* (1992) observed that the development to fourth stage larvae in a strain of *H. contortus* resistant to ivermectin was not different from one that was susceptible to the drug. Similarly DeVaney *et al.* (1992) did not observe differences in the pathogenic effects or prepatent period in ivermectin-resistant and susceptible strains of *H. contortus,* nor were there any differences in the survival of the free-living stages under laboratory conditions.

A comparison by Echevarria *et al.* (1993b) of an ivermectin-susceptible strain (S-IVM), an ivermectin resistant population (R-IVM) derived from S-IVM and an independent multi-resistant field strain (R-IVM/SA), under both field conditions and laboratory conditions indicated variable differences in survival and development. After incubation for one week at 22C about 30% and 20% of the eggs had developed to infective stage in the R-IVM and S-IVM respectively. In contrast at 27C, 60% of the eggs in the susceptible strain had developed to L3 stage while development of the R-IVM was less than 10%. However at both temperatures, the percent development of multi-resistant strain was very low and never exceeded 5%. A field study carried out by seeding pastures during each of the three summer months in Brazil, showed that there were no significant differences in larval recoveries of the three strains from pasture except in the first two months. However when contamination occurred during the third month, the IVM-resistant strain produced significantly higher recovery rates from both pasture and soil.

A major criticism of many of these and related studies is that the comparisons involve anthelmintic resistant and susceptible strains that were derived from different sources and thus they may have shown ectopic differences. In addition where resistant strains were obtained in the lab (Maingi

et al., 1990; Echevarria *et al.*, 1993b), the effect of the rapid passage from host to host during the drug selection protocol was not assessed. It has been shown that rapid passage itself increases the infectivity and decreases survival of *H. polygyrus* in mice (Dobson and Owen, 1977).

This study was thus designed to determine whether selection with ivermectin alters the fitness traits of the parasite population and whether these changes are related to ivermectin resistance per se or to the rapid passage associated with the drug selection regime. The fitness traits characterized were establishment, fecundity, short and long term survival of a murine trichostrongylid, *H. polygyrus.*

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CHAPTER 2

THE EFFICACY OF IVERMECTIN (IVM) AGAINST LABORATORY STRAINS OF *HELIGMOSOMOIDES POLYGRUS*(Nematoda)

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ABSTRACT

A susceptible "parent" strain (S) of the mouse parasite *Heligmosomoides polygyrus* was selected for fifteen generations by treatment at day 6 post-infection (pi) with increasing doses of ivermectin (0-6 mg/kg). A Passage strain was also developed in parallel with the ivermectin selected strain, to control for the selection of traits due to rapid passage of parasite from host to host at 28 day intervals. Dose titration trials were conducted to determine the efficacy of ivermectin against the parent strain (S) when compared with the 8th generation of the ivermectin selected (IVM-8) and Passage (P-8) strains, as well as the strains IVM-15 and P-15 obtained after 15 generations of ivermectin selection and passage respectively. Mice were treated with 0-10 mg/kg ivermectin either on day 6 pi at the L4 stage or 21 days pi at the adult stage. Worm burden was assessed 21 days after treatment and the doses of ivermectin required to kill 50% (LD50), 90% (LD90) and 95% (LD95) of the parasite were determined using probit analysis. The LD50, LD90 and LD 95 of the two ivermectin-selected strains (IVM-8 and IVM-15) were approximately 1.5 times that of the parent strain (S), indicating resistance to ivermectin had been selected. There were no significant differences in the LD50, LD90 and LD95 of the Passage strains (P-8 and P-15) and the parent strain (S). Ivermectin was found to have a reduced efficacy against the L4 stages in strains IVM-8, P-8 and S, compared with the adult stage of parasite.

INTRODUCTION

Ivermectin was introduced in the early 80's as a broad spectrum anthelmintic for the control of gastrointestinal (GI) nematodes in livestock. However the effective use of this drug is gradually becoming limited due to the evolution of ivermectin resistant nematode populations. Reports of ivermectin resistance have come from the field especially among GI nematodes in sheep and goat herds (Carmichael *et al.,* 1987); Van Wyk and Malan, 1988; Echevarria and Trindade, 1989; Craig and Miller, 1990; DeVaney *et al.,* 1992; Jackson *et al.,* 1992; Mwamachi *et al.,* 1993; Le Jambre, 1993; Miller and Barras, 1994). Recently ivermectin resistance has been reported in cattle nematodes (Vermunt *et al.,* 1995, Watson *et al.,* 1994). In addition to isolating field strains, laboratory studies have been used to select ivermectin resistant nematode strains (Egerton *et al.,* 1988; Giordano *et al.* 1988; Shoop *et al.,* 1990; Echevarria *et al.*, 1993). Johnson (1993) has also generated mutants of the free living nematode *Caenorhabditis elegans* which are resistant to ivermectin.

The murine GI trichostrongyle *Heligmosomoides polygyrus* has been found to be a useful laboratory model of chronic GI nematode infections (Monroy and Enriquez 1992). It has a life cycle which is very similar to that of parasites of medical and veterinary importance, and in most available mouse strains primary infections last several months (Ehrenford, 1954; Keymer and Hiorns, 1986). This parasite has also been exploited by pharmaceutical firms in anthelmintic screens (Howes and Lynch, 1967; Coles and McNeillie, 1977; Misra *et al.,* 1981; Burg and Stapely, 1989). Previous studies (Sayles and Jacobson, 1983; Rajasekariah *et al.,* 1986; Wahid *et al.,* 1989) have established that *H. polygyrus* infections can be abbreviated using ivermectin both at the L4 and adult stages. In the present investigation *H. polygyrus* was thus used to study the evolution of ivermectin resistance. The main objectives of the study were (1) to develop an ivermectin resistant strain of *H. polygyrus* by exerting increasing ivermectin selection pressure on a susceptible "parent"

strain, (2) to develop a parallel control strain by simultaneous rapid passage of the parent strain at 28 day intervals and (3) to determine the efficacy of ivermectin against the parent strain (S) and the 8th and 15th generations of the ivermectin-selected (IVM-8 and IVM-15) and Passage (P-8 and P-15) strains. The Passage parasite strains were derived as a control for the study of the life history traits of *H. polygyrus* undergoing ivermectin selection, described in the next manuscript.

MATERIALS AND METHODS

General methods

In all experiments 3 week old CD-1 outbred (Charles River, Canada) female Swiss mice were maintained in groups of four in standard caging under constant temperature, and 14h light and 10h dark cycle. They were given a conventional pelleted rodent diet and water *ad lib*. The mice were acclimatized 4-5 days before infection. The eggs of *H. polygyrus* were cultured to infective larvae (L3) following Burren's method (1980). Mice were intubated under light anaesthesia, with L3 suspended in 15-25μl distilled water using a pipetteman with disposable tips. The larvae used in all infections were less than 7 days old. During treatment the required drug concentrations were obtained by appropriate dilution of ivermectin (EQVALAN, 10mg/ml Merck & Co., Inc., Rahway, N.J.) in distilled water and the resulting suspension was given orally within minutes of preparation under light anaesthesia (METOFANE, Janssen pharmacetica, Missassuga, Ontario). Untreated mice were given distilled water of the same volume as that of the diluted drug.

Post-treatment net egg production of the parasites was determined by placing individual mice in a cage over a wire grid for 24 hours. The faeces collected after 24 hours in the water below were washed and the number of eggs produced per mouse per day estimated as described by Scott (1988). Worm burden was determined by killing the mice with carbon dioxide and counting and sexing the worms in the small intestine of each mouse. Per capita

fecundity was then estimated by dividing net egg production by the number of female worms.

Selection Protocol

A "parent" stock population (S) of *H. polygyrus* has been maintained at the Institute of Parasitology for the past 12 years by passage in CD-1 female mice every 3-4 months. Forty five CD-1 mice were infected with the 500 L3 of stock "parent" parasite strain (S). On day 6 post infection (pi) 30 of these mice were treated with 1.0 mg/kg ivermectin. Faecal cultures on day 28 pi from the treated mice provided larvae for the first ivermectin-selected generation (IVM-1) of parasite. Larval cultures from ten of the untreated mice provided larvae for the first unselected generation referred to as the Passage strain (P-1). The remaining five untreated mice were used as a basis of comparison to determine the efficacy of ivermectin at the first treatment.

The first generation larvae (IVM-1) of the ivermectin-selected parasite population were then used to infect another group of 35 mice of which 30 were also treated on day 6 pi and 5 remained untreated as controls for the efficacy test. Increasing doses of ivermectin were required to achieve approximately 80% efficacy in the ivermectin selected strains indicating that the parasite was developing resistance to ivermectin (Fig 2.1). Thus the selecting doses of ivermectin were gradually increased to 6.0 mg/kg.

The first generation larvae from the Passage parasite population (P-1) were similarly used to infect another group of 10 mice that remained untreated and the procedure was also repeated for 15 generations.

Dose Response Trials

The following dose titration experiments were done to assess the efficacy of ivermectin against the adult and the L4 stages of the stock parasite (S), the eighth and fifteenth generations of the ivermectin- selected (IVM-8 and IVM-15) and the Passage (P-8 and P-15) parasite populations.

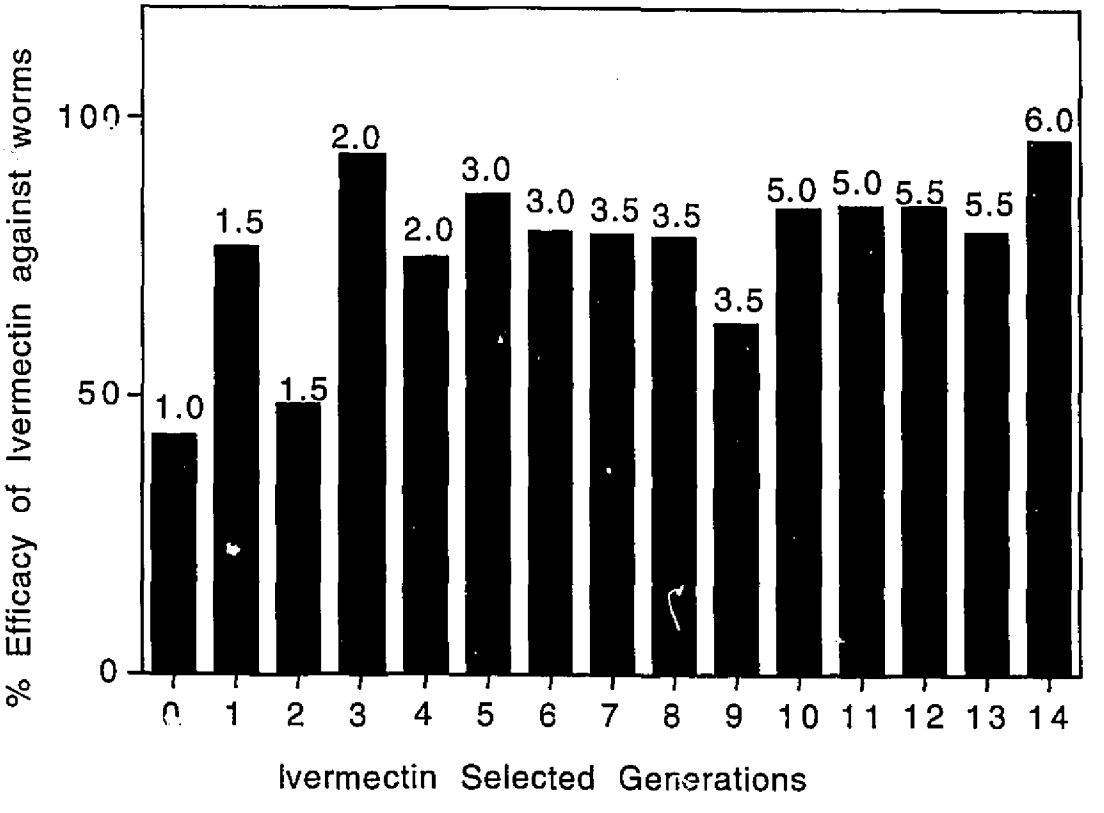

Fig 2.1: The efficacy of ivermectin against *Heligmosomoides polygyrus* during selection with increasing doses of ivermectin (mg/kg) as shown above each bar.

Efficacy Against the Adult Worms

Experiment 1

One hundred and twenty mice were randomly divided into 3 groups of 40 mice. Each group was infected by oral gavage with 150 L3 of either the S, IVM-8 or P-8 strains. Twenty one days pi, four mice per group were treated with varying doses of ivermectin (0, 0.25, 0.5, 1, 2, 3, 3.5, 4.5, 5, 7 and 10 mg/kg). Twenty one days post-treatment, the egg output of the surviving worms was quantified for each individual mouse. The mice were then killed the following day and the total number of female and male worms in each mouse was counted. The per capita fecundity was also estimated.

Experiment 2

The worm recovery in the control group of the stock parasite strain (S) in experiment 1 was low (Fig 2.2a), therefore the same experiment was repeated a few months later with the same parasite strains (S, IVM-8 and P-8). The sample size was increased to 10 mice/dose/strain. The mice were treated 21 days post-infection with various doses of ivermectin (0, 0.5, 1, 1.5, 2, 3, 4 and 5 mg/kg) and the worm burden was determined 21 days post-treatment.

Experiment 3

A similar protocol to experiment 2 was also used to test the efficacy of ivermectin against the S, IVM-15 and P-15 parasite strains. The sample size was 10 mice/dose/strain. The doses of ivermectin used were 0, 0.5, 0.75, 1, 1.5, 2, 2.5 and 3.5 mg/kg body weight. Treatment was at 21 days pi and mice were killed 21 days post-treatment.

Efficay Against L4

Experiment 4

Another group of 216 mice was divided randomly into three groups of 72 mice each. Each group was infected with 150L3 of either S, IVM-8 or P-8 strains. Eight mice/strain were then treated with ivermectin at 0.5, 1, 2, 3, 4, 5, 6 or 7 mg/kg body weight on day 6 pi. Twenty one days post-treatment the mice were killed and the worm burden assessed.

Data Analysis

The efficacy of ivermectin during the selection process was determined by percent reduction in worm burden at each dosage in the treated mice compared with the untreated mice (Fig 2.1). The percent efficacy was calculated using the arithmetic mean in the following formula

$$\{(C-T)*100\}/C$$

where C = arithmetic mean worm burden in control mice and T = arithmetic mean worm burden in treated mice. Probit analysis (SAS/STAT User's Guide, 1990) on the worm recovery data from the dose response trials, 21 days post-treatment was used to estimate the dose of ivermectin required to kill 50% (LD50), 90% (LD90) and 95% (LD95) of the parasites and the 95% confident limits (ci), (Finney 1971).

RESULTS

Efficacy Against the Adult Worms

The number of worms surviving the treatment decreased with increasing doses of ivermectin in all parasite strains. The ivermectin selected strains (IVM-8 and IVM-15) showed a higher worm burden at all doses compared with the Passage strains (P-8 and P-15) and stock (S) parasite strains (Fig 2.2). Post-treatment net egg output in strains IVM-8, P-8 and S showed a similar trend (Fig 2.3). The per capita fecundity (Fig 2.4) of worms surviving treatment was similar at all doses.

Table 2.1, shows the LD50, LD90 and LD95 and 95% CI of all strains (IVM-8, IVM-15, P-8, P-15 and S). Selection of the parent strain, S with ivermectin for eight generations significantly increased its LD50, LD90 and LD95, however an additional 7 generations of selection did not significantly change the level of resistance. The Passage strain (P-8) and the parent strains (S) had similar levels of LD50, LD90 and LD95. Similarly LD50, LD90 and LD95 for strains P-15 and S were not significantly different (Table 2.1). The LD50, LD90 and LD95 of the stock parasite in experiment 1 were not estimated due to low worm recovery in the untreated mice in this group.

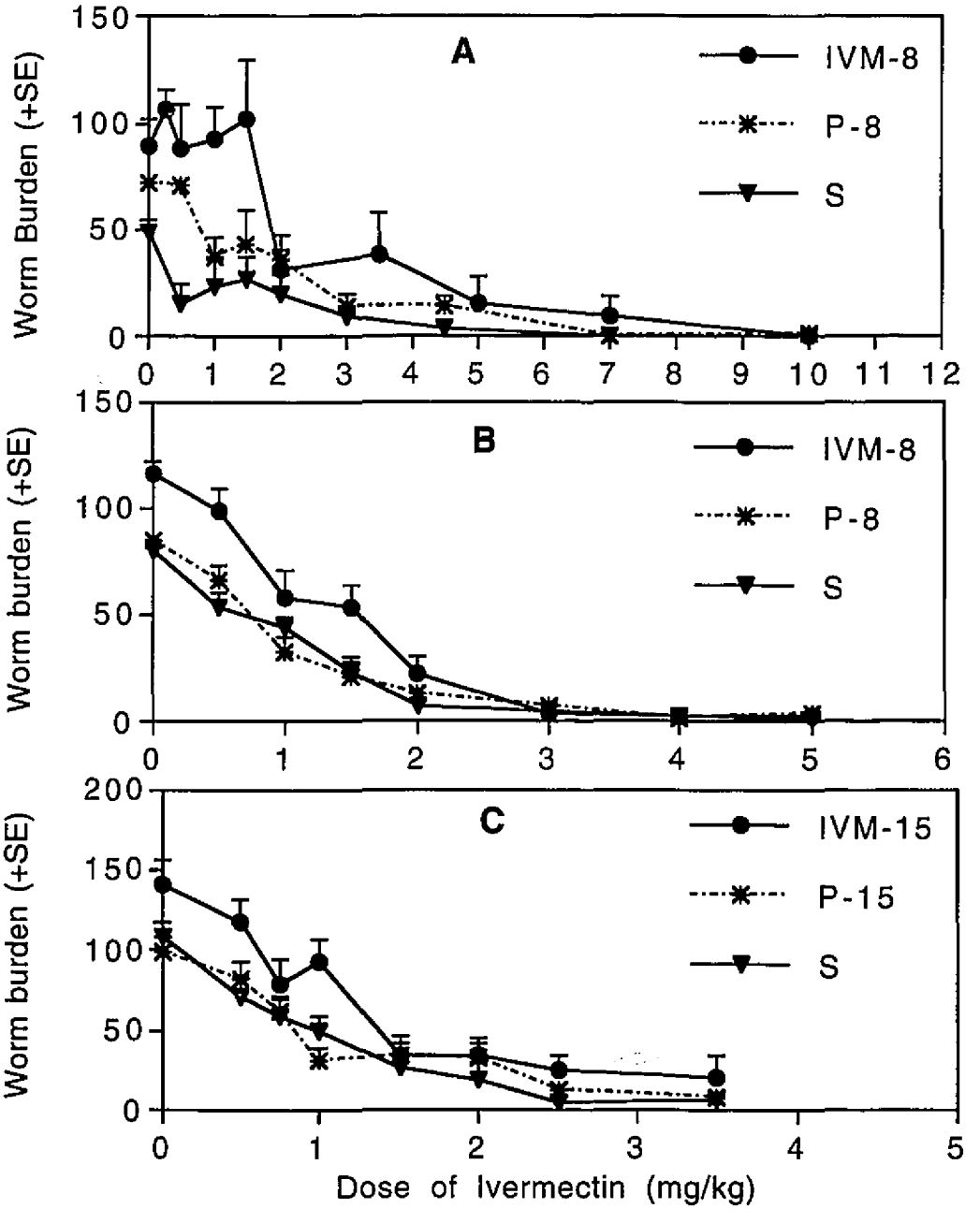

Fig 2.2: Number of worms (+se) 21 days post-treatment in mice infected with *Heligmosomoides polygyrus* and treated at 21 days post-infection (**A**) Strains IVM-8, P-8 and S, Experiment 1, (**B**) Strains, IVM-8, P-8 and S, Experiment 2, (**C**) Strains IVM-15, P-15 and S, Experiment 3.

Table 2.1: Dose response profiles of strains of *Heligmosomoides polygyrus* IVM-8, P-8, S, IVM-15 and P-15 at various dates of treatment.

Experiment	Day of treatment	Strain[1]	LD50 (95%ci)	LD90 (95%ci)	LD95 (95%ci)
1	21	IVM-8	0.531 (0.482-0.582)	2.578 (2.266-2.978)	4.411 (3.768-5.276)
		P-8	0.389 (0.339-0.429)	0.894 (0.758-1.089)	1.186 (0.993-1.573)
		S	not estimated	not estimated	not estimated
2	21	IVM-8	0.584 (0.560-0.607)	1.283 (1.208-1.371)	1.676 (1.557-1.821)
		P-8	0.377 (0.349-0.401)	0.752 (0.703-0.818)	0.950 (0.867-1.073)
		S	0.353 (0.326-0.379)	0.851 (0.795-0.919)	1.148 (1.053-1.270)
3	21	IVM-15	0.603 (0.579-0.629)	1.269 (1.197-1.357)	1.636 (1.520-1.780)
		P-15	0.469 (0.451-0.487)	0.842 (0.768-0.898)	1.028 (0.957-1.121)
		S	0.441 (0.419-0.462)	0.948 (0.897-1.010)	1.231 (1.147-1.337)
4	6	IVM-8	0.850 (0.813-0.889)	2.244 (2.109-2.401)	3.122 (2.895-3.391)
		P-8	0.561 (0.532-0.590)	1.465 (1.371-1.575)	2.031 (1.872-2.225)
		S	0.511 (0.486-0.536)	1.169 (1.090-1.257)	1.549 (1.429-1.698)

[1]S, Stock "parent" strain; IVM-8 and IVM-15, 8th and 15th generations of the ivermectin selected line; P-8 and P-15, 8th and 15th generations of control Passage, line.

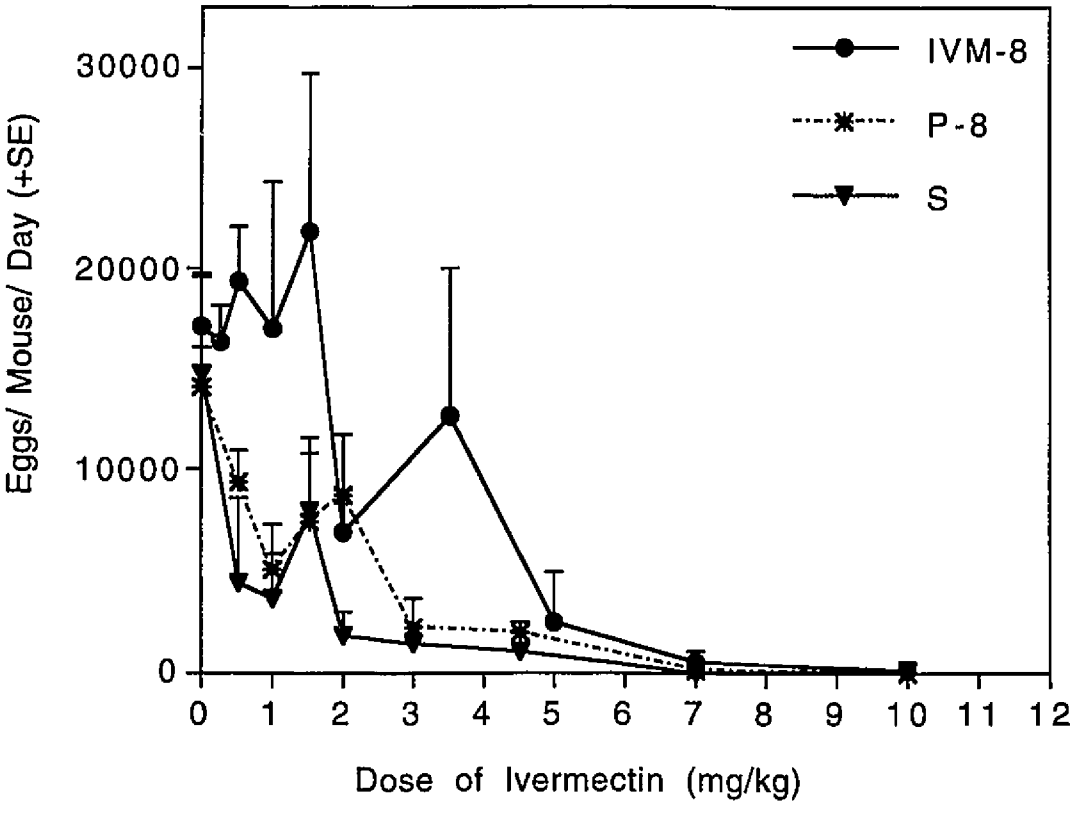

Fig 2.3: Net egg output (+se) 21 days post-treatment in mice infected with *Heligmosomoides polygyrus* strains, IVM-8, P-8 and S and treated at 21 days post-infection , Experiment 1.

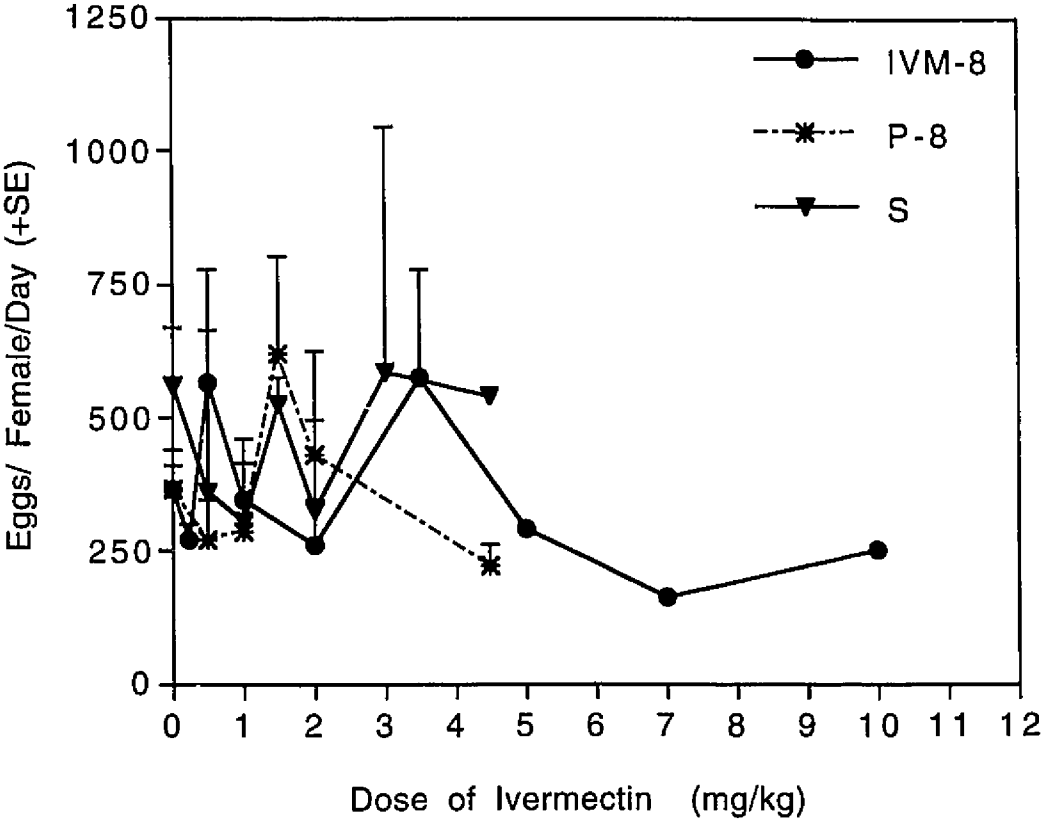

Fig 2.4: Per capita fecundity 21 days post-treatment in mice infected with *Heligmosomoides polygyrus* strains, IVM-8, P-8 and S and treated at 21 days post-infection , Experiment 1.

Efficacy Against the L4

The trend in worm recovery data of *H. polygyrus* strains IVM-8, P-8 and S when treated at L4 stage (Fig 2.5) was similar to when they were treated as adults. Higher worm recoveries were obtained in the ivermectin selected strain (IVM-8) when compared to the Passage (P-8) or the parent (S) parasite population. The LD50, LD90 and LD95 (Table 2.1) of IVM-8 was also significantly higher than that of P-8 or S parasites. The LD 50 of the parent strain was similar to that of the passage strain. However, the LD90 and LD 95 of the Passage strain, indicated an increase in tolerance to ivermectin. In all cases however higher dose of drug was needed to kill the L4 stage was compared to the adults.

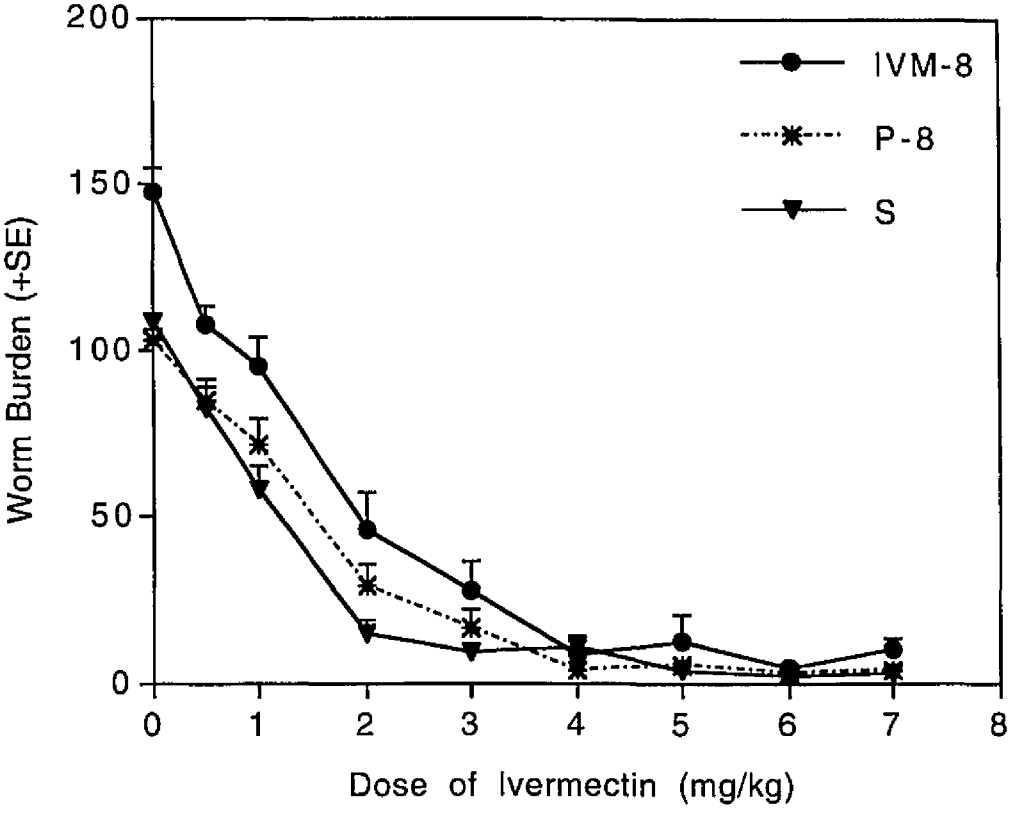

Fig 2.5: Worm recovery 21 days post-treatment in mice infected with Heligmosomoides polygyrus strains, IVM-8, P-8 and S, and treated at 6 days post-infection, Experiment 4.

DISCUSSION

Our results confirm that ivermectin resistance can be developed in a closed population of *H. polygyrus.* After 8 generations of selection, 1.5 fold resistance was obtained, however over a further 7 generations the level of ivermectin resistance did not increase significantly. The prime factors determining the rate at which resistance develops are the amount of genetic variation in the population and the intensity of drug selection pressure (Prichard *et al.,* 1980). Because our selection protocol involved approximately 80% efficacy at each generation (Fig 2.1), it is more likely that the genetic makeup of the parent population of *H. polygyrus* was the major factor in determining the rate at which resistance to ivermectin developed in this model,

High tolerance to ivermectin has been noted in a naive population of *H. polygrus* when compared to other GI nematodes suggesting a high frequency of ivermectin tolerance alleles in this nematode prior to ivermectin exposure. Rajasekariah *et al.* (1986) found that a dose of 0.2 mg/kg resulted in only 55% reduction in *H. polygyrus* when treatment was at adult stage. Other studies have also indicated a need for even higher doses necessary to eradicate 95% of a susceptible population of *H. polygyrus.* Sayles and Jacobson (1983) reported a dose of 5 mg/kg ivermectin and Wahid *et al.* (1989) reported a dose of 10 mg/kg IVM or higher (depending on the mouse strain) when treatment was done at the L4 stage. In our study the LD values indicate a dose of 1.0-1.3 mg/kg ivermectin is needed to eradicate 95% of the parent population of *H. polygyrus* when treated as adults and higher doses of 1.4-1.7 mg/kg ivermectin when treated at L4 stage (Table 2.1). In contrast the optimum dosage required for treating GI trichostrongylids in ruminants is about 0.1- 0.2 mg/kg (Campbell *et al.,* 1983).

Thus while high tolerance may partly explain the slow rate at which ivermectin resistance developed in this study, expression of resistance also depends upon the mode of action of a drug and its consequences upon a given

stage in a parasite life cycle in a given host species. The target site of ivermectin is a glutamate dependent chloride ion channel which has been well characterized in the free-living nematode *C. elegans* (Schaeffer and Haines, 1989; Arena *et al.,* 1991; 1992; Cully and Paress 1991; Rohrer., *et al* 1992). The opening of the chloride port leads to permeability to chloride ions which results in worm paralysis and eventually death. Survival of any individual may be influenced not only by the inherent degree of resistance but also by location within the host (Jackson *et al.,* 1992). The impairment of motor function may lead to serious consequences for luminal dwelling stages of nematodes when selection pressure is exerted on the adults. By way of contrast, the risk is much less for any histotrophic stages inherently capable of surviving exposure to drug that impairs motor function. Thus since the selection pressure in this trial was at the L4 stage of *H. polygyrus*, the requirement of a major genetic change to survive the effects of ivermectin selection pressure may have been less than if treatment had been administered when the worms were adults. The higher dose of ivermectin needed to kill the L4 stage when compared with adults in this and other studies (Wescott and LeaMaster, 1982; Giordano *et al.,* 1988; Echevarria *et al.,* 1992) suggests the effect of ivermectin may be immediate in adults due to the mode of action of drug as well as the location of parasite in host while it may be delayed in L4 stages, requring greater drug concentrations to achieve the same efficacy.

Previous lab selection studies have elicited different rates of resistance to ivermectin compared to our present result. An isolate of *Trichostrongylus colubriformis* had 4 fold resistance after only four generations of selection pressure with ivermectin exerted at the L4 stages (Giordiano *et al.,* 1988). Eight generations of selection with suboptimal doses of ivermectin at the adult stage of *Haemonchus contortus* elicited four fold resistance to ivermectin (Egerton *et al.,* 1988). On the other hand a strain of *T. colubriformis* which was also resistant to thiabendazole showed 20 fold resistant to ivermectin after 20 generations of selection (Shoop *et al.,* 1990) when drug pressure was also

applied at the adult stages. However caution should be exercised when comparing lab selection studies because there are differences in genetic makeup of the susceptible population among nematode species and because other factors such as dose used in selection and intensity of selection affect the rate at which drug resistance occurs.

As expected serial passage of the parasite had no effect on tolerance to ivermectin in adult worms. The increase in LD 90 and LD 95 against the L4 stage of Passage strain (P-8) was however not expected. A possible explanation is that passage process shifted the rate of development of the worms. Thus the parasite was more vigorous and drug tolerant at 6 days post-infection. Details of effects of rapid passage on life history trait of *H. polygyrus* are explained in Chapter 3.

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CONNECTING STATEMENT

Anthelmintic resistance is thought to be accompanied by changes in fitness characteristics of parasitic nematode populations. Therefore, having confirmed ivermectin resistance after 8 and 15 generations of ivermectin selection, we then proceeded to characterize and compare certain life history traits of the parent strain (S), the ivermectin selected strains (IVM-8 and IVM-15) and the Passage strains (P-8 and P-15) of the parasite *Heligmosomoides polygyrus*. The Passage strains were included to determine the effects of rapid passage of parasite from host to host at 28 day intervals. The fitness traits of interest were establishment at 8 days post-infection, worm survival, per capita fecundity, short and long term net egg output.

CHAPTER 3

THE EFFECT OF SELECTION WITH IVERMECTIN ON THE FITNESS OF *HELIGMOSOMOIDES POLYGYRUS*

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ABSTRACT

An ivermectin susceptible parent strain (S) of the mouse parasite *Heligmosomoides polygyrus* was selected for 15 generations with increasing doses of ivermectin. A Passage line was developed from the parent strain parallel with the ivermectin selected strain, to control for selection of life history traits due the effect of passage every 28 days during drug selection. A variety of life history traits were then characterized in five strains: the stock "parent" strain (S), the 8th and 15th parasite generations after selection with ivermectin (IVM-8 and IVM-15), and the 8th and 15th generations of control Passage strains (P-8 and P-15). Although the establishment of the ivermectin-selected strain, IVM-8, was significantly higher than that of the parent strain (S) its survival over four months was significantly lower. Strain P-8 had similar establishment and survival pattern to IVM-8. The egg production profile of three strains S, IVM-8 and P-8 were similar between 7 and 28 days post-infection (pi) but over the four months, net egg production was significantly higher in strain S than in IVM-8. At generation 15, the establishment and the net egg output profiles day 7-28 pi of strain IVM-15 were similar to those of the parent strain, S. However the Passage strain, P-15, had significantly higher establishment and developed to the adult stage more rapidly than either IVM-15 or S. This resulted in higher net egg output 7 to 28 days pi as well as a higher worm burden. The per capita fecundity did not differ among strains IVM-8, P-8, and S nor among strains IVM-15, P-15 and S. These data suggest that ivermectin selected strains of *H. polygyrus* had similar fitness with the parent strain. However the passage of the parent parasite strain from host to host increased the parasite's rate of establishment and development.

INTRODUCTION

Anthelmintic resistance of gastrointestinal (GI) nematodes can be a problem in sheep, goats, horses, cattle and swine (Prichard 1994). Yet surprisingly little is understood about the broader implications of anthelmintic resistance on the general biology of the parasites i.e the fitness of resistant phenotypes in absence of drug. The biological features important in multiplication, survival and transmission of nematodes define the fitness of an individual both in the host and in the environment. They also determine the contribution by an individual to the next generation (Anderson and May, 1982). It is important to understand whether parasite fitness does change with continued use of a drug if we are to control parasite populations effectively. It has always been assumed that a drug-resistant population will revert to drug susceptibility if the drug is withdrawn for a period of time (Jackson, 1993), but this may not be the case if the resistant parasite has an overall higher fitness.

Research comparing fitness of strains of GI nematodes that are resistant or susceptible to benzimidazole drugs (BZs) suggests that the biological features of the parasite population may change as a result of the drug selection pressure. For example, establishment, fecundity and survival of free-living stages were higher in BZ resistant than BZ susceptible *Haemonchus contortus* (Kelly *et al.*, 1978; Maingi *et al.*, 1990). However, the direction of change is not always consistent, as MacLean *et al.* (1987) reported decreased establishment of BZ-resistant *Trichostrongylus colubriformis* compared with a BZ-susceptible strain. A number of studies have also compared the fitness traits of ivermectin resistant and susceptible strains of GI nematode. As with the BZ studies, fitness changes were detected and included delayed onset of egg production (Scott *et al.*, 1990), and decreased survival of free-living stages (Scott and Armour, 1991; Echevarria *et al.*, 1993) of *H. contortus.*

Two general criticisms can be raised against several of these studies. Often the anthelmintic resistant and susceptible strains are derived from different sources (Kelly *et al.*, 1978; Maingi *et al.*, 1990; Scott *et al.*, 1990;

Scott and Armour, 1991). Thus, the observed fitness effects may be due to ectopic differences rather than resistance to the drug. When the resistant strain has been derived from a susceptible strain, a laboratory selection protocol is often used that involves rapid passage of the parasite from host to host (Maingi *et al.*, 1990; Echevarria *et al.*, 1993). The rapid passage itself may alter the life history traits of the parasite, and the effects attributed to drug selection pressure may be due to the rapid passage procedure instead. In fact, Dobson and Owen (1977) reported that frequent transfer of *Heligmosomoides polygyrus (=Nematospiroides dubius)* from mouse to mouse for 10 generations increased the infectivity but decreased the survival of the parasite.

The objectives of the present study were therefore to determine whether selection for ivermectin resistance alters the fitness traits the murine parasite *H. polygyrus* and whether these changes are related to drug selection *per se* or to the rapid passage associated with the drug selection regime. The fitness characteristics of interest were establishment, net egg output, per capita fecundity and survival of the parasite in the host.

MATERIALS AND METHODS

In all experiments, three week old CD-1 outbred (Charles River Canada) female Swiss mice were maintained in groups of four in standard caging in temperature controlled animal room (20-22C), 14day and 10h night cycle and provided with water and pelleted rodent diet *ad libitum*. The eggs of *H. polygyrus* were cultured to infective third-stage larvae (L3) following Burren's method (1980). The mice were acclimatized for 4-5 days. Mice were then inoculated under light anaesthesia with 150 L3 suspended in 15-25 μl distilled water using a pipetteman with disposable pipette tips. The number of eggs passed in the faeces and the worm burden in each mouse were determined as described by Scott (1988).

Parasite Strains

The details of the selection protocol for the *H. polygyrus* strains used in this study are described in the previous manuscript (Chapter 2). Briefly, a fully

susceptible stock (S) parasite population, which had an LD50 of 0.35 mg/kg ivermectin, was selected for 15 generations with increasing doses of ivermectin. After eight generations of selection, a 1.5 fold resistance was confirmed in strain IVM-8 (LD50 0.58 mg/kg). This did not change after a further seven generations of selection (LD50 of strain IVM-15, 0.6 mg/kg). The stock parasite (S) population was concurrently used to develop a second parasite line, referred to as the passage line, which served as a control for the effect of rapid passage of the parasite from host to host during the drug selection procedure. The passage parasite strains used in this study, P-8 and P-15, were found to be fully susceptible to ivermectin in the dose response trials (Chapter 2).

Characterization Experiments

Establishment

The eighth generation of ivermectin selected (IVM-8), Passage (P-8) or stock (S) parasite strains were used to infect 3 groups of 10 mice. The mice were killed at 8 days post-infection (pi) and the adult worms in the lumen and L4 in the mucosa were counted. The rate of development in was assessed as the percent adult worms in the parasite population 8 days pi. The same procedure was repeated with three groups of 20 mice each, infected with either S, IVM-15 or P-15 parasite strains.

Short-term Egg Production

Thirty mice were randomly divided into three groups of 10 mice each. Each group was infected with either IVM-8, P-8 or S parasite strains. The net egg output was monitored every other day from day 7 to day 28 pi in individual mice. The mice were then killed and the worm burden determined. The protocol was repeated using another 3 groups of 17 mice each that were infected with IVM-15, P-15 or S larvae.

Long-term Egg Production, Survival and Per Capita Fecundity

One hundred and twenty mice were intubated with infective larvae of either of S, IVM-8 or P-8 strains. The net egg production and the worm burden

were assessed in 10 mice per strain at 1, 2, 3, 4 months post-infection. The per capita fecundity was also estimated. The same procedure was repeated for IVM-15, P-15 and S, however the sample size was increased to 17 mice. In addition, egg output was monitored every two weeks for a period of four months. As an index of the cumulative reproduction effort over the four months, all data for net egg output per mouse were summed.

Data Analysis

All data were analyzed using SAS (Littell *et al.*, 1991). The data on rates of establishment were analyzed by one way analysis of variance. Tukeys multiple comparisons test was used to compare strains. Repeated measures analysis was performed on short-term net egg production profiles for all strains, as well as long-term net egg production profiles for strains IVM-15, P-15 and S. Two-way analysis of variance was used to determine the effect of time and strain on survival of the adult worms, monthly net egg production and per capita fecundity. When necessary, square root transformation was performed prior to analysis of variance to normalize variance. The level of significance was set at $\alpha = 0.05$.

RESULTS

Establishment

When generation 8 parasites were compared with the stock parasite, the number of worms in mice at 8 days pi was significantly higher ($P = 0.0244$) for the ivermectin-selected strain (IVM-8) than for the stock (S) parasite (Table 3.1). Establishment of the Passage strain (P-8) was intermediate and not significantly different from either IVM-8 or S. The rate of development, as measured by the percent of adult worms, was similar among all three strains (Table 3.1). After 15 generations, establishment of the Passage strain (P-15) was significantly higher ($P < 0.0001$) than that of either IVM-15 or S strains, as was the rate of development (Table 3.1). No significant differences were detected in establishment or rate of development between strains IVM-15 and S.

Table 3.1: The establishment and percentage of adult parasites at 8 days post-infection of *Heligmosomoides polygyrus* strains IVM-8, P-8, S, IVM-15, P-15 and S.

Strain[1]	Worm-Burden (SE)	% Adults
IVM-8	110.1 ± 8.2[a]	53.6±4.6[a]
P-8	103.1 ± 5.6[ab]	52.6±5.02[a]
S	81.5 ± 8.5[b]	63.4±4.0[a]
IVM-15	112.1 ± 4.9[b]	64.7±3.4[b]
P-15	141.5 ± 4.5[a]	73.8±2.9[a]
S	99.6 ± 4.6[b]	61.7±3.6[b]

Comparison is within a generation. Means with different subcripts are significantly different at $P<0.05$)

[1]S, Stock "parent" strain; IVM-8 and IVM-15, 8th and 15th generations of the ivermectin selected line; P-8 and P-15, 8th and 15th generations of control Passage, line.

Egg Production During First Month of Infection

Net egg production of strains IVM-8, P-8 and S increased rapidly between days 7 and 14 and remained relatively stable and comparable during the next three weeks of infection (Fig. 3.1a). At generation 15, the patterns were similar (Fig. 3.1b), but overall, strain P-15 had higher egg production than either IVM-15 or S strain ($P<0.0001$). This is likely due to the overall higher establishment success in mice infected with the P-15 strain.

Egg Production Over Four Months

Although, at generation 8, significant differences were detected among strains and with time, a significant interaction term was also detected in the analysis, limiting our interpretation of the data (Fig. 3.2a). Visual inspection suggest that egg production of IVM-8 dropped by 50% between months 1 and 2. Net egg output remained high in strain P-8 through the first two months of infection and then decreased by 50% at month 3. In the stock parasite (S), egg production did not decline until the 4th month pi.

Repeated measures analysis of variance was possible, using net egg production of IVM-15, P-15 and S strains, because the same mice were repeatedly sampled. As shown in Fig. 3.3, no significant differences were detected among strains, but changes did occur over time. Egg production increased until week 6 pi, then declined gradually in all three strains. Broadly comparable results were obtained from groups of mice killed monthly (Fig. 3.2b). Egg production declined significantly with time in all strains. However, the decline was slow and gradual for strain P-15, whereas egg production of strains IVM-15 and S remained constant for the first two months and then gradually declined. The total egg production estimated by summing the total net out put in mice repeatedly measured over four months indicated there were no significant difference among strains (Table 3.2).

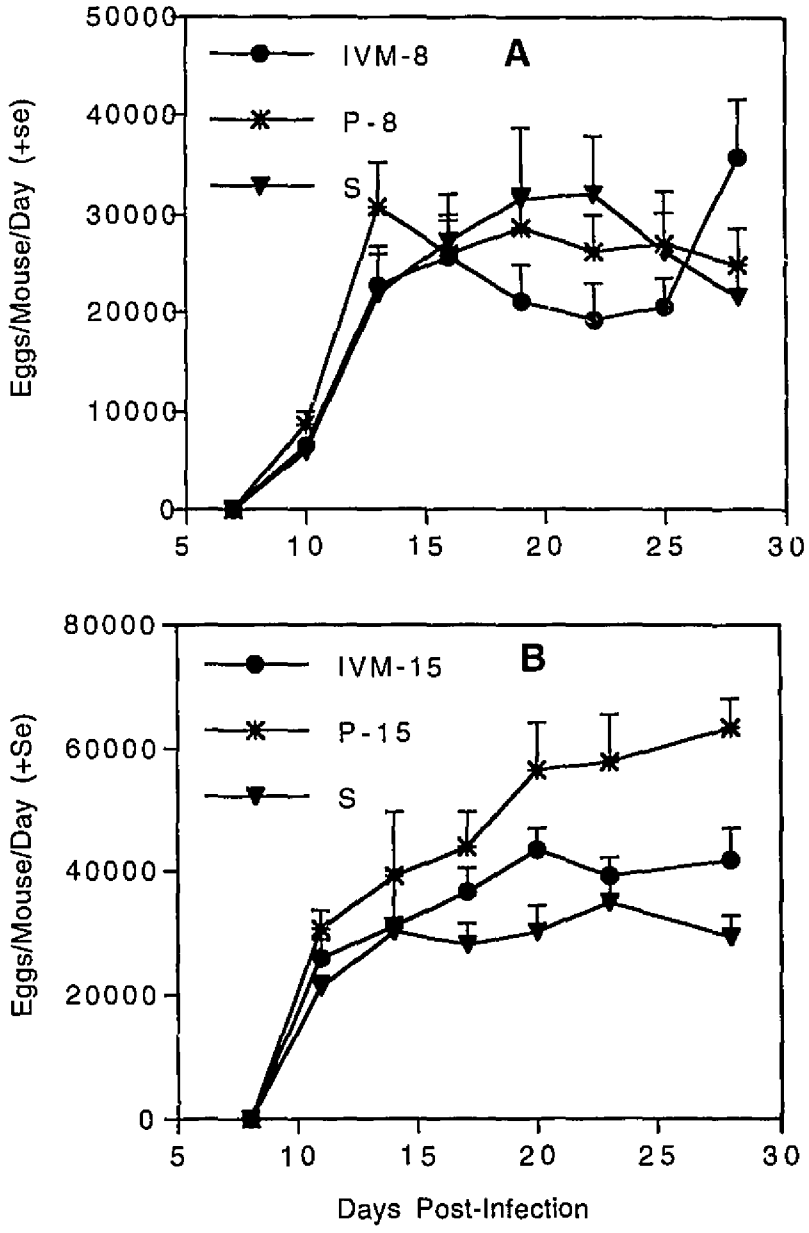

Fig 3.1: Short-term net egg output (+se) production profile 7-28 days post-infection in mice infected with *Heligmosomoides polygyrus* strains (**A**)IVM-8, P-8 and S (strain:$F_{2,27}$=0.51, P=0.6066; time:$F_{6,162}$=0.13, P=0.7331; strain*time:$F_{12,162}$, P=0.3522). (**B**) IVM-15, P-15 and S (strain:$F_{2,8}$, P<0.0001; time:$F_{5,240}$=6.68, P<0.0003; strain*time:$F_{10,240}$=1.14, P=0.3405).

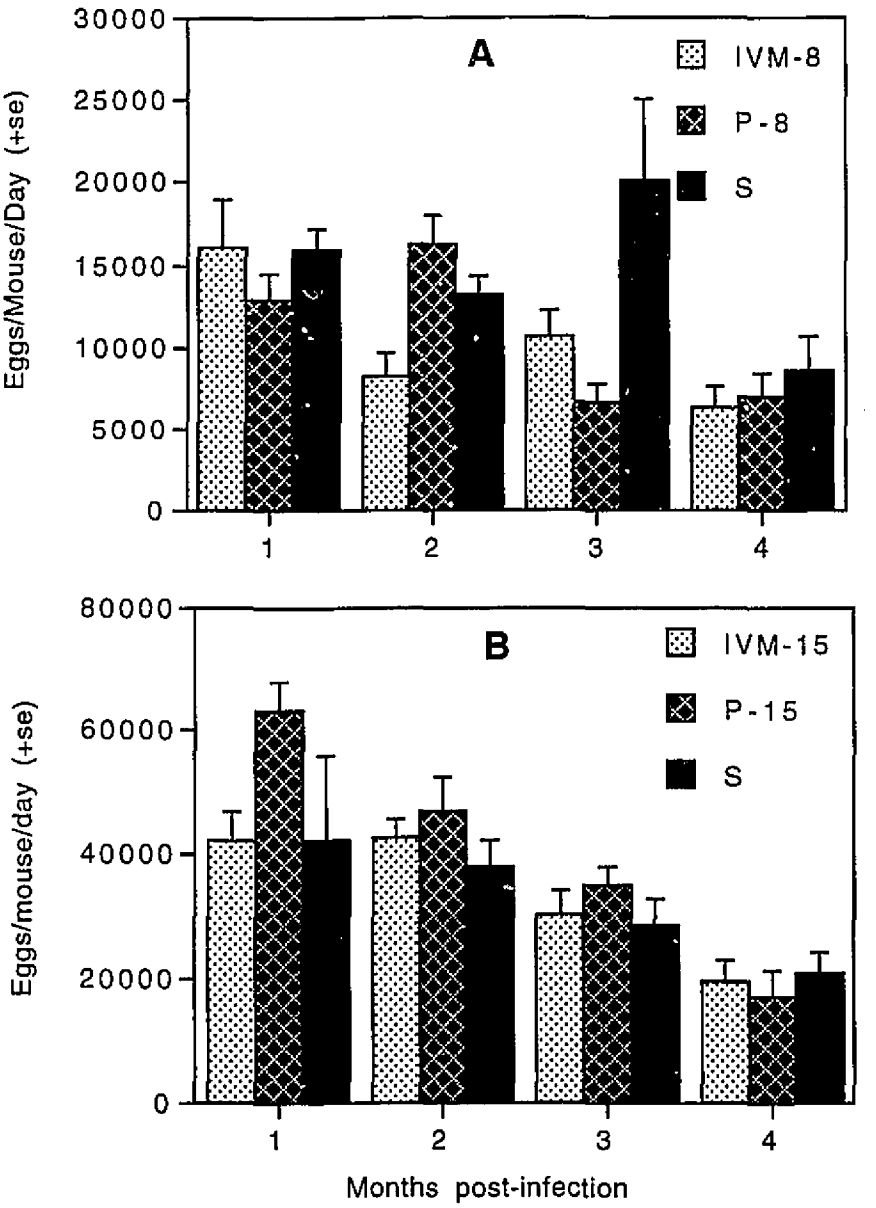

Fig 3.2: Net egg production (+se) in four months of *Heligmosomoides polygyrus* strains (**A**) IVM-8, P-8 and S (strain:$F_{2,108}$=4.24, P=0.0169 month:$F_{3,108}$=6.94, P=0.0003; strain*month:$F_{6,108}$, P=3.52, P=0.0032). (**B**) IVM-15, P-15 and S (strain:$F_{2,189}$=8.04, P=0.0004; month: $F_{3,189}$=25.94, P<0.0001; strain*month:$F_{6,189}$=3.82, P=0.0013).

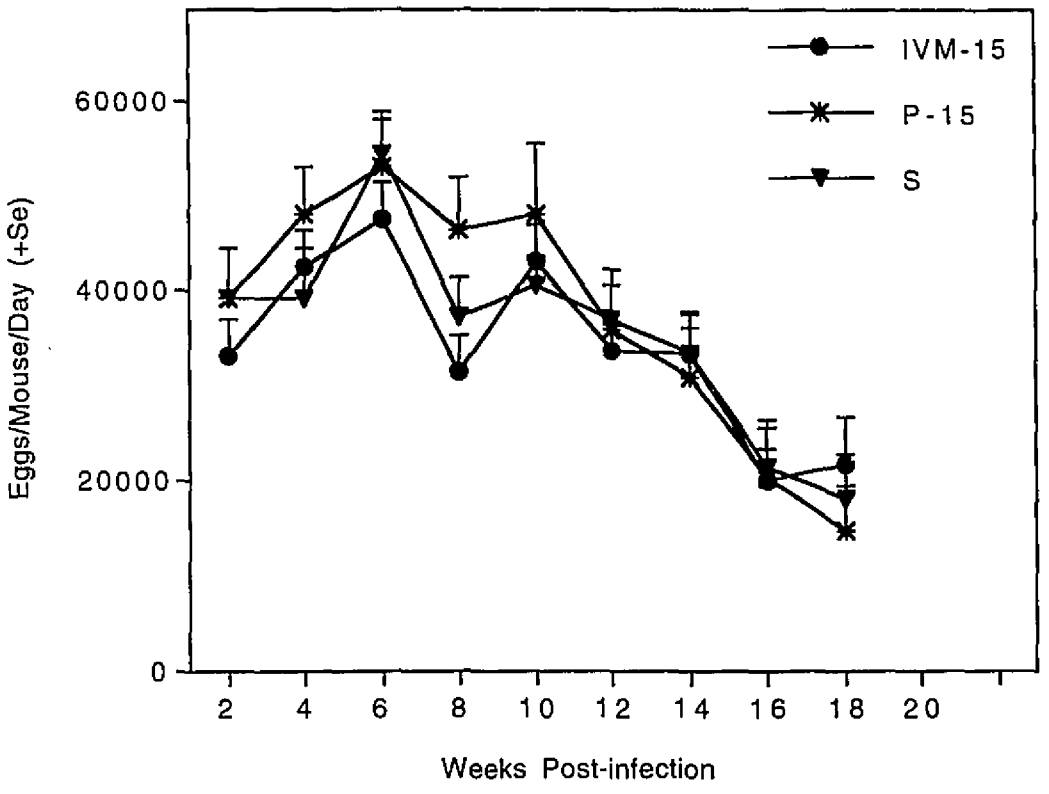

Fig 3.3: Long-term net egg production (+se) measured repeatedly over 4 months in mice infected with strains IVM-15, P-15 and S of *Heligmosomoides polygyrus* (strain:$F_{2,44}$=0.75, P=0.4776, time:$F_{8,352}$=11.81, P<0.0001; strain*time:$F_{16,352}$=1.2, P=0.2896).

Table 3.2: Mean total net egg reproductive effort of *Heligmosomoides polygyrus* strains IVM-15, P-15 and S , estimated by taking average total net egg output in mice measured repeatedly over four months

Strain[1]	Mean Total Net Egg Out-put (SE)
IVM-15	307623.8 ± 20277^{a}
P-15	337458.6 ± 26442^{a}
S	313635 ± 12902^{a}

Means with different subscripts are significantly different at $P<0.05$.

[1]S, Stock "parent" strain; IVM-8 and IVM-15, 8th and 15th generations of the ivermectin selected line; P-8 and P-15, 8th and 15th generations of control Passage, line.

Survival of Adult Worms

The profile of survivorship for strains IVM-8, P-8 and S is shown in Fig. 3.4a. Numbers of worms declined very gradually with time in all strains; overall worm recovery was highest in the stock parasite, intermediate in P-8 and lowest in IVM-8. These data suggest that rates of mortality between 1 and 4 months are comparable among strains.

At generation 15 (Fig. 3.4b), results were similar to those of generation 8. Worm numbers declined gradually until 3 months pi, then dropped substantially in all strains at 4 months pi. In this experiment, recovery of P-15 at each time point was higher than that of the other two strains, presumably reflecting the higher establishment of the strain (Table 3.1) rather than a higher survival rate.

Per Capita Fecundity

There were no significant differences in per capita fecundity among strains IVM-8, P-8 and S (Fig 3.5a) nor among strains IVM-15 P-15 and S (Fig 3.5b).

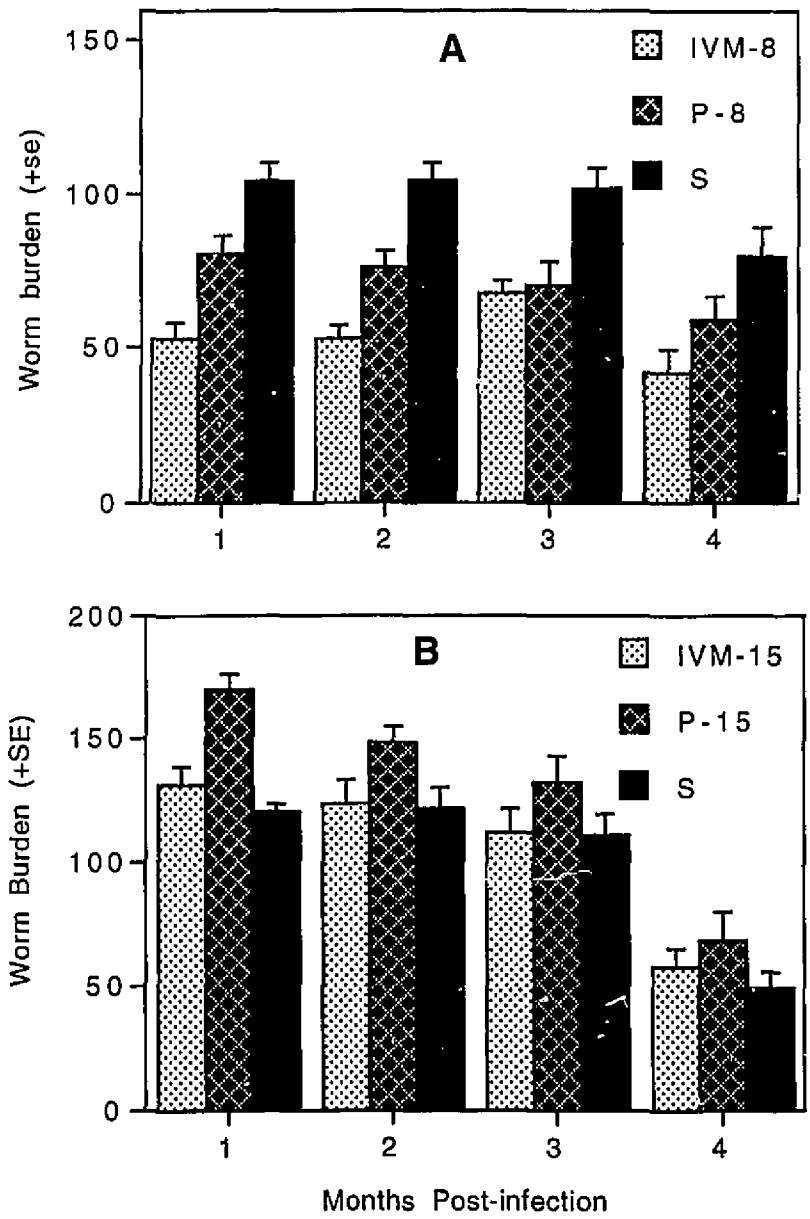

Fig 3.4: Mean worm burden (+se) in mice infected with *Heligmosomoides polygyrus* strains (**A**) IVM-8, P-8 and S (strain:$F_{2,108}$=43.05, P<0.0001; month:$F_{3,108}$=4.75, P=0.0038; strain*month:$F_{6,108}$, P=1.31, P=0.2573). (**B**) IVM-15, P-15 and S (strain:$F_{2,189}$=13.66, P<0.0001; month: $F_{3,189}$=56.93, P<0.0001; strain*month:$F_{6,189}$=0.81, P=0.5651).

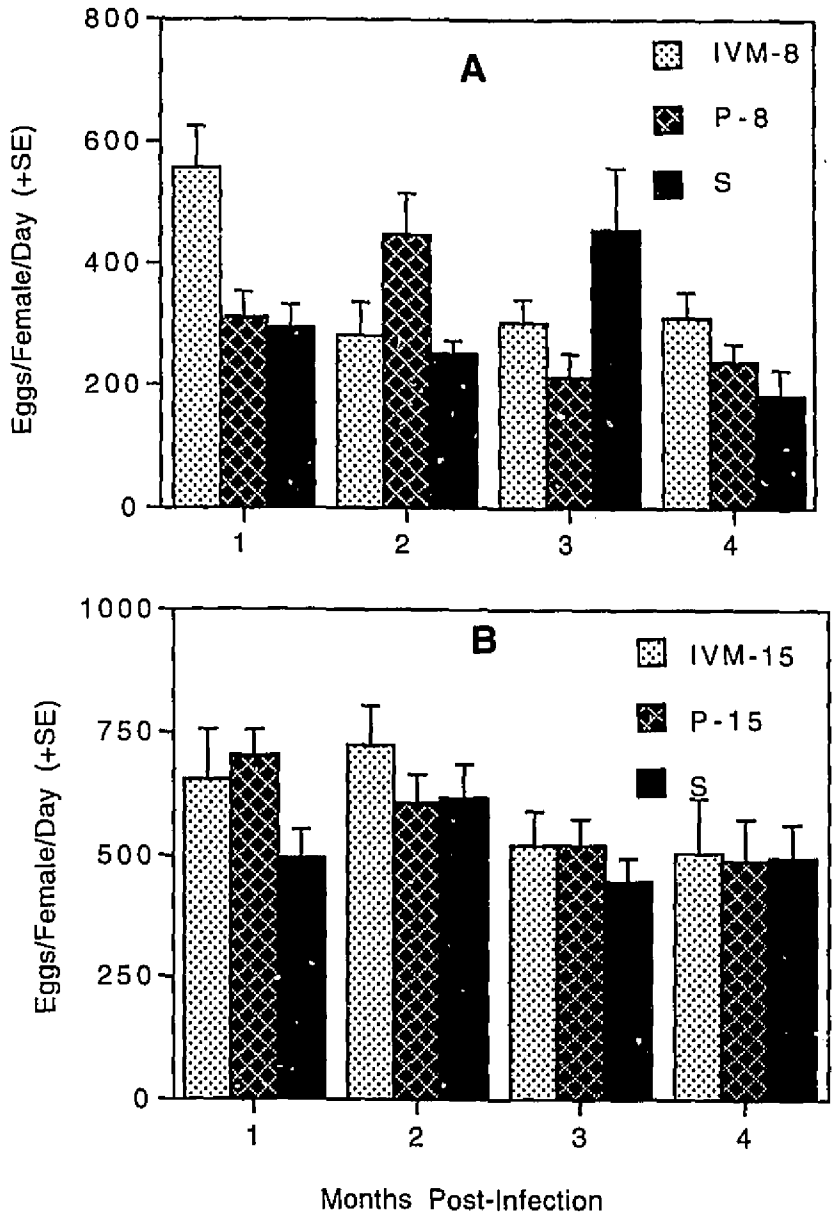

Fig 3.5:Per capita fecundity (+se) of *Heligmosomides polygyrus* strains (**A**) IVM-8, P-8 and S (strain:$F_{2,105}$=1.83 P=0.1481; month:$F_{3,105}$=3.46, P=0.0191; strain*month:$F_{6,105}$ P=5.22, P<0.0001). (**B**) IVM-15, P-15 and S (strain:$F_{2,180}$=0.48, P=0.6205 month: $F_{3,180}$=2.06, P=0.1066 strain*month:$F_{6,189}$=2.88, P=0.0106).

DISCUSSION

The purpose for undertaking this study was to determine whether selection for ivermectin resistance led to concomitant changes in parasite fitness. Data from generation 8 was suggestive that in fact changes had occurred. Although the percentage of larvae that established in mice was higher for IVM-8 larvae than for Stock larvae (Table 3.1), the IVM-8 worms appeared to have a high mortality over the first month of infection. This becomes clear when comparing data in Table 3.1 with that in Fig. 3.3a. Whereas the number of IVM-8 worms recovered was highest at 8 days pi, it was lowest at 1 month pi, suggesting mortality of IVM-8 worms between 1 and 4 weeks pi. Also the net egg production declined more rapidly in mice infected with the IVM-8 strain than the Stock parasite (Fig. 3.2a).

From our experimental design, we were also able to determine whether or not the observed changes were directly attributable to the drug selection pressure exerted on the parasite population, or to the monthly passage of parasite from mouse to mouse. At generation 8, the Passage parasite showed similar patterns to the IVM-8 parasite, but was intermediate in value. Establishment was intermediate between IVM-8 and S parasites, mortality over the first month was intermediate, and the subsequent decline in net egg production was also intermediate. The most parsimonious explanation of these results, overall, was that at least part of the observed shifts in the IVM-8 parasite were attributed to the monthly passage of the parasite.

We were further able to test these ideas, using parasites that had undergone an additional 7 generations of selection pressure. Here the results became much clearer. The IVM-15 strain was virtually identical to the Stock parasite in all fitness traits examined. However, the Passage strain was markedly different. Establishment of P-15 was significantly higher (Table 3.1), the rate of development to adult worms was higher (Table 3.1), net egg

production over the first month of infection was higher (Fig. 3.1b), and the pattern of decline in egg production over four months was more gradual than for either the IVM-15 or S parasite. These data clearly demonstrate that frequent passage of the parasite can have dramatic implications for the general biology of the parasite.

What is intriguing about the data from generation 15 is that similar effects were not observed in the IVM-selected strain, also exposed to the same frequency of transfer from mouse to mouse. Presumably the joint exposure to ivermectin and rapid passage in some way balances the response to rapid passage alone. For example, rapid passage may favour parasites that develop to the adult stage more quickly, whereas ivermectin treatment at day 6 pi may favour parasites whose development is delayed. The predicted net effect of rapid passage and ivermectin at day 6 would be no change in rate of development. Interestingly, Scott *et al.* (1990) observed a 5-6 day delay in onset of sexual maturity in ivermectin strain of *H. contortus*. Also, our prediction for *H. polygyrus* would be that ivermectin selection at 6 dpi would favour delayed development of the parasite. We reported, in Chapter 2, that the efficacy of ivermectin was consistently lower against the L4 stage. Therefore, parasites that had reached the adult stage by day 6 would presumably have a lower chance of surviving the drug than those that were still at the L4 stage.

Alternatively, the lack of effect in the ivermectin-selected line may relate to the proportion of the total parasite population that contributes to the next generation. In the Passage strain, any parasite whose eggs were passed in the faeces at 28 days post-infection had a chance to be part of the following parasite generation. Parasites that died before day 28, or whose egg production was very low at that time would not be represented in the future generation. While the same principle applies to the drug-selected line of parasites, a much smaller proportion of the total parasite population was represented in the day 28 faecal sample. Use of the drug at 6 days post-

infection killed, on average, 80% of the parasites in the mice (Chapter 2). Therefore, when parasite eggs were collected on day 28, a much smaller proportion of the parasite population was represented in the sample. Assuming that our interpretation of our data is correct, it is possible that, among the worms killed by ivermectin, are those who have genes for higher establishment, and early development. The consequence would be that eggs collected 28 dpi in the ivermectin-selected line have less possibility of showing the fitness shifts seen in the Passage line. We realize that this idea is very speculative.

The observed increase in establishment in the Passage line is consistent with a study by Dobson and Owen (1977). They examined the effect of serial passage of *H. polygyrus (=Nematospiroides dubius)* through the Quackenbush strain of mouse for 10 generations on numbers of parasite at 11, 21 and 28 days post-infection. Their data showed clearly an increase in numbers of worms at days 11 and 21, with successive generations, but a decrease in numbers of worms at 28 dpi. They attributed the increase in numbers early in the infection to greater infectivity with each successive generation. Unfortunately, the authors are not clear about the history of passage of the parasite prior to the study. Therefore, we do not know whether the parasite had been maintained in Quackenbush mice, or what the frequency of transfer had been. Shifts in life history traits in response to environmental pressures are commonly observed in free-living organisms as well. Perhaps the most parallel comparison is the effect of predation on life history traits of fish populations. It has been reported that the rate of development to sexual maturity increases and there is a higher reproductive effort when the predator preys upon sexually mature fish populations (Reznick *et al.*, 1990).

While on the surface, our interpretation seems appropriate, a few cautionary notes should be included. First, in contrast to many experimental studies where the same source of parasite larvae is used to infect different experimental groups, here each group of mice was infected with a different batch of larvae. The infectivity of larvae is known to vary with uncontrollable

variables of the culture conditions (Scott and Tanguay, 1994). Therefore, we cannot exclude the possibility that reported differences in establishment success of the different strains and generations of parasite may reflect differences in the larval culture conditions. We are not overly concerned about this, because, for each experiment, larvae were cultured simultaneously, and because the establishment of the Stock parasite when tested in two different experiments (generation 8 and generation 15) was similar (Table 3.1).

It should also be noted that the degree of resistance of the ivermectin-selected line of parasite had only increased by 1.5 fold (see Chapter 2). If, in fact, increased resistance to ivermectin is accompanied by changes in fitness traits, it is possible that we were unable to detect such an effect because of the relatively low degree of resistance, even in the generation 15 parasite. However, in comparison with other studies, ivermectin susceptible and resistant nematodes have also been reported to show similar egg production and establishment rates (Scott and Armour, 1991; Echevarria *et al.,* 1992; DeVaney, *et al.,* 1992).

Finally, all our measures of parasite fitness were done on the parasitic stage of the life cycle. Aside from the report by Scott *et al.* (1990) of delayed onset of sexual maturity in ivermectin-resistant *H. contortus*, all other reports of changes in fitness in response to ivermectin selection are for the free-living stages. Scott and Armour (1991) reported that survival of the free-living stages was lower in a strain of *H. contortus* with resistance to several anthelmintics including ivermectin. Echevarria *et al.* (1993) also reported that free-living stages of a multi-resistant strain had lower development and survival than a susceptible strain. We cannot comment on the effects of our procedure on the hatchability of the eggs or the survival of the free-living stage larvae of *H. polygyrus*.

The most important and consistent observation in this study is that the procedure of rapid passage increases the number of worms establishing in mice and their rate of early development. This in turn leads to higher net egg

production and higher numbers of worms over the first month of infection. Survival of worms appears not to be affected. Our only evidence that ivermectin selection itself leads to changes in fitness comes from the generation 8 data, but even at this time, somewhat similar changes were detected in the Passage line. On the basis of this study, we suggest that, whereas ivermectin selection does not alter the fitness of the parasitic stages of *H. polygyrus*, frequent passage of the parasite from mouse to mouse favours higher establishment and early development.

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CHAPTER 4

SUMMARY AND GENERAL DISCUSSION

GENERAL OVERVIEW

The main objectives of this study were (1) to subject a naive parent strain (S) of *Heligmosomoides polygyrus* to increasing ivermectin selection pressure and determine the level of resistance to ivermectin after 8 and 15 generations; (2) to develop a passage line in parallel with the ivermectin selected line by passaging the parent strain of *H. polygyrus* from mouse to mouse every 28 days, and (3) to characterize and compare the life history traits of the 8th and 15 generations of the ivermectin selected strains (IVM-8 and IVM-15) with the Passage strains (P-8 and P-15) and the parent strain (S). The passage line was included to determine the effect of the rapid passage itself on life history traits of the parasite during the drug selection process.

The results from the drug titration trial, Chapter 2, indicate that we successfully developed an ivermectin resistant strain of *H. polygyrus*, with 1.5 fold resistance after 8 generations of selection pressure. Further selection for a total of 15 generations did not increase the level of resistance to ivermectin (Table 2.1). The Passage strains (P-8 and P-15) remained susceptible to the drug.

The rate at which resistance to ivermectin developed in our study was slower that reported for laboratory selected strains of *Haemonchus contortus* (Egerton *et al.*, 1988; Echevarria *et al.*, 1993a) and *Trichostrongylus colubriformis* (Giordano *et al.*, 1988; Shoop *et al.*, 1990). It is however expected that the rate at which resistance to an anthelmintic develops will vary both within and among parasite species as the parent populations may differ in the amount of genetic variation and the intensity of the selection is also different from one trial to another.

It was interesting to note that the efficacy of ivermectin against the L4 stages was lower than for the adults (Table 2.1). Similar results were reported by Echevarria *et al.* (1992), whereby the efficacy of 0.2 mg/kg of ivermectin against the L4 stages of *H. contortus* was 96.2%, whereas the same dose of drug removed 98.7% of the adult parasites. Lower efficacy (46%) against the

Ostertagia circumcinta and *Cooperia curticei* (89%) L4 have also been reported when sheep were treated with 0.2mg/kg ivermectin. This dose of drug removed 99% of adult *O. circumcinta* and 92% of adult *C. curticei* (Westcott and LeaMaster, 1982). A decrease in efficacy of ivermectin at L4 stage compared to adult stage may be related to the mode of action of ivermectin. Ivermectin acts by opening a glutamate dependent chloride ion channel which leads to permeability of chloride ions resulting in worm paralysis and eventually death (Arena *et al.*, 1991; 1992; Cully *et al.*, 1993; Schaeffer *et al.*, 1993). Some parasites in the mucosa may survive ivermectin treatment as temporary paralysis may be less dangerous due to their to their location, however nematodes in gut lumen may not be able to avoid the paralytic consequences of ivermectin.

It is also apparent that *H. polygyrus* exhibits tolerance to ivermectin when compared to other gastrointestinal nematodes (GI) nematodes of sheep and goats. While 0.2 mg/kg has a 98-99% efficacy against adult GI nematodes in sheep and goats (Campbell *et al.*, 1983), *H. polygyrus* in our trial required 1.0-1.7 mg/kg ivermectin to achieve 95% clearance of the parasites both at adult and L4 stage. High tolerance of *H. polygyrus* to ivermectin has been reported in previous studies (Sayles and Jacobson 1983; Rajasekariah *et al.*, 1986; Wahid *et al.*, 1989). On the other hand the response of *H. polygyrus* to ivermectin may be also affected by its location in the small intestine. Lower efficacy (90.4% and 92%) of recommended dose of ivermectin against the small intestine nematode *Cooperia curticei* has been reported when compared to the abomasal nematode *H. contortus* (Westcott and LeaMaster, 1982; Bogan *et al.*, 1988).

Having confirmed ivermectin resistance after 8 and 15 generations of ivermectin selection we then proceeded, in Chapter 3, to characterize the life history traits of the drug selected strains (IVM-8 and IVM-15), the Passage strains, (P-8 and P-15) and the parent strain (S). The fitness attributes of interest were establishment, net egg production, per capita fecundity and

survival of the adult worms.

Our data indicate that the passage alone increased the rate of establishment and development of *H. polygyrus.* The effects were only marginal after 8 generations of passage (Table 3.1) as the Passage and ivermectin selected strains, P-8 and IVM-8 had similar establishment rates. They also had same net egg output pattern (Fig 3.1a) and worm burden over 4 months (Fig 3.4a). However after 15 generations of passage the effects of rapid passage were clearly detectable (Table 3.1). Strain P-15 had higher establishment ($P<0.0001$) and a higher rate of development ($P<0.0001$) when compared to the parent strain (S) or the ivermectin selected strain (IVM-15). Higher establishment of strain P-15 was also reflected in the higher short-term net egg output (Fig 3.1b) and higher worm recovery during the first month (Fig 3.4b). The increase in establishment of *H. polygyrus* due to passage as reported here has aslo ben observed by Dobson and Owen (1977). It an indication that life history traits of parasite populations may shift in response to environmental pressures. Alternations in life history traits due to environmental pressure has also been observed in guppies (*Peocilia reticulata*). *Crenichilia alta* (a cichlid) predominantly preys on large sexually mature guppies. *Rivulus hartii* (a Killifish), preys predominantly on small immature size classes. Guppies from localities with *C. alta* mature at an earlier age, have higher reproductive effort and have smaller brood than those from localities with just *R.hartii.* This pattern is caused by predation (Reznick *et al.,* 1990).

The effects of passage were however not observed when the drug was introduced into the system i.e. in the ivermectin selected strains. Ivermectin resistance has been shown to lower the rate of development of parasitic stages of *H. contortus* (Scott *et al.,* 1990). It is possible that ivermectin selection pressure exerted at L4 stage in our trial was selecting for parasite variants with a decreased the rate of development. Therefore passage effects that increased the rate of development were counter-balanced by ivermectin selection pressure and a compromise in fitness was reached resulting in an ivermectin selected

strain which had similar fitness to the parent strain after 15 generations of selection. The higher establishment seen at generation 8 in strain IVM-8, could be partly attributed to passage effects.

We realize however the level of resistance developed here was quite and this may account for no differences in the ivermectin selected and parent strains. Nevertheless comparable studies have indicated that parasitic stages of *H. contortus* resistant to ivermectin had no advantage or disadvantage in rate of egg output, establishment or pathogenicity when compared to susceptible strains (Scott and Armour, 1991; Echevarria *et al.,* 1992; DeVaney *et al.*, 1992). The only differences observed in life history traits were in the free-living stages. Development from egg to L3 stage was slower for the ivermectin resistant strain when compared to the drug susceptible strain (Scott and Armour 1991; Echevarria *et al.,* 1993b). However the response reported between these strains may have been due to ectopic differences or multi- drug resistance. Our work did not involve studies on the fitness of free living stages of *H. polygyrus*. Therefore there is a need to characterize the free living stages of ivermectin resistant and susceptible strains of *H. polygyrus*.

Overall this study indicates that an ivermectin resistant population of *H. polygrus* developed after 8 generations of drug selection pressure. Ivermectin selection affected the life history of the parasite at generation 8, however these effects may have been due to passage as the Passage strain at generation 8 showed similar characteristics. Indeed, after 15 generations of passage, the Passage strain (P-15) shows increased fitness while the ivermectin selected strain indicates similar fitness to that of the parent strain. Therefore passage is an important environmental pressure driving the selection of life history traits of *H. polygyrus.* These observations suggest that processes that disrupt the life cycle of parasites should be taken into consideration while planning management of nematode infections in livestock.

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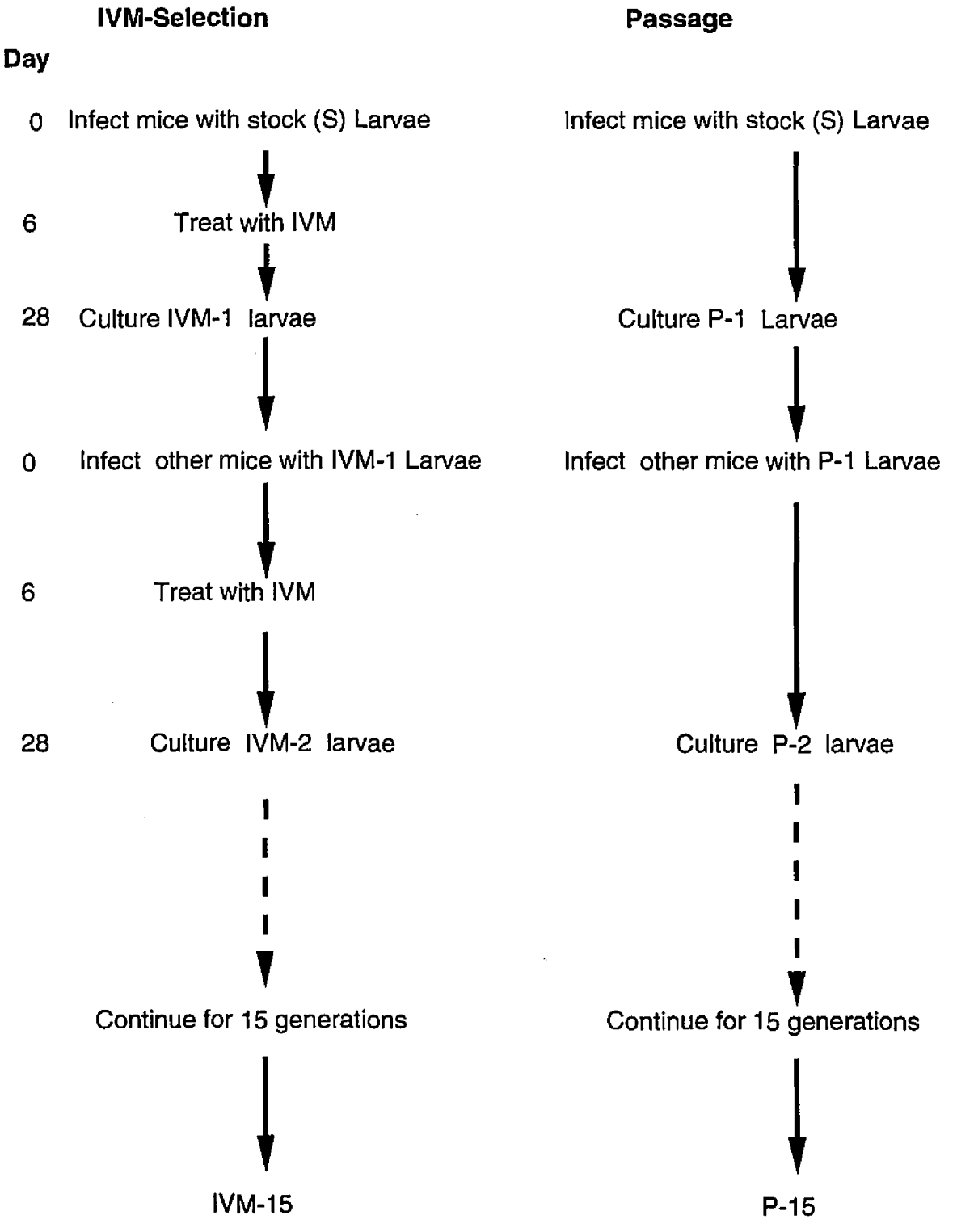
APPENDIX 1


IVM-Selection
Passage
Day
0
Infect mice with stock (S) Larvae
Infect mice with stock (S) Larvae
6
Treat with IVM
28
Culture IVM-1 larvae
Culture P-1 Larvae
0
Infect other mice with IVM-1 Larvae
Infect other mice with P-1 Larvae
6
Treat with IVM
28
Culture IVM-2 larvae
Culture P-2 larvae
Continue for 15 generations
Continue for 15 generations
IVM-15
P-15

APPENDIX 2

PROBIT ANALYSIS

Probit analysis is widely used in the analysis of data from toxicity tests for the assay of insecticides and 6 fungicides and also for data from other types of assay dependent upon quantal response. Ordinary least squares (OLS) regression has been shown to be inadequate when the dependent variable is discrete.

The data set used by PROC PROBIT must include a response variable giving the level of response for each observation. In some quantal-response situations the number of subjects exposed to a dose is unknown although the numbers of different doses are randomly selected from a definable distribution. For example in our dose response trials the actual number of parasite exposed to drug is unknown. This is because though we infected the mice with a given number of larvae the exact number present in the gut at the time of treatment is not known since the number of worms that establish and reach maturity differs slightly in each mouse and is subject to various factors. Therefore to overcome this problem direct information on the numbers parasites exposed to treatment were provided by the worm count in a parallel sample of untreated mice. Probit analysis was done using counts of worm survivors at each dose and the OPTC option used to control for the natural response rate C.

Specifications

; Proc probit log10 OPTC;

Model response/n = dose /d = logistic INVERSECL;

Log10 analyzes the data replacing the dose by common logarithm (Log to base 10)

OPTC - specified so that observations with dose zero are used in estimation as a control group.

The response is the number of worms recovered at each dose of drug.

n = is the number of larvae used to infect each mouse (150).

The INVERSECL option gives the estimated dose values and 95% fiducial limits for dose.

Example: Efficacy of ivermectin against adult *Heligmosomoides polygyrus* of strains, IVM-8, P-8 and S, Experiment 2.

```
data stock;
input dose n response;
cards;
   0   150   92
   0   150   81
   0   150   92
   0   150   88
   0   150   75
   0   150   93
   0   150   69
   0   150   63
   0   150   69
 0.5   150   62
 0.5   150   90
 0.5   150   61
 0.5   150   39
 0.5   150   64
 0.5   150   50
 0.5   150   28
 0.5   150   23
 0.5   150   65
   1   150   55
   1   150   21
   1   150   57
   1   150   40
   1   150   19
   1   150   71
   1   150   35
   1   150   59
   1   150   48
   1   150   33
 1.5   150   22
 1.5   150   15
 1.5   150   10
 1.5   150   20
 1.5   150    9
 1.5   150   67
```

1.5	150	15
1.5	150	29
1.5	150	0
1.5	150	48
2	150	3
2	150	6
2	150	2
2	150	7
2	150	12
2	150	6
2	150	11
2	150	6
2	150	4
2	150	10
3	150	1
3	150	7
3	150	13
3	150	2
3	150	9
3	150	2
3	150	1
3	150	8
3	150	2
3	150	6
4	150	1
4	150	0
4	150	0
4	150	0
4	150	2
4	150	4
4	150	6
4	150	4
4	150	2
4	150	1
5	150	0
5	150	0
5	150	1
5	150	0
5	150	2
5	150	0
5	150	4
5	150	6
5	150	3
5	150	1

```
;
proc probit log10 optc;
model response/n = dose / d = logistic inversecl;
run;
```

Output

Probit Procedure

Data Set = WORK.STOCK
Dependent Variable = RESPONSE
Dependent Variable = N Probit Procedure

Probit Analysis on DOSE

Probability	DOSE	95 Percent Fiducial Limits	
		Lower	Upper
0.01	2.22193	1.93338	2.62947
0.02	1.67691	1.49396	1.92655
0.03	1.41992	1.28196	1.60400
0.04	1.26028	1.14825	1.40716
0.05	1.14781	1.05290	1.27035
0.06	1.06254	0.97988	1.16781
0.07	0.99472	0.92127	1.08705
0.08	0.93889	0.87263	1.02117
0.09	0.89176	0.83125	0.96601
0.10	0.85117	0.79536	0.91887
0.15	0.70733	0.66582	0.75500
0.20	0.61531	0.58051	0.65333
0.25	0.54840	0.51702	0.58127
0.30	0.49593	0.46633	0.52590
0.35	0.45264	0.42399	0.48091
0.40	0.41557	0.38742	0.44280
0.45	0.38287	0.35501	0.40945
0.50	0.35333	0.32567	0.37946
0.55	0.32606	0.29860	0.35186
0.60	0.30041	0.27317	0.32590
0.65	0.27580	0.24885	0.30099
0.70	0.25173	0.22517	0.27655
0.75	0.22764	0.20164	0.25203
0.80	0.20289	0.17764	0.22668
0.85	0.17649	0.15232	0.19946
0.90	0.14667	0.12412	0.16836
0.91	0.13999	0.11788	0.16134
0.92	0.13297	0.11135	0.15392
0.93	0.12550	0.10444	0.14601
0.94	0.11749	0.09708	0.13748
0.95	0.10876	0.08911	0.12813

0.96	0.09906	0.08033	0.11766
0.97	0.08792	0.07037	0.10554
0.98	0.07445	0.05850	0.09070
0.99	0.05619	0.04279	0.07021