The Population Dynamics of Plasmid-Mediated Antibiotic Resistance in *Salmonella typhimurium* in Chickens

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Abstract

A model of growth and plasmid transfer between strains of *Escherichia coli* and *Salmonella typhimurium* was developed with reference to the literature. This was the organising principle for the collection of a complete set of *in vitro* life history parameters of one *S. typhimurium* and one *E. coli* strain. In the course of estimating these parameters two results of note were obtained. Fits of the Lotka-Volterra competition model were obtained for data on *S. typhimuiurm* growing in competition with *E. coli*. The first noteworthy discovery was the failure of this model to account for several characteristics of growth of these strains under competition. The growth rates of plasmid-bearing and plasmid-free strains were obtained. The second main result came from examination of the results of the growth rate data, which revealed that the cost to *S. typhimuiurm* 576 of bearing the resistance plasmid was low (4%). The model was also used to simulate the effect of antibiotic dose on the density of the donor, recipient and transconjugant populations over time. These simulations predicted that there would be a convex relationship between antibiotic dose and transconjugant density (i.e. that the density would first rise, then fall, with increasing dose). Following from this result, laboratory experiments and *in vivo* experiments in chickens were directed towards obtaining information on the relationship between these two variables. This convex relationship was not demonstrated within a single experiment, although some experimental environments produced an increase in transconjugant density with dose, and others, a decrease. Few transconjugants were formed *in vivo*. In order to investigate the low cost of resistance and low rate of *in vivo* transconjugant production, cost of resistance and plasmid transfer rate of this plasmid in several strain combinations of *E. coli* and *S. typhimuiurm* was evaluated.

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1. Introduction

Salmonella infection in farm animals may lead to animal disease, welfare and economic problems as well as health problems for farm workers, visitors and consumers of farm produce. *Salmonella* are intestinal bacteria that can be transmitted by all vertebrates, including humans and, in the case of poultry, there is the possibility of vertical transmission of infection in eggs. There are approximately 2000 different serotypes of *Salmonella*. Most do not normally cause clinical disease in poultry. However, virulent strains such as *Salmonella typhimurium* DT104 have been associated with illness and mortality in chicks although cases in chickens reared for meat have been falling. Currently about 200 *Salmonella* serotypes are associated with foodborne infections in humans in any one year in the UK. In 2001, vertical transmission from breeding flocks to commercial flocks of two of the most significant serotypes, *Salmonella enteritidis* and *Salmonella typhimurium*, has been substantially reduced in comparison with historical rates. Horizontal transmission, that is introduction of infection from the environment, including feed, hatchery equipment, staff movements and contaminated farm equipment, however, remains a key route for infection. If *Salmonella* is present in chickens reared for meat it increases the risk that the public may suffer food poisoning or acquire infections with *Salmonella*, some of which may be pathogenic strains resistant to antibiotics. It is important to reduce this hazard at all steps in the production and preparation of food. Small numbers of *Salmonella* organisms are widespread and their complete elimination from the environment in all but the primary breeder sector is unlikely to be economically feasible. Despite this, good management can reduce the risk of introduction and persistence of infection to minimal levels, particularly since improved *Salmonella*

control in the breeder sector and in feed production has greatly reduced the risk from these sources. The widespread presence of transferable antibiotic resistance elements in *Salmonella* and other gut bacteria is of great concern. R-plasmids may encode resistance to more than one antibiotic, and resistance transfer readily occurs in the environment. Multiple antibiotic resistance has become a serious problem in the treatment of other bacterial infections of humans, particularly in hospital-acquired infections (e.g. Methicillin Resistant Staphylococcus aureus (MRSA)). Transferable resistance elements pose a dual threat in that they may confer multiple drug resistance to their current host organism, and their increasing presence in the environment means that the likelihood of creating new antibiotic resistant transconjugants (i.e. resistant strains produced by acquisition of R-plasmids) increases also.

Mathematical modelling provides a useful framework with which to synthesise aspects of infectious disease epidemics, bacterial growth or other kinetic phenomena. Models have been successfully applied to the kinetics of growth and plasmid transfer by other authors. In this project, a within-host model incorporating aspects of bacterial growth, plasmid transfer and effect of antibiotic is used both to direct empirical work towards elucidating the parameters pertinent to the evolution of plasmid-mediated resistance in *S. typhimuiurm*, and to predict the outcome of varying dose of antibiotic on a system of donor, recipient and transconjugant bacteria.

The remainder of this thesis is laid out in ten chapters. Chapter 2 reviews the published literature on the epidemiology of Salmonellae in chickens. This review reveals a picture of high prevalence of Salmonellae infection in farmed chickens with, most alarmingly, rapidly decreasing sensitivity to a range of antibiotics. Our

understanding of the processes that lead to the spread of antibiotic resistance amongst Salmonellae is reviewed and shown to be quite well developed. One process that is highlighted in any qualitative description of such spread is conjugation in which resistance elements spread like an infection from donors to recipients and confer resistance on the resulting transconjugants. Despite well developed descriptions of the processes involved, quantitative data on the rates of growth of bacteria and on plasmid transfer rates is very patchy. There is no single coherent data-set describing the growth and plasmid transfer characteristics of a trio of donors, recipients and transconjugants. This thesis aims to generate just such a data-set, embedded within a coherent mathematical description of the growth of the three populations. Chapter 3 details the experimental materials and methods that were used in pursuit of this aim, whilst chapter 4 describes the modelling framework used as the organising principle for data collection and interpretation. Chapter 4 puts the model used here in its proper context with respect to other models of bacterial growth and the broader context of models of the transmission dynamics of infectious diseases. Chapter 5 combines theoretical and experimental approaches to first estimate a complete set of parameter values for the model presented in chapter 4 and then use those parameter values to generate model simulations. Those model simulations describe the evolution through time of the three bacterial populations (donors, recipients and transconjugants) at different doses of antibiotic. They allow simultaneous consideration of the processes of saturating growth, within- and between-species competition, transfer of antibiotic resistance and differential impact of antibiotic on resistant and sensitive bacterial populations. Chapter 6 serves as a point of transition from *in vitro* to *in vivo* work. In order to compare *in vitro* and *in vivo* experiments it was necessary to estimate doses of neomycin in chicken feed that would be equivalent to the doses used in *in vitro*

experiments. The relevant procedures and their results are described in chapter 6. Chapter 7 describes a pilot experiment designed to explore the *in vivo* behaviour of the two bacterial strains that were extensively characterised *in vitro* in chapter 5. The experiment described asks if these strains, which had been growing in the lab for many generations, will survive and conjugate *in vivo* in a manner comparable to their *in vitro* behaviour. Chapter 8 sets out to test the model-derived prediction that there will be a hump-shaped distribution between antibiotic dose and density of transconjugants produced. This is because at low dose the advantage in being antibiotic resistant does not outweigh the cost of bearing resistance, whilst at very high dose the recipient population is destroyed by antibiotic before a transconjugant population can emerge. Thus it is predicted that only at intermediate doses will transconjugants be observed. Chapter 8 presents an *in vivo* dose-ranging experiment that tests this prediction. Chapter 9 repeats this approach using *in vitro* experiments. Two sets of *in vitro* dose ranging experiments are presented, one set are in normal LB broth and the other are in caecal content. Chapter 10 returns to more fundamental questions about bacterial growth and the cost of bearing the resistance plasmid. Seeking to place the results found thus far in a broader context the chapter asks if the low cost of resistance observed directly in experiments presented in chapter 5 is a special characteristic of the plasmid/recipient pair used thus far or a more general phenomenon. The question is broadened out to include rates of plasmid transfer to several other recipients from the original donor. In chapter 11 the main results of the thesis are reviewed in the light of published information. No examples were found in the literature of the application of competition coefficients to describe bacterial competition. It is suggested that analyses of competition in bacteria might benefit from the use of mathematical models, with the proviso that more applicable models

should be developed. It is revealed that there are no published estimates of plasmid transfer rate to or from Salmonellae, but that the rates estimated in chapter 10 fit comfortably within established estimates of plasmid transfer rate between *E. coli* strains. Finally, the results of the dose-ranging experiments of chapters 7-9 and published estimates of the *in vivo* effect of antibiotics on *S. typhimurium* are examined. It is concluded that although these experiments did not demonstrate a convex relationship between dose and transconjugant production, it is likely that such a relationship would exist if chickens were administered doses of antibiotic more lethal to *S. typhimurium*, e.g. combinations of antibiotics which act in synergy to reduce *Salmonella* burden. The thesis ends with a concluding chapter summarizing all the findings.

2. Literature review

Salmonella is a gram negative, facultatively anaerobic bacterial genus in the family enterobacteriaceae. It can be carried by all vertebrates and is a frequent zoonotic pathogen of humans. It is often isolated from farm animals and common sources of human infection include food products from chickens, cattle and pigs.

2.1 Chicken production and *Salmonella*

2.1.1 Prevalence of *Salmonella* in broiler chickens

Chickens are farmed for meat (broilers), eggs for consumption (layers), and eggs to supply broiler and egg-producing establishments with chickens (breeders). Poultry meat is now produced on a vast scale; in 1997 it comprised 26% of world trade in meat. Genetic diversity in these broilers is low as most birds are supplied by only three or four international breeding companies. (Paul Gittings, 1999, notes from lecture 'World-wide trends in chicken production', Poultry Health course held at IAH, Compton). Although *Salmonella* infection prevalence is very low during parent production by breeding companies, prevalence is significant in commercial broilers. Large broiler producing plants in America (those that employ more than 500 people) are now subject to inspections by federal agents and must comply with new safety regulations. Data from 1998 inspections are available from the FSIS of the US Department of Agriculture on *Salmonella* prevalence (table 2.1), although these samples cannot be considered random. Of 5697 broiler carcass rinse samples, 621

were infected with some serotype of *Salmonella*. Some of the broiler establishments had zero incidence of *Salmonella*. These are grouped into the 0-5% bin in table 2.1.

Percent of 51 broiler carcass rinse samples with *Salmonella*	Number (%) of large broiler plants
0 – 5%	27 (36%)
5.1 – 10	20 (26%)
10.1 – 15	10 (13%)
15.1 – 20	8 (11%)
20.1 – 23.6	4 (5%)
23.7 – 30	5 (7%)
35 – 40	1 (1%)
45 – 50	1 (1%)
Total	76 (100%)

Table 2.1. Distribution of *Salmonella* prevalence from 76 large federally inspected broiler establishments. January 26, 1998 to January 25, 1999. From http://www.fsis.usda.gov/OPHS/salmdata.htm

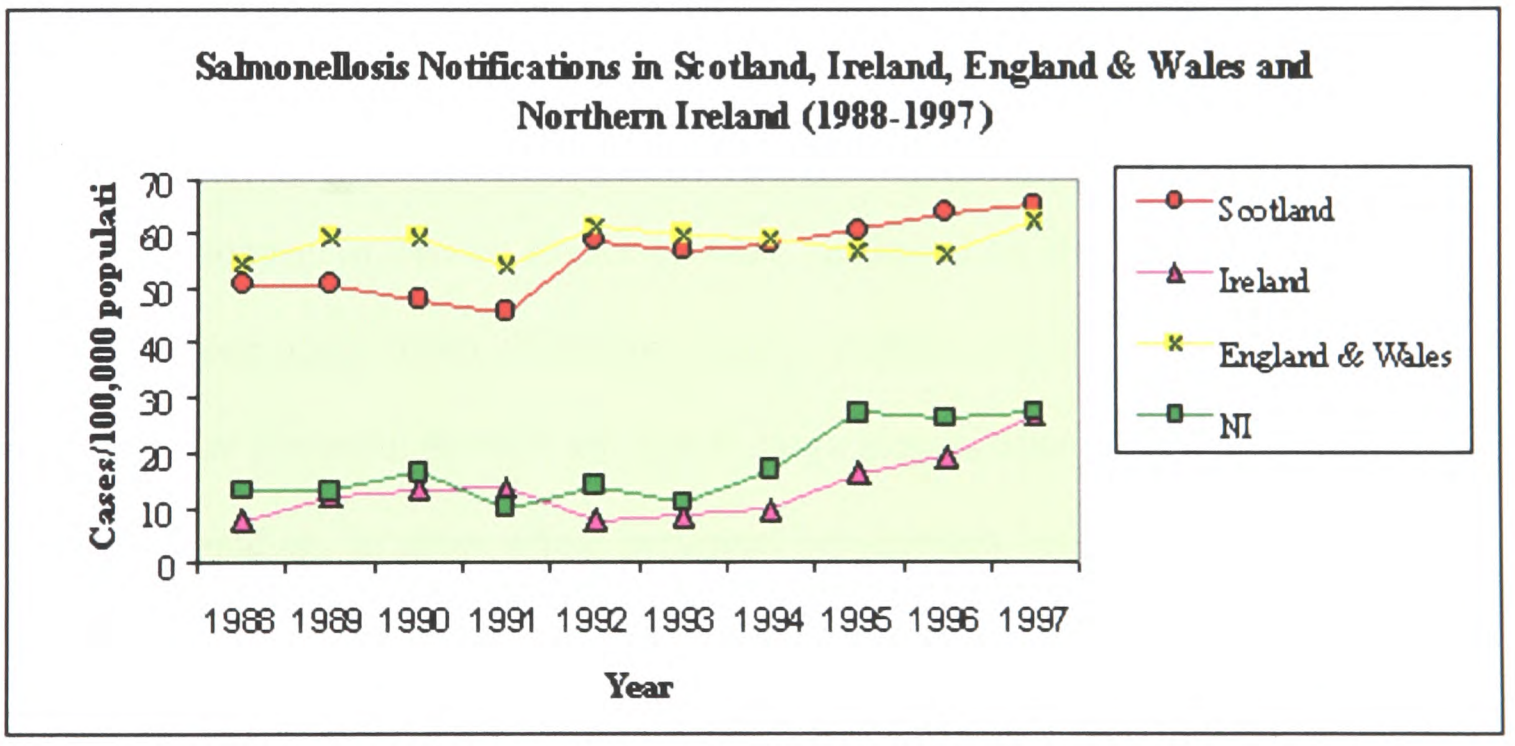


Figure 2.1. Incidence of Salmonellosis in the British Isles 1988 – 1997, URL: http://www.fsai.ie/research/data_10.htm .

Salmonella infection from food causes a serious problem in people: in 1997 there were more than 60 notified cases per 100,000 people in England and Wales. There

were many fewer notifications in Ireland and Northern Ireland (fig 2.1). This may be due to cultural differences.

2.1.2 *Salmonella* infection, transmission and control in farmed chickens

Layers

85% of layers are now housed in cages and most of the other 15% are free range. Of the caged birds, sites hold about 500,000 birds with each house holding 90,000 (O. Swarbrick, 1999, notes from lecture 'husbandry of commercial layers', Poultry Health course). Transmission of *Salmonella* is minimal as cages are arranged so that faecal transmission between chickens is prevented. Nevertheless, the aerosol transmissibility of *Salmonella* under such circumstances is unknown. As birds are kept away from the floor, the main sources of infection are contaminated feed and water (P.A. Barrow, pers. comm).

Broilers

Salmonella control in broiler flocks is often neglected in the face of economic pressure to keep prices down. Conditions highly conducive to infection and spread of *Salmonella* are common. Broilers are kept in large groups, often in wooden buildings with straw bedding, in areas where personnel are allowed between chicken houses without changing footwear or clothes. Recommended stocking density is 16-27 birds per m^2. Bedding is not changed throughout the birds' lives, and food is often unsterilised. Farms in Europe usually practice the 'all in-all out' system, where all birds are removed before the next batch of chicks arrive. In the US, transmission is exacerbated as farms commonly produce continuously. The former regime allows limited disease control by clearing the living space of infectious material between cohorts of chickens. Broilers under continuous production will have a higher infection

rate and will probably all be infected, regardless of age. Birds are usually killed at 7 weeks, although ongoing breeding for increased growth rate has meant that killing weight is reached increasingly early; Ross breeders have bred birds that reach a killing weight of 2kg in 5 ½ weeks. The sources of infection include infected feed, shoes or clothing of personnel and nearby rodents. The between-chicken transmission potential is enormous given high densities, large numbers and an environment which is difficult to clean and disinfect.

Free range

The term 'free range' is a broad term covering both small numbers of chickens living in an individual's garden, to large scale production operating within the legal limits for free range farms. Free range farming requires that the birds have continuous access to both open air and vegetation with density not exceeding one chicken per $10m^2$ and an indoor run with no more than 25 birds per m^2 and at least 25 cm of perch space (Report 1997). The most important generalisation to make is that chickens spending time outdoors will be exposed to greater numbers and/or diversity of Salmonellae from the faeces of wild birds. Treatment, where given, may only partially clear a mixed infection and thus may be less effective in free range birds.

2.1.3 *Salmonella* control measures.

The major sources of *Salmonella* infection are contaminated feed and water, faecal contamination of the environment, and contaminated or vertically infected hatchery eggs. The principal methods of control are therefore feed sanitation, isolation of groups of chickens and destruction of a group following *Salmonella* detection, provision of housing that is easily cleaned and disinfected, and control within chickens i.e. vaccination, chemotherapy and competitive exclusion (see below).

Monitoring of the success of the control strategy is important so that it may be altered if it is not working satisfactorily. Eradication has been achieved in this way in some establishments, particularly breeders, where market forces and legislation provide incentives for effective *Salmonella* control. There has been statutory monitoring and control of *S. enteritidis* and *S. typhimurium* in breeding flocks in the UK since 1989. When *S. enteritidis* or *S. typhimurium* is confirmed in a breeding flock no eggs may go for hatching and the flock is slaughtered.

There is less regulation of control of *Salmonella* in broiler flocks, and competition from countries with less stringent controls provide an economic disincentive for such control.

Antibiotic treatment

Chemotherapy is effective against some *Salmonella* infections, but this is likely to select for resistance, and as discussed below, many strains are already multiply resistant. Antimicrobial substances have been used in chickens to treat clinical infection in young birds; to increase nutrient availability and promote growth by killing anaerobic bacteria; to eliminate *Salmonella* infection from adults; and before competitive exclusion treatment to allow the best protection.

Vaccination

Initial killed vaccines against *Salmonella* were found to be of low efficacy. The 9R vaccine against *S. gallinarum* was developed in the 1950's; this is protective when used properly. There are also effective chicken vaccines developed against *S. typhimurium* and *S. enteritidis*, the major problem serotypes in human disease,

although these are not yet used commercially (Mead & Barrow 1990). An undefined vaccine is used in Germany, but its efficacy is unproven.

Competitive exclusion

The competitive exclusion technique involves feeding young chicks defined or undefined bacterial flora before potential exposure to *Salmonella*. The flora then provide a resistance to colonisation by subsequent infections. The mechanism of protection is not well known; competition for space and nutrients, and direct inhibition by bacteriocidal secretions may all play a role (Mead & Barrow 1990). Competitive exclusion is practised in Scandinavia (Hirn et al 1992). Treatment with undefined cultures has been shown to be effective under field conditions although defined cultures have met with less success (e.g., Aburuwaida et al 1995), and reviewed in Mead and Barrow (1990). Infection with one strain of *Salmonella* may prevent infection by another strain (and certainly does in *in vivo* experiments) and hence interestingly, the use of a live vaccine may competitively exclude challenge infections in addition to immunising the chicken against them (Barrow 1993, Methner et al 1997).

2.2 Important *Salmonella* serotypes

2.2.1. *S. gallinarum* and *S. pullorum*

This serotype comprises two closely related biotypes (*S. gallinarum* and *S. pullorum)* which produce different diseases. These types are transmitted faecal-orally and vertically in chickens. *S. pullorum* disease produces characteristic mortality in the first few weeks only. Day-old chicks often die of this disease where parental infection is a problem in breeding establishments. Fowl typhoid, also caused by *S. gallinarum*, is

characterised by the appearance of clinical disease in adult birds and the typical lesions of fowl typhoid. The source of these outbreaks is often traced to the hatchery (Sato et al 1997). The main sites of multiplication of these organisms are reticulo-endothelial tissue rather than the intestines, and organisms may not be excreted during the acute phase when infection is not extensively found in the gut.

2.2.2 *S. enteritidis*

After oral inoculation with *S. enteritidis* phage type 4, eggs infected by faecal contamination of shells are laid after a few weeks (Barrow & Lovell 1991). Egg infection has been shown to be predominantly on the shell (Barrow & Lovell 1991), but internally infected eggs are often found (Humphrey et al 1991). This appears to depend on the phage type, however; other studies cited in Barrow (1993) found contamination of eggs and clinical disease symptoms varied widely depending on the phage and strain type used. *S. enteritidis* is also capable of producing disease in young chicks. In older birds, no disease occurs but extensive faecal excretion results in carcass contamination and food poisoning.

2.2.3 *S. typhimurium*

S. typhimurium is transmitted both horizontally, and vertically by shell contamination. The progression of the disease differs widely in different strains of chicken (Smith & Tucker 1980b) and with antibiotic treatment (Smith & Tucker 1980a).

2.3 The problem of resistance

2.3.1 Extent of antibiotic resistance

Schrag and Perrot (1996) found that *Salmonella* strains can evolve resistance *in vitro* with little cost in terms of competitive fitness. This potential is now being realised; a recent survey of antibiotic resistance in *Salmonella* in farm animals in the UK during the years 1990-1997 (Report 1998) and an earlier report on the situation in 1984-1987 (Wray et al 1991) both indicate resistance is increasing. The Report (1998) summarises the results of the testing of *Salmonella* isolates sent to the Central Veterinary Laboratory and the Lasswade Veterinary Laboratory for serological identification. Given that the results are not a random sample of outbreaks (each isolate since 1994 represents one outbreak of *Salmonella* in livestock reported in England and Wales; before 1994 more than one isolate per outbreak may have been measured, and some companies have better monitoring than others do) the data show alarming increases in resistance in *Salmonella*. The data show the greatest decline in sensitivity of *S. typhimurium* of many Salmonellae tested in farmed animals, where sensitivity to all the 16 antibiotics tested fell from 44.2% to 11.4% of isolates from 1990 to 1997. Antibiotics they tested were Nalidixic acid, tetracycline, neomycin, ampicillin, furazolidone, cefuroxime, sulphamethoxazole/trimethoprim, chloramphenicol, amikacin, amoxicillin/clavulanic acid, gentamycin, streptomycin, sulphonamide compounds, cefoperazone, apramycin, and colistin. The decline of sensitivity in bacteria harboured by poultry has been particularly marked since 1993, declining from 90% complete sensitivity in 1993 to 30% in 1997. Resistance to most of the 16 antibiotics tested increased dramatically, although neomycin, furazolidone and apramycin resistance remained at a low level and tobramycin resistance declined steadily throughout this period. Those antibiotics to which resistance is plasmid

encoded (see table 2.2) are often those to which the largest proportions of total *S. typhimurium* isolates from chickens are resistant. Antibiotic resistance in potential human pathogens and commensals has worried government advisors enough that most growth promoting antibiotics in chickens have been banned. One of the reasons for the concern is the high transmissibility of the plasmids by conjugation.

Antibiotic	% *Salmonella* Isolates Resistant	Principal Mode of Action	Effect on Bacteria	Resistance carrying molecule	Main mechanisms of resistance
Streptomycin	53.2	Binds to 30S ribosomal subunit and interferes with protein synthesis	Death	Chromosome	30s rRNA mutations
Sulphonamides	71.5	Inhibits dihydrofolic acid synthesis	Growth arrest after several generations	Chromosome	Increased production of DHF precursor/mutant DHF producing enzyme with greater affinity for natural substrate than antibiotic.
Tetracyclines	78.9	Binds to ribosomes to inhibit protein synthesis	Stasis	Plasmid	Pumping out of tetracycline/reduction of tRNA sensitivity
Neomycin	3.3	Binds to 30S ribosomal subunit and interferes with protein synthesis	Death	Chromosome, Plasmid	30s rRNA mutations, inactivation, reduced accumulation
Ampicillin	44.3	Inhibits PBP's involved in peptidoglycan synthesis	Death	Plasmid, chromosome, transposon	Beta-lactamases (inactivation), Altered target, Reduced accumulation
Furazolidone	31.9				
Trimethoprim	32.7	Inhibits dihydrofolic acid reductase and THF reductase	Stasis	Chromosome	Increased production of DHF precursor/mutant DHF producing enzyme with greater affinity for natural substrate than antibiotic.
Chloramphenicol	36	Inhibits protein synthesis by blocking peptidyltransferase	Stasis	Plasmid	Inactivation, Altered target
Apramycin	2.8	Binds to 30S ribosmal subunit and interferes with protein synthesis	Death	Chromosome	30s rRNA mutations
Nalidixic acid	8	Binds DNA gyrase to stabilise double stranded break	Death	Chromosome	Reduced gyrase binding activity/reduced cell envelope permeability

Table 2.2. Characteristics of antimicrobial resistance in chickens, pigs, sheep and cows. Sources: Report (1998), Russell (1990). Information on the extent of antibiotic use would be valuable but UK data are not available. PBP = penicillin binding protein THF = trihydrofolate
See section 2.6 for more information on antibiotics and resistance.

2.3.2 Extent of resistance transfer.

Antibiotic resistance transfer in chickens can be easily demonstrated following experimental infection shortly after hatching (Walton 1966; Smith 1971a). Smith observed that resistance transfer is more likely where growth promoting antibiotics are administered to suppress lower gut anaerobes. This probably increases density of potential donors and potential recipients in addition to contacts between them. It appears that the caeca are the main sites of resistance transfer, perhaps because of the high density of bacteria there (Smith 1971a). Indirect evidence for this comes from the fact that chloramphenicol appears to have little effect on resistance transfer, and is absorbed anterior to the caeca in the gut: Smith (1970) investigated the site of action of chloramphenicol in chicks of age 1 day and 6 days. In both groups, he found antimicrobial activity in chyme from the crop and a smaller amount in the gizzard only. None was found in the small intestine, cloaca or caeca (see figure 2.2 for areas of the chicken gut these refer to).

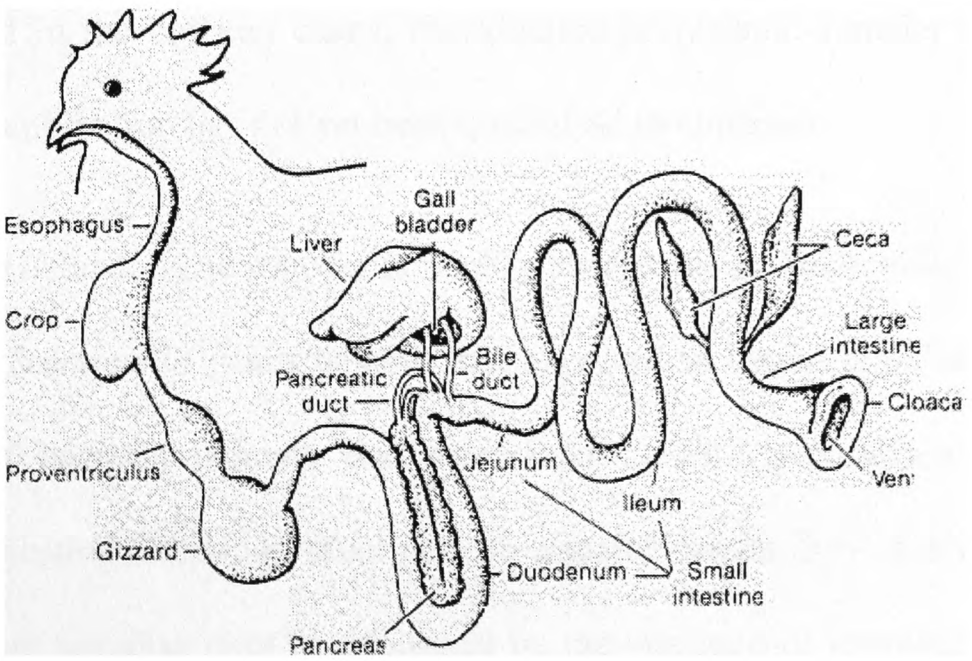


Figure 2.2. Diagram of the chicken digestive system (reproduced from Parkhurst & Mountney 1998).

There is good epidemiological (Balis et al 1996) and direct (Petrocheilou et al 1976, Platt et al 1984, Platt et al 1986) evidence that resistance transfer readily occurs between naturally acquired enterobacteria in the human gut, with or without treatment with antibiotics. It is safe to assume natural transfer is frequent in chickens as caecal contents have much higher concentrations of *Salmonella* and *E. coli* than the human gut. *In vivo* resistance transfer increases with concentration of donors and recipients as predicted by a mass action model (Freter et al 1983).

Bacteria also exchange genetic material through transduction and transformation. Transduction occurs by transfer of host genes during bacteriophage infection, whereas transformation involves passive uptake of DNA from the surroundings. Transduction in *Salmonella* was discovered by Zinder & Lederburg (1952). Transformation is not an important route of resistance acquisition as *Salmonella* are not naturally competent (i.e. they do not readily take up DNA from solution). Although transfer has been frequently documented in the chicken caeca, transduction or plasmid transfer rate for any donor-recipient combination has not yet been quantified in chickens.

Although resistance gene transfer is readily observable in *Salmonella*, globally it appears not to mix *Salmonella* genes beyond that expected in clonal populations. Studies of the clonality of bacteria indicate that natural *Salmonella* populations are predominantly clonal, suggesting inter-individual genetic transfer occurs only at a very low frequency. This apparent paradox may be explained by the presence of immune selection (Gupta et al 1996), or the existence of an epidemic population structure, similar to that of Pseudomonas (Maynard Smith et al 1993). Immune selection can result in the formation

of discrete subsets of the population each expressing antigens unique to that subset (Gupta et al 1996). This could give the appearance of clonality if the genes used to assess diversity encoded these antigens. An 'epidemic' population structure arises when a few strains become exceptionally abundant in a short space of time as occurs in species which cause disease epidemics. At any one moment in time most individuals will belong to one of several clones representing separate epidemics, no matter how readily gene transfer occurs between individuals.

2.2.3 Generation and persistence of antibiotic resistance plasmids

The cost of carrying antibiotic resistance plasmids is an important life history parameter and is a determinant of how quickly a population will evolve to resistance once treatment is started and regress once treatment is withdrawn. Studies suggest that there is frequently a cost of resistance to *E. coli* cells. Levin (1980) quantified the cost at >5% per bacterial generation; another study (reviewed in Levin et al 1997) found fitness costs of 1-6% for conjugative plasmids in *E. coli*. Schrag and Perrot (1996) studied costs of chromosomal resistance and found streptomycin resistance reduced fitness by up to 20%. There were lesser costs involved in resistance to rifampin and spectinomycin, whereas resistance to nalidixic acid appeared to carry no cost at all. Chromosomal resistance, or resistance encoded by plasmids integrated in the genome, will generally have less cost than plasmids carried in the cytoplasm. This is because bacteria can dispense with the time of copying the plasmid, or at least multiple copies of it. Bacteria do lose plasmids through segregation at a variable rate. In most enteric strains, segregation occurs at a rate of 10^{-4} per cell generation or less (Falkow 1975). Counteracting this loss are three mechanisms

which reduce the likelihood of reversion to a plasmid-free state in bacterial populations: co-adaptation between bacteria and plasmid; infectious transfer of the plasmid; and linkage selection. Studies on *E. coli* have shown that adaptation to the fitness cost of R-plasmids (i.e. plasmids encoding antibiotic resistance) can occur (Bouma & Lenski 1988, Modi & Adams 1990). Once the bacteria and plasmid are co-adapted, the removal of the plasmid may result in a drop in fitness of the bacterium (Schrag et al 1997). This idea can be expressed with the use of evolutionary landscapes. In the absence of antibiotic, a plot of fitness against bacterial adaptation to a resistance plasmid might look like figure 2.3.

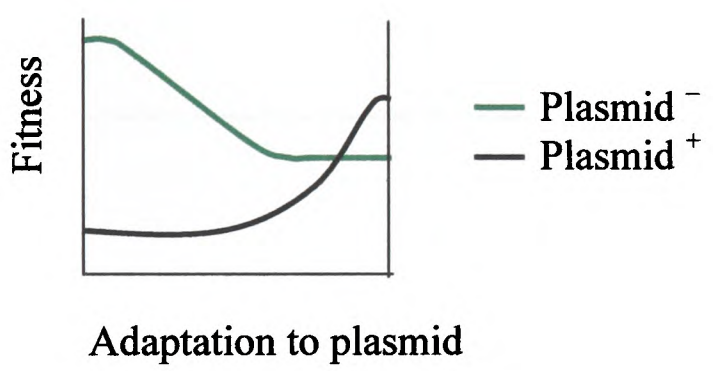


Figure 2.3. Representation of an evolutionary landscape demonstrating a drop in fitness when adapted bacteria lose their plasmids.

The plasmid$^+$ strain increases its fitness as it adapts to the plasmid, but these adaptations in a plasmid free strain would be expected to lower fitness, relative both to a non-adapted plasmid free strain and a plasmid carrying adapted strain. Therefore if an adapted plasmid$^+$ strain were to lose its plasmid, a temporary drop in fitness would result (from the black to the green line on the right hand side of figure 2.3). Plasmid$^-$ segregants would be outcompeted by individuals retaining plasmids and the population would maintain resistance. Thus reversion to susceptibility would be unlikely unless nonadapted plasmid$^-$ populations remained which would outcompete

both groups. The adaptation can take the form of mutations at the site of the resistance gene, or second site modifier mutations. Modi & Adams (1990) found that adaptation of both plasmid and host took place. A shift of the burden of adaptation from the host to the plasmid would mean that the fall in plasmid$^-$ strains' fitness with adaptation is less pronounced. Bouma and Lenski (1988) found that *E. coli* containing a resistance plasmid and selected by serial passage in medium containing chloramphenicol actually became more fit than the control strain after second site mutations even in the absence of chloramphenicol. This would imply a fitness landscape as in figure 2.4, where adapted plasmid$^+$ strains would directly outcompete their unadapted plasmid$^-$ relatives.

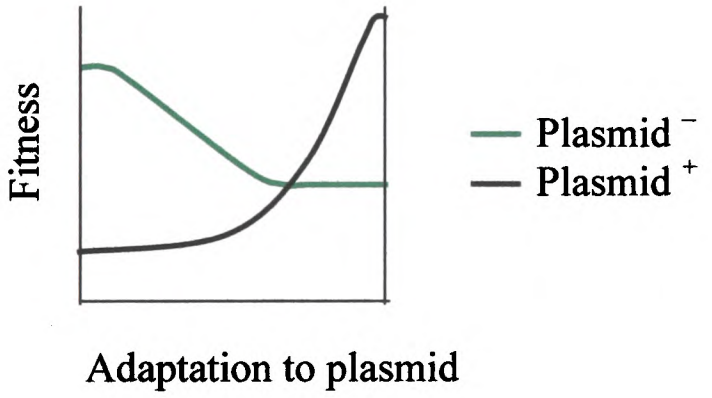


Figure 2.4. Possible fitness landscape when compensatory adaptations to the plasmid increase plasmid$^+$ fitness above that of the plasmid$^-$ strain in the absence of antibiotics.

Studies on chromosomal resistance mutants have also found that fitness compensation is possible here. Two different rspL mutants (rspL mutants are streptomycin resistant) had their fitness deficit halved after 180 generations of growth in selective medium.

Resistance could be maintained in bacterial populations despite fitness costs of plasmid carriage if the plasmid transfer rate is large enough. Plasmids of no known function can, in theory, be maintained in this manner in *E. coli* populations despite selective disadvantages. This is also rarely true of R-plasmids (Lundquist & Levin 1986, Levin 1980), where the cost of bearing them is low. This could occur if the generation of transconjugants, $\gamma DR + r_T T$, was greater than the division of recipients, $r_R R$, where D, R and T are the concentrations of donors, recipients and transconjugants respectively, γ is the rate of plasmid transmission and r_T and r_R are the growth rates of transconjugants and recipients respectively.

Linkage selection can also result in the persistence of R-plasmids. Plasmids that encode resistance to a particular antibiotic may encode resistance to several others, and other potentially favourable characteristics, such as virulence, resistance to ultraviolet light, mercury, and utilisation of unusual carbon energy sources. Summers et al (1993) found that selection for mercury resistance following dental treatment in *E. coli* of human beings maintained a high frequency of antibiotic resistant bacteria.

2.3.4 Experimental observations on resistance evolution.

Smith & Tucker (1975) measured the effect of feeding growth-promoting antibiotics on the faecal excretion of *S. typhimurium* in chickens. The four groups fed Nitrovin antibiotic excreted significantly larger quantities of *S. typhimurium* than the control groups. *S. typhimurium* in chickens fed sulphaquinoxaline, another antibiotic, appeared

unable to evolve resistance, with no resistant mutants formed and significantly decreased *Salmonella* excretion. Yet other antibiotics (Virginiamycin, Bacitracin, Flavomycin and Tylosin), appeared to produce only a slight increase in the number of *Salmonella* excreted. The likely explanation for these results is that, in chickens treated with antibiotics, the competition from other gut bacteria is reduced, leading to an increased opportunity for *Salmonella* survival. This can be modelled as an increased carrying capacity of the gut for *Salmonella.* However, treatment will lead to a reduction in *Salmonella* if the particular strain is sensitive to the antibiotic (Smith & Tucker 1975). There have been other experiments on different strains of *Salmonella*; Barrow (1998) investigated effects of avoparcin consumption on the excretion of *Salmonella* in chickens. It was found that reduced competition from lower gut anaerobes allowed increases in excretion of *S. typhimurium*, *S. cholerae-suis, S. dublin*, and *S. arizonae*, but did not increase excretion of *S. pullorum*, a non-intestinal *Salmonella.*

2.4 Growth rate

The growth rate of *Salmonella* and *E. coli* strains have been measured by several authors, in work designed to assess the influence of the environment on bacterial growth or assess bacterial fitness. These studies indicate that growth rate varies according to strain, substrate, temperature, pH, and water activity. The dependence of growth rate on medium and strain is illustrated in table 2.3.

Bacteria	Temp (^{0}C)	Medium	Growth rate (h^{-1})	Reference
E. coli B/r	37	LB	1.66	Tao et al. (1999)
E. coli B/r	37	MM	0.69	"
E. coli G1108E	37	LB	1.56	Rang et al. (1997)
E. coli 1452	37	LB	1.71	"
E. coli 604	37	LB	1.82	"
E. coli 2069	37	LB	1.66	"
E. coli 1491	37	LB	1.57	"
E. coli 563	37	LB	1.71	"
E. coli 27	37	LB	1.84	"
E. coli G1108E	37	MM	0.97	"
E. coli 1452	37	MM	0.90	"
E. coli 604	37	MM	0.90	"
E. coli 2069	37	MM	0.92	"
E. coli 1491	37	MM	0.81	"
E. coli 563	37	MM	0.90	"
E. coli 27	37	MM	0.96	"
E. coli O157: H7	37	HBI	3.38	Duffy et al (1999)
E. coli O157: H7	28	HBI	1.24	Buchanan and Bagi (1997)
E. coli BJ4	mouse intestine		0.53	
E. coli IAM1016	27	DNB	1.77	Mochizuki and Hattori (1987)
Salmonellae	30	TSB	2.16	Gibson et al (1988)

Table 2.3. Growth rates of various strains grown under different conditions. Media abbreviations: LB = Luria Bertani, MM = Minimal Medium, HBI = Heart Brain infusion DNB = Diluted Nutrient Broth TSB = Tryptone Soya Broth

Attempts to measure growth rates *in vivo* have also been made. Rang et al (1999) used fluorescent markers to assess the amount of bacterial mRNA in the mouse gut, thereby measuring growth rates. Smith et al (1978) used a temperature sensitive plasmid which segregated *in vivo* and therefore diluted out to measure *in vivo* growth.

As temperature affects growth rate so profoundly, attempts were made to convert the growth rates found at 27^{0}C and 28^{0}C (table 2.3) into equivalent growth rates at 37^{0}C. The descriptive model of Gibson et al (1988) was used which quantifies the effect of temperature among other factors on the growth rate of Salmonellae. This was found to be

inappropriate as the data used to derive the model used Salmonellae grown in temperatures up to 28^0C. Above these temperatures the quadratic component of the model effects a decrease in growth rate with time; when extrapolating from 27^0C to 37^0C the predicted growth rate decreases.

2.5 Stationary phase densities (K values)

The density at which individuals neither multiply nor decline, during the growth stage known as stationary phase, is the K value of ecological models. The K value is a product of the bacterial strain and its environment. Bacterial growth rate may decrease with time due to depletion of nutrients, as in glucose minimal broth cultures, or due to low pH, or the bacteria may limit their own growth, as happens in overnight nutrient broth cultures. The density at which growth ceases is dependent on which factor limits it. Recent research has indicated that self limitation may be the result of detection by bacteria of species specific molecules which, when their concentration is above a certain threshold, trigger growth slowdown in individuals. By this mechanism, many bacteria, including *S. typhimurium*, limit their density before starvation occurs. This system may have evolved to allow bacteria time to alter their physiology to starvation conditions while nutrients are still fairly abundant. This phenomenon is known as quorum sensing. Bacterial cells can 'sense' their population density because of an extracellular low molecular weight molecule (Swift et al 1996), produced constitutively by the organisms accumulating in the medium, which results in a linear relationship between concentration of the molecule

and the cell density. Quorum sensing was first observed in the bacterium *Vibrio* (*Photobacterium*) *fischeri*. They exist either as free living marine organisms or in the light-emitting organs of certain squids, where they are luminescent. If their density exceeds a certain level, they emit light as in nature they would only be present in high densities if they were living inside this organ. Other Gram-negative bacteria use similar molecules as autoinducers to regulate a variety of functions according to cell density rather than nutrient starvation. Searches have been made for equivalent genes in the *Enterobacteriaceae*, but without success. As *E. coli* and *Salmonella* do limit their growth before nutrient starvation, it seems likely that extracellular diffusable molecules cause this phenomenon, and the search continues to discover which molecules are responsible.

E. coli and *Salmonella* are facultative anaerobes and can reach higher densities where oxygen is present. Their stationary phase densities in a shaking incubator are higher, usually within an order of magnitude, than those in a static incubator due to the increased O_2 concentration under agitated conditions. The K-values of one *E. coli* strain and various *Salmonella* strains were estimated from data given in Berchieri & Barrow (1991) and are listed in table 2.4.

Strain	Mean stationary phase density
E. coli k12 proto	1.28E+09
S. heidelburg 17705	2.17E+09
S. infantis 1326/28	1.41E+09
S. tm F98	4.64E+08
S. enteritidis KMS	1.81E+09
S. montevideo KMS	2.02E+09
S. hadar butler	1.37E+09
S. verchow 1337/54	2.13E+09
S. kentucky 10935	1.64E+09

Table 2.4. Stationary phase densities of *Salmonella* and *E. coli* strains growing in LB broth at 37^0C in a shaking waterbath (average of 9 replicates; data from (Berchieri & Barrow 1991)).

2.6 Plasmid transfer rate

2.6.1 Methods of estimating plasmid transfer

The rate at which transconjugants are formed in liquid are modelled well with the mass action model of Levin (1979) which states that for a particular set of conditions, the rate of plasmid transfer is proportional to the both the donor and recipient concentrations. Transconjugant populations also increase due to the division of existing transconjugants. During exponential phase, then, the change in transconjugant cell density in a mixed population is modelled by

$$\frac{dT}{dt} = \gamma(D+T)R + rT \qquad 2.1$$

where D = donor cell concentration; R = recipient cell concentration; T = transconjugant cell concentration; γ = the plasmid transfer rate constant and r is the specific growth rate of transconjugants.

To estimate the plasmid transfer rate constant using this method, measurements of donor, recipient and transconjugant population sizes must be made at several time points during mating. The transconjugant growth rate r should also be measured, although the rT term may approximate to zero for a short time after mixing, so reasonable γ estimations may be made without it.

Simonsen et al (1990) derive a simple method of estimating plasmid transfer rate from a similar model to Levin's, which incorporates the slowing of growth at stationary phase in a batch culture due to depletion of resources. They derive an equation for plasmid transfer rate where only the start- and end- densities of donors, recipients and transconjugants, and their growth rate, needs to be measured (section 5.6). Freter et al (1983) model plasmid transfer in continuous flow cultures, and have separate parameters for the plasmid transfer rates of donors and transconjugants. They also make the assumption that the donor population does not multiply, as if an animal has ingested some donors from the environment which pass through the gut without colonisation. As they model two populations (donors and transconjugants) which are concurrently transmitting the plasmid at different rates, a derivation of a simple equation which gives

plasmid transfer rate is not possible. Instead they use a least squares method to evaluate donor conjugation rate and transconjugant conjugation rate from data on transconjugant densities at two time points. Simonsen's end-point method is used in this project as it is suitable for the estimation of plasmid transfer rate in batch cultures.

2.6.2 *In vivo* and qualitative data on plasmid transfer rate

Unfortunately, many authors attempting to estimate plasmid transfer rate (Venables et al, 1995; Watanabe 1963; Smith, 1970; Curtiss et al, 1969; Bale et al, 1987; Scott & Flint 1995) did not publish all the information required to estimate γ, so their estimates are not comparable with others. Data are comparable within studies, however, and these studies give information on the conditions under which plasmid transfer occurs. In particular, obtaining start- and end-point densities of donors, recipients and transconjugants in order to estimate plasmid transfer rate is often difficult *in vivo* or in nature. *In vivo* studies have usually obtained data on presence or absence of transconjugants only (Balis et al 1996, Nijsten et al 1995, Petrocheilou et al 1976, Platt et al 1986, Platt et al 1984, Smith 1970, Timoney & Linton 1982). *In vivo* transconjugant production is most easily demonstrated in the gut of gnotobiotic animals (whose alimentary tracts are free of microflora except the inoculated strains, (Freter et al 1983, Rang et al 1996, Sansonetti et al 1980)), or in very young animals with an undeveloped gut flora (Smith 1970, Smith 1972, Walton 1966). By contrast, where animals with a developed gut flora are used, much lower densities of transconjugants are usually isolated (Platt et al 1986, Smith 1978), although plasmid transfer in the transient presence of donor and in competition with high densities of background flora has been demonstrated (Duval-Iflah et al 1980). The adult gut flora

can however be altered with antibiotics to become more conducive to plasmid transfer (Anderson et al 1973, Licht et al 1999).

Scott & Flint (1995) investigated plasmid transfer rates in ex-vivo gut content. They carried out their experiments in ovine rumen fluid. They did not estimate the plasmid transfer rate coefficient, but they expressed transfer efficiency as transconjugants/recipients. They obtained an average transfer efficiency in whole rumen fluid of 2.44×10^{-6}.

Post-1979, several authors have used Levin's model or similar ones to obtain comparable estimates of γ. Levin's model has been used by Simonsen (1990) to describe transfer on surfaces, and the model without inclusion of transfer from transconjugants was used by Yao et al (1990). A modification of it including a dilution term to describe continuous culture has been used (Freter et al 1983, Toda et al 1990) and the dilution model has been used to describe bacteria growing in gut contents *ex vivo* (Licht et al 1999). Top et al (1992) compared two mathematical models based on competing ideas concerning the mechanics of plasmid mobilisation from recipients to donors using proteins encoded on donor plasmids (retrotransfer), and in the process modelled dT/dt as γDR during lag phase and measured γ on solid agar using *E. coli* to *E. coli* transmission. Top's model did not include a growth term as the populations were measured only over three hours, whereas Ohtake et al's (1989) stochastic model did not include a growth term as transfer was measured at stationary phase. Simonsen et al (1990) simplified Levin's model to make γ easier to estimate, and his method has been followed (Gordon 1992, Licht et al 1999). Among Simonsen's additional assumptions are that the growth rate of donors and

recipients is equal and the transconjugants' growth rate is not affected by the carriage of the plasmid; and that plasmid transfer does not occur at stationary phase.

These various assumptions seem appropriate for the conditions in each experiment, making their estimations comparable.

2.6.3 Estimations of plasmid transfer rate

The only published estimate of *Salmonella* plasmid transfer rate (Alcaide & Garay 1984) estimates it as density of transconjugants per initial number of donors, yet they do not estimate the initial concentration of parents or the growth rates of the strains, and furthermore the time of co-incubation before sampling varied from 4-18 hours. It is therefore impossible to obtain an estimate of γ from this.

Donor	Recipient	Plasmid	Plasmid transfer rate	Medium	Notes	Reference
E. coli K12 J53	*E. coli* CSH50 Nalr	R1drd19	$3.8x10^{-9}$	Still broth		Levin et al. (1979)
E. coli K12 J53	*E. coli* CSH50 Nalr	R1drd19	$5.0x10^{-9}$	Agar surface		Simonsen (1990)
E. coli LC102	*E. coli* DH1 Nalr	R100-1	$3.43x10^{-10}$	Still broth	Average of three	Yao et al. (1990)
"	"		$5.7x10^{-10}$	Rotary shaker		"
"	"		$7.4x10^{-10}$	Vibratory shaker		"
"	"		$3.1x10^{-11}$	Spinner flask		"
E. coli LC102	*E. coli* DH1 Nalr	R100-1	$8.4x10^{-12}$	Continuous culture	Average of two	Toda et al. (1990)
E. coli K12 J53	*E. coli* CSH50 Nalr	R1drd19	$6.51x10^{-9}$	Still broth	Average of donor and trans. rates	Freter et al. (1983)
"	"	"	$3.7x10^{-9}$	Still broth with F strains	"	"
"	"	"	$8.5x10^{-8}$	Still broth with caecal flora	"	"
"	"	"	$2.86x10^{-9}$	in vivo (mice)	"	"
E. coli HB101	*E. coli* X7 Nalr	RP1	$4.93x10^{-16}$	in vivo (mice)	growth rate from a different strain*	Rang et al. (1996)
Unknown	Unknown	Unknown	$1.5x10^{-11}$	in vivo (human)	"	"
E. coli LE392	*E. coli* CSH52	RP4	$1.03x10^{-10}$	Agar surface	Average of three	Top et al. (1992)
E. coli K12	*E. coli* K12	R100-1	$8x10^{-12}$	Gently shaken broth		Ohtake et al.(1989)
"	"	R100	$2x10^{-15}$	"		"
"	"	RP4	$3x10^{-12}$	"		"
"	"	RSF2124	$2x10^{-15}$	"		"
E. coli K12 CSH7	*E. coli* CSH7 Nalr	R1	$1x10^{-9}$	Broth shaken at 150 rpm		Simonsen et al. (1990)

Table 2.5. *E. coli* plasmid transfer rate estimates from the literature

* It is possible to work out an in vitro estimate for mouse transmission using Simonsen's method and donor, recipient and transconjugant densities from (Rang et al 1996) and growth rates from (Rang et al 1999). Growth rate is that of *E. coli* BJ4 in mice under identical conditions to those under which the plasmid transfer rate was measured.

2.6.4 *In vivo* dose-conjugation relationship

Simulations in this study (section 5.8) predict a convex relationship between antibiotic dose and transconjugant production. Where a bacterial infection is successfully treated with a course of antibiotics of high enough dose, potential recipients are eliminated before transconjugants are created. This accounts for the decline of transconjugants at high doses. Although some antibiotics impede conjugation (Burman 1977b), at lower concentrations, or where transconjugants have already become established, an antibiotic to which the transconjugant is resistant and the recipient is susceptible would be expected to increase the density of transconjugants relative to that of recipients. Duval-Iflah et al (1980) provide evidence that antibiotic administration can lead to an increase in transconjugant density. They investigated transmission from *Serratia liquefaciens* to *E. coli* in the mouse gut which had been colonised with human faecal flora. They sampled faeces every two days for sixty days. After 30 days they administered ampicillin at 500 $\mu g\ ml^{-1}$ in drinking water for the remainder of the experiment. *E. coli* transconjugants were formed in the absence of ampicillin and stabilised at a level four orders of magnitude lower than the recipient. During ampicillin administration, transconjugants replaced recipients and became the dominant strain in mice. Plasmid transfer is more difficult to demonstrate *in vivo* in the absence of antibiotic; Anderson et al (1973) found that R-plasmid transfer would only occur in the presence of chemotherapy.

2.7 Summary

It is now widely perceived that the emergence of antibiotic resistance in strains of *Salmonella* in chickens poses a significant threat to humans. This perception has lead to the gathering of large amounts of quantitative information about *Salmonella* and *E. coli* in chickens. These data reveal an epidemiological picture of high prevalence on contamination of broiler carcasses with *Salmonella* (64% of establishments tested in the US in the late 90s suffered contamination of more than 5% of their produce). This goes hand-in-hand with a continuing serious food poisoning problem posed by *Salmonella*: 50-60 cases per 100, 000 population was the norm throughout the 1990's in Great Britain. Although *Salmonella* infection in chickens can be controlled through antibiotic treatment, vaccination and competitive exclusion treatments, the economic pressures to produce cheap meat and eggs do not favour large expenditure on such control measures. Most worrying of the data on the epidemiology of *Salmonella* in chickens are those that point to rapidly declining sensitivity of farm-derived Salmonellae to antibiotics. Over 40% of samples taken from farms in 1990 were completely sensitive to a range of antibiotics, but in 1997 this figure had fallen to just 11%.

These alarming epidemiological data highlight a need to understand the mechanisms that allow antibiotic resistance elements to spread and persist amongst a population of Salmonellae. Such an understanding comes in two parts: on the one hand lies quantification of the rates at which each of the underlying processes occur. The underlying processes of importance can be encapsulated in a description of bacterial growth and transfer of resistance elements that is a simple extension of Monod's original

model of saturating growth. Such a model describes the growth of a population of transconjugant bacteria formed when a plasmid is transferred from a donor to a recipient. In the current context the plasmids of interest are those that carry antibiotic resistance. The model provides a framework within which to organise a disparate mass of data on life-history parameters of donors, recipients and transconjugants into a coherent description of what one would expect to happen for a given set of parameter values and starting conditions. The parameters of interest are the intrinsic growth rates and carrying capacities for donors, recipients and transconjugants along with the plasmid transfer rate. A review of the existing literature shows that whilst individual measurements of those parameters for different strains of *Salmonella* and *E. coli* abound, there is no single coherent set of measurements of all relevant parameters for a given donor-recipient-transconjugant trio. This thesis aims to fill that gap.

3. Materials and Methods

The following materials and methods are those used in several experiments. Methods specific to individual experiments are detailed in appropriate chapters.

3.1 Software

Modelling was carried out using Microsoft Excel and ModelMaker version 3.0.3 and 4 (Cherwell Scientific Publishing Ltd.); data were statistically analysed using Minitab (release11.11).

3.2 Bacterial strains

Donor bacteria were *Escherichia coli* 2442 (Institute for Animal Health, Compton [IAH] culture collection), resistant to ampicillin, chloramphenicol, tetracycline, sulphonamide, spectinomycin, and neomycin and partially resistant to streptomycin and furazolidone. This antibiotic resistance was encoded on several plasmids; the plasmid most frequently transferred to recipients carried resistance to sulphonamide, spectinomycin, ampicillin and neomycin.

Recipient bacteria were *Salmonella typhimurium* 576. This strain was chromosomally resistant to nalidixic acid and carries no resistance plasmids.

Transconjugant bacteria are the same strain of *S. typhimurium* 576 which have received the plasmid encoding resistance to sulphonamide, streptomycin, ampicillin and neomycin from the donor.

Bacterial population	**Resistance profile***	**Medium used to isolate**	
		Medium	**Antibiotics included**
Background *E. coli*	varies	MacConkey**	none
Donor *E. coli* 2442	Amp, C, OT, S3, SH, Neo pt Fr pt S	MacConkey**	Neo, OT
Recipient *S. typhimurium* 576	Nal	Brilliant Green†	Nov††, Nal
Transconjugant *S. typhimurium* 576	Nal, Amp, Neo, S3, pt S	Brilliant Green†	Nov††, Nal, Neo

Table 3.1. Media and antibiotics used to isolate different bacteria. Pt = partial resistance.
* Abbreviations of antibiotics:

Amp	Ampicillin
Neo	Neomycin sulphate
C	Chloramphenicol
OT	Tetracycline
S3	Sulphonamide
SH	Spectinomycin
Nal	Sodium nalidixate
S	Streptomycin

** MacConkey (CM7, Oxoid) agar represses the growth of most bacteria except enterobacteria (the group to which *Escherichia* and *Salmonella* belong)

† Brilliant Green (CM263, Oxoid) agar represses most bacteria but allows *Salmonella* colony growth.

†† Nov (Novobiocin) is added as *Salmonella* are intrinsically resistant to it and it represses other bacteria.

3.3 Experimental animals

Three-week-old *Salmonella*-free Light Sussex chickens obtained from a flock sustained at the IAH were used in all *in vivo* experiments. These animals are outbred and have a moderate susceptibility to *S. typhimurium*. Chickens were infected with 0.3 ml of undiluted broth culture containing approximately 10^8 organisms. The chickens were housed in wire pens held above a scroll of plastic which enabled collection of faeces. New plastic was pulled beneath each pen every morning 1-2 hours before sample collection to ensure the freshness of droppings. Caecal contents were sampled post mortem, and throughout experiments from beneath the cage and placed in universal bottles. Contents were diluted and plated as soon as possible after sampling. Where samples from individual birds were required, the cloaca was swabbed and the swab smeared on plates without enrichment for *Salmonella*.

3.4 Growth media

Strains were maintained on Luria Bertani (LB) agar slopes (Oxoid) at 4^0C inoculated from one colony purified on MacConkey agar. Strains were plated on MacConkey agar with antibiotic discs to ascertain their resistance profile before slopes were made. Slopes were renewed approximately each month. One colony was inoculated into 10 ml of LB broth (Difco, East Molesey, Surrey, UK) and grown overnight at 37^0C in a shaking incubator (100 strokes min^{-1}) to achieve an approximate density of 10^9 colony forming

units ml^{-1} to create the inoculum for experiments. Strains were frozen at -80^0C and are now kept in the IAH Compton frozen culture collection.

3.5 Estimation of bacterial density

Universal bottles for containing samples were pre-weighed, then weighed again once caecal content samples had been collected. Samples were then diluted 10-fold in their bottles and decimally diluted a further five times in dilution tubes. All dilutions were carried out using phosphate buffered saline (PBS) and volume was measured using Pasteur pipettes (3 drops = 0.1 ml). The method for obtaining bacterial counts was that of Miles and Misra (1938). Cultures in LB medium were decimally diluted six times in dilution tubes. Dilutions were plated onto plain MacConkey's agar, MacConkey containing 30 $\mu g\ ml^{-1}$ neomycin and 20 $\mu g\ ml^{-1}$ tetracycline, Brilliant Green containing 20 $\mu g\ ml^{-1}$ nalidixic acid and 1 $\mu g\ ml^{-1}$ novobiocin, and Brilliant Green containing 30 $\mu g\ ml^{-1}$ neomycin, 20 $\mu g\ ml^{-1}$ nalidixic acid and 1 $\mu g\ ml^{-1}$ novobiocin. Colonies were counted by hand if single drops were plated, or by machine if 0.9 ml were plated per dilution.

3.6 Antibiotic resistance testing

Strains and background flora were surveyed for antibiotic resistance to ampicillin, chloramphenicol, tetracycline, sulphonamide, spectinomycin, neomycin, streptomycin, furazolidone and nalidixic acid. This was carried out using the BSAC standardised disc sensitivity testing method (Brown, 1998) except where otherwise stated. Briefly, ISO sensitiest agar (Oxoid CM471) plates were made up and inoculated with bacteria diluted in enough distilled water to achieve semi-confluent growth. The inoculum contained either one of the test strains or a reference sensitive strain (*E. coli* K12 proto). After inoculation, paper discs containing the above antibiotics were pressed onto the agar surface and the plates were incubated for 18h. The diameters of the zones of inhibition were measured and those of the test strains compared to the reference strain. If a test strain zone of inhibition was smaller than that of the reference strain, the test strain was said to be resistant to that antibiotic.

3.7 Maintenance of antibiotic

The IAH's lab services department maintained stock solutions of some antibiotics. These are thawed at room temperature and diluted in distilled water to give lower concentrations which can be dispensed into the medium. Neomycin is not maintained as a stock solution, and this was made up as required by dissolving weighed powdered neomycin sulphate in distilled water, whilst wearing mask and gloves. The purity of the

antibiotic was taken into account to ensure concentrations were not overestimated. Care was taken to minimise exposure of antibiotic stock solutions to light and heat. Periodically, stock solutions were tested for effectiveness by placing drops of diluted antibiotic of known concentration onto paper discs of standard diameter and placing them on lawns of sensitive and resistant bacteria. The neomycin used in chicken feed was the same as that used in laboratory experiments. Powdered neomycin was mixed thoroughly with 1 kg of feed to give a premix which was then mixed with larger amounts of feed to give the specified dose.

3.8 MIC determination

The minimum inhibitory concentration (MIC) of various antibiotics for the strains was determined both in agar and in broth. Doubling dilutions of the antibiotic were prepared, using LB broth or molten autoclaved LB agar at 45^0C. To determine agar MICs, bacteria were diluted and 30µl containing approximately 100 colony forming units (CFUs) was plated on the antibiotic plates. Colony growth was assessed after 24h. In broth MIC determination, the bacteria were added at a density of approximately $10^5 ml^{-1}$ unless otherwise stated. The tubes were inspected after 24h and those visibly cloudy were taken to contain antibiotic below the MIC. The MIC in each case was taken to be the lowest concentration of antibiotic which prevented growth.

3.9 Replica-plating

Another technique used to facilitate bacterial identification was replica-plating. Colonies grown on one plate were transferred to another of different selectivity and re-incubated. Colonies were identifiable by their position on the plate. A square of sterilised velvet was placed on a metal cylinder of diameter just smaller than a Petri dish, velvet side up. It was secured taut across it with a close fitting metal ring which was placed over the top. Plates with colonies were pressed onto the velvet and removed, then a clean plate was inoculated with those colonies in the same way. The velvets were boiled then autoclaved to sterilise them.

3.10 Slide agglutination tests

Some organisms isolated from caecal content were difficult to identify. In the experiment on dose ranging *in vivo*, very few transconjugants were isolated, so it was important to ensure the bacteria were not misidentified. In these cases, slide agglutination tests were employed. Four small drops of saline were placed onto a new slide. Part of the colony to be identified was picked up with a sterile loop and the bacteria were suspended in all the saline drops. Colonies known to be *S. typhimurium* were suspended in drops on another slide in a similar manner. A small amount of serum was mixed into each drop. Sera mixed in were polyvalent-O, O-4, O-9 and acriflavine. Polyvalent-O agglutinates any *Salmonella*; O-4 agglutinates *S. typhimurium* only; O-9 agglutinates *S. enteritidis* only

and acriflavine agglutinates rough bacteria (those that have lost their LPS), which ensures the bacteria still possess lipopolysaccharide (LPS) and are still virulent.

3.11 Nomenclature

To differentiate between *E. coli* and *S. typhimurium*, the word species is used; and to differentiate between donors, recipients and transconjugants of a particular mating, the word population is used. 'Strain' is used to differentiate between the different strains acting as recipients regardless of species, and in the 'testing strains' section is also used to differentiate between the different strains acting as donors.

4. Mathematical models of the dynamics of plasmid mediated resistance

4.1 Introduction

Mathematical modelling is a formal process of simplifying complex processes in order to understand them better, or make predictions about how to change outcomes or how they will behave in the future. The modelling of the epidemic spread of infections amongst hosts can give examples of each of these uses of modelling.

An example of the use of mathematical models to understand disease processes was the malaria model of Ross (1915) and Macdonald (1952). Ross recognised from his models that a threshold number of mosquitoes existed below which malaria could not persist. Macdonald generalised from Ross' work to formulate the idea of R_0. An example of mathematical model used for planning control strategies is the modelling of rabies transmission by Brochier (1991). Theory predicts that there is a threshold number of individuals which must be vaccinated in order for a disease to be eradicated. This threshold exists because there is a minimum number of susceptibles required to maintain an epidemic. Modelling is used more rarely for prediction because as with any forecasting, the complexities which are not taken into account in the model may quickly divert the course of events in unpredictable ways. Measles is a good example of a disease which is more easily predictable than most, as it exhibits strong cyclic patterns. Roberts

& Tobias (2000) predicted the onset of a measles epidemic in New Zealand, 6 years after the previous epidemic. The outbreak occurred only a few months before predicted and as it was anticipated, was quickly brought under control. Mathematical models have been most recently employed to help plan the control of foot and mouth disease in the UK (Kao 2002). Whilst modelling of epidemics is a huge field, only a subset of the field is of direct relevance to this project: the models of bacterial growth and of the spread of antibiotic resistance.

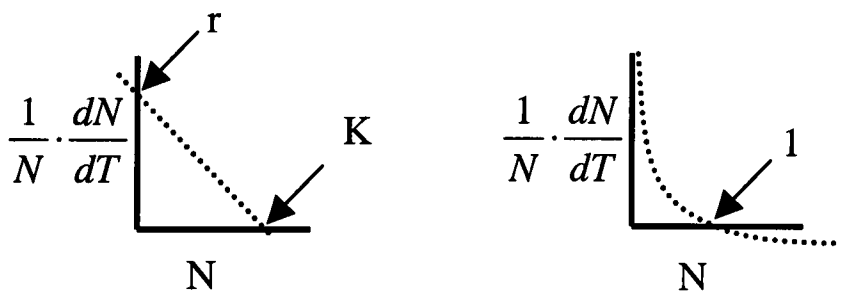
4.2 Models of bacterial growth

The process of bacterial growth lends itself to mathematical modelling used to describe and understand aspects of growth. Monod (1949) proposed the model of growth where bacteria at first grow exponentially, and then are limited by the degradation of substrate in the medium. The consumption of resources is modelled explicitly in Monod's model. His is therefore an example of a resource competition model (Tilman 1982). Bacterial growth may be described satisfactorily without the explicit inclusion of resources. The limiting of growth at stationary phase may be described by a reduction in growth rate with time or bacterial density. The Logistic model describes a linear decrease in growth rate with bacterial density. It has become the most widely used description of bacterial growth, as it is has few parameters and fits growth curves well.

$$\frac{dN}{dt} = rN\frac{K-N}{K} \qquad 4.1$$

Where N = population density, r = intrinsic growth rate and K = maximum density or carrying capacity.

Zwietering et al (1990) compare the fit of various models to *Lactobacillus plantarum* growth and favour the use of Gompertz model on criteria of goodness of fit and ease of use, although the logistic model fares well also. The difference between logistic and Gompertz lies in the way the approach to equilibrium is modelled; logistic has a linear decrease in per capita growth rate as bacteria increase, whereas in Gompertz this decrease is exponential:



Logistic Gompertz

Figure 4.1. Difference between logistic and Gompertz models in per capita growth rate with population size.

4.3 Existing models of antibiotic resistance evolution

4.3.1 Modelling the effect of antibiotics on bacteria

Lipsitch and Levin (1997) present and analyse a model of emergence of antibiotic resistance in humans. They consider only mutational resistance which appears by a Poisson process (in time), with rate μ. They do not specify μ; they merely choose different arbitrary values for μS, where S is the susceptible population size. They consider the realistic situation that antibiotic concentrations are not constant in time; they are administered, then the concentration decays until the next administration. Neither does antibiotic concentration determine death of bacteria linearly; it rises as a saturating function, the fraction of bacteria killed being given by:

$$f(C) = k_k \frac{C}{C + C_{50}} \qquad 4.2$$

where k_k is the maximal rate of killing, C is the antibiotic concentration, and C_{50} is the concentration required to give 50% maximal killing. This results in a decline in a growth rate-dose relationship which is steep at first but which tends to r-k_k where r is the intrinsic growth rate (figure 4.2).

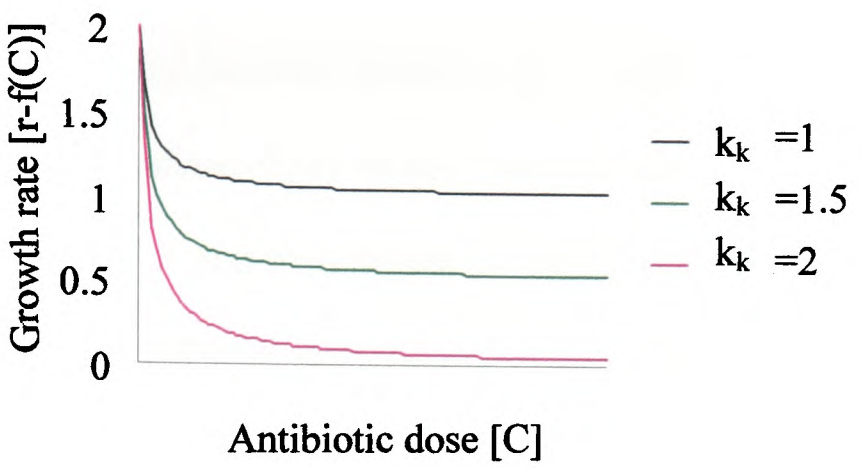


Figure 4.2. A model of the effect of antibiotic dose on bacterial growth rate (Lipsitch & Levin 1997b). r =2.

This model is extended to encompass multidrug resistance and different dosing schedules, and patient non-adherence to regime. The former two may be of relevance to the *Salmonella*-chicken interaction, but the latter is not unless farm workers stop treatment before the course of antibiotics is complete.

Levin et al (1997) use a different approach, where the fraction of bacteria exchanged between the host and the environment in each bacterial generation is modelled. They replaced the complicated saturating function of the effects of antibiotic treatment with the assumption that all antibiotic treatment immediately increases the proportion of resistant bacteria to 100%. The type of resistance modelled is plasmid-borne, although a transfer rate parameter is not included in the model; a proportion of the bacteria are resistant at the start of the simulation, and this changes only through selection.

Guerillot et al (1993) model bacterial density over time in the presence of antibiotic (killing curves). Their model is descriptive, and has four parameters, which enable close fits to time-density curves of antibiotic treated *E. coli*. The three parameters which might

be affected by antibiotic dose describe respectively (i) the lag before antibiotic killing, (ii) the minimal bacterial density and (iii) the steepness of the decline in density immediately after lag phase. They fit this model to a limited range of doses of five antibiotics, which are 1, 2, 3 and 5 multiples of the minimum inhibitory concentrations (MICs) of each antibiotic. The killing curves for each antibiotic are very similar across this dose range, such that they find a significant effect of dose only with three of the five antibiotics tested, and then only on the parameters describing lag and minimum number, not the rate of decline. These results do not indicate the effect of low or intermediate levels of antibiotic on bacterial growth parameters. Rather, they suggest that when concentration is much higher than the MIC, bacterial density initially declines then equilibriates at a low level which is fairly independent of dose. These results are in agreement with Lipsitch and Levin's model, which predicts insensitivity of growth rate to dose at high doses (figure 4.2).

4.3.2 Models incorporating resistance-transfer

Four models of plasmid transfer between bacteria are described below.

Levin et al (1979) developed a model describing the kinetics of plasmid transfer during the exponential phase of bacterial growth. It is a simple mass-action model with the term representing plasmid transfer equivalent to that used in infectious disease models to describe disease transfer (a constant multiplied by the density of donors (infecteds) and by the density of recipients (susceptibles)). They tested their model by measuring *in vitro* plasmid transfer. They inoculated liquid media with a concentration of just under 10^7 donors and recipients ml^{-1}, and measured the density of the three populations

approximately every hour for 9 hours. They found their model accurately predicted transfer rate during exponential phase, but transfer rate was over estimated at both lag phase and stationary phase. Transfer rate increased throughout lag phase until it matched that of the exponential phase. They suggest in their discussion that this model as it is will not apply directly to an *in vivo* situation, as the gut environment is likely to be heterogeneous and thus the mass action principle will not apply.

Simonsen et al (1990) extend this model to describe growth kinetics throughout exponential and stationary phases, in developing a simple method of estimating the plasmid transfer rate constant. Austin et al. (1997) also include infectious resistance plasmid transfer in their model and include between-host processes, in order to predict which strain eventually predominates in the host population. Bergstrom et al (2000) incorporate the loss of plasmids from cells due to segregation during division, and the cost of bearing a plasmid into their infectious transfer model, as a reduction in intrinsic growth rate. They go on to explore the existence conditions for bacterial plasmids. They assume that selection and segregation will eliminate plasmids in the long term if infectious transfer is the only process maintaining them. They conclude that beneficial genes do not result in plasmid maintenance. They argue that plasmids can theoretically exist only with the occurrence of selective sweeps or by virtue of the fact that they can be transmitted across species boundaries.

4.3.3 Models including immune interactions.

The other main selective pressure on *Salmonella* in chickens is the chicken immune system. This matures with age, and immunity to *S. typhimurium* is effective against *S.*

typhimurium to varying degrees; virulent strains are cleared from the gut more quickly than non-virulent strains (Barrow and Lovell 1988). There are over 2000 serotypes of *Salmonella* according to the Kauffman-white classification which share immunogenic antigens to lesser or greater extents. Hence the other models of relevance to *Salmonella* infections of chickens (not considered in this thesis) are strain competition/cross immunity models. Gupta and co-authors (1996, 1997) investigate the theory behind the dynamics of a recombining population with variable antigens and some cross-immunity. They model a system of four competing strain types, each with one of two alleles at two loci (i.e. ax, ay, bx, by). The outcome of immune selection (1996) or vaccination (1997) depends on the degree of cross-immunity (γ). Where cross-immunity is very high, vaccination against ax will protect well against strains ay and bx. Hence, in this case, only strain by will predominate. Where cross-immunity is less effective, the strains will equilibrate according to each R_0, where the number immunised + the number naturally immune = $1 - 1/R_0$. In the immune selection model, immunity above the critical level allows the persistence of only two strains in the population; despite recombination the other two strains cannot invade the population. Hence, immune selection can cause linkage disequilibrium in a recombining population of bacteria.

4.3.4 Evolution of resistance to vaccination.

McLean (1995) models evolution of resistance to vaccination in human populations. There are several possible interesting outcomes of vaccination against one strain in a system where two strains compete for hosts. Where no superinfection is allowed, vaccination against strain 1 with weak cross-protection against strain 2 may eventually

result in a strain 2 epidemic. This could be due to the build up of susceptibles, because of gradual decline in the immunity to strain 2 once natural cross-infection induced immunity is reduced. Alternatively, this could be prevented by low vaccine coverage, so that natural cross-protection is maintained, or using a more cross-reactive vaccine. If superinfection is allowed where one strain replaces another within hosts, there are particular cases where vaccination could have the disastrous result of increasing incidence. This is the case where the predominant strain has a lower R_0 but higher infectivity than the coexisting strain, such that the predominant strain 'wins' within-host battles. Vaccination against the lower R_0 strain will remove some of the within-host competition and allow the higher R_0 strain to equilibriate in the population, necessarily infecting more people than the equilibrium of the lower R_0 strain.

4.4 R_0

The fundamental concept in the epidemiology and control of infectious diseases, is that of R_0. R_0 in microparasitic epidemiology can be defined as "The average number of secondary infections caused by a single infected in an entirely susceptible population" (Anderson & May 1991). Once some susceptibles have become infected the effective reproductive rate (R_E) falls below R_0 due to increasing proportions of those individuals contacted being immune or infected. Pathogens with a high R_0 (e.g. measles R_0=6-10 (Mclean & Anderson 1988)) will infect a large proportion of a population, and where endemic, will have a very low average age of infection. R_0 is directly related to ease of

eradication from the population. Where vaccination is possible, it is theoretically possible to reduce R_E to below one. In such a population, each new case will on average not 'replace' itself with another infection; hence the infection will inevitably eventually die out. The proportion of the population that requires vaccination for this to be possible (p_c) may be calculated as follows:

$$p_c = 1 - \frac{1}{R_0} \qquad 4.3$$

Note that p_c refers to the proportion protected rather than vaccinated; if a vaccine is only 80% effective and the critical proportion was 75%, more than 93.75% of the population would have to be vaccinated to achieve elimination. Elimination of *Salmonella* in a chicken flock is desirable and possible, and could be done by vaccination if a vaccine effective enough to result in protection above the critical proportion was possible, or if it could be used in combination with other methods. The R_0 of a disease is also important in calculating how best to eliminate it with chemotherapeutic agents. This is because the R_0 of a disease is directly proportional to the duration and the extent of infectiousness. If antibiotics become completely effective at preventing *Salmonella* excretion half way through the normal course of an infection say, the R_0 of the disease is halved if all infections are treated. If treatment is prophylactic and is 100% effective, then the critical fraction of any population that needs to be treated for elimination (p_{ct}) is equal to the critical fraction effectively vaccinated. Where the untreated infectious period is D and treatment reduces the average infectious period to a duration d, the critical fraction which must be treated is

$$P_{ct} = \frac{R_0 - 1}{R_0 \cdot \left(1 - \frac{d}{D}\right)} \qquad 4.4$$

In Salmonellosis of chickens, R_0 is determined in part by the infectiousness of the strains, which in turn depends largely on the density of Salmonellae excreted in faeces or caecal droppings.

4.5 Relationship between virulence and R_0

Plasmids confer virulence factors, which often have implications for their survival. A convenient definition of virulence is the host mortality caused by an infection (May & Anderson 1983). This definition does not consider morbidity, however, which can be economically important. Morbidity is taken into account when virulence is defined as the influence of the pathogen on host fitness, but reproductive fitness is a redundant concept in broiler chickens, as they do not reproduce. In this context, morbidity should be incorporated in the definition of virulence from an economic perspective. Virulence in diseases of broilers should therefore be defined as the economic loss due to a disease resulting from mortality, reduced growth rate, and loss of meat quality.

The R_0 – virulence relationship is important for the purposes of modelling between-host interactions, as information on both virulence and R_0 of strains is necessary before the outcome of an epidemic can be predicted. If plasmids confer this virulence then it has implications for plasmid survival, also. Conventional wisdom dictates that parasite populations evolve to avirulence, thus avoiding elimination of its host population (Bull 1994). This view was challenged by Anderson & May (1982) and Stewart & Levin (1984), who showed that selection on pathogen populations acts merely to increase R_0. If virulence can increase R_0, then virulence will be indirectly selected for. Two examples of a relationship between virulence and high R_0 in plasmid-bearing Salmonellae are provided by the work of Barrow and other authors. Barrow et al. (1987) found two plasmids common to each of the four independent cases of fowl typhoid caused by *S. gallinarum* they investigated. Of these, the 85 Kilobase (Kb) plasmid conferred both a

large increase in virulence (host mortality) to plasmid$^+$ strains, and the ability to both invade and grow in the endothelial system in chickens. In 21-day old birds, excretion of *S. gallinarum* not carrying the 85 Kb plasmid from the caeca had ceased within 24 hours, whereas plasmid$^+$ strains were found beyond 60 hours. If it is assumed that increased excretion will lead to greater numbers of other individuals becoming infected, the R_0 of the plasmid-bearing strains is higher. One-day old chicks excreted both strains over the total duration of the experiment (two weeks), but this is probably due to the increased invasiveness of all strains in young chicks. In this example the most virulent strain is also the one with the highest R_0. Barrow & Lovell (1988) found similar results with the closely related serotype, *S. pullorum*. It was found that the most virulent type, that containing one 85Kb plasmid, both increased virulence significantly and was found in larger numbers and for longer in the caeca. Timoney & Linton (1982) also found that some plasmids can have a positive effect on colonisation.

Plasmids are not thought to be associated with virulence in *S. typhimurium* however; in this species pathogenicity islands in chromosomal genes are thought to effect virulence (Groisman & Ochman 1997).

4.6 Modelling plasmid transmission

4.6.1 Motivations for modelling plasmid transmission

The dynamics of bacterial infections in chickens are difficult to describe intuitively. Mathematical models may provide a formal description of the interactions between

bacteria infecting chickens. These models allow greater understanding of the dynamics of an infection as described by the underlying processes of bacterial growth, plasmid transfer, competition and infection.

In particular, models can be used for two purposes:

1. Defining the processes important in the kinetics of populations transferring antibiotic resistance plasmids and evaluating the parameter values for those processes. Parameter estimations are presented in sections 5.3-5.7.
2. Prediction of the effect of antibiotic dose on the density of donor, recipient and transconjugant bacteria in a system where donors and recipients are in co-culture (section 5.8). These predictions are based on the model presented here incorporating the parameter values estimated in section 5.3-5.7.

Modelling was carried out using the software ModelMaker and Excel.

4.6.2 Description of within-host models

The transfer of plasmids between bacteria occurs occasionally when eligible donors and recipients come into proximity with each other, and depending on plasmid and host, transconjugants resulting from such pairings can retransmit the plasmid. Plasmid transmission through a population of bacteria can be modelled as a mass action infection process (Anderson & May 1991). They use the term βXY to describe disease transmission where β = infectiousness, X = susceptibles and Y = infecteds. Bacterial growth and cessation of growth at stationary phase are described by the logistic model. In this model, at low densities a population of individuals N grows at its maximal rate, r.

Growth rate decreases linearly with bacterial density until the maximum density is reached, K, when growth ceases. This model was found to fit growth curve data with a high degree of statistical significance by Zwietering et al (1990). It is also very simple, with just two parameters (growth rate and carrying capacity).

The Logistic model is combined with the transmission term to comprise the basic plasmid transmission model which is very similar to that of Simonsen (1990). Simonsen's differs in that it explicitly models resource competition. It was not thought necessary to include this extra parameter as the logistic model fits so well to growth curve data (Zwietering et al 1990). The main assumptions of biological interest are listed below:

1. Bacteria have an equal probability of encountering each other for conjugation (the mass action assumption)
2. Plasmid transfer is a function of growth phase, i.e. stationary phase transfer is negligible.
3. Segregational loss of plasmids does not significantly affect dynamics.
4. Plasmid transfer rate from donors and transconjugants is equal
5. Lag phase is not considered

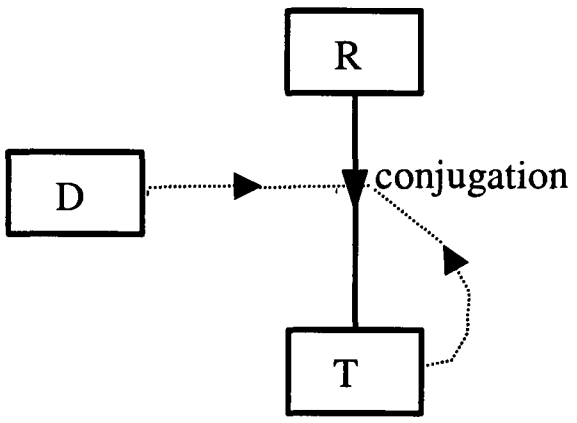


Figure 4.3. Flow diagram of the within-host model (equations 4.5-4.7). Recipient bacteria (R) are transformed into transconjugants (T) when they receive a plasmid from the donor (D). The dotted lines denote the influence of donors and transconjugants on transconjugant production.

$$\frac{dD}{dt} = rD\frac{K-N}{K} \qquad 4.5$$

$$\frac{dR}{dt} = (rR - \gamma(D+T)R)\frac{K-N}{K} \qquad 4.6$$

$$\frac{dT}{dt} = (rT + \gamma(D+T)R)\frac{K-N}{K} \qquad 4.7$$

where D = donor density R = recipient density T = transconjugant density (ml^{-1}) r = intrinsic growth rate ($hour^{-1}$) γ = plasmid transfer rate (ml $hour^{-1}$) K = carrying capacity (ml^{-1}) and $N \equiv D+R+T$.

This is the fundamental structure of the models studied here, but for different experimental situations, a number of modifications were required. The depressive effect

on growth rate of a population due to competition varies with the competing strain. In the model presented above the impact of within and between population competition is assumed to be equal. In some experimental situations this assumption is untenable, often within population competition is stronger than between-population competition, or in some cases, weaker. Lotka-Volterra competition coefficients were included and these parameters estimated (section 5.5). If the populations are subjected to antibiotic treatment, terms modifying growth rate are added which describe antibiotic killing and antibiotic resistance. When necessary different specific growth rates and carrying capacities of the populations are used, and a death or dilution rate is included.

A model incorporating all these terms is set out below. It assumes additionally that antibiotic resistance of transconjugants is equal to that of donors.

$$\frac{dD}{dt} = r_D(1-pz)D \cdot \frac{K_E - D - \alpha_{ES}(R+T)}{K_E} - m_E D \qquad 4.8$$

$$\frac{dR}{dt} = \left[r_R(1-z)R - \gamma(D+T)R\right] \cdot \frac{K_S - R - T - \alpha_{SE}D}{K_S} - m_S R \qquad 4.9$$

$$\frac{dT}{dt} = \left[r_T(1-pz)T + \gamma(D+T)R\right] \cdot \frac{K_S - R - T - \alpha_{SE}D}{K_S} - m_S T \qquad 4.10$$

where r_x = intrinsic growth rate of D, R or T ($hour^{-1}$), z = antibiotic killing, p = degree of resistance of resistant bacteria (i.e. those bearing a plasmid: D or T), K_E and K_S = carrying capacity parameter of *E. coli* and *S. typhimurium* respectively (ml^{-1}), α_{ES} = effect

of competition by *S. typhimurium* on *E. coli*, α_{SE} = effect of competition by *E. coli* on *S. typhimurium*, m_X = dilution or death parameter ($hour^{-1}$) of *E. coli* or *S. typhimurium*.

4.6.3 Discussion of the within-host model

This model describes bacterial growth and reduction of growth rate by antibiotic treatment and by competition, plasmid transfer, and removal of bacteria by death or dilution. This method of describing growth and plasmid transfer was chosen for its simplicity (i.e. minimum number of parameters). This model follows those of several authors (see Begon et al (1996) and Simonsen et al (1990)). Experimental data on plasmid transfer and competition were fitted to variants of the model and data on antibiotic action and resistance were compared with it. This model describes growth in laboratory broth culture, but it was hoped that the results will translate to an *in vivo* situation. The competition aspect of the model is the most important distinction between the *in vitro* and *in vivo* environments. *In vivo*, donors, recipients and transconjugants are in competition not only with each other, but also with large numbers of resident bacteria, e.g. enterococci. These competitors will limit the population densities of the bacteria of interest. If the in vivo situation were to be modelled, the effect of these populations could be represented explicitly by including their population densities, and respective competition coefficients in the model. A more workable model would assume that the effect of background flora population on the populations of interest is constant. This would be included in the models by reducing the carrying capacity parameter, K. The

carrying capacity in *ex vivo* caecal content for donors, recipients and transconjugants was measured (section 5.4). These allow simulations of in vivo dynamics by this method.

The growth rate of a bacterial strain depends on many factors e.g. growth medium, temperature and competition. The intrinsic growth rate is the fastest rate of growth achievable in a particular medium at a particular temperature. Intrinsic growth rates were measured in LB medium at 37^0C during exponential phase (section 5.3).

Antibiotics may act to reduce the growth rate (bacteriostatic antibiotics, e.g. tetracycline), or they may kill bacteria (bacteriocidal antibiotics e.g. ampicillin, neomycin). This model can describe either situation as the parameter combination r(1-z)-m represents the initial growth rate of the population, i.e. replication – removal and can be negative.

The parameter m can be included in the model where a death or dilution process is significant. It represents the possibility that a population may still turn over at equilibrium. Small changes in density during stationary phase (figure 10.2) indicate some turnover occurs. This is presumed to be because when individuals die, others divide to return the population density to the maximum. *In vitro* experiments were carried out in closed rather than continuous flow vessels, so m *in vitro* will just be due to natural turnover and is likely to be small. m has been assumed to be equal to 0.1, which results in a good visual fit to data, and this is the value used in generating model predictions. In chickens, *S. typhimurium* grow mainly in the blind-ended caeca. These are closed volumes that empty and refill approximately every eight hours, so m will be time dependent; it will be very large when the caecum empties and very small in-between. Although m is small, it is a significant parameter as it is the only check on transconjugant

density. This allows the calculation of threshold conditions for the dominance of resistant *Salmonella* (section 5.8.2). Although not considered in this project, other *Salmonella* strains (e.g. *enteritidis*) localise to other parts of the gut where a constant flow model is more appropriate, so when modelling these strains larger values for the parameter m would be appropriate. In all the comparisons made between models and data that follow a model with $m = 0$ is used. Only in section 5.8 where simulations are presented is the complicating factor of $m \neq 0$ considered.

4.6.4 Assumptions of the within-host model

The five main assumptions are listed above (4.6.2). The mass action assumption is justified when bacteria are growing and conjugating while suspended in the growth medium, as largely occurs in the *in vitro* experimental model. Levin (1979) stated the mass action assumption was likely to be violated in the gut, due to heterogeneity. However, *S. typhimurium* living in the chicken caecum experience a fairly homogenous environment. Bacteria may associate with the gut wall *in vivo*. As the plasmid transfer rate on surfaces is greater (Bradley et al 1980, Simonsen 1990) One might presume this would lead to a greater plasmid transfer rate *in vivo* than *in vitro*. In an *in vivo* infection *S. typhimurium* does not associate with the caecal lining, however (Barrow et al 1994). The assumption that bacteria encounter eachother with equal probability should therefore hold in this environment. Various authors have noted a sharp decline or ceasing of conjugation at stationary phase (Levin 1979) and this assumption was tested experimentally (section 10.2.3). Plasmids are lost from cells by segregation into one daughter during division. Bergstrom et al (2000) assumed that the segregant without plasmids would not be a

viable cell. In this case, segregation processes are accounted for in the measurement of intrinsic growth rate of transconjugants. In any case segregation rate is low; in most enteric strains segregation occurs at a rate of 10^{-4} per cell generation or less (Falkow 1975).

4.6.5 Estimation of parameter values for the within-host model

This model was simplified in various ways to allow estimation of parameters from data (section 5.3-5.7). The simplest example is that of the intrinsic growth rate, where the model used reduces to $\frac{dN}{dT} = rN$. Data was collected on density of populations grown in the absence of other strains between 1 and 6 hours without antibiotic to eliminate considerations of conjugation, competition, antibiotic killing, etc. Other parameters were estimated with similarly appropriate pared down models. Parameters were estimated in LB broth, and where possible in *ex vivo* caecal content (chapter 5).

Parameter estimates are useful to allow model predictions, and are sometimes interesting in their own right. For example, the cost of antibiotic resistance is an important issue (section 2.3), and a comparison of recipient and transconjugant growth rates gives information on this. Plasmid transfer rate and competition coefficient data is also interesting and values for these characteristics are not easily found in the literature for *Salmonella*.

4.6.6 Predicting *S. typhimurium* density using the within-host model

The dose-response relationship was investigated, in an *in vivo* co-infection with donors and recipients (chapters 7 and 8) and in co-culture of donors and recipients in laboratory medium and caecal content taken from chickens (chapter 9). In a real situation, transconjugants are likely to be formed if donors and recipients are detectable. If transconjugants are present, they grow at a rate governed by the strength of competition from other bacteria in addition to their intrinsic growth rate, r, and will cease growth when the stationary phase is reached. If antibiotic is present this favours transconjugants because antibiotic kills a greater proportion of recipients and background flora than donors and transconjugants, thus reducing competition for resistant populations. The results of (Duval-Iflah et al 1980), section 2.4, demonstrate this outcompetition of recipients by transconjugants in the presence of antibiotic.

If the turnover rate, m, is included in the model, the transconjugants will continue to increase in density at a low rate until an equilibrium is reached. This occurs when $T + R = K_S - \left(\frac{K_S \cdot m}{r}\right)$, and the value of T depends on the relative growth rates of R and T, γ, p (antibiotc resistance) and z. The relationship between antibiotic dose (z) and transconjugant density is discussed in chapter 5.9.

4.6.7 Tests of the within-host model

Data on the density of donors, recipients and transconjugants over time after co-incubation of donors and recipients was collected. These experiments were different to those used for parameter estimation, and were carried out solely for the purpose of testing (or verifying) the within-host model. Experiments were set up to collect density data in

both laboratory medium and chicken caecal content for this purpose. The predictions of the relationship between the density of transconjugants and antibiotic dose were tested with *in vivo* and *in vitro* dose-response experiments. These experiments are described in chapters 7-9.

4.7 Summary

Mathematical modelling of the spread of infectious disease is a huge field of which models of antibiotic resistance evolution within- or between- host form a subset. The modelling of bacterial growth has arisen separately and there are several competing models which describe it. They share two properties: an initial exponential growth rate and a saturation of growth at high densities. They vary in the degree of mechanistic detail they include. A number of models exist that include plasmid-transfer, which all represent the process as akin to infection where contact between a donor and a recipient results in the generation of a transconjugant 'infected' with the plasmid. The model to be used as the organising principle for all subsequent data collection is presented and its assumptions examined in some detail.

5. *In vitro* parameter estimation and model results

5.1 Introduction

Parameters of the model (equations 4.8-4.10) were estimated, and these values were put into the model in order to make predictions about the effect of antibiotics on transconjugant production. These predictions were then tested experimentally in chapters 7-9. The literature has been reviewed to provide parameter estimations (chapter 2), but some parameters are not adequately estimated for *Salmonella* strains in particular, and no complete set of parameters have been estimated from the same donor-recipient strain combination. Experiments were conducted to determine the following parameters: growth rate, plasmid transfer rate, carrying capacity, competition coefficients, the effect of antibiotic and the degree of resistance using one donor-recipient pair. The donor and recipient strains to be used in all the following experiments were chosen on the basis of the strength of their donation and reception (section 5.2). Parameters were estimated using standard methods where they are available, or with reference to the model (sections 5.3-5.7). These estimates were then used in generating model predictions (section 5.8). The transmission rate of donor *E. coli* 2442 and recipient *S. typhimurium* 576 from infected to uninfected birds was estimated from *in vivo* data in chapter 7.

5.2 Identification of a donor-recipient pair with a high conjugative transfer rate

Bacterial strains vary widely in their ability to donate and receive plasmids (e.g. Smith 1971b, Bale et al 1987, Gordon 1992). Some *Escherichia coli* can receive resistance-plasmids *in vivo* without the presence of antibiotics (Smith 1971b), and can donate plasmids encoding antibiotic resistance to several *Salmonella* serotypes. *Salmonella typhimurium* strains vary in their ability to transfer resistance to other *S. typhimurium* strains. Plasmid transfer that can be demonstrated between strains *in vitro* may not occur *in vivo* (Smith 1971b). Conjugation may be inhibited by anaerobiosis between some donor-recipient pairs, but not others (Burman 1977a). Three screens were carried out to select for strains that can transmit resistances to different antibiotics and are high transmitters in both aerobic and anaerobic conditions. Transfer has been documented both within and between populations of *E. coli* and *S. typhimurium*, and each of these combinations was tested.

5.2.1 Materials and methods

16 potential donor and 2 potential recipient bacteria from the IAH poultry isolate strain collection were chosen after advice (P. Barrow, pers. comm.) and tested for antibiotic resistance by the disc method (section 3.6) In this method, paper discs of various antibiotics are placed on lawns of the test bacteria, in addition to lawns of bacteria known to be sensitive, and the radii of inhibition compared. Plasmid transfer rates were quantified using Simonsen's (1990) end-point method (section 5.5.2). This method

provides an estimate of the rate of plasmid transfer per donor per recipient cell. It requires only end-concentrations of donors, recipients and transconjugants after donor and recipient co-incubation and growth rate of the combined population. Growth rate was taken to be the average *E. coli* growth rate in LB of those found in table 2.3.

First Screen

Strains were then screened for high transfer rates. One recipient and two donors were *Escherichia coli*; all other strains were *Salmonella typhimurium* (see table 5.1 below). The recipients (*E. coli* proto nal^r and *S. typhimurium* 576 nal^r) were the best two found by Smith (1971b). Nalidixic acid mutants were used as potential recipients because they were to be used *in vivo* and can usually be recovered in pure culture from caecal content containing very high densities of background flora on plates containing nalidixic acid. The strain numbers in table 5.1 identify donors within the IAH culture collection.

Resistance: Donors carried uncharacterised plasmids encoding multiple antibiotic resistance including resistance to ampicillin (amp), neomycin (neo), streptomycin (strep) and tetracycline (tet). Recipients had chromosomally encoded resistance to nalidixic acid; these nal^r mutants had been previously selected from a sensitive strain by other workers. Nalidixic acid resistance in this stain has previously been shown not to affect colonisation ability or virulence in chickens (Smith & Tucker 1980b).

Methods: The stock of donor and recipient strains were maintained in 10 ml overnight broth cultures in 25ml sample tubes kept at 4 ^{0}C for two weeks at a time. New stock was created by agitating the old sample tube, inoculating 0.1 ml (as measured by three drops from a Pasteur pipette) into fresh broth, incubating statically overnight at 37 ^{0}C and then

placed back at 4 ^{0}C. When strains were required 0.1 ml was taken from the stock culture, inoculated into another 10-ml nutrient broth and re-incubated overnight the day before conjugative experiments began. Each of the two strains of the 32 combinations were then inoculated into a sample tube and incubated as above for 16-19 hours, ensuring stationary phase densities. The starting density of donor and recipient were not measured but they are known to be approximately equal as equal volumes (0.1 ml) of cell suspension were used as inoculum, of approximate density 10^9 ml^{-1}. The donors, recipients and transconjugants in the mixture were counted the following day by serial decimal dilution followed by plating on selective MacConkey media (section 3.5). Donors were counted on MacConkey plates containing ampicillin at 30 μg ml^{-1} or neomycin at 15μg ml^{-1}. Recipients were selected using MacConkey nalidixic acid plates at 20μg ml^{-1}. Transconjugants were selected on ampicillin + nalidixic acid plates and neomycin + nalidixic acid plates at the above concentrations.

Second screen (various antibiotics)

The best seven combinations from the first screen (see below) were re-incubated together, and transfer of resistance to a further two antibiotics was sought. These transconjugants were counted on media containing streptomycin at 15 μg ml^{-1} and tetracycline at 15 μg ml^{-1} in addition to nalidixic acid at 20 μg ml^{-1}.

Third screen (anaerobic test)

The potential transmissibility of the best two of these donors' plasmids under *in vivo* conditions was investigated by incubation under anaerobic conditions to simulate the gut.

Bacterial plasmids may increase or decrease their transfer rate in conditions of anaerobiosis (Burman 1977a). Four 25ml sample tubes containing 10ml nutrient broth were inoculated with 0.1 ml of an overnight incubation of each strain as above. Two of these were placed in an anaerobic jar with a gas generating kit mixture (H_2 + CO_2 Oxoid anaerobic system BR 038B) and stood at 4 ^{0}C for several hours to allow removal of oxygen, before incubation overnight as above. The other two acted as controls and were chilled and incubated on the same schedule without being in an anaerobic chamber.

5.2.2 Results

Antibiotic resistance testing

	Antibiotic resistances*									
Donor Strain	**A**	**N**	**Fr**	**C**	**OT**	**S3**	**W**	**SH**	**S**	**Na**
E.c. 2428	✓		pt	✓	✓				✓	
E.c. 2442	✓	✓	pt	✓	✓	✓		✓	pt	
S.t. 283					✓	✓			pt	
S.t. 292			pt		✓	✓			✓	
S.t. 1725	✓	✓	✓	✓	✓	✓			✓	
S.t. 1727	✓	✓	✓	✓	✓	✓	✓	✓	pt	
S.t. 1730	✓	pt	✓	✓	✓	✓	✓	✓	pt	
S.t. 4342	✓				pt	✓				
S.t. 5325										
S.t. 204c pptA	✓			✓	✓	✓	✓		pt	
S.t. 204c pptH	✓	✓	✓	✓	✓	✓	✓	✓	✓	
S.t. 454/67	✓		pt		✓	✓			✓	
S.t. 5439/93	✓			✓	✓	✓		✓	✓	
S.t. 5611/94	✓		pt	✓	✓	✓	✓	✓		
S.t. AFF	✓		pt		✓	✓			✓	
S.t. jrw16	✓	✓	pt	✓	✓	✓			✓	
E. c. proto nal										✓
S.t. 576 nal										✓

Table 5.1. Results of antibiotic disc testing of strains. A tick (✓) indicates full resistance of the strain to the indicated antibiotic, and pt indicates partial resistance. *E.c.* = *Escherichia coli* and *S.t.* = *Salmonella typhimurium* *A = ampicillin, N = neomycin, Fr =

furazolidone, C = chloramphenicol, OT = tetracycline, S3 = sulphonamide, W = trimethoprim, SH = spectinomycin, S = streptomycin Na = nalidixic acid.

The results of the antibiotic resistance tests performed prior to the screens are given in table 5.1.

Screen 1

	Transfer rate with recipient			
	***S. t.* 576**		***E. c.* Proto nal r**	
Donor strain	**Neo**	**Amp**	**Neo**	**Amp**
E.c. 2428	7E-12*	7E-11*	3E-09	0
E.c. 2442	7E-11*	1E-10*	1E-09	0
S.t. 283				
S.t. 292				
S.t. 1725	1E-14	3E-14	4E-12	0
S.t. 1727	0	0	4E-14	2E-13
S.t. 1730	4E-15	0	1E-11	4E-14
S.t. 4342		0		0
S.t. 5325				
S.t. 204c pptA		0		0
S.t. 204c pptH	6E-16	2E-15	2E-12*	1E-12*
S.t. 454/67		4E-13		2E-12*
S.t. 5439/93	3E-14	3E-14	9E-16	0
S.t. 5611/94		4E-13		2E-12
S.t. AFF		4E-10*		9E-12*
S.t. jrw16	8E-14	2E-12	1E-17*	2E-12*

Table 5.2. Conjugation rate (as measured by the method of Simonsen et al (1990)) of donor-recipient pairs transmitting resistance to neomycin (neo) and ampicillin (amp). *S. t.* = *Salmonella typhimurium*; *E. c.* = *Escherichia coli*. Gaps in the table occur when no transconjugants were isolated because the donor is sensitive to that antibiotic. The transfer rates of the pairs which were chosen for inclusion in the second screen are marked with an asterisk*.

This experiment included control pairs in which the donor did not carry resistance to one of the antibiotics included in transconjugant isolation plates. In one example the results appear to show that resistance to neomycin can be transferred at a low rate from a donor sensitive to that antibiotic (strain *S.t.* 5439/93). The resistance profile of this donor strain was re-tested and found to be correct. The conjugation was repeated and no transconjugants were isolated on nalidixic acid neomycin plates. After discussion, it was realised that sensitive bacteria could grow on antibiotic plates because the plates were not properly 'dried in' before they were incubated, so some sensitive organisms could grow in suspension in the drop of PBS (phosphate buffered saline used for dilution) above the antibiotic media while the drop was drying in the incubator. This is not likely to affect the results of the pairs which transmitted at a high rate as this 'forcing through' does not usually result in high bacterial counts.

Screen 2

Several donor-recipient pairs which appeared to produce consistently high conjugation rates were then chosen for the second screen. *E. coli-E. coli* pairs were excluded as experiments on *S. typhimurium* were desired. The transfer rates of the high transmitting pairs denoted by asterisks in table 5.2 were re-incubated and population densities were counted, ensuring the plates were properly dried in. To enable selection based on high transferability of several antibiotic resistances, populations were isolated on plates containing four different antibiotics. The four transconjugant types were isolated on nalidixic acid + neomycin, nalidixic acid + ampicillin, nalidixic acid + tetracycline and

nalidixic acid + streptomycin plates, and the standard deviation across the four resistances in addition to the mean conjugation rate per strain was calculated.

Donor	Recipient	Mean transfer rate	Standard deviation
E.c. 2428	*S.t.* 576 nal	2.10E-12	2.84E-12
E.c. 2442	*S.t.* 576 nal	3.54E-11*	1.94E-11*
S.t AFF	*S.t.* 576 nal	1.28E-10	1.79E-10
S.t AFF	*E.c.* proto nal	1.87E-10	3.14E-10
S.t 204c pptH	*E.c.* proto nal	2.82E-11*	5.21E-11*
S.t 454/67	*E.c.* proto nal	1.18E-11	1.52E-11
S.t jrw16	*E.c.* proto nal	5.58E-12	9.40E-12

Table 5.3. Second round of screening for high transmission rates of plasmids. Mean and standard deviation were calculated from the transfer rate of the four antibiotic resistances. Asterisks* denote the results from the pairs selected for further screening.

Third screen

The pairs *E. coli* F18 2442/*S. typhimurium* 576 nal^r and *S. typhimurium* 204c pptH/*E. coli* proto nalidixic acid were chosen for further tests because they have fairly high average transmissibility and low standard deviation. Although *S.t.* AFF had a higher average transfer rate, it is resistant to fewer antibiotics (table 5.1).

	***S.t.* 204c pptH/*E.c.* proto nal^r**		***E.c.* 2442/*S.t.* 576 nal^r**	
	Mean	**Standard deviation**	**Mean**	**Standard deviation**
Aerobic1	2.85E-11	1.13E-11		
Aerobic2	8.81E-13	8.95E-13	2.63E-11	5.10E-12
Anaerobic1	2.06E-11	2.87E-11	3.21E-11	1.18E-11
Anaerobic2	1.35E-11	9.07E-12	8.94E-11	3.52E-11

Table 5.4. Mean and standard deviation of transfer rate of the four antibiotic resistances (measured using Simonsen's end point method) under aerobic and anaerobic conditions.

The donor *E. coli* 2442/recipient *S. typhimurium* 576 pair was chosen as its transfer rates are highest and least variable both in aerobic and anaerobic environments. To aid further experiments, the transfer rate of resistances to several antibiotics between these strains is given in table 5.5.

	Transfer rates of resistance to			
	neo	amp	strep	tet
Aerobic1				
Aerobic2	3.3E-11	2.5E-11	2.6E-11	2.1E-11
Anaerobic1	3.7E-11	4.6E-11	2.6E-11	2E-11
Anaerobic2	1.3E-10	8.5E-11	1E-10	4.3E-11

Table 5.5. Transfer rates (estimated with Simonsen's end-point method) of resistances to four antibiotics between the chosen donor and recipient strains in aerobic and anaerobic experiments. neo = neomycin, amp = ampicillin, strep = streptomycin, tet = tetracycline .

5.2.3 Summary

The pair *Escherichia coli* F18 2442 as a donor and *Salmonella typhimurium* 576 as a recipient demonstrate transfer of plasmid encoded resistance of four antibiotics consistently well under conditions of O_2 presence or absence. It is important to use all round high transmitters for four reasons:

1. These strains are to be used in animal experiments to assess the dynamics of plasmid transfer within chickens. As such they must be high transmitters to reduce the risk of no transfer occurring.

2. The gut is largely anaerobic, so high transmitting strains under anaerobic conditions may transmit well *in vivo*.
3. Various antibiotics are used in experiments (chapter 9) so strains were chosen that were high transmitters across several antibiotics.
4. Donors/recipients with high transfer rates would indicate a worst case scenario of the spread of resistance in *S. typhimurium*.

An *E. coli* donor and an *S. typhimurium* recipient represent the situation in which susceptible *S. typhimurium* infecting a chicken flock obtain resistance from the background *E. coli* in those birds. This scenario is both increasingly likely as resistance in gut flora increases (chapter 2), and of concern for the treatment of *Salmonella* in humans and chickens.

5.3 Evaluating the intrinsic growth rate of donors, recipients and transconjugants.

Altering the intrinsic growth rate parameter, r_x, of one strain with respect to another can reverse the equilibrium state of the model from resistance to susceptibility and vice-versa. It is essential, therefore, that the intrinsic growth rates of the strains modelled are quantified. The literature on growth rate estimation is reviewed in section 2.3. Growth rate is also one measure of fitness which has been used by other authors to assess the cost of antibiotic resistance (Bjorkman & Andersson 2000) The percent difference in intrinsic growth rate between recipients and transconjugants is one measure of this cost. Factors to be controlled for in a quantification experiment are substrate, temperature, mixing, aeration, and competition from background flora, as they may affect growth rate. Growth was measured in two media: Luria Bertani (LB), the standard laboratory medium, and minimal, as it was thought a more nutrient stressed environment might better reveal intrinsic growth rate differences. Aside from this, the effect of other factors e.g. temperature and aeration were not quantified separately as the model assumes that they are constant. Bacteria were grown alone in universal tubes incubated statically at 37^0C.

5.3.1 Materials and Methods

Four growth curves from each of the three populations (donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576) were obtained in LB medium and three in minimal medium. These were grown in an incubator, the temperature fluctuations within which were found to cause large variation between growth rate estimations. Three further curves from each population were obtained in a water bath. To

assess the cost of plasmid carriage, eight further curves were obtained for recipients and transconjugants from four strains (chapter 10). The data on *S. typhimurium* 576 from that experiment are analysed here. The plasmid transmitted by donors encoded resistance to ampicillin, neomycin, streptomycin and sulphonamide. The minimal medium was made up from equal parts of IN9 and distilled water with glucose added at 0.5%. IN9 contains the following substances in distilled water: K_2HPO_4: 0.0402 M, KH_2PO_4: 0.0220 M, NaCl 0.0854 M, ammonium sulphate: 0.0076 M, Magnesium Sulphate $7H_2O$: 0.00027 M. Overnight shaking cultures of donors, recipients and transconjugants were diluted into the media to an approximate density of 10,000 cells ml^{-1}, swirled for 30 seconds and 10ml measured into several universal tubes. These were placed into a static incubator or water bath at 37^0C and sampled at intervals (see table 5.6). The tubes were mixed before sampling by drawing the broth into and then expelling from a pipette several times. LB media tubes were serially diluted tenfold and counted on MacConkey plates. The cell density in minimal media tubes was estimated using a spectrophotometer using light at 600nm and calibrated with three manual counts for each strain. A spectrophotometer was used to measure density of cultures in minimal media as the 'time window' between the point where optical density becomes detectable and stationary phase is approached is more lengthy when minimal medium is used as growth is slower. Samples taken between one and five hours after inoculation were used to calculate intrinsic growth rate as the bacteria were clearly growing exponentially during this period (i.e. the plot of the logarithm of density is linear over this time). Cultures in minimal media were sampled every 30 minutes from time of inoculum, but the first two hours could not be recorded because density was too low to measure by light absorption.

Experiment	No.	Sampling times (hrs)	Medium	Repeat	Incubation	Density measurement
Growth rate quantification for modelling	1	every 30 mins for 6 hours	LB	1	Static incubator	Plate counts
		"	"	2	Static incubator	Plate counts
		"	"	3	Water bath	Plate counts
		"	"	4	Water bath	Plate counts
		"	Minimal	1, 2, 3	Water bath	Spectrophotometer
	2	1 2 3 4 5 6 26 30	LB	1, 2, 3	Water bath	Plate counts
Cost of plasmid carriage	3	1 3 5	"	1-8	Water bath	Plate counts

Table 5.6: Conditions under which bacteria were grown and measured varied between the three experiments. Growth was carried out in a water bath after the first two repeats of the first experiment because it was discovered that the varying temperature of the incubator affected the measurement of growth rate.

Statistical Analysis

The repeated measurements over time complicate the analysis of growth rate. This was overcome by calculating the growth rate (the slope of the regression of Ln density) of each repeat-strain combination separately, and using the GLM command in Minitab to test whether the values were different (Minitab session 5.1). This eliminates the need to include observations on the same populations at different time points.

Experiment 1 was performed in an incubator rather than a water bath, and its day to day variation (standard error) is much greater than that of the experiment 2 (figure 5.5). For this reason it was excluded from the analysis determining intrinsic growth rate

differences between recipients and transconjugants. It was included in the analysis of donors, however, because its exclusion would only leave three donor datapoints.

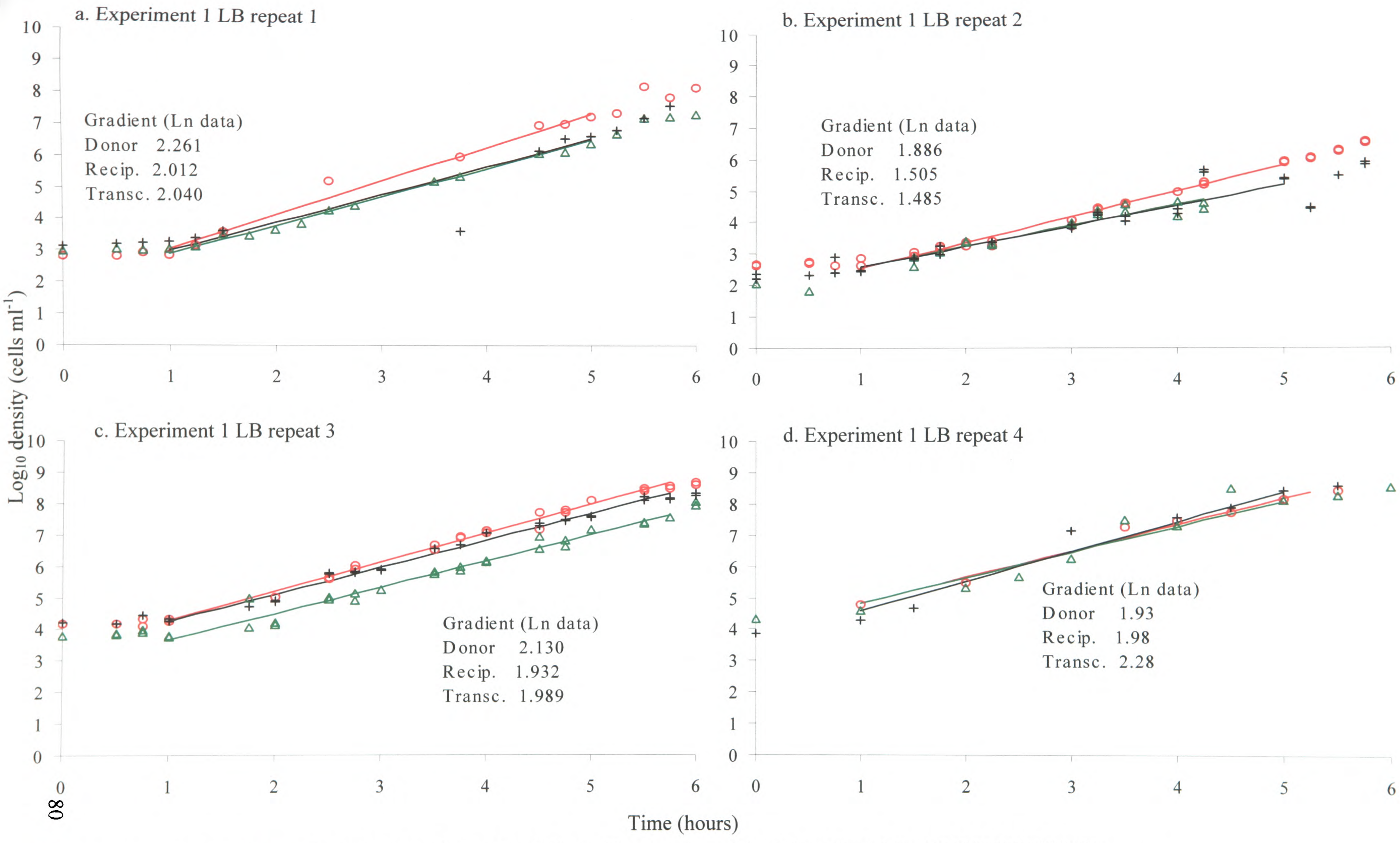
The analyses were performed to answer two questions: (i) Does *E. coli* 2442 have a different intrinsic growth rate to *S. typhimurium* 576? and (ii) Does the intrinsic growth rate of the recipient differ from that of the transconjugant? This was tested by carrying out three comparisons: Test 1, were there any differences at all between the three strains?; Test 2, did *E. coli* have a different intrinsic growth rate to *S. typhimurium*?; and Test 3, was the recipient intrinsic growth rate different to that of the transconjugant?

5.3.2 Results

The growth curves in LB are presented in figure 5.1 and 5.3; and those in minimal media in figure 5.2. A summary figure of the growth rates obtained is given in figure 5.4.

Further growth curves of recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576 are provided in chapter 10 (figure 10.1a) for the experiment comparing intrinsic growth rates of several different strains.

The intrinsic growth rates in LB are given in table 5.7.



○ –· Donor *E. coli* 2442 △ –· Recipient *S. typhimurium* 576 + – Transconjugant *S. typhimurium* 576 Lines are regressions.

Figure 5.1. Growth of donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576 in LB broth at 37^{0}C in a static incubator

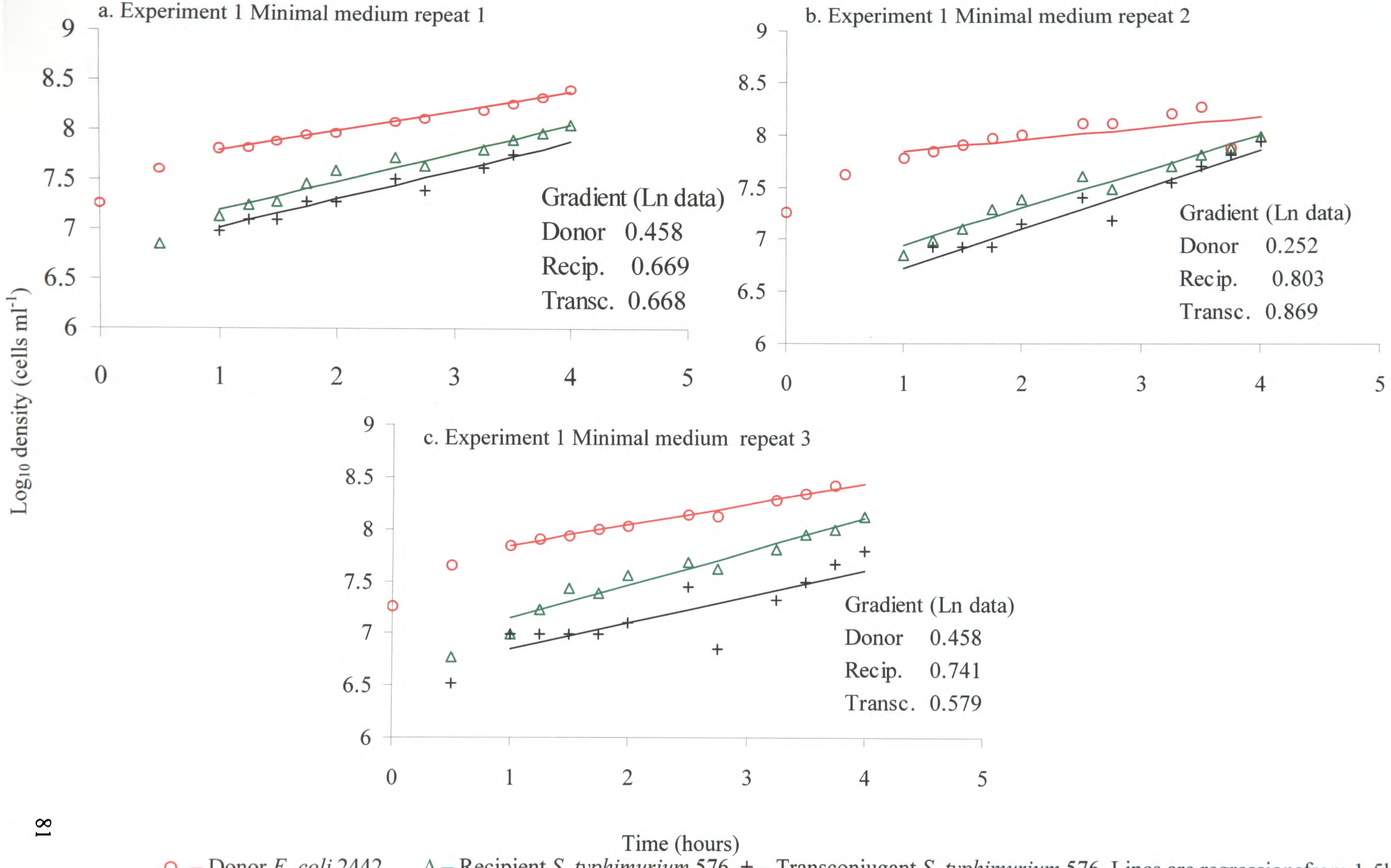
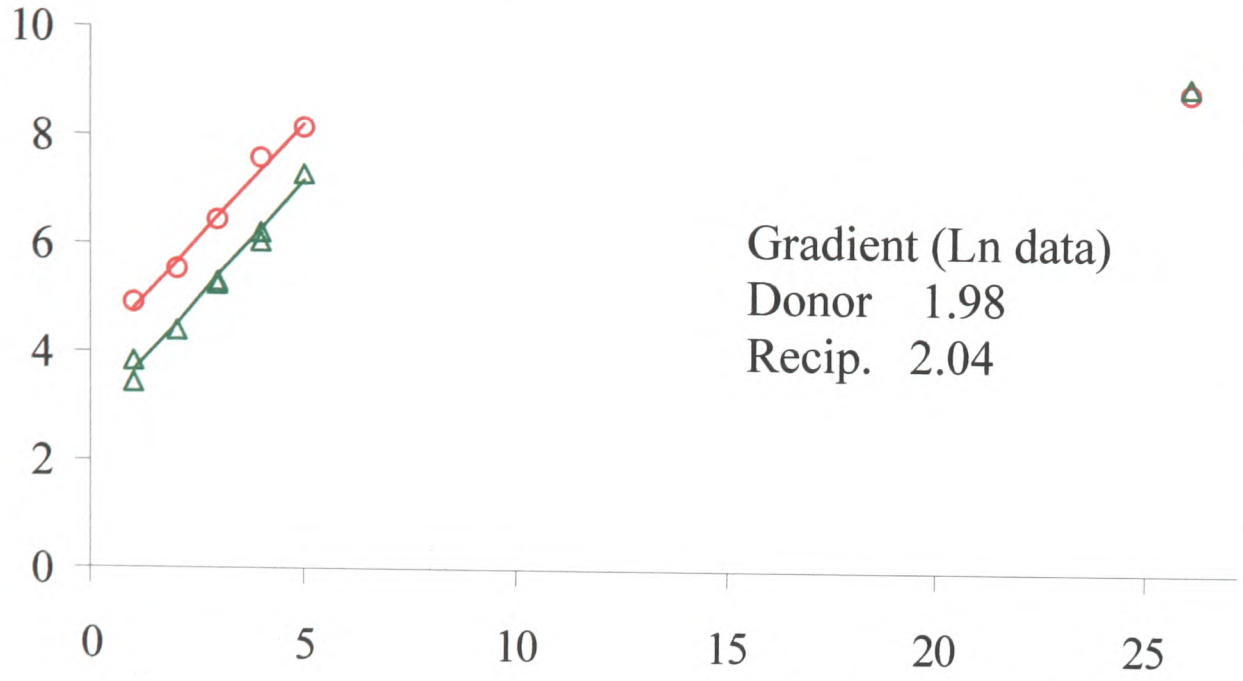
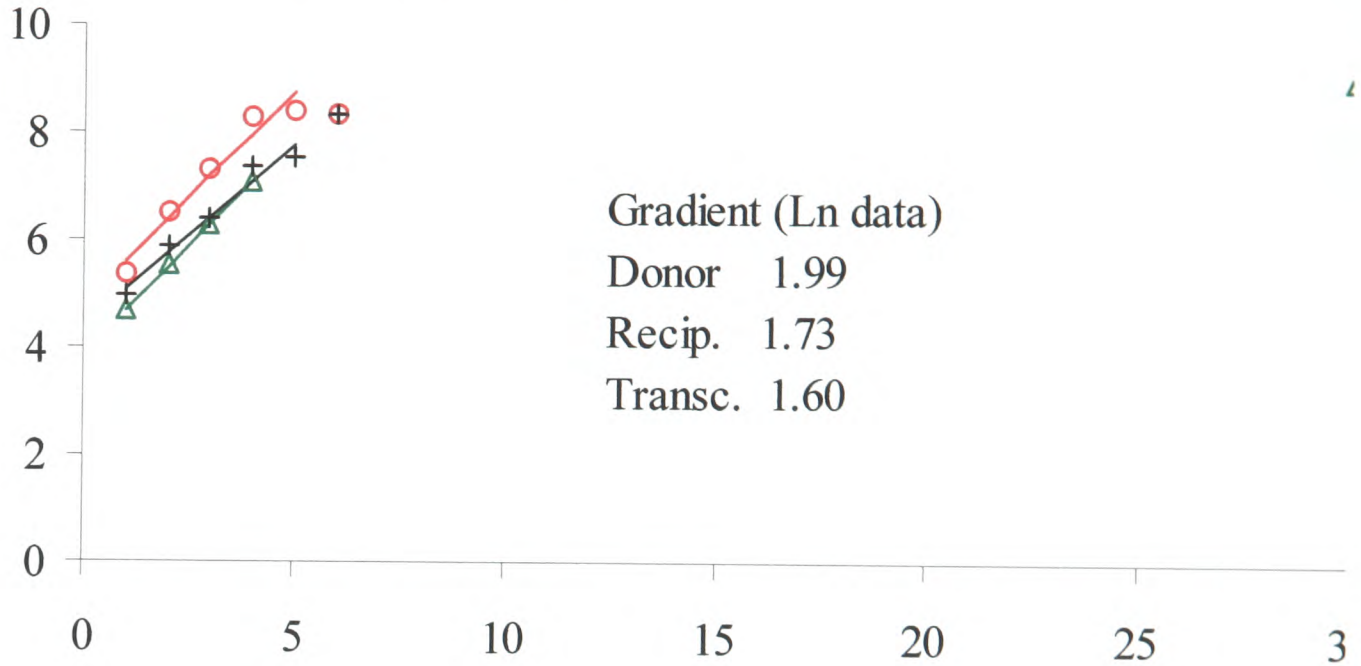


Figure 5.2 Growth of donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576 in LB at 37^0C in a waterbath.

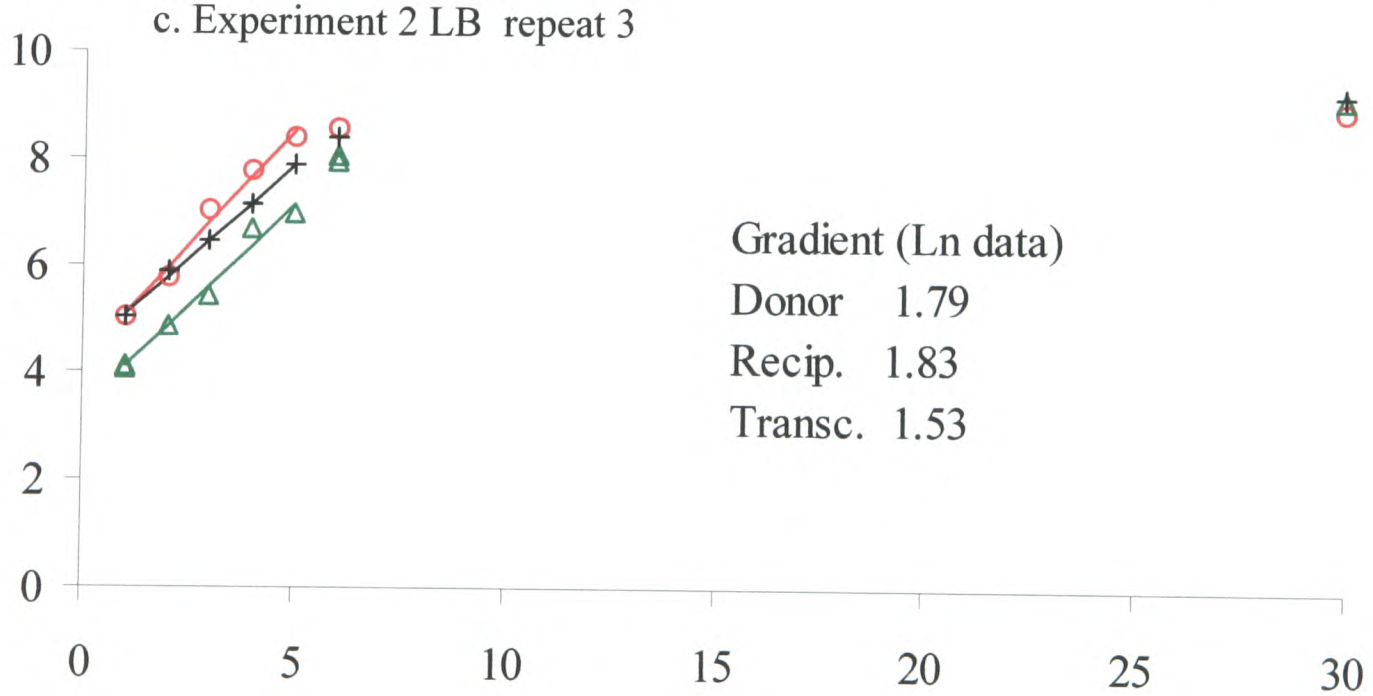
a. Experiment 2 LB repeat 1



b. Experiment 2 LB repeat 2



c. Experiment 2 LB repeat 3



○ – Donor *E. coli* 2442 Δ – Recipient *S. typhimurium* 576 + – Transconjugant *S. typhimurium* 576 Lines are regressions.

Figure 5.3 Growth of donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576 in minimal medium at 37^0C in a static incubator.

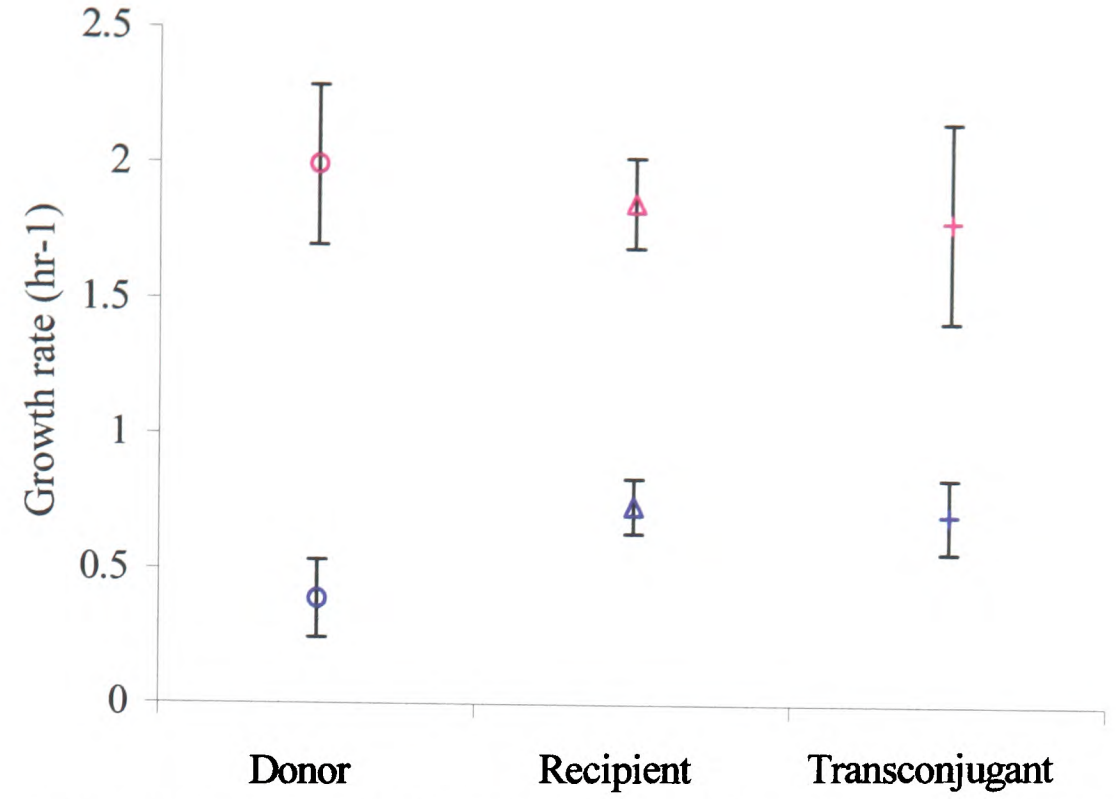


Figure 5.4. Summary of the estimations of growth rates of donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576 from experiment 1 (figures 5.1 and 5.2). Pink symbols indicate strains grown in LB broth, and blue symbols indicate strains grown in minimal medium. Confidence intervals are ∓ 4.3*standard error for minimal medium (df = 2) and ∓ 2.4*standard error for LB (df = 6)

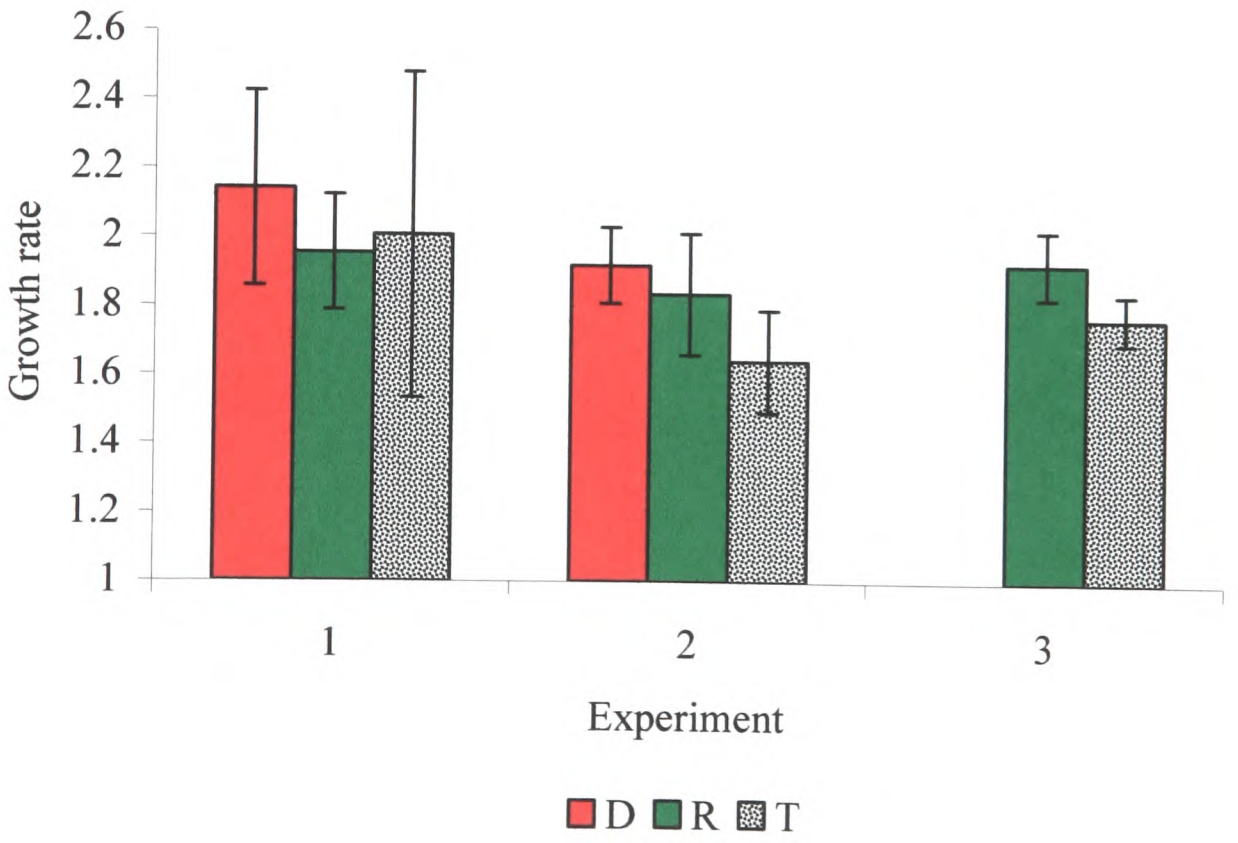


Figure 5.5. Intrinsic growth rate in LB medium of donor *E. coli* 2442 (D), recipient *S. typhimurium* 576 (R) and transconjugant *S. typhimurium* 576 (T) measured during three experiments. Confidence intervals are ∓ 3.2*standard error for experiment 1 (df = 3), ∓ 4.3*standard error for experiment 2 (df = 2) and ∓ 2.4*standard error for experiment 3 (df = 7)

Experiment	Day	Donor *E. coli* 2442	Recipient *S. typhimurium* 576	Transconjugant *S. typhimurium* 576
1	1	2.52	2.02	1.83
1	2	1.85	1.50	1.75
1	3	2.19	1.97	2.04
1	4	2.01	2.33	2.42
2	5	1.98	2.04	*
2	6	1.99	1.73	1.60
2	7	1.79	1.83	1.53
3	8	*	1.76	1.80
3	9	*	1.77	1.72
3	10	*	2.06	1.84
3	11	*	2.11	1.71
3	12	*	1.84	1.76
3	13	*	1.80	1.88
3	14	*	1.82	1.87
3	15	*	1.75	1.60
Average		**2.05**	**1.89**	**1.81**
Doubling time (mins)		**20.29**	**22.00**	**22.98**

Table 5.7. All intrinsic growth rates ($hour^{-1}$) estimated for the three populations, the average of these growth rates and a doubling time calculated from this average. *experiment not performed.

Figure 5.1 confirms that these strains are growing at their intrinsic growth rate between 1-5h because the logged data increase linearly over this time. The variability between intrinsic growth rate in LB measured in each experiment is shown in figure 5.5. The first experiment gave highly variable growth rate estimations. Eventually a large amount of data on growth rates of recipients and transconjugants were available (experiment 3 in table 5.6 and figure 10.1 in chapter 10). Only the data on donor growth rates from experiment 1 was used.

The analysis (see Minitab session 5.3 at the end of this section) showed no overall effect of population on intrinsic growth rate, but that donor *E. coli* 2442 grew significantly faster than the total *S. typhimurium* 576, and that recipient *S. typhimurium* 576 grew significantly faster than transconjugant *S. typhimurium* 576. The cost of resistance is calculated as $(1- r_T/r_R)$ and is 4.15%.

5.3.3 Summary

The cost of resistance in *S. typhimurium* 576 is only 4%. This is small when compared to the costs of resistance found by Schrag & Perrot (1996) (see literature review section 2.3.3), so it was decided to explore the cost of resistance plasmid when carried by other bacteria (chapter 10).

Estimation of bacterial density by spectrophotometer was found to be insufficient to measure intrinsic growth rate in minimal medium (figure 5.2). Growth rates before 1h of growth are greater than growth rates after. This may indicate that bacteria were on the approach to stationary phase after 1h of growth. The bacteria were not in lag phase before measurement started, as they were sampled for 2h before density reached recordable levels. These data cannot therefore provide estimates of intrinsic growth rate in minimal medium.

The large variability in intrinsic growth rate in LB in the first four repeats of experiment 1 (figure 5.5) indicates that an incubator does not have a constant enough temperature to provide reliable estimates of intrinsic growth rate at 37^0C.

When the donor *E. coli* 2442 intrinsic growth rates are compared with the published *E. coli* growth rates in LB (table 2.2) they are found to be slightly higher than average.

5.4 Estimating K values

The carrying capacity K, the maximum density a bacterial population can achieve in a given set of conditions, is assumed to be constant in the models studied here. Good model predictions, and estimates of the competition coefficients α_{SE} and α_{ES} (section 5.5) are highly dependent on a good measurement of the carrying capacity. K values were estimated in two sets of lab *in vitro* conditions, LB broth in normal conditions, and caecal content taken from birds and inoculated with laboratory bacteria. A full set of parameter values has only been estimated for LB medium, so these are the data used to provide estimates for modelling. Caecal content estimations were carried out to provide insight into the potential infection levels that may be expected *in vivo*.

5.4.1 Methods

LB broth

Diluted overnight incubation of donor *E. coli* 2442 or recipient *S. typhimurium* 576 without the resistance plasmid were inoculated into 10 ml LB to create a starting density of approximately 10^4 ml^{-1}. These were then incubated overnight in a shaking incubator at 37^0C and diluted, plated and counted as detailed in section 3.5.

Caecal content

Weighed caecal content samples taken from uninfected birds at PM were mixed with volumes of *E. coli* 2442 or *S. typhimurium* 576 dilutions to give a starting density of approximately 10^4 ml^{-1}. These samples were then statically incubated for 12 hours and the densities of the populations were estimated by dilution and plating on antibiotic containing media (section 3.5).

The model described above (section 4.6) explicitly models competition from the lab populations of interest, but avoids incorporating the contributions of diverse gut flora to growth and maximum density suppression *in vivo*. Estimations of K in LB are therefore made in the absence of competition, and in caecal content without any attempt at sterilisation.

5.4.2 Results

Estimation of carrying capacity are given in table 5.8

Medium	Strain	Mean	Standard deviation	N
LB	*E. coli* 2442	1.24E+09	5.50E+08	67
LB	*S. typhimurium* 576 nalr	1.04E+09	4.38E+08	66
Caecal content	*E. coli* 2442	1.03E+07	8.28E+06	3
Caecal content	*S. typhimurium* 576 nalr	8.80E+06	5.22E+06	3

Table 5.8 Mean and standard deviation of carrying capacity estimates of *S. typhimurium* 576 and *E. coli* 2442 in 10 ml of LB broth or caecal content medium. N = number of replicates.

5.4.3 Summary

The variation between estimates of carrying capacity in LB is not large, which is reassuring as an accurate estimate is required for modelling.

Many fewer replicate counts were carried out to estimate carrying capacity in caecal content but still the standard deviation of these estimates is smaller than their mean. A lower density of bacteria were found in caecal content, which is to be expected due to

the presence of competing bacteria. It is not known, however, how long these bacteria take in caecal content to reach stationary phase, so the caecal content estimates would strictly be best thought of as 12 hour densities rather than carrying capacities.

5.5 Estimating the degree of competition between *S. typhimurium* and *E. coli*

Bacteria of the same strain in the same locality compete with each other for resources and this results in the reduction of growth at high densities until the density reaches a maximum carrying capacity, K if m=0.

Individuals of different strains/species may also compete with each other for space, nutrients, oxygen etc, and they may affect each other's growth adversely. The reduction in growth of one strain may be caused by restricted access to resources, accumulation of toxic biproducts or the release of antimicrobials by the other strain, the latter an insignificant process in intrastrain interactions. The more similar the niches of the competing strains, the fiercer the competition.

The two classes of competition model are exemplified by the Lotka-Volterra competition model (described in Begon et al 1996), and the Tilman resource competition model (Tilman 1982). This work on competition does not consider resource competition and uses the Lotka-Volterra model. The Lotka-Volterra competition model is based on the Logistic. If the carrying capacity is K, and the density of organisms N, the simplest form of the logistic model states that growth will decrease linearly with population size and cease when N = K. For example, in a bacterial mating mixture (i.e. a co-culture of donors and recipients with the transconjugants they produce) where within-strain competition is equal to between-strain competition, growth will cease when D + R + T = K. Conversely, if different strains occupy non-overlapping niches within the habitat (e.g. because they utilise completely different nutrients), there would be no competition between strains. In the case of a mating mixture where the donor and recipient occupy such non-overlapping niches, growth of donors would cease when $D = K_D$ and growth of recipients and

transconjugants would cease when $R + T = K_R$ as recipients and transconjugants are always the same strain. If the amount of between-strain competition is somewhere in-between these extremes, extra parameters are required. In the Lotka-Volterra competition model, competition coefficients are added so that an equilibrium of, for example, donors, is reached when $D + \alpha_{SE}(R + T) = K_D$. The evidence suggests that such an intermediate level of competition is often experienced by competing bacterial strains in nature (e.g. Iba et al 1995). If there are several species present in the habitat, enumeration of the densities and competition coefficients of all of them would be required (equilibrium reached when $K_A = A + \alpha_{BA}B + \alpha_{CA}C\ldots$). As there have been 400-500 species of bacteria identified as living in the gut (Reid et al 2001) competing strains should only be included if the model fit is not good enough without them.

This section will examine competitive interactions between *Salmonella* and *E. coli* and determine whether the Lotka-Volterra competition model is good enough to describe them.

5.5.1 Materials and Methods

Bacterial Strains

I used the following strains in the competition experiments: *E. coli* 2442, *S. typhimurium* 576 Nal^r , *S. typhimurium* F98 Nal^r and *S. typhimurium* LB5010 Nal^r. The data provided by P.A. Barrow, published in Barrow et al (1996), used the strains *S. typhimurium* F98 nal^r and *E. coli* P4. Strains used by Bercherei are listed in table 5.9.

Datasets

Three different data sets were analysed:

1. Data collected by P. A Barrow (Barrow et al 1996) of *S. typhimurium* F98 growing in stationary phase *E. coli* P4 and vice-versa
2. Equivalent data on *E. coli* 2442 and *S. typhimurium* 576;
3. 0 and 24hr counts of various *Salmonella* strains growing in stationary phase *E. coli* and vice-versa collected by Bercherei (unpublished data)

Experimental design

The experiments aim to estimate the retardation of growth of one genus by another. A 10 ml overnight culture (approximate density 10^9 ml^{-1}) of one genus was prepared and to it was added 0.1 ml of a diluted culture at a density of 10^5 ml^{-1}.This mixed culture was allowed to grow in a static waterbath at 37^0C for 50 hours. Population densities were estimated at regular time intervals using dilution and plating (see section 3.5).

Nomenclature

To differentiate between the strain at stationary phase and the strain diluted into the strain at stationary phase, they are called the established strain and the introduced strain respectively.

Models

Estimation of α_{SE} and α_{ES} from datasets 1 and 2 was achieved in ModelMaker4 and by analysis of phase diagrams. The equations used to model datasets 1 and 2 were:

$$\frac{dE}{dT} = r_E E \cdot \frac{K_E - E - \alpha_{ES} S}{K_E} \qquad 5.1$$

$$\frac{dS}{dT} = r_S S \cdot \frac{K_S - S - \alpha_{SE} E}{K_S} \qquad 5.2$$

E = density of *E. coli*; S = density of *Salmonella*; r_X = growth rate of E or S; K_X = carrying capacity of E or S; α_{ES} = repression of *E. coli* by *S. typhimurium*; α_{SE} = repression of *S. typhimurium* by *E. coli*.

The "optimise" function in ModelMaker 4 was used to calculate α_{SE} and α_{ES} from datasets 1 and 2 (Modelkinetix 2000). The Marquardt procedure was used. For dataset 1, K_S and r_S were estimated from the first 28 hours of data in figure 5.6 (a). K_E and r_E were estimated from the first 28 hours of data in figure 5.6 (b). For dataset 2, K_E and r_E were estimated from the data shown in figure 5.3. r_S for the three strains was estimated from data shown in figure 10.1 (a), (b) and (d). K_S was estimated from 26-hour counts of the strains (data not shown).

	Dataset 1	Dataset 2		
***E. coli* strain**	P4	2442	2442	2442
***S. typhimurium* strain**	F98	576	F98	LB5010
K_E	2.29×10^9	1.24×10^9	1.24×10^9	1.24×10^9
K_S	4.71×10^9	1.04×10^9	1.40×10^9	1.42×10^9
r_E	1.81	2.05	2.05	2.05
r_S	1.72	1.89	1.81	1.74
s_E	1500	500	1100	400
s_S	1995	10400000	4113000	3100

Table 5.9. Parameter values used to estimate α_{SE} and α_{ES} by optimisation of the model to data. Values were estimated from data on strains growing in the absence of competition. Parameters are as in equations 5.1-5.2 except s_E and s_S which are the inoculum densities of *E. coli* and *S. typhimurium* respectively when they were the introduced strain.

To fit the model (equations 5.1 and 5.2) to the data (datasets 1 and 2) the following iterative process was used. The objective was to find values of α_{SE} and α_{ES} that would, along with the parameter values in table 5.9, provide the best agreement between model predictions and data. For each *E. coli–S. typhimurium* pair there were two sets of data, *S. typhimurium* growing in stationary phase *E. coli* (most informative about α_{SE}) and *E. coli* growing in stationary phase *S. typhimurium* (most informative about α_{ES}). At the start of the model run the density of the established culture was set at its carrying capacity, and that of the introduced culture was set at the s value (table 5.9). The procedure was to first guess a value for α_{SE} from the data on *S. typhimurium* growing in stationary phase *E. coli* and a value for α_{ES} from the data on *E. coli* growing in stationary phase *S. typhimurium*. The "optimise" function was then run on α_{ES} to obtain the best fit to the data on *S. typhimurium* growing in stationary phase *E. coli*. That estimated value of α_{SE} was then used in the optimisation of α_{ES} to obtain the best fit to the data on *E. coli* growing in stationary phase *S. typhimurium*. This process was reiterated until the values did not change between estimations. Modelmaker gives the goodness of fit parameter r^2, which is an estimate of the proportion of variation in the data which is explained by the model. These values were recorded (table 5.10).

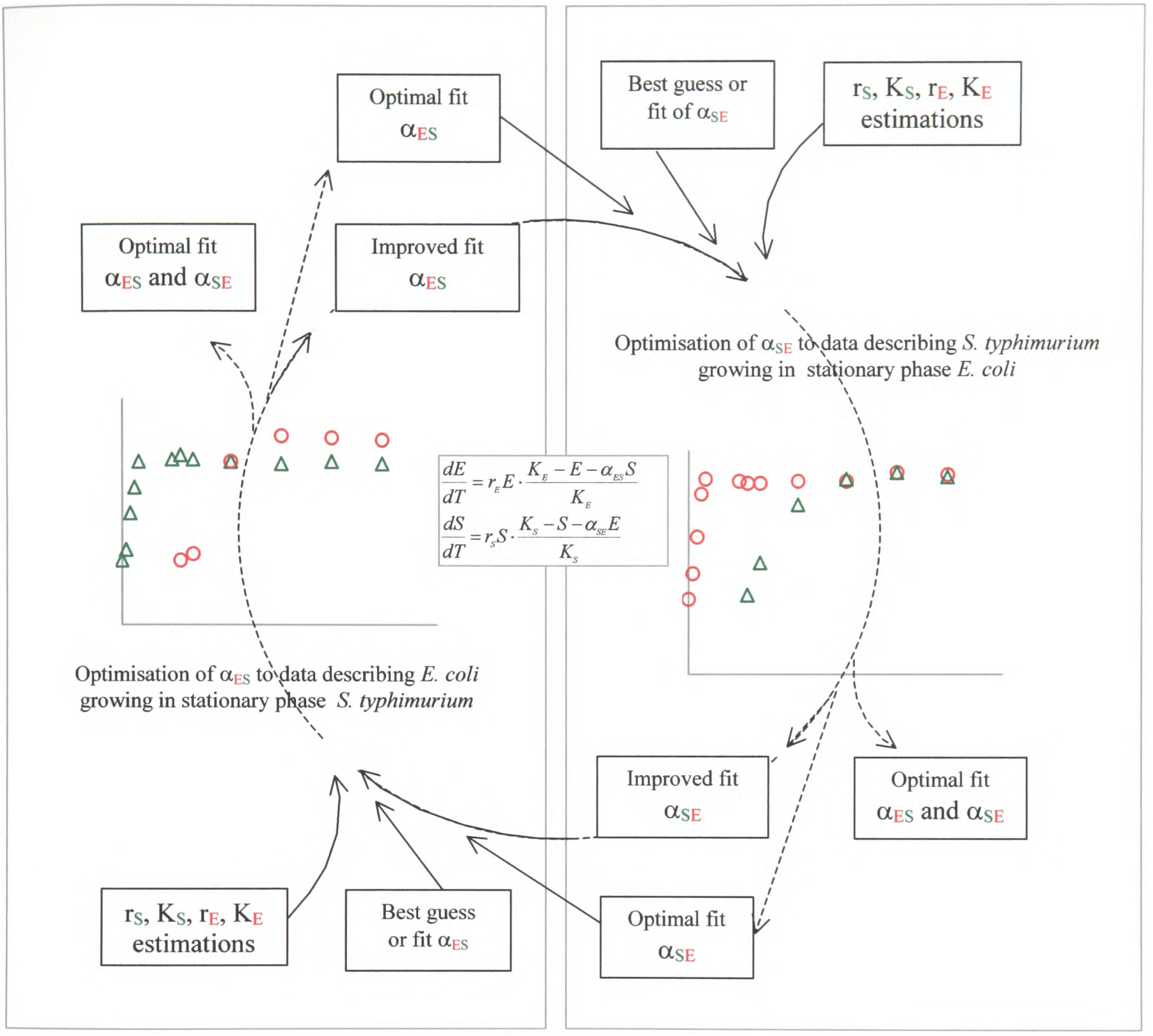


Figure 5.6. Method of calculating α_{SE} and α_{ES}. Values for α_{SE} and α_{ES} were guessed at, then the model incorporating these parameter values was optimised to the most appropriate data set alternately to until further optimisation failed to improve the fit of the model to the data (i.e. r^2 to 2dp was not altered).

According to theory (Begon et al. 1996), if the data conform to the Lotka-Volterra model, competition coefficients may be estimated from the phase diagrams. If the density of one population is plotted against the density of the competing population, at most points on the diagram each population is either growing or declining. For each

population, an isocline can be drawn where it is neither increasing nor decreasing. At low densities of population 1, the density at which population 2 does not change is high. Conversely, where the density of population 2 is high, the point of no change of population 1 is low. If the isoclines of each population cross, this may be a point of stable coexistence, where neither population increases nor decreases. Alternatively, it may be an unstable equilibrium (see Begon et al (1996) figure 4.25). If a stable equilibrium exists, it is only at this point. Carrying capacities can be estimated because the line through the carrying capacity of species 1 (K_1, 0) and the point of coexistence crosses the other axis at K_1/α_{12} and vice-versa.

Points of stable coexistence of each *E. coli* – *S. typhimurium* combination were plotted on phase diagrams to estimate α_{ES} and α_{SE} this way.

Dataset 3 (0 and 24 hour counts of competing populations) was also modelled using the Lotka-Volterra model. α_{SE} and α_{ES} were estimated using the following method. It was assumed that from 0-24 hours after inoculation, the density of the introduced strain is low such that if *E. coli* is the introduced strain, K_E-$E \approx K_E$ i.e. that only competition with the established strain causes retardation of growth of the introduced strain. The exponential rate of increase of the model of the introduced strain therefore does not change during this time. If the populations exhibit a lag phase or begin to be affected by intraspecific competition towards the end of the 24 hours α will be overestimated. A typical lag and degree of intraspecific competition would result in an overestimation of 4%. Furthermore it is assumed that the density of the established population equals the K-value of that population and does not change over the first 24 hours i.e. the density of the established strain is not adversely affected by the competition with the introduced strain. These assumptions appear to hold for the data

on strains from which several measurements were taken over time (figures 5.6-5.7). If these assumptions are made α_{SE} and α_{ES} can be estimated as follows:

$$\alpha_{SE} = \frac{K_S(r_S - G)}{r_S K_E} \qquad 5.3$$

$$\alpha_{ES} = \frac{K_E(r_E - G)}{r_E K_S} \qquad 5.4$$

where G is the gradient of the natural logarithm of introduced strain density with time i.e. (ln(24hr count)-ln(0hr count))/24. K values are known because the overnight cultures of the strains were diluted 10,000-fold before inoculation and immediately counted, and the r values are taken from the average r values of *Salmonella* and *E. coli* found in the literature review (chapter 2) and experimentally in section 5.3 and 10.3.

5.5.2 Results

The growth of *S. typhimurium* in stationary phase cultures of *E. coli* and of *E. coli* in *S. typhimurium* (datasets 1 and 2) is illustrated in figures 5.6 and 5.7 with the fits of the Lotka-Volterra model. The results of the optimisation of α_{SE} and α_{ES} for the Lotka-Volterra model are displayed in table 5.10, with the goodness of fit parameter r^2. The calculation of α_{SE} and α_{ES} from dataset 3 are shown in table 5.11.

***E. coli* strain**	***S. typhimurium* strain**	α_{ES}	r^2	α_{SE}	r^2
P4	F98	0.90	-2.23	0.38	0.86
2442	576	1.08	0.51	0.82	0.29
2442	F98	0.69	0.81	0.81	0.35
2442	LB5010	0.78	0.54	1.00	0.73

Table 5.10. Results of optimisation of α_{ES} and α_{SE} on time course data and the goodness of fit parameter r^2 (datasets 1 and 2).

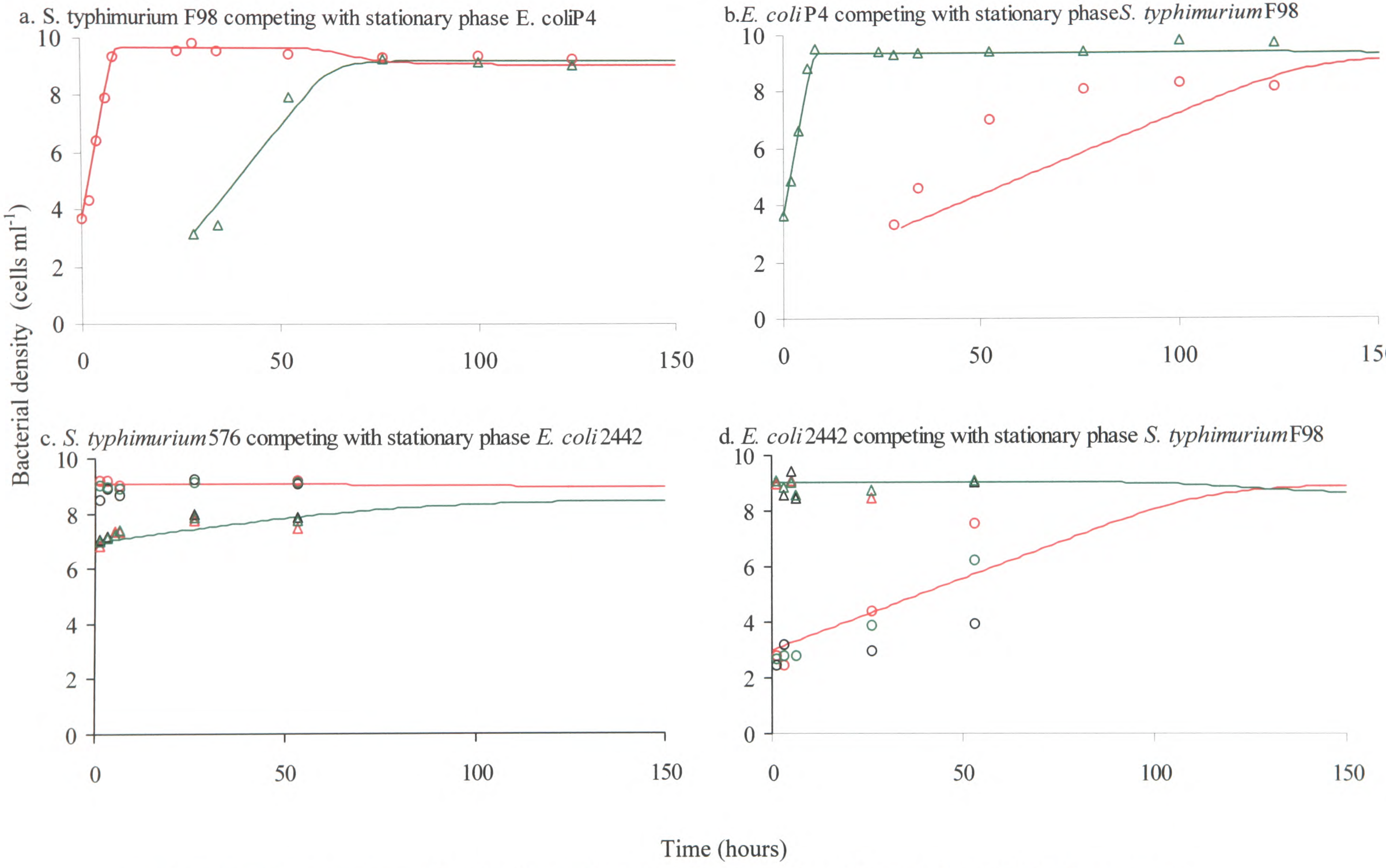
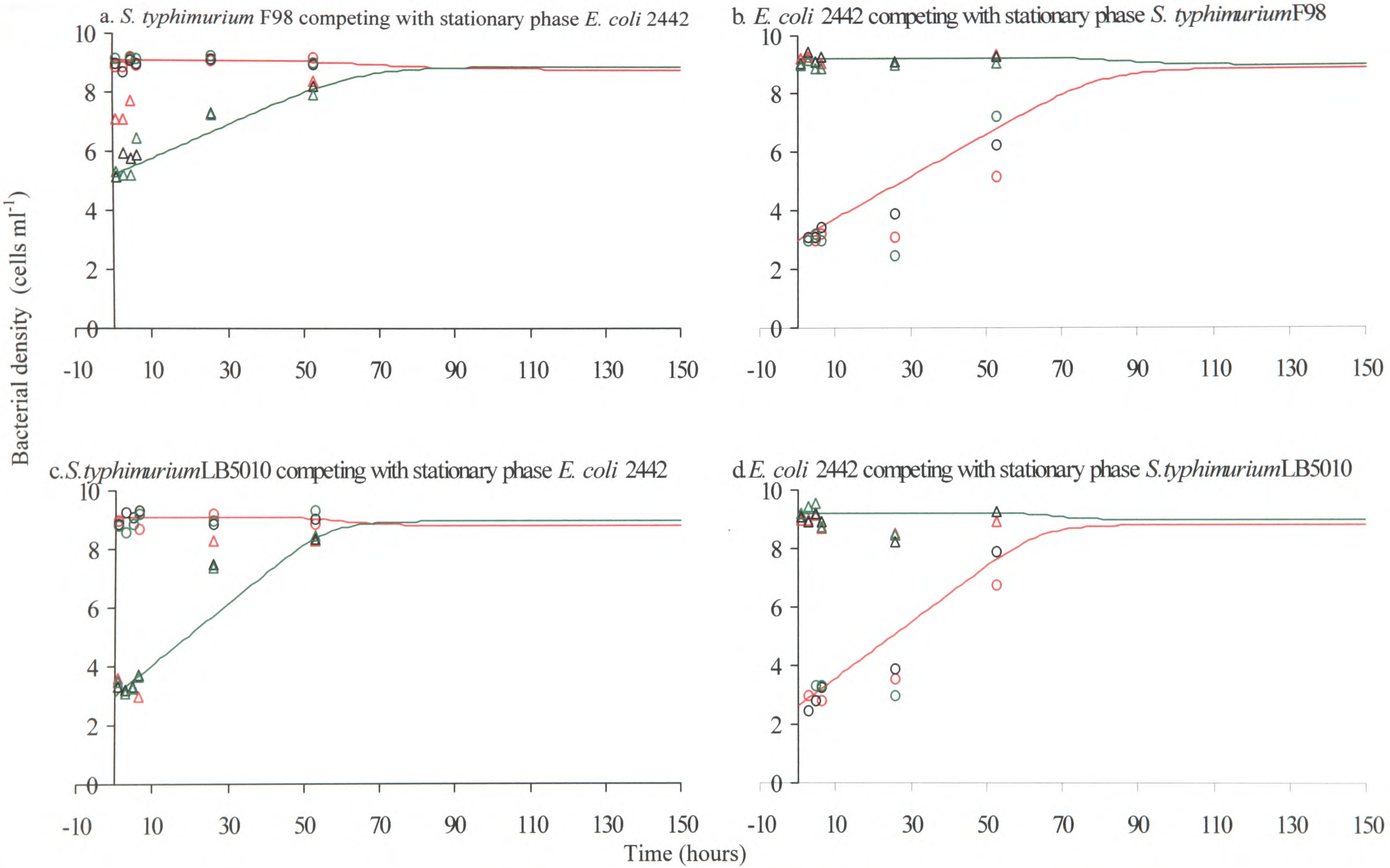


Figure 5.7. Data and model fits on *E. coli* growing in stationary phase *S. typhimurium* and vice versa. Fits are to Lotka-Volterra model.(a) and (b) are from dataset 1, from Barrow et al (1996). (c) and (d) are one pair from dataset 2.



○ –*E. coli* Δ –*S. typhimurium* Lines are the result of model optimisation. Colours of symbols indicate repeats.

Figure 5.8. Data and model fits on *E. coli* 2442 growing in stationary phase *S. typhimurium* of two strains and vice versa. Fits are to Lotka-Volterra model. Data is from two pairs belonging to dataset 2.

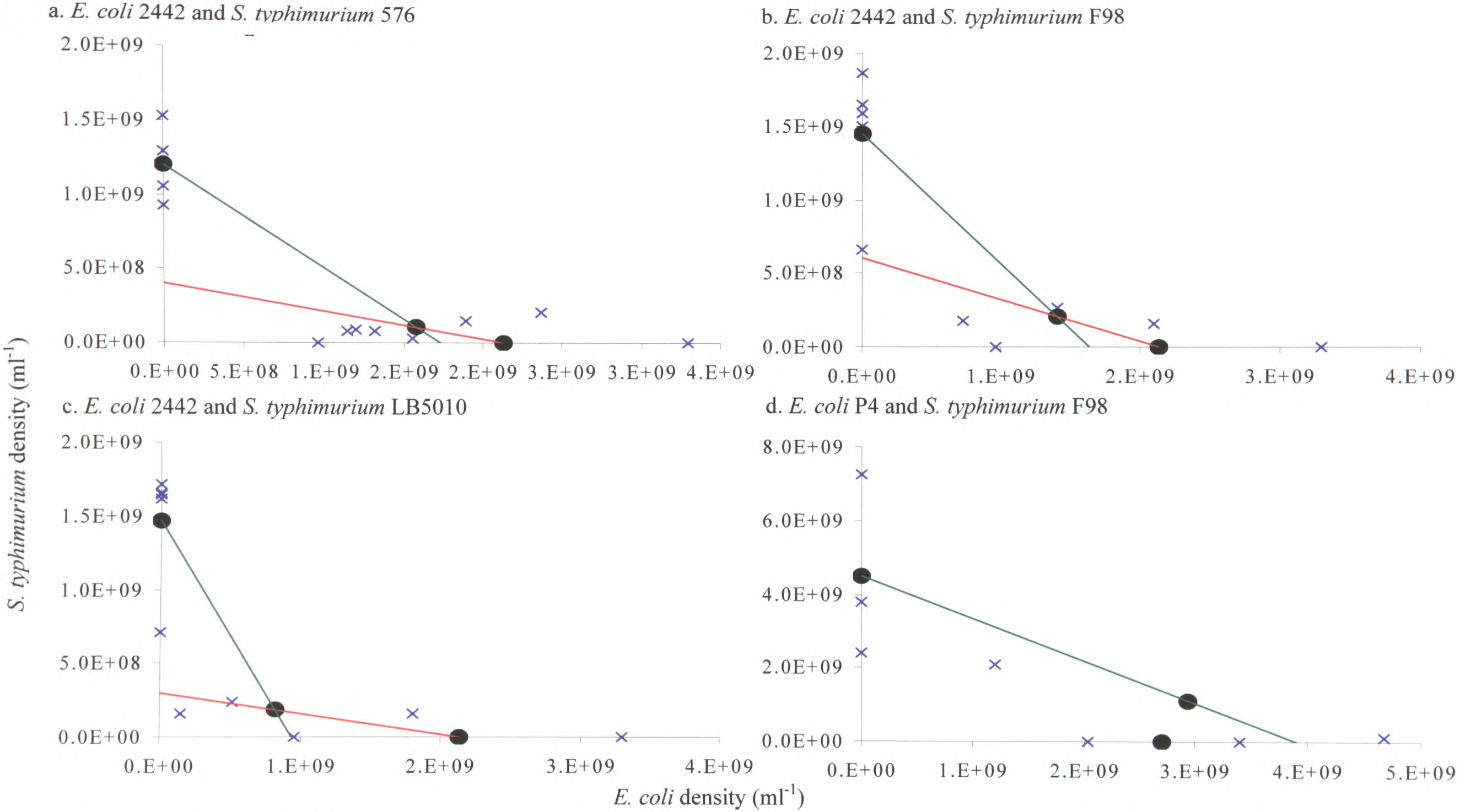


Figure 5.9. Competition maps of *E. coli* and *S. typhimurium* of different strains. The data points on the axes are the carrying capacities of the strains; other points are equilibrium densities at stable coexistence taken from data shown in figures 5.5-5.7.Where the average of the non-trivial equilibrium points is les than the carrying capacity this has been taken as the point of stable coexistence and Lotka-Volterra isoclines have been drawn.

Salmonella strain	Log 10 count of competing strain				α_{ES}	α_{SE}
	0hr (S)	24hr (S)	0hr (E)	24hr (E)		
S. heidelburg 17705	4.30	8.85	4.00	7.14	1.54	0.43
S. infantis 1326/28	4.45	8.83	4.04	7.28	2.00	0.33
S. typhimuiurm F98	4.18	5.70	4.04	8.51	1.20	0.57
S. enteritidis KMS	4.26	7.32	4.00	7.26	1.54	0.46
S. montevideo KMS	4.45	7.45	4.48	8.04	0.79	0.89
S. hadar butler	4.08	8.2	4.11	7.23	0.74	0.91
S. verchow 1337/54	4.38	8.43	3.92	7.08	2.29	0.29
S. kentucky 10935	4.20	8.04	4.04	7.53	1.17	0.58
				Mean	**1.41**	**0.56**
				Std dev	**0.55**	**0.23**

Table 5.11. Calculating competition coefficients from 0 and 24 hour counts of *E. coli* K12 in various *Salmonella* strains and vice-versa.

Results in table 5.11 were interrogated to address the question 'is the impact of *Salmonella* on *E. coli* greater than the impact of *E. coli* on *Salmonella*? ($\alpha_{ES} > \alpha_{SE}$)

A paired, one tailed TTest (see below) was carried out on the eight *Salmonella* strains grown with *E. coli* K12 proto.

```
MTB > TTest 0.0 C3;
SUBC>   Alternative 1.

T-Test of the Mean
Test of mu = 0.000 vs mu > 0.000

Variable      N       Mean     StDev    SE Mean          T
P
C3            8      0.850     0.773      0.273       3.11
0.0085
```

A significant difference was observed. ($P < 0.01$).

Results of model optimisation

Of the 8 estimations achieved by optimisation (table 5.10), only three provided a reasonable fit to data ($r^2 > 0.7$). At the worst fit, the optimisation procedure failed to give any sensible result (dataset 1, figure 5.7 (b) $r^2 = -2.23$, a nonsense figure). The reason for this can be seen from the visual fits of the model to the data (figures 5.7-5.9). As the equilibrium density of the strains in dataset 1 (figure 5.7 (a) and (b)) depended on which strain was present first, a compromise between the optimation of α_{ES} and α_{SE} could not be achieved. The model of the growth of one or other of the competing populations was always wildly inaccurate. For *S. typhimurium* growing in stationary phase *E. coli* 2442, if the optimised equilibrium level appeared to fit the data the growth rate was too low (figure 5.8 (a) and (c)). The growth of *E. coli* 2442 in *S. typhimuirum* appears to be in lag phase during the first 26 hours (figure 5.8 (b) and (d)), which cannot be described by the Lotka-Volterra model.

Results from phase diagrams

If the Lotka-Volterra model is appropriate, the points of stable coexistence of *E. coli* and *Salmonella* should lie above a line connecting the two carrying capacities (K_E, 0 and K_S, 0), (Begon et al 1996). Figure 5.9 (a), (b) & (c) show that this is not the case for *E. coli* 2442 competing with these three *S. typhimurium* strains. The average point of stable coexistence falls below this imaginary line. Figure 5.9 (d) shows a point of equilibrium at which the *E. coli* P4 density exceeds that of its carrying capacity. If this was correct for this strain combination, it might be concluded that they co-operate. It is more likely to be due to lack of data; only two estimates of carrying capacity and equilibrium densities were obtained for this strain combination. The large variability between the densities at equilibrium is also noteworthy; it suggests that there may be

more than one point of equilibrium. This may be due in part to an effect of precedence of the established strain.

The Lotka-Volterra competition model was created to describe competition between animals. One species can reduce the density or numbers of another, either directly by predation or aggression, or indirectly by consuming nutrients more efficiently than the other species that both need to maintain themselves. These processes are known as interference and exploitation competition respectively. It is not clear in a bacterial nutrient broth culture that one species would necessarily reduce the density of the other. Bacteria alter their gene expression at stationary phase to enable them to survive at very low nutrient levels, and would not die of starvation even if most of the remaining nutrients had been sequestered. Bacteria do sometimes release substances that are toxic to other bacterial species (bacteriocins and antibiotics), but it seems unlikely that this would be of much benefit in stationary phase; dead bacteria would not release many of their nutrients and thus the competing species would not be able to increase much. A better model of the competition between bacteria might be based on a growth model which explicitly includes nutrient availability (e.g. Tilman 1982). The data showing growth of *E. coli* 2442 in stationary phase *Salmonella* populations apparently shows an extended lag phase, which is not accounted for in the model. A lag parameter can be included in mathematical models of growth (Zwietering et al 1990) but the extended lag in competition with another strain is not explicable by current competition models.

As a test of the Lotka-Volterra model, one of its predictions is that stable coexistence should only occur when $Ke>Ks\alpha_{SE}$ and $Ks>Ke\alpha_{ES}$. These populations can clearly stably co-exist and in all cases these inequalities hold. Stable co-existence requires

some niche differentiation, i.e. utilisation of different nutrients, space, temperatures where these vary etc. In an environment as uniform as a nutrient broth culture, it is probably nutrient utilisation on which the bacterial species differ.

As donor and recipient bacteria are grown together in these experiments, some transconjugants will be formed. Transconjugants grow slightly more slowly than recipients, but the growth rate was not adjusted because the number of transconjugants is likely to be very small compared to the number of recipients (<1%), whichever way round the competition is carried out.

5.5.3 Summary

Lotka-Volterra competition coefficients were estimated for various strains of competing *Salmonella* and *E. coli*. The Lotka-Volterra model was found to produce only a poor fit to the data, for various reasons discussed above. Nevertheless, the competition coefficients estimated for *E. coli* 2442 in competition *with S. typhimuirum* 576 showed a reasonable fit to the data and were used in running simulations of the model (section 5.8).

5.6 Estimating the plasmid transfer rate constant

An estimation of the rate of plasmid transfer is essential to predict the spread of resistance. The literature on plasmid transfer rates has been reviewed (section 2.6). The plasmid transfer rates estimated in the literature vary over seven orders of magnitude in broth alone (table 2.5), highlighting the importance of quantification of plasmid transfer rate from the donor to the recipient strain used in the rest of the experiments. Population densities were estimated between 1-6 hr, to enable quantification of growth rate, and at 26 and 30 hr, to enable testing of the assumption that plasmid transfer does not occur at stationary phase. This last test is not carried out in this chapter; it is performed on data from these and other strains in chapter 10.

5.6.1 Materials and methods

Plasmid transfer rates were assessed using the end point method of Simonsen et al. (1990). They derive their equation for γ as follows. They show that if growth and conjugation cease when the population reaches stationary phase (i.e. $m = 0$), then the density of transconjugants produced is dependent on the start and finishing densities of the populations rather than the time elapsed. They therefore rearrange their model differentiating with respect to N. Their model is different to the one presented here, as they explicitly include resource concentration. Nevertheless, if the simplified within-host model is used (equations 5.5-5.7), differentiating with respect to N produces the same set of equations.

$$\frac{dD}{dt} = rD\frac{K-N}{K} \qquad 5.5$$

$$\frac{dR}{dt} = (rR - \gamma(D+T)R)\frac{K-N}{K} \qquad 5.6$$

$$\frac{dT}{dt} = (rT + \gamma(D+T)R)\frac{K-N}{K} \qquad 5.7$$

$$\frac{dN}{dt} = rN\frac{K-N}{K} \qquad 5.8$$

Dividing equations 5.5 to 5.7 by equation 5.8 gives

$$\frac{dD}{dN} = \frac{D}{N} \qquad 5.9$$

$$\frac{dR}{dN} = \frac{R}{N} - \frac{\gamma(D+T)R}{rN} \qquad 5.10$$

$$\frac{dT}{dN} = \frac{T}{N} + \frac{\gamma(D+T)R}{rN} \qquad 5.11$$

These are rearranged (Simonsen et al 1990) to produce the simple equation used to calculate plasmid transfer rate:

$$\gamma = r\ln\left(1+\frac{T}{R}.\frac{N}{D}\right).\frac{1}{N-N_0} \qquad 5.12$$

To calculate plasmid transfer rates using this method, initial and final densities of the populations are required, as is the growth rate of the mating culture (i.e. all bacteria., D + R + T growing together. Mating culture growth rate is r_N where N = D+R+T. The densities of the three populations were summed and a growth rate calculated from the gradient of naturally logged data in exponential phase.

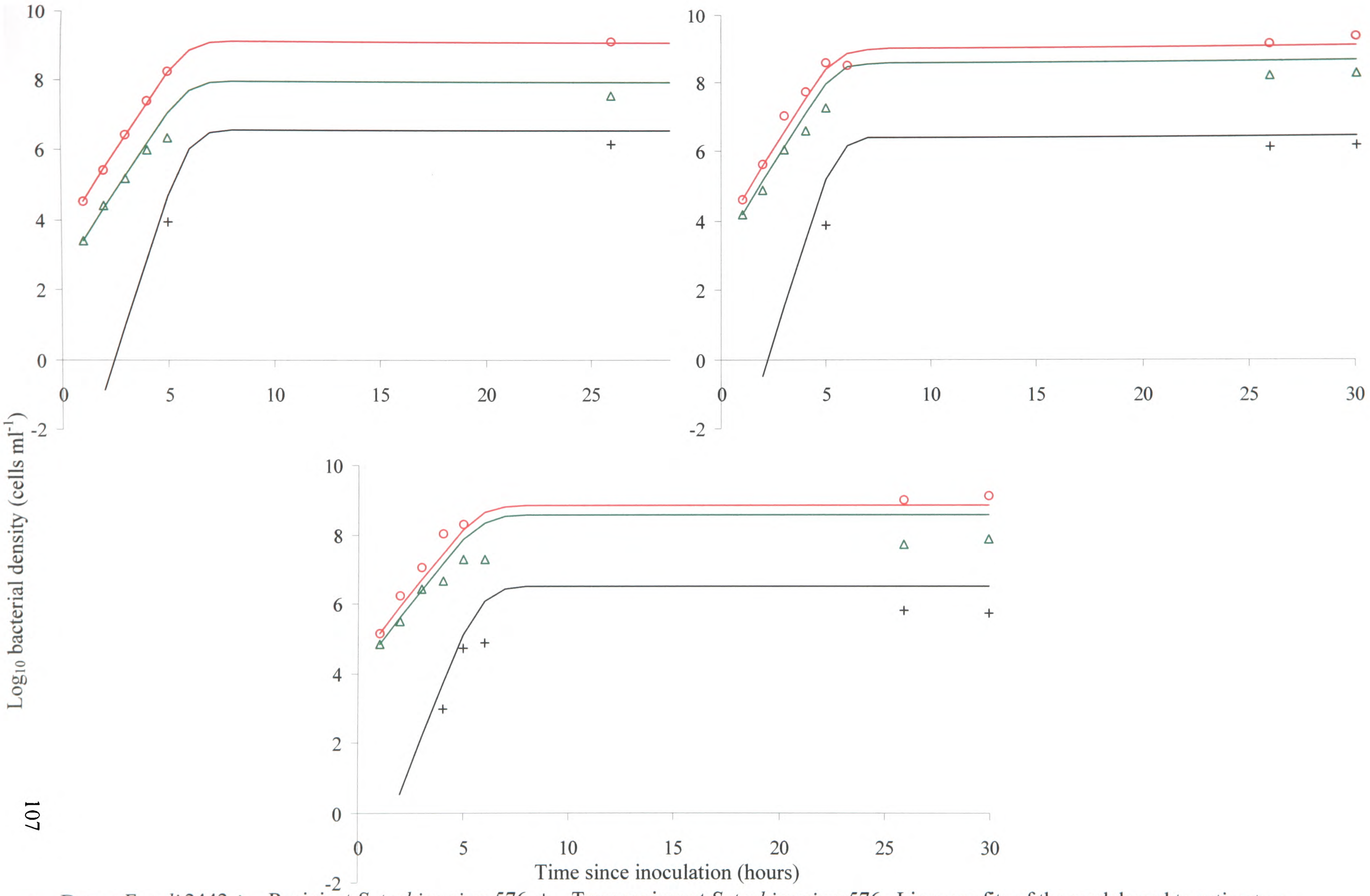
Overnight cultures of donors and recipients were diluted 10,000-fold into 10ml Luria broth, and the population densities were estimated at every hour post inoculation for six hours, then at 26 and 30 hours by the method described in chapter 2. The growth rate of the mating culture was determined by the regression slope of the natural log of N between 1-5 hours. The initial and final densities of each population used to calculate γ were those at 1 and 26 hours respectively. Three model runs of equations 5.1 to 5.3 were created to overlay graphs of the data. For this purpose, recipient *S. typhimurium* 576 and starting densities were taken from those used in γ calculation; γ was the calculated estimate and K was taken to be N (D + R + T) at 26 hours.

5.6.2 Results

The three repeats and the overlaid simplified model are displayed in figure 5.10. The plasmid transfer rates calculated are given in table 5.12.

Repeat:				
1	**2**	**3**	**Mean**	**Standard Deviation**
5.24E-11	1.45E-11	2.48E-11	3.05E-11	1.99E-11

Table 5.12. Plasmid transfer rates (γ) of donor *E. coli* 2442 transferring a resistance plasmid to recipient *S. typhimurium* 576.



○ – Donor *E. coli* 2442 Δ – Recipient *S. typhimurium* 576 + – Transconjugant *S. typhimurium* 576. Lines are fits of the model used to estimate γ.

Figure 5.10. Growth of donor *E. coli* 2442 and recipient *S. typhimurium* 576 co-grown in a water bath at 37^{0}C and production and growth of transconjugants.

A discrepancy between the model and the data can be observed in figure 5.10. Transconjugant densities are overestimated as are recipient growth rates. These are both largely a result of the assumption that each strain grows at the growth rate of the mating mixture. As donors are the most dense population (comprising 90% of the mating mixture) the growth rate of the mating mixture is close to the growth rate of the donors. The recipient's intrinsic growth rate is actually only 92% of the donor's; and the transconjugant's is 96% of the recipient's (table 5.7). The error in the γ estimates caused by these discrepancies can be assessed with reference to Simonsen et al. (1990). They calculated the error in plasmid transfer rate calculation caused by growth rate differences between the different populations. They showed that a 10% growth rate advantage of donors over recipients, and of recipients over transconjugants, at a plasmid transfer rate of 1×10^{-12}, results in a 10% underestimate of plasmid transfer rate. As the growth rate differences here are less than 10%, and plasmid transfer rate less that 1×10^{-12}, the error in these estimates is within 10%.

5.6.3 Summary

A plasmid transfer rate (3.05×10^{-11}) from *E. coli* 2442 to *S. typhimurium* 576 was estimated based on Simonsen et al (1990)'s method. The variation between the three estimates of this rate was not great, and the error in the estimation caused by inaccuracies in the method, looked up from tables in Simonsen et al (1990), was found to be <10%.

5.7 Estimation of the effect of antibiotic and the degree of resistance

If the conclusions drawn from this work are to be applied, it is essential that the relationship between the growth reduction caused by the antibiotic, z, and the dose given in terms of $\mu g\ ml^{-1}$, or $g\ tonne^{-1}$, is known. Chapter 6 explores the relationship between the laboratory antibiotic dose, $\mu g\ ml^{-1}$, and the dose in chicken feed, $g\ tonne^{-1}$. The first aim of this section is to establish the relationship between the laboratory antibiotic dose and its effect upon antibiotic sensitive bacterial populations, z. The second is to quantify the lessening of the effect of the antibiotic on resistant populations, i.e. to establish the degree of resistance conferred by the plasmid, p. Two published models of the effect of antibiotic are described in chapter 2. The main body of the experiments involving antibiotic in this project use neomycin. The aminoglycoside class of antibiotics, of which neomycin is a member, is important both in human and in veterinary medicine. Neomycin is commonly fed to chickens to treat bacterial enteritidis (NOAH 1998), and in 1997, 154 tonnes of aminoglycosides were used in veterinary medicine in the E. U. (Schwarz et al 2000). In human medicine it is cmmonly used to treat or prevent ear, nose, eye and skin infections (Source: British National Formulary; http://www.bnf.org).

5.7.1 Methods

MIC values were calculated on both agar and in LB broth (for methods see section 3.7). MICs in broth were calculated at different starting densities.

Growth curves were obtained from each of the three bacterial populations (donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576) grown in LB

broth at 37^0C in a shaking incubator at various doses of neomycin. 10ml universal tubes were inoculated to give a starting density of 10^5 ml^{-1}, and bacterial densities were assessed hourly between 1 and 6 hours using the same methods employed to assess intrinsic growth rate (section 5.3). The natural logarithm (Ln) transformation was applied to densities and the gradients of the exponential period of their growth was calculated by regression in Minitab to give growth rates. For details of method also see chapter 3.

Estimates of the parameters were made by assuming that during the period of exponential growth, changes in bacterial density can be modelled as follows:

$$\frac{dD}{dt} = r_D(1 - pz)D \qquad 5.13$$

$$\frac{dR}{dt} = r_R(1 - z)R \qquad 5.14$$

$$\frac{dT}{dt} = r_T(1 - pz)T \qquad 5.15$$

where D = density of donors ml^{-1}, R = density of recipients ml^{-1}, T = density of transconjugants ml^{-1}, r_x = intrinsic growth rate of D, R or T $hour^{-1}$, z = antibiotic effect, p = degree of resistance of resistant bacteria.

Note that p is an inverse measure of resistance; when p = 1, bacteria are susceptible and when p = 0, they are fully resistant.

r_D, r_R and r_T were estimated as the growth rate of the controls which were grown with no antibiotic. The relationship between z and antibiotic dose was estimated over a range of

doses, and z and p were estimated at one level of antibiotic. z was calculated as 1 – growth rate of recipients/ r_R and p was calculated for transconjugants and donors as (1- growth rate of transconjugants/ r_T)/z and as (1- growth rate of donors/ r_D)/z respectively.

5.7.2 Results

The MIC of the three strains was plotted against inoculum density (figure 5.10). As it is difficult to read off this log scale the data is given again in table 5.13. MICs of neomycin and other antibiotics on agar plates are given in table 5.14.

The growth rate of the three strains at different concentrations of neomycin was plotted against antibiotic dose and is displayed in figure 5.11.

Inoculum density	Donors	Recipients	Transconjugants
10	125	8	250
10	125	8	250
100	62.5	8	500
10000	500	16	500
10000	250	8	500
10000000	1500	250	3000

Table 5.13 Data from which figure 5.11 was created. MICs (μg ml^{-1}) of neomycin for donor *E. coli* 2442, recipient *S. typhimurium* 576, and transconjugant *S. typhimurium* 576 in LB broth at different starting densities (cells ml^{-1}).

MIC	Donor *E. coli* 2442	Recipient *S. typhimurium* 576
Neo	30	1.875
Amp	>400	1.5
Strep	25	12.5
Chlor	>50	3
Tet	>30	<1

Table 5.14 MIC (μg ml^{-1}) of various antibiotics on agar plates.

Figure 5.10

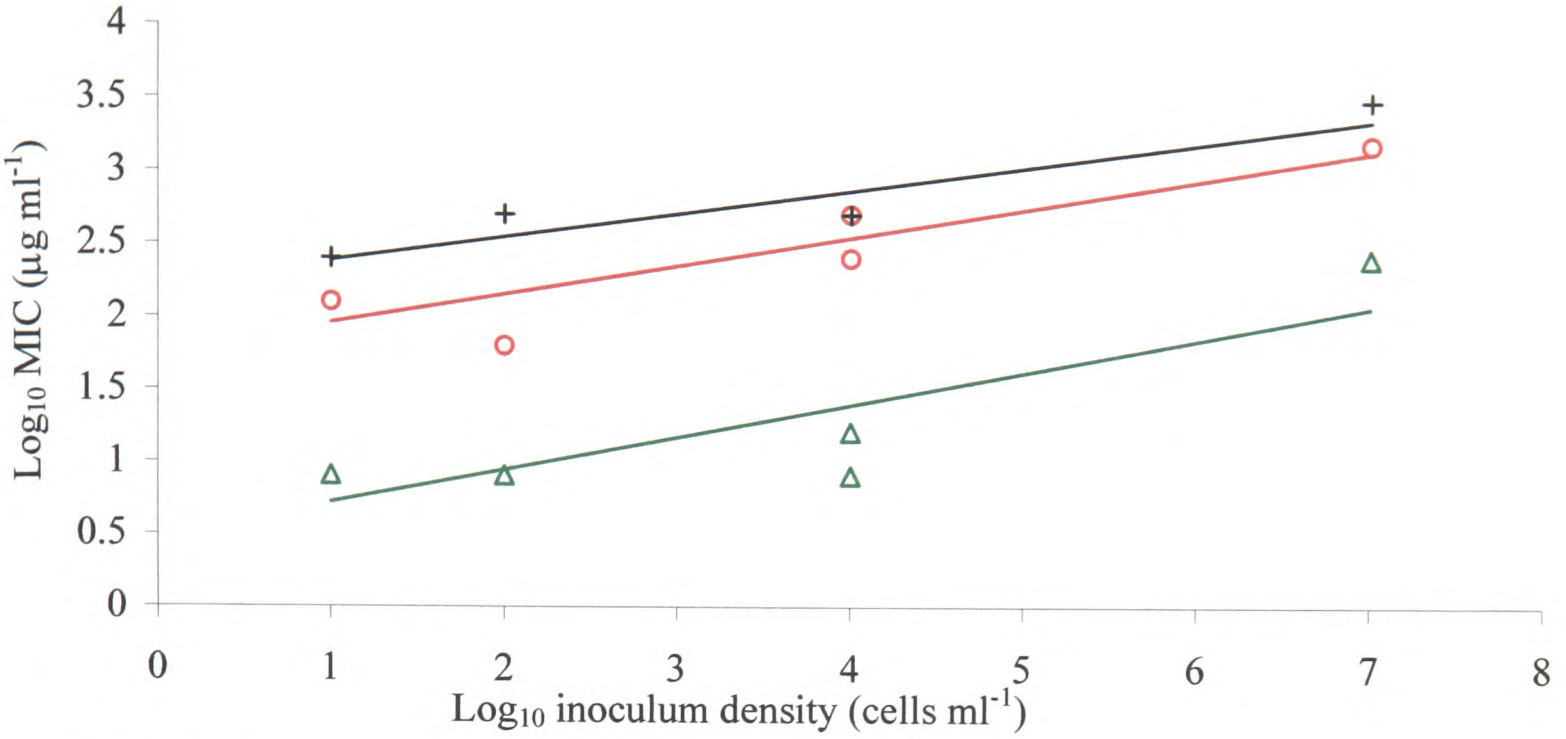
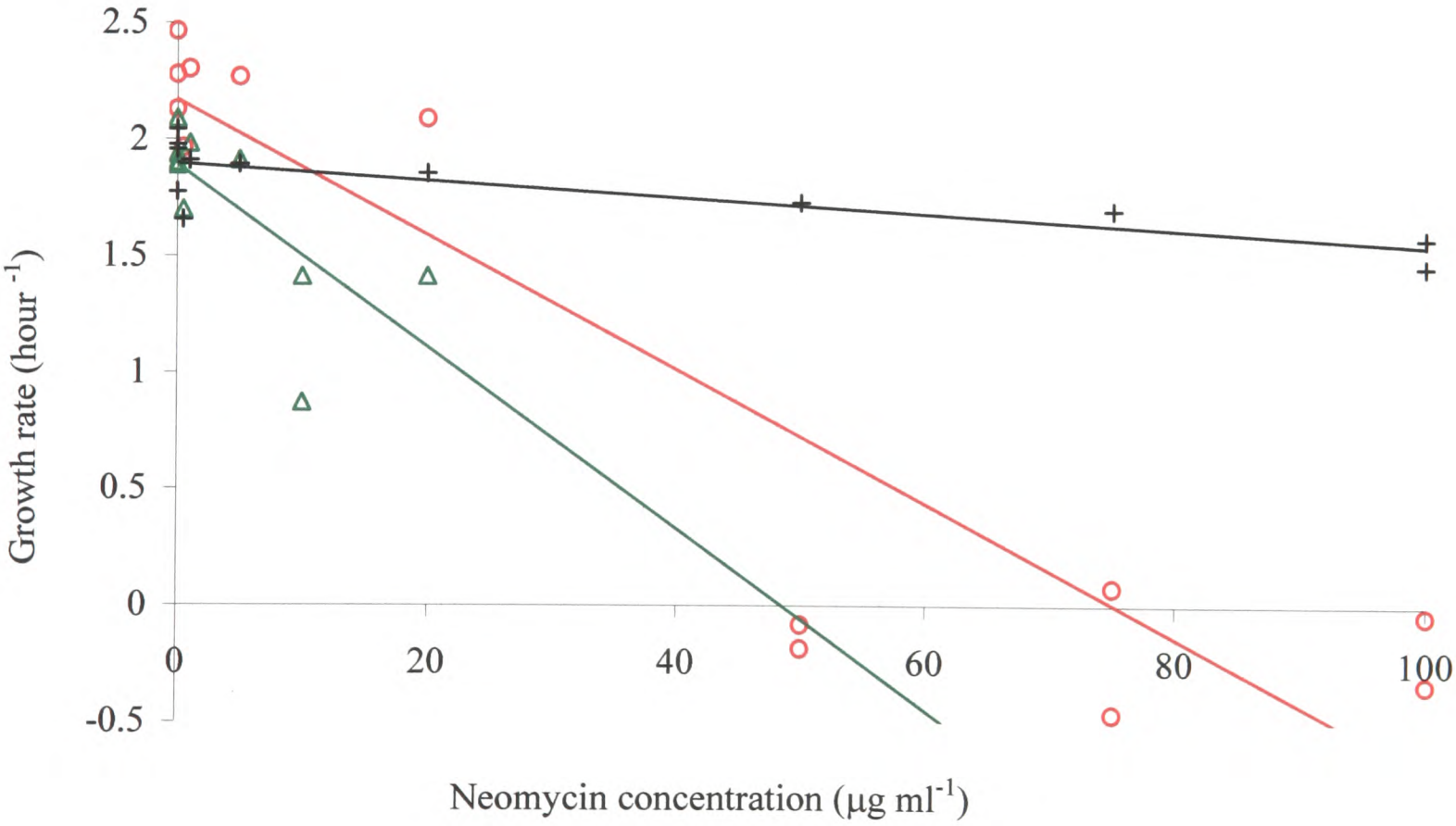


Figure 5.11



o – Donor *E. coli* 2442 Δ – Recipient *S. typhimurium* 576 + – Transconjugant *S. typhimurium* 576. Lines are regressions.

Figure 5.11 MIC of neomycin for donors, recipients and transconjugants at different starting densities.

Figure 5.12 Growth rate of donors, recipients and transconjugants at various doses of neomycin.

Growth rates

The growth rates of populations at different concentrations of antibiotic are shown in figure 5.12. No data exist for recipients at doses above 20 μg ml^{-1} because the population was killed so quickly at these doses that an assessment of growth rate was impossible. This biases the recipients' regression slope towards shallowness.

The growth rates at a dose of 20 μg ml^{-1} were used to estimate the parameters p and z as this provided a high enough dose to reduce growth rates below that of the control without making recipient growth rates impossible to measure. At 20 μg ml^{-1}, z = 0.27, p for donors is 0.31 and p for transconjugants is 0.15. As resistance in donor *E. coli* 2442 and transconjugant *S. typhimurium* 576 is modelled by the same parameter, the average of these resistances (0.23) was used in models.

Discussion

There is a striking difference in the resistance of donor *E. coli* 2442 and that of transconjugant *S. typhimurium* 576. This is probably because *Salmonella* are naturally more resistant to neomycin than *E. coli* is. (Neomycin is currently used to treat *E. coli* enteritis but not Salmonellosis of chickens (NOAH 1998))

The relationship between antibiotic effect, z and the dose given in μg ml^{-1} is non-linear. The growth rate of recipient *S. typhimurium* 576 steadily declines between 0 and 20 μg ml^{-1}, then drops off suddenly to a value so negative it is impossible to measure. Donor *E. coli* 2442 also demonstrate a small decline then a drop to just below 0. This response validates the concept of MIC, the measurement of resistance by giving one cut-off point

of an antibiotic on a particular population. The growth rate-dose relationship of donor *E. coli* 2442 is in accord with the killing curves found by Guerillot et al (1993) (section 4.3.1), who found that above the MIC, the density of *E. coli* declines then stabilises at a low level fairly independent of dose. The growth rate of transconjugants declines as antibiotic decreases, but does not fall off to zero or below zero in this range.

The results of MIC calculation and those of the growth rate – dose investigation do not entirely agree. A measurable growth rate can still be obtained for recipients at 20 $\mu g\ ml^{-1}$ neomycin, whereas at comparable starting densities, 8 or 16 $\mu g\ ml^{-1}$ neomycin prevents growth according to the MIC calculation. Conversely, the growth of donors was recorded as negative at 50 $\mu g\ ml^{-1}$, whereas its MIC calculation recorded growth up to 250 $\mu g\ ml^{-1}$ at lower starting densities. As MIC calculation in broth relies only on measurement by eye of presence or absence, any densities below about 10^7 cells ml^{-1} are recorded as absence of growth. Variation in the K-value (carrying capacity) of strains grown under conditions of antibiotic could lead to false negatives. K-values for these strains grown with antibiotic were found to vary, sometimes below 10^7 (data not shown), while growth was measurable. The discrepancy between the negative growth rate of donors when measured and the growth in the MIC calculations is more difficult to understand and may be the result of variation between populations of the donor strain.

There is a marked decrease in MIC values when measured on agar as opposed to in broth. This may be due to the fact that neomycin is not used up or degraded as much in agar as the bacteria are not dispersed throughout it. Antibiotic may diffuse through the agar and kill populations on the surface when the higher densities of bacteria in broth effectively lower the concentration of antibiotic. Although MIC values may be used as a guide to the

effect antibiotics have on populations, it is clear that detailed studies of the effect of antibiotic on growth rate and K values provide valuable extra information.

The estimations of z and p are provided from one set of growth curves: those at 20 μg ml^{-1} neomycin. This p estimate does not provide a full account of antibiotic resistance in these strains of *E. coli* and *S. typhimurium*, as donors are more resistant at lower concentrations (<20 μg ml^{-1}) and less resistant than transconjugants above this concentration. Figure 5.12 could be used as a reference to determine z and p values at concentrations between 0 and 100 μg ml^{-1} rather than presuming constant resistance and a linear relationship between dose and antibiotic effect.

5.7.3 Summary

MIC values of donors *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576 at different starting densities in LB broth and on agar plates were calculated. The growth rates of these strains in broth at different concentrations of neomycin were determined. The growth rate of transconjugants was found to decline much more slowly that the growth rate of donors as neomycin dose was increased. The decline in growth rate of recipients with dose was found to accelerate until it was impossible to measure at doses of > 20 μg ml^{-1}. The parameter values p and z were calculated at the only neomycin dose at which the calculation was feasible: 20 μg ml^{-1}. Some discrepancies between the estimates of MIC values and growth rate data were observed.

5.8 Model simulations

The models in this project were used to synthesise disparate aspects of bacterial dynamics. By combining assumptions about the relationship of bacterial processes to each other with numerical values for the extent of each process, a model was created which was used to simulate possible outcomes. The model described in chapter 4 is analysed here, and parameters estimated in sections 5.3-5.7 are put into the model in order to generate predictions. The analysis is directed at understanding the outcome of varying antibiotic dose on *Salmonella*, and antibiotic resistant *Salmonella*, populations.

5.8.1 Summary of parameter values

The parameter values to be used in the models are as given in table 5.15. All these parameters were calculated assuming $m = 0$. As m is used in the modelling results, some parameters were recalculated with the inclusion of m at $m = 0.1$. The parameters whose estimations change most when m is included are the r and K values. $r_D(m)$, $r_R(m)$ and $r_T(m)$ are calculated as r_D, r_R and $r_T + m$. $K_E(m)$ and $K_S(m)$ become $\frac{K_E}{1-\frac{m}{r_D(m)}}$ and $\frac{K_S}{1-\frac{m}{r_R(m)}}$ p and z are relative to the intrinsic growth rate and do not change when m is included in the model. The effect of leaving α_{SE}, α_{ES} and γ the same when m is included is minimal, so these parameters have not been recalculated.

Parameter	Value (m = 0)	Value (m = 0.1)
r_D	2.05	2.15
r_R	1.89	1.99
r_T	1.81	1.91
K_E	1.24×10^9	1.3×10^9
K_S	1.04×10^9	1.1×10^9
α_{ES}	1.08	1.08
α_{SE}	0.82	0.82
γ	3.05×10^{-11}	3.05×10^{-11}
p	0.23	0.23
m	0	0.1

Table 5.15. Parameter values estimated experimentally in this chapter, and a value for m which gives a reasonable fit to data. These values are used in modelling bacterial population densities.

The turnover of populations, m, is difficult to measure experimentally. This value allows a reasonable fit to data.

5.8.2 Analytical results

Competition between donor *E. coli* and recipient *S. typhimurium*, and between recipients and transconjugants, was analysed mathematically. These two comparisons provide an insight into whether *Salmonella* will persist in a system, and if it does, whether it will be resistant or sensitive to antibiotic. In the first comparison, conjugation is ignored; the plasmid transfer rate is set to zero. Resistant donor *E. coli* (E) competes against sensitive recipient *S. typhimurium* (S).

$$\frac{dE}{dt} = r_E(1-pz)E \cdot \frac{K_E - E - \alpha_{ES}S}{K_E} - mE \qquad 5.16$$

$$\frac{dS}{dt} = r_S(1-z)S \cdot \frac{K_S - S - \alpha_{SE}E}{K_S} - mS \qquad 5.17$$

where E = resistant donor *E. coli* density (ml^{-1}) S = sensitive *S. typhimurium* density (ml^{-1}) r_x = intrinsic growth rate of E or S (h^{-1}), K_x = carrying capacity of E or S (ml^{-1}), N ≡ E+S, z = antibiotic effect, p = degree of resistance to antibiotic, m = dilution or death.

R_0's (defined below) can be determined for *E. coli* and *S. typhimurium*:

$$R_0(E) = \frac{r_E(1-pz)}{m} \qquad 5.18$$

$$R_0(S) = \frac{r_S(1-z)}{m} \qquad 5.19$$

The value of R_0 determines whether populations will become established upon introduction into a naïve host, when densities are far from the equilibrium. For this reason the equilibrium term $\frac{K_X - X - \alpha_{XY}Y}{K_X}$ will be close to 1. R_0 is defined in infectious disease epidemiology of microparasites as 'the number of new infections resulting from one infected individual in an otherwise susceptible population', and its value determines whether a disease will take off in a population or die out. In the present model the 'cases' are individual *Salmonella* bacteria and it is bacterial division which produces new cases

as opposed to infection. If $R_0(S) > 1$, sensitive bacterial density will increase from its starting level, and if $R_0(E) > 1$, resistant bacterial density will increase.

If the effect of antibiotic, z, is below a critical level, E and S can coexist. At the parameter values estimated in sections 5.3, 5.4, 5.5 and 5.7, S never excludes E. If z is above the critical level, S are depleted until they go extinct. Figure 5.13 illustrates these two possibilities.

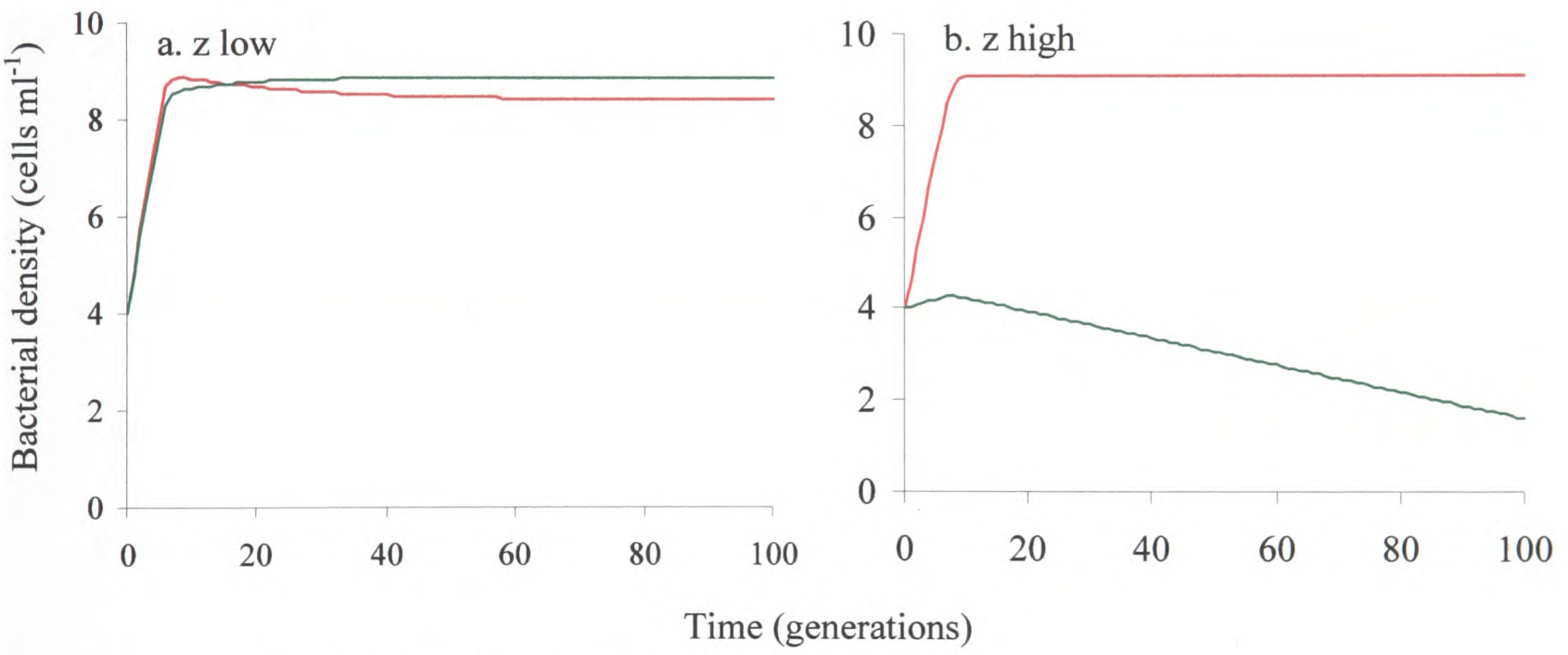


Figure 5.13. Diagrams showing the growth of antibiotic resistant *E. coli* and antibiotic sensitive *S. typhimurium* at two levels of antibiotic.

Phase diagrams were drawn, where the equilibrium densities of each population is plotted against the population of the other (Begon et al 1996, figure 5.13)

The line connecting the points of equilibrium density for each population is called an isocline. If the two isoclines cross, there is an equilibrium which may or may not be stable.

The diagrams were created by rearranging equations 5.16 and 5.17 set to zero:

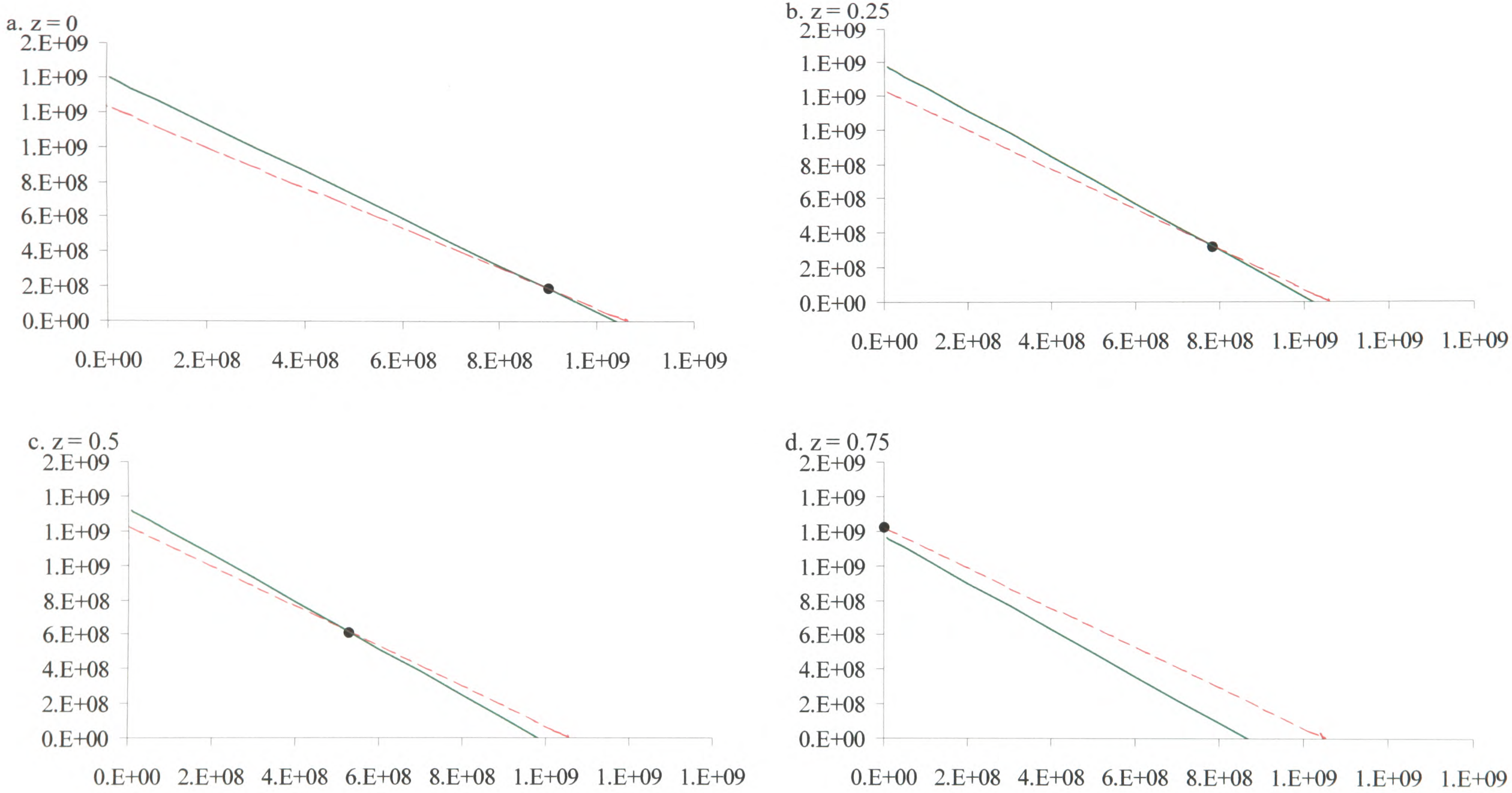
$$E^* = K_E - S\alpha_{SE} - \frac{K_E m}{r_E(1-pz)} \qquad \text{or} \qquad E^* = 0 \qquad 5.20$$

$$S^* = K_S - E\alpha_{ES} - \frac{K_S m}{r_S(1-z)} \qquad \text{or} \qquad S^* = 0 \qquad 5.21$$

These describe a straight line with gradient α_{SE} or α_{ES} and intercept $K_E - \frac{K_E m}{r_E(1-pz)}$ or vice versa. The normal Lotka-Volterra results follow, where the Lotka-Volterra $K_E \Rightarrow K_E\left[1 - \frac{m}{r_E(1-pz)}\right]$.

E. coli will exclude *S. typhimurium* when

$$K_E\left[1 - \frac{m}{r_E(1-pz)}\right] > \frac{K_S}{\alpha_{ES}}\left[1 - \frac{m}{r_S(1-z)}\right] \qquad 5.22$$



-- Equilibrium density of *E. coli* – Equilibrium density of *S. typhimurium* • = point at which dE/dt = 0 and dS/dt = 0.

Figure 5.14. Phase diagrams showing equilibrium values of *E. coli* density plotted against *S. typhimurium* density and vice-versa at different values of antibiotic concentration, z. Other parameter values are in table 5.15.

Figure 5.14 shows the effect of increasing antibiotic on the equilibrium values of *E. coli* and *S. typhimurium*. When z = 0, co-existence occurs at a level close to the maximum density of *S. typhimurium* (compare with figure 5.8). As z increases to 0.75, this equilibrium point increasingly favours *E. coli* over *S. typhimurium*, until the isoclines do not cross; *S. typhimurium* where present will decrease to zero. These isoclines were created when m = 0.1. The equilibrium point is affected very little by m when z = 0. When $z \neq 0$, increasing m favours *E. coli* at lower levels of z.

Recipient-transconjugant competition

Assume donors are in the background flora at a constant density. If $r_T(1\text{-}pz) = y$ and $r_R(1\text{-}z) = x$,

$$\frac{dT}{dt} = \left[yT + (\gamma R(T + D)\right] \cdot \frac{K - N}{K} - mT \text{ and} \qquad 5.23$$

$$\frac{dR}{dt} = \left[xR - (\gamma R(T + D)\right] \cdot \frac{K - N}{K} - mR. \qquad 5.24$$

If x(K-N)/K<m, R will decline. If x(K-N)/K is slightly larger than m, an equilibrium exists between R and T. These two families of equilibria are

$$R = 0,\ T = \frac{K(y - m)}{y} \qquad 5.25$$

and

$$R = \frac{1}{\gamma(T + D)}\left[\frac{TKm}{K - N} - yT\right], \qquad T = \frac{1}{\gamma}\left[x - \gamma D - \frac{Km}{K - N}\right] \qquad 5.26$$

5.8.3 Numerical simulations

The parameters estimated above (table 5.15, parameters estimated with m = 0.1) were put into the full model and it was run for 100 hours at different levels of antibiotic. The version of the model used was as follows:

$$\frac{dD}{dt} = r_D(1-pz)D \cdot \frac{K_E - D - \alpha_{SE}(R+T)}{K_E} - mD \qquad 5.27$$

$$\frac{dR}{dt} = \left[r_R(1-z)R - \gamma(D+T)R\right] \cdot \frac{K_S - R - T - \alpha_{ES}D}{K_S} - mR \qquad 5.28$$

$$\frac{dT}{dt} = \left[r_T(1-pz)T + \gamma(D+T)R\right] \cdot \frac{K_S - R - T - \alpha_{ES}D}{K_S} - mT \qquad 5.29$$

where r_x = intrinsic growth rate of D, R or T, z = antibiotic killing, p = degree of resistance of resistant bacteria (i.e. those bearing a plasmid: D or T), K_E and K_S = carrying capacity parameter of *E. coli* and *S. typhimurium* respectively, α_{SE} = effect of competition by *S. typhimurium* on *E. coli*, α_{ES} = effect of competition by *E. coli* on *S. typhimurium*, m = dilution or death parameter.

Two sets of model runs were produced. In the first, the antibiotic effect varies between 0 and 1.5 in linear steps. z may be > 1 because antibiotic can cause a negative growth rate. As the estimation of the effect of antibiotic on growth rates indicated the relationship may be more one of a steepening gradient rather than linear (figure 5.12; recipients), another set of model runs was carried out where the z was increased in logarithmic steps between 0.1 and 1000.

The effect of time and antibiotic effect on transconjugant density are shown in figures 5.15-5.20. Figures 5.15-5.17 display dose in linear steps, and 5.18-5.20 display dose in exponential steps. The starting densities were D: 10000, R: 10000, and T: 0. The parameter values are taken from table 5.15 (m = 0.1).

Donors

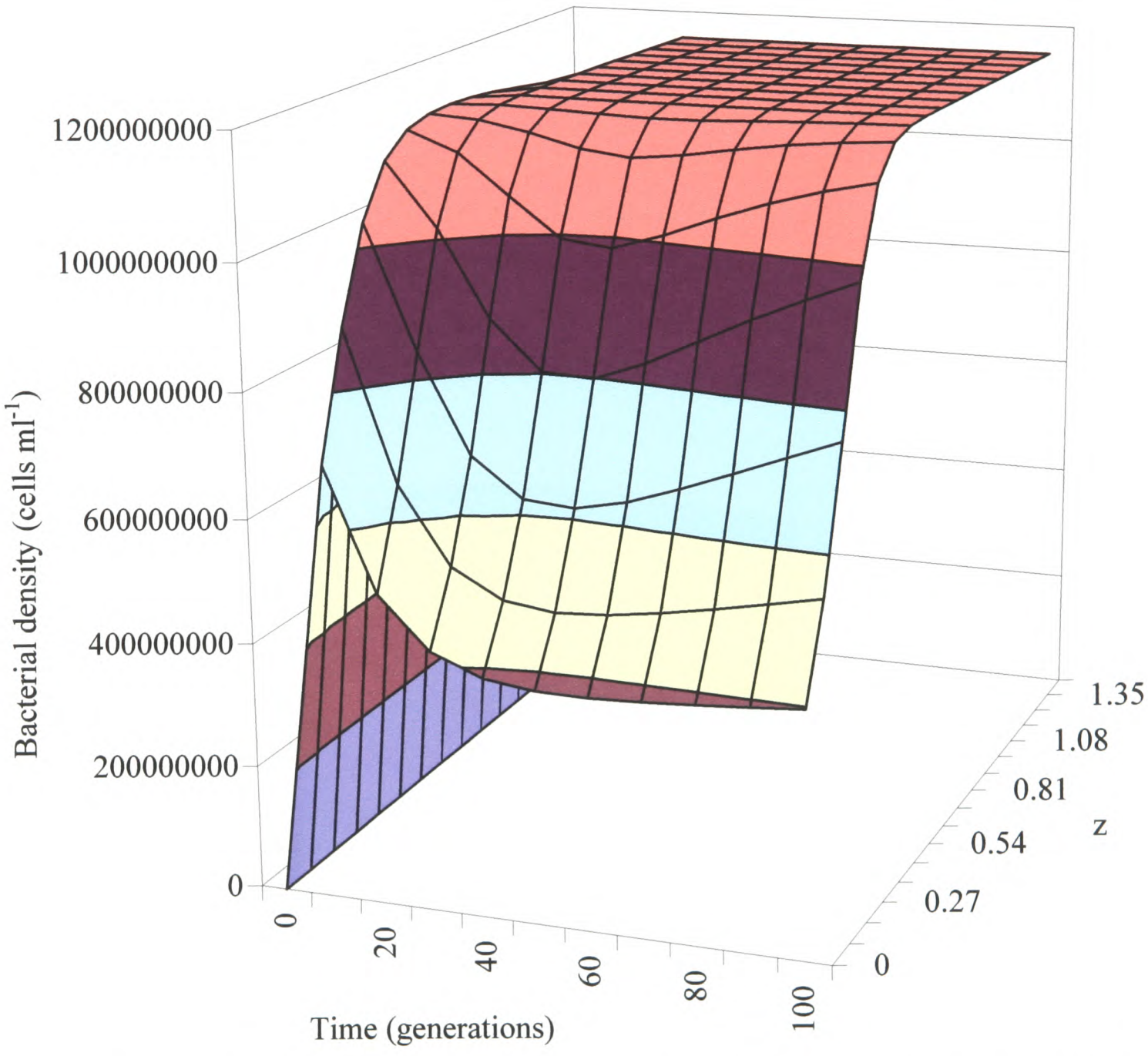


Figure 5.15. Density of donors with time and antibiotic dose in a continuous, deterministic model. z varies in linear steps from 0 to 1.5.

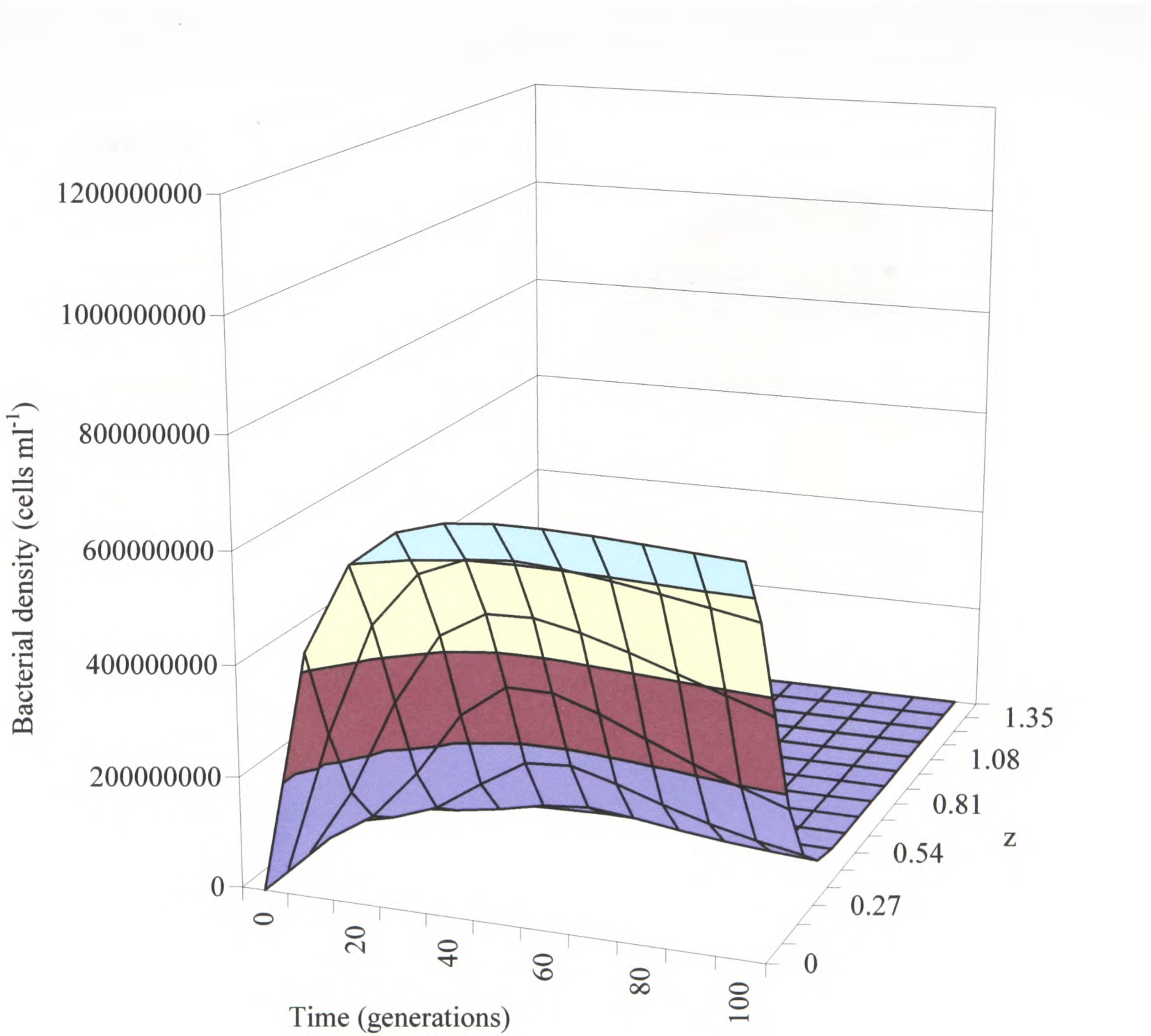


Figure 5.16. Density of recipients with time and antibiotic dose in a continuous, deterministic model. z varies in linear steps from 0 to 1.5

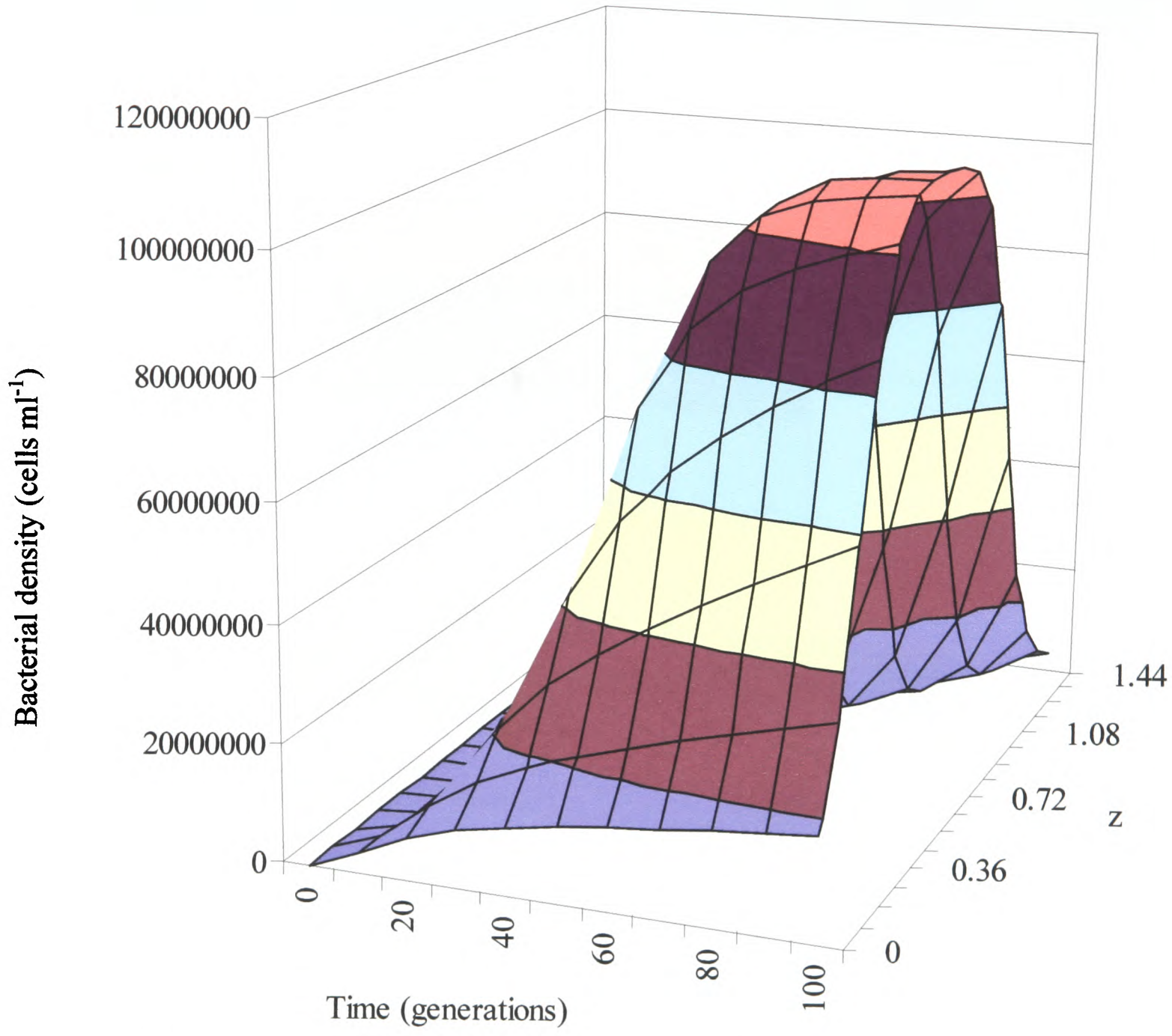


Figure 5.17. Density of transconjugants with time and antibiotic dose in a continuous, deterministic model. z varies in linear steps from 0 to 1. Note that the divisions on the scale are 10-fold smaller than the divisions on the donor and recipient graphs.

Donors

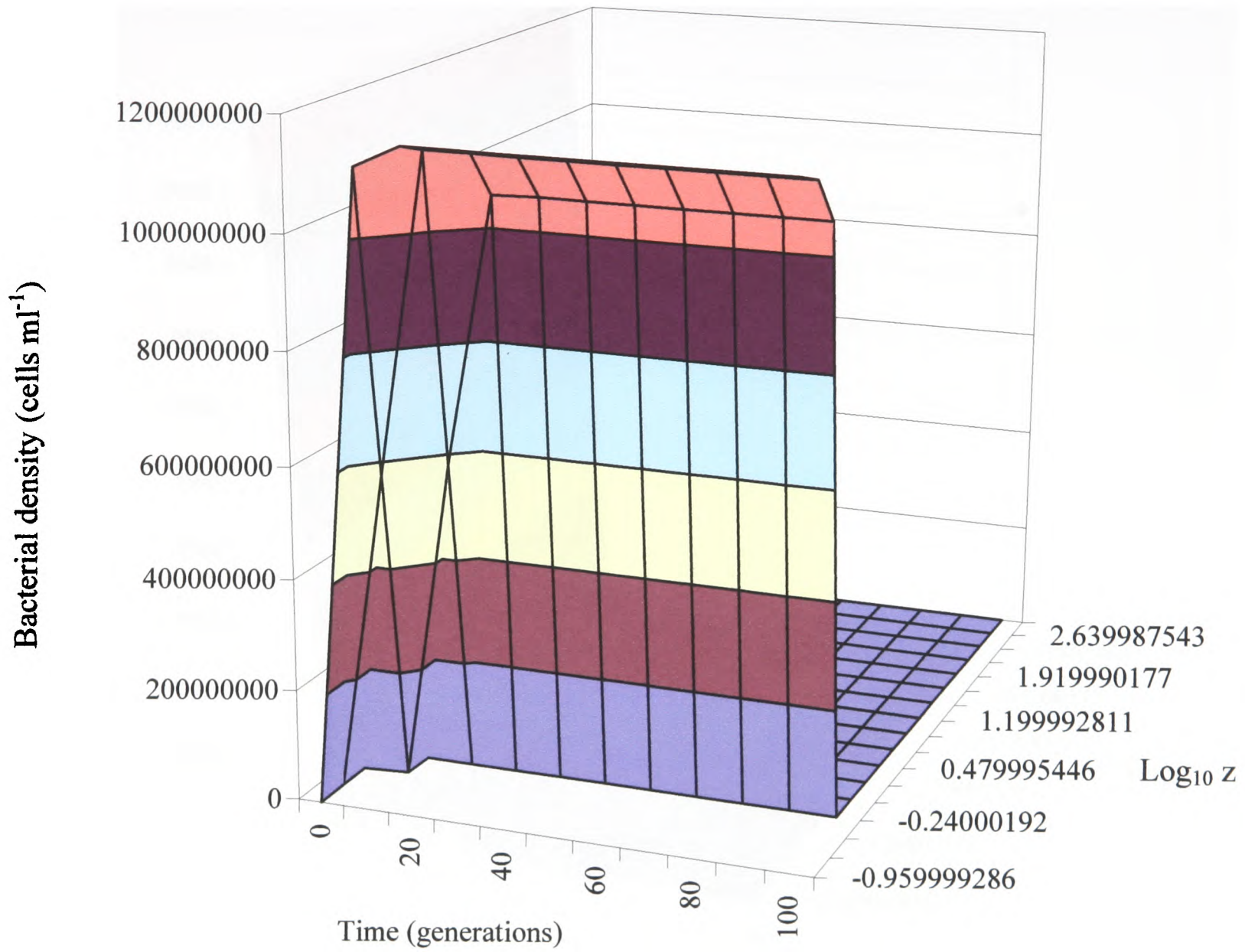


Figure 5.18. Density of donors with time and antibiotic dose in a continuous, deterministic model. z varies in logarithmic steps from 0.1 to 1000

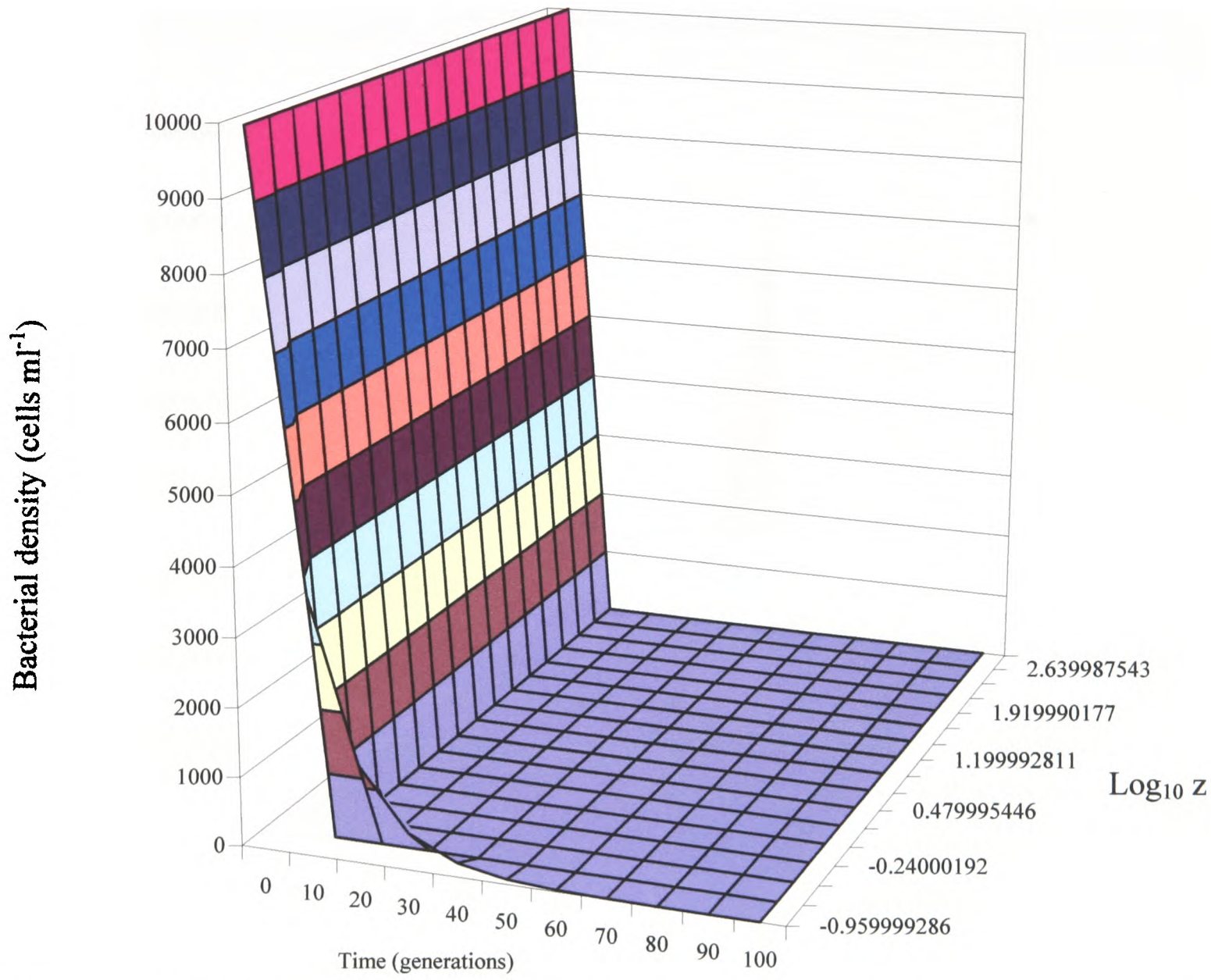


Figure 5.19. Density of recipients with time and antibiotic dose in a continuous, deterministic model. z varies in logarithmic steps from 0.1 to 1000

Transconjugants

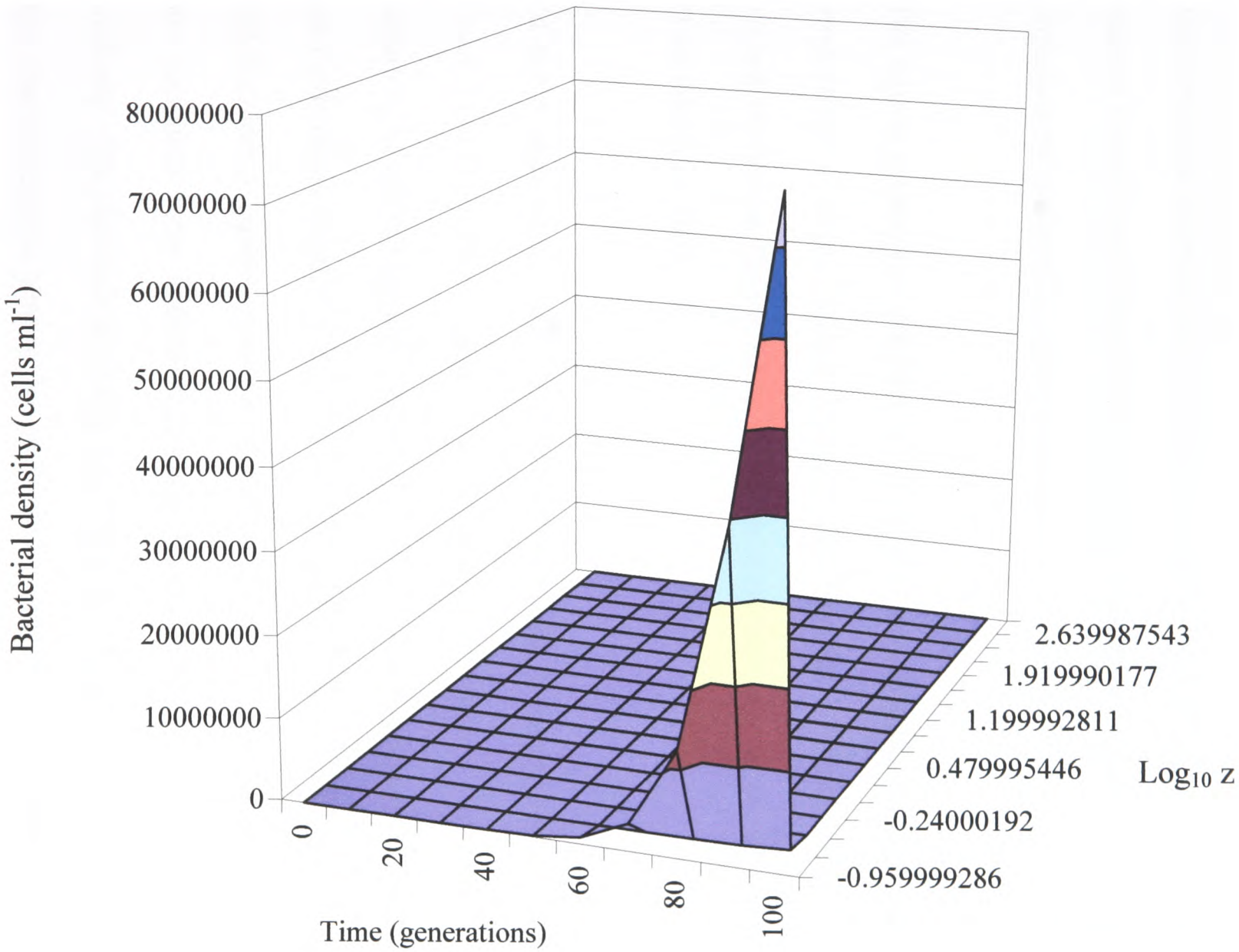


Figure 5.20. Density of transconjugants with time and antibiotic dose in a continuous, deterministic model. z varies in logarithmic steps from 0.1 to 1000.

5.8.4 Summary

There are threshold values of m and z above which recipients lose out to either donors or transconjugants when in direct and exclusive competition. The most important threshold is that which enables recipients to co-exist with donors. If these conditions are satisfied, transconjugant *Salmonella* will exist to a lesser or greater degree. The second threshold dictates whether equilibrium transconjugant populations can exist freed from population limitation by competition with recipients.

The figures display two possible relationships between antibiotic dose and transconjugant production; a large, gradual increase followed by a decline (figure 5.17), and an immediate decline (figure 5.20). The form of the relationship is explored through a range of antibiotcs in chapter 9.

Of the experimental work presented in this thesis, there are several anomalous results which do not seem to agree with the model predictions. The first is the infrequent isolations of transconjugants produced in the *in vivo* experiment despite a broad range of antibiotic doses (figure 8.1 d). This may be due to the low densities of donor *E. coli* 2442 and recipient *S. typhimurium* 576. Donors were isolated fairly frequently from all groups, and recipients were isolated frequently from the two groups fed the lowest dose of antibiotic. The densities are fairly low, however, of the order of 1000 cells ml^{-1}. Given that transconjugants tend to be produced at only 1% of parent densities overnight in laboratory conditions ideal for conjugation, that *in vivo* conditions are close to stationary phase (thus reducing growth and conjugation rate), and that the limit of detection in this

experiment was 30 cells ml^{-1}, perhaps it is not surprising the transconjugants were not often isolated. This result is in accordance with Freter et al (1983), who concluded that a low density of transconjugants in the mouse intestine can be explained solely by the density of donors and recipients and that suppression of transfer *in vivo* need not be invoked. Alternative explanations for the low density of transconjugants observed *in vivo* are given by Anderson (1975), who suggests that the *in vivo* environment might reduce plasmid transfer rate, and Licht et al (1999), who demonstrate a reduced plasmid transfer rate constant in the mouse intestine compared to *in vitro*.

6. Calculating the proportion of water and the amount of antibiotic in caecal content

6.1 Introduction

This study of the evolution of antibiotic resistance in *Salmonella* involves both *in vivo* and *in vitro* experimental parameter estimations and model testing. To make an accurate estimate of the relationship between *in vitro* and *in vivo* concentrations of antibiotic, the ratio of water to dry matter in the caecal content requires estimation. To make up the feed to give caecal content neomycin concentrations approximately corresponding to *in vitro* concentrations already investigated, estimations were made of caecal water content from the literature (Thornburn and Wilcox 1964). However, no estimations could be found for three to six week old Light Sussex chickens. Part of the *in vivo* study of the effect of neomycin dose on transconjugant production, then, was to estimate this proportion in these birds.

The estimate of proportion of water in caecal content was used to determine whether the antibiotic is concentrated in the solid part of the caecal content as compared to the feed.

Antibiotic can localise to different parts of the gut; it can become diluted or broken down in particular areas, or become concentrated to a level higher in gut contents than that of the feed (Barrow 1989, Smith 1970). An estimate of the neomycin concentration in the chicken caecum was necessary in order to correlate *in vivo* with *in vitro* studies, and no information could be found in the literature on the localisation and concentration of neomycin in the caecum of chickens. An estimate was therefore made using caecal content taken from the chickens involved in the study of the effect

of neomycin dose on transconjugant production reported in chapter 8. The work in chapters 6, 8 and 9 were performed more or less concurrently. Work on the determination of the peak *in vitro* transconjugant production had started before the main *in vivo* experiment, so the work described in this chapter was used to ensure that the doses used related to the *in vitro* work already carried out. The bulk of the experiments presented in chapter 9 was completed after the *in vivo* experiments.

6.2 Materials and Methods

6.2.1 Experimental procedure

Caecal content was taken at PM from birds used in the *in vivo* dose ranging experiment (chapter 8). For details of the housing and sampling of chickens see section 8.2. The birds were three weeks old when the first PM caecal samples were taken, and six weeks old when the last one was taken. Some birds were infected with *S. typhimuiurm*, and its density in caecal content samples from the same birds was measured and correlated with water content. Five samples from each treatment group were taken on each date. Samples from the first date were those used for estimation of bacterial population sizes, so the samples had already undergone one decimal dilution. Decimally diluted samples were separated from the glass beads used for mixing during population size estimates by shaking for 10 seconds and transferred to another weighed universal tube.

On day 10 and day 24, one caecum of each chicken was emptied into two weighed universal bottles, one for use in water content estimation and the other for use in

antibiotic concentration estimation. On the other dates, the contents of the second caecum were used solely for water content estimation. Water content was estimated by weighing and drying the sample containing bottles without lids at 75^0C. They were re-weighed five, ten and eleven days later ensuring that two successive weights were within 0.01g of each other.

6.2.2 Statistical tests

The proportion of water in the caecal content samples were first analysed (using GLM in Minitab) for any effect of chicken age, antibiotic dose and *Salmonella* infection level. The effect of initial weight of sample was also tested for significance, as it was thought that there might be a different water content in samples where only a very small amount of caecal content was available. As PM samples were used, there are no problems with lack of independence; each sample was taken from a different bird.

Methods of measuring antibiotic concentration are similar to those used by Smith (1970), and involve measuring the zones of inhibition produced by caecal content on lawns of sensitive bacteria. Samples used to measure antibiotic concentration were diluted 1:1 with PBS to allow measurement of volume by droplet. The attempts to measure the antibiotic concentration of day 1 samples diluted 1:9 failed because the suspension was too dilute to produce any zones of inhibition. Caecal content taken from chickens fed no antibiotic on day 10 was diluted 1:1 with known concentrations of neomycin solution in PBS in order to produce reference inhibition zones (see figure 6.1). A lawn of the neomycin sensitive bacterium *E. coli* K12 proto nal^r was made on LB nalidixic acid plates. Nalidixic acid was included at 20 $\mu g\ ml^{-1}$ to prevent growth of neomycin resistant bacteria from the caecal content, as it was thought this might

obscure the zones of inhibition around the caecal content drops. Populations fully resistant to both nalidixic acid and neomycin were probably not numerous enough to cause this problem (table 8.1).

The surfaces of dry LB nalidixic acid plates were covered with a 100-fold PBS dilution of overnight culture of the bacterium, and the excess removed. The drops of diluted caecal content were placed onto the plates along with reference caecal content as in figure 6.1.

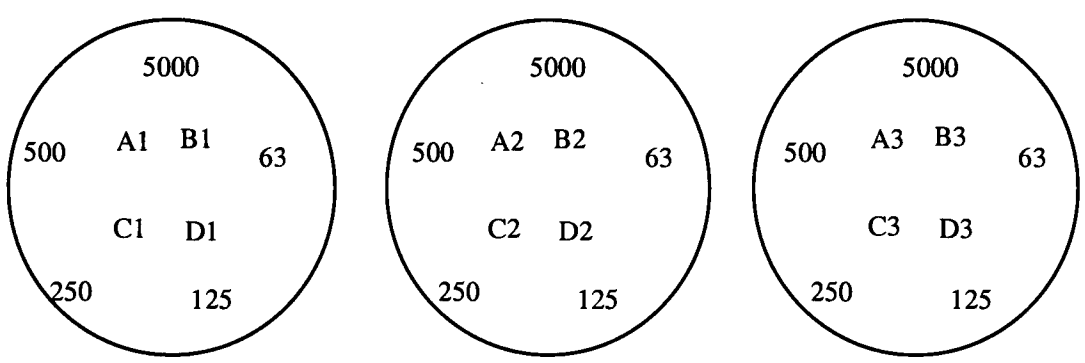


Figure 6.1: Position of drops of caecal content on lawns of sensitive *E. coli*. Letters A to D mark the positions of caecal content from each of the four groups of chickens, fed respectively 0, 64, 160 and 250 g tonne^{-1} neomycin, and the numbers 1-5 following them indicate which chicken from that group the caecal content sample was taken from. The numbers 63, 125, 250, 500 and 5000 indicate reference caecal content samples containing those concentrations in μg ml^{-1} fluid of neomycin. Replication is provided by the 5 different chickens (numbers 1-5).

6.3 Results

6.3.1 Water content estimation

The effect on water content of chicken age, antibiotic dose, *Salmonella* infection level and initial weight of sample are illustrated in figure 6.2. The average percentage of water in the caecal content was 69.8%. The only variable with a significant effect was antibiotic dose ($P<0.05$). The regression equation is:

Proportion of water = -0.0003*antibiotic dose + 0.7344.

The average percentage varies from 72.9% in chickens fed no antibiotic to 66.5% in chickens fed 250 $\mu g\ ml^{-1}$ neomycin.

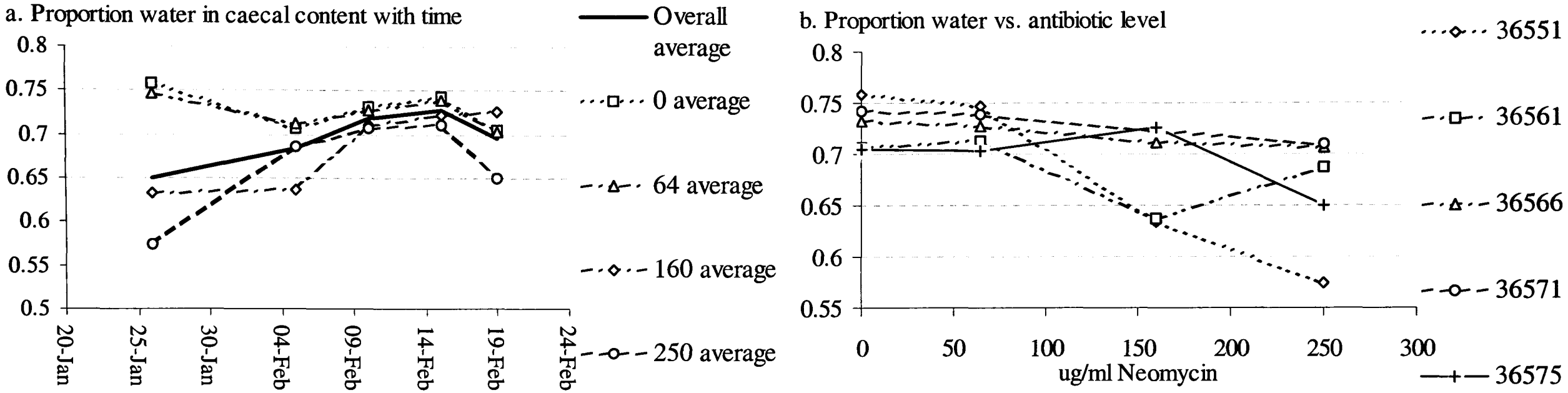


Figure 6.2. The relationship of four variables to percent water in caecal content.

6.3.2 Antibiotic concentration estimation

The zones of inhibition around the caecal content were not properly circular. It was therefore decided to record the widest and the narrowest point of the variable radius around each drop to achieve a range for each. The average of the ten radii around the caecal content mixed with standard amounts of antibiotic (i.e. five samples from the 0 g tonne^{-1} group on day 10, each with a maximum and minimum radius) were plotted against their concentrations so that the antibiotic content of the samples to be tested could be determined (figure 6.3 and table 6.1). The average water content of the caecal matter was used to translate the concentrations in the neomycin solution mixed with control caecal matter into concentrations per ml water and per g dry matter. These have been plotted on the y-axes on the standard curve to allow estimations of antibiotic level in the different components of caecal content.

Chicken group (neomycin g tonne^{-1})	Average radius of inhibition zone (mm) on		Equivalent g tonne^{-1} in caecal dry matter on	
	Day 10	Day 24	Day 10	Day 24
0	0	0	0	0
64	0.22	0.39	390	500
160	1.20	0.91	1235	875
250	1.46	1.75	1545	2220

Table 6.1. Concentrations of neomycin in the dry matter of caecal content as read off the standard curve in fig 8.2. The equivalent g tonne^{-1} numbers are the average of the estimations for the two samples, one on each date.

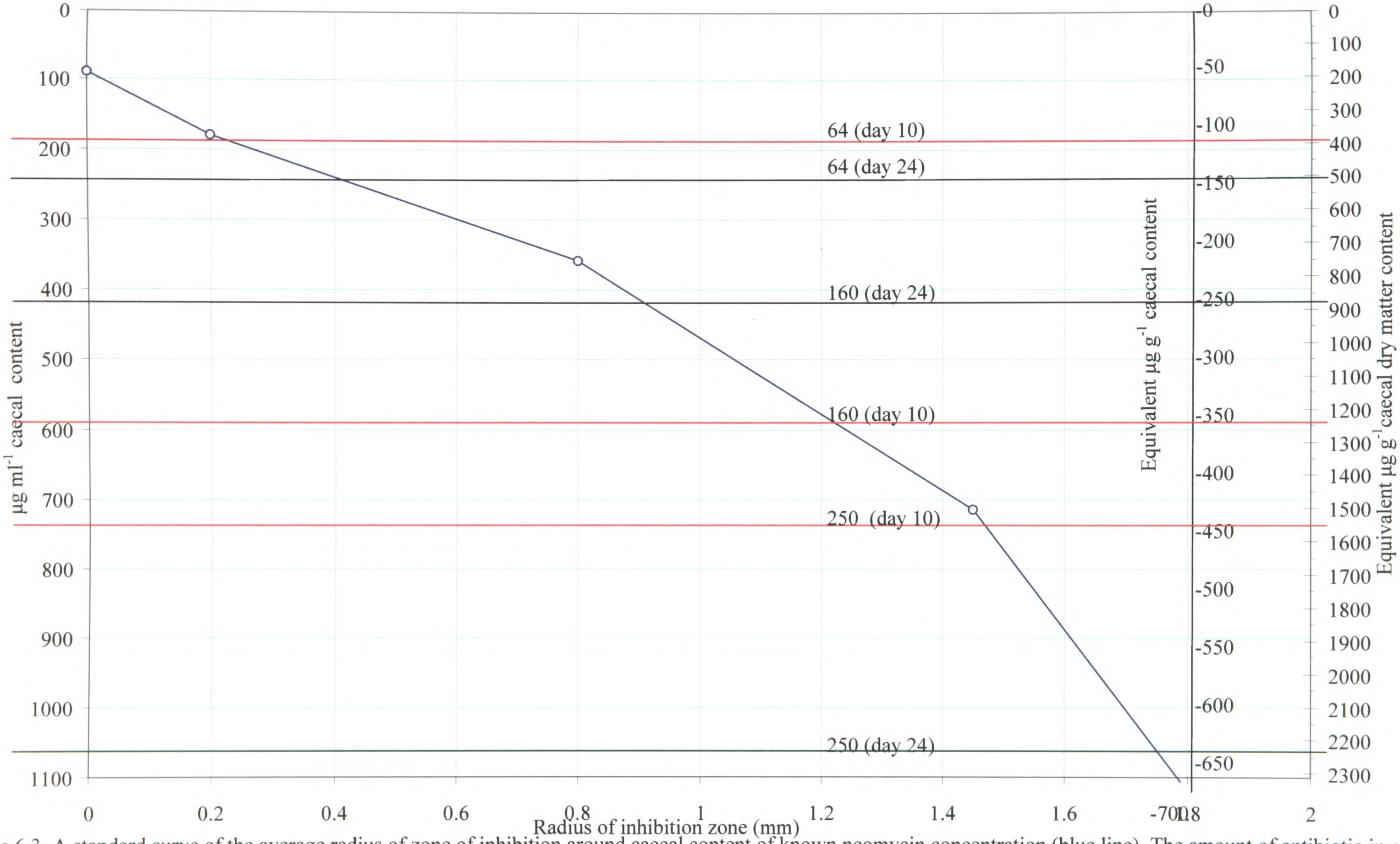


Figure 6.3. A standard curve of the average radius of zone of inhibition around caecal content of known neomycin concentration (blue line). The amount of antibiotic in caecal content of birds fed neomycin can be read off this curve when the radii of their zones of inhibition are known (horizontal dashed lines). Red horizontal lines indicate the sample taken on day 10; Black lines indicate the sample taken on the day 24. Numbers above the lines indicate the concentration of neomycin (g tonne^{-1}) in the feed of the chickens the sample came from.

6.4 Discussion

It is difficult to draw conclusions from the significance of the effect of antibiotic dose on water content (figure 6.2 (b)), as it is compounded with that of *Salmonella* infection. In the separate test on infection level, effect of *Salmonella* prevalence proved non-significant. This may indicate that neomycin affects caecal water content directly. Since *Salmonella* infection frequently causes watery faeces in chickens, it seems more likely that the high water content in the low dose chickens was due to a higher prevalence of *Salmonella* in the gut. As can be seen in the data from the experiment on the effect of dose on transconjugant production, very few *Salmonella* were isolated at all (figure 8.1). It is probably this lack of information that led to no significant effect being found of *Salmonella* on water content, i.e. the test is insufficiently sensitive to detect *Salmonella*.

Can neomycin concentrations in caecal contents be predicted by assuming that the solid component contains the same concentration as feed?

The concentration of neomycin in the caeca of chickens is far higher than predicted by the concentrations in feed. If the amount of antibiotic is expressed in terms of $\mu g\ g^{-1}$ (= g $tonne^{-1}$) of the solid component of caecal content, it is about 10 times greater than that of the feed. This large concentration could be explained if the neomycin passed through the gut to the caeca largely undegraded, and the digestible component of the feed was removed by the gut. Some of this high concentration might be artefactual; for example antibacterial effects of gut contents e.g. defensins can act synergistically with antibiotic (P Barrow, pers comm).

Measuring the killing effect of caecal content on sensitive lawns is a more direct way of estimating antibiotic activity in the caeca than predicting it from caecal water content and neomycin concentration in feed. It is fairly inaccurate, however, especially if carried out by someone not well practised at it. It is not easy to create perfectly circular drops of caecal content on the plate, and uneven drops result in uneven radiuses. Despite the large error, it can be seen that antibiotic becomes much more concentrated in the caeca than it is in the feed.

6.5 Summary

The water content of the chicken caecum were not unlike those found by Thornburn and Wilcox (1964) who found a water content percentage of 83%, although they were using 12 week old chickens of a different strain (Rhode Island Red not Light Sussex).

These results can be used to correlate the *in vivo* and *in vitro* concentrations of antibiotic used in the experiments detailed in chapters 7, 8 and 9 and to predict over which dose ranges model results will apply *in vivo*.

7. *In vivo* pilot study of bacterial infectiousness and the effects of neomycin dose and time on transconjugant production

7.1 Introduction

This chapter describes experiments preliminary to the main *in vivo* dose-ranging experiment (chapter 8). The main experiment was conducted using the same strains used *in vitro* (chapters 5 and 9). A pilot experiment was necessary to ensure these strains behave as expected *in vivo*. Although these strains were initially isolated from chickens, they have been grown for several generations *in vitro* and may consequently now be less fit *in vivo*. In addition, *in vivo* experiments often produce unpredictable results due to natural variation between animals and between the bacterial communities they host. The pilot experiment was therefore performed not long before the main experiment, in similar conditions and with chickens bred at the same facility.

The production of transconjugants depends on the concentration of recipient and donor in the system. The maximum production is achieved when recipient and donor are equally abundant at high densities, and in the exponential phase of growth (Levin 1979). One would expect that abundance in the chicken gut would depend on number of bacteria given, antibiotic concentration, intrinsic growth rate of the strains, carrying capacity, and competition from other bacteria as is assumed in the within-host model (equations 4.8-4.10). It is not known how great the influence of these factors on the test strains will be *in vivo*. Moreover, the methods of counting used here prohibit detection of population densities below 300 colony forming units (cfu) ml^{-1} in the

solution plated. The corresponding density in the live chicken will be slightly higher, as death of bacteria occurs between sampling from the chicken and final plating.

A level of neomycin of 225 g tonne^{-1} was chosen as this is the therapeutic level (NOAH 1998). Williams (1985) found that at a level of 250 g tonne^{-1} neomycin, an experimental infection of *S. typhimurium* declined sharply over two weeks in comparison to the control, but was not eliminated altogether. It was hoped that this level would provide some selective advantage for transconjugants without eliminating recipients before they could be produced by conjugation.

In this pilot experiment, data was gathered by three different methods and at two different levels of medication to assess the best method of data collection.

The model predicts a convex relationship between transconjugant production and antibiotic dose (figure 5.16). The eventual aim is to show this relationship *in vivo*. Further reasons to conduct this pilot experiment are the assessment of whether transconjugants would be produced at the test dose, and what duration of experiment might be necessary to produce this convex relationship. Similarly, in order to model the extent of a bacterial epidemic in a chicken population, quantification of infectiousness is required (β; Anderson and May, 1991). A between-host model would require this parameter.

This pilot study aims to answer the following questions:

1. Do the strains previously used in lab experiments survive in chickens?
2. What is their infectiousness from host to host (β)
3. Are transconjugants produced *in vivo* by these donor and recipient strains?

4. Are they produced at the normal therapeutic level of neomycin?
5. How does the duration of the experiment affect transconjugant production?

7.2 Materials and methods

7.2.1 Experimental design

Sixty *Salmonella*-free Light Sussex chickens numbered by wing band were to be divided between two pens, below which lay a scroll of plastic for easy collection of caecal droppings. The feed in one pen (for the medicated group) was combined with 225 g tonne^{-1} neomycin. Twenty birds from each cage were infected with donor *E. coli* 2442 by administering to them a 0.3 ml nutrient broth culture grown in a shaking incubator overnight by syringe into the crop, and three days later with an equal amount of recipient *S. typhimurium* 576 (approximate densities: 10^9cfu ml^{-1}). One chicken was dead on arrival from the poultry production unit (PPU) and so only nine were assigned to the medicated: uninfected group (thus the medicated group comprised 29 birds and the non-medicated group 30).

Pen	Group	Medicated?	Experimentally infected?	Birds	PM days
1	A	Y	Y	20	5, 10, 14, 18, 21
	B		N	9	10, 18, 21
2	C	N	Y	20	5, 10, 14, 18, 21
	D		N	10	10, 18, 21

Table 7.1. Information on the experimental chickens. They can be divided into four groups (A-D) between which the medication and infection status differ as detailed. Post mortem (PM) days are the days since *E. coli* dosing on which birds were killed and dissected.

7.2.2 Antibiotic resistance testing of background flora

Colonies isolated from swabs the day before infection were tested for antibiotic resistance. *E. coli* colonies were found that were partially resistant to neomycin and fully resistant to ampicillin, but no tetracycline resistance was found (table 7.2). Tetracycline was included in the donor isolation plates in order that no background *E. coli* would be mistaken for donor *E. coli* 2442, which is resistant to tetracycline (section 3.2).

7.2.3 Materials

Bacterial strains: donor *E. coli* 2442 harbouring plasmids with a resistance profile of Ap, C, TO, S_3, S, SH, Fr (partial) and N; and recipient *S. typhimurium* 576 chromosomally resistant to nalidixic acid. The birds were infected with donors on day 1 and with recipients on day 5. See section 3.3 for abbreviations of antibiotics and more information on the strains.

Birds: Three week old SPF Light Sussex chickens hatched at the Poultry production unit (PPU) at the IAH.

Conditions: Birds were housed in groups of 29 or 30 in square mesh pens and given continual access to either normal feed or feed containing 225 g $tonne^{-1}$ neomycin sulphate.

Media: Bacterial inoculum was grown overnight in 10 ml volumes of LB broth in 25 ml universal bottles in a shaking incubator (37^0C, 100 RPM). Viable bacterial counts were made by plating decimal dilutions on each of the agar media detailed in table 7.2.

Bacterial isolate		**Isolation plates used for detection**		
Population	**Resistances include**	**Media**	**Media selectivity**	**Antibiotics added**
Background *E. coli*	Various	MacConkey	Enterobacteria	none
Donor *E. coli* 2442	neo, tet (not nal)	MacConkey	"	neo, tet
Recipient *S. typhimurium* 576	nal only	Brilliant Green	Salmonellae	nov, nal
Transconjugant 1	nal, neo (not tet)	Brilliant Green	"	nov, nal, neo
Transconjugant 2	nal, tet (not neo)	Brilliant Green	"	nov, nal, tet

Table 7.2. Selective media used to isolate bacterial populations with resistance to different antibiotics. Abbreviations and concentrations of antibiotics in plates are as follows: Neomycin sulphate 30 μg ml^{-1} (neo); oxytetracycline 20 μg ml^{-1} (tet); novobiocin 1 μg ml^{-1} (nov); nalidixic acid 20 μg ml^{-1} (nal). *Salmonella* and *E. coli* can be distinguished on MacConkey media because their colonies are different colours. *Salmonella* is naturally resistant to novobiocin (nov); this is added to increase selectivity of the medium against other bacteria.

7.2.4 Data collection

Three methods were used to collect data: (i) daily sampling of caecal droppings (ii) samples of caecal content and spleens at post mortem (PM) and (iii) daily cloacal swabbing. For diagram of areas of the chicken gut see figure 2.2. Daily samples of swabs and caecal droppings were taken in order to detect excretion of donors, recipients and transconjugants as early as possible. The caecal content samples taken daily from the pen floor and at PM allow quantification of bacterial density and the daily cloacal swabs and the PM samples allow identification of the infected birds by the numbers in their wing bands.

1. Daily caecal samples were taken by removing five caecal droppings from the plastic scrolls under each cage (caecal droppings are distinguishable from non-caecal faeces by 'caramel' appearance). As droppings are discrete, each sample was known to be from one bird only. It was not possible to know whether two droppings were from the same or different birds, so samples were taken as far apart as possible to reduce this potential non-independence. Samples were placed in weighed universal tubes, diluted tenfold by weight, then serially decimally diluted by volume and plated out on selective media as soon as possible after collection.
2. Post mortem caeca and spleen samples were taken twice weekly (table 7.1). Each caecum was emptied into a universal tube, and diluted and plated as above. Spleens were crushed with pestle and mortar, diluted ten-fold by wieght into PBS, and serially decimally diluted as the caecal samples.
3. Daily cloacal swabs of all birds were taken from 15th April 1999 - 7th May 1999. Swabs were placed in test tubes with nutrient broth and chilled on return to the

laboratory to maintain bacterial density, and were plated as soon as possible. Each swab was smeared across each of the selective plates detailed in table 7.2. As an accurate count of the bacteria on a swab is not possible, a semi-quantitative measure of abundance was ascribed to each plate. A value of five was given where growth was confluent, three where colonies were crowded but largely separate; two for more than ten colonies and one for less than ten. The scoring is approximately logarithmic, where 10^score approximates to the number of colonies on each plate. As different swabs carried different amounts of faeces, a relative measure of abundance was devised where the score on any one particular selective plate was divided by the score on the non-selective MacConkey plate for that swab.

7.2.5 Statistical analysis

Daily swab results were graphed and analysed by eye as opposed to statistically, as these counts are not fully quantitative. Caecal count results (from samples obtained from the pen floor or at PM) were tested in Minitab using the GLM command for the effect of day, medication and infection, and some interactions between these variables. The questions answered by the analyses are listed in table 7.3.

Caecal sample type	Test number	Population	Question
Pen floor	1	Background *E. coli*	Does medication affect density?
	2	Donor *E. coli* 2442	Does time or medication affect density and is the slope of density against time affected by medication?
	3	Recipient *S. typhimurium* 576	
Post mortem	4	Background *E. coli*	Does medication affect density?
	5	Donor *E. coli* 2442	Does time, medication or experimental infection status affect density, is the slope of density against time affected by medication and does the effect of infection depend on medication?
	6	Recipient *S. typhimurium* 576	
	7	Transconjugant *S. typhimurium* 576	

Table 7.3. Questions answered by the analyses presented in Minitab output 7.1 (pen floor samples: tests 1-3) and 7.2 (post mortem samples: tests 4-7). Too few transconjugant *S. typhimurium* 576 were isolated from pen floor caecal samples to make analysis worthwhile.

7.2.6 Calculation of infectiousness, β

Infectiousness was calculated by examining the rate at which birds in the non-experimentally infected classes (groups B and D) became infected with donor *E. coli* 2442 or recipient *S. typhimurium* 576. The data from PM and swab samples (methods 2 and 3) were combined in order to calculate β. With those methods the infected birds can be identified. Swab sampling provides the best record of infection because all birds were swabbed daily. Swabbing does not always detect an infection, however, because birds may not be excreting although they are infected. *Salmonella* infections usually last several weeks, and non pathogenic *E. coli* strain can persist indefinitely. For these reasons it was assumed that once infection was detected, the bird remained

infected throughout the rest of the duration of the experiment regardless of whether infection was detected after the initial isolation.

The natural logarithm of the proportion of birds infected was plotted against time. The negative slope of this line gives the force of infection, λ. It was assumed that the force of infection was constant despite the slight increase in proportion infected throughout the experiment as groups B and D acquire infection. If the force of infection was increasing throughout the experiment (i.e. the number of individuals acquiring infection was accelerating) the gradient of Ln[proportion uninfected] against time would become more negative with time. To test this assumption the data were analysed to determine whether the relationship between time and Ln[proportion uninfected] was linear by testing for the significance of the interaction time*time (Minitab output 7.3). The gradient of the line was then determined by regression which was forced to go through 0,0 (Minitab output 7.4). This effectively means 1,0 for donors and 5,0 for recipients as these were the days on which they were administered. The negative of the gradient, λ, was divided by the average number of infected individuals over the course of the experiment to give infectiousness, β. The statistical tests performed to test for this interaction are listed in table 7.4.

Test	Transmission of population from group		Linearity	λ calculation
8, 11	A to B	Recipient *S. typhimurium* 576	Is the plot of Ln[proportion uninfected] against time linear?	What is the -gradient of Ln[proportion uninfected] against time when it is forced to go through 0,0?
9, 12	C to D	Recipient *S. typhimurium* 576		
10, 13	A to B	Donor *E. coli* 2442		

Table 7.4. Tests performed to assess the linearity of a plot of Ln[proportion uninfected] against time (Minitab output 7.3) and its gradient (Minitab output 7.4) carried out on the infection rates of the non-experimentally infected groups (B and D)

7.3 Results

Data from daily swabs and caecal content collection both from pen floor and at PM are presented in figures 7.1-7.4. Graphs are presented showing density on a $\log_{10}$ scale, but statistical analysis was carried out on Ln data. The plots of Ln [proportion uninfected] against time used in β estimation are presented in figure 7.5. No isolations of tetracycline resistant *Salmonella* (transconjugant 2, table 7.2) were made.

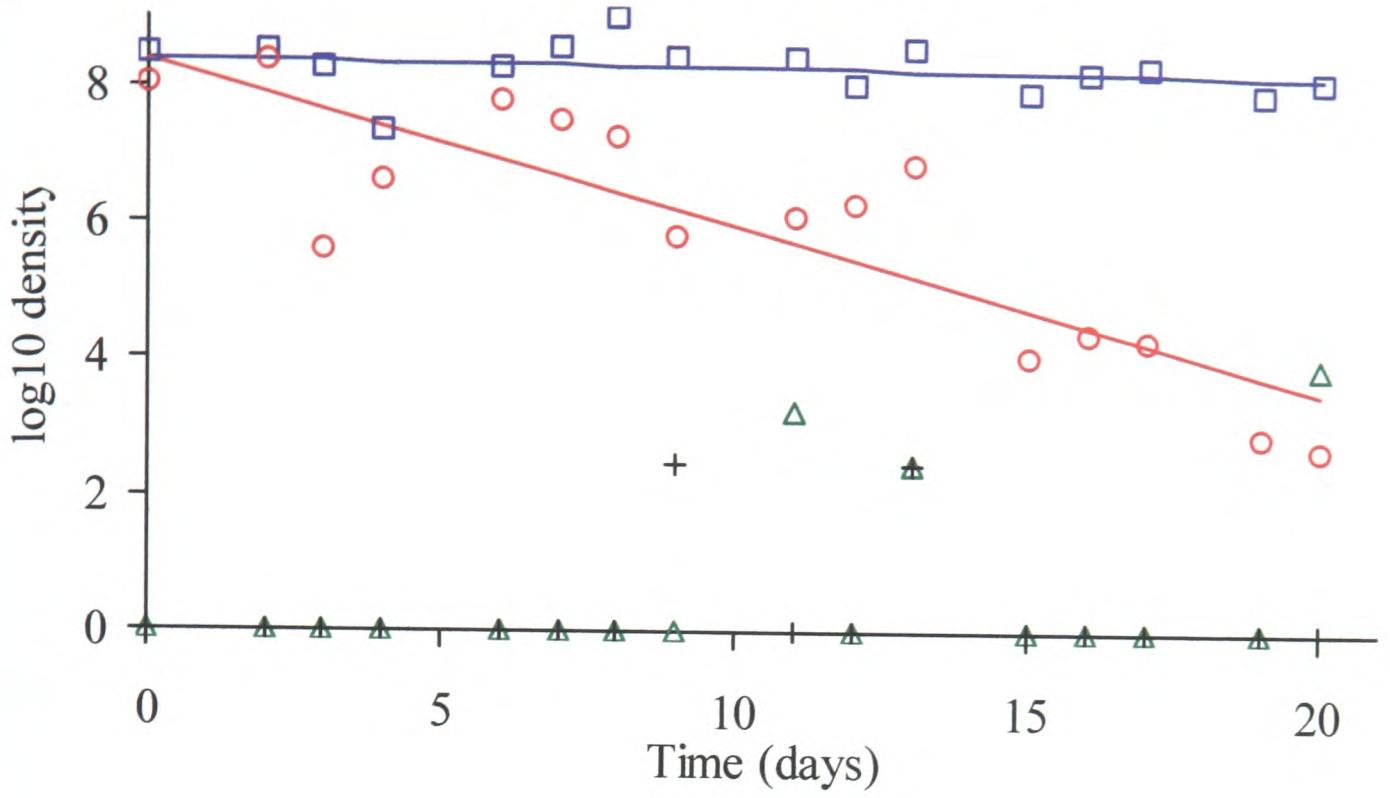
7.3.1 Isolations from spleen

Isolations of *Salmonella* from the spleen are given in table 7.5. *Salmonella* isolations were made from only five spleen samples. All the positive samples contained recipient *S. typhimurium* 576; no transconjugants were isolated from the spleen.

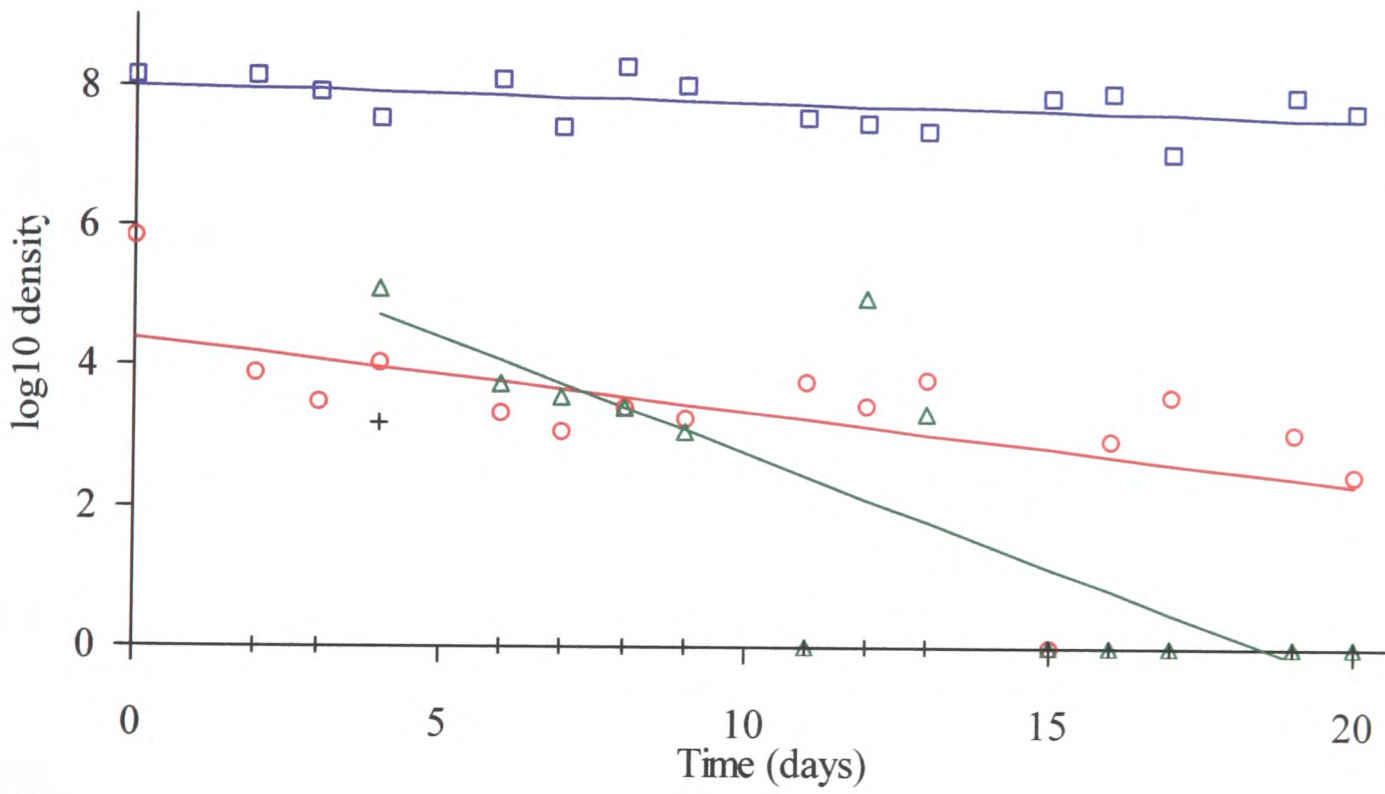
Group	Day	# Birds sampled	Positive samples in		Density in	
			spleen	caeca	spleen	caeca
A (medicated, infected)	5	5	1	0	1200	0
	10	5	1	1	1650	5×10^8
	14	5	0	0	0	0
	18	2	0	0	0	0
	21	3	0	0	0	0
B (medicated, uninfected)	5	0	*	*	*	*
	10	5	1	0	900	0
	14	0	*	*	*	*
	18	3	0	0	0	0
	21	1	0	0	0	0
C (unmedicated, infected)	5	5	0	4	0	224730
	10	4	1	2	600	712.5
	14	5	1	1	2100	333
	18	2	0	2	0	5400
	21	4	0	0	0	0
D (unmedicated, uninfected)	5	0	*	*	*	*
	10	6	0	0	0	0
	14	0	*	*	*	*
	18	3	0	0	0	0
	21	1	0	0	0	0

Table 7.5 Results of spleen sampling compared to the results of caecal sampling at PM. *Salmonella* were only ever detected in one spleen sample per group per day. The data given are the density of *S. typhimurium* found in the one positive spleen, and the average density of bacteria found in caecal contents. The caecal content data is a summary of figure 7.2. *no samples taken

a. Daily caecal counts of medicated birds (groups A and B)

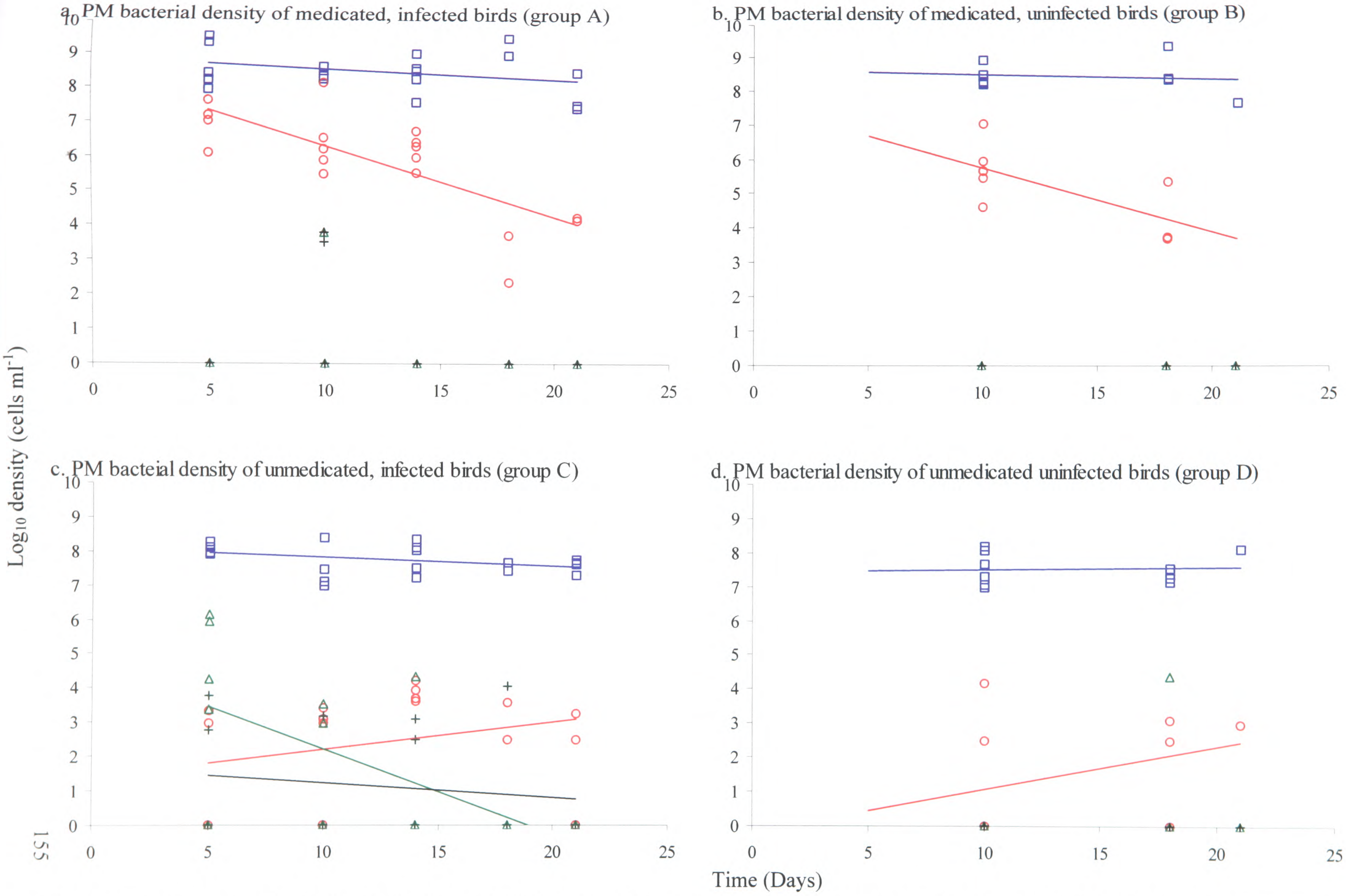


b. Daily caecal counts of unmedicated birds (groups C and D)



□ – Background *E. coli* ○ – Donor *E. coli* 2442 Δ – Recipient *S. typhimurium* 576
+ Transconjugant *S. typhimurium* 576. Lines are linear regressions.

Figure 7.1. Density of bacteria in caecal content taken daily from the pen floor. Since groups A and B were housed in the same pen (to allow transmission from A to B) it is impossible to know which of A or B any caecal dropping came from. The same applies to groups C and D.



□ – Background *E. coli* ○ – Donor *E. coli* 2442 Δ – Recipient *S. typhimurium* 576 + – Transconjugant *S. typhimurium* 576 Lines are linear regressions.

Figure 7.2. Density of bacteria in caecal content taken from chickens post mortem.

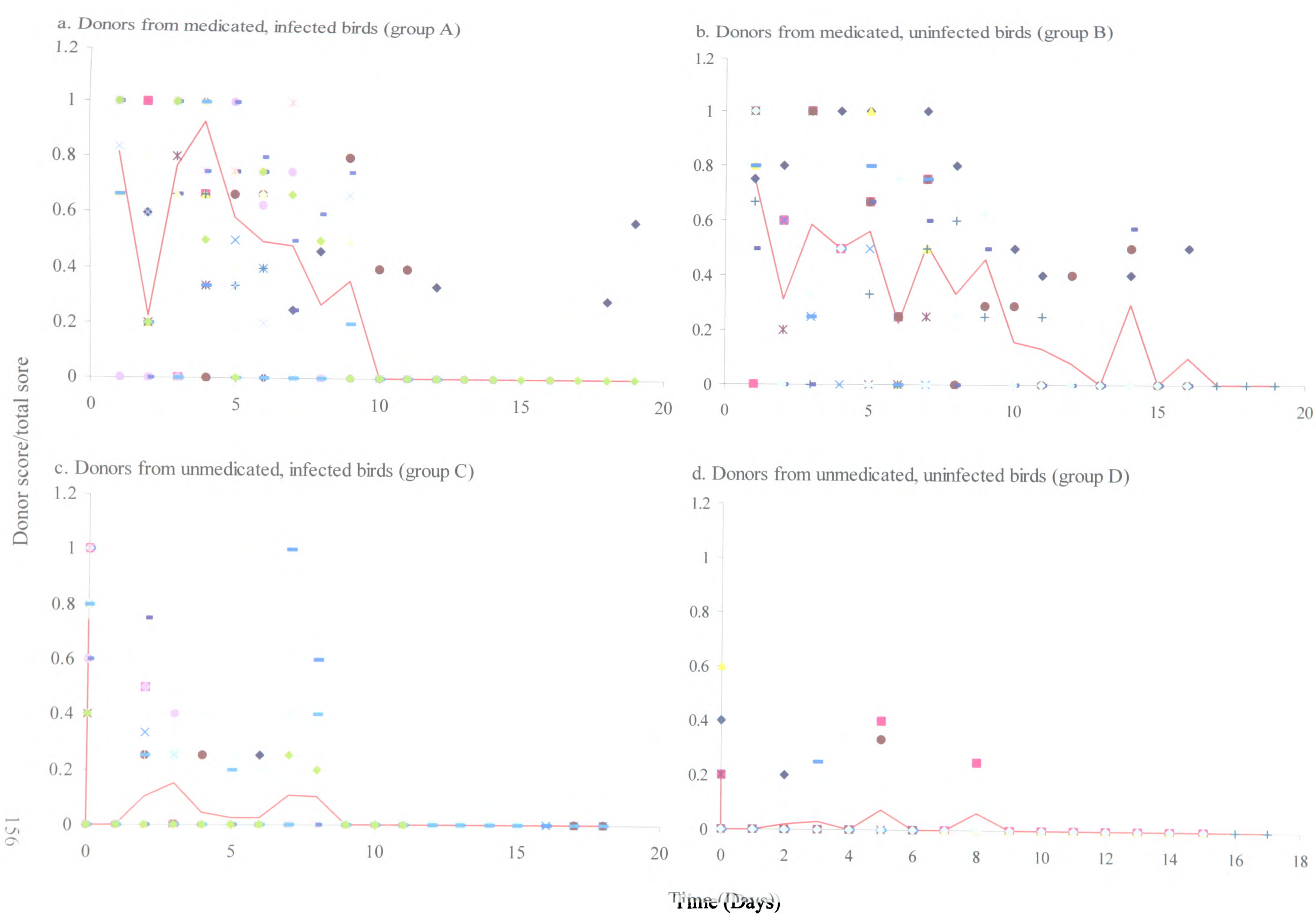


Figure 7.3. Relative abundance of donor *E. coli* 2442 on caecal swabs taken daily. Red line is the average score. Symbols represent individual birds.

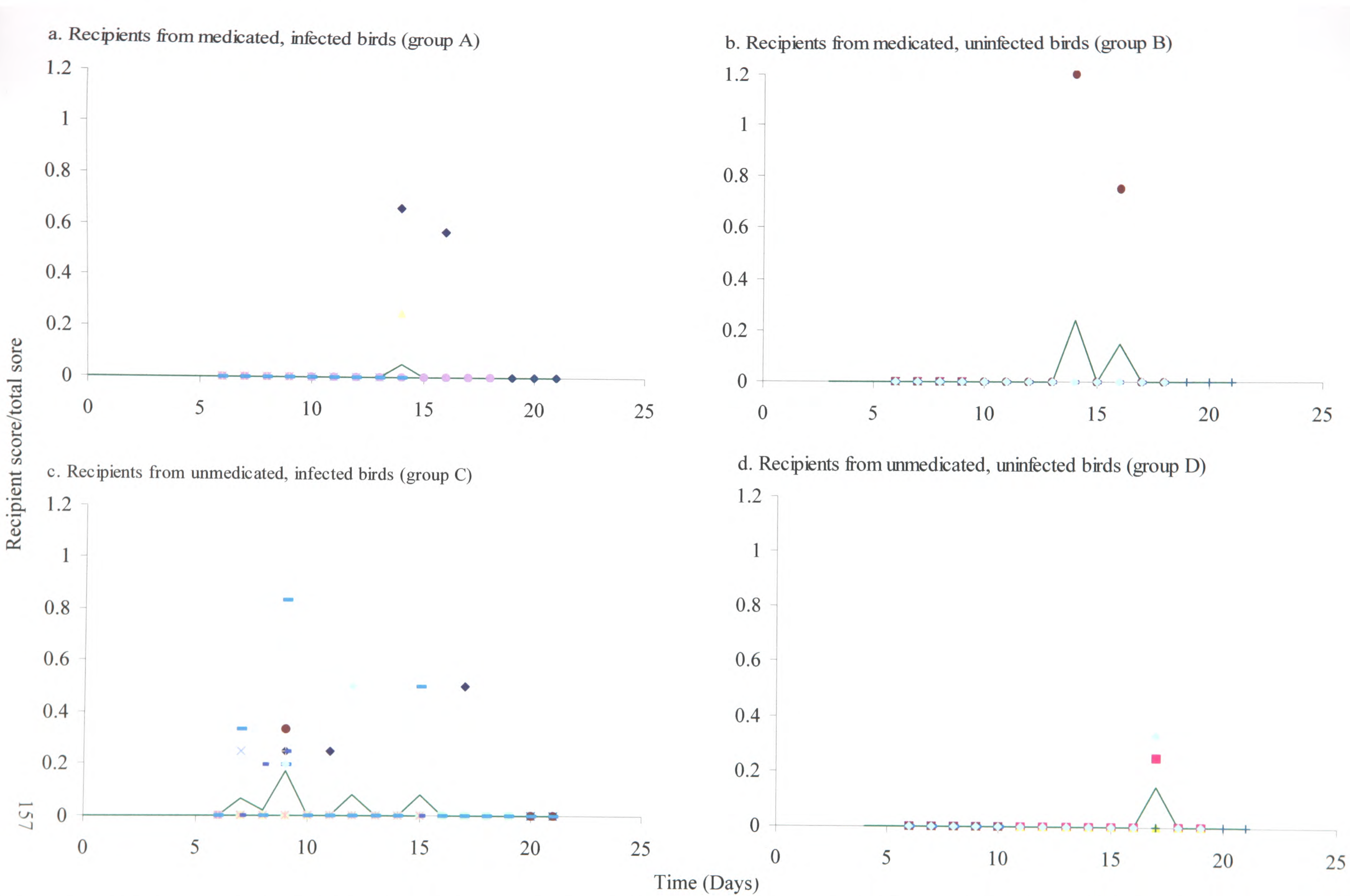
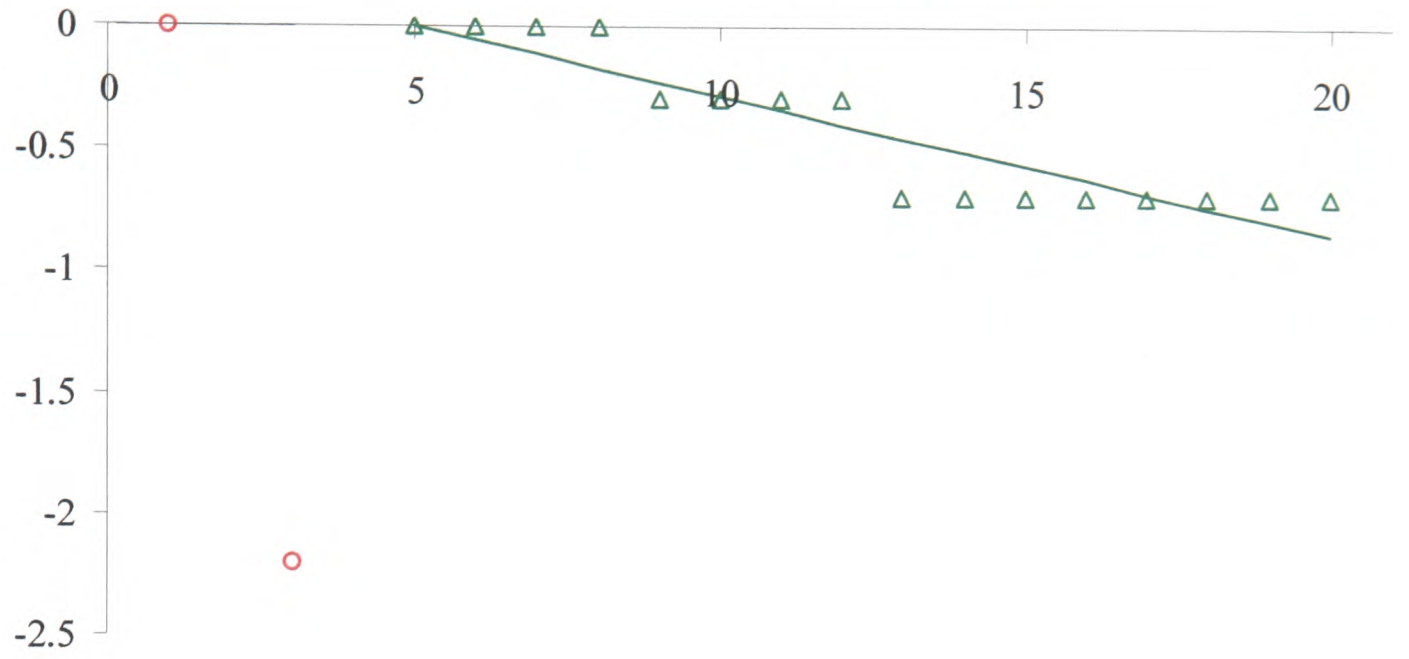
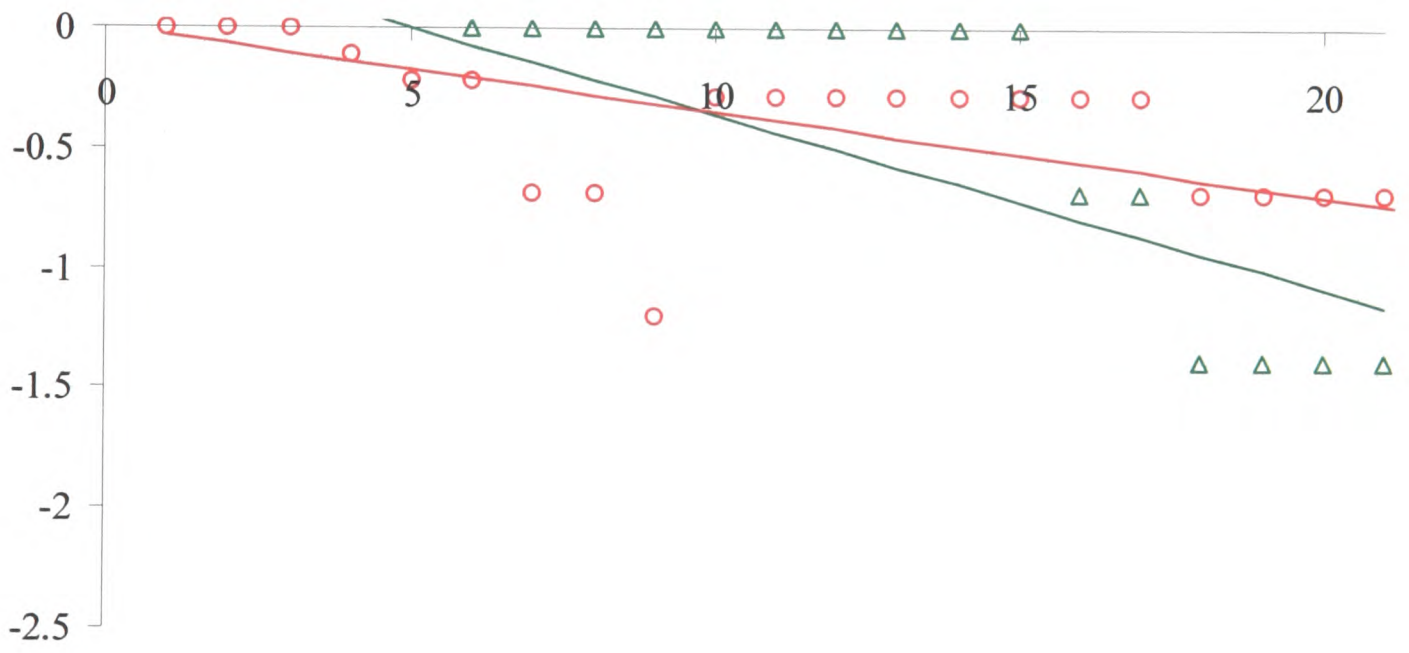


Figure 7.4. Relative abundance of recipient *S. typhimurium* 576 on caecal swabs taken daily. Green line is the average score. Symbols represent individual birc

a. Ln (proportion uninfected) in medicated, uninfected birds (group B)



b. Ln (proportion uninfected) in unmedicated, uninfected birds (group D)



o – Donor *E. coli* 2442 Δ – Recipient *S. typhimurium* 576. Lines are linear regressions

Figure 7.5. Calculation of force of infection (λ) from infection rates of the non-experimentally infected classes (groups B and D). The negative of the slope of regression of Ln [proportion uninfected] against time forced to go through 0,0 gives λ.

7.3.2 Description of results

Daily pen floor caecal samples (figure 7.1)

There was a decline in donors in both medicated and unmedicated treatments, and fewer donors were found in the unmedicated treatment. Background *E. coli* appears to remain at a constant level. Many fewer recipients were found in the medicated treatment, and they declined in the unmedicated treatment. Transconjugants were isolated in two samples from the medicated treatment and in one sample from the unmedicated treatment.

PM caecal samples (figure 7.2)

All these trends are observable in the PM samples, in addition to the following. Recipients and transconjugants were isolated from more samples and at higher densities from PM samples than the pen floor samples. Birds not experimentally infected acquired donor *E. coli* rapidly. Only one non-experimentally infected bird could be shown to have acquired recipient *S. typhimurium* 576 through sampling of caecal contents at PM; that bird was from the unmedicated treatment. Transconjugants were neither acquired by infection nor produced by conjugation within birds which were not experimentally infected to detectable levels.

Daily swabs (figures 7.3 and 7.4)

Of the trends shown by the pen floor and the PM samples the following are apparent in the daily swab results: the decline in donors; the higher density of donors in the medicated treatment; the lower density of recipients in the medicated treatment and the rapid infection by donors of the non-experimentally infected groups. In addition, these data demonstrate that the same birds are repeatedly identified as infected

throughout the experiment. Some birds score zero in-between two positive counts, indicating that excretion may be intermittent. These data have been used to assess transmission rate (section 7.3.4).

7.3.3 Analyses of caecal data (Minitab outputs 7.1 and 7.2)

The natural log transformation was found to be appropriate for all the populations because this was the weakest transformation that gave a nearly Normal histogram of residuals (there was a slight left skew, but the stronger transformation (square rooting) gave a pronounced right skew). The results of the 7 tests (table 7.3) are listed below.

Test 1. Does medication affect the density of background *E. coli*? (Figure 7.1; data in blue)

Yes, *E. coli* density is different in medicated and unmedicated birds. The average density in the medicated group was about 9.22×10^7 ml^{-1} and in the unmedicated 3.43×10^7 ml^{-1}. The difference of 0.99 in the naturally logged density implies that the background *E. coli* densities in the medicated birds were about 40% higher than in the unmedicated. This difference is significant at the $p = 0.001$ level.

Test 2. Does time or medication affect density of donor *E. coli* 2442 and is the slope of density against time affected by medication? (Figure 7.1; data in red)

The answer to each of these questions is yes. The average density of donor *E. coli* 2442 in medicated birds is 1.60×10^7 ml^{-1} and in unmedicated, 4.85×10^2 ml^{-1}. The difference of 10.4 between the Ln density in the two treatments implies that the density of donor *E. coli* 2442 in medicated birds was 33,000-fold higher than in the unmedicated group. The difference was significant ($P < 0.001$). In the medicated group donors declined at a growth rate of -0.000269 day^{-1}, and in the unmedicated,

-0.000115 day^{-1}. The difference in gradient of donor *E. coli* 2442 decline between the two treatments was significant (P = 0.003).

Test 3. Does time or medication affect density of recipient *S. typhimurium* 576 and is the slope of density against time affected by medication? (Figure 7.1; data in green).

Recipient *S. typhimurium* 576 does decline with time, but medication does not affect its density either overall or in its effect on the gradient of density with time. The average density of recipient *S. typhimurium* 576 is 5 cells ml^{-1}, and it declines at a rate of –0.00003 day^{-1}. The decline is significant (P = 0.043).

Test 4. Does medication affect the density of background *E. coli*? (Figure 7.2; data in blue)

The PM data also demonstrates that yes, medication does affect the density of background *E. coli*. The average density in the medicated group was about 19.48 cells ml^{-1} and in the unmedicated 17.78 cells ml^{-1}. The difference of 1.81 in the naturally logged density implies that the background *E. coli* densities in the medicated birds were about five-fold higher than in the unmedicated. This difference is significant at the p = 0.001 level.

Test 5. Does time, medication or infection affect density of donor *E. coli* 2442, is the slope of density against time affected by medication and is the effect of infection on density dependent on medication? (Figure 7.2; data in red)

The answer to the first four questions is yes, and the effect of infection on density is not affected by medication (interaction is not significant). The average density of donor *E. coli* 2442 in group A is 2.76 x 10^8 cells ml^{-1} in group B, 4.90 x 10^7 cells ml^{-1},

in group C 1.48 x 10^1 cells ml^{-1} and in group D, 2.63 cells ml^{-1}. The difference of 16.7 between the Ln density in the two treatments implies that the density of donor *E. coli* 2442 in medicated birds was 2 x10^7-fold higher than in the unmedicated group. The difference of 1.7 between the Ln density in the two infection statuses implies that the density of donor *E. coli* 2442 in infected birds 6-fold higher than in the unmedicated group. These differences were significant ($p = 0.05$). In the medicated group donors declined at a growth rate of –0.463 day^{-1}, and in the unmedicated it grew at 0.207 day^{-1}. The difference in gradient of donor *E. coli* 2442 decline between the two treatments was significant ($p < 0.001$).

Test 6. Does time, medication or infection affect density of recipient *S. typhimurium* 576, is the slope of density against time affected by medication and is the effect of infection on density dependent on medication? (Figure 7.2; data in green)

Medication and time affect density, and the slope of time against density is affected by medication, but infection does not affect recipient *S. typhimurium* 576 densities. The average density of recipient *S. typhimurium* 576 in medicated birds is 1.7 cells ml^{-1} and in unmedicated, 1627.8 cells ml^{-1}. The difference of 6.85 between the Ln density in the two treatments implies that the density of recipient *S. typhimurium* 576 in medicated birds was 943.9-fold lower than in the unmedicated group. This difference was significant ($P < 0.05$). In the medicated group recipient *S. typhimurium* 576 declined at a growth rate of –0.02 day^{-1}, and in the unmedicated, -0.38 day^{-1}. The difference in gradient of recipient *S. typhimurium* 576 decline between the two treatments was significant ($P < 0.025$). Note that the significance of the effect of medication on time found in these recipient data take precedence over the lack of significance found during the analysis of the pen floor samples (test 3).

Test 7. Does time, medication or infection affect density of transconjugant *S. typhimurium* 576, is the slope of density against time affected by medication and is the effect of infection on density dependent on medication?

Only infection status affects transconjugant density. The average density of transconjugant *S. typhimurium* 576 in experimentally infected birds is 5.23 cells ml^{-1} and in non-experimentally infected, 2.28 cells ml^{-1}. The difference of 0.832 between the Ln density in the two infection statuses implies that the density of transconjugant *S. typhimurium* 576 in infected birds was 43.5% higher than in the non-experimentally infected group. This difference was significant (P = 0.033).

The lack of significance are probably due to the small numbers of transconjugant *S. typhimurium* 576 isolated; see table 7.6.

N	**Population**			
Group	*E.c*	D	R	T
A (Med inf)	19	19	1	2
B (Med uninf)	9	8	0	0
C (Unmed inf)	20	13	7	7
D (Unmed uninf)	9	5	1	0

Table 7.6. Total number of positive isolations for each treatment-population combination.

7.3.4 Calculation of infectiousness, β

Test 8. Is the plot of Ln[proportion group B uninfected with recipient *S. typhimurium* 576] against time linear?

This significantly deviates from linearity. The interaction term is significant (P = 0.041) The interaction is positive, indicating a concave relationship.

Test 9. Is the plot of Ln[proportion group D uninfected with recipient *S. typhimurium* 576] against time linear?

It significantly deviates from linearity in group D also (P = 0.001). The interaction here is negative, indicating a convex relationship.

Test 10. Is the plot of Ln[proportion group D uninfected with donor *E. coli* 2442] against time linear?

There is no evidence to say this is not a linear relationship; the interaction is non – significant (P = 0.550). Neither is the test of the gradient significant, however (P = 0.210).

Despite the failure to demonstrate a significant linear relationship between Ln[proportion uninfected] and time in any case, the calculation of β was carried out. λ values were calculated as the negative of the slope of the regression of Ln [proprtion uninfected] against time (plotted on figure 7.5). β values were calculated by dividing these by the average number of chickens infected across the duration of the experiments. Chickens were taken for post mortem throughout the experiment, depleting their numbers. The average number infected was calculated e.g. for the donor transmission in the medicated group as: [number present in group A (infecteds) on day 1 + number infected in group B (uninfecteds) on day 1 + number present in group A on day 2 + number infected in group B on day 2.... + number present in group A on day 21 + number infected in group B] / 21 The calculations of recipient's λ and β were carried out only from the day they were infected, day 6. The results are given in table 7.7.

Population	Recipient *S. typhimurium* 576	Donor *E. coli* 2442	Recipient *S. typhimurium* 576	Donor *E. coli* 2442
Uninfected group	B	B	D	D
λ	0.057	2.197	0.072	0.035
β	0.0045	0.1761	0.0056	0.0027

Table 7.7. Estimates of the parameter β and λ values used to calculate them.

7.4 Discussion

7.4.1 General

The counts of background *E. coli* were higher in medicated birds than in unmedicated. This is surprising as neomycin is used to treat pathogenic *E. coli* infections in chickens. This effect may be due to the suppression of competing species of bacteria in the gut in the medicated birds, the obligate anaerobes, which may be more susceptible to neomycin than *E. coli*.

The donor *E. coli* counts of infected and uninfected birds are equivalent, whereas fewer recipient *Salmonella* were isolated from uninfected birds. This might be due to the fact that *E. coli* can reach much higher densities in the gut of a chicken than *Salmonella* (10^8 - 10^9 as opposed to 10^6 - 10^7), which would tend to increase the between-chicken transmission rate of *E. coli*. In terms of modelling, this may mean it is accurate to treat a group of chickens living in close proximity as a unit as regards their *E. coli* flora, but as individuals having separate *Salmonella* flora.

Few transconjugants were isolated. This could be due to the low numbers of recipients, which appear to be killed off by this level of neomycin before obtaining resistance. Transconjugants were undetectable from swab samples. This is probably due both to the smaller numbers collected by this method, and to the death of bacteria during exposure to the air between the chicken house and the laboratory.

The few transconjugants that were isolated occurred when recipient and donor were abundant, as would be expected. Transconjugants are created even in the absence of antibiotic selection pressure, indicating that the strength of donation is high. This is unsurprising given that these strains were selected on the basis of their donating and receiving ability.

Infection status was the only variable that was significant in the statistical tests on transconjugants; in fact transconjugants were not detected in birds which were not experimentally infected. As transconjugant production was low, excretion and therefore transmission was also low, so that birds in groups B and D were unlikely to become infected with transconjugants by ingestion.

It is interesting that no *Salmonella* were isolated on media containing nalidixic acid, novobiocin and tetracycline. Tetracycline resistance is encoded on a different plasmid to resistance to neo. Neomycin resistance was transferred in the absence of neomycin treatment, but no tetracycline resistance was transferred at all. This could be because the tet-R plasmid is less transferable (table 5.5), or that the unmedicated birds were exposed to some neomycin by being in the same room as those that were medicated, although this latter possibility is unlikely.

One problem with taking caecal samples at post mortem is that the time since caecal emptying is not known. Assuming bacterial density increases from the time of caecal refilling, the density of bacteria in the caecum will vary, as well as whether the population is in lag, exponential or stationary phase, all of which affect plasmid transfer rate. This will tend to increase the error variation of the PM samples.

7.4.2 Determination of infectiousness, β

The fact that the chicken population size underwent a stepped decline at each PM day affected the calculation of β. Birds which were not experimentally infected but which acquired infection were by chance taken disproportionately for PM, which led to a large increase in the proportion uninfected, which led to an underestimate of β (donor *E. coli* 2442, figure 7.3). As this calculation involved the infection rates of only 9 or 10 chickens per calculation, which chickens were removed at PM had a profound effect on the outcome. This effect caused the concave relationship between Ln[proportion recipient *S. typhimurium* 576 infected] in group B and time. The compounding of the data from daily swabbing and PM samples may affect outcome also as the two methods have different sensitivity. One PM caecal sample is more likely to identify an infected individual than one swab. This leads to the detection of infection on the PM day of birds which had previously been undetected, and an underestimate of β. Further problems were encountered because *E. coli* transmission from group C to Donor *E. coli* 2442 was so rapid that by day 3 all birds were infected. Analysis of infection could not be carried out on this data.

7.4.3 Comparison of methods used to detect infection

Overall, the swab method was not always successful at detecting excretion. Where birds were identified as excretors more than once, indicating a continuity of infection, there were days in-between positive samples where excretion was not detected. This is probably due to the fact that *S. typhimurium* mainly infects the caeca, so detection by cloacal swabbing may be dependent presence of caecal content in the droppings. In addition, this method did not detect transconjugants and is not quantitative enough for analysis of bacterial density in gut contents. If a full investigation into the spread of *S. typhimurium* in an experimental chicken population were to be conducted, chickens should be isolated periodically to enable collection of caecal droppings from numbered birds. This should ensure that excreting birds are not misidentified as non-excretors.

The spleen samples did provide some extra information on infection status of the birds. Some individuals which belonged to groups where no *S. typhimurium* isolations were made from the PM caecal counts on a particular day were positive for *S. typhimurium* in the spleen. However, given that spleen sampling only yielded five positive samples, it was decided that this method was not sufficiently useful to use in the main experiment. In addition, in this study of the spread of *Salmonella* and development of resistant *Salmonella* in chicken populations, knowledge of excretion is more valuable than knowledge of the severity of the disease in chickens. If animal welfare or *Salmonella* content of organs in carcasses were important, a spleen count should be made.

The PM and daily pen floor samples of caecal content proved to be the most useful indicators of infection status. Bacterial counts were lower in the samples taken from the pen floor, probably because the caecal content was less fresh and bacterial death had occurred before sampling. Nevertheless, daily sampling allows a better picture to be built up of infection status of the flock over time and isolations of recipients and transconjugants were made by this method. Daily sampling enabled detection of a significant effect of day on donor density, which the PM data failed to reveal.

It is not worth plating on nalidixic acid, novobiocin + tetracycline media when neomycin is the antibiotic fed to the birds, as no isolations were made by this method. It is important, however, to increase the sensitivity of the isolation method. The first dilution of the caecal content plated should be only just enough to allow droplet measurement of volume, in order that lower densities of bacteria can be detected. More drops should also be plated where the density of the target population is likely to be low. These alterations should ensure that more isolations of recipients and transconjugants are made.

7.5 Summary

7.5.1 Summary of results

1. *E. coli* transfer between chickens is extensive; donor counts between infected and non-infected birds were indistinguishable. This suggests the chicken population should be treated as a unit when modelling an *E. coli* infection.

2. The *Salmonella* transfer to the non-infected birds occurred at a low rate such that there were significant differences between the two infection groups. This indicates that a model incorporating β would be appropriate for *Salmonella*, rather than treating chickens as a unit for infection with this bacterium.
3. Donor *E. coli* 2442 declined in the chickens over the three weeks of the experiment whereas background *E. coli* remained at about the same density. It may be concluded that the donor is less fit than the background flora in this environment; this may be due to resistance plasmid carriage, or the fact that donor *E. coli* 2442 has been grown in the lab for several generations.

7.5.2 Answering the questions posed in section 7.1

1. Do the strains previously used in lab experiments survive in chickens?

They do survive in chickens, although they decline over the experimental period of three weeks. It appears that they could have survived for a few weeks longer in these chickens.

2. What is their infectiousness from host to host (β)?

The infectiousness of these strains of bacteria in this flock has been quantified, although with little data the error is large, and estimations from this small experimental flock cannot be applied to large commercial chicken flocks whose transmission rates would be expected to be greater.

3, 4 and 5. Are transconjugants produced *in vivo* by these donor and recipient strains, are they produced at the normal therapeutic level of neomycin, and how does the duration of the experiment affect transconjugant production?

Transconjugants were produced when no antibiotic is present and when neomycin dose was at the normal therapeutic level. Although relatively few samples were found to contain transconjugants, they were isolated throughout the duration of the experiment. The main experiment could therefore be conducted on the same or a longer time-scale and expect transconjugant isolation, especially if the sensitivity of detection is increased.

8. Determination of the dose of neomycin which maximises transconjugant production *in vivo*

8.1 Introduction

The within-host model described above predicts that there will be an intermediate antibiotic dose at which the production of transconjugant *S. typhimurium* 576 will be maximised. The reason for this is that at low doses, the transconjugant *S. typhimurium* 576 will be at a selective disadvantage over the recipient *S. typhimurium* 576, and will be outcompeted. Conversely, at very high doses of antibiotic, sensitive bacteria will decline and become extinct so that densities of recipient *S. typhimurium* 576 are never high enough to allow transconjugant production.

This dose-response relationship occurs only over particular time and dose ranges (figure 5.16). To test this model prediction, chickens were inoculated with donor *E. coli* 2442 and recipient *S. typhimurium* 576, and administered neomycin at various levels in their feed. Neomycin is an antibiotic which is commonly used to treat *E. coli* enteritidis in chickens (NOAH 1998). The density of the transconjugants produced was then measured over time. The model predictions would be supported if most transconjugant *S. typhimurium* 576 were found in chickens which had been fed an intermediate level of neomycin.

This full-scale experiment builds on other experiments: the *in vitro* equivalent (chapter 9) and a pilot *in vivo* experiment (chapter 7). The *in vivo* pilot indicated that: 1. caecal sampling is preferable to spleen sampling or swabbing; 2. transconjugant *S. typhimurium* 576 are produced at neomycin doses of 0 and 225 g $tonne^{-1}$; 3. transconjugant *S. typhimurium* 576 are found *in vivo* one day to two weeks after inoculation of recipients and 4. transconjugant *S. typhimurium* 576 are produced at low densities and sensitive methods of detection are therefore preferable. These findings have been used to direct the methods of this main experiment. Samples were taken from caecal content only; the range of antibiotic doses chosen was 0 to 250 g $tonne^{-1}$; caecal contents were sampled 1 – 18 days after infection and ten drops of the first dilution were used for counting, lowering the cut off for detection to 30 cfu ml^{-1} caecal content. Undiluted caecal content is too viscous to measure into drops itself.

Extrapolation from *in vitro* to *in vivo* requires information on the antibiotic concentration in caecal content. To find nutrient broth concentrations of antibiotic (measured in $\mu g\ ml^{-1}$) equivalent to *in vivo* concentrations when chickens are fed a certain concentration in feed (measured in g $tonne^{-1}$), the percentage of water in caecal content and any dilution or concentration of neomycin occurring in this region requires quantification. Published estimates (Thornburn and Wilcox 1964) were found to be inadequate (estimates refer to different chicken strain and age), so empirical estimations were made using the caecal content of these chickens (chapter 6).

Given that antibiotic resistance is prevalent in many bacteria (Report 1999), it was thought advisable to test the background flora of the chickens for resistance to antibiotics used in the isolation of donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576. This was carried out on MacConkey's

medium (selective for enterobacteria) with antibiotic discs to identify colonies which might be confused with laboratory strains. The likelihood of identifying false positives can thus be estimated.

8.2 Materials and Methods

8.2.1 Experimental design

Twenty *Salmonella*-free three-week-old Light Sussex chickens were housed in each of four cages in one room. Their cages were suspended above a roll of plastic to facilitate sample collection. Three days after housing, the caecal content of the birds was sampled for antibiotic resistance tests, their feed was supplemented with 0, 64, 160 and 250 g tonne^{-1} of neomycin respectively and the birds were all infected with 0.3ml of overnight shaking culture of donor *E. coli* 2442. Five days later they were infected with the same amount of recipient *S. typhimurium* 576.

8.2.2 Antibiotic resistance testing of background flora

Five samples of caecal droppings were taken from each cage before the infection of the birds with the experimental bacterial populations. These were diluted into PBS (phosphate buffered saline) and this suspension was tested for bacteria expressing resistance to neomycin, ampicillin, tetracycline and nalidixic acid using antibiotic discs and the standard method (chapter 3).

8.2.3 Sampling

Caecal contents were sampled in two ways throughout the experiment:

1. PM samples.

   Five birds were taken for PM (post mortem) examination from each group at 5, 10, 15 and 20 days after the start of the experiment. One caecum of each chicken was removed and the contents transferred to a universal bottle within one hour of death.

2. Pen floor samples.

   Caecal samples were taken every 1-4 days from the pen floor 1-2 hours after winding on plastic and removing feed, but not on days on which PM samples were taken. Five were taken when caecal droppings were numerous enough; towards the end of the experiment the few birds left did not always produce five during this time. Caecal samples were taken as far away from each other on the plastic as possible to reduce the frequency of samples from one day being taken from the same bird, thus minimising non-independent samples.

8.2.4 Preparation

Samples were weighed and diluted 1:9 by weight with chilled (4^0C) PBS. Glass beads of 2-4 mm diameter were added and the mixture shaken with a laboratory bench rotary shaker to fully suspend the caecal content. This was not always possible and the first dilution was frequently slightly 'lumpy'. This increases the error variation of the counts. Six further decimal dilutions were made, and one 0.033 ml drop of each dilution was placed onto each of the four plate types with a Pasteur pipette. Plates were either selective for either all enterobacteria (MacConkey), donor *E. coli* 2442 (MacConkey containing neomycin and tetracycline), recipient *S. typhimurium* 576 (Brilliant Green containing nalidixic acid and novobiocin) or transconjugant *S.*

typhimurium 576 (Brilliant Green containing nalidixic acid, neomycin and novobiocin). The drops on the plates were thoroughly dried in under a lamp before incubation. The process from sampling to drop drying took a maximum of three hours. This interval was recorded and tested for any significant effect of processing time. None was found (data not shown). Unplated samples were kept at 4^0C when possible to prevent *ex-vivo* division of bacteria. After overnight incubation of the plates at 37^0C the colonies arising from individual bacteria were counted on the lowest dilution at which they could be resolved.

8.2.5 Testing colonies

Several problems were found identifying colonies based on their ability to grow on one type of selective plate:

1. Approximately 0.01% of the background *E. coli* present in the caeca of two birds on day one, before any experimental infection, was already resistant to one or more antibiotics used in isolation of lab strains (table 8.1).
2. When large numbers of sensitive organisms are present they can 'force through' onto antibiotic plates, i.e. sensitive colonies grow where they should have been killed; similarly, *E. coli* can force through at lower dilutions in small numbers onto brilliant green plates, which normally prevent *E. coli* growth.
3. Some birds at the IAH's PPU were at the time infected at low levels with an unknown organism whose colonies look like *Salmonella* and can grow on brilliant green plates.

Slide agglutination tests (section 3.10) were carried out in order to unequivocally identify the colonies as *S. typhimurium*, *E. coli* or something else. In some cases, these were difficult to interpret, so replica-plating was employed (chapter 4) to transfer all

colonies to plain MacConkey plates, and MacConkey plates with 100 μg ml^{-1} ampicillin added. In this technique, sterilised velvet is held taut over a metal cylinder just smaller in diameter that a Petri dish. Plates on which there are colonies are pressed onto the velvet, followed by clean plates of different selectivity. In this way, individual colonies can be examined on different plates and a count can easily be made of the colonies of interest. Velvetting allowed better differentiation between the unknown organism, *Salmonella* and *E. coli*. All the *Salmonella* colonies were further checked by resistance profiling (chapter 3) to differentiate between recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576. The plates containing ampicillin were themselves tested for selectivity by putting a loop of donor *E. coli* 2442 and recipient *S. typhimurium* 576 on 25 ampicillin-containing MacConkey and 25 plain MacConkey plates used for testing. Both could grow on plain plates but only donor *E. coli* 2442 could grow on ampicillin-containing MacConkey, as would be expected.

8.2.6 Statistical tests

Each type of bacterium (background *E. coli*, donor E. coli 2442 and Recipient S. typhimurium 576) was analysed separately for the effect of day and neomycin dose on log_{10} count. Too few transconjugant *S. typhimurium* 576 were found to analyse. The pen floor samples are non-independent with respect to time, so they were not analysed. The following Minitab commands were used on each bacterial population of the PM data:

GLM 'Log Density' = Day + 'Neomycin Dose';
SUBC> covar Day 'Neomycin Dose'.

then

GLM 'Log Density' = Day + 'Neomycin Dose' + Day * 'Neomycin Dose';

SUBC> covar Day 'Neomycin Dose'.

The first command tests for the main effects of neomycin dose and time; whereas the second tests for interactions between neomycin dose and time.

Significance was taken to mean $P<0.05$.

8.3 Results

8.3.1 Resistance testing of background flora samples

Caecal sample	Resistances present in caecal content from pens dosed with: 0g tonne^{-1}	64g tonne^{-1}	160g tonne^{-1}	250g tonne^{-1}
1	Neomycin (pt) Nalidixic acid (pt)	Nalidixic acid (pt)	Ampicillin Neomycin (pt)	Ampicillin Nalidixic acid (pt)
2	Ampicillin Neomycin (pt) Tetracycline Nalidixic acid (pt)	Ampicillin Nalidixic acid (pt)	Ampicillin Neomycin (pt) Nalidixic acid (pt)	Ampicillin Nalidixic acid (pt)
3	Ampicillin Neomycin (pt) Tetracycline Nalidixic acid (pt)	Ampicillin Nalidixic acid (pt)	Ampicillin Nalidixic acid (pt)	Ampicillin Nalidixic acid (pt)
4	Ampicillin Neomycin (pt) Tetracycline Nalidixic acid (pt)	Ampicillin Neomycin (pt) Nalidixic acid (pt)	Neomycin (pt) Nalidixic acid (pt)	Ampicillin Nalidixic acid (pt)
5	Neomycin (pt) Nalidixic acid (pt)	Tetracycline Nalidixic acid (pt)	Nalidixic acid (pt)	Nalidixic acid (pt)

Table 8.1. Resistances found in background populations of bacteria isolated from caecal content before chickens were inoculated with laboratory strains. pt = partial resistance.

Each antibiotic that was used in the test was found to have some colonies that were partially or fully resistant to it. An accurate quantification of the proportion of

colonies which were resistant was not carried out; but around 10 colonies were found to be resistant to each antibiotic per plate. The method of dilution and plating used means that resistant bacteria can be estimated to occur at a frequency of the order of 0.01%. The bacteria were not formally identified, but they looked like *E. coli* on MacConkey plates, and could be mistaken for them. The concern was that these background bacteria could have grown on plates used for the isolation of donor *E. coli* 2442, which contain neomycin and tetracycline. One sample was fully tetracycline resistant, and many were partially neomycin resistant. None of these colonies could grow on the donor isolation plates as sampled on this day; and unless the neomycin resistance increased and became associated with tetracycline resistance in one individual, was unlikely to cause problems with misidentification as donor *E. coli* 2442.

8.3.2 Background *E. coli*, donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576 population dynamics.

The data are illustrated in figure 8.1. Transconjugant *S. typhimurium* 576 were isolated from only two samples. The hypothesis that transconjugant production peaks at intermediate levels of antibiotic can not therefore be tested. The two transconjugant-containing samples were from the pen floor samples of the two chicken groups fed the highest amounts of antibiotic (i.e. the 160 and 250 g tonne^{-1} groups). More than one cfu (colony forming unit) was isolated from each sample; five were found in the 160 g tonne^{-1} sample and 28 in the 250 g tonne^{-1} sample. Visually, the background *E. coli* and donor *E. coli* 2442 appear to maintain their density (donor *E. coli* 2442 at about seven orders of magnitude lower than the background *E. coli*), whereas the recipient *S. typhimurium* 576 decline throughout the experiment.

The Minitab output of the tests can be found in the appendix to this chapter. Test 1 showed that background *E. coli* was affected by both day and neomycin dose. Background *E. coli* declined with time but increased with neomycin dose. Donor *E. coli* 2442 was unaffected by the passage of time and the dose of neomycin to which it is exposed (test 2). Recipient *S. typhimurium* 576 declined with time and was negatively affected by the dose of neomycin (test 3). Using the second statistical test described above on PM data, interactions were found between day and neomycin dose on recipient *S. typhimurium* 576 (test 4) but not on background *E. coli* (test 5). This is borne out by a visual inspection of the data; at the two highest levels of neomycin the trendlines are horizontal, whereas recipient *S. typhimurium* 576 were fairly prevalent at the start of the experiment in the other two groups but quickly decline.

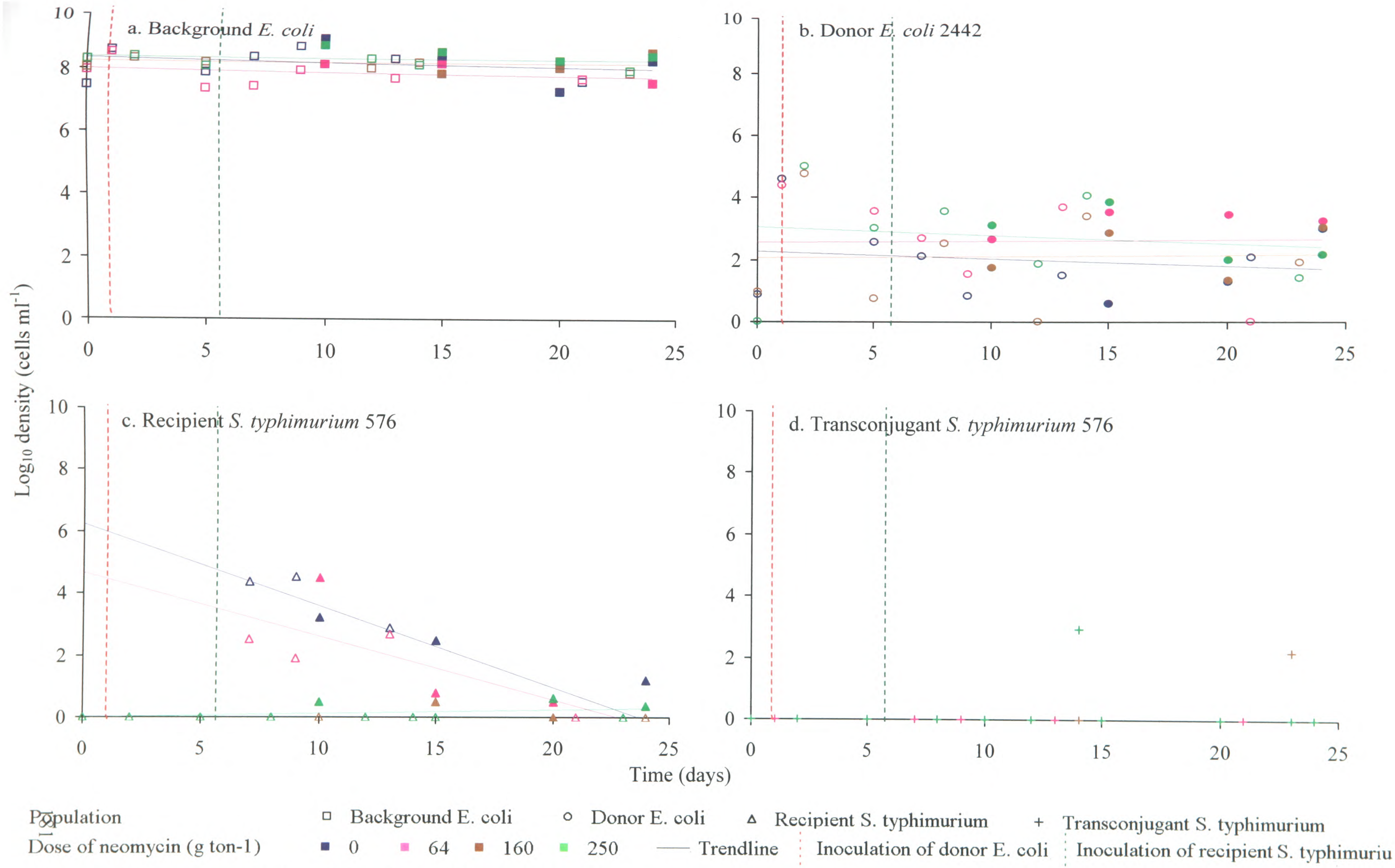


Figure 8.1. Counts through time of densities of different bacterial populations isolated from chicken caecal content. Open symbols represent data collected from the pen floor; filled symbols indicate post mortem samples. Datapoints in a. b. and c. are averages of one to five counts; points in d. display the values of the two counts obtained. The trendlines are used for visual purposes only and are not the result of statistical tests.

8.4 Discussion

The virtual absence of transconjugant *S. typhimurium* 576 is remarkable given three observations: 1. *in vitro* experiments performed to choose strains based on high transmission, this donor and recipient produced transconjugants of the order of 1% of the density of parents after 24 hour incubation; 2. the transconjugant has an *in vitro* growth rate not much less than that of the recipient (4%); 3. many more than two samples containing transconjugant *S. typhimurium* 576 were isolated during the pilot experiment. The discrepancy between transconjugant isolation in this experiment and the pilot may be due to the differences in establishment of donor *E. coli* 2442 and recipient *S. typhimurium* 576 in each case. Donor *E. coli* 2442 and recipient *S. typhimurium* 576 achieved population densities of about one order of magnitude higher in the pilot experiment. Given the *in vitro* production of transconjugant *S. typhimurium* 576 of 1%, the densities of donor *E. coli* 2442 and recipient *S. typhimurium* 576 established *in vivo* would be expected to produce transconjugants on the borderline of detection by these methods. The fact that the transconjugant *S. typhimurium* 576 did not increase their density, and were apparently cleared from whichever birds they were produced in before the bird was sampled post mortem, might have been due to increasing immunity. Immune clearance or colonisation failure may account for the fact that recipient *S. typhimurium* 576 declined throughout the experiment. *S. typhimurium* declined even in the untreated birds, in both this experiment and the pilot, which suggests that antibiotic is not necessary for clearance. The conditions were as similar as possible between each of the *in vivo* experiments, so the discrepancy in donor and recipient density could be due to uncontrollable factors. As the chickens used were not inbred, variation in the immunity of the birds to

Salmonella could explain the inconsistency. Additionally, the isolation procedures were slightly less rigorous in the pilot experiment, so some of the bacteria counted as transconjugant *S. typhimurium* 576 may have been misidentified. Other workers have found *in vivo* resistance plasmid transfer difficult to demonstrate, and when transconjugants are found, it is usually at low densities (Balis et al 1996, Corpet 1996, Licht et al 1999, Nijsten et al 1995, Petrocheilou et al 1976, Platt et al 1986, Rang et al 1996).

As the two samples from which transconjugant *S. typhimurium* 576 were isolated came from the two groups which were fed the most antibiotic, the result of the experiment is not inconsistent with the model describing a convex relationship between dose and transconjugant production. However, the dose at which peak transconjugant production occurs cannot be determined. As the density of transconjugant *S. typhimurium* 576 was higher in the sample from chickens fed the highest level of antibiotic, the peak may be outside the range of the current experiment. The fact that transconjugants were formed at the therapeutic dose of neomycin (250 g tonne^{-1}) is of concern.

Neomycin appears to kill both recipient *S. typhimurium* 576 and background *E. coli*, as recipient *S. typhimurium* 576 is strongly affected with dose and background *E. coli* declines with time. The role of background flora may be significant in the establishment of donor *E. coli* 2442. Counts of background *E. coli* were higher in chickens fed more neomycin according to the analysis of the PM data. This is probably due to the reduced competition from the gut anaerobes, which may be more

susceptible to neomycin than *E. coli*. The donor *E. coli* 2442 could presumably be shown to be affected in the same way if more data were available.

Table 8.1 indicates that antibiotic resistance was widespread in the background *E. coli* (or other bacteria able to grow on MacConkey plates) present in the caeca of the uninfected chickens. It is striking that most resistance occurs in the undosed (0 g tonne^{-1}) group. All birds were undosed prior to sampling for background resistance testing, and neither were the results of the background resistance tests were known before dosing; so the relative susceptibility of the background flora of the dosed birds is a co-incidence. It is a fortunate co-incidence, because the relative rarity of neomycin resistant background flora of dosed groups should mean that the neomycin used in the experiment should not cause much evolution of fully resistant bacteria. The combination of tetracycline and neomycin resistance was not found in any one sample. Two samples containing *E. coli* able to grow on plates containing neomycin and tetracycline were isolated from the first dilution one day before inoculation of donor *E. coli* 2442. This is probably a result of partially resistant or susceptible bacteria forcing through when present at high density, and this low-level growth on plates should have been masked by donor *E. coli* 2442 present at higher densities once donors were established in the caecal contents. The partially resistant isolates could not grow on selective plates, and flora was not exposed to antibiotic other than neomycin before being plated, making resistance evolution during the course of the experiment unlikely.

Concerns have been raised that transconjugants can form due to the occurrence of mating on the selective plates after sampling has occurred (Walter et al 1991). The

fact that several transconjugant colonies were isolated from each of the two samples, whereas none were isolated on other plates, suggests these transconjugants were formed *in vivo*. Post-sampling mating should not occur in this experiment because nalidixic acid was included in the sampling plates, which was found to eliminate conjugation (Burman 1977b).

The adequacy of the model must also be questioned. Sensitive *Salmonella* (recipients) were able to survive even at the highest dose of neomycin given to chickens in this experiment, which is higher than the clinical level of 225 g tonne^{-1}. It is possible that the level at which peak production lies is substantially higher than the 250 g tonne^{-1} concentration. It is also possible that the peak required more time in which to develop. *S. typhimurium* infection of chickens may last from 3 to 8 weeks (Barrow & Duchet-Suchaux 1997), so the experiment may have been more successful if the infection had been followed through to complete clearance. This is unlikely, however, due to the downward trend in *S. typhimurium* mentioned above. The accuracy of the predicted dose-response relationship cannot be judged using these data. The role of background flora in modifying the effect of antibiotic on the bacterial populations of interest appears to be important and has not been included in the model. Antibiotics kill gut anaerobes thus making nutrients more available in the gut (it is this action which allows the use of antibiotics as growth promoters in farm animals), and if other gut bacteria are relatively sensitive to the antibiotic this allows them to increase in density.

This experiment can unfortunately shed no light on the relationship between dose and transconjugant production. Few transconjugant *S. typhimurium* 576 were produced in

these chickens probably because the density of parent bacteria was low. Parental density could be increased by inoculating more bacteria and/or by using younger chickens, whose gut flora is not as highly developed. It might also be advisable to enrich caecal content samples for transconjugant *S. typhimurium* 576 by growing them with antibiotics to which they are resistant to in nutrient broth overnight. This would give an indication of the number of samples containing transconjugant *S. typhimurium* 576 at a level below that which quantitative methods can detect.

It would also be interesting to carry out the same experiment using different antibiotics, particularly those which are more lethal to *S. typhimurium*. The peak of transconjugant production might then be reduced to a level below the therapeutic dose and a convex dose-response relationship might be measured.

The inclusion of a growth promoting effect (the reduction of some components of background flora by antibiotic) in a predictive model would make it significantly more complex, and its inclusion should only be contemplated if the sensitivity of the conjugating bacteria involved made the model inadequate without it.

9. Exploring the effect of dose ranging *in vitro*

9.1 Introduction

The experiments detailed in this chapter consist of growth of donor *E. coli* 2442, recipient *S. typhimurium* 576 and their production of transconjugants in laboratory medium and *ex vivo* caecal content at various doses of neomycin and other antibiotics. The aim of this work was to determine whether a threshold dose of antibiotic exists above which the production of transconjugants declines. If so, doses of antibiotic given to animals and humans should be tailored to exceed this threshold. As antibiotic dose increases from zero, the production of transconjugants is expected to increase for three reasons. The first is that antibiotic resistant transconjugants gain a competitive advantage over non-plasmid bearing (antibiotic sensitive) recipients as antibiotic dose increases. The second is that potential donors gain a competitive advantage over background flora and increase their density, allowing more encounters with recipients, leading to more conjugation and formation of new transconjugants. The third is that donors increase relative to recipients as antibiotic increases from zero, and the maximum transconjugant counts are produced when donors and recipients are equally abundant. This last reason is why the model simulation shows this increase (figures 5.14-5.16). Counteracting this increase is the negative effect upon growth of potential recipients as dose increases. Depending on which process becomes significant first, either a decline of transconjugant production from zero dose is expected (figure 5.20), or a rise and then a decline. (figure 5.17) These

experiments were conducted to determine which of these situations arise, and if it is the latter, what is the threshold of antibiotic above which transconjugant production declines. Dose ranging was carried out *in vitro* in addition to *in vivo* primarily to ascertain what levels of antibiotic the groups of chickens should be given in the *in vivo* experiment, and secondarily as an inexpensive, easily repeatable alternative to the *in vivo* model testing.

9.2 Materials and methods

9.2.1 LB dose ranging

Three drops (0.1 ml) of overnight shaking culture of donors and recipients were co-inoculated into 10ml LB nutrient broth, resulting in an initial concentration of the order of 10^7 bacteria ml^{-1}. The universal tubes were incubated statically at 37^0C for 25 hours. Measured amounts of antibiotic solution (neomycin, ampicillin, tetracycline or chloramphenicol) were added to the LB broth after four hours of incubation which allowed populations to become established. The experiments were carried out on three different days. On the first, the MIC of the recipient for the antibiotic was taken as a mid-point of the range of concentrations; when this did not demonstrate a convex relationship, later concentration ranges included much larger amounts of antibiotic.

Sampling of populations was carried out at 0, 2, 18 and 21 hours post-dosing during the experiments on neomycin dose ranging. This demonstrated a convex relationship only after 21 hours, so the experiments involving other antibiotics were only sampled after this

time. Density estimations and media used were as in section 3.5 except for the tetracycline and chloramphenicol transconjugants. As resistance to these antibiotics is carried on a different plasmid to neomycin, these were counted on nalidixic acid + tetracycline plates.

9.2.2 Dose ranging in caecal content

Caecal content was used as a growth medium within one week of its removal from uninfected, unmedicated chickens at post mortem. It was kept sealed and chilled at 4^0C before use. Background flora of a sample from each caecum was enumerated on MacConkey's media and tested for antibiotic resistance likely to interfere with results (see section 3.6 for method). No resistance was found likely to interfere with isolation of populations of interest. Donors and recipients were added to give a density of approximately 10^7 colonies ml^{-1} each and it was diluted 1:1 w/w with PBS to allow droplet measurement of volume. Neomycin was added after 4 hours of incubation Six mixtures per day were incubated at 42^0C in a water bath and sampled hourly for eight hours. Initial neomycin concentrations spanned those which appeared to indicate a covex relationship in nutrient broth medium. Controls were diluted with distilled water at the same time and to the same extent as the other samples.

9.2.3 Statistical analysis

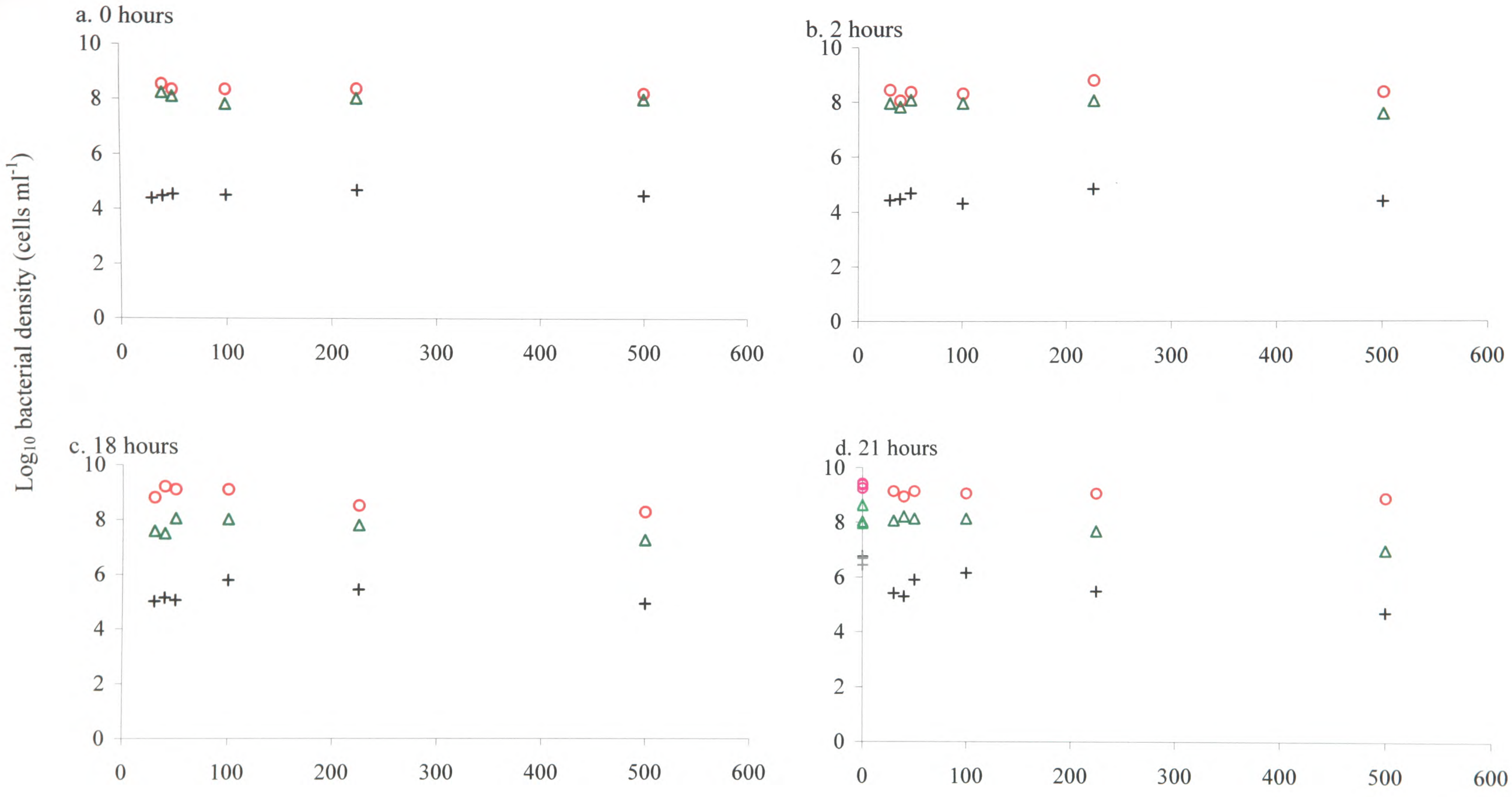
Donor and recipient densities were tested for a linear relationship with dose, and transconjugant densities were tested for a linear or a curved relationship. The latter was

achieved by including a dose*dose term in the GLM command, which tests for a change in direction of the relationship between the two variables as the dose increases.

9.3 Results

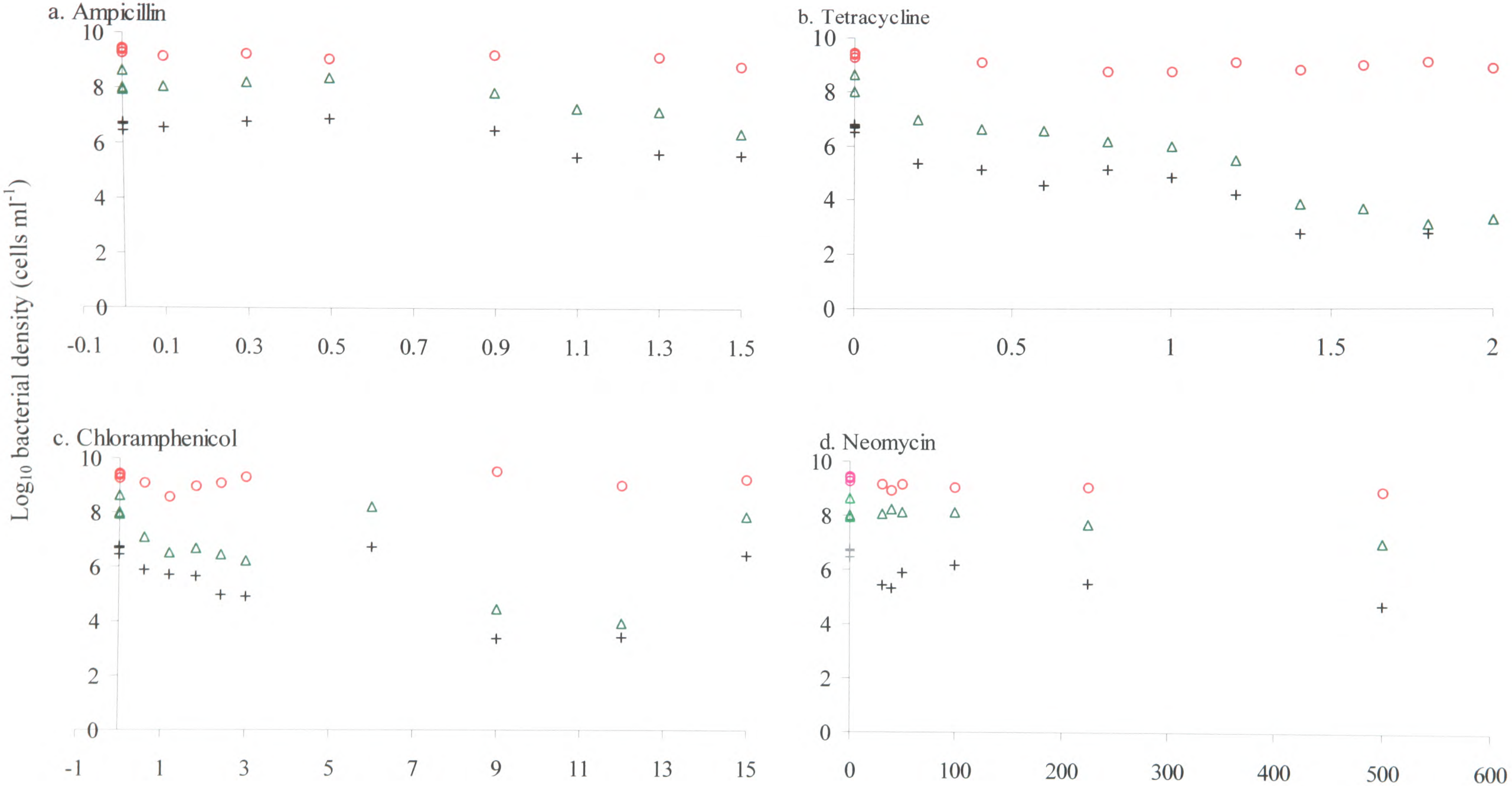
The density of donor *E. coli* 2442, recipient *S. typhimurium* 576 and transconjugant *S. typhimurium* 576 is plotted against dose of antibiotic in figures 9.1-9.3.

Figure 9.1 shows that a relationship between dose and transconjugant production does not become apparent until 18 hours after antibiotic is added to the cultures, and that this relationship becomes stronger at 21 hours. Following this result, the caecal content and other antibiotic experiments (figures 9.2 and 9.3) were only sampled at 21 hours post dosing. Of the four antibiotics tested, those populations dosed with neomycin and ampicillin show a slight convex relationship between dose and transconjugant production; transconjugant production is maintained at levels close to the control when dosed with tetracycline until levels reach 1.4 $\mu g\ ml^{-1}$, and increasing chloramphenicol dose causes a steady decline in transconjugant production. As neomycin dose increases in caecal content, donor levels rise, recipient levels do not alter much and neither do transconjugant levels. It is apparent from all three sets of data that transconjugant levels follow recipient levels fairly closely, with transconjugants typically being 1-2 orders of magnitude less dense than recipients are.



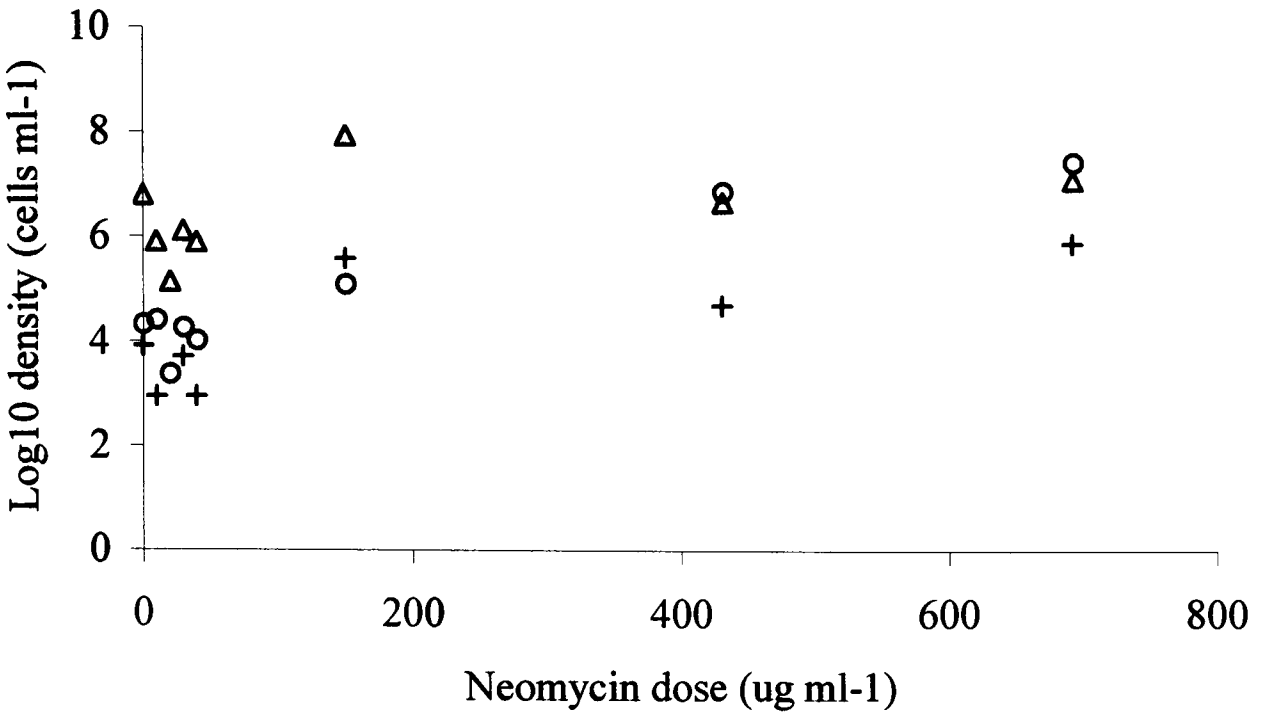
○ Donor *E. coli* 2442 △ recipient *S. typhimurium* 576 + transconjugant *S. typhimurium* 576.

Figure 9.1. Density of donor *E. coli* 2442 and recipient *S. typhimurium* 576 co-grown in a water bath at 37^0C for 4 hours in the absence of antibiotic and for the stated times with antibiotic, and density of transconjugant *S. typhimurium* 576 produced by the parent cells and by division. The 21 hour controls were grown a few days later and so are marked in lighter colours.



○ Donor *E. coli* 2442 △ recipient *S. typhimurium* 576 + transconjugant *S. typhimurium* 576.

Figure 9.2. Density of donor *E. coli* 2442 and recipient *S. typhimurium* 576 co-grown in a water bath at 37^0C for 4 hours in the absence of antibiotic and for 21 hours with the stated antibiotics, and density of transconjugant *S. typhimurium* 576 produced by the parent cells and by division.



o Donor *E. coli* 2442 Δ Recipient *S. typhimurium* 576
+ Transconjugant *S. typhimurium* 576.

Figure 9.3. Density of donor *E. coli* 2442 and recipient *S. typhimurium* 576 incubated in caecal content for 21 hours with various concentrations of neomycin, and the density of transconjugant *S. typhimurium* 576 produced by them and by division during this time.

9.3.2 Statistical analysis

LB results

The analyses produced non-significant results with the exception of the following. It was confirmed that increasing ampicillin or chloramphenicol dose correlates with less transconjugant production (Minitab session 9.2; test 7). In addition, a significant decline in recipient density with increasing neomycin, ampicillin or chloramphenicol dose (test 6), and a significant effect of the interaction term in the chloramphenicol test were demonstrated. This interaction indicates a concave rather than convex relationship, however; the coefficient is positive. The tests failed to demonstrate the significance of the

convex relationship between neomycin and ampicillin dose just observable in figures 9.2 (a) and 9.2 (d).

Caecal content results

These analyses (Minitab session 9.3) demonstrate that the increase in donor and transconjugant density with neomycin dose is significant. This relationship does not significantly deviate from linear.

9.4 Discussion

The data from caecal content dose ranging is the most interesting. The increase in donor density with increasing neomycin dose is probably due to the reduction in competition from background flora. The maintenance of recipient density could be due to the shielding effect of the background flora, which would presumably be removed if the dose of neomycin were to be increased. The increase in transconjugant density with neomycin dose could be predicted from the density of the parent populations. The model predicts that production is proportional to the product of donor and recipient densities, and as can be seen from figure 9.3, this increases with increasing neomycin dose. The doses used here are roughly equivalent to those used in the in vivo dose-ranging experiment (chapter 8). This can be read from figure 6.3; the highest dose used in the ex-vivo caecal content (700 μg) when read off the left-most scale, is close to the day 10 estimation of antibiotic effect for an in vivo dose level of 250 g $tonne^{-1}$.

Scott & Flint (1995) also estimated plasmid transfer in ex-vivo gut content (section 2.4). They calculated transfer efficiency as transconjugants/recipients after 6 hours of incubation. The average efficiency was 2.44×10^{-6}. When this calculation is performed on the data at 6 hours (displayed in figure 11.3), an estimate of 3.28×10^{-3} is obtained. This may reflect a higher plasmid transfer rate constant in the strains used in the present study; the 6h densities of donors and recipients used by Scott & Flint (1995) are of the order 10^{10} as opposed to those used here where 6h densities are of the order 10^{7}. The fact that the perceptible convex relationship between neomycin dose and transconjugant production is not significant may be due to lack of data.

The densities of donors, recipients and transconjugants used in these experiments might be unrealistically high compared to concentrations *in vivo*. *S. typhimurium* can reach densities of 10^{8} in the caecum. Potential donors are not likely to be present at such high densities initially, although after antibiotic treatment they could dominate the bacterial community.

The timing of the onset of antibiotic treatment with relation to a *S. typhimurium* infection could be crucial in relation to the evolution of plasmid-mediated resistance. If *S. typhimurium* are found at high densities in chicken caeca, then problems of resistance acquisition could occur when treatment commences.

9.5 Summary

The increase in transconjugant production with dose in caecal content is exciting evidence that increasing antibiotic dose can lead to an increase in the problem of resistance. Furthermore, the maintenance of recipient density indicates that resistance may arise without reducing the burden of pathogenic bacteria. The significant downturn in transconjugant density at high doses of ampicillin, and the decrease in recipient density with neomycin, ampicillin and chloramphenicol suggest that where antibacterials are effective against recipients they may also reduce transconjugant production.

The inadequacy of the results of the LB experiments in reflecting this trend highlights the importance of the choice of experimental model: it must be as similar to the *in vivo* environment as possible.

10. Investigating resistance and plasmid transfer

10.1 Introduction

To attempt to put this work in a wider context, it was decided to explore the effects of the plasmid on various bacteria in various conditions. This was seen as particularly important in view of the more unusual characteristics of transconjugants: their low rate of *in vivo* growth, their similar intrinsic growth rate to their parent recipient and their resistance to neomycin greater than that of donors (chapter 5). This chapter aims to determine whether other transconjugants share these characteristics. In particular it was asked:

1. How does the cost of resistance vary between strains
2. How does plasmid transfer rate vary between strains
3. Does stationary phase plasmid transfer occur and how does it vary between strains

Stationary phase transfer is measured because if it occurs it affects the measurement of plasmid transfer rate by Simonsen's end point method, the method of choice.

10.2 Materials and methods

The 2442 plasmid encoding resistance to ampicillin, neomycin, sulphonamide and spectinomycin was conjugated into the strains *S. typhimurium* 576, F98 nal, LB5010 and *E. coli* F77 nal. These strains were selected from the IAH culture collection. There were initially two more *E. coli* strains which became contaminated. Where the recipient was *Salmonella*, the usual donor (*E. coli* 2442) was used and the transconjugants produced were screened with antibiotic discs to ensure they had only

acquired the plasmid of interest. The *E. coli* recipient was conjugated with a 576 transconjugant. The intrinsic growth rate and plasmid transfer rate at exponential and stationary phases of the recipient and transconjuant populations of each strain were calculated. This was achieved by growing the strains alone or together and taking regular samples for density estimation. Plasmid transfer rate was calculated using Simonesen's (1990) end-point method (see section 5.6). 26 hour counts were used as the end-point density. To estimate the extent of plasmid transfer at stationary phase, samples were taken at two time points after stationary phase had been reached. If plasmid transfer occurs during stationary phase, recipients would be depleted over this time as they convert to transconjugants, with a corresponding increase in transconjugant density. For details of density estimation and antibiotic resistance testing methods, see chapter 3. For more detail on intrinsic growth rate and plasmid transfer rate estimation, see sections 5.3.2 and 5.6.2 respectively.

10.3 Results.

10.3.1 Cost of resistance.

Ten replicates of recipients and transconjugants of the four strains were grown over five hours and three samples for density estimation were taken during this time The two species' samples were analysed separately for simplicity's sake. The strain difference in the cost of resistance in *S. typhimurium* was tested for with the command:

GLM slope = population + strain + population*strain.

'Slope' is the gradient of the regression of the natural logarithm of density against time, i.e. intrinsic growth rate. The inclusion of 'population' tests for the difference between transconjugant and recipient intrinsic growth rate, i.e. it determines whether there is a cost of resistance. The interaction term 'population*strain' tests for differences in cost of resistance between strains.

The cost of resistance in each strain including the *E. coli* was tested for in separate tests of the form:

GLM slope strain1 = population.

The results are listed in table 10.1, shown in figure 10.1 and Minitab session 10.1 gives the details of the analyses.

	Growth rate: average of ten replicates		
Strain	**Recipients**	**Transconjugants**	**Cost of resistance**
***S. typhimurium* 576**	1.87	1.72	8.06%*
***S. typhimurium* F98**	1.81	1.61	10.64%*
***E. coli* F77**	1.80	1.74	3.09%
***S. typhimurium* LB5010**	1.74	1.87	-7.61%

Table 10.1 Intrinsic growth rate and derived cost of resistance of the strains listed where the transconjugant carries a plasmid from donor *E. coli* 2442. Cost of resistance is the percentage intrinsic growth rate reduction in transconjugant compared with recipients. * cost of resistance significant ($P<0.05$)

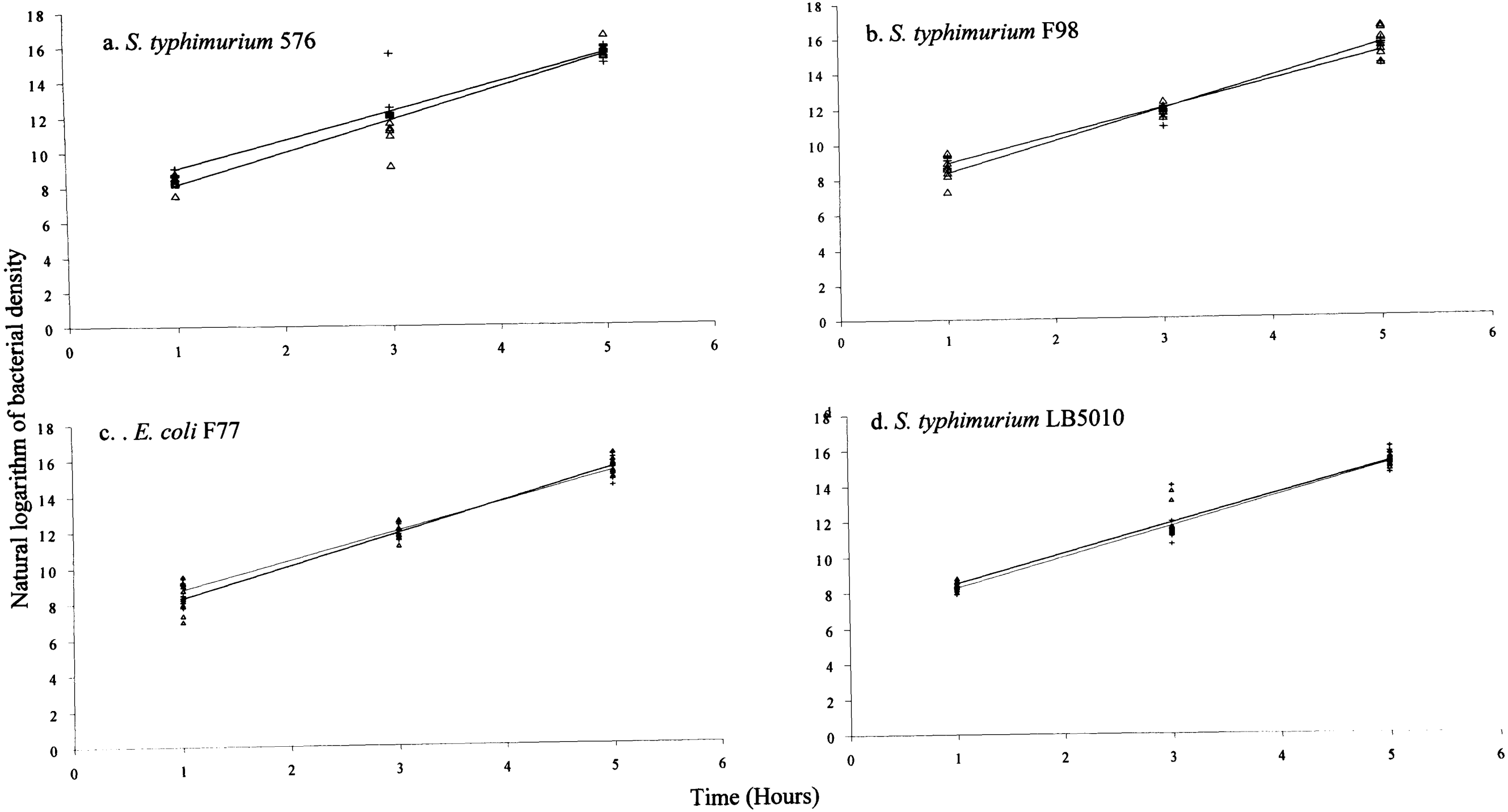


Figure 10.1. Growth of recipients and transconjugants of various strains. Green symbols indicate recipients and black, transconjugants. Lines are a regression through the points. Each graph depicts growth of ten replicates. Inoculum was produced by dilution of overnight shaking cultures 10,000 fold.

10.3.2 Plasmid transfer rate

Plasmid transfer rates (γ s) were estimated from these data using the end point method of Simonsen et al. (1990):

$$\gamma = r\ln\left(1+\frac{T}{R}.\frac{N}{D}\right).\frac{1}{N-N_0}$$

where r = average intrinsic growth rate of all strains, T R and D represent end point densities of the three populations; N ≡ D+R+T and N_0 is the starting density of donors and recipients. This equation is derived in section 5.6.2.

The growth rates and carrying capacities used to calculate γ are derived from data on lone growth of each strain for 26 hours carried out at the same time as the co-growth.

Differences between plasmid transfer rates of recipient strains were tested by logging the rates and using the GLM command (Minitab session 12.2) Firstly, GLM rate = recipient species, then, within the *S. typhimurium*, GLM rate = typhimurium strain. Logging of the rates before testing for differences was thought to be appropriate as the estimations varied over two orders of magnitude. A comparison of the plot of the residuals when analysis is performed with and without the transformation shows that the assumption of homogeneity of variance is batter satisfied with naturally logged (Ln) data.

Parameters for each strain were calculated using data from 3 replicates.

Results

The plasmid transfer rates are displayed in table 10.2; the growth trajectories are shown in figure 10.2 and the analyses are presented in Minitab session 10.2. The trends in figure 10.2 were calculated by fitting the simplified model from which Simonsen's end-point method of calculating plasmid transfer rate is derived from, with the parameter values used in this calculation. These equations of this simplified model are given in section 5.6.2.

Recipient	**Plasmid transfer rate**			**Mean**
***S. typhimurium* 576**	5.237E-11	1.448E-11	2.475E-11	3.053E-11
***S. typhimurium* F98**	1.659E-11		5.334E-12	1.096E-11
***E. coli* F77**	8.998E-12	1.371E-11	5.988E-12	9.564E-12
***S. typhimurium* LB5010**	1.101E-11	2.181E-11	4.172E-12	1.233E-11

Table 10.2. Plasmid transfer rates of a resistance plasmid transferred from *E. coli* 2442 to each of the recipient strains listed.

There were no significant differences between the plasmid transfer rates of the strains (Minitab session 10.2). This is probably due to lack of data, as (Gordon 1992) did find differences between the plasmid transfer rates of different recipient strains.

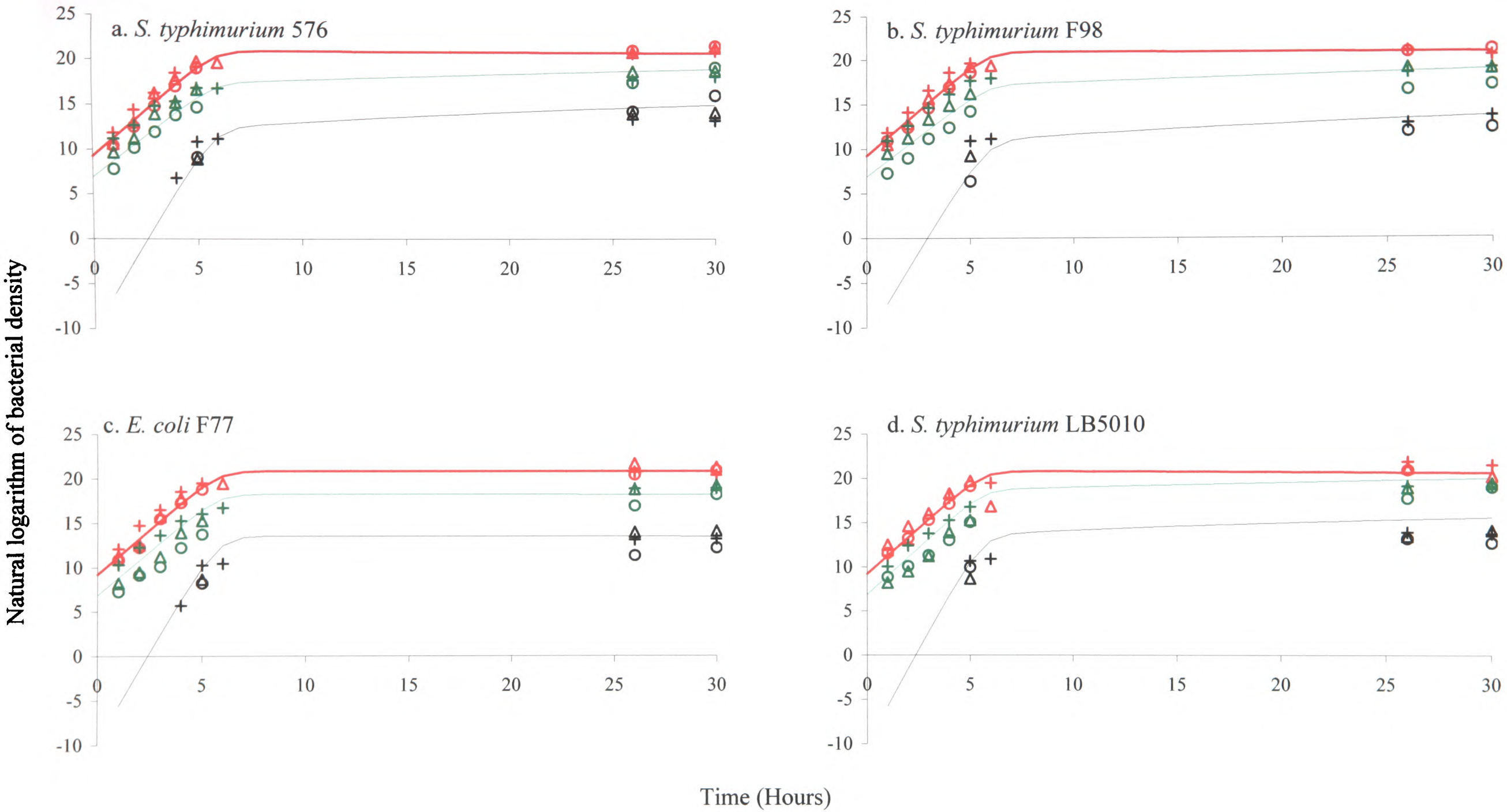


Figure 10.2. Growth and production of transconjugants by donor *E. coli* 2442 and various recipients in LB at 37^{0}C in a waterbath. Red symbols indicate donors, green indicate recipients and black, transconjugants. Lines are a model of the growth curves with growth rate and plasmid transfer rate parameters from each strain. Models used for the *Salmonella* recipients (a, b and d) allow growth of *Salmonella* when the donor *E. coli* is at stationary phase (section 5.5). Each graph depicts growth of three replicates. The different symbols (circle, triangle, cross) indicate which transconjugants were produced by which donors and recipients. Inoculum was produced by dilution of overnight shaking cultures 10,000 fold.

10.3.3 Stationary phase conjugation

Population densities were measured at 26 and 30 hours after inoculation, when donor and recipient populations had been at stationary phase for several hours (figure 10.2) to test the assumption that transfer does not occur after stationary phase has been reached. A paired, one tailed T-test was carried out (Minitab session 12.3) by taking the difference between the natural logarithms of the measurements at 30 and 26 hours and using a standard T-test to determine whether these were greater than zero. The density of recipients was measured to assess any decline (they would decline if they were transforming into transconjugants by conjugation); and that of donors was measured as a control of growth overall. The analysis (Minitab session 12.3 test 11) indicated there was a significant increase in transconjugant density over this time ($P = 0.049$). The average rate of transconjugant growth over this time was 0.144 hr^{-1}. There was also a significant increase in recipient densities over this time (test 10; $P = 0.004$, growth rate = 0.180 hr^{-1}) but not of donors (test 9; $P = 0.84$). This disparity is probably due to insufficient interspecific competition. Donor *E. coli* reach stationary phase about six hours after inoculation at these concentrations (figure 10.2), before the *S. typhimurium* have reached stationary phase densities. As competition is not complete between *E. coli* and *Salmonella* (section 5.5; Barrow et al (1996)) limited growth of recipients and transconjugants can occur. The fact that recipients increase at a comparable and slightly greater rate than transconjugants indicates that conjugation is probably not responsible for the increase.

10.4 Discussion

10.4.1 Cost of resistance

The percentage difference between recipient and transconjugant intrinsic growth rates was substantial in two of the *S. typhimurium* strains (about 10%) and not significant in the *E. coli* and third *S. typhimurium* strain. This result is in agreement with the literature, which demonstrates widely varying costs of resistance (chapter 2.)

10.4.2 Plasmid transfer rate

Gordon (1991) found significant differences in plasmid transfer rate between *E. coli* recipients when receiving the R1 plasmid from the same donor. The lack of significant differences between these strains is probably due to lack of data. Concerns have been raised that the numbers of transconjugants can be overestimated due to the occurrence of mating on the selective plates after sampling has occurred (Walter et al 1991b). Post-sampling mating should not occur in this experiment, however, because nalidixic acid was included in the sampling plates, which was found to eliminate conjugation (Walter et al 1991b).

10.4.3 Stationary phase plasmid transfer

Conflicting results have been found regarding the rate of transmission at stationary phase. Levin (1979) found that plasmid transmission was a function of resources i.e., at stationary phase both growth and conjugation should cease as resources are depleted. Simonsen et al. (1990) felt that the stationary phase plasmid transfer rate was small enough to be negligible, and his model for estimating transfer rate assumes that the densities as stationary phase is reached will not change. Conversely, Muela et

al (1994) found that plasmid transfer declined throughout exponential phase, reaching a minimum at mid log phase, then sharply increased at early stationary phase to a maximum, maintained from about 7 hours until experiment termination at 10 hours. This could be an experimental artefact of the plasmid transfer rate measurement, a description of which follows. Cells were harvested at hourly time points throughout lag, exponential and stationary phases, centrifuged and resuspended together at densities of about 10^8 ml^{-1} (recipient) and 10^7 ml^{-1} (donor). Two hours later the densities of donor and transconjugant cells were estimated and the transfer rate expressed as the number of transconjugants per donor. It is possible that the lag phase cells experience the most disruption during this process as they have been transplanted from an environment in which they can divide at the maximum growth rate to one in which cell densities are close to stationary phase. By contrast, the cells at stationary phase and those in exponential phase, recently transplanted from an overnight stationary phase culture, will not experience such a dramatic change in conditions. The cells transplanted from exponential phase may be adjusting to their change in environment and consequently not conjugating as rapidly as they are when undisturbed.

10.5 Summary

The bearing of these results on the answers to the questions posed at the beginning of the chapter will now be examined.

10.5.1 How does the cost of resistance vary between strains?

Strains 576 and F98 had a large cost of resistance of about 10%, whereas strains F77 and LB5010 had no detectable cost of resistance. F77, like the original donor, is a strain of *E. coli*. It seems reasonable to assume that the plasmid might have become adapted to existence within *E. coli* and not impose as much cost on other *E. coli* hosts. LB5010, however, is a *S. typhimurium*. The lack of any detectable cost of resistance in this strain has worrying implications for the survival of antibiotic resistance genes in pathogenic bacteria.

10.5.2 How does plasmid transfer rate vary between strains?

The plasmid transfer rates varied from 4e-12 to 5e-11. This variation is limited when compared to the huge variation over many orders of magnitude measured by other authors, even when using the same donor (chapter 2). These results indicate that the recipient may play a significant role in the regulation of plasmid transfer rate.

10.5.3 Does stationary phase plasmid transfer occur and how does it vary between strains?

These results indicate that transconjugant densities can increase slightly during stationary phase, but that it is probably due to division of existing transconjugants rather than creation of new transconjugants by plasmid transfer. The growth rate was small and the increase did not vary significantly between strains. The assumption made by modellers that conjugation does not occur at stationary phase is borne out by the results obtained here.

11. Discussion

In pursuing the aim of understanding the dynamics of plasmid mediated resistance in *S. typhimurium* infections of chickens this thesis has generated a coherent data-set describing all the *in vitro* life history parameters of one donor-recipient pair and of several other strains of *E. coli* and *S. typhimurium*, and some information on their behaviour *in vivo*. This chapter aims to put those experimental results in their context as established by the published literature. The growth rates of recipients and transconjugants estimated in chapter 10 give information about the cost of resistance which is discussed first. Next, the estimation of bacterial competition coefficients in this project are compared with two bodies of literature concerned with competition. The plasmid transfer rates estimated in chapter 10 are then considered, and finally the results of dose-ranging on transconjugant production are reevaluated in the light of studies on the effect of antibiotics on *Salmonella*.

The cost of bearing a plasmid was estimated as the percentage difference in recipient and transconjugant growth rates (table 10.1). These were found to be in accordance with published values of the costs of antibiotic resistance in *E. coli* cells (table 2.3). The evaluation of the cost of resistance in the experiments of this thesis did not determine competition coefficients between plasmid-containing, and plasmid-free strains of *S. typhimurium* 576. This is because transconjugant *S. typhimurium* 576 would be expected to increase their density due to plasmid transfer from transconjugants to recipients in addition to any effect of competition. It has been assumed that because recipients and

transconjugants are of the same strain, the competition coefficients of each to the other would equal 1.

The Lotka-Volterra model of competition is an extension of the logistic model, in which population density is at equilibrium when it is equal to the carrying capacilty (K):

$$\frac{dN}{dt} = rN\left(\frac{K-N}{K}\right)$$

Where N = density (ml^{-1}) r = growth rate ($hour^{-1}$) and K=carrying capacity (ml^{-1})

In the Lotka-Volterra model, the maximum density of one population (N_1) depends on the density of a competing population (N_2) where

$$\frac{dN_1}{dt} = r_1 N_1\left(\frac{K_1 - N_1 - \alpha_{12}N_2}{K_1}\right)$$

$$\frac{dN_2}{dt} = r_2 N_2\left(\frac{K_2 - N_2 - \alpha_{21}N_1}{K_2}\right)$$

The parameters α_{12} and α_{21} are called the competition coefficients of the Lotka-Volterra competition model. They describe the degree to which one population impinges upon the growth rate of the other. Lotka-Volterra competition coefficients were determined for four *E. coli-S. typhimurium* strain combinations (table 5.10). It was found that for one set of data (figure 5.6 (a) and (b)), this model was inappropriate because the point of stable coexistence between *E. coli* and *S. typhimurium* appeared to depend on which bacteria became established first. The competition coefficients of Lotka-Volterra, or other models of competition (Ayala et al 1973), provide a standard method of describing the outcome of competition between two species or strains provided they are allowed to equilibrate.

Despite this, the literature on bacterial competition does not make use of such models. Measurements on competing bacteria are used to evaluate bacterial fitness, and competition has also been investigated to determine possible candidates for competitive exclusion cultures to prevent infection by pathogenic strains of bacteria in chickens. The ways in which authors measuring bacterial fitness use the concept of competition is now examined. Bacterial fitness is usually measured in one of two ways: comparison of intrinsic growth rates (Bjorkman et al 1998, Bjorkman et al 1999, this thesis); or by pairwise competition experiments (Bouma & Lenski 1988, Filonov et al 1985, Schrag & Perrot 1996, Schrag et al 1997). Fitness in pairwise competition experiments is calculated as the regression coefficient of the natural logarithm of the ratio of sensitive:resistant strains over time, which implies that this ratio is constant. Bouma & Lenski (1988), Schrag & Perrot (1996) and Schrag et al (1997) measured fitness by serial transfer experiments where competing populations were inoculated daily into medium, incubated for several hours, then re-inoculated into fresh medium the next day. Fitness may be partitioned into three components: duration of lag phase, intrinsic growth rate, and invasiveness at stationary phase (a combination of intrinsic growth rates and competition coefficients). It can be seen that the changes in density observed during growth between serial transfers depend upon the duration spent in each growth phase. Changes in density between each inoculation within experiments, and differences in procedure between experiments, could lead to variation in the measurement of fitness both within and between estimations. It is clear that the calculated fitness differences between the two strains measured this way are a conflation of differences between lag phase duration, the intrinsic growth rates (the effects of which predominate during exponential phase) and

between competition coefficients of the two species (the effects of which predominate in stationary phase and the approach to it). For example, in the results presented in section 5.5, recipient *S. typhimurium* 576 has a competition coefficient >1 in competition with *E. coli* whilst their growth rate is less than that of *E. coli*. In this case, growth rates alone do not encapsulate the effect of competition between these *E. coli* and *S. typhimurium* strains.

Lenski et al (1998) also investigated competitive fitness of baseline and evolved *E. coli* strains in serial transfer experiments. They separated out two fitness components: duration of lag phase and the exponential phase growth rate. They concluded that the evolved strain was more fit than the baseline strain regarding each component. They did not, however, consider aspects of competitive fitness that might come into play during stationary phase (e.g. Lotka-Volterra competition coefficients). They observed that their evolved strains had larger cells than the baseline strain, which is independent of growth rate, and they could not explain why this might increase the competitive fitness of the evolved strain. It is interesting to speculate that the larger cell size could increase competitive fitness during the stationary phase of bacterial growth. They show that although stationary phase densities of the evolved strain are smaller than the baseline strain, the two do not exactly compensate each other so that the evolved strain occupies a greater volume at stationary phase than the baseline strain. Evolved strains could thus exclude more nutrients from use by the baseline strain at stationary phase, limiting their density.

In vitro measurements of bacterial fitness would benefit from separate consideration of each parameter which can affect fitness: duration of lag phase, intrinsic growth rate and

competition coefficients (the latter estimated from densities at points of stable equilibria). Several studies have assayed fitness *in vivo*. *In vivo* assays of fitness are of more practical use than *in vitro* assays, as they take into account more aspects of fitness encountered by bacteria in nature.

The other body of literature which addresses bacterial competition is the competitive exclusion (CE) literature. This focuses on competition between non-pathogenic bacteria and *Salmonella* strains pathogenic to humans or chickens. The goal of these studies is to isolate strains of bacteria which, when administered to chickens, could prevent subsequent colonisation of invading pathogenic strains. In contrast to the fitness literature, this work addresses competition at stationary phase to the exclusion of growth rate or lag phase differences, as it is presumed that the strain used for CE will be at stationary phase in the gut. Iba et al (1995) present data similar to dataset 2 in section 5.5 (table 5.11), where the 24 hour counts of strains in competition with stationary phase cultures are measured in addition to *in vivo* competition assays. They use *Salmonella* in competition with other *Salmonella* strains as they are interested in the strains which cause the most growth suppression. This is of less use for the evaluation of competition coefficients, which are more easily evaluated at intermediate levels of competition. Although an estimation of competition coefficients can be made with such data (section 5.5.2; dataset 3), 24 hour counts do not allow exact enumeration of competition coefficients as the invading strain might still be increasing in density after 24 hours (figures 5.6 and 5.7) and therefore the equilibrium densities are not known. Cosby et al (1997) investigated strains isolated from the chicken caecum for inhibitory activity against *S. typhimurium*. Several *E. coli* strains were found exhibiting inhibitory activity,

but their methods preclude calculation of competition coefficients. They did, however, compare five different methods of assessing inhibition, which measure the different aspects of bacterial fitness to varying degrees. Berchieri & Barrow (1991) investigated different factors that cause *in vitro* growth inhibition of *S. typhimurium* by *Salmonella* strains. They found that cell density alone does not account for inhibition; mid-exponential phase cultures which were spun and resuspended at stationary phase densities failed to inhibit a competing strain to the same degree as a stationary phase culture. Inhibition was also obtained when the established and introduced strains were separated by a dialysis membrane, indicating that direct cell to cell contact is not necessary to produce inhibition. The perception by bacteria of the 'inhibitory principle' excreted at stationary phase which limits density is known as quorum sensing. The family of molecules which enable communication between gram-negative cells has been identified as the *N*-acetylhomoserine lactone (AHL) group. The pathways by which reduction in growth due to quorum sensing is effected in different species are currently being elucidated (reviewed in Withers et al 2001). The emphasis placed on stationary phase competition is logical given that few nutrients are spare in the gut and populations are likely to be in stationary phase. The molecules which effect quorum sensing have been detected *in vivo* (Williams et al 2000). Given that Iba et al (1995) failed to detect a correlation between *in vitro* ability to invade stationary phase cultures and *in vivo* colonisation ability, perhaps if all aspects of competitive fitness were investigated separately *in vitro* there would be more likelihood of a correlation being discovered. The different methods of assessing inhibition used by Cosby et al (1997) revealed different aspects of the competition process and go some way to achieving this.

The data gathered in section 5.5 suggest that competition in bacteria does not conform well to the Lotka-Volterra model. In particular, (i) equilibrium points may depend on which population is established first and (ii) bacteria may exhibit an extended lag phase of adjustment to the competitive environment before growth as the introduced strain. Molecular studies on the mechanism of growth limitation of one bacterial population by another could form the basis of a new mathematical model describing competitive growth.

Rates of plasmid transfer from or to *Salmonella* strains that are independent of both donor and recipient densities (i.e. estimates of γ) are not well documented. The *in vitro* rates of plasmid transfer from donor *E. coli* 2442 to three *S. typhimurium* recipient strains (table 10.2) indicate that these pairs exchange the plasmid at a rate clearly within the established range of plasmid transfer rates estimated for *in vitro E. coli* to *E. coli* transmission (table 2.4). These estimates use strain combinations in which only the recipient varies. The recipient plays an important role in determining plasmid transfer rate. Gram negative bacteria including *S. typhimurium* and *E. coli* generally exchange plasmids via pili, as opposed to gram positive bacteria, whose recipients secrete a pheromone to ensure close contact with potential donors (Andrup 1998). There are two classes of pili in gram-negative organisms: long, flexible pili which allow transfer in liquid medium, and short, rigid pili which require a surface on which to effect plasmid transfer. The best studied system is the F-plasmid system in *E. coli* which is of the former type. At the recipient cell surface there are several factors which moderate whether F

plasmid transfer takes place, and at what rate (Sanderson 1996). There are three classes of mutants which are deficient or inefficient recipients. (i) ompA mutants of *E. coli* or *S. typhimurium* (omp for outer membrane protein) are inefficient recipients in liquid medium, but not on membrane filters. This suggests ompA has a role in stabilising the recipient-pilus join. (ii) *E. coli* and *S. typhimurium* mutants with heptose-deficient polysaccharide are also ineffective recipients. (iii) The presence of O (somatic) side chains on the lipopolysaccharide molecules reduce the frequency of conjugation. Thus, recipients can affect the plasmid transfer rate and vary in their receiving ability (demonstrated by Gordon, 1992). Although these estimates of plasmid transfer rate of donor *E. coli* 2442 to three *S. typhimurium* strains (chapter 10) were not found to vary between strains, they are valuable in that they provide the first estimates of the rates of plasmid transfer to *S. typhimuiurm* strains.

The work on plasmid transfer continued with estimations of the effect of varying the dose of antibiotic on the production of transconjugants by donors and recipients. The results of dose-ranging in caecal content (figure 9.3) reveal an increase in transconjugant density with neomycin dose, and the fact that transconjugants were only isolated from the groups administered the highest amount of antibiotic *in vivo* (figure 8.1 (d)) suggests that neomycin causes an increase in transconjugant production in the chicken caecum. Conversely, transconjugants decline with neomycin dose *in vitro* in the absence of background flora (figure 9.2 (d)). The positive relationship *in vivo* and in *ex vivo* caecal content is probably due to the reduction in density of background flora with increasing dose, which would in turn both increase contacts between donors and recipients and increase their density. Aminoglycoside antibiotics bind to the 30S ribosomal subunit and

interfere with protein synthesis, thus they are removed from the medium by their antibiotic action (Russell, 1990). Plasmid-encoded resistance to aminoglycosides involves their degradation rather than efflux, which further removes antibiotic molecules from the environment. Thus, high bacterial densities may act to absorb neomycin from the medium, allowing susceptible populations to grow despite the administration of antibiotic, *in vivo* or in *ex vivo* caecal content. *Salmonella* are more resistant to neomycin than other components of the flora are, meaning that more susceptible components of flora might absorb the neomycin before the *Salmonella.* Contradicting this reasoning is the finding that neomycin is present at high concentrations in the caecal content of chickens administered neomycin (chapter 6). Nevertheless, susceptible recipient *S. typhimurium* 576 attain higher densities in caecal content at high doses of neomycin than occurs in LB. This increase in transconjugant count with dose is worrying. What, then, would be the best treatment to reduce the formation of transconjugants *in vivo*? If an antibiotic was administered which was more lethal to *S. typhimurium*, the decline in recipient density with dose observed *in vitro* should eventually be apparent *in vivo* at high enough doses, which could lead to the reduction in transconjugant density with dose shown in figure 9.2 (a) and (c). Williams (1984) found that neomycin at 200 g ton^{-1} reduced the excretion of *S. typhimurium*, as did tetracycline at the same dose, but neither could eliminate *S. typhimurium* from the faeces alone during the two weeks of the experiment. In combination, however, they reduced *S. typhimuiurm* count below the level of detection in one week. *S. typhimurium* counts declined in the control group over this time, but to a lesser extent. MacKenzie & Bains (1974) studied administration of antibiotics singly to ten groups of birds, which were given oxytetracycline,

clortetracycline, sulphadimidine, furazolidone, neomycin, furaltadone, chloramphenicol, or ampicillin, or were left untreated or uninfected and untreated. They found that furaltadone, ampicillin and chloramphenicol significantly reduced infection above that of the control, whereas the others did not. Olesuik et al (1972) studied the efficacy of six antimicrobials including neomycin but not including furaltadone, ampicillin or chloramphenicol, and found that none were particularly effective at preventing acquisition of infection by susceptible birds or eliminating it in infected birds. Lastly, Gast et al (1988) studied the effect of kanamycin administration to turkey poults on the counts of recipient and transconjugant *S. typhimurium* in the liver, and then fed the turkey livers to rats to assess the rate of interspecies transfer. Groups of turkeys were either given donor *E. coli* and recipient *S. typhimurium* only, donor and recipient plus kanamycin, or transconjugant *S. typhimurium* without kanamycin. It was found that kanamycin increased both *S. typhimurium* invasion of the liver, and the ratio of transconjugant to recipient cells found there. These results mirror the *in vivo* results of this thesis in that antibiotic administration caused an increase in the transconjugant count, apparently due to the suppression of competing gut bacteria. Kanamycin is not commonly used to treat *Salmonella* in poultry and is not effective enough against it to cause a reduction in the *S. typhimurium* burden. Thus, combinations of antimicrobials would appear to be the best method of reducing counts of transconjugants, both because they lead to an effective reduction in sensitive *S. typhimurium in vivo* (Williams 1984), and because increasing the number of antibiotics administered decreases the likelihood of resistance being transferred on a single plasmid. For example, neomycin and tetracycline

resistance are found on separate plasmids in the donor *E. coli* 2442 used here (section 3.2).

In summary, the complete set of life-history parameters estimated for the donor-recipient-transconjugant trio in chapter 5 appears to be unique. Although the Lotka-Volterra competition equations are far from ideal for describing the dynamics of bacterial competition, they have the great advantage of highlighting the importance of finding ways to estimate rates of population growth and interaction that are independent of population densities. Such quantities arise very naturally as model parameters. Once they have been defined through the modelling process, designing experiments to measure them was, in this case, relatively straightforward.

Problems encountered and possible future directions

Mathematical models highlighted the important processes in evolution of plasmid-mediated resistance and predicted possible outcomes when a host was treated with antibiotic. The problems that arose consisted of experimental difficulties during data collection, and discrepancies between the model and data collected.

The predictions of the mathematical model deviated from the data in two main areas: the effects of between-strain competition and the effects of antibiotic on susceptible bacteria. The discrepancies between the model and competition data are discussed in section 5.5. A closer fit to data could be achieved by considering how well existing models of competition between species might apply to bacteria, and devising new models if

necessary. The choice of model should be made with the consideration that bacterial density in the caeca is high and populations are likely to be physiologically close to the stationary phase of their life-cycle. With this in mind, some deviation of the model from the data during the exponential and lag phases can be overlooked. The second discrepancy between the observed behaviour of the three bacterial populations and the model occurred in response to antibiotic administration. A dose of antibiotic is expected to reduce the growth rate of antibiotic-sensitive bacteria, and to a lesser extent, of antibiotic-resistant bacteria. Contrary results were obtained where antibiotic failed to decrease sensitive population density, in ex vivo caecal content (figure 9.3). It is thought that the antibiotic reduced the density of background bacteria with a consequent reduction in competition. This opportunity for increased growth would counteract the antibiotic killing effect experienced by donors, recipients and transconjugants to some extent. The model does not predict such an outcome because it does not account for competitive interactions between the populations of interest and populations of background bacteria. The additional complexity of such interations in a mathematical model could prove well worthwhile, as the model as it stands always predicts decline of susceptible populations following antibiotic treatment.

In addition to the failure of the model to account for every aspect of the data acquired by experiments in this work, other characteristics of naturally acquired *Salmonella* infections in chickens were not addressed. In particular, between-host transmission of *Salmonella* was not considered, which determines the extent of an outbreak of *Salmonella* infection in a chicken flock. The model formulated here describes a single host, and can represent a

flock only if between-host transmission is rapid enough to ensure gut flora does not differ between birds. In reality *Salmonella* transmission between chickens in a flock would not bear out this assumption; within each pen in the pilot experiment described here birds not experimentally infected picked up *Salmonella* over a period days and weeks (figure 7.5). A between-host model would follow standard infectious disease models (Anderson and May, 1991). The values of model parameters can be calculated from the within host model. Once it has been formulated, R_0s (defined in section 4.4) can be calculated for resistant and sensitive strains, and thus predictions made of the persistance of resistant or sensitive *Salmonella* in a chicken flock.

The other difficulties experienced were experimental; very few transconjugants were isolated *in vivo* and measuring growth parameters in *ex vivo* caecal content proved difficult. Better results might have been obtained *in vivo* if the following alterations were made. Firstly, the strain of recipient *Salmonella* could have been one freshly isolated from chickens. The strain 576 was originally isolated from chickens, but has been grown in the lab for many generations, and its poor persistance in these experiments suggests that it may have become less fit in the *in vivo* environment than it once was. Secondly, suspensions of caecal content could have been enriched for *Salmonella* using selective growth medium. This would have lowered the limit of detection for recipients and transconjguants, although this approach does not allow quantification of bacterial density. It is interesting to carry out experiments using *ex vivo* caecal content as a growth medium, as it is a much closer representation of the *in vivo* environment than LB broth is. Attempts were made to measure each of the growth parameters in caecal content, but

only the measurement of stationary phase density succeeded. Populations of donors, recipients and transcongjuants did not grow enough to obtain reliable parameter measurements, probably because the environment was saturated with background bacteria. It would be interesting to repeat these experiments using variations on these conditions. Caecal content could be partly or wholly sterilised before inoculation, with just the donors, recipients and transconjugants, or a defined background flora; and it could be incubated anaerobically and at different temperatures. It is surprising that *ex vivo* gut contents are not used more frequently to study gut bacteria, as this is one of the best models of the gut environment achievable in the laboratory.

12. Conclusion and future directions

The aim of this thesis was to develop a clearer understanding of the population dynamics of plasmid mediated resistance in *S. typhimuiurm* in chickens. The questions posed by the experiments in chapters 5-10 and the results obtained pertaining to those questions are summarised here.

Chapter 5 was concerned with the estimation of a complete set of parameter values for the model presented in chapter 4 in order to use those estimations in model simulations of the effect of time and antibiotic dose on transconjugant density. The estimations produced some surprising results: (i) the growth rates of recipient *S. typhimurium* 576 and its transconjugant differed only by 4% (this implies a very low cost to resistance); (ii) Competition between *E. coli* and *S. typhimurium* strains could not be modelled well with the Lotka-Volterra competition equations; (iii) *S. typhimuiurm* 576 carrying the same resistance plasmid as *E. coli* 2442 was much more resistant to neomycin than the donor *E. coli* (neomycin is one of the resistances encoded on the plasmid). In addition, growth rates of donors, recipients and transconjugants were estimated, as were their carrying capacities in LB and caecal content, and rates of plasmid transfer between donors and recipients in LB were obtained. These values were incorporated into model simulations that addressed the question of the form of the relationship between antibiotic dose and the densities of donors, recipients and transconjugants. It was concluded that the production of transconjugants first increases, then decreases, with antibiotic dose. Chapter 6 aimed to allow comparisons between *in vitro* work on bacteria growing in antibiotic, and *in vivo* work on chickens administered antibiotic by observing the relationship between neomycin dose administered to chickens and the effective concentration of antibiotic

found in their caecal contents. The proportion of water in caecal content was also estimated in order to establish whether this relationship could be estimated as the density of neomycin in feed diluted by the moisture content of the caecal contents. A standard curve of dose-effect was created from which *in vivo* doses can be tailored to correspond with *in vitro* concentrations or vice-versa. It was found that an estimate of the concentration of neomycin in the caecal content based only on the amount in feed diluted by the moisture content of caecal contents would underestimate concentration approximately 10-fold. In other words the chicken gut acts to greatly concentrate the dose of neomycin in the caecum compared to its concentration in feed given to the chicken. Chapter 7 attempted to address questions concerning the rate of between-chicken transfer and the *in vivo* growth, survival and plasmid transfer between the two strains which had been characterised *in vitro* in chapter 5. Several sampling methods were applied in order to select the best method of sampling for use in the main *in vivo* dose ranging experiment. It was found that both of these strains were able to colonise the chicken gut, and that plasmid transfer between them occurs there as transconjugants were isolated from many caecal samples. Tentative estimations of the rate of between-chicken transfer of donor *E. coli* 2442 and recipient *S. typhimuiurm* 576 were made. It was concluded that caecal content sampling, both at post mortem and daily from the pen floor, were the best methods of assessing infection status. Chapter 8 was an attempt to test, *in vivo*, the model-derived prediction that there will be a hump-shaped distribution between antibiotic dose and density of transconjugants produced. Neomycin was administered to three groups of chickens at different doses with one control group, and they were all fed donor *E. coli* 2442 and recipient *S. typhimurium* 576. Very few transconjugants were isolated from the caecal contents of these birds so no conclusions could be drawn about the shape of this relationship. The

two transconjugant isolations that were made were in the groups fed the highest two doses of neomycin. The failure of the *in vivo* experiment to reach conclusions on this relationship led to efforts to elucidate the dose-transconjugant production relationship *in vitro* in chapter 9. Two sets of *in vitro* dose ranging experiments were carried out, one set in normal laboratory LB broth and the other in caecal content taken from undosed, uninfected chickens at PM. A decrease in transconjugant production with dose was observed in LB, whereas the opposite was found in caecal content. The experiments in chapter 10 were designed in order to address questions which had arisen during the work for this thesis. This was achieved by measuring the plasmid transfer rate from donor *E. coli* 2442 to other strains acting as recipients, and measuring the cost to these other strains of bearing the resistance plasmid. The plasmid transfer rate was not highly variable between strains. It was found that the cost of resistance varies from 0 to 10%, where a zero cost of resistance was ascribed when the difference between growth rates of recipients and transconjugants was non-significant.

Through the use of a mathematical model describing growth, competition and plasmid transfer in a pair of interacting bacterial populations, the work presented here was able to frame a coherent description of those processes. Extensive experimental work allowed the estimation of all the parameters of this model. The details of the trajectories of the population dynamics of the two populations deviated widely from the predictions of the model, but this in itself is an interesting observation and calls for better models of competitive processes as they operate in bacteria. Through guiding experimental work and specifying exactly what one would expect to observe under a given set of assumptions the modelling work of this thesis allowed a clearer

understanding of the complex interacting dynamics of two populations of bacteria, sharing an environment and sharing genetic elements.

13. References

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Appendix: Minitab session outputs

Session number refers to the number of the corresponding chapter

Mintab session output 5

Test 1. Does intrinsic growth rate differ between populations?

MTB > glm slope = population.

General Linear Model

Factor Levels Values
populati 3 1 2 3

Analysis of Variance for Slope

Source	DF	Seq SS	Adj SS	Adj MS	F	P
populati	2	0.26292	0.26292	0.13146	2.81	**0.074**
Error	33	1.54213	1.54213	0.04673		
Total	35	1.80505				

Test 2. Does intrinsic growth rate differ between *E. coli* and *S. typhimurium*?

MTB > glm slope = species.

General Linear Model

Factor Levels Values
Species 2 1 2

Analysis of Variance for Slope

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Species	1	0.21852	0.21852	0.21852	4.68	**0.038**
Error	34	1.58653	1.58653	0.04666		
Total	35	1.80505				

Test 3. Does intrinsic growth rate differ between recipients and transconjugants?

MTB > glm slope = population.

General Linear Model

Factor Levels Values
Populati 2 1 2

Analysis of Variance for Slope

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Populati	1	0.09250	0.09250	0.09250	5.47	**0.030**
Error	19	0.32106	0.32106	0.01690		
Total	20	0.41357				

Minitab session 7.1: analysing daily pen floor sample densities.

Test 1. Background *E. coli*

```
MTB > glm ec = medication;
SUBC> brief 3.
```

General Linear Model

```
Factor   Levels Values
Medicati      2  1  2

Analysis of Variance for Ec
Source     DF    Seq SS    Adj SS    Adj MS      F      P
Medicati    1    33.628    33.628    33.628  11.01  0.001
Error     136   415.313   415.313     3.054
Total     137   448.941

Term          Coef    StDev       T      P
Constant   17.8479   0.1488  119.97  0.000
Medicati
1           0.4937   0.1488    3.32  0.001
```

Test 2. Donor *E. coli* 2442

```
MTB > glm d = day|medication;
SUBC> covar day;
SUBC> brief 3.
```

General Linear Model

```
Factor   Levels Values
Medicati      2  1  2

Analysis of Variance for D
Source        DF    Seq SS    Adj SS    Adj MS      F      P
Day            1     793.9     946.2     946.2   58.12  0.000
Medicati       1    2107.5    1415.8    1415.8   86.96  0.000
Medicati*Day   1     153.6     153.6     153.6    9.43  0.003
Error        140    2279.3    2279.3      16.3
Total        143    5334.3

Term             Coef    StDev       T      P
Constant      11.3864   0.5580   20.41  0.000
Day         -0.000192 0.000025   -7.62  0.000
Medicati
1            5.2032   0.5580    9.33  0.000
Day*Medicati
  1       -0.000077 0.000025   -3.07  0.003
```

Test 3. Recipient *S. typhimurium* 576

```
MTB > glm r = day|medication;
SUBC> covar day;
```

SUBC> brief 3.

General Linear Model

Factor Levels Values
Medicati 2 1 2

Analysis of Variance for R

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	31.022	29.260	29.260	4.18	0.043
Medicati	1	6.914	21.513	21.513	3.07	0.082
Medicati*Day	1	14.614	14.614	14.614	2.09	0.151
Error	118	826.749	826.749	7.006		
Total	121	879.299				

Term	Coef	StDev	T	P
Constant	1.6357	0.4078	4.01	0.000
Day	-0.000037	0.000018	-2.04	0.043
Medicati				
1	-0.7146	0.4078	-1.75	0.082
Day*Medicati				
1	0.000026	0.000018	1.44	0.151

Minitab session 7.2: analysing PM caecal sample densities.

Test 4. Background *E. coli*

MTB > glm ec = medication;
SUBC> brief 3.

General Linear Model

Factor Levels Values
Medicati 2 1 2

Analysis of Variance for Ec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Medicati	1	47.573	47.573	47.573	35.04	0.000
Error	56	76.025	76.025	1.358		
Total	57	123.599				

Term	Coef	StDev	T	P
Constant	18.5839	0.1531	121.40	0.000
Medicati				
1	0.9062	0.1531	5.92	0.000

Test 5. Donor *E. coli* 2442

MTB > glm d = day + medication + infection + medication* day + medication * infection;
SUBC> covar day;
SUBC> brief 3.

General Linear Model

Factor Levels Values
Medicati 2 1 2
Infectio 2 1 2

Analysis of Variance for D

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	18.31	23.69	23.69	2.68	0.108
Medicati	1	910.81	518.09	518.09	58.58	0.000
Infectio	1	31.61	35.60	35.60	4.03	0.050
Medicati*Day	1	159.18	161.83	161.83	18.30	0.000
Medicati*Infectio	1	3.52	3.52	3.52	0.40	0.531
Error	49	433.35	433.35	8.84		
Total	54	1556.78				

Term	Coef	StDev	T	P
Constant	10.201	1.094	9.33	0.000
Day	-0.12840	0.07846	-1.64	0.108
Medicati				
1	8.371	1.094	7.65	0.000
Infectio				
1	0.8640	0.4307	2.01	0.050
Day*Medicati				
1	-0.33561	0.07846	-4.28	0.000
Medicati*Infectio				
1 1	-0.2716	0.4307	-0.63	0.531

Test 6. Recipient *S. typhimurium* 576

MTB > glm r = day + medication + infection + medication* day + medication * infection;
SUBC> covar day;
SUBC> brief 3.

General Linear Model

Factor Levels Values
Medicati 2 1 2
Infectio 2 1 2

Analysis of Variance for R

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	79.10	70.50	70.50	6.91	0.011
Medicati	1	87.44	94.17	94.17	9.23	0.004
Infectio	1	19.99	21.58	21.58	2.12	0.152
Medicati*Day	1	60.71	54.62	54.62	5.35	0.025
Medicati*Infectio	1	10.43	10.43	10.43	1.02	0.317
Error	53	540.70	540.70	10.20		
Total	58	798.37				

Term	Coef	StDev	T	P
Constant	3.970	1.127	3.52	0.001
Day	-0.20808	0.07915	-2.63	0.011
Medicati				
1	-3.425	1.127	-3.04	0.004
Infectio				
1	0.6523	0.4485	1.45	0.152
Day*Medicati				
1	0.18315	0.07915	2.31	0.025
Medicati*Infectio				
1 1	-0.4535	0.4485	-1.01	0.317

Test 7. Transconjugant *S. typhimurium* 576

MTB > glm t = day + medication + infection + medication* day + medication * infection;
SUBC> covar day;
SUBC> brief 3.

General Linear Model

Factor	Levels	Values
Medicati	2	1 2
Infectio	2	1 2

Analysis of Variance for T

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	8.365	6.048	6.048	0.85	0.362
Medicati	1	19.761	2.959	2.959	0.41	0.523
Infectio	1	35.649	34.290	34.290	4.80	0.033
Medicati*Day	1	0.766	0.277	0.277	0.04	0.845
Medicati*Infectio	1	9.831	9.831	9.831	1.37	0.246
Error	53	378.994	378.994	7.151		
Total	58	453.366				

Term	Coef	StDev	T	P
Constant	1.6542	0.9437	1.75	0.085
Day	-0.06094	0.06627	-0.92	0.362

Medicati				
1	-0.6070	0.9437	-0.64	0.523
Infectio				
1	0.8222	0.3755	2.19	0.033
Day*Medicati				
1	0.01305	0.06627	0.20	0.845
Medicati*Infectio				
1 1	-0.4402	0.3755	-1.17	0.246

MTB >

Minitab session 7.3: testing whether force of infection (λ) data conforms to a straight line.

Test 8. Recipient *S. typhimurium* 576 transmission from A to B (medicated group)

MTB > glm rb = time|time;
SUBC> covar time.

General Linear Model

Analysis of Variance for rb

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	1	1.16231	0.16214	0.16214	13.98	0.002
Time*Time	1	0.05935	0.05935	0.05935	5.12	0.041
Error	13	0.15082	0.15082	0.01160		
Total	15	1.37247				

Term	Coef	StDev	T	P
Constant	0.8898	0.2441	3.65	0.003
Time	-0.14550	0.03892	-3.74	0.002
Time*Time	0.003223	0.001425	2.26	0.041

Test 9. Recipient *S. typhimurium* 576 transmission from C to D (unmedicated group)

MTB > glm rd = time|time;
SUBC> covar time.

General Linear Model

Analysis of Variance for rd

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	1	4.1206	0.4075	0.4075	8.61	0.012

Time*Time	1	0.9098	0.9098	0.9098	19.23	0.001
Error	13	0.6150	0.6150	0.0473		
Total	15	5.6453				

Term	Coef	StDev	T	P
Constant	-0.9789	0.4929	-1.99	0.069
Time	0.23066	0.07859	2.93	0.012
Time*Time	-0.012620	0.002878	-4.39	0.001

Test 10. Donor *E. coli* 2442 transmission from C to D (unmedicated group)

MTB > glm dd = time|time;
SUBC> covar time.

General Linear Model

Analysis of Variance for dd

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	1	0.54851	0.12404	0.12404	1.68	0.210
Time*Time	1	0.02730	0.02730	0.02730	0.37	0.550
Error	19	1.40186	1.40186	0.07378		
Total	21	1.97767				

Term	Coef	StDev	T	P
Constant	-0.0749	0.1591	-0.47	0.643
Time	-0.04550	0.03509	-1.30	0.210
Time*Time	0.000982	0.001614	0.61	0.550

Minitab session 7.4: Calculating force of infection (λ) from gradient of Ln [proportion uninfected] against time

Test 11. Recipient *S. typhimurium* 576 transmission from A to B (medicated group)

MTB > Regress 'rb' 1 'Time';
SUBC> NoConstant.

Regression Analysis

The regression equation is
rb = - 0.0565 Time

Predictor	Coef	StDev	T	P
Noconstant				
Time	-0.056531	0.003375	-16.75	0.000

S = 0.1189

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	3.9627	3.9627	280.49	0.000
Error	15	0.2119	0.0141		
Total	16	4.1747			

Test 12. Recipient *S. typhimurium* 576 transmission from C to D (unmedicated group)

MTB > Regress 'rd' 1 'Time';
SUBC> NoConstant.

Regression Analysis
The regression equation is
rd = - 0.0721 Time

Predictor	Coef	StDev	T	P
Noconstant				
Time	-0.07211	0.01088	-6.63	0.000

S = 0.3830
Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	6.4478	6.4478	43.95	0.000
Error	15	2.2004	0.1467		
Total	16	8.6482			

Test 13. Donor *E. coli* 2442 transmission from C to D (unmedicated group)
MTB > Regress 'dd' 1 'Time';
SUBC> NoConstant.

Regression Analysis
The regression equation is
dd = - 0.0349 Time

Predictor	Coef	StDev	T	P
Noconstant				
Time	-0.034909	0.004722	-7.39	0.000

S = 0.2717
Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	4.0350	4.0350	54.65	0.000
Error	21	1.5505	0.0738		
Total	22	5.5855			

Minitab session 8.1: Analysing PM data without testing for interactions

Test 1: Is the density of background *E. coli* affected by time or dose of neomycin?

```
MTB > glm 'background E. coli' = day + 'neomycin dose';
SUBC> covar day 'neomycin dose'.
```

General Linear Model

Analysis of Variance for Backgrou

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	3.4140	3.4140	3.4140	8.40	0.005
Neomycin	1	1.8471	1.8471	1.8471	4.55	0.036
Error	77	31.2840	31.2840	0.4063		
Total	79	36.5452				

Term	Coef	StDev	T	P
Constant	1472.5	505.2	2.91	0.005
Day	-0.03926	0.01354	-2.90	0.005
Neomycin	0.001601	0.000751	2.13	0.036

Test 2: Is the density of donor *E. coli* 2442 affected by time or dose of neomycin?

```
MTB > glm donors = day + 'neomycin dose';
SUBC> covar day 'neomycin dose'.
```

General Linear Model

Analysis of Variance for Donors

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	0.006	0.015	0.015	0.01	0.943
Neomycin	1	0.361	0.361	0.361	0.12	0.726
Error	65	189.894	189.894	2.921		
Total	67	190.261				

Term	Coef	StDev	T	P
Constant	106	1451	0.07	0.942
Day	-0.00278	0.03891	-0.07	0.943
Neomycin	0.000771	0.002193	0.35	0.726

Test 3: Is the density of recipient *S. typhimurium* 576 affected by time or dose of neomycin?

```
MTB > glm recipients = day + 'neomycin dose';
SUBC> covar day 'neomycin dose'.
```

General Linear Model

Analysis of Variance for Recipien

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	34.854	33.746	33.746	17.82	0.000
Neomycin	1	28.249	28.249	28.249	14.92	0.000
Error	75	142.038	142.038	1.894		
Total	77	205.141				

Term	Coef	StDev	T	P
Constant	4660	1104	4.22	0.000
Day	-0.12489	0.02959	-4.22	0.000
Neomycin	-0.006339	0.001641	-3.86	0.000

Minitab output 8.2: Is there an interaction between the effects of time and neomycin dose on bacterial populations?

Test 4: Does the decrease of background *E. coli* density with time depend on the dose of neomycin given?

MTB > glm 'background E. coli' = day+'neomycin dose' +day*'neomycin dose';
SUBC> covar 'day' 'neomycin dose'.

General Linear Model

Analysis of Variance for Backgrou

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	3.4140	3.2796	3.2796	8.15	0.006
Neomycin	1	1.8471	0.7059	0.7059	1.75	0.189
Day*Neomycin	1	0.7062	0.7062	0.7062	1.76	0.189
Error	76	30.5778	30.5778	0.4023		
Total	79	36.5452				

Term	Coef	StDev	T	P
Constant	2304.1	804.2	2.87	0.005
Day	-0.06155	0.02156	-2.86	0.006
Neomycin	-7.016	5.297	-1.32	0.189
Day*Neomycin	0.000188	0.000142	1.32	0.189

Test 5: Does the decrease of recipient *S. typhimurium* 576 density with time depend on the dose of neomycin given?

MTB > glm recipients = day+'neomycin dose' +day*'neomycin dose';
SUBC> covar 'day' 'neomycin dose'.

General Linear Model

Analysis of Variance for Recipien

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Day	1	34.854	56.376	56.376	35.62	0.000

Neomycin	1	28.249	24.936	24.936	15.76	0.000
Day*Neomycin	1	24.928	24.928	24.928	15.75	0.000
Error	74	117.110	117.110	1.583		
Total	77	205.141				

Term	Coef	StDev	T	P
Constant	9802	1642	5.97	0.000
Day	-0.26275	0.04402	-5.97	0.000
Neomycin	-42.44	10.69	-3.97	0.000
Day*Neomycin	0.001138	0.000287	3.97	0.000

Minitab session 9.1: Testing relationship between neomycin dose and 22 hour density.

Test 1

MTB > glm d = neo;
SUBC> covar neo.

General Linear Model

Analysis of Variance for D

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Neo	1	0.032885	0.032885	0.032885	4.29	**0.107**
Error	4	0.030692	0.030692	0.007673		
Total	5	0.063577				

Term	Coef	StDev	T	P
Constant	9.10167	0.04919	185.02	0.000
Neo	-0.000444	0.000214	-2.07	0.107

Test 2

MTB > glm r = neo;
SUBC> covar neo.

General Linear Model

Analysis of Variance for R

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Neo	1	1.1197	1.1197	1.1197	130.20	**0.000**
Error	4	0.0344	0.0344	0.0086		
Total	5	1.1541				

Term	Coef	StDev	T	P
Constant	8.27727	0.05208	158.93	0.000
Neo	-0.002591	0.000227	-11.41	0.000

Test 3

MTB > glm t = neo + neo*neo;
SUBC> covar neo.

General Linear Model

Analysis of Variance for T

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Neo	1	0.6168	0.0784	0.0784	0.53	0.520
Neo*Neo	1	0.2114	0.2114	0.2114	1.42	**0.318**
Error	3	0.4451	0.4451	0.1484		
Total	5	1.2732				

Term	Coef	StDev	T	P
Constant	5.5064	0.3282	16.78	0.000
Neo	0.003186	0.004383	0.73	0.520
Neo*Neo	-0.000010	0.000008	-1.19	0.318

Test 4

MTB > glm t = neo;
SUBC> covar neo.

General Linear Model

Analysis of Variance for T

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Neo	1	0.6168	0.6168	0.6168	3.76	**0.125**
Error	4	0.6564	0.6564	0.1641		
Total	5	1.2732				

Term	Coef	StDev	T	P
Constant	5.8011	0.2275	25.50	0.000
Neo	-0.001923	0.000992	-1.94	0.125

Mintab session 9.2: testing relationship between dose of other antibiotics and 22 hour density

Test 5

MTB > glm d = amp;
SUBC> covar amp.

General Linear Model

Analysis of Variance for D

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Amp	1	0.02230	0.02230	0.02230	0.90	**0.396**
Error	4	0.09872	0.09872	0.02468		
Total	5	0.12102				

Term	Coef	StDev	T	P
Constant	9.2154	0.1155	79.81	0.000
Amp	-0.1191	0.1252	-0.95	0.396

Test 6

MTB > glm r = amp;
SUBC> covar amp.

General Linear Model

Analysis of Variance for R

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Amp	1	2.3159	2.3159	2.3159	19.08	**0.007**
Error	5	0.6069	0.6069	0.1214		
Total	6	2.9228				

Term	Coef	StDev	T	P
Constant	8.5972	0.2561	33.57	0.000
Amp	-1.1781	0.2697	-4.37	0.007

Test 7

MTB > glm t = amp + amp*amp;
SUBC> covar amp.

General Linear Model

Analysis of Variance for T

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Amp	1	1.5309	0.0008	0.0008	0.01	0.938
Amp*Amp	1	0.0982	0.0982	0.0982	0.88	**0.401**
Error	4	0.4464	0.4464	0.1116		
Total	6	2.0755				

Term	Coef	StDev	T	P
Constant	6.7552	0.3696	18.28	0.000
Amp	0.095	1.152	0.08	0.938
Amp*Amp	-0.6639	0.7078	-0.94	0.401

Test 8

MTB > glm t = amp;
SUBC> covar amp.

General Linear Model

Analysis of Variance for T

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Amp	1	1.5309	1.5309	1.5309	14.06	**0.013**
Error	5	0.5446	0.5446	0.1089		
Total	6	2.0755				

Term	Coef	StDev	T	P
Constant	7.0143	0.2426	28.92	0.000
Amp	-0.9579	0.2555	-3.75	0.013

Test 9

MTB > glm d = chlor;
SUBC> covar chlor.

General Linear Model

Analysis of Variance for D

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Chlor	1	0.10503	0.10503	0.10503	1.37	**0.286**
Error	6	0.46025	0.46025	0.07671		
Total	7	0.56527				

Term	Coef	StDev	T	P
Constant	8.9749	0.1442	62.24	0.000
Chlor	0.02202	0.01882	1.17	0.286

Test 10

MTB > glm r = chlor;
SUBC> covar chlor.

General Linear Model

Analysis of Variance for R

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Chlor	1	8.5350	8.5350	8.5350	267.69	**0.000**

Error	5	0.1594	0.1594	0.0319
Total	6	8.6945		

Term	Coef	StDev	T	P
Constant	7.06955	0.09795	72.18	0.000
Chlor	-0.27099	0.01656	-16.36	0.000

Test 11

MTB > glm t= chlor + chlor*chlor;
SUBC> covar chlor.

General Linear Model

Analysis of Variance for T

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Chlor	1	6.2089	1.2127	1.2127	51.54	**0.002**
Chlor*Chlor	1	0.4399	0.4399	0.4399	18.70	**0.012**
Error	4	0.0941	0.0941	0.0235		
Total	6	6.7429				

Term	Coef	StDev	T	P
Constant	6.3535	0.1434	44.30	0.000
Chlor	-0.56707	0.07899	-7.18	0.002
Chlor*Chlor	0.026809	0.006200	4.32	0.012

Minitab session 9.3: testing relationship between neomycin dose and 22 hour density in caecal content

Test 12

MTB > glm d = neoCC;
SUBC> covar neoCC.

General Linear Model

Analysis of Variance for D

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NeoCC	1	13.147	13.147	13.147	62.53	**0.000**
Error	6	1.261	1.261	0.210		
Total	7	14.408				

Term	Coef	StDev	T	P
Constant	4.0546	0.1998	20.29	0.000
NeoCC	0.005394	0.000682	7.91	0.000

Test 13

MTB > glm r = neoCC;
SUBC> covar neoCC.

General Linear Model

Analysis of Variance for R

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NeoCC	1	1.1107	1.1107	1.1107	1.63	**0.249**
Error	6	4.0855	4.0855	0.6809		
Total	7	5.1962				

Term	Coef	StDev	T	P
Constant	6.1759	0.3596	17.17	0.000
NeoCC	0.001568	0.001228	1.28	0.249

Test 14

MTB > glm t = neocc + neocc*neocc;
SUBC> covar neocc.

General Linear Model

Analysis of Variance for T

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NeoCC	1	4.9854	1.1317	1.1317	1.43	0.298
NeoCC*NeoCC	1	0.2986	0.2986	0.2986	0.38	**0.573**
Error	4	3.1709	3.1709	0.7927		
Total	6	8.4549				

Term	Coef	StDev	T	P
Constant	3.4364	0.4903	7.01	0.002
NeoCC	0.006827	0.005714	1.19	0.298
NeoCC*NeoCC	-0.000005	0.000008	-0.61	0.573

Test 15

MTB > glm t = neocc;
SUBC> covar neocc.

General Linear Model

Analysis of Variance for T

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NeoCC	1	4.9854	4.9854	4.9854	7.18	**0.044**
Error	5	3.4694	3.4694	0.6939		
Total	6	8.4549				

Term	Coef	StDev	T	P
Constant	3.5841	0.3997	8.97	0.000
NeoCC	0.003422	0.001277	2.68	0.044

MTB >

Mintab session 10.1 Analysing the cost of resistance

Test 1. Is there a cost of resistance and does it vary witlı strain of *S. typhimurium* recipient?

MTB > glm salmslope = population + strain + population*strain.

General Linear Model

Factor Levels Values
populati 2 2 3
strain 3 1 2 4

Analysis of Variance for SALMSLOP

Source	DF	Seq SS	Adj SS	Adj MS	F	P
populati	1	0.07753	0.07753	0.07753	2.81	0.099
strain	2	0.10162	0.11275	0.05638	2.04	0.139
populati*strain	2	0.32621	0.32621	0.16310	5.90	**0.005**
Error	57	1.57444	1.57444	0.02762		
Total	62	2.07980				

Test 2. Is there a cost of resistance in an *E. coli* recipient?

MTB > glm ecolislope = population.

General Linear Model

Factor Levels Values
populati 2 2 3

Analysis of Variance for Ecolislo

Source	DF	Seq SS	Adj SS	Adj MS	F	P
populati	1	0.01611	0.01611	0.01611	0.44	**0.515**
Error	19	0.69548	0.69548	0.03660		
Total	20	0.71159				

Test 3. Which *S. typhimurium* strains have a significant cost of resistance when analysed separately?

Strain1:

MTB > glm salm1slope = population.

General Linear Model

Factor Levels Values
populati 2 2 3

Analysis of Variance for salm1slo

Source	DF	Seq SS	Adj SS	Adj MS	F	P
populati	1	0.11909	0.11909	0.11909	6.41	**0.020**
Error	19	0.35321	0.35321	0.01859		
Total	20	0.47230				

Strain2:

MTB > glm salm2slope = population.

General Linear Model

Factor	Levels	Values
populati	2	2 3

Analysis of Variance for salm2slo

Source	DF	Seq SS	Adj SS	Adj MS	F	P
populati	1	0.19322	0.19322	0.19322	5.44	**0.031**
Error	19	0.67465	0.67465	0.03551		
Total	20	0.86787				

Strain3:

MTB > glm salm4slope = population.

General Linear Model

Factor	Levels	Values
populati	2	2 3

Analysis of Variance for salm4slo

Source	DF	Seq SS	Adj SS	Adj MS	F	P
populati	1	0.09143	0.09143	0.09143	3.18	**0.091**
Error	19	0.54659	0.54659	0.02877		
Total	20	0.63802				

Mintab session 10.2: Analysing plasmid transfer rates

Test 4. Does the plasmid transfer rate vary with species?

MTB > glm ptr = species.

General Linear Model

Factor Levels Values
Species 2 1 2

Analysis of Variance for PTR

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Species	1	0.0843	0.0843	0.0843	0.79	**0.397**
Error	9	0.9596	0.9596	0.1066		
Total	10	1.0438				

Test 5. Does the plasmid transfer rate vary with strain within the *S. typhimurium* strains?

MTB > glm ptr = strain.

General Linear Model

Factor Levels Values
Strain 3 1 2 4

Analysis of Variance for PTR

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Strain	2	0.3556	0.3556	0.1778	1.65	**0.282**
Error	5	0.5393	0.5393	0.1079		
Total	7	0.8949				

Mintab session 10.3. Analysing plasmid transfer during stationary phase:

Test 6. Does the change in donor densities during stationary phase vary with strain?

MTB > glm d=strain.

General Linear Model

Factor Levels Values
strain 4 1 2 3 4

Analysis of Variance for d

Source	DF	Seq SS	Adj SS	Adj MS	F	P
strain	3	3.5088	3.5088	1.1696	3.85	**0.065**
Error	7	2.1291	2.1291	0.3042		
Total	10	5.6379				

Test 7. Does the change in recipient densities during stationary phase vary with strain?

MTB > glm r=strain.

General Linear Model

Factor Levels Values
strain 4 1 2 3 4

Analysis of Variance for r

Source	DF	Seq SS	Adj SS	Adj MS	F	P
strain	3	0.2583	0.2583	0.0861	0.21	**0.890**
Error	8	3.3508	3.3508	0.4188		
Total	11	3.6090				

Test 8. Does the change in transconjugant densities during stationary phase vary with strain?

MTB > glm t=strain.

General Linear Model

Factor Levels Values
strain 4 1 2 3 4

Analysis of Variance for t

Source	DF	Seq SS	Adj SS	Adj MS	F	P

strain	3	0.7110	0.7110	0.2370	0.48	**0.703**
Error	7	3.4210	3.4210	0.4887		
Total	10	4.1320				

It is concluded that there are no significant strain differences in growth during stationary phase and a nested model is not required.

Analysing plasmid transfer during stationary phase: Testing for increase in population density during stationary phase.

Test 9. Do donor bacteria increase during stationary phase?

MTB > TTest 0.0 'd';
SUBC> Alternative 1.

T-Test of the Mean

Test of mu = 0.000 vs mu > 0.000

Variable	N	Mean	StDev	SE Mean	T	P
d	11	-0.240	0.751	0.226	-1.06	**0.84**

Test 10. Do recipient bacteria increase during stationary phase?

MTB > TTest 0.0 'r';
SUBC> Alternative 1.

T-Test of the Mean

Test of mu = 0.000 vs mu > 0.000

Variable	N	Mean	StDev	SE Mean	T	P
r	12	0.535	0.573	0.165	3.24	**0.0040**

Test 11. Do transconjugant bacteria increase during stationary phase?

MTB > TTest 0.0 't';
SUBC> Alternative 1.

T-Test of the Mean

Test of mu = 0.000 vs mu > 0.000

Variable	N	Mean	StDev	SE Mean	T	P
t	11	0.354	0.643	0.194	1.83	**0.049**

MTB >

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