The differential transmissibility of myxoma virus strains of differing virulence grades by the rabbit flea *Spilopsyllus cuniculi* (Dale)*

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SUMMARY

Laboratory studies showed that few rabbit fleas (*Spilopsyllus cuniculi* (Dale)) transmitted myxomatosis after removal from wild rabbits (*Oryctolagus cuniculus* (L)) that had been infected for fewer than 10–12 days, irrespective of the virulence of the myxoma virus strain involved. Rabbits infected with fully virulent (Grade I) strains died within 10–15 days and few fleas from these hosts became infective; averaging all the samples taken, 12 % of the fleas were infective. Also, few fleas acquired infectivity on individual rabbits which recovered from infection with attenuated strains; the mean was 8 % infective. Rabbits which died between 17 and 44 days after infection had higher proportions of infective fleas at all sampling times; the mean was 42 % infective. Male and female fleas transmitted virus with equal efficiency.

For rabbits infected with any of the attenuated virus strains the mean percentage of infective fleas was inversely related to the survival time of the host. Rabbits infected with moderately attenuated strains (Grades IIIA and IIIB) had, on average, the highest proportion of infective fleas; hence such strains have a selective advantage and have become predominant under natural conditions in Britain. The changes that might occur if there is an increase in host resistance to myxomatosis are discussed.

INTRODUCTION

The strains of myxoma virus used to initiate the Australian and European epizootics were both of high virulence for European rabbits (*Oryctolagus cuniculus* (L)), producing > 99 % mortality in laboratory rabbits. In both continents attenuated strains of virus giving reduced kills were recovered from the field within two years of the initial outbreaks (Fenner, 1953; Fenner & Marshall, 1955, 1957; Hudson, Thompson & Mansi, 1955; Jacotot, Vallée & Virat, 1955; Mykytowycz, 1953). Subsequent large scale surveys have shown that moderately attenuated strains have come to predominate in the field in both Australia and

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Britain (Fenner & Chapple, 1965; Fenner & Ratcliffe, 1965; Marshall & Fenner, 1960).

Experiments by Fenner, Day & Woodroffe (1952) indicated that transmission of myxoma virus by mosquitoes, the principal vectors of myxomatosis in Australia, was an example of mechanical transmission. Virions adhering to the piercing mouth-parts of the vector when it ceased feeding on an infected rabbit could be deposited within the skin of another rabbit during a subsequent feed and produce an infection in the latter rabbit. Later Fenner, Day & Woodroffe (1956) showed how mosquitoes could play a major evolutionary role by favouring the selection of moderately attenuated field strains of virus. It was found that a high titre of virus, in the region of 10^7 RID 50 g^{-1} , was required in the skin of an infected rabbit before mosquitoes would transmit virus readily. Rabbits infected with highly virulent strains of myxoma virus have the shortest survival times and they die a few days after the skin virus titre has reached such values. Rabbits infected with highly attenuated strains have long survival times but the virus titre may reach high values only briefly, falling away as the lesions regress. By contrast, rabbits infected with moderately attenuated strains present a combination of extended survival times with a more prolonged high skin virus titre. Thus rabbits infected with moderately attenuated strains of virus provide feeding mosquitoes with the most persistent sources of infectivity and hence these are the strains most likely to be transmitted in the field and to become dominant.

In Britain the principal vector of myxomatosis is the rabbit flea (*Spilopsyllus cuniculi* (Dale)), although mosquitoes may play a part in some instances (Lockley, 1954; R. C. Muirhead-Thomson, unpublished 1956; Muirhead-Thomson, 1956; Service, 1971). The rabbit flea is a relatively sedentary species closely associated with a host for most of the time, but it was found by Mead-Briggs (1964) that there can be a considerable interchange of fleas between living hosts. This was contrary to earlier opinion which had held that the fleas usually left an individual rabbit only after the death of the host. This belief had led to the suggestion by Andrewes, Thompson & Mansi (1959) that in a situation where rabbit fleas were the principal vectors the spread of highly virulent virus, which killed virtually every rabbit and thus led to the release of their fleas, would be selectively favoured.

This paper reports observations on the effectiveness of the rabbit flea as a vector of strains of myxoma virus of differing virulence and discusses the influence the flea may have on the extent to which attenuation of the virus is likely to proceed under natural conditions in Britain.

MATERIALS AND METHODS

The basic experimental procedure consisted of the infection of individual wild rabbits with selected strains of myxoma virus, and their infestation with rabbit fleas. Batches of fleas were removed at selected times during the course of the disease and assayed by controlled feeding on domestic rabbits to determine the proportions of infective fleas in the samples.

Table 1. *The strains of myxoma virus used in flea transmission experiments*

Name	Virulence grade	Synonym and first description	Origin
Cornwall	I	England/Cornwall/4-54/1 (Fenner & Marshall, 1957)	Wild rabbit
Glenfield	I	Australia/Dubbo/2-51/1 (Fenner & Marshall, 1957)	Wild rabbit
Brecon	IIIA	4298 (Weybridge 1961) (Chapple & Bowen, 1963)	Wild rabbit
Ryston	IIIA	V11/8-68	Wild rabbit
Skokholm	IIIA	V4/11-67	Wild rabbit
Breamore	IIIA	V28-2/2-70	Wild rabbit
Breamore	IIIB	V28-1/2-70	Wild rabbit
FS 98	IIIB	Australia/-	Wild rabbit
Nottingham	V	England/Nottingham/4-55/1 (Fenner & Marshall, 1957)	Wild rabbit

Seven British and two Australian virus strains representing a range of virulence grades were used. Details of the virulence grade, full designation and first description, if published, of each strain are given in Table 1. All strains were obtained from lesion material taken from wild rabbits, passaged and assayed for virulence grade by the method of Fenner & Marshall (1957). Cornwall, Brecon and Nottingham strains are used in this laboratory as standard reference strains for their respective virulence grades. The Breamore, Ryston and Skokholm strains were collected as representative contemporary field strains in the localities from which most of the experimental wild rabbits were collected. The two Australian strains were kindly supplied by Dr W. R. Sobey.

Most of the wild rabbits required were collected as young animals 2-4 months old. The methods of capture used and the technique for acclimatizing and maintaining young wild rabbits in captivity were as described by Mead-Briggs & Vaughan (1973). As only rabbits susceptible to myxoma infection were suitable those possessing active immunity to myxomatosis, indicating recovery from infection in the wild, were eliminated from the collection after testing blood samples for the presence of myxoma antibody. Blood samples were taken from the marginal ear vein when the animals were more than 4 months of age; by this age any passive, maternally-derived antibody has disappeared. All domestic rabbits used were New Zealand Whites weighing 2.5-4 kg. and obtained from a commercial supplier.

Supplies of fleas were obtained either from wild rabbit nests sent to the laboratory and kept wrapped in paper towels inside polythene bags until adults emerged, or from healthy wild rabbits bagged up immediately after being shot. Fleas collected from the second source were starved at 4° C. 95% R.H. for at least 4 days before use.

For each experiment three wild rabbits were shaved on both earbases and on a portion of one flank. The ears were then enclosed in nylon organdie bags attached to the earbases with adhesive surgical tape in order to prevent access by fleas as the ears are a poor source of infectivity (Chapple & Muirhead-Thomson,

1964). The rabbits were inoculated intradermally on the shaved flank with 0.1 ml. of a suspension containing 10–100 RID 50 of a known strain of virus and infested with 75 ♂ 75 ♀ fleas released onto the head. If numbers of fleas on a rabbit appeared low during an experiment further batches of 25 ♂ 25 ♀ were released, but never within 3 days before any sampling.

The rabbits were inspected daily from the third day after inoculation and records kept of the time of appearance of a primary lesion at the site of inoculation and the subsequent appearance of secondary symptoms. The earbags were checked and renewed if they had been damaged by the rabbit or constricted the earbases.

Batches of 12 ♂ 12 ♀ fleas were combed from the head of each rabbit at intervals of 3 or 4 days, beginning 8 days after inoculation for Grade I strains and 12 days for attenuated strains. Preliminary experiments indicated that infective fleas were rarely found before these time intervals had elapsed. Batches were taken until 28 days after inoculation or earlier death of the rabbit. The fleas were starved at 4° C. 95% R.H. for 3 days then assayed for infectivity by allowing them to feed on the shaved flank of a domestic rabbit anaesthetized with 'Nembutal' (pentobarbitone sodium) administered through the marginal ear vein at 30–35 mg. kg.⁻¹. Twelve fleas could be fed simultaneously using a rack of 15 × 40 mm. transparent plastic tubes spaced in a 3 × 4 array by 10 × 10 × 15 mm. blocks of foam plastic stuck to the tubes. The array was held firmly but flexibly against the flank of the rabbit by a body belt made of a strip of fabric with three rubber bands attached at one end. The fabric was placed beneath the rabbit, the bands passed over a grid of hard plastic strips resting on the spacing blocks between the tubes and were then hooked to the free end of the belt. The apparatus is illustrated in Plate 1. One flea was placed in each tube and the tube capped. After 30 min. the fleas were removed to individual vials numbered to correspond with the feeding position on the flank. The feeding rack was removed from the rabbit and the feeding sites ringed using a felt-tip pen (Plate 2). The rabbit was given an additional dose of 5–20 mg. 'Nembutal' according to response, and the feeding rack fitted to the opposite flank. The second batch of twelve fleas was then treated similarly to the first. Usually all fleas fed on one flank were of the same sex.

After removal all fleas were checked for the presence of a fresh blood meal. To do this each flea was lightly anaesthetized with CO₂ and observed under a binocular dissecting microscope whilst gently compressed between a slide and cover-slip with a mounted needle. The fleas were again starved for 3 days at 4° C. 95% R.H. and then allowed the opportunity of feeding for 30 min. on a further rabbit, in the same relative positions as before; this procedure was required as not all fleas probed and fed on the first occasion. After recovery from anaesthesia the rabbits were returned to the animal house and checked daily from the third day after feeding for the presence of primary lesions at any of the flea feeding sites, indicating transmission by particular fleas. Lesions appearing within other marked sites up to 48 hr. after the first ones were also accepted as being positive transmissions. After this 48 hr. interval secondary lesions usually

Table 2. *The mean numbers ($\bar{x}$) of fleas transmitting infection, out of samples of 24 removed at various times from wild rabbits infected with different strains of myxoma virus*

(n = no. of replicate wild rabbits.)

Virus strain and grade		Flea sampling times (days after infection)					
		8	10-12	14-15	18	22	24-28
Cornwall I	$\bar{x}$	1.2	3.8	4.8	—	—	—
	n	6	6	5	—	—	—
Glenfield I	$\bar{x}$	1.0	2.5	—	—	—	—
	n	2	2	—	—	—	—
Brecon IIIA	$\bar{x}$	—	6.5	7.6	9.8	9.3	8.1
	n	—	11	11	11	7	7
Ryston IIIA	$\bar{x}$	—	5.2	6.0	5.2	4.0	3.8
	n	—	5	5	5	5	4
Skokholm IIIA	$\bar{x}$	—	5.5	9.0	11.8	13.5	8.8
	n	—	4	4	4	4	4
Breamore IIIA	$\bar{x}$	—	7.3	5.0	4.1	3.0	3.6
	n	—	7	7	7	7	7
Breamore IIIB	$\bar{x}$	—	7.0	3.0	5.7	0	1.7
	n	—	3	1	3	1	3
FS 98 IIIB	$\bar{x}$	—	3.3	4.5	0.3	0	0
	n	—	4	2	4	2	4
Nottingham V	$\bar{x}$	—	5.7	2.7	0.8	0	0
	n	—	3	3	3	3	3

began to appear and the rabbit was discarded. The results from the two assay rabbits for each batch of fleas were combined; a flea transmitting at both feeds was scored as one positive result. Feeding of successive batches of fleas from each wild rabbit gave a set of records of the proportions of fleas transmitting infection at various times during the course of the disease. Fleas transmitting infection are referred to subsequently as infective fleas.

RESULTS

Table 2 gives the means of the numbers of infective fleas found in the batches of 24 fleas taken at each sampling time from each of several replicate wild rabbits and for each strain of virus tested. In this table results from rabbits which died and from those which survived after infection with Brecon, Ryston, Skokholm, Breamore IIIA and FS98 strains have been combined in calculating the means. The two Grade I strains gave no survivors and all the rabbits infected with Breamore IIIB and Nottingham V strains survived. The results show that rabbits infected with the Grade I strains produced low mean numbers of infective fleas at each sampling time; these rabbits were all dead within 14-15 days of infection. FS98 IIIB and Nottingham V strains were also poorly transmitted, the number of infective fleas falling to zero by 22 days after infection. The four Grade IIIA strains all produced infective fleas at each sampling time. The results suggest

Table 3. *The mean numbers ($\bar{x}$) of fleas transmitting infection, out of samples of 24 removed at various times from wild rabbits infected with different strains of myxoma virus*

(Results grouped by survival times of rabbits. n = no. of replicate wild rabbits.)

Rabbit survival times (days)		Flea sampling times (days after infection)					
		8	10-12	14-15	18	22	24-28
10-16	$\bar{x}$	1.13	3.5	4.8	—	—	—
	n	8	8	5	—	—	—
17-23	$\bar{x}$	—	11.8	12.0	11.0	13	—
	n	—	4	4	4	1	—
24-30	$\bar{x}$	—	8.0	10.6	14.0	18.0	14.4
	n	—	5	5	5	4	5
31-37	$\bar{x}$	—	5.8	7.0	9.0	7.4	6.6
	n	—	5	5	5	5	5
38-44	$\bar{x}$	—	11.7	9.0	8.7	6.8	7.3
	n	—	3	3	3	3	3
Survivors	$\bar{x}$	—	3.5	2.6	2.3	0.5	0.5
	n	—	20	16	20	16	20

Table 4. *The mean percentages, over all samples, of fleas transmitting infection after removal at various times from myxoma-infected wild rabbits*

(Results grouped by survival times of rabbit.)

	Rabbit survival time groups (days)					Survivors
	10-16	17-23	24-30	31-37	38-44	
Mean % of infective fleas	12.0 ^a	49.6 ^b	52.8 ^b	29.8 ^b	36.1 ^b	8.3 ^a

Analysis of variance of arcsin transformed % of infective fleas in each survival time group

	ss	dF	MS	F	
Total	9927.5	44	—	—	
Between groups	7053.6	5	1410.7	19.1	$P < 0.001$
Error	2874.0	39	73.7	—	

a, b : means having the same suffix letter do not differ significantly (Scheffe's test).

that a peak was reached during the course of the infection, the timing of the maximum varying from strain to strain.

Table 3 shows the mean numbers of infective fleas at each sampling time, calculated after grouping the individual infected rabbits in survival time classes instead of by virus strains. Two classes contain low numbers of infective fleas, the 10-16 day class and the survivors class. The 10-16 day class contains only rabbits infected with Cornwall and Glenfield Grade I strains. The survivors class contains all rabbits surviving infection with any of the attenuated virus strains; in these experiments no rabbit died from myxomatosis more than 44 days after

Fig. 1. The relation between the survival time of a myxoma-infected wild rabbit and the percentage of infective fleas obtained by sampling at standardized times during the infection. The regression line (shown with its 95% confidence limits) excludes the results for rabbits infected with the Grade I Cornwall and Glenfield strains.

infection. Comparison of the means for the survivors class with those for the classes with survival times between 17 and 44 days shows that rabbits which survived produced much lower numbers of infective fleas throughout the course of the infection than did rabbits which died, regardless of the strain of virus used to initiate the infection.

In Table 4 the number of infective fleas, summed over all the sampling times, is expressed as a mean percentage of the total number of fleas assayed from each rabbit survival time group. The percentages are compared by analysis of variance after arcsin transformation. The results of the analysis show a highly significant difference between mean percentages of infective fleas for survival time classes ($P < 0.001$). It was found, by Scheffe's test, that all the significance arose from the contrast between the 10–16 day and survivors classes together and the remaining classes. Thus, significantly more fleas acquired infectivity on rabbits surviving between 17 and 44 days after infection than on rabbits which survived less than 17 days or which recovered from infection.

In Fig. 1 the percentage of infective fleas from each rabbit for all sampling times combined is plotted against the survival time of the rabbit. Animals which recovered were allotted an arbitrary survival time of 50 days, but in Fig. 1 their points are shown separated along the x -axis for clarity. Each virus strain tested is denoted by a different symbol. The regression line, which is shown with its 95% confidence limits, was fitted using results from rabbits surviving longer

than 17 days; the regression coefficient, $b = -1.501$. Fig. 1 shows that for animals infected with attenuated strains of virus the percentage of infective fleas produced is inversely related to the number of days the rabbit lives after infection. It also shows that most rabbits which recover from infection produce low numbers of infective fleas, irrespective of the strain of virus involved.

There was no significant difference between sexes of flea in the efficiency of transmission. In experiments replicated three times three rabbits were infected respectively with Cornwall I, Brecon IIIA and Nottingham V strains of virus and fleas assayed for infectivity. The results were: Cornwall, 20/96 ♂♂ and 17/96 ♀♀ infective, Brecon, 78/132 ♂♂ and 78/132 ♀♀ and Nottingham, 11/144 ♂♂ and 14/144 ♀♀.

DISCUSSION

Studies on the transmission of myxoma virus by the rabbit flea were made to ascertain what part the flea might play, actively or passively, in the selection of mutant field strains and thus its possible influence on the evolutionary course of myxomatosis in Britain. The results presented indicate how the flea has probably been a major factor in the selection and establishment of naturally arising, moderately attenuated strains of virus. During preliminary experiments it was found that few fleas acquired infectivity from infected wild rabbits earlier than 10–12 days after the infection of the rabbit, irrespective of the strain of virus involved. The data in Table 2 show that there were differences in efficiency of transmission both between strains and at different sampling times. Tables 3 and 4 and Fig. 1 show that the proportion of fleas acquiring infectivity on an infected rabbit was correlated with the survival time of that rabbit; this in turn is influenced by the virulence of the strain of virus initiating the infection. Wild rabbits infected with fully virulent strains survived 15 days or less and few fleas acquired infectivity on such rabbits; significantly fewer fleas were infective than was the case on rabbits infected with attenuated strains and dying between 17 and 44 days after infection. Also, significantly fewer fleas acquired infectivity on rabbits which survived infection with any of these attenuated strains. Groups of rabbits infected with moderately attenuated strains of Grade IIIA and IIIB virulence have, on average, shorter survival times and fewer survivors than rabbits infected with the more attenuated Grade IV and V strains (Fenner & Marshall, 1957). Therefore, when these attenuated variants arose in the field it is probable that the highest proportion of infective fleas came from those rabbits infected with the moderately attenuated strains, which thus had a selective advantage. By 1962 Grade III strains had become the most commonly occurring group in Britain (Fenner & Chapple, 1965).

Fenner *et al.* (1956) showed that the proportion of mosquitoes transmitting myxomatosis from an infected rabbit could be correlated with the virus titre in the skin lesions. Preliminary results from analogous studies indicate that the same relationship applies to the rabbit flea. Further experiments with a range of virus strains representing different virulence grades are in progress to extend these results.

At the present time, despite the continued presence in Britain of myxomatosis, wild rabbit numbers are increasing and causing concern. It is essential to have sufficient understanding of the factors involved, and of their interactions, in order to provide assessments of likely future trends and the need for increased use of conventional methods of rabbit control. It is not yet possible to make valid predictions but a brief review of the situation is relevant. The initial spread through Britain of highly virulent myxoma virus in 1953-5 caused extremely high mortalities, and the rabbit population was reduced to a very low figure. The appearance and establishment of mutant strains having reduced virulence probably prevented this host-specific disease from dying out at that time. The survey done in 1962 by Fenner & Chapple (1965) showed that of 222 randomly collected myxoma virus samples, 22% were of high virulence (Grades I and II), 64% were moderately attenuated (Grade III) and 14% were more highly attenuated (Grades IV and V).

When rabbit numbers are low casual field observations often fail to detect the disease, but as the population increases myxomatosis may develop as an epizootic. Such epizootics usually develop in the late summer or autumn and at intervals of 1-3 years. The frequency probably depends primarily upon the density of rabbits because rabbit fleas, infective and otherwise, are not efficient at locating and reaching healthy hosts when the rabbit population is too sparse. The impact of the outbreak on rabbit numbers will be influenced by several factors including the virulence of the virus circulating, the proportion of immune animals and the degree of any genetic resistance to the disease within the population. As rabbits are relatively short-lived the proportion of immune animals is likely to be highest in populations that experience frequent (annual) epizootics and lowest in ones that suffer infrequent outbreaks. Paradoxically, the frequent outbreaks are then likely to produce lower percentage kills than the infrequent ones. Insufficient is known as yet about possible changes in the susceptibility of wild rabbits in Britain to infection with myxoma virus. There is evidence from Australia that in the presence of annual outbreaks of myxomatosis natural selection had produced, within a few generations, rabbits with greatly increased resistance to the disease (Marshall & Fenner, 1958; Marshall & Douglas, 1961). The wild rabbits used for the experiments reported in this paper were caught before 1970. Up to that time studies in Britain had not indicated any substantial increase in heritable resistance (Vaughan & Vaughan, 1969; Ross, 1973), but this may no longer be the case (Ross, 1975). Any increase in resistance will be manifested initially as an increase in survival time. Provided that the relationship between survival time and the proportion of infective fleas, shown in Fig. 1, remains constant and that strains of high virulence still exist or can arise under natural conditions, then there would be a tendency for these strains of higher virulence to be most frequently transmitted and hence to become predominant in the field. The balance of the host-virus strain-vector system could change, therefore, if the level of host resistance changes. However, the overall mortality effect of more virulent virus strains on more resistant rabbits might not at first be greatly different from that experienced in recent years.

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EXPLANATION OF PLATES

PLATE 1

New Zealand White rabbit under 'Nembutal' sedation and fitted with a rack of 12 tubes in which fleas are placed and allowed to probe at individual areas of the shaved flank skin.

PLATE 2

Marking the skin after removal of fleas and feeding rack.