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Fluctuations in the titre of antibody to a soluble antigen of myxoma virus in field populations of rabbits, *Oryctolagus cuniculus* (L.), in Australia

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SUMMARY

Results are presented on tests carried out over the past 5 years to measure the antibody titre of various rabbit populations to a soluble antigen of myxoma virus. In an unexpectedly high percentage of immune rabbits the antibody fell below measurable titres, and later rose to measurable titres without the advent of an observable epizootic of myxomatosis. The re-stimulation of the immune response is discussed in terms of re-infection and virus reactivation.

INTRODUCTION

Little is known about the relation of myxoma virus to the soluble antigens associated with its multiplication. Lesion material invariably contains soluble antigens, and at least eight have been demonstrated (Fenner & Ratcliffe, 1965). Animals with an active viraemia often have the soluble antigens circulating in their serum, and animals which recover from the disease have circulating antibodies to the soluble antigens. A technique for collecting blood samples on filter-paper strips and measuring the presence of soluble antigens or the antibodies to them was developed by Sobey, Conolly & Adams (1966, 1970).

For several years, this technique has been used to test field and laboratory populations of rabbits for the presence of soluble myxoma antigens and their antibodies. This paper presents some of the results obtained over the past few years in various studies.

MATERIALS AND METHODS

Serological technique

Five soluble antigens of myxoma virus have been detected in $(NH_4)_2SO_4$ precipitates (75% saturation) from rabbit lesion extracts (Reisner, Sobey & Conolly, 1963; Conolly & Sobey, unpublished). The precipitate was dissolved in 0·01 M potassium sulphate buffer, pH 7·0, and applied to a DEAE Sephadex (A-50) column. Elution was performed with a 0–0·5 M-NaCl linear gradient. Five separate

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antigenic peaks, designated 'a' to 'e' respectively, were obtained. In all material prepared from 5-day lesions the 'd' antigen was most readily detected, and when run against recovery sera in two-dimensional gel diffusion plates always formed its precipitin band closest to the antigen source.

In the field, a spot of blood (obtained by puncturing the marginal ear vein of the wild rabbit with a lancet) was collected on a strip of filter paper. Care must be taken during the collection and storage of the blood samples to ensure that volatile substances, such as formalin, which denature proteins, are not allowed near them, otherwise the samples are spoilt. Any soluble antigens, or antibodies to them, in the blood were measured by their interference with the location of a precipitin band on an agar plate formed by the reaction between standard antigen and antibody solutions (Sobey *et al.* 1966, 1970). A rough estimate of the titre of the antibody in the blood sample was obtained by assessing the degree to which it interfered with the precipitin band when compared with a photograph of a $\log_2$ dilution series of a serum with a 1/128 titre. A grade 1 reaction was equivalent to a neat titre, a grade 2 reaction to 1/2, and grade 8 to 1/128. Zero on this scale indicates an absence or a low level of circulating anti-'d' antibody.

Management of enclosure

For one experiment, wild rabbits were maintained in a 0·5 acre enclosure. This was further surrounded by a rabbit-proof fence 6 ft. high, surmounted by electrified wires to prevent the entry of feral cats and foxes. Rabbits in the enclosure were examined every 3 weeks after being hand-captured in nets, or after being removed from their burrows. Adult females were palpated to see if they were pregnant and lactating, and the testes of males examined for size and position (either abdominal or scrotal). The size and location of any litters was noted; kittens were individually ear-tagged on reaching 3–4 weeks of age. Three blood samples were taken from each rabbit for serological examination.

Controlled-temperature experiment

One experiment involved exposing certain wild rabbits to various controlled temperatures. For this, use was made of a room in which the temperature could be maintained at any temperature from $-15°$ to $45° \pm 1°$ C. The humidity was unregulated and was not measured. The room housed 24 cages, each holding one rabbit. Water was supplied *ad libitum*, and food was given either as commercial pellets or green vegetables (cabbages, lucerne, etc.), or both.

Field experiments

The rabbit biology section of the Division of Wildlife Research, CSIRO has, for some 5 years, been intensively studying natural rabbit populations at three localities in New South Wales: Snowy Plains, in the subalpine region; Urana, in a Mediterranean-type climate; Canberra, on the slopes of the Dividing Range. These studies have involved the live-capture, tagging and watching of rabbits. As part of the routine, some rabbits which were live-trapped had a spot of blood taken for serological examination.

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Viruses

Two cloned strains of virus were used in this work, Lausanne (Lu) and a field strain (FS/98) collected from the Canowindra district of New South Wales in 1967.

RESULTS

Controlled-temperature experiment

On 20 March, 19 of the 21 surviving immune wild rabbits from a previous experiment (Williams, in preparation) were placed in individual cages in the temperature-controlled room. For serological examination three blood samples from each rabbit were obtained at one time each week. The rabbits remained in the room for 7 months (10 March until 16 October), during which time, except for three periods, each of 3 weeks, the temperature was maintained at 60° F. (15·6° C.), and water and vegetables were provided *ad libitum*. During the three periods the animals were subjected to various stresses, including heat stress (twice), cold stress (once), nutritional stress (each time) and the administration of ACTH (first period only), to determine if any of these factors influence the titre of circulating antibody.

Fig. 1 shows the fluctuations in the titre of circulating antibodies in ten animals injected with 2·5 i.u. of ACTH during the first period of heat stress (1A), and in the remaining nine injected with 10 i.u. of ACTH (1B), during the 7 months of the experiment.

There were variations in the antibody titre of each rabbit, but it was difficult to correlate any particular variation with a particular treatment. In the great majority of cases the variations were not large. In only a few cases did the titre fall to non-detectable levels. In the case of rabbit no. 13, which has been fully discussed elsewhere (Williams, Dunsmore & Parer, 1972), the antibodies rose from an extremely low initial titre to a medium one following the development of the overt sign of myxomatosis during the first period of heat stress. Rabbit no. 13 showed signs of myxomatosis for 6 weeks (from 1 May until mid-June 1970) and soluble antigens were found in her blood during the second and third weeks in May. She was the only rabbit during this experiment which showed signs of myxomatosis.

Enclosure experiment

During September and October 1970 two litters were obtained from each of six does which had shown circulating antibodies to myxoma antigens. Kittens from eight of the litters were challenged when 3 or 10 weeks old with a field strain (FS/98) of myxoma virus, and the remaining kittens in four litters were challenged at comparable ages with the Lu strain of the virus. The animals were held in cages in an animal house from 3 weeks of age. Details of the number of rabbits involved are shown in Table 1. All 35 kittens challenged with the field strain survived. These recovered during December 1970 and January 1971. When recovered, 18 were placed into a 0·5 acre enclosure. None of the 18 Lu-challenged kittens survived.

The fluctuations in the antibody titre of the 18 recovered animals during 1971

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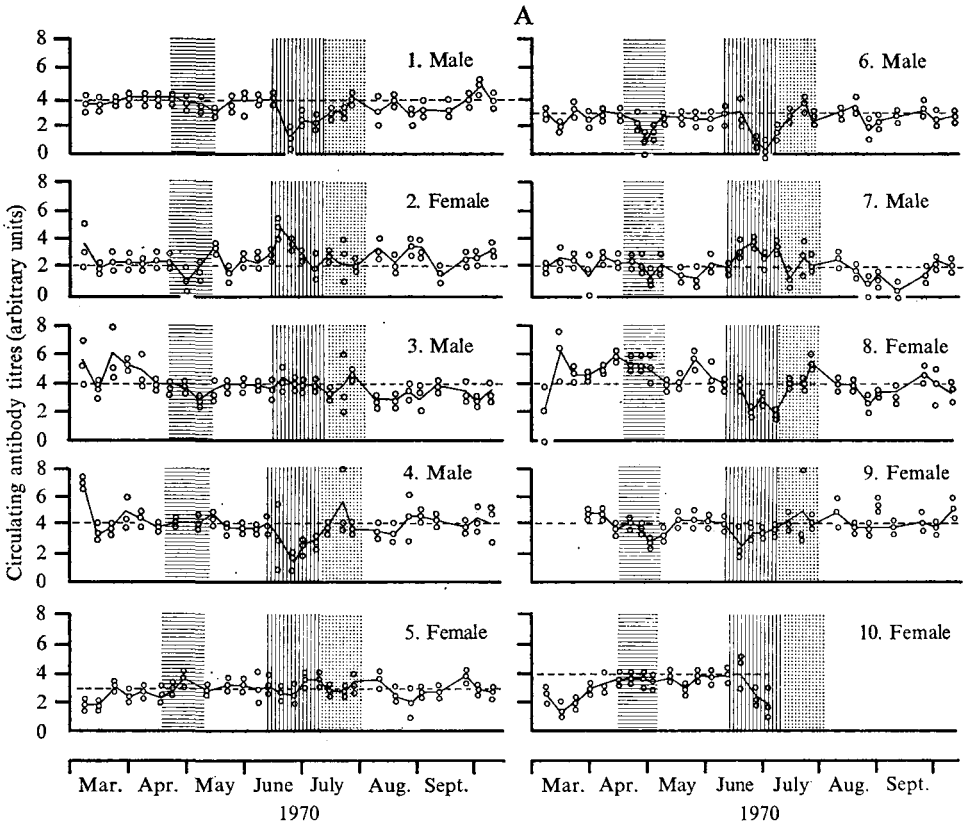

Fig. 1*a*. For legend see facing page.

Table 1. *Details of the number of young rabbits challenged at either* 3 *or* 10 *weeks of age with either a field strain or the Lausanne strain of myxoma virus*

	Age when challenged	
	3 weeks	10 weeks
Field strain		
No. challenged	19*	16†
No. survived	19	16
Lausanne strain		
No. challenged	11	7
No. survived	0	0
Mean survival time	12·0 days	13·0 days
Range	10–13 days	10–16 days

* Nine of the 3-week group and eight of the 10-week challenged group (†) either died on release into the enclosure following their recovery from myxomatosis or were used for other experiments.

and the early part of 1972 are shown in Fig. 2. Three of the ten rabbits (109, 136, 139) challenged at 3 weeks had a very low titre of antibody when released into the enclosure; all eight animals challenged at 10 weeks entered the enclosure with high antibody titres. From the time of being released most of the rabbits showed a decline in antibody titres until, by June 1971, all the rabbits challenged at 3 weeks and four of the eight challenged at 10 weeks had non-detectable antibody titres.

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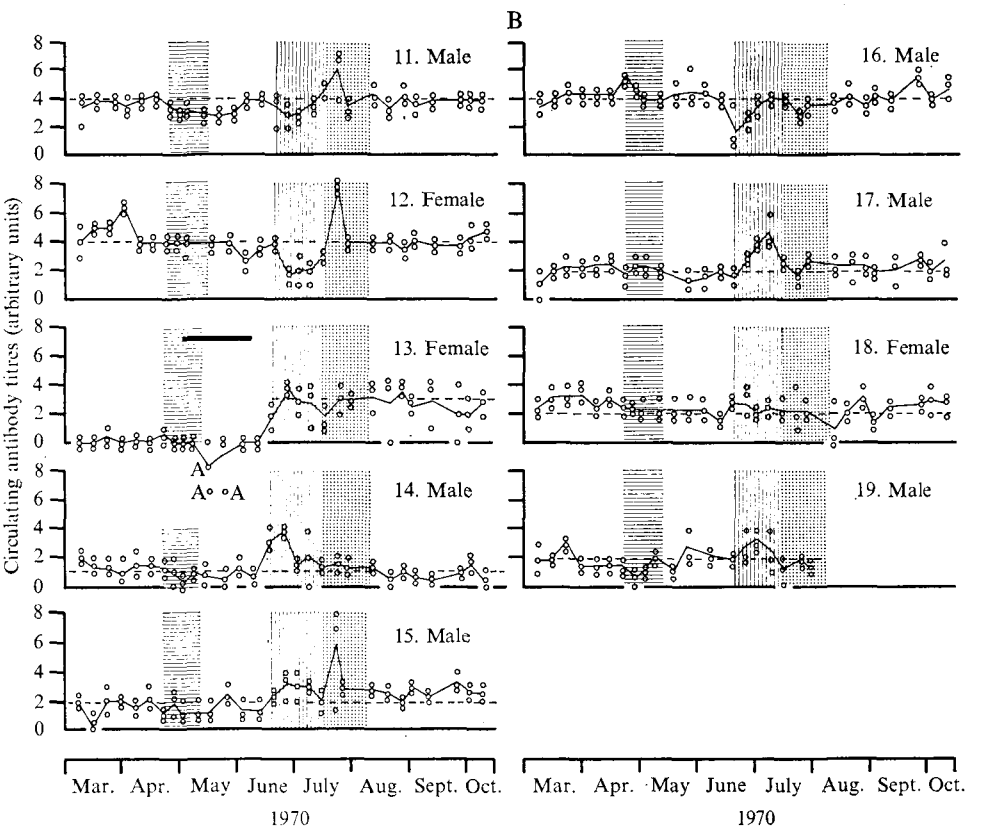

Fig. 1*b*

Temperature raised to 24°C, and vegetables withheld. Ten animals (*a*) received 10 i.u., and the remainder (*b*) 2·5 i.u. of ACTH daily.

Temperature dropped to 2°C, and vegetables withheld.

Temperature raised to 27°C, and straw only provided for food.

Fig. 1. The level of antibody to the 'd' soluble antigen of myxoma virus in 19 immune rabbits held in a constant temperature room from 10 March until 13 October, 1970. Temperature maintained at 16° C. and food (pellets and green vegetables) and water supplied *ad libitum*. The solid horizontal line denotes the period during which rabbit no. 13 was showing signs of myxomatosis and 'A' shows the presence of soluble antigens in her blood samples.

At the end of June the antibody titre of six rabbits (119, 132, 117, 128, 111, 112) rose sharply, whilst in the remainder of the animals it remained low. By late July 1971 the antibody titre of the former six animals had fallen, becoming undetectable in two cases (119, 132), and the other animals retained low antibody titres. In early August 1971 an outbreak of myxomatosis was first noticed amongst the young rabbits born in the enclosure; the last cases occurred in mid-September. Coinciding with the onset of myxomatosis the antibody titre of the adults rose, and remained high until the end of the epizootic.

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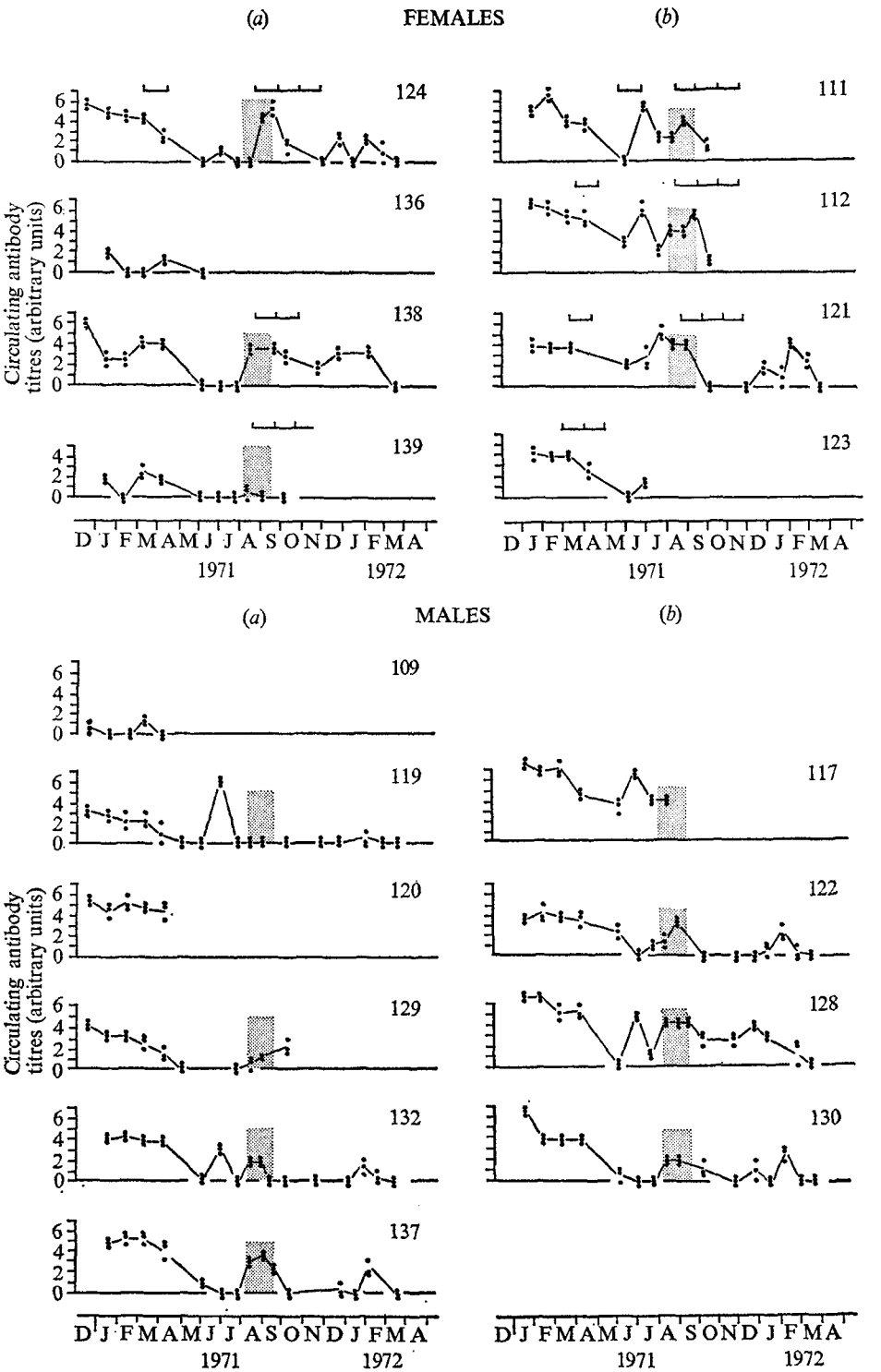

Fig. 2. The level of anti-'d' antibody in 18 rabbits which recovered from myxomatosis following challenge at either 3 (*a*) or 10 (*b*) weeks of age, which were maintained in a 0·5 acre enclosure from December 1970 until March 1972. The horizontal bars denote pregnancies in the females. The shaded area indicates the period of a myxomatosis outbreak which occurred amongst the young rabbits in the enclosure.

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Following the epizootic, the antibody titre in all the animals again declined, and in many cases reached non-detectable levels during October and November. In mid-November four of the adults were killed by vandals. However, in the remaining adults the antibody titre rose during December and January, in the absence of any known cases of myxomatosis in the immediate vicinity (within a radius of 1 mile). The antibody titre declined during February, and by March 1972 was not detectable in any of the animals.

Field studies

Table 2 sets out the results obtained at three study sites maintained by the CSIRO Division of Wildlife Research. Table 2(*a*) shows, for two sites, the percentage of rabbits showing antibody in their blood each month which were known to have recovered from myxomatosis or to have lived through an epizootic and shown anti-'d' antibody. Table 2(*b*) shows the percentage of rabbits never seen to have been challenged with myxoma virus which showed antibody each month, from the three sites. The results obtained from each site are as follows:

Urana

The most complete record of fluctuations in the presence or absence of antibody each month in animals which had recovered from the disease was obtained at Urana. The myxomatosis history at this site from 1967 until 1972 has been fully described elsewhere (Williams & Parer, 1972). At Urana, myxomatosis is epizootic in November and early December of most years, transmission is usually rapid, and very few animals die of the disease. In addition, the morbidity rate is high, such that nearly all animals which survive an epizootic have been seen with the disease, or showed antibody in their blood. The main breeding season at Urana lasts from June until October (Parer, pers. comm.). At any time of the year, therefore, the rabbit population can consist of animals which have survived an epizootic and are immune and the young of the year which have not been exposed to the disease.

Table 2(*a*) shows that each year the lowest percentage of recovered animals with anti-'d' antibody was found in September, 1 month before the end of the breeding season and immediately before the annual epizootic. Over all the years, only 56% of the immune animals showed antibody in September. The percentage showing antibody increased through October, November, December and January each year, during the period when myxomatosis was active. By January each year, from 87 to 89% of the immune animals showed antibody. The percentage showing antibody declined through February and March, in which month only 67% of the immune animals showed antibody, In April and May a high percentage (93 and 97%) of immune animals showed antibody. From this high level the percentage of immune rabbits with antibody declined through the breeding season, to reach the low in September previously referred to. These monthly differences in the overall percentage of animals showing antibody are highly significant on a χ^2 test ($P < 0{\cdot}01$).

Table 2(*b*) shows that a very high percentage (17% overall) of the 688 young susceptible rabbits at Urana, in the only years tested (1967, 1968), showed anti-

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Table 2. *The percentage of rabbits possessing anti-'d' antibody each month during various years from* 1967 *to* 1972 *at three sites*

(The figures in parentheses refer to the number in the sample each month.)

(*a*) Rabbits known to have recovered from myxomatosis, and/or to have possessed anti-'d' antibody following an epizootic

	J	F	M	A	M	J	J	A	S	O	N	D
Urana, N.S.W.												
1967	—	—	—	—	—	—	—	—	—	—	71 (41)*	50 (18)
1968	—	71 (104)	24 (25)	—	—	76 (17)	100 (2)	83 (12)	0 (4)	33 (9)	54 (13)	89 (9)
1969	88 (32)	90 (10)	77 (102)	93 (30)	97 (30)	—	—	—	—	—	—	—
1970	—	—	—	—	—	81 (64)	97 (34)	85 (46)	64 (47)	73 (30)	92 (26)	94 (51)
1971	87 (75)	—	—	—	—	79 (48)	57 (110)	49 (85)	50 (30)	69 (13)	65 (57)	73 (56)
1972	89 (113)	—	—	—	—	—	—	—	—	—	—	—
Total	88 (220)	73 (114)	67 (127)	93 (30)	97 (30)	80 (129)	67 (146)	64 (143)	56 (81)	65 (52)	76 (127)	83 (134)
Snowy Plains, N.S.W.												
1967	—	—	—	—	—	—	—	—	—	—	91 (23)	71 (14)
1968	100 (9)	100 (3)	50 (2)	100 (2)	0 (2)	—	—	88 (8)	80 (10)	50 (16)	9 (11)	50 (12)
1969	91 (11)	29 (7)	25 (4)	—	—	—	—	—	81 (26)	81 (16)	80 (5)	78 (9)
1970	100 (2)	—	67 (3)	100 (2)	—	0 (1)	100 (10)	—	100 (7)			
Total	95 (22)	50 (10)	44 (9)	100 (4)	0 (2)	0 (1)	100 (10)	88 (8)	84 (43)	66 (32)	67 (39)	66 (35)

(*b*) Rabbits known not to have been challenged with myxomatosis

Urana, N.S.W.												
1967	—	—	—	—	—	—	—	—	—	—	8 (127)	13 (56)
1968	—	—	—	—	—	—	23 (31)	12 (154)	20 (167)	23 (62)	28 (87)	50 (4)
Total	—	—	—	—	—	—	23 (31)	12 (154)	20 (167)	23 (62)	16 (214)	15 (60)
Snowy Plains, N.S.W.												
1967	—	—	—	—	—	—	—	—	—	—	24 (33)	6 (65)
1968	5 (114)	5 (61)	10 (41)	0 (26)	24 (21)	22 (9)	—	0 (33)	3 (38)	14 (64)	10 (176)	8 (237)
1969	4 (161)	3 (152)	5 (164)	9 (22)	—	—	—	—	0 (10)	23 (53)	2 (60)	1 (165)

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Table 2 (*cont.*)

	J	F	M	A	M	J	J	A	S	O	N	D
1970	4	—	7	10	—	50	36	—	0			
	(72)		(57)	(40)		(2)	(36)		(9)			
Total	4	4	6	7	24	30	36	0	2	18	10	5
	(347)	(213)	(262)	(88)	(21)	(11)	(36)	(33)	(57)	(117)	(93)	(467)
						Canberra, A.C.T.						
1967	—	—	—	—	—	—	—	—	—	0	2	0
										(45)	(50)	(4)
1968	0	0	—	—	—	—	0	6	3	6	2	2
	(20)	(5)					(5)	(50)	(38)	(96)	(113)	(51)
1969	—	13	0	2	0	0	0					
		(105)	(75)	(85)	(8)	(79)	(11)					
Total	0	13	0	2	0	0	0	6	3	4	2	2
	(20)	(110)	(75)	(85)	(8)	(79)	(16)	(50)	(38)	(141)	(163)	(55)

body. It should be remembered, however, that virtually all the animals tested were under 10 weeks of age, and the possibility exists that many of the kittens possessed maternal antibodies (Fenner & Marshall, 1954).

Snowy Plains

In contrast to the situation at Urana, myxomatosis at Snowy Plains does not appear to occur annually. Details of the myxomatosis history at Snowy Plains from 1967 until 1972 have been set out elsewhere (Dunsmore, Williams & Price, 1971; Dunsmore & Price, 1972). Briefly, an outbreak of the disease was in progress when the study began in March 1967, and all the animals entering the 1967 breeding season, which at Snowy Plains lasts only from October to December each year, were survivors of the outbreak. The next apparent outbreak of myxomatosis at this site was in the winter of 1969, and lasted from March until August, affecting susceptible animals born in 1967 and 1968. Few animals (a maximum of 14%) recovered from the disease, and fewer still (4% of the susceptible animals) avoided infection. Minor epizootics, affecting only a few animals, occurred in 1970 and 1971. The next major epizootic occurred in the summer of 1972, affecting animals born in 1969, 1970 and 1971. In this outbreak, recovery was much higher than in 1969, being about 67%.

Table 2(*a*) shows that the overall fluctuations in the percentage of immune animals showing antibody at Snowy Plains from 1967 to 1970 were similar to those obtained at Urana. Thus, a low percentage of immune animals (66%) showed antibody during the breeding period (October–December), increasing to 95% in January, and declining through February and March (50 and 44%). A total of only seven immune animals was trapped in April, May and June during the study period, which is an insufficient number to provide accurate information on antibody fluctuations. However, as at Urana, in the months immediately before the breeding season (July–September at Snowy Plains), there was a high but declining number of immune animals with antibody (100, 88 and 84%).

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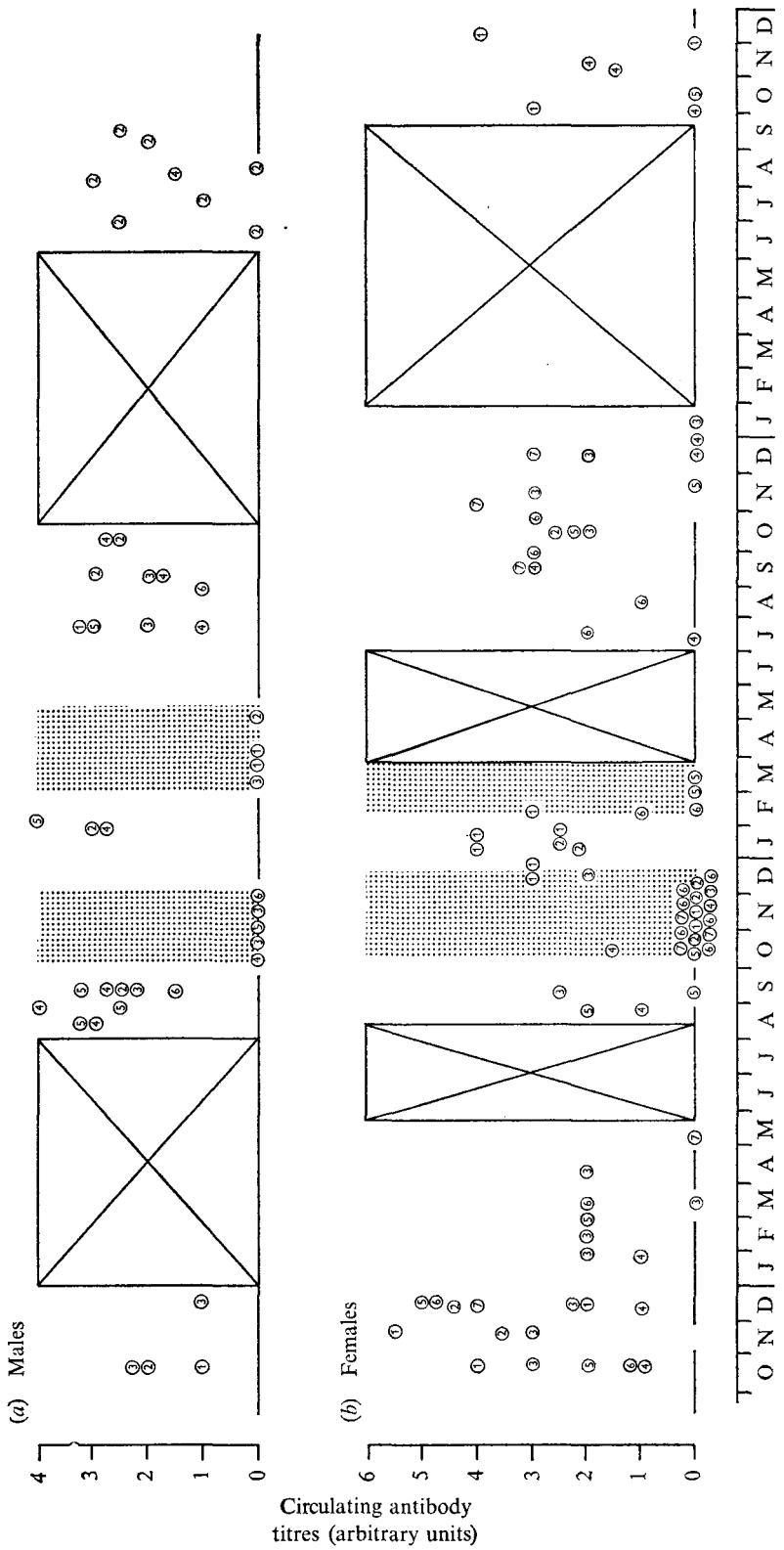

Fig. 3. The level of anti-'d' antibody in six male (*a*) and seven female (*b*) rabbits at Snowy Plains, New South Wales, which recovered from myxomatosis in March 1967, from October 1967 until December 1970. The shaded areas indicate the periods when antibodies decreased to non-detectable levels. The crossed-out portion shows the periods during which the rabbits were very difficult to trap. The solid horizontal line denotes the period of an epizootic of myxomatosis which occurred amongst the population.

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Unlike Urana, several animals at Snowy Plains were trapped sufficiently frequently to provide data on antibody titres in individual rabbits from 1967 to 1969. The results obtained from six male (Fig. 3*a*) and seven female (Fig. 3*b*) rabbits are shown in Fig. 3. This confirms that the fluctuations in antibodies demonstrated by the monthly samples at Snowy Plains are actually occurring in the case of individual rabbits.

Table 2(*b*) shows that, overall, 7% of the susceptible animals at Snowy Plains showed antibody. During the breeding season (October and November) many young rabbits (18 and 10%) showed antibody. In these cases, the antibody might have been derived from the mother. However, a very large proportion (from 24 to 36%) of the susceptible rabbits tested in May, June and July at Snowy Plains showed antibody. Since these animals would have been at least 6 months old, the antibody could not be of maternal origin (Fenner & Marshall, 1954).

Canberra

The history of myxomatosis in the Canberra population from 1966 until 1969 (Williams, Fullagar, Davey & Kogon, 1972; Williams, Fullagar, Kogon & Davey, 1973), is similar to that described for Snowy Plains. The population was started in mid-1966 with 36 adult rabbits, five of which had previously recovered from myxomatosis. None of the five immune rabbits survived past 1966. The rabbits bred in 1966 (September–January), 1967 (August–November), 1968 (July–December) and 1969 (March–April). An outbreak of myxomatosis occurred in the winter months (July–September) of 1969, as a result of which only two kittens, which avoided infection, survived.

Thus, in 1967, 1968 and 1969, when the sampling for antibodies was carried out, there were no immune animals in the population. Table 2(*b*) shows that of the 840 samples of Canberra rabbits tested, only 4% showed antibody. Further, all of these were scored as 1, which is approaching the limits of detectability of the antibody.

DISCUSSION

The results show that in field populations of rabbits the titre of antibody to the 'd' soluble antigen of myxoma virus varies in time after recovery from infection; at times it may fall below the level of sensitivity of the measurement technique. Most animals in this study had recovered from an attenuated ('field') strain of myxoma virus. Their chances of recovery had been increased by high temperatures (as at Snowy Plains in the summer of 1967), assisted by maternal antibodies (as with the enclosure rabbits) and genetic resistance (as at Urana each year). However, where field strains operate at low temperatures (i.e. in winter), amongst rabbits in which the genetic resistance is low, and maternal antibodies are absent, the chances of recovery are low (Dunsmore *et al.* 1971; Williams, Fullagar *et al.* 1972, Williams *et al.* 1973).

In many of the recovered animals the antibody titre decreased with time, and often fell below the threshold of measurement. It has often been observed that antibodies to a wide variety of antigens decline to low levels after a period following

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the initial challenge (Boyd, 1956). This has also been shown with the complement-fixing antibodies of myxoma virus in rabbits (Fenner, Marshall & Woodroofe, 1953). Sobey (unpublished) has observed a decline in the anti-'d' antibodies in recovered laboratory rabbits. In all these cases the antibodies to the various attenuated myxoma virus antigens had fallen to low titres within 6 months, which agrees reasonably closely with the declines observed in the field populations in this study.

The observation that high antibody titres may reappear after they have fallen very low suggests some form of stimulation. It seems likely that such stimulation is due to re-exposure to the 'd' antigen, which, since it is probably a by-product of virus multiplication, suggests that virus had multiplied in the animal. Virus can multiply in recovered animals, for a short time at least, if the animal is exposed to virus some months after recovery (Fenner *et al.* 1953). Stimulation of the anti-'d' antibodies may thus occur as a result of re-infection. Such is the probable explanation for the rise in antibody titre in the enclosed population at Canberra in August/September 1971, since myxoma virus was active amongst the susceptible animals in the enclosure, and the immune adults would have continually been exposed to the virus.

There are two possible explanations for the rise in antibody titres which, in the absence of any obvious virus activity, occurred in the populations in the enclosure and at Snowy Plains in summer, and at Urana in autumn. Firstly it is possible that the stimulation was caused by re-infection with a strain of myxoma virus which was so attenuated as to escape detection. This seems unlikely, because myxoma virus relies on mechanical transmission by arthropods from skin lesions for dissemmination; if the skin lesions were so slight as to escape intensive observation, the possibility of transmission would be low.

An alternative explanation is suggested by recent evidence (Williams, Dunsmore & Parer, 1972) that myxoma virus may survive in a latent phase. Latency is probably the result of a dynamic balance forming between the immune mechanism of the animal and the infecting virus. If the immune response falls below a certain level, the virus may multiply, re-activating the 'immunological memory', restoring the balance. It may be postulated that in most cases multiplication of virus after re-activation results in a subclinical infection. In a few cases, however, virus multiplication after reactivation may result in overt infection. This may be most likely to occur when the animal is under stress. Some other latent viruses, including *Herpes simplex* (Rasmussen, Marsh & Brill, 1957), *H. zoster* (Hope-Simpson, 1965), rabies (Soave, 1964) and psittacosis (Meyer, 1942), appear in various animals under a variety of stressful conditions. It seems likely that this stress-induced reactivation of virus leading to overt disease also applies to myxomatosis (Williams, Dunsmore & Parer, 1972).

There is evidence which supports this hypothesis. The times when animals in field populations have low antibody titres, and when presumably reactivation and multiplication of latent myxoma virus is more probable, are late spring and early autumn. These are both times when outbreaks of myxomatosis have been noted to start in field populations, resulting in either summer or winter epizootics.

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Additionally, in such summer and winter epizootics which have been studied in detail (Williams & Parer, 1972; Dunsmore *et al.* 1971; Williams, Fullagar *et al.* 1972, 1973) the re-activation of latent virus resulting in overt infection in certain rabbits was considered to be the source of virus initiating the epizootics. In these cases, climatic conditions and population density were such that they could be regarded as stressful in the periods immediately before the epizootics. In the same populations in intervening years when population densities were lower, no myxomatosis occurred.

Accepting this hypothesis, there remains unexplained the reason for the appearance of antibodies in a substantial percentage of the susceptible rabbits in the autumn at Snowy Plains. If the possibility that a highly attenuated strain of myxoma virus was present in the population is discounted, there remains the explanation that reactivation of latent virus occurred. If the latter is correct, it means that latent myxoma virus can pass from parent to offspring. A similar hypothesis was advanced, on other circumstantial evidence, to explain the appearance of myxoma virus in completely susceptible rabbit populations in which winter epizootics occurred (Williams, Fullagar *et al.* 1972, 1973).

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