LABORATORY STUDIES OF *ANOPHELES ATROPARVUS* IN RELATION TO MYXOMATOSIS

By C. H. ANDREWES, R. C. MUIRHEAD-THOMSON and J. P. STEVENSON*

National Institute for Medical Research, Mill Hill, London, N.W.7 and the Infestation Control Division, Ministry of Agriculture, Fisheries and Food, Tolworth, Surrey

Though rabbit fleas (*Spilopsyllus*) are the principal vectors of myxomatosis in Britain (Armour & Thompson, 1955), *Anopheles labranchiae atroparvus*† has been implicated in its transmission amongst domestic rabbits (Muirhead-Thomson, 1956). This mosquito was first shown to carry infection under laboratory conditions by Jacotot, Toumanoff, Vallée & Virat in France (1954). They found that infection was transmitted up to 21 days after the infective blood meal; that a single bite of the *Anopheles* was sufficient to produce infection and that a single mosquito could infect several rabbits one after the other at short intervals.

These basic observations have been confirmed. In addition, it has been shown that infection can be produced by insertion of the mosquito's mouthparts alone, without actual feeding; that, after the first few days, virus is present only in the mouthparts of the insect; and that infected mosquitoes can remain infective for several months, much longer than is reported for this insect by the French workers or for other mosquitoes by Australian workers (Fenner, Day & Woodroofe, 1952). The possibility that myxoma virus multiplies in *A. atroparvus* will be discussed.

METHODS

We used both wild and laboratory-reared *atroparvus*, most frequently wild caught semi-hibernating insects. Rabbits used for feeding experiments were first anaesthetized by intraperitoneal injection of nembutal.

Large batches of mosquitoes were first fed on a healthy rabbit to ensure that none was naturally infected. Those that gorged were put aside for critical transmission experiments, and when they were ready for a further feed they were exposed to advanced myxomatous rabbits in cages of 1 ft. cube; under these conditions the hungry *atroparvus* fed readily. *A. atroparvus* were reared in the laboratory by the method described by Shute (1936); we are much indebted to him for help and advice.

Cages of engorged mosquitoes were subsequently kept in conditions approxi-

* In receipt of an expenses grant from the Agricultural Research Council.

† This insect has been generally known as *A. maculipennis atroparvus*; according to Mattingly (1950) the correct name is *A. labranchiae* subsp. *atroparvus* van Thiel.

mating to those of the shelters in which they normally spent the winter. The temperatures at which they were kept were as follows:

November 1954	5–8° C.
December 1954	2–5° C.
January and February 1955	0–2° C.

When the blood meal was digested and the mosquitoes were ready for a further feed they were offered an anaesthetized healthy rabbit on which they usually fed readily. The experiment was continued on these lines throughout the winter and spring. At intervals batches of infected mosquitoes were removed for routine virus titration, as follows. The insects were immobilized by brief exposure to −10° C., then ground up in glass Ten Broeck grinders with saline containing 9 % Hartley's broth and 1 % horse serum (in all 1 ml./mosquito). The suspension was lightly centrifuged and 0·1 ml. quantities were inoculated intradermally in falling tenfold dilutions into closely clipped skins of rabbits.

RESULTS

(i) *Winter experiments*

The results of Exps. I and II are shown in the first two tables. It will be seen that mosquitoes remained infective for 149 days (Table 1) and 220 days (7 months) (Table 2). The virus titre (Table 1) did not fall from its level of 1/100 between the 14th and 79th days, but had done so by the 143rd day. It will be noted that infection was still transmitted after several feeds on normal rabbits. In Exp. III, not included in a table, mosquitoes fed on 28 and 29 October 1954, could still transmit infection 170 days later. Titres of ground mosquitoes were 10^{-2} after 64 days, 10^{0} after 181 days. On this occasion, pairs of mosquitoes were ground separately and tested; three of five pairs yielded virus.

(ii) *Summer experiments*

This long survival was in over-wintering semi-hibernating mosquitoes; the low temperatures doubtless favoured survival of the insects but would hardly be expected to be optimum for virus multiplication. The experiments were therefore repeated under summer conditions in 1955. In the Newhaven area *atroparvus* are difficult to find in April and May. Small numbers begin to appear between mid-June and early July, and from the end of July onwards large numbers may be taken regularly in pig-sheds and calf-sheds. Transmission experiments were made during August on the lines described above, the temperature range in the insectary being 16–24° C. At this summer temperature blood is digested more rapidly and the *Anopheles* require more frequent blood meals—every 5–6 days compared with 3 weeks or more in winter. In the summer brood, unlike the winter brood, the digestion of blood is accompanied by development of the ovaries. Many *Anopheles* with well-developed ovaries were reluctant to lay eggs in captivity but would nevertheless take a further partial blood meal, thus differing from semi-hibernating *atroparvus* which almost invariably take a full blood meal.

Table 1. *Recovery of virus from wild-caught* Anopheles maculipennis *fed on rabbit originally infected by bite of wild* A. maculipennis *from rabbit hutch, Newhaven, 17–18 October 1954*

Date	No. of days since infective feed	No. of mosquitoes feeding	Results of feed (with incubation period in days)	No. of intermediate feeds on normal rabbits	Titre of virus in ground-up mosquitoes
1. xi. 54	14	95	+5–6	0	10^{-2}
8. xi. 54	21	95	+5	0	No test
10. xii. 54	53	35	+5	0 2	+ +
5. i. 55	79	12	+8	3	10^{-2}
31. i. 55	105	5	+6	—	No test
10. iii. 55	143	—	—	2	10^{0}
16. iii. 55	149	1	—	—	—

Table 2. *Recovery of virus from wild-caught* Anopheles maculipennis. *Fed 5 November 1954 on diseased wild rabbit caught in Surrey*

Date	No. of days since infective feed	No. of mosquitoes feeding	Results of feed (with incubation period in days)
30. xii. 54	54	50	+5
19. ii. 55	105	5	+6
1. iii. 55	115	35	+6
22. iii. 55	136	24	+6
18. iv. 55	163	25	+7
9. v. 55	184	11	+5
23. v. 55	198	8	+11
31. v. 55	206	3	+12–13
7. vi. 55	213	3	—
14. vi. 55	220	2	+7
21. vi. 55	227	2	—
27. vi. 55	233	2	—

Experiment IV

A very large batch of wild-caught *atroparvus* was fed on an advanced myxomatous rabbit on 27 July 1955. Six of them were fed on a normal rabbit 6 days later and this animal showed symptoms after 4 days (possibly 3). Nineteen others transferred infection after 19 days, the incubation period in the latter rabbit again being very short, only 3 days. In previous experiments with *atroparvus* the incubation period (after biting) has as a rule been 6–7 days, only once less than 5 days. Ground-up mosquitoes proved infective at a 10^{-2} dilution after 3 days, and at 10^{0} and 10^{-1} respectively in two rabbits inoculated after 7 days. Tests on duplicate rabbits at 10, 15, 23, 27, 31 and 39 days all gave lesions at either 10^{0} or 10^{-1}, the virus titre thus remaining apparently constant for nearly 6 weeks. A test after 51 days yielded no virus.

Experiment V

In a similar experiment made in August 1955, ground-up mosquitoes were infective in the dilutions shown in Table 3. Again the virus titre remained constant for several weeks, and at summer temperatures.

A. atroparvus reared in the laboratory seemed to be less hardy and virus persisted in them for shorter periods than in the wild-caught.

Table 3. *Recovery of virus from infected* Anopheles atroparvus *during summer months*

Days after infection	Virus titres (in two rabbits)	
3	10^{-2}	10^{-2}
4	10^{-3}	10^{-3}
6	10^{-2}	10^{-2}
7	10^{-2}	10^{-2}
8	10^{-2}	10^{-3}
9	Neg. 10^{-1}	10^{-1}
10	10^{-2}	10^{-1}
13	10^{-2}	10^{-2}
14	10^{-2}	10^{-2}
15	10^{-1}	10^{-2}
17	10^{-1}	10^{-2}
18	10^{-1}	—
20	10^{-2}	10^{-2}
22	10^{-2}	—
36	10^{0}	10^{-1}

Experiment VI (26 *August* 1955)

In the best of four experiments, pools of five bred mosquitoes gave positive results on the day of infection at 10^{-2} dilution; subsequent titres on days 1, 3, 5, 6, 7 and 8 were 10^{0} or 10^{-1}; insects killed on days 9, 12 and 16 yielded no virus, though those tested after 13 and 14 days were positive at 10^{0} and 10^{-1} respectively. No insects survived to be tested after the 16th day.

Distribution of myxoma virus in mosquitoes

Tests were made for the presence of myxoma virus in head, thorax and abdomen respectively. In one instance, 3 days after an infective feed, abdomens alone were positive. In another experiment, again after 3 days, heads were positive at 10^{-2}, thoraces at 10^{0}. In all other tests, virus was present in heads only; and it may be safely assumed that the positive results obtained generally can be ascribed to the virus content of the mosquito heads. Kilham & Dalmat (1955) similarly found that fibroma virus in infected *Aëdes aegypti* was mainly confined to head and mouthparts. Multiplication of virus in the mosquito is suggested by its persistence over many months, yet the insect's head seems an unlikely place in which this could occur. Dr L. Whitman suggested to us that a possible site was the relatively soft labium which acts as a sheath for the probing and sucking mechanism, but does not itself penetrate the skin. In two experiments, the labia of infected mosquitoes were separated from other mouthparts under a dissecting microscope and ground up separately. On the first trial, involving lots of five mosquitoes, all parts were negative at 3 days; after 12 days labia were positive at 10^{-1} and 10^{0}, 'other mouthparts' at 10^{0} and $< 10^{0}$. This difference, roughly tenfold in favour of the labia, was not confirmed in a second test, in which the 'other mouthparts' took to a tenfold higher titre than did the labia (test after 4 days).

Survival of virus in live and dead mosquito heads

In hope of throwing light on the possibility that myxoma multiplies in mosquitoes, *Anopheles atroparvus* were infected and, after 3 days to allow establishment of virus, divided into two lots. Half were killed and their heads kept in test-tubes at room temperature for varying periods. The rest of the insects were kept alive and their heads tested after similar periods. In one experiment virus persisted in the 'live heads' for 14 days, but had disappeared from the 'dead heads' by the third day. In a second test, a minimum amount of virus remained on the 'dead heads' for 6 days only, while it persisted on 'live' but not 'dead heads' for 8 days—the latest time at which tests were made. In a third test, virus was present in 'live heads' at 7 days, though it had not been detected at 3 days. Seven-day 'dead heads' were negative.

Virus failed to persist in 'dead heads' whether these were kept in screw-capped bottles or in tubes plugged with cotton-wool. The results conform with those of Kilham & Dalmat on fibroma virus.

The Sussex strain of myxoma virus

The myxoma virus recovered from wild-caught mosquitoes at Newhaven (Sussex) (cf. Exp. I, Table 1) had properties different from those of other viruses isolated from wild-caught English rabbits. On intradermal inoculation the initial lesion was a perfectly flat erythema; only after several days did some thickening of the skin occur. Similarly, the secondary rash consisted of flat macules, not unlike a measles rash and quite different from the usual secondary 'pocks'. Histologically the flat skin lesions showed proliferative and destructive changes in

the epithelium, unaccompanied at first by the usual myxomatous thickening of underlying connective tissues.

The evolution of the disease was slower than that of our 'typical' strain myxoma, in which death usually followed 5–7 days after the appearance of generalized pocks or eye lesions. With the Sussex strain, the eye lesions developed much more slowly, and death was delayed; however, only two of sixty-three rabbits recovered.

Survival of rabbits infected with the Sussex virus was longer than that of a typical strain. Data on twenty-seven of the former and sixty-three of the latter were kindly analysed for us by Miss M. V. Mussett. Assuming that the few surviving rabbits actually died 1 day later than the date on which they were destroyed, we find the mean survival time for the typical strain to be 11·4 days (S.D. 2·8 days) and for the Sussex strain 16·5 days (S.D. 3·6 days). 50% of the deaths for the typical strain occurred in 10·5 days, for the Sussex strain in 15·3 days. If death or survival is regarded as a quantal response, the end of the thirteenth day being arbitrarily chosen as the time of survival, we find that fifty-seven out of sixty-three 'typical' and only five of twenty-seven 'Sussex' rabbits were dead. The probability that this is a chance result is less than one in a thousand ($\chi^2 = 43{\cdot}9$ with 1 degree of freedom, $P < 0{\cdot}001$).

Virus transmitted by *A. atroparvus* is not necessarily modified so as to resemble the Sussex strain. Virus recovered from mosquitoes in other experiments—including that in which virus persisted for 220 days—behaved in a typical manner. We cannot guess whether or not strains resembling our Sussex strain would have become numerous, if mosquito transmission had become a locally dominant factor; in fact, it did not do so and wild rabbits and myxomatosis both became rare around Newhaven and elsewhere on most of the south coast. Myxoma strains producing flat lesions are reported from Australia (Fenner, personal communication). The Sussex strain differs greatly from the attenuated strain described from Nottingham by Hudson, Thompson & Mansi (1955). This produced chronic, strongly raised, nodular lesions. Fenner, in correspondence, suggests that variant strains of myxoma should be named in a uniform manner. This strain from Newhaven in Sussex should accordingly be called England, Sussex/10.54/1, October 1954 being the month in which it was recovered.

Anopheles atroparvus *as a reservoir of myxomatosis in nature*

The results of our experiments show that *atroparvus* can retain its infectivity for periods up to 220 days after the infective blood meal, during which period it can feed on and infect a series of healthy rabbits. In terms of natural conditions this implies that *Anopheles* infected in the autumn are capable of carrying the infection over the winter to the following spring or early summer. Final proof that this actually happens in nature is lacking, however, as virus has not yet been recovered from any batch of wild-caught *A. atroparvus* semi-hibernating in farm buildings.

A somewhat similar state of affairs has been reported with another member of the pox group of viruses, namely, fowlpox (Bos, 1934). In that work, '*Anopheles*

maculipennis' (almost certainly *atroparvus*) were infected at the end of September and were still infective at various intervals up till the end of the following April—210 days. Another relevant observation is that of Kligler & Ashner (1931), who found natural infections in a batch of *Culex pipiens* caught in the vicinity of chickens infected with fowlpox at a time of the year—mid-November—when the mosquitoes were preparing to go into hibernation.

There is no reason to believe that the findings reported in this paper would not apply to *Anopheles atroparvus* in nature, as the conditions under which the mosquitoes were kept in captivity approximated closely to those in their natural shelters. It appears therefore that *A. atroparvus* is in a position to act as an unusually efficient reservoir of myxoma virus, capable in theory of bridging the gap between the virtual elimination of a rabbit population by disease, and the appearance of the odd survivors' healthy progeny many months later.

As described above, however, there is very little evidence that anything of this kind does in fact happen under natural conditions. For example, even under the favourable conditions provided at Newhaven, where many *atroparvus* were being infected from domestic stock in the autumn, not a single naturally infected mosquito was recorded in collections made during the winter in farm buildings in this locality. Although there are still (1955) many domestic rabbits in Newhaven, inquiries have failed to disclose any fresh cases of disease in domestic rabbits in 1955 even though many of these rabbits have not been immunized or protected in any way.

On the other hand, there are odd cases of the disease reappearing, both in wild and in domestic rabbits, at long intervals after the area had been originally cleared by myxomatosis. Two of these which occurred in areas of high *atroparvus* incidence have been investigated.

In the 'island' of Thanet on the extreme east coast of Kent, in one particular locality the disease was first recorded in October 1954, but one or two diseased wild rabbits were still being reported in the latter part of the following April. In the same area an isolated case occurred in February in one rabbitry in which there were two or three dozen rabbits; later collections—in June—confirmed that *atroparvus* were present in these hutches.

A rather more striking example comes from the island of Sheppey in the Thames estuary, off the north coast of Kent. The disease was active there in the last 3 months of 1954, and by the end of the year the rabbit population had practically been eliminated. A single diseased wild rabbit was seen at the beginning of April 1955, and about a month later, in the first week of May, a rabbit keeper in a locality 5 miles from this place lost one of his two domestic rabbits. The owner had moved earlier in the year from another part of the island where he had lost eighteen out of twenty-four rabbits during the October outbreak of 1954. Careful inquiries indicated that as far as domestic rabbits were concerned this isolated case in 1955 was unique. Occurring as it did at a time when semi-hibernating *atroparvus* would be leaving the strict confines of the winter shelters in farm buildings, the disease might well have been caused by the bite of a mosquito which had originally become infected in the outbreak of the previous autumn. Unfortunately, by the time

information about these odd cases comes to hand the rabbit hutches have either been disinfected or treated with insecticide, and any chance of confirming the presence of infected mosquitoes is lost. There is no doubt, however, that these isolated cases are rather rare, even in areas of high *atroparvus* density, and this is a further indication that this mosquito, despite its high vectorial capacity, does not play an important part in the general epizootiology of the disease.

With regard to isolated cases of disease among wild rabbits, theorizing is even less profitable as so little is known about the range of movement of normal healthy rabbits, much less diseased ones, and it is exceedingly difficult to say definitely whether or not the disease was actually contracted in the locality in which the diseased animal was first seen. In addition, diseased wild rabbits, unlike domestic ones, can easily escape notice and one can never be sure whether the isolated record really does represent a new case after a long interval of freedom from disease, or whether the disease has actually been spreading slowly in the interval among a very scanty wild rabbit population.

Possible multiplication of myxoma virus in mosquitoes

Workers in Australia offer cogent evidence that mosquitoes there transmit infection mechanically, acting as 'flying pins'. Their laboratory studies were carried out mainly with *Aëdes aegypti*, though this does not act as a vector in nature. Virus survival was for 25 days, a much shorter period than we have found. On the other hand, Kilham & Dalmat (1955), working with the closely related rabbit fibroma, found highly suggestive but not conclusive evidence that multiplication of that virus might occur in *A. aegypti*. Jacotot and his colleagues (1954) have also suggested that myxomatosis multiplies in '*Anopheles maculipennis*' though their evidence is, like ours, inconclusive.

Multiplication of myxoma in *A. atroparvus* is suggested by the long retention of infectivity by mosquitoes—up to 220 days. Even during summer months virus retained its titre in infected insects for several weeks, and although the virus is a relatively resistant one this result would hardly be expected if mere survival were concerned. It may be significant that we found survival of virus on mouthparts of dead mosquitoes only for a few days. On the other hand, actual multiplication has not, with myxoma, been demonstrable.

One thing is certain: if 'biological' transmission is concerned, it is of very different nature from that seen amongst yellow fever and insect-borne encephalitis viruses; there is no evidence for an extrinsic incubation period, nor for involvement of thoracic salivary glands. On the other hand, the virus is more stable than are those viruses; so some loss of infectivity over a few days, followed by a moderate rise, might well be missed. One must consider the possibility of relatively trivial multiplication occurring over several weeks or months, barely enough to balance virus loss from thermal inactivation; such might occur in the course of evolution when a virus was acquiring the ability to spread by means of vectors. It must be recalled that no adequate studies of this matter have been made in the natural host, *Sylvilagus brasiliensis*, in its native land, using natural vectors. Admittedly, one of the strongest arguments against multiplication is the presence of virus almost

wholly in mouthparts, apparently unsuitable tissues to support virus growth; our few experiments with *Anopheles* labia were unfortunately inconclusive.

SUMMARY

Anopheles labranchiae atroparvus which have gorged on myxoma-infected rabbits may retain their infectivity for as long as 220 days in a period covering the winter months. Virus titres in infected mosquitoes may also remain stable for several weeks at summer temperatures; virus has been recovered after 36 days in summer.

Virus in these *Anopheles* is, in most instances, to be found only in the head and mouthparts. Survival on mouthparts of killed mosquitoes, on the other hand, has been only for a few days.

One strain of virus (Newhaven strain) isolated from wild *A. atroparvus* produces flat erythematous lesions on intradermal inoculation into rabbits, and deaths occur later than with typical strains.

The possible role of over-wintering *atroparvus* as a reservoir of infection of myxomatosis is discussed.

The possibility is considered that transmission of infection by *Anopheles* is not purely mechanical, but that limited multiplication occurs in the insects.

REFERENCES

ARMOUR, C. J. & THOMPSON, H. V. (1955). *Ann. appl. Biol.* **43**, 511.
BOS, A. (1934). *Z. InfektKr. Haustiere*, **46**, 194.
FENNER, F., DAY, M. F. & WOODROOFE, G. M. (1952). *Aust. J. exp. Biol. med. Sci.* **30**, 139.
HUDSON, J. R., THOMPSON, H. V. & MANSI, W. (1955). *Nature, Lond.*, **176**, 783.
JACOTOT, H., TOUMANOFF, C., VALLÉE, A. & VIRAT, B. (1954). *Ann. Inst. Pasteur*, **87**, 477.
KILHAM, L. & DALMAT, H. T. (1955). *Amer. J. Hyg.* **61**, 45.
KLIGLER, I. J. & ASHNER, M. (1931). *Proc. Soc. exp. Biol., N.Y.*, **28**, 463.
MATTINGLY, P. F. (1950). *Handbooks for the Identification of British Insects*, vol. **9**, part 2. London: Roy. Entom. Soc.
MUIRHEAD-THOMSON, R. C. (1956). *J. Hyg., Camb.*, **54**, 472.
SHUTE, P. G. (1936). *J. Trop. med. Hyg.* **39**, 233.

(*MS. received for publication* 5. v. 56)