The Transmission of Louping Ill by Ticks (*Rhipicephalus appendiculatus*).

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In July, 1933, a preliminary report was published on the transmission of Louping Ill to sheep by *R. appendiculatus* nymphae that had picked up the virus infection by feeding on a reacting sheep in the previous larval stage. These initial results prompted a fairly extensive study of the mode of transmission of this virus by the brown tick. The outcome of this work is published in the hope that it may be of some value to workers in those areas where the disease is known to occur, in addition to being useful, should the disease be diagnosed in this country at some future date. In this connection it must be pointed out that a thorough knowledge of the infectivity of the various stages of a species of tick, after feeding on a variety of hosts, is not only of academic interest, but is of extreme practical importance because this knowledge must serve as a basis for the promulgation of those prophylactic measures which aim at the eradication of the disease by elimination of the infective arthropod vector. Bearing this in mind it became essential to determine the susceptibility of some domestic animals, other than sheep, which might serve as hosts and potential reservoirs of infection for the ticks. Therefore, while the tick breeding work was in progress, steps were taken to determine the susceptibility of cattle and of horses to Louping Ill.

THE SUSCEPTIBILITY OF CATTLE TO LOUPING ILL.

On 7th October, 1932, a calf (4535) was injected intrathecally with 2 c.c. of the turbid supernatant fluid obtained from centrifuging a 1 per cent. saline emulsion of desiccated infective mouse brain. The operation was performed with full aseptic precautions under chloral hydrate anaesthesia by inserting a long fine trocar and canula between the atlas and axis into the spinal canal.

On the afternoon of the third day after injection the temperature rose abruptly to 105·3°, but no clinical symptoms could be detected. The following morning the temperature was 105·6°, there was complete inappetence, the abdomen was markedly tucked up and there were signs of profuse watery diarrhoea. Salivation was fairly pronounced, respirations hurried and shallow and the animal either wandered aimlessly in an arch round the box, or stood in a semi stupor with wild, staring eyes and twitching muscles. During the course

of the day several violent fits, characterized by wild charging round the box, occurred. Towards evening these fits became much more frequent (2—3 per hour) and could be initiated by giving the animal a fright say by suddenly shouting or clapping the hands. Vision was now markedly impaired since the calf would charge, bellowing, with its head down into the walls or the manger. It would continue its mad stampede for several minutes before falling to the ground completely exhausted in a semi comatose condition. After some time it would recover sufficiently to stagger to its feet and after staring into space with a vacant expression would commence another wild charge. Any attempt at control was quite impossible and severe abrasions of the head, the supraorbital crest and the horns were sustained as a result of crashing into the walls. Later in the evening a paresis of the hindquarters developed so that the wild charging became less vigorous. The following morning the body temperature had fallen to 102·6 and the animal was unable to raise its hindquarters off the ground. Paralysis progressed rapidly from behind cranially so that by mid-day even a sternal position could not be maintained. The animal remained on its side but retained full conciousness as evidenced by flicking of the ears to chase away flies and obvious recognition of the approach of an attendant. Death occurred the same afternoon presumably as a result of paralysis of the diaphragm.

Post-mortem examination revealed no marked abnormality other than the severe abrasions due to traumata. The meningeal vessels showed slight engorgement but there was no evidence of a purulent meningitis or abcessation of the brain.

The brain was removed with aseptic precautions and stored overnight in the freezing chamber of an electric refrigerator. Next morning the surface of one cerebral hemisphere was washed with sterile saline and a small portion about 1 cm. square excised from the depth of the brain substance. A 1 per cent. saline emulsion of this material was injected intracerebrally in 0·03 c.c. amounts into each of three mice; all the mice died on the 6th day after showing typical symptoms of Louping Ill. This indicates that a considerable penetration and probably a considerable multiplication of the virus had taken place. The diagnosis of Louping Ill was confirmed by injecting 1 c.c. of a 0·2 per cent. emulsion of the brains of the mice subcutaneously into two susceptible sheep. Both sheep succumbed to typical Louping Ill.

In an attempt to confierm the susceptibility of cattle to the disease numerous *R. appendiculatus* nymphae from a batch (1297 B.c) known to be infected with Louping Ill virus were fed on the ears of a nine months old calf (5251). The ticks attached on 9.11.33 and all had engorged and detached by 17.11.33. Contrary to expectations the animal showed no febrile or clinical reaction, and after an interval of 17 days about 200 nymphae of the same batch were fed on the same calf. No reaction followed. Therefore, as an immunity test 42 days later a subcutaneous injection of 5 c.c. of a 1 per cent emulsion of infective mouse brain was given; at the same time two sheep and an additional calf (5412) were given a similar injection to serve as controls. Both sheep reacted typically and recovered. Both calves failed to exhibit any reaction. Two months later the original

calf (5251) and an additional heifer (5410) were given an intrathecal infection of 3 c.c. of a 1:300 saline emulsion of fresh infective mouse brain. The first calf showed no reaction. The temperature record of the control is shown in figure I.

Clinical symptoms.
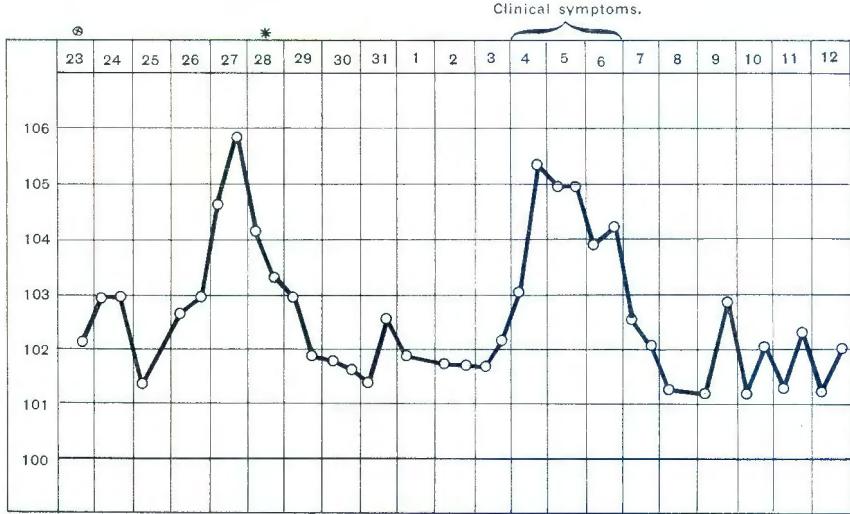
⊕ Intrthecally 1/300 saline emuls. Louping Ill Vs — Mouse brain.
✳ Defibrinated blood diluted 1/3 injected i.c. into 6 mice. *Negative.*

Fig. 1.

It will be noted that a diphasic febrile reaction occurred. During the first febrile period no symptoms of any kind were observed. During the second exacerbation the animal appeared somewhat excitable and nervous. There was spasmodic twitching of the muscles of the thighs, shoulders and neck. The ears continually twitched and there was a peculiar nodding of the head. These symptoms were noticed for about three days after which the habitus returned to normal.

An attempt was made to demonstrate the presence of circulating virus in the blood on 28.3.34 and 5 and 6.4.34, i.e. at the height of each febrile reaction, by the injection of defibrinated blood intracerebrally into mice (0·03 c.c.) and subcutaneosly into sheep (5 c.c. subcut.). No virus could be detected.

During the course of the above experiments serum had been collected from the animals as follows:—

 A. 5251 on 23.3.34, i.e. after the tick feeding and subcutaneous injection but prior to the intrathecal injection.

 B. 5412 on 24.3.34, i.e. 65 days after the subcutaneous injection.

 C. 5410 on 19.4.34, i.e. 30 days after the intrathecal injection.

In addition serum was obtained from a normal heifer, from a normal sheep and from a sheep which had recently recovered from an attack of Louping Ill initiated by a subcutaneous injection of virus.

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In vitro neutralization tests were set up with these sera using the following technique. To each of a series of agglutination tubes 1 c.c. of five-fold dilutions of a stock emulsion of infective brain in 1/10 horse-serum-saline was added, the dilutions varying from 1/5 to $1:5^9$. The sera to be tested for neutralising antibodies were diluted 1:3 in saline and added to the respective virus dilutions in 1 c.c. amounts. After incubation at 37° for two hours and overnight fixation in the ice box mice were injected with the following results.

Sera diluted 1 : 3 in saline.	Five-fold dilutions of stock virus emulsion in 1/10 horse-serum-saline.								
	$1 : 5$	$1 : 5^2$	$1 : 5^3$	$1 : 5^4$	$1 : 5^5$	$1 : 5^6$	$1 : 5^7$	$1 : 5^8$	$1 : 5^9$
Calf 5251 = A........	7 ; 8	7 ; 7	7 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0
5412 = B........	6 ; 7	8 ; 9	5 ; 8	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0
5410 = C........	7 ; 7	9 ; 0	9 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0
Normal sheep........	6 ; 6	6 ; 6	6 ; 6	6 ; 7	6 ; 6	7 ; 8	0 ; 0	0 ; 0	0 ; 0
Normal calf...........	6 ; 6	6 ; 6	6 ; 6	6 ; 6	6 ; 7	7 ; 8	7 ; 0	0 ; 0	0 ; 0
Immune sheep........	7 ; 7	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0

NOTE.—The numeral denotes the number of days after injection on which the injected mouse died. O indicated mouse still alive 14 days after injection. Two mice used for each solution and 0·05 c.c. injected intracerebrally, thus 7 ; 8 means two mice injected of which one died on day 7 and one on day 8.

A consideration of the table indicates that after contact with normal calf or sheep serum the stock virus emulsion used was infective for mice in doses of 0·05 c.c. in a dilution of $1:5^6$. After neutralization in vitro by the sera of the bovines being tested the same virus was infective in a dilution of $1:5^2$ or $1:5^3$. This indicates the presence of virucidal antibodies in the sera in a fairly high concentration though not so high as encountered in the serum of an immune sheep used as a control.

Conclusions.—From this series of experiments it must be concluded that the intrathecal injection of the virus of Louping Ill into bovines is followed by a febrile reaction which may or may not be accompanied by clinical symptoms of varying severity and may or may not be fatal. With recovery virucidal antibodies make their appearance in the serum. After subcutaneous injection of virus or the feeding of infective ticks no demonstrable reaction is to be expected but immune bodies are developed.

It is realized that a considerable amount of additional work is necessary to ascertain the full susceptibility of bovines and to determine whether there is a multiplication and circulation of virus in the blood stream after injection, but it is not unreasonable to believe that cattle may serve as a potential reservoir of infection for ticks in the field.

THE SUSCEPTIBILITY OF THE HORSE.

On 31st October, 1932, a horse (20308) was infested on the ears with numerous *R. appendiculatus* nymphae which had fed on a reacting sheep (34115) as larvae and represented part of a batch (1138 Ac) which were known to be infected with the virus of Louping Ill. The ticks attached readily and engorged well. As soon as they commenced to drop three individuals were emulsified in 5 c.c. of saline and injected subcutaneously into one sheep (34191). This sheep developed typical Louping Ill and was destroyed in extremis. The horse did not show any departure from normal health and after an interval of 18 days was given an intrathecal injection of 2 c.c. of a 0·5 per cent. emulsion of infective mouse brain. Three control mice injected intracerebrally with 0·05 c.c. of the same emulsion died on the 5th and 6th day. The horse showed no febrile reaction but after an interval of 14 days developed a slight posterior paresis which persisted for several weeks. At no time was it possible to associate the condition with Louping Ill since blood drawn on various occasions from the 7th day after injection was constantly non-infective for mice. On 17th March, 1933, the horse was again infested on the ears with numphae known to contain virus, but no constitutional disturbance was produced in the host after engorgement.

The results obtained from this one horse were so inconclusive that a second horse (20374) was infested on the ears with *R. appendiculatus* nymphae which had fed as larvae on a sheep (37412) during a Louping Ill reaction produced by subcutaneous injection of mouse virus. On the 5th day after infestation there occurred a slight rise in temperature to 102·2°, which persisted for 36 hours. On the 7th day the temperature rose abruptly to 104° and persisted at or above this level for 3 days after which it returned to normal by crisis. During this time the horse was markedly tucked up and showed a cyanotic, dirty mucous membrane; the respirations were hurried and shallow, the pulse exceedingly weak, rapid and thready; there was complete inappetence and a profuse diarrhoea. Recovery was uninterrupted and there were no nervous complications. Daily examination of blood smears stained by May-Grünewald-Giemsa failed to reveal the presence of any parasites, but a moderate monocytosis was apparent. Difibrinated blood drawn on the 8th day after tick infestation, i.e. at the height of the second febrile exacerbation produced typical Louping Ill in four mice all of which died on the 6th day after intracerebral injection. In addition 5 c.c. of the same blood injected subcutaneously into a sheep produced fatal Louping Ill, death occurring on the 8th day. The presence of virus could not be demonstrated in blood drawn two days later, either by intracerebral injection of mice or the subcutaneous injection of a sheep in 2·5 c.c. amount.

On 7th December, 1933 and 21st March, 1934, i.e. 57 and 140 days respectively after tick infestation blood was withdrawn from the horse and subsequently in vitro neutralization tests were carried out with the serum at the same time as those cited for the bovines above. The results obtained are given in Table II.

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TABLE II.

*In vitro neutralization of Louping Ill Virus by Serum of
Horse* 20374.

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Sera diluted 1 : 3 in saline.	Five fold dilutions of stock virus emulsion in 1/10 horse-serum-saline.								
	$1:5$	$1:5^2$	$1:5^3$	$1:5^4$	$1:5^5$	$1:5^6$	$1:5^7$	$1:5^8$	$1:5^9$
Serum of 7/12/33...	7 ; 8	7 ; 9	7 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0
Serum of 21/3/34...	6 ; 7	7 ; 8	11 ; 11	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0	0 ; 0
Serum of normal sheep	6 ; 6	6 ; 6	6 ; 6	6 ; 7	6 ; 6	7 ; 8	0 ; 0	0 ; 0	0 ; 0

It will be seen that virucidal antibodies to a fairly high titre had developed. Most unfortunately serum had not been collected from the first horse (20308) for inclusion in the test and in the meantime the animal had been destroyed.

Conclusions.—From this single positive case it may be concluded that the horse is susceptible to Louping Ill, and that after engorgement of infective ticks there is a multiplication of virus whose presence may be demonstrated in the blood stream.

No adequate explanation can be advanced for the failure of the first horse to react to the infestation of known infective ticks. It is suggested, however, that an ,, infection inapparante '' actually did occur, and that this produced an immunity sufficient to withstand the intrathecal injection of virulent brain emulsion.

Attention must be directed to the general similarity of the results obtained with the attempted infection of horses and cattle. No effort has been made to work out the course and nature of the disease in these species of animals, since the simple demonstration of susceptibility and the production of immune bodies in the serum was adequate for our purpose.

TICK TRANSMISSION.

A. TECHNIQUE.

For the sake of completeness it is considered necessary to give a short resumé of the methods employed at this Institution for routine tick breeding work.

During the summer months undipped cattle running on the farm Kaalplaas are inspected periodically and fully engorged female ticks are removed carefully. After identification they are placed in test tubes lightly stoppered with cotton wool. The tubes, suitably labelled, are placed in cylindrical glass jars about 22 c.m. high and 14 c.m. in diameter, in the bottom of each of which there is a small quantity of clean sand about 1 c.m. deep, kept moistened with a saturated solution of common salt in water. The jars are placed on·

the shelves of a cupboard in a room which happens to be fairly cool in summer and warm in winter. All the jars of ticks are inspected twice or three times weekly. Tubes containing numbers of dead individuals overgrown with moulds are discarded. As soon as oviposition, hatching or moulting from one stage to the next is noticed the date is recorded. It is found unnecessary to change ticks from one tube to another even though the number in a tube, particularly in the case of larvae, appear to be excessive.

Larvae are not fed until they have been hatched a week and subsequent stages are not fed until 10-14 days after moulting. For feeding purposes the ticks are shaken out of the tube into a stout calico bag fitted with a running noose at the mouth and having several lengths of tape attached to facilitate tying to the host. The bags are conveniently filled over a tray placed in a large sink containing disinfectant so that any ticks which escape may be captured or destroyed. Larvae are picked up by means of a camel hair brush and nymphae and adults are handled easily with forceps.

The bags are then placed over the ears or scrotum of the selected host. Sheep should have the area round the ears clipped short to minimize the risk of escape of ticks and the bags should be tied on fairly tightly since the passive hyperaemia induced seems to facilitate engorgement. For the first 48 hours the bags are left undisturbed. After that time the bags are changed daily; ticks which have failed to attach are destroyed; engorged ticks that have dropped to the bottom of the bag are collected and placed in tubes as before, about 100 engorged larvae, 50 engorged nymphae and a single engorged female per tube.

Except on rare occasions beyond the scope of this article no difficulty has been experienced in feeding and breeding ticks by this method and in the case of *Rhipicephalus appendiculatus* used in these experiments the procedure was highly satisfactory.

B. TRANSMISSION OF LOUPING ILL FROM LARVAE TO NYMPHAE.

On 31st August, 1932, 5 c.c. of a 1/500 saline emulsion of desiccated infective sheep brain was injected subcutaneously into a sheep (34115). On the third day, immediately the first rise in the febrile temperature curve was noticed, about 1/4 of the progeny of one clean *R. appendiculatus* female were placed on each ear. The larvae fed well and about 800 engorged specimens were collected between the 8th and the 12th day. The larvae commenced their moult to nymphae on 29.9.32.

On 7th October, 1932, a sheep (32241) was infested on one ear with a total of about 80 of these nymphae. The nymphae attached readily but the rate of engorgement was variable so that engorged nymphae continued to drop from 14 to 24.10.32. On the 5th day after tick infestation the temperature of the sheep rose abruptly to 106° F. The sheep showed no nervous symptoms of Louping Ill but the diphasic temperature curve was typical and blood drawn on the second day of fever proved highly infective for mice.

This experiment was repeated on three occasions with nymphae from the same batch (1238 A). One sheep (34787) infested on 19.10.32 commenced to react on the 4th day and died on the 10th day after showing typical nervous symptoms; a second sheep (34894) infested 26.1.33 showed a very mild reaction from the 5th day and recovered; a third sheep (26647) infested on 3.5.33 commenced a severe reaction on the 4th day, and after showing slight nervous symptoms recovered. These two sheep were given an immunity test consisting of the subcutaneous injection of 5 c.c. of a 1 per cent. emulsion of infective mouse brain and did not react, whereas two control sheep reacted severely but recovered.

To confirm this transmission from larvae to nymphae the entire experiment was repeated. A sheep (37412) was infested on the ears with numerous clean larvae (batch 1297) on 22nd July, 1933. Two days later a subcutaneous injection of 5 c.c. of a 1 per cent. dilution of infective mouse brain was given. A severe reaction, to which the sheep eventually succumbed, commenced after 48 hours and during the entire febrile reaction numerous engorged larvae continued to drop. These larvae commenced their moult to nymphae on 4th September, 1933. About 80 of these nymphae were fed on the ears of a susceptible sheep (37583) on 28th September, 1933. The ticks attached immediately and produced a mild attack of Louping Ill from the third to the eighth day. After recovery the sheep was solidly immune to the subcutaneous injection of a massive dose of mouse virus, the infectivity being controlled by subcutaneous injection into two controls and into mice.

Conclusion.—Rhipicephalus appendiculatus larvae become infected with the virus of Louping Ill after engorging on an animal undergoing a reaction. The resulting nymphae will pass on this infection when feeding on a susceptible animal.

C. TRANSMISSION FROM NYMPHAE TO ADULTS.

The nymphae used to pick up the infection in this experiment were obtained from larvae which had been fed on one of the susceptible calves used as routine tick feeders. At no time had there been any possible association with Louping Ill.

About 50 nymphae were placed on one ear of a sheep (32241) as soon as the first rise in temperature occurred after infective nymphae had been fed on the other ear. The ticks attached readily and were collected fully engorged from the 6th to the 10th day. At the time the ticks commenced to feed the presence of virus in the peripheral blood was demonstrated by the intracerebral injection of mice. By the time engorgement was complete virus could not be detected but specific immune bodies were demonstrable by in vitro neutralization of mouse virus.

On 14th November, 1932, the nymphae commenced their moult to adults and eleven days later 15 adults were placed on the ears of a susceptible sheep (34660). For some reason all the ticks except a single male refused to attach and eventually died in the ear bags, the sheep showing no deviation from normal health.

Subsequently, on 13th January, 1933, the same sheep was infested with ten adults from the same batch. On this occasion attachment and engorgement were rapid. A typical, severe febrile reaction commenced on the 4th day and lasted for six days. The sheep recovered and five weeks later was found to be immune to the subcutaneous injection of a test dose of virus which proved fatal to two controls.

Conclusion.—*R. appendiculatus* nymphae are capable of picking up the virus of Louping Ill while feeding on a reacting sheep, and of transmitting it to a susceptible sheep during engorgement as adults.

D. Transmission from adults through the egg to larvae of the next generation.

It is unnecessary to detail all the attempts that have been made to determine the possibility of transmission of the virus of Louping Ill through the egg from one generation to the next since every experiment has yielded negative results. Adults which had produced Louping Ill after picking up the infection as nymphae, and had engorged on a reacting host while free virus could be demonstrated in the peripheral blood, laid eggs which were found to be devoid of virus, and the larvae after hatching failed to transmit the disease. Similarly clean adults were fed on a reacting host and were shown to have ingested virus since an emulsion of a few engorged individuals were fully virulent, but no trace of virus could be found in either the eggs or the larvae after hatching.

Conclusion.—The eggs laid by *R. appendiculatus* adults harbouring the virus of Louping Ill are non-infective. Larvae hatched from these eggs do not transmit the disease. It is concluded that the virus is incapable of passing through the egg from one generation of tick to the next.

E. Transmission from larvae through nymphae to adults.

1. *Nymphae feeding on a susceptible sheep.*

In previous experiments reported above, a collection was made of numerous engorged nymphae which had produced Louping Ill in the sheep upon which they had fed. These ticks were used for the series of experiments to be described under this heading. For instance, one batch of nymphae (1238 Ac.) were fed on a sheep (34787) and produced a fatal case of the disease. The following injections and infestations were carried out with these nymphae or the resulting adults.

1. 24th October, 1932. Sheep 34874 injected subcutaneously with an emulsion of three engorged nymphae, which detached prior to the commencement of the febrile reaction in the host; injection carried out on the day the ticks dropped.

 Result.—Severe reaction; sheep died 6th November, 1932. Before death blood infective for mice.

2. 26th October, 1932. Sheep 31051 injected subcutaneously with an emulsion of three engorged nymphae which detached after the febrile reaction in the host had commenced. Injection carried out on the day the ticks dropped.

   Result.—Severe reaction; sheep died 6th November, 1932. Before death blood infective for mice.

3. 5th December, 1932. Sheep 33970 infested on ears with adults derived from engorged nymphae which detached after the commencement of the febrile reaction in the host.

   Result.—Severe reaction; sheep died on 24th December, 1932.

4. 13th January, 1933. Sheep 34202 injected subcutaneously with an emulsion of two adults derived from nymphae which detached during the febrile reaction in the host.

   Result.—Severe reaction; sheep died 25th January, 1933.

5. 13th January, 1933. Sheep 34358 infested on ears with adults which detached as nymphae during the febrile reaction in the host.

   Result.—Severe prolonged reaction. Sheep recovered and was immune to a test dose of virus given subcutaneously 38 days later.

From this series of experiments it would appear that *R. appendiculatus* which pick up an infection as larvae retain that infection through the nymphal to the adult stage after engorging on a susceptible sheep. Two series of confirmatory experiments were carried out on similar lines except that only the moulted adults were fed or emulsified and injected. Of five sheep which were injected with an emulsion of adults none reacted and subsequently all were found to be fully susceptible to a test dose of mouse virus. Of six sheep on which adults were fed (in each case six adults were placed on one ear) four did not react and two underwent mild reactions and recovered. On immunity test the non-reactors were found to be fully susceptible, the reactors solidly immune.

The significance of these results is discussed after considering the results obtained with adults derived from nymphae which fed on an immune sheep.

2. *Nymphae feeding on an immune sheep.*

The immune sheep (34497) used in this experiment had survived a typical Louping Ill reaction set up by tick infestation and had been shown to be immune to the subcutaneous injection of a massive dose of mouse brain virus.

On 13th January, 1933, numerous *R. appendiculatus* nymphae belonging to the same batch of infective ticks which had produced the disease in experiments cited above were placed in a bag over the

right ear. The ticks engorged readily and detached on the 6th, 7th, and 8th days. No reaction was produced in the sheep. The nymphae commenced their moult to adults on 17 February, 1933, and with the imagines the following experiments were carried out:—

1. 21st March, 1933. Sheep 35019 injected subcutaneously with an emulsion of three adults.

   Result.—No reaction and six weeks later the sheep reacted to an immunity test of mouse brain virus.

2. 21st March, 1933. Sheep 35024 infested on ears with six adults.

   Result.—Febrile reaction commenced on the 5th day and six weeks later the sheep resisted a test dose of mouse brain virus.

3. 19th April, 1933. Sheep 33110 injected subcutaneously with an emulsion of three adults.

   Result.—There were two rises in temperature to 106·2° and 106·4° F. on the afternoon of the third and the fifth day respectively. On immunity test six weeks later the sheep succumbed to typical Louping Ill.

4. 19th April, 1933. Sheep 33116 infested on ears with six adults.

   Result.—Typical febrile reaction commencing on the third day and six weeks later the sheep resisted a test dose of virus.

To confirm these results the entire experiment was repeated at a later date. A proved immune sheep was used as a host for feeding a different batch of nymphae whose infectivity was controlled by the production of Louping Ill in a susceptible sheep upon which a sample was fed. Two sheep were injected with an emulsion of three adults, the emulsions being prepared about two months after the ticks had moulted. No reaction was produced, and the sheep subsequently proved to be fully susceptible. On two sheep numerous adults were fed; the one developed a mild reaction followed by solid immunity, but the other did not react and was found subsequently to be susceptible.

Conclusions.—The results obtained from this series of experiments on feeding infective nymphae on susceptible and immune sheep are not perfectly clear cut but it is justifiable to direct attention to the following points.

1. Nymphae which picked up their infection as larvae still contain virus after the completion of engorgement on a susceptible sheep, and before moulting to adults.

2. The majority of adults derived from infective nymphae which engorged on a susceptible sheep, are infective, but it is not clear whether the nymphae did not clean themselves and then become re-infected by feeding at a time when free virus had made its appearance in the peripheral blood of the host.

3. The majority of adults derived from infective nymphae which fed on immune sheep are capable of transmitting the disease. Since the subcutaneous injection of an emulsion such adults usually does not set up the disease, either the virus titre is very low or the ingestion of blood is necessary to '' activate '' the virus contained.

F. TRANSMISSION FROM LARVA THROUGH NYMPHAE TO ADULTS.

1. *Nymphae feeding on a susceptible horse.*

On 31st October, 1932, a horse (20308) was infested on the ears with numerous infected nymphae which represented portion of a batch (1238) which had produced Louping Ill in susceptible sheep regularly.[*] The ticks attached readily and commenced to drop fully engorged on 3rd November, 1932. The moult to adults commenced on 30th November, 1932. The following feeding and injection experiments were carried out with these ticks.

1. 5th November, 1932. Sheep 34895 injected subcutaneously with an emulsion of three engorged nymphae, the injection being made two days after dropping.

> *Result.*—Severe Louping Ill reaction, the sheep being destroyed in extremis.

2. 13th January, 1933. Sheep 34191 injected subcutaneously with an emulsion of three adults.

> *Result.*—No reaction. On immunity test after 38 days the sheep proved susceptible and died on 7th March, 1933, after showing nervous symptoms of Louping Ill.

3. 12th December, 1932. Sheep 34653 infested on both ears with numerous adults.

> *Result.*—No reaction. After 70 days the sheep was susceptible to a test dose of mouse virus.

These results indicate that adults derived from nymphae which fed on a susceptible horse lose their infection even though the presence of virus may have been detected in the nymphae after engorgement.

From the point of view of the control of the disease in practice this finding is of such importance that it was decided to repeat the experiment on a more comprehensive scale.

A batch of larvae (1297), portion of the progeny of a single female were fed on a reacting sheep (37412) from 22nd to 27th July, 1933. These larvae commenced their moult to nymphae on 4th September, 1933. On 11th October, 1933, about 50 nymphae were placed on each ear of a horse (20374) which developed a febrile reaction, during which the presence of virus in the peripheral blood was demonstrated by the intra-cerebral injection of mice and the subcutaneous injection of sheep.[*]

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[*] c/f. Under heading '' susceptibility of the horse '' above.

The following feeding and injection experiments were carried out with these ticks.

1. 14th October, 1933. Sheep 37391 injected subcutaneously with an emulsion of six partially engorged nymphae forcibly removed after feeding for three days.

   Result.—No reaction. On immunity test after 46 days sheep developed a severe reaction but recovered.

2. 16th October, 1933. Sheep 35540 injected subcutaneously with an emulsion of six engorged nymphae which had just detached, i.e. before the febrile reaction in the horse commenced.

   Result.—No reaction. On immunity test after 46 days sheep developed a severe reaction and recovered.

3. 1st November, 1933. Sheep 36878 injected subcutaneously with an emulsion of six nymphae which had detached before the commencement of the febrile reaction in the horse.

   Result.—No reaction. On immunity test after 46 days developed a severe reaction and recovered.

4. 27th October, 1933. Sheep 36856 injected subcutaneously with an emulsion of six nymphae which had detached and were collected on the day on which the febrile reaction in the horse commenced.

   Result.—No reaction. On immunity test after 46 days the sheep developed a mild febrile reaction and recovered.

5. 24th October, 1933. Sheep 35532 injected subcutaneously with six engorged nymphae which dropped after commencement of the febrile reaction in the horse.

   Result.—Severe reaction; died 9th November, 1933.

6. 1st November, 1933. Sheep 36935 injected subcutaneously with six engorged nymphae which detached after the commencement of the febrile reaction in the horse.

   Result.—Severe reaction. On immunity test after 46 days the sheep was immune.

The remainder of the nymphae commenced their moult to adults on 23rd November, 1933, and subsequently the following experiments were carried out.

1. 16th January, 1934. Sheep 38204 infested on each ear with five adults which dropped as nymphae before the commencement of the reaction in the horse.

   Result.—No reaction.

2. 18th January, 1934. Sheep 38215 infested on each ear with five adults which dropped as nymphae after the reaction in the horse had commenced.

   Result.—Severe febrile reaction from which the sheep recovered.

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Conclusions.—The results obtained from this series of experiments indicate that most infective *R. appendiculatus* nymphae while feeding on a susceptible horse clean themselves, provided that engorgement is completed before the febrile reaction in the host commences, i.e. before the appearance of circulating virus in the peripheral blood stream. Some nymphae, after rapid engorgement in this way, contain virus when they detach but in the limited number of tests conducted this virus was not carried forward through the moult to the adult stage.

Nymphae which have not completed their feed by the time the febrile reaction commences naturally reacquire infection which is transmitted to the succeeding adults.

2. *Nymphae feeding on an immune horse.*

On 17th March, 1933, numerous infected nymphae were placed on the ears of a horse (20308) which had survived an intrathecal injection of 5 c.c. of a 2 per cent. emulsion of infective mouse brain. Engorged nymphae commenced to drop on 22nd March, 1933, and the moult to adults started on 29th April, 1933.

The following injection experiments were carried out with these ticks.

1. 24th March, 1933. Sheep 34510 injected subcutaneously with an emulsion of three engorged nymphae.

    Result.—Severe reaction. On immunity test after 40 days sheep was immune.

2. 1st Aprli, 1933. Sheep 32501 injected subcutaneously with an emulsion of three engorged nymphae.

    Result.—Severe reaction. Died 19th April, 1933. Louping Ill.

3. 22nd April, 1933. Sheep 34297 injected subcutaneously with an emulsion of three nymphae just prior to moulting.

    Result.—Severe reaction. Died Louping Ill 1st May, 1933.

4. 27th April, 1933. Sheep 32951 injected subcutaneously with an emulsion of three adults which had just moulted.

    Result.—No reaction. On immunity test after 40 days the sheep was fully susceptible.

A feeding experiment with the few remaining adults was attempted but the ticks refused to attach. For this reason and because the results were so interesting the entire experiment was repeated in the following way :—

On 27th February, 1934, numerous infected nymphae were placed on the ear of an immune horse (20374) and a susceptible sheep (37618). The ticks readily attached and engorged well on both animals. The sheep showed a typically severe reaction and was destroyed *in extremis* on the 14th day, thus demonstrating the infectivity of the batch of ticks used. The horse showed no departure

from normal health. The following injection and feeding experiments were carried out with the ticks obtained from the horse, the ticks commencing their moult on 28th March, 1934.

1. 6th March, 1934. Sheep 36881 injected subcutaneously with an emulsion of six nymphae, emulsification and injection being carried out on the day on which the ticks detached.

    Result.—A very mild reaction followed and on immunity test after an interval of 58 days the sheep was solidly immune to mouse virus.

2. 13th March, 1934. Sheep 38873 injected subcutaneously with an emulsion of six nymphae, emulsification and injection being carried out eight days after detachment of the ticks.

    Result.—No reaction. On immunity test the sheep was found to be fully susceptible.

3. 9th April, 1934. Sheep 38889 infested on ears with adults.

    Result.—Severe reaction to which the sheep succumbed on the 13th day.

4. 22nd September, 1934. Sheep 38885 injected subcutaneously with an emulsion of three adults.

    Result.—No reaction. On immunity test after 23 days sheep reacted severely.

5. 4th June, 1934, 27th June, 1934, 28th August, 1934. Sheep were infested on ears with numerous adults.

    Result.—On no occasion was any reaction produced though subsequent immunity test showed the sheep to be fully susceptible.

Conclusion.—From this series of experiments it must be concluded that infective nymphae after engorgement on an immune horse, still contain virus when they detach and drop from the host. The great majority of ticks loose this infection during and after the moult to adults, but an odd individual appears to retain sufficient virus to transmit infection during engorgement as an adult. This conclusion is based upon the finding that on only one out of four attempts did adults transmit infection during feeding, and though emulsions of engorged and moulting nymphae were shown to contain virus on two occasions the injection of emulsified adults produced no reaction.

G. Transmission from larvae through nymphae to adults.

1. Nymphae feeding on susceptible cattle.

2. Nymphae feeding on immune cattle.

This series of experiments was conducted in the same manner as those detailed for the corresponding work on horses and sheep.

Nymphae, whose infectivity was controlled by the production of the typical disease after feeding on a susceptible sheep, were placed on the ears of : —

1. A calf 5251 which had been reared under tick free conditions and could not have come into contact with the disease.

2. The same calf after it had survived feeding experiments by which time specific virucidal antibodies had made their appearance in the serum (see above).

With the engorged nymphae and the resulting adults the following injection and feeding experiments were carried out.

(a) *Engorgement on susceptible calf.*

1. 27th February, 1934. Sheep 37590 injected subcutaneously with an emulsion of three adults.

    Result.—No reaction. On immunity test after 24 days sheep reacted severely and was destroyed in extremis on the 11th day.

2. 27th February, 1934. Sheep 37518 infested on the ears with numerous adults.

    Result.—No reaction. On immunity test after 24 days sheep eventually recovered from a severe reaction.

3. 9th April, 1934. Sheep 38943 injected subcutaneously with emulsion of three adults.

    Result.—No reaction. On immunity test after 23 days sheep reacted severely and recovered.

4. 9th April, 1934. Sheep 38933 infested on ears with numerous adults.

    Result.—No reaction. On immunity test after 23 days sheep reacted severely and died on the 13th day.

Conclusion.—Adults derived from infective nymphae that engorged on a susceptible bovine rid themselves of infection.

(b) *Engorgement on an immune calf.*

1. 27th February, 1934. Sheep 32673 injected subcutaneously with an emulsion of three adults.

    Result.—No reaction. On immunity test after 24 days severe reaction.

2. 27th February, 1934. Sheep 37626 infested on ears with numerous adults.

    Result.—Severe reaction followed by recovery. On immunity test after 24 days no reaction.

3. 11th April, 1934. Sheep 38937 injected subcutaneously with an emulsion of three adults.

    Result.—No reaction. On immunity test after 23 days severe reaction.

4. 11th April, 1934. Sheep 38935 infested on ears with numerous adults.

Result.—No reaction. On immunity test after 23 days severe reaction.

Conclusion.—Infective nymphae after engorgement on an immune bovine may or may not carry the infection through the moult to the adult stage. This result is in keeping with those obtained from feeding on sheep and horses and is discussed below.

H. The infectivity of adults from " clean " nymphae which engorged on an immune sheep simultaneously with infected nymphae.

On 13th January, 1933, numerous *R. appendiculatus* nymphae which had been fed as larvae on one of the routine tick feeding calves were placed on the left ear of an immune sheep (34497). At the same time known infected nymphae were placed on the right ear. Both sets of ticks attached and engorged well and subsequently dropped at approximately the same time. The clean ticks commenced their moult to adults on 17th February, 1933, after which the following experiments were carried out.

22nd March, 1933. Sheep 35008 injected subcutaneously with an emulsion of five adults.

Result.—No reaction.

22nd March, 1933. Sheep 35007 infested on both ears with adults.

Result.—No reaction.

26th April, 1933. Sheep 33100 injected subcutaneously with an emulsion of five adults.

Result.—No reaction.

26th April, 1933. Sheep 33098 infested on both ears with adults.

Result.—No reaction.

Subsequently the four sheep were subjected to an immunity test by the subcutaneous injection of mouse virus. All reacted and one died after showing typical symptoms.

Conclusion.—From the above single series of experiments it may not be justifiable to conclude that ticks are incapable of picking up infection while feeding on an immune sheep together with infective ticks. But when it is remembered that the antibody content of the serum of an immune sheep is high, there appears little likelihood of the engorging ticks being able to take up free virus. Consequently it is believed that the immune animal is of no importance as a reservoir of infection.

DISCUSSION.

The primary object of the series of experiments described was to augment the preliminary report on the transmission of Louping Ill by *Rhipicephalus appendiculatus* (Alexander and Neitz, 1933), by

working out in detail the rôle of the various stages of the tick. The work has shown conclusively that larvae are capable of picking up infection and transmitting it as nymphae, and that nymphae may acquire infection for transmission as adults, but every attempt to demonstrate the passage of virus beyond the adult stage has failed.

Consequently with these investigations an attempt was made to obtain some information upon the tenacity of virus infection in the tick population by determining the effect upon the virus of engorgement upon susceptible and immune sheep. When the work was planned it was intended to carry out supplementary investigations to determine the effect upon the virus of feeding ticks on those unsusceptible species of domestic animals which might normally be encountered on a Louping Ill infected farm. The horse and the bovine were selected in the belief that these species were unsusceptible, and only during the course of the work was this assumption found to be incorrect. It is true that the pathogenesis of the disease in these animals has not been worked out but it has been established that virus may circulate in the peripheral blood of the horse, that a multiplication of virus may take place in the central nervous system of bovines, and that in both species injection of virus is followed by the appearance of virucidal antibodies in the serum to a considerable titre. Consequently there still remains to be worked out the effect upon the virus of tick engorgement on a completely unsusceptible species of animal.

A critical survey of the results obtained illustrates that in work of this nature it is not justifiable to conclude that every tick which ingests infective blood will pick up the infection. Naturally the danger of erroneous conclusions may be reduced to a minimum by feeding large numbers of ticks for each trial. This is a simple matter when larvae and nymphae are being handled, but usually it is impracticable to feed large numbers of adults so that additional significance must be attached to the smaller percentage of positive results obtained with this stage of the invertebrate host.

From a purely academical point of view it is of interest to note that infective nymphae after engorgement on immune sheep, cattle and horses still contain virus at the time of detachment but that this virus in the majority of instances has disappeared by the time the adult stage has been reached, a small percentage of adults only remaining a source of infection. This " cleansing " of the tick cannot be due entirely to the virucidal action of the immune bodies in the blood because a similar phenomenon was encountered in the work on susceptible animals. It is admitted that in the latter cases the results are more difficult to interpret since there always exists the chance that virus might have been circulating before the completion of engorgement, owing to the exceedingly short incubation period of the disease and the comparatively lengthy period of attachment of the ticks. Furthermore, attention must be directed to the low virus content of these adults derived from the nymphae as shown by the failure to produce the disease by subcutaneous injection of tick emulsions, though positive results were obtained by feeding. This activation of the virus has an analogy in Rocky Mountain Spotted Fever (Spencer and Parker, 1929), and Heartwater

(Alexander, 1931), two diseases caused by Rickettsia, but as far as the authors are aware it has not been noted in diseases due to filterable viruses. Whether this activation or multiplication represents the completion of a cyclical development of the virus is an interesting problem and an attractive hypothesis for which there is no direct experimental evidence.

From a practical point of view the work indicates that on an infected farm the disease may be controlled and eventually eliminated by running immune animals to serve as hosts for the ticks in order to hasten the development to a succeeding generation always providing that no susceptible animals are introduced during the time to serve as a potential reservoir of infection of this particular vector.

SUMMARY.

1. Experiments conducted to demonstrate the susceptibility of horses and cattle are described.

2. The appearance of virucidal antibodies in the serum of these animals after infection is demonstrated by a technique of *in vitro* neutralization of virus.

3. The technique of tick feeding investigations is briefly described.

4. It is shown that—

    (*a*) larvae of *R. appendiculatus* will pick up infection for transmission as nymphae;

    (*b*) nymphae will pick up infection for transmission as adults;

    (*c*) the virus does not pass through the egg to the next generation;

    (*d*) infective nymphae tend to lose their infection after feeding on immune animals though some of the resulting adults may still be infective;

    (*e*) the same occurrence was noted after feeding infective nymphae on susceptible animals but, particularly if sheep are the hosts, there is a danger of the nymphae reacquiring infection before detachment;

    (*f*) clean ticks do not acquire infection when feeding on an immune animal simultaneously with infective ticks.

5. The significance of the work is discussed.

REFERENCES.

ALEXANDER, R. A. (1931). Heartwater. The Present State of our Knowledge of the Disease. 17*th Rept. of D.V.S. and A.I.* Union of South Africa, pp. 89-150.

ALEXANDER, R. A., AND NEITZ, W. O. (1933). The Transmission of Louping Ill of Sheep by Ticks (*Rhipicephalus appendiculatus*). *Vet. Journ.*, Vol. 89, pp. 320-323.

SPENCER, R. R., AND PARKER, R. R. (1929). Rocky Mountain Spotted Fever. *J. of Infect. Diseases*, Vol. 44, No. 4, pp. 257-276.