

Investigations into the Transmission of Horse- sickness at Onderstepoort during the Season 1931-1932.

By

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THE natural transmission of horsesickness, an extremely important disease of equines in South Africa, is not known up to the present, although more than 30 years have elapsed since the first experiments were commenced. Although the horse has to a great extent been superseded by mechanical transport to-day, the practical importance of the disease has not been greatly diminished since those early days.

During the summer of 1931-1932 we started a new line of research into this problem, which had held the interest of the Onderstepoort Laboratories for more than 15 years.

There were only a few facts, representing general opinions about horsesickness, at our disposal which could serve as a guide for these investigations, viz., horsesickness is not contagious; the disease does not spread by close contact in stables; its occurrence under natural conditions is restricted normally to the summer months and the severity of the outbreaks depends mainly on the amount of rain, in dry seasons few cases occur, whereas in wet seasons the incidence of infection is very high; infection seems to occur mainly at night, round about sunset or sunrise; stabling of animals itself affords fairly good protection and, furthermore, the tops of hills may be regarded as fairly safe; after the first good frost the disease disappears suddenly and no new cases make their appearance.

This information is naturally very meagre wherewith to locate the natural transmitter of the disease. It serves to indicate to a great extent, however, that mosquitoes are the most probable transmitters, and with this theory a satisfactory explanation of all the facts known up to the present could be advanced. Whatever the actual vector may prove to be, any work on the transmission of horsesickness has to begin, in our opinion, with mosquitoes.

For the carrying out of this work we had at our disposal, to start with, only one summer, viz., that of 1931-1932. Six months, even under the most favourable conditions, is a very short period for work of this nature, and we could consequently not expect to finish in the time. A mosquito survey, the result of which has been described in the first paper of this series, had to be conducted in order to obtain some indication as to the most probable vectors, as it was obviously impossible to experiment with every species of mosquito known to occur in South Africa.

Suitable methods for feeding the mosquitoes on horses and also for keeping them alive for a sufficiently long time had to be devised, the former work in this direction having failed mainly on account of technical difficulties. The actual experiments had then to be carried out in which, apart from the vector being unknown, the length of the incubation period of the virus in the insect host, the range of temperature in which its development could take place, and the suitable time for feeding the probable transmitters on the infected animals, were also all unknown.

The strain of virus we had to do most of our experiments with was an old laboratory strain, and only towards the end of our work did we succeed in obtaining material from some natural cases.

Furthermore, the climatic conditions were so adverse that our difficulties were still further increased. It was very often impossible to obtain the material we wanted.

We were not successful in finding the actual transmitter of horsesickness. We believe, however, that the negative results are also of some value, and we wish, therefore, to give a detailed account of our experiments and observations as this work will serve as the basis for future work on the same lines, and may be of value to other workers interested in the same problems.

I. GENERAL OPINIONS CONCERNING THE EPIDEMIOLOGY OF HORSESICKNESS. REVIEW OF LITERATURE.

The main epidemiological facts known up to the present which can be of some assistance in trying to trace the transmitter of horsesickness, have already been mentioned briefly. They were regarded as all pointing to some species of mosquitoes as the most probable carriers.

The insect transmission theory does not find much support amongst the farming community mainly on account of the lack of knowledge on the biology of insects and the part played by them in the transmission of other diseases. The farmers' observations are, however, often remarkably accurate, and theories of particular interest formulated by them may be encountered. These theories all correlate to a greater or lesser extent the disease in question with climatic and telluric factors as may be seen from the following examples.

THE DEW THEORY.

Observations on the actual time at which infection is most likely to occur has revealed the fact that early morning or late evening must be regarded as particularly dangerous. At this time the grass is generally covered with dew during the horsesickness and bluetongue season, and many farmers have come to the conclusion that it is actually this dew which is responsible. This to some extent supports our present knowledge of the habits of the particular varieties of mosquitoes under suspicion which are largely confined to moist localities such as those afforded by dewy grass and which, furthermore, are most aggressive under these conditions coupled as they generally are with the absence of wind, which greatly facilitates matters for the mosquito who is not a strong flier.

TOXIN IN VLEI GRASS.

It has been noted that horsesickness and bluetongue are generally confined to low lying and marshy ground and that animals pastured on elevated ground are much less liable to infection. This has led to formulation of the theory that some toxin is developed in grasses in these low-lying areas under certain conditions of soil and climate, which is capable of setting up the diseases. This again bears out the mosquito theory, as these insects are to a very great extent confined to such localities as will be shown later.

WATER.

Water has frequently been blamed, vague referenes to contamination, presumably of animal or mineral origin, being advanced as the cause. Here again a comparison similar to the preceding can be drawn.

MORNING AND EVENING MISTS.

This is an extremely old conception, the cause of human malaria having been ascribed to such mists. Nevertheless, it still persists in the minds of many even to-day. Under such an environment again mosquitoes find particularly suitable conditions.

A complete review of these epidemiological facts has been given by *Knuth* and *du Toit* in their textbook on tropical diseases of animals, where further details may be found. These authors came to the conclusion as well that mosquitoes are the most probable transmitters.

As far as we are able to ascertain the only actual transmission experiments with mosquitoes were carried out some 30 years ago by *Pitchford* (1902) in Natal. He claims to have obtained six positive results with unidentified Anophelines. He placed an infected horse together with a number of Anopheles in an "infection box". After its death it was replaced by a normal horse. The resulting infection did not kill the horse but resembled a mild attack of horsesickness. It is not possible to obtain a very accurate idea of his experiments, as no satisfactory records are given. Only in one case is a temperature chart added. This horse had remained in contact with mosquitoes for 4 days, and 12 days later the temperature commenced to rise. Blood was subinoculated into another horse and three days

later the reaction appeared and the horse died on the 17th day, p.i. In another case the mosquitoes had been infected less than 48 hours previous to their feeding on this animal. This would suggest a mechanical transmission.

By some authors outside the Union arthropoda other than mosquitoes have been suggested as possible transmitters. No actual experiments have been carried out with them however. *Williams* (1913), *Leger* and *Teppas* (1922) and *Monfrais* (1923) regard *Lyperosia* species as probable transmitters, *van Saceghem* (1918) *Culicoides* and *Tabanus* species. The possibility of *Culicoides* acting as carriers is also discussed by *Patton* (1920). Furthermore, *Rockmann* (1911) and *Reinicke* (1912) for epidemiological reasons, regard ticks as the most probable transmitters, a theory which, however, does not agree well with those epidemiological facts mentioned in this paper and accepted for the time being as correct.

II. SCHEME OF EXPERIMENTS.

For reasons already stated in full, mosquitoes were regarded as the most probable transmitters of horsesickness and the mosquito survey carried out at Onderstepoort concurrently with the actual experiments, had pointed out that, disregarding Anophelines owing to lack of information, species of *Aedes* fulfilled most accurately the requirements of the known epidemiological factors. Representatives of this genus were, therefore, mainly used in the following experiments. The following were regarded as the most promising species; *Aedes caballus*, *A. lineatopennis*, *A. hirsutus* and to a somewhat lesser extent *A. vittatus* and *A. dentatus*.

When the experiments were commenced in the later part of the winter of 1931, *Aedes* species were not yet available and *Culex theileri* was the only mosquito obtainable in fair numbers. With this species a number of experiments were carried out, mainly to elaborate the experimental technique as far as possible before the actual season commenced. Furthermore, it seemed worth while experimentally to exclude this most common mosquito species from the list of possible transmitters. The transmission of horsesickness by insects might be direct, mechanical or indirect, requiring a certain multiplication or development of the virus in the insect's body.

That the direct or mechanical transmission is possible has been proved by *Schuberg* and *Vuhn* (1912), who succeeded in transmitting the disease in this way by *Stomoxys calcitrans*. Although mechanical infections may occur in nature, it is very unlikely that this is the ordinary way; e.g. daylight biting insects, especially *tabanids* and *Stomoxys* seem to be the most capable mechanical transmitters according to our knowledge of other diseases. Horsesickness is, however, normally not transmitted during day-time, as we have seen before.

Assuming the necessity for a multiplication or an actual development of the virus, a certain period will elapse before the insect is capable of transmitting an infection. In Yellow Fever, a human disease transmitted chiefly by *Aedes* species, which closely resembles horsesickness in many respects, this period is at least 8 days and on an average about 12 days.

In our experiments the mosquitoes were injected into susceptible horses five days and longer after their infective feed on a virus horse, or fed after a period of at least 14 days. During the interval they were fed on sugar water in the laboratory.

A positive result after injection of mosquitoes would merely show the presence of virus in some part of the insect's body, but does not reveal anything about its capacity for transmitting the disease actually. This can only be proved by feeding experiments. On the other hand, a negative result obtained by injection is more definite than is the case in a feeding experiment, especially if the latter is carried out with a limited number of specimens.

The injection experiments therefore give quite a lot of useful preliminary information about the suitability of a species for transmission. A negative result obtained with mosquitoes 5-7 days after their feeding on a virus horse, is a strong indication that the species used is not a transmitter, whereas a positive result, although requiring confirmation by feeding experiments, may be regarded only as promising. The one method therefore lends a sense of completeness to the other.

In the feeding experiments a period of 14 days, after feeding on a virus horse, was regarded as sufficient to allow for the necessary development or multiplication of the virus in the mosquito.

It was not possible in our work to adhere to a programme planned beforehand, as we were not sure about the suitability of the strains of horsesickness at our disposal for this work, and as we depended to a great extent in the choice of our material upon the climatic conditions.

III. STRAINS OF VIRUS AND ANIMALS USED IN THE EXPERIMENTS.

In all, the following four strains of horsesickness virus were used in our experiments.

(a) *O-virus*.—The strain of virus used in the first series of our experiments consisted of the laboratory vaccine strain known as *O-virus*. This strain had originally been isolated from a case of horsesickness by Sir Arnold Theiler in the year 1901 and was subsequently chosen by him as the strain most suitable for immunization purposes.

In August, 1901, the first injection was made into horse No. 96, blood from this horse constituting the first generation of the virus. By repeated subinoculations into susceptible horses the strain was preserved and is to-day used in the preparation of the horsesickness vaccine being now in its 225th generation. The virus has proved to be of a very high grade of virulence, resulting almost invariably in fatal cases of horsesickness upon inoculation. From a perusal of the temperature charts of the horses used in its maintenance it appears that slight though definite alterations in its potency have taken place. The incubation period of the reactions produced by it have shortened somewhat, viz., from 4 to 6 days to the usual 2 to 3 days, as is the case to-day. The duration of the disease itself,

which was originally in the neighbourhood of 6 to even 10 days has been reduced to an average of 3 days and in some cases even less. It would appear, therefore, that some alteration in the virus itself has taken place, and coupled with the fact that this virus has had no opportunity of developing outside the body of horses throughout the 31 years from its recovery from a natural case of horsesickness, it seems quite reasonable to assume that some biological change has taken place which may have affected its propensity for developing in the body of the supposed invertebrate host.

(b) *Losperfontein Virus*.—This virus was obtained on 3rd March, 1932, on the Government Irrigation Settlement at Losperfontein, approximately 40 miles west of Pretoria, from a fatal case of horsesickness in a mule. Owing to some confusion in the records of the settlement office, a certain amount of doubt exists as to the accurate history of this case. So far as can be ascertained the mule

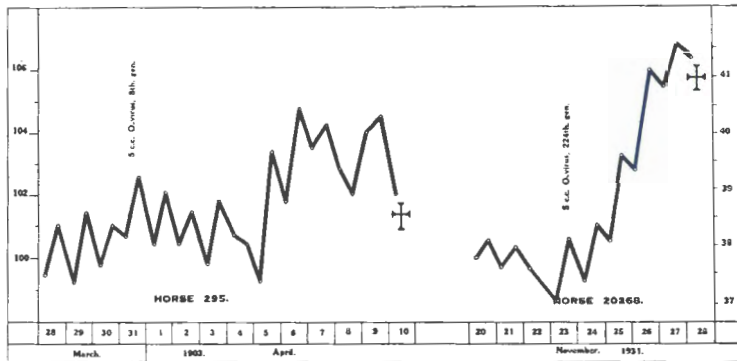


Fig. 1.—Comparison between horsesickness reactions produced by O-virus: 8th and 224th generations.

was immunized against horsesickness during the month of December, 1931, by the Onderstepoort method of hyperimmune serum and virus, one of the viruses used being the O-strain. The possibility therefore exists that we were dealing with a case of relapse after a period of three months, and had recovered the O-strain virus where-with the mule had been immunized in December. It must be pointed out, however, that had this mule not acquired immunity or not been immunized in the first instance, the latter also being possible on account of the confusion amongst the records, chances for natural infection on Losperfontein were excellent. A perusal of the meteorological records revealed the fact that fairly good rains had fallen early in February, i.e. about one month previously. The mules had been running in a paddock containing a stream where the chances for mosquitoes breeding out were very good indeed. Natural infection can, therefore, not be lost sight of and is in fact extremely likely. However, the possibility does exist that a relapse might have occurred, in which case O-virus would have been recovered, and its difficult propagation in our experimental mosquitoes would then not be surprising assuming that O-virus has become biologically altered.

(c) *Eshowe virus*.—This strain of virus was obtained by the Government Veterinary Officer of Eshowe, Natal, from a mule suffering from horsesickness which ended in recovery. This mule had been immunized against horsesickness on the 25th August, 1931, by the serum-virus method.

In this case again the possibility of a relapse exists, after an interval of 6 months. The fact that this mule recovered seems to point to some other virus strain being responsible for setting up the infection, as O-virus is almost invariably fatal. This does not, however, constitute sufficient evidence to exclude the relapse possibility as cases of recovery from O-virus are known and further the mule may have had some natural immunity.

(d) *Kaalplaas virus*.—The Kaalplaas virus used in our experiments was obtained from a horse No. 20031 owned by the laboratory which had been running on the laboratory farm Kaalplaas, about 5 miles from Onderstepoort. This horse had been hyperimmunized against horsesickness, O-virus strain, 225th generation, in September, 1931, receiving 10,000 c.c. virulent blood from the donor, horse No. 20154. The animal was then sent to Kaalplaas where it was exposed to natural infection from 6th October, 1931, to 13th April, 1932, and contracted horsesickness on the latter date. It was returned to Onderstepoort, where a typical case of Dikkop horsesickness, which ended fatally, was noted. Considering that this was the only case of horsesickness amongst a large number of immunized horses with which this animal was running, itself hyperimmunized against the disease, it appears extremely likely that we were dealing with a case of relapse to O-virus.

It will be realized from the above that in the case of the three viruses collected from the field for experimental purposes, viz., Losperfontein, Eshowe and Kaalplaas, a history of immunization against horsesickness accompanies each case so that the possibility of our having dealt with O-virus throughout our experiments can, therefore, not be ignored.

(e) *Experimental Animals*.—The horses used in our experiments consisted of old cast animals of little commercial value bought by the laboratory for experimental purposes and for the production of horsesickness vaccine. The animals are recruited principally from the large towns, viz., Johannesburg and Pretoria, where they had been used for various purposes, e.g. hauling commercial vehicles, etc. No guarantee of any sort can be obtained as to their susceptibility or resistance to horsesickness nor is anything of their past histories known. The fact that the greater number of these animals have spent their lives in towns indicates that they have probably not been exposed to infection and from past experience it is known that only very occasionally is an immune horse encountered.

IV. EXPERIMENTAL TECHNIQUE.

The technique used for feeding mosquitoes on horse and keeping them alive in the laboratory for the periods required by the experiments, have been described in the second paper of this series, and we, therefore, refer the reader to it for full particulars.

From this paper it will be seen that the technique was gradually altered and improved. Different methods were therefore used in the different sections of this work. In the introduction to each section we will briefly refer to the methods actually applied in that particular part.

V. EXPERIMENTS WITH O-VIRUS.

We commenced our experiments with the O-virus laboratory strain, which was isolated, as previously stated, more than 30 years ago, and had been transmitted through about 200 generations by intrajugular injection from horse to horse.

As mentioned previously, this strain has changed during these 30 years, especially the incubation period, which has considerably shortened, being at present only 2-3 days. The strain has virtually acquired the character of a virus fixe, and it is, therefore, not unreasonable to suspect a change in other biological respects as well.

From the commencement we had some doubt about the suitability of the strain for transmission experiments. It seemed possible that the exceptionally long direct transmission of the strain from horse to horse could have affected the developmental capacity of the virus in its natural insect host.

In the literature no reference is given to similar biological alterations in respect of other virus diseases of man or animals transmitted by insects, but no other virus, to our knowledge, has ever been transmitted through a series of direct transmissions by means of the syringe only. We know, however, that in trypanosomiasis the developmental capacity of the parasites in tsetse flies is very quickly reduced and lost through direct transmission. On the other hand, the human malaria parasite is not affected by such a procedure.

In connection with the theory of a reduced developmental capacity of the virus in its invertebrate host, a recent outbreak of horsesickness at Wellington, Cape Province, may serve to throw some light upon the subject. For a number of years this area was regarded as free from horsesickness. On 3rd March, 1931, some horses were immunized against horsesickness with the Onderstepoort strain. On 29th March one of the non-immunized contact horses died from horsesickness and shortly afterwards several others followed.

These cases seemed to indicate that the virus was still capable of being transmitted in the natural way as we are forced to exclude the possibility of natural horsesickness in this area. The whole outbreak was considered by the veterinary authorities to be something very exceptional, as they had never previously observed similar cases. There is the possibility, of course, of the disease not having been transmitted in the supposedly normal manner, i.e. by mosquitoes, in which case our contention of a possible biological alteration in the virus is supported. The time between the inoculation of the horses for immunization and the first death of one of the contact horses was 27 days. If we allow a period of three days for the appearance of the virus in the blood of the inoculated horses,

and a further period of 10 days for the interval between the infection of the contact horses by insects and their death, then there would remain a maximum of 14 days for the incubation period of the virus in the insect. This must be regarded, under our climatic conditions, with calm cool nights, as a relatively short incubation period in comparison with what we know about Yellow Fever and Dengue, i.e. if we accept at all the necessity for a certain period of development of the virus to render the insect infectious. The conditions for an insect transmission in this case must, therefore have been absolutely optimal. As an entomological survey was not successful, we do not know if this was the case. The transmission might possibly have been effected mechanically, but in any case the conditions under which the infection spread were apparently not normal, and it is, therefore, difficult to arrive at any definite conclusion as to the capacity of the strain developing normally in the insect host.

Our experiments were commenced in September, 1931. At that time we had only the O-virus strain at our disposal. We had hoped to obtain a normal field strain early in the season, but unfortunately one was not obtainable until towards the end of February, 1932.

In conducting the experiments discussed here it was our intention, firstly, to ascertain the most suitable time for feeding mosquitoes on infected horses. Secondly, to carry out some experiments in September or October with mosquitoes common in winter, i.e. before the commencement of the horsesickness season and the appearance of *Aedes* species, and thirdly, to start preliminary experiments in November, when the first *Aedes* mosquitoes generally appear, using those species most likely to be the natural vectors.

For these tests we used relatively small numbers of mosquitoes, having in mind the high infection index encountered in the Yellow Fever and Dengue work. Moreover, a positive result with a few specimens of a certain species would have been a more valuable indication than one with large numbers, as the method of injecting crushed mosquitoes was used. This method appeared to us to be the most suitable for preliminary work, for the reason that the virus being present in some part of the body in a virulent form, its presence would be revealed by injection of the whole mosquito even if it had not reached the salivary glands. The earliest interval chosen for injection was 5 days. The digestion of the blood in the intestines of the mosquitoes was usually completed after three days at the temperature we kept them, and there remained, therefore, no undigested blood to harbour the virus after five days. While blood was present in the intestines the presence of virus in emulsified mosquitoes would have been of no significance.

When all the above-mentioned experiments proved to be negative a further experiment was undertaken, using a large number of mosquitoes simultaneously. This concluded this series of tests.

A. EXPERIMENTAL TECHNIQUE.

The mosquitoes used in these experiments were as a rule fed in the small cages described in the second paper, which were fastened to the skin of the horse with strips of plaster. Only in the last test

were they liberated in a mosquito-proof tent containing the infected horse. Except in the last experiment, the mosquitoes were fed during the night on horses kept in one of the ordinary stables.

During September and October, when working with *Culex*, the percentage of engorged specimens was reasonably high (up to 90 per cent.), without making any arrangements for increasing the humidity. Unsatisfactory results were only obtained with horses that were very sensitive to the mosquito bites. The feeding results with the *Aedes* species were, on the whole satisfactory during November and December. In some cases, however, the percentage of engorged specimens was markedly less than in the experiments with *Culex*. Moreover a greater mortality was observed in the cages. Later it was found that the humidity was at fault, not being sufficiently high. The experiments with free mosquitoes in the fly-proof tent in the field, also yielded good results, without any precautions being taken. However, the night in question happened to be wet.

After feeding, the mosquitoes were kept in jam jars standing on wet cotton wool in larger glass jars, as has been described in the general section on technique. They were fed on 10 per cent. sugar water. This method proved satisfactory, although in some cases there was a considerable mortality.

In these experiments all mosquitoes were injected into normal horses not fed on them. They were first stunned by knocking the test tube containing them against the palm of the hand, after which they were crushed in normal horse serum in all cases, and injected subcutaneously into the neck of the horse. To commence with, ether was used to render them inert, and thus facilitate crushing, but its use was soon discontinued.

B. MOSQUITOES.

In this group of experiments 807 infected mosquitoes were injected into susceptible horses. These mosquitoes included the following species:—

<i>Culex theileri</i>	254 specimens.
<i>Anopheles squamosus</i>	5 specimens.
<i>Aedes caballus</i>	206 specimens.
<i>Aedes dentatus</i>	18 specimens.
<i>Aedes hirsutus</i>	145 specimens.
<i>Aedes lineatopennis</i>	127 specimens.
<i>Aedes vittatus</i>	52 specimens.

Whenever possible mosquitoes reared from larvae in the laboratory were used, and only when larvae were unobtainable had we to make use of adult specimens caught either in the field or in traps. The larvae of *Culex theileri* were very numerous in September and October, and could be caught in sufficient numbers amongst reeds growing near the banks of the Aapies River where the water was sluggish. Adults were very common during this period in the mosquito traps. Adults of *Anopheles squamosus* were only occasionally caught in the traps, and their larvae were rarely found in the Aapies River. The above-mentioned five species of *Aedes* commenced to appear in November. At first they were relatively common, but

later on more difficult to find, and during the first half of January became comparatively rare. As there was very little hope of the climatic conditions improving, we artificially flooded the breeding places of *A. hirsutus*, *A. caballus* and *A. lineatopennis*, and were then able to catch these species in large numbers. The breeding place of *A. vittatus* had regularly been infested with dragon-fly larvae since January, and did not yield any further mosquitoes. Catching the dragon-fly larvae was not attended with success, as fresh eggs were continually being deposited. In the case of *A. hirsutus* and *A. vittatus* only specimens reared from larvae and pupae were used, whereas with *A. caballus* and *A. lineatopennis* adults caught on their breeding grounds or in the mosquito traps were also used in addition to bred specimens.

C. VIRUS HORSES.

For this series of experiments we used a total of nine virus horses. By the term virus horse must be understood those infected horses used for feeding mosquitoes on. For simplifying later references, a short description of the course of the infection in each case will be given below, with an enumeration of the groups of mosquitoes fed on the respective horses.

Virus Horse 1 (No. 20181).—Injected on 7th September, 1931, intrajugularly with 5 c.c. O-virus (224th generation of 8th August, 1931).

Result: On 10th September p.m. first rise of temperature* up to 104°. The horse died 2½ days later. Fed *C. theileri*, group 1.

Virus Horse 2 (No. 20125).—Horse of experiment 1 (*Culex theileri* injection after ½ day). Fed *C. theileri*, group 2. For temperature see experiment 1.

Virus Horse 3 (No. 20183).—Injected intrajugularly on 21st September, 1931, with 5 c.c. O-virus (224th generation of 12th August, 1931).

Result: Temperature up to 23rd September a.m. normal, p.m. 102.5°; on 24th, 101.0° and 104.5°; on 25th, 103.4° and 105.0°; on 26th, 104.4° and 104.7°; and on 27th a.m., 105.0°. The horse died on the 27th p.m. Fed *C. theileri*, group 3.

Virus Horse 4 (No. 20261).—Injected on October 10th, with the same virus as the previous horse.

Result: Temperature normal up to 12th October a.m., p.m. 103.1°; on 13th, 103.3° and 106.1°; on 14th, 104.0° and 106.1°; on 15th, 104.0° and 106.0°. The horse died during the following night. Fed *C. theileri*, groups 4 and 5, and *A. squamosus*, group 1.

Virus Horse 5 (No. 20196).—This horse had been used for experiment 3 (*C. theileri* injection after 16 days). It had also been infected some months earlier for other reasons with the Tzaneen strain of horsesickness. On 2nd November 5 c.c. O-virus (191st generation of 27th September, 1931) were injected intrajugularly.

Result: Temperature normal up to 9th November a.m., p.m. first rise up to 103.3°. Temperature on 10th, 102.4° and 104.0°; on 11th, 102.0° and 103.2°; on 12th, 102.0° and 103.9°; on 13th, 101.2° and 102.5°; on 14th, 101.3° and 102.0°. Thereafter the temperature was normal. The course of the disease was somewhat attenuated, and it is very likely that there was a certain amount of immunity against O-virus present, due to the earlier injections with T-virus. On this horse were fed *Aedes caballus*, group 1; *A. dentatus*, group 1; and *Anopheles squamosus*, group 2.

Virus Horse 6 (No. 20184).—Used before in a negative experiment (experiment 2, *C. theileri*, 5 days). Injected 13th November with the same material of O-virus as virus horse 1.

* The temperatures of all experimental animals were taken twice daily; in the morning between 6.30 and 7, and in the afternoon between 3.30 and 4.

Result: Temperature normal up to 16th November a.m., p.m. rise to 104.4°. Temperature on 17th, 103.4° and 106.0°; on 18th a.m., 102.6°. The horse died the same afternoon. Fed *Aedes caballus*, group 2-3, and *A. lineatopennis*, group 1.

Virus Horse 7 (No. 20268).—Used for subinoculation in experiment 5 (*Culex theileri*, direct transmission). Injected on 23rd November with the same virus as the previous horse.

Result: Temperature normal up to 25th November a.m., p.m. 103.4°; on 26th, 102.8° and 106.0°; on 27th, 105.4° and 107.0°; and on 28th a.m., 106.5°. The horse died the same day. Fed *Aedes caballus*, groups 4-6; *A. dentatus*, group 3; *A. hirsutus*, groups 2-3; and *A. lineatopennis*, group 2.

Virus Horse 8 (No. 20287).—Injected 5th December, 1931, with 5 c.c. O-virus (191st generation of 27th September).

Result: Temperature normal up to 7th December a.m., p.m. 103.6°; on 8th, 104.0° and 104.2° on 9th, 105.0° and 106.4°; and on 10th, 105.4° and 106.4°. The horse died during the following night. Fed *Aedes caballus*, groups 7-10; *A. dentatus*, groups 4-5; *A. hirsutus*, group 4; *A. lineatopennis*, groups 4-7; and *A. vittatus*, group 1-2.

Virus Horse 9 (No. 20269).—Used previously for experiment 7 (*Aedes caballus* injection after 5 days). Injected on 29th January intrajugularly with 5 c.c. O-virus (225th generation of 11th September, 1931).

Result: The temperature on 31st January a.m. was 102.0°; on 1st February, 103.4° and 106.1°; on 2nd February a.m., 105.1°. The same day the horse was killed for other purposes. Fed *Aedes caballus*, group 11; *A. hirsutus*, group 5; and *A. lineatopennis*, group 8.

All these 9 cases developed the typical Dunkop form of horsesickness. Dikkop or mixed infections did not occur.

D. EXPERIMENTS WITH *Culex theileri*.

To obtain an approximate idea of the amount of virus present in an infected horse during the fever reaction in connection with our mosquito work, we fed, as a preliminary experiment, a few specimens of *Culex theileri* (at that time other species were not at our disposal) on an infected horse shortly after the first rise of temperature. They were injected shortly afterwards (the following morning) into a normal horse. A series of experiments with several lots of mosquitoes fed at different times of the horsesickness reaction would certainly have been highly interesting, but on account of the expense we limited ourselves to this one test. With the same species four further experiments were made, using a total of 249 specimens. The interval varied between one minute and 25 days.

Virus: O-virus 192nd and 225th generations. Virus horses 1-4 and mosquito groups:—

Group 1.—Fed on virus horse 1 from 11th p.m. to 12th a.m. Second day of fever. Temperature 104.0°. 46 Specimens engorged (reared from larvae). Used for experiments 1-4.

Group 2.—Fed on virus horse 2 on 16th September p.m. Second day of fever. Temperature 105.4°. 18 Specimens engorged (reared from larvae). Used for experiments 3 and 4.

Group 3.—Fed on virus horse 3 from 25th September p.m. to the following morning. Second to third day of fever. Temperature 105.0°. About 100 specimens engorged (reared from larvae) used for experiments 2-4.

Group 4.—Some specimens fed on virus horse 4 on 13th and 14th October. First and second day of fever. Temperature 103.3° and 106.1°. Mosquitoes reared or caught. Used for experiment 5.

Group 5.—Fed on same horse from 13th October p.m. till the following morning. Second day of fever. Temperature 106.1°-104°. 135 Specimens engorged (reared from larvae). Used for experiments 2 and 4.

*Experiment 1 (H.S. 1). 5 Culex theileri. Injection. Interval $\frac{1}{2}$ day.
Horse 20175.*

On 12th September, 1931, 5 *C. theileri*, group 1, which had fed the night before on an infected horse, were injected subcutaneously.

Reaction: The temperature of the experimental horse on 15th September was 101.4° and 103.8°; the following day: 103.4° and 105.4°. The horse was very wild, and it was difficult to take the temperature regularly. The horse died on the night of 17th-18th September. The post-mortem showed typical horsesickness, dunkop.

The result of the experiment was thus *positive*.

*Experiment 2 (H.S. 2). 50 Culex theileri. Injection. Interval 5 days.
Horse 20184.*

This horse was injected with 50 specimens of *C. theileri*, which had fed 5 days previously on an infected horse, viz., on 17th September with 10 specimens of group 1, on 1st October with 15 specimens of group 3, and on 19th October with 25 specimens of group 5. All the mosquitoes had fed on infected horses during the first or second day of fever.

Reaction: The temperature of horse 20184 remained normal, between 99° and 101°, up to 13th November, 25 days after the injection of the last batch of mosquitoes. Once the temperature rose to 102°, but only for half a day.

Immunity Test: The horse was subinoculated on 13th November with O-virus (224th generation of 17th August, 1931). It showed a typical horsesickness reaction and died 5 days after injection (*vide* virus horse 6).

The result of this experiment was thus *negative*.

*Experiment 3 (H.S. 3). 50 Culex theileri. Injection. Interval 16 days.
Horse 20196.*

Into this horse 50 mosquitoes were injected which had been fed 16 days previously on an infected animal. On 28th September, 1931, 10 specimens of group 1 were injected, on 3rd October 15 of group 2, and on 12th October 25 specimens of group 3. These mosquitoes were fed on the infected horse during the second day of fever.

Reaction: The temperature of horse 20196 remained normal up to 2nd November, three weeks after the injection of the last group of mosquitoes, and during this time did not exceed 101.4°.

Immunity Test: On 2nd November the horse was injected intrajugularly with 5 c.c. of O-virus (191st generation of 27th September, 1931). Seven days later the temperature rose up to 103.3° F. and remained above 102.0° (afternoon temperatures) for five days. Thereafter it returned to normal. It was thus only a mild attack of horsesickness. The horse had been injected some time previous to this experiment with T-strain of horsesickness and had probably acquired a certain amount of immunity against O-virus. This is, however, rather exceptional.

Result: As there was no temperature reaction at all after the injection of the mosquitoes, we do not think that the result of the experiment is doubtful owing to the partial resistance of the animal against horsesickness. We consider we are justified, therefore, in regarding this experiment as *negative*.

*Experiment 4 (H.S. 4). 140 Culex theileri. Injection. Interval 25 days.
Horse 20259.*

In this experiment 140 mosquitoes which had fed on infected horses 25 days before were used. On 7th October 14 specimens of group 1 were injected, on the 12th 2 of group 2, on the 21st 51 of group 3, and on 9th November 73 specimens of group 5. All these mosquitoes had been fed on virus horses during the first and second day of fever.

Reaction: The temperature of the horse did not exceed 101.2° up to 8th December, one month after the last injection of mosquitoes. It thus remained absolutely normal.

Immunity Test: On 15th February, 1932, the horse, whose temperature still remained normal, was injected with 5 c.c. blood of horse 20289 from experiment 16 (O-virus, 1st passage through mosquitoes). The fever reaction commenced the following day and the animal died 4 days later.

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Result: Horse 20259 was thus susceptible and the experiment therefore *negative*.

*Experiment 5 (H.S. 5). 9 Culex theileri. Direct transmission.
Horse 20277.*

On 13th October, 1931, 6 *Culex theileri* (group 4) were fed on virus horse 4. After they had taken up about half the normal amount of blood, the feeding was interrupted and the mosquitoes were immediately transferred to horse 20277 to complete their feeding on that animal. The interval was 85, 35, 40, 40, 60, and 50 seconds. The following day three more *C. theileri* of the same group were fed on both horses in the same manner. The interval was 95, 30, and 90 seconds.

Reaction: The temperature of the horse was normal for about three weeks, up to 5th November, when it rose to 102.5°. The maximum temperatures for the following days were: 101.2°, 101.0°, 101.9°, 99.3°, 101.6°, 104.1°, and 101.2°. During this period the horse showed muco-catarrhal discharges from both nostrils and an infection of one of the hind legs. The general appearance was very bad and it was killed on 12th November. The post-mortem was doubtful so far as horsesickness was concerned.

Subinoculation: 5 c.c. blood of this horse, taken on 7th November, was injected into horse 20268 on 11th November. The temperature of this second horse remained normal up to 23rd November; the maximum was 101.6°. On this day horse 20268 was injected with 5 c.c. O-virus (224th generation of 17th August, 1930). Two days later the temperature began to rise and the horse died 5 days later (*vide* virus horse 7).

Result: The observation period of the subinoculated horse, *viz.*, 12 days, was certainly very short, and it is therefore not possible to give a definite statement about the result of this experiment. On the other hand, there is a strong indication that this experiment was also *negative*.

E. EXPERIMENTS WITH *Anopheles squamosus*.

We were only able to make one test with a few specimens of *Anopheles squamosus*, a species which was exceptionally rare this season at Onderstepoort.

Virus: O-virus, 192nd and 225th generation. Virus horse 4 and 5 and mosquito groups:—

Group 1.—Fed on virus horse 4 on 15th October, third day of fever. Temperature 104.0°-106.0°. Three specimens engorged (caught as adults).

Group 2.—On 11th November two specimens (caught as adults) fed on virus horse 5. Third day of fever. Temperature 102.0°-103.2°.

*Experiment 6 (H.S. 6). 5 Anopheles squamosus. Injection.
Interval 5 days. Horse 20262.*

On 20th October three specimens of *A. squamosus*, group 1, were injected into horse 20262, and on 17th November the two specimens of group 2. These mosquitoes had fed 5 days before the injection on infected horses.

Reaction: The temperature of the horse up to 17th December, one month after the injection of the last mosquitoes, varied between 98.5° and 101°, and was thus normal.

Immunity Test: On 4th March the horse was injected with blood from a natural case of horsesickness and died nine days later (*vide* virus horse 10, Losperfontein virus).

Result: The horse was thus susceptible and the experiment proved to be *negative*.

F. EXPERIMENTS WITH *Aedes caballus*.

Aedes caballus, as we have stated before, is one of the common species of *Aedes* round Onderstepoort, and in the later stages of the work it was quite regularly obtained for experiments.

Two experiments were undertaken, using a total of 122 specimens at intervals of 5 and 15-16 days.

Virus: O-virus, 192nd and 225th generation. Virus horses 5, 6, 7, and 8, and mosquito groups:--

Group 1.—Fed on virus horse 5 on 11th November p.m. till the following morning. Third day of fever. Temperature 103.2°-102°. Twenty specimens engorged (hatched from pupae). Used for experiment 7.

Group 2.—Fed on virus horse 6 during the night, 17th-18th November. Second day of fever. Temperature 106.0°-102.6°. Six specimens engorged (reared from larvae). Used for experiment 7.

Group 3.—Fed on the same virus horse on 18th November a.m. Second day of fever. Temperature 102.6°. One specimen engorged (reared from larva). Used for experiment 7.

Group 4.—Fed on virus horse 7 on 27th November p.m. First to second day of fever. Temperature 102.8°-106.0°. Eighty specimens engorged (hatched from pupae and caught as adults). Used for experiments 7 and 8.

Group 5.—Fed on the same virus horse during the following night. Second day of fever. Temperature 106.0°-105.4°. Twenty-eight specimens engorged (hatched from pupae and caught as adults). Used for experiment 8.

Group 6.—Fed on the same virus horse on 27th November. Third day of fever. Temperature 105.4° and 107.0°. Thirty specimens engorged (caught as adults). Used for experiment 8.

Group 7.—Fed on virus horse 8 on 7th December p.m. First day of fever. Temperature 103.6°. Twelve specimens engorged (caught as adults). Used for experiment 7.

Group 8.—Fed on the same virus horse on 8th December p.m. Second day of fever. Temperature 104.2°. Twenty specimens engorged (caught as adults). Used for experiment 7.

Group 10.—Fed on the same virus horse one day later. Third day of fever. Temperature 105.0°-106.4°. Twenty-five specimens engorged (caught as adults). Used for experiment 7.

*Experiment 7 (H.S. 7). 91 Aedes caballus. Injection. Interval 5 days.
Horse 20269.*

In this experiment ninety-four specimens of *A. caballus*, which had fed about five days previously on experimentally infected horses, were injected in all. On 17th November ten specimens of group 1 were injected, on the 23rd six of group 2 and one of group 3, on 1st December thirty-three of group 4, on the 12th ten of group 7, on the 14th eleven of group 8 and twenty-three of group 10. The mosquitoes had fed on the virus horses during the first to third days of fever, except group 1, which had fed on the first or second day.

Reaction: The temperature showed a few slight rises after the injection of the mosquitoes, but otherwise remained within the normal limits during an observation period of 1½ months.

After the first injection, a slight rise up to 101.6° on the fourth day occurred. The second injection was followed by a rise up to 101.3° one day later. After the third injection of thirty-three specimens the temperature went up to 104.0° on the day of injection, but fell the next day down to 101.0°, and did not exceed 101.2° during the following ten days. The last two injections of 44 specimens followed one another directly. The temperature rose up to 102.0° a few days later, remained on the same level the next day, and then dropped again to normal. Except for these fluctuations, the temperature remained between 99.3° and 101.2° up to 28th January, 1932.

Immunity Test: On 29th January, 1932, the horse was injected with 5 c.c. O-virus (225th generation of 11th September, 1930). The first signs of fever began to develop two days later. The horse was killed four days p.i. and the post-mortem showed typical signs of the dunkop form of horsesickness (*vide* virus horse 9).

Result: The horse was thus susceptible to horsesickness and the experiment has to be looked upon as *negative*. The temperature reactions were not high enough to indicate even a very mild attack of horsesickness.

Experiment 8 (H.S. 13). Aedes caballus. Injection. Interval 15-16 days.
Horse 20291.

This horse was injected with twenty-eight specimens of *A. caballus* which had fed 15 or 16 days before on an infected horse. On 12th December, 1931, ten specimens of group 4, five of group 5, and thirteen of group 6 were injected. These mosquitoes had fed on a virus horse during the end of the first until the beginning of the third day of fever.

Result: The temperature of the horse remained normal, varying during the first month following the injection between 98.4° and 101.4°. The animal was kept under observation for a further 70 days. Between 26th January and 10th March, the temperature was somewhat irregular, rising on six days to above 102°, with 104.8° as maximum. On one occasion only did the temperature remain above 102° for two consecutive days, otherwise elevated temperatures were not maintained for longer than half a day at a time. On 23rd March, 1932, the horse was used in experiment 32 (Eshowe virus, *A. dentatus*, fed, 15 days and longer). No definite febrile reaction resulted.

Immunity Test: On 3rd June, 1 c.c. Eshowe virus was injected subcutaneously into this horse. The temperature commenced rising five days later and the horse died from dunkop four days later.

Result: The immunity test showed that the horse was normally susceptible to Eshowe virus. There is every reason to believe that it would have been susceptible to O-virus, as well as have been all horses tested from the same lot. As no typical fever reaction followed the injection of mosquitoes, the experiment has to be regarded as *negative*.

G. EXPERIMENTS WITH *Aedes lineatopennis*.

Two experiments, using 33 specimens, were carried out with this species.

Virus: O-virus (192nd and 225th generations). Virus horses 6, 7, and 8, and mosquito groups:—

Group 1.—Five specimens (reared from larvae) fed on virus horse 6 on 17th November, 1931. Second day of fever. Temperature 106°-102.6°. Used for experiment 9.

Group 2.—Thirteen specimens (reared from larvae) fed on virus horse 7 on 26th November. Second day of fever. Temperature 106°-105.4°. Used for experiment 10.

Group 3.—Thirty-three specimens (caught as adults) fed on virus horse 7 on 27th November. Third day of fever. Temperature 105.4°-107°. Used for experiment 10.

Group 4.—Three specimens (caught as adults) fed on virus horse 8 on 7th December. First day of fever. Temperature 103.6°. Used for experiment 10.

Group 5.—Five specimens (caught as adults) fed on virus horse 8 on 8th December. Second day of fever. Temperature 104.2°. Used for experiment 10.

Group 7.—One specimen (caught as adult) fed on same virus horse on 9th December. Third day of fever. Temperature 105°-106.4°. Used for experiment 10.

Experiment 9 (H.S. 8). 5 Aedes lineatopennis. Injection. Interval 5 days.
Horse 20270.

This horse was injected on 23rd November, 1931, with 5 *Aedes lineatopennis* (group 1), which had fed about 5 days previously on an infected horse during the second day of fever.

Result: The temperature of the horse remained normal (98.4°-101.4°) up to 1st December, when it was injected with *A. hirsutus* (fed on a horse, O-virus, 5 days previously, experiment 11; result: negative). On 20th February, 1932, the animal was injected with *Aedes* spp. (which had fed on a horse, O-virus after mosquito passage, 7-9 days previously, experiment 11; result: negative).

Immunity Test: On 19th March the horse was injected with Eshowe virus (1st generation), and died of horsesickness 5-6 days later.

Result: The experiment was therefore *negative*, as the horse proved to be susceptible.

Experiment 10 (H.S. 12). 28 Aedes lineatopennis. Injection.
Interval 5 days. Horse 20288.

Twenty *A. lineatopennis* (eleven of group 2 and nine of group 3) were injected into the horse on 12th December, and two days later three of group 4, four of group 5, and one of group 7.

Reaction: The temperature of the horse remained normal (99°-101°) for nearly three months, except for short rises directly after the injections of the mosquitoes, when the temperature reached 102·1° one afternoon.

On 9th March the horse was used for experiment 24, and was injected with *A. caballus* and *A. lineatopennis* which had fed on a horse (Eshowe virus) 7 days previously. This experiment was positive and proved that the horse was susceptible to horsesickness.

Result: The experiment was therefore *negative*.

H. EXPERIMENTS WITH *Aedes hirsutus*.

It was only possible to carry out one test, using 30 specimens, with this species.

Virus: O-virus (192nd and 225th generations). Virus horses 7 and 8, and mosquito groups:—

Group 2.—Seventeen specimens (reared from larvae) fed on virus horse 7 on 26th November, 1931, p.m. First and second day of fever. Temperature 102·8-106°. Used for experiment 11.

Group 3.—Ten specimens (reared from larvae) fed on the same virus horse from 26th November p.m. until the following morning. Second day of fever. Temperature 106°-105·4°. Used for experiment 11.

Group 4.—Fifteen specimens (reared from larvae) fed on virus horse 8 on December 9th. Third day of fever. Temperature 105°-106·4°. Used for experiment 11.

Experiment 11 (H.S. 10). 30 Aedes hirsutus. Injection.
Interval 5 days. Horse 20270.

Fifteen specimens (group 2) were injected into the horse on 1st December, 1931, five of group 3 the following day, and ten (group 4) on 14th December. They had fed on the virus horses during the first to third days of fever.

Reaction: The temperature varied between 97·4° and 101·6° up to 19th February, more than a month after the last injection. It thus remained normal. On 20th February, 1932, the horse was injected, in experiment 17, with 17 *Aedes* spp. which had fed on a horse (O-virus after mosquito passage) 7-8 days previously. There was no marked temperature reaction during that month.

Immunity Test: On 19th March the horse was injected intrajugularly with E-virus (1st generation). Three days later the temperature began to rise, and the horse died of horsesickness 5-6 days later.

Result: The horse thus proved to be susceptible, and the experiment must be regarded as *negative*.

I. EXPERIMENTS WITH *Aedes dentatus*.

Two experiments, using 18 specimens, were carried out with this species.

Virus: O-virus (192nd and 225th generation). Virus horse 7 and 8, and mosquito groups:—

Group 3.—Thirteen specimens (caught as adults) fed on virus horse 7 on 26th November p.m. First to second day of fever. Temperature 102·8°-106°. Used for experiment 12.

Group 4.—Three specimens (reared from larvae) fed on virus horse 8 on 7th December p.m. First day of fever. Temperature 103·6°. Used for experiment 13.

Group 5.—Twelve specimens (reared from larvae) fed on virus horse 8 on 9th December. Third day of fever. Temperature 105°-106·4°. Used for experiment 13.

*Experiment 12 (H.S. 11). 6 Aedes dentatus. Injection. Interval 5 days.
Horse 20271.*

This horse was injected on 2nd December, 1931, with 6 *A. dentatus* (group 3) which had fed 5 days previously on an infected horse.

Reaction: The temperature of the horse remained normal during the first 11 days p.i., showing a maximum of 101.4°. On 14th December the temperature rose to 103° and on the following seven days was: 100.4° and 102.3°, 101.3° (a.m.), 101 and 102°, 102° (a.m.), 98° (a.m.), 98° (a.m.), and 103° (a.m.). During this reaction the horse showed discharges from the nostrils and swelling of the glands, due to an attack of rhinitis. On 23rd December the temperature was again normal and remained below 101° for more than two months, i.e. up to 26th February, 1932.

On 27th February the horse was injected with *Aedes* spp. which had fed on a horse (O-virus after mosquito passage) 8-9 days previously (experiment 18). During an observation period of 40 days no reaction resulted. On 8th April the horse was used as a control in experiment 31 (Eshowe virus, *A. hirsutus*, feeding). No temperature reaction occurred during a period of more than a month.

The result of this experiment must be regarded as negative. We did not consider it necessary to test this animal for immunity, as all animals from the same source, so far as they were tested, proved to be susceptible.

*Experiment 13 (H.S. 9). 12 Aedes dentatus. Injection. Interval 16 days.
Horse 20280.*

Three specimens (group 4) were injected into this horse on 23rd December, 1931, and 9 (group 5) on the following day. The mosquitoes had fed about 16 days previously on an infected horse during the first and third days of the fever reaction.

Reaction: The horse did not show any fever reaction during the month following the last injection of the mosquitoes. The temperature remained below 101°, except on a few separate days. The highest temperature registered was 101.8°. The horse was kept under observation for a further 14 days up to 8th March. Three times during this period the temperature surpassed 102°, but only half a day in each case. The maximum temperature was 102.5°. On 9th March the horse was used for experiment 30 (Eshowe virus, *A. hirsutus*, injection). No temperature reaction occurred in this experiment either.

Immunity Test: The horse was injected with Eshowe virus on 27th May. Four days later the temperature commenced rising, and on the 8th day p.i. the horse died from dunkop horsesickness.

Result: The horse did not show any temperature after the injection of mosquitoes, but later proved to be susceptible to another strain of horsesickness. For reasons already stated in other experiments, we may regard the horse as susceptible to O-virus, and the experiment was therefore *negative*.

J. EXPERIMENTS WITH *Aedes vittatus*.

Two experiments, using 52 specimens, were carried out with this species.

Virus: O-virus (192nd generation). Virus horse 8, and mosquito groups:—

Group 1.—Thirty-one specimens (reared from larvae) fed on virus horse 8 on 7th December, 1931. First day of fever. Temperature 103.6° used for experiments 14 and 15.

Group 2.—Forty specimens (reared from larvae) fed on the same horse on 9th December. Third day of fever. Temperature 105°-106.4°. Used for experiments 14 and 15.

*Experiment 14 (H.S. 14). 20 Aedes vittatus. Injection. Interval 5 days.
Horse 20289.*

Ten specimens (group 1) were injected into the horse on 2nd December, and two days later ten more of group 2. The mosquitoes had fed on an infected horse during the first and third days of fever.

Reaction: The temperature remained normal (98.2°-101.6°) up to 8th February, 1932, nearly two months after the last injection of the mosquitoes, except on one occasion, about a month after the injections, when it rose to 103.4°, but dropped again the next day to 99.3°.

Immunity Test: On 8th February, 1932, the horse was used for experiment 16., and was injected with a large number of *Aedes* spp., 6 days after they had fed on an infected horse. This experiment was positive and proved the susceptibility of the horse for horsesickness.

Result: The experiment must, therefore, be regarded as *negative*.

Experiment 15 (H.S. 15). 32 *Aedes vittatus*. *Injection. Interval 15 days.*
Horse 20297.

Fifteen specimens (group 1) were injected into the horse on 23rd December and 17 (group 2) on the following day. These mosquitoes had fed on a virus horse approximately 15 days before during the first and second days of fever.

Reaction: The temperature of the horse remained normal, the highest temperature registered during the first month after the injection being 101°. The horse was kept under observation for almost a further two months, and during this period the temperature only reached 102° for half a day.

On 23rd March the horse was used for experiment 19 (Losperfontein virus, *A. caballus*, feeding). In this experiment also no fever reaction occurred.

Immunity Test: On 11th May, 1932, the horse was injected with 5 c.c. Losperfontein virus (from original case). After an incubation period of 7 days the temperature commenced to rise. The horse died 4 days later, the maximum temperature being 105°.

Result: The horse was thus susceptible to the Losperfontein strain of horsesickness and would very likely have reacted equally well to O-virus. The experiment has therefore to be regarded as *negative*.

K. EXPERIMENTS WITH LARGER NUMBERS OF DIFFERENT *Aedes* SPECIES.

In the last experiment of this series we injected a larger number of specimens of the three most important species of mosquitoes, *Aedes caballus*, *A. hirsutus* and *A. lineatopennis* simultaneously, hoping to obtain a positive result in this manner. The mosquitoes were injected one week after their having fed on the infected horse, thus giving the virus a certain time in which to multiply or develop.

Virus: 225th generation of O-virus. Virus horse 8, and mosquitoes:—

Aedes caballus, group 11. On 1st February a large number of newly hatched mosquitoes were brought into contact with virus horse 9 in a mosquito-proof tent, standing in the veld near the laboratories. Second to third day of fever. Temperature 106.1°-105.1°. The engorged mosquitoes were caught the following morning and kept in the warm room.

A. lineatopennis, group 6. Mosquitoes from the same source as *A. caballus*, group 11, and treated in the same manner.

A. hirsutus, group 5. A large number of hatched adults fed on the same virus horse at the same time as the other two groups.

Experiment 16 (H.S. 16). 294 *Aedes* spp. *Injection. Interval 6 days.*
Horse 20289.

The horse in this experiment had been used previously in experiment 14 *Aedes vittatus*, injection after 5 days), but it had not shown any temperature reaction during an observation period of 56 days. It was in poor condition and showed filling of the hind legs.

On 8th February, 1932, the horse was injected subcutaneously with 294 mosquitoes. 85 *Aedes caballus* (group 11), 115 *A. hirsutus* (group 5), and 94 *A. lineatopennis* (group 8). These mosquitoes had fed on the night of 1st to 2nd February, thus about 6½ days previously, during the first to third day of fever. The injections were made on both sides of the neck, and marked swellings developed at the sites of injection, no doubt due to the very large number of mosquitoes used, which naturally were not sterile.

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Reaction (see fig. 2): The temperature during the week preceding the injection was 99.0°-101.4°; on the day of injection 99° and 100.6°. The following day the temperature rose to 104.0° in the morning, in the afternoon to 104.4°, and on 10th February, the second day after injection, 102.0° and 103.0°, and on the 11th (third day a.m.) 101.6° was registered. The swellings at the sites of injection were still more marked the day after the injection, but a slight reduction was noticeable on the second day, followed, however, by further enlargement on the third day. The high rise of temperature shortly after the injections was undoubtedly due to a local reaction set up by the injected mosquitoes. When the swellings decreased the temperature also went down.

On 11th February p.m. (third day) the temperature rose from 101.6° up to 104.0°, reaching, on the 12th (fourth day) 105.4° and 105.8°, and on the 13th a.m. (fifth day) 105.3°. During the afternoon of the same day the temperature came down to 102.5°, and the horse died during the following night. The post-mortem was unfortunately not altogether clear cut as to the cause of death, owing to the advanced state of decomposition of the carcass.

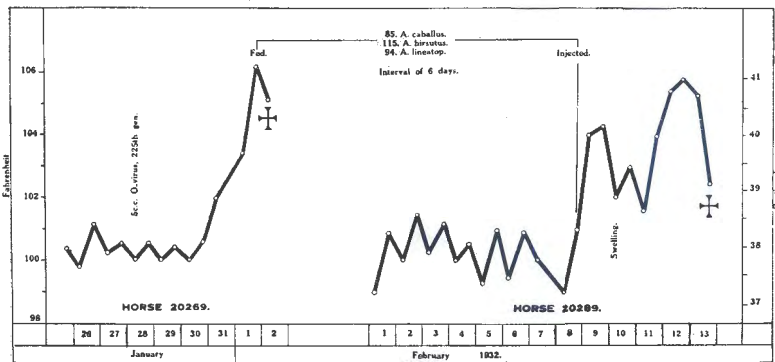


Fig. 2.

According to the course the reaction took, the indications pointed strongly to horsesickness being the cause of death. In order to establish this fact, another animal was subinoculated with blood from this horse.

Subinoculation: On 15th February horse 20259 was injected intrajugularly with 5 c.c. blood of horse 20289, bled on 12th February.

This new horse had been used before in experiment 4 (*Culex theileri*, injection after 25 days). During an observation period of more than 3 months it had not shown any significant rise in temperature. The temperature during the last week before the injection was 98.8° to 101.1°.

On the day after injection, 16th February, the temperature was 100.0° in the morning, but rose in the afternoon up to 104.5°. On the second day it was 105.0° and 105.4°, on the third day 105.7° and 106.6°, and on the fourth day 106.3° and 105.4°. The horse died during the night. The post-mortem showed the typical lesions of the dunkop form of horsesickness.

The very short incubation period of only 1½ days in this case was remarkable.

The result of the main experiment was thus positive as confirmed by subinoculation of blood.

L. RESULTS OF THE EXPERIMENTS WITH PURE O-VIRUS.

In the first preliminary experiment a positive result was obtained by the injection of five mosquitoes which had fed the night before on an infected horse 24 hours after the first rise of temperature, during a horsesickness reaction. At that time, therefore, the concentration of virus was so high, that it must have been taken up by at least one of the five mosquitoes. To determine exactly the

optimum range for transmission experiments more tests of this kind would have been of value, but they seemed to us not to be absolutely justified considering the costs involved. We considered ourselves as safe, by analogy with the results obtained in the Yellow Fever and Dengue work, by commencing the experiments after the first rise of temperature above 102.5° and finishing them after the third day of fever.

In these experiments, as in all the others of this series, the mosquitoes were crushed and injected subcutaneously into susceptible horses, not fed on them. We thought that this method would give better and quicker results in a preliminary investigation into the actual transmitters. A negative result would indicate that there was no virus present in any part of the insect's body, but a positive test naturally would need confirmation through feeding experiments.

As a rule we began with the injection of the mosquitoes 5 days after their feeding on an infected horse. Normally all the blood in the intestines of a fully engorged mosquito was digested after 2-3 days at the temperature they were kept at in the warm room.

The experiments with *Culex theileri*, which were carried out in September and October, when no other mosquitoes could be obtained, were planned mainly to develop the experimental technique. This species is as common in winter as in summer, and does not depend on rain as it breeds in permanent water. From an epidemiological point of view, therefore, it does not fit in with the transmission at all.

All experiments with *C. theileri* were negative. The number of specimens used was certainly sufficient. After five days 50 specimens were injected, after 16 days 50 and after 25 days 140, altogether 240 specimens. We therefore considered that *C. theileri* could be excluded from the experimental point of view as a transmitter of horsesickness. However, as will be seen later, this conclusion is not absolutely justified.

In the further experiments 5 species of *Aedes* were used, viz., *A. caballus*, *A. dentatus*, *A. hirsutus*, *A. lineatopennis* and *A. vittatus*. The entomological survey, undertaken under adverse climatic conditions, had indicated that these species had to be regarded as potential transmitters.

The results of the first set of experiments were certainly unexpected, all of them, as it happened, being negative. We used the following numbers of mosquitoes:—

- A. caballus* interval, 5 days, 94 specimens; 15 days, 28 specimens.
- A. dentatus*, interval 5 days, 6 specimens; 15 days, 12 specimens.
- A. hirsutus*, interval 5 days, 30 specimens.
- A. lineatopennis*, interval 5 days, 33 specimens.
- A. vittatus*, interval 5 days, 20 specimens; 15 days, 32 specimens.

Of these 5 species, 183 specimens were injected after 5 days and 72 after 15 days. This number is certainly not insignificant when we compare it with the high percentage of infections in Yellow Fever and Dengue.

What were the reasons for the negative results? Naturally, the first possibility to be considered is that the species used were not the natural transmitters. We have already laid stress upon the fact that the entomological survey pointed clearly to these species as the potential transmitters. Further, almost daily observations in the field after these unexpected results could not give us any other indication of importance.

Another possibility, already mentioned above was, that either the virus had lost its developmental capacity in mosquitoes, or that this was at least reduced to a certain extent. We used the 192nd and 225th generations of the O-virus strain which had been isolated more than 30 years previously from a natural case of horsesickness. It was, therefore, reasonable to suppose that the strain, never having been in contact with mosquitoes for such a long time, would have lost its normal capacity of developing in mosquitoes.

In considering the following facts the results are doubly strange. In contrast to the virus of yellow fever and dengue, the horsesickness virus, *in vitro*, is very resistant. It remains virulent in blood at ordinary room temperature for years and even in a putrefactive state its virulence is not easily reduced. In the mosquitoes, however, its virulence was completely destroyed after 5 days. If we had any reason to doubt the results of our entomological survey, we would have given up the further work with these *Aedes* species, regarding them either as non-transmitters or in any case not as transmitters of importance.

Undoubtedly the best thing we could do was to abandon the O-virus and take another strain derived from a fresh case of horsesickness. Every possible effort was, however, made in this direction for more than a month, without success. The result was merely a loss of valuable time.

We decided thereupon to make at least one more effort with the O-virus strain, this time injecting simultaneously a large number of mosquitoes into one horse after an interval of 6-7 days. Supposing that the normal developmental capacity of the virus in the mosquitoes was diminished, there would be a better chance of getting positive results when using a large number of insects. In fact, this experiment proved positive without any marked lengthening of the incubation period. We injected 85 *A. caballus*, 115 *A. hirsutus* and 94 *A. lineatopennis*, altogether 294 specimens, or nearly 40 specimens more than in all the previous experiments together. It was thus possible to keep the virus virulent in the bodies of mosquitoes for at least 6-7 days. We had hoped that this short passage of the strain would enable us to carry on with the work. In the following chapter the results of the further experiments with this strain will be described.

VI. EXPERIMENTS WITH O-VIRUS AFTER ONE PASSAGE THROUGH MOSQUITOES.

In the preceding chapter we have recorded one positive experiment obtained by the injection of about 300 mosquitoes belonging to three different species (experiment 16). The virus had remained in the mosquitoes for about 6-7 days. We hoped that this would constitute a beginning for the adaptation of the virus to live in the mosquito transmitters, and that by a series of similar short passages through mosquitoes the virus would regain its normal developmental capacity in these insects. We still believed that we were right in our choice of the natural invertebrate host of the virus.

As the results were not satisfactory, we conducted two experiments.

A. MOSQUITOES: EXPERIMENTAL TECHNIQUE AND VIRUS HORSES.

Mosquitoes.—For these experiments we chose mainly three species of *Aedes*, viz., *A. caballus*, *A. hirsutus* and *A. lineatopennis*. These we regarded as the most probable transmitters of horsesickness, on grounds stated in detail in one of the preceding chapters.

In the two experiments together we injected the following mosquitoes:—

Aedes caballus, 185 specimens; *A. lineatopennis*, 121 specimens.

A. dentatus, 11 specimens; *Aedes* spp., 3 specimens.

A. hirsutus, 37 specimens.

Most of these mosquitoes were reared from larvae; only a small percentage (31 specimens) were caught as adults.

Experimental Technique.—In the first experiment the mosquitoes were brought into contact with the infected horse in the same mosquito-proof tent we used in experiment 16 of the preceding chapter. Certainly, more than 1,000 specimens were liberated in this tent, but only 95 engorged themselves on the horse. A very large number died without taking any food. It was a dry day and night, and we are convinced that the low humidity was the reason for the bad results. We therefore never used this tent again.

For the next experiment we built another tent (described in the paper on technique) near one of the buildings of the laboratory, and during the feeding of the mosquitoes we had water running down from the roof to keep the air inside the tent sufficiently moist. The mosquitoes were again liberated in the tent, and the result was much better. Nearly 350 mosquitoes engorged themselves on the horse.

The engorged mosquitoes of the first experiment were kept in the warm room in small jars, those of the second partly in jars and partly in special cages surrounded by wet hessian. As virus horses we used horse 20289 from experiment 16 and horse 20259 which had been injected with blood of the preceding horse.

B. EXPERIMENTS.

Experiment 17 (H.S. 17). 57 *Aedes* spp. Injection. Interval 7-8 days.
Horse 20270.

The mosquitoes used in this experiment were fed on horse 20289 of experiment 16, which had reacted after an injection of crushed mosquitoes belonging to three different *Aedes* spp.

TRANSMISSION OF HORSESICKNESS AT ONDERSTEPSPOORT DURING 1931-32.

Mosquitoes: A. caballus, group 12. Fed on horse 20289 on 12th February. Five specimens engorged. First to second day of fever. Temperature 105.4°-105.8°. Mosquitoes caught as adults.

A. caballus, group 13. Fed on the same horse between 12th February p.m. and the next morning. Twenty-seven specimens engorged. Second day of fever. Temperature 105.8°-105.3°. Mosquitoes reared from larvae.

A. lineatopennis, group 9. Fed on the same horse during the night of 11th to 12th February. First day of fever. Temperature 104°-105.4°. Forty-one specimens engorged. Mosquito caught as adults.

A. hirsutus, group 6. Fed on the horse, together with *A. caballus*, group 13. Two specimens, reared from larvae, engorged.

The horse in this experiment had been used before in experiment 11 (*Aedes hirsutus*, injected after 5 days). During more than two months after the last injection of mosquitoes the temperature of the horse had remained normal (between 97.4° and 101.6°), and the result of the test had been negative.

On 20th February, 1932, the horse was injected with 4 *A. caballus*, group 12, twenty-four of group 13, with 2 *A. hirsutus* of group 6, and 27 *A. lineatopennis* of group 9, altogether with fifty-seven specimens. The mosquitoes had fed 7-8 days before on the infected horse during the first and second day of fever. There was a swelling at the site of injection, which decreased after a few days and disappeared totally later on.

Reaction: The day after the injection the temperature rose up to 102.6°, but fell the following morning to 99.6° and did not surpass 101.7° during the next 5 days. The horse was kept under observation for 28 days. During this period the temperature was never higher than 101.5°, ordinarily remaining below 100.5°. There was therefore no temperature reaction.

Immunity Test: On 19th March the horse was injected intrajugularly with 5 c.c. E-virus, 2nd generation (see virus horse 12). Three days later the temperature began to rise and after two more days the horse died.

Result: The horse died of horsesickness, and the experiment proved to be negative. The immunity test was actually made with another strain of virus, but nevertheless the horse may be regarded as having been susceptible to O-virus also, as all immunity tests carried out on other horses of the same lot of animals were successful.

Experiment 18 (H.S. 18). 300 Aedes spp. Injection. Interval 8-9 days.
Horse 20271.

The virus horse used for feeding the mosquitoes on was horse 20259 of experiment 16. It was inoculated with blood from horse 20289, which reacted after an injection of nearly 300 mosquitoes previously fed on a horse infected with O-virus.

The mosquitoes fed on this horse belonged to six different species, *A. caballus*, *A. dentatus*, *A. hirsutus*, *A. lineatopennis*, the common mosquitoes used regularly in these experiments, and *A. cumminsi* and *A. punctothoracis*, two species which are usually rare at Onderstepoort.

Mosquitoes: A. caballus, group 14. Fed on horse 20259 between 17th February and the next morning. Second day of fever. Temperature 105.4°-105.7°. Forty-three specimens (reared from larvae) engorged.

A. caballus, group 15. Fed on the same horse the following night. Third day of fever. Temperature 106.6°-106.3°. 133 Specimens (reared from larvae) engorged.

A. cumminsi, group 1. Fed on the same horse, together with *A. caballus*, group 14. One specimen engorged (reared from larva).

A. dentatus, group 7. Fed on the same horse, together with *A. caballus*, group 14. Eleven specimens engorged (reared from larvae).

A. hirsutus, group 7. Fed on the same horse, together with *A. caballus*, group 14. Seventeen specimens engorged (reared from larvae).

A. hirsutus, group 8. Fed on the same horse, together with *A. caballus*, group 15. Nineteen specimens engorged (reared from larvae).

A. lineatopennis, group 11. Fed on the same horse, together with *A. caballus*, group 14. Twenty-seven specimens engorged (reared from larvae).

A. lineatopennis, group 12. Fed on horse, together with *A. caballus*, group 15. Ninety-one specimens engorged (reared from larvae).

A. punctothoracis, groups 1 and 2. Group 1 fed with *A. caballus*, group 14; group 2 with 15. Two specimens engorged (reared in the laboratory).

The horse for this test had been used before in experiment 12 (*Aedes dentatus*, injection after 5 days). 12-19 days after the injection of the mosquito it had shown an irregular temperature reaction accompanied by discharges from the nostrils, due very probably to an ordinary rhinitis. It was kept under observation for nearly 3 months (87 days). The temperature only passed the 101° limit for one afternoon. The experiment had, therefore, to be regarded as negative.

On 27th February, 1932, the horse was injected subcutaneously with the following 300 mosquitoes:—

157 *A. caballus*: 31 of group 14, 126 of group 15.

1 *A. cumminsi*.

11 *A. dentatus*, group 7.

35 *A. hirsutus*: 16 of group 7, 19 of group 8.

94 *A. lineatopennis*: 22 of group 11, 72 of group 12.

2 *A. punctothoracis*: 1 of group 1, 1 of group 2.

These mosquitoes had fed on the infected horse 20259 on the second and third day of fever between 17th February p.m. and 19th a.m., thus 8-9 days previously.

Reaction: The day after the injection of the mosquitoes the temperature rose up to 103·2° in the morning, but fell to 101·2° in the afternoon. It was 101° the next morning, and rose again in the afternoon to 102·8°. The following day the temperature was nearly normal (100°-100·8°). The horse was kept under observation for 40 days, but no fever reaction followed, the temperature remaining between 98·3° and 101·8°, exceeding 101° only once for half a day.

On 8th April the horse was used for experiment 31 (Eshowe-virus, *Aedes hirsutus*, feeding, subinoculation). During an observation period of more than one month 101° was the highest temperature recorded.

Result: As no fever reaction resulted after the injection of the mosquitoes, the experiment has to be regarded as *negative*. An immunity test was not carried out on this horse. It could be omitted, as quite a number of horses of the same origin always proved to be susceptible when tested.

C. RESULTS OF EXPERIMENTS.

In the experiments described in the foregoing section we had our positive result after injection of a large number of mosquitoes belonging to different species of *Aedes* which had been injected about 6 days after their having fed on a horse experimentally infected with O-virus. On this horse and on other animals which had been injected with blood of the previous horse, we fed a large number of *Aedes* mosquitoes.

In two experiments these mosquitoes were injected after an interval of 7-8 and 8-9 days into two susceptible horses. Altogether we used 357 specimens, viz., 185 *A. caballus*, 121 *A. lineatopennis*, 37 *A. hirsutus*, 11 *A. dentatus*, 2 *A. punctothoracis* and 1 *A. cumminsi*. According to our mosquito survey the first three species had to be regarded as very promising possible transmitters. Both experiments were negative, proving that after a maximum interval of 9 days there was no virulent virus left in any of the mosquitoes.

Our hope, that through a relatively short sojourn in mosquitoes, the virus would regain at least part of its normal developmental capacity, was not fulfilled. It is possible that, if still larger numbers of mosquitoes were used, positive results could be obtained, but the chances of modifying the virus successfully by this method do not seem to be very great. We therefore ended these attempts, as we had obtained two new strains of horsesickness in the meantime which seemed to be suitable for our experiments.

VII. EXPERIMENTS WITH LOSPERFONTEIN VIRUS.

At the commencement of March, 1932, we obtained fresh material from a case of horsesickness which had occurred in a mule at Losperfontein, near Brits (Transvaal), it being a typical case of the "Dikkop" form of the disease. We were unable to trace the history of this case with certainty, but it was ascertained that most or all of the mules at Losperfontein, which is a Government Irrigation Settlement, had been immunized against horsesickness in December, 1931, by means of the Onderstepoort O-virus strain. A certain number of deaths had occurred following upon the immunization and throughout February, 1932, a few further horsesickness deaths were encountered, of which the above-mentioned mule formed one, from which our material had been obtained. As certain indications pointed to this being a naturally contracted case of horsesickness, we injected one of our horses with this material and used it for feeding mosquitoes on. Except for a somewhat longer incubation period the course of the disease closely resembled a typical O-virus infection.

For reasons outlined below, we did not regard this strain as very suitable for our purpose and, therefore, injected only one horse with it.

A. VIRUS HORSE.

The following horse was injected with this strain for the purpose of feeding mosquitoes:—

Virus horse 10 (= horse 20262). Injected on 4th March, 1932, intrajugularly with 5 c.c. blood taken the previous day from a mule at Losperfontein, showing typical symptoms of dikkop.

[This virus horse had been used in October, 1931, for experiment 6 (O-virus, *Anopheles squamosus*, injection), but had not shown any fever reaction.]

After injection the temperature remained normal for the four days up to 8th March. On 9th March the temperature was 100.2° (a.m.) and 103.7° (p.m.), the 10th 102.5° and 103.0°, the 11th 104.6° and 106.3°, the 12th 105.3° and 106.6°, and on the 13th 106.0° (a.m.). The same day the horse died, the post-mortem showing typical symptoms of dunkop.

B. MOSQUITOES AND EXPERIMENTAL TECHNIQUE.

Mosquitoes.—At the beginning of March, 1932, when the experiments with the Losperfontein strain were being conducted, *Aedes caballus* was present in large numbers. They were hatching out in the laboratory from larvae caught in the field and were also present as adults in their breeding grounds. *Aedes lineatopennis* could be

obtained from the same source but were not present in such large numbers. Besides these two species, *A. dentatus* and *A. vittatus*, hatched from captured larvae, were available, the former species in sufficiently large numbers but the latter being represented by only a few specimens.

We were able to feed the following mosquitoes on the virus horse:—

<i>Aedes caballus</i>	816 specimens.
<i>Aedes lineatopennis</i>	151 specimens.
<i>Aedes dentatus</i>	61 specimens.
<i>Aedes vittatus</i>	7 specimens.

From the epidemiological point of view the former two species had to be regarded as the more probable natural transmitters.

Experimental Technique.—During the course of these experiments the finally adopted technique was already being employed as far as possible. The mosquitoes were fed on the virus horse in the mosquito tent, which was kept wet by two showers running all night throughout the duration of the experiments. The special stable was still under construction at that time, but the saddle for keeping the small mosquito cages in position, described elsewhere, had already been completed.

When the time arrived for the mosquitoes to be fed on the susceptible horses the special stable had been completed and we used it in preference to the mosquito tent.

A. caballus was, as a rule, difficult to keep alive in the laboratory for long periods; especially was this the case with the fertilized specimens caught as adults, large numbers of which died after oviposition. We were, furthermore, exceptionally unlucky at this time on account of our losing hundreds of specimens as a result of an unforeseen invasion of ants into the cages. Of more than 800 specimens only 100 were left after two weeks, and at the end of the following week this number had been reduced to not more than about 10.

In the case of *Aedes lineatopennis* the results were much better. Of 151 specimens which had fed on the virus horse, 71 or nearly 50 per cent. were alive after 19-21 days, 38 specimens after slightly more than a month (33-35 days), and 11 specimens were still present after as long an interval as 60-62 days. This may certainly be regarded as satisfactory.

The original material of *A. dentatus* consisted of 61 specimens, of which 22 specimens survived after 25-27 days, and 6 after 35-37 days.

Only 7 specimens of *A. vittatus* had fed on the virus horse and these proved to be very resistant to the experimental conditions.

Only in the case of *A. caballus* were the feeding results not very satisfactory and this could be attributed to the horse, which was in rather poor condition, having an extremely hollow back, which resulted in the under surface of the cages not coming into proper contact with skin and, therefore, not allowing of the mosquitoes getting at the animal properly.

In the experiments with *A. lineatopennis*, for example, 51 out of 71 fed after an interval of 19-21 days, 33 out of 42 after 25-27 days, 33 out of 38 after 33-35 days, and 10 out of 11 specimens after 60-62 days. In the case of *A. dentatus* 20 out of 22 specimens fed after 25-27 days.

C. EXPERIMENTS WITH *Aedes caballus*.

We succeeded in feeding more than 800 specimens of *A. caballus* on the virus horse but, owing to a large mortality already referred to above, only 100 specimens remained at the end of a fortnight, of which about 70 could be induced to feed again on our experimental horse. The results must, therefore, be regarded as fairly satisfactory.

Virus horse 10 was used, and mosquito groups:—

Group 20.—Fed on virus horse 10 during the night of 9th to 10th March, 1932. First day of fever. Temperature 103·7°-102·5°. 181 specimens engorged (reared from larvae). Used for experiment 19.

Group 21.—Fed together with group 20. 26 specimens engorged (caught as adults). Used for experiment 19.

Group 22.—Fed on the same virus horse during the following night (10th-11th March). Second day of fever. Temperature 103·0°-104·6°. 156 specimens engorged (reared from larvae). Used for experiment 19.

Group 23.—Fed together with group 22. 242 specimens engorged (caught as adults). Used for experiment 19.

Group 25.—Fed on the same virus horse one night later (11th to 12th March). Third day of fever. Temperature 106·3°-105·3°. 211 specimens engorged (caught as adults). Used for experiment 19.

Experiment 19 (H.S. 24). Aedes caballus. Feeding. Interval 14-20 days.

Horse 20297.

This horse had been used about 4 months previously in experiment 5 (O-virus, *Aedes vittatus*, injection, interval 15 days), but had shown no temperature reaction following the injection of mosquitoes.

On 23rd March, 1932, the combined groups 20 and 21 of *A. caballus* were put on to the horse, and during the night 33 (out of 44) specimens engorged themselves. The next day the unfed mosquitoes of these groups were allowed to feed again during the daytime and five more specimens took up blood. During the following night (24th to 25th March) groups 22 and 23 were allowed to feed, and 27 (out of 42) specimens engorged themselves. On 26th March six (out of 19) specimens of group 25 fed. On the night of 29th to 30th March the remainder of group 20 and 21 were put on to the horse again and six (out of 10) specimens fed.

In all, the horse was bitten 77 times by mosquitoes as follows:—

- 33 specimens (group 20 and 21) after an interval of 14 days.
- 5 specimens (group 20 and 21) after an interval of 15 days.
- 27 specimens (group 22 and 23) after an interval of 14 days.
- 6 specimens (group 25) after an interval of 14 days.
- 6 specimens (group 20 and 21) after an interval of 20 days.

Or, in other words, 71 specimens after 14-15 days and by six after 20 days. These mosquitoes had fed on the virus horse during the first to third day of the fever reaction.

Reaction: The horse was kept under observation until 10th April, i.e. 42 days after the last group of mosquitoes had fed. It showed no fever reaction whatsoever the highest temperature recorded during this period being 101°.

Immunity Test: On 11th April the horse was injected intrajugularly with 5 c.c. blood of the original Losperfontein virus. The temperature remained normal for 5 days, up to 16th April, when it was followed on the ensuing day by a slight rise up to 101°. Only the day after did the definite fever reaction commence, temperatures of 100·6° and 103·8° being recorded on 18th April and 103·2° and 104·2° on the 19th. The following night, 8-9 days p.i., the horse died.

Result: This experiment must be regarded as negative. No temperature reaction followed the feeding by the mosquitoes, but after the intrajugular injection of the same strain of virus the horse succumbed to horsesickness within 9 days. The incubation period of 6 days in this case was relatively long, especially when compared with that following an injection of the O-virus strain, but this prolongation cannot be regarded as an indication of a slight immunity, as we experienced a similar long incubation period (5 days) in the case of the virus horse injected with the same material.

D. EXPERIMENTS WITH *Aedes lineatopennis*.

Of *A. lineatopennis* a smaller number than was the case with *A. caballus* was available for feeding on the virus horse, viz., only about 150 specimens. On the other hand, we were more successful in keeping them alive as, after 20 days 71 specimens survived and after more than two months 11 specimens were still living.

Virus horse 10 was used, and the following mosquito groups:—

Group 17.—Fed on virus horse 10 during the night of 9th-10th March, 1932. First day of fever. Temperature 103·7° and 102·5°. 26 specimens engorged (reared from larvae). Used for experiments 20 and 21.

Group 18.—Fed together with group 17. 69 specimens (caught as adults) engorged. Used for experiments 20 and 21.

Group 19.—Fed on the same virus horse during the following night (10th-11th). Second day of fever. Temperature 103·0°-104·6°. 22 specimens engorged (reared from larvae). Used for experiments 20 and 21.

Group 20.—Fed on the same virus horse one night later (11th-12th March). Third day of fever. Temperature 106·3° and 105·3°. 10 specimens engorged (caught as adults). Used for experiments 20 and 21.

Group 21.—Fed together with group 20. 11 Specimens engorged (reared from larvae). Used for experiments 20 and 21.

Experiment 20 (H.S. 22). Aedes lineatopennis. Feeding. Interval 13-62 days. Horse 20276.

On 22nd March, 1932, the mosquitoes of group 18 were put on to this horse for feeding and 35 (out of 55) specimens engorged themselves during the night. The unfed specimens of this group were allowed to feed again on 26th March, during the day and 3 (out of 8) specimens engorged themselves. On 30th March the remaining mosquitoes of the combined groups 17 to 21 were put on to the horse and during the night, of the 71 specimens, 50 fed. The same mosquitoes were fed again during the night of 6th to 7th April and 33 (out of 42) took up blood. Eight days later, on 13th April, the 38 mosquitoes still alive were fed and once more 33 specimens engorged themselves during the ensuing night. Almost one month later we still had 11 specimens, and of these, 10 fed for the last time during the night of 10th to 11th May.

In all, this horse was bitten 165 times by the mosquitoes as follows:—

- 35 specimens (group 18) after an interval of 13 days;
- 3 specimens (group 18) after an interval of 17 days;
- 51 specimens (group 17-21) after an interval of 19-21 days;
- 33 specimens (group 17-21) after an interval of 25-27 days;
- 33 specimens (group 17-21) after an interval of 33-35 days;
- 10 specimens (group 17-21) after an interval of 60-62 days;

or in other words by 38 specimens after 13-17 days, 51 after 19-21 days, 66 after 25-35 days, and by 10 after 60-62 days. These mosquitoes had all fed on the virus horse during the first to the third day of fever.

Reaction: After the first feed of the mosquitoes on 22nd March the temperature, to start with, remained normal for 22 days, up to 13th April, the maximum being 101°. On 14th April the temperature rose suddenly from 99° in the morning to 103·2° in the afternoon. The following day, however, it returned to normal, where it remained for the succeeding 2 days. During the next 4 days irregular temperatures were recorded, viz., on the 18th, 98 and 101°, the 19th, 98·4 and 100·5°, the 20th, 98·6 and 102°, and on the 21st, 98·4 and 101·6°. Thereafter the temperature remained normal (below 100·5°) for 42 days, up to 3rd June, 23 days after the feeding of the last group of mosquitoes.

Immunity test: On 3rd June the horse received 1 c.c. blood of the original Losperfontein case subcutaneously. Up to 17th June, 14 days after the injection, no fever reaction occurred, the highest temperature recorded being 100·8°.

On 17th June the horse received a further injection of the same material, but this time 2 c.c. intrajugularly. The temperature remained normal for 6 days following the second injection (maximum 100°). On the 7th day, 24th June, temperatures of 99 and 101° were recorded, the 25th 101 and 102°, the 26th (a.m.) 103·6°, and the 27th 104·4 and 104·2°. The horse was then transferred to another experiment wherein it received an intravenous injection of mercurochrome. It died the following day with typical symptoms of dunkop.

Result: There seems little doubt but that this experiment must be regarded as *negative*. Notwithstanding the fact that this horse was bitten 165 times by mosquitoes of different groups, no definite fever reaction occurred. Except for the one brief rise to 103·2° the temperature was slightly higher than normal only between 18th and 21st April (maximum 102°) and this was not sufficient to justify its being regarded as a weak reaction. The result of the immunity test was undoubtedly unusual. The first injection of 1 c.c. of infected blood, performed subcutaneously, produced no result, at any rate during the observation period of 14 days, which was perhaps somewhat short. The following injection of 2 c.c. of blood intrajugularly was followed by the death of the horse, the incubation period of the disease of 7-8 days being relatively long. It is not impossible that the horse possessed a slight natural immunity or an immunity acquired following the mosquito bites and that this immunity was sufficient to protect it against a subcutaneous injection of 1 c.c. of virus, but was subsequently broken down by the intravenous injection of 2 c.c. of virus, only the lengthened incubation period being indicative of this initial immunity. However, the observation period following the subcutaneous injection was unfortunately somewhat short and, moreover, there may be other explanations, e.g. failure of the injection with stored blood to set up the disease which is occasionally met with, to account for the apparent failure of this initial injection, so that we, therefore, prefer, as already stated, to regard this experiment as *negative*.

Experiment 21 (H.S. 36). 6 Aedes lineatopennis. Injection. Interval 63-65 days. Horse 20236.

In the preceding experiment it was noted that of the combined groups 17-21 of *Aedes lineatopennis* 11 specimens were still alive more than two months after their original feed on an infected virus horse. Of these mosquitoes 10 specimens fed for the last time during the night of 10th to 11th May.

On 13th May, 1932, there were still 6 specimens of these groups alive. These were emulsified in normal horse serum and injected into horse 20236. On the date of injection 63 to 65 days had elapsed since these mosquitoes had had their initial feed on the infected virus horse.

Reaction: The morning following the injection the temperature rose to 102° but regained normal the next day. The horse was kept under observation for 31 days, up to 13th June, but no typical fever reaction resulted throughout this period. On ~~two~~ occasions only did the temperature exceed 101° but these fluctuations were not maintained for more than 24 hours in each case.

Result: The experiment must be regarded as *negative* as the injection of the mosquitoes was not followed by a temperature reaction. The immunity test of this horse by virus injection was not considered to be necessary.

E. EXPERIMENTS WITH *Aedes dentatus*.

In addition to the *Aedes caballus* and *A. lineatopennis*, mentioned above, 61 specimens of *A. dentatus* were fed on the virus horse infected with the Losperfontein strain virus and used in the previous experiments. The mortality amongst these mosquitoes was not too high, one third of the original number surviving after 25-27 days. For this experiment the same virus horse was used, viz.:—

Virus horse 10 and mosquito groups:—

Group 11.—Fed on virus horse 10 during the night of 9th to 10th March, 1932. First day of fever. Temperature 103.7-102.5°. 21 specimens engorged (reared from larvae). Used for experiment 22.

Group 12.—Fed on the same virus horse during the following night (10th-11th March). Second day of fever. Temperature 103.0-104.6°. 28 specimens engorged (reared from larvae). Used for experiment 22.

Group 13.—Fed on the same virus horse one night later (11th-12th March). Third day of fever. Temperature 106.3-105.3°. 12 specimens engorged (reared from larvae). Used for experiment 22.

Experiment 22 (H.S. 28). Aedes dentatus. Feeding. Interval 25-37 days.
Horse 20314.

On 5th April, 1932, the combined groups 11 to 13 of *Aedes dentatus* were put on to the horse, and during the following night 20 (out of 22) specimens engorged themselves. The remaining mosquitoes of the same groups were fed again on the same horse 10 days later, during the night of 15th-16th April, and 6 specimens took up blood.

The horse was therefore bitten 26 times by the mosquitoes as follows:—

20 specimens (group 11-13) after an interval of 25-27 days.

6 specimens (group 11-13) after an interval of 35-37 days.

These mosquitoes had had their initial feed on a virus horse during the first to third day of fever.

Reaction: The horse was kept under observation up to 16th May, i.e. one month after the last feeding of the mosquitoes. The temperature exceeded 101° on each of the two days on which the mosquitoes fed, viz., on 9th and 19th April, but was not maintained for longer than half a day in each case, and the highest temperature registered was only 101.4°. Apart from these small fluctuations the temperature remained between 98 and 101°.

Result: The experiment has to be regarded as *negative*, as no temperature reaction of any consequence followed the feeding of the mosquitoes during an observation period of one month. In this case as well, the horse was not tested for immunity.

F. EXPERIMENTS WITH *Aedes vittatus*.

A. vittatus was only obtainable by us in very small numbers at the time of these experiments. Not more than 7 specimens fed on the virus horse but the mortality amongst them was low and 4 mosquitoes were still alive at the end of one month.

In this experiment we used virus horse 10 and mosquito group:—

Group 3.—Fed on virus horse 10 during the night of 9th-10th March, 1932. First day of fever. Temperature 103.7-102.5°. 7 specimens engorged (reared from larvae). Used for experiment 23.

Experiment 23 (H.S. 26). Aedes vittatus. Feeding. Interval 23-37 days.
Horse 20298.

On 1st April, 1932, *Aedes vittatus* group 3 was put on to the horse of this experiment, 5 specimens taking a blood feed during the night. The same mosquitoes were fed again on this horse during the night of 15th-16th April, when 4 specimens engorged themselves.

The horse was bitten therefore 9 times, viz., by 5 specimens after an interval of 23 days, 4 specimens after an interval of 37 days. The mosquitoes had fed on the virus horse during the first day of its fever reaction.

Reaction: The horse was kept under observation until 16th May, i.e. one month after the feeding of the last mosquitoes. During this period only once, viz., on the day following the second feed of the mosquitoes, was there a rise in temperature exceeding 101°, when 101.5° was recorded, but this was not maintained for longer than half a day. With the exception of this insignificant elevation the temperature varied between 98.4 and 100.9°.

Result: As no fever reaction whatsoever followed the feeding of the mosquitoes, the experiment must be regarded as *negative*. The immunity test was omitted in this case, as in the preceding experiment.

G. DISCUSSION OF THE RESULTS OBTAINED WITH LOSPERFONTEIN VIRUS.

On a horse infected by means of a blood inoculation from a mule at Losperfontein showing typical symptoms of dikkop, 816 *Aedes caballus*, 151 *A. lineatopennis*, 61 *A. dentatus* and 7 *A. vittatus* were fed. These mosquitoes were kept in a room during the experiment in which the temperature varied between 20 and 30° C. with a probable mean of 24° C. They were fed on susceptible horses commencing at 13-14 days after their initial feed and this feeding was continued at intervals, in some cases up to more than two months. Injection of emulsified mosquitoes was only exceptionally employed in this series of experiments.

The results of these experiments were as follows:—

Aedes caballus (experiment 19). Interval 14-15 days: 71 specimens. Interval 20 days: 6 specimens. Result negative.

Aedes lineatopennis (experiment 20):

Interval 13-17 days: 38 specimens.

Interval 19-21 days: 51 specimens.

Interval 25-27 days: 33 specimens.

Interval 33-35 days: 33 specimens.

Interval 60-62 days: 10 specimens.

TOTAL, 165 specimens.

Result: Negative.

Aedes lineatopennis (experiment 21) injection.

Interval 63-65 days: 6 specimens.

Result: Negative.

Aedes dentatus (experiment 22).

Interval 25-27 days: 20 specimens.

Interval 35-37 days: 6 specimens.

Result: Negative.

Aedes vittatus (experiment 23).

Interval 23 days: 5 specimens.

Interval 37 days: 4 specimens.

Result: Negative.

The four horses together were bitten 277 times by the four *Aedes* species after the following intervals:—

Interval 13-17 days: 109 specimens.

Interval 19-21 days: 57 specimens.

Interval 23-27 days: 58 specimens.

Interval 33-37 days: 43 specimens.

Interval 60-62 days: 10 specimens.

In none of these experiments were positive results or fever reactions, which might be suspected of being possible reactions, obtained. In the first two experiments the horses were tested for immunity by injection of the same strain. In the first case the injection was performed intrajugularly and followed in almost the normal time by the death of the horse with typical symptoms. In the second case a subcutaneous injection of 1 c.c. of blood containing virus was apparently a failure, but a second, and in this case intrajugular injection, produced a fatal infection after a somewhat prolonged incubation period. However, we regard this horse also as being normally susceptible, the reasons for which were given under the discussion of the results of the experiment in question. The other three horses were not tested for immunity, this not being regarded as absolutely necessary in view of the fact that the other horses from the same lot tested in various experiments proved to be susceptible.

What was the cause of the failure of these experiments? There is first of all naturally the possibility that none of the mosquito species used was the actual transmitter. We have already, on several occasions stated why these species were chosen for our experiments.

The numbers of mosquitoes used may have been insufficient. This, however, does not appear to be the case when the data of our experiments is taken into consideration, and it is furthermore improbable that the natural transmitter would have a very low infection index.

The time allowed for the extrinsic incubation period may have been insufficient, but, in our experiments, in the case of quite a number of mosquitoes used for feeding at any rate, it was certainly more than sufficient as the horses were bitten more than 100 times by mosquitoes which had fed more than three weeks previously, and 10 times where the interval exceeded two months.

The temperature at which the mosquitoes were kept is also considered to have been high enough for a normal development of the virus in the mosquitoes. Neither in other questions of minor importance could a reason for the failure of these experiments be found. One important possibility, however, still remains, as already pointed out in the introductory chapter, viz., that the Losperfontein virus, derived from a mule previously immunized with the O-virus strain, was nothing else than O-virus. We will leave the question of the possibility of a relapse in horsesickness out of the discussion at this stage and only lay stress on the fact that there existed a very striking resemblance between the fever reactions set up by our Losperfontein strain and the O-virus used in the immunization of the mules. The strain was obtained in a place where quite a large number of animals had been injected previously with O-virus and should the two strains be identical the objections against the use of O-virus for transmission experiments, discussed fully in one of the previous chapters, would apply equally well in the case of Losperfontein virus.

VIII. EXPERIMENTS WITH ESHOWE-VIRUS.

Towards the end of February, 1932, almost at the same time that the Losperfontein strain was obtained, Mr. Franz, the Government Veterinary Officer of Eshowe, Zululand, sent us blood from a mule, which had died from horsesickness. We are very much indebted to Mr. Franz for the trouble he took in forwarding us a fresh strain from his area.

The mule from which the strain was derived, referred to here as the Eshowe strain, suffered from a typical attack of "dikkop", followed by recovery. It had been immunized in August, 1931, that is about 6 months previously, with the O-virus strain always used for this purpose.

The first horse (20302) injected with this strain showed a course of infection somewhat different from that of the ordinary O-virus type; a longer incubation period and a fever more of the remittent type. By the injection of mosquitoes fed on this animal into a susceptible horse (20288) an infection was produced which, except for a longer incubation period, to be expected, resembled more closely the O-virus course. Besides these two horses 9 more were injected with the Eshowe-virus, 3 as virus horses, 5 for testing the immunity, and 1 for a reason outside our work.

In the case of the three virus horses, viz., 20270, 20299 and 20310, the first showed an acute infection commencing two days after injection with death on the fifth day, of mixed horsesickness. In the second case a reaction set in on the second day after injection with death on the afternoon of the seventh day of mixed horsesickness. The third animal commenced reacting on the afternoon of the fifth day after injection with death on the tenth day, again in this case of mixed horsesickness. The 5 horses of which the immunity was tested by means of injections of Eshowe-virus all reacted positively and died of acute horsesickness. These horses were: 20300, 20315, 20291, 20280, 20130. The incubation periods varied between 4 and 5 days, and the duration of the reactions between 3 and 4 days, three of the horses dying from the "dikkop" and 2 from the "dunkop" form of the disease. The clinical picture of the reactions in the above 8 horses is undoubtedly somewhat different from what is experienced in the case of O-virus where after an incubation period of 2 to 3 days and a duration of an additional 2 days horses almost invariably succumb to the "dunkop" form of the disease. However, in comparing these reactions with those set up by most "field" viruses distinct differences are noticeable. Whereas the incubation period of the "field" virus varies as a rule between 6 to 8 days and the peak period of the reaction is reached in 3 to 4 days by a series of remissions, the above reactions apart from a slightly increased incubation period incline more closely to the O-virus type of reaction with a rapid rise to the height of the reaction followed by virtual collapse. This strain was not what we really wanted as it was derived from an animal previously immunized, as was also the case with the Losperfontein strain. We therefore still had to wait for a genuine natural strain which, however, was not obtained until May, when there was scarcely any opportunity left of conducting any more

experiments. In the meantime we completed as many experiments as possible with the Eshowe-strain which we regarded as the best strain we had at our disposal, as it showed at any rate some difference from the O-virus.

In all, 9 experiments were made, in which *Aedes caballus*, *A. lineatopennis*, *A. hirsutus* and *A. dentatus* were used. 280 specimens were injected after an interval of 7-30 days and 379 mosquitoes induced to feed after an interval of 15-22 days on the susceptible horses.

A. VIRUS HORSES.

In the experiments with the Eshowe strain 4 horses were used for feeding mosquitoes on; one injected with the original material, 2 with the first and one with the second laboratory generation.

Virus horse 11 (horse 20302).—Injected on 25th February, 1932, intrajugularly with 5 c.c. blood of a natural case of horsesickness in an immunized mule at Eshowe.

Result: Temperature normal up to 29th February p.m. Temperature on 1st March, 101.2 and 103.0°; the 2nd, 102.0 and 104.8°; the 3rd, 103.2 and 105.5°; the 4th, 103.9 and 103.3°; and the 5th (a.m.), 99.2°. The horse died during the same day. Post-mortem diagnosis: Horsesickness, dunkop. It was an acute infection with a relatively long incubation period of 5 days.

Virus horse 12 (horse 20288).—Infected in experiment 24 by injection of mosquitoes (for temperature see under experiment 24).

Virus horse 13 (horse 20276).—Injected on 19th March, 1932, intrajugularly with 5 c.c. blood of virus horse 11.

Horse 13 had been used one month before in experiment 17 (O-virus, *Aedes* spp., injection, interval 7-8 days), with negative results.

Result: Temperature normal up to 21st March p.m. Temperature on 22nd March, 100.6 and 103.1°; the 23rd, 99.5 and 104.5°; and the 24th, 103.0° (a.m.). The horse died during the following night. Post-mortem diagnosis: Horsesickness, mixed. The course of the disease was very acute, resulting in death within 5 days p.i., with the temperature relatively low. The incubation period was only 2 days in this case.

Virus horse 14 (horse 20299).—Injected on 16th April intrajugularly with 5 c.c. blood of virus horse 11.

Result: Temperature normal for two days p.i.: on 19th April, 100 and 103.2°; the 20th, 101.0 and 102.0°; the 21st, 102.0 and 101.4°; the 22nd, 101.7 and 103.4°; the 23rd, 103.0 and 104.6°. The horse died during the following night. Post-mortem diagnosis: Horsesickness, mixed. The case was acute with an incubation period of 3 days.

Virus horse 15 (horse 20310).—Injected on 18th April with 10 c.c. serum of virus horse 12, which had been cataphorized by the passage of an electrical current of 9 milliamperes at 250 volts for 4 hours.

Result: Temperature rose to 104° on the day of injection, but returned to normal the next day. On 23rd April, 101.2° was registered. This was maintained on the 24th. The 25th, 102.6 and 104° were recorded; the 26th, 104.6 and 105°; the 27th, 104 and 106°. On the 28th the temperature dropped to 103° and the horse died during the day, the post-mortem findings revealing the mixed form of horsesickness. Acute horsesickness with an incubation period of 6 days had resulted. It is doubtful whether the passage of the electrical current had any effect upon the potency of the virus and the somewhat lengthened incubation period is therefore somewhat difficult to account for.

B. MOSQUITOES AND EXPERIMENTAL TECHNIQUE.

Mosquitoes.—From the beginning of March, when these experiments were commenced, till the end of April, notwithstanding the dry season, *Aedes caballus* could be obtained in quite large numbers, either as larvae or adults, by artificial flooding of their breeding

places. *Aedes lineatopennis*, the species we were most anxious to test thoroughly, was still present in sufficient numbers at the beginning of March. They then, however, became more rare, being outnumbered in their breeding places by *A. caballus*. By the end of March our supply was practically exhausted. During the first experiment with this strain *Aedes hirsutus* was fortunately hatching out in large numbers from one of the breeding places, but thereafter this species also became too scarce for experimental purposes. *Aedes dentatus* was never really common during the period we were working with this strain, but a fairly regular supply could at any rate always be obtained.

On the virus horse the following mosquitoes were fed:—

<i>Aedes caballus</i>	1,068 specimens.
<i>Aedes lineatopennis</i>	272 specimens.
<i>Aedes hirsutus</i>	211 specimens.
<i>Aedes dentatus</i>	129 specimens.

In our opinion, as arrived at during the mosquito survey, the probable transmitters of horsesickness had to be looked for amongst the three first-named species.

Experimental Technique.—The technique described in the second paper as that finally adopted, was already in use as far as possible from the commencement of these experiments.

The fresh mosquitoes were fed on virus horses 11, 13 and 15 in the mosquito tent, the provisional arrangement for feeding mosquitoes, whereas in the case of virus horses 12 and 14 the specially constructed stable was used.

For feeding the mosquitoes on susceptible horses our special stable was used for preference and the tent only resorted to when the stable was already in use for another experiment.

After their blood-meal on one of the virus horses the mosquitoes were kept in our warm room, *A. caballus*, *A. lineatopennis*, and *A. hirsutus* in the larger cages covered with wet hessian, and *A. dentatus* mainly in small jars.

Aedes caballus was again the most sensitive to experimental conditions, showing the highest mortality of all the species. Out of 132 specimens used for injection about 50 per cent. were alive one week after the first feeding, and out of more than 900 specimens, which were used in feeding experiments, only about 10 per cent. survived 15-21 days. The highest mortality occurred as usual at the end of the first week after the initial feeding when eggs were deposited, although on this occasion most of the groups consisted of specimens reared from larvae, eggs were frequently laid. Females and males had hatched out together in the large cages, and before being used had remained there together for some time during which copulation must have taken place.

The results with *A. lineatopennis* after longer intervals were as usual better than with the former species. Out of 163 specimens used for injection 66 (or 10 per cent.) were alive after one week, showing, therefore, a mortality slightly higher than in the case of *A. caballus*. However, out of 109 engorged females from the feeding

experiment 49 specimens, or nearly 50 per cent., were alive 16-18 days after their initial feeding. This species does not seem to be affected to the same extent by artificial conditions as *A. caballus*.

Aedes hirsutus once more proved to be very resistant to the experimental conditions under which they were kept. In fact, it was the easiest species to handle. Out of a total of 311 specimens 100 were injected after 7 days. Of the remaining 211 specimens 107 were alive at the end of 20-21 days, and 46 after 29-30 days, thus giving results that may be looked upon as very satisfactory. In this case copulation had not taken place.

With *Aedes dentatus* the results were quite good, although not as good as with *A. hirsutus*. Originally 129 specimens had fed and of these nearly 50 per cent. were still alive at the end of 14-21 days.

C. EXPERIMENT WITH *A. caballus* AND *A. lineatopennis*.

In the first experiment with the Eshowe virus a number of *A. caballus* and *A. lineatopennis* were injected together into the same horse after an interval of 7 days. Another horse was injected under the same conditions with *A. hirsutus*.

At the time of these experiments no larvae of *A. caballus* or *A. lineatopennis* were at our disposal, and we had to make use of specimens freshly caught in the field where they could be obtained in sufficient numbers.

Virus horse 11, injected with the original Eshowe material, was used for feeding mosquitoes on, and mosquito groups:—

A. caballus group 17.—Fed on virus horse 11 during the night of 1st to 2nd March. First day of fever. Temperature 103.0-102.0°. 21 specimens (caught as adults) engorged. Used for experiment 24.

A. caballus group 18.—Fed on the same virus horse during the following night. Second day of fever. Temperature 104.0-103.2°. 34 specimens engorged (caught as adults). Used for experiment 24.

A. caballus group 19.—Fed on the same virus horse one night later (3rd to 4th March). Third day of fever. Temperature 105.5-103.9°. 77 specimens engorged (caught as adults). Used for experiment 24.

A. lineatopennis group 12a.—Fed together with *A. caballus* group 17. 35 specimens engorged (caught as adults). Used for experiment 24.

A. lineatopennis group 13.—Fed together with *A. caballus* group 18. 57 specimens engorged (caught as adults). Used for experiment 24.

A. lineatopennis group 14.—Fed together with *A. caballus* group 19. 71 specimens engorged (caught as adults). Used for experiment 24.

Experiment 24 (H.S. 20). 68 *A. caballus* and 66 *A. lineatopennis*. Injection. Interval 7 days. Horse 20288.

This horse was injected with 68 *A. caballus* and 66 *A. lineatopennis*, altogether 134 specimens. On 9th March, 1932, 14 *A. caballus* group 17 were injected and 16 *A. lineatopennis* group 12a; on 10th March, 16 *A. caballus* group 18 and 22 *A. lineatopennis* group 13; on 11th March, 38 *A. caballus* group 19 and 28 *A. lineatopennis* group 14. All these mosquitoes had fed on an infected horse 7 days before, during the first to third days of its fever reaction.

TRANSMISSION OF HORSESICKNESS AT ONDERSTEEPOORT DURING 1931-32.

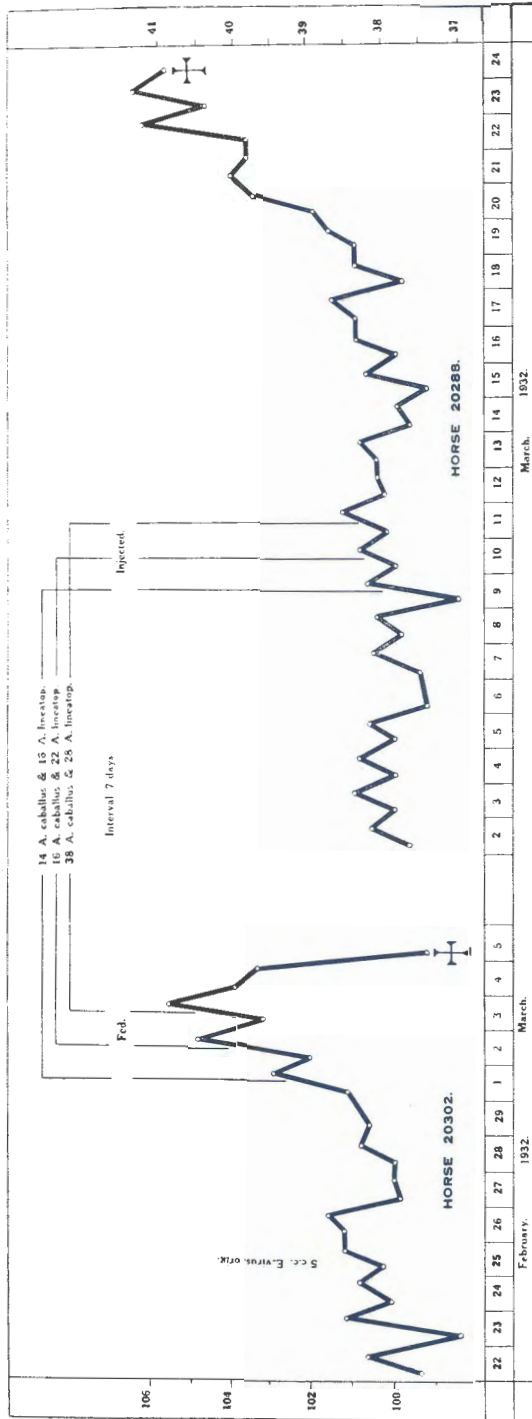


Fig. 3.
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Reaction (vide fig. 3): The temperature remained practically normal up to 18th March, 9 days after the injection of the first group of mosquitoes and 7 days after the last. On the following days the temperature was: 19th March, 101.0 and 101.7°; the 20th, 102.0 and 103.5°; the 21st, 104.0 and 103.6°; the 22nd, 103.6 and 106.2°; the 23rd, 104.5 and 106.5°; and the 24th (a.m.), 105.5°. During the afternoon of the same day the horse died. The post-mortem diagnosis was: horsesickness, dunkop.

The result of this experiment was *positive*. Virulent virus was, therefore, present in one or more of 134 *A. caballus* and *A. lineatopennis* 7 days after their having fed on an infected horse.

D. EXPERIMENTS WITH *Aedes caballus*.

The experiments with this species were carried out from the latter part of March until the middle of May, 1932. *Aedes caballus* could be obtained relatively easily, much more easily in any case than the other species. Altogether more than 900 specimens were fed on the virus horses. Owing to a considerable mortality following upon oviposition, however, only 83 specimens fed for a second time on the susceptible horses.

Four experiments were made with this species; experiment 25, with mosquitoes fed on virus horse 12, which had been infected by the injection of mosquitoes after an interval of one week; experiment 26, with specimens from virus horse 14; experiment 27, with specimens from virus horse 15, and experiment 28, with mosquitoes fed on horse 20300 from experiment 25, during a fever reaction which might have been of the nature of horsesickness fever.

The mosquitoes were:—

Group 25a.—Fed on virus horse 12 during the night of 21st to 22nd March, 1932. Second day of fever. Temperature 103.6-103.6°. 260 specimens engorged (caught as adults). Used for experiment 25.

Group 27.—Fed on the same horse the following night. Third day of fever. Temperature 106.2-104.5°. 192 specimens engorged (caught as adults). Used for experiment 25.

Group 31.—Fed during the night of 18th-19th April on horse 20300 of experiment 25. Temperature 102.4-101.2°. 86 specimens engorged (reared from larvae). Used for experiment 28.

Group 32.—Fed on virus horse 14 during the night of 19th-20th April. First day of fever. Temperature 103.2 and 101°. 104 specimens engorged (reared from larvae). Used for experiment 26.

Group 33.—Fed during the night of 19th-20th April on horse 20300 of experiment 25. Temperature 103.6-101.4°. 130 specimens engorged (reared from larvae). Used for experiment 28.

Group 35.—Fed on virus horse 14 during the night of 21st and 22nd April. Third day of fever. Temperature 101.4-101.7°. 107 specimens engorged (reared from larvae). Used for experiment 26.

Group 36.—Fed on virus horse 15 during the night of 26th-27th April. Fourth day of fever. Temperature 104-106°. 57 specimens engorged (reared from larvae). Used for experiment 27.

Experiment 25 (H.S. 30). 10 *Aedes caballus*. *Feeding*. *Interval* 16-17 days.
Horse 20300.

On 7th April, 1932, *A. caballus* groups 25a and 27 were put on to this horse and 10 specimens fed during the night. These mosquitoes had had their initial feed 16-17 days previously, during the second and third days of fever, on a horse infected by the injection of mosquitoes.

Reaction: This horse normally showed a fairly high temperature, which varied during the week preceding the application of the mosquitoes between 100 and 102°, averaging 101° for the period.

The day after the feeding of the mosquitoes an attack of colic occurred and the temperature rose to 104.6° , but dropped back to normal (100°) the following day. It remained below 102° for three more days when a febrile reaction commenced. The temperature on 13th April was 101.2 and 102.4° ; the 14th, 100 and 102.0° ; the 15th, 101.1 and 101.2° ; the 16th, 102.4 and 104.3° ; the 17th (a.m.) 102° ; the 18th, 103 and 102.4° ; the 19th, 101.2 and 103.6° ; the 20th 101.4 and 102.2° . From 21st April to 10th May the temperature only once reached 102° for half a day, viz., on 7th May, for the rest it remained between 100 and 101.5° .

Subinoculation: On 19th April, 1932, 5 c.c. blood of horse 20300, taken the day before, during the slight febrile reaction, was injected intrajugularly into horse 20309. During an observation period of one month no febrile reaction occurred, the highest temperature registered being 100.4° , excluding the day of the injection and the day following thereupon. It was not thought necessary to test the susceptibility of this horse by virus injection.

Testing by means of mosquito feeding: During the febrile reaction two groups of *A. caballus* were fed on the horse and about 20 days after were again fed on a susceptible horse, but with negative results. A full account will be given under experiment 28.

Immunity test: On 11th May, 33 days after the feeding of the mosquitoes and 21 days after the end of the febrile reaction, the horse was injected intrajugularly with 5 c.c. blood of horse 20288 (virus horse 12). Five days later, on 16th May, the temperature rose to 105.4° and remained between 105 and 105.8° during the following 2 days. On the next morning, 19th May, the temperature was 102.2° and the horse died the same day.

Result: This experiment must also be regarded as negative. Eight days after the feeding of the group of mosquitoes a fever reaction occurred which might have been due to a slight attack of horsesickness fever. A subinoculation of blood into a normal horse was negative, however, and 3 weeks after this reaction the horse proved to be normally susceptible when injected intrajugularly with the same strain.

Experiment 26 (H.S. 34). Aedes caballus. Feeding. Interval 18-20 days.
Horse 20331.

On 9th May, 1932, *A. caballus* groups 32 and 35 were put on to horse 20331 and during the ensuing night 14 specimens of group 32 and 4 of group 35 engorged themselves. In total, therefore, the horse was bitten by 18 specimens, viz., by—

- 4 specimens (group 35) after an interval of 18 days;
- 14 specimens (group 32) after an interval of 20 days.

These mosquitoes had fed on the infected horse during the first and third days of fever.

Reaction: The horse was kept under observation up to 12th June, 35 days after the feeding of the mosquitoes. Only once during this period did the temperature reach 101.0° , and then only for half a day.

Result: As no temperature reaction whatsoever followed the biting of the mosquitoes the experiment has to be regarded as *negative*. The test for immunity was omitted in this case.

Experiment 27 (H.S. 35). Aedes caballus. Feeding. Interval 15 days.
Horse 20333.

On 11th May, 1932, *A. caballus* group 36 was put on to this horse, but only three specimens took up blood. These mosquitoes had fed 15 days previously on virus horse 15, which had been infected with a strain of virus obtained from virus horse 12. This virus prior to injection, had been subjected to cataphorisation by the passage of an electrical current of 9 milliamperes under a pressure of 250 volts for 4 hours.

Reaction: The temperature of this horse remained normal for 33 days, after the feeding of the mosquitoes (up to 13th June), when the horse was discharged from observation. Only once did the temperature exceed 101° for half a day.

The result of this experiment was negative, as no reaction at all occurred after the feeding of the mosquitoes. A test of the susceptibility of the horse was not thought necessary.

Experiment 28 (H.S. 33). Aedes caballus. Feeding. Interval 20-21 days. Horse 20235.

On 9th May, 1932, *Aedes caballus* groups 31 and 33 were put on to this horse and during the following night 52 (out of 66) specimens engorged themselves. These mosquitoes had fed 20-21 days previously on horse 20300 of experiment 25 during a fever reaction which, as far as one could see at the time, might have been connected with horsesickness fever.

Reaction: The temperature at first remained normal for 14 days. A slight fever reaction then commenced. The temperature on 21th May was 101·4 and 101·6°; the 25th, 102 and 101°; the 26th, 103·4 and 103·6°; the 27th, 102·8 and 102·4°. From the 28th onwards the temperature was normal again up to 13th June, when the horse was discharged from observation.

Result: We will regard this experiment also as *negative*. About two weeks after the feeding of the mosquitoes a slight temperature reaction occurred, but we cannot regard this as an attack of horsesickness fever as the horse on which these mosquitoes had had their initial feed was not infected with horsesickness as shown by the immunity test in experiment 25. For the same reasons a test of the susceptibility of this horse was not thought necessary.

E. EXPERIMENT WITH *Aedes lineatopennis*.

Together with the first groups of *Aedes caballus* referred to in the preceding section, 89 *Aedes lineatopennis* were fed on virus horse 12, which had been infected by the injection of mosquitoes after an interval of one week. On virus horse 13, 20 more specimens engorged themselves. The mosquitoes were caught partly as adults, partly reared from larvae or pupae. They survived better than those of *A. caballus* and after two weeks 49 specimens or nearly 50 per cent. were still alive, of which 34 fed again.

On the other virus horses which were used in the preceding experiments with *A. caballus*, no *A. lineatopennis* could be fed as at that time no more specimens of this species, adults or larvae, could be obtained.

Virus horses 12 and 13 were used and mosquito groups:—

Group 21a.—Fed on virus horse 12 during the night of 21st to 22nd March, 1932. Second day of fever. Temperature 103·6-103·6°. 24 specimens engorged (caught as adults). Used for experiment 29.

Group 22.—Fed together with group 21a. 27 specimens engorged (reared from larvae). Used for experiment 29.

Group 23.—Fed on the same virus horse during the following night. Third day of fever. Temperature 106·2-104·5°. 38 specimens engorged (reared from larvae). Used for experiment 29.

Group 24.—Fed on virus horse 13 during the night of 24th-25th March. Third day of fever. 20 specimens engorged (caught as adults). Used for experiment 29.

Experiment 29 (H.S. 29). 42 Aedes lineatopennis. Feeding. Interval 16-22 days. Horse 20315.

On 6th April, 1932, *A. lineatopennis* groups 21A and 22 were put on to this horse and 22 specimens (out of 25) fed during the following night. Group 23 was fed on the same horse during the night of 8th to 9th April, and 9 specimens (out of 21) engorged themselves. Group 24 was fed during the night of 11th-12th April, and 3 specimens took up blood. Finally, the combined groups 21A-23 were again put on to the horse on the 12th, and 8 specimens fed during the night.

The horse was, therefore, bitten 12 times in all by:—

- 22 specimens (groups 21A-22) after an interval of 16 days.
- 9 specimens (group 23) after an interval of 17 days.
- 3 specimens (group 24) after an interval of 18 days.
- 8 specimens (groups 21A-23) after an interval of 21-22 days.

These mosquitoes had fed on the infected horse during the second and third days of fever.

Reaction: A brief elevation of the temperature up to 103° occurred on 9th April, one day after the feeding of the second group of mosquitoes, but it lasted only half a day. During an observation period of 48 days, up to 27th May, no real temperature reaction occurred, only 6 times was 101° reached or slightly exceeded, but on each occasion for not longer than 12 or 24 hours.

Immunity test: On 27th May the horse was injected subcutaneously with 1 c.c. blood of horse 20302 (virus horse 11). Five days later the temperature began to rise. On 1st June it was 103·8 and 105°; the 2nd, 105 and 105°; and the 3rd, 103·6 and 100·4°. The following night the horse died, the post-mortem showing typical signs of horsesickness.

Result: The experiment was *negative*, no temperature reaction occurring after the feeding of the mosquitoes, and the horse was normally susceptible to the subcutaneous injection of 1 c.c. virus blood.

F. EXPERIMENTS WITH *Aedes hirsutus*.

At the commencement of our experiments with the Eshowe strain we had at our disposal quite a large supply of *Aedes hirsutus* from one of our breeding places. We succeeded in feeding 311 specimens on the first virus horse injected with the original material of this strain. The mortality amongst these mosquitoes was very low. After a week 100 mosquitoes were injected and after 20-21 days there were still 107 of the remaining specimens alive.

Two experiments were made with these mosquitoes. In the first, experiment 30, 100 specimens were injected after an interval of one week. This experiment was run in conjunction with experiment 24 in which combined groups of *Aedes caballus* and *A. lineatopennis* were injected after the same interval. The two experiments were intended to serve as controls to one another. In the second experiment, No. 31, the mosquitoes were fed, in the same manner as in the experiments with *A. caballus* and *A. lineatopennis*, on a susceptible horse, and the remaining specimens were injected into the horse of experiment 30 after an interval of nearly one month.

Only one virus horse, No. 11, was used, and mosquito groups:—

Group 10.—Fed on virus horse 11 during the night of 1st to 2nd March, 1932. First day of fever. Temperature 103·0-102·0°. 201 specimens engorged (reared from larvae). Used for experiments 30 and 31.

Group 11.—Fed on the same virus horse the following night. Second day of fever. Temperature 104·8-103·2°. 110 specimens engorged (reared from larvae). Used for experiments 30 and 31.

Experiment 30 (H.S. 19). 146 *Aedes hirsutus*. *Injection.* Interval 7-30 days. *Horse* 20280.

The horse of this experiment had previously been used in December, 1931, for experiment 13 (O-virus, *A. dentatus*, injection interval 16 days), but no reaction had followed the injection of these mosquitoes.

On 9th March, 1932, 50 *Aedes hirsutus* group 10 were injected and the following day 50 specimens of group 11. After this the remaining specimens of both groups were used in the following experiment for feeding. Of these, 46 specimens were still alive on 31st March and injected on that date into the horse of this experiment.

Altogether 146 specimens were injected into the horse:—

100 specimens after an interval of 7 days.

46 specimens after an interval of 20-30 days.

These mosquitoes had fed on the virus horse during the first and second days of fever.

Reaction: Directly after the injection of the second lot of mosquitoes a slight rise in temperature occurred (101.4°), and 10 days later another, up to 102°, lasting, however, only half a day. After the third injection the temperature rose to 102°, but by the next morning it had already commenced falling again. The mosquito injections were, therefore, tolerated very well. Up to 27th May, 57 days after the last and 79 days after the first injection of mosquitoes, no fever reaction had occurred, the highest temperature recorded, except for the days mentioned above, being 101°.

Immunity test: On 27th May, the horse was injected subcutaneously with 1 c.c. blood of horse 20302 (virus horse 11). Five days later, on 1st June, the temperature rose to 104.4 and 105°, remaining the following day at 105°. On 3rd June, it was 103.8 and 105°, and the following morning 104.2°. The same day the horse died from horsesickness.

The result of the experiment was absolutely *negative*. After the injection of 146 mosquitoes, fed 7-30 days before on an infected horse, no temperature reaction at all followed, whereas the horse proved to be fully susceptible when injected subcutaneously with 1 c.c. of the same strain. It may be remembered that with *Aedes caballus* and *A. lineatopennis* under the same conditions a positive result had been obtained.

Experiment 31 (H.S. 21). Aedes hirsutus. Feeding. Interval 15-21 days.
Horse 20130.

During the night of 16th to 17th March, 1932, *Aedes hirsutus* group 10 was allowed to feed on this horse, and out of 114 specimens 72 took up blood. During the following night group 11 was put on to the horse and 19 (out of 43) specimens engorged themselves. Both groups were put on to the same horse again during the night of 22nd-23rd March, and 96 specimens (out of 107) fed. On 31st March, 9 days later, the remaining 46 specimens of these two groups were injected into horse 20280, as described in the preceding experiment.

The horse of this experiment was bitten 187 times by the mosquitoes, viz. :—

91 specimens after an interval of 15 days.
96 specimens after an interval of 20-21 days.

These mosquitoes had fed on the virus horse during the first and second days of the fever.

Reaction: The first afternoon, when the mosquitoes were put on to the horse, the temperature rose to 101°. This was a frequent occurrence when the horses would not willingly enter our mosquito stables and force had to be used. After this, the temperature remained between 98.8 and 100.7°, thus normal up to 5th April, i.e. 20 days after the first and 14 days after the last feeding of mosquitoes. A short fever reaction then set in, the following temperatures being registered: 6th April, 99.4 and 101.9°; the 7th, 99 and 102.8°; the 8th, 99.2 and 103.8°; the 9th, 99.4 and 101.5°. After this the temperature dropped to normal and remained so for a further observation period of 18 days, during which period 100.4° was the highest temperature noted.

Subinoculation: On 8th April, during the short febrile reaction, 5 c.c. blood of horse 20130 was injected into horse 20271.

This horse had previously been used in December, 1931, in experiment 12 (O-virus, *Aedes dentatus*, injection, interval 5 days), and afterwards in February, 1932, in experiment 18 (O-virus after mosquito passage, *Aedes* spp., injection after 8-9 days). In both experiments no reaction resembling horsesickness fever followed the injection of the mosquitoes.

The horse was kept under observation for 35 days, up to 13th April. The day after the injection the temperature rose up to 101°, but returned to normal the following day and remained so until the end of the experiment, 100.2° being the highest temperature registered.

Immunity test: On 28th April, 42 days after the first, 37 days after the last feeding of the mosquitoes, and 19 days after the end of the short febrile reaction, the horse (20130) of the main experiment was injected subcutaneously with 1 c.c. blood of horse 20302 (virus horse 11).

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The temperature remained normal for three days. On 2nd May, the fourth day p.i., it was 100·5 and 103·6°; the 3rd, 101·4 and 105°; the 4th, 103·8 and 106°; the 5th (a.m.), 104·4; and the 6th (a.m.), 102°. During the same day the horse died. The post-mortem confirmed the diagnosis of horsesickness.

Result: The original horse, on which the mosquitoes had fed, showed a brief febrile reaction of a remittent character, lasting 4 days and showing 103·8° as maximum temperature. This reaction occurred 14-20 days after the feeding of the mosquitoes. Tested with the same virus 19 days later, the horse proved to be normally susceptible. A subinoculation of blood taken during the fever period and injected into another horse, failed to give any reaction. The febrile reaction in the first horse was, therefore, not due to horsesickness fever, and the whole experiment has to be regarded as *negative*.

G. EXPERIMENTS WITH *Aedes dentatus*.

Aedes dentatus were not present in large numbers amongst our material during these experiments, nor were they actually rare. In all, 129 specimens could be fed on the five virus horses injected with the Eshowe strain, 21 on virus horse 11, 73 on horse 12, 7 on horse 13, 9 on horse 14 and 18 on horse 15. Of these mosquitoes at least 52 specimens could be induced to feed 67 times on the susceptible horse. The result was, therefore, quite satisfactory.

The virus horses 11 to 15 were used and mosquito groups:—

Group 9.—Fed on virus horse 11 during the night of 2nd to 3rd March, 1932. Second day of fever. Temperature 103·0-102·0°. 13 specimens engorged (reared from larvae). Used for experiment 32.

Group 10.—Fed on the same horse the following night. Third day of fever. Temperature 105·5-103·9°. 8 specimens engorged (reared from larvae). Used for experiment 32.

Group 14.—Fed on virus horse 12 during the night of 21st to 22nd March. Second day of fever. Temperature 103·6-103·6°. 6 specimens engorged (caught as adults). Used for experiment 32.

Group 15.—Fed on the same horse together with group 14. 67 specimens engorged (reared from larvae). Used, for experiment 32.

Group 16.—Fed on virus horse 13 during the night of 24th to 25th March. Third day of fever. 7 specimens engorged (caught as adults). Used for experiment 32.

Group 18.—Fed on virus horse 14 during the night of 19th to 20th April. First day of fever. Temperature 103·2 and 101°. 9 specimens engorged (reared from larvae). Used for experiment 32.

Group 19.—Fed on virus horse 15 during the night of 26th to 27th April. Fourth day of fever. Temperature 104 and 106°. 18 specimens engorged (reared from larvae). Used for experiment 32.

Experiment 32 (H.S. 23). Aedes dentatus. Feeding. Interval 15-22 days.
Horse 20291.

This horse had been used before in December, 1931, in experiment 8 (O-virus, *Aedes caballus*, injection, interval 15-16 days). No typical reaction had followed the injection of the mosquitoes.

On 23rd March, 1932, the mosquito groups 9 and 10 were put on to this horse and during the following night 4 (out of 15) specimens engorged themselves. During the night of 6th-7th April the groups 14 and 15 were allowed to feed and 36 (out of 37) specimens took up blood. On 11th April group 16 was put on to the horse resulting in the feeding of 3 specimens during the ensuing night. Groups 14 and 15 were again put on to the same horse during the night of 12th-13th April and 15 specimens engorged themselves. During the night of 10th-11th May 4 specimens of group 18 were fed and lastly, during the following night 5 of group 19.

Altogether the horse was bitten 67 times by these mosquitoes, viz.:—

- 5 specimens (group 19) after an interval of 15 days.
- 36 specimens (group 14-15) after an interval of 16 days.
- 3 specimens (group 16) after an interval of 18 days.
- 4 specimens (group 9-10) after an interval of 20-21 days.
- 4 specimens (group 18) after an interval of 21 days.
- 15 specimens (group 14-15) after an interval of 22 days.

These different mosquitoes had fed on four different virus horses during the first to third days of fever.

On 28th April and 6th May *Aedes dentatus* infected with the Kaalplaas virus were fed on the same horse. This will be referred to under experiment 35.

Reaction: The horse was kept under observation up to 3rd June, 72 days after the feeding of the first batch of mosquitoes and 23 days after the last group. During this period no fever reaction occurred, the highest temperature registered being 101.2°.

Immunity test: On 3rd June the horse was injected with 1 c.c. blood of horse 20302 (virus horse 11) subcutaneously. Five days later, on 8th June, the temperature commenced rising and 102° was recorded. On the 9th it was 102.4 and 104°; the 10th, uncertain (anus open, intravenous injection of .002 gm. Akiron per Kg., body-weight); the 11th, 103 and 104°; and the 12th (a.m.), 104°. The same day the animal died from horsesickness (dunkop).

Result: The experiment has to be regarded as *negative* as no temperature reaction was shown after the application of the mosquitoes, whereas the horse proved to be normally susceptible when injected subcutaneously with material of the same strain.

H. DISCUSSION OF THE RESULTS WITH THE ESHOWE VIRUS.

A fresh virus strain obtained from a mule previously immunized against O-virus at Eshowe was designated Eshowe virus. Its relationship to O-virus was thus the same as was the case with the Losperfontein strain, and it was, therefore, not the type of strain we really wanted.

With the original material we injected one horse and with its blood two other animals. Another horse was injected after the virus had been subjected to cataphorization by the passage of an electrical current of 9 milliamperes at 250 volts for 4 hours, and a fourth by the injection of mosquitoes. Mosquitoes were fed on all these horses for our experiments.

Altogether 1,650 mosquitoes were fed on the virus horses, 1,068 *Aedes caballus*, 272 *A. lineatopennis*, 211 *A. hirsutus* and 129 *A. dentatus*. The mosquitoes were caught partly as adults in the fields, partly reared in the laboratory from larvae. Of *A. hirsutus* only specimens reared in captivity were used. During the experiments the mosquitoes were kept in jars or cages in the warm room of the laboratory, in which the temperature varied between 20°, 30° C. with a probable mean of 24°. As a rule, during these experiments, the room was only heated during the day-time, as there was no automatic control. At night the room was actually warmer than out of doors, whereas during the day-time it was cooler. As usual, between the two feedings on the infected and normal horses, the mosquitoes were fed on 10 per cent. sugar water, renewed every day or every second day.

In this series of experiments the mosquitoes were partly injected and partly fed on the susceptible horses. We will discuss first of all the two experiments by means of mosquito injection.

In the first experiment (No. 24) 68 *Aedes caballus* and 66 *A. lineatopennis*, thus 134 specimens, were injected into a horse in the form of an emulsion on three consecutive days. In this horse a fever reaction made its appearance 8 days after the last, and 10 days after the first injection. Four days later the animal died from horsesickness. All these mosquitoes had fed on an experimentally infected horse 7 days before being injected. In one or more specimens the virus must, therefore, have been present after this lapse of time.

In the second experiment (No. 30), *Aedes hirsutus* were used. They had fed together with *A. caballus* and *A. lineatopennis* of the preceding experiment. Seven days after their feed on an infected horse 100 specimens were injected in the form of an emulsion on two consecutive days, and 46 more specimens of the same groups after an interval of 29-30 days. No temperature reaction whatsoever followed these injections however, and later the horse proved to be normally susceptible when injected subcutaneously with material from the same strain.

In these two experiments, therefore, virus could be demonstrated by means of the injection of mosquitoes in a combined lot of 134 *A. caballus* and *A. lineatopennis* after an interval of 7 days, whereas in 100 *A. hirsutus* after 7 days and in 46 after nearly a month no virus was present.

By feeding the mosquitoes the following experiments were carried out:—

- Aedes caballus* (experiment 25). Interval 16-17 days: 10 specimens. Result, negative.
- Aedes caballus* (experiment 26). Interval 18 days: 4 specimens. Interval 20 days: 14 specimens. Total, 18 specimens. Result, negative.
- Aedes caballus* (experiment 27). Interval 15 days: 3 specimens. Result, negative.
- Aedes lineatopennis* (experiment 29). Interval 16-18 days: 34 specimens. Interval 21-22 days: 8 specimens. Total 42 specimens. Result, negative.
- Aedes hirsutus* (experiment 31). Interval 15 days: 91 specimens. Interval 20-21 days: 96 specimens. Total 187 specimens. Result, negative.
- Aedes dentatus* (experiment 32). Interval 15-18 days: 44 specimens. Interval 20-22 days: 23 specimens. Total 67 specimens. Result, negative.

In these 6 experiments together the horses were bitten 327 times by the four *Aedes* species after the following intervals:—

Interval 15 days	99 specimens
Interval 16-18 days	87 specimens.
Interval 20-22 days	141 specimens.

In none of these experiments in which mosquitoes were fed on a normal horse after an interval of 15 days or longer, did we succeed in transmitting the infection. Four of the six normal horses were tested for immunity after a sufficiently long incubation period, 3 by

subcutaneous injection of 1 c.c. and the other by 5 c.c. virus intrajugularly and all reacted to horsesickness followed by death. There is every reason to believe that the other horses were normally susceptible as well.

The transmission experiments with this strain were, therefore, also a complete failure.

As was the case with O-virus, this virus may be present in mosquitoes after an interval of one week. In the positive experiment with O-virus *A. caballus*, *A. hirsutus* and *A. lineatopennis* were used, with the Eshowe strain *A. caballus* and *A. lineatopennis*; these two species being common to both experiments. We are unable to say, however, whether this survival of the virus in the mosquitoes up to one week is normal or exceptional. It seems rather to be somewhat exceptional. In any case, the results of the feeding experiments do not permit of our regarding it as the beginning of an actual development of the virus in a suitable insect transmitter.

With regard to the possible reasons for the obvious failure of our transmission experiments we may refer to the discussion of the Losperfontein strain in the preceding chapter. The choice of the mosquito species may have been at fault or the strain of virus may have been the old O-virus, which has perhaps lost its capacity for developing in insects. Other factors, or faults in the experimental technique, cannot have been, in our opinion, of any real importance.

IX. EXPERIMENTS WITH KAALPLAAS VIRUS.

On 13th April, 1932, a horse (No. 20031) showing typical and very advanced symptoms of the dikkop form of horsesickness was brought into the Onderstepoort Laboratories from Kaalplaas, a farm belonging to Onderstepoort and almost contiguous with it. This animal had a temperature of 102.2° and died during the following night. It had been hyperimmunized in September, 1931, of the previous year against horsesickness by means of O-virus.

We were able to feed only one large batch of *A. caballus* and a few *A. dentatus* on this horse before it died. The experimental technique was the same as that adopted in the preceding experiments with the Eshowe strain.

A. EXPERIMENTS WITH *Aedes caballus*.

On account of our having succeeded in feeding a fair number of *A. caballus* on this horse notwithstanding the short time at our disposal between its receipt and death, we decided to perform two experiments by (a) injecting a certain number of the mosquitoes after an interval of one week, and (b) allowing the remainder to feed on a second horse, commencing at an interval of 15 days.

Mosquito group 30.—Fed on virus horse 16 during the night of 13th-14th April, 1932. Temperature 102.2°. (Day of commencement of fever reaction unknown.) 365 specimens engorged (reared from larvae). Used for experiments 33 and 34.

*Experiment 33 (H.S. 31). 23 Aedes caballus. Injection. Interval 7 days.
Horse 20318.*

On 21st April, 1932, 25 *A. caballus* (group 25), which had fed 7 days before on a horse suffering from horsesickness, which had contracted the disease spontaneously on the farm Kaalplaas and was then in a moribund condition, were injected subcutaneously after thorough emulsification into horse No. 20318.

Reaction: The horse showed a somewhat irregular temperature, varying normally between 99° and 101°. Eleven days after the injection the temperature rose to 102.2°, remaining elevated, however, for only half a day. Nine days of normal temperature followed, then a rise to 101.2° occurred, and two days later (14th May) 103.4° was recorded, both these elevations, however, lasted less than 24 hours. A further period of normal temperature followed but on 27th May some other mosquitoes were injected and this was followed by a slight rise to just over 101° for a few days. The horse was kept under observation for a further 31 days, but no temperature above 101.2° was registered.

Result: The experiment was *negative* as no temperature reaction of any importance followed the injection of the mosquitoes during an observation period of 67 days. An immunity test was not considered necessary. Another horse from the same lot was tested and found to be fully susceptible.

Experiment 34. Aedes caballus. Feeding. Interval 15-23 days. Horse 20323.

On 27th April, 1932, group 30 (*Aedes caballus*) was put on to this horse and during the following night 75 specimens engorged themselves. The remaining specimens of this group were fed on the same horse on the night of 6th to 7th May, and on this occasion 7 specimens took up blood.

The horse was therefore bitten 82 times by these mosquitoes, after an interval of 15 days by 75 specimens, and after an interval of 25 days by 7 specimens. The mosquitoes had been fed on a spontaneous case of horsesickness shortly before the death of the animal.

Reaction: No temperature reaction at all followed the feeding of the mosquitoes, during an observation period of 36 days, the highest temperature registered during this period being 100.8°.

Immunity test: On 3rd June, 36 days after the first and 29 days after the last feeding of the mosquitoes, the horse was injected subcutaneously with 1 c.c. preserved blood of horse 20031, the virus horse used in these experiments. On 8th June, 5 days after the injection, the temperature rose to 105.2° and remained between 105 and 106° for three days. The following morning it had fallen to 100° and the horse died the same day, the post-mortem confirming the diagnosis of horsesickness.

Result: This experiment was clearly *negative*. No temperature reaction had followed the feeding of 82 mosquitoes which had taken a feed of infected blood. The horse subsequently proved to be normally susceptible when injected subcutaneously with 1 c.c. preserved blood of the same strain.

B. EXPERIMENT WITH *Aedes dentatus*.

On the afternoon on which we received the infected horse from Kaalplaas we had at our disposal only a few mosquitoes of this species, of which 14 only could be induced to feed.

Mosquito group 17.—Fed on virus horse 16 during the night of 13th-14th April, 1932. Temperature 102.2°. Day of commencement of fever reaction unknown. 14 specimens engorged (reared from larvae). Used for experiment 35.

*Experiment 35 (H.S. 23). Aedes dentatus. Feeding. Interval 15-23 days.
Horse 20291.*

This horse had been used previously, in December, 1931, in experiment 8 (O-virus, *A. caballus*, injection, interval 15-16 days), which had been negative.

From 23rd March, 1932, until the end of this experiment it was used also for experiment 32 (Eshove virus, *A. dentatus*, feeding).

On 28th April, *Aedes dentatus* group 17 was put on to this horse and during the following night 5 (out of 8) specimens engorged. The remaining mosquitoes were fed on the same horse again during the night of 6th-7th May and 4 (out of 6) specimens fed on this occasion. The mosquitoes, therefore, fed 9 times on this horse as follows:—

5 specimens after an interval of 15 days.

4 specimens after an interval of 23 days.

Reaction: The horse was kept under observation up to 3rd June, without any reaction being noticed.

Immunity test: The horse was tested for immunity by the subcutaneous injection of Eshowe virus and died 9 days later from horsesickness (*vide* experiment 32).

The result of this experiment therefore was *negative*.

C. DISCUSSION OF THE RESULTS WITH THE KAAALPLAAS VIRUS.

About the middle of April, 1932, we received a horse previously hyperimmunized against O-virus, which had contracted horsesickness spontaneously at Kaalplaas. The horse was in a moribund condition when received and had to be made use of for our purpose that same afternoon. One large batch of *Aedes caballus* and a few *A. dentatus* were fed on this animal before it died. The mosquitoes were kept in our warm room during the experiment under the same conditions as was the case with those of the Eshowe strain.

Three experiments were conducted. In the first (No. 33) 25 *A. caballus* were injected into a horse after an interval of 7 days. No temperature reaction followed this injection so that no virulent virus could have been present.

The remaining specimens of the same group were fed on a second horse (experiment 34), in which 75 specimens engorged themselves after an interval of 15 days and 7 specimens after 23 days. No temperature reaction followed, while the horse proved to be susceptible when inoculated later with the same strain. This experiment was also negative.

Only one experiment was conducted with *Aedes dentatus* 5 specimens fed after 15 and 4 after 23 days, without conveying infection to a horse.

No positive results were, therefore, obtained with the Kaalplaas virus. This strain was identical, in certain respects, with those received from Eshowe and Losperfontein. All three were derived from animals previously immunized against horsesickness by O-virus. The possible explanation of the failure to transmit the disease, already advanced in the case of the other two strains, may apply equally well in this case and we therefore refer here to these previous discussions.

X. DISCUSSION OF THE RESULTS OBTAINED IN TRANSMISSION EXPERIMENTS WITH HORSESICKNESS.

During the latter part of the winter of 1931 and the summer 1931-1932 experiments were carried out with the object of finding the natural transmitter of horsesickness, a virus disease of great economic importance in South Africa.

Pitchford (1902) only has carried out actual transmission experiments, and he claims to have transmitted the disease by means of Anophelines. From his account, however, it is not possible to obtain a clear idea as to how his results were obtained and they cannot be regarded as proof that Anophelines are the actual transmitters.

Taking into account the known epidemiological evidence mosquitoes must be regarded as the most probable transmitters. The disease is caused by a virus not transmitted by ordinary contact. In its appearance it is practically limited to the summer months, heavy rainfall being followed by a high percentage of infection. In dry seasons relatively few cases appear ordinarily. The infection is practically only transmitted during the night or near sunset or sunrise, and stables afford a relatively good protection. This evidence is sufficient to allow of horsesickness being regarded as an insect-borne disease. However, it is in itself not sufficient to enable the potential transmitters to be narrowed down to a certain group of insects with certainty, although, assuming the epidemiological facts as being correct, mosquitoes must then be regarded as the most probable transmitters. At any rate, none of the facts mentioned oppose the theory of mosquitoes being the natural transmitters, and on this theory we have based our work.

Together with these experiments a mosquito survey was carried out at Onderstepoort, covering the latter part of the winter of 1931, and the whole summer 1931-1932. Mosquitoes were caught in traps containing horses as bait animals, and as thorough a search as possible was made for breeding places. The season at our disposal was very dry and all the information desired could, therefore, not be gained. A review of the results of this survey has already been given in the second paper of this series in which we arrived at the conclusion that certain *Aedes* species accurately fulfilled the requirements of the potential carriers as laid down by the known epidemiological evidence already mentioned. Amongst the species of this genus, *Aedes caballus*, *A. lineatopennis*, and *A. hirsutus*, and *Mucidus scatophagooides* must be regarded as the most suitable transmitters, with *Aedes vittatus* and *A. dentatus* as good potential transmitters, although probably of secondary importance. Owing to lack of experimental information, no definite conclusion could be arrived at regarding the possible rôle of Anophelines.

Our experimental work was based mainly on these conclusions. The majority of the experiments were carried out with the above-mentioned *Aedes* species. *Mucidus scatophagooides* was discovered to be a blood-sucking species (contrary to the statements in the literature) too late in the season to enable us to carry out experimental work. During the commencement of the work, when no other species were available, a number of experiments were carried out with *Culex theileri*, mainly to obtain technical experience.

STRAINS OF HORSESICKNESS VIRUS.

For our experiments four different strains of virus were used. Most of the experiments were carried out with the Onderstepoort vaccine strain known as O-virus. This strain had been isolated in 1901 from a spontaneous case of horsesickness. From that date on

it had been transmitted by direct inoculation repeatedly from horse to horse, the 192nd and 225th generations being used in our experiments. Almost invariably the inoculation results in death, the incubation period being usually 2-3 days and the duration of the disease itself generally 3-4 days. For more than 30 years this virus had been transmitted without the normal passage through insects. From the point of view of our work this strain was not very suitable, as, on account of these long years of direct transmissions the developmental capacity of the virus in the insect might have been altered (reduced), thus affecting in particular the value of negative transmission results.

In March, 1932, virus was obtained from a fatal case of horsesickness in a mule at Losperfontein (Transvaal), and from a mule at Eshowe (Natal), both suffering from horsesickness which ended in recovery, and in April from a horse which had contracted a fatal infection at Kaalplaas (Pretoria district). In all these cases a history of immunization against horsesickness with O-virus existed and the possibility cannot be excluded that we have dealt throughout our experiments with O-virus which had appeared with the second attack of horsesickness in the form of a relapse.

EXPERIMENTAL ANIMALS.

The horses used in our experiments were animals of little commercial value, recruited principally from the larger towns in the Transvaal. From the large number of experiments on other phases of horsesickness conducted at Onderstepoort and embodying hundreds of horses it is known that only very exceptionally is an immune horse encountered amongst them.

EXPERIMENTAL TECHNIQUE.

The experimental technique employed in this work has been discussed in full in the second paper of this series.

The mosquitoes were generally fed on the infected and susceptible horses in small cages enclosed with mosquito netting. In the technique finally adopted these cages were held in place on the backs of the horses by means of a specially constructed saddle which prevented any movement of them even on the more sensitive animals. The mosquitoes were put on to the animals late in the afternoon and removed again the next morning.

Special provision had to be made, at any rate during the summer months, to ensure a sufficiently humid atmosphere for the mosquitoes during their feeding on the horses. In dry surroundings the mortality was extremely high. Very good results were obtained in the specially constructed stable, which was surrounded on all sides by hessian kept wet by a constant flow of water.

In the laboratory the mosquitoes were kept in small jars or in cages consisting of a wooden framework covered with mosquito netting. In these cages a sufficient degree of humidity had also to be provided for. The jars were placed on wet cotton wool in slightly larger jars and the cages on shelves, surrounded on all sides by wet hessian. 10% Sugar solution on cotton wool was provided as food.

The mosquitoes were kept in a room which could be heated electrically. The heating apparatus had to be regulated by hand, however, and variations in the temperature could therefore not be avoided. The average temperature was 24-26° C. but varied from time to time between 20 and 30° C.

Experiments with O-virus.

With O-virus, a strain isolated about 30 years ago, 757 mosquitoes were infected on 9 virus horses, 308 *Culex theileri*, 5 *Anopheles squamosus*, 198 *Aedes caballus*, 62 *A. lineatopennis*, 43 *A. hirsutus*, 70 *A. dentatus* and 71 *A. vittatus*. Furthermore, between 400 and 500 mosquitoes were fed in one experiment in which the actual number was not ascertained.

With *Culex theileri* the following five experiments were carried out.

- Experiment 1. 5 specimens injected after $\frac{1}{2}$ day. Result positive.
- Experiment 2. 50 specimens injected after 5 days. Result negative.
- Experiment 3. 50 specimens injected after 16 days. Result negative.
- Experiment 4. 140 specimens injected after 25 days. Result negative.
- Experiment 5. 9 specimens fed, direct transmission. Result negative.

The first experiment, in which 5 *C. theileri* were injected approximately 12 hours after their initial feed, was positive. Sufficient virus was taken up, therefore, by 5 specimens to cause a mortal infection of horsesickness. No conclusions can be drawn from this result as to the ability of *C. theileri* acting as a transmitter, but it indicates that the method of infecting mosquitoes was effective.

In the following three experiments 240 specimens were injected after 5-25 days, 50 of them after 5 days, but in no case was a positive result obtained.

One experiment, by direct transmission with 9 specimens, was also apparently negative. A temperature reaction developed but it could not be traced to horsesickness.

Anopheles squamosus was only used in one experiment.

- Experiment 6. 5 specimens injected after 5 days. Result negative.

With *Aedes caballus* two experiments were carried out.

- Experiment 7. 94 specimens injected after 5 days. Result negative.
- Experiment 8. 28 specimens injected after 15-16 days. Result negative.

Both experiments, in which 122 specimens were injected, proved to be negative.

Aedes lineatopennis was also used in two experiments.

- Experiment 9. 5 specimens injected after 5 days. Result negative.
- Experiment 10. 25 specimens injected after 5 days. Result negative.

In 30 specimens no virus could be detected, therefore, after an interval of 5 days.

Aedes hirsutus was only used in one experiment, which was negative.

- Experiment 11. 30 specimens injected after 5 days. Result negative.

With *A. dentatus* two experiments were made.

- Experiment 12. 6 specimens injected after 5 days. Result negative.
 Experiment 13. 12 specimens injected after 16 days. Result negative.

In 18 specimens no virus was present after 5-16 days.

With *A. vittatus* two experiments were also carried out.

- Experiment 14. 20 specimens injected after 5 days. Result negative.
 Experiment 15. 32 specimens injected after 15 days. Result negative.

Both experiments with 52 specimens were negative.

Finally, an experiment was carried out in which a large number, 294 mosquitoes, consisting of 85 *A. caballus*, 115 *A. hirsutus*, and 94 *A. lineatopennis* were injected together after an interval of 6 days. This experiment was *positive* and the result could be confirmed by subinoculation.

With *Aedes* species 10 experiments were carried out. Only one experiment was positive, in which almost 300 specimens belonging to three different species were injected after an interval of 6 days. In the remaining 9 negative experiments 94 *A. caballus* were injected after 5 days and 28 after 15 days, 6 *A. dentatus* after 5 and 12 after 16 days, 30 *A. hirsutus* after 5 days, 30 *A. lineatopennis* after 5 days and 20 *A. vittatus* after 5 and 32 after 15 days. In all, 180 specimens were injected after 5 and 72 after 15 days.

Experiments with O-virus after one short passage through mosquitoes.

In the preceding series of experiments one positive result was obtained by injecting almost 300 specimens of *Aedes* 6 days after their having fed on an infected horse. We hoped that the virus had adapted itself to the development in mosquitoes and therefore used this strain in the following two experiments.

- Experiment 17. 28 *A. caballus* injected after 7-8 days. Result negative.
 2 *A. hirsutus* injected after 7-8 days. Result negative.
 27 *A. lineatopennis* injected after 7-8 days. Result negative.
 Experiment 18. 157 *A. caballus* injected after 8-9 days. Result negative.
 94 *A. lineatopennis* injected after 8-9 days. Result negative.
 35 *A. hirsutus* injected after 8-9 days. Result negative.
 11 *A. dentatus* injected after 8-9 days. Result negative.
 2 *A. punctothoracis* injected after 8-9 days. Result negative.
 1 *A. cummingsi* injected after 8-9 days. Result negative.

Both experiments were negative. Altogether 357 specimens had been injected after an interval of 7-9 days, 185 *A. caballus*, 121 *A. lineatopennis*, 37 *A. hirsutus*, 11 *A. dentatus* and three specimens of two other species.

There was no sign that the developmental capacity of the virus in the *Aedes* species used had increased by the short passage in mosquitoes, and further work with this strain was abandoned.

Experiments with Losperfontein Virus.

With a strain of virus derived from a mule, which had been immunised with O-virus some months before, five experiments were conducted. One virus horse was used and on it 1,035 mosquitoes were fed, 816 *A. caballus*, 151 *A. lineatopennis*, 61 *A. dentatus* and 7 *A. vittatus*.

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With *A. caballus* the following experiment was carried out:—

- Experiment 19. 71 specimens fed after 14-15 days. Result negative.
6 specimens fed after 20 days. Result negative.

77 specimens, refed after 14-20 days, failed to transmit the disease.

A. lineatopennis was made use of in two experiments:—

- Experiment 20. 38 specimens refed after 13-17 days. Result negative.
51 specimens refed after 19-21 days. Result negative.
66 specimens refed after 33-35 days. Result negative.
10 specimens refed after 60-62 days. Result negative.
Experiment 21. 6 specimens injected after 63-65 days. Result negative.

Both experiments were negative. In the first the horse was bitten 165 times by batches of *A. lineatopennis* infected 13-62 days previously. In the remaining six specimens of the same batches no virus could be traced by injection after 63-65 days.

With *A. dentatus* the following experiment was made:—

- Experiment 22. 20 specimens refed after 25-27 days. Result negative.
6 specimens refed after 35-37 days. Result negative.

A. vittatus was made use of in the last experiment:—

- Experiment 23. 5 specimens refed after 23 days. Result negative.
4 specimens refed after 37 days. Result negative.

Altogether four horses were bitten 277 times by specimens of *A. caballus*, *A. lineatopennis*, *A. dentatus* and *A. vittatus* as follows: After 13-17 days by 109 specimens, after 19-21 by 57, after 23-27 by 58, after 33-37 by 43 and after 60-62 by 10 specimens. Furthermore, six specimens were injected after 63-65 days. In none of these experiments were positive results or reactions, which could be suspected of being horsesickness fever, obtained.

Experiments with Eshowe Virus.

Towards the end of February another strain was received, isolated from a spontaneous case of horsesickness in a mule, which, however, had also been immunised previously with O-virus.

In all, four virus horses were used in which 1,680 mosquitoes were fed, 1,068 *A. caballus*, 272 *A. lineatopennis*, 211 *A. hirsutus* and 129 *A. dentatus*.

In the first experiment a number of *A. caballus* and *A. lineatopennis* were injected together a week after their having fed on a virus horse.

- Experiment 24. 68 *A. caballus* injected after 7 days. Result positive.
66 *A. lineatopennis* injected after 7 days. Result positive.

The temperature of the horse commenced to rise 9 days after the first and 7 days after the last injection. The disease itself, which ended in death, lasted 6 days. The diagnosis of horsesickness was confirmed on post-mortem.

With *A. caballus* alone three experiments were made:—

- Experiment 25. 10 specimens refed after 16-17 days. Result negative.
Experiment 26. 18 specimens refed after 18-20 days. Result negative.
Experiment 27. 3 specimens refed after 15 days. Result negative.

In all, 31 specimens fed after 15-20 days without transmitting the infection.

A. lineatopennis was used in the following experiment, which also was negative:—

- Experiment 29. 34 specimens refed after 16-18 days. Result negative.
8 specimens refed after 21-22 days. Result negative.

With *A. hirsutus* the following two experiments were carried out:—

- Experiment 30. 100 specimens injected after 7 days. Result negative.
46 specimens injected after 29-30 days. Result negative.
Experiment 31. 91 specimens refed after 15 days. Result negative.
96 specimens refed after 20-21 days. Result negative.

Both experiments were negative. The mosquitoes from the second experiment which were still alive after a month, were used for the second injection in the first experiment. In the second experiment a short febrile reaction appeared 14-20 days after the injection of the mosquitoes. The horse, however, later proved to be normally susceptible and a subinoculation of blood, taken during the febrile reaction, failed to infect another horse.

In the last experiment *A. dentatus* was used, also with negative results:—

- Experiment 32. 44 specimens refed after 15-18 days. Result negative.
23 specimens refed after 20-22 days. Result negative.

The first experiment with this strain, in which a combined lot of 134 *A. caballus* and *A. lineatopennis* was injected gave a positive result. We were therefore quite hopeful as to the suitability of this strain. All subsequent experiments were however, negative.

In these negative experiments 146 *A. hirsutus* were injected after 7-30 days and 31 *A. cabullus* refed after 15-20 days, 42 *A. lineatopennis* after 16-22 days, 187 *A. hirsutus* after 15-21 days and 67 *A. dentatus* after 15-22 days. In all the horses were bitten by 327 specimens of these four species after intervals ranging between 7 and 30 days.

Experiments with Kaalplaats Virus.

In April, 1932, a horse suffering from horsesickness which had previously been hyperimmunised against O-virus, was received from the farm Kaalplaats. It died the following morning, but in the interim between its receipt and death we succeeded in feeding 365 *A. caballus* and 14 *A. dentatus* on it.

With *A. caballus* two experiments were carried out, which yielded negative results:—

- Experiment 33. 25 specimens injected after 7 days. Result negative.
Experiment 34. 75 specimens refed after 15 days. Result negative.
7 specimens refed after 23 days. Result negative.

In the last experiment *A. dentatus* were fed, also without any result:—

- Experiment 35. 5 specimens refed after 15 days. Result negative.
4 specimens refed after 23 days. Result negative.

In all, 25 *A. caballus*, which had fed on a spontaneous case of horsesickness, were injected after 7 days, 82 specimens of the same species refed after 15-23 days and 9 *A. dentatus* refed after 15-23 days. In none of the experiments was a positive result obtained.

GENERAL DISCUSSION OF THE RESULTS.

On horses infected with four different strains of horsesickness, the O-virus vaccine strain and one strain each from Losperfontein, Eshowe and Kaalplaas, 4,254 mosquitoes were fed, 308 *Culex theileri*, 5 *Anopheles squamosus*, 2,655 *Aedes caballus*, 628 *A. lineatopennis*, 292 *A. hirsutus*, 285 *A. dentatus*, 78 *A. vittatus*, 2 *A. punctothoracis* and 1 *A. cumminsi*. Furthermore, in one experiment, in which the actual number was not ascertained, at least 400-500 specimens had fed. To obtain these results, several times this number of mosquitoes, at a rough estimation, 10,000 specimens, had to be collected and handled.

With these mosquitoes 34 experiments were carried out in all (excluding experiment 28, in which the mosquitoes fed during a fever reaction, which, at first, was regarded as horsesickness fever, a diagnosis which, however, was not confirmed by the further experiments). In these experiments 1,434 mosquitoes were injected after $\frac{1}{2}$ -65 days, viz.: 245 *Culex theileri*, 5 *Anopheles squamosus*, 485 *Aedes caballus*, 287 *A. lineatopennis*, 328 *A. hirsutus*, 29 *A. dentatus*, 52 *A. vittatus*, 2 *A. punctothoracis* and 1 *A. cumminsi*. A large number of specimens was re-fed on susceptible horses at intervals varying from about 1 minute to 62 days, 704 feedings actually taking place, viz.: 9 feedings with *Culex theileri*, 190 with *Aedes caballus*, 207 with *A. lineatopennis*, 187 with *A. hirsutus*, 102 with *A. dentatus* and 9 with *A. vittatus*.

Positive results were obtained only by injections of mosquitoes. In the first case (experiment 1), five *Culex theileri* were injected the morning after having fed on an infected horse. This result only demonstrated, however, that sufficient virus to produce an infection had been taken up by 5 mosquitoes. In the second experiment (No. 16), 294 mosquitoes, 85 *A. caballus*, 115 *A. hirsutus* and 94 *A. lineatopennis*, which had fed 6 days before on a horse infected with O-virus, were injected. In the third positive case (experiment 24), 68 *A. caballus* and 66 *A. lineatopennis* had been injected. These mosquitoes had fed 7 days previously on a horse infected with Eshowe virus.

The horsesickness virus can therefore retain its full virulence in Aedes species up to 7 days. This result was obtained with two strains of virus, and *A. caballus*, *A. lineatopennis* and *A. hirsutus* were the species concerned, the former two species being common to both experiments.

The remaining 31 experiments were negative. We will combine the experiments made with the different virus strains according to the species used.

With *Culex theileri* four experiments were carried out with O-virus (Nos. 2-5). 240 specimens were injected after 5-25 days, viz.: 50 after 5, 50 after 16 and 140 after 25 days. Direct transmission was also attempted by partially feeding 9 specimens on an infected horse and immediately thereafter on a susceptible horse.

With *Anopheles squamosus* one experiment was conducted with O-virus in which 5 specimens were injected after 5 days (experiment 6).

Aedes caballus was used in 10 experiments, four with O-virus (experiments 7-8, 17-18), one with Losperfontein virus (experiment 19), three with Eshowe virus (experiments 25-27) and two with Kaalplaas virus (experiments 33-34). 332 specimens were injected after 5-16 days, viz., 94 after 5, 210 after 7-9 and 28 after 15-16 days. Mosquitoes were refed 190 times on susceptible horses after 14-23 days, viz., 159 after 14-17, 24 after 18-20 and 7 after 23 days.

Aedes lineatopennis was used in 7 experiments, four with O-virus (experiments 9-10, 17-18), two with Losperfontein virus (experiments 20-21) and one with Eshowe virus (experiment 29). 157 specimens were injected after 5-65 days, viz., 30 after 5, 121 after 7-9 and 6 after 63-65 days. 207 specimens were refed on susceptible horses as follows:—72 after 13-18, 59 after 19-22, 66 after 33-35 and 10 after 60-62 days.

Aedes hirsutus were used in five experiments, three with O-virus (experiments 11 and 17-18) and two with Eshowe virus (experiments 30-31). 213 specimens were injected after 5-30 days, viz., 30 after 5, 137 after 7-9 and 46 after 29-30 days. 187 specimens refed after 15-21 days, viz.: 91 after 15 and 96 after 20-21 days.

Aedes dentatus was used in 5 experiments, three with O-virus (experiments 12, 13 and 18), one with Losperfontein virus (experiment 32) and one with Kaalplaas virus (experiment 35). 29 specimens were injected after 5-16 days, viz., 6 after 5, 11 after 8-9 and 12 after 15 days. 102 specimens refed after 15-37 days, viz.: 49 after 15-18, 27 after 20-23, 20 after 25-27 and 6 after 35-37 days.

Aedes vittatus was used in three experiments, two with O-virus (experiments 14-15) and one with Losperfontein virus (experiment 23). 53 specimens were injected after 5-13 days, viz.: 20 after 5 and 32 after 15 days. 9 specimens refed after 23-37 days, viz.: 5 after 23 and 4 after 37 days.

Aedes punctothoracis and *A. cumminsi* were used in the experiment with O-virus (experiment 18) in which three specimens were injected after 8-9 days.

Amongst the various *Aedes* species employed the following number of specimens was used:—786 specimens were injected after 5-65 days, viz.: 18 after 5, 482 after 7-9, 72 after 15-16, 46 after 29-30 and 6 after 63-65 days. 695 mosquitoes refed on susceptible horses after 13-62 days, viz.: 375 after 13-18 days, 214 after 9-23, 20 after 25-27 days, 76 after 33-57 and 10 after 60-62 days.

Thirty-one experiments, in which (apart from 240 Culex and 5 Anopheles), 786 specimens belonging to Aedes species were injected after 5-65 days and 695 refed after 13-62 days, were thus negative. We cannot but regard these negative results as significant, in fact even more significant than the few positive results, the more so as these positive results were only obtained by injections of mosquitoes within a short period after their having fed.

Notwithstanding the, undoubtedly, sufficiently large amount of material used, it is not possible to arrive at a definite conclusion as to the results. On several occasions we have laid stress upon the fact

that the strains of virus used in our experiments were not really suitable. Most of the experiments were carried out with O-virus, the laboratory vaccine strain which had been isolated more than 30 years ago and had been transmitted about 200 times from horse to horse by direct inoculation. It must be regarded as possible that this strain, not having been in contact with its invertebrate host for such a long time, has lost its developmental capacity in insects either totally or partially. Furthermore, in each of the strains derived from spontaneous cases a history of immunization with O-virus existed. We therefore might have been dealing throughout the whole course of the experiments with O-virus and if this supposed reduction in its developmental capacity were an actual fact, the value of the negative results would naturally also be reduced.

The best, and perhaps correct, reason for our negative results might be the fact that we were not dealing with the real transmitters. We had this possibility in mind throughout the work. The results of the mosquito survey, based on epidemiological evidence, assumed to be correct but being beyond our control, pointed clearly to certain *Aedes* species as the most promising transmitters, and, almost daily observations in the field, yielded no further information of importance. It must be remembered, however, that the season at our disposal was not a suitable horsesickness season.

There is, furthermore, the possibility that errors in our methods were responsible for the failures. In the event of the infection index being low, a small number of specimens might be insufficient, but in this case it cannot be an important factor, considering the amount of material we used. The extrinsic incubation period allowed should have been long enough and the temperature at which the mosquitoes were kept was certainly high enough to allow of a rapid development or multiplication of the virus. So far as we can judge there do not appear to exist other factors of any importance which could be regarded as responsible for the failures. Further strong evidence in favour of the view that *Aedes* species are not the real transmitters is contained in the fact that 662 "infected" specimens belonging to this genus were injected without causing an infection. Horsesickness virus is very resistant and retains its virulence for a number of years at ordinary room temperature nor is it easily destroyed by putrefaction. Yellow Fever virus, on the other hand, which bears a close resemblance to horsesickness virus in many respects, loses its virulence very rapidly at room temperature, yet it remains alive for a considerable time in a large variety of mosquitoes more or less related to its natural transmitter. It appears strange, therefore, that horsesickness virus was so easily destroyed in all the species of mosquitoes used.

Taking every argument into consideration, we must come to the conclusion that *Aedes* species are in all probability not the transmitters of horsesickness. The nature of the strains used, however, makes a definite conclusion not possible.

In future work, experiments with spontaneous virus strains and *Aedes* species will, first of all, have to be carried out to ascertain the exact importance of these species, and should these fail, *Anophelines*

will then have to be tested. Furthermore, a thorough mosquito survey, during a suitable season, is of the utmost importance in order to obtain more comprehensive epidemiological data.

SUMMARY.

During the latter part of the summer of 1931-1932 and during the winter of 1932 experiments in connection with the natural transmission of horsesickness were carried out at Onderstepoort. The season was unfavourable on account of a shortage of rain.

The result of a mosquito survey, carried out at the same time and described in the first paper of this series, had pointed out that, taking into consideration the epidemiological evidence generally accepted as correct, certain *Aedes* species are the most promising transmitters of horsesickness amongst the flying insects. On this assumption mainly was our work based.

Four strains of virus were used, O-virus, the laboratory vaccine strain, and three strains derived from field cases of the disease.

The ordinary experimental technique in mosquito transmission work had to be modified and adapted to the special requirements (horses as experimental animals) and to the South African climatic conditions. The methods used were described in the second paper of this series.

Altogether over 4,500 clean mosquitoes, belonging principally to different species of the genus *Aedes*, were fed on experimentally infected horses. Over 10,000 specimens had to be caught or reared and handled.

In all, 35 experiments were carried out, in which the mosquitoes were either injected into susceptible horses or refed on them after different intervals.

1,434 specimens were injected $\frac{1}{2}$ -65 days, 245 *Culex theileri*, 5 *Anopheles squamosus*, 485 *Aedes caballus*, 287 *A. lineatopennis*, 328 *A. hirsutus*, 28 *A. dentatus*, 52 *A. vittatus*, 2 *A. punctothoracis* and 1 *A. cummingsi*.

704 mosquitoes were refed at from 1 minute to 62 days after their having fed on infected horses, 9 *Culex theileri*, 190 *A. caballus*, 207 *A. lineatopennis*, 187 *A. hirsutus*, 102 *A. dentatus* and 9 *A. vittatus*.

Three experiments only were positive. In the first of these experiments 5 *Culex theileri* were injected about $\frac{1}{2}$ day after their initial feed, indicating that sufficient virus had been taken up by this number of mosquitoes. In the second positive experiment 85 *A. caballus*, 94 *A. lineatopennis* and 115 *A. hirsutus* were injected after 6 days, and in the third experiment 68 *A. caballus* and 66 *A. lineatopennis* after 7 days. In these mosquitoes it was therefore demonstrated that the virus might remain alive for periods of up to one week.

The remaining experiments were all negative. 240 *Culex theileri* were injected after 5-25 days, 5 *Anopheles squamosus* after 5 days, 332 *Aedes caballus* after 5-16 days, 157 *A. lineatopennis* after

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5-65 days, 213 *A. hirsutus* after 5-30 days, 29 *A. dentatus* after 5-16 days, 52 *A. vittatus* after 5-15 days, 2 *A. punctothoracis* and 1 *A. cummingsi* after 8-9 days.

190 *A. caballus* refed after 14-23 days, 207 *A. lineatopennis* after 13-62 days, 187 *A. hirsutus* after 15-21 days, 102 *A. dentatus* after 15-37 days and 9 *A. vittatus* after 23-37 days.

In all, 786 *Aedes* species were injected after 5-65 days and 695 *Aedes* species refed after 13-62 days.

By injections of mosquitoes at intervals of up to 9 days, two positive results were obtained by using 428 specimens, whereas with 662 further specimens only negative results were procured. The virus is usually quickly destroyed, therefore, in the *Aedes* species, although it is normally very resistant, often remaining virulent at ordinary room temperature for a number of years.

The virus strains themselves were not very suitable for our work. The O-virus strain, with which most of the experiments were carried out, had been isolated about 30 years ago and transmitted through almost 200 generations from horse to horse without any passage through the natural transmitters. We must take into account therefore, the possibility of the virus having lost at least part of its developmental capacity in insects. In the other strains at our disposal, derived from field cases, a history of immunisation with O-virus against horsesickness existed, and it is thus possible that we were dealing throughout our work with O-virus.

From these experiments we arrive at the final conclusion, that *Aedes* species are very probably not the transmitters of horsesickness. It is, however, impossible to come to a definite conclusion owing to the nature of the strains used.

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