

Dr. Daniel's report to the secretary of the Malaria Investigation Committee of the Royal Society, London, on the results of investigations made by him in Calcutta in conjunction with Major Ross, I.M.S.

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CALCUTTA:

January 23rd, 1899.

SIR,

I have the honour to report the results of my observations since my arrival here on December 21st, 1898.

2. Major Ross, I.M.S., first demonstrated and explained to me his method of dissection of the mosquito and the structures normally met with. From prepared specimens he then showed me the bodies met with after feeding these mosquitoes on birds infected with the proteosoma and the change day by day which they showed, ending with a demonstration of the germinal threads in cysts in the stomach wall, as seen in the fluids of the body, and in cells in the salivary gland.

3. On my arrival there were in the laboratory in test tubes series of mosquitoes fed on birds infected with Proteosoma on the night of November 30th, December 10th, December 12th, December 15th, and December 20th.

Of each of these series Major Ross dissected specimens for me after killing the mosquitoes with chloroform, and again demonstrated in these the same bodies that he had already shown me in prepared specimens. Pointing out and demonstrating as he went on that in the older mosquitoes it was possible on cutting the thorax the nature of the contents both of the coccidia in the stomach and of those of the cells of the salivary glands.

The points showed to me I readily observed.

4. From series of mosquitoes before mentioned I day by day examined both those which died and others I killed, and was as readily able myself to repeat the observations and in the earlier series to trace the changes in the size and nature of contents of the coccidia.

I also examined a large number of mosquitoes caught about the laboratory and others which had been raised from larvæ. In no case did I find either coccidia in the stomach wall, germinal threads in the body fluids, or in the cells in the salivary gland; nor did I find "black spores" in them.

5. Major Ross informed me that his published results were based on observations made in the hot season when the temperature was 80°F., or more; and that now I should find the changes considerably slower as it was the cool season, but that the sequence of events was the same.

My observations on the mosquitoes fed on December 20th, and December 15th, showed that this was the case and that the coccidia advanced more slowly than the published results indicated. He also informed me that mosquitoes fed less readily and more difficulty was met with in rearing them to a spore bearing age.

These difficulties the use of the incubator was only partially successful in obviating.

6. On the evening of January 1st, following exactly in Major Ross' lines, I commenced a repetition of his main experiment. Two mosquito nets, free from rents, were taken, and in them were released a large number of grey mosquitoes reared from larvæ.

In the one, four birds were placed; in three of them on December 31st, I had found proteosoma in large numbers and in the fourth a moderate number.

In the other net two birds, in whose blood no proteosoma had been found, were placed; these two died, two and three weeks later, and no pigment was found in their organs and repeated examinations of their blood had failed to show proteosoma.

On January 2nd, none of the mosquitoes had fed, and on January 3rd, only 2 in the first net and 8 in the second. On January 4th, a warm night with a minimum temperature of 59.2°F ., 63 mosquitoes were found gorged with blood in the morning and were caught in separate test tubes plugged with wool and placed in the incubator. Eighteen in the other net, where the non-infected birds were placed, the control series, were similarly collected; these were caught in the same manner and treated in the same way.

On the following two evenings with minimum temperatures of 60.7 and 63.2 , 68 and 46 mosquitoes were fed on the infected birds and were kept for the preparation of specimens. Twelve mosquitoes were fed on the non-infected birds and were used as additional controls so as to bring the number of the control on Blue Jay with numerous Halteridia.

On the third day the 63, with exception of those killed for examination or dead, were released inside a clean net free from mosquitoes, and birds free from proteosoma were also placed in it.

In the morning all mosquitoes found inside were collected and most of them had fed well; the minimum temperature was 63.2°F .

This is the method Ross employs to refeed the mosquitoes. If infected birds are used you get a younger generation of coccidia; so I used sterile birds. The method works fairly well in warm weather; but there is always some loss, as the full number are not collected again in the morning. As the process is repeated over and over again, this loss becomes serious, the more so the longer the period required for maturation. In a frequently repeated process of this kind there is always the possibility of an outside mosquito getting in.

The mosquitoes were not fed on the following night as they were full of blood, but most of them voided it during the night and many died next day.

The remainder were given the opportunity of refeeding every night after this; but a spell of cold weather ensued with minimum temperatures of 44°F .— 49°F .; only on one night did it exceed 50°F .; and on these nights few fed well or at all and there was a consequent continued heavy mortality, only one being alive on the 10th day, and that subsequently escaped in the night.

This method of feeding is very unsatisfactory in exceptional weather of this kind; the mosquitoes in the day are kept warm in the incubator and rapidly digest their food whilst at night the cold renders them torpid and they do not feed.

The control mosquitoes were treated in exactly the same manner and fed on birds free from proteosoma. The last died on the 13th day.

7. The results of the two series are as follows:—

Sixty-three fed on proteosomal birds.

49 examined, 3 reserved for sections, 1 too much decomposed for satisfactory examination.

10 not accounted for, lost in the nets.

Of the 49 examined, two were killed on the first day, that is, under 24 hours, and possibly under 12 hours after they had fed. No coccidia were found in these. Two more were examined the following morning under 36 and possibly under 24 hours after they had fed, no coccidia were found in these.

In two examined about 4 P.M., the minute pigmented coccidia were found; that is, under 46 and possibly not more than 34 hours after they had fed on the infected birds.

The remainder were examined on the following days, the largest numbers 18 on the fourth day and 12 on the seventh day, as on these two days those 1 number died.

In every mosquito examined, with one exception, the coccidia were found; usually in numbers, but in one there was only one coccidia.

The exception occurred on the 9th day; as by then they had been refed several times, it may have been an outside one which had effected an entrance.

So that out of 45 mosquitoes fed on the infected birds and examined more than 34 hours after, 44 contained coccidia.

This I may say is a more successful result than in the other series I have seen.

The other two sets of mosquitoes were used by all of us for preparation of specimens and no record was kept of the number of failures. From my own examination only about $\frac{2}{3}$ of them developed coccidia.

The treatment was a little different and half of them were not incubated for several days.

Of the controls fed on birds free from *Proteosoma*, 38 in number and treated in the same manner, 29 were examined and 9 are unaccounted for—"lost in the nets". None of the 29 were examined on the first day, but one was in the afternoon of the second day. The largest number examined were on what would correspond to the fourth and seventh days, *i.e.*, 7 and 5; but there were 4 each on the 5th and 6th days.

It will be observed that these control mosquitoes were not, as the other series, collected on one, but on three nights. A very slight difference in breeze and light seems to affect the number who bite; or any extra restlessness on the part of the birds would have the same result.

In none of these 29 were coccidia found. Of the 18 fed on the Blue Jay with *Halteridia*, 12 were examined from 2 to 6 days after feeding and none contained coccidia.

8. The forms found on the second day measured 6-7 μ , some of them a little more. They were oval bodies containing scattered granules of black pigment, and had a sharp, clear outline.

I incised the stomach and by repeated washing and compression with a coverglass was able not only to wash out the contents of the stomach but even to express the loosely attached epithelium, so as to leave the stomach as a transparent clear bag. To this outer wall the majority of coccidia remained fixed, though in one of the mosquitoes I observed some to escape with the epithelium. At no subsequent date could I ever detach any by this process, though some coccidia would be ruptured.

The next morning the smallest measured 10 μ ; some were 12 μ . On the sixth day they were met with up to 30 μ ; by this time the pigment had absolutely as well as relatively diminished.

In another three days some of them reached 60 μ ; and in the last of the series examined (10th day) there were coccidia measuring 70 μ .

The coccidia could now be seen to project from the outer wall of the stomach; very few contained pigment and that in small amount.

Some of the coccidia were clear and others had a granular appearance; but in none were there either black spores or germinal threads to be seen.

9. For the further development the early deaths of the mosquitoes from the inclemency of the weather rendered this series useless.

One of those which were infected on the night of January 5th, and another infected on January 7th, did reach this stage; and in the last of those first fed on January 5th, which died on January 22nd, ruptured cysts were found by me in the stomach wall, as well as numerous cysts containing mature germinal threads, and these threads were also found in the body fluids and in cells in the salivary glands.

My observations are, therefore, mainly based on those infected November 30th, and subsequent dates before my arrival, and on some infected December 22nd. The one infected on January 5th died on January 19th, and the coccidia in it had an appearance of striation.

On adding salt solution (Gr. XV to the ounce) and pressing on the cover glass, a projecting coccidium was ruptured; and the contents poured into the fluid, leaving the cyst wall still attached to the stomach.

The contents were seen to consist of a mass of shrivelled threads. This appearance in the other series mentioned I have frequently seen.

These threads, Ross' germinal threads, are sickle shaped bodies about 14-15 μ in length, they stain with logwood or methyl blue but not strongly; on adding water or Farrant's solution they lose their shrivelled appearance and become more rounded. Nearer one end than the other is an unstained portion (? Nucleus).

They show no signs of movement; but as they are invisible in water and only become visible when shrivelled by the salt or stained, it may be doubted if they have been seen alive.

If a mosquito has its thorax incised when rather older than this, similar threads are found in the fluid exuded if salt solution is added to it.

In such a case ruptured cysts are found in the stomach wall.

The position as regards the salivary gland involves a difficulty which is not met with in any other part of the examination.

The dissection of the stomach is easy; that of the salivary gland in its entirety is not, and for some reason appears to be more difficult in the old infected mosquitoes. Any rough manipulation results in the detachment of the cells and little more than the duct is left. In most cases however one entire gland, or portions of both, can be exposed in fair condition even in old infected mosquitoes.

In every case where this was done and germinal threads were found in the body-fluids, the germinal threads were also found in some of the cells in the salivary gland; and no similar threads have I found in a large number of salivary glands examined by me in mosquitoes bred from larvæ, free about the laboratory; or in the earlier stages of coccidial infection.

The affected cells can be distinguished with a low power, as they have a granular appearance, whilst the unaffected cells are quite clear.

With a high power, if not very numerous, the isolated germinal threads can be clearly distinguished and recognised by their peculiar shape and shrivelled appearance (the examination must be made in salt solution). If numerous, the individual threads can be no more distinguished than in the coccidia, but, as in those, pressure on the cover glass will rupture the cell and the germinal threads are then poured out.

The threads do not fill the cell. There is a faintly granular crescentic portion on the side most remote from the duct which in many cases at least is free. The part of the cell in which the threads lie must be nearly fluid as it permits oscillations of the threads to take place.

On these points I have satisfied myself by repeated examinations, though the appearances are by no means difficult to make out; and have gone at some length into the question, as so far we have found no satisfactory method of making permanent preparation. All the preservatives at our disposal wrinkle up the delicate cells, with the exception to some extent of weak formalin solution; and I have no confidence in that as a means of making permanent specimens.

The whole gland is never involved. In one dissection made by Ross the cells in both middle lobes and in no other part of the gland contained the threads. In several cases where one gland has been exposed entire, the middle lobe alone has been involved; but in the majority all that can be stated with certainty is that the cells in one portion of the gland contain threads and in other portions they do not.

The following specific observations made by myself on mosquitoes dissected by Major Ross, Dr. Rivenberg of the American Mission who is working with Dr. Ross, and myself may be of interest.

- (a) Coccidial cysts full of apparently mature germinal threads, no ruptured cysts, no germinal threads in the body fluids or salivary glands. 2 observations.

- (b) Cysts full of germinal threads, other ruptured empty cysts, germinal threads in body fluids, germinal threads in salivary glands. Over 20 observations.
- (c) Empty cysts in stomach wall, germinal threads in body fluids of thorax. Germinal threads in salivary glands. No cysts still containing germinal threads. 2 observations.
- (d) Empty cysts only in stomach wall, no germinal threads in body cavity, no germinal threads in well exposed salivary glands. One observation, the mosquito had been infected four weeks before death.

These observations fully confirm Ross' statements in every point. They indicate that the threads are formed in the coccidia, that they escape on the rupture of these into the body cavity and are again collected in the salivary glands. I should have liked to extend the series, but the continued cold weather renders it improbable that I shall be able to do so before I leave.

10. With your permission I should like to publish an abstract of this, confirming Ross' work; and to this Major Ross consents.

In case you should consider this advisable I am, to avoid delay, forwarding an abstract to Dr. Manson, with a request to him to forward it to the British Medical Journal, if your consent is granted.

11. The infection of birds free from proteosoma by the bites of mosquitoes.

On December 20th the day before my arrival 22 birds were examined and found free from proteosoma. On that night some of these were used for feeding the mosquitoes which had been infected on November 30th and on the 24th and subsequent days; the remainder of the birds were used for feeding the mosquitoes first infected on November 30th and December 10th, 12th and 15th. In other mosquitoes of this series germinal threads were found in the salivary glands; and the ones which fed, when examined later, gave the results indicated in paragraph 9.

On December 30th Dr. Rivenberg and myself examined these birds; 3 of them had proteosoma, 2 in large numbers.

On January 4th I examined them all except one which died on January 2nd; in that the heart's blood contained no proteosoma and the organs were free from pigment.

Five more of these had now proteosoma, all very numerous. On January 6th and 7th I again examined them; 3 more had proteosoma all very numerous.

On January 9th no more cases had developed. But on January 18th one of them had numerous proteosoma; whilst many of the ones which had been infected had recovered, and the others now showed few proteosoma.

Thus 12 out of 22 birds became infected or 54 per cent. This compares unfavourably with Ross' earlier results, as, in his published series, 22 out of 28 were infected, or 79 per cent. But it is to be remembered that at the time he was working the germinal threads were found in a week; whilst in December the development was much slower, and now takes at least twice the time. It is much easier to keep the mosquitoes alive for one week than longer, while in the hot weather mosquitoes bite more readily.

These results are less unfavourable, if compared with the normal proportion of birds infected with proteosoma at this season. Thus Ross out of 111 wild birds found proteosoma in 15 or 13.5 per cent; whilst I find at this season only one with proteosoma out of 30 or 3.3 per cent.

It is possible that in the cold season the birds have a greater power of resistance; and this is rendered more probable by the short duration of the proteosomal attack in my infected birds. Of these 12, 5 died within the first week. In 3, in which also the proteosoma had been very numerous, none could be found 10 days after the invasion; in one in which they were never numerous none could be found on the fifth day.

In the other 3, very few are now found; though at first they were numerous

The recovery of these birds and the death of the mosquitoes fed on them diminishes the chances of much future work on this line in the time remaining to me here.

12. Mention has been made of the differentiation of the contents of the coccidia previous to the formation of the germinal threads into clear and granular; the second of these can be traced day by day into those forming the germinal threads. This differentiation was clearly visible in my series. Instead of germinal threads in a minority of the coccidia, in most mosquitoes, when the germinal threads are mature black tubular bodies are found in cysts with otherwise clear contents.

These were met with frequently in the series of mosquitoes infected in November and December. Most of these contained some coccidia with black spores; though in few all the cysts contained germinal threads. In some cysts these black spores are numerous and occupy the entire cyst; in other cysts there are only a few. In most cases germinal threads are not found in the same cyst; but there have been a few cysts in which it has been doubtful whether there are germinal threads also in the cyst, or whether there are overlying escaped threads from a neighbouring capsule.

These black spores are very resistant; I have seen some kept in water for months by Ross with no visible change, and they will withstand irrigation with Liquor Potassæ.

When the cysts are ruptured the spores are found all over the body, but not in cells; nor do they seem to accumulate in any one part of the body.

The most plausible view of the nature of these black spores seems to be that held by Major Ross, *viz.*, that they are "resting spores," and that through them by another cyst the proteosoma can be propagated in conditions unfavourable for direct propagation by injection into a warm blooded animal.

In that case three courses suggest themselves:

a. From them arise bodies capable of non-parasitic life and possibly of reproduction, but capable at certain stages of their existence of introduction into a warm blooded host by inhalation through drinking water, or even by injection by a mosquito or other blood sucker transferring them from the medium in which they live directly.

b. That they may be ingested by mosquito larvæ and in them undergo such development as will result in the formation of germinal threads in the adult, which in turn might be injected into the bird.

c. That they may when swallowed or inhaled by a warm blooded host so develop as to reach the circulation and pass into the sporulating phase.

Such experiments as have been made are inconclusive; and it is obvious that till the nature of these "black spores" is determined we cannot exclude, even for the proteosoma of sparrows, the possibility of some one of the many alternative possible channels of infection, some of which would only require the occasional intervention of an intermediate host.

Still less are we justified in concluding that Malaria in man can only be acquired from the mosquito or devoting our exclusive attention to that channel.



