No. 57.

(*NEW SERIES.*)

# SCIENTIFIC MEMOIRS

BY

OFFICERS OF THE MEDICAL AND SANITARY DEPARTMENTS

OF THE

GOVERNMENT OF INDIA

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**No. I.**

***The Morphology and Life History of Herpetomonas culicis, Novy, MacNeal and Torrey.***

BY

CAPTAIN W. S. PATTON, M.B., I.M.S.,

*Assistant to Director, King Institute of Preventive Medicine, Madras.*

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# Studies on the Flagellates of the Genera Herpetomonas, Crithidia and Rhynchoidomonas.

## No. I.

### The Morphology and Life History of Herpetomonas culicis, Novy, MacNeal and Torrey.

IN several earlier papers I have described in detail certain flagellates of the genera Herpetomonas, Crithidia, and Rhynchoidomonas, and it was then pointed out that the knowledge of the life histories of these important protozoa would not only help in solving the problems connected with the methods of transmission of the herpetomonads of Kala Azar and Oriental Sore, but would also lead to a better understanding of the morphology and life histories of the pathogenic and non-pathogenic trypanosomes.

In a recent paper on the life history of *Herpetomonas muscæ domesticæ* in *Musca nebulo*, bred and infected in the laboratory, it was shewn that in nature this fly becomes infected with this flagellate in one of three ways: (1) By ingesting the long flagellates which are passed out in large numbers in the fluid excreta of heavily infected flies. (2) By ingesting the pre-encysting forms, also discharged in the fæces of *Musca nebulo*, and (3) by ingesting the ripe cysts.

The study of these flagellates, outlined in my paper on *Herpetomonas muscæ domesticæ*, opens up an entirely new field of research in connection with these parasites, and it may well be compared with Novy's ingenious devices for studying the trypanosomes of vertebrates in pure cultures. It differs however from cultural methods in that the observer has the advantage of following in the insect host, on each successive day, those vital and important changes which culminate in the preparation of the parasites for their brief stay outside the alimentary tracts of their hosts; these changes cannot be studied in the test tube.

All previous observations on *Herpetomonas muscæ domesticæ* have been made on caught flies, and in them it is quite impossible to form any idea, either of the method by which the fly becomes infected, or the stage in which the parasites were at the time of examination. It is therefore easy to understand why my views regarding the structure of *Herpetomonas muscæ domesticæ* differ so considerably from those of other observers, who have only studied this herpetomonas in flies caught at large; this is a point which is frequently overlooked.

In my laboratory experiments with *Herpetomonas muscæ domesticæ* in *Musca nebulo*, the flies were first bred, and hereditary transmission was never found. Next, the bred flies were infected and then placed in glass jars which were further surrounded by a fly proof cage, and in this way it was quite impossible for the flies to have become infected with any other flagellate. It was then found that *Herpetomonas muscæ domesticæ*, having passed through its stage of longitudinal division (the stage in which it is said to be a bi-flagellate), ceased to divide, and then it exhibited all the characters of a typical herpetomonas, with a single flagellum, the so-called *Leptomonas*. Strange to say, it has been suggested that my flies had at this time become infected with a second flagellate, a *Leptomonas*, and thus the finding of the uniflagellate. Those who believe that this actually happened cannot have read my paper describing the experiments in question, for it was physically impossible for any other insect to have come in contact with the flies. I am therefore at a loss to understand the object for such statements. There could be no doubt whatever that the flagellates, which appeared in the flies towards the end of the experiment, were none other than those which had resulted from the division of the earlier forms. These experiments conclusively proved that *Herpetomonas muscæ domesticæ* is a uniflagellate protozoon and the generic name *Leptomonas* can therefore only be a synonym. The methods which have been adopted in studying the flagellates of insects have undoubtedly led to the present confusion, and the introduction of numerous generic names such as *Leptomonas*, *Leptotrypanosoma*, etc., has now been carried to such an extent, that the main points at issue, namely, the true morphology, life histories, and phylogeny of these parasites, have been overlooked.

Apart from these considerations, it is very necessary to study the flagellates of insects, for, as they are so little understood, they have in the past been confused with pathogenic forms which the insects may have ingested. Many erroneous conclusions have been drawn regarding the true origin of these flagellates, especially those which are parasitic in blood sucking insects. The methods by which insects become infected are so diverse, that even with first hand knowledge, it is often difficult to distinguish them from pathogenic ones. For these reasons I propose in the present paper (the first of a series), taking up the study of the flagellates of the genera *Herpetomonas*, *Crithidia* and *Rhynchoidomonas* parasitic in several species of *Diptera*, *Siphonaptera*, *Rhynchota* and *Ixodidæ*, which I have had the opportunity of studying during the last seven years.

I do not however intend entering into any further controversy with those who believe that the genus *Herpetomonas*, Kent, should be split into two, retaining the genus *Herpetomonas*, *sensu stricto* for those flagellates which are

supposed to possess two flagella, and erecting the genus *Leptomonas* for those which have only a single flagellum ; nor do I intend discussing any further as to whether the flagellates of blood sucking insects are merely developmental stages of known or unknown vertebrate trypanosomes. Nothing further can be gained by continuing such controversies ; those who hold these views have either never studied these parasites, or they have never attempted to work out their life histories in insects bred and infected in the laboratory.

## The Flagellates of Mosquitoes.

It is now well known that mosquitoes in different parts of the world are infected with flagellates of the genera *Herpetomonas* and *Crithidia*. In 1898 Ross encountered them while conducting his researches into the etiology of malaria, and in 1906, he again called attention to these early studies. Ross correctly states that these flagellates had originated from earlier stages present in the larvæ, and that they are therefore present in the adult insects before they have fed on blood. In 1900 Durham found flagellates in a specimen of *Stegomyia fasciata* which had fed on a small bat ; and notes that they were quite different in structure from the trypanosomes found in the blood of vertebrates. In 1901 Chatterjee found a flagellate in an anopheles in Calcutta, which he assumes was related to *Trypanosoma evansi ;* there can be little doubt however that this was one of the harmless flagellates of mosquitoes. In the same year Christophers records the presence of swarms of flagellates in the rectum and hind gut, in a large proportion of anophelines and culicines he had occasion to examine. In their book on Malaria, Stephens and Christophers draw attention to these flagellates and clearly depict them.

Léger, in 1902, is the next observer who has studied the flagellates of mosquitoes, and he describes one from the alimentary tract of *Anopheles maculipennis*, which he considered to belong to a new genus, *Crithidia*, and which on account of certain morphological characteristics, he rightly believed to be intermediate between *Herpetomonas* and *Trypanosoma*. In the same year, Léger, in collaboration with Duboscq, found this flagellate in the alimentary tracts of anopheles larvæ, as well as in those of the hibernating insects; this is an observation of the utmost importance, and if it had been fully realised by subsequent observers, there would to-day have been fewer erroneous statements regarding the nature of these parasites. Still later, the brothers Sergent record the presence of a herpetomonas in *Culex pipiens* and in *Stegomyia fasciata* both in the larvæ, pupæ, and adult insects. In 1907, Novy, MacNeal, and Torrey recorded the presence of *Herpetomonas culicis* and *Crithidia fasciculata* in *Culex pungens*, *Culex sylvestris*, and *Culex triseriatus ;*

they found 6·2 per cent. of the mosquitoes to be infected with *Crithidia* alone, and 6 per cent. with *Herpetomonas*, while 2·1 per cent. contained both *Crithidia* and *Herpetomonas*. It is most important to note that mosquitoes may be infected with *Herpetomonas* as well as with *Crithidia*. The writer has recorded the presence of these flagellates in *Culex fatigans*, *Culex sp.* and *Neocellia stephensi*, in Madras ; Adie has found them in several species of anopheles mosquitoes in North India. Lastly Wenyon describes a herpetomonas from the malpighian tubes of the larvæ of *Stegomyia fasciata* in Bagdad.

It is unnecessary to make any further reference to Schaudinn's observations on the development of the blood parasites of the little owl in *Culex pipiens*, they have been fully discussed by Novy as well as by the writer.

Now let us suppose that an investigator in Madras, suspecting that the parasite of Kala Azar is transmitted by some species of mosquito, collects a large number of the common forms, either by catching the adult insects, or by breeding them out from the larvæ. Let us suppose, for instance, he selects *Culex fatigans*, *Neocellia stephensi*, and *Myzomyia rossi*. After feeding these mosquitoes on Kala Azar patients he would most certainly find flagellates in a varying percentage of those he examined; and would naturally conclude that they represented the developmental forms of the human parasite. This is exactly the conclusion the writer came to in 1905. Precisely the same results would be obtained if the observer fed the mosquitoes on cultures of the human parasite, here however the mosquito may at the same time contain three different species of flagellates in its stomach, a possible double infection of herpetomonas and crithidia, as well as the flagellate of the parasite of Kala Azar. When the mosquitoes were only fed on the peripheral blood there would be less chance of their containing the flagellates of the human parasite.

The observer who only has a slight acquaintance with the flagellates of mosquitoes would be further led astray on finding that there was a great variation in the number of infected mosquitoes (females). He would, for instance, find that in a batch of mosquitoes caught, say in a particular well or house, or bred out from larvæ collected from a certain tank, pond or well, that not a single mosquito after being fed on the Kala Azar patient contained flagellates ; and might conclude that this species was not the suitable host of the human parasite, or that none of the female mosquitoes sucked up parasites. On the other hand he may find almost every female mosquito infected, and if it was of a different species, he may conclude that he had at last found the true invertebrate host of the parasite ; the explanation would most probably be, that this particular mosquito was taken from a well where it readily becomes infected with its herpetomonas or crithidia. The fact that a particular mosquito, when collected

from a well, is nearly always infected with flagellates, and that the same mosquito bred out from larvæ from a tank is never infected is of the first importance in connection with such feeding experiments.

If the observer carried his observations further he would find that an infected mosquito often passes out the flagellates in its excreta, and that in addition to the long mature forms he would find many short and round forms, closely simulating similar stages of the parasite of Kala Azar. In the mid-guts of the mosquitoes he would find flagellates, as well as non-flagellated forms, and all the intermediate stages between the two. In some of the mosquitoes he would find only a few parasites, while in others he would find vast numbers. All these observations would support his view that the parasites were none other than developing parasites of Kala Azar.

In a recent paper Franchini claims to have observed the multiplication of the parasite of Italian Kala Azar in the alimentary tracts of *Anopheles maculipennis* and *Stegomyia fasciata*. In this case the mosquitoes were fed on cultures of the human parasite, and were examined at varying intervals. From half to two hours after the mosquitoes fed, flagellates were abundant, but after eight hours they became rare, and then appeared more globular. Twelve hours after the insects fed, fewer parasites were found, and they were now without flagella. After an interval of from twenty-four to forty-eight hours, the parasites had all rounded up and were exactly similar to the characteristic intracellular stage of the parasite of Kala Azar. Franchini concludes that the flagellated stage of the human parasite can live, and develop, in these mosquitoes, and that it passes on to its round or oval form, which is then ready to be transmitted to man. It is not quite clear where these various stages were found, but presumably the rounding up forms were most abundant in the hind guts of the insects. Franchini makes the extraordinary statement that, in spite of the presence of bacteria in the mosquitoes, the flagellates persisted and continued to develop. It is well known that the parasite of Indian Kala Azar and that of Italian Kala Azar will never flagellate in any medium infected with bacteria, it is therefore difficult to understand how they were able to maintain themselves in these mosquitoes.

In a still more recent paper Franchini again draws attention to his earlier observations, this time depicting the various stages of the parasites. In view of my failures to infect mosquitoes with the parasite of Indian Kala Azar, and knowing that many species are infected with very similar flagellates I find it necessary to carefully analyse Franchini's observations. At the outset he states he has been unable to recover any stages of the parasite of Italian Kala Azar in the alimentary tracts of *Cimex lectularius*, *Ctenocephalus canis* (*P. serraticeps*) and *Pulex irritans* though they had fed on cultures containing

numerous parasites, similar results were obtained in the case of *Pediculus capitis*. Several hundred experiments were carried out with *Anopheles maculipennis* fed on cultures, and numerous controls were examined. He states that the parasites found in the mosquitoes fed on cultures, could not be mistaken for any natural flagellates they may contain, for the latter can be distinguished by their nuclei, different staining and much larger size. After years of study of the flagellates of mosquitoes, especially *Herpetomonas culicis*, I would feel extremely diffident in expressing an opinion as to the true nature of a herpetomonad occurring in a stained smear of the midgut of any mosquito which had fed on cultures of the parasite of Kala Azar. I must confess I know of no differences in the structure of their nuclei or of their reactions to stains, especially to Romanowsky's or Giemsa's stain. It is true that, in the majority of instances, the flagellates of mosquitoes are larger than the corresponding stage of the human parasite. Franchini's diagrams however do not in my opinion bear out even this point. In his plate 2, the figures are said to be drawn with a Zeiss objective 6, and compensating ocular 8, presumably with a camera lucida, though this is not stated; this would represent a magnification of about 500 diameters. The longest mature flagellate depicted in Figure I may well be compared with figure 15 in the plate accompanying this paper. My figures of the mature flagellate stage of *Herpetomonas culicis* were drawn by the aid of a camera lucida with a Leitz 1/16 oil immersion and compensating ocular 8 and are therefore magnified about 1260 diameters. The flagellate noted in Franchini's plate 2, proves then, on comparison, to be the same size as that depicted in figure 15, and only slightly shorter than the very long forms, figures 30 to 37. Precisely the same will be noted on comparing the figures of the round forms of the parasite from *A. maculipennis* with the round forms depicted in my plate. This would be exactly in accordance with my observations, for as stated above though most of the flagellated forms of *H. culicis* are larger than the corresponding stage of the parasite of Indian Kala Azar, many are actually smaller.

It is evident that Franchini has encountered the natural flagellates of *A. maculipennis*, for he states that they are rather scarce; he does not however mention whether his mosquitoes were bred from larvæ, or caught as mature insects. Nor does he tell us whether these natural flagellates of *A. maculipennis* were commoner in certain batches of these insects, and rare in others; points which I have laid stress on above. There can be very little doubt that Franchini has actually seen the flagellated forms of the parasite of Kala Azar in the mosquitoes, but the proof that all the forms he depicts, are those of the human parasite, are in my opinion based on very slender evidence. It is unfortunate that no precise statements are made as to the exact part of

the alimentary tract in which they were found. The nature of a herpetomonad, based on staining reactions, will not hold in the case of these parasites, neither are structural details of much value. A parasite from the alimentary tract of a blood sucking insect, be it exactly like the parasite of Kala Azar as it occurs in man, is no proof as to its true nature, yet I find Franchini relies to a great extent on this point. The granular structure as depicted by Franchini in the parasites of *A. maculipennis* are extremely well seen in *H. culicis* as will be seen on referring to my figures of this flagellate. Karyokinetic figures in the nuclei of the parasites of Kala Azar as seen by Franchini, are just as well seen in the nuclei of *Herpetomonas culicis*, so that these appearances cannot be used in distinguishing the two parasites. It is definitely known that both *Anopheles maculipennis* and *Stegomyia* are naturally infected with flagellates, and I can find no proof in Franchini's paper that he has conclusively excluded the possible presence of these parasites in his mosquitoes. I may also point out that control experiments are really of very little value, because the number of infected mosquitoes varies according to the opportunities their larvæ have of becoming infected. I have repeatedly proved this in the case of *Herpetomonas culicis* in *Culex fatigans*. In specimens of this mosquito, bred out from larvæ collected from tanks, I have never found this flagellate, whereas out of those hatching from larvæ breeding in wells, from 50 to 90 per cent., are usually infected.

I am therefore of opinion that though Franchini undoubtedly has seen the parasites of Italian Kala Azar in his mosquitoes, he has omitted to rigidly exclude other flagellates, and that these forms were present in the hind guts and fæces of his insects in their post-flagellate stages; this stage is extremely like the human parasite in every way. *Whether the parasite of Italian Kala Azar will actually pass from its pre-flagellate to its flagellate stage, after being ingested by the mosquito in a leucocyte from the peripheral blood of a patient, has yet to be demonstrated; herein will lie the proof of the mosquito, transmission hypothesis.*

## Material and Methods.

The *Herpetomonas* which forms the subject of this paper is parasitic in the alimentary tract of the larva, nymph, and imago of *Culex fatigans*, and it is in all probability identical with a similar flagellate, described from America by Novy and his Collaborators. I therefore propose adopting the specific name *culicis*. There can be little doubt that this herpetomonas was encountered by Ross when conducting his classical researches into malaria, and he has already described some of its stages. *Culex fatigans* is abundant in Madras at all times

of the year, and breeds in wells and tanks ; it is particularly fond of breeding in the septic tank of this Institute, and over 95 per cent. of the larvæ taken from this situation were found to be infected. With this rich material close at hand, there was no difficulty in working out its complete life history.

A large number of larvæ, of all ages, were collected, and placed in large stoneware trays, and on selecting a particular specimen, its alimentary tract was dissected out in ·6 per cent. saline solution on a glass slide, and then examined with a No. 3 objective. After a time it became possible to detect infected larvæ by examining their alimentary tracts with a low objective, for the parasites are only found at the junction of the malpighian tubes with the mid-gut, and if infected, this portion has a characteristic glistening white appearance. It was also found that the malpighian tubes of an infected larva were never swollen, and packed with granules, but were shrunken and contained fewer granules. On dissecting out the alimentary tract, that portion of it containing the parasites was transferred to a clean slide in a drop of saline solution, and before placing a cover slip over it, a hair was fixed at the side and the cover slip edged with vaseline ; the parasites could then be examined in the fresh condition, and as the hair prevented undue pressure they could be studied for a considerable time without the gut rupturing. If it was desired to stain them, the part containing the parasites was still further isolated, the malpighian tubes being carefully removed, the alimentary tract was then teased up with fine needles by the aid of a dissecting microscope. The emulsion containing the parasites was next exposed to the vapour of osmic acid, and with a small piece of square cover glass it was lightly smeared out on a clean slide, immediately fixed with absolute alcohol, and later stained with Romanowsky's stain ; the same technique was employed in the case of the parasites in the nymph and imago.

The adult mosquitoes were also bred out and were fed on bananas and human blood, and the behaviour of the parasites under these conditions was studied. There can be no question as to the origin of this herpetomonas, for the larvæ could obtain the parasites from no other source than the water in which they were living. In the tank mentioned above, many thousands of mosquitoes were resting just above the surface of the water, and would naturally pass out their excreta on to it, and the larvæ when feeding could not do otherwise than ingest the postflagellate stages. It is interesting to note that the mature flagellated form of the parasite was never seen in the larvæ, yet the mosquitoes frequently pass out this stage in their excreta ; the flagellates do not appear to be able to live in the water or in the alimentary tracts of the larvæ. The intestines of the larvæ frequently swarm with bacteria, and this would probably explain the absence of the flagellated forms in them. It is true that the pre-

flagellate stage may flagellate in the larval gut, but this only occurs in the mature larvæ, which have by this time cleared themselves of most of the bacteria.

Two other mosquitoes, *Culex concolor*, and a species of *Joblotia*, which were breeding in the same tank, were never infected with *Herpetomonas culicis ;* the larvæ of the former were feeding on the larvæ of *Culex fatigans*, and they must have ingested the pre-flagellate stage, yet the parasite was never found in them. This fact points to *Culex fatigans* being the specific host of this parasite in Madras.

There is a small fly, *Ephrydid Sp.*, which is commonly found on the surface of stagnant water in Madras, and it was abundant in the septic tank ; I have long known that it is infected with a species of herpetomonas, but I am unable at present to say whether it is identical with *Herpetomonas culicis*. A large number of experiments would have to be carried out before this question could be decided ; owing to the great pleomorphism exhibited by these insect flagellates, and also to the fact that many of them are indistinguishable, it is futile to attempt to make any definite statement on this point. I would however like to point out that accurate experiments in this direction would undoubtedly help towards settling the question, as to whether these flagellates occurring in different insects belong to one and the same species. I have already carried out some experiments to try and see whether *Herpetomonas muscæ domesticæ*, which is parasitic in *Musca nebulo*, will live and develop equally well in *Lucilia serenissima*, but have found that the pre-flagellates and flagellates of this herpetomonas are unable to live in the alimentary tract of this fly; both *Musca nebulo* and *Lucilia serenissima* feed side by side on the meat in the bazaar shops. In the same way the herpetomonas of *Lucilia serenissima* will not live in the alimentary tract of *Musca nebulo ;* all these experiments were carried out with bred flies. These observations will be referred to in detail in the papers dealing with these parasites.

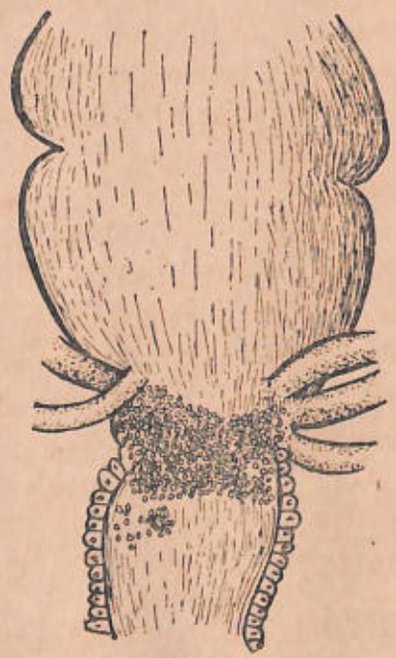
## THE STRUCTURE AND LIFE HISTORY OF THE PARASITE.

### The Pre-flagellate Stage.

The pre-flagellate stage of *Herpetomonas culicis* is almost entirely confined to the larval gut, but may also be found in the nymph, and even in the imago ; it is in this latter situation that this stage may lead the observer astray. It was pointed out that on dissecting out the mid and hind gut, the parasites will be seen massed together, and under a dissecting lens they appear as a

glistening patch, just posterior to the junction of the malpighian tubes with the mid-gut; they were never seen in the urinary tubules.

On examining the mass of parasites under a 1-12 objective in the fresh condition the individual parasites can be readily studied; they appear as round, oval, or elongated bodies, depending upon their degree of development, the elongated forms represent the gregarine or resting stage of earlier authors. Their protoplasm may contain many granules grouped together at one or other end; their nuclei appear round, and frequently the small rod-shaped blepharoplasts may be detected. If such a group of pre-flagellates is watched for some time, the individual parasites will be seen dividing, a process which may occupy an hour or more. There can be little doubt that these young forms adhere together by some sticky substance, for considerable pressure is required to dislodge them from the intestinal wall, and when this is accomplished, two, four, or more, may be seen adhering together, and the coverslip must be further compressed before the individual parasites can be separated. It is very probable that this secretion is necessary in order that the parasites may remain attached to the intestinal wall, otherwise they might be expelled by the powerful peristaltic waves which pass along the mid and hind gut.



Text Fig. I.

In the medium sized and mature larvæ, the parasites may be seen in large numbers packed together in many layers, so that they extend into the upper part of the hind gut. Text Fig. I. is a diagramatic illustration of an infected larval gut as seen under a low power. On smearing out the

part of the gut containing the parasites, and staining it with Romanowsky's stain, all the changes, undergone by the parasites during the pre-flagellate stage, can be well studied; I will now describe the minute structure of these forms.

Figure I. on the plate represents the early pre-flagellate stage, which is found in the small larvæ. These forms measure about 4$\mu$ in breadth and are almost globular in shape; their protoplasm stains blue, is vacuolated, and may contain a number of large, dark pink staining granules, either scattered, or close together. The outer layer of the protoplasm, or ectoplasm is of the nature of a closely fitting sheath. The nuclei of these forms are as a rule situated centrally, but may lie to one or other side; they stain a light pink, and may contain a dark central granule or a number grouped together. The nuclei frequently shew a well marked nuclear membrane. Lying at some little distance from the nucleus will be seen the rod-shaped blepharoplast. In deeply stained specimens, and more particularly in those which have ruptured, a delicate pink filament may be seen arising close to the blepharoplast and stretching across the cell. This is the flagellar myoneme, and it is from this structure that the flagellum arises later on; it has been noted in my paper on *Herpetomonas donovani*.

The pink staining granules noted above are clearly connected with the growth of the parasite, for if infected larvæ are placed in clean water for several days, and the parasites they contain are then examined, the granules will be found to be fewer in number, and may even disappear altogether. They are therefore of the nature of food granules, but as there is no satisfactory means of analysing them chemically I am unable to express a definite opinion regarding their true nature.

In the fresh condition, these young parasites do not shew any movement, their shape remains the same, although as growth proceeds, they slowly enlarge, their protoplasm becoming more voluminous and they now exhibit the changes preceding simple biniary fission, figures 2 and 3. Their nuclei at this time increase in size, and appear more granular, they then become dumbell shaped, figure 4. At the same time their blepharoplasts elongate, preparatory to division. Later the nuclei divide and the two halves separate, and this is followed by the division of the blepharoplast, figures 5, 6 and 7. The parasites now become constricted at one end, and later spilt into two, and many of them may be seen still attached at one or other pole. This process of division appears to be the common one, and as it is repeated several times, the parasites become massed together in layers. They adhere to each other, as well as to the intestinal wall. A careful search was made as to whether the parasites ever became intracellular, but they were never seen inside the intestinal cells.

Figure 8 depicts a much enlarged parasite about to divide into two. Many of these forms, however, instead of immediately dividing up, go on enlarging. Their nuclei and blepharoplasts divide again, so that eventually they may contain three or four nuclei and an equal number of blepharoplasts, figures 9, 10 and 11. Such a parasite on ceasing to enlarge splits up into three or four smaller ones, figure 12, the protoplasm collecting around each nucleus and blepharoplast. This process of multiple segmentation in the pre-flagellate stage is uncommon, the parasites usually splitting into two before they have attained any great size. This method of multiple segmentation in the pre-flagellate stage is similar to that which takes place in the case of *Herpetomonas donovani* in the human body. It will be seen then that the pre-flagellate stage of *Herpetomonas culicis* is characterised by the growth of the parasite, and its subsequent division, either by simple biniary fission or multiple segmentation. This stage is found in the alimentary tract of the larva, but may also be seen in the nymph, and even in the adult insect. As a rule, however, the parasite has flagellated when the larva becomes a nymph.

When the larvæ were removed from the water in which they became infected, and were placed in clean water, which contained practically no food, the development of the pre-flagellate stage was so retarded that when the larvæ turned into nymphs, the parasites were still without flagella, and when the adult insects hatched out of such nymphs, the parasites were still in their pre-flagellate stage. Figure 29 depicts a young pre-flagellate as seen in an adult mosquito, and figure 30 shews a pair of young flagellates from a mosquito. It is clear then, that if the larvæ are unable to obtain sufficient food, the growth and development of the parasites are so retarded, that they remain practically unchanged throughout the larval stage and are then seen as pre-flagellates in the mosquitoes which emerge later. This is a point worth noting, for it shews the great precaution which must be observed when dealing with the life histories of these parasites. It further emphasises the truth that the food of the insect has a direct bearing on the life history of its herpetomonas. An observer who is utilising a particular mosquito for feeding experiments on the blood of some vertebrate, in order to see whether its blood parasite will develop in the mosquito, would naturally collect a large number of larvæ, and place them in jars or dishes in the laboratory. Now under these conditions, many of the larvæ would certainly be starved, and if they contained a herpetomonas, its development would be retarted, so that when the mature insects hatched out, they would still contain the pre-flagellate stage of the parasite. After feeding such mosquitoes, and on dissecting them, the observer would find the early stages, many round forms and young flagellates ; he might assume that these had originated from the blood parasite which the insects had ingested. Such a conclusion

would obviously be erroneous, and would not only mislead the observer, but also those who were not in a position to repeat his experiments.

## The Flagellate Stage.

In my recent paper on the development of *Herpetomonas donovani* in the bed bug, I advanced the view that the flagellum was of ectoplasmic origin, and that it develops from a myoneme, which can nearly always be demonstrated in stained preparations of the pre-flagellate stage. In support of this hypothesis it was shewn that just prior to the extrusion of the flagellum, a pink staining area appears in close proximity to the blepharoplast, and that the flagellum may be seen to grow out directly from it. It was at first thought that this structure was of the nature of a vacuole, a view which was entirely based upon its appearance in stained specimens; but on studying a large number of this stage of herpetomonads, I came to the conclusion that the vacuole was in reality a thickened myoneme, or rhizoplast of other authors, which had merely become stretched and thus simulated a vacuole. I have drawn attention to the presence of the flagellar myoneme in the pre-flagellate stage of *Herpetomonas culicis*, and it is depicted in figures 13 to 15; here however the characteristic vacuole-like appearance is not usually seen, the myoneme appearing as a thickened strand, which on further growth, results in the formation of the flagellum, figure 16. I have been unable to trace any direct connection between the flagellum and the blepharoplast.

Under normal food conditions, *Herpetomonas culicis* begins to flagellate in the larval gut just prior to its becoming a nymph. One of two changes may now take place; (1) the parasites may either flagellate singly, or (2), they may enlarge further, and then divide up into a rosette of four or more young flagellates. I will first describe the changes undergone by the single flagellates. As the flagellum is about to be extruded, it appears to be surrounded by a sheath, figures 18, 19 and 23; this in my opinion further supports the view that the flagellum is in reality a part of the ectoplasm. At the same time the nucleus and blepharoplast become enlarged prior to division. The flagellum may now divide along its attached portion, and later split throughout its entire extent. The parasite begins now to lose its globular appearance, its posterior end growing out, resulting in the formation of a spindle shaped flagellate. There are two views regarding the origin of the second flagellum, one that it is an entirely new growth from the original flagellum, and the other that it is formed by the splitting of the first flagellum. Whichever is the correct interpretation, there can be no doubt that the second flagellum arises in close proximity to the first one; I am inclined to consider it to be the result of the

division of, and the subsequent growth from, the parent flagellum. On the division of the nuclei and blepharoplasts of these round or oval flagellates, they split up longitudinally into two smaller forms, which later elongate and produce the typical flagellates. The second form of multiplication in the flagellate stage is characterised by the formation of a number of round or oval flagellates. A young parasite which is just flagellating enlarges, its nucleus and blepharoplast dividing several times, the former passing towards the periphery, while the latter is situated more centrally. As the flagella become extruded they are directed towards a common centre, thus producing a typical rosette, figures 20, 21, 22 and 24. As these rosettes increase in size, the individual parasites begin to split off, and the lines of cleavage are clearly seen passing down between them, figure 21. The flagella now begin to grow out between the parasites, and the anterior ends of each individual cell becomes clearly defined, figure 22. Later the parasites break away, and then develop further as has been described in the case of the single round or oval forms.

In the fresh condition these rosettes are extremely striking objects, for on being freed from the intestinal contents, they may be seen whirling round like the well known Catherine Wheels of pyrotechnical displays. They are usually seen on the first and second day of the nymphal stage but may also be found in the adult insect. Figure 24 depicts a rosette of twelve parasites from the mid-gut of a mosquito. Many of the rosettes may be seen breaking up before the individual parasites have elongated, and figures 27 and 28 depict several of these forms. Their flagella are extremely active, in the fresh condition, and their nuclei and blepharoplasts may be seen in all stages of division, figure 30. These round or oval flagellates later enlarge, and become spindle shaped as described above. It will be seen from this account of this stage of *Herpetomonas culicis* that it is exactly similar to the same stage of *Herpetomonas donovani* in *Cimex rotundatus*, multiple segmentation however being better marked in the mosquito herpetomonad.

As in the case of *Herpetomonas donovani* the typical mature flagellate stage may be produced in one of two ways, either by the single round parasites, which on flagellating, elongate, or they may be produced as a result of multiple segmentation and further growth of the young flagellates.

The typical flagellate stage of *Herpetomonas culicis* is best seen in the mosquito as it emerges from the nymph ; they will then be seen actively moving about in a greenish substance, the remains of the larval food, in the lower part of the mid-gut of the insect. They may however be found all up the mid-gut, even as far as the proventriculus. In the fresh condition they will be seen to be actively dividing. This stage of the parasite measures from 15 $\mu$ to 25 $\mu$ in length and from 3 $\mu$ to 5 $\mu$ in breadth ; on staining them with Romanowsky's

stain, their structural details will be well seen, figures 31 to 38. The blepharoplast usually lies about 2·5 $\mu$ from the anterior end, which is always slightly rounded ; the flagellum, which arises close to it, at once projects freely, and may measure from 25 $\mu$ to 40 $\mu$ in length. It consists of a deeply stained filament which appears to lie in a close fitting sheath, the ectoplasm of the parasite. The nucleus usually lies from 8 $\mu$ to 10 $\mu$ from the anterior end ; it is nearly always oval in shape, measuring from 2 $\mu$ to 2·5 $\mu$ in length and from 1 $\mu$ to 1·5 $\mu$ in breadth. The protoplasm of the parasite stains a light pink and contains a number of vacuoles, and large pink granules which are usually situated towards the posterior end which is more or less pointed. These forms divide by simple longitudinal fission. Just prior to division, the nucleus and blepharoplast enlarge, and the flagellum begins to split at its basal end, a line of division now begins to form, and on the separation of the nucleus and blepharoplast into two, the parasite splits longitudinally. In the fresh condition these flagellates exhibit extremely active movements, they dart across the field of vision, the flagellum vibrating and drawing them along ; they are able to turn with great rapidity.

Several experiments were carried out to see the effect of varying foods on the flagellate stage of *Herpetomonas culicis*. Mosquitoes (*C. fatigans*) were bred out from larvæ taken from the septic tank, they were placed in small glass jars and small pieces of banana were given them to feed on ; they were then dissected and examined in the fresh condition at varying intervals. Owing to the fact that yeasts readily developed on the bananas, it was not found possible to carry this experiment to its conclusion ; as soon as a mosquito became infected with these yeasts, the flagellates disappeared and could not be found in any part of the alimentary tract of the insect. In a few instances it was noted that the adult flagellates soon left the upper part of the alimentary tract of the mosquito, and passed down into the hind gut and rectum, where they were beginning to shew the earliest changes towards the formation of the post-flagellate stage. This experiment recalls a very similar one with *Herpetomonas muscæ domesticæ* in *Musca nebulo*. It will be remembered that it was found that when infected flies were fed on jam, the post-flagellate stage of the parasite was produced at an earlier stage than when the flies were fed on meat juice. This experiment again points to the important bearing the food of an insect has on the life processes of its herpetomonas.

In another experiment a number of recently hatched female mosquitoes were allowed to feed on human blood, and they were dissected at varying intervals. Although most of the controls were found to be heavily infected with the flagellates, not a single one of those which had fed on blood, and been dissected 24 hours later contained any flagellates. The experiment was repeated

and practically the same result was found, except that in a few of the insects there were a considerable number of flagellates. It was noted that here again the flagellates tended to pass on to their post-flagellate stage, but there could be no doubt that human blood had some injurious effect on the flagellated forms of *Herpetomonas culicis*. It was this experiment which led me to discover the important fact that the flagellate stage of *Herpetomonas donovani* in *Cimex rotundatus* is readily destroyed when brought in contact with fresh human blood. I had hoped to have carried out a long series of experiments with *H. culicis* in order to discover the true explanation of the phenomenon noted above, but as this work would necessarily entail a long series of observations, it had to be abandoned. I hope however to again return to the study of this important problem, for I am convinced that the study of the action of human blood on such a flagellate as *Herpetomonas culicis* will help us to understand the origin of those forms which are now known to have passed from the insect to man ; in short it will give us the clue as to how a harmless flagellate may evolve into a pathogenic one.

The herpetomonads of mosquitoes throughout the first part of their life histories, are accustomed to living in a medium, consisting chiefly of the animal matter, found in water. In the case of the male mosquito the remaining part of their life histories are spent in a medium consisting of plant juices. In the female mosquito, on the other hand, they are suddenly brought in contact with blood which must necessarily have a profound effect on their life processes.

## The Post-flagellate stage.

This stage is characterised by the formation of the resting phase of the parasite, which is destined to be passed on to another insect. It is best studied in the male mosquito for it will live in captivity for a short time with little or no food.

Text Fig. 2.

Text figure 2 depicts the alimentary tract of an infected male mosquito as seen under a low power. The mid-gut contains the mature flagellates while the hind gut is packed with layers of short oval bodies, the post-flagellate stage ; scattered here and there are islands of the final encysted stage. This appearance is very characteristic, and the intestine, when it is dissected out, has a stiff appearance and it readily breaks. The flagellates may be seen actively moving about in all parts of the hind gut and large numbers will be seen in the rectum between the papillæ ; they are thus readily passed out by the mosquito in its excreta. The figure was drawn from an actual preparation.

The early phase of the post-flagellate stage is characterised by the flagellates collecting all along the hind gut. They fix themselves by their flagellar ends to the intestinal wall, and on coming to rest, divide in such a regular manner that a typical palisade, consisting of rows upon rows of spindle shaped bodies, is produced. This regular formation then is the result of the division of the flagellates. It should be remembered that it is not in every mosquito, that the parasites are found in such enormous numbers as is depicted in the text figure. In the majority, they will be found to only occupy that segment of the hind gut just above the rectum, in others they will be seen collected high up ; in a few they will be seen scattered about in small patches or islands. They may therefore be readily overlooked. On staining such a preparation as that depicted in the text figure all the various changes undergone by the parasites at this time can be easily studied. Figure 39 shews a flagellate about to round up, its posterior end is losing its rounded appearance, the protoplasm collecting about the nucleus. The flagellum soon begins to have a shredded appearance, and its basal part becomes indistinct, so that it appears to be lying loosely in its sheath. In a still later stage it seems to be just attached to the margin of the cell, figures 40 to 43. The nucleus and blepharoplast now divide, and the parasite splits into two, and these in their turn divide again till the final encysted form is reached, figures 34 to 47. The cysts are round or oval bodies with a light pink staining protoplasm, and granular nuclei, their blepharoplasts are still rod shaped and usually lie well towards the centre of their bodies. The ectoplasm consists of a pink staining rim, ensheathing the parasite. These post-flagellates adhere together in masses, and they are then passed out *en masse*.

## Conclusions.

*Herpetomonas culicis* is a natural flagellate of *Culex fatigans*, passing its complete life history in the larva, nymph, and imago ; it is in no way connected with any vertebrate blood parasite. It, or any of its allied forms, should be

rigidly excluded in the case of experiments carried out with mosquitoes and the parasites of Kala Azar, and Oriental Sore. These flagellates of mosquitoes should be carefully studied, for as they are parasitic in insects which suck man's blood their life histories may become so modified that they may in time be transmitted to the human body and become pathogenic.

## Explanation of Figures on Plate.

Figure 1.—An early pre-flagellate form, note the flagellar myoneme extending across the body of the parasite.

Figure 2.—An enlarged pre-flagellate, the flagellar myoneme and the pink granules are well seen.

Figure 3.—A much enlarged pre-flagellate, the blepharoplast is about to divide, the nucleus is enlarged; the protoplasm contains many pink staining granules.

Figure 4.—An oval pre-flagellate, the blepharoplast is thickened, and the nucleus is just about to divide.

Figure 5.—A pre-flagellate which has split into two.

Figure 6.—An enlarged pre-flagellate, the result of the further growth of the form depicted in figure 4; the parasite does not yet shew any signs of splitting.

Figure 7.—A similar parasite, but here the posterior end shews the early appearance preceding simple biniary fission.

Figure 8.—A much enlarged pre-flagellate about to divide, the blepharoplasts are already thickened ready for the next division.

Figure 9.—A pre-flagellate shewing the early changes towards multiple segmentation.

Figure 10.—Another similar parasite, the nucleus and blepharoplast have divided once, and one of the daughter nuclei and blepharoplasts have divided again.

Figure 11.—A still further stage of the same.

Figure 12.—Multiple segmentation in the pre-flagellate stage.

Figure 13.—The early changes towards flagellation, the flagellar myoneme is well marked.

Figure 14.—A further stage of the same, there is just the faintest suspicion of the vacuole-like appearance.

Figure 15.—A very similar parasite, the vacuole-like area is better marked and the myoneme is considerably thickened.

Figure 16.—A similar parasite, the nucleus is much enlarged and the blepharoplast is thickened.

Figure 17.—A young flagellate, the flagellum is just protruding freely, the blepharoplast is about to divide.

Figure 18.—Another young flagellate, note the flagellum has the sheath well seen at its base, the nucleus is about to divide.

Figure 19.—A young flagellate with well developed flagellum, which is splitting at its base.

Figure 20.—A rosette of four parasites, shewing multiple segmentation in the flagellate stage.

Figure 21.—A typical rosette, note the position of the nuclei and blepharoplasts; the flagella are all passing out at one point and the anterior ends of the parasites are not clearly defined.

Figure 22.—A well developed rosette, the parasites are now quite separate, the flagella have grown out between each parasite.

Figure 23.—A young oval flagellate, the flagellum has divided throughout its extent, it is surrounded by a sheath.

Figure 24.—Another rosette.

Figure 25.—A spindle shaped flagellate with well developed flagellum.

Figure 26.—A round flagellate, separated from a rosette.

Figure 27.—A group of four round flagellates, shewing evidences of further division.

Figure 28.—Another group of four round flagellates, with flagella all passing out in one direction.

Figure 29.—An early pre-flagellate from the mid-gut of a mosquito, note the absence of granules.

Figure 30.—Two young flagellates from the mid-gut of a mosquito, both are dividing. It is these forms, in the adult insect, which may lead to confusion.

Figure 31.—The mature flagellate stage of *Herpetomonas culicis* with a very long and stout flagellum.

Figure 32.—Longitudinal division in the flagellate stage, the parasites only being attached by their posterior ends.

Figure 33.—Another typical mature flagellate.

Figure 34.—A very long flagellate.

Figure 35.—A long flagellate shewing the early phase of longitudinal division.

Figure 36.—A similar flagellate.

Figure 37.—A short flagellate dividing.

Figure 38.—Another similar flagellate.

Figure 39.—The early post-flagellate stage, the parasite is becoming globular.

Figure 40.—Another early post-flagellate, the blepharoplast is thickened and the flagellum is seen to be attached to the margin of the body of the parasite, the basal portion is faintly seen.

Figure 41.—Another post-flagellate, the nucleus and blepharoplast are about to divide.

Figure 42.—Another similar form.

Figure 43.—A more advanced post-flagellate, the flagellum is broken off and has a shredded appearance; the ectoplasmic layer is growing round the parasite.

Figure 44.—A post-flagellate dividing.

Figures 45, 46, and 47.—The final post-flagellate stage.

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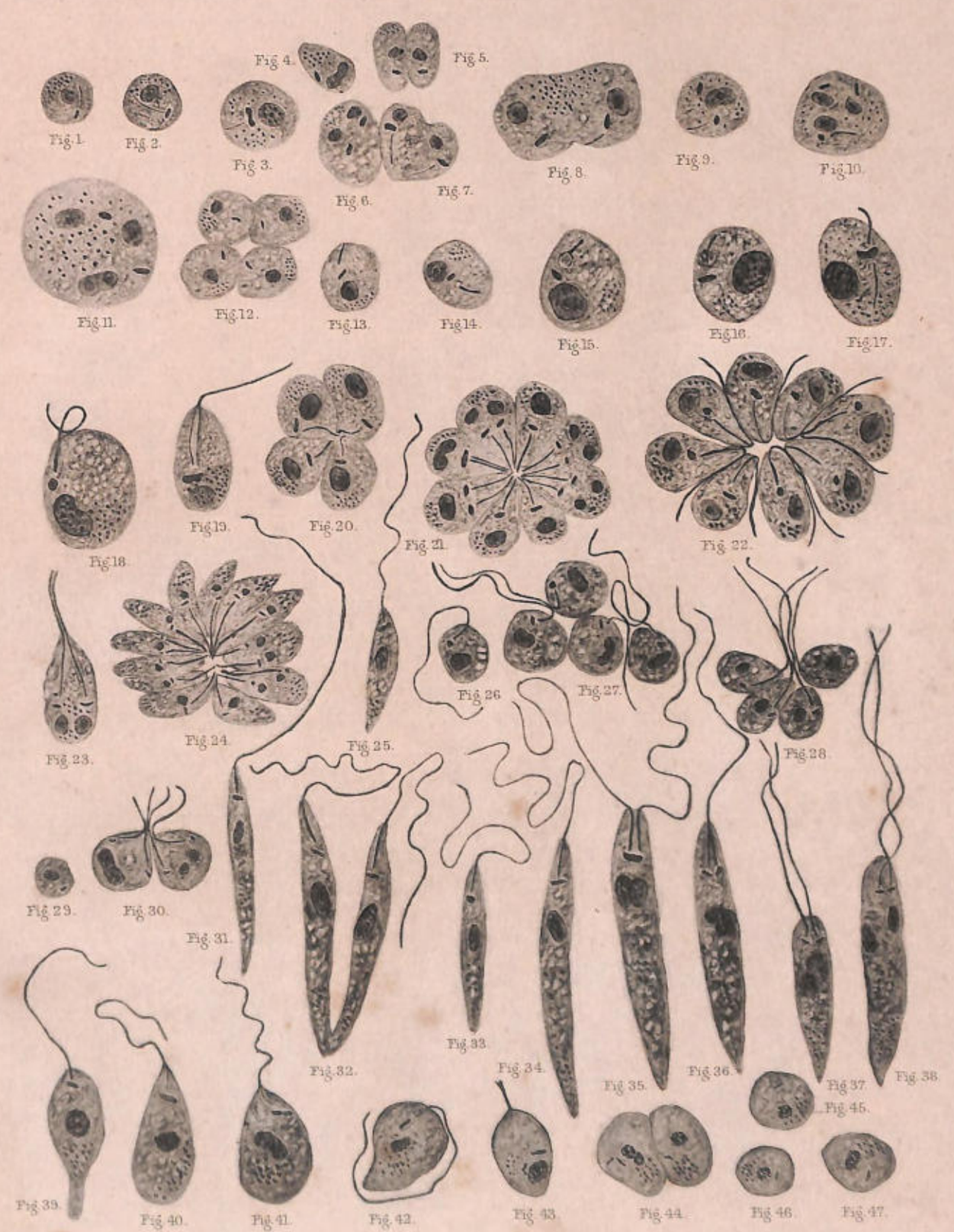


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