A GREGARINE, *MONOCYSTIS CHAGASI* n. sp., OF *PHLEBOTOMUS LONGIPALPIS*. REMARKS ON THE ACCESSORY GLANDS OF *P. LONGIPALPIS*

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SUMMARY

A gregarine of the genus *Monocystis* is recorded from the sandfly *Phlebotomus longipalpis*, male and female, wild and laboratory bred.

Oocysts were observed in the accessory glands in 20 per cent of all infections in females. They are injected into the lumen of the accessory glands by mature cysts attached to the glands. They are discharged from the glands by repeated contractions of the latter.

During oviposition eggs are exposed to the secretion of the accessory glands. This secretion sets and forms a layer on the surface of the egg which protects them against invasion by micro-organisms.

During oviposition the surface of eggs becomes contaminated with oocysts discharged from infected glands; these oocysts are glued to the surface of the eggs by the secretion of the glands. Larvae emerging from contaminated eggs are thus exposed to infection.

The name *Monocystis chagasi* is suggested for the gregarine of *Phlebotomus longipalpis*.

INTRODUCTION

During routine dissections of sandflies caught in Lapinha near Belo Horizonte, Minas Gerais, we found 10% of specimens of *P. longipalpis* infected with a gregarine of the genus *Monocystis*. This gregarine would call for no special attention beyond the fact that it is a parasite of *P. longipalpis*, the vector of visceral leishmaniasis in Brazil, were it not for an interesting adaptation, *viz.* infection of the accessory glands with oocysts and, as a consequence, contamination of the surface of eggs during oviposition. We found no gregarines in other species of *Phlebotomus* caught in Lapinha (*P. renei, P. migonei, P. quinquifer, P. pessoai*). The parasite was also found in laboratory bred male and female *P. longipalpis* (from 20% to 80% in various batches). The relatively dense population bred under laboratory con-ditions obviously favour a higher infection rate than that found in nature because of the increased opportunities for larval infection. The gregarine is not pathogenic and the heaviest infections (up to 80 gregarines per sandfly) did not interfere with egg development or oviposition.

The smallest gregarines seen in adult sandflies were 32 μ in size and were round, oval or pear shaped. Larger parasites were round or oval. There is a narrow clear rim between the wall and the endoplasm. The latter contains brownish granules, which are smaller, darker and more densely packed in the larger and more mature specimens than in the smaller ones. The granules are basophilic in Giemsa stained preparations. In individuals up to 60 μ in diameter a blunt

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conical proboscis-like projection about 10 μ at its base, containing clear agranular ectoplasma, is occasionally seen. This structure which projects and retracts very slowly was suspected to act as an organellum of attachment although active attachment to tissue was not observed.

The nucleus attains a size of 28 μ in the largest adults and contains a relatively large nucleolus (8 to 10 μ in diameter). The majority of mature adults (Plate 1, Fig. 1), i.e., individuals capable of participating in pseudoconjugation, vary in size between 72 μ and 108 μ, but a few exceptional individuals attain a size of 120 μ.

Pseudoconjugation begins by contact between two adults; the point of contacts becomes flattened and the area of contact extends till the two pseudoconjugants are separated by a straight narrow partition continuous with the outer wall of both participants. The partition dissolves and the two pseudoconjugants are enclosed in a common gametocyst (Plate 1, Fig. 2) which varies from 72 μ to 108 μ in diameter but, as in the case of the adult exceptional individuals, attain 120 μ. Two small indents between the wall and the outer border of the endoplasma may be seen at opposite sides of the gametocyst; one of them is probably the site of rupture of the cyst wall during the liberation of oocysts.

Nuclear division is not always synchronous in the two individual conjugants; there may be a considerable number of nuclei (16 or more) in one, before nuclear division has commenced in the other. Observations on the course of development of the gametocyst in laboratory-bred sandflies indicate that nuclear multiplication proceeds for one to two days before production of gametes which conjugate and form zygotes. The latter grow and become elliptical or spindle-shaped (10.0 μ-11.9 $\mu \times 5.8$ μ when fully grown) with a nucleus 4 μ in diameter. The nucleolus is relatively large (2 μ in diameter) and the protoplasm contains grayish lipid granules. Occasionally fully grown zygotes which are triangular in shape are found (Plate 1, Fig. 4); this accounts for the rare triangular oocysts found in heavy infections. Each fully grown zygote secretes a tightly fitting shell and becomes a biconical symmetrical oocyst (10.9 μ-11.9 $\mu \times 5.8$ μ) with a small projection at each extremity (Plate 1, Fig. 5; Plate 3, Figs. 1 and 2). The nucleus of the oocyst in the mononuclear stage is 4 μ to 5 μ in diameter and contains a relatively large nucleolus, up to 2 μ in diameter; chromatin is distributed in small masses on and near the nuclear wall. A relatively large nucleolus is characteristic of this gregarine in all its developmental stages.

The large majority of oocysts found in the lumen of the accessory glands or free in the abdominal cavity were uninucleate. Nuclear division (up to three corresponding to 8 sporozoites) occurred in some oocysts after 24 hours in a moist chamber but many oocysts remained uninucleate even after three days.

Prior to the development of oocysts the gametocyst is resistant to pressure and considerable force is necessary to rupture its wall. With the appearance of the oocysts, the consistency of the gametocyst wall changes, the internal pressure rises and the wall ruptures under the slightest external pressure, liberating oocysts through one aperture. During this process the outer cyst wall shrinks rapidly without folding and finally assumes the shape of a horse-shoe with an aperture at the narrow end (Plate 2, Fig. 4). The inner wall of this structure is corrugated and encloses a patchy matrix often containing residual oocysts, but the brown granules characteristic of the previous stages are absent. This structure is very characteristic and may well be mistaken for tissue of the host, particularly when residual oocysts are absent, but its origin and mode of formation from a cyst which has liberated oocysts is quite clear. In fresh dissections this typical structure is found either free or attached by its narrow end to the wall of an accessory gland and in some instances its lumen appears to be continuous with that of the gland. The above structures were found only in females which contained oocysts free in the haemocele or in the lumen of the accessory glands, or both.

The oocysts are not resistant to desiccation; after drying in air on a slide, one side collapses and the contents shrink.
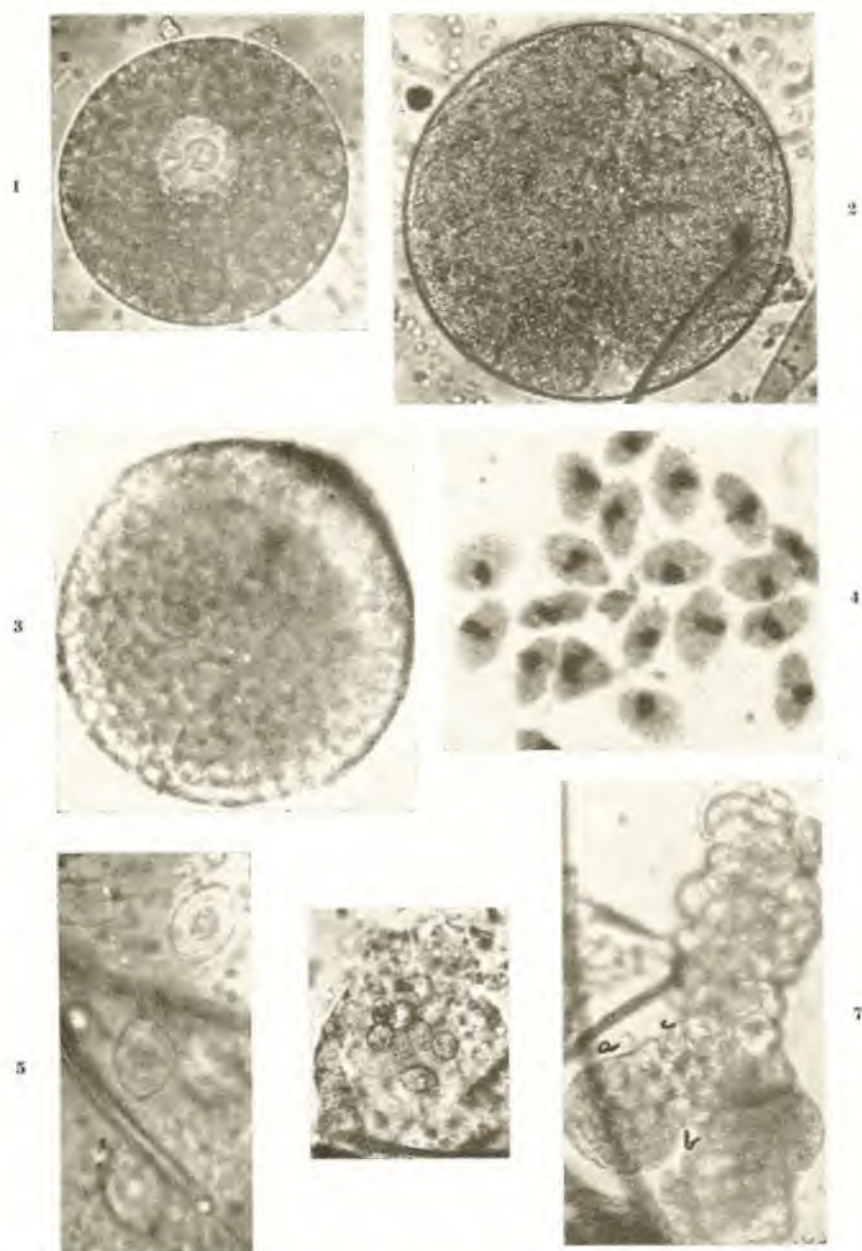
PLATE 1.

1. Adult Gregarine. Fresh preparation ×500.
2. Pseudo-conjugation. Fresh preparation ×500.
3. Gametocyst containing fully grown zygotes ×500.
4. Fully grown zygotes. One zygote is triangular. Giemsa stain ×1,300.
5. Oocysts in uninucleate stage. Fresh preparation ×1,300.
6. Part of accessory gland containing residual oocysts. Fresh preparation ×500.
7. Accessory gland with appearance of herniation. (a) Cyst which has ruptured and discharged its oocysts into the lumen of an accessory gland. (b) The wall of the gland is continued on to that of the cyst. (c) An oocyst at junction of cyst and gland. Fresh preparation ×300.
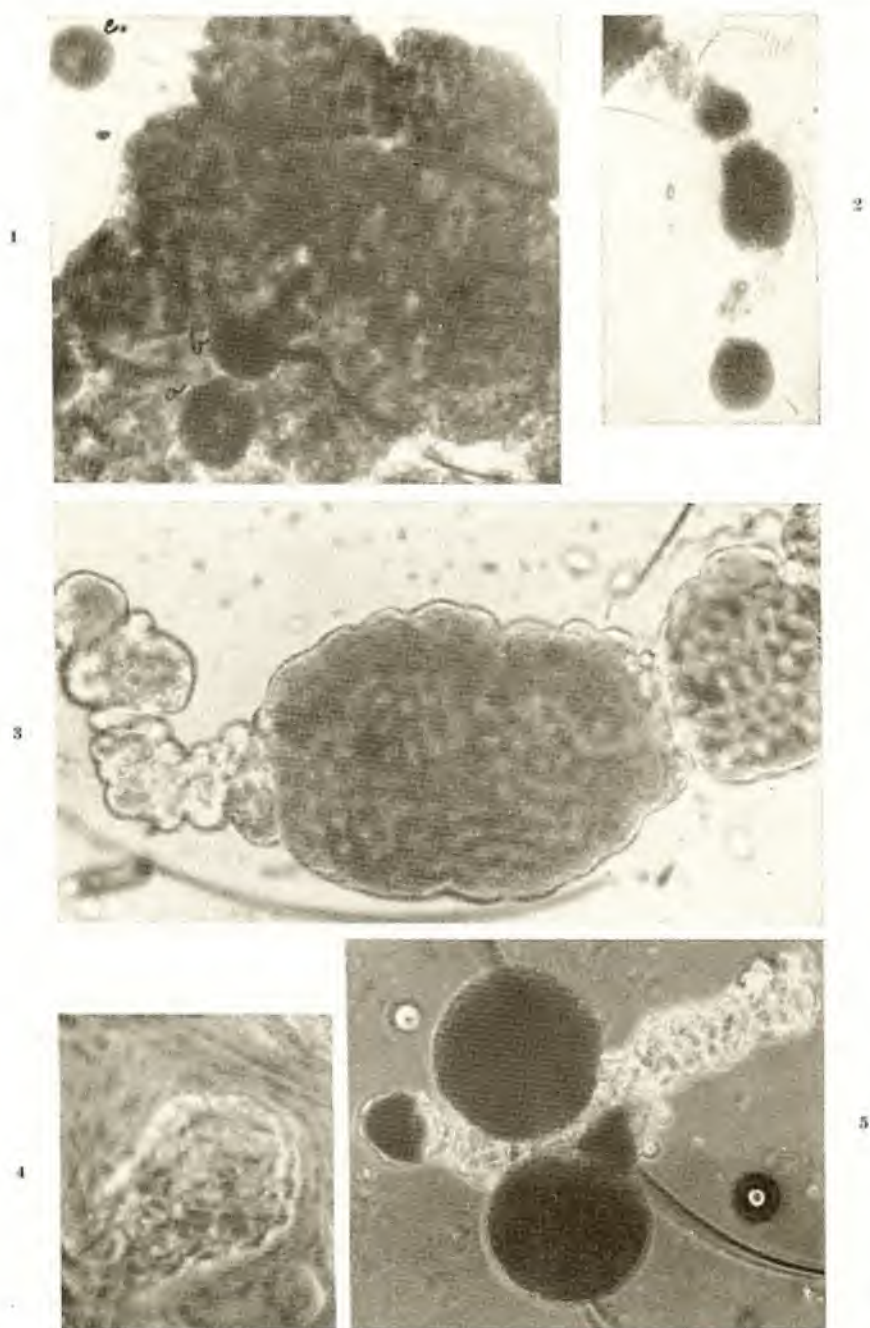
PLATE 2.

1. Part of ovary of *P. longipalpis*. (a), (b) Two gametocysts lying among ova. (c) An adult gregarine lying outside the ovary. Fresh preparation (2 days in ice chest before photography) ×200.
2. Fresh dissection of an active accessory gland showing dilated and constricted parts. This gland contained numerous oocysts ×200.
3. Portion of same gland showing numerous oocysts in the lumen ×650.
4. Remnants of a cyst which has discharged oocysts. Note corrugated inner wall ×700.
5. Part of an accessory gland with two gametocysts lying on its surface. The upper cyst contained zygotes. Fresh dissection ×300.

Both pseudo-conjugation and gametocysts containing zygotes were found in laboratory-bred sandflies two to four days after a blood meal. The above data apparently represent the shortest time necessary for the completion of the sexual cycle but the development of individuals in a single sandfly is not synchronous. It is not unusual to find parasites of all sizes from 32 μ upwards and fully developed cysts containing oocysts in the same sandfly.

It is interesting to note that we did not see pseudo-conjugation or any subsequent part of the sexual cycle, in infected laboratory-bred males, although gametocytes up to 80 μ in diameter were found and some infections were relatively heavy (20 to 40 parasites in the abdominal cavity). This may be due to the fact that the life span of laboratory-bred males is too short to include the sexual cycle of the gregarine.

The shortest time required for the maturation of sandfly ova approximates to that required for the development of oocysts of the gregarine, but the two events do not always coincide. We noted instances of oviposition by heavily infected females in which the most advanced cysts had not discharged oocysts. On the other hand we found oocysts in both accessory glands in a female in which the ova had only attained a length of 140 μ, i.e., one to two days before maturation. These discrepancies may be due to variations in the interval between hatching and the opportunity for the first blood meal without which ova of P. longipalpis cannot attain full development, and to variations in the opportunities for pseudo-conjugation between mature gametocytes.

The above brief description indicates that the life cycle of the gregarine of P. longipalpis conforms to that of Monocystis sp. in general and resembles that of Monocystis mackiei in P. argentipes as described by SHORTT & SWAMINATH (1927). There are, however, some differences between Monocystis mackiei and the gregarine of P. longipalpis. The gametocytes of P. mackiei are 166.0 $\mu \times 66.3$ μ; those found in P. longipalpis are 72 μ to 108 μ in diameter though rare individuals attain a size of 120 μ. In P. mackiei adults are found in the pupae of P. argentipes; in P. longipalpis full development of the gametocyte from 32 μ to full size occurs in the adult sandfly (we did not study the course of development in larvae or pupae). The nucleus of the adult M. mackiei is slightly larger (30 μ) than in our material (28 μ). The oocysts of P. mackiei are 9.8 $\mu \times 5.8$ μ in size, those in our material are 10.9 to 11.9 $\mu \times 5.8$ μ. Infections of P. argentipes with P. mackiei were much heavier than in P. longipalpis, in which the number of parasites in an infected sandfly ranged between 1 and 80, and in most cases was less than 20. P. mackiei invades all parts of the sandfly (head, thorax, abdomen, legs) but parasites were not recorded from the accessory glands; in our material the parasites were found in the abdominal cavity, accessory glands and occasionally in the ovaries. A solitary adult was found only on one occasion in the thorax of a male sandfly, but no gregarines were found in the head. The accessory glands (one or both) were infested with oocysts in 20% of all individuals. This figure should be considered as an underestimate of the potential infection rate in accessory glands which are never infected before the cycle of the gregarine has advanced to the formation of oocysts.

We propose the name Monocystis shagasi for the above described gregarine of P. longipalpis.

The surface of the eggs deposited by a wild sandfly P. longipalpis was found to be contaminated with firmly adhering oocysts and we observed this condition (Plate 3, Figs. 1 and 2) in the eggs of infected laboratory-bred sandflies; in the latter the number of oocysts per egg varied from 1 to 50. In one batch of eggs from a single female the number of oocysts per egg varied from 1 to 10 and some eggs apparently escaped contamination. In another batch they were arranged in rows of 8 to 10 at right angles to the long axis of the egg. The eggs were examined from almost immediately after oviposition up to 6 days later, and no differences were noted in the distribution and number of oocysts attached to their surface. (It is interesting to note that the optical properties of the shell permit observation of the developing embryo in intact egg of P. longipalpis.)
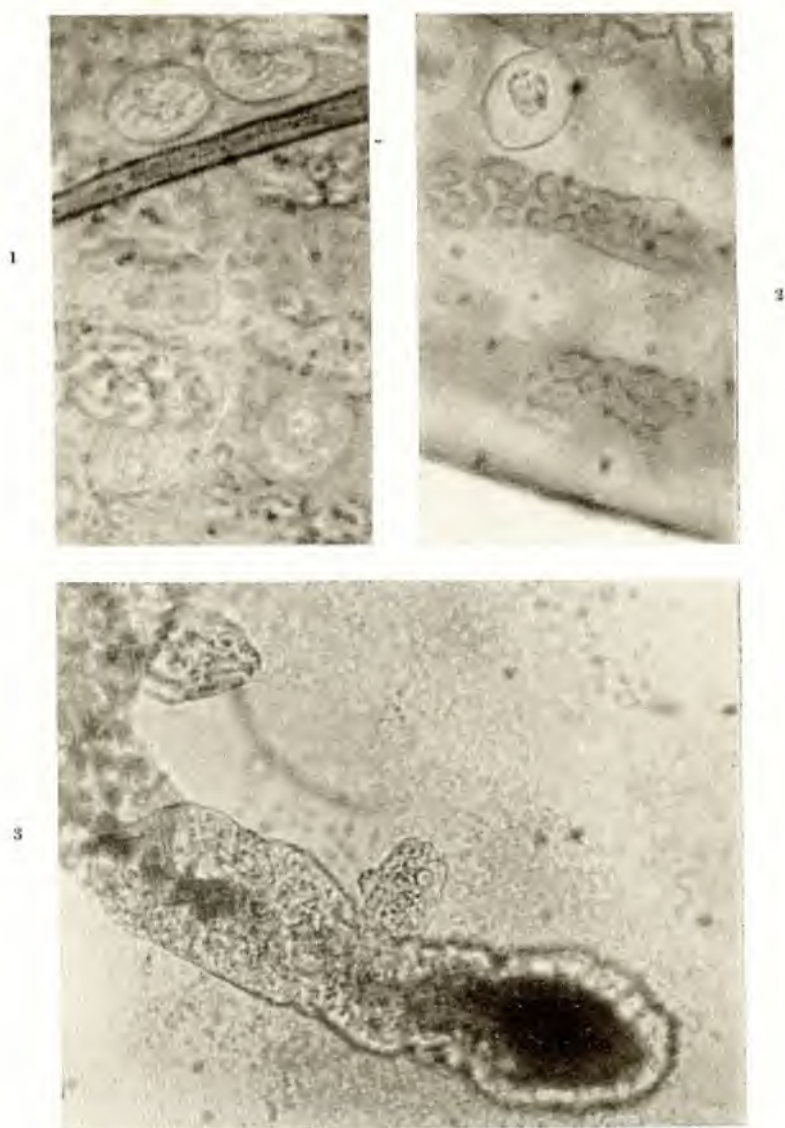
PLATE 3.

1 and 2. Oocysts of *Monocystis chagasi* on the surface of eggs of *P. longipalpis* ×1,300.
3. Part of an accessory gland of wild sandfly *P. longipalpis*. Two cysts which have discharged their oocysts are attached to the gland. Fresh dissection ×300. The dark areas at the apex and near centre of the gland contain granules secreted by the gland.

Five instances were found, four in laboratory-bred sandflies and one in a wild sandfly, in which oocysts were restricted entirely to the lumen of the accessory glands, *i.e.*, none were free in the abdomen. In two of the above sandflies no stages of the parasite were seen apart from oocysts in the accessory glands; in one the infection was heavy and in the other we found only 20 to 30 oocysts, apparently the residues of a population the majority of which had been discharged through the genital aperture. A dissection of this sandfly revealed three typical structures which were obviously residues of

gametocytes which had discharged their oocysts. These structures which contained no residual oocysts were attached to an accessory gland. The abdomen of this sandfly was full of almost mature eggs.

In two other of the above five sandflies, the abdomen contained adults, pseudo-conjugants, and gametocytes with zygotes but no oocysts in their interior. The fifth sandfly will be discussed later in some detail because it illustrated the mechanism whereby oocysts are introduced into the accessory glands.

We were at first puzzled by the relatively frequent infestation of one or both accessory glands with oocysts, particularly as we found no pseudo-conjugation and no gametocytes in the lumen of the accessory glands in more than 700 dissections and it was also clear that oocysts could not enter a gland by their own efforts. This point was cleared up by a study of the accessory glands of *P. longipalpis,* and the behaviour of gametocysts of *M. chagasi* in relation to these glands.

In newborn laboratory-bred females the accessory glands are grayish-white in color and contain no granules in their lumen. After a blood meal granules together with a dark brown fluid appear in the lumen.

It may be noted that wild sandflies *P. longipalpis, P. renei, P. quinquifer, P. migonei, P. pessoai* and *P. cavernicolus* without a trace of a blood meal in the alimentary tract are of two types, one without and the other with granules in the lumen of the accessory glands. The former are therefore considered to be recently hatched specimens which have not yet taken their first blood meal, and the latter are considered to be individuals which have previously taken a blood meal, developed mature ova, oviposited, and emerged for a refeed after complete digestion of the previous blood meal.

In females sacrificed at any stage of their life the glands are found to be mechanically active; segments contract and dilate slowly and repeatedly while the adjacent part may be constricted until its lumen is almost obliterated (Plate 2, Fig. 2). The movements in the ative segment create a slow current first in one and then in the opposite direction and as a result its contents are churned and homogenised. Different segments become active and their contents hemogenised at various times.

In their passage through the vagina, during oviposition, eggs are exposed to the secretion of the active accessory glands and are simultaneously brought into contact with oocysts discharged from infected glands. The secretion of the glands sets quickly and thus glues oocysts on to the surface of contaminated eggs.

The secretion of the glands is essential for the protection of the developing embryo; out of 200 fully grown apparently mature eggs removed from the abdomen by dissection and therefore not exposed to the secretion of the glands, the interior of the large majority became infected with fungi, yeasts or bacteria after being kept on moist filter paper for a few days. The thin layer on the surface of the egg contributed by the accessory glands serves as an impenetrable barried against invasion of the interior of the egg by micro-organisms.

The accessory glands like the ovaries and hindgut continue to contracts and dilate up to thirty hours after the death of the sandfly if the latter is protected from drying by being placed in saline. (The heart continues beating up to 90 times a minute for several hours after death.)

A network of fine fibrils continuous with the boundary of each ovary divides the latter into compartments each containing an ovum and its respective nurse cells. This system of fibrils and the terminal filament of each ovary contract and relax continuously, thereby causing slight changes in shape and a slow agitation of the contents of the ova until they are at least 140 μ in length. The nurse cells are also affected by the above-mentioned movements of the ovaries and in addition change their shape spontaneously. *P. longipalpis* usually deposits all its mature eggs in a single laying but occasionally one or a few mature ova remain in the abdomen; in such cases one or both of the new ovaries grow round and include the residual mature ova.

We found three cases of infestation of ovaries with the gregarine. In one instance an adult gregarine lying in an ovary became

slightly deformed and displaced with every contraction of the organ from which it was eventually expelled. In two other instances several gametocysts were lying among developing ova almost half the size of mature ones (Plate 2, Fig. 1).

P. longipalpis is occasionally infected with a parasitic fungus and it is interesting that even heavy infestation of the ovaries do not affect the movements of the fibrils but may cause premature oviposition. A moribund sandfly deposited eggs which were considerably smaller than normal. Dissection revealed that both ovaries were heavily infected with fungus.

Gametocysts in an advanced stage of development were frequently found continuous with the accessory glands (Plate 2, Fig. 5) and cases were noted in which the outer border of the gland was intimately connected with a part of the wall of one or more cysts. We do not know whether this relationship is established directly by the previously mentioned transient conical projection from the gregarine or by the adhesion of mature cysts to the wall of the gland.

During the dissection of a laboratory-bred sandfly (the last of the above-mentioned five in which oocysts were restricted to the accessory glands), fed three days previously on a guinea pig, we observed the inoculation of oocysts from a fully developed cyst into an accessory gland to which it was attached. The cyst contracted and injected oocysts into the lumen of the gland through a breach at the point of union between the two. After inoculating hundreds of oocysts into the lumen of the gland the cyst shrank and became transformed into the typical horse-hoe-shaped structure previously described. A few minutes later the shrunken cyst still containing residual oocysts turned upwards and lay on the upper surface of the gland to which it was still attached by its narrow end. During the whole of this process, segments of the glands were contracting and dilating and numerous oocysts were pouring out through the vagina. The hindgut and the soft tissue enclosing the spermathecae and their ducts were also actively contracting and dilating. It was easy to follow the whole process in this dissection because the infection in the sandfly was relatively mild and, apart from

the abovementioned cyst, only a solitary adult 80 μ in diameter was found lying in the abdomen. The source of the oocysts and the mechanism of their introduction into the accessory glands were quite obvious in this case, and this mechanism is probably responsible for all cases of infestation of the glands with oocysts. Structures typical of cysts which have liberated their oocysts remain attached to the accessory glands for some time (Plate 3, Fig. 2) but they eventually separate and may infected glands are found without them. While attached to the gland they may produce the impression of a hernia and in one case (Plate 1, Fig. 7) we found what appeared to be definite herniation of a gland, the wall of the cyst forming the distal part of the hernia. The lumen of the gland in this particular case was continuous with the cavity of the cyst which contained residual oocysts. The other accessory gland of the same sandfly (Plate 2, Figs. 2 and 3) contained numerous oocysts but no residues of gametocysts were found on its surface.

Gregarines in advanced stages of development tend to be deposited in the hindmost part of the abdomen and the most mature cysts are therefore brought into close proximity with the hindgut, rectum, spermathecae and accessory glands. We found no instances of attachment of cysts to the wall of the hindgut or to any object apart from the walls of one or both accessory glands. Oocysts were found either free in the abdominal cavity or inside the lumen of the accessory glands or both, and nowhere else, except for occasional contaminations of the inner surface of the terminalia to which they were glued by the secretion of the accessory glands. The fact that oocysts may be restricted to accessory glands even in mild infections indicates a predilection on the part of gametocysts for attachment to the wall of the glands. We know of no other instance in the genus *Phlebotomus* of infestation of the accessory glands with a parasite.

Infestation of the haemocele with oocysts may be sufficient for perpetuating the gregarine through chance ingestion of oocysts by larvae feeding on the carcasses of infected females. The opportunities for this type of infection are obviously greater under condi-

tions of laboratory breeding than in nature and the infection rates in laboratory-bred sandflies, *P. argentipes* and *P. longipalpis* are accordingly much higher than those found in nature, but the probability of infecting the offspring of an infected female in nature by this method is obviously less than in the case of infestation of the accessory glands. Oocysts lying free in the abdominal cavity may well mingle with mature eggs as noted by SHORTT & SWAMINATH in the case of *M. marckiei*. We did not however observe the passage of oocysts directly from the haematocele to the oviduct in our material. This point requires further investigation.

The injection of oocysts into the accessory glands, their expulsion from the glands by the contraction of the latter, the contamination of eggs with oocysts glued to their surface by the secretion of the glands, and the consequent opportunity of infecting the emerging larvae, altogether constitute an efficient mechanism for the perpetuation of *Monocystis chagasi* in spite of the fact that oviposition and dicharge of oocysts from the gland are not always synchronous. This mode of dissemination which does not necessarily involve the death of the sandfly *P. longipalpis* since the latter frequently survives oviposition, is the only one possible in those cases where oocysts are restricted to the accessory glands.

RESUMO

Sôbre uma gregarina, "Monocystis chagasi" n. sp., de "Phlebotomus longipalpis". Notas sôbre as glândulas acessórias de "P. longipalpis".

Os autores encontraram e descrevem uma gregarina do gênero *Monocystis* em machos e fêmeas de *Phlebotomus longipalpis* (Diptera, Psychodidae), apanhados na natureza ou criados em laboratório.

Verificaram a presença de oocistos nas glândulas acessórias de 20% das infecções nas fêmeas; êles são injetados no lume das glândulas acessórias por cistos maduros prêsos às referidas glândulas e são eliminados destas por contrações repetidas das mesmas.

Durante a ovoposição os ovos são revestidos pela secreção das glândulas acessórias, que forma uma camada protetora contra a invasão de microorganismos.

Por ocasião da postura a superfície dos ovos contamina-se com oocistos eliminados das glândulas infectadas e que poderão contaminar as larvas quando da sua eclosão.

A gregarina do *P. longipalpis* é considerada uma nova espécie, para a qual os autores propõem o nome de *Monocystis chagasi*.

REFERENCES

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