PART I

MORPHOLOGY, LIFE HISTORY, SYSTEMATICS AND SEASONAL INTENSITY OF TWENTY TWO NEW SPECIES OF SEPTATE GREGARINES FROM INSECT PESTS OF WEST BENGAL

WITH A STUDY OF THE SCANNING ELECTRON MICROSCOPY OF A SPECIES OF STYLOCEPHALUS
Linnaeus (1758) first enlisted about 9000 species of plants and animals and gave explicit explanation and systematic recognition of diversity of life. The investigated species have been carried on 1.5 to 1.8 million. Though the most number of species is uncertain, according to some scientists, the number ranges from 3 millions to 80 millions or more (Gaston and May, 1992).

The most numbers of animals may be regarded as free living as they are not dependent upon the occurrence in the same area or any particular species or group of species. However, some organisms have varying degrees of cooperation with members of one or more species. Such relationships are known as interspecific relationships. Protozoa display an amazing variety of interspecific relationships among which parasitism is the most common in the class Conoidasida of the phylum Apicomplexa. These apicomplexan protozoans remain associated as parasites in the digestive tract and appended organs of invertebrate hosts. Among them, mainly the eugregarines, also known as septate or cephaline gregarines, generally harbour the arthropod gut.

The adaptive capability of the eugregarines is found to have a wide range of hosts belonging to the following phyla. Coelenterata, Platyhelminthes, Coelhelminthes (Archiannelidae, Gephyrea, Hirudinea, Annelida, Polychaeta and Oligochaeta), Arthropoda. (Crustacea, Onycophora, Myriapoda, Hexapoda and Arachnida), Mollusca, Echinochordata and Chordata (Enteropneusta and Tunicata) (Watson, 1916a).

Location of the gregarines is most commonly observed in the mid gut of the host, to a lesser extent in the hepatic caeca and to a still lesser extent in the pyloric caeca. Generally the parasites are not recovered from the oesophagus, crop or rectum except in case of heavy infection. Cysts are usually recovered from the rectum but often from the hind gut.

According to Mackinnon and Hawes (1961) the eugregarines which are the most numerous and widely distributed gregarines, “depend entirely on sexual reproduction and sporogony, lack the schizontogenic phase completely, produce oocysts which always contain eight sporozoites. These are divided naturally into two tribes, the Acaphalinina, which are predominantly coelozoic with the simple body undivided, and the Cephalina, which are gut parasites in which the body is typically divided into different regions, of which the anterior develops an organ of fixation, called the epimerite. The epimerite may be a simple knob or it may be stalked, frilled, proboscis-like, with recurved cuticular hooks, or it may produce root-like processes which ramify within the host cell.”

**Life Cycle**

The life cycle of gregarines presents problems of special interest, for many are correlated both with the life cycle and external environment of the host. After ingestion of oocysts the hatching takes place and the released sporozoites either enter the host cell or they may remain inside the gut lumen. Within the gut lumen of the host the sporozoites obtain their nourishment directly from the host’s digested food. After attainment of a reasonable size the growth of the anterior region slows down and that of the posterior...
region increases, eventually becoming divided into the protomente and the deutomente (Harry, 1965) At this stage it is known as fully formed trophozoite.

However, in cases where the sporozoites enter the epithelial cells of the host gut, they develop into young trophozoites at the expense of the host cells. The trophozoites after emergence into the lumen, remain attached to the inner cell layer of the gut by the attachment organ called the epimente. Eventually, they break away from their epimentes and live freely in the gut lumen as sporadms or sporonts. In some cases the sporadms are solitary while in others they are able to form a sexual association of the two or more individuals, termed syzygy. Sporonts associated in syzygy by their anterior or posterior ends, laterally, or head to tail, or permit the formation of chain of individuals, or several small ones may become attached to a larger anterior partner, whatever the combinations, the anterior member is called the primate, the posterior member, the satellite. They often move around in this joined condition. Then these associating sporadms gradually bend upon about 180° and encyst to form a thick gelatinous cyst wall around themselves, the whole forming a gametocyst. In each organism, the protomente and the deutomente merge and become gametocytes which bud off gametes. The enclosed gametocytes may be equal or unequal in size.

The gametocysts then leave the mid gut and are expelled along with the faecal pellets of the host. Within the cyst membrane, the nucleus of each gametocyte undergoes repeated divisions forming large numbers of nuclei. Gametes are ultimately formed by the addition of cytoplasm around each nucleus. By this time, the partition wall between the two gametocytes disappears. Union of male and female gametes, one from each gametocyte, results in the formation of a zygote which in turn encloses itself in a resistant membrane and develops into a sporocyst or an oocyst. The oocysts are released either by simple dehiscence of cyst or through formation of sporoducts on the cyst surface. Eventually eight sporozoites are formed inside each oocyst. The oocyst germinates when it is swallowed by the specific host organism along with the ingested food into the digestive tract and are ready to repeat the cycle. Only the zygotes of the gregannes are always diploid although there is disagreement about when meiosis occurs, which may vary in different species (Watson, 1916a, Mackinnon and Hawes, 1961; Kudo, 1966, Nelson, 1970; Sokoloff, 1972; Manwell, 1977; Smyth, 1982, Levine, 1988).

Ultrastructure

The ultrastructural studies of the septate gregannes have compelled the systematists for a complete taxonomic revision of the group. The presence of the apical complex itself has been sufficient enough for the creation of a new phylum Apicomplexa to accommodate them.

About the turn of the last century, the Apicomplexa plus some other groups were called Sporozoa because it was thought they all have oocysts. They do not. With the advent of the electron microscope, it was realized that most Sporozoa have an apical complex. Those which do not, like the Microspora, Myxozoa and Ascetospora were shifted out and the group became natural and not artificial.

The apical complex is present at the anterior end of some stage or other. It consists of a number of structures, some of which have been lost by some groups.
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1. The first structure is one or more electron dense polar rings.

2. Inside the polar rings is a conoid, a hollow truncated cone, composed of a number of spirally coiled microtubules.

3. Passing through the conoid are the necks of a number of rhoptnes (for the Greek word for club), electron dense long necked bags of uncertain function which perhaps may secrete enzymes used for cell penetration.

4. Alongside the rhoptnes, in the cytoplasm are a number of rod-like micronemes, which some workers believed are attached to rhoptnes.

5. Running backward from one of the polar rings are subpellicular microtubules whose number varies with the group and which probably have to do with both locomotion and support.

Tronchin and Schrével (1977) studied the sporozoite to trophozoite development of the cephaline gregarine, *Greganna blaberae* in the larvae of the cockroach, *Blaberus craniifer*. According to them, the sporozoite transforms into a cephaline trophozoite affixed to the intestinal epithelium within 18 days. The sporozoite's ultrastructure is not different from that of the sporozoites of other conoidasians. The conoid and dense bodies are present. The pellicle consists of 3 membranes but there are some interruptions in the internal membrane complex. The first dictyosomes are formed from the nuclear envelope. The migration of the nucleus and of the dense bodies followed by the regression of all the structures characteristic of the sporozoites and the establishment of the critical zone that comes to cover the epimente take place within 48 hrs and mark the transformations of the sporozoite into the trophozoite.

Development of the cephaline involves the formation of the epicytic folds which occurs at the base of the deutormente starting on the 3rd day of development. A regular system of longitudinal or epicytic folds is formed over the entire surface of the gregarine. On the 4th and 5th days of development a vacuolar system and a chondnome become differentiated in the epimente, while a fibrillar septum separates the protomente from the deutomente. The next stage is characterized by distribution of polysacchande reserves between these two segments. The studies of Tronchin and Schrevel allows us to determine the role of the epimente in the parasite's nutrition as well as the development of the chondnome and of the cortical membranes in the course of the vegetative growth phase of the cephaline gregannes. Studies with Scanning Electron Microscopy was conducted to find an answer to the mechanism of the gliding movement in the eugregannes by Walker *et al.* (1979). Eugregannes, both single and paired, have the ability to glide through their surrounding media without any deformation in shape. This unique gliding movement has aroused particular interest because at the light microscope level there are no visible signs of the mechanism while Schultze in 1886 attributed gliding over a mucus strain left behind the organism, Schewia koff (1894) suggested that the secreted slime built up and solidified behind the gregarine which was pushed forward. Crawley (1902) implicated a well developed muscular system considering the propulsion to be internally based. After the introduction of electron microscopy to the study of eugregannes it was suggested that various ultrastructural system are involved in greganne-movement. These include fibrils, fibres, tubular structure, tubules and myonemes, all of which may overlap in their application. Both SEM and TEM studies have led to wide speculation of the role of the longitudinal folds in the mobility of eugregannes.
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In view of current interest in non-muscle motile system, gliding in eugregarines is an intriguing system. There are several factors which may be involved

1. The surface of the organisms is covered with epicytic folds. Lateral undulation of the epicyte folds have been implicated in this gliding movement.

2. A trail of mucus thread is left behind the organism as it moves.

3. An ectoplasmic network is present. The function of the network is unknown but it was suggested that the ectoplasmic network coupled with the myonemes provided the force for locomotion, although no explanation was given of how the force is generated. Walker et al. believed that the lateral undulations of the epicyte folds are involved in gliding movement. Sufficient displacement of paraglycogen occurs so that in some areas the folds can be clearly observed by phase contrast microscopy during gliding. However, no lateral undulations of the epicyte folds during gliding were observed which are compared to the undulation observed by the SEM. The folds appear straight on both the upper and lower surfaces of the gregarines as they glide, although there is an apparently periodic pattern of swelling along the length of the folds. The ectoplasmic network and the presence of microtubules in the ectoplasm have been reported by many workers. However, the role of the ectoplasmic network has not been established.

Although there are several reports on the ultrastructure of sporadins and trophozoites, practically nothing was known of the gametocysts and oocysts in this regard because of the difficulty of infiltration of fixative solution and embedding regions into these stages. Hoshide and Todd (1993) for the first time successfully did their ultrastructure studies. According to them, the mature gametocyte consists of five parts, a wall, a homogeneous layer, spongioform layer, centrosphere and sporoduct. Numerous oocysts are formed in the centrosphere. The oocysts are covered with thick homogeneous walls and there are three openings in the walls. These have numerous small projections on the surface and 14 to 15 concentric folds at both ends of the longitudinal axis. Eight sporozoites are arranged longitudinally within the oocysts.

Pathogenicity caused by the parasites

The cephaline gregarines may be intercellular, intracellular or extracellular. According to Théodontès (1988) there is no real intracellular development in eugregarines, the young trophozoite being always surrounded by a membrane and lying in-between and not in the host's cell. The epimentes of all intracellular gregarines are designed so as to enable them to penetrate into the host tissue. The epimente reaches in the vicinity of nucleus of the affected cell and causes breaking and rearrangement of chromatin material (Léger and Duboscq, 1909). The cytoplasm stains less deeply and hence may be regarded to be chemically affected. According to some authors the toxic secretions of the parasites cause atrophy to the host cells. The epithelium is mechanically but physiologically damaged.

Lipa (1967a) believes that nonpathogenicity of eugregarines may not always be true, especially for species inhabiting the gastric caeca. He points out that the presence of the parasites often causes great hypertrophy of the tissues involved, and this may cause rupture of the caecal wall. When this occurs, bacteria enter, often inducing septicemia and consequent death of the host. The mechanism of injury, Lipa
Haidar and Chakraborty (1975a), Haldar (1978) and Ghosh et al (1986) reported some pathogenic eugregarines of insects. The oocysts after being ingested enter the deepest layer of gut epithelium. Histopathological changes occur like shrinkage of the nucleus, vacuolated cytoplasm, enlarged cell size, ultimately leading to its degeneration. Ghosh et al (1986) and Haldar et al. (1988) found that due to infection the hosts become very much lethargic, lay fewer eggs and die within a short period. Haldar et al (1988) in a biotechnological paper remarked the gregarines might be used as control agents for suppressing the populations of harmful and noxious insects.

According to Janardanan and Ramachandran (1982), the infected midgut epithelial cells of Trigonulus wenti and the trophozoite of the harboring parasite Stenodactylus trigoniuli demonstrate trace amounts of lipids. With progressive growth of the trophozoite the host cell is hypertrophied. A fully grown trophozoite is almost completely embedded in lipid globules in the host cell. The lipid globules could be the end products of an anaerobic metabolism as suggested by Brand (1966).

Léger and Duboscq (1909) believe that hypertrophy of the infected cells is initiated by the prolonged mechanical irritation and chemical action by the parasite.

Problems of dispersal

Although it is clear in eugregarines that host infection results from the ingestion of cysts containing sporozoites, which may be produced in great number (two examples are mentioned by Corbel (1968) of species producing, in one case, about 5,12,000 sporozoites, and in another, 8,00,000), it seems likely that things are not always so simple (Manwell, 1977). Watson (1916a) explains the dispersal of gregarines in this way "the oocysts are liberated from the residual protoplasm of the cyst. They are scattered over the grass and ground by the wind and rain and are eaten by some host along with its food. Parasitism is thus accidental." Lipa (1967a) remarks that gregarine infection is indeed more common among ground-dwelling host, such as centipedes and millipedes, and less among flying insects. Coprophagous insects show a high incidence of infection due, perhaps, largely to the frequent density of their populations.

In species in which the food habits of larvae and adults are similar, gregarine infections naturally tend to be heavier, but when dietary requirements differ greatly from those of the adult, larval infections...
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are often lost at metamorphosis. Even then cysts may be carried over in or the adult, and thus parasite dispersal is facilitated (Manwell, 1977)

Classification

Taxonomists differed greatly for a long period in the classification of this group of parasites. The inclusion of gregarines under the phylum Apicomplexa Levine, 1980, and to the class Conoidasida Levine, 1988 has now been accepted by the taxonomists. A thorough survey of literature, in connection with the classification of gregarines, may be elucidated here.

Watson (1916a) in her monograph considered the Cephalina (named Cephalinae) as a tribe under the suborder Schizogregarinae, class Teleosporidia of the subphylum Sporozoa.

Wenyon (1926) regarded Gregarina (Gregarina according to him) the rank of a subclass and included the order Eugregarinida and Schizogregarinida under it.

Grasse (1953) named the group Gregarina as Gregarnomorpha and classified it into three orders, viz., Archigregarina, Eugregarina and Neogregarina. He placed all the families of monocystid and polycystid gregarines under the order Eugregarina without dividing them into Acephalina and Cephalina.

Hall (1953) gave Sporozoa the rank of a subphylum and divided it into three classes viz., Teleosporidia, Cnidosporidia and Acnidosporidia. Teleosporidia was further divided into three subclasses, Gregarina, Coccidia and Haemosporidia. The subclass Gregarina comprised of two orders, Eugregarinida and Schizogregarinida. The order Eugregarinida was further subdivided into two superfamilies - Acephalinoidea and Cephalinoidea.

Chakravarty (1959) divided the subphylum Sporozoa into three classes viz., Teleosporidia, Cnidosporidia and Acnidosporidia. The class Teleosporidia was further divided into two subclasses Gregarina and Coccidia. Gregarina comprised of two orders, Eugregarinida and Schizogregarinida. Acephalina and Cephalina were placed as two suborders of the order Eugregarinida. Cephalina was divided into two infraorders - A septata and Septata. Homberg et al. (1964) introduced a new classification, where Sporozoa was considered as a subphylum. Levine (1970) placed class Sporozoa under the new subphylum Apicomplexa and divided the class Sporozoa into two subclasses viz., Gregarina and Coccidia.

Kudo (1966), however, considered Sporozoa as a class under the phylum Protozoa and divided it into four orders viz., Gregarina, Coccidia, Haemosporida and Haplosporida.

In 1980, the Committee of Systematists and Evolution of the Society of Protozoologists under the chairmanship of Prof. N. D. Levine proposed a newly revised classification of the Protozoa where seven phyla were accepted, viz., Sarcomastigophora, Labyrinthomorpha, Apicomplexa, Microspora, Ascetospora, Myxozoa and Ciliophora. The phylum Apicomplexa was further divided into two classes, Perkinsea and Conoidasida. Three subclasses were included under the class Conoidasida, viz., Gregarina, Coccidia and Piroplasma. The septate gregarines were placed in suborder Septatina of the order Eugregarinida under the subclass Gregarina. Ultimately Levine (1988) introduced a new class Conoidasida to include the gregarines. The same system of classification up to the level of suborder has been followed here.
**Introduction**

**Aim and objective of the present investigation**

The present dissertation deals with the description of 24 species of septate gregannes, all of which are new to science. These gregannes have been obtained from various insect pests infesting a number of economically important plants and some stored products that are collected from different districts of West Bengal, India. Observations on their morphology and life cycle have been given. In defining their systematic positions, attempts have been made to consider the different features of life cycle; characters and measurements of the trophozoites, the sporadms, the cysts and the oocysts, the nature of cyst dehiscence as well as the type of host. The effects of various ecological factors like temperature, humidity and rainfall on the incidence of infection of the gregannes have also been incorporated. Comprehensive discussions on some aspects of biology of the gregannes have been made. The surface morphology of one of the species of septate gregannes belonging to the genus *Stylocephalus* has been worked out with the scanning electron microscopy.

The present thesis has been divided into two parts for convenience in describing the various aspects of the biology of the septate gregannes.

**Importance of the present study**

Although a large number of septate gregarines have been reported from various parts of the world infesting the various insect hosts, more work has to be done in this respect in India. It is hoped that the present investigation will remove some gap of our knowledge in this regard. Almost all economically important plants (vegetables and stored grains) in India are known to be infested with insect pests. The outbreak of these pests quite often becomes a major concern of the forester, the farmer, or the public health (Alwal, 1976). It is an established fact that these insect pests cause immense damage to the standing crops as well as to stored products. Researches on the systematics, biology, life history and control of these pests have been carried out extensively.

For keeping these pests suppressed a number of devices, principles and practices have been developed by scientists. It was noticed by many agriculturists that by the application of pesticides there was a regular resurgence of the pests which was owing to the fact that the natural predators and parasites were also killed. The importance of ecology was then felt and integrated approaches to pest control are now being made in order to achieve more lasting and economical control.

Protozoologists are now giving top priority to the biocontrol of these insect pests through Protozoa (Haldar et al., 1987; 1988). Researchers on the parasitic infections in the insect pests have revealed that many of them are parasitized by members of the subkingdom Protozoa, particularly the eugregarines and microporans. The pathogenicity of eugregarines for their hosts is not generally accepted. Since the gregannes never cause serious injury to their hosts, that is why, perhaps, they have not been studied as thoroughly as other pathogenic coniodasidans (Nelson, 1970) As they live in the gut lumen, many others assume that they are rather harmless commensals (Théodontidès, 1988). On the other hand, schizogregarines have long been recognized as lethal to their hosts.
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It also appears that under certain conditions eugreganne species, not ordinarily pathogenic, may become so. Such is the case with meat-worm larvae infected with Greganna polymorpha. They are usually not significantly affected by the parasite, but are harmed when fed a suboptimal diet (Harry, 1967).

Leonova (1937) found no effect of G. polymorpha or G. minuta on Tribolium confusum. On the other hand, Sumner (1936) found that the development of Tenebrio molitor is much better when it is parasitized with gregannes. Harry (1967) states that larvae of Tenebrio molitor harbour considerable populations of gregannes in the mid gut. They are not necessary for the normal growth of the larvae. Ashford (1970) showed that when Tribolium castaneum larvae were parasitized with Lymphotropha trboli, the larval growth rate was greatly reduced, but there was no visible effect on activity. As such the pathogenicity of eugregannes is very complex. There is very little factual information illustrating the specific mechanisms and there are even contradictory data on the pathogenicity of these protozoans.

A few workers have worked and succeeded in artificially reinfecting the gregannes to their natural insect hosts. Allegre (1948) was successful in artificially reinfecting Greganna rigida (Hall) Ellis to its grasshopper host, Melanoplus differentialis. Narasimhamurti (1964a) was also successful in reinfecting the host Gammarus pulex by its two greganne parasites Heliospora longissima and Retundula gammadri. In 1985, Patil et al. studied the cross infection of cephaline gregannes of tenebroid insects but were unable to establish sporozoites in the lumen of the recipient hosts due to the innate immunity of the insects as reported by Steinhaus (1967).

Attempts in the line of artificial reinfection and cross-infection to various insects is of certain importance as the knowledge will enable us to understand the degree of host-parasite relationship. Researches on the bionomics and cross-infection experiments may thus also throw some light on the possible biological control of injurious insect pests by the eugreganne protozoans.

According to a recent study by Gaston and May (1992), the numbers of researchers in taxonomy and systematics have not decreased considerably although funds and researches in other fields have increased markedly and giving a look at the total number of named species (1.5 - 1.8 millions) and probable number of existing species which may be 3 millions to 80 millions or even more, it becomes apparent that the present investigation will remove some gap of our knowledge. In the same commentary Gaston and May (1992) say, "A crude estimate of the number of taxonomists shows a striking mismatch between the geographical location of practitioners and biological diversity." Thus, a large number of septate gregannes have been reported from various parts of the world infesting various arthropod hosts and a lot of work has to be done in this respect in India.