PREFACE
General Introduction:

A cursory review of the literature, on "Gregarines", reveals that mostly these parasites infect a large number/non-chordate hosts, although a few species are reported from the protocordates, viz. the ascidians (Hyman, 1940). They do not seem to infect the craniate vertebrates. In the invertebrate group they have been reported from the following Phyla: Coelenterata, Platyhelminthes, Annelida, Arthropoda, Mollusca and Echinodermata (Kamm, 1922). Among these, the animals that most commonly harbour the gregarines are the arthropod hosts.

In the present study, we have studied the different stages of the life-cycle of four species of cephaline gregarines, viz. Gregarina cuneata (Stein, 1848) from the larva of beetle Tenebrio molitor, Hirmocystis speculitermis (Uttangi and Desai, 1962) from the worker caste of the non-xylophagous termite Speculitermes cyclops sinhalesis (Roonwal and Sen Serma), Hirmocystis incola (Uttangi and Desai, 1961) from the worker caste of another non-xylophagous termite Capritermes incola (Wasmann) and Steinina termitis (Uttangi and Desai, 1962) from the worker caste of the termite Speculitermes cyclops sinhalesis (Roonwal and Sen Serma).

The above mentioned hosts are abundantly available around Dharwad, 15° 17' N and 75° 3' E (Karnataka State, India), located at an altitude of about 2000' to 2400' MSL in the transitory belt between the Western Ghats and the Eastern plains of the Deccan plateau. The annual rain fall is about 800 to 900 mm. Temperature fluctuations during the summer and winter are from 45° to 20°C.
As we are dealing with different stages of the life-history of the above mentioned gregarines, viz. trophozoites, gamonts and gametes, it is considered appropriate to describe in brief the general life-cycle of the gregarines in the preface.

The salient features of the life-cycle of the cephaline gregarines are:

1) Parasitisation of a single host
2) Absence of schizogony or asexual multiplication
3) Completion of trophic growth within the body of host, while the reproductive stages are completed outside the host's body.
4) The initial stages during the trophic phase are cytozoic (inside the cell of the gut epithelium of the host) and the later ones are coelozoic (inside the lumen of gut).

When the food material contaminated with spores of gregarine is ingested by the natural host, the spores dehisce and liberate the sporozoites in the fore-gut of the host. These sporozoites soon invade the fore-gut epithelium and derive nourishment from this tissue and start growing. Gradually the tiny sporozoites develop into cephalonts in which three regions, the epimerite, protomerite and deutomerite are differentiated. Gradually, the cephalonts make their way out of the gut epithelium but they remain suspended to the latter by means of their epimerite. At this stage they are known as trophozoites. The trophozoite is a conspicuous stage in the life-cycle of gregarines.
and contains the same three parts as cephalonts with the nucleus in the deutomertie. In most of the species the epimerite is lost in the grown up trophozoites. These trophozoites in a majority of the species and genera remain in between the peritrophic membrane of gut lumen for an unknown period and complete their trophic growth in order to prepare for encystment and sexual reproduction. The trophozoites usually occur in biassociation (rarely multiple associations), a condition known as syzygy. In a syzygy the anterior end of one trophozoite adheres to the posterior end of another trophozoite. In some genera such as *Stylocephalus*, the association is seen just prior to encystment and it is between the protomerites of gamonts. In a syzygy the anterior one is known as the primite and the posterior one is the satellite. In some genera such as *Hirmocystis*, three or more trophozoites may form a chain. Such chains appear to be the temporary associations and they will break into pairs as the trophic growth ensues. Development of mature trophozoites into gamonts often involves noticeable changes. The epimerite (of the primite) undergoes complete resorption. The associated gamonts prepare for the encystment. After reaching maturity, they become sluggish and inactive, and slowly start exhibiting the bending and rotatory movements. After sometime they bend in such a way that the tail end of the satellite embraces the head end of the primite and form a rounded mass. In the mean while a cuticular transparent cyst wall is secreted by them so as to form a perfectly spherical or oval cyst called as gamontocyst or gametocyst. Within the gametocyst each gamont undergoes gamogony. During this process the septum between the protomerite and deutomerte
of each gamont disappears, representing some advanced stage. However, the line of association between the gamonts is still present and the cysts at this stage are extruded out of the host's alimentary canal along with faecal matter and further development occurs outside the host's body.

Gradually the line of association between the two gamonts the cyst disappears indicating the beginning of formation of gametes. During this stage the nuclei of both gamonts/gametocytes undergo repeated divisions, giving rise to many daughter nuclei, which soon form the gametes. Each gamont produces one type of gametes i.e. either the macrogametes or the microgametes. Then the partition wall disappears allowing the syngamy and formation of zygotes. Soon each zygote envelops itself by a transparent and resistant covering and becomes a spore. The spores are of different shapes such as barrel-shaped as in Gregarina cuneata, or biconical as in Stemina termitis, or hat-shaped as in Stylocephalus conoides, etc. Subsequently within each spore develop eight sporozoites. The spores are liberated free either by the dehiscence of the cyst or through the spore ducts developed on the cyst. The spores get mixed up with soil, humus etc., or water in case of the cysts voided by the aquatic hosts. When a natural host ingests such a contaminated matter, the spores get entry into the latter's alimentary canal where they dehisce and set free the tiny sporozoites. The sporozoites eventually pave their way into the gut epithelium. From this onwards the life-cycle of the parasite is repeated.
The Subphylum Apicomplexa is characterised by the zygotic meiosis. Only the zygote stage is diploid while all other stages of the organism are haploid (Raikov, 1982).

**Specific Introduction:**

In metazoa, differences in gametes are reduced to practically those between eggs and spermatozoa. In majority of higher organisms where sexes are separate, the sexual dimorphism is not restricted to the gametes alone, it is also seen in the trophic forms. In Protozoa, there is no common type of difference but all gradations may be found here from apparently similar individuals to well differentiated ova and spermatozoa. This has lead to attempts to classify the gametes for purpose of description, into those which are similar (isogametes) and those which are dissimilar (anisogametes) (Calkins, 1933). The female gamete is usually spherical or oval in shape, larger in size and non-motile and stores considerable amount of nutrients and the male shows the opposite characteristics. In protozoa also, the larger receptive cells with dense cytoplasm are conventionally designated as the females and the smaller and motile gametes as the males (Curtis, 1969).

Among the protozoans, this phenomenon of sexual dimorphism is noticed in the vegetative stages also in some ciliates such as *Vorticella nebulifera* (Mapusa, 1889, cited in Wenyon, 1926) and *V. microstomata* (Finley, 1952, cited in Mackinnon and Hawes, 1961) and in *Ephelota gemmipora* (Grell and Meister, 1984), in Mastigophora, such as *Volox spermatospharea*, etc. (Powers, 1908, cited in Kudo,
1977) and in Coccidians (Wenyon, 1926).

Hyman (1940), Hall (1953), Mackinnon and Hawes (1961) and Kudo (1977) opine that in Gregarina genera, even in the apparent isogamety two types of gametes can be distinguished on the basis of morphological features such as the body size and the cytoplasmic inclusions. Careful ultra-structural/cytological studies should reveal that conspicuous differences between the sexes are commoner than usually stated. Nevertheless it is true that marked anisogamy of Coccidia is never seen in this group. The primitive anisogamy apart from the biochemical differentiation may involve differences in shape and slight differences in size of the gametes and this trend culminated in the development of microgametes resembling spermatozoa in their low cytoplasmic content and relatively large gametes (macrogametes) containing appreciable amount of stored nutrients.

Among the Gregarina genera although anisogamety has been described in Monocystis (Cuenot, 1901, cited in Wenyon, 1926), Stylocephalus (Léger, 1904, cited in Wenyon, 1926, Desai, 1966 and 1980), Monoductus (Ray and Charkravarty, 1933, cited in Bhatia, 1938) and Stenophora (Karandikar and Rodgi, 1955 and Kudo, 1977) on the basis of morphological features such as the presence of flagella or flagellum in one type of gametes and the absences of it in the other, the anisogamous gamonts have not been described. Wenyon (1911a), (cited in Wenyon, 1926), has described anisogamety in Lankesteria culis on the basis of the difference in size of nuclei of gametes but no reference is made to anisogamony. It is difficult to distinguish
the male and female gamonts as they are typically solitary until they are about to encyst. However, in *Schaudinnella henleae* (Nusbaum, 1903, cited in Kudo, 1977) and *Stylocephalus bahli* (Mishra, 1941) anisogamous gamonts have been described on the basis of unequal sizes of gamonts, i.e., the larger gamont as the female and the smaller one as the male. So far, to the best of our knowledge no systematic attempt is made to study the problem of sexual dimorphism in gregarines with reference to the morphological features such as the body size of the trophozoites and gamonts in syzygies and the size of their nuclei and the morphology of the gametes. Hence there is a need to study the morphological features of gregarines to know whether they would give any clue to the identification of sexes in a conventional manner.

In recent years apart from the traditional ways of tracing the sexual dimorphism on the basis of morphological/structural features of the trophozoites, gamonts and gametes, Canning (1961 and 1962) and Klimes et al. (1972) have adopted some cytochemical techniques in differentiating one sex from the other on the basis of differences in the reserved nutritive material in the two sexes in coccidians Adelidae and Eimeridae. By adopting these cytochemical techniques Stein (1951), Bobyleva (1963) and Desai (1980) have observed the probable existence of sexual dimorphism at the cytochemical level in a few gregarines such as *Geneiorhynchus aeshnae*, *Enterocystis ensis* and *Stylocephalus conoides* respectively. Thus it is evident from the available literature that information on the
sexual dimorphism in gregarines based on cytochemical studies is also rather very limited. Similar studies extended to a few more gregarines might help in corroborating the findings of earlier workers.

The biosynthesis of steroids including the sex-steroids and their *in vitro* conversion have been studied in a number of metazoan invertebrates, viz. in nematodes (Brigs, 1972); insects (Lehoux and Sandor, 1969 and 1970; Sandor et al., 1975 and Sandor and Medhi, 1979; Trandabura and Tasca, 1976; Hurkadli, 1982, De Clerck et al., 1983 and 1984; Ohnishi et al., 1985 and Ogiso et al., 1986); in molluscs (Gottfried and Dorfman, 1970a, 1970b & 1970c; De Longcamp et al., 1974; Carreau and Dorsdowsky, 1974, and Gouder et al., 1977) and in echinoderms (Hathway, 1965; Schoenmakers, 1979; and Schoenmakers and Voogt, 1980). The pathways involved in the process are shown to be similar to those operating in the vertebrates (Barrington, 1972, and Sandor and Medhi, 1979).

Likewise, biosynthesis of steroids and sterols have also been reported in a few protozoans other than sporozoans (Sebeck and Michaels, 1957; Conner et al., 1966; and reviews Wagtendonk, 1955; Wright, 1964, Shorb, 1964; Charney and Herzog, 1967; Dewey, 1967 and Von Brand, 1973). In gregarine species neutral lipids and cholesterol in *Stylocephalus meomorphi* (Amoji, 1975) and neutral lipids, cholesterol and certain key steroid dehydrogenases involved in the biosynthesis/metabolism of steroids in *S. conoïds* (Desai and Nadkarni, 1987) have been reported.
On the basis of biochemical studies two 17β-hydroxy-steroid dehydrogenase entities have been inferred in the insect Gryllus domesticus (Lehoux and Sandor, 1970) and in the bivalve mollusc Mytilus edulis (De Longcamp et al., 1974). Histochemical studies on the gonads of the insects Philosamia ricini, Antitheria mylitta and Bombyx mori have also revealed two separate specific entities of 17β-hydroxysteroid dehydrogenase, namely, testosterone 17β-hydroxysteroid dehydrogenase and estradiol 17β-hydroxysteroid dehydrogenase (Hurkadli, 1982). Further the ovaries of the insect Bombyx mori convert estrone to estradiol and vice versa but testosterone is not appreciably changed. The testes convert testosterone to several metabolites but estradiol is not converted (Ogiso et al., 1986). No such studies so far are made in the case of gregarnines, even though the presence of some steroid dehydrogenases are reported in S. conoides (Desai and Nadkarni, 1987). It would be interesting to find out whether in gregarnines also occur similar entities of the sex steroid dehydrogenase, viz. 17β-hydroxysteroid dehydrogenase (17β-HSDH), on the basis of the preferential utilisation of sex steroid substrates - testosterone and 17β-estradiol. The results might help in strengthening the concept of sexual dimorphism in these protozoans.

The female sex-specific yolk proteins (vitellogenins) have been identified in the haemolymph of a number of insects (Englemann, 1970 and 1972; a review Doane, 1973). The female sex-specific yolk proteins have been identified electrophoretically in Gryllus domesticus (Kunz and Petzelt, 1970) and in Acheta domesticus (Bradley and Edwards, 1978).
Among the protozoans the electrophoretic studies with reference to proteins have been carried out in Amoeba, sp. (Kates and Goldstein, 1964), in Plasmodium knowlesi (Williamson, 1967)*, in Trypanosoma brucei (Humphreys, 1967)*, in T. cruzi, (Afchain and Capron, 1969)* and in Leishmania maxicana (Crook et al., 1969)*. In Paramecia it has been shown that the activity of a soluble protein which is similar to immarturin in terms of molecular size and net charge is responsible for mating activity (Nobuyuki and Karina, 1986). In plasmodia both microgametogenesis and macrogametogenesis are shown to be dependent on de novo protein synthesis (Toye et al., 1977 and Kumar et al., 1983). Proteins with reference to sex differentiation in gregarines have not been studied so far. Quantitative estimation of proteins and their electrophoretic studies in gregarines might help to understand whether sexual dimorphism can be elicited at the protein level also.

**Broad outline of the present work:**

In view of the facts mentioned above a sort of comprehensive study has been taken up in four cephaline gregarine species mentioned already to understand the extent of sexual dimorphism from the morphological, cytochemical and bio-chemical aspects. The methodology adopted, the findings and the discussions, etc. have been presented in this thesis in four chapters. Following are some excerpts of the aims and methodology adopted in each chapter.

Chapter I:

Chapter I deals with morphometric studies of the primites and the satellites of the trophozoites and gamonts in syzygies and their nuclei, of the four species of gregarines, Gregarina cuneata, Hirmocystis speculitermis, Hirmocystis incola and Steinina termitis. These studies have been extended to the gametes of only two species viz. Gregarina cuneata and Hirmocystis speculitermis, since the cysts of only these species are available in plenty. The results are statistically analysed by applying the Student's 't' test.

Chapter II:

Chapter II deals with the study of sexual dimorphism by applying the cytochemical techniques such as Periodic Acid Schiff (PAS), Oil Red O (O.O) and Fettrot 7B, to the primites and the satellites in the syzygies in trophozoite and gamont conditions of all the above mentioned four species of gregarines, to find out whether there is any difference in the amount of stored reserve nutritive materials and which would indicate the probable sex of the individual. In addition, these studies have been extended to the gametes of G. cuneata and H. speculitermis, and the results are compared with those noted in the trophic forms.

Chapter III:

Chapter III deals with the histochemical demonstration of certain important steroid dehydrogenases involved in the synthesis/metabolism of sex steroids in the primites and the satellites in syzygies in gamont condition to know any preponderance of either of the two
sex hormones over the other in the primites and the satellites of the above mentioned four species of gregarines, as might be indicated by the difference in the intensities of the activity of sex steroid dehydrogenase by using different substances. The results would further give some clue to the sexual dimorphism in these organisms.

Chapter IV:

Chapter IV deals with Sodium Dodecyl Sulphate (SDS) gel electrophoretic studies on soluble protein fractions of the primites and the satellites (separated from the syzygies) of the gregarine Hirmocystis incola to know whether there are any qualitative differences in the soluble proteins and in the number of protein fractions of the primites and the satellites which would reveal the sexual dimorphism at the protein level also.
Limitations of the present work:

Our knowledge on sexual dimorphism in gregarines is rather scanty as evidenced by only a few reports available so far. Some authors (Nusbaum, 1903*; Misra, 1941' and Wenyon, 1911a**) have considered a few morphological features of these organisms, while others (Stein, 1961; Bobyleva, 1963 and Desai, 1980) have taken into consideration only some cytochemical features. The findings of each group naturally turn out to be a one sided approach to the problem. Under circumstances this area of gregarine study was taken up in our laboratory for investigation from four different aspects, viz. the morphological aspects, the cytochemical aspects to study the storage of nutrients, the preferential utilization of testosterone and estradiol-17β during the synthesis/metabolism of sex steroids and the biochemical aspect at the protein level. The present project though has yielded some interesting data in each aspect of our approaches, we cannot claim ours as an exhaustive study. At least two more aspects which could not be taken up by us are, (a) karyological aspect, and (b) the environmental factors that influence the sex differentiation in these organisms. Owing to the extremely diffused state of the chromatin material, the gregarine nucleus is totally unsuitable for study in the trophic and gamont conditions (Raikov, 1982). Further the gametes are too small

* Vide; Kudo, 1977
** Vide, Wenyon, 1926.
in the species taken up for the present study and hence they too are unsuitable for their karyological study. Recent studies on *Toxoplasma gondii* Cornelissen and Overdulve (1985) have shown that environmental factors have a decisive role in influencing the differentiation of sex in these protozoans. These techniques cannot be applied to the gregarines as the gregarine cultures cannot be raised since there is no asexual method of their propagation in their life-cycle. Even the extensive biochemical studies could not be undertaken in these organisms only due to this reason. Any way these areas of sexual dimorphism will be taken up in our laboratory as and when better facilities are made available.