A COMPARATIVE STUDY OF EGG DEVELOPMENT IN TWO SPECIES OF SIPHONARIID LIMPETS WITH CONTRASTING DEVELOPMENTAL MODES

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Abstract

The family Siphonariidae is considered primitive amongst the basommatophorans although the ancestry and evolutionary relationships of these marine pulmonates are far from settled. This thesis investigates and compares different aspects of egg development and the female reproductive system in two sympatric species of *Siphonaria* with different developmental mode (*S. capensis*, a planktonic developer and *S. serrata*, a direct developer). The study on the seasonality of gametogenesis and spawning shows that they are both spring/ summer spawners with continuous sperm production. The egg production is highest in spring and summer with a brief interruption in winter months. The gametogenic cycle when examinated, reveals that both species are simultaneous hermaphrodites once sexually mature. Amongst various factors that are investigated, density of animals has a positive effect on the number of spawn only during peak spawning. Larger individuals of *S. capensis* and *S. serrata* contains more mature oocytes in the gonad indicating that the shell length and fecundity of these two limpets are positively correlated. By contrast, parasitism by trematodes has a drastic effect on the reproductive output of these limpets leaving them completely castrated. Egg development in *S. capensis* and *S. serrata* shows that both produce yolk autosynthetically (with the help of organelles like RER, Golgi bodies) but *S. serrata* also incorporates some high molecular weight precursors via endocytotis. The structure as well as the biochemical composition of the egg ribbons is also different between these two species with higher carbohydrate and protein content of collar shaped spawn of *S. serrata*. *S. capensis* produces egg ribbons of less fibrous nature containing thinner egg capsules compared to the direct developer. A comparison of both the glandular complex and spermatheca

between these two limpets shows no inter specific difference in the structure although the glandular complex of the siphonariids shows fine structural and histochemical similarities with the albumen gland and membrane gland of the opisthobranchs. The structure of the spermatheca suggests that in both species the organ most possibly receives sperm (for degradation only?) and may transport them via the spermathecal duct (for fertilization?). Finally, it is suggested that *S. capensis* and *S. serrata* exhibit primitive features (e.g., an autosynthetic mode of vitellogenesis in *S. capensis* and a single glandular complex composed of an albumen and a mucous gland) compared to other basommatophorans, which should be considered in future phylogenetic investigations.

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I have wandered far,

reaching a cross-road and walking

straight ahead, smiling.

JE

Chapter 1

General introduction

Molluscs are second only to the arthropods in the number of species discovered, diversity of forms, and habitats they occupy (Hyman, 1967; Ruppert & Barnes, 1994; Baur, 1998; Saleuddin, 1998; Hickman *et al.*, 2000). Molluscs originated in the sea (Hyman, 1967) and it is in marine habitats where they dominate other animal phyla (Giese & Pearse, 1977). Many molluscs now occupy terrestrial habitats, although the majority of these that is the stylommatophorans, are partially dependent on a moist environment (Fretter & Peake, 1975; Hickman *et al.*, 2000). Within the phylum Mollusca the Gastropoda is the largest class, containing at least 60% of the described species (Salvini-Plawen, 1985; Ruppert & Barnes, 1994). Gastropods are therefore considered one of the most diverse groups of animals (Ponder & Lindberg, 1997). Three subclasses of Gastropoda are recognised, the Prosobranchia and Opisthobranchia, which are primarily marine and the Pulmonata, which are mainly terrestrial, although some live in aquatic and semi-aquatic habitats (Hyman, 1967; Fretter & Peake, 1975; Solem, 1985). Whilst there is vast literature available for the terrestrial and freshwater pulmonates, marine pulmonates are a less studied group.

Although the phylogeny of the Pulmonata is unresolved, 2 superorders are currently recognised, the Basommatophora and Eupulmonata (which includes the orders Stylommatophora and Systellomatophora) (Nordsieck, 1992). More recently however, the pulmonates have been placed together with some 10 opisthobranch groups under the monophyletic group Euthyneura (Fig. 1) (Dayrat & Tillier, 2002), which was originally proposed by Spengel (1881, cited in Hyman, 1967) and later supported by Hyman (1967). Fretter & Peake (1975) suggested that the opisthobranchs and pulmonates had a similar marine ancestry. Whereas the opisthobranchs have continued to inhabit the marine

environment only a few primitive basommatophoran pulmonates are marine.

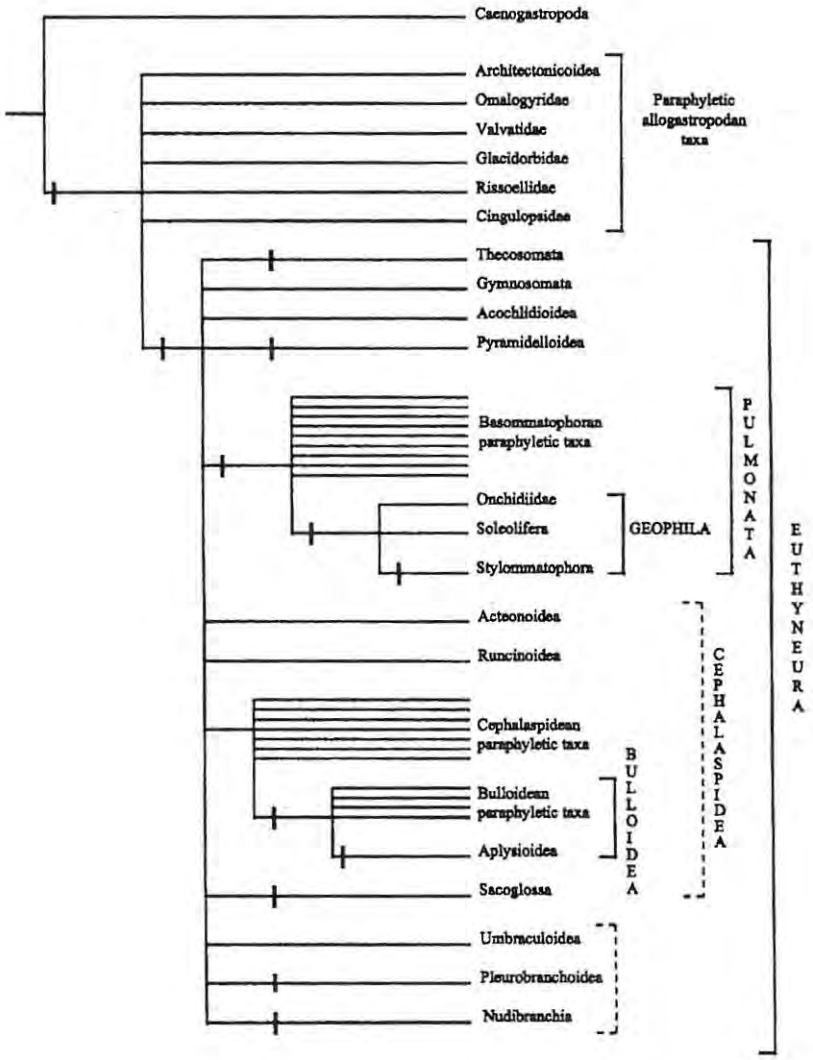


Fig. 1. Phylogeny of Heterobranchia (based on the morphological characters) showing the relationships within Euthyneura and paraphyly of Basommatophora. The interrupted lines indicate possible phylogenetic relationship. Reproduced with permission from the author (B. Dayrat, pers. comm.). (from Dayrat & Tillier, 2002)

The monophyly of the Pulmonata has been confirmed by morphological as well as molecular data, although within the Pulmonata the Basommatophora and Geophila (Onchidiidae, Soleolifera and Stylommatophora) are considered paraphyletic taxa

(Dayrat *et al.*, 2001; Dayrat & Tillier, 2002) (Fig. 1). Hubendick (1978) had previously suggested that whilst the Basommatophora consisted of "clearly defined groups", their relationships were uncertain. Within the Basommatophora the Thalassophila (which includes Amphiboloidei and Siphonarioidei) and Hygrophila (which includes Chilinoidei and Branchiopulmonata) have been recognised as orders (Nordsieck, 1992). The Siphonariidae and Amphibolidae are considered the most primitive families of the Basommatophora as they show monauly (Geraerts & Joosse, 1984). Nordsieck (1992) stated that within basommatophorans, the family Siphonariidae shows more apomorphic characters such as a patelliform shell, reduced chromosome number n= 16, sperm transferred in spermatophores and bursa copulatrix when compared to the Amphibolidae (e.g., jaws absent, bursa copulatrix absent). Several authors (e.g., Hyman, 1967; Purchon, 1977; Dayrat & Tillier, 2002; G. Haszprunar, pers. comm.) however, have considered *Siphonaria* to be the most primitive extant pulmonate genus (Fig. 2). Four genera *Siphonaria*, *Williamia*, *Kerguelenella* and *Benhamina* belong to the family Siphonariidae and 60 species have been recognised to date (Hodgson, 1999). If the phylogeny of pulmonates is to be resolved, (especially of the basommatophorans) and the evolutionary relationships between different clades understood, it is crucial that primitive groups such as the Siphonariidae are studied.

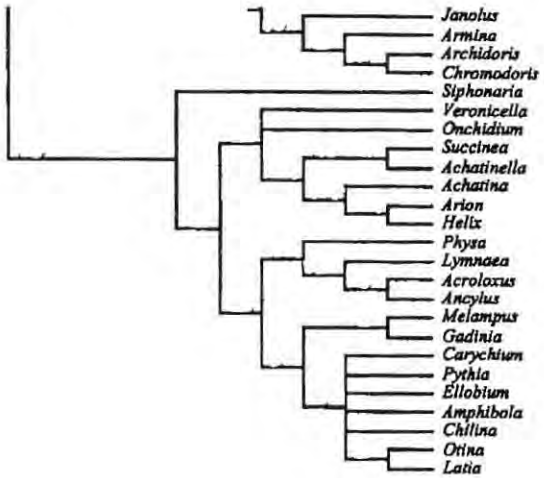


Fig. 2. Strict consensus tree of 3446 equally parsimonious trees showing the genus *Siphonaria* at the base of Basommatophora. Reproduced with permission from the author (B. Dayrat, pers. comm.). (from Dayrat & Tillier, 2002)

Siphonariids are widely distributed globally and most diverse in warm waters of the Southern Hemisphere (Hodgson, 1999). These marine limpets, which are primarily intertidal, feed mainly on macroalgae (Hodgson, 1999). The success of siphonariid limpets has been attributed to many of their physiological (anaerobiosis, metabolic rate depression) and behavioural adaptations (foraging activity, homing) (Marshall & McQuaid, 1991; Gray & Hodgson, 1997; Hodgson, 1999). Siphonariid limpets possess a secondary gill, which allows them to respire both in air and water (Hodgson, 1999), a feature that has raised considerable debate about their ancestry (Yonge, 1952; Solem, 1985). According to Eckelbarger (1994), studying closely related animals with differing life history strategies can provide evolutionary insights, a view supported by Stearns & Hoekstra (2000). The presence of planktonic larvae in some siphonariids implies that they have a marine ancestry (Hyman, 1967; Ruppert & Barnes, 1994; Hodgson, 1999; Pechenik, 1999) although some biologists have also advocated the possibility of a

terrestrial origin i.e., siphonariids stem from land pulmonates that have re-invaded marine habitats (Yonge, 1952; Chambers, 1994). Despite recent molecular studies (using techniques such as estimation of total cellular protein and RAPD), the origin of siphonariids remains unsolved (Chambers *et al.*, 1996, 1998). It has been suggested that resolving the relationships of siphonariids to other primitive pulmonates may help clarify the origin of siphonariids (Hubendick, 1978; Solem, 1985; Nordsieck, 1992; Chambers, 1994).

Like all basommatophorans, siphonariids are hermaphrodites and lay benthic egg masses (except *S. tasmanica* and *S. virgulata* which produce pelagic egg masses) after internal fertilization (Hubendick, 1978; Creese, 1980; Quinn, 1983; Chambers & McQuaid, 1994a; Hodgson, 1999). Chambers & McQuaid (1994b) recognised three types of developmental strategy in siphonariid limpets: direct development in which embryos complete their development inside the egg capsule and, on hatching, juveniles emerge with a crawling foot; planktonic development whereby larvae hatch as free swimming veligers; and intermediate development (larvae possess both a velar apparatus and foot). Although the term "direct development" has been used by Chambers & McQuaid (1994a & b), it is technically incorrect as the veliger stage is present and the mode of development should be termed "intracapsular". In this thesis, I have discarded the term proposed by Chambers (1994) and used the term "intracapsular development" instead. Chambers (1994) proposed both adaptive and phylogenetic models to explain the occurrence of these developmental modes in *Siphonaria*. It has been further proposed that except for 2 subgenera (*Patellopsis* and *Sacculosiphonaria*), to which most South African siphonariids belong, there is a systematic basis to developmental mode (Table 1)

(Chambers & McQuaid, 1994b) even though only 21 species were considered. Although Chambers (1994) attempted to combine both a phylogenetic and adaptive approach to explain the presence of different life history strategies in *Siphonaria* he essentially dealt with reproductive traits like developmental mode, shape of the egg masses, body size, fecundity and the relationship of these traits with developmental mode. A life history strategy is a combination of several different life history traits such as age at first reproduction, life time fecundity, mortality and reproduction, which deal directly with reproduction and survival of an organism (Stearns, 1992; Stearns & Hoekstra, 2000). Life history studies describe and explain the variation in life history traits, which is a key to understanding how the organisms have evolved under certain ancestral constraints (Stearns, 1992).

Table 1. Presence of direct and planktonic developers among eight subgenera of *Siphonaria* (Reproduced with permission; Chambers & McQuaid, 1994b).

Subgenus	Number of direct developing species	Number of planktonic developing species
Benhamina	0	1
Ductosiphonaria	0	2
Heterosiphonaria	0	1
Kerguelenella	2	0
Pachysiphonaria	0	3
Patellopsis	4	4
Sacculosiphonaria	1	1
Siphonaria	2	0

Nine species of siphonariids are currently recognised along the South African coast with the greater species diversity along the subtropical to warm temperate east and south-east coast (eight species) (Chambers & McQuaid, 1994a). By contrast only three species [*S. capensis*, *S. serrata* and *S. compressa* (the latter found in Langebaan lagoon only)] are found on the cool temperate Atlantic coast (Chambers & McQuaid, 1994a). Of these nine species, which have either direct (*S. serrata*, *S. anneae*, *S. compressa*, *S. dayi*, *S. nigerrima*, *S. tenuicostulata*) or planktonic (*S. capensis*, *S. concinna*, *S. oculus*) development, *S. capensis* and *S. serrata* occur on both the east and west coast of South Africa (Chambers & McQuaid, 1994a & b). Research on South African siphonariids has included a study of their systematics (Allanson, 1959; Chambers & McQuaid, 1994a), distribution (Allanson, 1959), anatomy (Allanson, 1959; De Villiers & Hodgson, 1987), parasitism (Hodgson *et al.*, 1993), physiology (Marshall & McQuaid, 1991), foraging behaviour (Branch & Cherry, 1985; Gray & Hodgson, 1997) and population genetics (Seaman, 2002). Studies on their reproductive biology, however are limited to a description of basic reproductive anatomy (Allanson, 1959), an investigation of sperm morphology (Hartley *et. al.*, 1985; Hodgson *et. al.*, 1991), a review of the type of larval development as well as some information on reproductive seasonality in two species (Chambers, 1994) and a description of oogenesis in two species of siphonariids (Pal & Hodgson, 2002). In recent years morphological studies on metazoan ovaries and eggs have been used to resolve systematic and phylogenetic questions (see Eckelbarger, 1994 for review). Such studies are lacking in *Siphonaria* and therefore the goal of this research was to provide the first detailed comparative description of gametogenesis, structure of

the ovary, oogenesis, structure of the spawn and some of the accessory reproductive organs in siphonariid limpets with different modes of development. In addition, aspects of the seasonality of reproduction not studied by Chambers (1994) will be dealt with.

Two species *Siphonaria serrata* (Fischer, 1807) and *S. capensis* (Quoy & Gaimard, 1833) were chosen as representatives of intracapsular and planktonic developers. These species are not only abundant on South African rocky shores, they also have a wide geographic distribution (*S. capensis* is found from Maputo on the east coast to the Orange river on the Atlantic coast and *S. serrata* has a continuous distribution from Durban to Cape Town and, after a short geographical break, can again be seen northwards from the Orange river) much of which is overlapping (Chambers & McQuaid, 1994b). *S. serrata* is an intracapsular developer with encapsulated veliger larvae whereas *S. capensis* has planktonic development (Chambers & McQuaid, 1994b).

Before a detailed study of egg formation could be undertaken it was necessary to establish the reproductive cycle of both species so that tissues could be sampled at the appropriate time of the year. Whilst Chambers (1994) had documented some aspects of reproductive seasonality in *S. serrata* (gonad index and egg mass counts), his study did not include any histological examination of the gonad and gametogenesis. Furthermore, there was no published data on *S. capensis*. Chapter 2, therefore, examines the seasonality of reproduction (gametogenesis and spawning of *S. capensis* and *S. serrata*) in the Eastern Cape of South Africa. Chapter 3 expands on the spawning results and investigates the variability in the fecundity of *S. capensis* at a population level. This is followed by a light and ultrastructural investigation of oogenesis in *S. capensis* and *S. serrata* including a consideration of the implications of the mode of vitellogenesis with

respect to the different developmental strategies. The fine structure of the egg masses of *S. capensis* and *S. serrata* are compared in Chapter 5, the results being related to the adaptive physiology of the egg mass. Finally in Chapter 6 the ultrastructure of other regions of the reproductive system, which may be involved in egg mass formation e. g., the glandular complex of *S. capensis* and *S. serrata* is compared and its role in the egg development is evaluated. Chapter 6 also examines the ultrastructure of the spermatheca and discusses the possible role(s) of this organ. The conclusions on gametogenesis, hermaphroditism, seasonality of reproduction and functional morphology of accessory sex organs are drawn in Chapter 7 and presented in the light of siphonariid ancestry.

With the exception of first and final chapters, each chapter in this thesis comes with its own introduction, methodology, results, discussion and a list of references.

References

ALLANSON, B. R. 1959. On the systematics and distribution of the molluscan genus *Siphonaria* in South Africa. *Hydrobiologia*, **12**: 149- 180.

BAUR, B. 1998. Sperm competition in molluscs. In: *Sperm Competition and Sexual Selection* (T. R. Birkhead & A. P. Møller, eds), 255- 290. Academic Press, London.

BRANCH, G. M. & CHERRY. M. I. 1985. Activity rhythms of the pulmonate limpet *Siphonaria capensis* Q. & G. as an adaptation to osmotic stress, predation and wave action. *Journal of Experimental Marine Biology and Ecology*, **87**: 153- 168.

CHAMBERS, R. J. 1994. *The conflict between adaptation and constraint: the case of the siphonariid limpets*. Ph.D thesis. Rhodes University.

CHAMBERS, R. J. & MCQUAID, C. D. 1994a. Notes on the taxonomy, spawn and larval development of South African species of the intertidal pulmonate limpet genus *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **60**: 263- 275.

CHAMBERS, R. J. & MCQUAID, C. D. 1994b. A review of larval development in the intertidal limpet genus *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **60**: 415- 423.

CHAMBERS, R. J., MCQUAID, C. D. & KIRBY, R. 1996. Determination of genetic diversity of South African intertidal limpets (Gastropoda: *Siphonaria*) with different reproductive modes using polyacrylamide gel electrophoresis of total cellular proteins. *Journal of Experimental Marine Biology and Ecology*, **201**: 1- 11.

CHAMBERS, R. J., MCQUAID, C. D. & KIRBY, R. 1998. The use of randomly amplified polymorphic DNA to analyze the genetic diversity, the systematic relationships

and the evolution of intertidal limpets, *Siphonaria* spp. (Pulmonata: Gastropoda), with different reproductive modes. *Journal of Experimental Marine Biology and Ecology*, **227**: 49- 66.

CREESE, R. J. 1980. Reproductive cycles and fecundities of two species *Siphonaria* (Mollusca: Pulmonata) in South-eastern Australia. *Australian Journal of Marine and Freshwater Research*, **31**: 37- 47.

DAYRAT, B., TILLIER, A., LECOINTRE, G. & TILLIER, S. 2001. New clades of euthyneuran gastropods (Mollusca) from 28S rRNA sequences. *Molecular Phylogenetics and Evolution*, **19**: 225- 235.

DAYRAT, B. & TILLIER, S. 2002. Evolutionary relationships of euthyneuran gastropods (Mollusca): a cladistic re-evaluation of morphological characters. *Zoological Journal of the Linnean Society*, **135**: 403- 470.

DE VILLIERS, C. J. & HODGSON, A. N. 1987. The structure of the secondary gills of *Siphonaria capensis* (Mollusca: Pulmonata). *Journal of Molluscan Studies*, **53**: 129- 138.

ECKELBARGER, K. J. 1994. Diversity of metazoan ovaries and vitellogenic mechanisms: implications of life history theory. *Proceedings of the Biological Society of Washington*, **107**: 193- 218.

FRETTER, V. & PEAKE, J. 1975. *Pulmonates*. Vol, I. Functional Anatomy and Physiology. Academic Press, London.

GERAERTS, W. P. M. & JOOSSE, J. 1984. Freshwater snails (Basommatophora). In: *The Mollusca*. (A. S. Tompa, N. H. Verdonk & J. A. M. van den Biggelaar, eds), **7**: 141- 207. Academic Press, London.

GIESE, A. C. & PEARSE, J. S. 1977. *Reproduction of Marine Invertebrates. Molluscs:*

Gastropods and Cephalopods. Vol. **4**. Academic Press, London.

GRAY, D. R. & HODGSON, A. N. 1997. Temporal variation in foraging behaviour of *Patella granularis* and (Patellogastropoda) and *Siphonaria concinna* (Basommatophora) on a South African shore. *Journal of Molluscan Studies*, **63**: 121- 130.

HARTLEY, A., DE VILLIERS, C. J. & HODGSON, A. N. 1985. A study of the structure of the spermatozoon of four species of *Siphonaria* (Gastropoda: Pulmonata). *Electron Microscopy Society of Southern Africa, Proceedings*, **15:** 147- 148.

HICKMAN, C. P., ROBERTS, L. S. & LARSON, A. 2000. Molluscs. In: *Animal Diversity*. 154- 179. McGraw Hill, New York.

HODGSON, A. N., BERNARD, R. T. F. & LINDLEY, D. S. 1991. Comparative spermatology of four sympatric species of *Siphonaria* (Pulmonata: Basommatophora). *Journal of Molluscan Studies*, **57**: 309- 322.

HODGSON, A. N., FARQUHAR, M. R. & DE VILLIERS, C. J. 1993. Variations in infestations of *Siphonaria capensis* and *S. concinna* (Pulmonata) by larval trematodes in southern Africa. *Journal of Molluscan Studies*, **59**: 83- 93.

HODGSON, A. N. 1999. The biology of siphonariid limpets (Gastropoda: Pulmonata). *Oceanography and Marine Biology: an annual review*, **37**: 245- 314.

HUBENDICK, B. 1978. Systematics and comparative morphology of the Basommatophora. In: *Pulmonates*. (V. Fretter & J. Peake, eds), **2A**: 1- 47. Academic Press, London.

HYMAN, L. H. 1967. *The Invertebrates. Mollusca I*, Vol. **6**. McGraw Hill, New York.

MARSHALL, D. J. & MCQUAID, C. D. 1991. Metabolic rate depression in a marine pulmonate snail: pre-adaptation for a terrestrial existence? *Oecologia*, **88**: 274- 276.

NORDSIECK, H. 1992. Phylogeny and system of the Pulmonata. *Archiv für Molluskenkunde*, **121**: 31- 52.

PAL, P. & HODGSON, A. N. 2002. An ultrastuctural study of oogenesis in a planktonic and a direct developing species of *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **68**: 337- 344.

PECHENIK, J. A. 1999. On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. *Marine Ecology Progress Series*, **177**: 269- 297.

PONDER. W. F. & LINDBERG, D. R. 1997. Towards a phylogeny of gastropod molluscs: an analysis using morphological characters. *Zoological Journal of the Linnean Society*, **119**: 83- 265.

PURCHON, R. D. 1977. *The Biology of the Mollusca*. Pergamon Press, Oxford.

QUINN, G. P. 1983. Spawning and egg masses of *Siphonaria tasmanica* Tenison Woods, 1876 from Victoria. *Journal of the Malacological Society of Australia*, **6**: 81- 82.

RUPPERT, E. E. & BARNES, R. D. 1994. The Molluscs. In: *Invertebrate Zoology*. 361-486. Saunders College Publishing, Florida.

SALEUDDIN, A. S. M. 1998. Mollusca. In: *Encyclopedia of Reproduction*. (E. Knobil & J. D. Neill, eds), **3**: 276- 283. Academic Press, London.

SALVINI-PLAWEN, LV. 1985. Early evolution and the primitive groups. In: *The Mollusca*: *Evolution* (E. R. Trueman & M. R. Clarke, eds), **10**: 59- 150. Academic Press, London.

SEAMAN, J. A. 2002. *Genetic variation within two pulmonate limpet species, Siphonaria capensis and S. serrata along the South African coast*. Ph.D thesis. Rhodes University.

SOLEM, A. 1985. Origin and diversification of pulmonate land snails. In: *The Mollusca: Evolution* (E. R. Trueman & M. R. Clarke, eds), **10**: 269- 293. Academic Press, London.

STEARNS, S. C. 1992. *The Evolution of Life Histories*. Oxford University Press, Oxford.

STEARNS, S. C. & HOEKSTRA. R. F. 2000. *Evolution: an introduction*. Oxford University Press, Oxford.

YONGE, C. M. 1952. The mantle cavity in *Siphonaria alternata* (Say). *Proceedings of the Malacological Society*, **29**: 190- 199.

Chapter 2

Seasonality of gametogenesis and spawning

Introduction

The pattern of annual reproductive cycles (gametogenesis and spawning) in marine invertebrates is highly variable (Giese, 1959; Branch, 1981; Wourms, 1987). Giese (1959) suggested that although reproductive cycles can be categorized into several stages, these might not always be discrete. Furthermore, gametogenesis may terminate in either distinct (complete) or protracted (partial) spawning events, both of which are common in marine invertebrates (Lasiak, 1986b; Wourms, 1987; Ramofafia *et al.*, 2000).

In gonochorists, males and females become sexually mature simultaneously, and in external fertilizers, spawning is generally synchronized to maximize fertilization success (Wourms, 1987). By contrast, hermaphrodites can produce male and female gametes either simultaneously or sequentially and fertilization (if internal) can be both reciprocal and non-reciprocal (Heller, 1993). Nevertheless for external fertilizing hermaphrodites the situation is similar to gonochorists in that reproductive synchronization is necessary.

Various environmental factors can influence reproductive cycles of organisms (Mendes & Woodley, 2002). For example, food availability, temperature and photoperiod can all affect gonad maturation (Byrne *et al.*, 1997). Similarly, spawning, and settlement of larvae can be affected by sea temperature, photoperiod, wind, tide, tidal range, lunar phase and food in the water (if larvae are planktotrophic) (Wourms, 1987; Currie *et al.*, 2000; Flores *et al.*, 2002).

Whilst there are numerous investigations on the seasonality of reproduction in South African molluscs (see Table 1), the majority of these are on broadcast spawners with only two studies on egg laying gastropods, [McGwynne & Van Der Horst (1985) on *Bullia*

and Chambers (1994) on *Siphonaria*]. Species of *Siphonaria* form one of the most abundant egg laying groups of gastropods on the intertidal rocky shores of South Africa. Whilst a great deal is known about the biology of *Siphonaria* (see Hodgson, 1999, for review) there are some prominent gaps in our understanding of siphonariid reproduction.

Siphonariids are hermaphrodites with a single gonad that lies next to the digestive gland (Allanson, 1959; Marcus & Marcus, 1960; Hyman, 1967; Berry, 1977; Hubendick, 1978). The gonad is composed of numerous acini in which both male and female gametes develop (Berry, 1977). Although there have been no studies on the seasonality of gametogenesis in *Siphonaria*, a number of authors have suggested that both eggs and sperm are always present in the gonad (Marcus & Marcus, 1960; Berry, 1977; Hodgson *et al.*, 1991). Hodgson (1999) also commented that siphonariids with an annual spawning cycle would probably produce gametes seasonally.

Giese (1959) suggested that an effective study of invertebrate reproductive cycles should include field observations on spawning, gonad index estimates and a histological evaluation of the gonad. In hermaphrodite animals like *Siphonaria* spp. studies on the gametogenic cycle would provide valuable information on the sequence of maleness and femaleness.

The aim of this study was to determine the reproductive seasonality (gametogenesis and spawning) in *S. capensis* and *S. serrata*. As Chambers (1994) had already described the reproductive cycle of *S. serrata* in terms of gonad index and seasonality of egg laying, this was not repeated in the current study. Gametogenesis in both species was studied by a histological examination of the gonad. The work on *S. serrata* was confined to a gametogenic study with a view to correlating results with the findings of Chambers

(1994). Finally, the relationship between photoperiod and sea surface temperature and reproductive seasonality in *S. capensis* and *S. serrata* was also investigated.

Table 1. Summary of seasonality of reproduction in some South African gastropods.

Species	*Type of fertilization*	*Location*	*Season of spawning*	*Source*
Turbo sarmaticus	External	Port Elizabeth	Nov-Mar	Foster, *et al.*, 1999
T. coronatus	External	Former Transkei	Dec-Feb	Lasiak, 1986a
Oxystele tabularis	External	Former Transkei	Continuous	Lasiak, 1987a
O. variegata	External	Former Transkei	Continuous	Lasiak, 1987a
Monodonta australis	External	Former Transkei	Feb-Jun	Lasiak, 1987a
Haliotis midae	External	West coast	Oct-Dec and Mar-May	Newman, 1967
H. midae	External	Eastern Cape	Apr-Jun	Wood & Buxton, 1996
H. spadicea	External	Port Elizabeth	Oct-Jan	Muller, 1984
Patella concolor	External	Former Transkei	Sep-Nov and Feb-Mar	Lasiak, 1987b
P. aphanes	External	Natal	Jan-Feb and Apr-Jun	Robson, 1986
P. argenvillei, P. barbara and P. granatina	External	Western Cape	May-Jun	Branch, 1974
P. oculus	External	False Bay	Sep	Branch, 1974
P. longicosta	External	False Bay	Oct-Nov	Branch, 1974
P. granularis	External	Western Cape	May-Jun	Branch, 1974
P. granularis	External	Cannon Rocks	May-Jul/ Aug	Vat, 2000
P. cochlear	External	Western Cape	May-Jun	Branch, 1974
Helcion pectunculus	External	Bloubergstrand (West coast)	Apr-May and Nov-Dec	Gray, 1996
H. pruinosus	External	Kommetjie (Atlantic coast) and Gonubie (North of EL)	May-Aug and Nov-Jan (Kommetjie); Apr- Jun and Nov-Dec (Gonubie)	Henninger, 1998
Cellana capensis	External	Former Transkei	Sep-Oct and Feb-Apr	Lasiak, 1987b
Siphonaria concinna	Internal	Waterloo Bay	Nov-Dec	Chambers, 1994
S. serrata	Internal	Waterloo Bay	Oct and Mar	Chambers, 1994
S. capensis	Internal	Kenton-on- Sea	Nov-Jan and Mar	Present study
Littorina kraussi	Internal	Former Transkei	Dec-Mar	Lasiak, 1987c
Nodilittorina natalensis	Internal	Natal	Dec-Feb	Potter, 1984
L. africana africana	Internal	Natal	Continuous	Potter, 1984

Materials and methods

All animals were collected from Kenton-on-Sea (33°42' S, 26°41' E) in the Eastern Cape, South Africa.

Gonad index of *S. capensis*

Mean monthly gonad index was estimated from 30 animals (shell length 15-24 mm) collected once every month from September 1999 to December 2000 (16 month sampling period). Animals were brought back to the laboratory, dissected and the blotted wet weight of both the somatic (excluding shell) and gonadal tissue was measured to the nearest 0.01g. Gonad index (GI) was calculated using the following formula:

GI= (Wet gonad weight / Wet somatic weight) X 100.

Gametogenesis in *S. capensis* and *S. serrata*

To determine the gametogenic condition of *Siphonaria capensis* and *S. serrata* the gonad was dissected out and fixed in 10 % aqueous Bouin's fluid for at least 7 days at room temperature. Following fixation, the tissues were dehydrated in a graded ethanol series (50 to 100%) and embedded in Paraplast (via xylene). 5 µm thick serial sections were cut on a Leica RM 2035 microtome and stained with haematoxylin and eosin (Humason, 1981). The seminal vesicle region of the hermaphrodite duct was also examined under light microscope from August 1999 to November 2000. Tissue samples from five animals (that have been used for taking gonad tissue) of each species were prepared following the method described above.

For a more quantitative assessment of gametogenesis, both oogenesis and spermatogenesis were classified into various stages of development.

I. Oogenesis

Oogenesis was found to be asynchronous between acini (Pal & Hodgson, 2002) and different stages of oogenesis could be found within an acinus. Therefore to examine the pattern of oogenesis, the number of oocytes in an acinus at different stages of maturity was counted. Five histological sections were selected randomly to cover different regions of the gonad for each individual of each species and five acini in each section were marked at random for counting the number of oocytes of each developmental stage. For each species the total number of oocytes (at different stages) were calculated for five animals and the mean was estimated for each class of oocyte and plotted with the standard error of the mean against time (in months). Stages of oogenesis quantified were: (1) previtellogenic oocytes (which included both early oocytes with a relatively small cytoplasmic area compared to the nuclear area, and previtellogenic oocytes containing more than one nucleolus); (2) early vitellogenic oocytes (characterized by a large germinal vesicle) (3) late vitellogenic oocytes with marked eosinophilia; (4) mature oocytes (Fig. 1).

II. Spermatogenesis

For spermatogenesis, acini were also classified as: Stage A (early), containing mainly spermatocytes; B (mixed), showing the presence of spermatocytes as well as Sertoli cells with spermatids; C (late or mature) where spermatids were seen with or without Sertoli

cells as well as spermatozoa and D (spent or partially spent) (Fig. 2). For spermatogenesis the sampling method was similar to that for oogenesis (i.e., five animals; five sections per gonad and five acini per section) except that the occurrence of different stages were expressed as a percentage for five animals in each month.

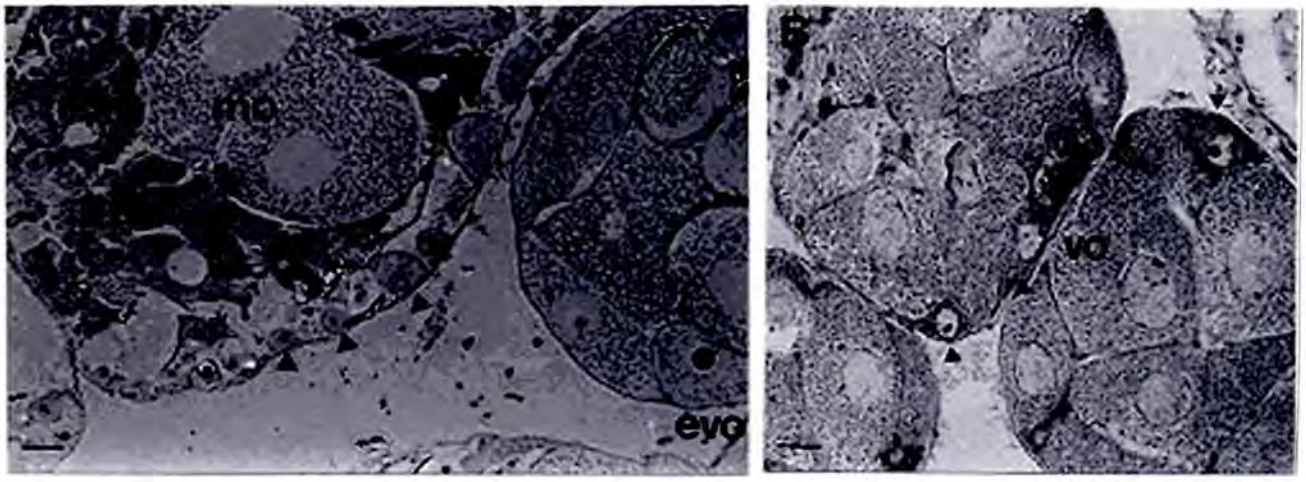


Fig. 1A. Different stages of egg development in the toluidene blue stained section of the gonad of *S. serrata*.
Small arrowheads, early oocytes; large arrowhead, previtellogenic oocyte with more than one nucleolus; evo, early vitellogenic oocytes and mo, mature oocytes. Scale bar = 0.1 mm.
Fig. 1B. Different stages of egg development in the haematoxylin and eosin stained section of gonad of *S. capensis*.
Small arrowheads, early vitellogenic oocytes; vo, late vitellogenic oocytes. Scale bar = 0.1 mm.

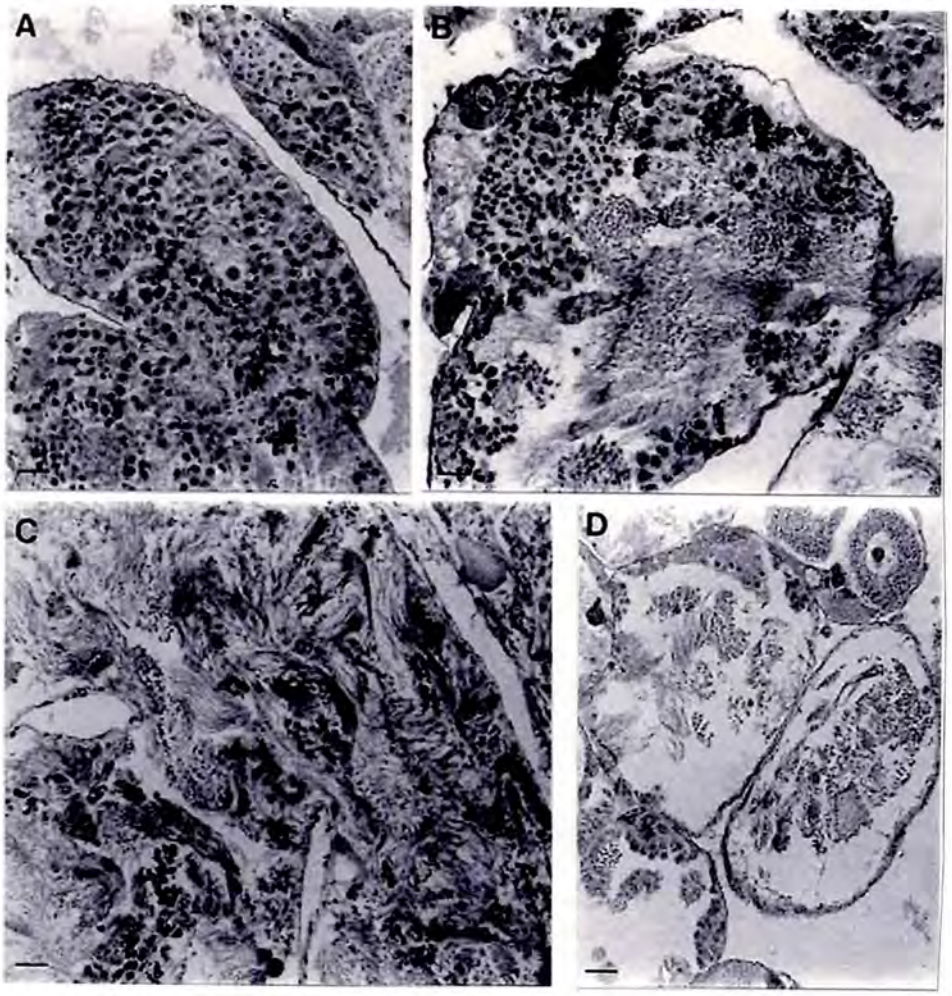


Fig. 2. Different stages of spermatogenesis in the haematoxylin and eosin stained sections of the gonad of *S. capensis.* **A**. Stage A, spermatocytes only; **B**. Stage B, spermatocytes and spermatids; C. Stage **C**, spermatids and spermatozoa and **D**. Stage D, spent or partially spent. Scale bars = 0.1 mm.

Sexual maturity of *S. capensis*

To determine the size of *S.capensis* at reproductive maturity, 30 animals (10 animals per site) with shell lengths between 7-18 mm were collected in summer, 2000 (December) from three sites in the Eastern Cape: Kenton-on-Sea (33°42' S, 26°41' E); High Rocks (33° 41' S, 26° 42' E); Three Sisters (33° 33' S, 27° 03' E). Limpets were grouped into 1 mm size classes. Animals were dissected and the gonad was processed for histology (as described previously). Sections were cut, stained with haematoxylin and eosin, and examined under a light microscope for the presence of male and/ or female gametes (both mature and immature).

Seasonality of spawning in *S. capensis*

To determine whether egg laying or spawning was seasonal in *S. capensis*, egg masses were counted once a month (at spring low tide, either new or full moon) for 16 months from September 1999 to December 2000 at Kenton-on-Sea. *S. capensis* lays eggs in rock pools, vertical walls and wave-cut flat platforms. As it was not known whether microhabitat could influence the timing of spawning, this study was restricted to horizontal platforms only. Sampling was undertaken at two sites (hereafter referred to as sites A & B), which were about 65 m apart. The number of egg masses were counted in 25 random quadrats (0.0625 m^2) at each of four sub-sites, two nested in site A (I & II) and two nested in site B (III & IV). Sub sites I & II covered an area of approximately 25 m^2 each and they were about 5 metres apart. Sub-sites III and IV were approximately 15 metres apart and covered areas of 20 and 24 m^2 respectively. A 3- factor nested ANOVA (time and site as fixed factors, sub-site as random factor nested in site) was used to

analyze the data. To meet the assumptions of normality and homogeneity of variances, data were transformed [log (x+1)] and Cochran's test was used to check for homogeneity of variances (Underwood, 1997). Data were analysed using Statistica Statsoft (version 6).

Number of egg masses in relation to density of *S. capensis*

As reproductive output (number of egg masses) of *S. capensis* varied between sites A & B during the 16-months sampling (see results), it was decided to assess the density of sexually mature (shell length ≥ 10 mm, see results) *S. capensis* at each sub-site. The number of egg masses was also counted to determine whether there was a link between the density of animals and number of egg masses. A 6-month study was undertaken during the main spawning period (September 2001 to February 2002) and 25 random quadrats (0.0625 m^2) were sampled at sites A & B. Two 3- factor nested ANOVAs (time and site as fixed factors, sub-site as the random factor nested in site) were done with the density of individuals and number of egg masses as the dependent variables. To investigate the temporal pattern of egg laying with respect to density of animals, a 3-factor nested ANOVA was conducted (time and site as fixed and sub-sites as random factors) with mean number of egg masses per individual as the dependent variable. Cochran's test was used to check homogeneity of variances and transformations [log (x+1)] were done when needed (Underwood, 1997). Data were analysed using Statistica Statsoft (version 6).

Environmental parameters

Data on mean monthly daylength was calculated from the daily sunrise and sunset table (at Kenton-on-Sea) provided by United States Naval Observatory. Daily sea surface temperature recorded at Port Elizabeth weather station from August 1999 to February 2002 was used to estimate mean monthly fluctuations in sea temperature.

Results

Reproductive cycle of *S. capensis*

I. Sexual maturity of *S. capensis*

At all three sites (Kenton-on-Sea, Three Sisters and High Rocks) animals sampled in summer with a shell length ≥ 10 mm always contained both vitellogenic oocytes and sperm whereas individuals of 9-10 mm shell length contained spermatids only, although in some (three 9 mm long animals) a few previtellogenic oocytes were observed. In animals of < 9 mm shell length, the gonad could not be discerned.

II. Estimation of gonad index (GI) and pattern of gametogenesis

The gonad index of *S. capensis* was highest in spring (October 1999) and summer (December 1999 to February 2000) (Fig. 3). During early spring the gonads mainly contained a large number of previtellogenic oocytes and early vitellogenic oocytes (Fig. 4a). By mid spring the number of late vitellogenic and mature oocytes had begun to

increase reaching a peak in summer (December to February) (Fig. 4b). From February to May 2000 there was a decline in the GI (Fig. 3) suggesting that spawning was occurring during this time. The decline in GI was accompanied by a decrease in the number of late vitellogenic as well as mature oocytes in the gonad acini (Fig. 4b). The gonad index remained very low throughout the autumn and winter months (Fig. 3). During this time except for a few previtellogenic oocytes the gonad of *S. capensis* was devoid of any oogenic activity (Fig. 4a & b).

Sperm production was continuous throughout the sampling period although the animals were spermatogenically most active in the autumn and winter months (Fig. 5a & b). A very low frequency (around 1 %) of spent acini was found in August and Sepetmber 2000 only (Fig. 5b). Sperm was observed in the seminal vesicle region of the hermaphrodite duct throughout the year.

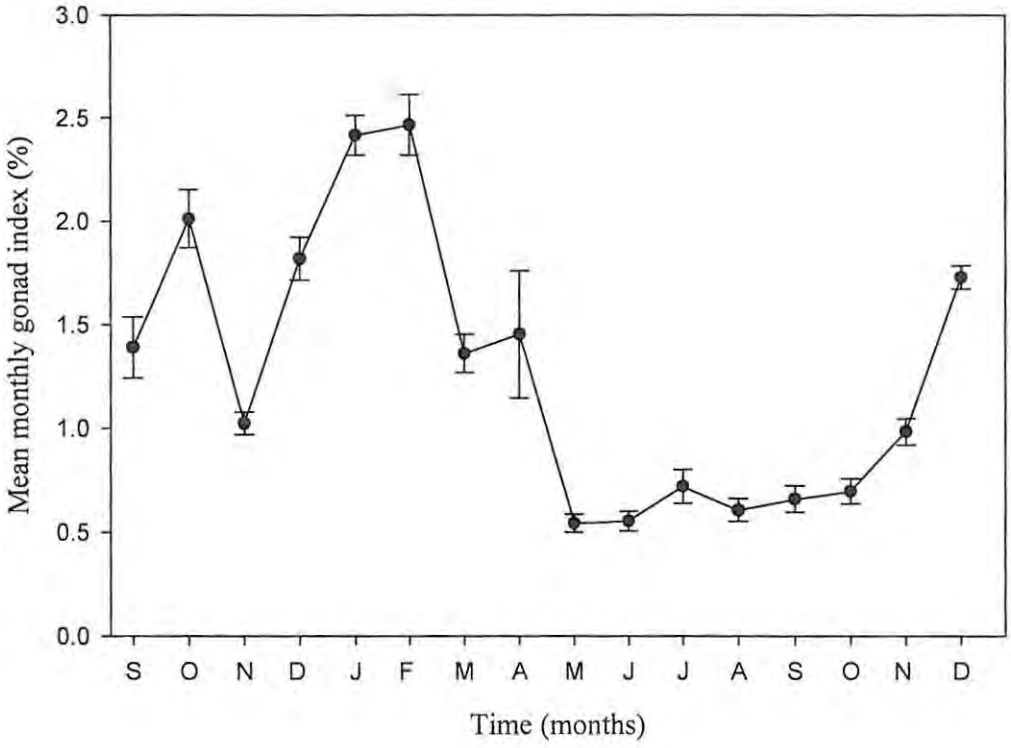


Fig. 3. Mean (± SE) monthly gonad index of *S. capensis* from September 1999 to December 2000.

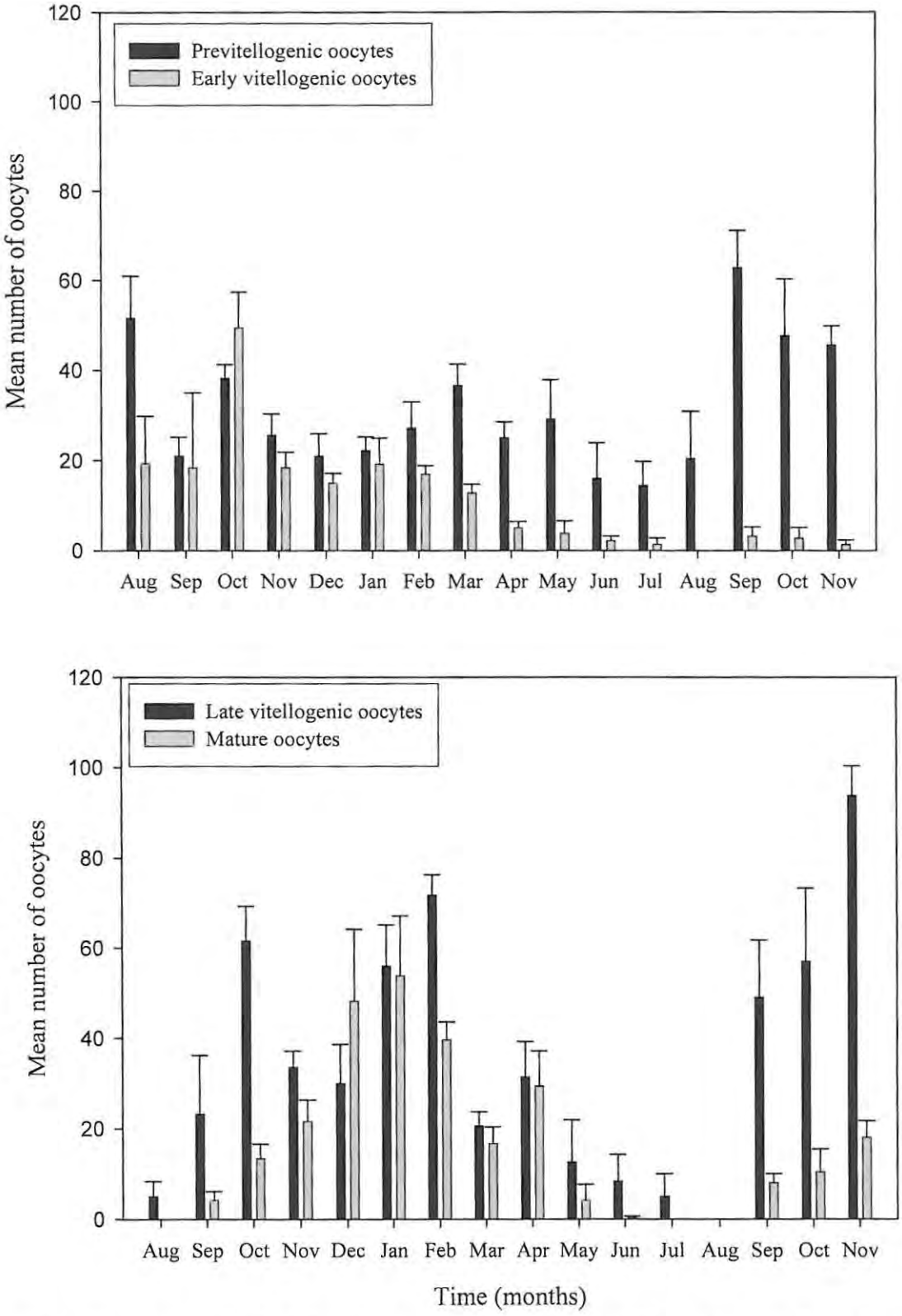
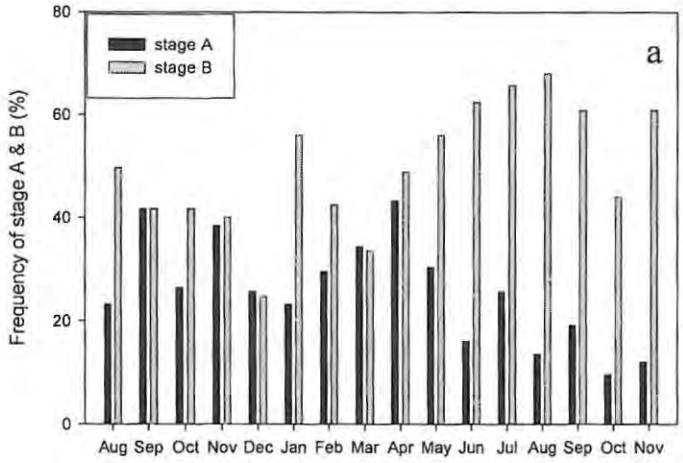


Fig. 4. Mean number (± SE) of oocytes of *S. capensis* at different stages of development from August 1999 to November 2000.



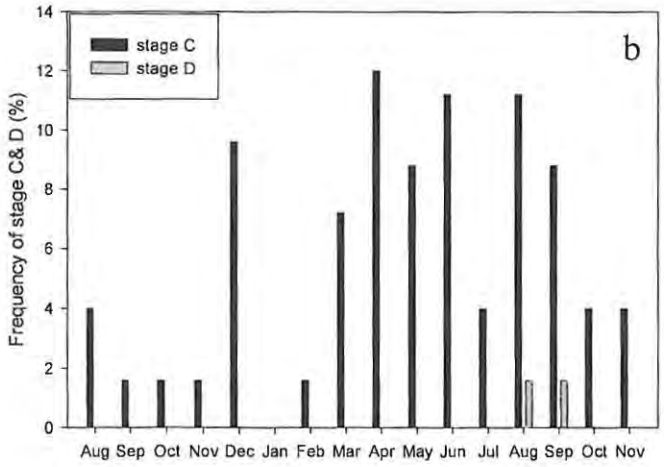


Fig. 5. Histograms showing percentage of different spermatogenic stages in *S. capensis* from August 1999 to November 2000. Stage A, mainly spermatocytes; Stage B, spermatocytes and early spermatids; Stage C, late or advanced spermatids and spermatozoa; Stage D, spent or partially spent.

III. Spawning

At both sites A & B, *S. capensis* mainly spawned in the summer months (Fig. 6). In 1999 spawning commenced in spring (October) with the mean number of egg masses peaking in November and December (Fig. 6). Very few egg ribbons were found for the rest of the year although at site B egg masses were relatively abundant in March 2000 (Fig. 6). An analysis of variance revealed that number of egg masses differed significantly over time (Table 2), with the mean number of egg masses being lowest in February, June and August 2000 and highest in November and December 1999 (Fig. 6) (see Appendix I for the results of a post-hoc test). The number of spawn differed significantly within sites, i.e., between sub-sites (Table 2).

There was a significant interaction between time and site (Table 2). Whilst the temporal pattern (timing) of spawning by *S. capensis* was similar at both sites, the spawning peaks lasted longer and the magnitude of the spawning peaks was higher at site B than site A (Fig. 6).

Table 2. A 3- factor nested ANOVA of number of egg masses at two different sites A & B (with two subsites each) from September 1999 to December 2000. Data log (x+1) transformed. Cochran's test $p > 0.05$.(Zar, 1984). Significant p values (< 0.05) are in bold.

Sources	*df*	*MS*	*F*	*p*
Time	15	17.890	95.466	**<0.0005**
Site	1	44.689	9.950	>0.10
Subsite (Site)	2	4.491	23.967	**<0.0005**
Time* site	15	4.251	22.686	**<0.0005**
Residual	1566	0.187		

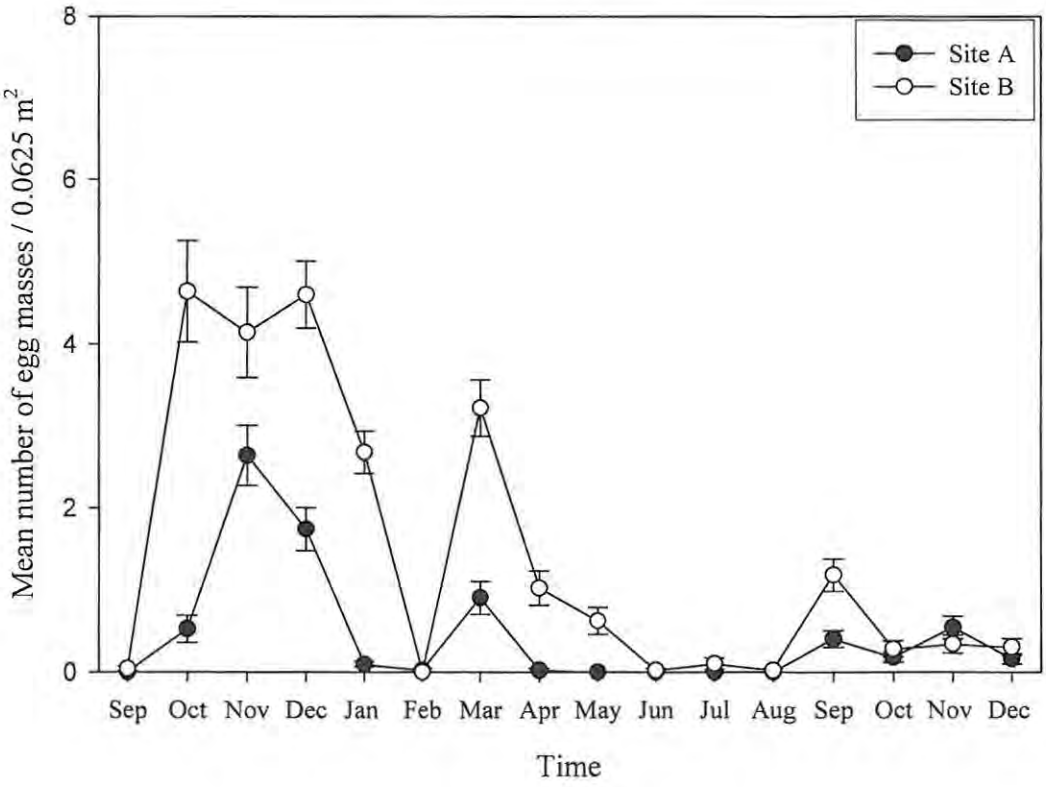


Fig. 6. Seasonality of spawning in *S. capensis* at two sites From September 1999 to February 2000. The number of egg masses is presented as mean ±SE.

IV. Number of spawn in relation to density of adults

During the six month (September 2001 to February 2002) study of spawning in *S. capensis*, egg laying began in spring (September/ October) with a peak in November and an increase in February (Fig. 7a). The mean number of egg masses differed significantly within sites but not between sites (Table 3). The pattern of spawning was similar to that of the previous study in 1999- 2000, with the density of egg masses being higher at site B during peak spawning. The density of sexually mature individuals (≥ 10 mm shell length) showed a significant temporal pattern, with the density of animals decreasing at site A and increasing at site B over time (Table 4, Fig. 7b). The temporal pattern of animal density however, did not correspond to the pattern of spawning (Fig. 7a). The density of limpets differed significantly between sites with a greater number of animals at site B (6-10 animals per 0.0625 m^2) as well as within sites (Table 4, Fig. 7b).

Using the data on number of egg masses and animal density, the number of egg masses per individual was calculated. The highest number of spawn per individuals was observed in November (0.4 per 0.0625 m^2) similar to that of the spawning cycle (Figs. 8 & 7a), which indicates that the peak of spawning was in November. When number of egg masses per individual was selected as a dependent variable there was no significant difference between the sites and also no significant interaction between the sites and time, although the sub sites were still significantly different (Table 5).

All post-hoc test results (number of egg masses, density of animals and number of egg masses per individual as dependent variable respectively) are shown in the Appendix II.

Table 3. A 3 factor nested analysis of number of egg masses at two different sites from September 2001 to February 2002. Data log (x+1) transformed, Cochran's test p> 0.05. (Zar, 1984). Significant p values
(< 0.05) are in bold.

Sources	*df*	*MS*	*F*	*p*
Time	5	1.082	26.186	**<0.0005**
Site	1	1.754	5.883	>0.2
Subsite (Site)	2	0.298	7.22	**<0.001**
Time* site	5	0.193	4.671	**<0.0005**
Residual	586	0.041		

Table 4. A 3-factor nested analysis of density of animals (> 10 mm) at two different sites from September 2001 to February 2002. Data log (x+1) transformed, Cochran's test p> 0.05. (Zar, 1984). Significant p values (< 0.05) are in bold.

Sources	*df*	*MS*	*F*	*p*
Time	5	1.757	3.803	**<0.005**
Site	1	113.595	20.538	**<0.05**
Subsite (Site)	2	5.531	11.972	**>0.0005**
Time* site	5	1.588	3.437	**<0.01**
Residual	586	0.462		

Table 5. A 3-factor analysis of number of egg masses/ individual at two sites from September 2001 to February 2002. (Zar, 1984). Significant p values (< 0.05) are in bold.

Sources	*df*	*MS*	*F*	*p*
Time	5	1.126	9.105	**<0.0005**
Site	1	0.167	0.291	>0.5
Subsite (Site)	2	0.574	4.637	**<0.02**
Time* site	5	0.112	0.908	>0.5
Residual	586	0.124		

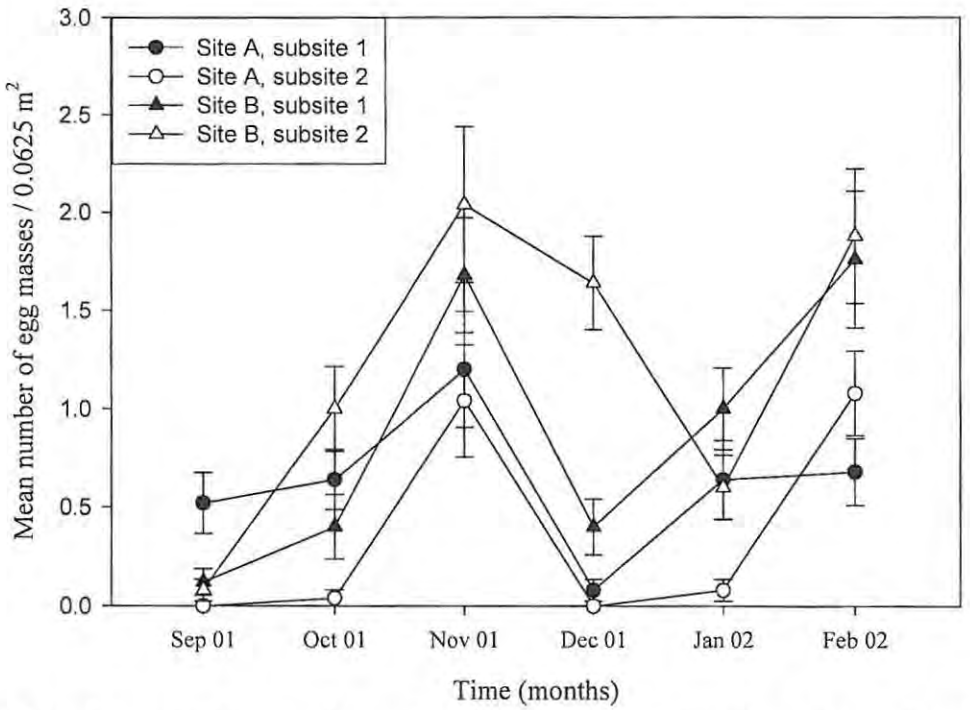


Fig. 7a. Mean (± SE) number of egg masses at two sites from September 2001 to February 2002.

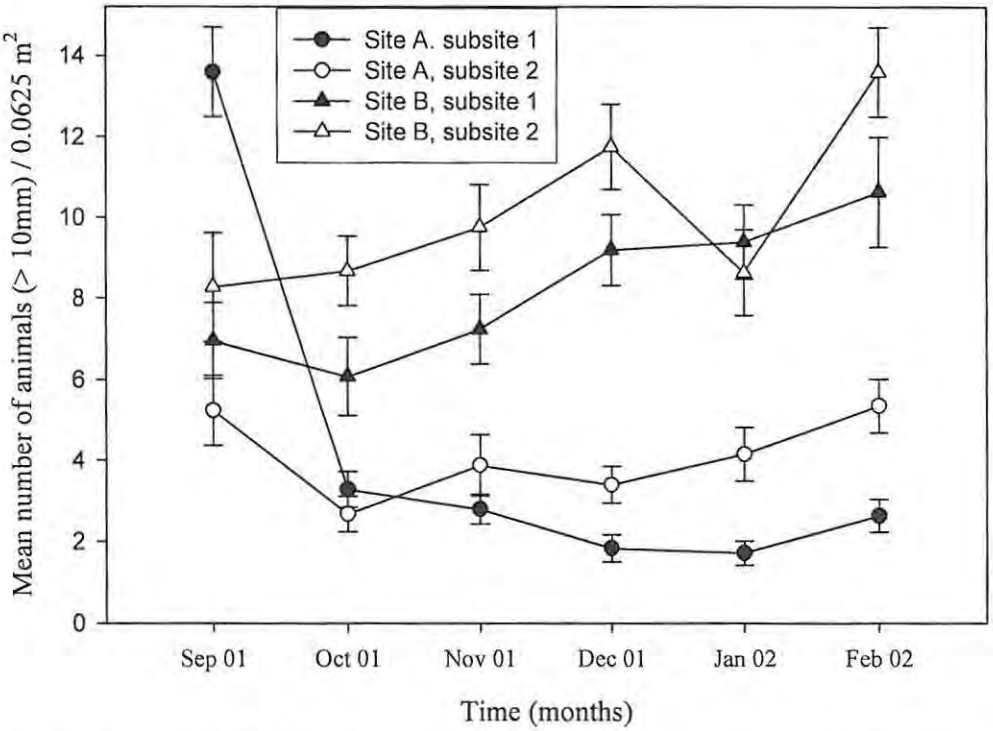


Fig. 7b. Density of animals (mean ± SE) at two sites from September 2001 to February 2002.

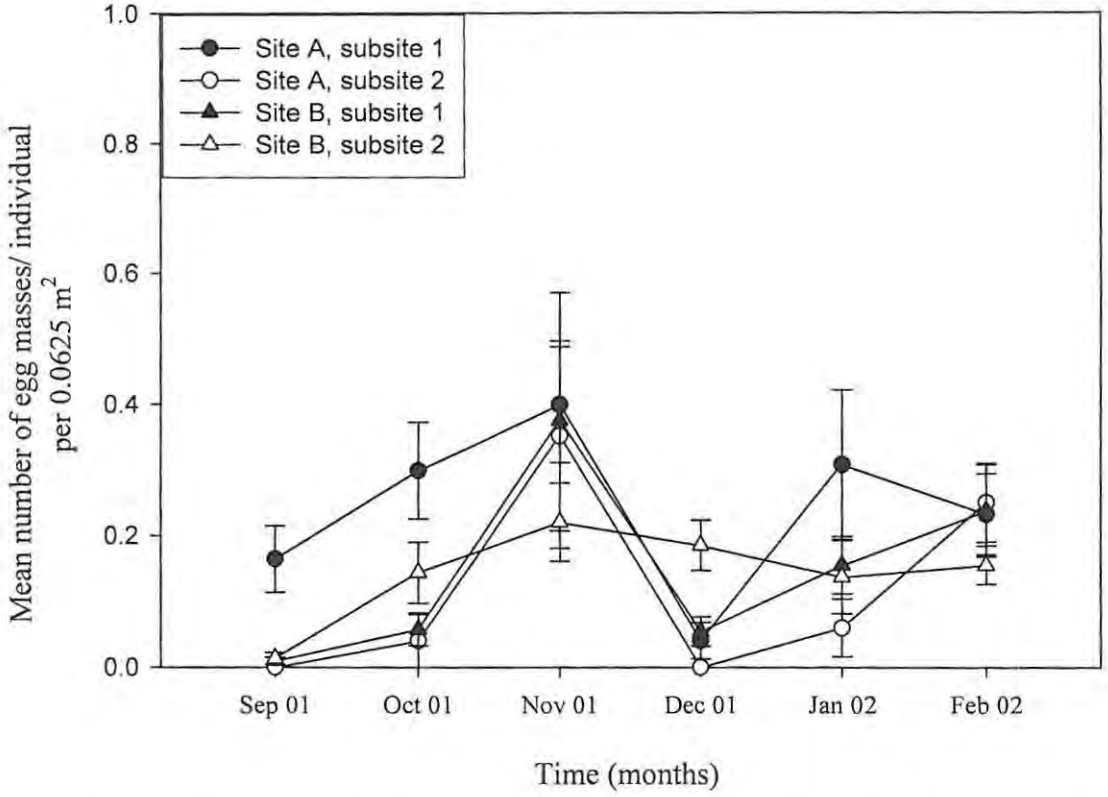


Fig. 8. Mean (± SE) number of egg masses of *S. capensis* per individual at two sites during September 2001 to February 2002.

Gametogenesis in *S. serrata*

I. Gametogenesis

Siphonaria serrata had a similar gametogenic cycle to that of *S. capensis*. Previtellogenic oocytes were present in spring (August/September), with greater numbers of late vitellogenic and mature oocytes throughout the summer (December) (Fig. 9a & b). In the winter months few late vitellogenic and mature oocytes were present in the gonad acini (Fig. 9b). Spermatogenesis occurred throughout the year although the acini showed greater activity during the winter months (Fig. 10a & b). A few acini were found spent or

partially spent in March, September and November 2000. Like *S. capensis* sperm was always found in the seminal vesicle region of the animals.

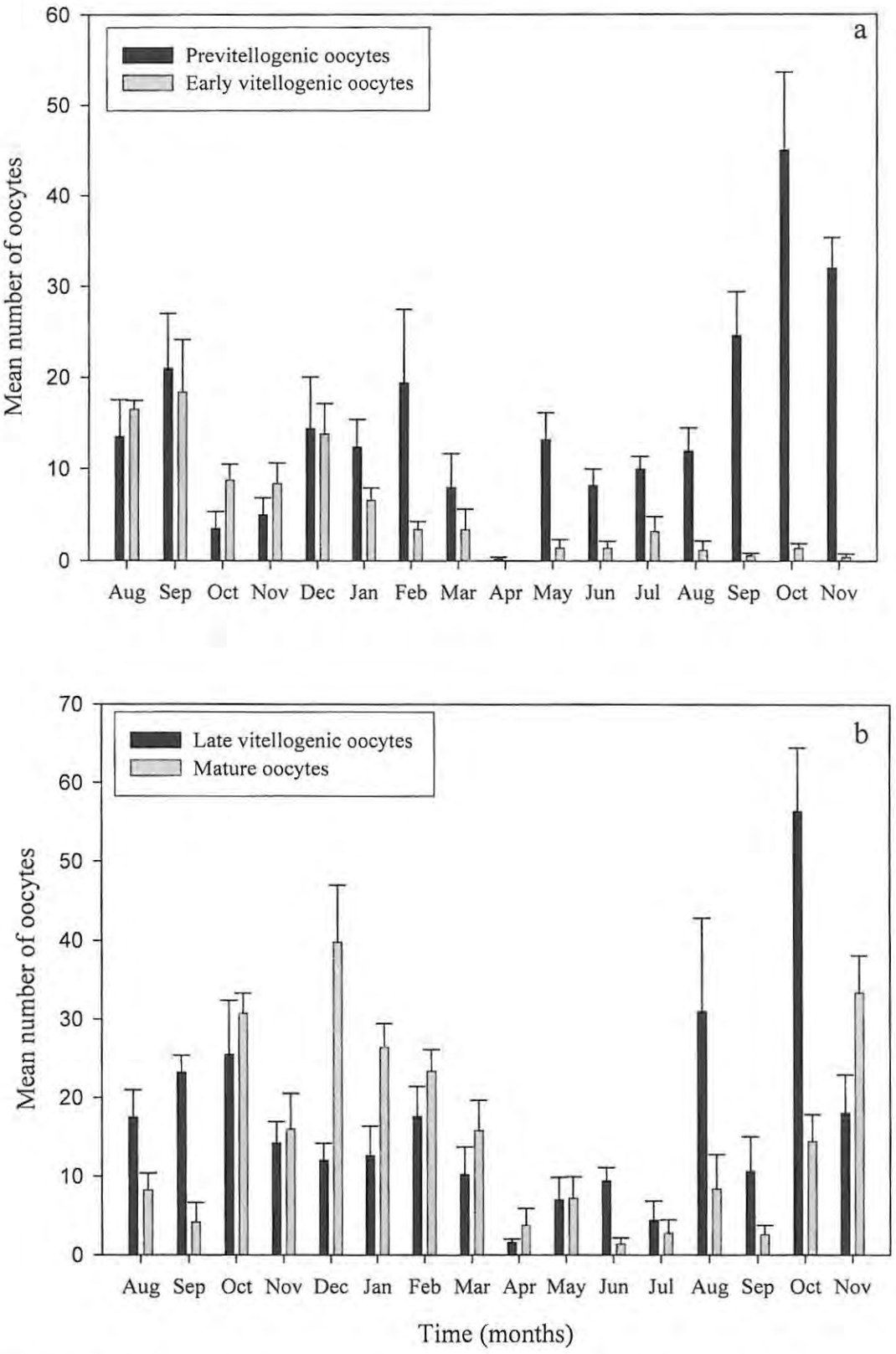
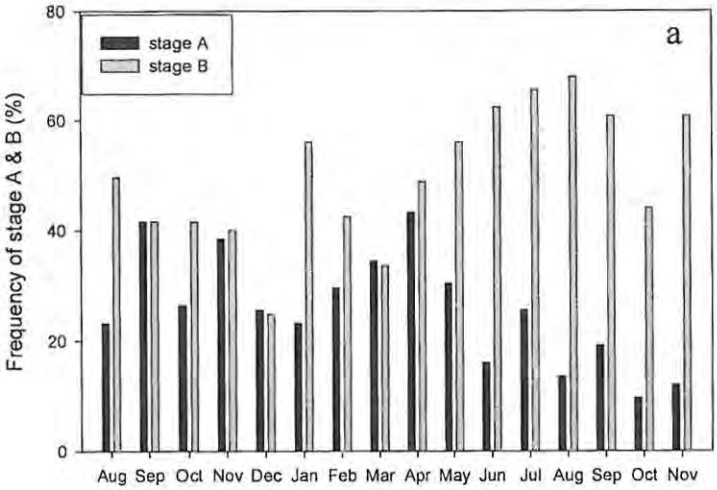


Fig.9. Mean number (± SE) of oocytes of *S. serrata* at different stages of development from August 1999 to November 2000.



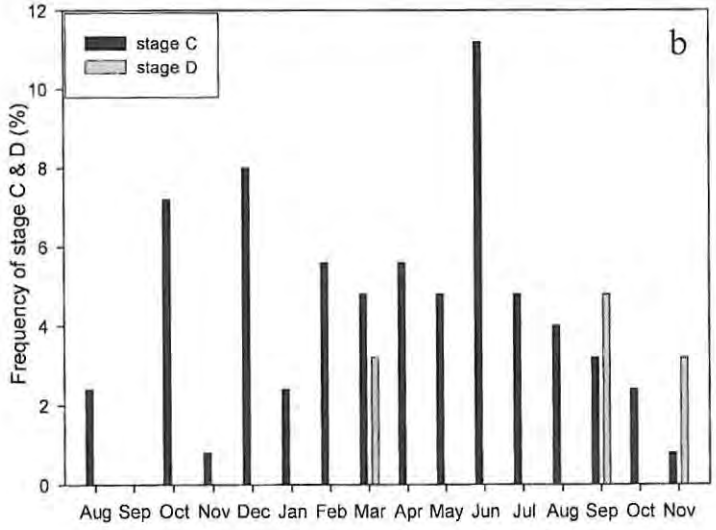


Fig. 10. Histograms showing percentage of different spermatogenic stages in *S. serrata* from August 1999 to November 2000. Stage A, mainly spermatocytes; Stage B, spermatocytes and early spermatids; Stage C, late or advanced spermatids and spermatozoa; Stage D, spent or partially spent.

Relationship between spawning and environmental parameters

The peak spawning of *S. capensis* (November-January) corresponded with the mean maximum daylength in summer (14 hrs) (Fig. 11). Mean minimum daylength was recorded in winter (9 hrs) when the gonad index, number of mature oocytes in the gonad and spawning in the field were low (Fig. 11). Mean monthly sea surface temperature was more variable when compared to photoperiod. The mean monthly sea temperature in October, November and December 1999 was 16, 17.8 & 18.4 ° C (Fig. 12) respectively, when spawning was observed in the field. A small peak in spawning of *S. capensis* was observed in March when the mean sea temperature was 11.3 ° C. The number of egg masses also increased in November 2001 and February 2002 when the sea temperature was recorded 15. 7 & 19. 7 ° C respectively (Fig. 12).

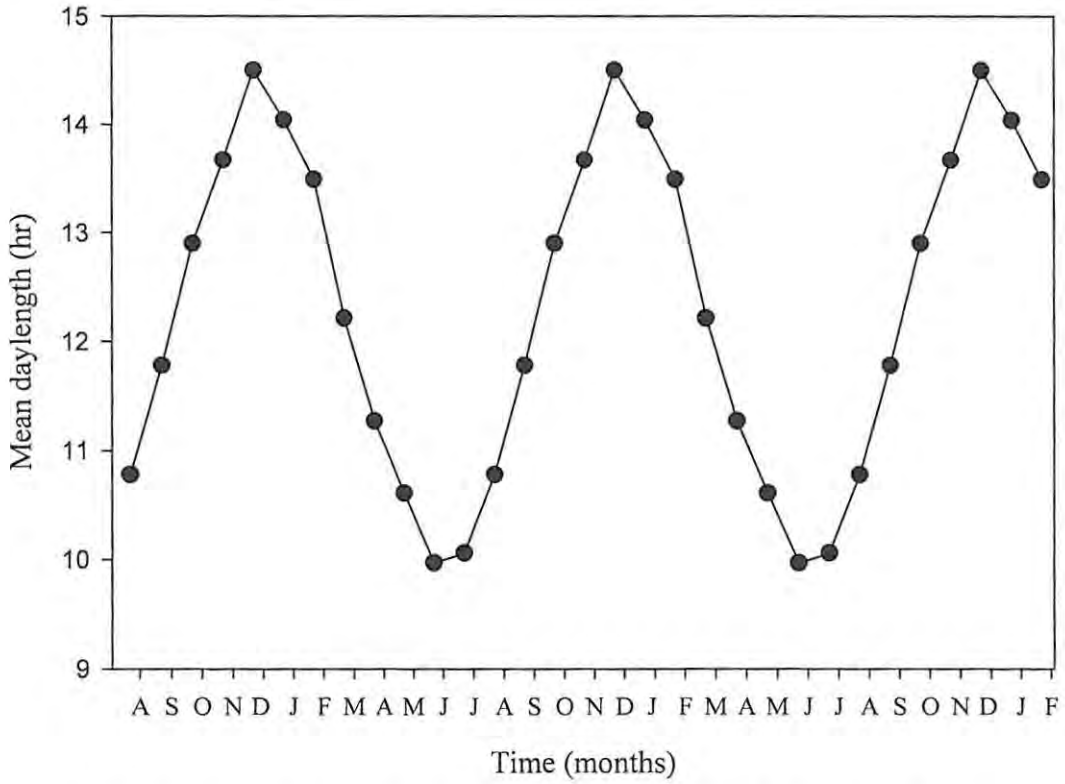


Fig. 11. Mean monthly daylength from August 1999 to February 2002.

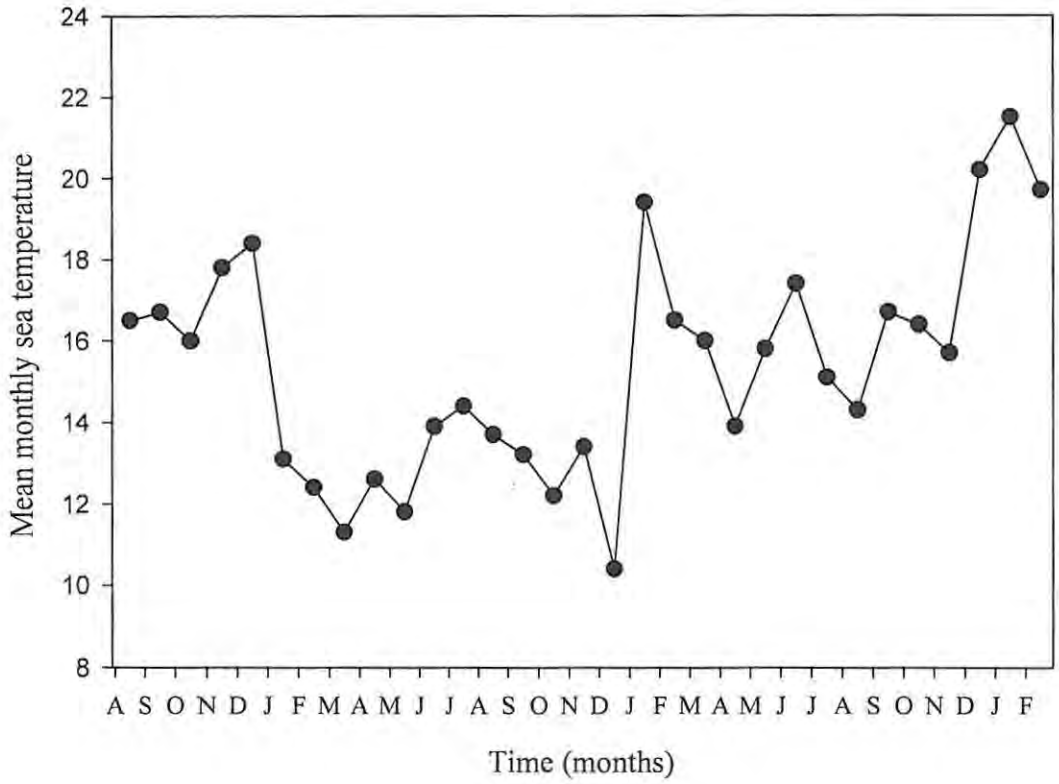


Fig. 12. Mean monthly sea temperature from August 1999 to February 2002.

Discussion

The present study on reproductive seasonality and that of Chambers (1994) have revealed that spawning in *Siphonaria capensis* and *S. serrata* commences in late spring and peaks in summer. During summer, the gonad index of the limpets declines, probably due to loss of mature oocytes from the gonad. This is supported by the increase in the number of egg masses on the shore. Generally molluscs from the south and south-east coast of South Africa reproduce in spring/summer (see Table 1). By contrast, molluscs on the west coast are autumn and/or winter breeders (see Table 1). From the present study it is clear that like other South African molluscs from the south- east coast, *S. capensis* and

S. serrata also follow the pattern of spring/summer reproduction. Both *S. capensis* and *S. serrata* however occur on the west coast of South Africa and it is not known whether the populations from the west coast also reproduce in summer as has been shown in some other molluscs with a broad geographic distribution (Gray, 1996; Henninger, 1998; Vat, 2000).

Although gonad index is a useful tool to assess the reproductive seasonality of an animal it does not give any information about gametogenesis (Webber & Giese, 1969). In both *S. capensis* and *S. serrata* sperm were present in the gonad all year round indicating that spermatogenesis is a continuous process. Thus the number of spent acini in the gonad of both species was extremely low. Continuous sperm production has also been recorded in *S. hispida* (Marcus & Marcus, 1960). In *S. hispida* sperm production was greatest after egg laying (Marcus & Marcus, 1960). This also was the case in *S. capensis* and *S. serrata*. Although spermatogenesis occurred throughout the year, activity was lower in summer (December 1999 to February 2000) when compared to late winter/ early spring (August & September 1999; July & August 2000). The conclusion that sperm production is continuous is supported by observations of the hermaphrodite duct of *S. capensis* and *S. serrata*. In both species sperm were present in the duct throughout the year with lesser abundance in February 2000.

In *S. pectinata*, mature oocytes in the gonad were observed throughout the breeding season, but spent gonads were found after spawning (Ocaña & Emson, 1999). By contrast, no degenerating oocytes or recovering gonads were observed in *S. capensis* and *S. serrata*. Although a few previtellogenic oocytes were found in the gonads of *S. serrata*, egg production in both species of *Siphonaria* studied was interrupted in the winter

months.

As it was not possible to monitor spawning in individual limpets, whether an individual underwent partial or complete spawning could not be determined. The gonad index (GI) results show that the decline in GI took place over three months and during this time the number of mature oocytes in the gonads also decreased gradually. This suggests that the limpets undergo partial spawning and will lay eggs more than once during the breeding season.

Marcus & Marcus (1960) and Chambers (1994) noted that egg masses of *S. hispida*, *S. concinna* and *S. serrata* were present on shores all year round (although in *S. serrata* number of egg masses per individual was low in the winter months). By contrast, in this study the spawn of *S. capensis* was found in spring and summer only. Joska & Branch (1983) in a study on the trochoidean prosobranch *Oxystele variegata* commented that there will always be some individuals in the population that spawn out of the cycle, but they cannot be taken as representative. One advantage of haphazard spawning by some individuals is that the new recruits may contribute to the existing population at any time of the year if they survive (Williamson & Steinberg, 2002). It has been suggested for *S. diemenensis* that the number of egg masses laid in the first half of the spawning season was higher than that in the second half (Quinn, 1988). This was also recorded for *S. capensis* in the present study with more egg masses laid in November when compared to March. This could also mean that *S. capensis* is a partial spawner. The number of mature oocytes in the gonads of *S. capensis* was still low in November 2000, which indicates that animals were not ready to spawn as they have previous year. The annual variability was also observed in the way GI was increasing in November 2000. Sampling was finished

before the next spawning season started.

During the 16 months of this investigation the mean number of egg masses of *S. capensis* differed significantly between two study sites A & B (Site B > Site A) during spawning peaks (see Table 2). This was also the case in 2001 when the study on spawning was repeated. When the variability in density of animals was examined at these two sites, density was found significantly higher at site B than site A, but the number of egg masses per individual was similar between sites. This suggests that the density of animals was responsible for the greater number of spawn at site B but only at peak spawning. The number of egg masses per individual showed the same temporal pattern (peak in November 2001) as the total number of egg masses, which means that density of animals had no effect on the temporal pattern of spawning. The variability in mean number of spawn within sites might be due to the number of sexually mature animals, food availability or fecundity of individuals. The analyses as well as personal observations on egg mass counts and density of animals showed that there is small-scale variability or patchiness of animals within sites. During sampling it was noted that animals are often aggregated in groups within sites. It is difficult to explain why more animals were found at site B as this study did not look at food availability or size of *S. capensis* individuals at different sites. Site B was often inundated with sand (pers. obs.) and was less wave exposed than site A. Because of their low tenacity, Hodgson (1999) commented that siphonariids either avoid or suffer higher mortality in areas with strong wave action. This may explain the decline in animal density at site A from September to October 2001 (see Fig. 7b).

A number of studies have shown that siphonariids spawn during a particular phase of

the moon or time of the day (Zischke, 1974; Creese, 1980; Hirano & Inaba, 1980; Branch, 1981; Levings & Garrity, 1986; Chambers, 1994; Iwasaki, 1995). Although the present study did not examine the relationship between the timing of spawning in *S. capensis* and the lunar cycle or day/night cycle, it was found that egg masses were present on the shore both at new and full moon. A more detailed experimental design is needed to look at any relationship that might exist between spawning and lunar rhythm in siphonariids.

Although it has been suggested that both exogenous and endogenous factors influence the reproductive cycles of marine invertebrates, very little is known how these factors actually work (Giese, 1959; Wourms, 1987). It is possible that copulation in *Siphonaria* plays a role in initiation of egg maturation and/or breeding. Although the effect of exogenous factors on siphonariid reproduction is unknown, it has been shown that secretions from the dorsal bodies of the cerebral ganglia controls egg development in *Siphonaria* (Saleuddin *et al.*, 1997).

The relationship between environmental factors and reproduction is difficult to predict without controlled experiments (Webber & Giese, 1969). The factors that initiate gametogenesis and vitellogenesis might be different from those that induce spawning (Underwood, 1974; Henninger, 1998; Vat, 2000). It is difficult to draw firm conclusions on the influence of daylight on the spawning or gametogenesis of *S. capensis* and *S. serrata*. Although gonad maturation of *S. capensis* and *S. serrata* and spawning of *S. capensis* increased with the increase in daylength, it is not clear whether the animals would continue laying eggs if the daylength is still long after the first spawning. Joska & Branch (1983) considered temperature as the most important exogenous factor that

affects reproduction of marine invertebrates. The data on sea surface temperature is insufficient to conclude that *S. capensis* and *S. serrata* reproduce at warmer temperatures, although this was the case on most occasions. There may be a weak correlation between temperature and gonad maturation as a threshold temperature for initiation of egg production might exist in marine organisms. It is more likely that an interaction of exogenous parameters, which are seasonal in nature control reproduction, as noted by Griffiths (1977) in bivalves. Webber & Giese (1969) suggested that exogenous parameters may also work in accordance to endogenous factors which once initiated can influence gamete production without further environmental input.

Heller (1993) noted that the majority of pulmonates are simultaneous hermaphrodites and he also concluded that the simultaneous presence of eggs and sperm in the gonad is determined by ancestry rather than an adaptation to the local environment. Hubendick (1978), however, stated that siphonariids are protandrous hermaphrodites, a conclusion based on the observations of Marcus & Marcus (1960) and Zischke (1974) on *S. hispida* and *S. pectinata* respectively. Furthermore, Zischke (1974) determined that in *S. pectinata*, the majority of limpets of > 20 mm shell length were functional females unlike *S. capensis and S. serrata* that possess eggs and sperm simultaneously. The histological examination of gonads of *S. capensis* revealed that the adult animals ≥ 10 mm shell size are simultaneous hermaphrodites i.e., eggs and sperm are always present in the gonad (except for a brief period in winter when eggs are absent). Nine millimetre long individuals of *S. capensis* produce sperm only and therefore *S. capensis* could be regarded as a protandrous hermaphrodite albeit for a short period only.

In summary, *S. capensis* and *S. serrata* are spring/ summer spawners on the east coast

of South Africa. They produce both sperm and eggs simultaneously in the gonad once they reach certain shell length (≥ 10 mm for *S. capensis*). During the spawning season they may lay eggs more than once i.e., they could be partial spawners. They produce sperm all year round although spermatogenic activity is highest in winter. Egg production ceases for a brief period in winter in both species. Density of animals in Kenton-on-Sea can affect the difference in the number of spawn between two sites, only at peak spawning.

References

ALLANSON, B. R. 1959. On the systematics and distribution of the molluscan genus *Siphonaria* in South Africa. *Hydrobiologia,* **12**: 149- 180.

BERRY, A. J. 1977. Gastropoda: Pulmonata. In: *Reproduction of Marine Invertebrates.* (A. C. Giese & J. S. Pearse eds), **4**: 181- 226. Academic Press, New York.

BERRY, A. J. 1978. Reproduction, growth and production in the mussel *Perna perna* (Linnaeus) on the east coast of South Africa. *Investigational Report of the Oceanographic Research Institute*, **48**: 1- 28.

BRANCH, G. M. 1974. The ecology of *Patella* Linnaeus from the Cape Peninsula, South Africa. 2. Reproductive cycles. *Transactions of the Royal Society of South Africa*, **41** (2): 111- 160.

BRANCH, G. M. 1981. The biology of limpets: physical factors, energy flow, and ecological interactions. *Oceanography and Marine Biology: an Annual Review*, **19**: 235- 380.

BYRNE, M., MORRICE, M. G. & WOLF, B. 1997. Introduction of the northern Pacific asteroid *Asterias amurensis* to Tasmania: reproduction and current distribution. *Marine Biology*, **127**: 673- 685.

CHAMBERS, R. J. 1994. *The conflict between adaptation and constraint: the case of the siphonariid limpets*. Ph.D thesis, Rhodes University.

CREESE, R. G. 1980. Reproductive cycles and fecundities of two species of *Siphonaria* (Mollusca: Pulmonata) in south-eastern Australia. *Australian Journal of Marine and Freshwater Research*, **31**: 37- 47.

CURRIE, D. R., MCARTHUR, M. A. & COHEN, B. F. 2000. Reproduction and distribution of the invasive European fanworm *Sabella spallanzani* (Polychaeta: Sabellidae) in Port Phillip Bay, Victoria, Australia. *Marine Biology*, **136**: 645- 656.

FLORES, A. A. V., CRUZ, J. & PAULA, J. 2002. Temporal and spatial patterns of settlement of brachyuran crab megalopae at a rocky coast in Central Purtugal. *Marine Ecology Progress Series*, **229**: 207- 220.

FOSTER, G. G., HODGSON, A. N. & BALARIN. 1999. Effect of diet on growth and reproductive fitness of *Turbo sarmaticus* (Mollusca: Vetigastropoda: Turbinidae). *Marine Biology*, **134**: 307- 315.

GIESE, A. C. 1959. Comparative physiology: annual reproductive cycles of marine invertebrates. *Annual Review of Physiology*, **21**: 547- 576.

GRAY, D. R. 1996. *Studies of the biology and ecology of the high shore South African limpet, Helcion pectunculus (Mollusca: Patellogastropoda)*. Ph.D thesis, Rhodes University.

GRIFFITHS, R. J. 1977. Reproductive cycles in littoral populations of *Choromytilus meridionalis* (Kr.) and *Aulacomya ater* (Molina) with a quantitative assessment of gamete production in the former. *Journal of Experimental Marine Biology and Ecology*, **30**: 53- 71.

HELLER, J. 1993. Hermaphroditism in molluscs. *Biological Journal of Linnean Society*, **48**: 19- 42.

HENNINGER, T. O. 1998. *Aspects of the ecology and reproductive biology of the limpet, Helcion pruinosus (Gastropoda : prosobranchia)*. M. Sc. Thesis, Rhodes University.

HIRANO, Y. & INABA, A. 1980. *Siphonaria* (pulmonate limpet) survey of Japan. I.

Observations on the behaviour of *Siphonaria japonica* during breeding season. *Publications of Seto Marine Biological Lab.*, **25**: 323- 334.

HODGSON, A. N., BERNARD, R. T. F. & LINDLEY, D. S. 1991. Comparative spermatology of four sympatric species of *Siphonaria* (Pulmonata: Basommatophora). *Journal of Molluscan Studies*, **57**: 309- 322.

HODGSON, A. N. 1999. The biology of siphonariid limpets (Gastropoda: Pulmonata). *Oceanography and Marine Biology: an Annual Review*, **37**: 245- 314.

HUBENDICK, B. 1978. Systematics and comparative morphology of the Basommatophora. In: *Pulmonates* (V. Fretter & J. Peake, eds), **2A**: 1- 47. Academic Press, London.

HUMASON, G. L. 1981. *Animal Tissue Technique*. W. H. Freeman. New York.

HYMAN, L. H. 1967. *The Invertebrates. Mollusca I.* Vol. **6**. McGraw- Hill, New York.

IWASAKI, K. 1995. Foraging and spawning rhythms of the pulmonate limpet *Siphonaria sirius* (Pilsbry): switching of activity period by a diurnal forager. *Journal of Molluscan Studies*, **61**: 275- 288.

JOSKA, M. A. P. & BRANCH, G. M. 1983. The reproductive cycle of the trochid gastropod *Oxystele variegata* (Anton, 1839). *The Veliger*, **26** (1): 47- 51.

LASIAK, T. 1986a. The reproductive cycle of the intertidal gastropod *Turbo coronatus* Gmelin 1791, on the Transkei coast. *South African Journal of Zoology*, **21**: 153- 155.

LASIAK, T. 1986b. The reproductive cycles of the intertidal bivalves *Crassostrea cucullata* (Born, 1778) and *Perna perna* (Linnaeus, 1758) from the Transkei coast, Southern Africa. *The Veliger*, **29** (2): 226- 230.

LASIAK, T. 1987a. The reproductive cycles of three trochid gastropods from the

Transkei coast, Southern Africa. *Journal of Molluscan Studies*, **53**: 24- 32.

LASIAK, T. 1987b. Observations on the reproductive cycles of *Cellana capensis* (Gmelin, 1791) and *Patella concolor* (Krauss, 1848) (Gastropoda: Patellidae). *Southern African Journal of Zoology*, **22** (3): 195- 199.

LASIAK, T. 1987c. Reproductive cycle of the supralittoral gastropod *Littorina kraussi* (Rosewater, 1970). *Southern African Journal of Zoology*, **22** (2): 170- 172.

LEVINGS, S. C. & GARRITY, S. D. 1986. Notes on reproduction of a tropical pulmonate limpet, *Siphonaria gigas* (Sowerby). *The Veliger*, **29**: 86- 90.

MARCUS, E. & MARCUS, E. 1960. On *Siphonaria hispida. Universidade de Sao Paulo. Faculdade de Filosofia, Ciencias e Letras. Boletim de Zoologia*, **23**: 107- 139.

MCGWYNNE, L. E. & VAN DER HORST, G. 1985. Patterns of reproduction in three sandy beach whelks of the genus *Bullia* Griffith. *Journal of Molluscan Studies*, **51**: 190- 197.

MENDES, J. M. & WOODLEY, J. D. 2002. Timing of reproduction in *Montastraea annularis*: relationship to environmental variables. *Marine Ecology Progress Series*, **227**: 241- 251.

MULLER, S. 1984. *Studies on the ecology, feeding, growth and reproduction of Haliotis spadicea Donovan, 1808 Mollusca: Gastropoda.* M. Sc. Thesis, Port Elizabeth University.

NEWMAN, G. G. 1967. Reproduction of the South African abalone *Haliotis midae. Investigational Reproductive Division of Sea Fisheries of South Africa*, **64**: 1- 24.

OCAÑA, T. M. J. & EMSON, R. H. 1999. Maturation, spawning and development in *Siphonaria pectinata* Linnaeus (Gastropoda: Pulmonata) at Gibraltar. *Journal of*

Molluscan Studies, **65**: 185- 193.

PAL, P. & HODGSON, A. N. 2002. An ultrastuctural study of oogenesis in a planktonic and a direct developing species of *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **68**: 337- 344.

POTTER, C. M. 1984. *Aspects of the biology of the Natal Littorinidae*. M. Sc. Thesis, Natal University.

QUINN, G. P. 1988. Ecology of the intertidal pulmonate limpet *Siphonaria diemenensis* Quoy et Gaimard. II Reproductive patterns and energetics. *Journal of Experimental Marine Biology and Ecology*, **117**: 137- 156.

RAMOFAFIA, C., BATTAGLENE, S. C., BELL, J. D. & BYRNE, M. 2000. Reproductive biology of the commercial sea cucumber *Holothuria fuscogilva* in the Solomon Islands. *Marine Biology*, **136**: 1045- 1056.

ROBSON, G. 1986. *Aspects of biology of a new species of South African Patella (Mollusca: Gastropoda: Patellidae)*. M. Sc. Thesis, Natal University.

SALEUDDIN, A. S. M., ASHTON, M. L. & KAHN, H. R. 1997. An electron microscope study of the endocrine dorsal bodies in reproductively active and inactive *Siphonaria pectinata* (Pulmonata: Mollusca). *Tissue and Cell*, **29**: 267- 275.

UNDERWOOD, A. J. 1974. The reproductive cycles and geographical distribution of some common eastern Australian prosobranchs (Mollusca: Gastropoda). *Australian Journal of Marine Biology and Freshwater Research*, **25**: 63- 88.

UNDERWOOD, A. J. 1997. *Experiments in Ecology: Their Logical Design and Interpretation Using Analysis of Variance*. Cambridge University Press, London.

VAT, L. S. 2000. *The growth and reproduction of Patella granularis (Mollusca:*

Patellogastropoda) on the south-east coast of South Africa. Ph. D thesis, Rhodes University.

WEBBER, H. H. & GIESE, A. C. 1969. Reproductive cycle and gametogenesis in the black abalone *Haliotis cracheroidii* (Gastropoda: Prosobranchia). *Marine Biology*, **4**: 152- 159.

WILLIAMSON, J. E. & STEINBERG, P. D. 2002. Reproductive cycle of the sea urchin *Holopneustes purpurascens* (Temnopleuridae: Echinodermata). *Marine Biology,* **140**: 519- 532.

WOOD, A. D. & BUXTON, C. D. 1996. Aspects of the biology of the abalone *Haliotis midae* (Linne, 1758) on the east coast of South Africa. 2. Reproduction. *South African Journal of Marine Science*, **17**: 69- 78.

WOURMS, J. P. 1987. *Reproduction of Marine Invertebrates.* (A. C. Giese, J. S. Pearse & V. B. Pearse, eds.), Vol. **IX**. Blackwell Scientific Publications, California.

ZAR, J. H. 1984. *Biostatistical analysis*. Prentice Hall, New Jersey.

ZISCHKE, J. A. 1974. Spawning, development and growth in the pulmonate limpets *Siphonaria pectinata* Linnaeus, 1758 and *Siphonaria alternata* Say, 1882. *The Veliger*, **16**: 399- 404.

Appendix I

Table I. Results of the Student- Newman- Keuls test (post-hoc test) to determine the differences in the mean number of egg masses between the months from September 1999 to December 2000. (Zar, 1984)

Months	*Mean*	*1*	*2*	*3*	*4*	*5*	*6*
Feb 2000	0.007	X					
Jun 2000	0.007	X					
Aug 2000	0.007	X					
Sep 1999	0.014	X					
Jul 2000	0.025	X					
Dec 2000	0.139	X	X				
Oct 2000	0.142	X	X				
May 2000	0.158	X	X				
Nov 2000	0.246		X				
Apr 2000	0.253		X				
Sep 2000	0.421			X			
Jan 2000	0.621				X		
Mar 2000	0.843					X	
Oct 1999	0.858					X	
Dec 1999	1.185						X
Nov 1999	1.190						X

Appendix II

Table II. Results of the Student- Newman- Keuls test (post- hoc test) to determine the differences in the mean number of egg masses between the months from September 2001 to February 2002. (Zar, 1984)

Months	*Mean*	*1*	*2*	*3*
Sep 2001	0.050	X		
Dec 2001	0.124		X	
Oct 2001	0.131		X	
Jan 2002	0.146		X	
Feb 2002	0.297			X
Nov 2001	0.310			X

Table III. Results of the Student- Newman- Keuls test (post- hoc test) to determine the differences in the density of animals between the months from September 2001 to February 2002. (Zar, 1984)

Months	*Mean*	*1*	*2*
Oct 2001	1.536	X	
Nov 2001	1.575	X	
Sep 2001	1.662	X	
Jan 2002	1.702	X	X
Dec 2001	1.710	X	
Feb 2002	1.913		X

Table IV. Results of the Student- Newman- Keuls test (post- hoc test) to determine the differences in the number of egg masses per individual between the months from September 2001 to February 2002. (Zar, 1984)

Months	*Mean*	*1*	*2*	*3*
Sep 2001	0.047	X		
Dec 2001	0.070	X		
Oct 2001	0.135	X	X	
Jan 2002	0.165	X	X	
Feb 2002	0.219		X	
Nov 2001	0.337			X

Chapter 3

Within- and between-site variability in fecundity of *S. capensis* during the spawning season, with a note on the effect of size on fecundity of *S. capensis* and *S. serrata*

Introduction

Fecundity or reproductive effort can be considered as one of several fundamental life history traits (Stearns, 1992; Stearns & Hoekstra, 2000) and therefore life history studies are often centred on fecundity. The development of life history models of an organism is based on the answers to questions such as how fecund the animal is, when it starts reproducing and how many times it reproduces in its life (Llodra, 2002). According to Llodra (2002) fecundity is a plastic character, which changes within the limits of the life history strategy of an organism. Fecundity can be affected by a number of different parameters including age, size, mortality, population density (Stearns, 1992) and environmental factors e.g., food availability, temperature and salinity (Cheung & Lam, 1999; Prevedelli & Simonini, 2000; Llodra, 2002).

Except for *Siphonaria tasmanica* and *S. virgulata* (Creese, 1980; Quinn, 1983) all other siphonariids lay benthic egg masses (Chambers & McQuaid, 1994a; Hodgson, 1999) and each embryo is protected by an egg capsule, which in turn is embedded in a gelatinous matrix (Chambers & McQuaid, 1994b; Hodgson, 1999; see Chapter 5). In siphonariid limpets with different developmental strategies fecundity is highly variable (Chambers & McQuaid, 1994a & b). Chambers & McQuaid (1994a & b) and later Hodgson (1999) summarized data on fecundity of several species of siphonariids. In general, intracapsular developers lay nine (in *Kerguelenella stewartiana*) to 2500 (in *Siphonaria serrata*) eggs per egg mass whereas planktonic developing species have approximately 9000 (in *S. atra*) to 75000 (in *S. gigas*) eggs within an egg mass (Hodgson, 1999). Studies on the variation in fecundity at the population level in a single species of

Siphonaria have mainly been neglected, with the exception of studies by Creese (1980) and Quinn (1983).

This study mainly deals with *S. capensis*, because of its abundance on aeolian platforms (where the study was carried out) compared to *S. serrata* (which occurs in very low densities on this rock type, pers. obs.). The intertidal pulmonate *Siphonaria capensis* Q. & G. has planktonic development, and inhabits mid to high regions of the rocky shores of South Africa (Chambers & McQuaid, 1994a & b) and it has been observed that *S. capensis* successfully dwells in both sand covered and non-sand covered sites (Marshall & McQuaid, 1989). Chambers & McQuaid (1994b) determined that *S. capensis* lays gelatinous egg ribbons containing about 20,000 egg capsules, each containing a single developing embryo (Chapter 5). During the study on the seasonality of reproduction in *S. capensis* (Chapter 2) high variability in the number of egg masses within a site was observed. The aim of this study was to look further at variability in fecundity of *S. capensis*, both between and within sites, and also examine whether the size of individual limpets could be a factor affecting the number of spawn in *S. capensis*. It is difficult in siphonariids to detect which individual has laid the egg mass (as they do not lay egg masses at the home scar, pers. obs.) and also how many times a particular limpet spawns unless it is a controlled laboratory experiment. Therefore, it is impossible to relate the size of an individual siphonariid to fecundity unlike other marine organisms e.g., some polychaetes (Cassai & Prevedelli, 1999). It is for these reasons that this study focused on the fecundity of populations rather than on individual limpets. In addition to investigating how the fecundity of populations of *S. capensis* varied between different sites and habitats (sand covered and non-sand covered) at different times during the breeding

season, the effect of adult size on fecundity in *S. capensis* and *S. serrata* was also examined. The results are compared to those presented in Chapter 2 as the current study is a more detailed approach towards fecundity of limpet populations.

Materials and methods

Three study sites (all aeolian platforms), Three Sisters (33° 33' S, 27° 03' E), Kenton- on- Sea (33°42' S, 26°41' E) and High Rocks (33° 41' S, 26° 42' E), were visited at the beginning of November (16/11), end of November (26/11) and beginning of December (10/12) during spring low tides in 2000. These summer months were chosen for sampling because *S. capensis* spawns in summer (see Chapter 2). At each site two different habitats, one non-sand covered (NS) and one sand- covered (S) region of the shore were identified. Non-sand covered habitats were never covered with sand whereas the sand-covered habitats were often inundated with a few centimeters of sand for short periods (up to a week). During each sampling trip, 15 random quadrats (each 0.0625 m^2) were photographed at each habitat using a Nikon N6006 AF camera with 35 mm colour slide film. A graduated ruler (in mm) was included in each photo to provide a scale. The slides were projected and the number of egg masses, number and size of animals in each quadrat were estimated from the slides. To determine the number of egg capsules within the egg masses, five egg ribbons were collected randomly from each habitat during each sampling session. The egg ribbons were fixed and stored in 70 % ethanol at 4 °C for subsequent processing. Each egg mass was measured (to 1 mm accuracy) and two 1 mm thick sections were removed and the number of egg capsules was counted using a Nikon light microscope.

Variability in population fecundity of *S. capensis*

To compare the variability in fecundity between and within sites and its relationship with the density of adult animals (≥ 10 mm shell length) a 3-factor ANCOVA (site, habitat and time as independent factors, number of adult animals as a covariate and number of egg masses as the dependent variable) was used. A 3-factor ANOVA was also used with density of animals as a dependent variable and site, habitat and time as the independent factors. Also, the effect of size of adult animals (> 10 mm) on the reproductive output of the population was analyzed using a 3-factor ANCOVA (site, habitat and time as the independent factors, number of egg masses as the dependent factor). The covariate used in this analysis was the ratio ([L+1]/ [S+1]) between large animals (L) {> 20 mm (i.e., 21 mm onwards)} and small animals (S) {> 10 mm (i.e., 11-20 mm)}. All data were analysed using Statistica Statsoft (version 6).

Variability in the density of egg capsules per mm of egg mass, total number of egg capsules and the relationship between density of capsules and length of egg mass

The relationship between the length of egg masses and the mean number of egg capsules per mm of an egg mass was assessed by two 3-factor ANCOVAs (one without Kenton-on-Sea and one without early November data or Time 1). This was done because of the absence of egg masses at Kenton during the first sampling period. These analyses used site, habitat and time as independent factors; length of an egg mass as a covariate

and density of egg capsules as the dependent variable. Two tables are presented in the results section with Bonferroni-corrected p values (pB), as parts of the same data set were used twice in the analyses.

Two 3-factor ANOVAs (site, habitat, time as independent factors and total number of egg capsules as the dependent variable) were also used to examine the variability in the total number of egg capsules in an egg mass in a similar analytical way as described before. Total number of egg capsules was calculated by multiplying the length of an egg mass by the mean number of egg capsules per mm of the egg mass.

Heterogeneity of variances for all analyses was checked by Cochran's test and as they were homogeneous no transformation was necessary (Underwood, 1997). All data were analysed using Statistica Statsoft (Version 6).

Relationship between shell length and number of eggs in the gonads of *S. capensis* and *S. serrata*

The effect of size on the fecundity of *S. capensis* and *S. serrata* was determined by collecting five adults of each species (15- 24 mm shell length) from Kenton-on-Sea each time during the spring and summer months (October 1999- February 2000). In the laboratory, after measuring the shell length of the limpets to the nearest mm, they were dissected to remove their gonads, which were fixed in 10 % aqueous Bouin's fluid. The gonads of both species were processed for subsequent histological examination (for details of the method used see Chapter 2). Five sections of each gonad were observed under a light microscope to count the number of mature oocytes in five randomly chosen

gonad acini. Linear regressions were done (for those months when the proportion of mature oocytes was high) to examine the relationship between size of the animal (shell length) and the number of mature oocytes per 25 acini in the gonad of both species separately.

Results

Variability in population fecundity of *S. capensis*

Variability in the density of egg masses (between and within sites)

A 3-factor ANCOVA with site, habitat and time as independent factors showed that the mean number of egg masses differed significantly between sites and over time (Table 1). Although the mean number of egg masses was not significantly different between the habitats, both site and habitat showed variability in the number of spawn over time (Table 1). For example, both habitats at Three Sisters had the highest number of egg masses at time 3 (early December), (about 12 egg masses/ 0.0625 m^2) whereas at Kenton-on-Sea and High Rocks the densities were never greater than 1egg mass/ 0.0625 m^2 (Fig. 1).

The density of adult individuals as a covariate showed no significant relationship with the number of egg masses found in the field (Table 1). Linear regressions were determined to examine the relationship between density of animals and number of spawn for each site and time, as all three sites were significantly different from each other over time (i.e., site* time interaction was significant), (Table 1). Except for High Rocks at time 2 (late November) and 3 (early December) (n= 30 quadrats; $R^2 = 0.256$ and 0.186; p= 0.004 and 0.017 respectively; Figs. 2 & 3) the number of egg masses did not show any

relationship with the density of animals. The density of adult animals was not consistently different between sites and times (Table 2). However, there was a highly significant 3- way interaction between the factors site, habitat and time (Table 2) with Kenton-on-Sea always having a greater density of animals at the sand-covered habitat and High Rocks showing a higher density of animals in non-sand covered habitats (Fig. 4). Animal density at Three Sisters increased and decreased in sand-covered and non-sand covered respectively at time 3 (Fig. 4).

The ratio between large and small individuals (L/S) showed no significant relationship with the variability in the number of egg masses (Table 3) although the increase in the L/S ratio coincided with the increase in the mean number of spawn in the non-sand covered habitat of Three Sisters at time 3 (early December), (Figs. 1 & 5). As all three sites showed significantly different number of spawn over the three sampling sessions, linear regressions between number of egg masses and the ratio between large and small animals were done for each site and time. A majority of regressions showed no relationship, except for Three Sisters at time 2 (late November), (Fig. 6), Kenton-on-Sea at time 3 (Fig. 7) and High Rocks at time 3 (early December), (Fig. 8) which exhibited weak positive relationships (n= 30 quadrats; R^2: 0.135-0.227; p: 0.0078-0.046).

Table 1. A 3-factor analysis of covariance at three sites (two habitats and three sampling trips) with number of egg masses as the dependent variable and the total number of animals as a covariate. Significant p values ($p < 0.05$) are in bold.

Sources	*df*	*MS*	*F*	*p*
Total # of animals	1	18.031	2.676	0.103
Site	2	897.888	133.273	**0.000001**
Habitat	1	3.167	0.470	0.494
Time	2	212.378	31.523	**0.000001**
Site* habitat	2	4.138	0.614	0.542
Site* time	4	182.920	27.151	**0.000001**
Habitat* time	2	25.517	3.787	**0.024**
Site* habitat* time	4	15.999	2.375	0.052
Residual	251	6.737		

Table 2. A 3-factor analysis of variance at three sites (two habitats and three sampling trips) with density of animals as the dependent variable. Significant p values ($p < 0.05$) are in bold.

Sources	*df*	*MS*	*F*	*P*
Site	2	4.19E+02	0.153	0.865
Habitat	1	4.26E+02	0.178	0.714
Time	2	8.50E+02	1.305	0.366
Site* habitat	2	2.39E+03	7.862	**0.041**
Site* time	4	6.51E+02	2.145	0.239
Habitat* time	2	5.70E+02	1.88	0.266
Site* habitat* time	4	3.04E+02	5.59	**0.0003**
Residual	252	5.43E+01		

Table 3. A 3-factor analysis of covariance at three sites (two habitats and three sampling trips) with number of egg masses as the dependent factor and the ratio between large and small animals (L/S) as a covariate. Significant p values ($p < 0.05$) are in bold.

Sources	*df*	*MS*	*F*	*p*
L+1/S+1	1	3.527	0.519	0.472
Site	2	775.743	114.164	**0.000001**
Habitat	1	8.206	1.208	0.2273
Time	2	265.931	39.136	**0.000001**
Site* habitat	2	3.536	0.521	0.595
Site* time	4	197.081	29.004	**0.000001**
Habitat* time	2	26.658	3.923	**0.021**
Site* habitat* time	4	18.448	2.715	**0.030**
Residual	251	6.795		

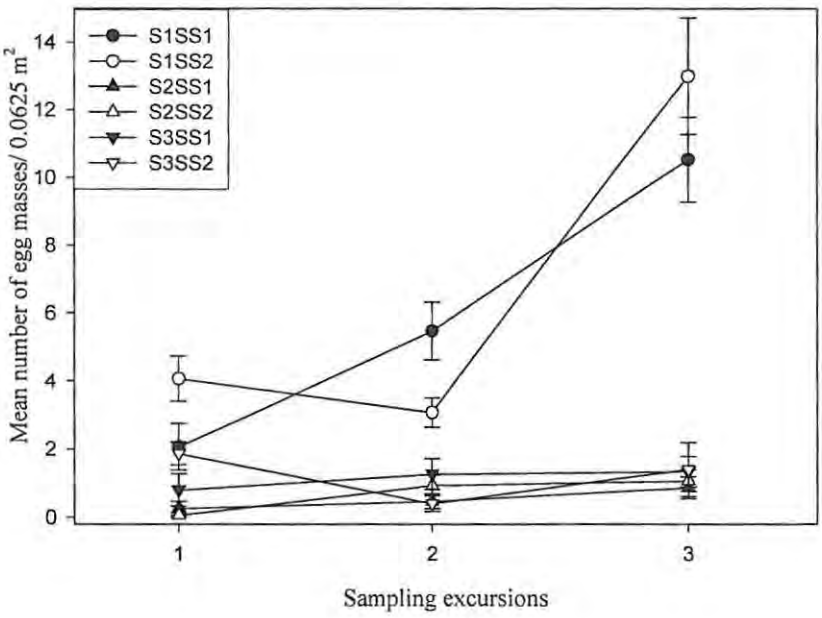


Fig. 1. Mean number of egg masses (± SE) at three sites at three sampling excursions. S1= Three Sisters; S2= Kenton-on-Sea and S3= High Rocks; SS1= non-sand and SS2= sand.

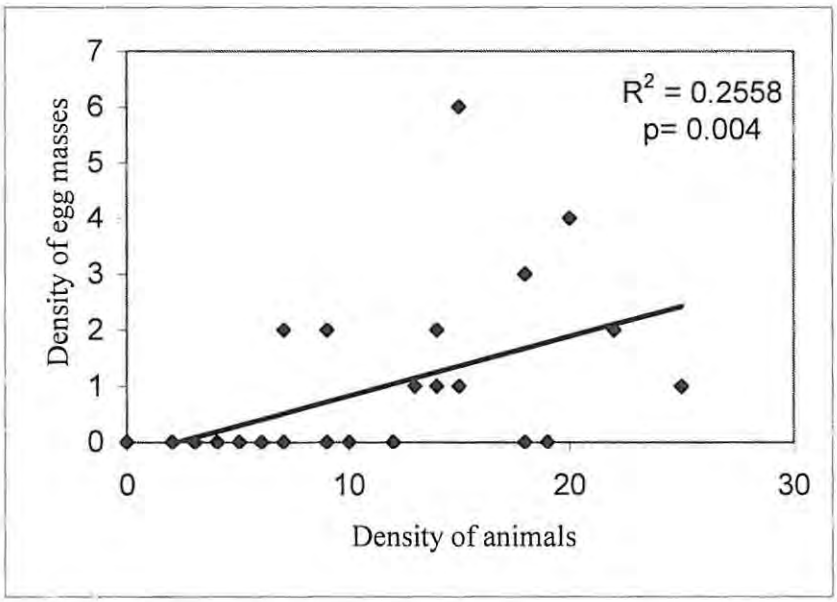


Fig. 2. Relationship between density of animals and density of egg masses at High rocks at Time 2 (late November). Data represented per 0.0625 m^2.

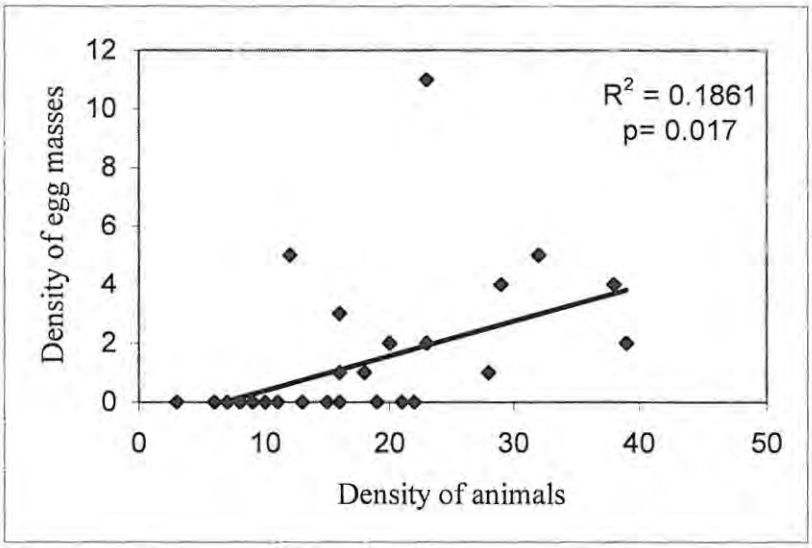


Fig. 3. Relationship between density of animals and density of egg masses at High Rocks at Time 3 (early December). Data represented per 0.0625 m^2.

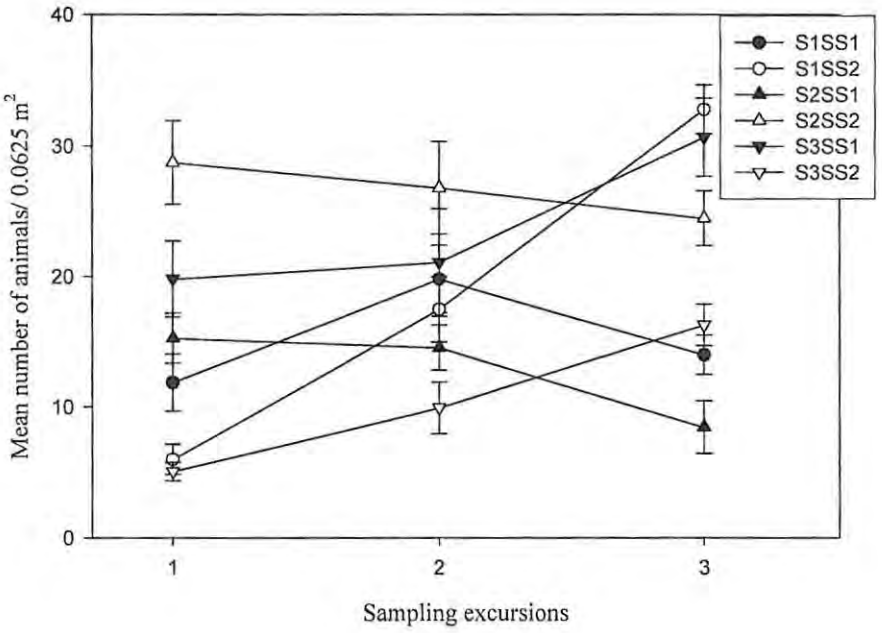


Fig. 4. Density of animals (mean ± SE) (*S. capensis*) at three sites at three sampling excursions. S1= Three Sisters; S2= Kenton-on-Sea and S3= High Rocks, SS1= non-sand covered and SS2= sand covered.

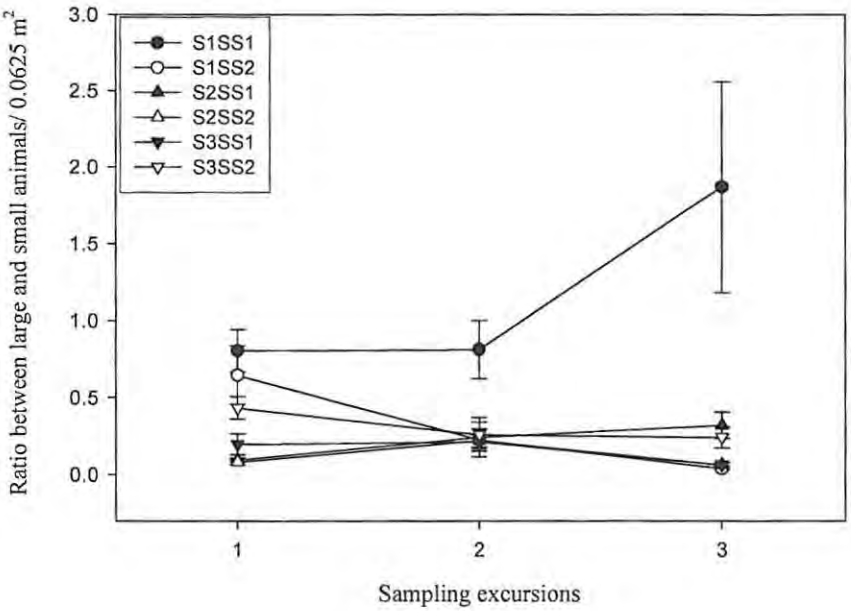


Fig. 5. Ratio between large and small individuals (mean ± SE) of *S. capensis* at three sites at three sampling excursions. S1= Three Sisters, S2= Kenton-on-Sea and S3= High Rocks; SS1= non-sand and SS2= sand.

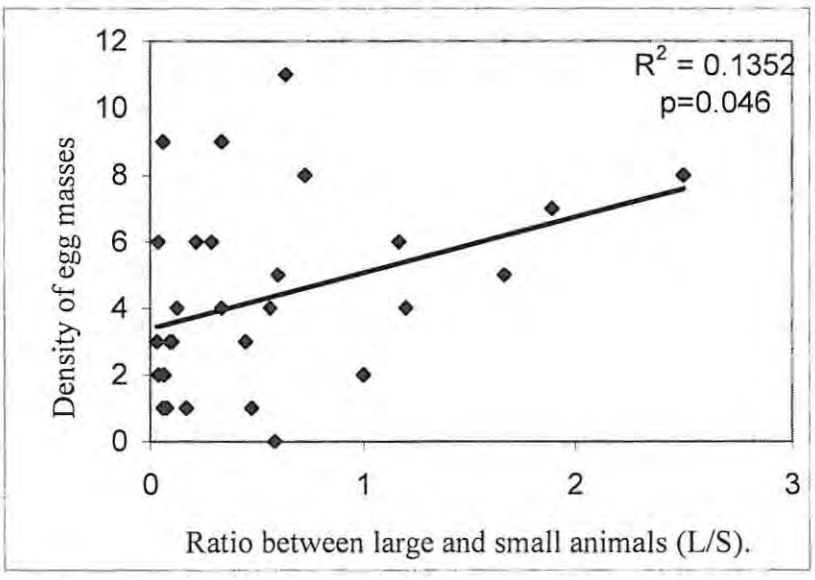


Fig. 6. Relationship between L/S and density of egg masses at Three Sisters at Time 2 (late November). Data represented per 0.0625 m^2.

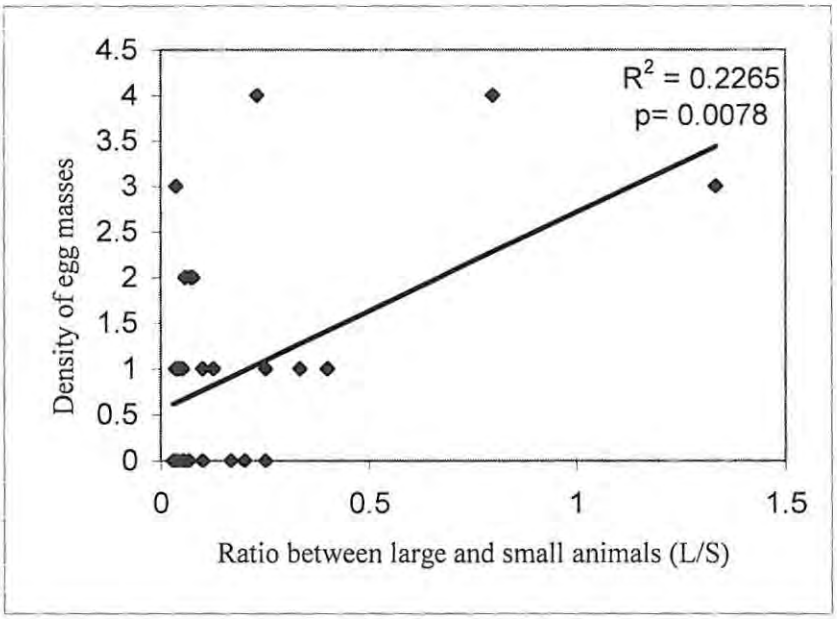


Fig. 7. Relationship between L/S and density of egg masses at Kenton-on-Sea at Time 3 (early December). Data represented per 0.0625 m^2.

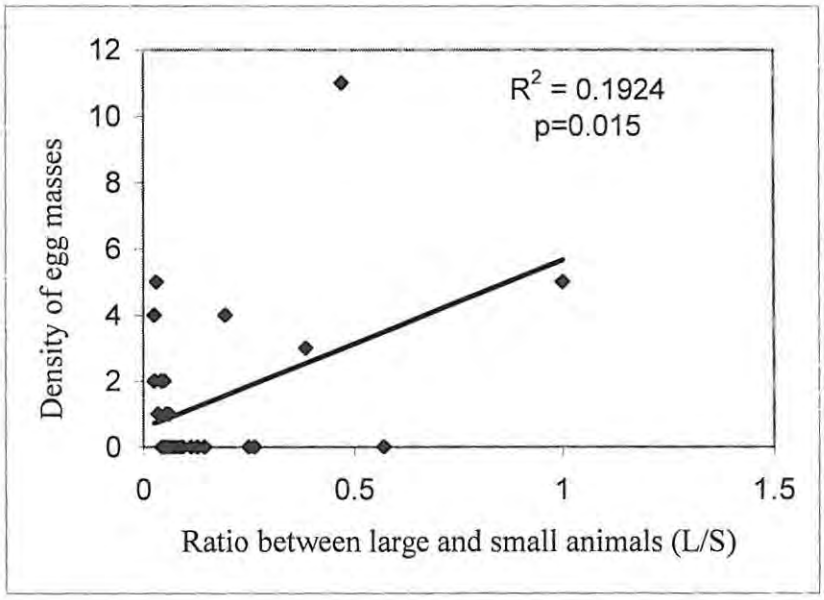


Fig. 8. Relationship between L/S and density of egg masses at High Rocks at Time 3 (early December). Data represented per 0.0625 m^2.

Variability in the density of egg capsules per mm of egg mass, total number of egg capsules and the relationship between density of capsules and length of egg mass

In both analyses (excluding Kenton-on-Sea and without Time 1 or early November data) the length of an egg mass as a covariate had no significant effect on the mean number of egg capsules/ mm i.e., the density of egg capsules present in the egg mass (Table 4 & 5). When Kenton-on-Sea was excluded from the analysis, both Three Sisters and High Rocks had significantly different density of egg capsules over time (site * time interaction significant, Table 4). The density of egg capsules in the egg masses at Three sisters was greatest in early December (Time 3) at the sand covered habitat (about 850 egg capsules per mm of an egg mass) whereas at the non-sand covered habitat the density of egg capsules remained similar at all times (about 700 egg capsules per mm of an egg mass), (Fig. 9). At High Rocks the egg masses from the non-sand covered habitat had a density of more than 600 egg capsules per mm of an egg mass at time 1 (early November) and 3 (early December) while egg masses from the sand covered habitat had a density of almost 1000 egg capsules per mm of an egg mass at time 1 and much lower at time 2 and 3 (Fig. 9). Without time 1 (early November data) the results of an ANCOVA showed that the sites had significantly different densities of egg capsules in the egg masses (Table 5) with Three Sisters having the highest number of egg capsules per mm of an egg mass compared to the other two sites (Fig. 10). Both habitats in Kenton-on-Sea had higher density of egg capsules in the egg masses at time 3 (early December), (Fig. 10). The variations in the length of egg masses at different sites over the

three sampling sessions are presented in Figs. 11 & 12.

Table 4. A 3-factor analysis of covariance at two sites (Kenton is excluded) (two habitats and three sampling trips) with mean number of egg capsules as the dependent variable and length of egg mass as a covariate. pB= Bonferroni-corrected p value (only presented when significant). Significant p values ($p < 0.05$) are in bold.

Sources	*df*	*MS*	*F*	*p*	*pB*
Length	1	9233.8	0.463	0.503	
Site	1	39079.5	1.962	0.175	
Habitat	1	80164.1	4.024	**0.057**	
Time	2	22005.5	1.105	0.348	
Site* habitat	1	11999.9	0.602	0.446	
Site* time	2	87143.4	4.374	**0.025**	**0.05**
Habitat* time	2	8797.4	0.442	0.648	
Site* habitat* time	2	60193.6	3.021	0.068	
Residual	23	19922.2			

Table 5. A 3-factor analysis of covariance at three sites (two habitats and two sampling trips) with mean number of egg capsules as the dependent variable and length of egg mass as a covariate. pB= Bonferroni-corrected p value (only presented when significant). Time 1 is excluded. Significant p values ($p < 0.05$) are in bold.

Sources	*df*	*MS*	*F*	*p*	*pB*
Length	1	18624.4	1.075	0.311	
Site	2	110269.7	6.363	**0.006**	**0.013**
Habitat	1	10868.9	0.627	0.436	
Time	1	52909.3	3.053	0.094	
Site* habitat	2	10725.1	0.619	0.547	
Site* time	2	23271.5	1.343	0.281	
Habitat* time	1	516.1	0.030	0.865	
Site* habitat* time	2	7707.2	0.445	0.646	
Residual	23	17328.9			

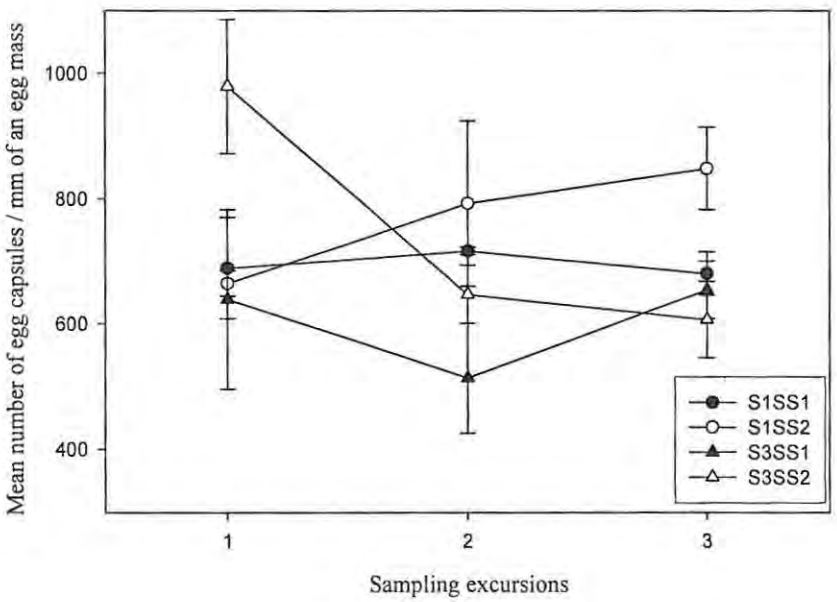


Fig. 9. Mean number of egg capsules (± SE) in the egg masses of *S. capensis* at two sites (Kenton-on-Sea excluded) at three sampling excursions. S1= Three Sisters and S3= High Rocks; SS1= non-sand and SS2= sand.

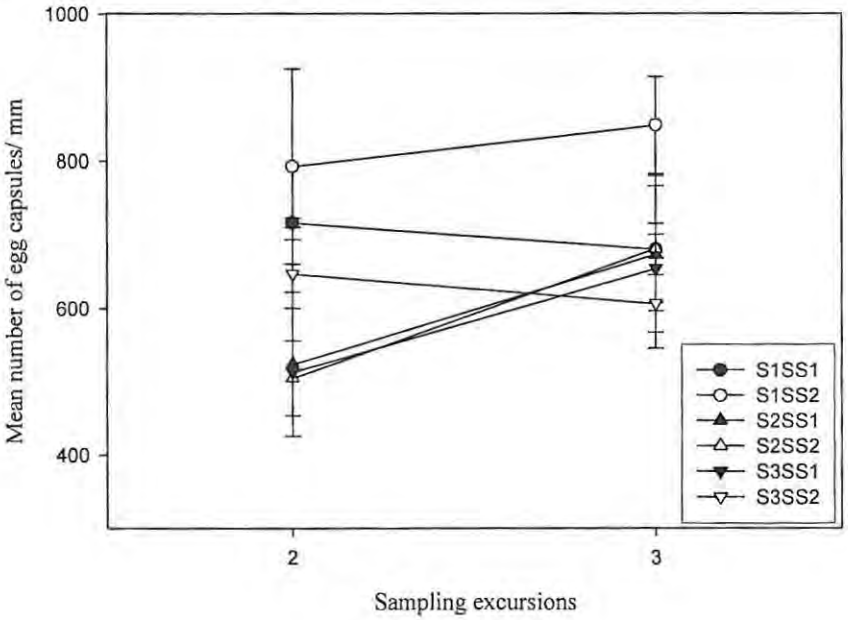


Fig. 10. Mean number of egg capsules (± SE) in the egg masses of *S. capensis* at three sites (Time 1 excluded) at two sampling excursions. S1= Three Sisters, S2= Kenton-on-Sea and S3= High Rocks; SS1= non-sand and SS2= sand.

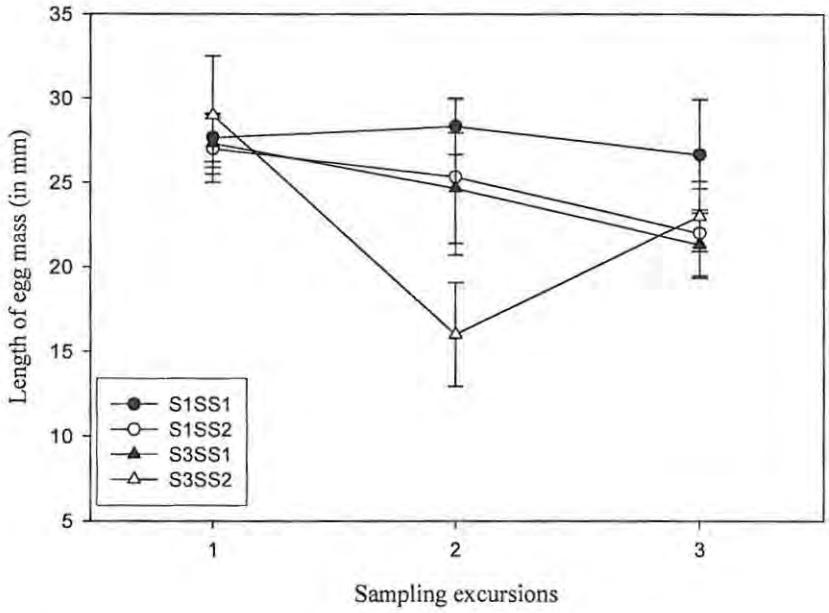


Fig. 11. Length of egg masses (mean ± SE) of *S. capensis* at two sites (Kenton-on-Sea excluded) at three sampling excursions. S1= Three Sisters and S3= High Rocks; SS1= non-sand and SS2= sand.

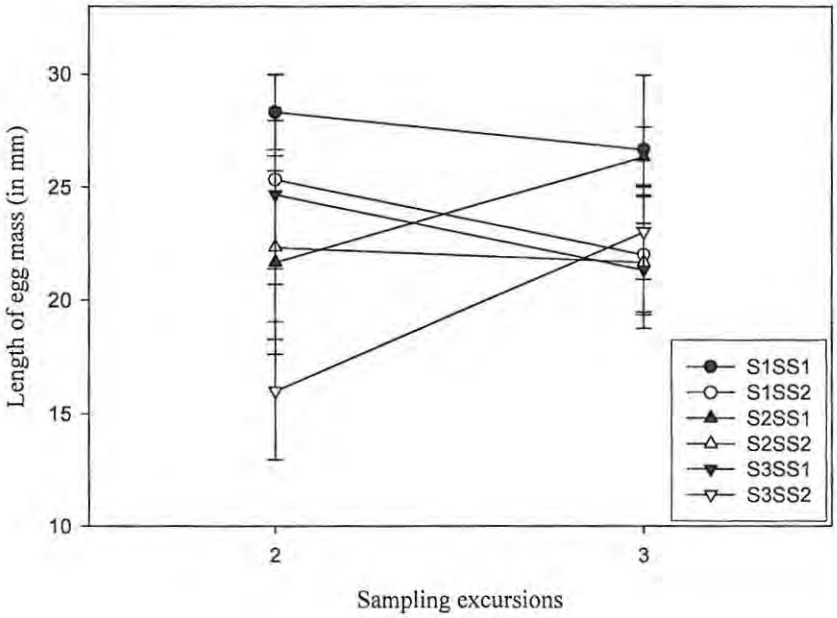


Fig. 12. Length of egg masses (mean ± SE) of *S. capensis* at three sites (Time 1 excluded) at two sampling excursions. S1= Three Sisters, S2= Kenton-on-Sea and S3= High Rocks; SS1= non-sand and SS2= sand.

A 3-factor ANOVA with the estimated total number of egg capsules (in an egg mass) as a dependent variable showed that there was a significant variation over time (when Kenton-on-Sea was excluded), (Table 6). Both High Rocks and Three Sisters showed significant differences in the total number of egg capsules within egg masses over the three sampling excursions (Table 6). The egg masses from Three Sisters had similar numbers of egg capsules at all times (about 18000-20000 egg capsules) whereas the egg masses from High Rocks had the highest number of egg capsules at time 1 (early November) (Fig. 13). The total number of egg capsules in an egg mass did not differ significantly between the habitats within a site although the egg masses from the sand covered habitat in High Rocks at time 1 (early November) had more egg capsules (> 25000) than the non-sand covered habitat (about 18000 egg capsules) (Fig. 13). When time 1 (early November data) was excluded from the analysis only sites were significantly different from each other (Table 7) with Three Sisters having the highest total number of egg capsules (around 20000) in the egg masses compared to Kenton-on-Sea (around 15000) and High Rocks (less than 15000), (Fig. 14).

Table 6. A 3-factor analysis of variance at two sites (Kenton excluded) (two habitats and three sampling sessions) with total number of egg capsules as the dependent variable. pB= Bonferroni-corrected p value (only presented when significant).

Sources	*df*	*MS*	*F*	*p*	*pB*
Site	1	6.16E+07	3.047	0.094	
Habitat	1	9.10E+06	0.450	0.509	
Time	2	9.02E+07	4.462	**0.023**	**0.045**
Site* habitat	1	2.26E+07	1.115	0.301	
Site* time	2	1.20E+08	5.919	**0.008**	**0.016**
Habitat* time	2	3.03E+07	1.497	0.244	
Site* habitat* time	2	3.59E+07	1.776	0.191	
Residual	24	2.02E+07			

Table 7. A 3-factor analysis of variance at three sites (two habitats and two sampling sessions) with total number of egg capsules as the dependent variable. pB= Bonferroni-corrected p value (only presented when significant).Time 1 is excluded. Significant p values ($p < 0.05$) are in bold.

Sources	*df*	*MS*	*F*	*p*	*pB*
Site	2	1.33E+08	8.306	**0.002**	**0.004**
Habitat	1	8.96E+06	0.561	0.461	
Time	1	3.55E+07	2.222	0.149	
Site* habitat	2	1.23E+06	0.077	0.926	
Site* time	2	3.46E+07	2.166	0.137	
Habitat* time	1	4.99E+05	0.031	0.861	
Site* habitat* time	2	7.33E+06	0.459	0.637	
Residual	24	1.60E+07			

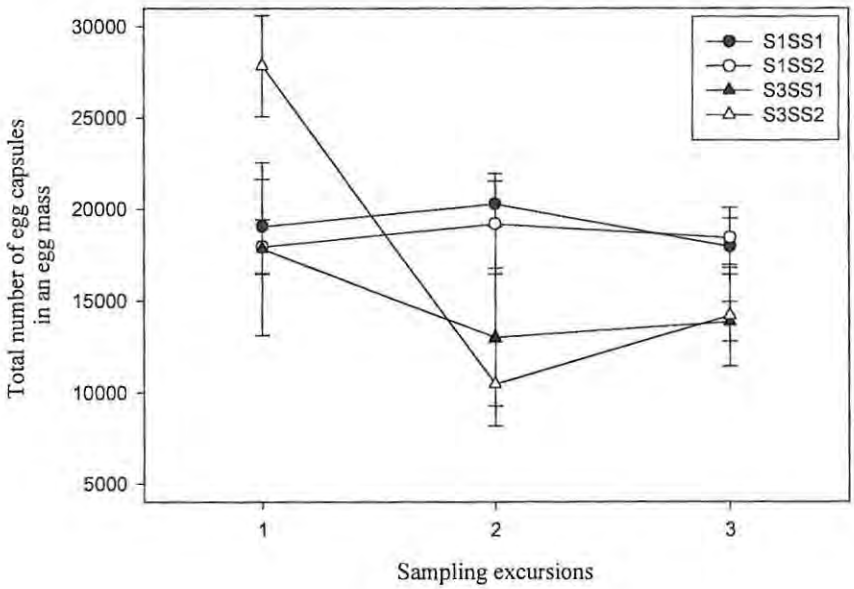


Fig. 13. Total number of egg capsules (mean ± SE) in the egg masses of *S. capensis* at two sites (Kenton-on-Sea excluded) at three sampling excursions. S1= Three Sisters and S3= High Rocks; SS1= non- sand and SS2= sand.

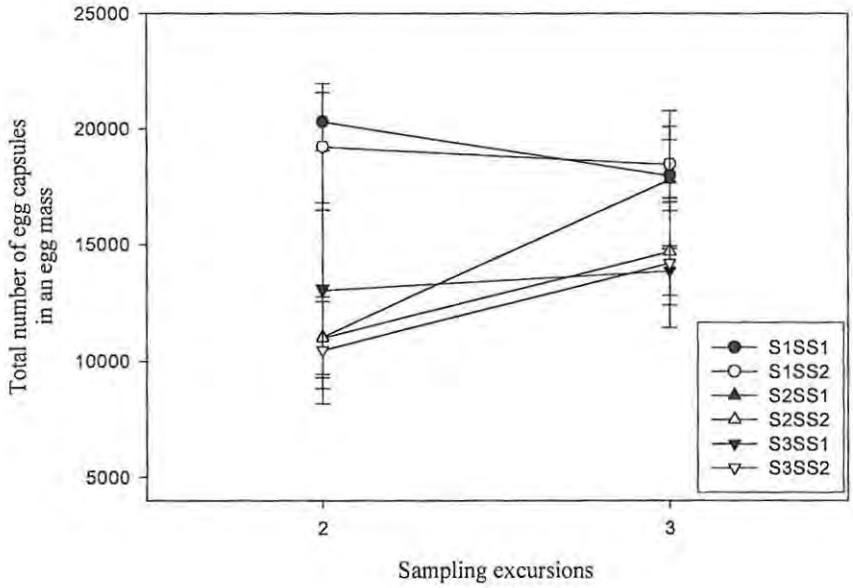


Fig. 14. Total number of egg capsules (mean ± SE) in the egg masses of *S. capensis* at three sites (Time 1 excluded) at two sampling excursions. S1= Three Sisters, S2= Kenton-on-Sea and S3= High Rocks; SS1= non-sand and SS2= sand.

Relationship between size (shell length) and number of oocytes in the gonad of *S. capensis* and *S. serrata*

Linear regressions between the shell length of *S. capensis* and *S. serrata* and number of mature oocytes in the gonad showed that there was a positive relationship. In the case of *S. capensis* the relationship was significant [n= 20; R^2= 0.1952; p= 0.051] (Fig. 15) whereas in *S. serrata* there was a tendency towards a positive relationship [n=24; R^2= 0.1264; p= 0.088] (Fig. 16).

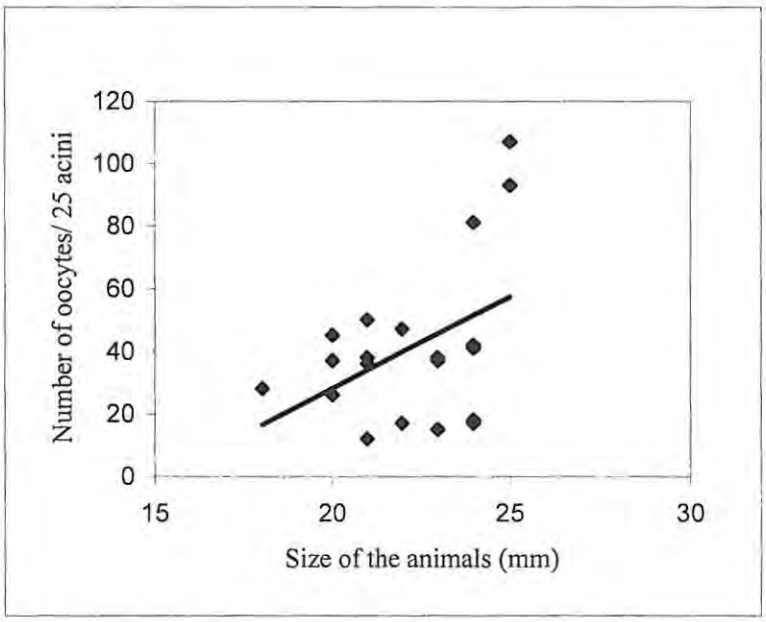


Fig. 15. Relationship between shell length of *S. capensis* and number of mature oocytes in the gonad (November to February) (n= 20; R^2 =0.195; p= 0.051).

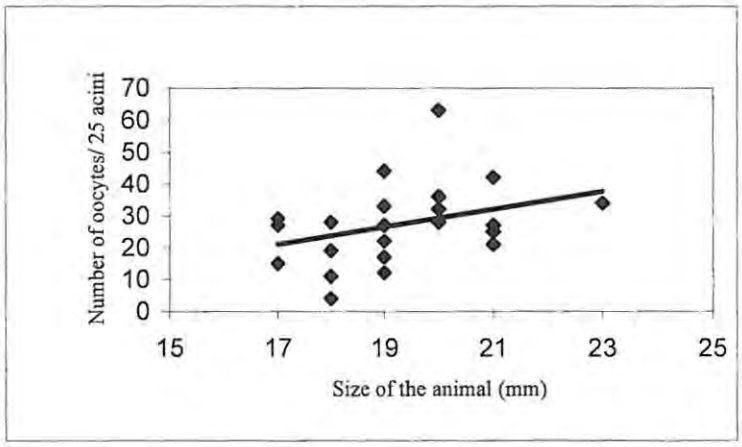


Fig. 16. Relationship between shell length of *S. serrata* and number of mature oocytes in the gonad (October to February) (n= 24; R^2 = 0.126; p= 0.088).

Discussion

Density of egg masses in relation to the density of animals

Quantitative data on the variability in fecundity of populations within a species of *Siphonaria* are scarce (see review by Hodgson, 1999). This study estimated the variability in the number of egg masses laid by individuals in populations of *S. capensis* at three different sites during the breeding season. Each site consisted of two habitats namely sand-covered and non-sand covered. The data collected during a study on the seasonality of spawning in *S. capensis* in Kenton-on-Sea, showed that density of animals was higher in sand-covered than the non-sand covered habitats (Chapter 2). Also the number of spawn during peaks of spawning was higher in the sand-covered than that of the non-sand covered habitat, but the individual fecundity remained the same in both habitats (see Chapter 2), which indicated that differences between the habitats in the density of adult animals affected the number of spawn during peaks. In the current study, the density of animals was again higher in the sand-covered habitat in Kenton but the opposite pattern was found in High Rocks. Three Sisters did not show any consistent pattern in the variation of animal density. The present study found very few egg masses on the shore at Kenton-on-Sea and High Rocks during the three sampling times, which probably explains why there was no relationship between animal density and number of spawn. The analysis of covariance did not show any effect of the covariate (in this case density of limpets) on the number of egg masses, and the linear regressions (for each site and time) showed that only at High Rocks (in late November and early December) the number of spawn was positively correlated to the density of animals. In contrast to *S.*

capensis, increase in the density of animals had a negative effect on the number of egg masses of *S. diemenensis* due to the seasonal supply of food (Quinn, 1988).

Density of egg masses in relation to the size of adult limpets, and variability in the number of egg capsules

Although the present study showed that the relationship between the density of adult individuals and number of spawn is not usually significant, the increase in the ratio between large and small animals (L/S) (which could be the result of either loss of small animals or presence of more large animals) at Three Sisters (late November), Kenton-on-Sea (early December) and High Rocks (early December) correlated to the greater number of spawn. In the study on the spawning seasonality of *S. capensis* at Kenton-on-Sea (Chapter 2), the size differences of limpets between non-sand and sand-covered habitats was not examined and it might be that more large animals were present at the sand-covered than the non-sand covered habitat, leading to higher density of spawn at the sand-covered habitat. At Three Sisters in early December, the higher density of animals at the sand-covered than the non-sand covered habitat may explain the differences in the density of egg masses between these two habitats. However, the ratio between large and small animals (L/S) increased on the non-sand covered habitat of this site in early December, perhaps explaining why the difference in egg mass density was small between habitats at this site and time.

It is clear from the analyses that the mean number of spawn differed between different habitats at these three sites over time (site* time interaction significant) as it did in the initial study (see Chapter 2). Although this study did not aim to investigate the

reasons behind the variability in population fecundity of *S. capensis*, it may be a result of populations at Three Sisters, High Rocks and Kenton-on-Sea spawning at different times.

Generally, both the number of egg masses (approximately 12 egg masses/ 0.0625 m^2) and number of egg capsules (around 20000 per egg mass) was greater at Three Sisters, especially in December (time 3). Even though the number of egg masses was quite low at High Rocks at all times, the number of egg capsules in the egg masses was high at time 1 (> 25000 egg capsules per egg mass; sand-covered habitat, early November). The effect of food availability or quality on the variability in fecundity of *S. capensis*, which is considered important for the fecundity of many invertebrates including *Siphonaria* (Fletcher, 1984; Quinn, 1988; Cassai & Prevedelli, 1999; Cheung & Lam, 1999; Prevedelli & Simonini, 2000; Valentinsson, 2002), cannot be commented upon as this study did not examine the difference in food availability between the three sites. All three sites studied here are aeolian platforms and it is not known whether food availability differs between aeolian shores in the Eastern Cape (Vat, 2000).

Number of egg capsules and length of egg masses

There was no relationship (from the ANCOVA) between the length of an egg mass and mean number of egg capsules/ mm of an egg mass of *S. capensis*. This indicates that the density of egg capsules within small egg masses of *S. capensis* is probably no different than that of longer egg masses. What causes the variation in the length of an egg mass has not been investigated in this study. Observations on egg laying of *S. capensis* in laboratory aquaria suggest that during the breeding season the animals lay eggs more than once and the egg mass laid first is usually longer than the following ones.

Relationship between shell length and fecundity

Both the size-dependent (as shown here) (Creese, 1980; Hughes & Roberts, 1980; Perron, 1983; Bayne *et al.*, 1983; Quinn, 1988) and size-independent fecundity (Perron, 1982; Fletcher, 1984) are common in invertebrates, especially in molluscs. Although preliminary, the positive relationship between the number of mature oocytes in the gonad and the shell length of *S. capensis* and *S. serrata* indicates that larger animals may be more fecund, although there is also a greater possibility of them being parasitized (Hodgson *et al.*, 1993). Fecundity of other intertidal gastropods such as littorinids and subtidal gastropods such as the neogastropod *Buccinum* has also been shown to increase with shell height (e.g. Janson, 1985; Hughes & Answer, 1982; Valentinsson, 2002). This kind of positive relationship between size and fecundity may be due to the fact that energy allocated to reproduction increases with size whereas the energy required for somatic functions remains unaltered as suggested by Quinn (1988) for *S. diemenensis*. This has previously been proposed by many other authors studying molluscs (e.g., Parry, 1982; Perron, 1982; Perron, 1983). The presence of a greater number of spawn on the shore when the L/S ratio was higher may also indicate that large individuals of *S. capensis* are more fecund. It will be interesting to examine whether larger animals also produce more egg capsules within the egg masses compared to their small congeners. Another possibility is that larger animals spawn more frequently compared to small individuals, which was reported by Creese (1980) for *S. denticulata*.

To summarize, *S. capensis* at the three sites studied here (Three Sisters, Kenton-on-

Sea and High Rocks) laid different number of egg masses over three spring tides in November and December 2000. The density of spawn was highly variable within sites (between habitats) over time. The number of spawn may be related to the density of adult limpets only in certain habitats during spawning peaks. A higher ratio between large and small individuals positively affects the number of spawn on the shore in some populations. The length of egg masses vary inconsistently between sites and times but has no effect on the density of egg capsules within an egg mass. In both *S. capensis* and *S. serrata*, preliminary observations suggest larger animals tend to be more fecund as they contain more mature oocytes in the gonad.

References

BAYNE, B. L., SALKELD, P. N. & WORRALL, C. M. 1983. Reproductive effort and value in different populations of the marine mussel, *Mytilus edulis* L. *Oecologia*, **59**: 18-26.

CASSAI, C. & PREVEDELLI, D. 1999. Fecundity and reproductive effort in *Ophryotrocha labronica* (Polychaeta: Dorvilleidae). *Marine Biology*, **133**: 489- 494.

CHAMBERS, R. J. & MCQUAID, C. D. 1994a. Notes on the taxonomy, spawn and larval development of South African species of the intertidal pulmonate limpet genus *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **60**: 263- 275.

CHAMBERS, R. J. & MCQUAID, C. D. 1994b. A review of larval development in the intertidal limpet genus *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **60**: 415- 423.

CHEUNG, S. G. & LAM, S. 1999. Effect of food availability on egg production and packaging in the intertidal scavenging gastropod *Nassarius festivus*. *Marine Biology*, **135**: 281- 287.

CREESE, R. J. 1980. Reproductive cycles and fecundities of two species of *Siphonaria* (Mollusca: Pulmonata) in South-eastern Australia. *Australian Journal of Marine and Freshwater Research*, **31**: 37- 47.

FLETCHER, W. J. 1984. Variability in the reproductive effort of the limpet, *Cellana tramoserica*. *Oecologia*, **61**: 259- 264.

HODGSON, A. N., FARQUHAR, M. R. & DE VILLIERS, C. J. 1993. Variations in infestations of *Siphonaria capensis* and *S. concinna* (Pulmonata) by larval trematodes in

southern Africa. *Journal of Molluscan Studies*, **59**: 83- 93.

HODGSON, A. N. 1999. The biology of siphonariid limpets (Gastropoda: Pulmonata). *Oceanography and Marine Biology: an annual review*, **37**: 245- 314.

HUGHES, R. N. & ANSWER, P. 1982. Growth, spawning and trematode infection of *Littorina littorea* (L.) from an exposed shore in north Wales. *Journal of Molluscan Studies*, **48**: 321- 330.

HUGHES, R. N. & ROBERTS, D. J. 1980. Reproductive effort of winkles (*Littorina* spp.) with contrasted methods of reproduction. *Oecologia*, **47**: 130- 136.

JANSON, K. 1985. Variation in the occurrence of abnormal embryos in females of the intertidal gastropod species *Littorina saxatilis* Olivi. *Journal of Molluscan Studies*, **51**: 64- 68.

LLODRA, E. R. 2002. Fecundity and life history strategies in marine invertebrates. *Advances in Marine Biology*, **43**: 88- 170.

MARSHALL, D. J. & MCQUAID, C. M. 1989. The influence of respiratory responses on the tolerance to sand inundation of the limpets *Patella granularis* L. (Prosobranchia) and *Siphonaria capensis* Q. et G. (Pulmonata). *Journal of Experimental Marine Biology and Ecology*, **128**: 191-201.

PARRY, G. D. 1982. Reproductive effort in four species of intertidal limpets. *Marine Biology*, **67**: 267- 282.

PERRON, F. E. 1982. Inter- and intraspecific patterns of reproductive effort in four species of cone shells (*Conus* spp.). *Marine Biology*, **68**: 161-167.

PERRON, F. E. 1983. Growth, fecundity and mortality of *Conus pennaceus* in Hawaii. *Ecology*, **64**: 53- 62.

PREVEDELLI, D. & SIMONINI, R. 2000. Effects of salinity and two food regimes on survival, fecundity and sex ratio in two groups of *Dinophilus gyrociliatus* (Polychaeta: Dinophilidae). *Marine Biology*, **137**: 23- 29.

QUINN, G. P. 1983. Spawning and egg masses of *Siphonaria tasmanica* Tenison Woods, 1876 from Victoria. *Journal of the Malacological Society of Australia*, **6**: 81- 82.

QUINN, G. P. 1988. Ecology of the intertidal pulmonate limpet *Siphonaria diemenensis* Quoy et Gaimard. II Reproductive patterns and energetics. *Journal of Experimental Marine Biology and Ecology*, **117**: 137- 156.

STEARNS, S. C. 1992. *The evolution of life histories*. Oxford University Press, Oxford.

STEARNS, S. C. & HOEKSTRA. R. F. 2000. *Evolution: an introduction*. Oxford University Press. Oxford.

UNDERWOOD, A. J. 1997. *Experiments in ecology: their logical design and interpretation using analysis of variance*. Cambridge University Press, London.

VALENTINSSON, D. 2002. Reproductive cycle and maternal effects on offspring size and number in the neogastropod *Buccinum undatum* (L.). *Marine Biology*, **140**: 1139- 1147.

VAT, L. S. 2000. *The growth and reproduction of Patella granularis (Mollusca: patellogastropoda) on the south-east coast of South Africa*. Ph. D. thesis. Rhodes University.

Chapter 4

Oogenesis and modes of vitellogenesis

[This chapter published as:
Pal, P. & Hodgson, A. N. 2002. An ultrastructural study of oogenesis in a planktonic and a direct developing species of *Siphonaria* (Gastropoda: Pulmonata). Journal of Molluscan Studies, 68: 337-344.]

Introduction

Siphonariid limpets are very common pulmonates in the intertidal regions of warm temperate to tropical rocky shores, especially in the southern hemisphere (Hodgson, 1999). As basommatophorans they are considered primitive pulmonates (Hubendick, 1978) but their ancestry is uncertain. The presence of planktonic development in many species of *Siphonaria* and their lack of tentacles, has led some authors to argue for a marine ancestry (Hyman, 1967; Purchon, 1979). By contrast others have suggested that siphonariids have a terrestrial origin and they re-invaded marine environments (e.g. Hubendick, 1947; Borland, 1950; Yonge, 1952). The more recent work of Chambers *et al.*, (1996, 1998) did not clarify matters. Their results from analysis of total proteins suggested that planktonic development and marine ancestry (Chambers *et al.*, 1996) was primitive, whereas the later information from DNA fingerprinting (Chambers *et al.*, 1998) indicated the oppostite i.e., siphonariids are descended from terrestrial ancestors with direct development.

Like all pulmonates, siphonariids are hermaphrodites with internal fertilization. They lay fertilized eggs on rocks, the eggs being protected by capsules, which are embedded in a jelly matrix (see Hodgson, 1999 for review). Although these limpets have been studied extensively, many aspects of their reproductive biology are still unknown (Hodgson, 1999). Two main larval developmental patterns, planktonic and intracapsular, have been recorded for the genus *Siphonaria* (Chambers and McQuaid, 1994 a, b). Planktonic developers lay large numbers of small eggs, which hatch after 4-5 days as veliger larvae, whereas intracapsular developers lay smaller numbers of larger eggs from which

crawling juveniles emerge after 3-4 weeks (Chambers, 1994; Chambers & McQuaid, 1994 a, b; Hodgson, 1999). Species with both forms of development can be found in sympatry on South African shores (Allanson, 1958; Chambers, 1994).

The occurrence of different life history strategies in siphonariids has been explained in terms of adaptation and ancestry (Chambers, 1994). Although the type of development and size of egg or egg capsule is well known for numerous species of *Siphonaria*, there have been no descriptions of egg formation (oogenesis and vitellogenesis) (Hodgson, 1999). Whilst the life history strategy or developmental mode is constrained by ancestry (as has been shown in littorinids, Reid, 1990) some studies have shown that there is a close correlation between the type of oogenesis and life history pattern (Eckelbarger, 1994). Oogenesis and associated modes of vitellogenesis also set interspecific differences during egg development and for this reason *Siphonaria* presents an opportunity to compare the process of oogenesis in closely-related species from the same habitat, but with different modes of development. The aim of this work was to compare oogenesis and vitellogenesis in species of *Siphonaria* with planktonic (*S. capensis* Quoy & Gaimard, 1988) and intracapsular development (*S. serrata* Fischer, 1807), testing the hypothesis that different modes of development (with the production of eggs of different sizes) would involve differences in oogenesis.

Materials and methods

Specimens of *Siphonaria capensis* and *S. serrata* were collected from intertidal rocks at Kenton-on-Sea (33°42' S, 26°41' E) in the Eastern Cape, South Africa. To ensure that all stages of oogenesis were obtained, three specimens of each species were collected

seasonally. Samples were collected twice in spring (September and October, 1999) and summer (December, 1999; February, 2000), once in autumn (May, 2000) and winter (July, 2000). Animals were transported back to the laboratory where the gonad was removed. Small portions of the gonad were fixed for approximately 12 hours in cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and filtered sea-water (pH.7.2). After fixation, tissues were washed in 0.2 M sodium cacodylate buffer (pH. 7.0) and postfixed in 1% OsO_4 in sodium cacodylate buffer for 90 minutes at room temperature. After rinsing the tissues in two changes of buffer they were dehydrated in ascending concentrations of ethanol to 100%. Tissues were infiltrated (via propylene oxide) and embedded in an Araldite / Taab mixture (Cross, 1989). Both semi-thin and ultra-thin sections were cut using glass knives on a RMC MT7 ultramicrotome. Semi-thin sections, stained in 1% toluidene blue dissolved in 2.5% sodium carbonate, were observed and photographed with a light microscope. Ultra thin sections were stained in 5% aqueous uranyl acetate (30 minutes) and Reynolds' lead citrate (5 minutes) (Reynolds, 1963) and the grids were viewed with a JEOL 1210 transmission electron microscope at 100 kV.

Results

The gonad of siphonariids, including the species studied here, is composed of numerous closely grouped acini in which both eggs and sperm develop (Marcus & Marcus, 1960; Berry, 1977; Hodgson *et al.*, 1991; Luchtel *et al.*, 1997). Except for winter months (June / July), most stages of oogenesis could be found all year round within a gonad, but oogenesis was asynchronous between acini (Figs. 1 & 2). Early oocytes lie next to the wall of the acinus and as they grow and mature they gradually fill its lumen

(Figs. 1 & 2).

The wall of each acinus is about 0.8-1.0 μm thick and consists of a layer of thin cells, which often contain pigment granules, a small amount of smooth muscle and a band of fibrous connective tissue (Figs. 6, 7 & 8).

Previtellogenic oocytes

The structure of the previtellogenic oocytes in both species is very similar (Figs. 3 & 4). The smallest oocytes observed were about 15 x 12 μm in size. Early previtellogenic oocytes possess a large, round nucleus (about 8-10 μm diameter) with scattered heterochromatin and a prominent nucleolus (Fig. 3). The ooplasm contains a few round mitochondria (about 0.5 μm diameter) with prominent cristae, and small amounts of endoplasmic reticulum (Fig. 4). As the oocytes grow, the nucleus develops more than one nucleolus [one large amphinucleolus (about 9 x 8μm) and a small eunucleolus (about 4 μm diameter)] (Fig. 5).

Previtellogenic oocytes are separated from the wall of the acinus by follicle cells (Fig. 3) that completely surround each oocyte. Each follicle cell has a large nucleus (about 7 x 3 μm) with scattered heterochromatin and a single nucleolus (Figs. 23 & 3). The cytoplasm contains scattered rough endoplasmic reticulum, Golgi bodies and elongate mitochondria with prominent lamellar cristae (Figs. 24 & 25). The Golgi bodies produce small vesicles, which are membrane-bound and contain electron-dense material (Fig. 25). Some dense bodies are also found in the cytoplasm (Figs. 24 & 25). Cell junctions connect adjacent follicle cells as well as the oocytes to the follicle cells (Figs. 28 & 30). Follicle cells lie adjacent to the connective tissue wall of the acinus but do not appear to

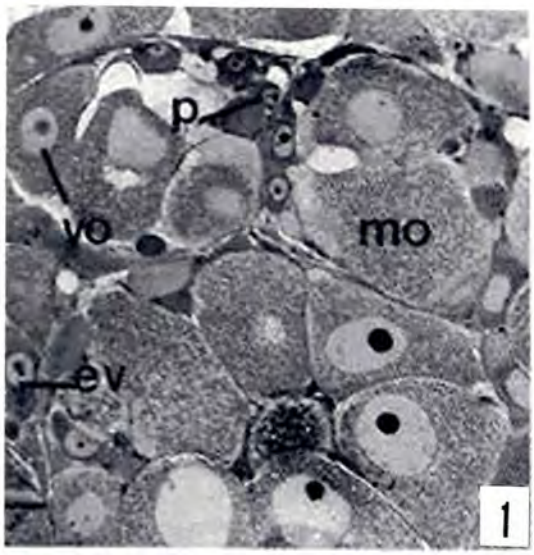
be attached to it by cell junctions (Fig. 7).

Figs. 1 & 2. Light micrographs of toluidene blue stained sections showing differents stages of oogenesis in the gonad of *S. capensis*. Scale bar= 35 μm.

Figs. 3 & 4. Previtellogenic oocytes of *S. serrata* and *S. capensis* respectively. The ooplasm contains a spherical nucleus (n), a few small mitochondria (m), rough endoplasmic reticulum (rer) and in *S. serrata* a few lipid droplets (l). Scale bar= 2 μm and 500 nm respectively.
The oocytes are surrounded by follicle cells (f), containing spherical nucleus (n) with a nucleolus (arrowhead).

Fig. 5. Previtellogenic oocyte of *S. capensis* in which the nucleus (n) contains a large amphinucleolus (an) and a small eunucleolus (en). Note the band of mitochondria (m) around the nuclear membrane (arrowhead). Scale bar= 1 μm.

Figs. 6, 7 & 8. Electron micrographs of the wall of gonad acini. The wall (w) consists of a layer of thin cells containing pigment granules (pg) with an irregularly- shaped nucleus (n), connective tissue (ct) and muscle (mu). Scale bar= 2 μm, 1 μm and 200 nm respectively. ev, early vitellogenic oocyte; h, heterochromatin; mo, mature oocyte; p, previtellogenic oocyte; vo, vitellogenic oocyte.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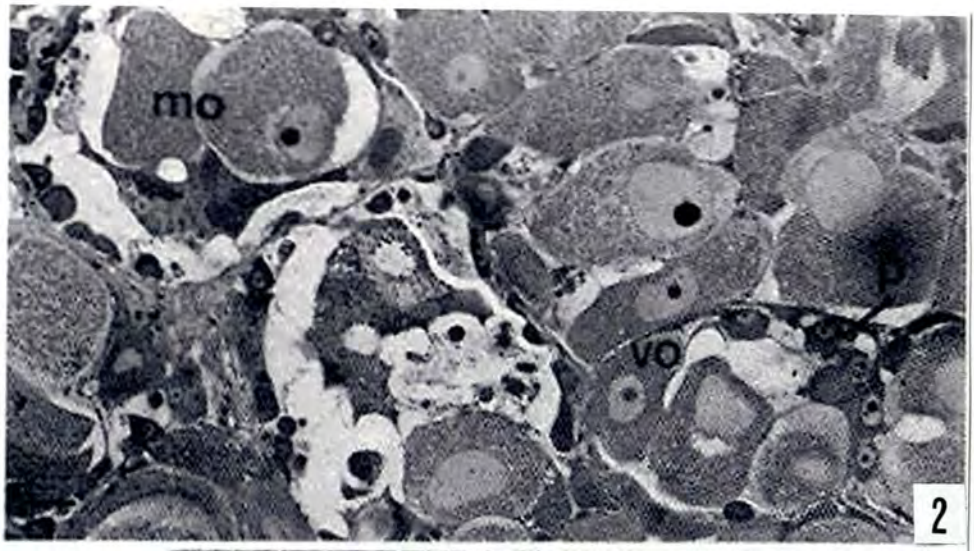
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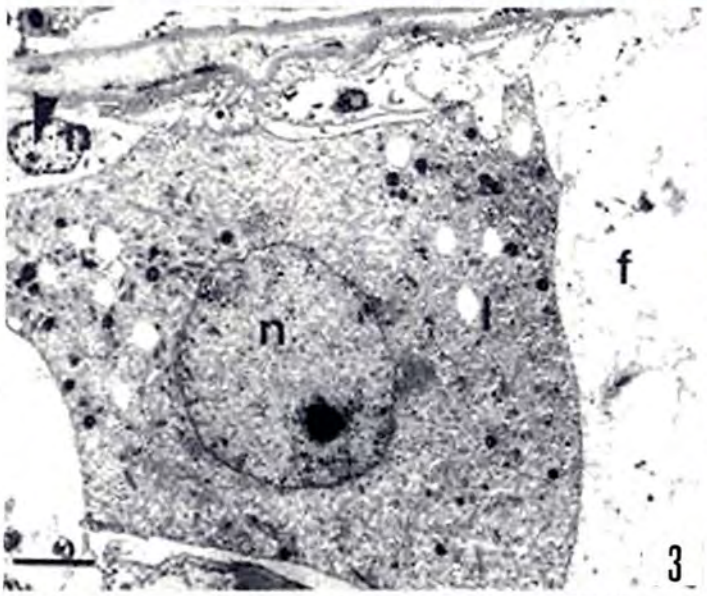
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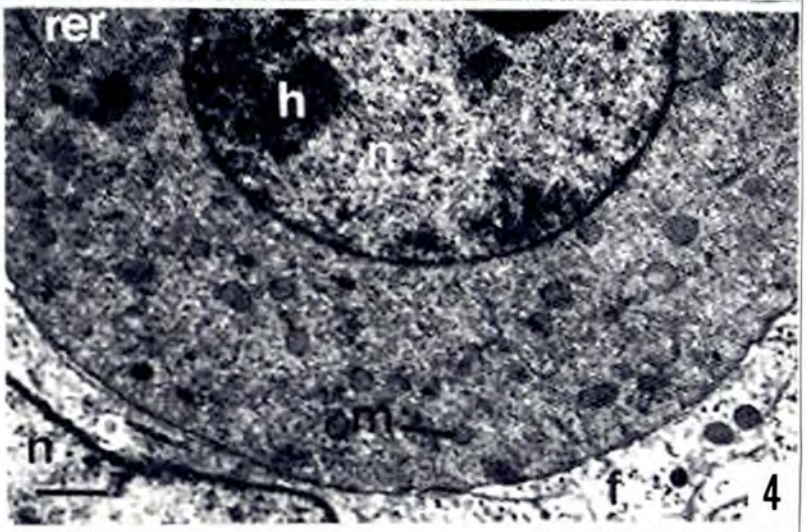
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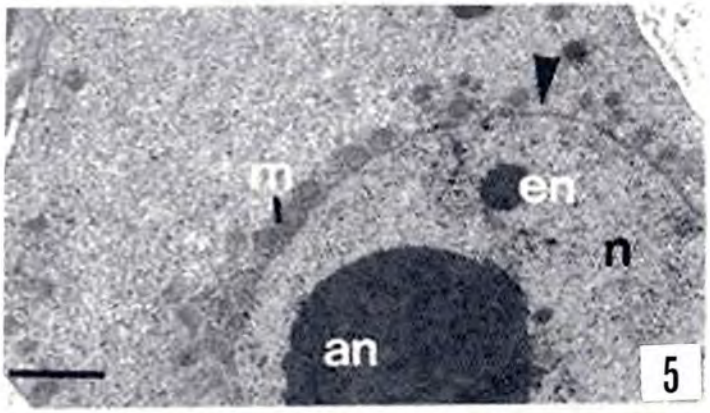
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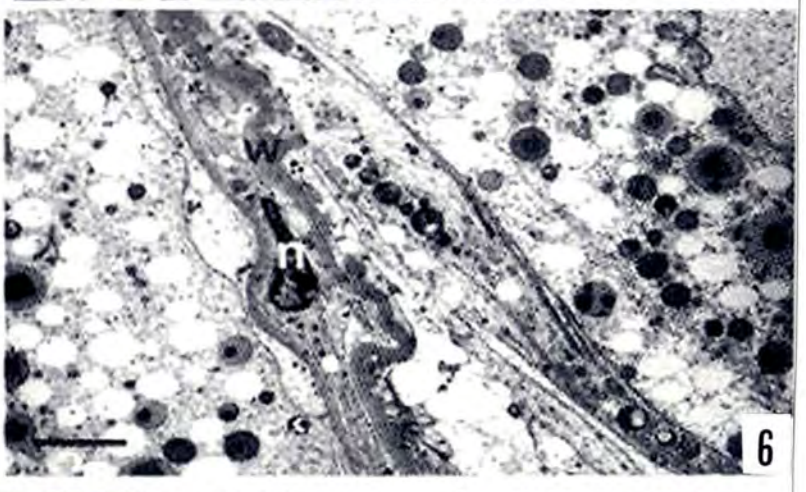
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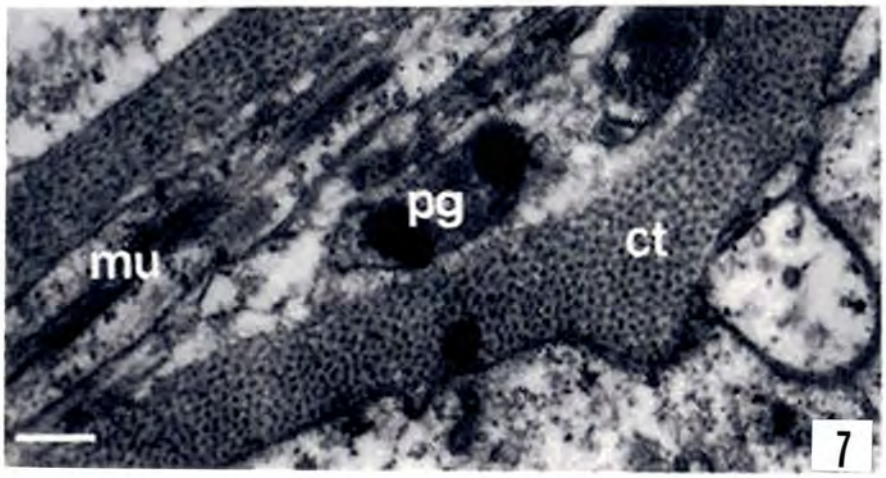
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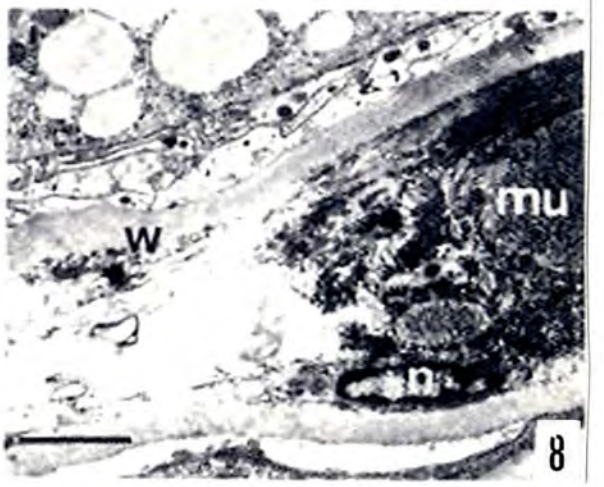
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Vitellogenic oocytes

As vitellogenesis commences the nucleus of the oocytes of both species increases in size to approximately 30 x 40μm and becomes lobular in shape (Fig. 9). There is also an increase in the number of proteosynthetic organelles (Figs. 9 & 10). In *S. capensis* small, spherical mitochondria accumulate close to the nuclear membrane along with arrays of rough endoplasmic reticulum, some Golgi bodies and nuage-like material (Fig. 12). Vitellogenesis starts perinuclearly with the appearance of small electron-dense vesicles (about 0.8-1.0 μm diameter), which are possibly nascent yolk granules, forming close to the rough endoplasmic reticulum (Figs. 10, 11 & 12). Other small vesicles, which appear to be produced by Golgi bodies, fuse with the nascent yolk granules (Fig. 16). Once yolk granule formation is underway, lipid droplets about 1μm in diameter begin to accumulate in the ooplasm (Fig. 11).

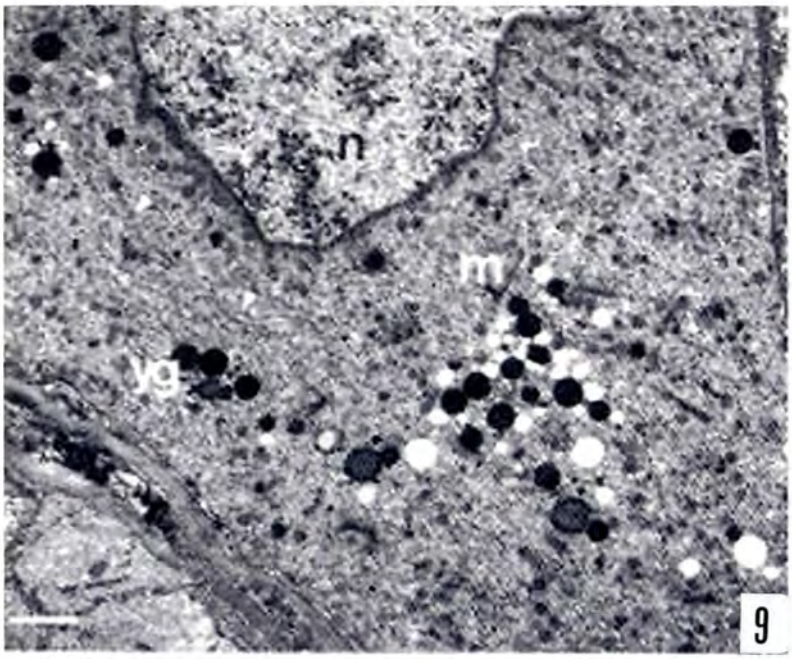
In *S. serrata*, early vitellogensis is characterized by proliferation of smooth endoplasmic reticulum and mitochondria, both of which are closely associated with lipid (Fig. 13). Yolk granules begin to appear once lipid formation is underway (insert to Fig. 14).

As vitellogenesis proceeds in both species, the proteosynthetic organelles increase in number, the mitochondria proliferate and elongate (especially in *S. serrata*) (Fig. 14). There is an increase in the number and size of the yolk granules, which gradually fill the ooplasm from the centre outwards, eventually reaching a size of about 2-5 μm in diameter (Figs. 11, 14, 17 & 22). In addition they also show changes in their structure. In both *S. capensis* and *S. serrata* the contents of the electron-dense yolk granules begin to

differentiate into a crystalline core surrounded by an electron-lucent cortex (Figs. 17, 18 & 22).

Fig. 9. Early vitellogenic oocyte of *S. capensis* with rough endoplasmic reticulum (arrowheads), a few yolk granules (yg). Scale bar= 1 μm. **Fig. 10.** Early vitellogenic oocyte of *S. capensis* showing dense bodies (db), which are probably nascent yolk granules and a band of mitochondria (m) close to the nucleus (n). Scale bar= 1 μm. **Fig. 11.** Vitellogenic oocyte of *S. capensis* in which yolk synthesis is underway and lipid droplets (l) have begun to appear in the ooplasm. Extensive arrays of rough endoplasmic reticulum (arrowhead) are seen in the perinuclear cytoplasm. Scale bar= 1 μm. **Fig. 12.** Early vitellogenic oocyte of *S. capensis* showing rough endoplasmic reticulum (rer), a nascent yolk granule (yg) and nuage (ng). Scale bar= 200nm.

Fig. 13. Early vitellogenic oocyte of *S. serrata* with lipid droplets (l) and smooth endoplasmic reticulum (ser). Scale bar= 1 μm. **Fig. 14.** Perinuclear ooplasm of a vitellogenic oocyte of *S. serrata* showing lipid droplets (l), elongated mitochondria (m), lobular nucleus (n) and yolk granules (yg). Scale bar= 1μm. Insert: Nuage like material (ng) in *S. serrata*. Scale bar= 1μm. **Fig. 15.** Early vitellogenic oocytes of *S. capensis* showing Golgi body (g) producing small vesicles (v). Scale bar= 200nm. **Fig. 16.** Vesicles (v) fusing to form mature yolk granule (yg) in early vitellogenic oocyte of *S. capensis*. Scale bar= 200nm. f, follicle cell ; l, lipid; m, mitochondria; n, nucleus; rer, rough endoplasmic reticulum.



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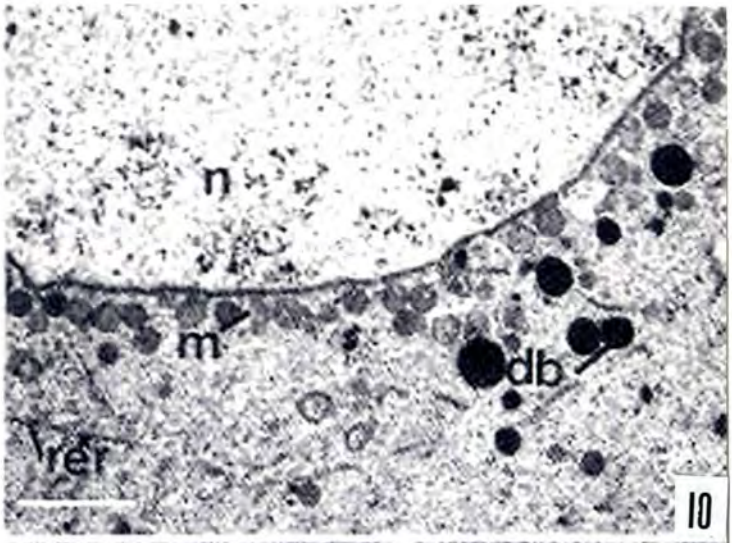

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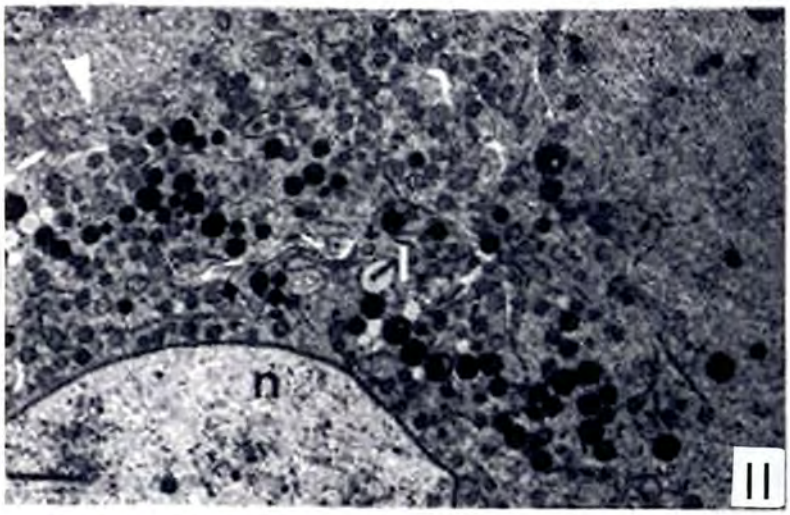

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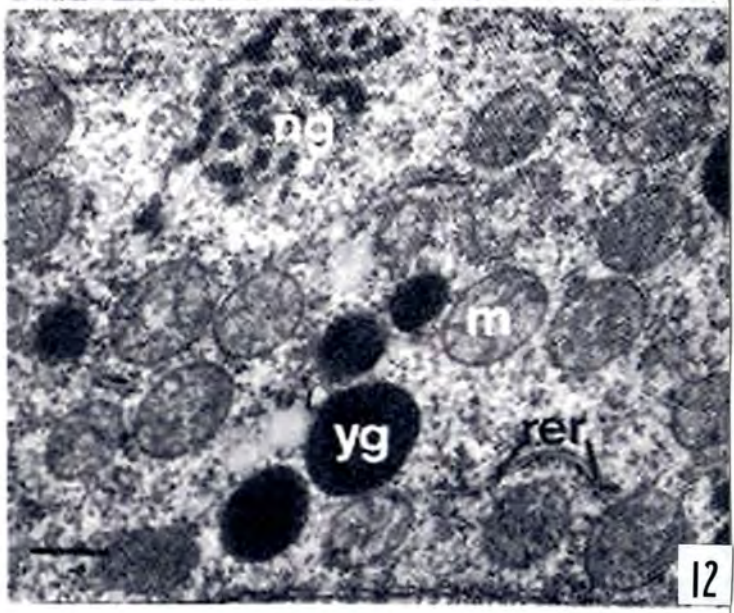

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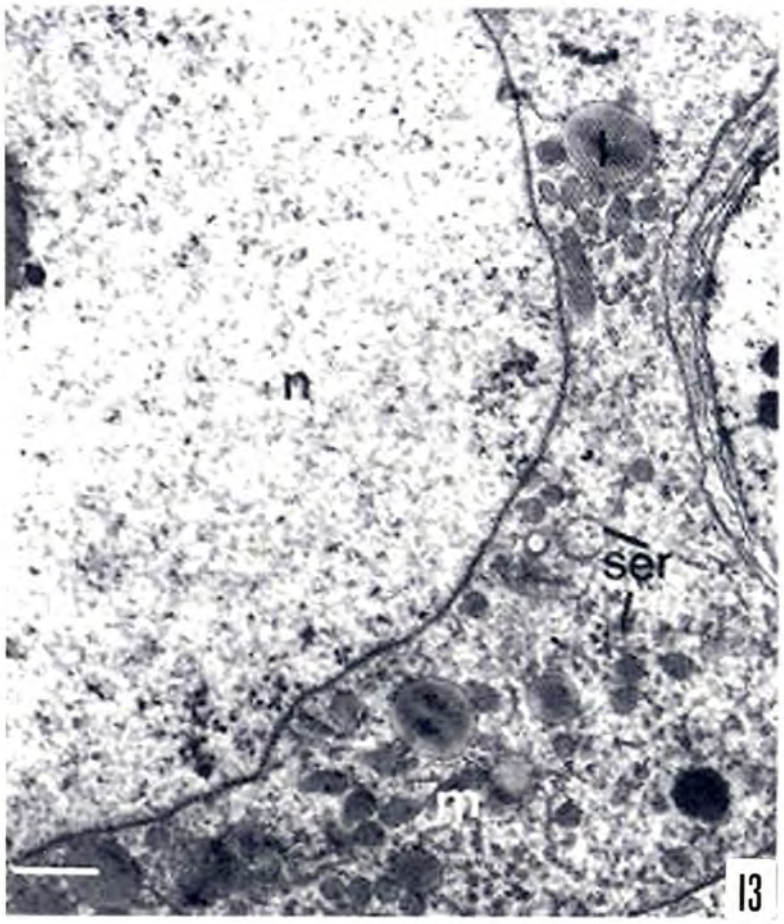

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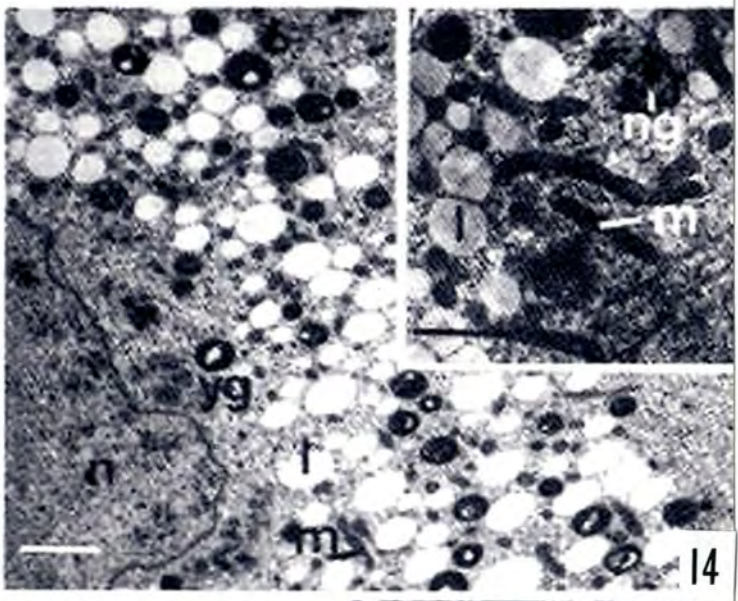

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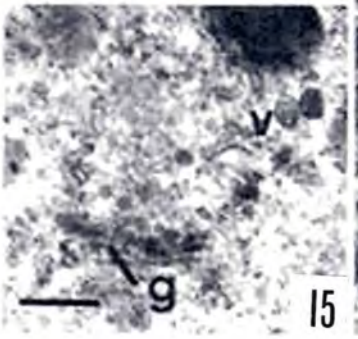

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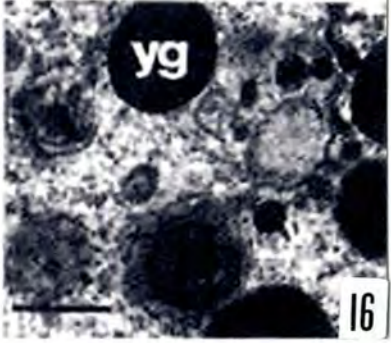

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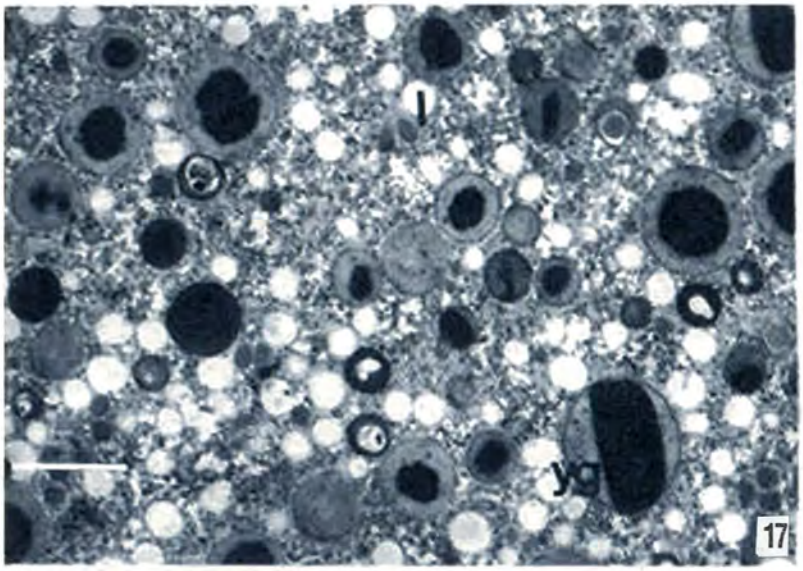
Fig. 17. Late vitellogenic oocyte of *S. capensis* showing lipid (l) and yolk granules (yg) with bipartite structure. Scale bar= 1 μm. **Fig. 18.** Higher magnification of yolk granules from *S. capensis* shows the crystalline core (c) and electron lucent cortex (co). Scale bar= 100nm. **Fig. 19.** Late vitellogenic oocyte of *S. serrata* showing lipid droplet (l) in close contact with smooth endoplasmic reticulum (ser) in the cortical ooplasm. Scale bar= 200nm. **Fig. 20.** Endocytotic pit (p) forming along the oolemma (ol) in a late vitellogenic oocytes of *S. serrata*. Scale bar= 200nm. **Fig. 21.** Vesicles fusing (arrowed) to form yolk granule (yg) in the cortical ooplasm of *S. serrata* oocytes. Scale bar= 200nm.
Fig. 22. Late vitellogenic oocyte of *S. serrata* showing the prevalence of lipid (l) in the ooplasm. Scale bar= 2μm.
f, follicle cell; is, intercellular space; l, lipid; mv, microvilli; m, mitochondria; w, wall of acinus.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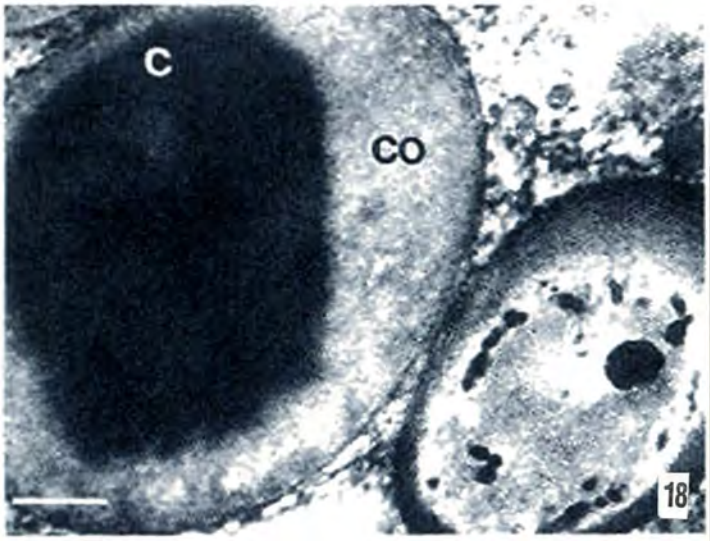
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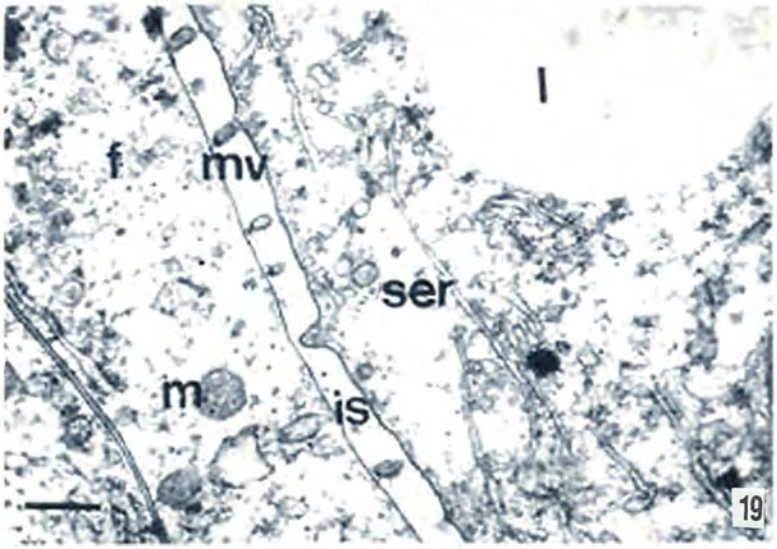
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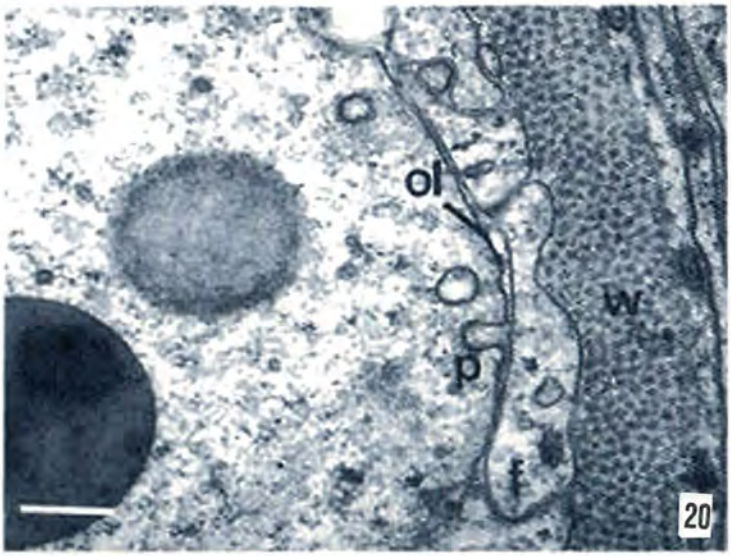
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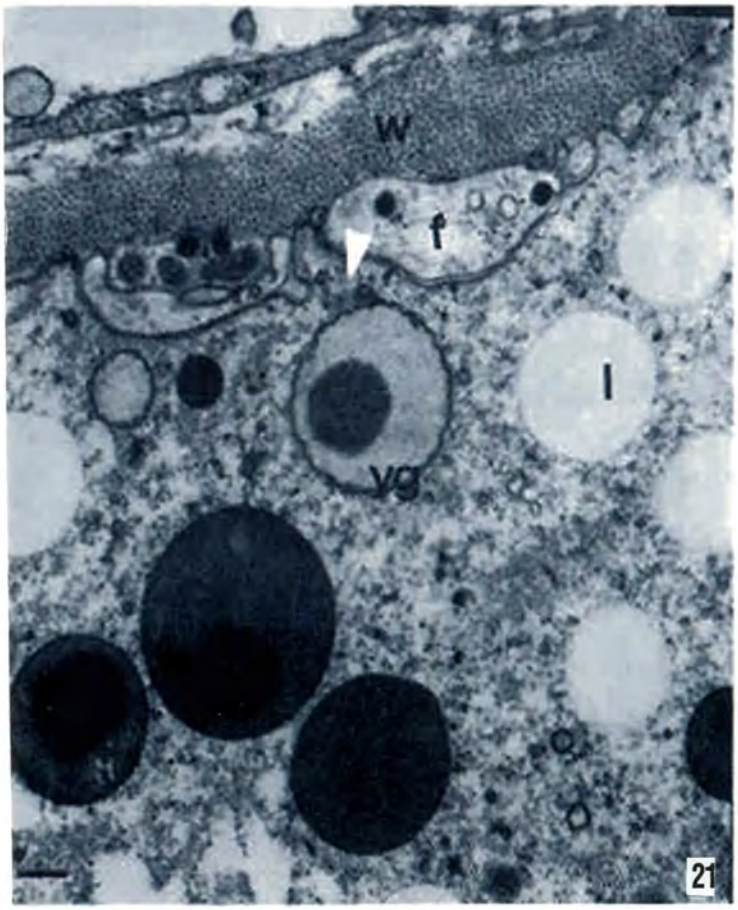
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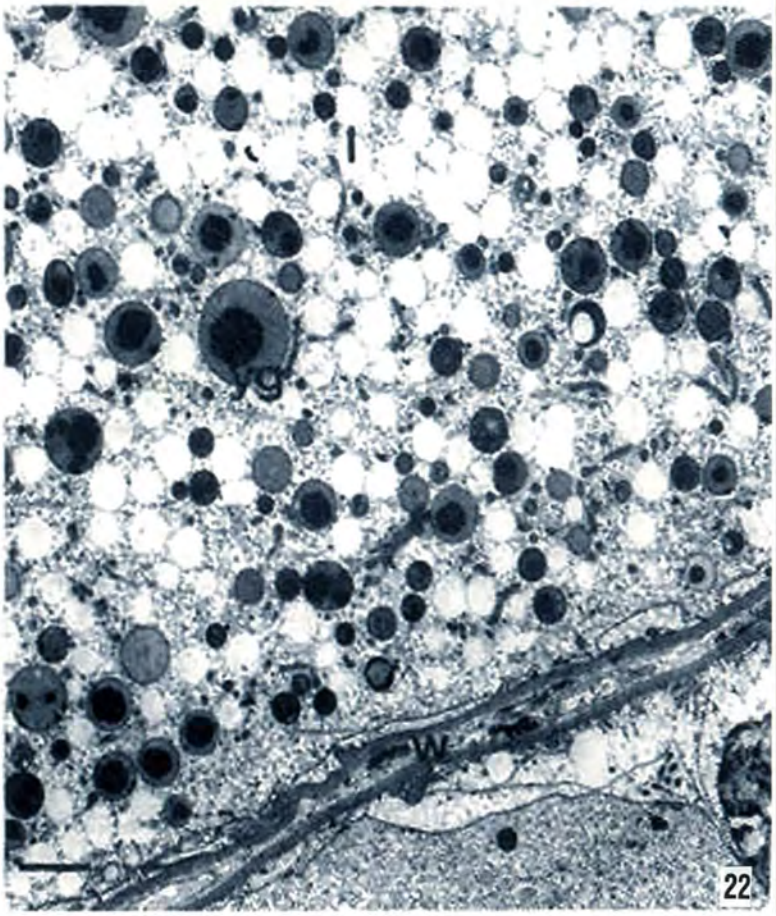
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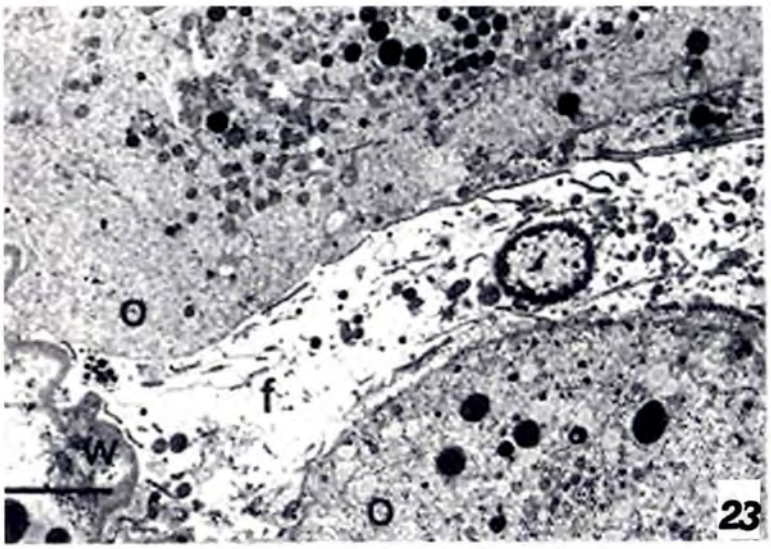
In the mid- to late vitellogenic oocytes of *S. serrata* endocytotic pits form along the oolemma (Fig. 20). From this endocytotic activity vesicles are produced which fuse to produce yolk granules (about 1.5- 2 μm diameter) in the cortical region of the oocyte (Figs. 21 & 22). These granules, which reach a maximum size of 2 μm in diameter, have an electron lucent cortex with a granular core (Fig. 21). They are restricted to the cortical region of the egg and may represent a second type of yolk granule. Also present in the cortical region are arrays of smooth ER, which are closely associated with lipid droplets (Fig. 19). In both species glycogen granules appear in the ooplasm at this stage (not illustrated). The yolk granules reach a size of approximately 4- 5 μm in *S. capensis* and 2- 3 μm in *S. serrata.*

The oocytes, which reach a maximum diameter of 70 -100 μm in *S. capensis* and 90 - 150 μm in *S. serrata,* are surrounded by follicle cells throughout oogenesis. During vitellogenesis, the follicle cells show an increase in the number of proteosynthetic organelles especially arrays of rough endoplasmic reticulum, Golgi bodies, and some lysosomes (Figs. 23, 24, 25 & 26). In addition the follicle cells accumulate electron-dense granules, which are presumed to be glycogen (Fig. 29). Follicle cells seem to contain microtubules at all stages of oogenesis (Fig. 27). By late vitellogenesis, the follicle cells are squamous and are becoming detached from the oocytes creating an intercellular gap between the follicle cell and oocyte (insert to Fig. 27 & Fig. 28). The oolemma of the oocytes then begins to form simple microvilli with glycocalyx (Fig. 26 & 28).

Fig. 23. Follicle cell (f) between two early vitellogenic oocytes of *S. capensis*. Scale bar= 2 μm.
Fig. 24. Follicle cell (f) surrounding an early vitellogenic oocyte of *S. serrata*. Scale bar= 500 nm.
Fig. 25. Follicle cell cytoplasm showing Golgi bodies (g) producing small vesicles (v). Scale bar= 200 nm.
Fig. 26. Two mid vitellogenic oocytes (mvo) and follicle cells (f) with extensive arrays of rough endoplasmic reticulum (rer). Scale bar= 1μm. **Fig. 27.** Follicle cell (f) shows presence of microtubules (mt). Scale bar= 500 nm. Insert: Squamous follicle cells (f) between two mid vitellogenic oocytes (mvo) of *S. capensis*. Scale bar= 500 nm. **Fig. 28.** Micrograph showing cell junction (arrowhead) between two follicle cells (f). Microvilli (mv) of an oocyte of *S. serrata* can also be seen. Scale bar= 500 nm. **Fig. 29.** Follicle cell cytoplasm showing glycogen (gl) in *S. capensis*. Scale bar= 200 nm. **Fig. 30.** Cell junction (arrowhead) between a follicle cell (f) and an oocyte (o) of *S.serrata*. Scale bar= 200 nm.
w, acinar wall; db, dense bodies ; f, follicle cell ; ly, lysosome ; m, mitochondria ; mv, microvill; o, oocyte ; rer, rough endoplasmic reticulum ; yg, yolk granule.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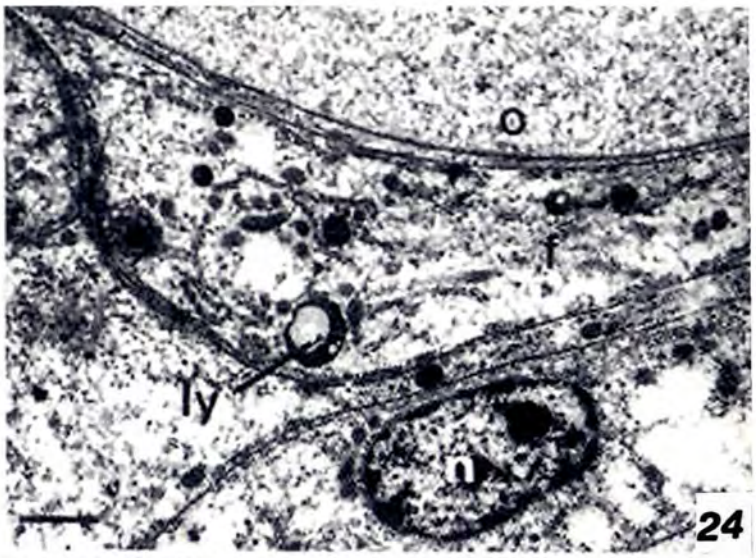
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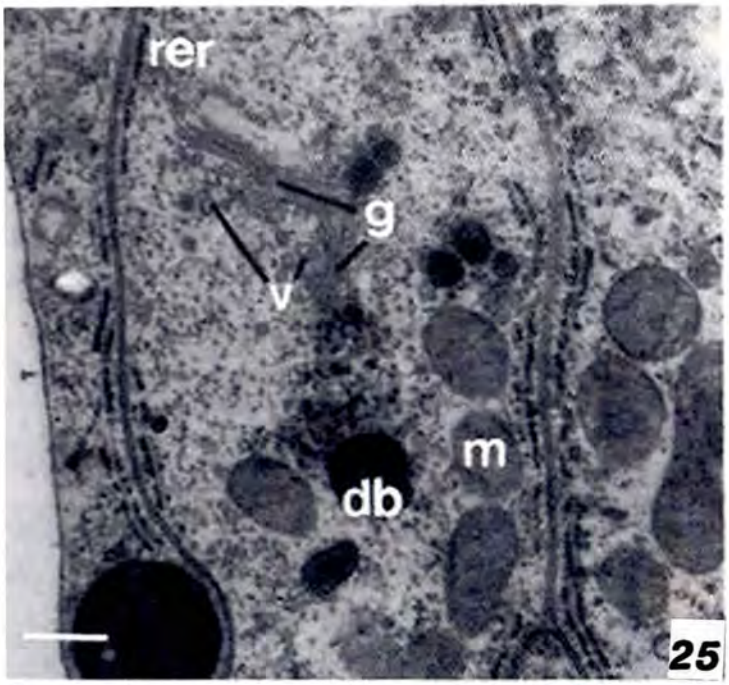
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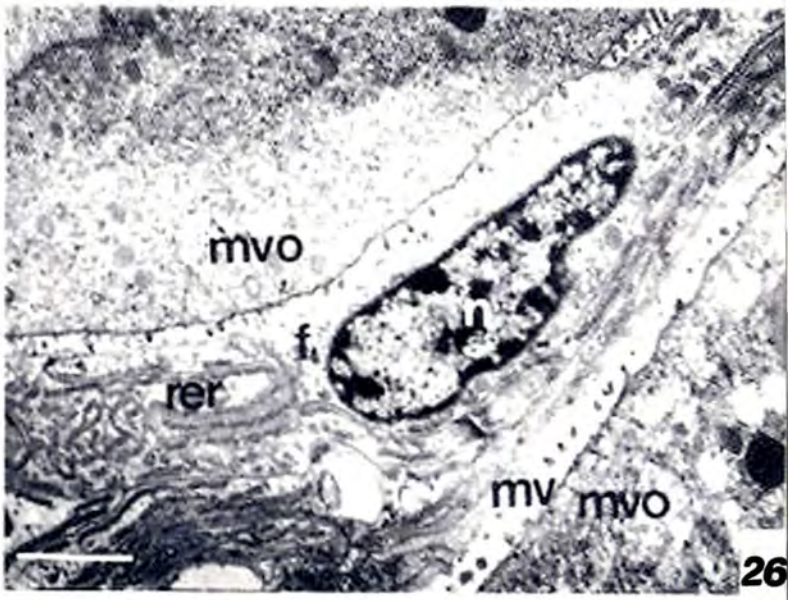
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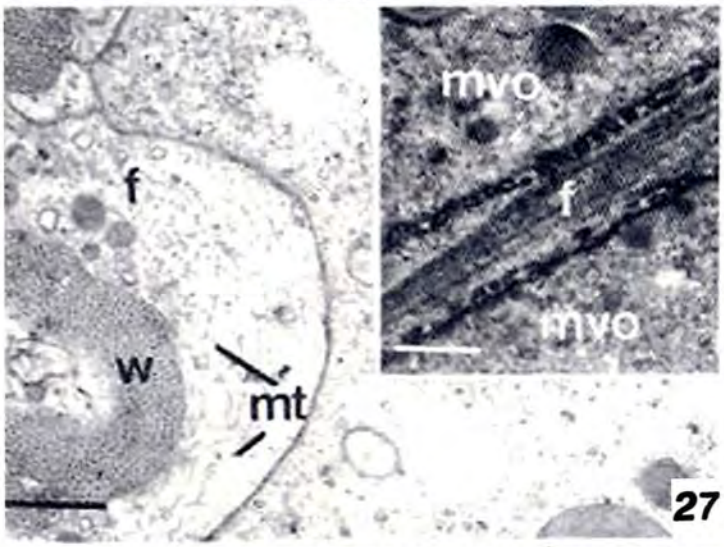
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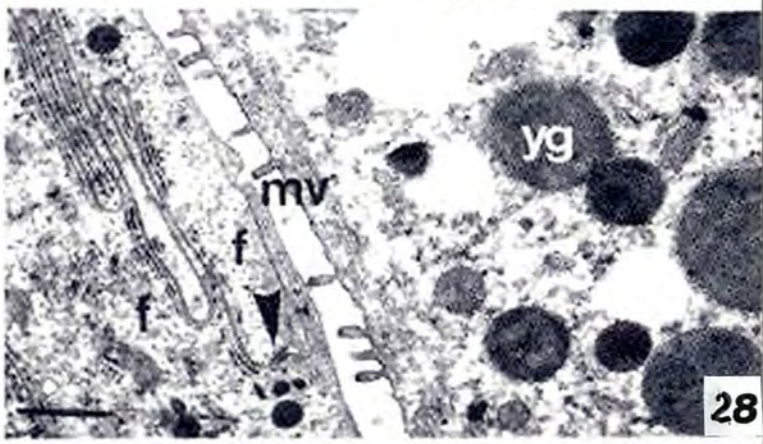
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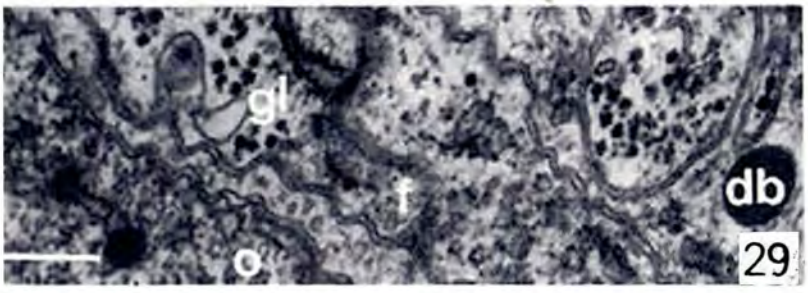
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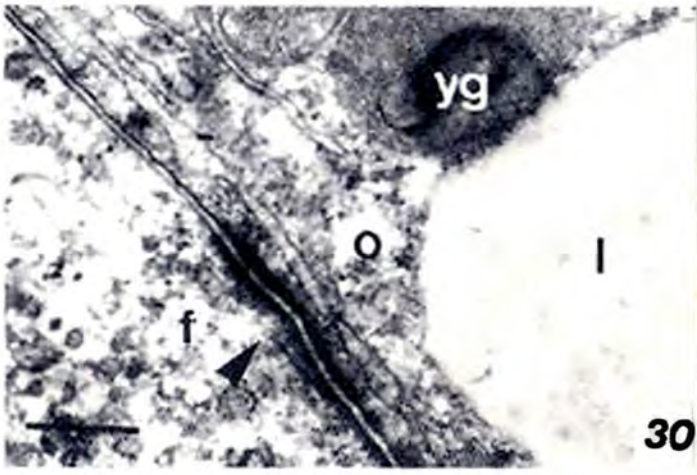
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Discussion

The general structure of gonad of *Siphonaria capensis* and *S. serrata* does not differ from that of other pulmonates (De Jong-Brink *et al.*, 1983; Luchtel *et al.*, 1997). It consists of numerous acini in which both male and female gametes develop. The oocytes develop peripherally next to the wall of the acinus and as they mature they gradually fill its lumen. In siphonariid limpets the acinar wall is composed of connective tissue, muscles and squamous cells with pigment granules, which is in agreement to what has previously been described in other pulmonates (Sabelli & Sabelli-Scanabissi, 1982; Luchtel *et al.*, 1997).

The formation of yolk by autosynthesis is widespread in molluscs (Wourms, 1987; Eckelbarger & Davis, 1996), and it has been suggested that this is the primary yolk-forming means in this phylum (De Jong-Brink *et al.*, 1983; Medina *et al.*, 1986). Nevertheless the incorporation of exogenous yolk precursors by endocytosis is also well documented (Bottke 1973, 1986; Hill & Bowen, 1976; Selman & Wallace, 1978; West, 1981; Bottke *et al.*, 1982; Khan & Saleuddin, 1983; Eckelbarger & Blades-Eckelbarger, 1989; Eckelbarger & Young, 1997; Hodgson & Ecklebarger, 2000). Results of the present study indicate that the planktonic developer, *S. capensis* produces membrane-bound yolk granules by autosynthesis only, which was manifested by the increase in the number of proteosynthetic organelles (eg. rough endoplasmic reticulum and Golgi bodies). The involvement of RER and Golgi bodies in yolk production is well documented (Bedford, 1966; Taylor & Anderson, 1969; De Jong-Brink *et al.*, 1976; West, 1981; Kress, 1986; Wourms, 1987; Eckelbarger & Davis, 1996). By contrast, in *S.*

serrata yolk appears to be formed both auto- and heterosynthetically. Like *S. capensis*, the majority of the yolk granules are formed autosynthetically at the beginning of vitellogenesis in the perinuclear cytoplasm. As vitellogenesis proceeds some yolk develops in the cortical region of the oocyte. These yolk granules (which may represent a second type) appear to be formed by heterosynthesis with coated pits developing along the oolemma. These in turn form vesicles, which fuse to form the yolk granules. Yolk formation from endocytotic activity during mid- to late vitellogenesis has been observed in other molluscs (e.g. *Spurilla* and *Bathynerita*; Eckelbarger & Blades-Eckelbarger, 1989; Eckelbarger & Young, 1997). The presence of these pits in the cortical ooplasm indicates possible uptake of large molecular weight extra-ovarian substances. The source and nature of these extra-oocytic substances needs to be established but it is possible the follicle cells (which are proteosynthetically active) may be their source. In the polychaete worm *Capitella jonesi* the follicle cells were suggested to be most probable site for extra-oocytic substances (Eckelbarger & Grassle, 1982). In siphonariids, apart from RER and Golgi bodies no other organelle was involved in producing yolk autosynthetically, unlike in *Planorbis corneus* where mitochondria participate in producing protein yolk (Favard & Carasso, 1958).

The yolk granules in both species have a bipartite structure, a common feature of mollusc yolk as well that of other invertebrates (Nørrevang, 1968; Wourms, 1987; Eckelbarger & Blades-Eckelbarger, 1989). Such yolk granules or yolk platelets typically consist of a protein yolk core surrounded by other carbohydrate moieties (Gérin, 1976; Wourms, 1987).

Apart from membrane-bound yolk granules, lipid accumulates in the ooplasm of both

species of *Siphonaria* during early vitellogenesis. It was not possible to determine how the lipid might be formed but in *S. serrata* lipid droplets developed in close association with mitochondria and smooth endoplasmic reticulum. Kessel (1982) suggested that in the patellogastropod *Acmaea digitalis,* annulate lamellae and mitochondria play a role in lipid formation. To date annulate lamellae in the oocytes of *Siphonaria* have not been observed.

Cortical granules are a prominent feature of the oocytes of many invertebrates (Nørrevang, 1968; Wourms, 1987). Such structures were not observed in the caenogastropods *Ilyanassa obsoleta* (Taylor & Anderson, 1969), *Melanopsis buccinoidea* and *Melanoides tuberculata* (Hodgson *et al.*, 2002). The cortical regions of the eggs of the two species of *Siphonaria* studied were also devoid of cortical granules, which appears to be a feature of pulmonate oocytes (Bottke, 1973; Terakado, 1974; Hill & Bowen, 1976; De Jong-Brink *et al.*, 1976).

In late oocytes the oolemma develop unbranched microvilli. Apart from forming junctions with accessory cells and neighbouring oocytes, it has been suggested that the microvilli help in absorption, transportation, secretion of egg envelopes (Nørrevang, 1968; Wourms, 1987). It is interesting to note however that not all mollusc oocytes develop microvilli such as the methane- seep mollusc *Bathynerita naticoidea* (Eckelbarger & Young, 1997).

Several functions have been assigned to follicle cells including transportation of oocytes and ovulation, hormone production, the provision of nutrients and yolk precursors, and phagocytosis (Taylor & Anderson, 1969; Bottke, 1973; De Jong-Brink *et al.*, 1976, West, 1981; Eckelbarger & Young, 1997). Although a study of follicle cell

function was beyond the scope of this study, the proliferation of proteosynthetic organelles during vitellogenesis in *S. serrata* and *S. capensis* would suggest that they play a key role in oocyte formation.

Oogenesis, life history and the evolution of siphonariids

Although both *S. capensis* and *S. serrata* have eggs, which contain yolk, lipid and glycogen, the oocytes of *S. serrata* (the direct developer) are larger and clearly must contain a greater quantity of storage product. This difference in size of the eggs of planktonic and direct developers confirms earlier observations on siphonariids (Hodgson, 1999). The qualitative observations, however, suggest that the type and proportion of the storage product is not the same in these species, with oocytes of *S. serrata* containing more lipid. Presumably these additional energy reserves are required to sustain intra-capsular development in this species. Whether the embryos of *S. serrata* obtain any nutrients from capsule fluids as has been shown for *Littorina* (Moran, 1999), has still to be determined (see Chapter 5). Similar differences in the quantity of storage products within oocytes have also been observed between oviparous (egg-laying) and ovoviviparous (brooding) freshwater gastropods (Hodgson *et al.*, 2002).

I suggest that provisioning the *S. serrata* egg with additional energy reserves is facilitated by the use of more than one mode of vitellogenesis. Unlike in *S. capensis* where evidence for autosynthesis was found, yolk is formed by both auto- and heterosynthesis (mixed synthesis). Autosynthesis is considered to be the primitive mode of vitellogenesis in monotelic breeders (Eckelbarger 1994). The fact that *S. capensis* has this mode of synthesis only, supports the hypothesis that planktonic development is primitive and that siphonariids, therefore, could have had a marine ancestry. However

further studies should be undertaken to ascertain whether the vitellogenic modes described for *S. capensis* and *S. serrata* are typical of planktonic and intracapsular developers respectively.

References

ALLANSON, B. R. 1958. Some aspects of the ecology of the molluscan genus *Siphonaria* in South Africa. *Portugaliae Acta Biologica*, **6**: 179-212.

BEDFORD, L. 1966. The electron microscopy and cytochemistry of oogenesis and the cytochemistry of embryonic development of the prosobranch gastropod *Bembicium nanum* L. *Journal of Embryology and Experimental Morphology*, **15**: 15-37.

BERRY, A. J. 1977. Gastropoda: Pulmonata. In: *Reproduction of marine invertebrates* (A. C. Giese & J. S. Pearse, eds), **IV**: 181-226. Academic Press, New York.

BORLAND, C. 1950. Ecological studies of *Benhamina obliquata* (Sowerby), a basommatophorous pulmonate in Otago harbour. *Transactions of the Royal Society of New Zealand*, **78**: 385-393.

BOTTKE, W. 1973. Zur Ultrastruktur des Ovars von *Viviparus contectus* (Millet, 1813) (Gastropoda: Prosobranchia). II. Die Oocyten. *Zeitschrift für Zellforschung und mikroskopische Anatomie*, **138**: 239-259.

BOTTKE, W. 1986. Immuno-localization of ferritin polypeptides in oocytes and somatic tissue of the freshwater snails, *Lymnaea stagnalis* L. and *Planorbrarius corneus*. *Cell Tissue Research*, **243**: 397- 404.

BOTTKE, W., SINHA, I & KEIL, I. 1982. Coated vesicle-mediated transport and deposition of vitellogenic ferritin in the rapid growth phase of snail oocytes. *Journal of Cell Science*, **53**: 173-191.

CHAMBERS, R. J. 1994. *The conflict between adaptation and constraint: The case of the siphonariid limpets*. Ph.D. Thesis, Rhodes University.

CHAMBERS, R.J. & MCQUAID, C.D.1994a. Notes on the taxonomy, spawn and larval development of South African species of the intertidal limpet *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **60:** 263-275.

CHAMBERS, R.J. & MCQUAID, C.D. 1994b. A review of larval development in the intertidal limpet genus *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **60:** 415-423.

CHAMBERS, R.J., MCQUAID, C.D. & KIRBY, R. 1996. Determination of genetic diversity of South African intertidal limpets (Gastropoda: *Siphonaria*) with different reproductive modes using polyacrylamide gel electrophoresis of total cellular proteins. *Journal of Experimental Marine Biology and Ecology*, **201:** 1-11.

CHAMBERS, R.J., MCQUAID, C.D. & KIRBY, R. 1998. The use of randomly amplified polymorphic DNA to analyze the genetic diversity, the systematic relationships and the evolution of intertidal limpets, *Siphonaria* spp. (Pulmonata: Gastropoda), with different reproductive modes. *Journal of Experimental Marine Biology and Ecology,* **227:** 49-66.

CROSS, R. H. M. 1989. A reliable epoxy resin mixture and its application in routine electron microscopy. *Micron and Microscopica Acta*, **20**: 1-7.

DE JONG- BRINK, M., BOER, H. H., JOOSSE, J. 1983. Mollusca. In: *Reproductive Biology of Invertebrates* (K. G. Adiyodi & R. G. Adiyodi eds), **I**: 297-355. John Wiley Publication, New York.

DE JONG-BRINK, M., WIT, A. DE, KRAAL, G., BOER. H. H. 1976. A light and electron microscope study on oogenesis in the freshwater pulmonate snail *Biomphalaria glabrata. Cell and Tissue Research*, **171**: 195-219.

ECKELBARGER, K. J. 1994. Diversity of metazoan ovaries and vitellogenic mechanisms: Implications for life history theory. *Proceedings of the Biological Society Washington*, **107**: 193-218.

ECKELBARGER, K. J. & BLADES- ECKELBARGER, P. 1989. Structure of the ovotestis and evidence for heterosynthetic incorporation of yolk precursors in the oocytes of the nudibranch mollusc, *Spurilla neapolitana*. *Journal of Morphology*, **201**: 105-118.

ECKELBARGER, K. & DAVIS, C. V. 1996. Ultrastructure of the gonad and gametogenesis in the eastern oyster, *Crassostrea virginica*. I Ovary & oogenesis. *Marine Biology*, **127**: 79- 87.

ECKELBARGER, K. J. & GRASSLE, J. P. 1982. Ultrastructure of the ovary and oogenesis in the polychaete *Capitella jonesi* (Hartman, 1959). *Journal of Morphology*, **171**: 305-320.

ECKELBARGER, K. J. & YOUNG, C. M. 1997. Ultrastructure of the ovary and oogenesis in the methane-seep mollusc *Bathynerita naticoidea* (Gastropoda: Neritidae) from the Louisiana slope. *Invertebrate Biology,* **116**: 299-312.

FAVARD, P & CARASSO, N. 1958. Origine et ultrastructure des plaquettes vitellines de la planorbe. *Archives D' Anatomie Microscopique et Morphologie Experimental*, **47**: 211-219.

GÉRIN, Y. 1976. Origin and evolution of some organelles during oogenesis in the mudsnail *Ilynassa obsoleta*. I. The yolk platelets. *Acta Embryologiae Experimentalis*, **1**: 15-35.

HILL, R. S. & BOWEN, I. D. 1976. Studies on the ovotestis of the slug *Agriolimax reticulatus* (Müller). 1. The oocyte. *Cell and Tissue Research*, **173**: 465- 482.

HODGSON, A. N. 1999. The biology of siphonariid limpets (Gastropoda: Pulmonata). *Oceanography and Marine Biology: An Annual Review*, **37**: 245-314.

HODGSON, A. N. & ECKELBARGER, K. J. 2000. Ultrastructure of the ovary and oogenesis in six species of patellid limpets (Gastropoda: Patellogastropoda) from South Africa. *Invertebrate Biology*, **119**: 265-277.

HODGSON, A.N., BERNARD, R.T.F. & LINDLEY, D. 1991. Comparative spermatology of four sympatric species of *Siphonaria* (Pulmonata: Basommatophora). *Journal of Molluscan Studies*, **57:** 309-322.

HODGSON, A. N., BEN-AMI. F. & HELLER, J. 2002. Ultrastructure of oogenesis in an ovoviviparous and an oviparous freshwater snail (Caenogastropoda). *Invertebrate Reproduction and Development*, **41**: 1-3.

HUBENDICK, B. 1947. Phylogenie und Tiergeographie der Siphonariidae Zur Kentnis der Phylogenie in der Ordnung Basommatophora und des Ursprungs der Pulmonatengruppe. *Zoolgiska Bibrag Fran Uppsala*, **24:** 1-216.

HUBENDICK, B. 1978. Systematics and comparative morphology of the Basommatophora. In: *Pulmonates* (V. Fretter & J. Peake, eds), **2A**: 1- 47. Academic Press, London.

HYMAN, L. H. 1967. *The Invertebrates. Mollusca* I, Vol. **VI**. McGraw Hill, New York.

KHAN, H. P & SALEUDDIN, A. S. M. 1983. Cell contacts between follicle cells and the oocyte in *Heliosoma* (Mollusca: Pulmonata). *Journal of Morphology*, **177**: 319- 328.

KESSEL, R. G. 1982. Differentiation of *Acmaea digitalis* oocytes with special reference to lipid- endoplasmic reticulum- annulate lamellae- polyribosome relationships. *Journal of Morphology*, **171**: 225-243.

KRESS, A. 1986. Ultrastructural study of oogenesis and yolk formation in an opisthobranch mollusc, *Runcina*. *Tissue and Cell*, **18**: 915-935.

LUCHTEL, D. L., MARTIN, A. W., DEYRUP-OLSEN, I., BOER, H. H. 1997. Gastropoda: Pulmonata. In: *Microscopic Anatomy of Invertebrates* (F. W. Harrison & A. J. Kohn, eds), **6B**: 459-718. Wiley-Liss, New York.

MARCUS, E. & MARCUS, E. 1960. On *Siphonaria hispida*. *Universidade de Sao Paulo. Faculdade de Filisofia, Ciencias e Letras. Boletim de Zoologia,* **23:** 107-139.

MEDINA, A., GARCIA, J. C., MORENO, F. G. & LOPEZ- CAMPOS, J. L. 1986. Comparative studies on the histology of the ovotestis in *Hypselodoris tricolor* and *Godiva banyulensis* (Gastropoda: Opisthobranchia), with special reference to yolk formation. *Journal of Morphology*, **188**: 105-118.

MORAN, A. L. 1999. Intracapsular feeding by embryos of the gastropod genus *Littorina*. *Biological Bulletin*, **196**: 229-244.

NØRREVANG, A. 1968. Electron microscopic morphology of oogenesis. *International Review of Cytology*, **23**: 113-186.

PURCHON, R. D. 1979. *The Biology of the Mollusca*. Oxford: Pergamon Press.

REID, D. G. 1990. A cladistic phylogeny of the genus *Littorina* (Gastropoda): implications for evolution of reproductive strategies and for classification. *Hydrobiologia*, **193**: 1-19.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology*, **17**: 208-212.

SABELLI, B & SABELLI-SCANABISSI, F. 1982. The structure of the acini of the gonad of *Ovatella myosotis* (Draparnaud) (Gastropoda: Pulmonata: Basommatophora).

Monitore Zoologica Italiano, **16**: 333-339.

SELMAN, K & WALLACE, R. A. 1978. An autoradiographic study of vitellogenesis in the squid *Loligo pealei. Tissue & Cell*, **10**: 599- 608.

TAYLOR, G. T. & ANDERSON, E. 1969. Cytochemical and fine structural analysis of oogenesis in the gastropod *Ilyanassa obsoleta. Journal of Morphology*, **129**: 211-248.

TERAKADO, K. 1974. Origin of yolk granules and their development in the snail, *Physa acuta. Journal of Electron Microscopy*, **23**: 99-106.

WEST, D. L. 1981. Reproductive biology of *Colus stimpsoni* (Prosobranchia: Buccinidae). IV. Oogenesis. *Veliger*, **24**: 28-38.

WOURMS, J. P. 1987. Oogenesis. In: *Reproduction of Marine Invertebrates* (A. C.Giese, J. S. Pearse & V. B. Pearse, eds), **IX**: 50-178. Blackwell Scientific Publications, California.

YONGE, C. M. 1952. The mantle cavity of *Siphonaria alternata* Say. *Proceedings of the Malacological Society of London*, **29**: 190-199.

Chapter 5

Structure of the egg masses and biochemical composition

Introduction

Siphonariid limpets are marine intertidal pulmonates, which belong to the primitive superorder Basommatophora. They play an important role as grazers in intertidal ecosystems especially in the warmer waters of the Southern hemisphere (Hodgson, 1999). Siphonariid limpets have been investigated largely to understand their behaviour, physiology, ecology and some aspects of their reproduction (see Hodgson, 1999 for review). Despite being successful inhabitants of rocky shores, little is known about their gametogenic cycles (but now see Chapter 2), embryonic development, nutrition and larval biology (Hodgson, 1999). Siphonariids are hermaphrodites with internal fertilization, and after copulation they lay gelatinous egg masses as ribbons on rocks, embryos developing inside capsules, which in turn are embedded in a jelly matrix.

According to Chambers and McQuaid (1994a, b) siphonariids have one of two main types of life history strategy. A number of species have planktonic development whereby large numbers of small eggs are laid, which take only 4-5 days to hatch as free-swimming veliger larvae. Others have direct (= intracapsular) development laying smaller numbers of larger eggs, which hatch as crawling juveniles after 3- 4 weeks. Chambers (1994) determined that the rate of water loss of the egg ribbons of *Siphonaria capensis* (Quoy & Gaimard, 1833), a planktonic developer, was twice as rapid as that of a direct (= intracapsular) developer *S. serrata* (Fischer, 1807). Furthermore Chambers (1994) noted that the egg ribbons of *S. serrata* were "tougher" than those of *S. capensis* and with a lower surface to volume ratio. Together this suggests that there may be structural and/ or biochemical differences in the composition of the egg ribbons of planktonic and

intracapsular developers.

Although there are now a number of studies on the structure and composition of gastropod egg capsules or egg masses (e.g. Bayne, 1966 & 1968; Eyster, 1986; Hawkins & Hutchinson, 1988; Wägele, 1996; Klussmann-Kolb & Wägele, 2001; Miles & Clark, 2002) to date there has been only one detailed study on the spawn of a basommatophoran pulmonate - that of Bayne (1968) who investigated the freshwater pulmonate *Lymnaea stagnalis*. The aim of the present study was to examine and compare the structural and fundamental biochemical composition of the egg ribbons of a planktonic and an intracapsular developing species of siphonariid limpet in an attempt to explain the physiological results of Chambers (1994). Eggmasses, which vary in size and shape between species, consist of egg capsules (containing a single embryo) embedded evenly throughout the jelly matrix (Marcus & Marcus, 1960; Mapstone, 1978; Chambers & McQuaid, 1994a). Two species were chosen for this comparative study, *S. capensis*, which lays egg ribbons on rocks as well as in rock pools and *S. serrata* with a collar shaped egg mass. These are not only common animals on the rocky shores along the southeast coast of South Africa but they are also sympatric in their distribution.

Material and methods

Newly laid egg ribbons were collected from Kenton- on- Sea (33° 42' S, 26° 41' E) in the Eastern Cape Province of South Africa during the summer months (December, 2000 to March, 2001 and February to April, 2002) and from animals kept in laboratory aquaria. The egg ribbons were processed for light microscopy, transmission and scanning electron

microscopy, as well as for biochemical analyses.

Histochemistry, transmission and scanning electron microscopy

For histochemistry small pieces of the egg masses of each species were fixed in either Rossman's fluid, Carnoy's fluid, formal calcium (40 % formaldehyde in 10 % calcium chloride) or formal saline for 12 hrs (Bancroft & Stevens, 1990). Fixation was followed by dehydration in a graded ethanol series and embedding in Paraplast via xylene. 7 μm thick sections were cut on a Leica RM 2035 microtome. Tissues preserved in Rossman's fluid were stained in PAS (periodic acid- Schiff reaction) to identify neutral polysaccharides and Alcian Blue (pH 2.5)-PAS for acidic mucopolysaccharides. Carnoy's fluid fixed tissues were stained in bromophenol blue for proteins, whereas egg masses preserved in formal calcium were stained in Sudan Black B for unsaturated fats and phospholipids. To further characterize the structure of the egg masses, formal saline fixed tissues were stained in Masson' s trichrome (Humason, 1967; Pearse, 1968; Bancroft & Stevens, 1990).

For TEM, small portions of egg ribbons were fixed for 12 hrs in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.0) or by Eisenman & Alfert's (1981) method (pH 7.4) at 4° C followed by embedding in one of the three media, Spurr's low viscosity resin, araldite/ taab mixture (Cross, 1989) or LR White. Both semi- thin (1μm thick) and ultra-thin (100- 120 nm thick) sections were cut using glass knives on a RMC MT7 ultramicrotome. Semi-thin sections were stained with 1% toluidene blue dissolved in 2.5% sodium carbonate and viewed and photographed using an Olympus BX 40 light microscope. Ultra thin sections were stained in 5% aqueous uranyl acetate (30 minutes) and Reynolds' lead citrate (5 minutes) and observed with a JEOL 1210 transmission

electron microscope at 100 kV.

For SEM the egg masses were prepared following the O-D-O technique (Tanaka, 1981). After fixation in 2.5 % glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.0) overnight at 4° C, the samples were rinsed with cacodylate buffer (pH 7.0) and post-fixed in 0.2 M cacodylate buffered 1 % osmium tetroxide for 2 hrs at 4° C. After infiltration in DMSO and freezing (in liquid nitrogen) the samples were freeze-fractured. Post fixation was repeated in 1 % buffered osmium after 28 hrs of etching in 0.1 % buffered osmium at room temperature. Dehydration and infiltration were followed by critical point drying. The samples were sputter-coated with gold and observed in a JEOL JSM 840 scanning electron microscope at 12 kV.

Biochemistry

To investigate biochemical composition of egg masses, egg ribbons (n= 10 of each species) were pooled and dried in the oven at 60° C overnight and weighed to 0.001g accuracy and stored in 1.5 ml eppendorf tubes. The dried mass was subdivided into three replicates for subsequent analyses for carbohydrate, protein and lipid.

For carbohydrate, dried egg masses were left overnight at room temperature in 1.5 ml of 5 % trichloroacetic acid (TCA). The samples were then homogenized using a mortar and pestle and a further 0.5 ml of 5 % TCA was added. After centrifugation at 9000 rpm for 5 mins the supernatant was retained for analysis. Two different volumes (20 and 40 µl) of the supernatant were taken and the volume was adjusted to 2 ml with distilled water. 2 ml distilled water was used as a blank. 50 µl of phenol solution and 5 ml of H_2SO_4 (95- 97 %) were added to each test tube and they were allowed to stand for 30 mins. Readings were taken at 485 nm using Beckman DU 530 spectrophotometer and

concentrations of TCA soluble carbohydrate were calculated from a glucose standard curve (Kochert, 1978; Foster & Hodgson, 1998).

For protein determination, the dried egg masses were left overnight in 1.5 ml of 0.5 N NaOH at room temperature and then homogenized and centrifuged at 9000 rpm for 5 mins. Two different volumes (20 and 40 µl) of the supernatant were taken and each adjusted to 0.1 ml with distilled water. The blank consisted of 0.1 ml distilled water. 5 ml of Coomassie Brilliant Blue G-250 (Sigma) was added to each tube and mixed by a vortex mixer. Absorbance was read after 2 mins at 595 nm and concentrations were calculated from a Bovine Serum Albumen standard curve (Kochert, 1978; Miloslavich, 1996; Foster & Hodgson, 1998).

Total lipid content was measured by lipid extraction in 2 ml of 2:1 chloroform: methanol mixture at room temperature. After centrifugation, the supernatant was stored in a pre- weighed eppendorf and left to evaporate at room temperature. After evaporation the eppendorfs were re-weighed again (to 0.0001g accuracy) to determine the total lipid content (Foster & Hodgson, 1998).

Results

Structure of the egg ribbon and histochemistry

The egg ribbons of *Siphonaria capensis* and *S. serrata* consist of a jelly matrix made of several layers in which egg capsules containing embryos are housed (Fig. 1). As the general morphology of the egg mass of both siphonariids was found to be similar to that of the opisthobranch egg masses described by Klussmann-Kolb & Wägele (2001), the

structural terminology used by them has been adopted in this chapter. In this study the structure of the egg ribbon will be described from the outer most layer to the developing embryo inside the capsule.

The egg ribbon has an outer layer, the outer mucous cover (Fig. 2), parts of which were in contact with the substratum. Beneath this is a mucous matrix, which has a compartment-like organization, compartments containing egg capsules (Figs. 2, 3 & 5). Each egg capsule is surrounded by a mucous strand and an inner mucous layer (Fig. 3). In both species the capsule wall is separated from the perivitelline fluid by an envelope (Fig. 4). Both the mucous strand and the envelope were difficult to distinguish by light microscopy but were clearly visible with the electron microscope (Figs. 3, 4 & 14). The capsule of each species contains a single developing embryo surrounded by perivitelline fluid (Figs. 3 & 5). Within an egg mass in both species all embryos appeared to be at the same stage of development (Fig. 19).

In both species the outer mucous cover of the egg mass (approximately 5.5 μm thick in *S. serrata* and 4.7 μm thick in *S. capensis*) consists of layers of condensed mucoid fibres (Figs. 6 & 7). In egg masses of *S. capensis*, which have been collected from the shore, this layer often contains bacteria (Fig. 8). In both species the outer mucous layer stains positively in toluidene blue and PAS suggesting the presence of both basophilic and neutral mucopolysaccharides (Table 1).

In both *S. capensis* and *S. serrata* the mucous matrix has two regions (i and ii) with different staining intensities (Fig. 9). Region i lies just beneath the outer mucous cover and both regions are composed of muco-substances (Figs. 10 & 11; Table 1). Furthermore, region i in *S. serrata* probably contains acid mucins as it stains blue with

Alcian Blue- PAS whereas in *S. capensis* this area stains for neutral mucins (Table 1). Ultrastructurally the mucous matrix is a mosaic of tubular or cylindrical units where the fibres are compacted at the periphery and loosely arranged in the centre (Figs. 10 & 11). In *S. capensis* parts of the matrix, which traverse the egg mass to create compartments, are composed of very few tubular units (Fig. 12) whereas in *S. serrata* they are highly fibrous and contain many larger tubular units (Fig. 13).

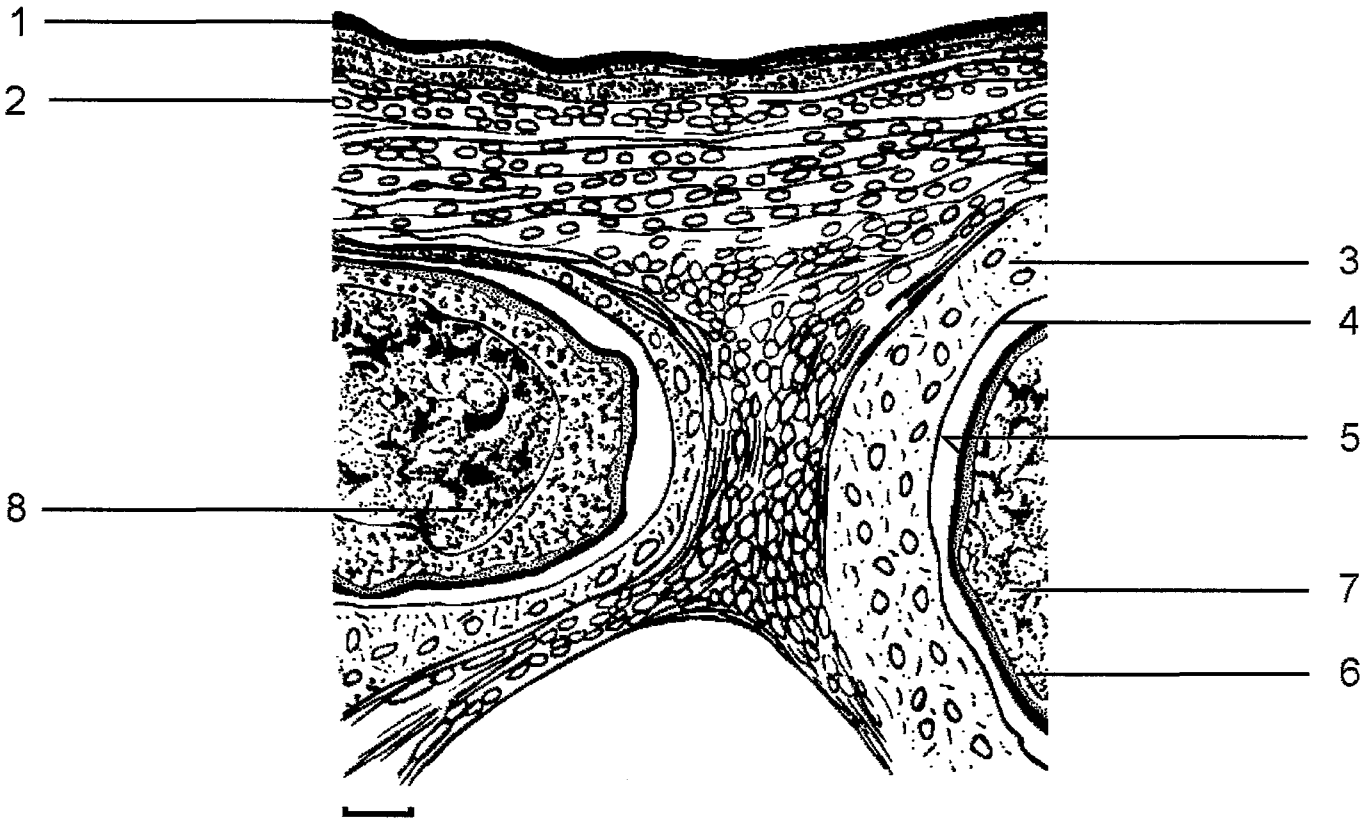


Fig. 1. Schematic diagram showing fundamental structure of a siphonariid egg mass. Scale bar= 70 μm (*S. serrata*) and 28 μm (*S. capensis*). 1, outer mucous cover (omc); 2, mucous matrix (mma); 3, inner mucous layer (iml); 4, mucous strand (ms); 5, capsule wall (cw); 6, envelope (en); 7, perivitelline fluid (pvf); 8, developing embryo (de).

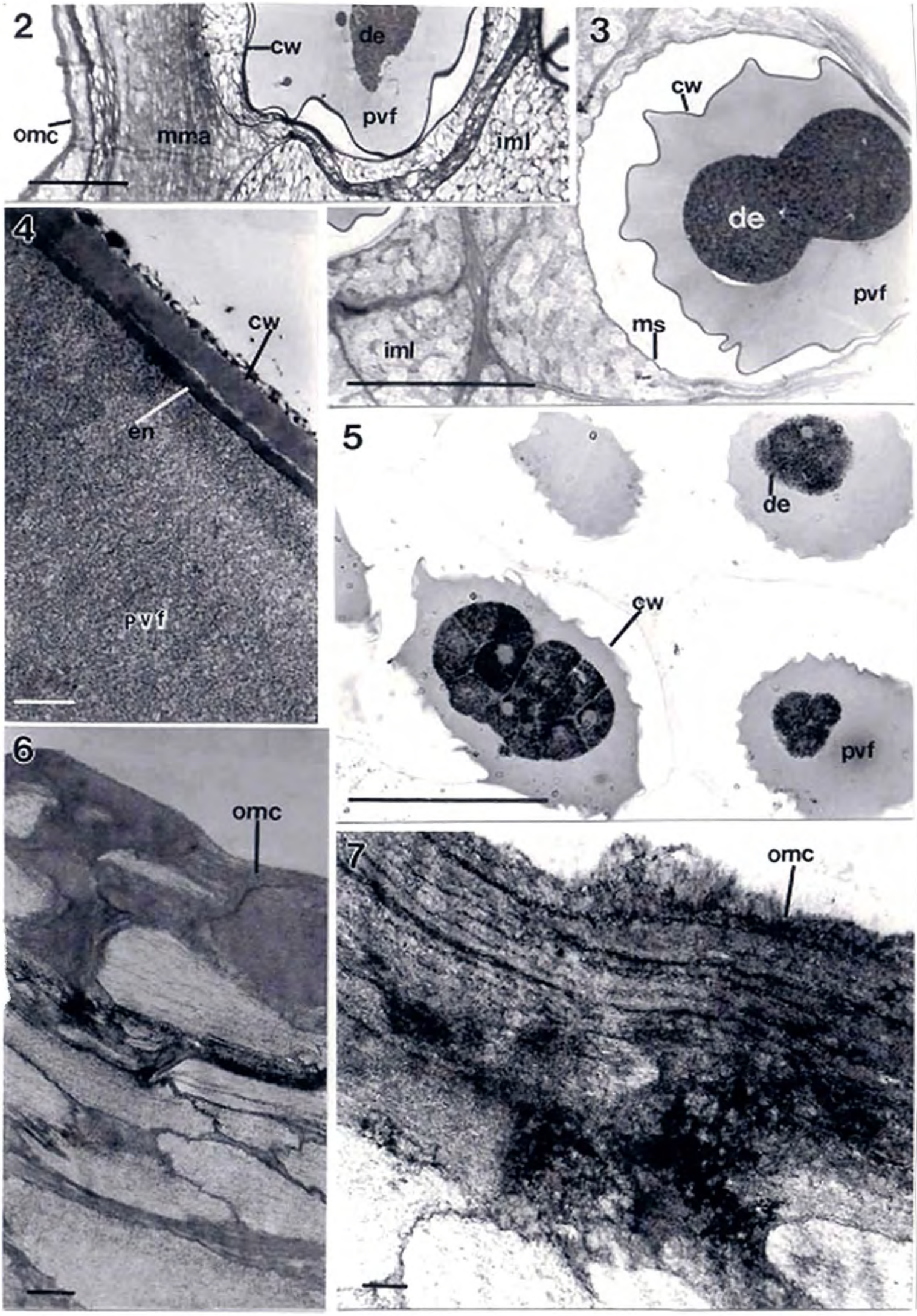
Fig. 2. Light micrograph of toluidene blue stained section showing part of *S. serrata* egg mass, which contains a developing embryo (de) bathed in perivitelline fluid (pvf) enclosed by a capsule wall (cw). The outer most layer, outer mucous cover (omc) is followed by mucous matrix (mma). iml, inner mucous layer. Scale bar= 100 μm.

Fig. 3. A light micrograph of the egg masses of *S. capensis* stained with toluidene blue. cw, capsule wall; de, developing embryo; iml, inner mucous layer; ms, mucous strand; pvf, perivitelline fluid. Scale bar= 100 μm.

Fig. 4. A transmission electron micrograph of the egg masses of *S. capensis* showing the capsule wall (cw) separated by an envelope (en) from the perivitelline fluid (pvf). Scale bar= 200 nm.

Fig. 5. A light micrograph of toluidene blue stained section of *S. capensis* egg mass showing a single embryo in each capsule (cw). de, developing embryo. Scale bar= 100 μm.

Figs. 6 & 7. Transmission electron micrographs of the egg masses of *S. serrata* and *S. capensis* respectively, showing the outer mucous cover (omc). Scale bars= 1 μm and 500 nm respectively.


2
cw
de
omc
mma
pvf
iml
3
cw
de
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en
pvf
5
de
cw
pvf
6
omc
7
omc

Table 1. Histochemical analyses of the egg masses of *S. capensis* (Sc) and *S. serrata* (Ss) egg masses. OMC= Outer mucous cover, MMA= Mucous matrix, IML= Inner mucous layer, CAP= Capsule wall, PVF= Perivitelline fluid, DE= Developing embryo. ++++ Very strong; +++ strong; ++ medium; + weak; - no reaction; AB-PAS Alcian Blue- periodic acid Schiff'; SBB Sudan Black B.

STAIN	OMC	MMA Region i	MMA Region ii	IML	CAP	PVF	DE
	Sc	Sc	Sc	Sc	Sc	Sc	Sc
	Ss	Ss	Ss	Ss	Ss	Ss	Ss
PAS	+	+	+	+	+	++	++
	++++	+	+	+++	++	+	+
AB- PAS	Blue	Pink	Pink	Pink	Purple	Purple	Pink
	Blue	Blue	Pink	Blue	Purple	Pink	Pink
SBB	-	-	-	-	-	-	+
	-	-	-	-	-	-	+
Masson's	-	-	-	-	Blue	Blue	Purple
trichrome stain	-	-	-	-	Blue	Blue	Pink
Bromophenol	-	-	-	-	-	+	++
Blue	-	-	-	-	-	++	++
Toluidene	+	++	++	++	++++	+++	++++
Blue	+++	+	++	+	+	++++	++++

The inner mucous layer in *S. serrata* consists of highly compact fibres containing tubular units, the contents of which are floccular (Figs. 14 & 15). In *S. capensis* the same layer has no definite structure with the fibres being organized haphazardly (Fig. 16). Histochemically, this layer contains a mixture of both acid and neutral mucins (Table 1).

A mucous strand delimits the inner mucous layer from the capsule wall in both species (Fig. 3). The mucous strand is composed of fibres, which are more tightly packed in *S. serrata* than in *S. capensis* (Figs. 14, 15 & 16). Unlike *S. serrata* (Fig. 15) the fibres in *S. capensis* are not organized as a well-defined layer (Fig. 16).

Ultrastructurally the capsule wall (approximately 0.9 μm thick in *S. serrata* and 0.5 μm thick in *S. capensis*) has an amorphous appearance in both species (Figs. 14 & 16). In *S. capensis* the capsule wall stains for neutral mucins whereas in *S. serrata* it is composed of both neutral and acid mucins (Table 1). SEM revealed that the wall has a smooth appearance (Figs. 17 & 18).

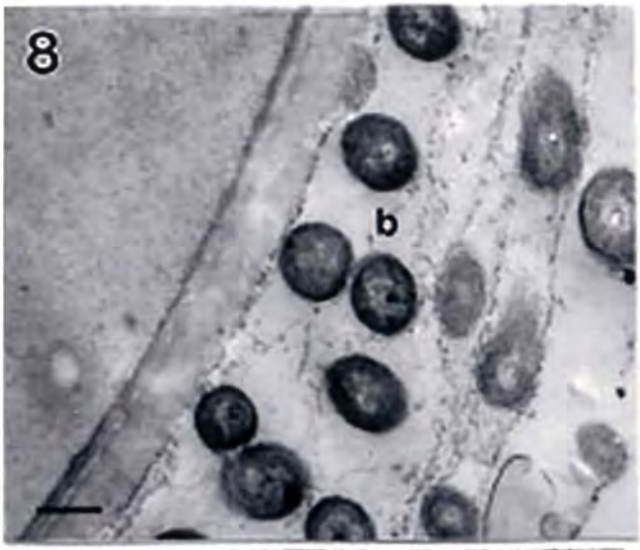
The developing embryo is surrounded by perivitelline fluid, which in turn is enclosed by an electron-dense envelope (Figs. 3, 4 & 5). This envelope could not be observed with the SEM probably because it is closely associated with the inner capsule wall (Figs. 17 & 18). In both species endocytotic pits along the embryonic cell membrane were observed suggesting the uptake of nutrients from the perivitelline fluid at different stages of embryonic development (Fig. 20, insert to Fig. 20 & Fig. 21). The developing embryo and the surrounding perivitelline fluid in both species showed basophilia when stained with toluidene blue and the presence of neutral mucins when stained with Alcian Blue-PAS (Table 1). In the whole egg mass, only the growing embryo stained positively for lipid (Table 1).

Fig. 8. Part of the outer mucous cover (omc) of the *S. capensis* egg mass. Note presence of bacteria (b) in the gelatinous matrix. Scale bar= 200 nm.

Fig. 9. Light micrograph of parts of the mucous matrix (mma) from a toluidene blue strained section of *S. serrata* egg mass. Note region i and ii with different staining intensity. Scale bar= 100 μm.

Figs. 10 & 11. The mucous matrix of the egg masses of *S. capensis* (Fig. 10) and *S. serrata* (Fig. 11). Note the presence of tubular units (*) in both. Scale bars= 500 nm and 2 μm.

Figs. 12 & 13. Transmission electron micrographs of the egg masses of *S. capensis* (Fig. 12) and *S. serrata* (Fig. 13) showing the structural differences in the inner mucous layer (iml) and part of the mucous matrix, which forms compartments (arrowheads) for egg capsules. Scale bars= 500 nm and 2 μm.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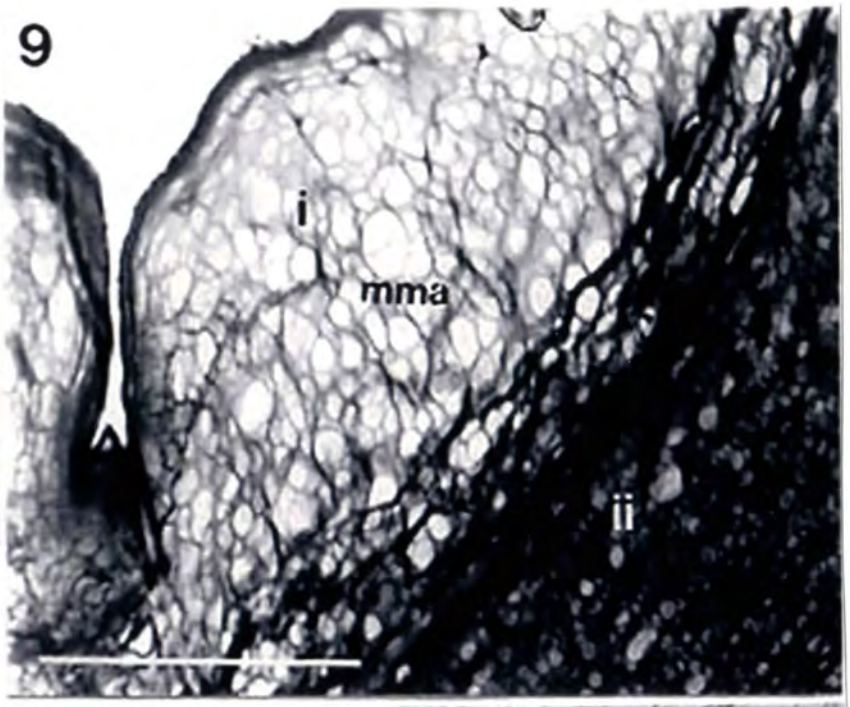
b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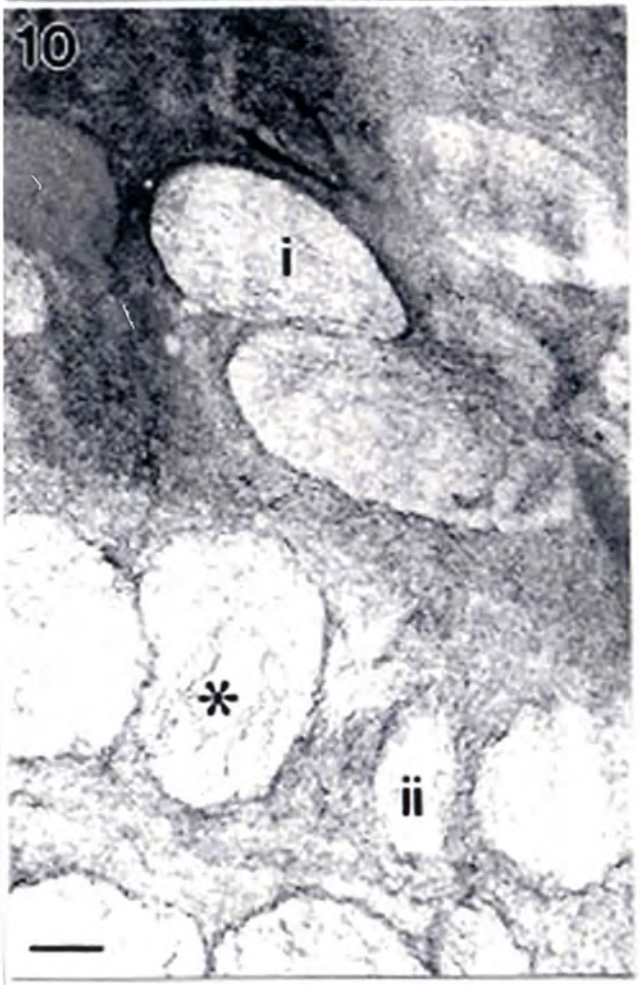
i
mma
ii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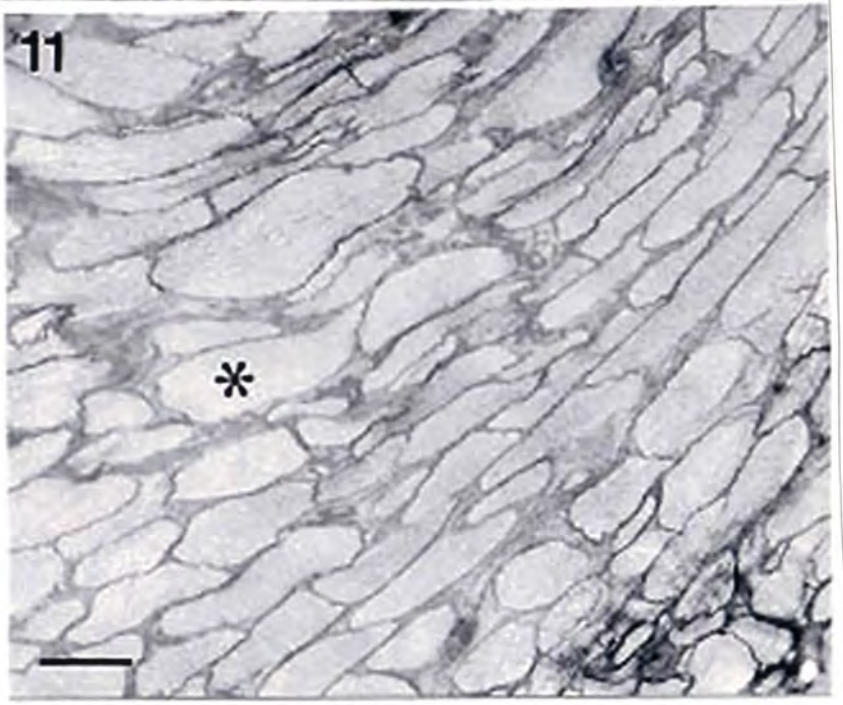
i
*
ii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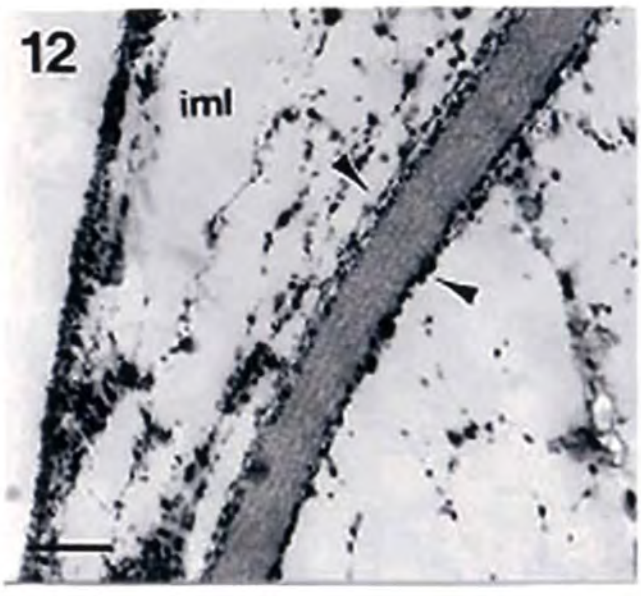
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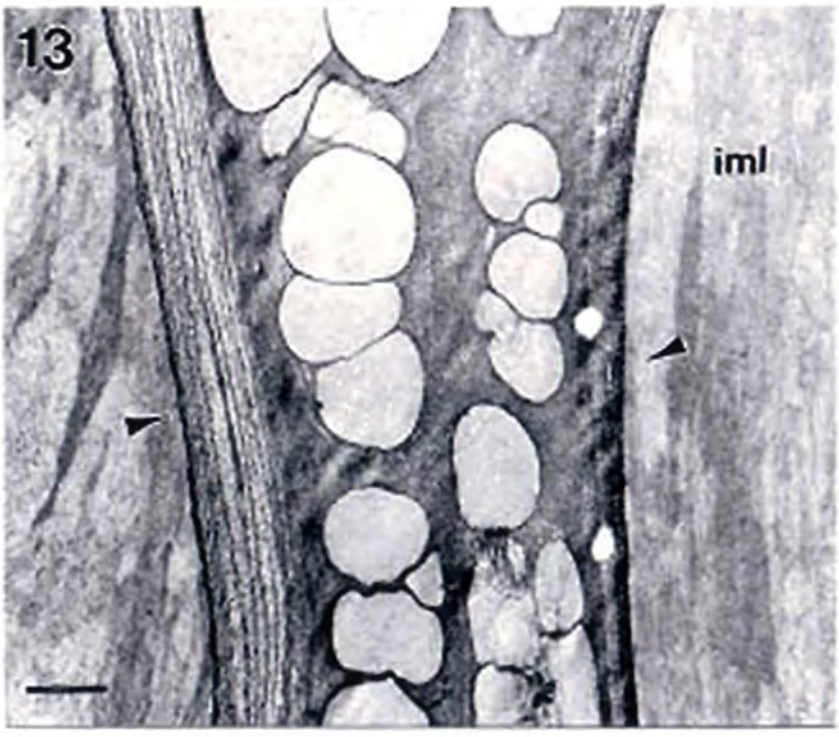
iml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iml

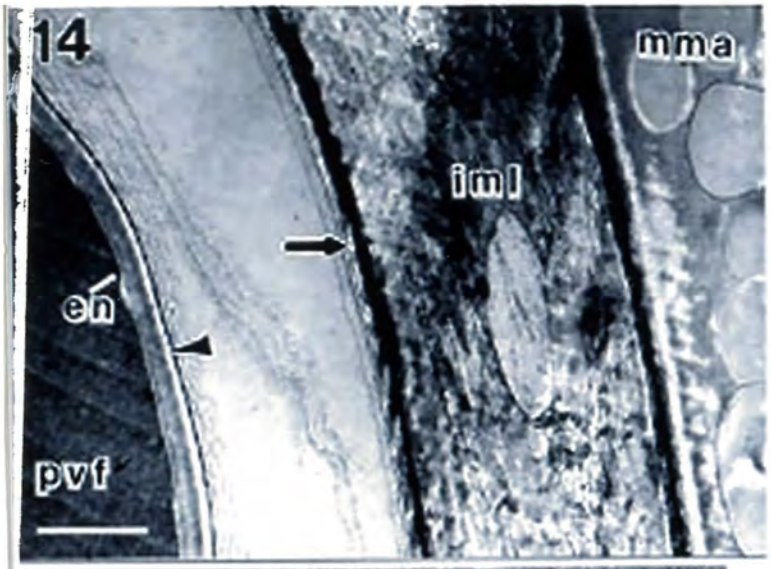
Fig. 14. An electron micrograph of the egg mass of *S. serrata* showing part of capsule wall (arrowhead), envelope (en), inner mucous layer (iml), mucous matrix (mma), mucous strand (arrow) and perivitelline fluid (pvf). Scale bar= 2 μm.

Figs. 15 & 16. The structure of mucous strand region (arrowhead) in *S. serrata* and *S. capensis* respectively. Note the difference in the inner mucous layer (iml) between these two species. cw, capsule wall. Scale bar= 500 nm each.

Fig. 17. Scanning electron micrograph of part of the egg masses of *S. serrata* showing capsule wall (cw), inner mucous layer (iml), mucous matrix (mma) with tubular units (*) and perivitelline fluid (pvf). Note that both the envelope (en) and the mucous strand (ms) are difficult to locate. Scale bar= 10 μm.

Fig. 18. Scanning electron micrograph of the egg capsule of *S. serrata* showing the smooth capsule wall (cw) surrounding granular perivitelline fluid (pvf). Scale bar= 10 μm.

Fig. 19. A light micrograph showing toluidene blue stained section of siphonariid egg mass showing all embryos (de) at the same developmental stage. Scale bar= 100 μm.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mma
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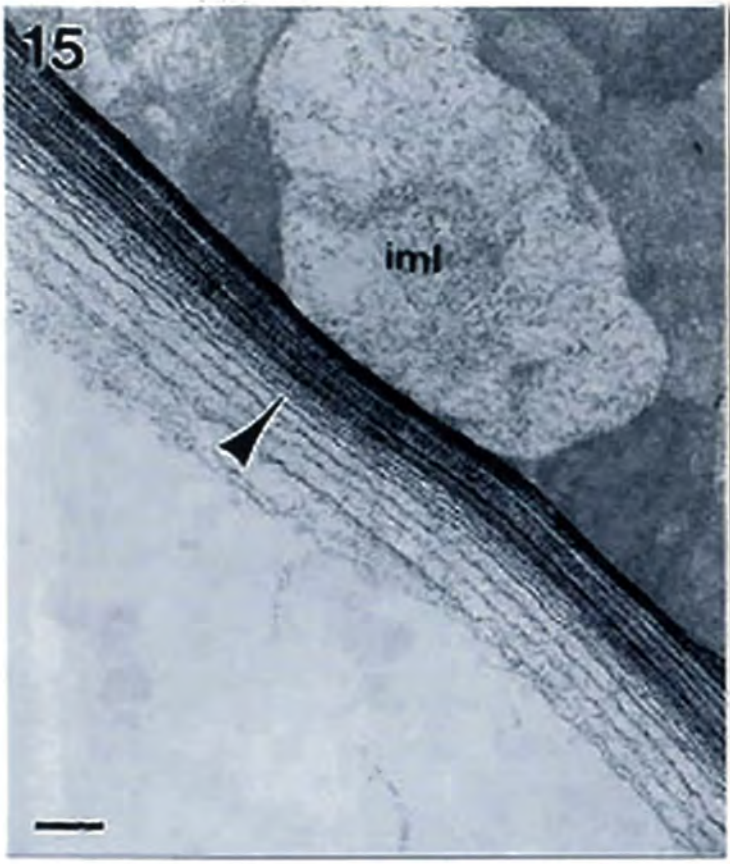
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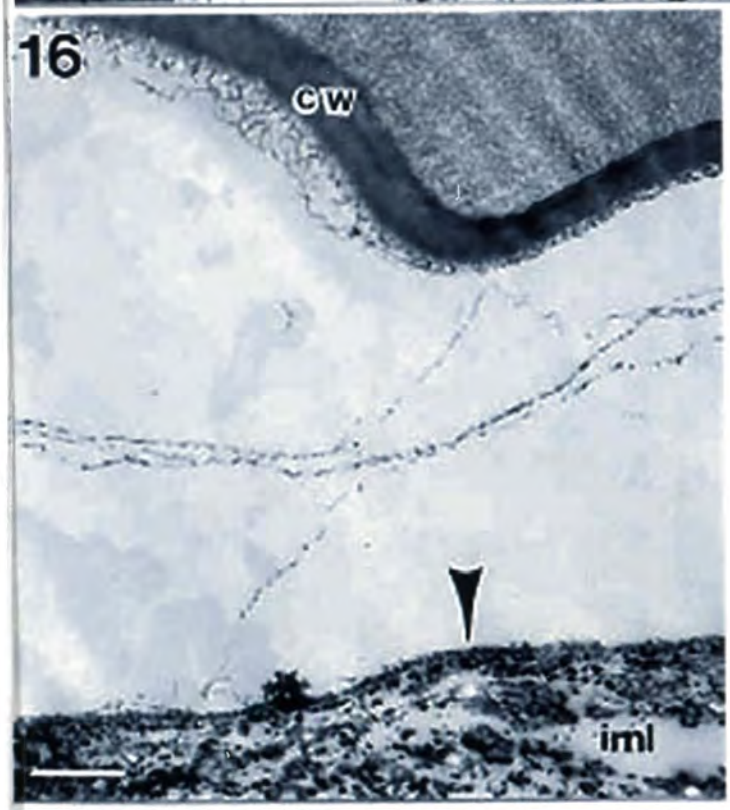
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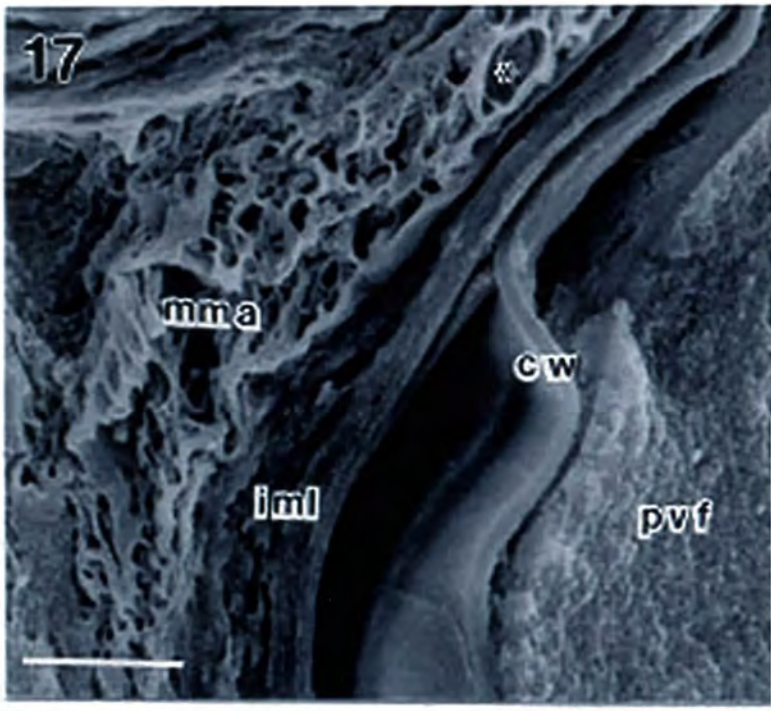
cw
iml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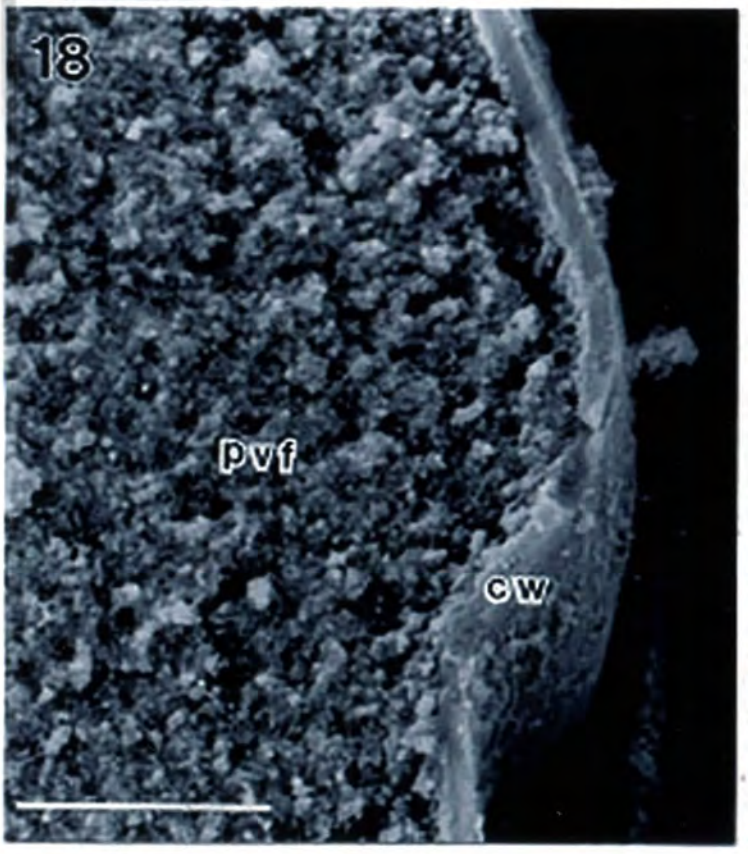
18
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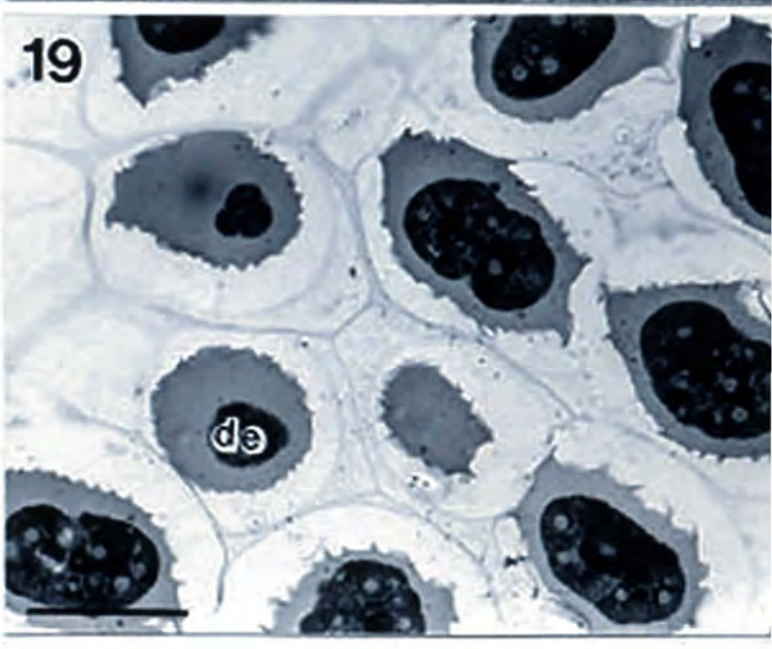
cw


19
de

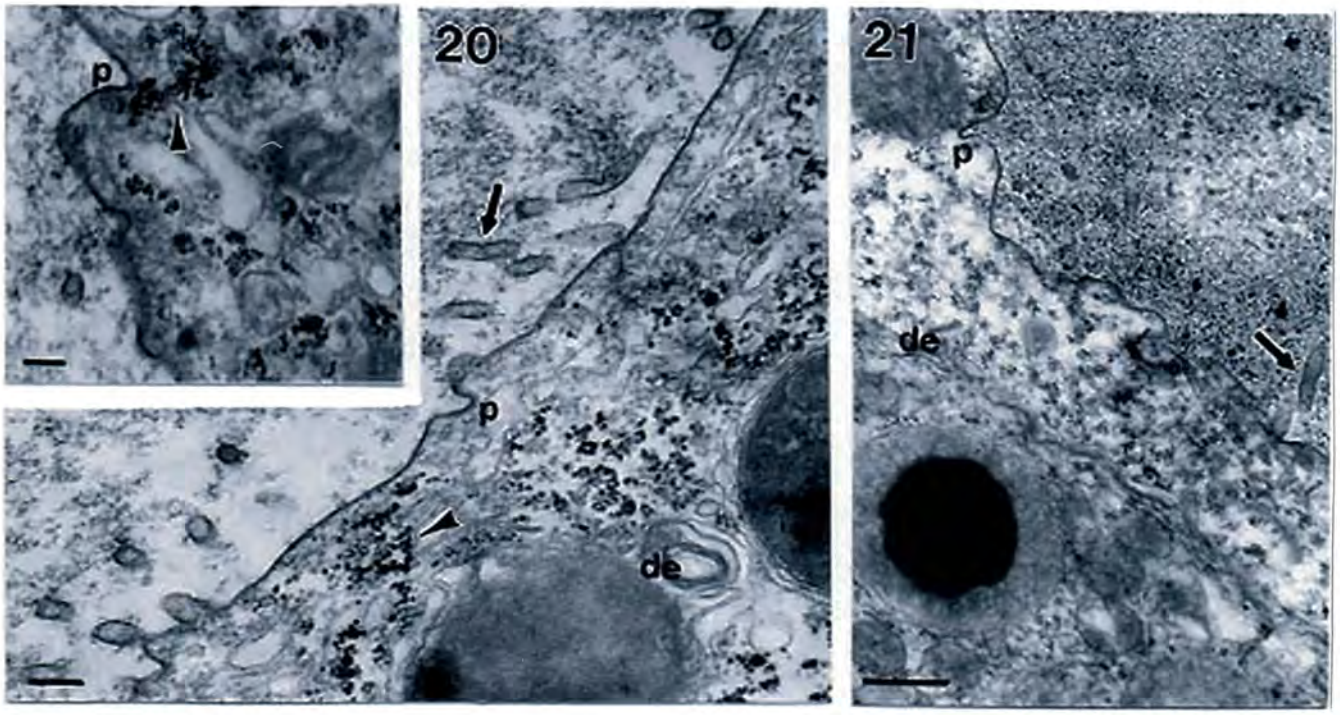


Fig. 20 and insert to Fig. 20. Transmission electron micrographs of *S. serrata* embryos (de) inside the capsule. Note the presence of endocytotic pits (p), microvilli (arrow) along the embryonic cell membrane and putative glycogen (arrowhead). Scale bar= 200 nm each.
Fig. 21. A transmission electron micrograph of developing embryo (de) of *S. capensis* inside the egg capsule showing presence of an endocytotic pit (p) along the embryonic cell membrane with microvilli (arrow). Scale bar= 500 nm.

Biochemical composition of the egg ribbon

The egg mass of *S. serrata* contained 8.45 % more carbohydrate and 11.55 % more protein than *S. capensis* (Table 2). By contrast, *S. capensis* had more lipid than *S. serrata*, which must be from the developing embryos, as the jelly matrix does not contain lipid (Table 1).

Table 2. Organic composition of siphonariid egg masses.

Species (n= 10 egg masses for each species)	Protein content (µg / mg dry weight)	Soluble carbohydrate content (µg / mg dry weight)	Total lipid content (µg / mg dry weight)
S. serrata	63.63	22.98	170
S. capensis	52.08	14.53	250

Discussion

The fundamental structure of the egg ribbons of *Siphonaria capensis* (a planktonic developer) and *S. serrata* (an intracapsular developer) was very similar. In both species the egg capsules are surrounded by a jelly matrix consisting of fibrous material organized as a number of layers. Furthermore, the jelly matrix in both species is composed largely of mucopolysaccharides, although the concentration of both protein and carbohydrate is higher in the intracapsular developer, *S. serrata*. The greater concentration of these components may provide the egg ribbons of *S. serrata* with the "toughness" needed to endure 3- 4 weeks on the shore. By contrast the egg ribbons of *S. capensis* need only remain intact for 4-5 days. In comparison to siphonariids more protein and carbohydrate was found in the egg masses of *Costasiella* sp., which has planktonic development when compared to spawn of *C. ocellifera* (an encapsulated developer) (Miles & Clark, 2002). Although the functional role of mucins could not be confirmed in the present study, it has been suggested by Klussmann-Kolb & Wägele (2001) that the mucous matrix of opisthobranch egg masses provides "stability". According to previous studies on other gastropods, carbohydrate is an integral part of the jelly, capsule wall and the intra-capsular fluid, which was also apparent in this study (Bayne, 1966 & 1968; Plesch *et al.*, 1971; Sullivan & Maugel, 1984; Hawkins & Hutchinson, 1988; Miloslavich, 1996; Klussmann-Kolb & Wägele, 2001).

During the present study microorganisms were observed in *S. capensis* spawn but not *S. serrata*. It is hard to explain how the egg masses of *S. serrata* resist any bacterial invasion as the spawn is exposed for longer periods. As the outer mucous cover of both

siphonariids contain acid mucopolysaccharides, which are suggested to play a protective role in gastropod egg masses (Klussmann-Kolb & Wägele, 2001), it is difficult to clarify why *S. capensis* only had microorganisms. It is possible that the more fibrous nature of *S. serrata* spawn (or antimicrobial agents?) prevents infection by bacteria.

Only the perivitelline fluid and the developing embryo stained positively for protein which implies that protein does not contribute significantly towards the structure of the egg mass in siphonariids. By contrast in some other gastropods protein has been found in the capsule wall and it has been suggested that protein alone, or together with carbohydrate, imparts structural integrity (Eylar, 1965; Bayne, 1968; Plesch *et. al.*, 1971; Sullivan & Maugel, 1984; Hawkins & Hutchinson, 1988; Miloslavich, 1996; Klussmann-Kolb & Wägele, 2001). Although lipid was found in other gastropod egg masses (Bayne, 1968; Miloslavich, 1996) in the siphonariids studied lipid was found in the developing embryo only.

The ultrastructural observations on siphonariid egg masses revealed that there are some structural differences between the direct and the planktonic developer. The mucous matrix in both siphonariids is composed of tubule-like units, which are particularly well developed in *S. serrata*. These tubule-like sub-structures have been observed in the outer mucous cover of the egg masses of the opisthobranchs *Aplysia* and *Dermatobranchus* (Klussmann-Kolb & Wägele, 2001) as well as other gastropod egg capsules (Tamarin & Carriker, 1967; Sullivan & Maugel, 1984). The function of these structures is unknown, but they may facilitate gaseous exchange between the embryos and its external environment.

The number of layers and the density of fibres in the jelly matrix of an egg mass vary

between different gastropod species and may reflect phylogeny, or the life history strategy (Wägele, 1996; Klussmann-Kolb & Wägele, 2001; present study). The difference in the structure of the inner mucous layer of *S. serrata* and *S. capensis* is hard to explain. In both species the structure of the capsule and the envelope surrounding the intra-capsular fluid or perivitelline fluid was similar. Although in opisthobranchs and in the prosobranch gastropods *Urosalpinx* and *Ocenebra* the capsule wall has been reported to have more than one layer (Tamarin & Carriker, 1967; Hawkins & Hutchinson, 1988; Klussmann-Kolb & Wägele, 2001), in the siphonariids studied here only one amorphous layer could be discerned. In most studies the perivitelline fluid has been referred to as "albumen" and the envelope as the "albuminous layer" or "perivitelline membrane" although it is not certain whether this membrane or the layer is comparable to the envelope of siphonariid eggs (Bayne, 1966; Klussmann-Kolb & Wägele, 2001). Son & Hong (1998) also observed such an inner envelope adjacent to the capsule wall in *Littorina brevicula*. In opisthobranchs this layer is dissolved during embryonic growth (Klussmann-Kolb & Wägele, 2001). As temporal changes in siphonariid egg masses has yet to be studied, dissolution of the envelope surrounding perivitelline fluid has still to be determined.

According to Bayne (1966) the perivitelline fluid, of *Agriolimax* egg capsules is very complex in nature containing proteins, galactogen, glycoprotein, water, calcium and some free amino acids. In a review on nudibranch embryonic capsules Eyster (1986) commented that no periodate-reactive carbohydrates were present in the intracapsular fluid. In contrast the current study on siphonariid egg capsules revealed the presence of neutral carbohydrates and protein in the perivitelline fluid, which is in agreement with the

work of Bayne (1968) on other gastropods including the basommatophoran gastropod *Lymnaea*. Eyster (1986) commented that a thorough investigation on the chemical composition of intracapsular fluid is needed to understand its adaptive value. In a study on intra-capsular feeding, littorinid embryos with nonplanktonic development were shown to take up labeled protein from the intra-capsular fluid (Moran, 1999). Although endocytotic uptake of nutrients during embryonic development needs to be confirmed in siphonariids, both *S. serrata* and *S. capensis* embryos showed the presence of endocytotic pits along the embryonic cell membrane suggesting a nutritive role of perivitelline fluid. In the terrestrial slug, *Agriolimax* it was suggested that the membrane surrounding perivitelline fluid was formed by albumen coagulation (Bayne, 1966). As the envelope in siphonariids was difficult to distinguish from the capsule wall, it is difficult to comment on its composition. In *Siphonaria* the envelope stained positive with toluidene blue, which suggests presence of the basophilic substances.

Chambers (1994) found that *S. serrata* spawn has better water retention ability than that of *S. capensis* and he suggested that the collared shape of the egg ribbon of *S. serrata* might be an important adaptation to reduce desiccation by retention of water. In addition to the shape of the spawn it appears that the thickness of the egg capsule and fibrous nature of the egg mass also contribute to a reduction in water loss. Pechenik (1979) and Perron (1981) have advocated that thick walled capsules also provide more protection to the early stages of embryonic development when they are more vulnerable to environmental stress and predation.

Although this study did not investigate the energetic cost of producing thick-walled capsules in *S. serrata* it may be more than the planktonic developer. The total cost of

producing spawn however may be similar in the direct and planktonic developer as the latter produces more eggs (approximately 20000 eggs per spawn compared to 2000 in *S. serrata*). The energy allocation for capsule production not only depends on developmental time but also on whether there is parental care involved during early stages of development (Perron, 1981; Chaparro *et al.*, 1999; Chaparro & Flores, 2002).

References

BANCROFT, J. D & STEVENS, A. 1990. *Theory and Practice of Histological Techniques*. Churchill Livingstone, New York.

BAYNE, C. J. 1966. Observations on the composition of the layers of the egg of *Agriolimax reticulatus*, the grey field slug (Pulmonata: Stylommatophora). *Comparative Biochemistry and Physiology*, **19** : 317- 338.

BAYNE, C. J. 1968. Histochemical studies on the egg capsules of eight gastropod molluscs. *Proceedings of the Malacological Society of London*, **38**: 199- 212.

CHAMBERS, R. J. 1994. *The conflict between adaptation and constraint: the case of the siphonariid limpets*. PhD thesis, Rhodes University.

CHAMBERS, R. J & MCQUAID, C. D. 1994a. Notes on taxonomy, spawn and larval development of South African species of the intertidal limpet *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **60**: 263- 275.

CHAMBERS, R. J & MCQUAID, C. D. 1994b. A review of larval development in the intertidal limpet genus *Siphonaria* (Gastropoda: Pulmonata). *Journal of Molluscan Studies*, **60**: 415- 423.

CHAPARRO, O. R. & FLORES, M. L. 2002. Reproductive output of *Crepidula fecunda* females: distribution of energy in the production of gametes and capsular walls. *New Zealand Journal of Marine and Freshwater Research*, **36**: 661- 673.

CHAPARRO, O. R., OYARZUN, R. F., VERGARA, A. M. & THOMPSON, R. J. 1999. Energy investment in nurse eggs and egg capsules in *Crepidula dilatata* Lamarck (Gastropoda: Calyptraeidae) and its influence on the hatching size of the juvenile.

Journal of Experimental Marine Biology and Ecology, **232**: 261- 274.

CROSS, R. H. M. 1989. A reliable epoxy resin mixture and its application in routine electron microscopy. *Micron Microscopica Acta*, **20**: 1-7.

EISENMAN, E. A. & ALFERT, M. 1981. A new fixation procedure for preserving the ultrastructure of marine invertebrate tissues. *Journal of Microscopy*, **125** : 117-120.

EYLAR, E. H. 1965. On the biological role of glycoproteins. *Journal of Theoretical Biology*, **10** : 89-113.

EYSTER, L. S. 1986. The embryonic capsules of nudibranch molluscs: literature review and new studies on albumen and capsule wall ultrastrastructure. *American Malacological Bulletin*, **4**: 205- 216.

FOSTER, G. G. & HODGSON, A. N. 1998. Consumption and apparent dry matter digestibility of six intertidal macroalgae by *Turbo sarmaticus* (Mollusca: Vetigastropoda: Turbinidae). *Aquaculture*, **167**: 211- 227.

HAWKINS, L. E. & HUTCHINSON, S. 1988. Egg capsule structure and hatching mechanism of *Ocenebra erinacea* (L.) (Prosobranchia: Muricidae). *Journal of Experimental Marine Biology and Ecology*, **119**: 269- 283.

HODGSON, A. N. 1999. The biology of siphonariid limpets (Gastropoda: Pulmonata). *Oceanography and Marine Biology: an Annual Review*, **37**: 245- 314.

HUMASON, G. L. 1967. *Animal Tissue Technique*, W. H. Freeman, New York,

KLUSSMANN-KOLB, A. & WÄGELE, H. 2001. On the fine structure of opisthobranch egg masses (Mollusca, Gastropoda). *Zoologischer Anzeiger*, **240**: 101- 118.

KOCHERT, G. 1978. Protein determination by dye binding and carbohydrate determination by phenol- sulphuric acid method. In: *The Handbook of Phycological*

Methods: Physiological and biochemical methods. (Hellebust, J. A & Craigie, J. S. eds). Cambridge University Press, London.

MARCUS, E. & MARCUS, E. 1960. On *Siphonaria hispida. Universidade de Sao Paulo. Faculdade de filosofia, Ciencias e Letras. Boletim de Zoologia,* **23**: 107-139.

MAPSTONE, G. 1978. Egg capsules and early development in *Siphonaria diemenensis* (Quoy & Gaimard, 1833) and *Siphonaria baconi* (Reeve, 1856). *Journal of Malacological Society of Australia*, **4**: 85- 92.

MILES, C. M. & CLARK, K. B. 2002. Comparison of biochemical composition and developmental mode in two populations of *Costasiella* [Opisthobranchia: Ascoglossa (=Sacoglossa)]. *Journal of Molluscan Studies*, **68**: 101- 109.

MILOSLAVICH, P. 1996. Biochemical composition of prosobranch egg capsules. *Journal of Molluscan Studies,* **62**: 133- 135.

MORAN, A. L. 1999. Intracapsular feeding by embryos of the gastropod genus *Littorina. Biological Bulletin*, **196**: 229- 244.

PEARSE, A. G. E. 1968. *Histochemistry: theoretical and applied*, Churchill, London,

PECHENIK, J. A. 1979. Role of encapsulation in invertebrates with mixed life histories. *American Naturalist*, **114**: 859- 867.

PERRON, F. E. 1981. The partitioning of reproductive energy between ova and protective capsules in marine gastropods of the genus *Conus. American Naturalist*, **118**: 110- 118.

PLESCH, B., JONG-BRINK, M. DE. & BOER, H. H. 1971. Histological and histochemical observations on the reproductive tract of the hermaphrodite pond snail *Lymnaea stagnalis* (L.). *Netherlands Journal of Zoology*, **21**: 180- 201.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology*, **17**: 208-212.

SON, M. H. & HONG, S. Y. 1998. Reproduction of *Littorina brevicula* in Korean waters. *Marine Ecology Progress Series*, **172**: 215- 223.

SULLIVAN, C. H. & MAUGEL, T. K. 1984. Formation, organization, and composition of the egg capsule of the marine gastropod, *Ilyanassa obsoleta*. *Biological Bulletin,* **167**: 378- 389.

TAMARIN, A. & CARRIKER, M. R. 1967. The egg capsule of the muricid gastropod *Urosalpinx cinerea*: An integrated study of the wall by ordinary light, polarized light and electron microscopy. *Journal of Ultrastructural Research*, **21**: 26- 40.

TANAKA, K. 1981. Demonstration of intracellular structures by high resolution scanning electron microscopy. *Scanning Electron Microscopy*, **II**: 1- 8.

WÄGELE, H. 1996. On egg clutches of some Antarctic Opisthobranchia. *Malacological Review Supplement*, **6**: 21- 30.

Chapter 6

Structure and function of the glandular complex and spermatheca

Introduction

The structure of the reproductive system of pulmonates is complex and varies between and within the subgroups (Hyman, 1967; Duncan, 1975). The diversity of accessory reproductive organs and the distal genitalia in this group has therefore formed the basis for a number of taxonomic and phylogenetic studies (Duncan, 1975; Luchtel *et al.*, 1997). The anatomy, histology and ultrastructure of the reproductive tract and associated glands as well as the terminal genitalia of pulmonates have been reviewed by several authors (Duncan, 1975; Berry, 1977; Geraerts & Joosse, 1984; Runham, 1988; Luchtel *et al.*, 1997). Two of the important structures that branch off the reproductive tract of pulmonates are the albumen gland and spermatheca (also known as the bursa copulatrix or gametolytic gland) (Luchtel *et al.*, 1997).

Unlike other euthyneurans (Klussmann-Kolb, 2001a), detailed morphological studies of the reproductive organs in most basommatophorans are scarce, and with the notable exception of the light microscopic account on *Siphonaria hispida* by Marcus & Marcus (1960) there are no investigations on siphonariids. The gross anatomy of the reproductive system of siphonariids, however, has been described by a number of authors (Allanson, 1959; Marcus & Marcus, 1960; Berry, 1977; Hubendick, 1978) (Fig. 1). The reproductive system consists of numerous organs including a simple gonad, hermaphrodite duct, spermoviduct, glandular complex, bursa copulatrix and an epiphallus gland (Marcus & Marcus, 1960; Berry, 1977). The hermaphrodite gland (also known as the ovotestis) of *Siphonaria* lies next to the digestive gland. A hermaphrodite duct (which includes the seminal vesicle) leads to an area where a fertilization pouch and glandular complex open.

Both the epiphallus gland and spermatheca open into the genital tract or spermoviduct close to the common genital pore (Hyman, 1967; Marcus & Marcus, 1960; Berry, 1977; Hubendick, 1978; Geraerts & Joosse, 1984; Hodgson, 1999; pers. obs.) (Fig. 1).

In opisthobranchs, Klussmann-Kolb (2001a) has identified and described, using light and electron microscopy and histochemistry, the structure of the albumen gland, membrane gland, mucous gland and capsule gland. By contrast, in pulmonates, especially in basommatophorans, only an albumen gland, posterior mucous gland (= membrane gland by Dayrat & Tillier, 2002) and prostate have been recognized (Berry, 1977; Runham, 1988). Except for *Biomphalaria* and *Lymnaea*, a detailed study of the structure of the glands (albumen and mucous gland) that may have a role in egg mass formation, has not been undertaken in basommatophorans. Like most basommatophorans, siphonariids also lay gelatinous egg masses on rocky shores and each embryo is enclosed in a tough capsule (see Chapter 5). The present investigation of the structure and function of glandular complex of *S. capensis* and *S. serrata* was carried out with the aim of examining the possible roles of this organ in relation to egg mass formation.

The role of spermatheca in both auto- and allosperm storage and possible post-copulatory sexual selection in terrestrial pulmonates has been debated by several authors in recent years (e. g., Haase & Baur, 1995; Bojat *et al.*, 2001a & b; Bojat *et al.*, 2002). No such studies have been carried out on marine pulmonates and there is no information on spermathecal structure. The presence of a spermatheca or bursa is an important character in phylogenetic analyses of pulmonates especially basommatophorans (see Nordsieck, 1992) although its role is still unclear. Although data on promiscuity of siphonariids are scarce (an exception being Hirano & Inaba, 1980) it may be crucial to examine the fine

structure of spermatheca of siphonariids in an attempt to answer questions on sperm storage in these intertidal limpets.

The present study combines histology, histochemistry and electron and fluorescence microscopy to investigate the morphology, function and possible phylogenetic significance of glandular complex and spermatheca of *S. capensis* and *S. serrata*.

Material and methods

Transmission electron microscopy and light microscopy

Individuals of *Siphonaria capensis* and *S. serrata* were collected from Kenton-on-Sea (33°42' S, 26°41' E) in Eastern Cape during summer (January), autumn (March), winter (June) and spring (September) 2001. In the laboratory, animals were dissected and parts of the glandular complex and the entire spermatheca were fixed and processed for transmisson electron microscopy (as described in Chapter 4). For histochemical investigations small pieces of tissue were prepared for periodic acid-Schiff's test (neutral carbohydrates), combined Alcian Blue- PAS test (acidic mucopolysaccharides) and bromophenol blue (protein) (for details of the method see Chapter 5).

Epifluorescence microscopy

Spermathecae from fresh specimens (of both species) were placed on a clean glass slide coated with gelatin (0.1 % gelatin in 0.01% chromium potassium sulphate) and the contents were removed by rupturing the organs in a drop of phosphate buffered saline (PBS) (pH 7.2). The slide was then dried in an oven at 40° C before fixation in methyl alcohol: glacial acetic acid (3:1) for 4 mins. After a rinse in PBS for 1 min the slides were

stained with Hoechst 33258 (0.01 g of stain in 100 ml of PBS) (Aldrich Chemicals) in PBS for 1 min. The slides were then rinsed in three changes of PBS and dried carefully with filter paper. A coverslip was placed on the slide after adding a drop of glycerol: PBS (9:1) solution. The slides were viewed with an Olympus BX series light microscope set for epifluorescence microscopy. A G365 exciter filter, FT420 chromatic beamsplitter and LP418 barrier filter were used for the observation and images were taken by a digital camera (Olympus Camedia). The stain binds only to DNA, enabling estimation of the numbers of sperm or eggs stored in the organ (Sakaluk & O' Day, 1984; Pitnick & Markow, 1994).

Scanning electron microscopy

Spermathecae from both species were dissected out from fresh specimens and the contents were dried on a clean coverslip at 40 °C in a drop of poly- L- lysine added with a drop of 0.1 M phosphate buffer (pH 7.0). Spermathecae were fixed in 2.5 % glutaraldehyde [prepared in 0. 1 M phosphate buffer (pH 7.0)] for 12 hrs at 4 °C. A graded series of acetone, was used for dehydration and samples were dried in a critical point dryer, coated with gold and viewed with a JEOL JSM 840 at 12 kV (Báo *et al.*, 1996).

Variability in size of spermatheca between *S. capensis* and *S. serrata*

To estimate the size of spermatheca of both species, spermethecae of six animals of different shell length (15- 25 mm) were collected and the major and minor axes were

measured (to a precision of 1 μm) under a light microscope with an ocular scale. A 1-factor analysis of covariance was used to analyze the data. The surface area of each spermatheca was calculated and used as a dependent variable while the shell length of the animals was the covariate (Underwood, 1997). As the variances were homogeneous in Cochran's test no transformation was necessary (Underwood, 1997).

Results

Glandular complex of *S. capensis* and *S. serrata*

I. Gross anatomy

The gross anatomy of the reproductive system of *S. capensis* and *S. serrata* differs in the structure of the genitalia only (Fig. 1). *S. capensis* lacks penis as well as a flagellum (Fig. 1), which has also been shown by a previous account on South African siphonariids (Allanson, 1959).

The glandular complex of *S. capensis* and *S. serrata*, which includes a fertilization chamber, joins the hermaphrodite duct proximally and spermoviduct distally (Fig. 1). In both species the glandular complex is tubular (Fig. 2) and consists of two glands that could not be identified on the basis of external anatomy only. Based on the toluidene blue staining, two components of the glandular complex can be recognized, an albumen gland and a mucous gland (Fig. 2). As the histology, histochemistry and ultrastructure of the glands of *S. capensis* and *S. serrata* were similar, a single description only is presented.

II. Histochemistry

The albumen gland stains blue in toluidene blue indicating basophilia whereas, the

mucous gland is pink, which indicates acidophila (Fig. 2). Both glands are composed of columnar secretory cells [approximately 0.42-0.54 (l) x 0.01-0.04 mm (w) in the albumen gland and 0.12-0.24 (l) x 0.05-0.06 mm (w) in the mucous gland], which show different staining properties (note the secretory cells of the albumen gland) (Fig. 3). The albumen gland stains positively with PAS (Fig. 4) and bromophenol blue (Fig. 5) suggesting the presence of neutral polysaccharides and protein respectively whereas, the mucous gland does not stain with bromophenol blue (Fig. 6). Both glands stain differentially with AB-PAS confirming the presence of both neutral and acid-mucopolysaccharides (Fig. 7).

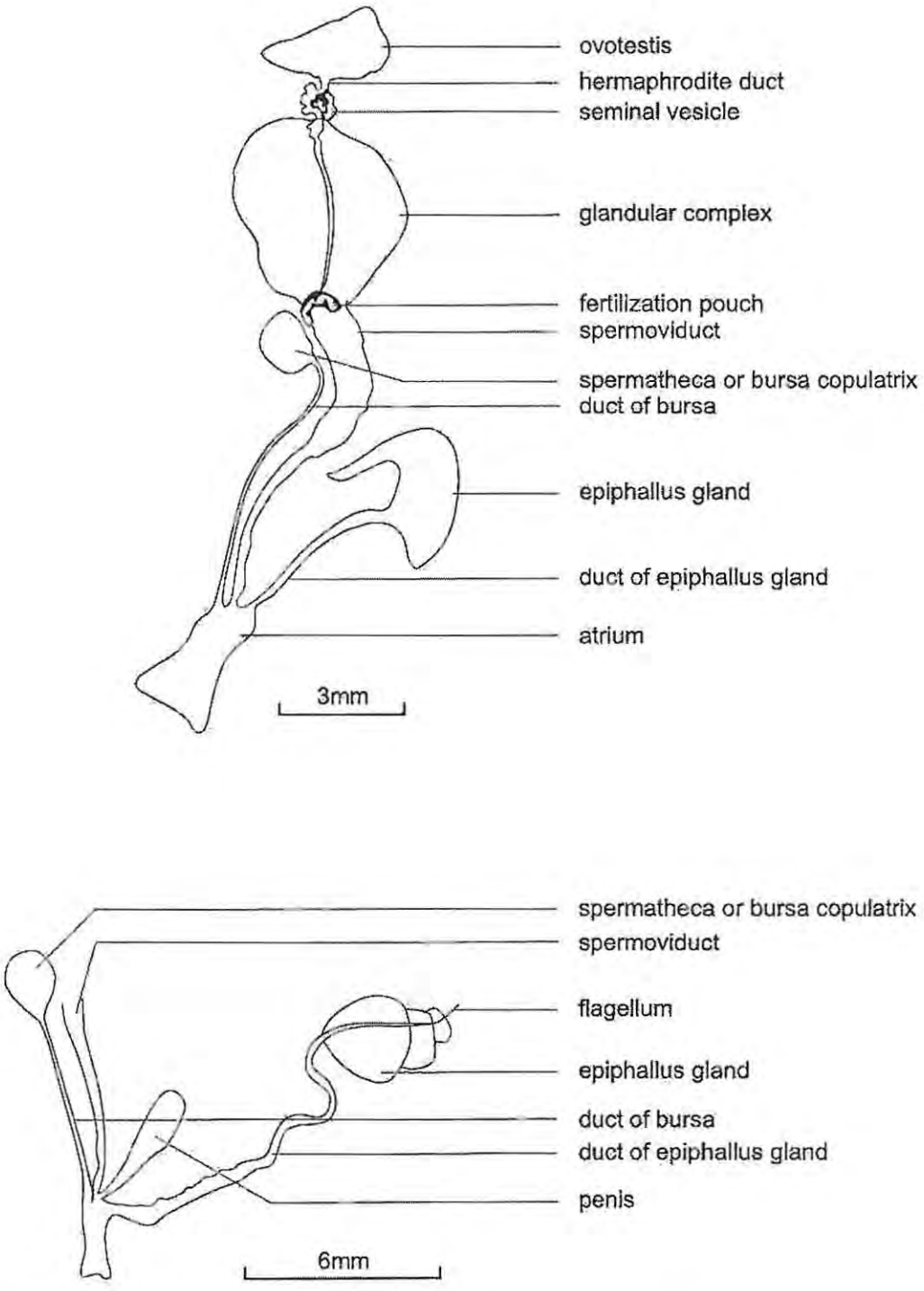


Fig. 1. The reproductive anatomy of *S. capensis* (top diagram) and *S. serrata* (bottom diagram). The glandular complex, hermaphrodite duct and ovotestis are not illustrated in *S. serrata*.

III. Ultrastructure of the albumen gland

The albumen gland is composed of columnar secretory cells and wedge-shaped ciliated cells (Fig. 8). The secretory cells, which stain positive for protein, contain a centrally located lobular nucleus with a prominent nucleolus (Fig. 9). The cytoplasm of these glandular cells is full of secretory material in the form of spherical electron-dense vesicles (of about 1.75-5.25 μm diameter) (non-membrane bound) embedded in an irregular mass of mucous material (Fig. 9). Extensive arrays of rough endoplasmic reticulum with some product in the swollen cisternae are in close proximity to these vesicles (Fig. 10). Golgi bodies are observed, producing numerous small vesicles (Fig. 11). In the cytoplasm mitochondria are also present (Figs. 10 & 15). The apical cytoplasm of the secretory cells is devoid of organelles (Fig. 12).

The secretory cells that stain positive for polysaccharides and mucopolysaccharides only, are filled with fine filamentous mucous material and putative glycogen granules (Figs. 13 & 14). Apart from the Golgi bodies no other organelles can be seen in the cytoplasm of these cells (Fig. 13).

Some cells were seen to have both spherical electron-dense vesicles as well as filamentous mucous secretions (Fig. 16). It is therefore possible that the albumen gland only possesses one type of secretory cell, which is able to produce more than one type of secretion.

IV. Ultrastructure of the mucous gland

The mucous gland also possesses columnar secretory cells although they are shorter than the albumen gland cells (Fig. 17). The fine structure of these secretory cells is simple. They possess an irregularly shaped nucleus with a small nucleolus as well as a

few round mitochondria (Fig. 18), numerous Golgi bodies (Fig. 20) and aggregates of putative glycogen granules (Figs. 21). The cells are packed with fine filamentous secretory material, which is aggregated into irregular shapes (Fig. 19).

Based on the histological (Fig. 26) and ultrastructural observations (Figs. 19 & 22), both glands appear to release their content in the form of droplets.

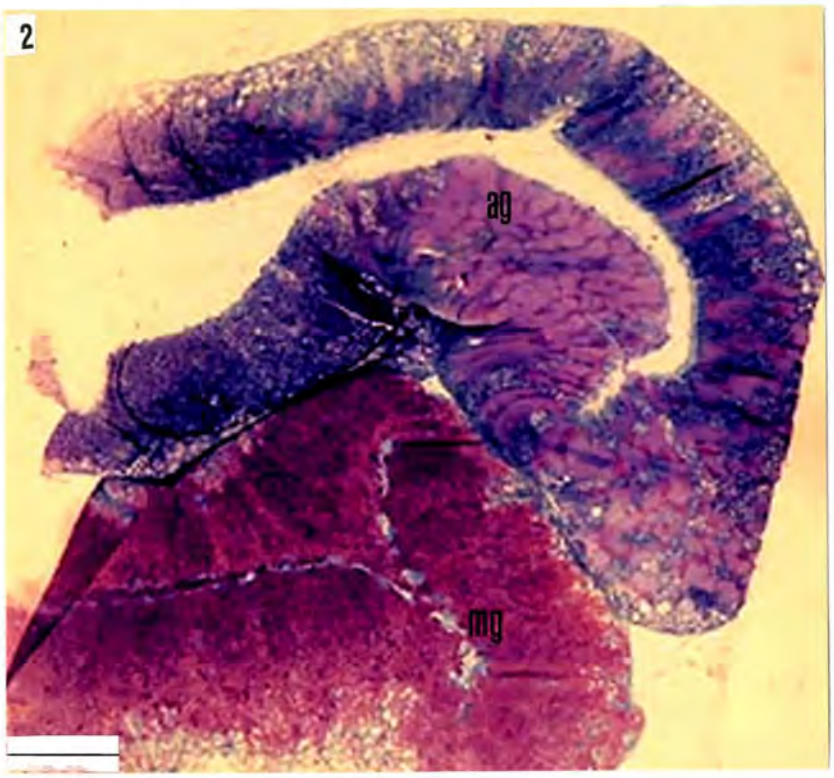
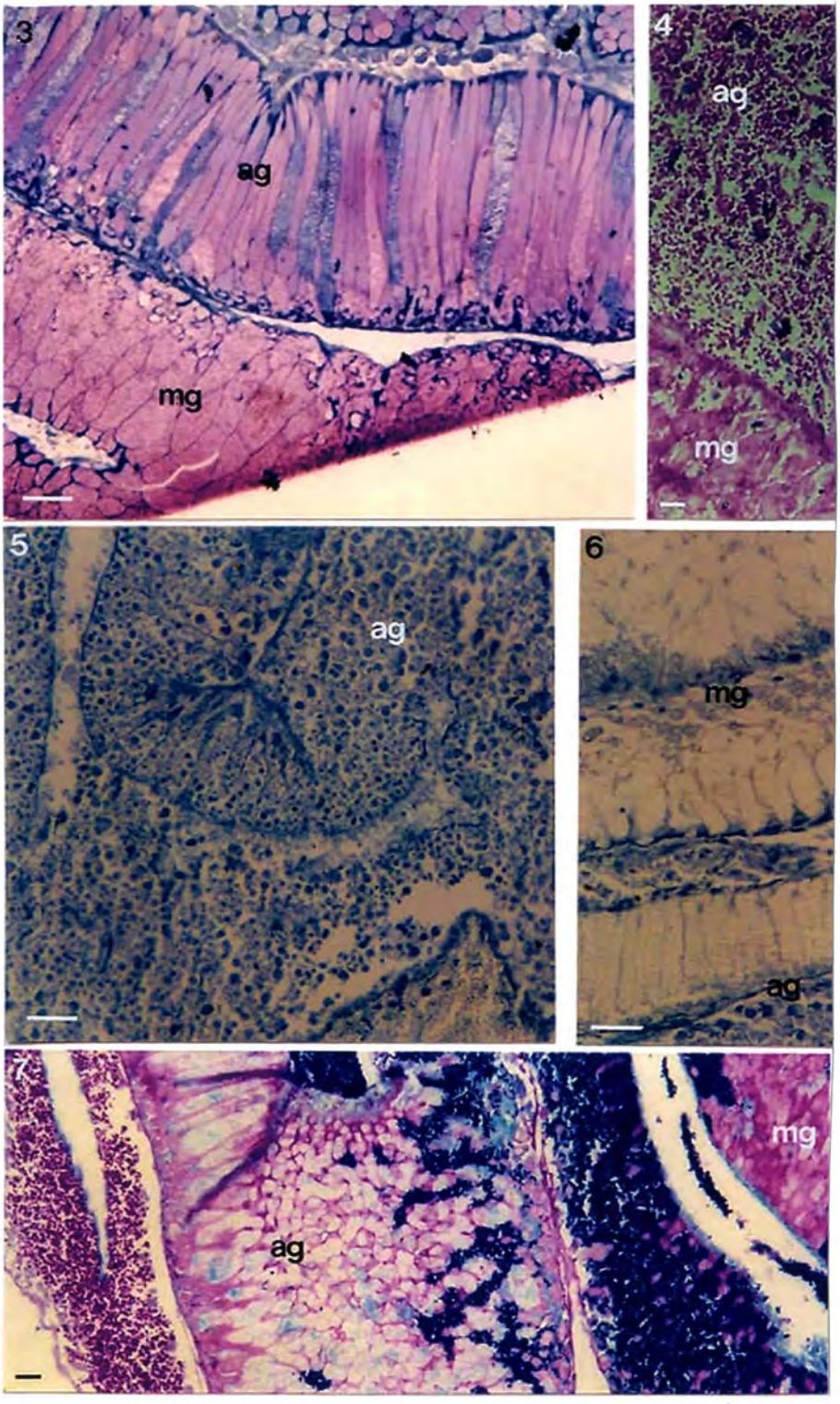


Fig. 2. A toluidene blue stained section of the glandular complex of *S. serrata* showing basophilic albumen gland (ag) and acidophilic mucous gland (mg). Scale bar = 0.1 mm.

Fig. 3. A toluidene blue stained section of the glandular complex of *S. serrata* showing differentially stained cells of the albumen gland (ag). mg, mucous gland. Scale bar = 0.1 mm.
Fig. 4. A section of the glandular complex of *S. capensis* stained with periodic acid-Schiff's reagent. ag, albumen gland; mg, mucous gland. Scale bar = 0.1 mm.
Fig. 5. Bromophenol blue stained section of the albumen gland (ag) of *S. serrata*. Scale bar = 0.1 mm.
Fig. 6. A section stained with bromophenol blue of the glandular complex of *S. serrata* showing unstained mucous gland (mg) and parts of the albumen gland (ag). Scale bar = 0.1 mm.
Fig. 7. A section of the glandular complex of *S. serrata* stained with Alcian blue- PAS showing a presence of both acid and neutral mucopolysaccharides in the albumen gland (ag) and mucous gland (mg). Scale bar = 0.1 mm.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mg
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mg

Fig. 8. An electron micrograph showing secretory cells (sc) and ciliated cells (cc) of the glandular complex of *S.serrata*. ci, cilia. Scale bar = 2 μm.

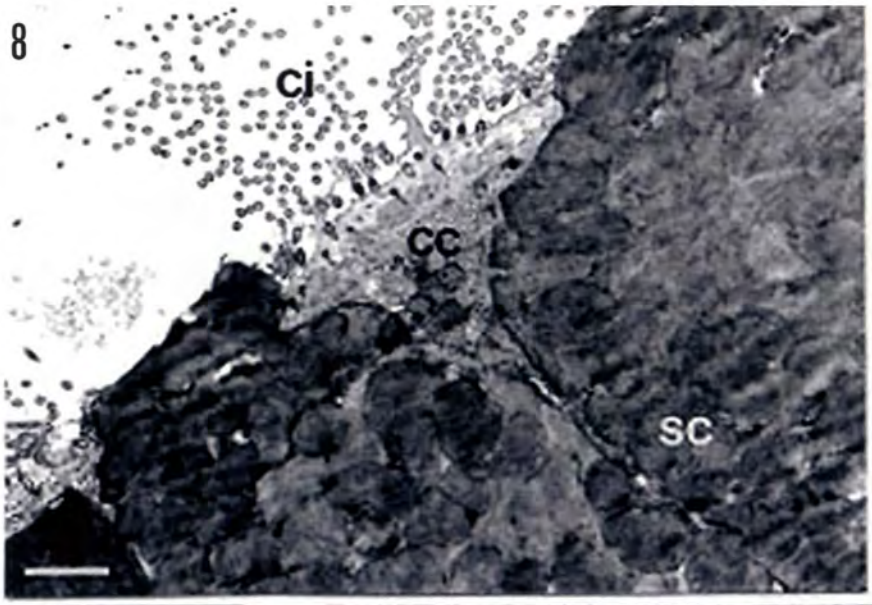
Fig. 9. The secretory cells of the albumen gland of *S. capensis* with a prominent nucleus (n) and secretory material which contains a dense vesicle (c) embedded in a mucous matrix (mu). nu, nucleolus. Scale bar = 2 μm.

Fig. 10. The secretory cells with protein secretion in the albumen gland of *S. serrata*, showing a presence of extensive arrays of rough endoplasmic reticulum (rer) with swollen cisternae (arrowheads). c, dense vesicle; mu, mucous matrix. Scale bar = 1 μm.

Fig. 11. A micrograph showing Golgi body (g) producing small vesicles (v) in the secretory cells of the albumen gland of *S. capensis*. arrowheads, rer. Scale bar = 200 nm.

Fig. 12. The apical cytoplasm of the secretory (sc) as well as ciliated cells (cc) of the albumen gland of *S. serrata*. Scale bar = 1 μm.

Fig. 13. The secretory cells of the albumen gland of *S. serrata* with mucous material (mu) in the cytoplasm. g, Golgi body; n, nucleus. Scale bar = 1 μm.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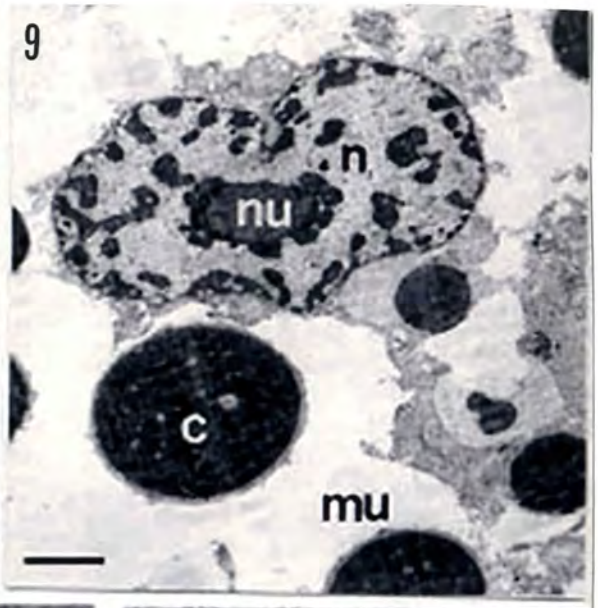
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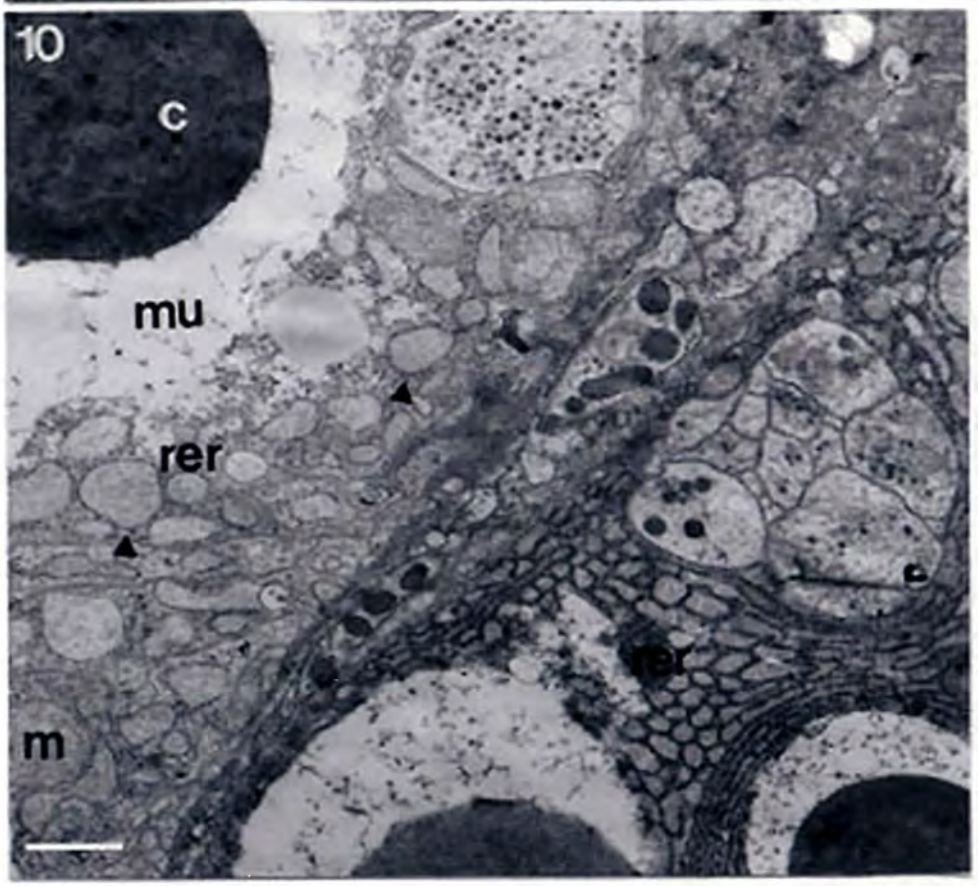
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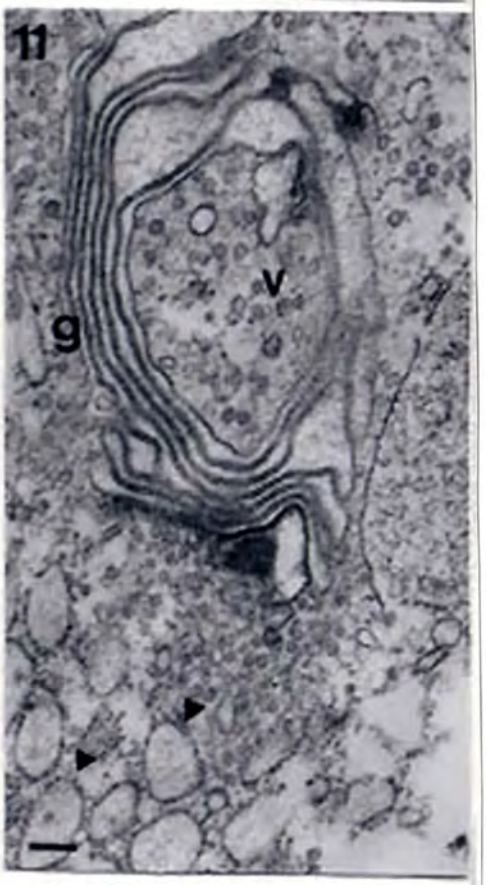
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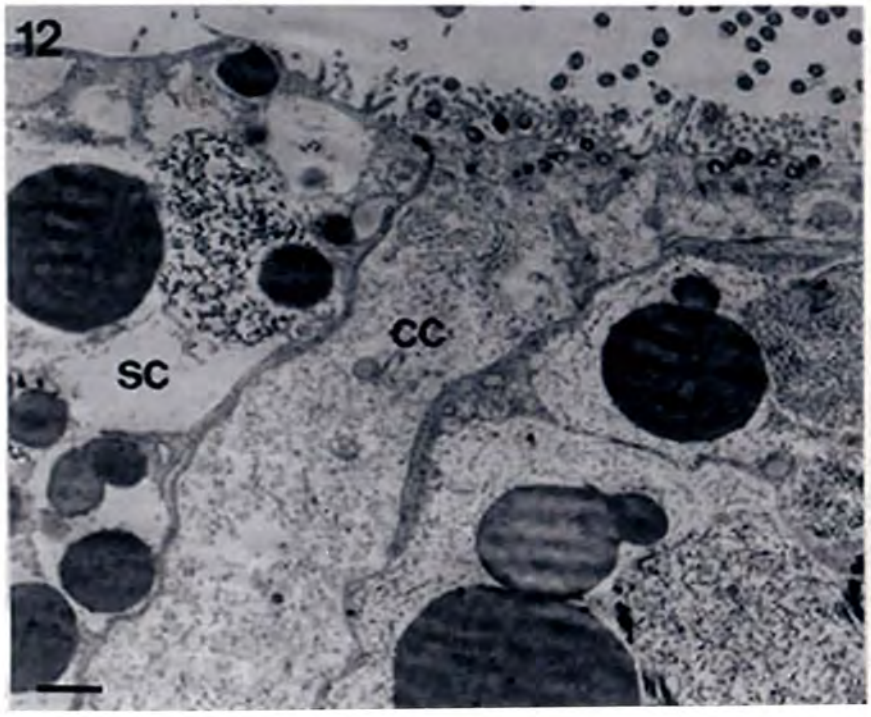
g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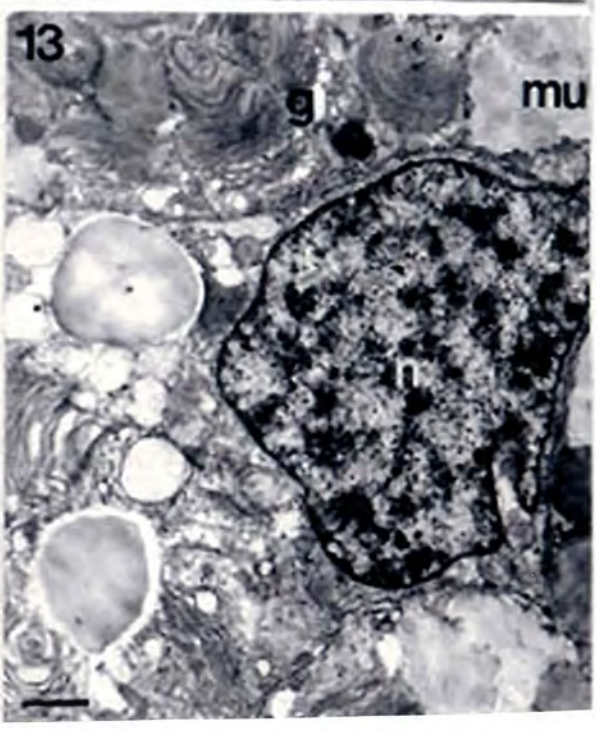
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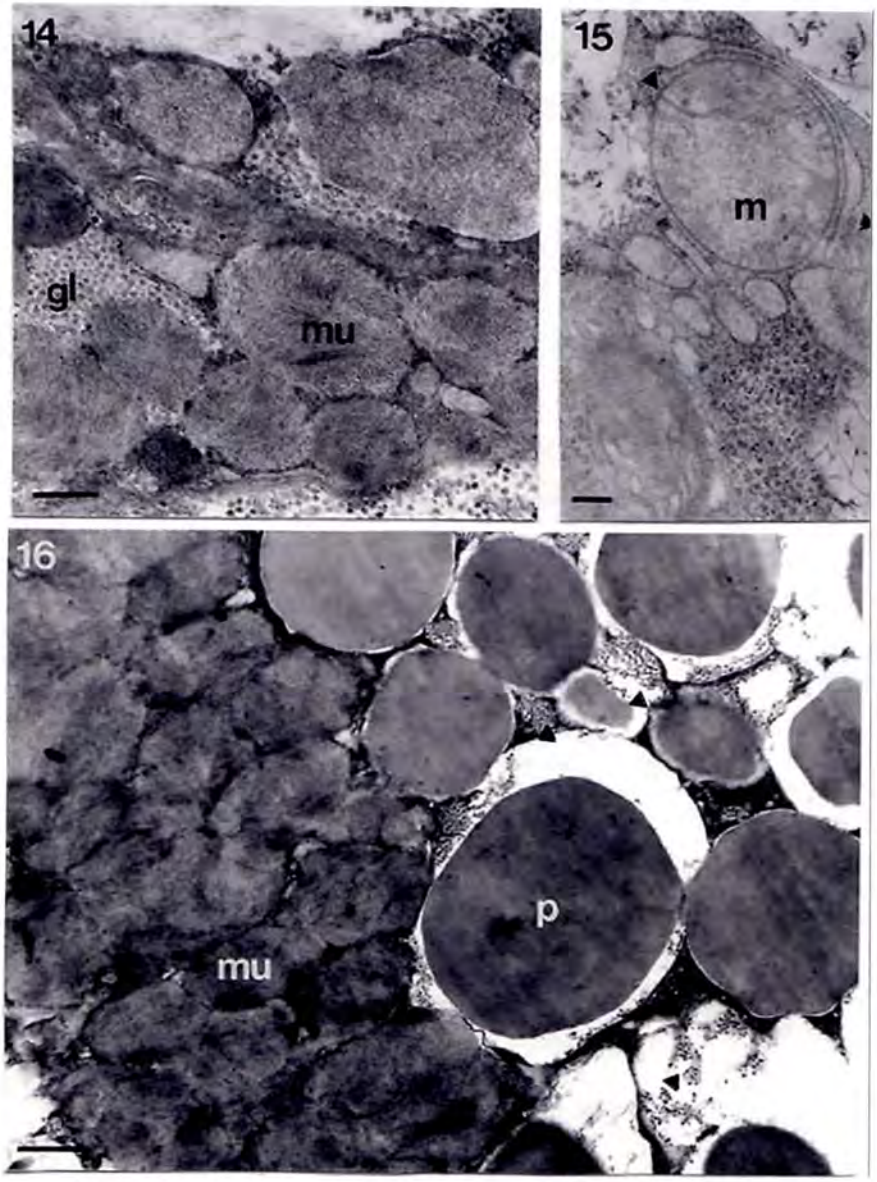


Fig. 14. An electron micrograph of the secretory cells of the albumen gland of *S. capensis* with mucous secretion (mu) and putative glycogen (gl) in the cytoplasm. Scale bar = 500 nm.
Fig. 15. The secretory cells of the albumen gland of *S. serrata* showing mitochondria (m) surrounded by RER (arrowheads). Scale bar =200 nm.
Fig. 16. The secretory cells of the albumen gland of *S. serrata* showing the presence of both protein (p) and mucous secretion (mu). arrowheads, putative glycogen. Scale bar = 1 μm.

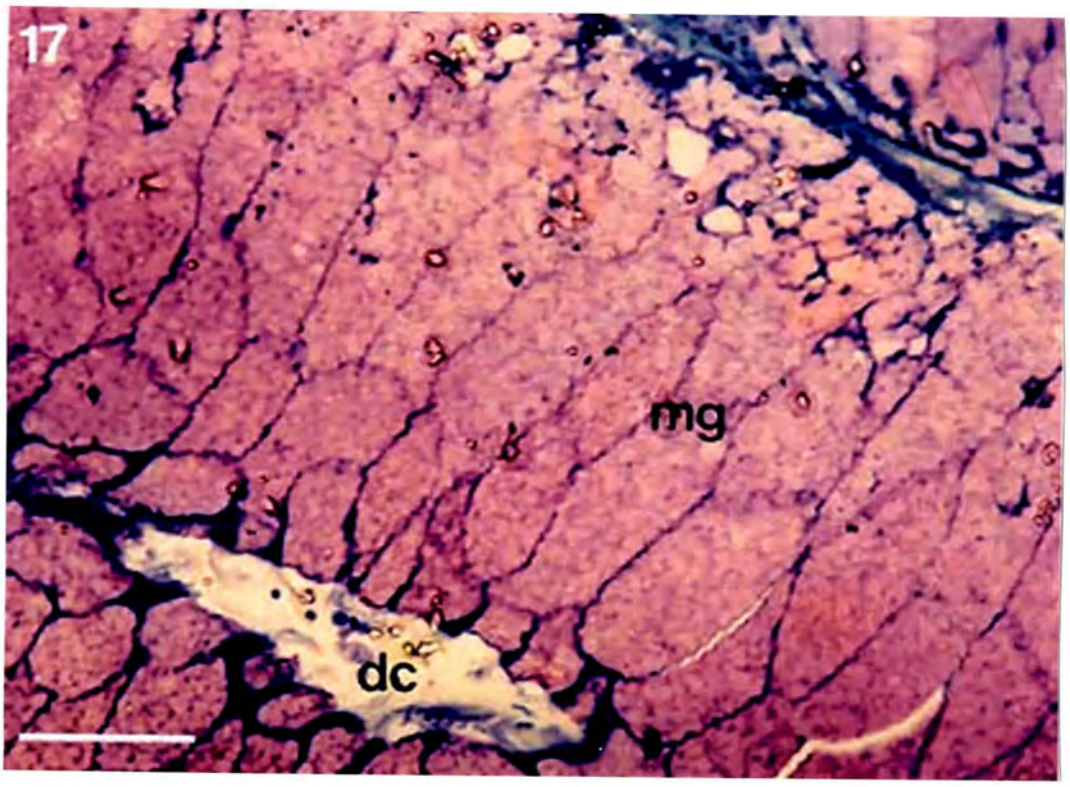
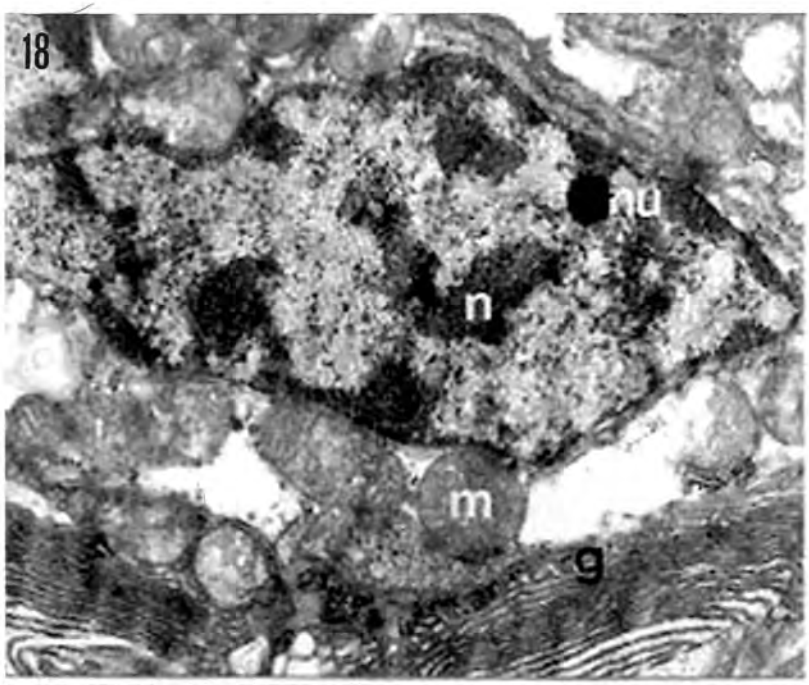


Fig. 17. A toluidene blue stained section of the glandular complex of *S. serrata* showing the arrangement of secretory cells of the
mucous gland (mg). dc, duct. Scale bar = 0.1 mm.

Fig. 18. The secretory cells of the mucous gland of *S. serrata* with a prominent nucleus (n) and some mitochondria (m) in the cytoplasm. g, Golgi body; nu, nucleolus. Scale bar = 500 nm.
Fig. 19. The secretory cells of the mucous gland of *S. serrata* with irregular-shaped mucous secretions (mu). Scale bar = 2 μm.
Fig. 20. Numerous Golgi bodies (g) in the secretory cells of the mucous gland of *S. serrata*. Scale bar = 1 μm.
Fig. 21. Putative glycogen granules (gl) and mucous material (mu) in the secretory cells of the mucous gland of *S. serrata*. Scale bar = 500 nm.
Fig. 22. The ciliated cells (cc) are interspersed between the secretory cells (sc) of the glandular complex of *S. serrata*. Scale bar = 2 μm.
Fig. 23. A wedge-shaped ciliated cell of *S. capensis* with a central nucleus (n), dense bodies (db), lipid (l) and mitochondria (m). Note numerous cilia (ci) with rootlets (rt) at the apex of the cell. Scale bar = 1 μm.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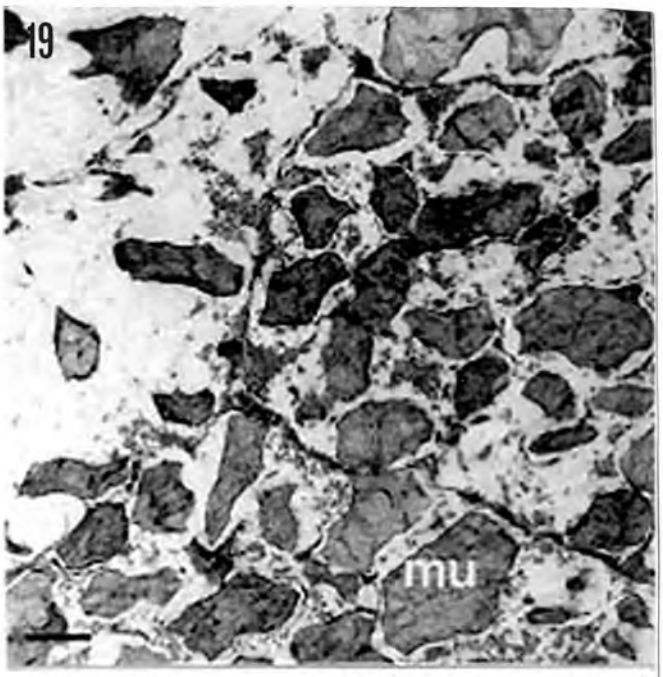
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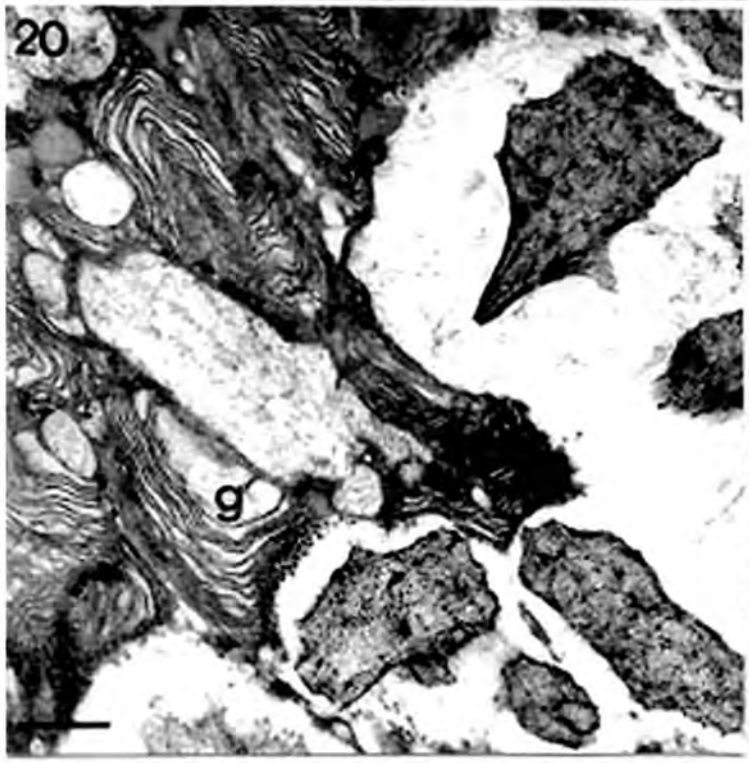
mu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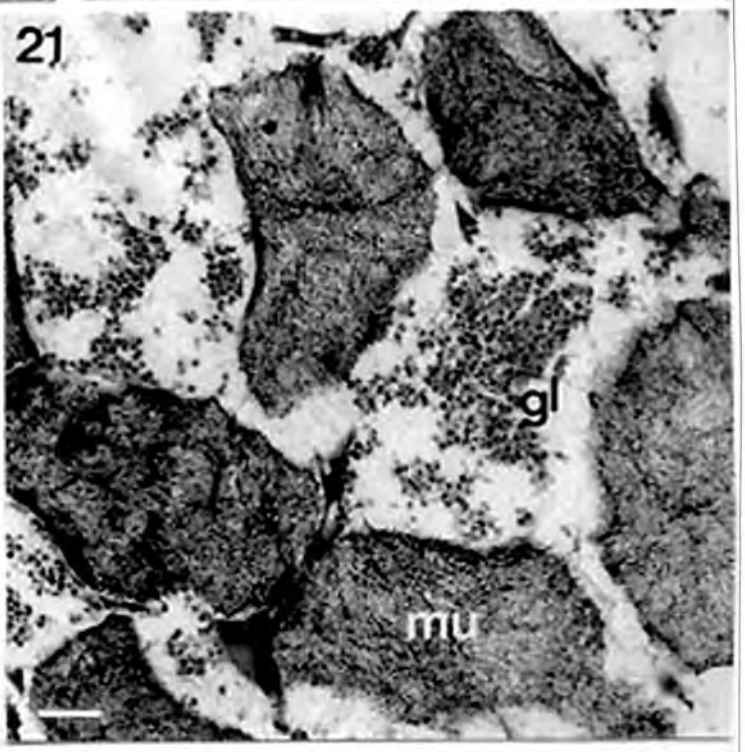
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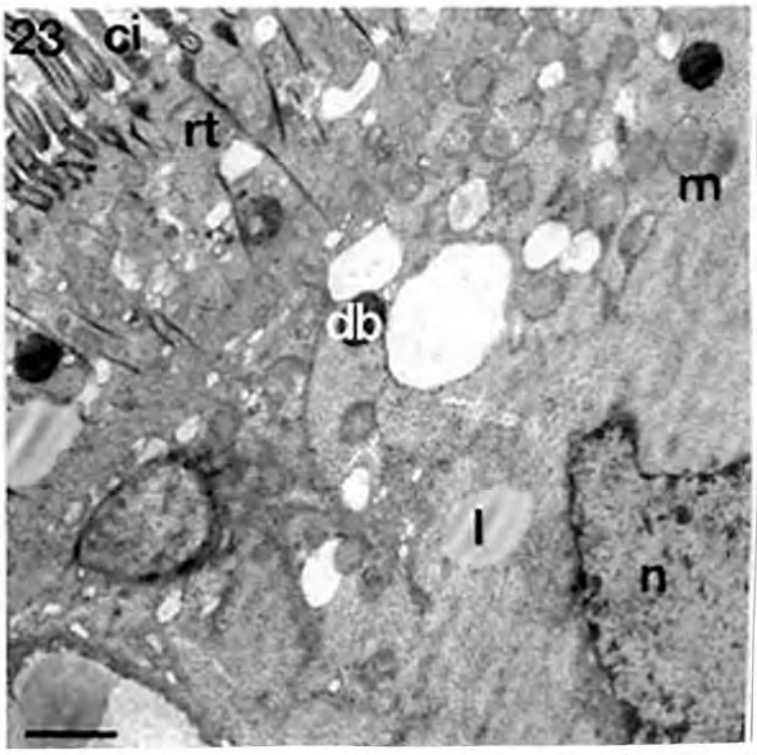
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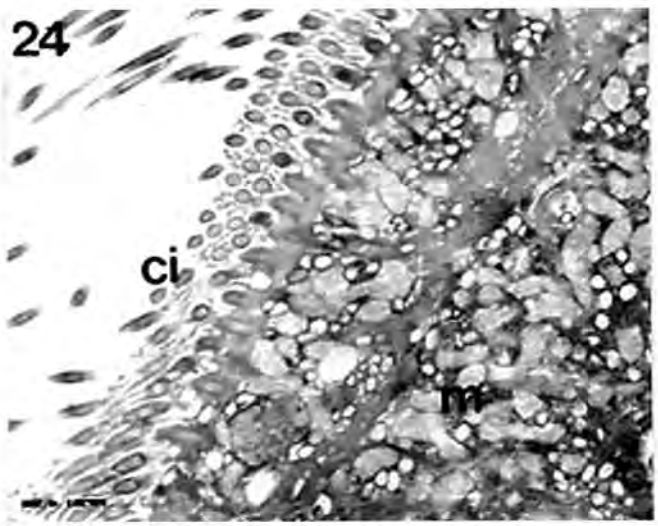
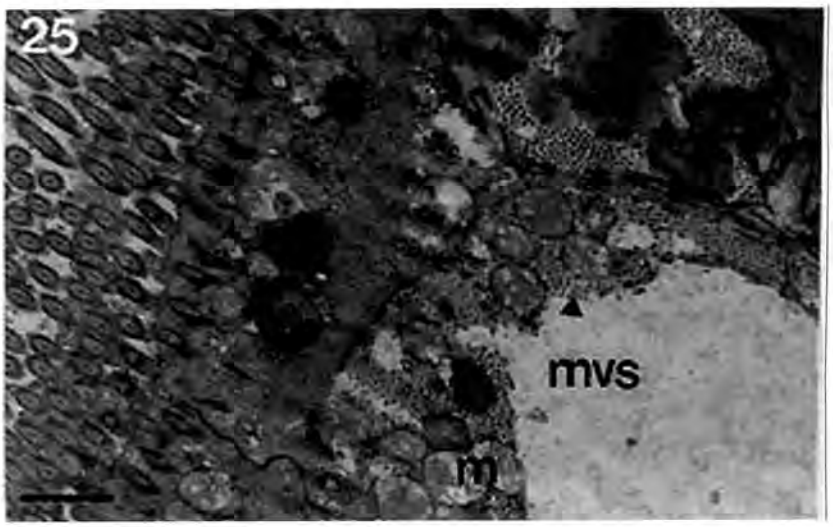
V. Ultrastructure of the supporting cells

In both glands the supporting cells are highly ciliated and interspersed between the gland cells (Fig. 22). They are usually triangular in shape and possess a centrally positioned prominent nucleus (Fig. 23). The cytoplasm contains numerous mitochondria, glycogen granules as well as a few lipid droplets (Figs. 23, 24 & 25). The cilia have long striated rootlets and mucous vesicles in the cytoplasm (Figs. 23 & 25).





Figs. 24 & 25 The ciliated cells with numerous mitochondria (m) in the apical cytoplasm, mucous vesicle (mvs) and putative glycogen granules (arrowheads). ci, cilia. Scale bars = 1μm.

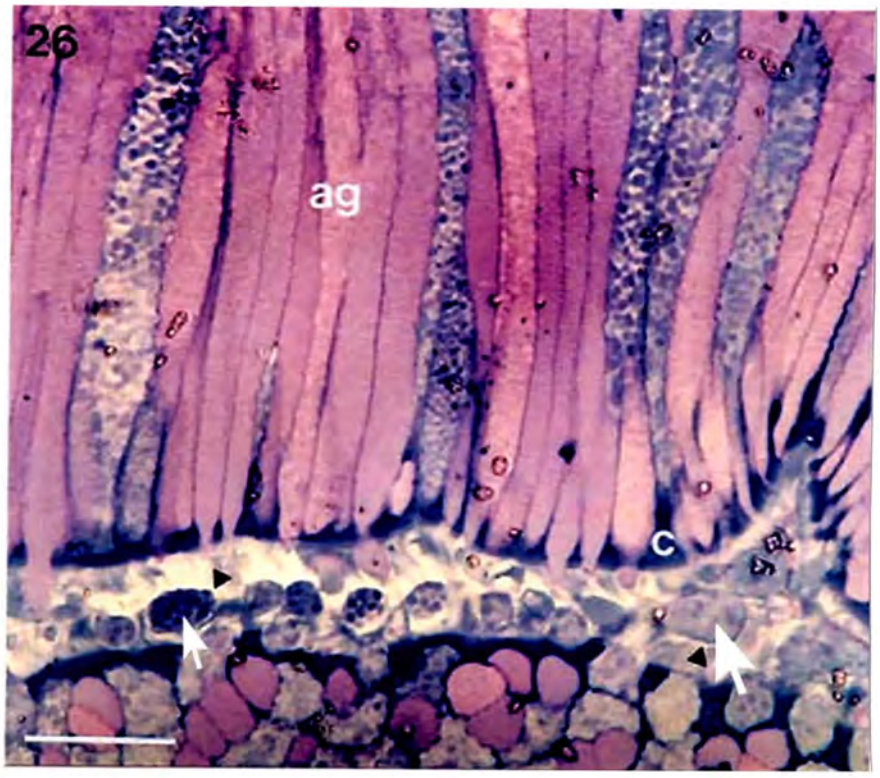
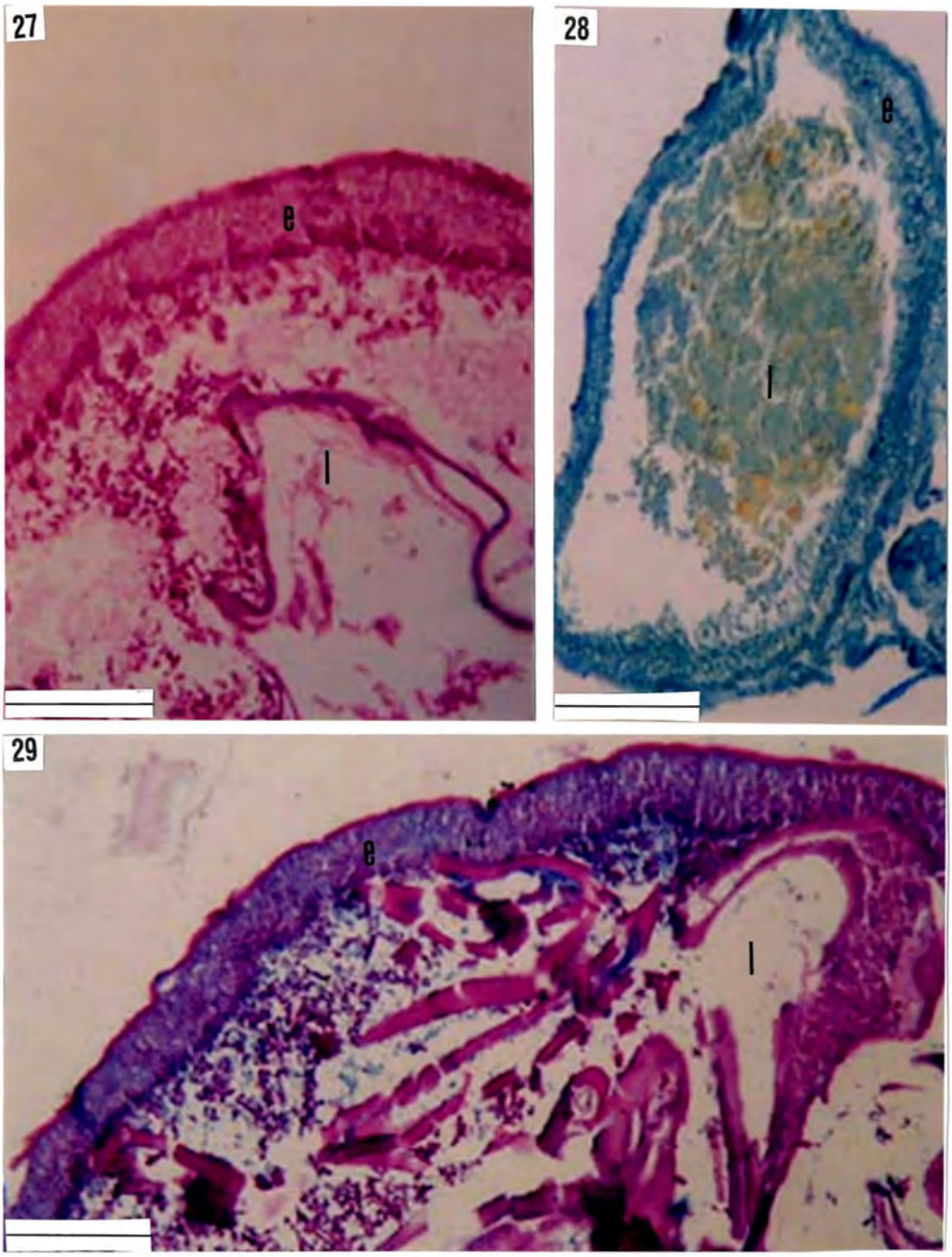


Fig. 26. A toluidene blue stained section of the albumen gland (ag) of *S. serrata* showing glandular secretion in the form of droplets (arrows) in the duct. c, ciliate cell. Scale bar = 0.1 mm.

The spermatheca of *S. capensis* and *S. serrata*

The spermathecae of *S. capensis* and *S. serrata* are round to oval, sac-like structures [mid-cross sectional area of 6.2-24.9 mm^2 (in *S. serrata*) and 2.4-8.4 mm^2 (in *S. capensis*)], located next to the junction of the glandular complex and the proximal end of the spermoviduct (Fig. 1). The spermathecal duct is long and it joins the spermoviduct just before the genital opening (Fig. 1).

The spermathecae are composed of an epithelium surrounding a lumen (Fig. 27). The sections of the spermathecae (epithelium and content of the lumen) of both species stained positively for polysaccharides with PAS (Fig. 28) and protein with bromophenol blue (Fig. 27). With AB-PAS the epithelium stained blue indicating the presence of acid-mucopolysaccharides (Fig. 29). Ultrastructurally, no difference was observed between the two species and therefore a single description only is presented here.



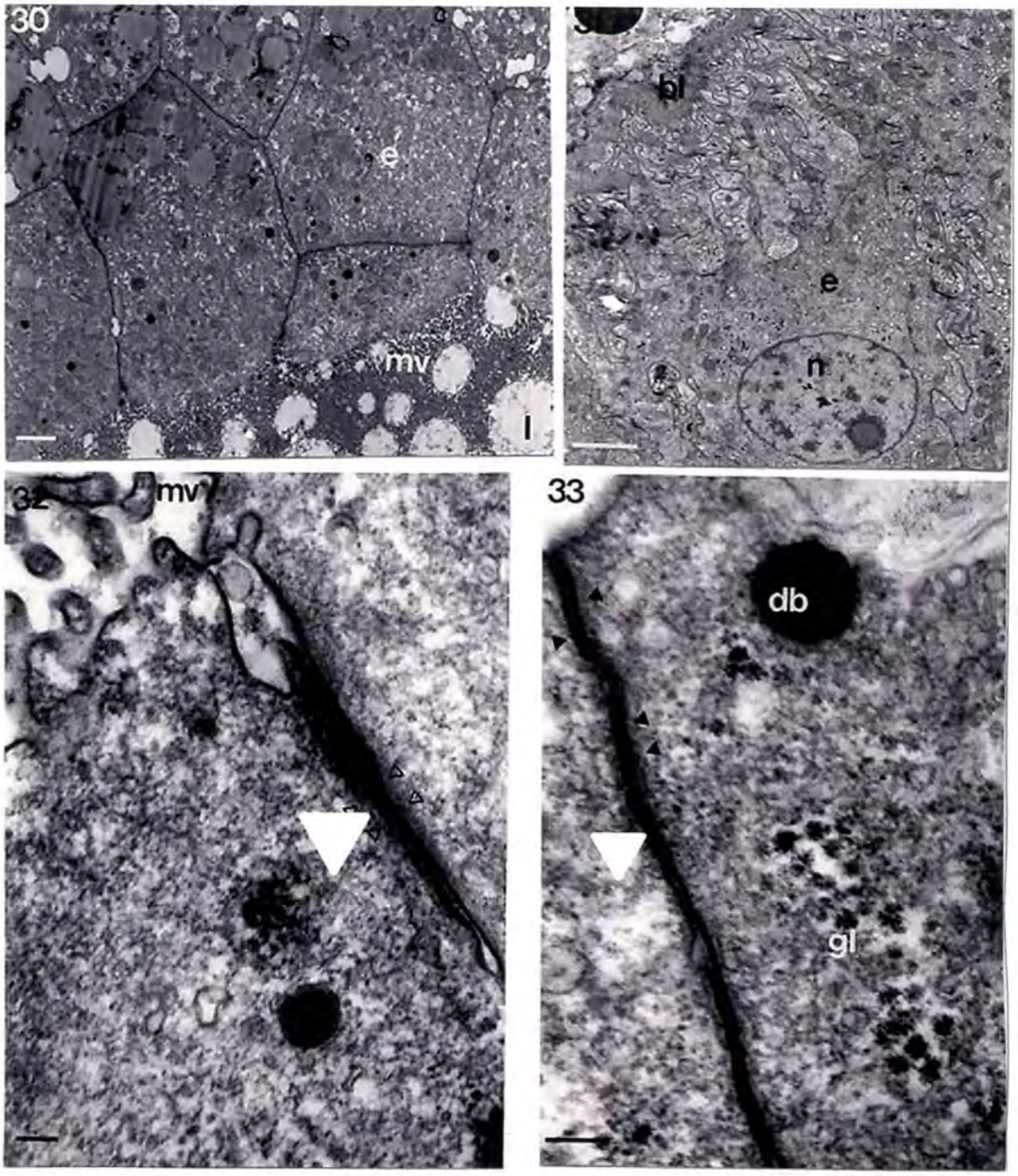
Figs. 27 & 28. The sections of the spermathecae of *S. serrata* (27) stained with PAS and *S. capensis* (28) stained with bromophenol blue. e, epithelium; l, lumen. Scale bars = 0.1 mm.
Fig. 29. A section of spermatheca of *S. serrata* stained with Alcian blue-PAS. e, epithelium; l, lumen. Scale bar = 0.1 mm.

The spermathecal epithelium is lined with a single layer of cuboidal to prismatic cells (Figs. 30 & 31). The apical part of the epithelial cells possesses long, unbranched microvilli, which form a brush border encircling the lumen of the spermatheca (Fig. 30). The epithelia of both species features only one type of non-ciliated cell (Figs. 30 & 31), the cells being joined apically as well as along their length by zonulae adhaerens (Figs. 32 & 33). Numerous intercellular spaces separate the cells laterally (Fig. 34). The epithelial cells, which rest on the basement membrane have highly folded basal cell membranes (Fig. 35). They contain a round, basal nucleus (approximately 4.1 x 4.7 μm in diameter) with a prominent nucleolus (Figs. 35). Present within the cell cytoplasm long to dumb-bell shaped mitochondria (about 285 x 357 nm) with prominent lamellar cristae (Fig. 36), rough endoplasmic reticular cisternae, which surround the nucleus, are numerous (Fig. 37). Electron-dense granules (Fig. 33), multivesicular bodies (Fig. 38), lipid (Fig. 34) and putative glycogen granules (Fig. 33) are also present. Numerous small vesicles with an electron-lucent content are seen throughout the cell cytoplasm (Fig. 36) whereas the Golgi bodies were only observed in the basal cytoplasm (Fig. 39).

The spermathecal lumen in both species occasionally contains sperm as well as cellular debris (Figs. 40-43). Once some cellular bodies with numerous processes were observed in the spermathecal lumen of *S. capensis* in the close proximity of the epithelial layer as well as sloughing off the epithelium (Fig. 44). These cellular structures contained abundant mitochondria and putative glycogen granules, which were aggregated in the form of rosettes (Fig. 44).

Smooth muscle cells and pore cells constitute the wall around the epithelium of the

spermatheca (Fig. 45). The smooth muscle cells are arranged in layers forming tight units underneath the basal lamina (Figs. 46). An elongated nucleus (Fig. 47) and mitochondria with longitudinal cristae are also present in the muscle cells (Fig. 48).



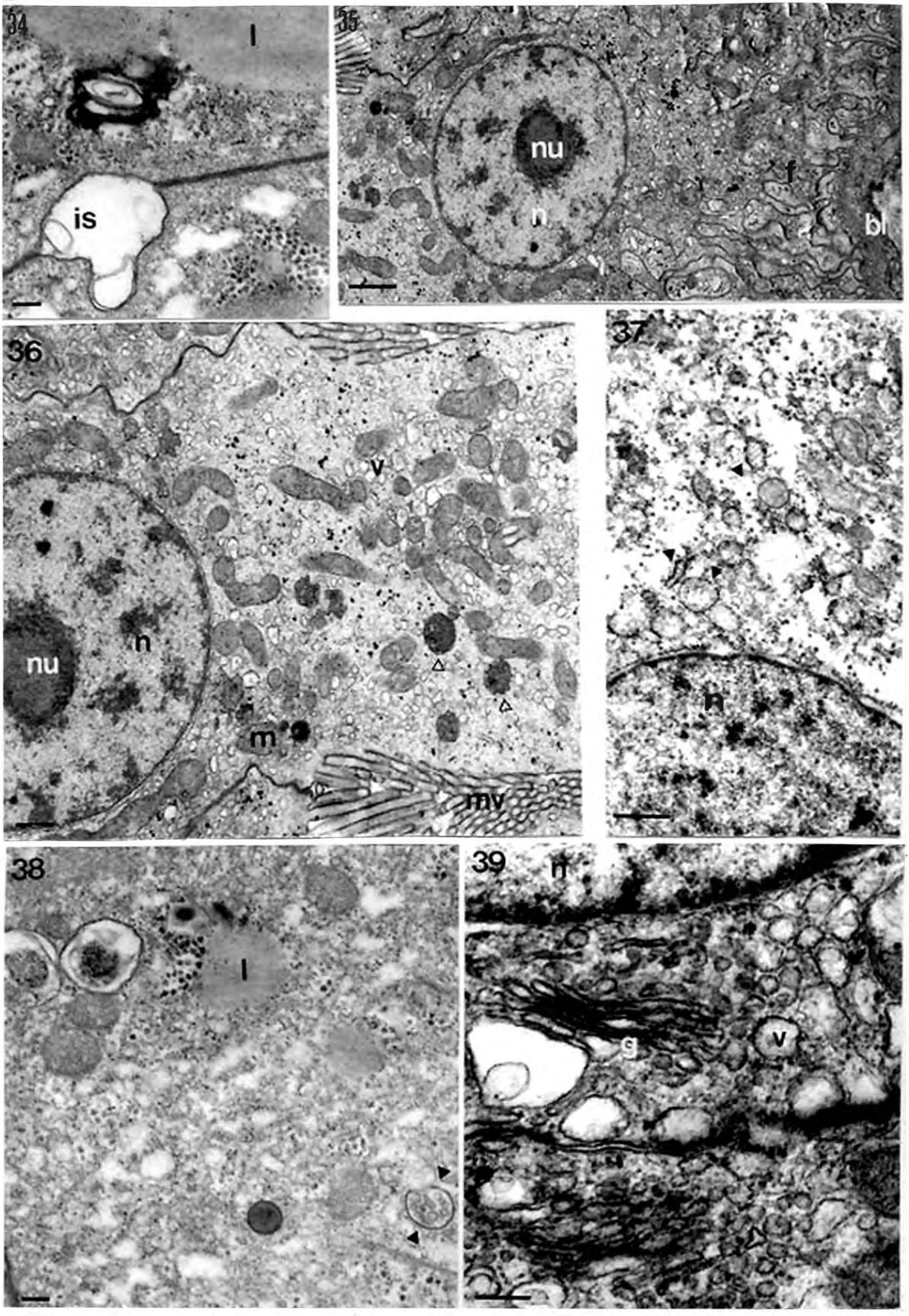
Figs. 30 & 31. Electron micrographs of spermatheca of *S.* capensis (30) and *S. serrata* (31) showing mono-layered epithelial cells (e) with numerous microvilli (mv). Bl, basal lamina; l, lumen; n, nucleus. Scale bars = 2 μm.
Figs. 32 & 33. The epithelial cells of spermatheca of *S. serrata* are joined by zonulae adhearens both apically (arrowhead) and laterally (arrowhead). db, dense body; gl, putative glycogen; mv, microvilli. Scale bars = 200 nm.

Fig. 34. An electron micrograph of spermatheca of *S.capensis* showing an intercellular space (is) between two epithelial cells. l, lipid. Scale bar = 200 nm.

Figs. 35 & 36. The epithelial cells of spermatheca of *S.capensis* (35) and *S. serrata* (36) with basal in-foldings (f) resting on the basal lamina (bl). clear arrowheads, dense bodies; m, mitochondria; mv, microvilli; n, nucleus; nu, nucleolus; v, vesicles. Scale bars = 1 μm & 500 nm respectively.

Fig. 37. The epithelial cells of *S. serrata* show a presence of RER (arrowheads) around the nucleus (n). Scale bar = 500 nm.

Fig. 38 & 39. A presence of multivesicular body (arrowheads), lipid (l), Golgi bodies (g) and vesicles (v) in the epithelial cell cytoplasm. N, nucleus. Scale bars = 200 nm. The micrographs are representatives of *S. capensis* and *S. serrata* respectively.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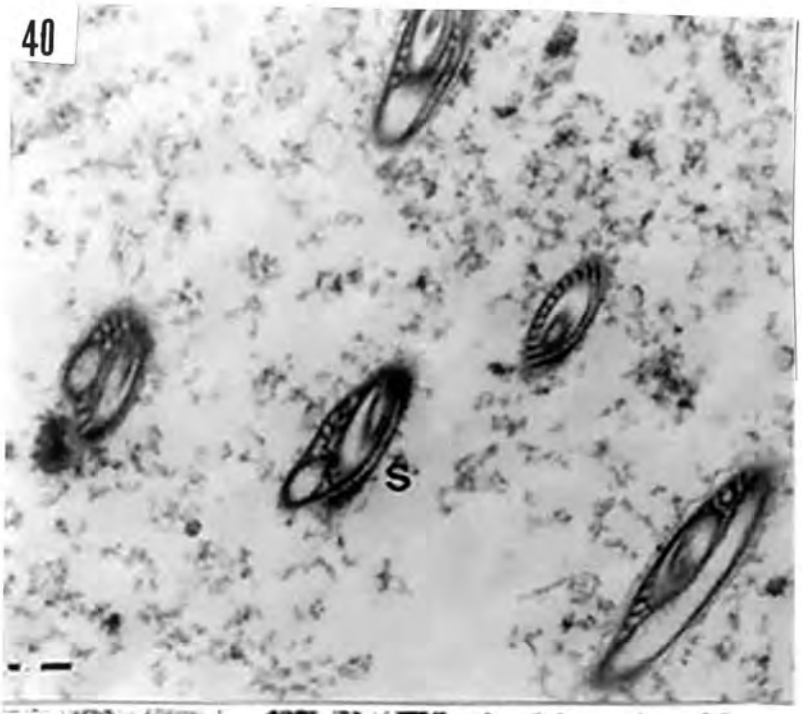
Figs. 40-43. The spermathecal lumen of *S. serrata* showing the presence of sperm (s) and cellular debris. Scale bars= 500 nm, 200 nm, 1 & 2 μm respectively.
Fig. 44. The cellular bodies (cb) in the spermathecal lumen of *S. capensis* containing putative glycogen (gl) and mitochondria (arrow). arrowheads, mitochondria; e, epithelial cells. Scale bar = 2 μm.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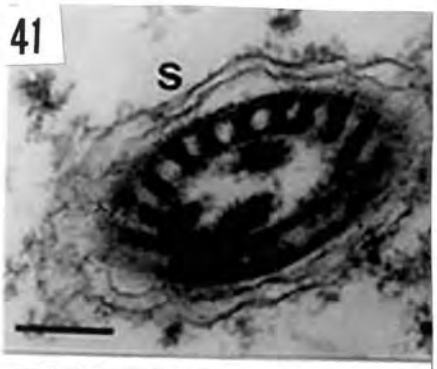
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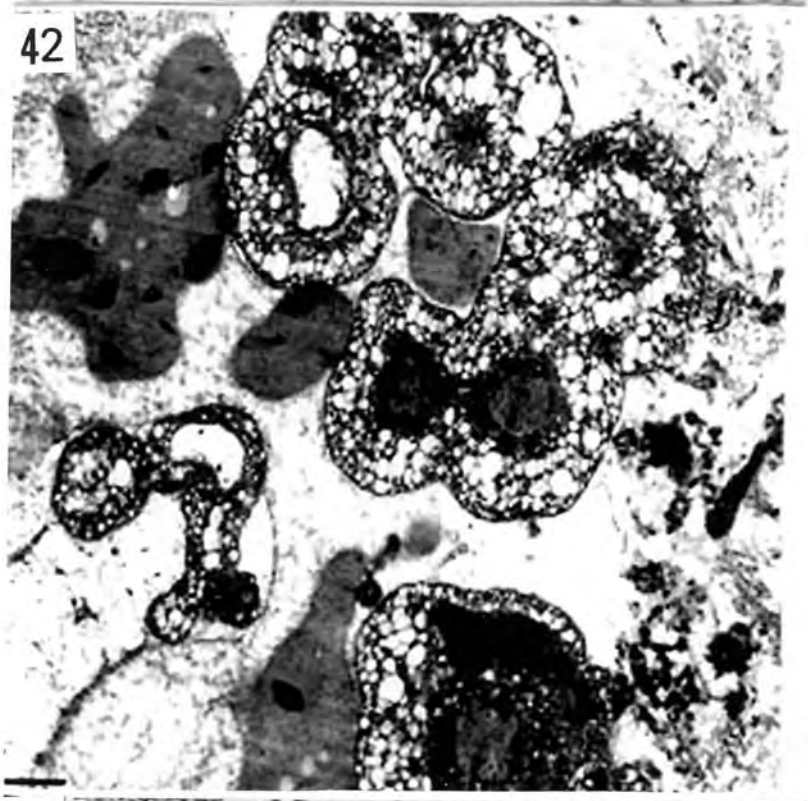
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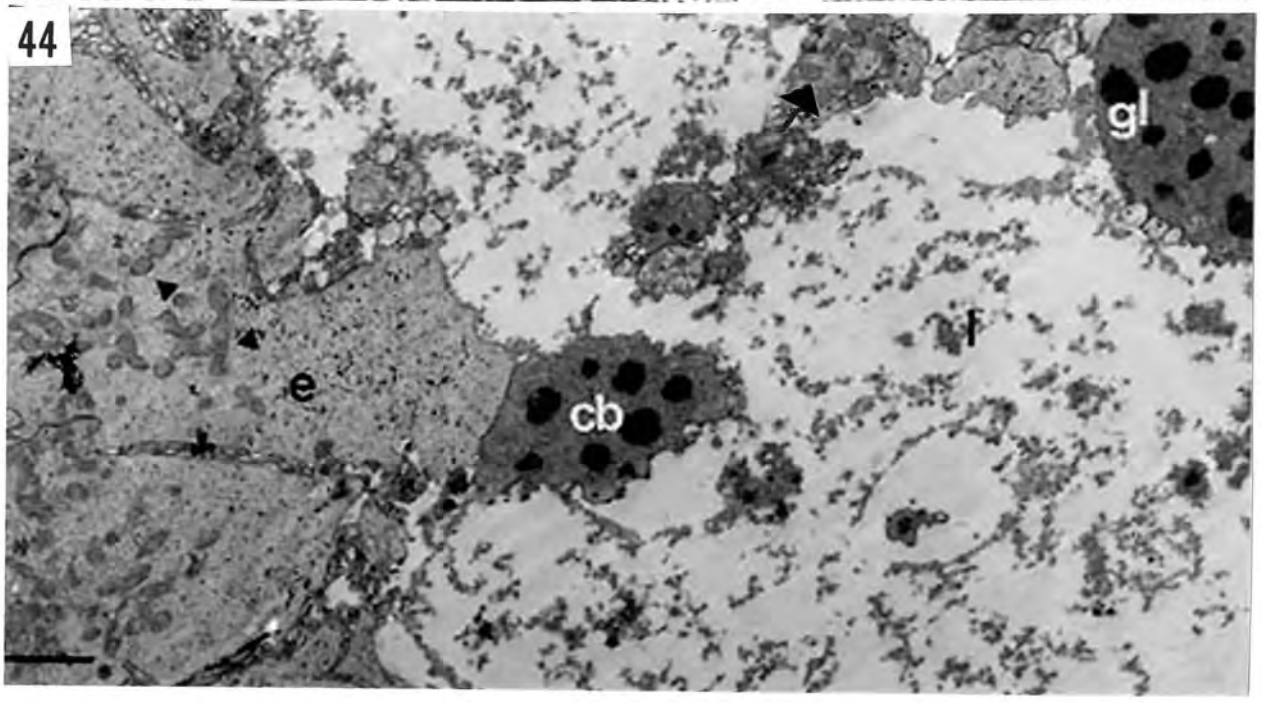
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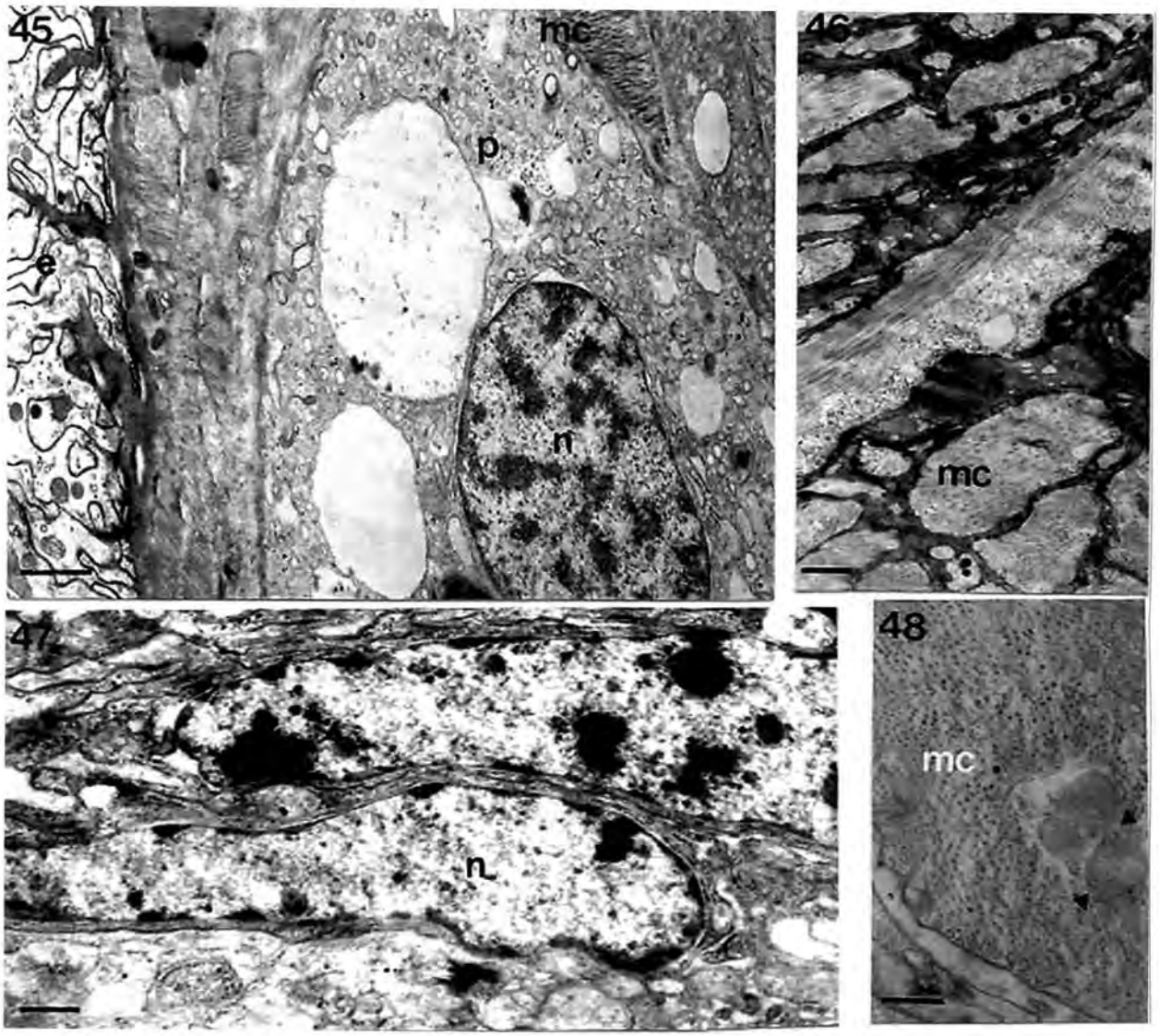
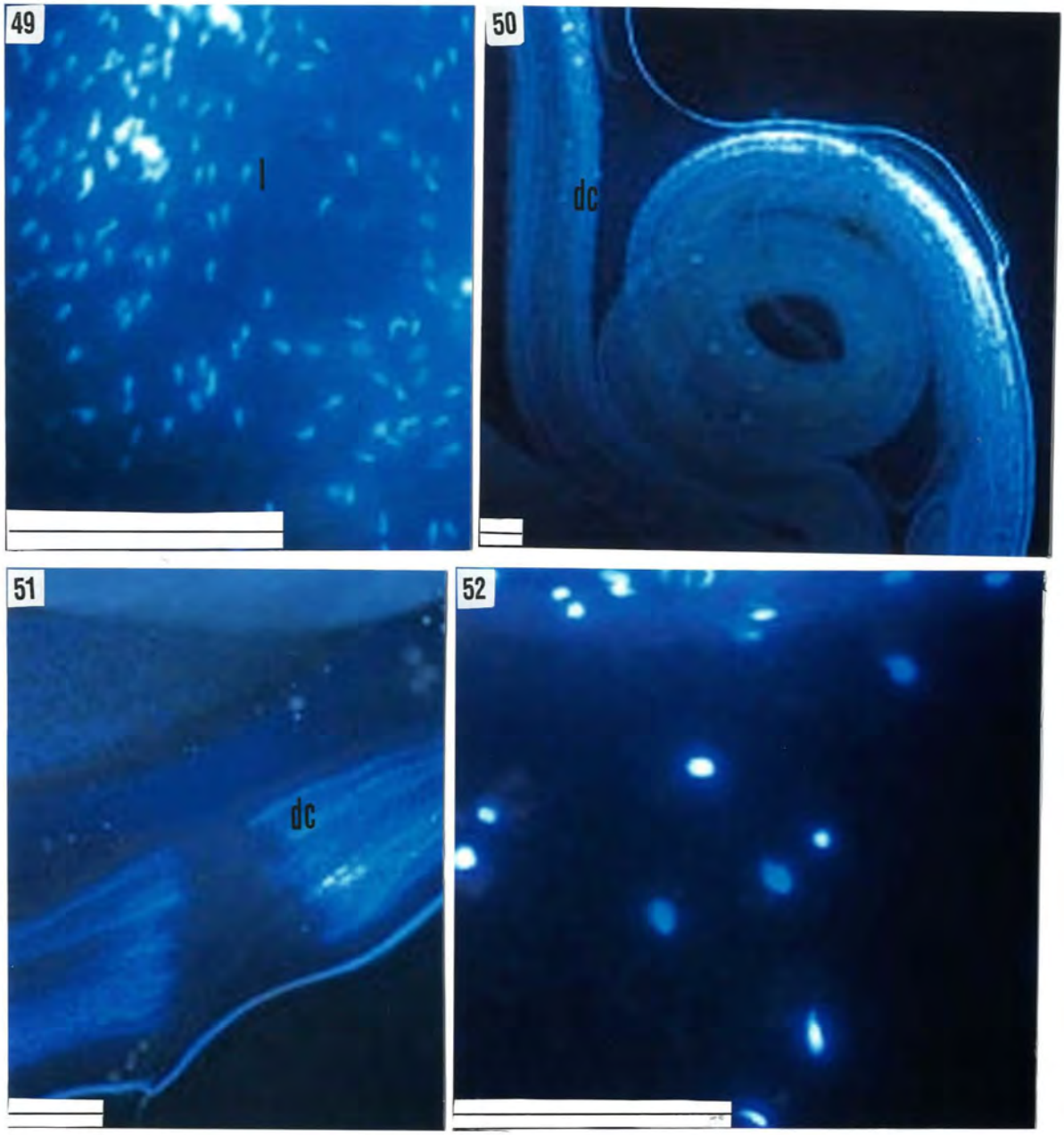


Figs. 45-48. The connective tissue around the spermatheca of *S. serrata* (45, 46 & 48) and *S. capensis* (47) showing the presence of muscle cells (mc) and pore cells (p). arrowheads, mitochondria; e, epithelium; n, nucleus. Scale bars = 1 μm, 1 μm, 500 nm & 500 nm respectively.

In the lumen of spermatheca and the spermathecal duct, sperm were observed with fluorescence microscopy (Figs. 49-52). The scanning electron microscope did not provide any useful information in this area of the investigation.



Figs. 49-52. Fluorescence micrographs of the spermathecal lumen (l) and duct (dc) of *S. capensis* and *S. serrata* showing sperm nuclei. Scale bars = 0.1 mm. Figs. 49-51 represent *S. serrata* and Fig. 52 shows sperm in the spermathecal lumen of *S. capensis*.

The size of spermathecae differed significantly between *S. capensis* and *S. serrata* (Table 1). The size of the spermathecae was positively correlated with the shell length of the animals (Table 1) although the linear regression between the shell length of *S. capensis* and the size of the spermatheca was not significant (Fig. 53). By contrast, in *S. serrata* the linear regression between the shell length and size of spermatheca was significant (Fig. 54). The spermathecae of *S. serrata* were significantly bigger than those of *S. capensis* (Fig. 55).

Table 1. An analysis of covariance showing the size difference of spermatheca between *S. capensis* and *S. serrata*. Significant p values ($p < 0.05$) are in bold.

Sources	***df***	***MS***	***F***	***p***
Shell length	1	187.048	19.008	**0.002**
Species	1	157.560	16.011	**0.003**
Residual	9	9.841		

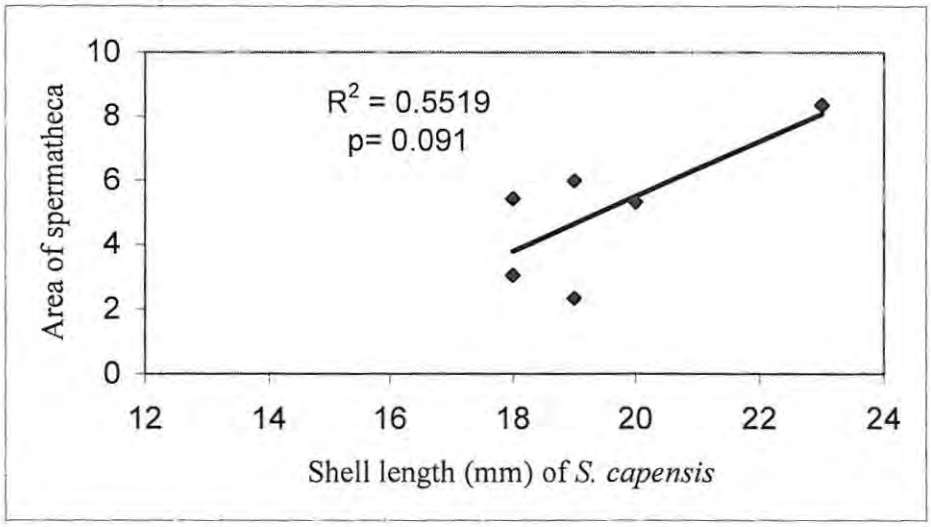


Fig 53. Linear relationship between the shell length of *S. capensis* and the area (in mm^2) of spermathecae.

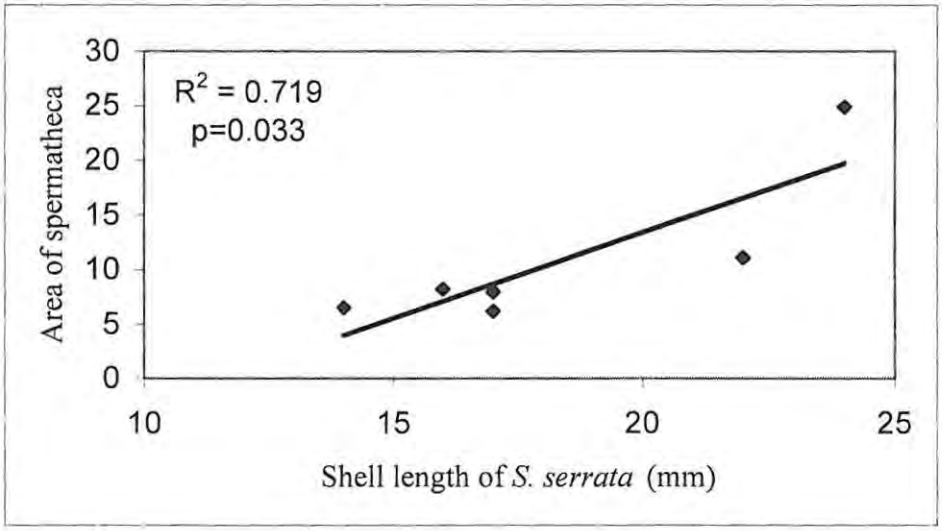


Fig. 54. Linear relationship between the shell length of *S. serrata* and the area (in mm^2) of spermathecae.

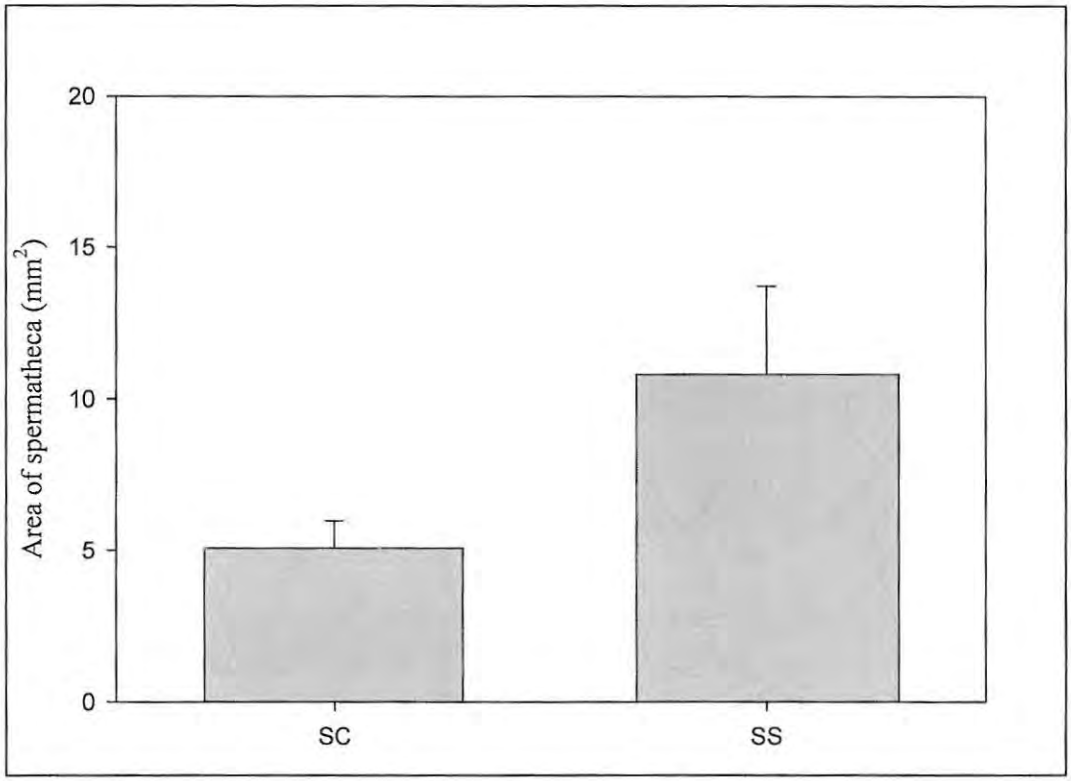


Fig. 55. The difference in size of spermathecae (n = 6) between *S. capensis* (SC) and *S. serrata* (SS). Data presented with standard error bars.

Discussion

The general structure of the glandular complex and spermatheca of *S. capensis* and *S. serrata* is similar. The glandular complex consists of a highly folded tubular albumen gland and a mucous gland, which is embedded amongst the folds of the albumen gland as also reported by Berry (1977) for *S. atra* and *S. exigua*. Hubendick (1947) cited in Berry (1977) suggested that the mucous gland (= posterior mucous gland) of *Siphonaria* sp. is similar to that of some ellobiids in the way the mucous gland is coiled and extended further along the reproductive tract. The tubular form of the albumen gland, with its characteristic columnar secretory cells and highly ciliated supporting cells, seems to be universal among the subclass Pulmonata with the exception of *Pythia* (Duncan, 1975; Berry, 1977; Geraerts & Joosse, 1984; Runham, 1988).

The ultrastructure of the secretory cells of the albumen and mucous glands is similar to that observed in opisthobranchs (Klussmann- Kolb, 2001a). The secretory cells of the albumen gland possess Golgi bodies and RER, whereas in the mucous gland (which is called the membrane gland in opisthobranchs) the cells contain Golgi bodies only (Klussmann- Kolb, 2001a & present study on siphonariids). It is difficult to ascertain whether the albumen gland of *S. capensis* and *S. serrata* possesses two types of secretory cells (one stains positive for polysaccharides and another for protein), even though it is suggested in the current study that only one cell type is present as both type of secretion was noted in some cells (based on histology and electron microscopy). The only histochemical difference between the albumen and mucous gland of siphonariid limpets was the presence of protein in the albumen gland. The secretory granules of the albumen

gland cells of *S. capensis* and *S. serrata* stain positively with bromophenol blue suggesting that the granules have more than one component as they also stained positive with PAS (present study). This has also been observed in the secretory cells of the albumen gland of the freshwater basommatophoran, *Biomphalaria* (De Jong-Brink, 1969).

In other pulmonates (both aquatic and terrestrial) the albumen gland has been shown to be the source of perivitelline fluid, which provides nutrition to the developing embryo in addition to the yolk that forms during vitellogenesis (Runham, 1988). Several authors have speculated about the function of the albumen gland on the basis of histochemistry (for example, De Jong- Brink, 1969; Plesch *et al.*, 1971). According to De Jong- Brink (1969) and Duncan (1975) the albumen glands of pulmonates contain galactogen and protein. The current observations (both histochemical and ultrastructural) of the albumen glands of *Siphonaria* (*S. capensis* and *S. serrata*) suggest that they secrete neutral polysaccharides, mucopolysaccharides (mainly neutral) and protein. This secretion possibly contributes towards the perivitelline fluid, which surrounds the developing embryo, as this fluid contains neutral polysaccharides, basic to neutral mucins and protein (see Chapter 5).

Although egg mass formation along the spermoviduct in siphonariids has not been investigated here, it can be hypothesized that the mucous gland plays some role in the formation of mucous membranes and the matrix around the egg capsules. As the egg masses of *S. capensis and S. serrata* are composed largely of mucopolysaccharides (see Chapter 5) it is probable that the mucous gland has an important role in egg mass formation in these species. Plesch *et al.*, (1971) also suggested the same for the

muciparous gland of *Lymnaea*, a freshwater basommatophoran. The reproductive tract of pulmonates, especially the glandular sections, are known to contain more than one cell type which could not be confirmed in the siphonariids studied here (De Jong-Brink, 1969; Plesch *et. al.*, 1971; Rudolph, 1983). Different cell types of the albumen gland and mucous gland could be different physiological stages of the same cell type. Klussmann-Kolb (2001b) has also expressed similar views in a study of the nidamental glands of nudibranchs. Both glands of the glandular complex in siphonariids release their secretions in the form of droplets, which presumably follow the common spermoviduct to reach the carrefour region. Both the albumen gland and the mucous gland are merocrine. Klussmann-Kolb (2001a) has also suggested a merocrine mode of secretion for albumen glands of opisthobranchs whereas the mucous and membrane glands stop secreting once mature. Although the function of supporting cells has not been determined in this study it can be proposed that they facilitate transporting the glandular secretion with the aid of cilia.

The other structure examined also associated with the female reproductive system, was the spermatheca or bursa copulatrix. Although there have been few investigations regarding sperm storage in the spermatheca (a structure, that is associated with fertilization chamber of terrestrial pulmonates) (Bojat *et al.*, 2001a & b), there is no clear consensus on sperm storage in marine pulmonates. Duncan (1975) stated that fertilization and the fate of both autosperm and allosperm is highly variable in pulmonates. All the previous studies on the spermatheca in basommatophoran pulmonates had suggested it plays a role in the disintegration of excess foreign gametes (De Jong- Brink, 1969; Plesch *et al.*, 1971; Németh & Kovács, 1972; Duncan, 1975; Berry, 1977; Rogers *et al.*, 1980;

Gomez *et al.*, 1991).

The present study shows that the spermatheca of *S. capensis* and *S. serrata* is a simple sac-like structure with a long duct, which joins the spermoviduct. The presence of sperm in the lumen of both the spermathecae and spermathecal duct suggests that the organ has the capability of transporting sperm along its duct to the lumen of the organ. The ultrastructure of the organ also showed a presence of a smooth muscle system that may support the change in the volume after reproductive activities (pers. obs) although the role of the musculature needs further investigation as it has been carried out in *Arianta* (Bojat *et al.*, 2001a). The sperm that were found in the lumen or in the duct could only be allosperm as it is unlikely in siphonariids that autosperm would travel to the spermathecae for disintegration. Another role has been attributed to the spermatheca, which entails sperm maturation while allosperm are being stored in the lumen (Duncan, 1975). Although Berry (1977) has stated that in many siphonariids sperm are transferred via spermatophores, it is not known whether the spermathecae of *S. capensis* and *S. serrata* also help in sperm maturation as spermatophores have not been observed in this study. The cellular bodies observed in the spermatheca lumen of *S. capensis* are packed with glycogen, which might be used for sperm maintenance (A. Hodgson pers. comm.). An abundance of mitochondria in the epithelial cells of *S. capensis* suggested that sloughing off cells might be an energetically expensive process. Mitochondria might also be supporting any metabolic activity that is carried out by the epithelial cells (presence of Golgi bodies and small vesicles in the cytoplasm, lipid synthesis).

The results from the ultrastructural study of the epithelial cells and the connective tissue as well as the histochemical and fluorescence microscopy, all indicate that this

organ in *S. capensis and S. serrata* can receive and store sperm before degeneration (by pseudopodial cells as in *Leucopythia*? Morton, 1955 cited in Berry, 1977) as sperm were often observed in the spermathecal lumen and the duct of the animals. Although the question remains whether this is the only organ that acts as a sperm storage site in siphonariids or the sperm can also be stored and manipulated elsewhere as shown in other stylommatophorans (Haase & Baur, 1995; Bojat *et al.*, 2001a & b). The morphology of the fertilization pouch, seminal vesicle of the hermaphrodite duct should also be investigated in the future as these sites might influence sperm competition (Baur, 1998), which may lead to post-copulatory female choice influencing paternity (Eberhard, 1996 cited in Baur, 1998; Bojat *et al.*, 2001a). The difference in the size of spermatheca between two limpet species was significant although the reason behind this variation and its significance in sperm storage remains unclear.

Ponder & Lindberg (1997) stated that phylogeny of the clade Euthyneura needs special attention as biologists still render opisthobranchs and pulmonates equal rank. They also suggested paraphyly or polyphyly of opisthobranchs and pulmonates. Although monophyly of the clade Euthyneura has been confirmed, the evolutionary relationships within Euthyneurans are still unresolved (Dayrat & Tillier, 2002). The present study showed a proximally situated tubular albumen gland and a mucous gland in *S. capensis* and *S. serrata,* which is similar to the observations made on opisthobranchs (Klussmann-Kolb, 2001a). The ultrastructure of the mucous gland cells of *S. capensis* and *S. serrata* is comparable to the ultrastructure of membrane gland cells of opisthobranchs (Klussmann-Kolb, 2001a). Although the terminologies of nidamental glands are confusing in both opisthobranchs and pulmonates, the current study did not examine the glands further

down the reproductive tract, so the presence or absence of a membrane gland cannot be commented upon here. The proximal albumen gland, distal membrane or mucous gland and tubular structure of glands, both the arrangement and the structure have been considered plesiomorphic by Klussmann-Kolb (2001a) on the basis of comparison between the sister groups of Opisthobranchia, which cannot be confirmed here as the present study only investigated two species. Nevertheless, within pulmonates especially basommatophorans, *S. capensis* and *S. serrata* showed a median glandular complex with incomplete separation between the albumen gland and mucous gland, which may be the most primitive condition.

References

ALLANSON, B. R. 1959. On the systematics and distribution of the molluscan genus *Siphonaria* in South Africa. *Hydrobiologia*, **12**: 149- 180.

BÁO, S. N., KITAJIMA, E. W., CALLAINI, G., LUPETTI, P. & DALLAI, R. 1996. Spermiogenesis in three species of whitefly (Homoptera, Aleyrodidae). *Acta Zoologica* (Stockholm), **78**: 163- 170.

BAUR, B. 1998. *Sperm competition in molluscs*. In: *Sperm competition and sexual selection*. T. R. Birkhead & A. P. Møller (eds.), pp. 255- 305. Academic Press. New York.

BERRY, A. J. 1977. *Gastropoda: Pulmonata*. In *Reproduction of Marine Invertebrates*. IV. A. C. Giese & J. S. Pearse (eds), pp. 181- 226. Academic Press. New York.

BOJAT, N. C., DÜRRENBERGER, M. & HAASE, M. 2001a. The spermatheca of the land snail *Arianta arbustorum* (Pulmonata: Stylommatophora): muscle system and potential role in sexual selection. *Invertebrate Biology*, **120**: 217- 226.

BOJAT, N. C., SAUDER, U. & HAASE, M. 2001b. The spermathecal epithelium, sperm and their interactions in the hermaphroditic land snail *Arianta arbustorum* (Pulmonata: Stylommatophora). *Zoomorphology*, **120**: 149- 157.

BOJAT, N. C., SAUDER, U. & HAASE, M. 2002. Functional anatomy of the sperm storage organs in Pulmonata: the simple spermatheca of *Bradybaena fruticum* (Gastropoda: Stylommatophora). *Zoomorphology*, **121**: 243- 255.

DAYRAT, B. & TILLIER, S. 2002. Evolutionary relationships of euthyneuran gastropods (Mollusca): a cladistic re-evaluation of morphological characters. *Zoological*

Journal of the Linnean Society, **135**: 403- 470.

DE JONG- BRINK, M. 1969. Histochemical and electron microscope observations on the reproductive tract of *Biomphalaria glabrata* (*Australorbis glabratus*), intermediate host of *Schistosoma mansoni*. *Z. Zellforsch.*, **102**: 507- 542.

DUNCAN, C. J. 1975. *Reproduction*. In: *Pulmonates*. Vol. 1 (V. Fretter & J. Peake eds.), pp. 309- 365. Academic Press. London.

GERAERTS, W. P. M. & JOOSSE, J. 1984. *Freshwater snails (Basommatophora)*. In: *The Mollusca*. Vol, VII (A. S. Tompa, N. H. Verdonk & J. A. M. van den Biggelaar, eds), pp. 141- 207. Academic Press. London.

GOMEZ, B. J., ANGULO, E. & ZUBIAGA, A. M. 1991. Ultrastructural analysis of the morphology and function of the spermatheca of the pulmonate slug *Arion subfuscus*. *Tissue and Cell*, **23**: 357- 365.

HAASE, M. & BAUR, B. 1995. Variation in spermathecal morphology and storage of spermatozoa in the simultaneously hermaphroditic land snail *Arianta arbustorum* (Gastropoda: Pulmonata: Stylommatophora). *Invertebrate Reproduction and Development*, **28**: 33- 41.

HIRANO, Y. & INABA, A. 1980. *Siphonaria* (Pulmonate limpet) survey of Japan. I. Observations on the bahaviour of *Siphonaria japonica* during breeding season. *Publications of Seto Marine Biological Lab.*, **25**: 323- 334.

HODGSON, A. N. 1999. The biology of siphonariid limpets (Gastropoda: Pulmonata). *Oceanography and Marine Biology: an annual review*, **37**: 245- 314.

HUBENDICK, B. 1978. *Systematics and comparative morphology of the Basommatophora*. In *Pulmonates* 2A, V. Fretter & J. Peake (eds). London: Academic

Press.1- 47.

HYMAN, L. H. 1967. *The Invertebrates*, Volume VI, Mollusca I. New York: McGraw-Hill.

KLUSSMANN- KOLB, A. 2001a. Comparative investigation of the genital systems in the Opisthobranchia (Mollusca: Gastropoda) with special emphasis on the nidamental glandular system. *Zoomorphology*, **120**: 215- 235.

KLUSSMANN- KOLB, A. 2001b. The reproductive systems of the Nudibranchia (Gastropoda: Opisthobranchia): comparative histology and ultrastructure of the nidamental glands with aspects of functional morphology. *Zoologischer Anzeiger*, **240**: 119- 136.

LUCHTEL, D. L., MARTIN, A. W., DEYRUP- OLSEN, I. & BOER, H. H. 1997. *Gastropoda: Pulmonata*. In: *Microscopic Anatomy of Invertebrates*. Vol. 6B (F. W. Harrison & A. J. Kohn, eds.), pp. 459- 718. Wiley- Liss. New York.

MARCUS, E. & MARCUS, E. 1960. On *Siphonaria hispida*. Universidade de Sao Paulo. Faculdade de Filosofia, Ciencias e Letras. Boletim de Zoologia, 23: 107- 139.

NÉMETH, A. & KOVÁCS, J. 1972. The ultrastructure of the epithelial cells of seminal receptacle in the snail *Helix pomatia* with special reference to the lysosomal system. *Acta Biologica Academiae Scientiarum Hungaricae*, **23**: 299- 308.

PITNICK, S. & MARKOW, T. A. 1994. Male gametic strategies: sperm size, testes size and the allocation of ejaculate among successive mates by thee sperm-limited fly *Drosophila pachea* and its relatives. *The American Naturalist*, **143**: 785- 819.

PLESCH, B., DE JONG- BRINK, M. & BOER, H. H. 1971. Histological and histochemical observations on the reproductive tract of the hermaphrodite pond snail

Lymnaea stagnalis (L.). *Netherlands Journal of Zoology*, **21**: 180- 201.

PONDER. W. F. & LINDBERG, D. R. 1997. Towards a phylogeny of gastropod molluscs: an analysis using morphological characters. *Zoological Journal of the Linnean Society*, **119**: 83- 265.

ROGERS, S. H., REEDER, R. L. & SHANNON, W. A. 1980. Ultrastructural analysis of the morphology and function of the spermatheca of the pulmonate snail, *Sonorella santaritana*. *Journal of Morphology*, **163**: 319- 329.

RUDOLPH, P. H. 1983. Histochemistry of the reproductive tracts of *Stagnicola elodes* (Basommatophora: Lymnaeidae). *Malacological Review*, **16**: 43- 57.

RUNHAM, N. W. 1988. *Mollusca*. In: *Reproductive Biology of Invertebrates*. Vol. III (K. G. Adiyodi & R. Adiyodi eds.), pp. 113- 188. John Wiley & Sons. New York.

SAKALUK, S. K. & O' DAY, D. H. 1984. Hoechst staining and quantification of sperm in the spermatophore and spermatheca of the decorated cricket, *Gryllodes supplicans* (Orthoptera: Gryllidae). *The Canadian Entomologist*, **116**: 1585- 1589.

UNDERWOOD, A. J. 1997. *Experiments in ecology: their logical design and interpretation using analysis of variance*. Cambridge University Press.

Chapter 7

General discussion

This thesis investigated and compared different aspects of the reproductive biology of two sympatric species of pulmonate limpet *Siphonaria*, which belong to the Superorder Basommatophora (see Table 1 for summary of results). One of the aims of this investigation was to gain an insight into the life history, ancestry and evolutionary relationships of siphonariids and then compare the findings with other euthyneurans. Siphonariid limpets are considered to be the most primitive basommatophorans (see Chapter 1), because they lack tentacles and a majority of them produce planktonic larvae (Hyman, 1967; Dayrat & Tillier, 2002; G. Haszprunar, pers. comm.). The current study focused on two siphonariids with contrasting developmental modes, *Siphonaria capensis*, a planktonic developer, which belongs to the subgenus *Patellopsis* and *S. serrata* with intracapsular development belonging to the subgenus *Siphonaria s. s.*

The recent development of phylogenetic methods has changed the approach of marine biologists towards the study of evolution as well as the shifts between developmental modes (McHugh & Rouse, 1998). According to Poulin *et al.*, (2002) developmental modes can be classified on the basis of spatial location, trophic level and extent of parental involvement. Although there are a few examples (Rouse & Fitzhugh, 1994) where aplanktonic development is thought to be the primitive condition, a wide distribution of planktonic development in marine invertebrates still raises the question of why then planktonic development is so common in marine invertebrates if the loss of larvae (feeding or non-feeding) has more advantages (Pechenik, 1999) An advantage of having pelagic larvae is greater dispersal and therefore higher gene flow between populations, enabling the species to survive local extinctions compared to the direct development. The larvae in the plankton also suffer high pelagic mortality (Pechenik,

1999) and planktonic developers are often more fecund than their congeners with direct development which is energetically expensive (Chia, 1974). Direct developers however, also have a benefit such as rapid colonisation of a new habitat (Johannesson, 1988).

In siphonariids a wide distribution of both intracapsular and planktonic development has also intrigued biologists who tried to address the issue in terms of adaptation and phylogeny (Marshall & McQuaid, 1991; Chambers, 1994; Hodgson, 1999). Even the use of advanced techniques such as DNA fingerprinting did not resolve the problem as to which mode is the primitive condition (Chambers *et al.*, 1998).

The ultrastructural investigation of oogenesis and modes of vitellogenesis in these limpets showed that *S. capensis* produces yolk with the help of intracellular organelles (autosynthesis) whereas *S. serrata* not only produces yolk granules autosynthetically, but also most probably takes up high molecular weight precursors via the endocytotic vesicles from outside the cell to synthesize yolk (heterosynthesis) (Chapter 4). Whilst Eckelbarger (1994) has suggested that autosynthesis is a primitive mode of yolk synthesis compared to heterosynthesis or mixed synthesis, it is not known whether other planktonic developing siphonariids also use a similar mode of vitellogenesis. If the autosynthetic mode of vitellogenesis is characteristic of "primitiveness" (Eckelbarger, 1994) then *S. capensis* with planktonic development is primitive and hence siphonariids may have had marine ancestry. If planktonic development has evolved more than once in the genera *Siphonaria* and *Williamia* (as suggested by Chambers, 1994) then this character cannot be used to resolve the relationships within the family Siphonariidae.

Many authors have suggested planktrophy to be primitive in invertebrates (Reid, 1989; Strathmann, 1986; Nielsen, 1998) as often it is difficult if not impossible to regain

the structures possessed by the pelagic feeding larvae, once lost (e.g., Todd, 1991; Nielson, 1998; Pechenik, 1999). In the Phylum Mollusca, lecithotrophy has been recognised as a plesiomorphic condition by Haszprunar (1988) and later again by Haszprunar *et al.*, (1995) and planktotrophy may have evolved twice (once in Caenogastropods and once in Heterobranchia) (Ponder & Lindberg, 1997). According to Nielsen (1998) however, planktotrophy in gastropods is the ancestral condition as it has been lost several times but regained rarely, and lecithotrophy can be found in all clades. Within Heterobranchia, the clade Euthyneura (Opisthobranchia and Pulmonata) emerges basally and it is considered monophyletic (Dayrat & Tillier, 2002). To date nothing has been said about the evolution of developmental mode in euthyneurans especially pulmonates.

Although it is not known whether siphonariids with planktonic development have feeding larvae it has been suggested by Chambers (1994) that the veliger larvae of *S. concinna*, which spend up to two months in the plankton, most probably feed. Unfortunately as there is no published information on larval feeding organs of siphonariid limpets (A. Hodgson, pers. comm.), it is not possible to comment on the occurrence of planktotrophy or lecithotrophy in *Siphonaria*. However, in the close relative *Williamia radiata* facultative planktotrophy has been observed (Ruthensteiner & Schaefer, 2002). Despite having an intracellular reserve of yolk, both *S. capensis* and *S. serrata* utilise perivitelline fluid as a source of nutrition during embryonic development inside the capsule (Chapter 5).

The histological examination of the gametogenesis in *S. capensis* and *S. serrata* revealed that both species are simultaneous hermaphrodites (once they are sexually

mature) although Hubendick (1978) had previously suggested protandry for all siphonariids. *S. capensis* becomes sexually mature when they attain 10 mm shell length (present study) compared to 14 mm for *S. serrata* (Chambers, 1994). Occasionally, sperm can be found in *S. capensis* individuals of less than 10 mm shell length, which indicates protandry for these animals for a very short period of their lives. Unfortunately, no detailed information is available regarding sexual maturity of *S. serrata*. Both Duncan (1975) and Hubendick (1978) had suggested that protandry is a characteristic condition in primitive pulmonates. However, in recent phylogenetic studies hermaphroditism has not been used as a character as it may be a convergent character in many non-euthyneuran taxa (Haszprunar, 1988; Dayrat & Tillier, 2002).

Even though the structure of the egg ribbons in *S. capensis* and *S. serrata* was different, the accessory reproductive gland i.e., the glandular complex, not only had a similar external morphology, but the tissue structure and histochemistry was also the same. The albumen gland and the mucous gland that makes up this complex are not clearly defined on the basis of external morphology. Both these glands that are similar in ultrastructure to those of opisthobranchs, probably play a role in the egg mass formation in siphonariids especially the production of perivitelline fluid (albumen gland) and the mucous matrix or mucous membranes (mucous gland), but this needs further investigation. Compared to the other basommatophorans, the condition of the glandular complex in these two siphonariids may indicate the primitiveness of these basommatophorans, thus supporting the position of *Siphonaria* at the base of the tree of basommatophorans as suggested by Dayrat & Tillier (2002) (see Chapter 1).

The ultrastructure of the distal spermatheca of both species was also similar

indicating that this organ plays a similar kind of role in both the intracapsular and planktonic developer even though there is a statistically significant difference in the size of the organ between the two species (Chapter 6). It is likely that the spermatheca of siphonariids does not store sperm but receives them during copulation for further degradation (?). Both auto and allosperm might be stored either in the seminal vesicle of the hermaphrodite duct or in the fertilization chamber (pers. obs.) as in terrestrial pulmonates. Unfortunately, the structure of the spermatheca or bursa copulatrix has not been studied in many aquatic pulmonates from a functional aspect, which makes it difficult to comment on its role in sperm storage. A distal bursa has also been observed in a member of the genus *Williamia* and thought to function as a gametolytic organ (B. Ruthensteiner, pers. comm.).

Table 1. Summary of the results of the current study on *S. capensis* (planktonic developer) and *S. serrata* (intracapsular developer).

Aspects	*S. capensis*	*S. serrata*
Spawning cycle	Spring/ summer	Spring/ summer
Gametogenic cycle	Oogenesis- interrupted in winter; Spermatogenesis- continuous. (activity higher after egg production).	Oogenesis- interrupted in winter; Spermatogenesis- continuous. (activity higher after egg production).
Hermaphrodite duct	Sperm present all year.	Sperm present all year.
Hermaphoditism	Simultaneous with a slight tendency of protandry (?) in early life.	Simultaneous.
Fecundity	Increases with size.	Increases with size.
Oogenesis and vitellogenesis	Autosynthetic.	Auto and heterosynthetic.
Structure and composition of egg ribbons	Less fibrous, less carbohydrate and protein.	More fibrous, greater amounts of carbohydrate and protein.
Feeding by embryo	Yes (source yolk + perivitelline fluid).	Yes (source yolk + perivitelline fluid).
Glandular complex	Albumen and mucous gland. Similar structure in both species.	
Spermatheca and sperm storage.	Distal. Similar structure in both species although size is greater in *S. serrata*. Seminal vesicle/ fertilization pouch for sperm storage?	

Considering the debate on the systematic position and phylogenetic relationship of the Superorder Basommatophora (Dayrat & Tillier, 2002), it must be stressed that characters associated with the female reproductive system, egg development and spawn can be useful in the phylogenetic analyses of euthyneurans just as the characters associated with sperm morphology have proved to be. The process of egg development and the morphology of glandular complex (presence of a tubular albumen and mucous gland together) and spermatheca (which is distinct from the seminal vesicle) in siphonariids as well as their fine structural similarities (alternation of columnar secretory cells with ciliated supporting cells of glandular complex) with the opisthobranchs (see review by Haszprunuar, 1988) studied here suggest a primitive condition in *Siphonaria* compared to other pulmonates although the concept of "apomorphy-plesiomorphy" is relative (Haszprunar, 1988). In other words, the similarities between these two siphonariids and opisthobranchs once again points towards a common ancestry of these two groups. In the light of siphonariids possessing many apomorphic characters (Nordsieck, 1992) and possible marine ancestry (Hyman, 1967; Hodgson, 1999; G. Haszprunar, pers. comm.) in contrast to terrestrial ancestry (Marshall & McQuaid, 1991; Chambers *et al.*, 1998), a detailed comparative study of distal genitalia and larval structures will be the next step to resolve the relationships within the four genera of the Siphonariidae.

Co-occurrence of more than one type of larval strategy amongst the congeners has been noted in marine animals in different taxa and drawn the attention of many invertebrate biologists (Christiansen & Fenchel, 1979; Todd, 1979; Todd, 1991; Nielsen, 1998). Co-existence of two developmental modes in closely related organisms is

determined by many factors such as energy allocation to reproduction (gametogensis, capsule production, size of the ribbon, jelly production) and number of metamorphosed larvae, length of development and predation (both pelagic and benthic) (Vance, 1973; Todd, 1979; Gibson & Chia, 1991) as well as size of the adult (Christiansen & Fenchel, 1979; Gallardo & Perron, 1982). To understand the distribution, evolutionary shifts of developmental mode in siphonariid limpets, large sets of molecular data (including the species with mixed mode of larval development) should be used in the phylogenetic studies, as transitions in developmental mode can control the number of species with planktonic and aplanktonic development which may lead to species selection (Duda & Palumbi, 1999).

References

CHAMBERS, R. J. 1994. *The conflict between adaptation and constraint: the case of the siphonariid limpets*. Ph.D thesis. Rhodes University.

CHAMBERS, R. J., MCQUAID, C. D. & KIRBY, R. 1998. The use of randomly amplified polymorphic DNA to analyze the genetic diversity, the systematic relationships and the evolution of intertidal limpets, *Siphonaria* spp. (Pulmonata: Gastropoda), with different reproductive modes. *Journal of Experimental Marine Biology and Ecology*, **227**: 49- 66.

CHIA, F-S. 1974. Classification and adaptive significance of developmental patterns in marine invertebrates. *Thalassia Jugoslavica,* **10**: 121- 130.

CHRISTIANSEN, F. B. & FENCHEL, T. M. 1979. Evolution of marine invertebrate reproductive patterns. *Theoretical Population Biology*, **16**: 267- 282.

DAYRAT, B. & TILLIER, S. 2002. Evolutionary relationships of euthyneuran gastropods (Mollusca): a cladistic re-evaluation of morphological characters. *Zoological Journal of the Linnean Society*, **135**: 403- 470.

DUNCAN, C. J. 1975. *Reproduction.* In: *Pulmonates.* Vol. 1 (V. Fretter & J. Peake eds.), pp. 309- 365. Academic Press. London.

DUDA, T. F. & PALUMBI, S. R. 1999. Developmental shifts and species selection in gastropods. *Proceedings of the National Academy of Sciences of the United States,* **96**: 10272- 10277.

ECKELBARGER, K. J. 1994. Diversity of metazoan ovaries and vitellogenic mechanisms: implications of life history theory. *Proceedings of the Biological Society of*

Washington, **107**: 193- 218.

GALLARDO, C. S. & PERRON, F. E. 1982. Evolutionary ecology of reproduction in marine benthic molluscs. *Malacologia*, **22**: 109- 114.

GIBSON, G. D. & CHIA, F-S. 1991. Contrasting reproductive modes in two sympatric species of *Haminea* (Opisthobranchia: Cephalaspidea). *Journal of Molluscan Studies*, **57**: 49- 60.

HASZPRUNAR, G. 1988. On the origin and evolution of major gastropod groups with special reference to the Streptoneura. *Journal of Molluscan Studies,* **54**: 367- 441.

HASZPRUNAR, G., SALVINI-PLAWEN, L. V. & RIEGER, R. M. 1995. Larval planktrophy- a primitive trait in the Bilateria? *Acta Zoologica*, **76**: 141- 154.

HODGSON, A. N., FARQUHAR, M. R. & DE VILLIERS, C. J. 1993. Variations in infestations of *Siphonaria capensis* and *S. concinna* (Pulmonata) by larval trematodes in southern Africa. *Journal of Molluscan Studies*, **59**: 83- 93.

HODGSON, A. N. 1999. The biology of siphonariid limpets (Gastropoda: Pulmonata). *Oceanography and Marine Biology: an annual review*, **37**: 245- 314.

HUBENDICK, B. 1978. Systematics and comparative morphology of the Basommatophora. In: *Pulmonates*. (V. Fretter & J. Peake, eds), **2A**: 1- 47. Academic Press, London.

HYMAN, L. H. 1967. *The Invertebrates. Mollusca I*, Vol. **6**. McGraw Hill, New York.

JOHANNESSON, K. 1988. The paradox of Rockall: why is a brooding gastropod (*Littorina saxatilis*) more widespread than one having a planktonic larval dispersal stage (*L. littorea*)? *Marine Biology*, **99**: 507- 513.

MARSHALL, D. J. & MCQUAID, C. D. 1991. Metabolic rate depression in a marine

pulmonate snail: pre-adaptation for a terrestrial existence? *Oecologia*, **88**: 274- 276.

MCHUGH, D. & ROUSE, G. W. 1998. Life history evolution of marine invertebrates: new views from phylogenetic systematics. *Trends in Ecology and Evolution*, **13**: 182-186.

NIELSEN, C. 1998. Origin and evolution of animal life cycles. *Biological Reviews*, **73**: 125- 155.

NORDSIECK, H. 1992. Phylogeny and system of the Pulmonata. *Archiv für Molluskenkunde*, **121**: 31- 52.

PECHENIK, J. A. 1999. On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. *Marine Ecology Progress Series,* **177**: 269- 297.

PONDER, W. F. & LINDBERG, D. R. 1997. Towards a phylogeny of gastropod molluscs: an analysis using morphological characters. *Zoological Journal of the Linnean Society,* **119**: 83- 265.

POULIN, E., PALMA, A. T. & FÉRAL, J- P. 2002. Evolutionary versus ecological success in Antarctic benthic invertebrates. *Trends in Ecology & Evolution*, **17**: 218- 222.

REID, D. G. 1989. The comparative morphology, phylogeny and evolution of the gastropod family Littorinidae. *Philosophical Transactions of Royal Society of London Series B*, **324**: 1-110.

ROUSE, G. W. & FITZHUGH, K. 1994. Broadcasting fables: is external fertilization really primitive? Sex, size and larvae in sabellid polychaetes. *Zoologica Scripta*, **23**: 271-312.

RUTHENSTEINER, B. & SCHAEFER, K. 2002. The cephalic sensory organ in the veliger larvae of Pulmonates (Gastropoda: Mollusca). *Journal of Morphology*, **251**: 93-181

102.

STRATHMANN, R. R. 1986. What controls the type of larval development? Summary statement for the evolution session. *Bulletin of Marine Science,* **39**: 616- 622.

TODD, C. D. 1979. Reproductive energetics of two species of Dorid Nudibranchs with planktotrophic and lecithotrophic larval strategies. *Marine Biology,* **53**: 57- 68.

TODD, C. D. 1991. Larval Strategies of nudibranch molluscs: similar means to the same end? *Malacologia,* **32**: 273- 289.

VANCE, R. R. 1973. On reproductive strategies in marine benthic invertebrates. *The American Naturalist*, **107**: 339- 352.