REPRODUCTION AND BACTERIAL SYMBIOSIS IN CARIBBEAN COMMERCIAL SPONGES (PORIFERA: DEMOSPONGIAE: DICTYOCERATIDA)

by

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A Dissertation submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy (Oceanography).

REPRODUCTION AND BACTERIAL SYMBIOSIS IN CARIBBEAN COMMERCIAL SPONGES.

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7760

DEDICATED TO

Michelle, Jason, Matthew and Megan

who were born during the course of this study

your love of life and learning kept me going

It Couldn't Be Done

Somebody said that it couldn't be done, But he with a chuckle replied That "maybe it couldn't," but he would be one Who wouldn't say so till he'd tried. So he buckled right in with the trace of a grin On his face. If he worried he hid it. He started to sing, as he tackled the thing That couldn't be done, and he did it.

Somebody scoffed: "Oh, you'll never do that; At least no one ever has done it"; But he took off his coat and he took off his hat, And the first thing we knew he'd begun it. With a lift of his chin and a bit of a grin, Without any doubting or quiddit, He started to sing as he tackled the thing That couldn't be done, and he did it.

There are thousands to tell you it cannot be done, There are thousands to prophesy failure; There are thousands to point out to you one by one, The dangers that wait to assail you. But just buckle in with a bit of a grin, Just take off your coat and go to it; Just start in to sing as you tackle the thing That "cannot be done," and you'll do it.

Edgar Guest

ABSTRACT

The genera <u>Spongia</u> and <u>Hippospongia</u> include all of the commercially important bath sponges of the Caribbean fishery. This study examined the uniform presence of immense symbiotic bacterial populations of four of these species: <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>S. cheiris and S. graminea</u>. The nutritional characteristics and antibiotic sensitivities of bacteria isolated from the four species were also examined. A combined light and transmission electron microscopic investigation outlined the reproductive processes and larval development, behaviour, settlement and metamorphosis in these four species.

Symbiotic bacteria in these four sponge species are specific to the sponges and different from ambient seawater bacteria. Populations of intercellular bacteria within the tissues of these sponges are greater than those of ambient seawater. A variety of morphologically different types of sponge-specific bacteria were observed. The symbionts are not fastidious organisms but utilize a variety of amino acids, carbohydrates and tricarboxylic acid cycle intermediates as sole carbon and energy sources for growth. The bacteria showed sensitivities to a variety of antibiotics but were not susceptible to fluid from the sponges.

These sponges are viviparous and probably dioecious. Egg production and larval development are localized in patches or "nurseries" of endosomal tissue. Statistical analyses of specific morphological characteristics of female reproductive elements have identified four specific stages in the process of oogenesis. Umbilici connect young and maturing embryos to the maternal mesohyl and are the pathway for extracellular transfer of intercellular symbiotic bacteria and other mesohyl substances. These

4

bacteria were observed in the embryos and larvae of all four species. Oocytes and embryos develop asynchronously within a given individual. Spermatogenesis occurs synchronously within cysts by transformation of entire choanocyte chambers. Cysts develop asynchronously within an individual. Male gametes exhibit a bright yellow-white autofluorescence when excited with blue light (460-485 nm). Spermatozoa do not possess intermediate segments or acrosomes.

The incubated parenchymella larvae of these four species are ovoid with dark grey pigmentation and enlarged posterior regions encircled by a black pigmented ring of cells bearing long cilia. Laboratory behavioural studies indicate that free-swimming larvae display directional swimming with constant rotation and negative phototaxis. Larval behaviour probably reflects the ecological situation of adult populations. Larval settlement occurs 26-56 hours after release and involves the rapid formation of a basal lamella between the larvae and substrate. There is no evidence of substrate selection or orientation by larvae. Precocious development of choanocytes does not occur in the larvae or post-larvae or these four species.

RESUME

Les genres <u>Spongia</u> et <u>Hippospongia</u> comportent toutes les éponges commercialement importantes pêchées dans les Antilles. Cette étude examine la présence généralisée d'immenses populations de bactéries symbiontes parmi quatre de leurs espèces: <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>S. cheiris</u> et <u>S. graminea</u>. Les exigences nutritives et la sensibilité aux antibiotiques des bactéries isolées de ces quatre espèces furent aussi considérées. Une étude combinée aux microscopes optique et électronique mit en évidence les mécanismes de reproduction et de développement larvaire, et le comportement, la colonisation du substrat et la métamorphose chez ces quatre espèces.

Les bactéries symbiontes de ces quatre espèces d'éponges leur sont spécifiques et diffèrent des bactéries de l'eau de mer ambiante. Les populations de bactéries intercellulaires des tissus de ces éponges sont plus abondantes que celles de l'eau de mer ambiante. Une variété de types morphologiques différents de bactéries spécifiques aux éponges fut obscrvée. Ces bactéries symbiontes robustes utilisent une variété d'acides aminés, d'hydrates de carbone et d'intermédiaires d'acide tricarboxylique cyclique comme seules sources de carbone et d'énergie pour la croissance. Les bactéries sensibles à une variété d'antibiotiques ne l'étaient pas au fluide interne des éponges.

Ces éponges sont vivipares et probablement dioïques. La production d'oeufs et le développement larvaire apparaissent par taches ou "pouponnières" dans l'endosome. Des analyses statistiques des caractéristiques morphologiques spécifiques des éléments reproducteurs femelles ont identifiés quatre stades distincts du processus d'oogénèse. Un

-vi-

cordon ombilical relie le jeune et l'embryon en maturation au mésohyle maternel et est le chemin du transfert extracellulaire des bactéries symbiontes intercellulaires et d'autres substances du mésohyle. Ces bactéries furent observées dans les embryons et les larves des quatre espèces. Les oocytes et les embryons ont un développement asynchrone à l'intérieur d'un individu donné. Une spermatogénèse synchrone se produit à l'intérieur de kystes par une transformation complète des chambres à choanocytes. Les kystes ont un développement asynchrone à l'intérieur d'un individu. Les gamètes mâles produisent une lumière autofluorescente d'un jaune blanc vif lorsque excités par une lumière bleue (460-485 nm). Les spermatozoïdes ne possèdent ni de segments intermédiaires ou d'acrosomes.

Les larves parenchymellaires incubées de ces quatre espèces sont ovoïdes avec une pigmentation grise et une région postérieure élargie encerclée d'un anneau de cellules pigmentées noires porteuses de cils longs. Les études de comportement en laboratoire indiquent que les larves libres sont capables d'une nage dirigée en rotation constante et montrent une phototaxie négative. Le comportement larvaire représente probablement la situation écologique des populations adultes. La colonisation larvaire se produit 26 à 56 heures après leur libération et comporte la formation rapide de lamelles basales entre la larve et le substrat. Il n'y a aucun indice de sélection du substrat ou d'orientation par les larves. Un développement précoce des choanocytes n'apparaît ni dans les larves ni dans les postlarves de ces quatre espèces.

TABLE OF CONTENTS

ABSTRACT	1v vi ix xii xii1 xi11
GENERAL INTRODUCTION LITERATURE CITED	1 5
CHAPTER I. NUTRITIONAL CHARACTERISTICS AND ANTIBIOTIC SENSITIVITIES OF BACTERIA ISOLATED FROM FOUR CARIBBEAN COMMERCIAL SPONGE SPECIES.	
ABSTRACT	10 11 13 19 30 33 34
CHAPTER II. A STUDY OF SEXUAL REPRODUCTION IN FOUR CARIBBEAN COMMERCIAL SPONGE SPECIES. I. OOGENESIS, TRANSFER OF BACTERIAL SYMBIONTS, AND LARVAL DEVELOPMENT, BEHAVIOUR, SETTLEMENT AND METAMORPHOSIS.	
ABSTRACT INTRODUCTION MATERIALS AND METHODS OBSERVATIONS AND RESULTS	39 40 43 53

MATERIALS AND METHODS	43
OBSERVATIONS AND RESULTS	53
DISCUSSION	71
CONCLUSIONS	94
ACKNOWLEDGEMENTS	96
LITERATURE CITED	97

CHAPTER III. A STUDY OF SEXUAL REPRODUCTION IN FOUR CARIBBEAN COMMERCIAL SPONGE SPECIES. II. REPRODUCTIVE CYCLES AND SPERMATOGENESIS.

1.30

ABSTRACT	107
INTRODUCTION	108
MATERIALS AND METHODS	111
OBSERVATIONS AND RESULTS	115
DISCUSSION	128
ACKNOWLEDGEMENTS	136
LITERATURE CITED	137
CONCLUDING REMARKS	141

Page

ACKNOWLEDGEMENTS

The success of conducting field work depends to a large degree on the cooperation and help of a number of people. I express sincere thanks to: Mr. Dick Conant and Mr. Jim Tilmant of the National Park Service, Florida for their help and advice on locating the study site, the Biscayne National Park supervisors and Rangers for their assistance in transportation to and from the collecting area, and Mr. John Stevely and Mr. Don Sweat of the Florida Cooperative Extension Program, Sea Grant Program for their assistance in obtaining all available information concerning the commercial sponge populations in the waters around Florida.

I would like to thank the University of Miami, Rosenstiel School of Marine and Atmospheric Science for the use of their facilities, and a special thanks to Dr. Barrie Taylor for his helpful advice and providing laboratory facilities. Sincere thanks to Ms. Mary Jane Crowe and Ms. Barb Mazurkiewiez for accomodations during the various trips to Miami and for your support and friendship.

I am grateful to Dr. Wayne Hunte for providing research facilities at the Bellairs Research Institute where I had initially planned to carry out the research. I wish to express my gratitude to Dr. Brian Marcotte for many stimulating conversations and suggestions, and critical review of the manuscripts. I would like to thank Dr. Joan Marsden for helpful comments and critical review of portions of the thesis. I wish to acknowledge the aid of Dr. Maria Neuwirth of the Electron Microscope Centre, McGill University for her guidance and help with electron micrograph interpretations.

Working in the laboratory at Macdonald College, Ste.-Anne-de-Bellevue

was a rewarding and enlightening experience. My thanks to all of you for supporting me during my time spent there. Pierre Martin, your guidance and assistance helped me learn the techniques of microbiological analyses.

Thank you to all my friends for their help and moral support through the years: Dr. John Boers, Ms. Tina Ortiz, Dr. Paula Scott, Ms. Carolyn Rymes, Dr. Tom Tomascik, Ms. Anmarie Mah, Mr. Mitchell Sisak, Dr. Julie Horrocks, Dr. Hazel Oxenford, Mr. Paul Snelgrove, and special thanks to Mr. Sam Salley for his assistance with the statistical analyses and Mr. Luc Rainville for his time spent on the french translation of the abstract. You are a unique group of people that will always be remembered. Together you commemorate the Institute of Oceanography, McGill University.

I would like to thank Dr. Robert A. MacLeod and Dr. Patricia MacLeod of the Microbiology Department at Macdonald College, McGill University who introduced me to the methods and techniques of microbiology, and offered invaluable information and guidance which contributed greatly to the completion of this study. For the knowledge I gained and the many hours you spent encouraging and enlightening me in this subject area, I am indebted.

I would like to express my deep gratitude to my advisor, Dr. Henry M. Reiswig. I was blessed with a winning combination of patience and perception. You taught me how to do science. Your talent for understanding and being able to explain <u>everything</u> helped me produce a thesis I am proud of. Your interest in the area of sponge biology and knowledge of science constantly provided new challenges and served as valuable incentives. I am indeed grateful for the many hours you spent in reviewing the thesis, your financial support, your invaluable comments and advice, and must importantly for the knowledge I have gained from you through the years. Your compassion and understanding have inspired me.

-X-

I would like to thank Dr. John Fernandes who helped me make a decision eleven years ago that directed me on a course that has finally culminated in this dissertation. Thanks for being there for me. Finally, I would like to express my gratitude to, and my love for, my family. You always understood the pressures and never demanded more from me than I could give. Thank you for understanding when I couldn't be there. Your love kept me going. And yes Mom, I'm finally finished my thesis.

LIST OF TABLES

Page

CHAPTER I. Nutritional characterisitcs and antibiotic sensitivities
of bacteria isolated from four Caribbean commercial sponge
species.
Table 1: Average bacterial counts per gram of sponge tissue and per
millilitre of ambient seawater 21
Table 2: Morphological characteristics of sponge bacterial
strains
Table 3: Capacity to use organic compounds for growth 24
Table 4: Antibiotic sensitivities 29
CHAPTER II. A study of sexual reproduction in four Caribbean
commercial sponge species. I. Oogenesis, transfer of
bacterial symbionts, and larval development, behaviour,
settlement and metamorphosis.
Table 1: Results of the discriminant analysis
Table 2: Results of the principal component analysis 57
Table 3: Results of Tukey's studentized range test
Table 4: Morphological characteristics of the stages in the process
of oogenesis in <u>H. lachne</u> 62
Table 5: Number of larvae settling on various substrate types 71
CHAPTER III. A study of sexual reproduction in four Caribbean
commercial sponge species. II. Reproductive cycles
and spermatogenesis.
Table 1: Reproductive status of individuals 116
Table 2: Morphological characteristics of the stages in the process of
spermatogenesis

~

LIST OF FIGURES

ł

(

	Page
CHAPTER I. Nutritional characterisitics and antibiotic sensitivities	
of bacteria isolated from four Caribbean commercial sponge	
species.	
Figure 1: Electron micrograph	. 20
Figure 2: Capacity of organisms to maintain viability or grow in	
various media	. 26
CHAPTER II. A study of sexual reproduction in four Caribbean	
commercial sponge species. I. Oogenesis, transfer of	
bacterial symbionts, and larval development, behaviour,	
' settlement and metamorphosis.	
Figure 1: Larval catching net	, 47
Figure 2: Dendrogram of reproductive elements	54
Figure 3: Micrographs	63
Figure 4: Micrographs	66
Figure 5: Micrographs	69
Figure 6: Micrographs	73
CHAPTER III. A study of Sexual reproduction in four Caribbean	
commercial sponge species. II. Reproductive cycles	
and spermatogenesis.	
Figure 1: Histogram of percentage of reproductive individuals	
throughout the study period	117
Figure 2: Micrographs	121
Figure 3: Micrographs	124
Figure 4: Micrographs	126

PREFACE

1.) Statement of Originality:

This study presents the first comprehensive and comparative investigation of the processes involved in sexual reproduction in four sponge species belonging to taxonomically and economically important genera that have previously been neglected in biological studies. The use of the light and transmission electron microscope has permitted a combined gross and ultrastructural analysis of morphological characteristics occurring in the sequence of events during sexual reproductive processes. In addition, this study presents the first application of statistical analyses to determine specific morphological characteristics that distinguish stages in the process of oogenesis.

This study presents the first record of the transfer of bacterial symbionts between generations in viviparous invertebrates. Furthermore, a unique mode of extracellular transfer of symbiotic bacteria in sponges has been demonstrated. The serendipitous observation of autofluorescence in male reproductive elements of the four sponge species investigated is the first record of a definitive label that can be used to identify and follow the development of male gametes. In addition, the use of this label may have important implications in targeting cells involved in the process of fertilization in sponges.

2.) Historical background:

An extensive historical background can be found in the General Introduction, and in the Introduction and throughout the text of each chapter.

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-xiv-

3.) Declaration of assistance:

The candidate acknowledges the contributions of Dr. Henry M. Reiswig and Dr. Robert A. MacLeod for supervision, guidance and advice during the relevant studies, their financial support and their critical review of the relevant manuscripts. In accordance with Section 7 of the McGill Faculty of Graduate Studies and Research Thesis Regulations and Guidelines the candidate declares that the study design, field and laboratory work, data anlayses and interpretation, and writing of the manuscripts was done by the candidate alone, as is clearly reflected in the candidate's position as sole or senior author of the manuscripts contained within this thesis.

4.) Thesis format:

This thesis has been prepared as a series of manuscripts suitable for submission to referred scientific journals for publication. For this reason each chapter contains its own Abstract, Introduction, Materials and Methods, Observations and Results, Discussion and Conclusion, Acknowledgements and Literature Cited; it understandably contains a certain amount of repetition. The present thesis format has been approved by the thesis committee and the Chairman of the Department.

Chapter I is presented almost entirely in the format in which it was published. Minor changes were made to respect thesis format. Capter III is presently being submitted. Chapter II is made up of two manuscripts, one has been accepted for publication and the other is presently being submitted. The relevant journal titles have been indicated on the title page of each chapter.

- X V -

GENERAL INTRODUCTION

Sponges are, based upon all available evidence, the most primitive Metazoans. With the exception of the epithelial cells, they lack discrete tissues and do not develop any true organs. They consist of three basic cell types: pinacocytes, choanocytes and the totipotent archaeocytes/amoebocytes; cell differentiation in these animals is highly labile. As a phylum, the Porifera is relatively small, containing about 5,500 described species and an unknown number of undescribed species. They are aquatic animals, mostly marine; there are a few freshwater species that inhabit ponds, lakes and streams in most areas of the world. Sponges are capable of sexual reproduction by the formation of gametes from somatic cells, and they have incredible potentialities for asexual reproduction.

At the beginning of this century, research on sponges was primarily taxonomic and histological (Minchin, 1900; Sollas, 1909; Hyman, 1940). Discovery of sponge cell reaggregation by Wilson (1907) turned attention towards the concept of cell recognition in the Porifera and, today, research on this phenomenon is at the fundamental, molecular level. However, most other aspects of the biology and ecology of sponges remain in their infancy for even the most frequently studied taxa. One of the greatest obstacles to gaining an understanding of some of the most basic biological systems in the Porifera is the difficulty of maintaining marine species in controlled laboratory conditions.

The genera <u>Spongia</u> Linnaeus, 1759 and <u>Hippospongia</u> Schulze, 1879 (Dictyoceratida) include all of the commercially important bath sponges (ca. 10 spp.) of the world's fisheries. In spite of their economic significance, few investigations have focused on the biology and ecology of these species.

-1-

Most of the available literature on commercial sponges deals with gross morphology and fisheries aspects. There is a glaring lack of information on their reproduction and development and the significance of immense bacterial populations harboured in the tissues of these species.

Electron microscopic studies have confirmed the existence of large numbers of apparently symbiotic bacteria within the tissues of many marine sponges. The symbionts occur extracellularly and intracellularly within large vacuoles of archaeocytes (Bertrand and Vacelet, 1971; Vacelet, 1975; Wilkinson, 1978a). Sponge-specific bacteria have been isolated (Wilkinson, 1978b) and some marine sponges are capable of distinguishing between bacteria apparently in symbiotic association and bacteria consumed as food (Wilkinson, et al, 1984). The relationship appears to be beneficial to both the host and associate, evidenced by the idea that sponge-specific bacteria are thought to have been associated with sponges since the Precambrian (Wilkinson, 1984). At the very least, the symbiont has a niche but may also receive nutritional benefit from the host. The effect on the sponge is less clear and probably varies with the bacteria. All species of dictyoceratids, examined and reported to date, have been observed to harbour massive populations of a variety of morphologically different types of intercellular bacteria. However, as yet, there is little information available on the exact role of the hosts and their symbionts in this association.

Chapter I of this thesis looks at the nutritional characteristics and antibiotic sensitivities of bacteria isolated from four commercial Caribbean sponge species: <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>Spongia cheiris</u> and and <u>Spongia graminea</u>. This study had two objectives: 1) to discover if the symbionts have specific nutritional requirements that may be met by the host sponge and, 2) to discover if the host produces antimicrobial substances

-2-

which do not interfere with their symbionts but do inhibit other microorganisms.

Recent and extensive literature reviews have reported numerous studies dealing with sexual reproduction in sponges (Fell, 1983; Reiswig, 1983; Simpson, 1984). In all of these reviews, however, there is a consistent lack of information on the reproductive processes in the dictyoceratids. What little is known about these processes has been inferred from gross structural observations in the course of general studies on reproduction in three species: <u>Hippospongia communis</u> (Tuzet and Pavans de Ceccatty, 1958), <u>H. lachne</u> (Storr, 1964) and <u>Spongia officinalis</u> (Liaci, <u>et al</u>, 1971). The use of the electron microscope has provided more detailed information on specific events during reproductive processes in other sponges. However, there is no available ultrastructural information on oogenesis, embryogenesis or larval development in the dictyoceratids; and only one ultrastructural study has investigated spermatogenesis in one of these species (Gaino, <u>et al</u>, 1984, <u>S. officinalis</u>).

There is a lack of available information on the sequential events in morphogenesis, behaviour, settlement and metamorphosis of sponge larvae in general. This information is essential to interpreting the results of ecological and biological studies of adult populations. A number of studies have investigated isolated aspects of larval biology in some marine sponges (Lévi, 1956; Warburton, 1966; Bergquist and Sinclair, 1968; Simpson, 1968; Fell, 1976; Evans, 1977) but there are only two studies that have reported on the specific sequence of events in the life histories of larvae (Bergquist and Green, 1977; Bergquist, et al, 1979). However, the information gleaned from many of these studies has been used in discussion of systematics and evolution, few have attempted ecological interpretations

of possible relevance to the adult populations.

Chapter II and III of this thesis examine the sexual reproductive processes in four commercial sponge species: H. lachne, S. barbara, S. cheiris and S graminea. Chapter II looks at oogenesis, the transfer of bacterial symbionts, and larval development, behaviour, settlement and metamorphosis in these four species. This study had three objectives: 1) to statistically group female reproductive elements into stages based on specific morphological characteristics, and to subsequently describe the sequence of events in the process of oogenesis and larval development, 2) to discover how intercellular bacterial symbionts are transferred to developing embryos, and 3) to describe the sequential events in the life histories of larvae, and to ecologically interpret the strategies as relevant to the adult populations. Chapter III looks at the reproductive cycles and the process of spermatogenesis in these same four species. This study had three objectives: 1) to describe the reproductive cycles and condition of sexual differentiation, 2) to discover whether temperature and salinity are factors that influence gametogenesis, and 3) to describe the sequence of events in the process of spermatogenesis.

This thesis, as a whole, provides some of the basic information on the life histories of four species of dictyoceratids and, at the same time, considers their symbiotic bacterial populations as major biotic components of an integrated community. For the most part, the studies have not tested hypotheses but reported basic scientific observations from which hypotheses can now be formulated and tested. The status of our current knowledge on reproduction and development in dictyoceratids is rudimentary. My intention was to provide a solid foundation upon which future studies can build.

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CHAPTER I

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NUTRITIONAL CHARACTERISTICS AND ANTIBIOTIC SENSITIVITIES OF BACTERIA ISOLATED FROM FOUR CARIBBEAN COMMERCIAL SPONGE SPECIES.

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Nutritional characterisitics and antibiotic sensitivities of bacteria isolated from four commercial West Indian sponge species.

Proceedings of the Fifth International Coral Reef Congress, Tahiti, 5:153-158 (1985).

ABSTRACT

Large populations of intercellular bacteria have been found to be associated with the tissues of four commercial Caribbean sponge species. Several of these organisms were isolated and their nutritional characteristics and antibiotic sensitivities investigated in relation to those of marine and terrestrial bacteria. The sponge isolates are not fastidious organisms but utilize a variety of amino acids, carbohydrates and tricarboxylic acid cycle intermediates as sole carbon and energy sources for growth in a mineral salts medium. The sponge must supply one or more carbon and energy sources to support such relatively large populations of organisms. The organisms did not demonstrate a capacity to grow chemoautotrophically in the basal salts medium containing NaHCO3 as a source of carbon and oxidizable inorganic compounds as energy sources. Seventy percent of the sponge associated bacteria and all of the marine and terrestrial bacteria survived in the basal salts medium and grew when a carbon source was added. The bacteria showed sensitivities to a variety of antibiotics but were not susceptible to fluid from the sponges. It is not evident why large populations of specific bacteria are found in the mesohyl of the sponges investigated in this study, however some suggestions are offered.

INTRODUCTION

Electron microscopic studies have confirmed the existence of large numbers of apparently symbiotic bacteria within the tissues of many marine sponges. In some of the sponges studied, the bacteria were found to constitute up to 40% of the living material and a variety of morphologically different types of bacteria were observed (Vacelet, 1975; Wilkinson, 1978a; 1978c). In other sponges the bacteria were scarce and each sponge species possessed one, and occasionally two morphologically different bacterial types (Vacelet and Donadey, 1977).

It is not possible however, to gain an understanding of the specificity of these symbiotic associations or the role of the hosts and their symbionts in this association by structural studies alone. The possible roles of these symbiotic sponge bacteria have been investigated through extensive bacterial characterization studies. Wilkinson (1978b) found that there are some heterotrophic bacteria that are specific to sponges and are not present in ambient water. It has also been established that at least some marine sponges are capable of distinguishing between bacteria apparently in symbiotic association and bacteria consumed as food (Wilkinson <u>et al</u>, 1984).

Recent studies have led to the discovery of a symbiosis between chemoautotrophic bacteria and marine invertebrates (Cavanaugh <u>et al</u>, 1981; Felbeck <u>et al</u>, 1981; 1983; Southward <u>et al</u>, 1981). These findings have suggested that the bacteria are important to the nutrition, distribution and productivity of the host organisms and their communities (Cavanaugh, 1983).

Studies on antimicrobial activities of marine sponges have shown that some sponges produce substances which frequently inhibit the growth of marine bacteria (Burkholder and Ruetzler, 1969; Bergquist and Bedford, 1978; Amade, <u>et al</u>, 1987). Most of the sponges screened for biologically active substances also contain populations of bacteria within their tissues. However, few studies have considered the ecological and physiological significance of the symbiotic bacterial populations within the sponges. Interpretations of the antimicrobial activities have been related to the relevance of such substances to the ecology of the sponges.

The symbiotic relationship between sponges and their associates appears to be beneficial to both the host and associate, evidenced by the idea that sponge-specific bacteria are thought to have been associated with sponges since the Precambrian (Wilkinson, 1984). At the very least, the symbiont has a niche but may also receive nutritional benefit from the host. The effect on the sponge is less clear and probably varies with the bacteria.

In the present study the tissues of four species of commercial Caribbean sponges: <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>S. cheiris</u> and <u>S.</u> <u>graminea</u> were examined for the presence of large populations of symbiotic bacteria. Some of the symbionts were then isolated from the highest dilutions of homogenates of these sponge tissues and examined for their nutritional characteristics and antibiotic sensitivities.

It was hoped that this study would provide added information required for an understanding of the symbiotic association between sponges and their bacteria, and the possible roles of the hosts and their symbionts in this association.

-12-

MATERIALS AND METHODS

Sponges:

Four commercial sponge species: <u>Hippospongia lachne</u>, <u>Spongia graminea</u>, <u>Spongia barbara</u>, and <u>Spongia cheiris</u> were examined. These demosponges are common members of the fauna of Biscayne Bay, Florida (average depth 2.5 m) which has intermittent periods of turbulence related to seasonal weather conditions. The collection site (lat. 25°38'N; long. 80°12'W) is characterized by a coarse sandy bottom and has extensive areas covered in eel and turtle grass, small scattered corals, gorgonians and other sponge species.

Sampling and Preparation for Microbiological Analyses:

Using SCUBA, tissues samples were removed <u>in situ</u> from specimens of the four sponge species (located at a depth of 1 to 1.5 m) using presterilized scalpel blades. The samples were placed in collecting bottles of sterile ambient seawater to "flush" the tissues of ambient seawater bacteria. Sterile 100 ml syringes were used to collect ambient seawater samples at the same time. All collections were returned to the laboratory at the University of Miami for immediate processing.

Sampling and Preparation of Specimens for Microscopy:

Using SCUBA, endosomal tissue samples were removed <u>in situ</u> from specimens of the four sponge species. The samples were then fixed <u>in situ</u> by placing the tissues in a syringe, egressing the ambient seawater and ingressing 2.5% glutaraldehyde in seawater (fixative). After returning to the laboratory, samples were placed in vials of fresh fixative for 16 hours, then rinsed in three changes of fresh seawater for 10 min each, post-fixed

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for one hour in 1% osmium tetroxide in seawater, dehydrated in an alcohol series, cleared in propylene oxide and embedded in Spurr epoxy resin.

Microscopy:

Epoxy blocks were sectioned on a Sorvall 'Porter-Blum' MT-2B ultramicrotome at 0.1 μ m. Sections were floated onto formvar and carbon coated single-slot grids and stained for 20 min in saturated aqueous uranyl acetate and for 15 min in lead citrate. Sections were viewed and photographed with a Philips 410 transmission electron microscope operating at 80 kV.

Bacteria:

In addition to the organisms isolated from the sponges the following organisms (from the Macdonald College, McGill University collection) were also examined: <u>Pseudomonas doudoroffii</u> (ATCC 27123), <u>Alteromonas haloplanktis</u> 214 (ATCC 19855), and <u>Alcaligenes aestus</u> 134 (ATCC 27128) which are marine bacteria, and two terrestrial bacteria, <u>Pseudomonas aeruginosa</u> (ATCC 9027) and Escherichia coli K-12.

Media Used:

All of the bacteria examined in this study were initially isolated and then maintained on Difco marine agar 2216. The basal salts medium used in this study contained: NaCl, 300 mM; KCl, 10 mM; MgSO₄, 50 mM; KH₂PO₄, 0.33 mM; NH₄Cl, 10 mM; Fe(NH₄)₂(SO₄)₂, 0.005 mM; morpholinepropanesulfonic acid (MOPS buffer), 10 mM; CaCl₂, 2 mM (Martin and MacLeod, 1984).

Small sub-samples, ca. 5 cm below the dermal surface, from the freshly harvested sponge tissue samples in sterile seawater were aseptically removed and blotted dry (to remove as much excess canal water as possible), weighed and then homogenized using a sterile mortar and pestle. The volume of the resulting liquid suspensions was measured. The smaller sub-samples were used to avoid contamination from epizoic organisms. It should be noted that there will be an error associated with the determination of wet weight and its relation to sponge biomass due to the water within canals of the sponge tissue. Recorded weights probably reflect about 80 to 90% of the true weight of the sponge tissue.

Sponge homogenates, prepared within 30 minutes after collecting the sponges, and ambient seawater samples freshly collected in sterile syringes were serially diluted in sterile seawater and spread-plated in duplicate on marine agar. The remainder of each homogenate from 1 specimen of each sponge species was collected in a sterile tube and frozen for later use. The spread plates were incubated at 25°C and counted with the aid of a Québec colony counter after 2, 4, 7, and 14 days. The bacterial populations, expressed as colony forming units (cfu) per gram wet weight of sponge tissue and per millilitre of ambient seawater, were averaged and the standard errors calculated. Comparisons of the most commonly occurring bacterial colonies in the four sponge species and those in ambient seawater were made. Only those sponge bacteria distinguishable by Gram stain, colony and cellular morphology from the ambient seawater bacteria were examined in this study. These sponge-specific colonies were streaked for isolation on marine agar plates, incubated at 25°C for 2 days and returned to the laboratory at Macdonald College, McGill University for use in the remainder of the study.

-15-

Examination of Nutritional Requirements:

A) Capacity to use organic compounds for growth:

Sufficient double-strength basal salts medium (adjusted to pH 7.4 with KOH) and distilled water were added to 50-ml Erlenmeyer flasks so that after any further addition of supplements, the volume of the final medium would be 10 ml. Supplements of yeast extract and Bacto-casitone were added where indicated to give a final concentration of 0.0025% of each in the final The flask contents were then sterilized by autoclaving (120°C, 15 medium. Solutions of CaCl₂, and various carbon sources (glucose, minutes). galactose, glycine, glutamate, succinate, aspartate, and proline) were prepared, adjusted to pH 7.4 with KOH (except glucose and galactose), sterilized separately by autoclaving (proline was filter sterilized), and added aseptically to the flasks as required. The carbon sources were added to yield a concentration of 1000 mgC/i in the final medium. The flasks were inoculated with a single colony from freshiy incubated marine agar plates and incubated on a rotary shaker at 25°C. Cultures were examined daily for 14 days and growth was recorded when the cultures became visibly turbid as compared to an uninoculated control.

B) Capacity to grow chemoautotrophically:

The basal salts medium was modified to contain: NaCl at 240 mM to maintain a final concentration of sodium at 300 mM, and MOPS buffer was added at 30 mM for pH control. The media were prepared (yeast extract and Bacto-casitone were added to give a final concentration of 0.0025% of each in the final organic medium) and autoclaved as previously described, and solutions of CaCl₂, succinate (for <u>P. doudoroffii</u>, <u>A. haloplanktis</u>, and <u>P. aeruginosa</u>), glucose, sodium thiosulphate (Na₂S₂O₃), sodium bicarbonate

(NaHCO3) and supplemental sodium chloride (to bring the final sodium concentration to 300 mM) were prepared, adjusted to pH 7.4 with KOH (except glucose), sterilized separately, and added aseptically to the flasks as required. The final concentrations of Na₂S₂O₃ and NaHCO₃ were 50 mM and 10 mM respectively. Glucose and succinate were added where indicated to yield a concentration of 1000 mgC/l in the final medium. The flasks were inoculated with 1 ml of a suspension containing a single colony from a freshly incubated marine agar plate suspended in 5 ml of sterile basal salts medium (pH adjusted to 7.4 with KOH), and incubated on a rotary shaker at 25°C (37°C for P. aeruginosa and E. coli). The pH values of all cultures and controls were recorded. Changes in the bacterial populations (cfu/ml of medium) were compared to the initial inoculum populations on days 4, 7, 10, 14 and 21 by plating dilutions of 0.5 ml subsamples (in sterile basal salts medium) on marine agar and incubating at 25°C (37°C for P. aeruginosa and E. coli) for 36 hours. Colonies were counted at 18 and 36 hours with the aid of a stereomicroscope. The pH values of all cultures and controls were again recorded on the last day (21) of the experiment.

Examination of Antibiotic Sensitivities:

A) Preparation of sponge fluid:

Frozen sponge suspensions from previous isolations (see above) stored at -20°C for 3 months were slowly thawed at 4°C and then centrifuged (34K at 4°C for 40 minutes). The supernatants were collected and filter sterilzed ($0.45\mu m$) at 4°C to be tested for antibiotic properties.

B) Disk-plate technique:

The organisms were inoculated evenly and entirely on marine agar plates using sterile cotton swabs dipped into a suspension containing a single colony from a freshly incubated marine agar plate suspended in 2 ml of sterile basal salts medium (pH adjusted to 7.4 with KOH). Commercial antibiotic disks and sterile disks dipped in the two filter sterilized sponge supernatants under examination, were placed (5 disks/plate) on the inoculated surfaces of the marine agar plates. Duplicate plates were incubated at 25°C for 24 to 48 hours, and then examined for zones of inhibition (clear areas) around the disks.

RESULTS AND DISCUSSION

Microscopy:

Immense populations of apparently symbiotic bacteria were observed in the endosomal tissues of all four sponge species. Several morphologically distinst intercellular bacterial types were present within the mesohyl of all the specimens examined (Fig. 1).

Isolation of Organisms from Sponges and Ambient Seawater:

The average bacterial counts from sponge tissue and ambient seawater are presented in Table 1. These numbers represent counts recorded on day 4 of the incubation; the numbers had increased from day 2 but remained the same on day 7 but by day 14, colonies had spread over the plate making isolated colony counts impossible. The bacterial populations of the two sponge species with the lowest counts, <u>S. graminea</u> and <u>S. cheiris</u>, are approximately 7.5 times that of the ambient water populations.

To isolate organisms specifically associated with sponge tissue and to avoid organisms present as contaminants from seawater, sponge tissue was removed from the sponges aseptically and rinsed in sterile seawater. At dilutions of 10^5 or 10^6 a limited number of colony types distinctive for each sponge species could be detected, and the most characteristic colonies from each sponge were isolated and maintained on marine agar slants. Ten of the isolates could not be maintained on the marine agar medium used. The remainder of the study was conducted using the 10 strains which could be maintained on the medium. The morphological characteristics of these sponge bacterial strains are summarized in Table 2.

It must be cautioned that the colony forming units (cfu) measured in this study always reflect only a portion of the bacterial population being FIGURE 1: Transmission electron micrograph showing a typical bacterial population in the endosomal tissue of <u>Hippospongia lachne</u>. Note the different morphological types of intercellular bacteria (b) present in the mesohyl (m). c, choanocyte; fc, flagellated chamber.


8

TABLE 1: Average bacterial counts per gram of sponge tissue and per millilitre of ambient seawater.

Expressed as colony forming units (cfu) \pm standard error; N= number of sample pairs.

SAMPLE	COUNTS (cfu/g or /ml)	N
Spongia barbara	$1.28 (+0.21) \times 10^6$	3
Hippospongia lachne	2.62 (<u>+</u> 0.31) × 10 ⁵	5
Spongia cheiris	1.39 (<u>+</u> 0.20) × 10^5	3
Spongia graminea	1.37 (<u>+</u> 0.12) × 10 ⁵	3
Ambient seawater	1.83 (<u>+</u> 0.26) × 10^4	4

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TABLE 2: Morphological characteristics of sponge bacterial strains.

+= positive; -= negative; S.g.= <u>Spongia graminea</u>; H.1.= <u>Hippospongia lachne</u>; S.b.= <u>Spongia barbara</u>; c= cocci; r= rods.

BACTERIAL STRAIN	HOST S ponge	GRAM Stain	CELLULAR Morphology	COLONY Morphology			
1	S.g.	+	c; pairs & small clusters	2 mm; white; raised; entire; smooth			
2	S.g.	-	r; long & thin; short chains; motile; capsule	10 mm; cream: flat; irregular; mucoid; spreading			
3	S.g.	+	c; pairs & double chains; small clusters	0.5 mm; white; raised; entire; smooth			
4	S.g.	-	r; long & thin; motile	1 mm; white; raised; entire; smooth			
5	H.1.	+	c; large; pairs & small clusters	1 mm; yellow; raised; entire; smooth			
6	н.1.	-	r; small; short & oval; motile	3 mm; opalescent; flat; entire; spreading			
7	Н.1.	-	r; long & thin; motile; capsule; refractile granules	10 mm; cream; flat; irregular; mucoid; spreading			
8	S.b.	-	r; short & thin; motile	5 mm; beige; flat; entire; spreading			
9	S.b.	-	r; short & oval; motile; capsule; refractile granules	2 mm; opalescent; raised; entire; rough; mucoid			
10	S.b.	+	c; pairs & small clusters	1 mm; pale yellow; raised; entire; smooth			

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sampled. Whether the strains selected for study are truly representative of the bacterial population remains uncertain. The density of symbionts (#/gm wet weight) is an underestimate and more accurate assessments will require the use of transmission electron microscopic counts and calculations.

Nutritional Requirements:

When the 10 sponge bacterial strains were tested for growth on various individual amino acids, carbohydrates and tricarboxylic acid cycle intermediates as sole carbon and energy sources in the test medium, the results shown in Table 3 were obtained. These sources of carbon and energy were also tested for growth with supplements of yeast and Bacto-casitone as sources of growth factors. The results indicate that all except 2 of the organisms could utilize glucose and galactose as the sole source of carbon and energy in a basal salts medium. Of the two which failed to grow, one grew on adding a supplement of yeast extract and Bacto-casitone suggesting that additional growth factor(s) may be required for this organism. Later results indicated that sponge bacterial strain 3 has a lag period of 14 days preceding growth in a basal salts medium supplemented with glucose, yeast extract, and Bacto-casitone. Therefore growth could not be observed in this organism during the 14 day period of this experiment. Of the 10 organisms: 7 used succinate, 5 glutamate, 4 proline, 2 glycine and 1 aspartate as sole sources of carbon and energy in the medium. The further addition of growth factors in most cases decreased the lag period preceding the appearance of growth.

It is of particular interest that the organisms found associated with the sponges had such relatively simple organic growth requirements. They were not fastidious at all. Their presence in the sponge cannot be related

-23-

TABLE 3: Capacity to use organic compounds for growth.

+= growth; -= no growth; number of days of incubation included

COMPOUND ¹ (1000 mgC/l) <u>+</u> GROWTH FACTORS ²														
STRAIN Gluc NO	cose	Galactose		Gly cine		Proline		Glut am ate		Succinate		Aspartate		
	-	+	-	+	-	+	-	+	-	+	-	+	-	+
1	_ 14	+ 5	14	+ 5	- 14	+ 5	 14	_ 14	_ 14	 14	14	- 14	_ 14	- 14
2	+ 4	+ 3	+ 3	+ 3	+ 4	+ 3	+ 6	+ 3	+ 3	+ 3	+ 3	+ 3	- 14	- 14
3	- 14	- 14	- 14	_ 14	- 14	- 14	- 14	- 14	-14	14	14	- 14	- 14	- 14
4	+ 4	+ 3	+ 4	+ 3	- 14	 14	+ 6	+ 3	+ 6	+ 3	+ 4	+ 3	_ 14	+ 3
5	+ 2	+ 2	+ 2	+ 2	- 14	+ 2	+ 6	+ 6	- 14	+ 2	+ 4	+ 4	- 14	+ 6
6	+ 3	+ 3	+ 3	+ 3	- 14	- 14	- 14	- 14	+ 6	+ 3	+ 3	+ 3	14	+ 6
7	+ 4	+ 3	+ 3	+ 3	+ 4	+ 4	+ 6	+ 3	+3 3	+3 3	+ 3	+ 3	14	+ 6
8	+4 4	+4 4	+ 3	+ 3	 14	- 14	_ 14	- 14	- 14	+4 6	- 14	+4 8	14	14
9	+4 2	+4 2	+4 5	+4 2	- 14	- 14	- 14	+4 3	14	+4 6	+4 5	+4 5	- 14	14
10	+ 2	+ 2	+ 3	+ 2	- 14	+ 4	- 14	+ 6	+ 6	+ 3	+ 3	+ 3	+ 6	+ 6

1 glutamate, succinate, aspartate added as Na⁺ salts; 2 0.0025% yeast + 0.0025% Bacto-casitone; ³ rust colour; ⁴ clumps & flocs;

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to the capacity of the sponge to supply complex mixtures of factors required for growth. However, the sponge must be able to either directly supply or indirectly augment supply of one or more carbon and energy sources to support such relatively large populations of organisms.

Other studies have suggested that in some marine organisms chemoautotrophic bacteria grow symbiotically with, and supply nutrients to, the host animal (Cavanaugh <u>et al</u>, 1981; Cavanaugh, 1983; Felbeck <u>et al</u>, 1981; 1983; Southward <u>et al</u>, 1981). Are the microorganisms in the sponges facultatively chemoautotrophic? Can the sponge bacteria grow on an inorganic medium containing an oxidizable inorganic compound as the sole source of energy and use carbon dioxide (CO_2) as a source of carbon? To test this possibility the organisms were examined for their capacity to grow on the basal salts medium to which carbon as NaHCO₃ was supplied and various oxidizable inorganic compounds, namely ammonia, nitrite and thiosulfate were added as energy sources. Preliminary results indicated that some of the organisms were able to grow chemoautotrophically in such media but on subculture into fresh medium of the same composition growth could not be maintained.

The relative capacity of the organisms to maintain their viability, or to grow when inoculated into the basal salts medium, the basal salts medium supplemented with glucose (succinate for <u>P. doudoroffii</u>, <u>A. haloplanktis</u>, and <u>P. aeruginosa</u>) and growth factors (yeast extract and Bacto-casitone), and the basal salts medium with $Na_2S_2O_3$ added as an inorganic energy source (with and without NaHCO₃ added as a carbon source) was tested. Figure 2 demonstrates the various types of responses obtained. The response of sponge strain 1 illustrates one type of response; neither the basal salts medium nor the basal salts medium to which thiosulfate (with or without FIGURE 2: The capacity of organisms to maintain viability or grow in various media.

----basal salts medium (bsm) -----bsm + $Na_2S_2O_3$ + $NaHCO_3$ ••• bsm + $Na_2S_2O_3$ ------bsm + glucose/succinate + growth factors

1 Response of sponge bacterial strains 1 & 3.
2 Response of sponge bacterial strains 2, 5, 6, 7 & 8, marine bacteria
<u>P. doudoroffii, A. haloplanktis & A. aestus, & terrestrial bacteria</u>
3 Response of sponge bacterial strains 4 & 10.
4 Response of sponge bacterial strain 9.

Succinate (Na⁺ salts for <u>P. doudoroffii</u>, <u>A. haloplanktis</u> and <u>P. aeruginosa</u>) and glucose (for all others) at a concentration of 1000 mgC/T. Growth factors (yeast extract & Bacto-casitone) at concentrations of 0.0025%.



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NaHCO₃) had been added could maintain the viability of the cells on incubation. The presence of an organic carbon source and/or additional growth factors was necessary for growth and to maintain viability. This same type of response is illustrated by sponge strain 3. A second type of response is illustrated by sponge strain 2. For this organism, viability was maintained in the basal salts medium but an organic carbon source and/or additional growth factors was required for growth. This same type of response was observed in four other sponge bacterial strains and in the marine and terrestrial bacterial species examined. Sponge bacterial strains 4 and 10 represent a third type of response. Viability of these organisms was also maintained in the basal salts medium, and an organic carbon source and/or additional growth factors was required for growth. However, after 7 days incubation the organic medium could no longer maintain the viability of these organisms. The response of sponge strain 9 illustrates the final type of response that could be distinguished. Neither the basal salts medium nor the basal salts medium to which thiosulfate alone had been added could maintain the viability of the cells on incubation. However, slight growth occurred and viability was maintained in the basal salts medium to which thiosulfate and NaHCO3 had been added. Growth of this organism required an organic carbon source and/or additional growth factors but after 4 days incubation the organic medium could no longer maintain viability of the cells.

Seventy percent of the sponge associated organisms survived in the basal salts medium and all of them grew when a carbon source was added. Since this was similar to the response of other bacteria present in the marine and terrestrial environments it is not at all obvious why the particular organisms isolated should be present in the sponge in such large

-27-

numbers particularly when their nutritional requirements are so simple.

Antibiotic Sensitivities:

Studies have shown that some marine sponges produce antimicrobial substances which frequently inhibit the growth of marine bacteria (Burkholder and Ruetzler, 1969; Bergquist and Bedford, 1978; Amade, et al, 1987). Do the sponges in this study contain antimicrobial substances which do not interfere with their associated bacteria but do inhibit other microorganisms? To test this possibility the organisms were examined for their susceptibility to different antibiotics and to fluid from the sponges. The antibiotic sensitivities of the sponge associated organisms and two marine bacteria isolated from other sources (P. doudoroffii and A. haloplanktis) are presented in Table 4. A 1 of the organisms showed sensitivities to various antibiotics as would be expected from the spectrum of antimicrobial agents tested. However, neither the marine bacteria from other sources nor the bacteria from the sponges were inhibited by the fluid from the two sponges tested. Therefore, the presence of bacteria specific to the sponge is not likely to result from resistance to antimicrobial substances produced by the specific sponge hosts.

TABLE 4: Antibiotic sensitivities.

TE= tetracycline ($30\mu g$); SxT= sulfamethoxazole ($25\mu g$); CR= cephalothin ($30\mu g$); P= penicillin ($10\mu g$); K= kanamycin ($30\mu g$); C= chloromycetin ($30\mu g$); AM= ampicillin ($10\mu g$); S= streptomycin ($10\mu g$); S.b.= supernatant from Spongia barbara; S.g.= supernatant from Spongia graminea; A.h.= Alteromonas haloplanktis; P.d.= Pseudomonas doudoroff11; += inhibition; -= no inhibition

		SENSITIVITY TO ANTIBIOTICS								
STRAIN	TE	SxT	CR	P	ĸ	С	AM	S	S.b.	S.g.
1	+	-	+	+	-	+	+	+	-	-
2	+	-	-	-	+	+	-	+	-	-
3	+	-	+	+	-	+	+	+	-	-
4	-	-	-	-	+	+	-	+	-	-
5	+	-	+	+	-	+	+	+	-	-
6	+	+	+	+	+	+	+	+	-	-
7	+	-	-	•	+	+	-	+	-	-
8	-	-	+	-	+	-	-	+	-	-
9	-	+	+	+	+	+	+	+	-	-
10	+	+	+	+	-	+	+	+	-	-
A.h.	-	+	-	+	+	+	+	+	-	-
P.d.	+	-	+	+	+	+	+	+	-	-

CONCLUSIONS

The presence of large populations of intercellular bacteria in the endosomal tissue of four commercial sponge species has been confirmed. The results of the present study on tropical Caribbean sponges provide further evidence to support the conclusions of Wilkinson (1978b) and Wilkinson, <u>et</u> <u>al</u> (1981) based on studies of Great Barrier Reef and Mediterranean sponges. Wilkinson concluded that the bacterial symbionts of sponges which harbour large populations of bacteria are specific to that habitat and different from ambient seawater bacteria. Furthermore, bacterial populations of the sponges are higher than those of the ambient seawater.

The present study has demonstrated that: 1) the specific bacteria associated with the sponges metabolize a wide range of compounds and have relatively simple growth requirements, and 2) the sponges do not appear to possess antimicrobial substances which inhibit marine bacteria. A recent study by Amade, <u>et al</u> (1987) has also found that extracts from sponge species of the same genus and closely related genera do not possess antimicrobial substances active against marine bacteria. All of the commercial sponge species of the order Dictyoceratida, examined and reported to date, have been observed to harbour large populations of intercellular bacteria. Some of those same species have also been tested for antimicrobial substances and, in all cases, sponge extracts were not active against marine bacteria. Is there a correlation between the lack of antimicrobial activity in these sponges and the fact that they also harbour large populations of bacterial symbionts within their tissues?

The nature of the association between symbiotic bacteria and sponges is not known, and two questions still remain: Why are there large populations of specific bacteria present in the tissues of many marine sponges that are not present in the ambient seawater? And what, if any, role do these symbionts and their hosts have in this association?

The sponges probably directly supply or indirectly augment supply of one or more carbon and energy sources to support the massive populations of intercellular bacteria within their tissues. In turn, the sponges probably derive a part of their own energy requirements by incorporating symbiont byproducts into host mesohyl. The bacteria may also play a role in providing trace elements and vitamins which cannot be synthesized by the sponges themselves. Some of the symbionts may be capable of digesting sponge collagen, thereby modifying the insoluble collagenous fibrils and producing soluble products that the sponge may then utilize in reorganizing and constructing its structural network. If this is indeed the case, then the symbiotic association that has been established between bacteria and sponges may be a significant element in the taxonomic differentiation of the Dictyoceratida. The bacterial symbionts may, directly or indirectly, function in the construction of the spongin skeleton, a major feature of Poriferan classification.

Another important aspect that should be considered in this symbiotic association between bacterial populations and commercial sponges is the possible significance of these associated bacteria in the epidemic diseases that devastated many of the Caribbean commercial sponge beds four and five decades ago (Smith, 1941). The 'agent' of disease, very tentatively identified as a filamentous fungus, may not have been an invasive pathogen to the sponge host per se but may alternatively have been any factor affecting the associated bacterial population or the balance of the association itself, thus resulting in the death of these commercial sponge populations. Whatever the scenario, it is clear that studies of the dictyoceratids must include consideration of the bacterial populations harboured within them as a major biotic component and treat this intimately balanced association as an integrated but potentially disturbable community.

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ACKNOWLEDGEMENTS

We wish to thank Dr. Barrie Taylor of the Rosenstiel School of Marine and Atmospheric Sciences, University of Miami for his helpful advice and for the use of laboratory facilities. This study was supported by a grant from the Royal Bank/McGill International/Caribbean Development Fund, and an NSERC (Canada) operating grant to H.M. Reiswig, and an NSERC (Canada) operating grant to R.A. MacLeod.

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CHAPTER II

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A STUDY OF SEXUAL REPRODUCTION IN FOUR CARIBBEAN COMMERCIAL SPONGE SPECIES.

I. Oogenesis, transfer of bacterial symbionts, and larval development, behaviour, settlement and metamorphosis.

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in press, Oogenesis and transfer of bacterial symbionts. Proceedings of the Third International Conference of Sponges.

in submission, Larval development, behaviour, settlement and metamorphosis.
International Journal of Invertebrate Reproduction and Development.

ABSTRACT

-39-

Oogenesis in four commercial Caribbean sponge species, Hippospongia lachne, Spongia barbara, S. cheiris and S. graminea, was studied using light and transmission electron microscopy. Eggs and embryos develop asynchronously in localized endosomal nurseries of these viviparous and gonochoristic species. Statistical analyses of specific morphological characteristics of reproductive elements have unequivocally identified four specific stages in the process of oogenesis. Oogonia undergo mitotic division to produce primary oocytes. Meiotic division occurs producing secondary oocytes which are fertilized before zygotes undergo major by phagocytosis and the transfer of nutrients growth through cytoplasmic bridges. During cleavage of the zygote, umbilici form between embryos and nurse cell layers and these function in the transfer of symbiotic bacteria and other mesohyl substances from the maternal parent to the embryo. These symbionts were observed dispersed between blastomeres of all young and maturing embryos and between internal cells of cytodifferentiated parenchymella larvae. Extracellular transfer of symbiotic bacteria from maternal tissues to developing embryos has not been reported in any other viviparous invertebrate. Laboratory behavioural studies indicate that free-swimming larvae display directional swimming with constant rotation and negative phototaxis. Larval settlement occurs 26-56 hours after release from the parent sponge and involves the formation of a basal lamella between the post-larvae and substrate. There is no evidence of substrate selection or orientation by larvae. Larval behaviour is probably an important factor in the habitat specializations of adults.

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INTRODUCTION

The genera <u>Spongia</u> Linnaeus, 1759 and <u>Hippospongia</u> Schulze, 1879 (Dictyoceratida) include all of the commercially important bath sponges of the Caribbean (ca. 7spp.) and Mediterranean/Red Sea (ca. 3spp.) fisheries. Epidemic diseases devastated entire populations of the Caribbean species four and five decades ago and, in spite of the resurgence of these populations, few recent investigations have focused on the biology and ecology of these once extremely valuable economic commodities. Most of the available literature on commercial sponge species deals with gross morphology and fisheries aspects. Little is known of their biology including reproduction, embryology, and larval development and metamorphosis.

Although recent and extensive literature reviews have reported numerous studies dealing with sexual reproduction in sponges (Fell, 1983; Reiswig, 1983; Simpson, 1984), there are relatively few studies on reproductive processes in the dictyoceratids (Tuzet & Pavans de Ceccatty, 1958; Liaci, <u>et al</u>, 1971). Much of what is known about these processes has been inferred from gross structural observations in the course of general studies on reproduction. Crucial events such as those dealing with gamete differentiation, fertilization and incorporation of symbionts into developing embryos still require elucidation. The use of the electron microscope (EM) has provided more detailed information on specific events during reproductive processes in sponges. However, there is no available ultrastructural information on oogenesis or embryogenesis in the dictyoceratids and only one EM study has investigated spermatogenesis in these sponges (Gaino, <u>et al</u>, 1984).

The occurrence of symbiotic bacterial associations with marine invertebrates is a fairly widespread phenomenon. These have been reported in at least a few members of most of the major invertebrate phyla. Symbiotic bacteria have been reported in all sponge species of the order Dictyoceratida which have been carefully examined (Wilkinson, 1984). However, there is a glaring lack of appreciation of the uniform presence and possible significance of immense symbiotic bacterial populations in these animals. Recent electron microscopic studies have revealed the existence of large populations of intercellular symbiotic bacteria in the tissues of four commercial Caribbean sponge species: <u>Hippospongia lachne, Spongia</u> <u>barbara, Spongia cheiris</u> and <u>Spongia graminea</u>. Bacteria isolated from these sponges are specific sponge symbionts and different from ambient seawater bacteria (Kaye, <u>et al</u>, 1985). Intercellular bacterial symbionts were also observed in the embryos and brooded larvae of these species.

There is a lack of information available in the literature on sequential events in the life histories of sponge larvae (morphogenesis, behaviour, settlement and metamorphosis). This information is essential to interpreting the results of ecological and biological studies of adult populations. A number of studies have investigated isolated aspects of larval biology in some sponge species (Lévi, 1956; Warburton, 1966; Bergquist & Sinclair, 1968; Simpson, 1968; Evans, 1977; Fell, 1976) but there are only two studies that have reported on the specific sequence of events in the life histories of larvae (Bergquist & Green, 1977; Bergquist, et al, 1979). The information gleaned from many of these studies has been used in discussion of systematics and evolution. However, few have attempted ecological interpretations of possible relevance to the adult populations.

-41-

The present study was undertaken as a first step in the analyses of specific reproductive processes in four species of Caribbean commercial sponges: <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>Spongia cheiris</u> and <u>Spongia graminea</u>. The following aspects of their reproduction have been addressed: oogenesis, embryonic and larval development, transfer of bacterial symbionts, and larval morphology, behaviour, settlement and metamorphosis. Other investigations in progress will focus on reproductive cycles and spermatogenesis in these four species.

Morphological characteristics of reproductive elements have been analyzed statistically to objectively identify specific stages in oogenesis before attempting to describe the dynamics of this process. Light and transmission electron microscope studies, together with laboratory experiments have been employed to help elucidate the reproductive processes of these economically important members of the phylum Porifera.

MATERIALS AND METHODS

Sponges:

Four Caribbean commercial sponge species: <u>Hippospongia lachne</u> de Laubenfels, 1936, <u>Spongia barbara</u> Duchassaing & Michelotti, 1864, <u>Spongia</u> <u>cheiris</u> de Laubenfels & Storr, 1958, and <u>Spongia graminea</u> Hyatt, 1877 were investigated. These rounded, black to dark grey demosponges are common members of the fauna of Biscayne Bay, Florida. This shallow bay (average depth 2.5 m) has intermittent periods of turbulence related to seasonal weather conditions. The collection site (25°38'N; 80°12'W) is characterized by a coarse sandy bottom with extensive areas covered in eel and turtle grasses, small scattered corals, gorgonians and other sponge species. A total of 177 specimens were collected over a three year period (December 3, 1981; February 12, 1982; June 27, 1982; September 1, 1982; January 18, 1983; May 23, 1983; August 24, 1983) and analyzed histologically for reproductive activity.

Sampling and Preparation of Specimens for Microscopy:

Using SCUBA, endosomal tissue samples were removed <u>in situ</u> from specimens of the four sponge species (located at a depth of 1 to 1.5 m) using a large filet knife. Large triangular sections (ca. 60 mm on each side) were removed from the lower two-thirds of the individuals. A smaller tissue sample (ca. 20 mm³) was cut from that portion of the triangle that had come from the central region of the sponge. This procedure was employed to insure that if the sponge was reproductively active, tissue samples collected would contain reproductive elements. This smaller sample was then fixed <u>in situ</u> by placing the tissue in a syringe, egressing the ambient seawater and ingressing 2.5% glutaraldehyde in seawater (fixative). After returning to the laboratory, samples were placed in vials of fresh fixative for 16 hours, and then rinsed in three changes of fresh seawater for 10 min each. A smaller sub-sample (ca. 5mm³) from each specimen was post-fixed for one hour in 1% osmium tetroxide in seawater, dehydrated in an alcohol series, cleared in propylene oxide, and embedded in Spurr epoxy resin. The remaining fixed tissue from each specimen was dehydrated in an alcohol series, cleared in xylene, and embedded in paraffin.

Microscopy:

Epoxy blocks were sectioned on a Sorvall 'Porter-Blum' MT-2B ultramicrotome at 1µm (glass knife) and 0.1µm (diamond knife). Semi-thin sections were mounted on glass slides and stained for 1 min at 60°C with a mixture (equal parts by volume) of methylene blue (1% in 1% Na-borate solution) and azure II (1% in distilled water). Ultrathin sections were floated onto formvar and carbon coated single-slot copper grids and stained for 20 min in saturated aqueous uranyl acetate and for 15 min in lead citrate. Sections were viewed and photographed with a Philips 410 transmission electron microscope operating at 80 kV. Paraffin blocks were sectioned at 10µm, and the sections were viewed, and measurements of reproductive elements recorded under a compound microscope with an ocular micrometer accurate to 0.5µm. Light micrographs were taken on a Zeiss photomicroscope.

Larval Collection:

Once, during the reproductive period (May to Sept.), large pieces of tissue from reproductively active specimens of the four sponge species were collected, using SCUBA, placed in plastic bags and immediately returned to

-44-

the laboratory where they were placed in running seawater aquaria. Larvae released from the tissue samples were collected, observed and photographed under a WILD M5A dissecting microscope. In the field, a larval collecting net (Fig. 1) was placed over reproductively active specimens to aid in the collection of released larvae for observations of behaviour, settlement patterns, and metamorphosis. Sponges which were found to be reproductively active when tissue samples were collected were removed from the substrate and attached to a cement block by a monofilament line. The collecting net was then placed over the sponge and cement block to collect released larvae. Free-swimming larvae were fixed, embedded, and sectioned (as previously described) for electron microscopy and ultrastructural analyses of morphology and metamorphosis.

Behavioural Studies:

Separate studies were conducted on the larvae of <u>H. lachne</u>, <u>S. barbara</u>, S. cheiris and S. graminea at water temperatures of 28-29°C.

1) Phototaxis:

Fifty free-swimming larvae were collected from the running seawater aquaria and placed in an opaque black plexiglass experimental chamber (32.5 x 17.5 x 10cm) filled with seawater from the aquaria. A light source (tungsten lamp) was placed 5 cm from the surface of the water at an angle of ca. 45° to the bottom of the chamber. Irradiance ranged from 0.037 μ ein cm⁻² sec⁻¹ just below the surface of the water to 0.035 μ ein cm⁻² sec⁻¹ just off the bottom of the chamber. Larvae were exposed to the unidirectional light source under 3 separate trials. To eliminate behaviour modifications due to habituation and temperature differences, the location of the light source was varied (from end to end and side to side of the experimental chamber) between trials and applied for 30-60 seconds. A positive phototactic response was scored if the larvae were observed to swim into or remain in the path of light and a negative response was scored if the larvae were observed to swim out of or away from the light path, and remain in the darkened regions of the chamber. The experiments were conducted in the laboratory at night with minimal extraneous light.

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FIGURE 1: Larval catching net used in the field to collect larvae released from reproductively active sponges. The screen cloth used was Nitex high capacity nylon monofilament screen cloth No. HC3-190.

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2) Substrate Selection:

Substrate selection was tested by presenting glass and asbestos cement (transite) slides (25 x 75 x 1mm) as potential settlement surfaces. Freeswimming larvae were offered the upper and under surfaces of clean or "aged" glass and clean or "aged" transite. Four slides (clean-glass, "aged"-glass, clean-transite, and "aged"-transite) were placed in clear plexiglass chambers (8.5 x 12.5 x 8cm) filled with seawater from the running seawater aquaria. Larvae were placed in the chambers which were then maintained in running seawater tables. The top 2 cm of the chambers remained above the level of the water in the seawater tables and chamber water was changed twice daily (07:30 and 19:30). Slides were "aged" by placing them in racks which were suspended from wires in running seawater tables for two days until a thin film of bacteria and algae covered the slides. Settlement surfaces were raised off the bottom of the chambers by rubber discs (0.5×10^{-5}) 0.5cm). The discs were attached to the ends of the slides on both the upper and under surfaces by a silicone sealant. Fluorescent overhead lights in the laboratory supplied illumination during the day (07:30 - 19:30) and the laboratory remained dark during the night.

Larval Metamorphosis:

Settled larvae were observed daily over a period of three weeks. During this time water temperatures ranged from 28°C to 29°C. Macroscopic observations of morphological changes in metamorphosing juvenile sponges were recorded under a dissecting microscope. Larvae that had settled for 24 hours were easily loosened and removed from glass slides by sliding a razor blade under the metamorphosing sponges. Minimal disruption occured and intact specimens were fixed, embedded, and sectioned (as previously

described) for electron microscopy and ultrastructural analyses of morphology.

Data Collection and Analyses of Reproductive Elements:

A total of 170 reproductive elements from two specimens of H. lachne were investigated using light microscopy. Morphological characteristics (parameters) of these reproductive elements were statistically analysed to objectively identify stages in the process of oogenesis prior to embryo formation. The parameters considered in these analyses included: cell diameter, nucleus diameter, nucleolus diameter, ratio of cell diameter to nucleus diameter, ratio of nucleus diameter to nucleolus diameter, number of nucleoli, type of cytoplasm (scored as vesicular, vesicular-dense or dense), number of nurse cells surrounding reproductive elements, and evidence of phagocytic activity (scored as present or absent) by reproductive elements. Measurements of diameters were recorded as the maximum diameter measurement from serial sections when the same reproductive element could be followed in serial sections. The other parameters were also scored from observations of seril sections whenever possible. Measurements and observations of the same parameters in two specimens of each of the other three species S. barbara, S. cheiris, and S. graminea) investigated were also recorded. Reproductive elements were also investigated using electron microscopy. A detailed description of each of the stages in the process of oogenesis was made from light and electron microscopic observations of 9 specimens of H. lachne, 14 specimens of S. barbara, 5 specimens of S. cheiris, and 12 specimens of S. graminea.

The data collected from measurements and observations of the parameters of reproductive elements were analysed using the Statistical Analysis System (SAS) software computer package at McGill University. The basic assumptions of homogeneity of variance and normality of the data were checked with Bartlett's test and the Kolmogorov-Smirnov (D) statistic, respectively. As a result of violations of both assumptions after numerous transformations of the data, nonparametric statistics were applied to raw data and parametric statistics to the normalized ranks of the data (Conover, 1980). Data were ranked using the SAS Rank (Blom) procedure (Ray, 1985).

A classification analysis using the SAS Cluster (Ward's minimum variance method) procedure (Ray, 1985) was performed on the data to group the reproductive elements into relatively homogeneous groups that could be interpreted as specific stages in the process of oogenesis. A discriminant analysis using the SAS Discrim procedure (Ray, 1985) was performed on the data using the group memberships given in the cluster analysis as the dependent variables. This procedure fits a discriminant function to the data which is then used to see how correctly the discriminant function identifies each reproductive element's group. The percentage of correct predictions is used as a measure of the secondary validity of the group memberships (Romesburg, 1984).

A principal component analysis (PCA) using the SAS Princomp procedure (Ray, 1985) was used to interpret the pattern obtained from the classification analysis and to summarize the data in terms of new components, a small number of which account for most of the variation of the original data. The data was standardized because of the varying units of measure and the principal components were then computed from a correlation matrix to remove differences due to both the mean and dispersion of the parameters. These procedures make the parameters directly comparable (Dillon and Goldstein, 1984).

-50-

According to Mardia (1971), Ibanez (1972) and Marriott (1974) it is permissible to perform these procedures even though the data does not satisfy the assumptions of parametric statistics. However, as suggested by these authors, the same analyses were carried out on both ranked and raw data and the results were compared.

Since the data did not meet the basic assumptions of parametric analysis of variance (ANOVA), a nonparametric ANOVA using the SAS NPAR1WAY procedure (Kruskal-Wallis Chi-square approximation; Ray, 1985) was used to test the null hypothesis; H_0 : there are no differences in parameter (x) between specimens, against an alternate, Hi: there are differences in parameter (x) between specimens. This same procedure was used to test the null hypothesis; H_0 : there are no differences in parameter (x) among stages, against an alternate H1: there are differences in parameter (x) among stages. The same hypotheses were tested on ranked data using a parametric ANOVA (GLM procedure for unbalanced data; Ray, 1985). Where the ANOVA rejected the null hypothesis, the Tukey multiple means comparison test (at P=0.05 level, on the normalized ranks) was used to determine between which of the stages significant differences occurred. The Tukey test was chosen because it is a conservative test in that it is more likely to find no statistical differences between the multiple means being compared and tested (Zar, 1984).

Data Analyses of Substrate Selection:

The data collected were tested for the assumptions of normality and homogeneity of variance. Univariate analysis (SAS) showed no departures from normality, and Bartlett's test (SAS) showed homogeneity of variance between substrates. Therefore parametric statistics were used to analyze

the data. A four-factor ANOVA, including interaction effects, was performed to test the following null hypotheses; 1) Ho: there are no differences in the number of larvae settling on the various proffered substrates (glass vs. transite, clean vs. aged, and upper vs. under); 2) H_o: there are no differences in the number of larvae settling among species; and 3) H_0 : there are no interactions of substrate types (4 possible combinations) and substrate types and species (5 possible combinations) on the number of larvae settling. Replicate samples were not possible, and therefore the mean square of the highest order interaction (interaction effects for all factors) was used as the error term in the ANOVA (Zar, 1984).

OBSERVATIONS AND RESULTS

All of the samples collected (177) contained either male (5%), female (23%) or no (72%) reproductive elements. Male and female elements were never observed in the same specimen. Oocytes and embryos develop asynchronously within maternal tissues during the reproductive cycle as do spermiocysts within paternal tissues (Chapter 3). These observations strongly suggest that these species are dioecious (gonochoric), although successive hermaphroditism cannot be ruled out as the same individuals were not sampled consecutively. Clearly, such a regular sampling programme is neccessary to provide more information on sexual differentiation in these four species.

Data Analyses of Reproductive Elements:

Figure 2 is a dendrogram generated from the Ward's classification analysis illustrating the subdivisions of the normalized ranks of morphological characteristics (parameters) of the reproductive elements. These subdivisions are based on the parameters of cell diameter (\underline{c}), nucleus diameter (\underline{n}), nucleolus diameter (\underline{nu}), ratio of cell diameter to nucleus diameter ($\underline{c:n}$), ratio of nucleus diameter to nucleolus diameter ($\underline{n:nu}$), number of nucleoli (\underline{nonu}), type of cytoplasm (\underline{cy}), number of nurse cells surrounding reproductive elements (\underline{nurse}), and evidence of phagocytic activity by reproductive elements (\underline{p}). Four main clusters of observations are distinguished at an arbitrary similarity level of R²=0.5. This means that 50% of the variance between clusters can be explained by the four observed clusters. This suggests that reproductive elements in <u>H</u>. <u>lachne</u> can be grouped into four major stages.

The results of the discriminant analysis on the normalized ranks of
FIGURE 2: Dendrogram showing the classification of 125 reproductive elements (RE) of <u>H. lachne</u> based on cell diameter, nucleus diameter, nucleolus diameter, ratio of cell diameter to nucleus diameter, ratio of nucleus diameter to nucleolus diameter, number of nucleoli,type of cytoplasm, number of nurse cells surrounding RE, and evidence of phagocytic activity by RE. The normalized ranks of all parameters were used in the comparison of RE (classified using Ward's minimum variance; Ray, 1985). The RE split into 4 main clusters (1, 2, 3, 4) at an arbitrary similarity level of R² = 0.5 (R² is the sum of squares between all clusters divided by the corrected total sum of squares).

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parameters are presented in Table 1. The group memberships from the cluster analysis have been used together with the parameters to predict the accuracy of the grouping of reproductive elements. It can be seen from these results that: 86% of the time a reproductive element will be classified correctly as stage 1 and 14% of the time incorrectly as stage 2, and 100% of the time the element will be identified correctly as stage 2, 3 or 4. This analysis supports the suggestion of the classification analysis that products of oogenesis can be grouped into four major stages. The fact that a stage 1 reproductive element was incorrectly classified may be due to the limited number of these elements available for the analysis (N=7).

The results of the PCA on the normalized ranks of morphological characteristics are presented in Table 2. The parameters have been analyzed to assist in the interpretation of specific groups of reproductive elements and to summarize the data in terms of new components which account for most of the variation of the original data. According to Ibanez (1972) all parameters being analyzed must be independent and, when using a correlation matrix based on standardized data, only those principal components (PC) that have eigenvalues ≥ 1 can be interpreted. Therefore, the ratio of cell diameter to nucleus diameter (c:n) and the ratio of nucleus diameter to nucleolus diameter (n:nu) were not included in the PCA and, PC1 and PC2 are the only components considered in the interpretation of the analysis. It can be seen from these results that: 1) PC1 accounts for 70% of the variation of the original data and of the parameters tested c, n, nu, cy, nurse, and p load equally on PC1, and 2) PC2 accounts for 15% of the variation and the parameter nonu loads heavily on PC2. This analysis suggests that cell diameter, nucleus diameter, nucleolus diameter, type of cytoplasm, number of nurse cells surrounding reproductive elements, and

-55-

F rom Stage	NUMBER OF REPRODUCTIVE ELEMENTS (N) AND PERCENTS CLASSIFIED INTO STAGE							
	1	2	3	4	TOTAL			
1	N=6	N=1	N=0	N=0	N=7			
	85.7	14.3	0.0	0.0	100.0			
2	N=0	N=26	N=0	N=0	N=26			
	0.0	100.0	0.0	0.0	100.0			
3	N=0	N=0	N=57	N=0	N=57			
	0.0	0.0	100.0	0.0	100.0			
4	N=0	N=0	N=0	N=35	N=35			
	0.0	0.0	0.0	100.0	100.0			
TOTAL	N=6	N=27	N=57	N=35	N=125			
	4.8	21.6	45.6	28.0	100.0			

TABLE 1: Results of the Discriminant Analysis on the normalized ranks of morphological characterisitics of reproductive elements in <u>H. lachne</u>.

-56-

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PRINCIPAL COMPONENT	EIGENVALUE	PROPORTION	CUMULATIVE
 PR I N1	4.88144	0.697349	0.69735
PR I N2	1.01885	0.145550	0.84290
PR IN3	0.45700	0.065286	0.90818
PR IN4	0.31874	0.045534	0.95372
PR I N5	0.18465	0.026379	0.98010
PRIN6	0.09301	0.013287	0.99338
PR I N7	0.04631	0.006616	1.00000

TABLE 2:	Results of the Principal Component Analysis on the
	normalized ranks of morphological characteristics of
	reproductive elements in H. lachne.

-57-

PARAMETER	EIGENVECTORS PRIN1	PR I N2
Cell diameter (<u>c</u>)	0.437102	0.016161
Nucleus diameter (<u>n</u>)	0.419811	034824
Nucleolus diameter (<u>nu</u>)	0.393827	250751
# of nucleoli (<u>nonu</u>)	0.081212	0.966319
Type of cytoplasm (<u>cy</u>)	0.389878	0.020020
<pre># of nurse cells (nurse)</pre>	0.422984	0.036366
Phagocytic activity (<u>p</u>)	0.374280	0.012407

evidence of phagocytic activity by reproductive elements are equally important in distinguishing between groups of elements. The number of nucleoli is also an important parameter that should be considered in the grouping of reproductive elements.

It should be noted that the results of these procedures (classification, discriminant and PCA) on both ranked and raw data were the same. Therefore, for consistency with subsequent analyses, the results of the tests on the normalized ranks of parameters have been presented.

On the basis of the results of these three procedures, the data were analyzed according to the four identified stages. The results of the nonparametric ANOVA (NPARIWAY) and the parametric ANOVA (GLM) on ranked data showed no significant differences (P > 0.05) in between-specimen comparisons. Drawing conclusions from these tests with respect to the parameters tested ($\underline{c}, \underline{n}, \underline{nu}, \underline{c:n}, \underline{n:nu}, \underline{nonu}, \underline{cy}, \underline{nurse}, and \underline{p}$), the two specimens are assumed to come from populations having identical means. Therefore, the data from both specimens were pooled for further statistical analyses.

The results of the nonparametric ANOVA (NPAR1WAY) and the parametric ANOVA (GLM) on ranked data indicated that: 1) the means of <u>c</u>, <u>n</u>, <u>nu</u>, <u>c:n</u>, <u>nonu</u>, <u>cy</u>, <u>nurse</u>, and <u>p</u> were statistically different (P<0.05) among stages in oogenesis and 2) the means of <u>n:nu</u> were not statistically different (P > 0.05) among stages in oogenesis. To determine where the differences lie in the means, the Tukey test was performed on the normalized ranks of morphological characteristics and the results are presented in Table 3. Conclusions that can be drawn from this test are: 1) cell diameters (<u>c</u>), nucleus diameters (<u>n</u>), ratios of cell diameter to nucleus diameter (<u>c:n</u>), the type of cytoplasm (<u>cy</u>), and the number of nurse cells surrounding the reproductive elements (<u>nurse</u>) of all four stages are assumed to come from

- 58-

TABLE 3: Results of Tukey's Studentized Range Test on the normalized ranks of morphological characterisitics of reproductive elements in <u>H. lachne</u>.

Comparisons significant at the 0.05 level are indicated by $_$. N= number of observations.

PARAMETER	1	STAGE COMP 2	PARISON 3	4
 Cell diameter (<u>c</u>) (N=170)				
Nucleus diameter (<u>n</u>) (N=146)				
Nucleolus diameter (<u>nu</u>) (N=131)				
Ratio of C to N (<u>c:n</u>) (N=146)				_
Ratio of N to Nu (<u>n:nu</u>) (N=131)		,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		
# of nucleo!i (<u>nonu</u>) (N=141)			·····	
Type of cytoplasm (<u>cy</u>) (N=169)			_	
# of nurse cells (<u>nurse</u>) (N=164)				
Phagocytic activity (<u>p</u>) (N=170)	<u> </u>			

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populations having means different from each other, 2) nucleolus diameters (\underline{nu}) of stages 1 and 2 are assumed to come from populations having identical means but having different means from the other two stages which also have means different from each other, 3) the number of nucleoli (<u>nonu</u>) of stages 2, 3, and 4 are assumed to come from populations having identical means but having different means from stage 1, and 4) the evidence of phagccytic activity by reproductive elements (<u>p</u>) of stages 1, 2, and 3 are assumed to come from populations having means different from stage 4. It seems apparent that to distinguish all four stages as being different from one another the parameters of <u>C</u>, <u>n</u>, <u>C:n</u>, <u>Cy</u>, and <u>nurse</u> are required. The <u>n:nu</u> parameter does not distinguish stages as being different from one another; and the remaining parameters, <u>nu</u>, <u>nonu</u>, and <u>p</u>, only distinguish 3, 2, and 2 stages respectively as being different from one another.

When these results are compared to the results of the PCA it can be seen that the parameters <u>c</u>, <u>n</u>, <u>cy</u>, and <u>nurse</u> which each show statistically significant differences between all stages in the Tukey test, also load highly on PC1 which accounts for 70% of the variation of the data. Therefore, if reproductive elements are to be classified according to their stage of development, the required morphological characteristics to accurately classify the elements in this study would be: cell diameter, nucleus diameter, type of cytoplasm, and the number of nurse cells surrounding the reproductive elements. It is possible that any one of these parameters alone could be used to classify a reproductive element into one of the four stages identified; however, as in all scientific studies, it is in the best interest of the study to collect and analyze as much information as possible in order to gain a more complete understanding of a system. It has been shown statistically that, based on specific parameters, the reproductive elements of <u>H</u>. <u>lachne</u> examined in this study, can be grouped into four stages prior to embryo formation. Measurements and observations of the same parameters in the other three species (<u>S. barbara, S. cheiris</u>, and <u>S. graminea</u>) investigated, did not differ from those of <u>H</u>. <u>lachne</u>. The stages which were statistically identified as 1, 2, 3, and 4 have been interpreted biologically as the oogonium, prir <u>y</u> oocyte, secondary oocyte, and zygote stages of oogenesis prior to embryogenesis. Table 4 provides a summary of the morphological characterisitics of these four stages in <u>H</u>. <u>lachne</u>. The description that follows is based on these parameters and applies to all four sponge species examined unless otherwise stated.

The sequence of events in oogenesis and development reported here, is inferred from linking together static images clearly similar or very nearly identical to one another in series. The processes are described as active, for converience.

Microscopy:

Gamete production and embryo development are localized in patches or "nurseries" of endosomal tissue. These "nurseries", containing 20 or more oogeneic elements (Fig. 3a), are located in the lower two-thirds and towards the central part of the sponges where a major portion of the tissue is involved. In the immediate vicinity of the "nurseries" the mesohyl of individuals undergoing oogenesis is disrupted with an observed decrease in cell numbers, flagellated chambers and symbiotic bacteria. Endosomal tissue outside nursery areas and the ectodermal tissue remain undisturbed.

Light and transmission electron microscopic analyses of specimens of Hippospongia lachne, Spongia barbara, S. cheiris and S. graminea undergoing

-61-

	STAGES IN OOGENESIS							
MORPHOLOGICAL CHARACTERISTIC	oogonium (1)	primery oocyte (2)	secondary oocyte (3)	zygote (4)	embryo			
cell diameter (µm)	10-15 N=16 X=13.94 (<u>+</u> 1.73)	15-25 N=30 X=21.65 (<u>+</u> 2.92)	25-40 N=72 X=33.54 (<u>+</u> 5.09)	40-300 N=51 X=76.69 (<u>+</u> 68.09)	375-525 X 335-485 N=16 X=473.13 X 394.38 (+47.74 X 46.61)			
shape	triangular	ovoid to spherical	ovoid to spherical	ovoid to spherical	ovoid			
cytoplasm	vesicular	vesicular to dense	dense	dense with phagosomes	blastomeres (190 - 30µm)			
nucleus (diameter in µm)	en]arged, distinct 4-6 N=15 X=5.17 (<u>+</u> 0.70)	distinct, dense 6-8 N=27 X=6.61 (<u>+</u> 0.85)	distinct, dense 8-12 N=67 X=9.28 (<u>+</u> 1.47)	distinct, often a clear zone around membrane 10-22 N=37 X=12.99 (<u>+</u> 3,34)	could not be clearly distinguished in blastomeres			
nucleolus (diameter in µm)	single, prominent 1.5-2 N=7 X=1.79 (<u>+</u> 0.27)	often 2, distinct & eccentric 2-3 N=26 X=2.27 (<u>+</u> 0.47)	often 2, distinct & eccentric 3-4 N=63 X=3.06 (<u>+</u> 0.62)	usually 2, distinct & eccentric 3-8 N=35 X=4.31 (<u>+</u> 1.38)	-			
ratio of cell to nucleus diameter	N=15 X=2.77 (<u>+</u> 0. 43)	N=27 X=3.29 (<u>+</u> 0.39)	N=67 X=3.65 (<u>+</u> 0.46)	N=37 X=4.78 (<u>+</u> 1.16)	-			
ratio of nucleus to nucleolous liameter	N≠7 X=2.97 (<u>+</u> 0.55)	N=26 X=3.01 (<u>+</u> 0.60)	N=63 X=3.21 (<u>+</u> 0.55)	N=35 X=3.13 (<u>+</u> 0.51)	-			
phagocytosis	absent	absent	absent	present	absent			
urse cell	absent	1-3 cells	l layer	3-4 layers	3-5 layers, 1 closely apposed			

TABLE 4: Morphological characteristics of the stages in the process of oogenesis in <u>H. lachne</u>.

N= number of observations; \vec{x} = mean + standard deviation

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FIGURE 3:

- a) Nursery in endosomal tissue of <u>H. lachne</u>. bl, blastomeres of young embryo; me, mature embryo; pl, parenchymella larva.
- b) Light micrograph showing the separation of chromatids in late anaphase stage of mitosis in an oogonium in the endosomal tissue of <u>H. lachne</u>. Note the triangular shape of the oogonium and its vesicular cytoplasm.
- c) Electron micrograph of a primary oocyte in <u>S. barbara</u>. A portion of another primary oocyte (po) is visible in the bottom right hand corner. 1, lipid; m, cluster of mitochondria; n, nucleolate nucleus; y, yolk. Arrow indicates dense granular material around the periphery of the nuclear membrane.
- d) Light micrograph showing separation of four ova in late telophase II of meiosis in an endosomal nursery of <u>H. lachne</u>. ca, cavities remaining after release of differentiated parenchymella larvae.
- e) Light micrograph of male (\mathcal{O}) and female (\mathcal{P}) pronuclei in a secondary oocyte (ovum) of H. lachne. Transmitted light.
- f) Fluorescence micrograph of e). Note the distinction between the two pronuclei. Arrowhead indicates the male pronucleus. The bright structure at the bottom of the micrograph is part of a spongin fibre. Spongin exhibited autofluorescence as bright as that of the male reproductive elements (Chapter 3).



-63-

oogenesis suggest that presumptive oogonia differentiate directly from archaeocytes within the mesohyl of adults. These cells are initially triangular in shape, $10-15\mu$ m in diameter and contain a distinctly enlarged nucleus (4-6 μ m) with a single prominent nucleolus (ca. 2 μ m) and vesicular cytoplasm. Figure 3b shows an oogonium believed to be undergoing mitosis to produce primary oocytes. These cells become more or less ovoid or spherical in form and increase in size to $20-25\mu$ m. Primary oocytes maintain the distinct nucleolate (2-3 μ m) nucleus (6-8 μ m) of the oogonia, but the cytoplasm has now become dense. Often two discrete nucleoli were observed, as well as dense granular material around the periphery of the nuclear membrane. One or two archaeocytes were seen in close association with these oocytes, and it is believed that the archaeocytes form a limiting layer of nurse cells around the oocyte.

The cytoplasm of these primary oocytes already contains yolk granules, glycogen and lipid (Fig. 3c). However, evidence of phagocytosis and transfer of material from cells in the mesohyl was not seen in any of these cells examined. Tetrads of cells were frequently observed in the "nurseries" of all reproductively active specimens examined. Figure 3d shows a typical arrangement of these four closely associated cells which are believed to be secondary oocytes (ova) derived from the final phase (telophase II) of the maturational divisions of a primary oocyte. Polar body formation was never seen. Fertilization was not observed, but male and female pronuclei were found in secondary oocytes suggesting that fertilization occurs shortly after the second meiotic divisior (fig. 3e). During the study of spermatogenesis in these same four sponge species (Chapter 3) spermatids were found to be autofluorescent. When secondary oocytes containing the two pronuclei were examined under a fluorescence

- 64 -

microscope (see Materials and Methods, Chapter 3) one of the two nuclei demonstrated a bright yellow-white autofluorescence, similar to that of the spermatids (Chapter 3), while the other nucleus was not visible and the cytoplasm of the oocyte showed very low levels of fluorescence (Fig. 3f).

The zygote grows to about 300 μ m in diameter before cleavage occurs. During the growth of the zygote there has been subsequent apposition of nurse cell layers (2-3) to the initial limiting layer observed surrounding secondary oocytes. Nurse cells were observed actively phagocytosing bacteria, collagen fibrils and other cells from the mesohyl of the adult (Fig. 4a). The nurse cells are approximately 10 μ m in length with a dense nucleus (ca. 4 μ m) and cytoplasmic inclusions of yolk, lipid, phagosomes and glycogen. These cells make intimate contact, by means of microvilli, with the surface of the zygote and often form cytoplamic bridges in which the membranes of both lose their integrity (Fig. 4b). Phagosomes, glycogen, lipid and large yolk spheres accumulate within the growing zygote. In the latter stages, as the zygote increases in size, nurse cells are phagocytosed directly, and the remaining cells become progressively elongated and flattened.

A difference was observed in the zygote stage of the four sponge species investigated. Zygotes and nurse cells in specimens of only <u>S. cheiris</u> contain extremely electron dense cytoplasmic inclusions that could not be detected in thick sections. These inclusions are concentrated around the membrane of the zygote and are about the same size as the yolk granules, $1-2\mu$ m (Fig. 4c). They are also dispersed in the blastomeres of embryos, and the cells of differentiating larvae. These inclusions are very similar in appearance to the pigments observed in the flagellated epithelial layer of differentiating larvae in all four species.

-65-

FIGURE 4:

- a) Electron micrograph of nurse cells phagocytosing bacteria (b) and collagen fibrils (cf) from the mesohyl of <u>S. graminea</u>. 1, lipid; y, yolk; z, zygote.
- b) Electron micrograph of a nurse cell feeding a zygote in <u>S. barbara</u> by the formation of microvilli (mv) and cytoplasmic bridges (cb) which transfer material to the growing zygote (z). n, nucleus of nurse cell.
- c) Electron micrograph of a zygote in the endosomal tissue of <u>S</u>.
 <u>cheiris</u>. Note the presence of extremely electron dense inclusions
 (i) in the surrounding nurse cells (nc) and concentrated around the membrane of the zygote (z). mm, maternal mesohyl; ph, phagosomes.
- d) Light micrograph of an umbilicus connecting an embryo of <u>H. lachne</u> to parental tissue. Bacteria (b) are extracellularly transferred from the maternal mesohyl (mm) to the cleaving embryo (e). bl, blastomeres; ca, cavity in the mesohyl accommodating growth of the cleaving embryo.

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- e) Electron micrograph of symbiotic bacteria (b) from the maternal parent outside of blastomeres (bl) in a cleaving embryo of <u>H.</u><u>lachne</u>.
- f) Electron micrograph of an incubating cytodifferentiated parenchymella larva of <u>S. barbara</u> showing the peripheral region consisting of small uniflagellated cells of the pigmented epithelial layer (ep) and underlying layers of larger amoeboid cells (am) actively undergoing mitosis. The central region contains symbiotic bacteria (b) and collagen fibrils (cf) dispersed between archaeocytes (ar) containing small lipid and yolk granules. Note the flattened layer of nurse cells around the larva. cr, collagen-like fibril ring.



-66-

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Cleavage was total and equal resulting in the formation of solid, translucent-white stereoblastulae. As cleavage proceeds a single layer of flattened nurse cells becomes apposed to the embryonic membrane. Each of these cells has a large nucleus (many are binucleated) and dense cytoplasm. Collagenous-like connections (umbilici) were observed between this single layer of nurse cells and the other nurse cell layers which were separated by a cavity to accomodate the increase in volume of the cleaving embryo (Fig. 4d). Symbiotic bacteria were seen dispersed between blastomeres and inside the embryonic membrane of all of the young and maturing embryos (Fig. 4e). The symbionts, which have the same appearance as those in the mesohyl of adults, showed no signs of digestion and many were undergoing division. No other cells were found in the embryos. During cleavage the large yolk granules are broken down and become less distinct.

At the end of cleavage the blastomeres differentiate, resulting in the development of a cytodifferentiated parenchymella larva possessing two distinct regions: a central low density cell mass, and a peripheral region of high cell density. The central cellular mass consists of: a loose arrangement of collagen fibrils, symbiotic bacteria, archaeocytes and what appear to be collagen-like fibril rings. These rings are closely aligned with the archaeocytes and there is no matrix material within the space enclosed by the ring. Archaeocytes (ca. 10 μ m) contain a diffuse nucleus, numerous phagusomes, small yolk and lipid granules and, in larvae of <u>S. cheiris</u>, the extremely electron dense inclusions noted earlier. The peripheral region consists of: small uniflagellate cells (ca. 4 μ m in length) forming a tightly packed and distinctly pigmented columnar epithelial layer, and underlying layers of large amoeboid cells (ca. 5 μ m) which contain densely staining nuclei, phagosomes and small yolk and lipid

granules (Fig. 4f). The amoeboid cells in the larvae of <u>S. cheiris</u> also contain the dense inclusions reported earlier. Umbilici were not observed in differentiated larvae.

Parenchymella larvae of <u>H. lachne</u>, <u>S. barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u> have an average size of 420 μ m by 350 μ m when released (Fig. 5a). The free-swimming larvae are ovoid with dark grey pigmentation and posterior regions encircled by a black pigmented ring of cells bearing long cilia (ca. 80 μ m). Shorter cilia (ca. 16 μ m) are dispersed over the entire surface of the larvae. Released larvae, directed by the long posterior cilia, displayed directional swimming with constant lateral rotation for a period of 24-48 hours.

Ultrastructural examination of parenchymella larvae, of all four sponge species, that had been free-swimming for 24-36 hours also revealed two distinct regions (Fig. 5b). The peripheral region and the central cell mass are similar to those of the incubating larvae with the following exceptions. The epithelial cells possess elongated cytoplasmic extensions surrounding the single flagellum, and phagocytic vacuoles are often present at the tips of these extensions (Fig. 5c). Some of the amoeboid cells now contain pigment granules indistinguishable from those of the epithelial cells. The shape of the amoeboid cells, their subsequent occurrence at the surface of metamorphosing larvae, and the deformation of collagen fibrils in the vicinity of these cells suggest that these cells are mobile, streaming towards the periphery of the larvae. The archaeocytes in the central region also contain pigment granules (Fig. 5d). The main volume of the larvae consists of loose mesohyl where collagen secretion and phagocytosis were observed. However, definitive cell types such as exopinacocytes, basopinacocytes and collencytes present in adult sponges were not recognized

FIGURE 5:

- a) Live parenchymella larvae of <u>S. graminea</u>. The posterior ends (p) are encircled by a black pigmented ring of cells bearing long cilia (lc) which steer the actively swimming larva, and short cilia (sc) cover the remainder of the larval surface.
- b) Electron micrograph of the surface of a parenchymella larva of <u>S</u>. <u>barbara</u> which had been swimming for 24-36 hours. The two regions of the swimming larva are similar to those of the incubating larva (Fig. 4f). Anoeboid cells (am) in the peripheral region appear to be streaming towards the periphery of the larva. Archaeocytes (ar) in the central region of the larva are closely associated with collagen-like fibril rings (cr) also observed in the incubating larvae. Pigment granules (pg) are present in some of the amoeboid cells and archaeocytes as well as the ciliated epithelial cells (ep). b, bacteria.

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- c) Electron micrograph of the ciliated epithelium of a swimming larva of <u>S. graminea</u>. These cells possess elongated cytoplasmic extensions (ce) surrounding the single flagellum (f). Phagocytic vacuoles (pv) are often present at the tips of these extensions.
- d) Electron micrograph of the internal mesohyl of a swimming parenchymella larva of <u>S. barbara</u>. The loose mesohyl consists of: collagen fibrils (cf), symbiotic bacteria (b), archaeocytes (ar) containing pigment granules (pg) and collagen-like fibril rings (cr).



in the larvae.

Behavioural Studies:

1) Phototaxis:

All fifty of the larvae from each of the four sponge species demonstrated a negative phototactic response to a unidirectional light source applied directly to the experimental chamber. When the light was applied the larvae would immediately swim out of or away from the path of light and actively swim in the darkened regions of the chamber. This same response was recorded for all larvae on all three trials. No geotactic behaviour was observed; larvae would actively swim at all depths in the experimental chamber. Prior to settlement and metamorphosis, the larvae entered a creeping phase which was interrupted by sporadic episodes of swimming. This phase lasted for 2-8 hours during which time the larvae demonstrated no particular taxis to a unidirectional light source applied directly to the settlement chamber.

2) Substrate Selection:

There was no evidence of substrate selection, by type or orientation, by the larvae of <u>H</u>. <u>lachne</u>, <u>S</u>. <u>barbara</u>, <u>S</u>. <u>cheiris</u>, and <u>S</u>. <u>graminea</u> (Table 5). The results of the four-factor ANOVA, including interaction effects, showed no significant difference in: 1) the number of larvae settling on the various proffered substrates (P > 0.15), 2) the number of larvae settling among the four species (P=1.00), or 3) the various interactions of substrate types (P > 0.59) and substrate types and species (P > 0.47) on the number of larvae settling. This study strongly suggests that there is no selection of a specific settlement surface by the larvae of these four species. However, additional studies offering different substrate types

TABLE 5: Number of	larvae	settling	0 n	v ari o us	substrate	types.
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-71-

N= total number of larvae settling on proffered substrates.

6 95 0150	N	GLASS				TRANSITE			
SPECIES		CLEAN		AGED		CLEAN		AGED	
		UPPER	UNDER	UPPER	UNDER	UPPER	UNDER	UPPER	UNDER
H. lachne	17	3	2	2	2	3	2	2	1
<u>S. barbara</u>	19	3	2	2	3	2	2	3	2
<u>S. cheiris</u>	22	3	3	3	2	3	3	2	3
S. graminea	22	3	4	3	2	3	3	3	1

SUBSTRATE TYPE

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with more larvae and replicate testing are necessary to substantiate these findings.

A few larvae metamorphosed on the surface film of the water, but did not survive beyond their initial attachment to the air-water interface. A specific study on the proximity of settled larvae was not conducted. However, it should be noted that larvae tended to settle within about 2 cm of each other if they chose the same substrate type and orientation as another.

Larval Metamorphosis:

Attachment to a substrate is the beginning of the metamorphosis from a free-swimming larval form to the organized structure of a young sponge, and is a continuous process. Rapid and simultaneous changes take place externally and internally. In order to follow the events in this process it is convenient to report macroscopic and microscopic transitions separately. A post-larval metamorph is defined as the stage from substrate attachment to the manifestation of adult cell types and basic organization.

1) Macroscopic Events:

In each of the four species, settlement of the larvae occurs 26-56 hours after release from the parent sponge. The settling larvae come to rest vertically or obliquely on the anterior pole, often spinning slowly about the attachment point (Fig. 6a). If the settling larvae are disturbed during this stage they will settle on a new area of substrate in the same orientation as before. The original site of attachment is covered in an amorphous mucous-like secretion containing a few scattered sponge cells. As metamorphosis proceeds the cells in the region of attachment spread out over the substrate, progressively flattening the post-larval metamorphos. This

FIGURE 6:

- a) A live parenchymella larva of <u>S. graminea</u> spinning on its anterior pole prior to attachment to a glass slide. Note the black pigmented ring on the posterior pole of the larva. The ring consists of pigmented cells bearing long cilia which aid in the directed movement of the larva.
- b) A live post-larva of <u>S</u>. graminea 24 hours after settlement on a glass slide. Cells have spread out over the substrate progressively flattening the juvenile sponge. However, most of the cells remain centrally located. The dark grey pigmentation has paled.
- c) A live post-larva of <u>S. graminea</u> 5 days after settlement on a glass slide. The arrow indicates discrete black spots on the surface of the metamorphosing sponge. These later developed into the oscules. Most of the cells have become consolidated into a rounded disc.
- d) Electron micrograph of the free surface of a post-larva of <u>S</u>. <u>barbara</u> that had been removed from a glass slide 24 hours after settlement. Exopinacocytes (ep) and archaeocytes (ar) were observed secreting collagen fibrils (cf). A variety of cellular inclusions can be seen in both cell types including: pigment granules (pg) and phagocytic vacuoles (pv). There are no obvious larval ciliated epithelial cells present. cr, collagen-like fibril ring.
- e) Electron micrograph of the attachment surface of a post-larva of <u>S</u>. <u>barbara</u> that had been removed from a glass slide 24 hours after settlement. Archaeocytes are differentiating into basopinacocytes (bp) which contain many small vesicles (ve). A basal lamella (bl) consisting of collagen fibrils and an amorphous substance underlies the basopinacocytes. There are no obvious larval ciliated epithelial cells present. cr, collagen-like fibril ring. INSET: Electron micrograph of basal lamella of <u>S</u>. barbara. cf, collagen fibrils.
- f) Electron micrograph of the central region of a post-larva of <u>S</u>. <u>barbara</u> that had been removed from a glass slide 24 hours after settlement. Symbiotic bacteria (b) and archaeocytes (ar) are present in high density. Archaeocytes are engaged in phagocytosis and the production of the collagen matrix. Note the persistence of the collagen-like fibril rings (cr) first observed in the incubated larvae. pg, pigment granules.



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rapid reorganization changes the settling larvae from an ovoid shape to the rounded disc of the post-larvae in about 8 hours. The main mass of cells is centrally located. After 18 hours, the cilia that covered the surface of the free-swimming larvae are no longer present, and the dark grey pigmentation has paled. At this time the metamorphs are approximately 1 mm in diameter (Fig. 6b). During the next four days the metamorphosing sponges continue to spread out over the substrate exhibiting a high degree of pleomorphism. However, by five days post-settlement the metamorphs are once again disc-shaped and discrete black spots appear on the surface of the young sponges (Fig. 6c). Six days after settlement lacunae (cavities) develop beneath the epithelial layer and within the central mass of cells. These are the early stages in the formation of the canal system. Eleven days post-settlement the canals have increased in number and the discrete black spots have become miniature volcano-like structures, the oscules. By the end of the observation period (21 days), the young sponges are hemispherical in shape and approximately 1.5 mm in diameter. Connections have formed between the canals and the oscules are more prominent.

2) Microscopic Events:

The following description is based on ultrastructural examinations of post-larval metamorphs of <u>S. barbara</u> and <u>S. graminea</u> that had been removed from glass slides 24 hours after settlement. Observations of these specimens revealed little disruption of the attachment surface. It would appear that adhesion to the substrate at this time is tenuous, and occurs in only a few areas along the surface of the spreading metamorphs.

The absence of the ciliated epithelial layer present in incubated and free-swimming larvae substantiates macroscopic observations. There is a marked depletion of the cell population in the peripheral regions of the

metamorphs. The ciliated cells on the external surface of larval forms have been replaced, in the post-larvae, by archaeocytes (ca. 10-12 μ m) which contain a variety of cellular inclusions and large prominent nuclei. However, on the free surface the archaeocytes have differentiated into exopinacocytes. This is evidenced by the fusiform shape and very thin, elongated cytoplasmic extensions of these cells (Fig. 6d). There is no evidence of a uniform cell coat, however, an external amorphous "fuzz" covers portions of the exopinacocytes which were observed releasing collagen fibrils.

On the basal (attachment) surface of the post-larvae extracellular material underlies the archaeocytes. This basal lamella is 100-150 nm thick and consists of what appears to be collagen fibrils within an amorphous substance. Fibrils also extend into the sponge matrix and provide anchorage points which bind the basal lamella and matrix (Fig. 6e, inset). Archaeocytes have started to differentiate into basopinacocytes, evidenced by the flattened appearance of these cells and the presence of many small vesicles within the cytoplasm (Fig. 6e). However, the definitive adult basopinacocyte is not yet obvious. These cells were observed extruding collagen fibrils and vesicles into the surrounding matrix, suggesting their involvement in the synthesis of the basal lamella.

The central region of the post-larvae contains symbiotic bacteria and mesohyl cells in high density. Archaeocytes are engaged in the production of the collagen matrix and phagocytosis. At this stage in metamorphosis the young sponges are 75-100µm thick and show no evidence of canal or choanocyte formation (Fig. 6f). The collagen-like fibril rings first observed in the incubated larvae persist in the post-larvae. However, fewer fibril rings were observed in the post-larvae and they were more often observed in the

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region between the central cellular mass and the exopinacocyte layer of the metamorphs.

Intracellular pigments are not concentrated in the surface layer of post-larvae as they were in the larval forms, and this is paralleled by the fading in pigmentation reported macroscopically. Exopinacocytes, basal archaeocytes and mesohyl cells all contain these electron dense cytoplasmic inclusions. Pigment granules were often observed in intracellular vacuoles of these cells.

DISCUSSION

There have been relatively few detailed investigations of oogenesis in sponges (Fell, 1983). Many accounts of this process are part of more general descriptive studies on reproduction. The present study undertook detailed light and transmission electron microscopic examinations of oogeneic elements in four sponge species. Morphological characteristics of these elements were statistically analyzed to objectively identify specific stages in obgenesis prior to describing the dynamics of this process. Results indicate that it may be possible to stage elements in the process of oogenesis based on a few key structural features. This would reduce the time required in the histological examinations of serial sections, and suggest target cells to examine in greater detail for events such as gamete differentiation, meiosis and fertilization. There is a need for similar morphological studies, especially ultrastructural ones, on other sponges to provide more information on the details of oogenesis and oogeneic structures. However, to determine the factors and elucidate the mechanisms regulating oogenesis and other reproductive processes, biochemical and experimental approaches to investigations of these processes are urgently required.

Recent and extensive literature reviews on reproduction in sponges indicate a great variability in sexual differentiation among demosponges (Fell, 1983; Reiswig, 1983; Simpson, 1984), but from the variability two consistent patterns emerge. Demosponges that broadcast eggs or embryos (oviparous) are generally dioecious (gonochoristic). Only two exceptions to this pattern have been reported, <u>Tetilla</u> sp. (Liaci, <u>et al</u>, 1976) and <u>Verongia (Aplysina) aerophoba</u> (Liaci, <u>et al</u>, 1971); both of these species are oviparous and hermaphroditic but belong to genera in which other species are oviparous and gonochoristic (Reiswig, 1973; Watanabe, 1978). All of the tetractinomorphs where the mode of sexual reproduction is known (with the exception of <u>Tetilla</u> sp.) exemplify this form of sexuality. The second pattern that emerges is a tendency for demosponges which brood embryos and larvae (viviparous) to be hermaphroditic (<u>Halisarca dujardini</u>, Lévi, 1956; <u>Microciona prolifera</u>, Simpson, 1968; <u>Ircinia fasiculata</u>, <u>I. variabilis</u>, <u>Spongia officinalis</u>, Liaci, <u>et al</u>, 1971). Within the ceractinomorphs, and specifically among the keratose sponges, viviparity with gonochorism is considered to be an exception.

The present study demonstrates that <u>H. lachne</u>, <u>S. barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u> are viviparous, and all available evidence suggests that these species are dioecious. However, increasing evidence of sex reversal and a high degree of interspecific variability in sexual differentiation among sponges (Fell, 1970; Diaz, 1973; Van de Vyver and Willenz, 1975; Elvin, 1976; Gilbert and Simpson, 1976; Fell and Jacob, 1979) suggests that intrinsic interactions between genetic and/or environmental factors may determine sexual differences. Therefore, successive hermaphroditism in these four species cannot be ruled out. Further investigations of sponge populations over an extended period of time and under different environmental regimes are required to gain a better understanding of the process of sexual differentiation.

Sponges lack localized discrete reproductive organs and in most viviparous and oviparous individuals large areas of mesohyl are involved in reproduction. However, as in another viviparous species which produces large yolky eggs (<u>Halisarca dujardini</u>, Lévi, 1951), oocytes and embryos of the four species studied here develop asynchronously in clusters or nurseries within the basal or central zone of endosomal tissue. Similar asynchronous development of oogeneic elements grouped in small clusters has been reported in other sponges (<u>Oscarella lobularis</u>, <u>Halisarca dujardini</u>, Lévi, 1951; 1956; <u>Halichondria</u> sp., Fell and Jacob, 1979). The general pattern of oogenesis and larval development in <u>H. lachne</u>, <u>S. barbara</u>, <u>S.</u> <u>cheiris</u> and <u>S. graminea</u> is similar to that reported for other brooding demosponges (<u>Halisarca dujardini</u>, Lévi, 1956; <u>Hippospongia communis</u>, Tuzet and Pavans de Ceccatty, 1958; <u>Microciona prolifera</u>, Simpson, 1963).

OOGENESIS, MEIOSIS AND FERTILIZATION:

The present study, and light microscope studies of oogenesis in other viviparous demosponges (Leveaux, 1941; Lévi, 1956; Simpson, 1968) suggest that archaeocytes are the anlagen of oogonia. On the other hand, ultrastructural studies of oogenesis in an oviparous demosponge, Suberites massa (Diaz, et al, 1973; 1975; Diaz, 1979), and a viviparous demosponge, Halisarca dujardini (Korotkova and Aisenstadt, 1976), have reported that oogonia are derived from choanocytes. However, transformation or differentiation of choanocytes and choanocyte chambers into oogonia was not observed in light and transmission electron microscopic examinations of the sponges in this study. It should be noted that all conclusions regarding the origin of female germ cells are based upon interpretation of fixed tissues. Conclusive experimental work has not yet been possible but is desirable. Based upon present knowledge of oogonial genealogies, great variability among almost all of the species which have been investigated makes it impossible to predict detailed patterns of oogenesis in any group of sponges which has not been studied specifically. Therefore, generalizations concerning the process of oogenesis should be made

cautiously as it is likely that species and/or habitat differences may occur in this process similar to other biological and ecological phenomena (histocompatability responses, recognition, competition) in these lower metazoans.

The dense granular material observed around the nuclear membrane of developing oocytes has also been reported in a number of other demosponges (<u>Reniera elegans</u>, Tuzet, 1947; <u>Stelleta grubii</u>, Liaci and Sciscioli, 1967; <u>Suberites massa</u>, Diaz, <u>et al</u>, 1975; <u>Aplysina (Verongia) cavernicola</u>, Gallissian and Vacelet, 1976). The nature of the material is not known but it has been suggested that its presence may serve as a useful marker for developing oocytes (Simpson, 1984). The presence of two distinct nucleoli in nuclei of developing oocytes has also been reported in an oviparous demosponge (<u>Tethya citrina</u>, Gaino, <u>et al</u>, 1987). If further studies reveal this to be a consistent feature perhaps it too can serve as a marker for developing oocytes.

Two phases of growth in oocytes were first described in spongillids by Leveaux (1941), and have since been reported in a number of viviparous sponges (<u>Halisarca dujardini</u>, Lévi, 1956; <u>Hippospongia communis</u>, Tuzet and Pavans de Ceccatty, 1958; <u>Halisarca dujardini</u>, Aisenstadt and Korotkova, 1976). The first phase involves the uptake of soluble materials, and the second involves the phagocytosis of nurse cells. In the present study, the first phase, "le petite accroissement" does not involve the transfer of material from cells in the mesohyl. The small yolk granules, glycogen and lipid are probably synthesized by the oocytes themselves utilizing dissolved substances acquired through pinocytosis and/or diffusion. The second phase of growth, "le grand accroissment", initially involves transfer of materials

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of nurse cells by zygotes.

Phagocytic nutrition of oocytes/zygotes has been reported in other viviparous demosponges (Hippospongia communis, Tuzet and Pavans de Ceccatty, 1958; Haliclona ecbasis, Fell, 1969; Halisarca dujardini, Aisenstadt and Korotkova, 1976). Alternatively, transfer of material via cytoplasmic bridges has not been previously documented in viviparous demosponges. However, this nonphagocytic growth process has been reported in oocytes of oviparous demosponges (Aplysina (Verongia) cavernicola, Gallissian and Vacelet, 1976; Chondrosia reniformis, Lévi and Lévi, 1976; Suberites massa, Diaz, 1979) and is considered to play the major role in the nutrition of these oocytes. These nutritional differences may reflect different strategies for the apportioning of resources to reduce energy loss in gametic wastage. For example, viviparous sponges may allocate energy to the phagocytic nurturing of zygotes while oviparous species direct energy toward the production of large numbers of small unfed oocytes. Crucial information on the growth and nutrition of oocytes and zygotes will only be gained through autoradiographic and biochemical studies.

Although all phases of meiosis were not observed, the persistence of tetrads of dividing oocytes in the first phase of growth suggests that meiosis occurs without the formation of polar bodies and before the apposition of extensive layers of nurse cells. Fertilization was not observed, however the observation of presumed male and female pronuclei in oocytes of the size and appearance of those having completed meiosis suggests that after initial growth and meiosis, fertilization occurs and then zygotes undergo "le grand accroissment". The observation of autoflourescence in one of these nuclei, which is similar to the autoflourescence of spermatids in these sponge species, strongly supports

-81-

the suggestion that fertilization occurs after meiosis and then the zygote undergoes the major phase of growth. This is the first record of a definitive label that could be used to identify fertilization in the sequence of events leading to embryogenesis. Further studies investigating autoflourescence in other sponge species might ultimately lead to a clear picture of the process of fertilization in the sequence of events in sexual reproduction in sponges. The use of such a label may also be helpful in selecting cells for isolation and culturing experiments.

Other studies of relationships between meiosis and fertilization have offered different interpretations of the order of this sequence of events. In the calcareous sponge, Clathrina (Leucosolenia) coriacea (Tuzet, 1947) and the demosponge, Octavella galangaui (Tuzet and Paris, 1964) the primary oocyte undergoes initial growth followed by fertilization, major growth and meiosis respectively. Alternatively, in Hippospongia communis (Dictyoceratida), Tuzet and Pavans de Ceccatty (1958) have reported that the primary oocyte undergoes small growth followed by fertilization, meiosis, and "le grand accroissment" respectively. However, in none of these studies has meiosis been clearly documented. Gallissian (1980, Grantia compressa) has presented the only ultrastructural evidence of sperm cell transfer (via a carrier cell), and to date, no ultrastructural studies have documented meiosis. The greatest obstacle to gaining a clear picture of these two very important events in the sexual process is that of correctly interpreting dynamic events, that occur in a brief moment of time, from static images. More ultrastructural studies are required to provide the fine details, but biochemical and experimental approaches involving cell isolation and selective labelling are urgently needed to complete our understanding of these events.

42

EMBRYOGENESIS:

In the present study, the term embryonic membrane is defined as a thin membranous structure that completely envelopes the embryo. In the literature concerned with the embryology of sponges this structure is either never discussed or vaguely referred to as an envelope or membrane. The nature and function of the structure have not been investigated in this study or other studies, but this does not preclude its existence. Therefore, the term has been defined and used in this study to establish a reference. Further studies on the structure of the embryonic membrane may provide information on its function in embryogenesis.

The collagenous-like connections that are established during cleavage of the embryo have been referred to as umbilici in the context of links which serve to tether and/or supply a substance to a dependent component of a system. In the literature to date, there are no reports of similar structures in other sponge embryos. Although similar connections have been observed in two other viviparous sponges, their structure was not clear and their presence in these sponges was therefore overlooked (De Vos and Vacelet, pers. comm.).

Symbiotic bacteria were observed dispersed between blastomeres and inside the embryonic membrane of all the young and maturing embryos, and within the central mass of cells in larvae and post-larvae of all four sponge species. Oocytes and zygotes however, were devoid of these elements. The presence of symbiotic bacteria in the embryos together with the establishment of umbilici at this stage of development, suggests that the umbilici apparently function as the pathway by which maternal intercellular symbionts are extracellularly transferred to progeny. Thus, the reference to umbilici in the context of the present study appears to be appropriate. In a study on spermatogenesis in these same four sponge species (Chapter 3) symbiotic bacteria were never observed in any of the reproductive elements. Therefore, it would appear, symbionts in these sponges are maternally inherited.

There are only two other studies which have clearly demonstrated the transfer of sponge bacterial symbionts from one generation to the next. In these oviparous species where embryogenesis occurs externally, transfer took place directly by oocyte incorporation of bacteria from parental mesohyl (<u>Aplysina (Verongia) cavernicola</u>, Gallissian and Vacelet, 1976), or indirectly by embryo incorporation of somatic cells containing bacteria (<u>Chondrosia reniformis</u>, Lévi and Lévi, 1976). In both cases extracellular parental bacteria were intracellularly translocated to the next generation either in vacuoles within the oocyte itself or in vacuoles of incorporated somatic cells. Extracellular transfer of symbiotic mycoplasma-like organisms between generations has been reported in bryozoans (Zimmer and Woollacott, 1983). Mucous strands established during development appear to be involved in transmission of these symbionts from parent to larva.

The present study is the first record of: 1) the transfer of bacterial symbionts between generations in viviparous invertebrates and, 2) a unique mode of extracellular transfer of symbiotic bacteria in sponges in general. Specific investigations will be required to provide more information on the structure, formation and role of these umbilici in the development of embryos. They may perform an important function in the transfer of other cells and soluble substances involved in embryogenesis and/or larval differentiation. Substances required for the synthesis of pigments present in many parenchymella larvae, and other symbionts such as cyanobacteria and zooxanthellae may be genealogically translocated via umbilici.
LARVAL DEVELOPMENT AND METAMORPHOSIS:

The morphology and development of larvae in the sponges examined in this study is typical of parenchymella larvae in many viviparous demosponges (for review see Simpson, 1984). However, as in most other studies, the specific processes involved in cellular differentiation and organization are not clearly understood. The presence of symbiotic bacteria has only been reported in the larvae of four other sponge species which are also known to possess populations of bacterial symbionts in the mesohyl of adults (<u>Oscarella lobularis</u>, Lévi and Porte, 1962; <u>Hamigera hamigera</u>, Boury-Esnault, 1976; <u>Chondrosia reniformis</u>, Lévi and Lévi, 1976; <u>Neocoelia crypta</u>, Vacelet, 1979). However, the method by which larvae acquire these symbionts is known only for one of these species (Lévi and Lévi, 1976) and has already been discussed.

Collagen-like fibril rings have been reported in parenchymella larvae of three other demosponges: <u>Halichondria moorei</u>, <u>Ulosa</u> sp. and <u>Microciona</u> <u>rubens</u> (Bergquist and Green, 1977). They were first observed in these larvae 60 hours after settlement and their close association with archaeocytes suggested their involvement in canal formation. In the present study, the first appearance of these structures early in larval differentiation suggests that they may also play a role in cellular organization and matrix development. These fibril rings may give form and flexibility to the larvae allowing the organisms to modify their shape in response to pressure differences during release and settlement. They may also function in the movement of soluble substances into and/or out of the mesohyl.

The cilia of the free-swimming larvae of four other demosponges have been reported to be surrounded by a continuous collar formed from an

-85-

extension of the cytoplasm of epithelial cells (<u>Mycale contarenii</u>, Lévi, 1964; <u>Halichondria moorei</u>, <u>Ulosa sp.</u>, <u>Microciona rubens</u>, Bergquist and Green, 1977). In the present study, the presence of similar structures in free-swimming larvae, and their absence in incubating larvae may indicate a preparation for ensuing attachment. The existence of vacuoles at the tips of these extensions and the absence of any special secretory cells in the epithelial layer suggests that the ciliated cells may release substances involved in the preliminary events of larval-substrate interactions.

The first event in larval metamorphosis is the rapid formation of the epithelial surfaces (pinacoderm) of the young sponges. Within 24 hours of settlement archaeocytes appear to have migrated to the periphery of postlarvae and differentiated into exopinacocytes in the upper epithelial layer and basopinacocytes in the region of substrate attachment. Little information is available at the present time on the mechanism of substrate adhesion. However, it is clear from ultrastructural observations of postlarvae that attachment does not involve secretions from specialized gland cells since no such structures were obvious. The absence of specific cells and the presence of a basal lamella between the sponge and substrate implies that adhesion is a function of basopinacocytes, and mediated by a groundmat secreted by these cells. Attachment of the basal lamella was not observed but the lack of disruption of the attachment surface and the ease with which the post-larvae were removed suggests that, initially, attachment is tenuous and achieved by point adhesions of the basal lamella and substrate. Similar studies on larval settlement and metamorphosis in other species of Demospongiae have also reported the formation of a lamella secreted by basopinacocytes (Mycale contarenii, Borojevic and Lévi, 1965; Hamigera hamigera, Boury-Esnault, 1976; Halichondria moorei, Ulosa sp., Microciona

-86-

<u>rubens</u>, Bergquist and Green, 1977; <u>Halichondria moorei</u>, Evans, 1977). Considering that larval attachment is essential for the occurrence of other events in the metamorphosis of sponges it is surprising that, to date, no studies have examined the nature of the basal lamella or the mechanisms involved in larval-substrate adhesion.

Some adult sponges mount a non-aggressive defense to foreign substances by depositing a collagen-like lamella in the zone of contact between the sponge ('self') and other sponges or foreign material ('non-self') (Ephydatia fluviatilis, Van de Vyver and De Vos, 1979; Verongia longissima, Kaye and Ortiz, 1981; Axinella verrucosa, A. damicornis, Buscema and Van de Vyver, 1983). The remarkable similarity in the responses to 'non-self' contact of two very different stages in the life history of sponges suggests that the mechansisms involved in larval-substrate interactions are analagous to those which control the histoincomaptability responses of adult sponge tissue. There is a need for further ultrastructural studies on larval settlement and metamorphosis in other sponge species to provide more information on the details of these processes. However, to determine the factors and elucidate 'he mechanisms regulating larval-substrate attachment, biochemical and experimental approaches to investigations of this process are urgently required. In turn, the results of such studies may also help provide an understanding of the mechanisms that lie at the basis of 'self/non-self' recognition in sponges.

The fate of the ciliated epithelial cells of parenchymella sponge larvae at settlement have been a topic of contention for many years. Some workers suggest that these cells are exclusively larval structures, and at metamorphosis they are either shed or phagocytosed (Meewis, 1939; Bergquist and Green, 1977; Bergquist and Glasgow, 1986). Others suggest that these

-87-

cells play a direct role in the development of the adult and, at settlement they lose their cilia and migrate into the central cell mass of the juveniles where they differentiate into choanocytes (Lévi, 1956; Borojevic and Lévi, 1965; Borojevic, 1966; Boury-Esnault, 1976).

In the present study, ultrastructural observations of free-swimming larvae and post-larvae revealed numerous small (ca. 4µm X 1.5µm) ciliated cells in the larval epithelium and fewer large (ca. 10-12µm) cells in the pinacoderm and central cell mass of juveniles. If the ciliated cells of the larvae were to dedifferentiate and become components of the post-larvae cell population they would have to double in size and decrease their numbers in a relatively short period of time. This seems highly unlikely without evidence of a marked increase in the phagocytic activity of these cells. Phagocytosis was observed in post-larvae but not at a rate that would account for the incorporation of the larval epithelium. There were no recognizable ciliated cells in the young metamorphosing sponges nor was there any evidence of choanocyte formation. Therefore a continuity between these two cell types does not appear to exist. Macroscopic observations revealed the rapid loss of ciliated cells early in the metamorphosis of larvae (18 hours after settlement) and the presence of canal lacunae much later in development (6 days following settlement). This delay between the loss of the larval epithelial layer and the initial appearance of the canal system in metamorphosing sponges also suggests that these ciliated cells are probably not involved in the formation of choanocytes which are an integral component of the canal system in adult sponges.

One of the distinguishing characteristics of the larval epithelium in the sponges examined in this study is the presence of intracellular pigments concentrated in the cells of this layer. If these electron dense granules

-88-

could be used as a natural marker to follow the movements of the ciliated cells then an inward migration of these cells should result in a concomittant shift in the localization of pigments. However, 24 hours after settlement there was a macroscopic and microscopic attenuation in pigmentation and, ultrastructural examinations revealed the presence of pigments within vacuoles of cells scattered throughout the metamorphosing sponges. These observations also seem to suggest that the larval epithelium is not incorporated into metamorphosing sponges and, some of the ciliated cells are phagocytosed. The latter might account for the random presence of pigments in the young sponges.

Although the present study did not specifically address the issue of the fate of cillated epithelial cells, the results reported here provide evidence to support the view that these cells are larval structures only and represent a terminal differentiation. The process by which the ciliated epithelium is lost is not known. Some of the cells are undoubtedly phagocytosed however, it is suggested that they are also shed into the surrounding medium. Clearly more studies are required to resolve the question of the fate of ciliated cells. A sequence of ultrastructural observations including initial larval-substrate interactions and various stages of settlement prior to complete loss of the ciliated cells may provide some answers. However, a resolution to the question will require a technique for specifically labelling these cells and subsequently following their behaviour during settlement and metamorphosis of post-larvae. It is tempting to speculate on the function of the cells beyond that of locomotion and, to consider their involvement in larval release and substrate interactions. Future studies in this direction may elucidate the mechanisms involved in development and metamorphosis of parenchymella larvae.

-89-

LARVAL BEHAVIOUR:

A variety of behavioural patterns (negative and positive phototaxis, negative and positive geotaxis and gregariousness) and preferences for substrate type and orientation have been reported for sponge larvae. However, in most instances these patterns have been used in discussion of systematics and evolution; few studies have attempted to relate larval behaviour to the ecology of adult populations (for reviews see Bergquist, <u>et</u> <u>al</u>, 1970 and Fell, 1974). It should be emphasized that larval behaviour has only been studied in the laboratory and that responses in this artificial setting may differ somewhat from those in the natural environment.

Most sponges produce swimming larvae which creep over the substrate prior to settlement. In the present study, the swimming phase of the larvae lasted for one to two days and indeed this is the case for most larvae (Cliona celata, Microciona prolifera, Warburton, 1966; Microciona prolifera, Simpson, 1968; Haliclona ecbasis, Fell, 1969; Ophlitaspongia seriata, Fry, 1971; Hamigera hamigera, Boury-Esnault, 1976; Chondrosia reniformis, Lévi and Lévi, 1976). However, swimming phases lasting as little as 3 or 4 hours have been reported (Microciona coccinea, Ophlitaspongia seriata, Bergquist, et al, 1970). Some sponge larvae are benthic and creep over the substrate for one to 20 days before settling (Polymastia robusta, Borojevic, 1967; Polymastia granulosa, Bergquist, et al, 1970). These differences between pelagic and benthic larvae and their longevity may represent behaviours programmed to increase survival and effect habitat specializations in the adults. Attempts should be made to relate larval responses to adult situations.

Phototactic behaviour of sponge larvae may be an important factor in determining the distribution of sessile adults. The larvae of a number of

sponges, including those examined in this study, are negatively phototactic, while others are postively phototactic and some show no phototaxis (for reviews see Bergquist, <u>et al</u>, 1970 and Fell, 1974). However, these responses to light may also change just prior to settlement as was observed in the present study. It is possible that the habitat of adult sponges is dependent, in part, on the phototactic behaviour of larvae.

The type and orientation of the substrate on which the larvae of this study settled appears to vary considerably even within a species. In general, it appears that sponge larvae show no preference for any specific substrate except perhaps for those of boring sponges which may require some form of calcareous material. In situ, larvae have been reported to settle on mollusc shells, wood, rocks, concrete, algae, sea grasses, gorgonians, corals and other sponges. Under laboratory conditions sponge larvae will also attach to glass, paraffin, agar and epon (for review see Fell, 1974). In the course of behavioural studies several authors have incidentally suggested that larvae may aggregate during settlement (Lévi, 1956, Halisarca dujardini; Borojevic, 1967, Polymastia robusta). Specific investigations on gregarious behaviour of settling larvae might provide information on the mechanisms of habitat specializations and insight into the distribution of adult populations.

The behaviours of sponge larvae appear to be relatively complex and many other factors such as currents, turbidity, temperature and moon phases probably influence larval movement and settlement. Although larval behaviour is undoubtedly an important factor in effecting habitat specialization of adult sponges, selective mortality and differential survival of larvae and juveniles are probably also important determinants; however, little is known concerning these factors (Fell, 1974).

-91-

The negative phototaxis of the larvae of the sponges investigated in the present study could ensure proximity to benthic settlement surfaces and areas shaded by sea grasses and other sessile organisms. The lack of a response to light during the short creeping phase could allow the larvae to attach to any available substrate instead of having to delay settlement until contact with a specific surface. The lack of a preference for substrate type and orientation by larvae in the laboratory is consistent with the observations that the adult sponges are found attached to or in association with a variety of substrates including dead coral, gorgonians, sea grasses, rocks and concrete. This nonspecificity for substrate may be related to the basal lamella which is formed at the time of settlement. This layer might function as a buffer to ensure that the larvae do not actually contact the substrate, thereby allowing nonselective settlement. The relatively long swimming phase could allow for larval dispersal to areas some distance from the parent population. Indeed, these four commercial sponge species have been found in similar habitats along the coasts of Florida, the Bahamas, Cuba and in the deeper waters of the Gulf of Mexico (Dawson & Smith, 1953; Storr, 1964; Sweat & Stevely, 1981). Further investigations on the relationships between larval behaviour and adult habitat together with studies on the distribution and survival of larvae and juveniles are required to gain an understanding of the ecology and biology of these sponges. It does appear, however, that specific larval behaviours probably represent mechanisms of habitat specialization and niche partitioning of adult populations. These in turn probably reflect the reproductive strategies adopted by those populations.

This study represents the first step in the analyses of specific reproductive processes in Hippospongia lachne, Spongia barbara, Spongia

- 92 -

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cheiris and Spongia graminea. Together with a study on reproductive cycles and spermatogenesis, already in progress, an understanding of sexual reproduction and development in these four commercial sponges may prove useful in future ecological and biological investigations of these economically important species.

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CONCLUSIONS

A combined light and transmission electron microscope study of <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u> has outlined the process of oogenesis and larval development and metamorphosis in these commercial species. They are viviparous and probably gonochoristic. Gametes and embryos develop asynchronously within localized nurseries of endosomal tissue. Presumptive oogonia differentiate directly from archaeocytes and undergo a single oogonial division to produce primary oocytes. These oocytes autosynthesize yolk and lipid utilizing soluble substances acquired through pinocytosis and/or diffusion and then undergo meiosis, producing four secondary oocytes. Presumed male and female pronuclei found in secondary oocytes suggests that fertilization occurs after meiosis and before the major growth of zygotes.

Archaeocytes move from the adult mesohyl and form extensive layers of nurse cells around growing zygotes which increase in size through transfer of nutrients via cytoplasmic bridges and phagocytosis of nurse cells. Cleavage is total and equal, and during this process umbilicit are formed between the embryo and nurse cell layers. These apparently function as the pathway by which maternal intercellular symbiotic bacteria are extracellularly transferred to progeny. At the end of cleavage blastomeres differentiate, resulting in the development of a pigmented parenchymella larva with a ciliated epithelium and a mass of internal cells and symbiotic bacteria.

The ciliated free-swimming larvae possess a ring of cells bearing long flagella which aids in directional swimming with constant lateral rotation. The ciliated cells of the epithelium are probably larval structures only and

represent a terminal differentiation. The larvae demonstrated negative phototaxis until shortly before they entered a creeping phase during which time they showed no particular taxis to light. There is no evidence for substrate selection or orientation. Larval behaviour probably reflects the ecological situations of adult populations. Settlement of larvae occurred 26-56 hours after release and attachment to the substrate involved the rapid formation of a basal lamella. This layer may function as a buffer to ensure that the larval cells do not actually contact the substrate, thereby allowing nonselective settlement. Precocious development of choanocytes was not observed in ultrastructural examinations of free-Swimming larvae or young metamorphosing sponges.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Maria Neuwirth, for her assistance with electron micrograph interpretations, Dr. H.M. Reiswig for his help and advice on the manuscript, and Rosenstiel School of Marine and Atmospheric Sciences, University of Miami for use of laboratory facilities. This study was supported by a grant from the Royal Bank/McGill International/Caribbean Development Fund to Dr. H.M. Reiswig and the author, and an NSERC (Canada) operating grant to H.M. Reiswig.

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CHAPTER III

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ABSTRACT

The reproductive cycles of four Caribbean commercial sponge species. Hippospongia lachne, Spongia barbara, S. cheiris and S. graminea were determined. Spermatogenesis in these species was studied using light and transmission electron microscopy. The production of male gametes occurs in cysts within the endosomal tissue of mature specimens. Reproductive elements within an individual cyst develop synchronously while development between cysts is asynchronous. All available evidence suggests that these species are dioecious. Spermatogonia differentiate directly from choanocytes in situ. All cells of the chamber lose their collars and flagella and undergo mitosis to produce primary spermatocytes each possessing a single flagellum. The ratio of nucleus to cell diameter in these cells is almost double that for choanocytes. Primary spermatocytes undergo meiosis to produce four spermatids. The final stage in the process of spermatogenesis is the differentiation of spermatozoa which do not possess intermediate segments or acrosomes. Male gametes displayed a bright yellow-white autofluorescence when excited with blue light (460-485 nm).

INTRODUCTION

Members of the genera <u>Spongia</u> Linnaeus, 1759 and <u>Hippospongia</u> Schulze, 1879 (Dictyoceratida) include all of the commercially important bath sponges of the Caribbean (ca. 7spp.) and Mediterranean/Red Sea (ca. 3spp.) fisheries. Most of the available literature on commercial sponge species deals with gross morphology and fisheries aspects. Few investigations have focused on the biology and ecology of these once extremely valuable economic commodities. Little is known regarding their reproduction, embryology, and larval development and metamorphosis.

Sexual reproduction in the Porifera is a well documented phenomenon, although the processes vary considerably from species to species. Most sponges reproduce seasonally during a specific time of year. However, there is great variability in the onset and duration of the reproductive period (for review see Fell, 1974, 1983; Reiswig, 1983). Several factors can account for this variation: 1) the specific gametogenic process under investigation (oogenesis vs. spermatogenesis); 2) the sex phenotype (dioecious vs. hermaphroditic) of the species; 3) whether eggs and embryos are broadcast or brooded (oviparous vs. viviparous); 4) exogenous and endogenous factors affecting gametogenesis; and 5) asynchrony within individuals and within populations in the reproductive process. These factors coupled with the lack of localized discrete gonads in sponges and the limited size of local populations for repetitive sampling have resulted in few comprehensive studies on the reproductive biology of any sponge.

Although recent and extensive literature reviews have reported numerous studies dealing with sexual reproduction in sponges (Fell, 1983; Reiswig, 1983; Simpson, 1984), there are relatively few studies on reproductive

-108-

processes in the dictyoceratids (Tuzet & Pavans de Ceccatty, 1958; Liaci, <u>et</u> <u>al</u>, 1971). Much of what is known about these processes has been inferred from gross structural observations in the course of general studies on reproduction. The use of the electron microscope has provided more detailed information on specific events during reproductive processes in sponges. However, there is only one ultrastructural study that has investigated spermatogenesis in the dictyoceratids (Gaino, <u>et al</u>, 1984).

The process of spermatogenesis involves the differentiation of spermatogonia, the formation of spermatic cysts and the development of mature spermatozoa. Available data concerning the origin of spermatogonia in sponges suggests that these elements may derive from choanocytes (Tuzet and Pavans de Ceccatty, 1958; Tuzet and Paris, 1964; Tuzet, et al, 1970; Diaz, et al, 1973; Vacelet, 1979; Diaz and Connes, 1980; Gaino, et al, 1984; Gaino, et al, 1986; Efremova, et al, 1987) or amoeboid cells of the mesohyl (Gatenby, 1920; Fincher, 1940; Leveaux, 1942; Lévi, 1956). To date, ultrastructural studies (fuzet, et al, 1970; Diaz and Connes, 1980; Gaino, et al, 1984; Gaino, et al, 1986; Efremova, et al, 1987) have supported the view that male reproductive elements derive from choanocytes, however an amoebocyte (archaeocyte) origin of spermatogonia should not be discounted without reinvestigation. Fell (1974) has suggested that differentiation of spermatozoa within individual sponges may follow one of three patterns: 1) synchronously in all spermatic cysts of a specimen, or 2) synchronously only within a single spermatic cyst of an individual, or 3) asychronously within and between cysts of the sponge.

The present study was undertaken as a second step in the analyses of specific reproductive processes in four species of Caribbean commercial sponges: <u>Hippospongia lachne</u>, <u>Spongia</u> barbara, <u>Spongia</u> cheiris and Spongia

graminea. The following aspects of their reproduction are addressed here: reproductive cycles and spermatogenesis. Previous investigations focused on oogenesis, embryonic and larval development, transfer of bacterial symbionts, and larval morphology, behaviour, settlement and metamorphosis in these four species (Chapter 2).

Light and transmission electron microscope studies have been employed to help elucidate the process of spermatogenesis in these economically important members of the phylum Porifera.

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MATERIALS AND METHODS

Sponges:

Four Caribbean commercial sponge species: <u>Hippospongia lachne</u> de Laubenfels, 1936, <u>Spongia barbara</u> Duchassaing & Michelotti, 1864, <u>Spongia</u> <u>cheiris</u> de Laubenfels & Storr, 1958, and <u>Spongia graminea</u> Hyatt, 1877 were investigated. These rounded, black to dark grey demosponges are common members of the fauna of Biscayne Bay, Florida. This shallow bay (average depth 2.5 m) has intermittent periods of turbulence related to seasonal weather conditions. The collection site (25°38'N; 80°12'W) is characterized by a coarse sandy bottom with extensive areas covered in eel and turtle grasses, small scattered corals, gorgonians and other sponge species.

A total of 177 specimens were collected over a three year period (December 3, 1981; February 12, 1982; June 27, 1982; September 1, 1982; January 18, 1983; May 23, 1983; August 24, 1983) and analyzed histologically for reproductive activity. Water temperature and salinity data were recorded during the collection periods. Salinity was measured to the nearest $0.1^{\circ}/_{\circ\circ}$ in the field with a Yellow Springs Inc. salinity and temperature meter, and temperature was measured to the nearest $0.1^{\circ}/_{\circ\circ}$ with the same instrument.

Sampling and Preparation of Specimens for Microscopy:

Using SCUBA, endosomal tissue samples were removed in situ from specimens of the four sponge species (located at a depth of 1 to 1.5 m) using a large filet knife. Large triangular sections (ca. 60 mm on each side) were removed from the lower two-thirds of the individuals. A smaller tissue sample (ca. 20 mm³) was cut from that portion of the triangle that had come from the central region of the sponge. This procedure was employed

-111-

to insure that, if the sponge was reproductively active, tissue samples collected would contain reproductive elements. This smaller sample was then fixed <u>in situ</u> by placing the tissue in a syringe, egressing the ambient seawater and ingressing 2.5% glutaraldehyde in seawater (fixative). After returning to the laboratory, samples were placed in vials of fresh fixative for 16 hours, and then rinsed in three changes of fresh seawater for 10 min each. A smaller sub-sample (ca. 5mm³) from each specimen was post-fixed for one hour in 1% osmium tetroxide in seawater, dehydrated in an alcohol series, cleared in propylene oxide, and embedded in Spurr epoxy resin. The remaining fixed tissue from each specimen was dehydrated in an alcohol series, cleared in xylene, and embedded in paraffin.

Microscopy:

Epoxy blocks were sectioned on a Sorvall 'Porter-Blum' MT-2B ultramicrotome at lµm (glass knife) and 0.1µm (diamond knife). Semi-thin sections were mounted on glass slides and stained for 1 min at 60°C with a mixture (equal parts by volume) of methylene blue (1% in 1% Na-borate solution) and azure II (1% in distilled water). Ultrathin sections were floated onto formvar and carbon coated single-slot copper grids and stained for 20 min in saturated aqueous uranyl acetate and for 15 min in lead citrate. Sections were viewed and photographed with a Philips 410 transmission electron microscope operating at 80 kV. Paraffin blocks were sectioned at 10µm, and the sections were viewed, and measurements of reproductive elements recorded under a compound microscope with an ocular micrometer accurate to 0.5µm. Light micrographs were taken on a Zeiss photomicroscope.

-112-

Fluorescence Microscopy:

Stained sections were also examined for autofluorescence with a Nikon LABOPHOT compound photomicroscope equipped for bright field and fluorescence microscopy. An HBO 100W/2 high pressure mercury lamp supplied the incident-light source, a Nikon B2 excitation filter provided blue light with wavelengths between 460-485 nm, a 520W barrier filter blocked out all light with wavelengths of 520 nm or greater, and the dichroic mirror split the light beam at 510 nm. Photographs were taken with Kodak 100ASA technical film and Kodak 400ASA T-Max film.

Data Collection and Analyses of Reproductive Elements:

All specimens of the four sponge species undergoing spermatogenesis were investigated. Measurements of cell diameter, nucleus diameter and spermatogenic cyst diameter of each stage in the development of sperm cells were analyzed using the Statistical Analysis System (SAS) software computer package at McGill University (Ray, 1985). Measurements of cyst diameters were recorded while viewing sections under the compound microscope. Cell diameter and nucleus diameter were measured from transmission electron micrographs. Measurements were recorded as the maximum diameter measurement from serial sections when the same element could be followed in serial sections. The data collected were tested for the assumptions of normality (Univariate analysis) and homogeneity of variance (Bartlett's test). Since there was no violation of either assumption a parametric ANOVA (GLM for unbalanced data; Ray, 1985) was used to test the null hypothesis; Ho: there are no differences in parameter (x) between specimens of the same species, against an alternate, H1: there are differences in parameter (x) between specimens of the same species. This same procedure was used to test the

null hypothesis; H_0 : there are no differences in parameter (x) between sponge species, against an alternate H_1 : there are differences in parameter (x) between sponge species. Where the ANOVA rejected the null hypothesis, the Tukey multiple means comparison test (at P=0.05 level) was used to determine between which of the specimens and/or species significant differences occurred. The Tukey test was chosen because it is a conservative test in that it is more likely to find no statistical differences between the multiple means being compared and tested (Zar, 1984).

OBSERVATIONS AND RESULTS

Sexual Differentiation:

All of the samples collected (177) contained either male (5%), female (23%) or no (72%) reproductive elements. It was not possible to consecutively sample all of the same individuals during the three year study period. However, between the consecutive sampling dates of December 1981 and September 1982 several of the sponge specimens were examined in more than one collection period (Table 1). Nineteen individuals were sampled twice over a two to three month period and one individual was sampled three times over a six month interval. One individual of Spongia cheiris which was in the process of oogenesis in June 1982 was not reproductively active when sampled in September 1982; and one individual of S. barbara which was not reproductively active in December 1981 was in the process of spermatogenesis in February 1982 and that same individual was not reproductively active when sampled in June 1982. All other individuals were in the same reproductive/non-reproductive condition during the second sampling as they had been when they were first sampled. Male and female reproductive elements were never observed in the same specimen.

These observations strongly suggest that these four sponge species are dioecious (gonochoric), although successive hermaphroditism cannot be ruled out as all of the individuals were not sampled consecutively during the three year study. Clearly, such a regular sampling programme is necessary before any definitive conclusions on sexual differentiation can be made.

Reproductive Cycles:

Figure 1 is an histogram of the percentage of individuals, of each species, containing either male, female or no reproductive elements on each

TABLE 1:	Reproductive status of consecutively sampled individuals of fo	our
	sponge species.	

f= female; m= male; n.r= not reproductively active

	SPONGE SPECIES				
DATE	H. lachne	S. barbara	<u>S.</u> <u>cheiris</u>	<u>S. graminea</u>	
	fmn.r	fmn.r	f m rr	f m n.r	
Dec. 1981	5		4	4	
Feb. 1982	★ 5		↓ 4	4	
June 1982		1	1	2	
Sept. 1982			1	2	

4.0

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FIGURE 1: Histogram of the percentage of individuals of each species containing either male, female or no reproductive elements on each collection date. Salinity and temperature have been plotted. The numbers recorded at the top of the bars indicate the total number of specimens of each species analyzed on each of the collection dates.

male female	no reproductive elements
Hippospongia lachne	<u>Spongia</u> graminea
<u>Spongia</u> <u>cheiris</u>	<u>Spongia barbara</u>



(Å.-)

collection date over the three year study period (December 1981 - August 1984). The results indicate that <u>S. barbara</u> was reproductively active throughout the study period. However, the other three species demonstrated a distinct cycle of reproductive activity which occurred between: May and June for <u>Hippospongia lachne</u>, May and September for <u>S. graminea</u> and, June and August for <u>S. cheiris</u>. The data show that, during the reproductive seasons, there were always some individuals within the populations of each of the species that were not reproductively active and, there was also a greater percentage of females of all four species than there was males. With the exception of <u>S. barbara</u> in June 1982, male individuals of the four sponge species were always observed in the presence of female individuals. However, female members of all four species were observed in the absence of male members.

The reproductive period of <u>H. lachne</u>, <u>S. graminea</u> and <u>S. cheiris</u> coincided with an increase in water temperatures. None of the three species were reproductively active at temperatures below 25°C, and all three were active at temperatures between 25°C and 29°C. However, at temperatures above 29°C <u>H. lachne</u> was not reproductively active whereas <u>S. graminea</u> and <u>S. cheiris</u> were active. In the case of <u>S. barbara</u>, there appears to be no relationship between water temperatures and gametogenesis. Reproductively active members of this species were found throughout the study period when temperatures ranged from a low of 17.2°C to a high of 29.7°C.

There appears to be no relationship between reproductive activity and salinity in any of the four sponge species.

Data Analyses of Reproductive Elements:

The results of the ANOVA on the measurement data showed no significant

differences (P>0.05) when comparisons were made between specimens of the same species. Therefore, the three specimens of <u>S. graminea</u> are assumed to come from populations having identical means and the three specimens of <u>S. barbara</u> are also assumed to come from populations having identical means. Data from the specimens of each species were pooled for further statistical analyses on differences between species. Only one specimen of each of <u>H. lachne</u> and <u>S. cheiris</u> was available, therefore between specimen comparisons could not be made for these two species.

The results of the ANOVA (GLM) also indicated no significant differences (P > 0.05) when comparisons were made between species. Therefore, the four sponge species are assumed to come from populations having identical means. Table 2 provides a summary of the parameters measured and other morphological characteristics for each stage in the development of sperm cells in the sponges investigated. The description that follows is based on these parameters and applies to all four species.

The sequence of events in spermatogenesis reported here, is inferred from linking together static images clearly or very nearly identical to one another in series. The processes are described as active for convenience.

Microscopy:

The production of male gametes occurs in cysts within the endosome of mature specimens. The spermatic cysts are formed during the reproductive season when groups of cells in various stages of spermatogenesis are surrounded by a single, thin layer of flattened cells. The cells within an individual cyst are synchronized in their development however, development between cysts is asynchronous. Clusters of cysts at various stages of differentiation are localized in central tissues of the sponges (Fig. 2a).
TABLE 2: Morphological characteristics of the stages in the process of spermatogenesis.

MORPHOLOGICAL Characteristic	STAGES IN SPERMATOGENESIS			
	spermatogonium	pri mary spe rma to cy te	secondary spermatocyte	spermatid
cell diameter (µm)	3.5-6.1 N=12 X=5.03 <u>+</u> 0.21	3.5-5.0 N=23 X=4.14 <u>+</u> 0.55	1.7-2.1 N≈17 X=1.98 <u>+</u> 0.10	1.5-1.9 N=15 X=1.68 <u>+</u> 0.13
shape	ovoid to spherical; no flagellum	spherical; single flagellum	sperical; single flagellum	ovoid; single flagellum
cytoplasm	dense	dense; many mitochondrıa	dense; many mitochondria; glycogen	vesicles; mıtochondria; glycogen
nucleus (diameter in µm)	3.1-3.5 N=11 X=3.21 +0.17 nucleolated	2.5-3.5 N=23 X=3.00 ±0.39 dispersed chromatin filaments	1.0-1.4 №=17 X=1.18 <u>+</u> 0.10 dense	0.8-1.1 N=14 X=0.98 <u>+</u> 0.13 electron dense centre
ratio of cell to nucleus diameter	N=11 X=1.55 <u>+</u> 0.05	N*23 X*1.39 <u>+</u> 0.09	N=17 X=1.68 <u>+</u> 0.10	N=14 X=1.73 <u>+</u> 0.16
cyst (diameter in µm)	23-36 N=80 X=31.33+3.15 surrounded by follicle	36-45 №80 X=43.09+3.52 surrounded by follicle	45-70 N=80 X=55.64+5.60 surrounded by follicle	60-78 №=18 X=67.33+4.54 surrounded by follicle

N= number of observations; \overline{X} = mean <u>+</u> standard deviation

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FIGURE 2:

- a) Light micrograph of sperm cysts in encosomal tissue of <u>S. barbara</u>.
 Note the synchronous development of cells within each cyst.
 1, primary spermatocyst; 2, cyst containing spermatids.
- b) Electron micrograph showing typical choanocytes in endosomal tissue of <u>S. graminea</u>. Note the presence of symbiotic bacteria (b) and collagen fibrils (cf) in the mesohyl surrounding the cells. Choanocytes possess a single flagellum (f) directed toward the lumen of the chamber (on the right hand side), a basal anucleolate nucleus (n), pseudopodial processes (p) extending into the mesohyl and vesicular cytoplasm containing phagosomes (ph).
- c) Electron micrograph of differentiating spermatogonia within a cyst in the endosomal tissue of <u>S. graminea</u>. Symbiotic bacteria (b) are present in the mesohyl and a thin layer of flattened, pinacocyte-like follicle cells (fc) surround the germ cells. The spermatogonia possess a nucleolate nucleus (n) and dense cytoplasm containing mitochondria and various other inclusions. Arrowhead indicates the sloughing of cytoplasm and phagosomes at the base of cells.
- d) Electron micrograph of a spermatogonium of <u>S. barbara</u> in the metaphase stage of mitosis. Arrowhead indicates condensed chromosomes arranged at the equator of the cell.
 INSET: Electron micrograph of <u>S. barbara</u> in interphase containing a nucleolate nucleus (n) and dense cytoplasm containing mitochondria (m).



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Normal somatic tissues in the immediate vicinity of the cysts are disrupted with an observed decrease in cell numbers, flagellated chambers and symbiotic bacteria. Endosomal tissue in peripheral areas and the ectosomal tissue remain undisturbed.

Light and transmission electron microscopic analyses of specimens of <u>H. lachne</u>, <u>S. barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u> undergoing spermatogenesis suggest that presumptive spermatogonia differentiate directly from choanocytes <u>in situ</u>. Choanocytes are triangular in shape measuring about 3µm by 5µm in cross-section (Fig. 2b). They possess an anucleolate nucleus (ca. 2µm) located in the basal region of the cell (towards the mesohyl). Pseudopodial processes were often observed extending from the bases of the cells into the surrounding mesohyl which contains collagen fibrils and symbiotic bacteria. A single flagellum arises from the apical region of the cell (towards the lumen of the choanocyte chamber) and is encircled by a microvillus collar. The vesicular cytoplasm contains many phagosomes.

The differentiation of choanocytes into spermatogonia involves: loss of collars and flagella, migration of the nucleus into the apical region of cells, a sloughing of cytoplasm and phagosomes at the base of cells and, the migration of cells into the lumen of the chamber (Fig. 2c). These cells become more or less ovoid or spherical in form and measure about 5 μ m in diameter. They contain a distinct nucleolate nucleus (ca. 3.5 μ m) and dense cytoplasm with mitochondria and various other inclusions (Fig. 2d, inset). The spermatogonia are delineated by a single layer of flattened pinacocyte-like follicular cells. This limiting layer of cells is not present around choanocyte chambers prior to their transformation into spermatogonial cysts, but persists throughout the stages in the development of sperm cells. The germ cells undergo mitosis before differentiation to primary spermatocytes

(Fig. 2d).

These primary spermatocytes are spherical, about $4\mu m$ in diameter, and contain a distinctly enlarged n_cleus (ca. $3\mu m$) and dense cytoplasm with many small mitochondria (Fig.3a). The ratio of nucleus to cell diameter is almost double that for choanocytes. Primary spermatocytes are distinguished by the presence of a single flagellum and chromatin in the form of thread-like structures. A thickening of the chromatin filaments causes the formation of electron dense chromosomal masses.

Figure 3b shows two secondary spermatocytes which are believed to be derived from telophase I of the maturation divisions of a primary spermatocyte. Secondary spermatocytes are 2 μ m in diameter and possess a nucleus (ca. 1 μ m) and a single flagellum. The cytoplasm, similar to that of the primary spermatocyte, is dense and contains mitochondria and glycogen. These cells are often connected by cytoplasmic bridges.

Tetrads of cells were frequently observed in a chain formation within spermatogenic cysts. Figure 3c shows a typical arrangement of these four cells which are believed to be spermatids derived from the final phase (telophase II) of the maturation divisions. This observation together with the distinct change in the nature of the chromatin in primary spermatocytes, and the measured decrease in cell and nucleus size suggests that meiosis does occur in the development of sperm in these four species.

Spermatids are somewhat smaller than secondary spermatocytes, measuring about 1.7 μ m in diameter. They possess a slightly compressed nucleus (ca. 1 μ m) of condensed chromatin and a region of highly electron dense granules. The nuclear membrane cannot be clearly distinguished. The cytoplasm contains many vesicles, a few large oval-shaped mitochondria and glycogen. A single flagellum extends from one of a pair of centrioles

FIGURE 3:

- a) Electron micrograph of primary spermatocytes within a cyst in the endosomal tissue of <u>S. graminea</u>. Symbiotic bacteria (b) are present in the mesohyl and a layer of follicle cells (fl) surround the reproductive elements. Primary spermatocytes possess an enlarged nucleus (n) and dense cytoplasm with many, small mitochondria (m). The lumen of the cyst is towards the top of the micrograph.
- b) Electron micrograph of two secondary spermatocytes of <u>S. barbara</u> derived from telophase I of the maturation divisions of a primary spermatocyte. Secondary spermatocytes possess a nucleus (n), a single flagellum (f) arising from one of a pair of centrioles, and dense cytoplasm containing mitochondria (m) and golgi bodies (g). These cells are connected by cytoplasmic bridges (cb) probably resulting from incomplete cytokinesis.
- c) Electron micrograph of spermatids within a cyst in the endosomal tissue of <u>H. lachne</u>. Arrow indicates tetrads of cells (in chain formation) believed to be spermatids derived from telophase II of the maturation divisions.
 INSET: Electron micrograph of a spermatid of <u>H. lachne</u> containing a slightly compressed nucleus (n) with a region of highly electron dense granules (arrowhead), mitochondria (m) and a single flagellum arising from one of a pair of centrioles (c).
- d) Electron micrograph of a spermatozoan of <u>S. cheiris</u> containing a kidney-shaped nucleus (n), large mitochondria (m) and a single flagellum.
- e) Light micrograph of a cyst containing spermatozoa in the endosomal tissue of <u>S. barbara</u>. The cyst is surrounded by follicle cells (fc) and the flagella (f) of the male gametes are oriented towards the centre of the cyst.



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-124-

positioned close to the nucleus and opposite the mitochondria (Fig. 3c, inset). These cells often remain connected to one another by cytoplasmic bridges.

The final stage in the development of male gametes is the differentiation of a spermatozoan. This cell possesses a slightly elongated head and a single flagellum. The head, about 1.8 μ m long and 1.4 μ m wide in maximum diameter, contains an electron dense kidney-shaped nucleus (ca. 1 μ m) on one side and mitochondria and glycogen on the other (Fig. 3d). No acrosome or intermediate segment is observed in any of the sperm cells examined. The flagellum arises from one of a pair of centrioles close to the nucleus and extends latero-posteriorly from the head. Spermatozoa are oriented with their flagella directed towards the centre of the cyst (Fig. 3e).

During the process of spermatogenesis the cells of the follicle layer become more and more elongate as the spermatic cysts progressively increase in size from 25 μ m to 75 μ m. Fusion of cysts was not observed in any of the specimens examined.

Stained sections of specimens undergoing spermatogenesis were examined with fluorescence microscopy. The tissues autofluoresced an opaque yellowwhite when excited with blue light (460-485 nm). After the field of view was quenched for one minute, spongin fibres and the cytoplasm of sperm cells displayed a translucent yellow-white autofluorescence (Fig. 4b). During the study of oogenesis in these same four sponge species (Chapter 2) secondary oocytes considered to be in the process of fertilization were also examined with fluorescence microscopy. The cytoplasm of the oocyte exhibited low levels of fluorescence, one of the two nuclei was no longer visible and the other nucleus displayed a bright yellow-white autofluorescence similar to

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FIGURE 4:

- a) Light micrograph of a secondary spermatocyst in the endosomal tissue of <u>H. lachne</u>. The cyst is surrounded by a layer of follicle cells (fl). Transmitted light.
- b) Same as a). Fluorescent light. Note the bright yellow-white autofluorescence displayed by the secondary spermatocytes when excited with blue light (460-485 nm).



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that of the sperm cells in the present study (see Chapter 2, Fig. 3f).

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DISCUSSION

There have been relatively few detailed investigations of spermatogenesis in the Porifera (Reiswig, 1983). Many accounts of this process are part of more general descriptive studies on reproduction. The present study focused on reproductive cycles and spermatogenesis in four species of Caribbean commercial sponges. The investigation involved detailed light and transmission electron microscopic examinations of spermatogenic elements in <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u>. Morphological characteristics and dimensions of these elements were recorded and the dynamics of spermatogenesis has been described. An earlier investigation outlined the details of: oogenesis, the transfer of bacterial symbionts from one generation to the next, and larval development and metamorphosis in the same four species.

Recent and extensive reviews on reproduction in sponges indicate a great variability in sexual differentiation among demosponges (Fell, 1983; Reiswig, 1983; Simpson, 1984), but from the variability two consistent patterns emerge. Demosponges that broadcast eggs or embryos (oviparous) are generally dioecious (gonochoristic) and those that brood embryos and larvae (viviparous) are generally hermaphroditic. Within the ceractinomorphs, and especially among the dictyoceratids, viviparity with gonochorism is considered to be an exception. A previous investigation on oogenesis in the same four sponge species examined in the present study established that these sponges are viviparous (Chapter 2). All available evidence from both studies suggests that these species are dioecious. However, increasing evidence of sex reversal and a high degree of interspecific variability in sexual differentiation among sponges (Fell, 1970; Diaz, 1973; Van de Vyver and Willenz, 1975; Elvin, 1976; Gilbert and Simpson, 1976; Fell and Jacob, 1979) suggests that intrinsic interactions between genetic and/or environmental factors may determine sexual differences. Therefore, successive hermaphroditism in these four species cannot be ruled out. Further investigations of sponge populations over an extended period of time and under different environmental regimes are required to gain a better understanding of the process of sexual differentiation.

Most sponges have a repetitive seasonal reproductive period with oocyte and spermatozoan differentiation occurring in asynchronous cycles once a year. Generally, sexual reproduction is more prevalent in the warm summer months but there are species that reproduce in late autumn or early winter and, there are some species that produce gametes all year (for review see Simpson, 1984).

Little is known concerning the temporal aspects of gametogenesis. In some oviparous species, spermatogenesis and/or oogenesis may be precisely synchronized within individuals or throughout local populations. In such cases the time course of these processes can be estimated. In Polymastia mammillaris (Sara, 1961), Axinella damicornis and A. verrucosa (Siribelli, 1962) spermatogenesis is highly synchronized within individuals and populations, and differentiation of spermatozoa from primary spermatocytes occurs over a two week period. It appears that the process of oogenesis often occurs earlier in the reproductive cycle and over a longer period of In A. damicornis and A. verrucosa (Siribelli, 1962) differentiation time. of oocytes occurs over several months and, only after the female gametes are fully developed do male specimens begin the process of spermatogenesis. This pattern of gamete production in oviparous sponges appears to be an efficient method of ensuring reproductive success. In the four species

investigated in this study, and many other viviparous demosponges, spermatogenesis and oogenesis are asynchronous within individuals and within populations. Gametes are produced continuously during the reproductive period and not all individuals in a population are involved in gametogenesis. Therefore, it becomes extremely difficult to estimate the time span for these processes (Fell, 1974).

There is a lack of information concerning the factors that influence gametogenesis. However, for those species that exhibit repetitive seasonal reproductive periods, it seems likely that exogenous factors play an important role in controlling these processes. In a number of cases it appears that water temperature may have a direct effect on the development of gametes (Microciona prolifera, Simpson, 1968; Suberites massa, Diaz, 1973; Haliclona loosanoffi, Fell, 1976). In a study on Hippospongia lachne (Storr, 1964) the production of female reproductive elements was followed at monthly intervals in three different localities and, the occurrence of peaks in oocyte and embryo production was correlated with water temperature. The greatest proportion of specimens containing gametes was found when the water temperature was between 23°C and 29°C. Results of the present study support the suggestion that water temperature may play an important role in maintaining and/or initiating gametogenesis in H. lachne. The data of this study also suggests water temperature as a possible factor in sexual reproduction of Spongia cheiris and S. graminea. Reproductive specimens of these three species were never observed at temperatures below 25°C. However, water temperature does not appear to directly influence gametogenesis in S. barbara which produces gametes all year when temperatures as low as 17°C were recorded.

While exogenous factors may play an important role in reproductive

-130-

activity, endogenous factors have also been suggested as regulators of gametogenesis in sponges. Changes in metabolic state have been associated with reproductive condition in <u>Hymeniacidon perleve</u> (Stone, 1970) and <u>Haliclona permollis</u> (Elvin, 1979). Overwintering in gemmules is a necessary prerequisite to sexual reproduction the following spring in <u>Spongilla</u> <u>lacustris</u> (Simpson and Gilbert, 1974), <u>Ephydatia fluviatilis</u> (Van de Vyver and Willenz, 1975) and <u>Haliclona loosanoffi</u> (Fell, 1976). It is probably a combination of exogenous and endogenous factors that control reproduction. However, to determine the specific factors and elucidate the mechanisms regulating reproductive processes, biochemical and experimental approaches to investigations of these processes are urgently required.

Sponges lack localized discrete reproductive organs and in most individuals large areas of mesohyl are involved in reproduction. The production of male gametes occurs in spermatic cysts which are formed during the reproductive season when groups of cells in various stages of spermatogenesis are surrounded by a single, thin layer of flattened cells. The general pattern of spermatogenesis in the four sponge species investigated in the present study is similar to that reported for other viviparous demosponges (<u>Hippospongia communis</u>, Tuzet and Pavans de Ceccatty, 1958; <u>Aplysilla rosea</u>, Tuzet, <u>et al</u>, 1970; <u>Spongia officinalis</u>, Gaino, <u>et al</u>, 1984).

Investigations based on the appearance of different types of cells in histological sections have indicated that male gametes may have two different origins. The present study, and ultrastructural studies of spermatogenesis in other demosponges (<u>Aplysilla rosea</u>, Tuzet, <u>et al</u>, 1970; <u>Suberites massa</u>, Diaz and Connes, 1980; <u>Spongia officinalis</u>, Gaino, <u>et al</u>, 1984; <u>Oscarella lobularis</u>, Gaino, <u>et al</u>, 1986; <u>Myxilla incrustans</u> and <u>Iophon</u> piceus, Efremova, <u>et al</u>, 1987) suggest that choanocytes are the anlagen of spermatogonia. In all but one of these species, the entire choanocyte chamber transforms into a spermatic cyst containing the differentiated spermatogonia. The follicular cells that surround developing gametes are derived from mesenchymal amoebocytes or archaeocytes. Ultimately, after meiosis and differentiation these cysts contain the mature spermatozoa. In <u>Suberites massa</u> (Diaz, <u>et al</u>, 1973) individual choanocytes within chambers undergo transformation into spermatogonia and migrate into the mesohyl where they then form cysts. On the other hand, light microscope studies of spermatogenesis in other sponges (<u>Hymeniacidon</u> (<u>Stylotella</u>) <u>heliophila</u>, Fincher, 1940; <u>Halisarca dujardini</u>, <u>H. metschnikovi</u>, <u>Tethya aurantia</u> and <u>Polymastia mammillaris</u>, Lévi, 1956) have suggested that amoebocytes or archaeocytes are the origin of spermatogonia.

It should be noted that all conclusions regarding the origin of male germ cells are based upon interpretation of fixed tissues. Conclusive experimental work has not yet been possible but is desirable. Based upon present knowledge of spermatogonial genealogies, great variability among almost all of the species which have been investigated makes it impossible to predict detailed patterns of spermatogenesis in any group of sponges which has not been studied specifically.

The observation of synchronous differentiation of cells within spermatic cysts and asynchronous development between cysts has also been reported in a number of other viviparous demosponges (<u>Hippospongia communis</u>, Tuzet and Pavans de Ceccatty, 1958; <u>Aplysilla rosea</u>, Tuzet, <u>et al</u>, 1970; <u>Spongia officinalis</u>, Gaino, <u>et al</u>, 1984). The presence of cytoplasmic bridges between developing germ cells is also characteristic of spermatogenesis in many sponges (Tuzet, <u>et al</u>, 1970; Gaino, <u>et al</u>, 1984;

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Gaino, et al, 1986; Efremova, et al, 1987). These connections are probably the result of incomplete cytokinesis during cleavage of the cells, and may play a role in the contemporaneous differentiation of male gametes within cysts. Synchronous development of germ cells within a cyst could reduce the number of immature elements leaving the cyst when the mature gametes are broadcast.

There is only one other ultrastructural study that has investigated spermatogenesis in dictyoceratids (Gaino, et al, 1984, Spongia officinalis). In all species the differentiation of spermatogonia from choanocytes involves the loss of collars and flagella, migration of the nuclei toward the opposite ends of the cells and a sloughing of cytoplasm at the bases of the cells. However, unlike S. officinalis (Gaino, et al, 1984), the spermatogonia of H. lachne, S. barbara, S. cheiris and S. graminea undergo mitosis. Primary spermatocytes are characterized by a decrease in the ratio of cell to nucleus diameter and a modification in the organization of the chromatin. A decrease in cell and nuclear size between primary spermatocytes and spermatids also occurs. Similar changes have been reported in Aplysilla rosea (Tuzet, et al, 1970), Suberites massa (Diaz and Connes, 1980), Spongia officinalis (Gaino, et al, 1984) and Oscarella lobularis (Gaino, et al, 1986). These events strongly suggest that spermatogenesis in sponges involves two meiotic divisions to secondary spermatocytes and spermatids. Simpson (1984) emphasizes that no study has established chromosomal reduction or reported tetrad [chromatid] formation and, he suggests that the decrease in cell and nuclear sizes may be the result of two mitotic divisions. This study and one other recent study (Efremova, et al, 1987) are the only ones to have reported spermatids in tetrad (cell) formation. To date, there are still no reported studies that

have documented a halving of chromosome numbers during spermatogenesis. The greatest obstacle to gaining a clear picture of this very important event in the sexual process is that of correctly interpreting dynamic events, that occur in a brief moment of time, from static images. Biochemical and experimental approaches involving cell isolation and selective labelling are urgently needed to complete our understanding of this and other events in spermatogenesis.

The spermatozoa of the four sponge species investigated in the present study lack a true intermediate segment containing mitochondria. In the male gametes of <u>H. lachne</u>, <u>S. barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u> the mitochondria are grouped around the nucleus which is positioned to one side of the slightly elongated head. These sponges do not possess an acrosome or acrosome vesicles. Only one ultrastructural study has reported the presence of an acrosome in the sperm of sponges (Baccetti, <u>et al</u>, 1986, <u>Oscarella</u> <u>lobularis</u>). The absence of this organelle is consistent with a mechanism of fertilization through inactive sperm transfer. Gallissian (1980) reported the involvement of a carrier cell in the fertilization of <u>Grantia</u> <u>compressa</u>. In sponges that do possess an acrosome, fertilization may occur directly by penetration of the sperm into the egg. More studies on spermatozoan morphology and fertilization are required to elucidate our understanding of sexual reproduction in the Porifera.

The observation of autofluorescence in sperm cells of <u>H. lachne</u>, <u>S.</u> <u>barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u> is the first record of a definitive label that could be used to identify and follow the development of spermatic cells. In a previous study on oogenesis in these same four sponges (Chapter 2) autofluorescence of one of two nuclei in a secondary oocyte helped to identify the probable process of fertilization in the sequence of events

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This study represents the second step in the analyses of specific reproductive processes in <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>Spongia</u> <u>cheiris</u> and <u>Spongia graminea</u>. Together with the study on oogenesis, transfer of bacterial symbionts, and larval development, behaviour, settlement and metamorphosis (Chapter 2) an understanding of sexual reproduction and development in these four commercial sponges may prove useful in future ecological and biological investigations of these economically important species.

-136-

The authors wish to thank Dr. Maria Neuwirth, for her assistance with electron micrograph interpretations and Rosenstiel School of Marine and Atmospheric Sciences, University of Miami for use of laboratory facilities. This study was supported by a grant from the Royal Bank/McGill International/Caribbean Development Fund to the authors, and an NSERC (Canada) operating grant to H.M. Reiswig.

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CONCLUDING REMARKS

The presence of large populations of intercellular symbiotic bacteria in the endosomal tissues of <u>Hippospongia lachne</u>, <u>Spongia barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u> has been noted and several morphologically distinct types occur in all four species. These symbionts are specific to the sponges and different from ambient seawater bacteria. Populations of sponge specific bacteria are considerably higher than populations of ambient seawater bacteria.

Bacteria isolated from their hosts were capable of utilizing a variety of individual amino acids, carbohydrates and tricarboxylic acid cycle intermediates as sole carbon and energy sources in the test medium. Such relatively simple organic growth requirements implies that the symbiotic association with the sponges is not related to specific and complex nutritional factors that the sponge supplies. Two of the sponge species in the present study were tested for antimicrobial activity against their own symbionts and those of the other species, as well as two other marine bacteria. Fluid extracts from the sponge specific bacteria or other marine bacteria.

The nature of the relationship between sponges and their associates is not known. However, it is clear that studies of these taxonomically and economically important species must critically consider the bacterial populations within these species as major biotic components and, treat this intimately balanced association as an integrated but potentially disturbable community.

A comprehensive and comparative investigation of the processes involved

-141-

in sexual reproduction in <u>H. lachne</u>, <u>S. barbara</u>, <u>S. cheiris</u> and <u>S. graminea</u> has shown that these species are viviparous and probably dioecious. In three of the four sponge species gametogenesis occurs in synchronous cycles once a year, during the warm summer months. In <u>S. barbara</u>, gametes are asynchronously produced throughout the year; there is no specific seasonality in the reproductive processes. Water temperature appears to be a factor influencing gametogenesis in the three species that produce gametes during the warm summer months. Reproductive specimens of these three species were never observed at water temperatures below 25°C. However, water temperature does not appear to directly influence gametogenesis in <u>S.</u> <u>barbara</u>.

Statistical analyses have identified specific morphological features that characterize stages in the development of female reproductive elements Presumptive oogonia differentiate directly from in these sponges. archaeocytes and undergo a single mitotic division to produce primary oocytes. The production of male gametes occurs in cysts within the endosome of these sponges. The reproductive elements within an individual cyst are synchronized in their development however, development between cysts is asynchronous. Presumptive spermatogonia differentiate directly from choanocytes in situ. All cells of the chamber lose their collars and flagella and undergo mitosis to produce primary spermatocytes which then undergo meiosis to produce secondary spermatocytes and finally four spermatids. These cells differentiate into spermatozoa which do not possess intermediate segments or acrosomes. Presumed male and female pronuclei in secondary occytes suggests that fertilization occurs in these cells and then zygotes undergo major growth. During the process of cleavage connections are formed between the embryo and surrounding nurse cell layers. These

umbilici apparently function as the pathway by which maternal, intercellular symbiotic bacteria are extracellularly transferred to progeny. At the end of cleavage blastomeres differentiate, resulting in the development of pigmented parenchymella larvae.

The ciliated free-swimming larvae possess a posterior ring of cells bearing long cilia. The ciliated cells of the epithelium are probably larval structures only and represent a terminal differentiation. In laboratory studies the larvae demonstrated negative phototaxis until shortly before settlement during which time they showed no particlar taxis to light. There is no evidence for substrate selection or orientation. Larval behaviour probably reflects the ecological situation of adult populations. Settlement of larvae occurs 26-56 hours after release and attachment to the substrate involves the rapid formation of a basal lamella. This layer may function as a buffer to ensure that larval cells do not actually contact the substrate, thereby allowing non-selective settlement. Precocious development of choanocytes does not occur in the larvae or post-larvae of these four species.

Knowledge of the sexual reproductive processes and development in the four sponge species examined in this study will be useful in future ecological and biological investigations of these taxonomically and economically important genera.

-143-