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Spermatogenesis in Limnodrilus Hoffmeisteri (Clap); A Morphological and Environmental Study of the Development of Two Sperm Types

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SPERMATOGENESIS IN LIMNODRILUS HOFFMEISTERI (CLAP): A MORPHOLOGICAL AND ENVIRONMENTAL STUDY OF THE DEVELOPMENT OF TWO SPERM TYPES

by

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Edward Michael Block

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INTRODUCTION

Annelid worms belonging to the family Tubificidae are an abundant component of the fauna of mud bottomed streams throughout the world (Brinkhurst and Jamieson, 1971). These worms have interested students in a number of ways; for example, they have been used in experiments to determine their mobility in the presence of antibiotics in pharmacological research (Rice, 1939; Coler, *et al.*, 1968). These and other studies have identified species specific worms capable of digesting specific strains of bacteria (Wavre and Brinkhurst, 1971; Brinkhurst, 1969; Appelby and Brinkhurst, 1970; Brinkhurst and Chua, 1969; Coler, *et al.*, 1967). Preliminary studies of samples of crushed worms placed on selected areas of bacterial cultures suggested bacterial growth inhibition and/or bacterial destruction (C. J. Goodnight, personal communication). The physiological pathways involved in digesting or inhibiting the growth of selective bacterial strains as well as the effect of antibiotics on these worms have not been identified.

Today, researchers have observed that these organisms, as well as others, have been useful in identifying the degrees of organic pollution in lakes and streams (Goodnight and Whitely, 1960; Goodnight, 1973). Students of the environmental sciences as well as students having a sincere interest in the ever present environmental problems have been introduced to the concept of indicator organisms and degrees

of organic pollution in water systems. Students have become acquainted with this concept of indicator organisms through field experiences, controlled laboratory experiences, and/or through the use of the literature--text books and scientific publications.

In order to further knowledge of this concept, it is necessary to continue studying the life histories of indicator organisms. The tubificid worm *Limnodrilus hoffmeisteri* is probably the most abundant oligochaete found in organically enriched benthic communities. Despite the relative abundance of *L. hoffmeisteri*, the number of detailed studies of its ecology, life history, reproduction, and physiology have been limited (Kennedy, 1965, 1966; Brinkhurst and Kennedy, 1965; Block and Goodnight, 1976). Studies of the reproductive system of tubificids have been reported by a number of people, including Gathy (1900), Gatenby (1916), Stephenson (1930), Hess (1959), Inase (1960, 1968), Hirao (1967), Aston (1968), Ferraguti and Lanazavecchio (1971).

An unexpected observation involving the life history of *L. hoffmeisteri* was observed by Block and Goodnight (1973, 1976). They observed the development of two mature sperm types--one larger than the other. Such observations in related genera included Dixon (1915), Mehra (1926) for *Tubifex*; and an unpublished master's thesis on *Peloscolex* (Correll, 1969). These investigators did not identify the stages of spermatogenesis morphologically as they were related to the development of the two sperm types. These observations were, probably, chance findings apart from the main intent of

their investigations. From these and other studies, it appears that tubificids live under a wide variety of environmental conditions, exhibit some unusual physiological activity, and their period of reproductive activity is governed in some way by local conditions (Gabrilov, 1935, 1948; Kennedy, 1966; Block and Goodnight, 1972). Much remains to be learned as to how various environmental conditions control reproductive behavior and, perhaps, those physiological processes cited above.

The present study was designed to contribute additional information to the understanding of the life history of *L. hoffmeisteri* by describing the sequences of spermatogenesis from worms collected in the field. The following objectives indicate the scope and magnitude of the present investigation.

1. To investigate the reproductive activity of *L. hoffmeisteri* while under field conditions by comparing water chemistry and selected physical parameters with observed morphological development of the reproductive organs.
2. To describe in detail the morphological changes that occur during spermatogenesis by means of light microscopy, transmission electron microscopy, and scanning electron microscopy.
3. To compare morphologically the two pathways of sperm development and to determine how the resulting sperm are different.

REVIEW OF LITERATURE

Members of the genus *Limnodrilus* have been observed throughout the major continents of the world (Brinkhurst and Jamieson, 1971). The genus was first described by Claparède (1862) from the European continent. This genus is known not to survive high salinity and has become known as a cosmopolitan organism that is distributed throughout the world with greatest population densities near metropolitan areas (Block and Goodnight, 1972; Brinkhurst and Jamieson, 1971). In recent years, *Limnodrilus* has been of interest to researchers because the worm is closely associated with waterways enriched with organic materials (Goodnight and Whitely, 1960; Kennedy, 1966a, 1966b; Brinkhurst, 1964, 1965, 1966). Numerous studies have been undertaken to analyze this relationship. It is known that polysaprobic areas will support the life of one or two genera of Tubificinae (Brinkhurst, 1964; Goodnight, 1973) in great numbers (excess of 1000 per square meter assuming a depth of around 5 cm). This relationship is not well understood.

Numerous studies have been reported describing the population of worms by density, stratification, relationships to other organisms, survival with toxic substances and so on (Moreno, 1978). However, few attempts have been undertaken to study thoroughly and understand the life history of the family Tubificidae.

Life History of Members of the Family Tubificidae

The family Tubificidae consisting of annelids has not been investigated thoroughly. Certainly, the life history of this family of worms is not well understood. The genera *Limnodrilus*, *Tubifex* and *Branchiura* have been studied but conclusive evidence concerning their life histories is lacking. There is evidence that suggests that one or more of the genera may require from one to two years to complete a life cycle. Their breeding activity may take place at different time periods throughout the year and their breeding periods may differ from one geographical location to the next. It has also been suggested that water temperature may play a prominent role in breeding activity.

Kennedy (1966) suggested that the life cycle of *L. hoffmeisteri* varied from one to two years with the variation apparently associated with the geographical location in England. Sexual maturation and activity was measured by the thickened clitellum between segments 9 and 13. This thickening was easily observed with the developing sperm and egg sacs that appeared white through the semitransparent brownish cuticle in the live specimens. The appearance of cocoons marked the end of a given cycle. Kennedy was able to show that *L. hoffmeisteri* evidenced sexual maturity at decreased levels throughout the year. He observed single intense breeding activity in certain study areas in early spring. Poddubnaya (1959) suggested a single reproductive peak from late May to early June or July as measured by the appearance of cocoons. She also supported the

premise in the Rybinsk Reservoir in Russia that the life cycle was completed within one year. This interpretation was made difficult by the presence of *T. newaensis* which she stated had a peak breeding period at the same time as *L. hoffmeisteri*. The breeding activity was measured by the abundance of cocoons. However, a morphological separation of cocoons between the two genera is not possible at this time. Brinkhurst (1971) took issue with the one year life cycle concept since, in part, there was a lack of mature worms prior to the observance of the cocoons. One may note from the literature that the cycles for other similar genera are also open to conjecture. *B. sowerbyi* was reported to have a one year breeding cycle (Aston, 1968). Kennedy (1966) suggested that *L. udekemianus* took two years to mature and breed. He reported peak fecundity in late winter to early spring with variation from one collecting site to another.

Grigelis (1961), Poddubnaya (1959), and Timm (1962) have reported that breeding is initiated in countries in northern Europe after the winter months once the water temperature has increased to between 12°C and 15°C. Kennedy (1966) noted that low water temperature interrupted the reproductive process but that reproduction would still occur at temperatures below 12°C in the autumn or early spring.

Dixon (1915) observed sexual maturation in the autumn for *T. rivulorum* (=*tubifex*). She noted the appearance of cocoons in November from the estuarine Thames in England. Mehra (1926) observed large numbers of sexually mature *T. tubifex* from September

to December and from March to May. His specimens were from the River Cam and from an aquarium from the University of Cambridge laboratory in England.

Morphologic Characteristics Used to Identify *Limnodrilus hoffmeisteri*

The genus *Limnodrilus* like all the genera in the subfamily Tubificinae is identified by the male genitalia. The criteria used in this identification assume sexual maturity. This assumption causes numerous difficulties since there is evidence that shows that sexual maturity varies throughout the year (Block, 1972; Brinkhurst, 1971; Kennedy, 1966; Poddubnaya, 1959). Brinkhurst's key to the subfamily Tubificinae is currently used by most investigators. The subfamily Tubificinae is identified by an "atria with a single solid prostate attached by a narrow stalk" and by having spermatozeugma (=spermatophores) within the spermatheca. Such a description as well as the proper identification requires a trained eye and the use of a light microscope. The genus *Limnodrilus* is identified by the atria that are distinctly wider than the vasa deferentia. The vasa are longer than the atria. Additionally, the cylindrical usually thick walled cuticular penis sheath is much longer than broad. Species recognition of Tubificinae has throughout the historical recognition of this group of annelids been made on the genitalia. The greater variation found in the male genitalia and for the most part species recognition or identification is made by the size and shape of the penis. Descriptions of the genitalia have

been available since the use of the bright field microscope. *L. hoffmeisteri* was first described by Claparède (1862). His descriptions and drawings of the penis sheath of this worm are classic. It should be understood that these identification features do not take into account the developmental periods of the worm. For example, the size and shape of the penis do not mature at once. A growth period is required as is the situation for any organ. Such differences within the developmental process have left numerous questions for this investigator as to the validity of some species based solely on the penis sheath. This will be developed further in the Discussion.

As a historical note, it may be observed that many of the anatomical structures were identified with names associated with vertebrates. The functions of these structures and organs may or may not be homologous (Calkins, 1895). It is noteworthy that many of the earliest investigations, descriptions and drawings were done by renowned physicians. For the most part these drawings and the morphologic identifications have been accepted as valid.

Spermatogenesis Within the Clitellate Oligochaetes

Certain aspects of spermatogenesis have been investigated intensively within the oligochaete group. In general, reviews summarizing the current thinking within this research area are lacking. However, the maturation of the sperm during the process of spermatogenesis has been described (Franzen, 1956, 1974, 1977; Afzelius,

1972; Fawcett, *et al.*, 1971; Yasuzumi, 1974). The clitellate oligochaetes, which include the subfamily Tubificinae, appear to follow a uniform process in spermatogenesis. Part of this uniformity may be due to the reproductive process that includes sperm transfer during a copulatory act. A testicle or testicular-like tissue (germ tissue) initiates the formation of spermatogonia that are released in the form of several cells within a morula structure into a sperm sac within the coelom. The exact number of cells that are released as a morula structure has not been clearly established. Subsequent cell divisions lead up to the 128 cell morula structure at which point spermiogenesis is said to occur (Anderson, *et al.*, 1967; Grassé, 1959; Ferraguti and Lanzavecchia, 1971). Little is known about the testicular-like tissue or the release and development process of the spermatogonia as a morula structure within the sperm sac. Likewise, evidence for the 128 cell stage that typifies the last developmental spermatogonia stage and the beginning of spermiogenesis has not been sufficiently supported (Carter, 1858; Chatton and Tuzet, 1942, 1943a, 1943b; Tuzet, 1945; Bugnion and Popoff, 1905; Calkins, 1895; Bloomfield, 1880; Grassé, 1959; Anderson, *et al.*, 1967; Ferraguti and Lanzavecchia, 1971). The development and use of the electron microscope as a research tool have allowed researchers to investigate the morphology of cells and subcellular structures. In recent years, this tool has been used to investigate spermiogenesis within the clitellate oligochaetes.

Gatenby and Dalton (1959) were among the first to study spermiogenesis in *Lumbricus herculeus* (=*terrestris*) at the ultrastructural level. Their cytodifferentiation studies of the spermatid have led others to investigate some of the complex morphological changes that occur during the maturation of the sperm (Bradke, 1963; Anderson, *et al.*, 1967; Anderson and Ellis, 1968; Webster and Richards, 1977; Lanzavecchia and Donin, 1972). It has been established from these studies that the metamorphosis of the spermatid into a mature sperm occurs in three stages: (1) cell elongation, (2) reduction of both nucleus and cytoplasm volume, and (3) the formation of distinct cell compartments within the mature sperm-head, midpiece, and tail.

A few attempts to describe spermatogenesis in the subfamily Tubificinae have been reported. These reports include *T. rivulorum* (=*tubifex*) (Dixon, 1915), *T. tubifex*, *Ilyodrilus* sp. (Mehra, 1926), and *Peloscolex multisetosus* (Correll, 1969). More recently, Ferraguti and Lanzavecchia (1971) described selected phases of spermiogenesis in *T. tubifex* and *L. udekemianus*. Additional reports described sperm products or a certain stage in sperm development for the subfamily Tubificinae (Nasse, 1882; D'Udekem, 1855). The findings of these reports are the basis for the present study. Dixon's (1915) original attempts at describing the sequences of spermatogenesis in *Tubifex* were remarkable. But, attempts failed to describe the number of cell divisions associated with the process. She reported that a group of cells known as a morula structure, containing spermatogonia, were shed from a testis into the sperm sac.

Several nuclear divisions followed. The morula structure enlarged with each nuclear division. The nuclei were reported to align on the periphery of the morula (=blastophore, central nutritive mass, cytophorus) formation. Dixon suggested that two additional nuclear divisions followed. These nuclei were identified as spermatocytes. At this stage spermiogenesis occurred and the spermatids elongated and formed the mature spermatozoa.

Dixon observed an atypical sperm type that developed within the sperm sac along with the so-called normal sperm. The two sperm types were easily distinguishable. The atypical sperm was larger than the normal sperm. Dixon suggested that the two sperm types were truly a process of dimorphism. Her success to describe the morphogenesis of the atypical sperm eluded her as she was unable to establish conclusively a sequence of developmental stages. However, both mature sperm types had a head, midpiece and tail.

Mehra (1926) reported that approximately 6 - 10 spermatogonia cells were released from the testes into the sperm sac. These clumps of cells undergo a series of mitotic divisions that form the morulae composed of "spermatocytes of the first order". Spermatocytes were recognizable when they aligned peripherally within the morula structure leaving a central cytoplasmic mass (=blastophore) exposed.

"Spermatocytes of the second order" are a further stage of development. This stage was not well defined except that the blastophore was much larger than Spermatocyte I. At this stage,

Mehra suggested that the morula contained anywhere from 40 to 80 or more spermatids. The exact number was not clearly established.

Mehra (1926) reported an atypical sperm (apyrene) that he believed was different from that reported by Dixon (1915). His account suggests that the atypical sperm is giant in relation to the normal (eupyrene) sperm that he described. He suggests that this giant sperm does not mature into a sperm with the typical three morphological regions: head, midpiece, and tail.

Correll (1969) was one of the first investigators to use the light microscope in conjunction with the electron microscope for determining the sequences of spermatogenesis within the subfamily Tubificinae. Correll observed in *P. multisetosis* that an 8 cell morula sheds from the testis into the sperm sac. Individual morula undergo a series of cellular divisions recognizable as the 16, 32 and 64 cell stages. Beyond this point, Correll hypothesized that a 128, 256, and a 500+ cell stage would be observed before spermiogenesis. These latter stages were not distinguishable due to an inability to count accurately the different cells within an individual morula. The 8, 16, 32, 64 cell stages were classified spermatogonia I, II, III, IV respectively. The formation of the blastophore (Dixon, 1915; Mehra, 1926) was observed in the 16 cell stage. Correll determined the 128 cell stage to be primary spermatocytes. The meiotic process began with the 256 cell stage that was designated as secondary spermatocytes. Once the morula structure

reached the 500+ cell stage, the resulting spermatid metamorphosed into mature sperm.

Correll also reported the occurrence of an atypical giant sperm observed at the light microscopy level. He did not investigate this observation further but suggested that this was probably a polyploidy phenomenon rather than dimorphism.

Block (1973) indicated that two types of sperm were observed in *L. hoffmeisteri*. However, the observation was not pursued.

Ferraguti and Lanzavecchia (1971) published the only paper describing in some detail spermiogenesis in *L. udedemianus* and *T. tubifex* at the ultrastructural level. Their descriptions detailed the formation of the manchette, chromatin condensation and the role of microtubules in the metamorphosis of the spermatids. They did not attempt to investigate the developmental stages preceding spermatogenesis but relied on published accounts (Grassé, 1959). They noted from these published accounts that the 128 cell stage was commonly associated with spermatid formation for annelids in general. However, they discounted this premise for the tubificid worms that they investigated. They observed a cytophorus (=morula) containing much greater numbers of nuclei differentiating into mature sperm.

Other than these few papers, major attempts to establish the sequence of events in the development of mature sperm within this group of organisms were not discovered in the literature. Likewise, attempts that bring to light convincing evidence of the occurrence of the two types of sperm that have been briefly mentioned were not

discovered. The review of the literature also suggests that studies have not been made that follow the maturation sequence while under field conditions.

MATERIALS AND METHODS

The Study Area and Sampling Procedures

The Little Calument River (41° 34' 22" N, 87° 28' 30" W) near Hammond, Indiana was chosen as a suitable study area (Figure 1). This field site was selected due to the natural abundance of tubificid worms that have been observed over the last 20 years (C. J. Goodnight, personal communication). The population of worms in this area has remained relatively stable over the study period. Exceptions to the stability have been caused by periodic flooding that has scoured the benthos and redistributed the worms throughout the entire river system (Figure 2). However, the worms quickly re-inhabit the benthic areas of the stream and sufficient numbers of worms can be collected on a year round basis. This river system has been subjected to various sources of pollution including sewage, industrial effluents, and storm sewer drainage systems (Moreno, 1978).

Worm samples and water samples were collected monthly from September, 1977 through August, 1978 (Table 1). Additional samples were taken prior to, and after, these monthly collections in order to supplement information gathered and/or to ascertain the usefulness of histological methods described below (Table 1). Water chemistry and physical data were taken following the tests and methods described by Moreno (1978). Water samples were analyzed in

Figure 1

LITTLE CALUMET RIVER SHOWING LOCATION OF THE STUDY AREA

SAMPLES COLLECTED MONTHLY SEPTEMBER,1977 - AUGUST,1978

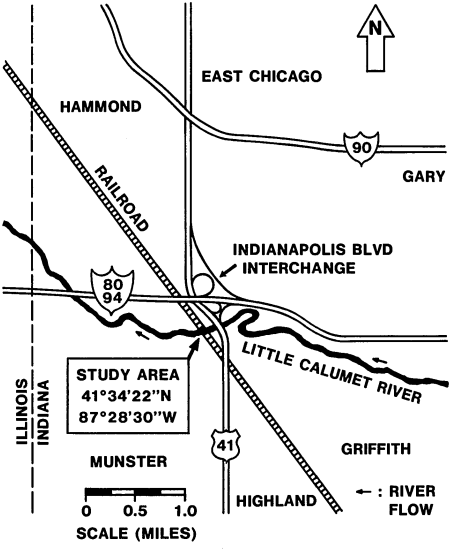


Figure 2

MAP OF THE LITTLE CALUMET RIVER AND SURROUNDING WATER SYSTEMS

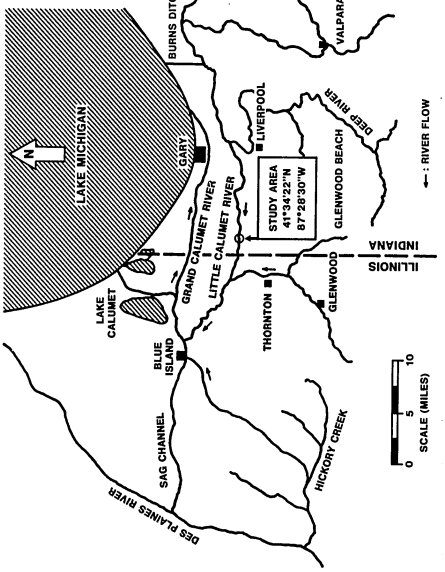


Table 1

DATES USED FOR FIELD COLLECTIONS OF Limnodrilus hoffmeisteri
FROM THE LITTLE CALUMET RIVER - HAMMOND, INDIANA
(41° 34′ 22″ N, 87° 28′ 30″ W)

1976						1977						1978					
	NO'S OF WORMS PROCESSED FOR						NO'S OF WORMS PROCESSED FOR						NO'S OF WORMS PROCESSED FOR				
DATE	SS	LM	TEM	SEM	COC	DATE	SS	LM	TEM	SEM	COC	DATE	SS	LM	TEM	SEM	COC
JUNE	0	72	52	0	0	MARCH	0	0	0	5	0	JANUARY 14	20	20	20	0	0
NOVEMBER	0	73	75	0	0	SEPTEMBER 24	20	20	20	0	0	FEBRUARY 11	20	20	20	0	0
						OCTOBER 22	20	20	20	0	0	MARCH 11	20	20	20	0	0
						NOVEMBER 19	20	20	20	0	0	APRIL 14	20	20	24	5	30
						DECEMBER 10	20	20	20	0	0	MAY 12	20	20	20	10	0
												JUNE 9	20	20	20	4	0
												JULY 7	20	20	20	0	0
												AUGUST 4	20	20	20	14	0
												OCTOBER 21	0	0	0	9	0

SS: SPERM SMEARS
LM: LIGHT MICROSCOPY
TEM: TRANSMISSION ELECTRON MICROSCOPY
SEM: SCANNING ELECTRON MICROSCOPY
COC: COCOONS WITH DEVELOPING EGGS AND/OR EMBRYOS

the laboratory using the Hach colorimeter kit. When a given test, e.g., oxygen, was subject to deterioration leading to a false result, the test was conducted in the field. Benthic samples were taken using a flat rectangular shovel with a blade measuring 18 cm by 40 cm. Normally the first 5 cm of the benthos was sampled. A single sample was approximately 3600 cm^3. Samples were placed into sealable plastic containers with sufficient moisture so that they would not dry out for the trip back to the laboratory located on the campus of Western Michigan University. All samples were processed within the laboratory since field processing was difficult during the winter months because samples would quickly freeze thus destroying them.

Benthic samples were placed in Standard No. 30 sieves and washed with water. Exposed worms were collected and placed into sediment free bowls of water. Individual worms were identified by observing them with a dissecting microscope for gross features including bifid setae, 5-6 setae per bundle, shape of the prostomium, size, clitellum swelling. These gross features were sufficient to identify *Limnodrilus hoffmeisteri*. Mature *L. hoffmeisteri* were not always observed. When mature worms were not found, the most mature looking *L. hoffmeisteri* were collected and processed. The gross characteristics noted of these worms were size, 2-4 cm long, constricted state, and cuticle diameter 0.5 to 1.0 mm. Confirmations of identifications were made later using the histological procedures described below.

Histological Procedures

Sixty *L. hoffmeisteri* were processed for light microscopy and transmission electron microscopy after each sampling. Twenty worms were used for sperm smears. This procedure has been described by Block, 1973; Block and Goodnight, 1976. All sperm smears were stained with Wrights stain and counterstained with Giemsa (Block, 1972). Ten worms were fixed in Neutral Buffered Formalin (Table 2). Ten worms were fixed in 5% Bouins Picro-Formol fixative (Table 2). The Neutral Formalin fixed, and the Bouin fixed, worms were embedded in paraffin and sectioned serially (5μm thickness). The slides containing the sectioned worms were then stained with Hematoxylin/Eosin and coverslipped using Permount mounting fluid.

Worms used for transmission electron microscopy were fixed by immersion in 2% Glutaraldehyde (Table 2) and post fixed in 1% Osmium Tetroxide (Table 2). The worms then were dehydrated in a graded alcohol series and embedded in Epon 812. Sections were made using a Porter Blum MT2-B ultramicrotome. 1 μm thick sections were made using glass knives. These sections were placed on glass slides and stained with Toluidine Blue O (CI 52040) (Table 3), then viewed with a light microscope in order to select the desired area for thin sectioning. Thin sections were made using a diamond knife. Thin sections, 800 to 1000 Å thick were placed on 200 mesh copper grids and stained with Uranyl Acetate and Lead Citrate (Table 4). Sections were viewed using a Philip's 201 electron microscope.

Table 2

NEUTRAL BUFFERED FORMALIN FIXATIVE

DIBASIC ANHYDROUS SODIUM PHOSPHATE (Na_2HPO_4)	6.5 gm
MONOBASIC POTASSIUM PHOSPHATE (KH_2PO_4)	4.0 gm
FORMALDEHYDE (36-40% HCHO BY ASSAY)	100.0 ml
DISTILLED WATER	900.0 ml

– MIX THOROUGHLY –

5% BOUINS PICRO-FORMOL FIXATIVE

SATURATED AQUEOUS PICRIC ACID	750.0 ml
FORMALDEHYDE (36-40% HCHO BY ASSAY)	250.0 ml

ADD 5% GLACIAL ACETIC ACID FIXATIVE JUST PRIOR TO USE

2% GLUTARALDEHYDE

NOTE: USE GLASS BOTTLE WITH GLASS STOPPER TO FIT. GLASSWARE MUST BE VERY CLEAN.

MILLONIG'S BUFFER (SINGLE STRENGTH)	68 ml
70% GLUTARALDEHYDE	2 ml

pH WILL BE 7.4. KEEP REFRIGERATED.

1% OSMIUM TETROXIDE

DISTILLED WATER	50 ml
MILLONIG'S BUFFER (DOUBLE STRENGTH)	50 ml
OSMIUM TETROXIDE (OsO_4)	1 gm

MIX WELL. STORE IN REFRIGERATOR.

Table 3

TOLUIDINE STAIN FOR EPOXY RESINS

STOCK SOLUTIONS: A. 1% TOLUIDINE BLUE 0 (CI 52040)

TOLUIDINE BLUE 0 (CI 52040)	1 gm
DISTILLED WATER	100 ml

STIR UNTIL DISSOLVED; FILTER

B. 1% SODIUM BORATE

SODIUM BORATE ($Na_2B_4O_7 \cdot 10 \cdot H_2O$)	1 gm
DISTILLED WATER	100 ml

WORKING SOLUTION: MIX 1 PART (A) WITH 9 PARTS (B)

Table 4

2% URANYL ACETATE

URANYL ACETATE	1 gm
DISTILLED WATER	50 ml

MIX THOROUGHLY UNTIL DISSOLVED.

LEAD CITRATE

LEAD NITRATE [$Pb(NO_3)_2$]	1.33 gm
SODIUM BORATE [$Na_3(C_6H_5O_7) \cdot 2H_2O$]	1.76 gm
DISTILLED WATER	30.00 ml

SHAKE FOR SEVERAL HOURS. ADD 8ml 1N SODIUM HYDROXIDE AND DILUTE TO 50ml WITH DISTILLED WATER. THE SOLUTION IS READY FOR USE AFTER IT IS COMPLETELY CLEAR. pH WILL BE 12.0.

Several *L. hoffmeisteri* were processed for scanning electron microscopy. Whole worms were placed in 2% glutaraldehyde for a minimum of 24 hours. The worms were then dehydrated in graded alcohols and graded amyl acetate prior to critical point drying. Whole worms were critical point dried with carbon dioxide using a Pelco Model "H" critical point dryer. The dried samples were then mounted on metal stubs and placed in a Denton DV-502 vacuum evaporator for sputter coating of the sample with gold. Subsequent sample examinations were completed using the Cambridge Stereoscan 150 scanning electron microscope.

Sperm smears were prepared for scanning electron microscopy following the same procedures outlined above with one exception. Smears were collected in a drop of Millonig's Buffer (Table 5) on a 12 mm diameter cover slip. The contents on the coverslip were allowed to set from one to three minutes prior to immersion in 2% glutaraldehyde.

Transmission electron microscope photographs were taken using Kodak #4463 electron image film. The film was processed using standard procedures. Photographic prints were made using Kodak Kodabromide F2 through F5 paper. Magnifications were determined by use of a Fullam #1002 carbon grating (.463 μm/line). The negatives were exposed periodically to the Fullam #1002 grating grid at selected magnification steps on the electron microscope in order to assure proper recording of magnification. Scanning electron microscope photographs were taken using Polaroid 4X5 Land Film Type 55/Positive-Negative film.

Table 5

MILLONIG'S BUFFER (DOUBLE STRENGTH)

MONOBASIC SODIUM PHOSPHATE ($NaH_2PO_4 \cdot H_2O$)	13.504 gm
DEXTROSE (D-GLUCOSE)	4.320 gm
2N SODIUM HYDROXIDE	38.552 ml
DISTILLED WATER	400.000 ml

WORKING SOLUTION: MIX 1 PART DOUBLE STRENGTH MILLONIGS BUFFER TO 1 PART DISTILLED WATER

STORE IN REFRIGERATOR. SOMETIMES A PRECIPITATE WILL FORM. PLACE BOTTLE IN WARM BATH AND REDISSOLVE PRECIPITATE.

Sperm Smear Count Procedures

Sperm smears were used, in part, to assess the numbers of the two types of sperm observed within individual worms. Additional observations were made of the developmental stages of the two sperm types from one collection to the next. These observations helped establish the spermatogenesis cycle throughout the year. Type I and Type II morula were counted (see Results section, p.). These morula structures were counted as follows. Individual slides were placed under a 10X wide field objective lens. Three fields were selected that contained sperm products. Counts were made from these observations and the following classification used:

Average Count per 3 Fields	Classification	Letter Code
1 through 10	Scarce	S
11 through 20	Few	F
21 through 30	Many	M
31 and above	Numerous	N

In addition this classification was used to determine the approximate numbers of mitotic (or meiotic) processes, maturation of the egg sac represented by the appearance of the nutritive granules, and appearance of non-differentiated sperm morula. The stages of spermatogenesis include germ tissue and morula stages that cannot be morphologically distinguished between Type I and Type II sperm. Therefore, the term non-differentiated sperm is used. This

information was collected and placed in tabular form (see Results section, p. 38).

Measurements of Cell, Cell Organelles

Size determinations were made using a TALOS general software program integrated with a Hewlett-Packard HP 9815A calculator. The procedure allows for computing area, distances, point to point contact by tracing a given cell, and cell organelle with a digitizer. All measurements were converted and recorded to actual cell or cell organelle size.

RESULTS

General Statements and Comments Observed During Collection

The major effort in examining the life history of *L. hoffmeisteri* as well as in examining possible environmental factors that influence the worm's life history was completed from September 1977 through August 1978. The study site (Figure 1, p. 16) undergoes seasonal changes from the bitter cold of winter to the hot humid days of summer. This study area, located in northwest Indiana, is part of a complex drainage system that occurs naturally and/or has been dredged for commercial reasons (Figure 2, p. 17). The surrounding areas are densely populated. Commercial businesses, light to heavy industry, and residential areas with associated services all combine to have an effect on the study site. Pollution, defined and used in the broadest sense, has left its mark on the area while the population of tubificid worms has thrived. Even a casual glance indicates that the study site is a recipient of pollution from several sources such as storm sewers that open into the stream; garbage of all imaginable sorts, at one time or another, can be observed; industrial wastes including polymerized plastics and sundry manmade objects including prophylactics, tires, tire rims, shopping carts, and disposable bottles-cans. These pollutants were always present. At times during the monthly collections the smell of human sewage permeated the area. Natural debris such as fallen

trees, probably brought down by area residents as evidenced by hatchet marks; branches; leaves; and plant materials were observed decomposing in or near the stream. Decomposing animals such as muskrats and other rodents were observed.

The melting of ice and snow brought seasonal flooding during the spring. Also, heavy rains preceded the May sample data. The river was 1 m to 2 m above its normal depths but had reversed the normal westerly flow (Figure 2, p. 17) to a swift easterly flow. During the summer and winter months the water levels remained fairly uniform. It should be noted that during the coldest months—December, January, February and March—ice often 7 cm to 10 cm thick would cover all but the central portions of the stream where the current was swiftest. In January and February, a thin coating of ice 0.5 cm to 1.0 cm thick, was observed across the entire stream. It was suspected that this thin coating was not permanent and would melt on sunny days and reappear during the cold of night.

The Study Site — Benthos Composition

The benthos consisted of sand with variable sizes of pebbles, stones, clay and silt. A major component of the benthos was a thick, black mud ooze in which the tubificid worms could easily be found. This mud ranged from 6 cm to approximately 24 cm in depth and probably contained enriched decomposing organic matter as well as a microflora that would aid in the decomposition process. The thickness varied due probably to the changing currents of the stream from one side of the stream to the other.

The Study Site — Water Chemistry

The chemical parameters that were measured during the September 1977 through the August 1978 collections are presented in Tables 6 through 9. These measurements were taken in collaboration with G. Moreno (1978). The dissolved oxygen ranged from <1 mg/l to 6.2 mg/l throughout the course of the study (Table 6). The lowest oxygen levels were recorded during the winter months of January, February and March with readings of <1 mg/l, <1 mg/l and 1 mg/l respectively. Water temperature varied from 0°C to 28°C (Table 6). As expected, the coldest temperatures were recorded from December through March with a range of 0°C to 2°C.

The study area was observed to be alkaline with the pH range varying from 7.4 to 8.2 (Table 6). Likewise, the values for alkalinity (Table 7) and carbon dioxide (Table 6) varied in accord with the changes in pH. The total hardness of the water was high throughout the study ranging from 271.6 mg/l to 325.0 mg/l (Table 7). As seen in Table 6 the values for oxygen varied inversely with the values for carbon dioxide.

Nutrient sources such as nitrogen in the form of nitrites and nitrates, as well as phosphorous in the form of meta- or ortho-phosphate, varied in concentration throughout the year (Table 7). The amount of chloride in solution remained high throughout the course of the investigation ranging from 85 mg/l to 95 mg/l (Table 8). There appeared to be no major fluctuations in chloride concentration due to seasonal variation. The concentration of chlorine

Table 6

CARBON DIOXIDE, pH, DISSOLVED OXYGEN, AND WATER TEMPERATURE MEASUREMENTS TAKEN FROM THE LITTLE CALUMET RIVER – HAMMOND, INDIANA (41° 34' 22" N, 87° 28' 30" W) SEPTEMBER, 1977 THROUGH AUGUST, 1978

	DATE	CARBON DIOXIDE (mg/l)	pH	DISSOLVED OXYGEN (mg/l)	WATER TEMPERATURE (C°)
1977	SEPTEMBER 24	62.3	7.9	5.2	22
	OCTOBER 22	78.3	7.5	3.4	12
	NOVEMBER 19	65.7	7.5	3.0	6
	DECEMBER 10	80.4	7.4	1.8	2
1978	JANUARY 14	90.5	7.3	<1.0	1
	FEBRUARY 11	90.0	7.4	<1.0	0
	MARCH 11	88.3	7.7	1.0	2
	APRIL 14	65.5	8.1	3.4	10
	MAY 12	45.5	7.7	6.2	17
	JUNE 9	46.6	7.7	6.0	23
	JULY 7	45.5	8.0	5.0	28
	AUGUST 4	40.0	8.2	6.0	N.C.

N.C.: NOT COMPLETED

Table 7

ALKALINITY, TOTAL HARDNESS, NITROGEN (NITRITE, NITRATE), AND PHOSPHATE (ORTHO-, META-) LEVELS MEASURED FROM THE LITTLE CALUMET RIVER – HAMMOND, INDIANA (41° 34' 22" N, 87° 28' 30" W) SEPTEMBER, 1977 THROUGH AUGUST, 1978

	DATE	ALKALINITY (mg/l)	TOTAL HARDNESS (mg/l)	NITROGEN NITRITE (mg/l)	NITROGEN NITRATE (mg/l)	PHOSPHATE ORTHO (mg/l)	PHOSPHATE META (mg/l)
1977	SEPTEMBER 24	320.5	323.3	.01	2.0	3.0	1.5
	OCTOBER 22	270.3	321.6	.02	2.1	3.4	1.3
	NOVEMBER 19	262.0	325.0	.01	1.8	3.2	2.8
	DECEMBER 10	196.0	280.0	.02	2.3	3.4	2.9
1978	JANUARY 14	200.0	320.0	.04	2.0	2.9	2.9
	FEBRUARY 11	193.3	321.6	.03	2.2	3.4	2.0
	MARCH 11	194.0	281.6	.04	1.7	2.9	1.6
	APRIL 14	174.0	273.0	.02	1.9	2.9	2.0
	MAY 12	230.5	271.6	.03	1.5	2.4	1.8
	JUNE 9	270.0	280.0	.04	1.0	2.3	1.3
	JULY 7	272.0	299.5	.04	1.1	2.6	1.8
	AUGUST 4	279.3	288.3	.03	0.8	2.3	1.3

Table 8

CHLORIDE, CHLORINE, SILICA, SULPHATE AND HYDROGEN SULPHIDE LEVELS MEASURED FROM THE LITTLE CALUMET RIVER – HAMMOND, INDIANA (41° 34' 22" N, 87° 28' 30" W) SEPTEMBER, 1977 THROUGH AUGUST, 1978

	DATE	CHLORIDE (mg/l)	CHLORINE (mg/l)	SILICA (mg/l)	SULPHATE (mg/l)	HYDROGEN SULFIDE (mg/l)
1977	SEPTEMBER 24	90.3	.01	35	140.0	.1
	OCTOBER 22	87.3	.02	33	140.5	.1
	NOVEMBER 19	85.0	.01	30	125.0	.1
	DECEMBER 10	88.3	.02	25	140.0	.1
1978	JANUARY 14	89.0	.02	28	120.4	.1
	FEBRUARY 11	95.0	.01	29	130.3	.1
	MARCH 11	87.0	.02	35	140.5	.1
	APRIL 14	94.0	.02	34	130.5	.1
	MAY 12	95.0	.02	38	130.0	.1
	JUNE 9	89.3	.02	29	140.8	.1
	JULY 7	95.0	.02	31	150.3	.1
	AUGUST 4	90.5	.02	29	150.0	.1

was not a consequential factor as the levels were low ranging from .01 mg/l to .02 mg/l (Table 8). Likewise, hydrogen sulfide levels were not in sufficient concentrations to merit consideration.

Silica and sulphate concentrations were relatively high but fairly uniform throughout the investigation (Table 8). Silica ranged from 25 mg/l to 38 mg/l from September through July. Sulphates ranged in concentration from 120.4 mg/l to 150.3 mg/l (Table 8). The low and high sulphate values were in January and July respectively. However, variations in levels before and after these collection dates failed to establish a noticeable seasonal pattern.

The concentration of the metals chromium, copper, iron and manganese were all low throughout the course of the investigation (Table 9). Since these levels were not unusual, it was assumed that these metals had little, if any, effect on the life cycle of *L. hoffmeisteri*.

The Study Site — Micro Flora

An extensive analysis of the micro flora was not attempted. Fecal coliform, total coliform and yeast/mold were the three parameters chosen for study. These should indicate the diversity and abundance of the overall micro flora population. The results of this analysis are listed in Table 10. The data in Table 10 were completed in conjunction with G. Moreno (1978). The data suggest that there is a seasonal fluctuation in numbers of fecal coliform, total coliform and yeast/mold between the winter months of December,

Table 9

CHROMIUM, COPPER, IRON, AND MANGANESE LEVELS MEASURED FROM THE LITTLE CALUMET RIVER – HAMMOND, INDIANA.
(41° 34' 22" N, 87° 28' 30" W)
SEPTEMBER, 1977 THROUGH AUGUST, 1978

DATE		CHROMIUM (mg/l)	COPPER (mg/l)	IRON (mg/l)	MANGANESE (mg/l)
1977	SEPTEMBER 24	.02	.01	.5	.02
	OCTOBER 22	.02	.01	.3	.02
	NOVEMBER 19	.03	.02	.5	.02
	DECEMBER 10	.02	.01	.4	.02
1978	JANUARY 14	.02	.02	.5	.01
	FEBRUARY 11	.01	.02	.3	.01
	MARCH 11	.02	.01	.5	.01
	APRIL 14	.02	.02	.4	.02
	MAY 12	.01	.01	.4	.02
	JUNE 9	.01	.01	.3	.01
	JULY 7	.01	.01	.3	.02
	AUGUST 4	.01	.01	.3	.01

Table 10

MEAN NUMBERS OF FECAL COLIFORM, TOTAL COLIFORM, AND YEAST/MOLD
FROM THE LITTLE CALUMET RIVER – HAMMOND, INDIANA
(41° 34' 22" N, 87° 28' 30" W)
SEPTEMBER, 1977 THROUGH AUGUST, 1978

	DATE	FECAL COLIFORM (COLONIES/100 ml) ± S.D.	TOTAL COLIFORM (COLONIES/100 ml) ± S.D.	YEAST/MOLD (COLONIES/100 ml) ± S.D.
1977	SEPTEMBER 24	100,000 ± 7,071	174,000 ± 2,450	88,000 ± 3,742
	OCTOBER 22	126,000 ± 2,550	162,000 ± 1,871	91,000 ± 707
	NOVEMBER 19	133,000 ± 7,071	194,000 ± 1,225	81,000 ± 1,633
	DECEMBER 10	72,000 ± 816	146,000 ± 707	73,000 ± 707
1978	JANUARY 14	64,000 ± 1,225	141,000 ± 1,225	73,000 ± 707
	FEBRUARY 11	56,000 ± 707	127,000 ± 1,225	61,000 ± 707
	MARCH 11	92,000 ± 707	175,000 ± 1,225	87,000 ± 707
	APRIL 14	110,000 ± 7,071	183,000 ± 707	93,000 ± 1,225
	MAY 12	115,000 ± 3,536	197,000 ± 707	92,000 ± 707
	JUNE 9	139,000 ± 816	194,000 ± 1,225	96,000 ± 1,225
	JULY 7	124,000 ± 1,225	192,000 ± 1,414	96,000 ± 1,871
	AUGUST 4	121,000 ± 1,871	185,000 ± 708	96,000 ± 707

S.D.: STANDARD DEVIATION

January, February and March and the summer months of June, July, August and September. The highest levels of all three microorganisms were observed during the summer months with the single exception of total coliform recorded for late spring (May collection), whereas the lowest numbers of these microorganisms were recorded during the winter months (Table 10). The range for the fecal coliform was 56,000 ± 707 colonies/100 ml for February to 139,000 ± 816 colonies/100 ml for June. Total coliform ranged from 127,000 ± 1,225 colonies/100 ml for February to 197,000 ± 707 colonies/100 ml for May. The numbers of yeast/mold ranged from 61,000 ± 707 colonies/100 ml for February to 96,000 ± 1,871 colonies/100 ml for the June, July and August collections.

Sampling Methods/Processing Methodology for *L. hoffmeisteri*

Sampling dates, tissue processing procedures, and numbers of *L. hoffmeisteri* used are listed in Table 1 (p. 18). Minimal difficulty was encountered using the sampling procedures listed in the Materials and Methods section. The procedures described in the same section for processing of sperm smears and whole organisms for light and electron microscopy were satisfactory. A few notes are necessary in order to understand better the interpretations that are described later concerning the events of spermatogenesis as well as life history of *L. hoffmeisteri*.

Two general procedures were used for light microscopy. They were 1) whole worm fixation, and 2) sperm smear fixation. The whole worm fixation procedure by immersion using Bouin's fixative or neutral formalin fixative was found to be satisfactory for studying whole organ development, such as the male reproductive structures. Observations of serially sectioned worms suggested that the sperm products within the sperm sac were subjected to shrinkage and compacting which made it difficult if not impossible to view and sort out the different stages of sperm development.

The use of these fixatives is not satisfactory for the study of the different stages of spermatogenesis. The sperm smear technique minimized this distortion and allowed for excellent viewing of the sperm stages. Staining procedures for serially sectioned worms and sperm smears were satisfactory.

The procedures used for electron microscopy were satisfactory. Organs, tissues, cells and cell organelles were identified easily.

Study Site — Population of Tubificidae

Two members of the family Tubificidae were observed throughout the study. They were *Limnodrilus hoffmeisteri* and *Tubifex tubifex*. A third member, *Limnodrilus profundicola*, was seen in the late fall collections but this identification was dismissed as a developmental stage of the penis sheath for *L. hoffmeisteri*. This phenomenon will be dealt with in the discussion.

Summarized data listing the numbers of worms and percent of *L. hoffmeisteri* to *T. tubifex* are listed in Table 11. The data in Table 11 were completed in collaboration with G. Moreno (1978). The average numbers of worms ranged from 503 per cubic meter in February to 2167 per cubic meter in April (Table 11). The lowest numbers of worms occurred during the January and February collections. As seen in Table 6 (p. 30), oxygen levels and water temperatures were at their lowest recorded levels during those months. Notes recorded at the time of sampling included the observations that worms appeared to be very mature, but in general appeared to be dying--posterior end necrotic and anterior end with well developed clittellum. Additional notes suggested that many worms had little or no benthic material passing through their alimentary canal.

The larger percentage of worms was identified as *L. hoffmeisteri*. *L. hoffmeisteri* represented 79% to 95% of the tubificid population with the remaining worms identified as *T. tubifex* (Table 11). On the average, *L. hoffmeisteri* represented 87% and *T. tubifex* the remaining 13%.

Observations of Type I and Type II Sperm Morula

An attempt was made to count the numbers of Type I and Type II sperm from individual worms through the course of the study. Since exact counts of sperm and sperm stages were not possible, a method was used that separated the two sperm types and approximate numbers of the two sperm types were determined. This procedure described

Table 11

AVERAGE NUMBERS OF TUBIFICIDAE, PERCENT OF *Limnodrilus hoffmeisteri* AND PERCENT OF OTHER TUBIFICIDAE OBSERVED FROM THE LITTLE CALUMET RIVER – HAMMOND, INDIANA (41° 34′ 22″ N, 87° 28′ 30″ W) SEPTEMBER, 1977 THROUGH AUGUST, 1978

	DATE	AVERAGE NO.'S TUBIFICIDAE/M^3	PERCENT *L. hoffmeisteri*	PERCENT *Tubifex tubifex*	PERCENT OTHER TUBIFICIDAE
1977	SEPTEMBER 24	1460	88	12	0
	OCTOBER 22	1673	89	11	0
	NOVEMBER 19	1420	91	9	0
	DECEMBER 10	1593	90	10	0
1978	JANUARY 14	863	95	5	0
	FEBRUARY 11	503	84	16	0
	MARCH 11	1247	86	14	0
	APRIL 14	2167	83	17	0
	MAY 12	1823	88	12	0
	JUNE 9	1663	84	16	0
	JULY 7	1680	83	17	0
	AUGUST 4	1637	79	21	0

previously worked effectively. The data collected from this procedure have been compiled in Table 12.

The data in Table 12 suggest that there is a seasonal pattern in the development of the sperm products for *L. hoffmeisteri*. This activity is marked by an increase in the Type I and Type II sperm morula during the fall months. This developmental activity peaks between January and April. The late spring and summer months were marked by decreased numbers of the two sperm.

Type I and Type II sperm morula are shown in Figure 7 (p. 57). Several morula stages containing the developing sperm are grouped for the counting and categorization shown in Table 12. The very early stages of morula development including 4, 8, 16, 32 cell stages have for the most part been excluded in order to make sure that proper recognition of the two sperm types were made. Therefore, the stages depicted in Figure 7 (p. 57) were used in the actual counts. All counts were made using the sperm smear technique.

In the fall months of October and November there was a dramatic increase in the numbers of Type I and Type II sperm morulae. Forty to 55% of the worms were classified as having numerous, or many Type I and Type II, morulae. Witness that only 15% of the worms observed in September had Type I and Type II sperm morulae whereas almost 75% of the worms were not observed to have the Type I and Type II sperm. Less than 35% of the worms observed were without the two types of sperm in the October or November collections.

Table 12

OBSERVATIONS OF TYPE I / TYPE II SPERM MORULA, NUTRITIVE GRANULES, AND NON-DIFFERENTIATED SPERM PRODUCTS FROM Limnodrilus hoffmeisteri TAKEN FROM THE LITTLE CALUMET RIVER – HAMMOND, INDIANA SEPTEMBER, 1977 through AUGUST, 1978

SAMPLE DATE	MORULA STRUCTURE										NUTRITIVE GRANULES		NON-DIFFERENTIATED SPERM				
	TYPE I					TYPE II											
	N	M	F	S	NO	N	M	F	S	NO	N	NO	N	M	F	S	N/T
SEPTEMBER 18, 1977	2	1	1	2	14	2	0	1	2	15	1	19	1	2	1	7	11/20
OCTOBER 22, 1977	8	0	4	4	4	8	0	4	3	5	1	19	3	4	2	1	10/20
NOVEMBER 19, 1977	10	1	2	0	7	10	1	3	1	5	0	20		1	3	1	4/20
DECEMBER 10, 1977	2	1	2	8	7	5	3	5	4	3	7	13	7	6	2	2	17/20
JANUARY 14, 1978	11	5	2	0	2	12	4	2	0	2	8	12	3		2		5/20
FEBRUARY 11, 1978	13	3	1	0	3	14	2	1	1	2	4	16				1	1/20
MARCH 11, 1978	10	2	2	3	3	11	2	2	3	2	5	15	2	2			4/20
APRIL 8, 1978	16	0	2	2	0	16	0	1	3	0	13	7		1			1/20
MAY 12, 1978	3	1	2	9	5	4	1	3	7	5	14	6			1	2	3/20
JUNE 9, 1978	0	0	2	1	17	0	0	2	0	18	4	16			2	6	8/20
JULY 7, 1978	2	3	1	2	12	2	3	0	2	13	0	20		1	5	2	8/20
AUGUST 4, 1978	1	1	2	2	14	1	1	1	2	15	4	20		1	1	4	6/20

N = NUMEROUS, M = MANY, F = FEW, S = SCARCE, NO = NONE, N/T = NUMBER OF WORMS OBSERVED PER TOTAL WORM SAMPLE. DATA BASED ON 20 WORM SPERM SMEARS MADE PER SAMPLE DATE.

The December counts were somewhat puzzling as there was a dramatic decrease in the numbers of Type I and Type II sperm morula. However, even though there were decreased numbers of the two sperm types there were, as found in previous collections, less than 35% of the worms that did not have the sperm products. Overall for this month there were only 15% of the worms that did not have non-differentiated sperm (Table 12). Consequently, the evidence would suggest that the worms were active in developing sperm products at this time of year.

From January through April approximately 80% of the worms had many Type I and Type II sperm. These months were representative of the peak numbers of sperm morula counted.

The worms from the remaining monthly collections were found to have greatly reduced numbers of the two sperm morula. Approximately 60 to 90% of the worms observed in these collections did not evidence Type I or Type II sperm morula. Less than 40% of the worms were observed to have non-differentiated sperm products.

This information suggests that *L. hoffmeisteri* had a single peak activity in the development of sperm that occurs from January through April. The data suggest that the process begins in the early part of the fall and continues through to spring. The data also suggest that the summer months do not appear to have an influence on the development of the sperm since low numbers of sperm morula were observed. It should also be noted that even though

there is a peak productivity period for sperm development, the production of sperm occurred at reduced levels throughout the year.

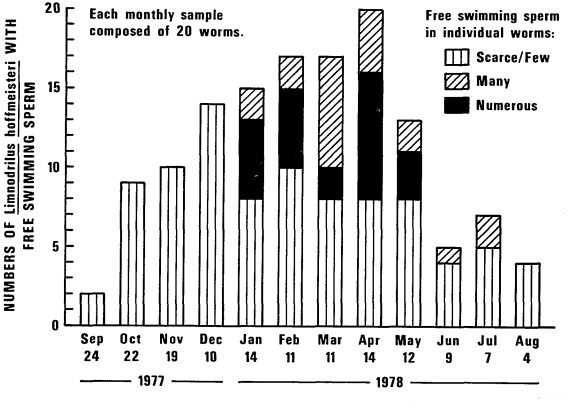
Since the sperm morula counts did not consider the numbers of mature free swimming sperm (mature Type I and Type II sperm unattached to a morula structure), it was decided to gather this information from the same sperm smears used for making the sperm morula counts. This information is presented in Figure 3. The numbers of mature sperm followed the same pattern as seen in Table 12. Low levels of mature sperm were found in June, July, August and September. Increased numbers of worms with mature sperm were observed during the fall months of October, November and December. Increased numbers of mature sperm as well as increased numbers of worms with matured sperm were seen in the peak months of January through April. A comparison of the May observations of the numbers of sperm morula with those of the previous four months reveals interesting information (Table 12). This comparison suggests that the breeding activity for the worms may subside around this period of time since the numbers of both sperm morula types are very low and the remaining numbers of mature sperm are lower than the previous four months. Since immature sperm are not so abundant as in the form of the Type I and Type II morula it may suggest that the reproductive period has subsided.

Another source of evidence in support of a single reproductive period is in observing the development of the egg sac and the formation of the nutritive granules within the egg sac. Again, similar

Figure 3

OCCURRENCE OF FREE SWIMMING SPERM (UNATTACHED TO MORULA STRUCTURE) FROM Limnodrilus hoffmeisteri AS OBSERVED FROM MONTHLY COLLECTIONS TAKEN FROM THE LITTLE CALUMET RIVER - HAMMOND, INDIANA.

September, 1977 through August, 1978.



counts were made in order to make comparative analyses. This information is summarized in Table 12 and Figure 4. Reduced numbers of worms containing nutritive granules were observed throughout the year except during April and May when 65% or greater numbers of worms were observed to have numerous nutritive granules. The preceding months of December, January, February and March had higher numbers of worms with nutritive granules as compared with those of the remaining months of the year. Again, nutritive granules were observed throughout the duration of the study.

Cell division or nuclear division represented by mitosis or meiosis was considered important in trying to identify the reproductive activity of *L. hoffmeisteri*. Cell division counts of the two types of morulae as well as undifferentiated sperm from individual worms were made following the previously described methods. These results are listed in Table 13. Low numbers of cell divisions were recorded in September, June, July and August. Forty-five to 60% of the worms observed did not appear to have sperm morulae or undifferentiated sperm undergoing cell division. This contrasts with the results for the months of October through May during which 5 to 15% of the worms observed did not have morulae or undifferentiated sperm actively dividing.

Cell division activity was observed in greater numbers of worms during the October sample in which 40% of the worms had many to numerous (see p. 25 for definition of terms) numbers of cells undergoing cell or nuclear division. Forty-five percent of the

Figure 4

OCCURRENCE OF NUTRITIVE GRANULES FROM Limnodrilus hoffmeisteri AS OBSERVED FROM MONTHLY COLLECTIONS TAKEN FROM THE LITTLE CALUMET RIVER - HAMMOND, INDIANA.

September, 1977 through August, 1978.

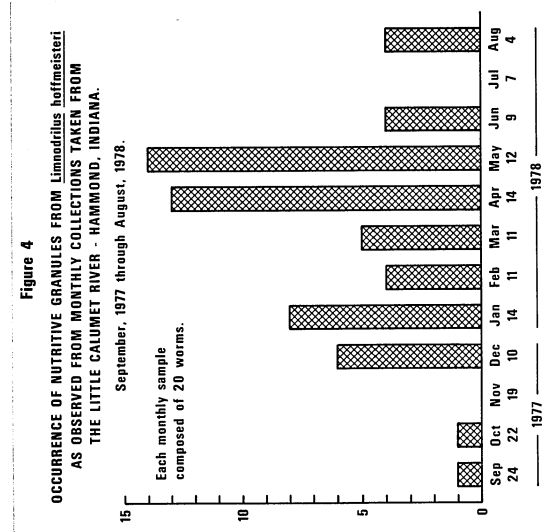


Table 13

MITOTIC (MEIOTIC) ACTIVITY OBSERVED IN SPERM MORULA FROM Limnodrilus hoffmeisteri TAKEN FROM THE LITTLE CALUMET RIVER – HAMMOND, INDIANA SEPTEMBER, 1977 THROUGH AUGUST, 1978

SAMPLE	MITOTIC (MEIOTIC) SPERM MORULA*					COMMENT(S)
DATE	N	M	F	S	NO	
SEPT. 24, 1977	3	2	2	1	12	FREE SWIMMING SPERM 2/20+
OCT. 22, 1977	1	7	7	2	3	FREE SWIMMING SPERM 9/20
NOV. 19, 1977	6	7	4	1	2	FREE SWIMMING SPERM 10/20
DEC. 10, 1977[A]	3	5	10	2	0	FREE SWIMMING SPERM 14/20
JAN. 14, 1978[A]	1	2	9	6	2	FREE SWIMMING SPERM 15/20
FEB. 11, 1978[A]	3	6	8	0	3	FREE SWIMMING SPERM 17/20
MAR. 11, 1978	5	4	8	0	2	FREE SWIMMING SPERM 17/20
APR. 14, 1978	0	9	8	2	1	FREE SWIMMING SPERM 20/20
MAY 12, 1978	0	6	3	5	6	FREE SWIMMING SPERM 13/20
JUNE 9, 1978	0	6	1	1	12	FREE SWIMMING SPERM 3/20
JULY 7, 1978	1	2	7	1	9	FREE SWIMMING SPERM 7/20
AUG. 4, 1978	3	3	2	2	10	FREE SWIMMING SPERM 4/20

* BASED ON 20 WORM SPERM SMEARS MADE PER SAMPLE DATE
N = NUMEROUS, M = MANY, F = FEW, S = SCARCE, NO = NONE
+ NUMBER OF INDIVIDUAL WORMS/20 WORMS
[A] COLDEST MONTHS OF THE SAMPLING YEAR.

worms were characterized as having scarce to few numbers of cell divisions. In November the number of meiotic or mitotic divisions observed in the many (see p. 25 for definition of terms) category increased to 65% of the worms observed. Twenty percent of the worms were categorized as having scarce to few numbers of dividing cells. The December and January collections were marked by decreasing recorded levels of oxygen as well as water temperature (Table 6, p. 30) and were found to have a decrease in the mitotic and meiotic activity. Forty percent of the observed worms in the December collections were classified in the many to numerous group whereas in January only 15% of the observed worms were classified in the many to numerous group. Sixty percent of the worms in the December collections had scarce to few numbers of dividing cells. This increased to 75% of the worms having scarce to few numbers of dividing cells in the January collections. It should be noted that the numbers of undifferentiated sperm were high for the December collections (Table 12) followed by low numbers of undifferentiated sperm from January through April. Worms from the January through April collections had for the most part recognizable Type I and Type II morulae structures (Table 12).

In February, cell division activity increased with 45% of the worms classified in the many to numerous groupings. This level of activity was maintained for the March and April collections. However, the increase was not accompanied with increases in oxygen levels or water temperature for February or March (Table 12).

The data suggest that water temperature and oxygen levels may have an effect on the development of undifferentiated sperm cells, Type I morulae, and Type II morulae, by retarding cell division activity. However, this effect appears to be limited to the primary germ cells and the Type I and Type II morula structures since the maturation of mature sperm as measured by the appearance of free swimming sperm continued to increase throughout this same period of time (Table 13, Figure 3). At the same time the effects of lower oxygen levels and low water temperature on the ability of germ cells and morula structures to divide are not clear since it was shown that these structures can continue to divide initially in low numbers and in increasing numbers as in this situation two months after a reduction in cell division.

Additional information of these developmental patterns can be illustrated with the appearance of nutritive granules (Table 12 and Figure 4). An increase in nutritive granules is recorded for the months of December and January whereas a decrease in numbers of these granules is seen in February and March. It is possible to suggest that an inverse relationship may exist under conditions of low oxygen and/or low water temperature in the gonadal development of these worms. That is, the development of nutritive granules within the worm may occur at the expense of continuing the development of primary sperm tissue and sperm morulae all in a subtle way to assure the necessary numbers of mature eggs and sperm at the time of copulation and the development of cocoons in early spring.

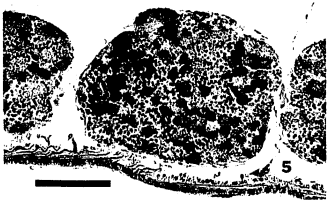
Oxygen levels and water temperatures increased during April and May (Table 6, p. 30) as did the cell dividing activities of the sperm products and the production of nutritive granules.

Perhaps, the single most convincing piece of evidence gathered during this investigation that supports a single reproductive cycle in a year for *L. hoffmeisteri* in the Little Calumet River is the appearance of cocoons with eggs surrounded by a cellular membrane containing numerous nutritive granules as well as developing young worms (Table 1, p. 18, Figures 78 and 80, p. 144). These observations were recorded for the month of April. Cocoons were numerous and easily identified from the substrate. Cocoons containing young worms were identified as *L. hoffmeisteri* if there was a lack of hair setae on the dorsal and/or ventral anterior segments of the worms. Cocoons containing young worms with hair setae were identified as *T. tubifex*.

The Development of Type I and Type II Sperm Light Microscopical Observations

Maturation of released groupings of cells formed into a morula structure occurs within a cellular membrane sac located within the coelom between segments 9 through 14. This sac is identified simply as the sperm sac (Figure 5). The maturation of the morulae structures towards mature sperm occurs without an observable pattern initially (Figure 5). The different stages of development from a 4 or 8 cell morula are often observed in close proximity. The close proximity of the many cell developmental stages to one another is

Figure 5. The sperm sac with developing sperm morulae is located within the coelom of *Limnodrilus hoffmeisteri*. (Sample Date: October, 1977; Photo Magnification: 200 X; Stain: Hematoxylin/Eosin) Marker = 100 μm.



easily explained since the process occurs within the sperm sac. The sperm sac is subjected to movements of the worm brought on, for example, by the contractions or relaxation of cuticular smooth muscles. Such movements probably exert pressure changes on the coelomic fluids which in turn exert pressures on the sperm sac. These changes, as an example, probably contribute to the mixing effect of the different developmental sperm stages from one segment to the next.

The developing morulae are released from primordial germ tissue that will eventually differentiate into the limiting cellular membrane of the sperm sac (Figure 6). Both Type I and Type II sperm morulae are released from this tissue. This mixing effect that occurs naturally within the sperm sac has made the task of separating the different developmental stages of the two sperm difficult. However, the data suggest that there are in fact two sperm types that develop concurrently and coexist in a similar environment, that is, the sperm sac. The data also support the findings that the two sperm type have a common origin from the primordial germ tissue (see section on development of male structures). There are marked morphological differences between the two sperm types. Difficulty in recognizing these differences is related to the time of the year, a factor that appears to regulate the reproductive period of the worm. This also affects certain stages of the Type II sperm which are not observed in great numbers. This is shown in the following sections.

Figure 6. Developing sperm morulae are released from a primordial germ tissue. Both Type I and Type II sperm morulae are released from this tissue. (Sample Date: October, 1977; Photo Magnification: 200 X; Stain: Hematoxylin/ Eosin) Marker = 100 μm.

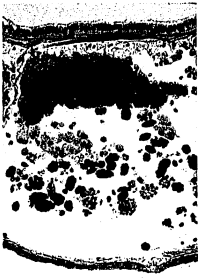


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Figure 7 represents the overall developmental stages of both Type I and Type II sperm. Similar stages of both Type I and Type II sperm have been placed within the same figure in order to make an overall comparative analysis. These drawings summarize the light microscopical findings that are described for the developmental stages of Type I and Type II sperm in the following sections.

The Developmental Stages of Type I Sperm

The mature Type I sperm is easily distinguished from the Type II sperm. Grossly, mature Type I sperm are larger than the mature Type II sperm (Figure 8). The length of the mature Type I sperm is approximately 55 μm. The width measured from the midsection of the sperm is approximately .800 μm.

The Type I morula structure is released from the primordial germ tissue generally as a 4 cell cluster (Figure 9). Two cell morula structures have been observed but these do not appear to be abundant. Subsequent cell or nuclear divisions occur until the morula structure has approximately 128 nuclei that will differentiate into the mature sperm. It is assumed that the 4 cell morula structure represents the first stage in sperm development within the sperm sac. Subsequent examination of the morula structures suggest that there are six developmental stages prior to the maturation of the Type I sperm.

FIGURE 7
DEVELOPMENTAL STAGES OF TYPE I SPERM AND TYPE II SPERM FROM Limnodrilus hoffmeisteri - A COMPARISON

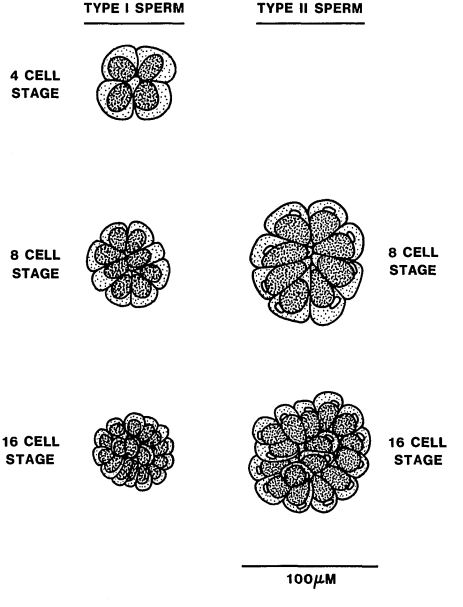


FIGURE 7 cont'd
DEVELOPMENTAL STAGES OF TYPE I SPERM AND TYPE II SPERM FROM Limnodrilus hoffmeisteri - A COMPARISON

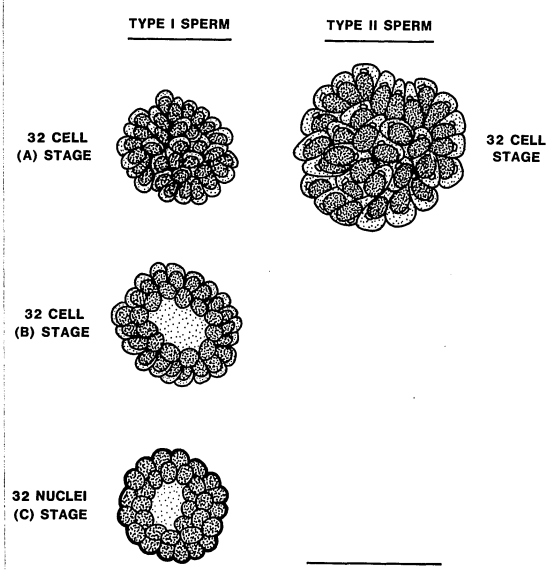


FIGURE 7 cont'd
DEVELOPMENTAL STAGES OF TYPE I SPERM AND TYPE II SPERM FROM Limnodrilus hoffmeisteri - A COMPARISON

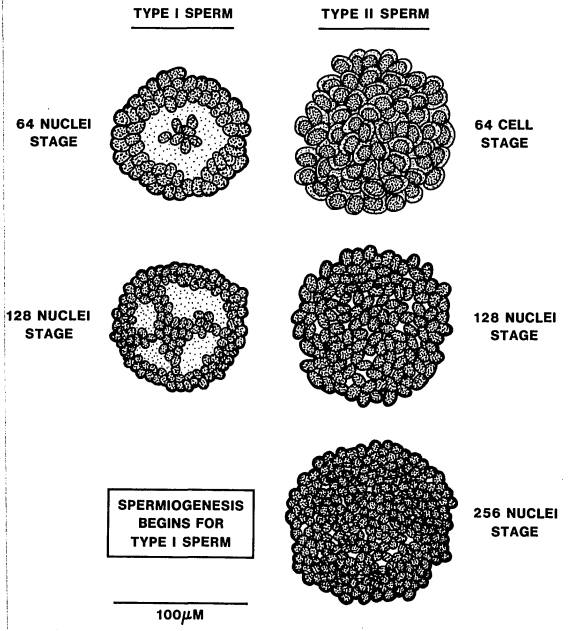


FIGURE 7 cont'd
DEVELOPMENTAL STAGES OF TYPE I SPERM AND TYPE II SPERM FROM Limnodrilus hoffmeisteri - A COMPARISON

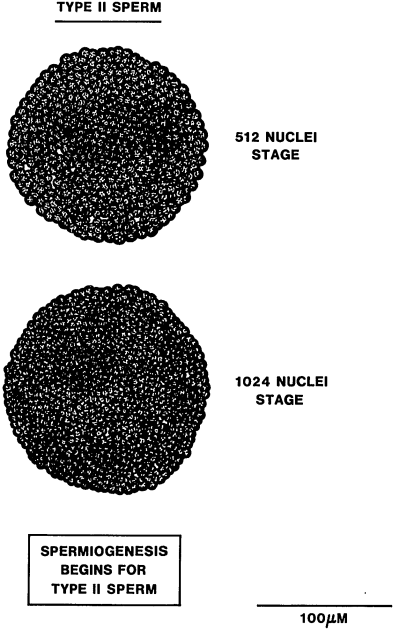
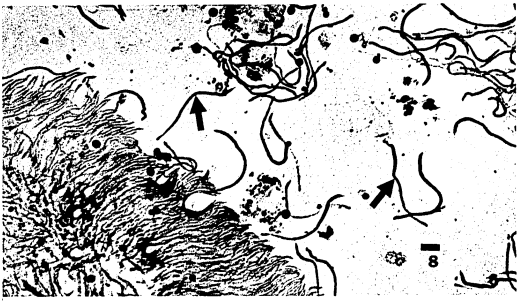


Figure 8. Light microscopical depiction of Type I and Type II sperm. Arrow points to Type I sperm which is larger in width than the Type II sperm. Type II sperm are in the lower left hand portions of the photo. (Sample Date: January, 1978; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

Figure 9. The 4 cell and 8 cell stage of the Type I sperm morulae are shown. (Sample Date: November, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.



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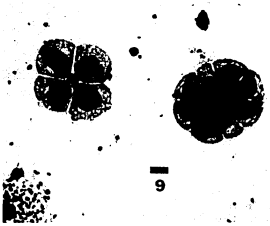

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4 Cell Stage

This 4 cell cluster (Figure 9) is the first observable morula structure within the sperm sac. The morula is composed of 4 distinct cells with cell membranes. The 4 cells measure approximately 50 μm in diameter. Each cell appears to occupy 1/4 of the total area of the structure. Each cell is approximately 25 μm in diameter. The nucleus is approximately 17 μm in diameter. The cell cytoplasm stains basophilic, that is, blue, and the nucleus stains acidophilic or red.

8 Cell Stage

It was not difficult to observe the 4 cell morula undergoing cell division. The process of division appeared to be synchronized within the morula structure and is affected little by surrounding different developmental stages of the sperm. All cells within the morula appeared to be undergoing the process at the same time. This division resulted in the 8 cell stage, a stage marked by 8 distinct cells of equal size all tightly arranged (Figure 9). The morula measured approximately 50 μm in diameter. Each cell measures approximately 16 μm in diameter. An average nucleus was 13 μm in diameter. No distinct differences were observed in structure from the first stage on except for the doubling of the number of cells. This stage was easily observed.

16 Cell Stage

The developmental process continued with the doubling of the number of cells from 8 to 16 (Figure 10). The 16 resulting cells were tightly arranged within the enlarging morula structure. Each cell was distinct and was distinguished easily from neighboring cells. Again, this stage developed in synchrony with each cell dividing at the same time. No distinct differences were observed from the preceding stage except the doubling of the number of cells within the morula structure. The diameter of the morula is approximately 50 μm. The approximate diameter of the cell and nucleus is 12 μm and 10 μm, respectively. This stage was easily observed.

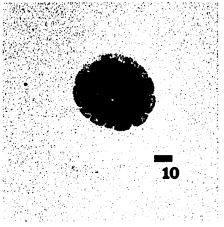
32 Cell Stage

The appearance of the 32 cell stage was simply a doubling of the number of cells from the previous stage. This development was in synchrony with the number. The cells were distinct from each other and were observed throughout the morula structure (Figure 11). It was at this stage that differences could be observed from the previous stages. The evidence suggested that the cell membranes that, for the most part, distinguished one cell from the next were absorbed within the morula structure. It was at this stage that nuclei were observed to migrate to the periphery of the morula structure leaving the central area of the morula with basophilic staining cytoplasm (Figure 12). The approximate diameter of the

Figure 10. The 16 cell stage of the Type I sperm is shown. (Sample Date: December, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

Figure 11. The 32 cell stage of the Type I sperm is marked initially by 32 distinct cells throughout the morula structure. (Sample Date: December, 1977; Photo Magnification: 480 X; Wright's/Giemsa) Marker = 10 μm

Figure 12. Further differentiation of the 32 cell Type I sperm morula shows the nuclei located on the periphery of the morula with an exposed central area of the morula. There are now 32 nuclei. (Sample Date: December, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm



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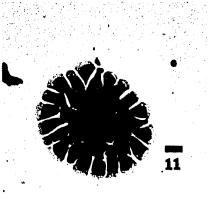

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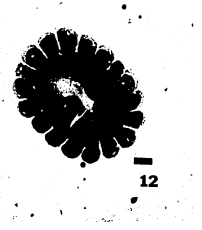

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morula was 70 μm. The diameter of the nucleus was approximately 8 μm.

64 Nuclei Stage

The distinguishing feature of this stage is that only nuclei were observed to divide from the previous stage to this one. In the preceding stages entire cells were observed to divide. The nuclei were observed on the periphery of the morula structure with the central areas of the morula structure containing basophilic staining cytoplasm (Figure 13). The approximate diameter of the morula was 70 μm. The diameter of the nucleus was approximately 8 μm.

This stage was not observed easily, a fact that would suggest that the morula structure does not remain in this stage for long but proceeds to undergo another nuclear division, leading to the next stage.

128 Nuclei Stage

The overall size of the morula structure at this stage is approximately 100 μm in diameter (Figure 14). The nuclei measured approximately 4.5 μm in diameter (Figure 14) and concentrate along the periphery of the morula sphere. They seem to align in a way suggesting a north and south pole concentration of nuclei as well as a band of nuclei occupying the equatorial periphery of the morula structure. For whatever reason, this alignment of the

Figure 13. The 64 nuclei stage of the Type I sperm is shown, The nuclei, some partially obscured, are located on the periphery of the morula. (Sample Date: November, 1977; Photo Magnification: 480 X; Stain: Wright's/ Giemsa) Marker = 10 μm.

Figure 14. The 128 nuclei stage of the Type I sperm depicts the arrangement of nuclei around the morula prior to the beginning of spermiogenesis. (Sample Date: November, 1977; Photo Magnification: 480 X; Stain: Wright's/ Giemsa) Marker = 10 μm.

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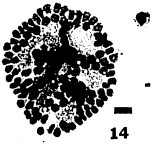

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nuclei is characteristic of the Type I sperm as observed from the light microscopical level. The stage was easily observed and marked the final developmental stage prior to the maturation of the mature sperm.

The evidence in support of the observation that the 128 nuclei morula is the final stage preceding Type I sperm maturation is seen in Figure 15. Early stages of nuclei elongation are observed within the morula structure. This elongation as observed in Figure 15 is likely to be, in part, an artifact of the fixation process. In sperm differentiation, the formation of the tail portion of the sperm comes from the surrounding cytoplasm near the nuclear material. This is the same with the Type I sperm and the evidence for this is in the electron microscopy section. The induced artifact, partly seen in Figure 15, brought on by air fixation has presented additional information suggesting that the 128 nuclei stage precedes Type I sperm maturation. The air fixation procedure allows the sperm products to circulate within the surrounding fluids on a glass slide prior to drying. This trauma appears to have an effect on the nuclei of certain 128 nuclei morula by causing the premature elongation of these nuclei. The association of the 128 nuclei stage as the final stage prior to sperm maturation is given further support in the fact that the number of mature sperm attached to the Type I morula structure is approximately 128 (Figure 16).

The Type I sperm goes through 6 distinct developmental stages within the sperm sac, assuming that the 4 cell morula is the first (Figure 7, p. 57).

Figure 15. This 128 nuclei stage of the Type I sperm shows the beginning of the nuclear elongation process. (Sample Date: November, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

Figure 16. This morula structure is representative of the nearly matured Type I sperm that are still attached to the morula. The number of maturing sperm is approximately 128. (Sample Date: November, 1977; Photo Magnification: 480 X; Stain: Wright's/ Giemsa) Marker = 10 μm.

15

16

The Developmental Stages of Type II Sperm

The developmental stages of the Type II sperm follow a pattern similar to that of the Type I sperm. The Type II sperm is easily distinguished from the Type I sperm as seen in Figure 8 (p. 62). The Type II sperm is approximately 85 μm long and approximately .435 μm wide as measured from a point halfway from the head and tail regions of the sperm. The developmental stages, as found in a morula structure, occur within the sperm sac. Each morula structure appears to be regulated as a single unit with little or no observable influences from surrounding developmental sperm stages (Type I or Type II).

The Type II sperm is released from a primordial germ as an 8 cell cluster representative of the morula structure (Figure 17). This grouping of cells was observed without much difficulty. Four cell clusters were observed but were not common. Subsequent cell and nuclei divisions occur that eventually become the 1024 nuclei morula structure. The developmental stages are simply identified by the number of cells or nuclei that make up the morula structure. All stages appear to mix freely within the sperm sac. The Type II morula structures are markedly different from the Type I morula structures. The Type II morulae are larger than the Type I and acquire a deeper basophilic (deep blue) appearance of the cytoplasm as well as a deeper acidophilic (deep red) appearance of the nuclei. The eight developmental stages are:

8 Cell Stage

Eight uniform sized cells clustered tightly together in a morula formation are easily observed within the sperm sac (Figure 17). The morula is approximately 70 μm in diameter. An individual cell within the morula measures approximately 27 μm in diameter. The nucleus is approximately 18 μm in diameter. A fairly distinct vacuole-like structure can be observed in the basal area of the cytoplasm near the nucleus (Figure 17). The basal area is the area nearest the perimeter of the morula structure. The vacuole-like structure is pale in coloration since it did not readily take the Wright's or Giemsa stain. This structure is a distinctive feature that further distinguishes between the two sperm types.

The cells of this stage are distinct with well defined cell membranes. As was the case with the Type I sperm stages, the cell division actively occurs in unison, with all cells undergoing the process at once.

16 Cell Stage

The 16 cell stage is not markedly different from the 8 cell stage excepting for the doubling in number of cells and change in size of the morula structure (Figure 18). The morula structure measures approximately 80 μm in diameter. Individual cells are approximately 21 μm in diameter. Individual nuclei measure approximately 14 μm in diameter. The cells tend to be rounded on the

Figure 17. The 8 cell stage of the Type II sperm is shown. (Sample Date: December, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

Figure 18. The 16 cell stage of the Type II sperm is shown. (Sample Date: December, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

17

18

periphery of the morula to somewhat conical or pointed as they face the center of the morula structure. This stage is easily observed.

32 Cell Stage

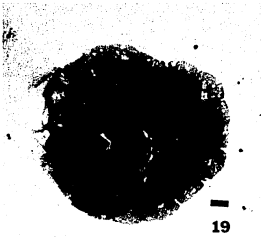
This stage is not markedly different from the preceding stage. The size of the morula structure is approximately 100 μm in diameter (Figure 19). All cells are distinct with cell membranes and are uniform in size. Approximate cell size is 21 μm in diameter, and the approximate nucleus size is 12 μm in diameter. This stage, like the others, proceeds with cell divisions in a uniform manner leading to the next developmental stage.

64 Cell Stage

The overall size of the morula is approximately 104 μm in diameter (Figure 20). This stage is not easily observed and suggests that the morula structure does not remain in this stage for a long period of time. During this stage the cell membranes that in previous stages have made the nuclei appear as distinct cellular units appear to be replaced by a single membrane surrounding the entire morula structure. The nuclei are approximately 8 μm in diameter. The nuclei do not appear to be located in the periphery of the morula structure but seem to be distributed throughout the entire structure.

Figure 19. The 32 cell stage of the Type II sperm is shown. (Sample Date: December, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

Figure 20. The 64 cell stage of the Type II sperm is observed initially as 64 distinct cells. During this stage the cells become distinguishable as 64 nuclei within the morula structure. (Sample Date: December, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.



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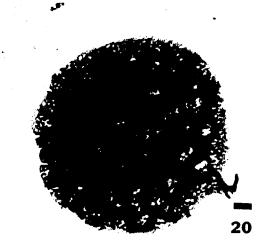

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128 Nuclei Stage

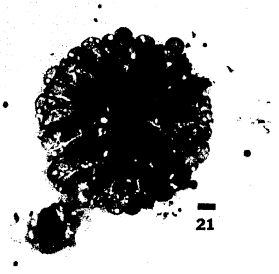
This stage was not observed in great numbers. Likewise, the 64 cell stage was not observed easily. This may again suggest that this stage does not last long but represents a series of rather quick nuclear divisions in the sperm maturation process. The morula structure is approximately 103 μm in diameter (Figure 21). The nuclei appear to be evenly distributed throughout the morula structure. An individual nucleus measures approximately 9 μm in diameter.

256 Nuclei Stage

Again, this stage is not common and may represent a single stage that may not be present for a long period of time. The morula structure is approximately 8 μm in diameter (Figure 22). An individual nucleus is approximately 3 μm in diameter. During this stage it appears that the nuclei are located near the periphery of the morula structure. No distinct central mass is observed probably due to the large numbers of nuclei that seem to be located equidistant from one another throughout the structure. The number of nuclei becomes difficult to count from this stage on. Careful examination of the different morula with large numbers of nuclei was needed in order to present the findings. Exact numbers such as 256 nuclei were never counted but only numbers that approximated this figure. It was assumed that the change from one developmental stage to another meant in effect a doubling of the previous number

Figure 21. The 128 nuclei stage of the Type II sperm is simply seen as a doubling of nuclei from the previous stage. The nuclei appear to be arranged around the periphery of the morula. (Sample Date: November, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

Figure 22. The 256 nuclei stage of the Type II sperm is shown. (Sample Date: November, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.



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of cells or nuclei. Although this may not be exactly true for the 256 nuclei stage and up, the data of the early stages as well as Type I sperm stages indicate that this is the pattern. Information to counter this viewpoint was not observed. The problem of counting nuclei in large numbers from a 3 dimensional structure that can only be viewed in 2 dimensions compounds the problem. Obviously, certain nuclei would mask others as seen in Figure 7 (p. 57).

512 Nuclei Stage

All the problems mentioned in the previous stage are doubled when viewing this stage with the light microscope. The morula structure measures approximately 80 μm in diameter and individual nuclei measure approximately 3 μm in diameter (Figure 23). Nuclei counts were very difficult but could be made on selective morula structures when the structures had been flattened somewhat revealing the majority of nuclei. Again, exact counts of 512 nuclei were not observed but counts close to these figures suggested the occurrence of this stage. This stage was more easily observed than the previous two stages. The nuclei were observed to be located around the periphery of the morula (Figure 23). The large numbers of nuclei prevented any observations of a central mass of cytoplasm free from nuclei within the morula sphere.

1024 Nuclei Stage

The stage is marked by the doubling of the numbers of nuclei from the previous stage. The morula is approximately 103 μm in

Figure 23. The 512 nuclei stage of the Type II sperm is shown. Exact numbers of nuclei representing this stage are not easily counted since a number of nuclei may occlude other nuclei. (Sample Date: December, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

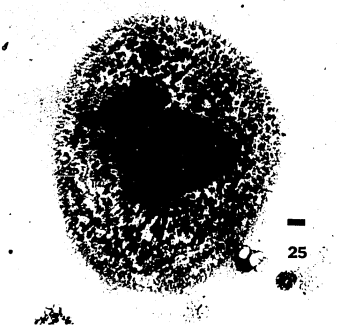
Figure 24. The 1024 nuclei stage is shown prior to the beginning of spermiogenesis. (Sample Date: November, 1977; Photo Magnification: 480 X; Stain: Wright's/Giemsa) Marker = 10 μm.

diameter and the individual nucleus is approximately 1.5 μm in diameter (Figure 24). Again, exact counts of 1024 nuclei were never achieved but numbers close to this number were observed. The same problems discussed in the previous two stages were also observed in this stage. This was the last stage before maturation of the sperm. Therefore, this stage was usually found in great numbers provided the worm was in the reproductive time period. The nuclei were observed to be peripherally located within the morula. Nuclear elongation, as suggested previously, partly an artifact of fixation, was observed. This premature elongation further strengthened the observation that this is the final stage before maturation of the sperm. This evidence is presented in Figure 25.

Spermiogenesis of Type I Sperm

Spermiogenesis begins after the Type I morula has reached the 128 nuclei stage. Mitochondria are seen to aggregate at the basal part of the nucleus (Figure 26). These mitochondria are characterized by either an elongated shape with cristae running along the long axis or by a small and rounded appearance (Figure 26). It is from this region that the flagellum develops. Shortly after the appearance of the mitochondria, this region near the nucleus begins to elongate and the mitochondria are observed to be centrally located along the developing flagellum (Figure 27). The flagellum continues to develop with the disappearance of the mitochondria and the appearance of the central core of microtubules. The flagellum

Figure 25. Spermiogenesis has begun and developing flagellum can be observed radiating out of this 1024 nuclei stage of the Type II sperm. (Sample Date: December, 1977; Photo Magnification: 480 X; Stain: Wright's/ Giemsa) Marker = 10 μm.



microtubules are characterized by the 9:2 pattern with 9 pairs of microtubules located on the periphery of the core and a pair of microtubules centrally located (Figure 28). A spindle process was observed that seems to connect the outer microtubules with the central pair of microtubules. The flagellum measures approximately .240 μm during this developmental stage (Figure 29).

As the flagellum continues to develop, a golgi complex is observed in the remaining basal cytoplasmic region (Figure 30). Mitochondria are observed adjacent to the golgi complex. As many as 4 mitochondria have been observed in this region. However, this observation varies depending on the region of the developing sperm that is being viewed with the electron microscope. In Figure 30 no mitochondria are observed around the golgi complex but in Figure 31 mitochondria are observed.

During the early sperm morphogenesis, the size and shape of the nucleus begins to change. The nucleus is circular or spheroidal in shape during the early phases of spermiogenesis (Figure 32). The diameter of the nucleus in the initial stages of flagellum development is slightly more than 4 μm (Figure 26). By the time the flagellum has developed and with the appearance of the golgi complex, the nucleus has reduced in size to about 2.5 μm in diameter (Figure 30). The chromatin remains fairly diffuse throughout the nucleus during these developmental changes.

It is at this point that the head region begins to undergo its morphogenesis. Nuclear condensation and elongation appear to be

Figure 26. Type I Sperm Spermiogenesis: Mitochondria (M) are seen to aggregate at the basal region of the cell near the nucleus (N). (Sample Date: November, 1977; Photo Magnification: 4,196 X; Negative #: 213) Marker = 1 μm.

Figure 27. Type I Sperm Spermiogenesis: Elongation and development of the flagellum (arrow) is seen with mitochondria (M) centrally located. Nucleus (N). (Sample Date: November, 1977; Photo Magnification: 4,196 X; Negative #: 233) Marker = 1 μm.

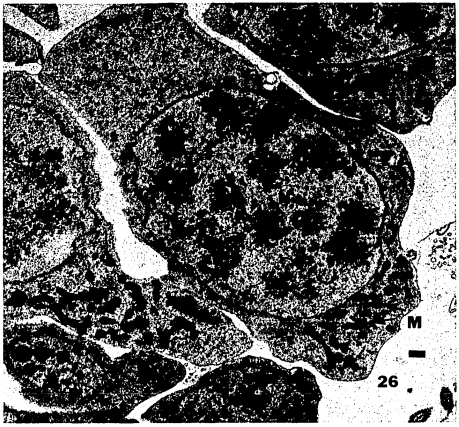
Figure 28. Type I Sperm Spermiogenesis: The development of the flagellum (F) is one of the first regions to develop. Note the extrusion of cell membranes in this condensation process (arrow). Insert: A transverse section of the flagellum shows the typical 9:2 arrangement of microtubules. (Sample Date: March, 1978; Photo Magnification: 4,464 X; Negative #: 10052) (Insert: Sample Date: September, 1977; Photo Magnification: 19,654 X; Negative #: 2655) Marker = 1 μm.

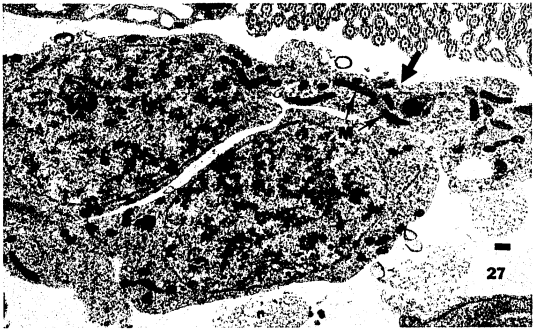
Figure 29. Type I Sperm Spermiogenesis: This scanning electron micrograph shows the early stages of the flagellum development. Note that the developing sperm are attached to the central core of the morula. (Sample Date: June, 1978; Photo Magnification: 1,650 X; Negative #: 101)

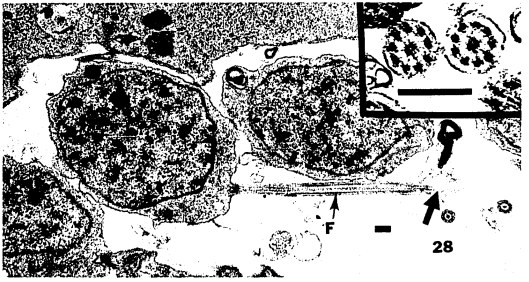
Figure 30. Type I Sperm Spermiogenesis: The golgi complex (G) becomes distinct around the same time that the flagellum (F) is developed. Nucleus (N). (Sample Date: March, 1978; Photo Magnification: 8,493 X; Negative #: 10053) Marker = 1 μm.

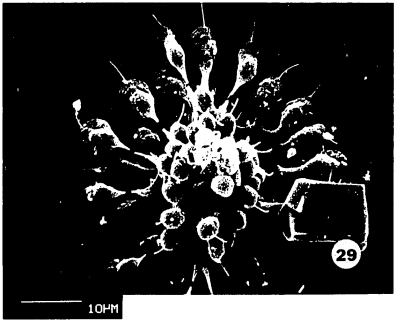
Figure 31. Type I Sperm Spermiogenesis: Mitochondria (M) are often seen near the basal region of the maturing sperm. Golgi complex (G), flagellum (F). (Sample Date: March, 1978; Photo Magnification: 19,654 X; Negative #: 2533) Marker = 1 μm.

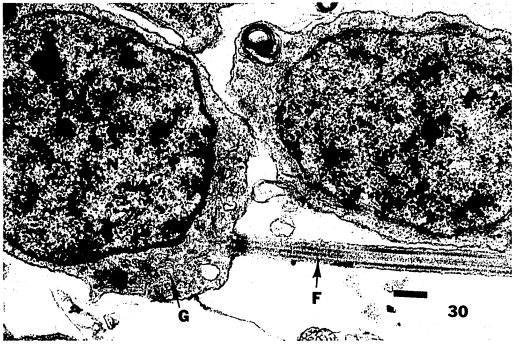
Figure 32. Type I Sperm Spermiogenesis: The nucleus (arrow) is spheroid in shape and remains attached to the central nutritive mass (CNM) or morula by a short narrow piece of cytoplasm. (Sample Date: October, 1978; Photo Magnification: 2,000 X; Negative #: 201)

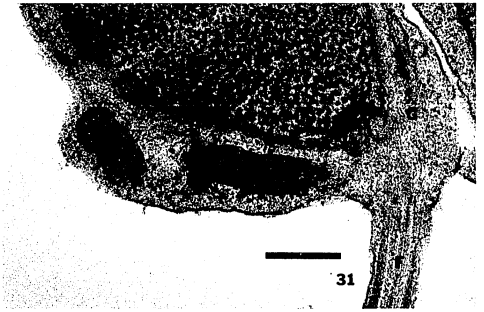


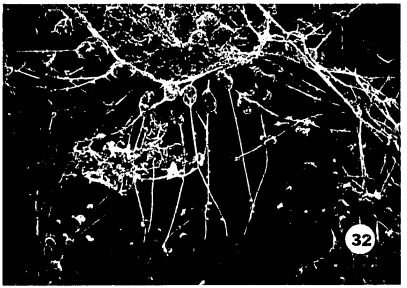












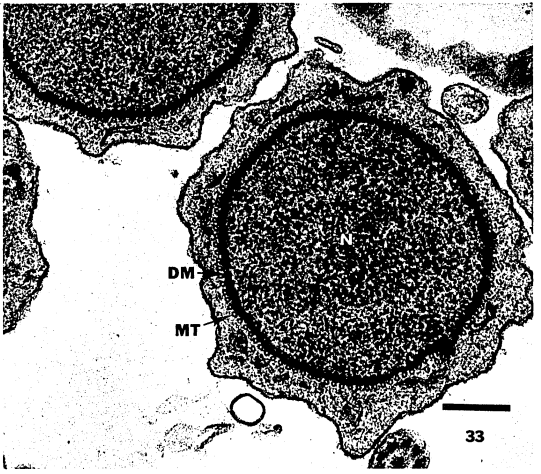
involved with two sets of microtubule formations that make up the manchette. The first set of microtubules are observed adjacent to the outer nuclear membrane. The microtubules do not appear to be connected or attached to the nuclear membrane (Figure 33). These microtubules are approximately 27.5 nm in diameter and are fairly uniformly spaced around the nucleus at approximate distances of 12.0 nm. The central core of these microtubules is clearly visible (Figure 33). The second set of microtubules is located adjacent to, and assumed to be in contact with, the inner nuclear membrane surfaces. These tubules are markedly different from the first set of microtubules due to their electron dense appearance. These tubules will be referred to as the dense microtubules while the remaining set will be referred to simply as microtubules. The dense microtubules are approximately the same size as their counterparts. They appear to be arranged parallel with their counterparts and are similarly spaced around the nuclear membrane (Figure 33). Figure 34 depicts the dense microtubules as they run along the long axis of the nucleus.

Changes in chromatin appearance have taken place during the manchette development. The chromatin has become finely granulated and uniform throughout the nucleus (Figures 33 and 34).

As the nucleus continues to elongate and condense, the dense microtubules become obscure, forming an electron dense layer around the periphery of the nucleus (Figure 35). The elongation process proceeds until the nucleus is approximately 14 μm long (Figure 36).

Figure 33. Type I Sperm Spermiogenesis: Dense microtubules (DM) are observed near the inner nuclear membrane. Microtubules (MT) form the manchette around the outside of the nuclear membrane. Note the uniform granulation of the chromatin within the nucleus (N). (Sample Date: March, 1978; Photo Magnification: 17,927 X; Negative #: 10128) Marker = 1 μm.

Figure 34. Type I Sperm Spermiogenesis: Dense microtubules (DM) are observed from a long section through the nucleus (N). Microtubules (MT) of the manchette are also seen. (Sample Date: March, 1978; Photo Magnification: 17,927 X; Negative #: 10081) Marker = 1 μm.



The apex of the nucleus is pointed (Figure 35). From this point the nucleus widens to approximately .655 μm in diameter. The nucleus appears circular in shape when viewed in transverse section (Figure 35). The nucleus further differentiates in that its diameter is reduced to about .340 μm and changes from the fine granular appearance of the chromatin to an electron dense appearance (Figure 37). This change appears to signal the last stage of nuclear differentiation. The nucleus begins to twist and take the form of a helix (Figure 38). This change is marked by similar coiling of the microtubules around the nucleus (Figure 39). The diameter of the microtubules during this process is approximately .0254 μm. The microtubules do not appear to have intimate contact with the nucleus as a thin layer of the tubules is observed through a long section of the head region (Figure 40). This is confirmed in a transverse section through the same region (Figure 41). The appearance of the coiled nucleus marks the maturation of the head region of the Type I sperm.

An acrosome has not been observed.

The neck region consists of the centriole and a single mitochondrion located adjacent to the basal part of the nucleus (Figure 42). Evidence confirming a single mitochondrion in the neck region is seen in Figure 43 showing a transverse section with a single mitochondrium present.

The formation of the midpiece occurs during the last stages of the head maturation. Electron dense granules begin to appear

Figure 35. Type I Sperm Spermiogenesis: Nuclear condensation and elongation is marked by the length of the nucleus (N) and the formation of an electron dense layer around the periphery of the nucleus. (Sample Date: November, 1976; Photo Magnification; 4,196 X; Negative #: 110) Marker = 1 μm.

Figure 36. Type I Sperm Spermiogenesis: The scanning electron micrograph shows the elongation process of the nucleus (N). (Sample Date: October, 1978; Photo Magnification: 1,850 X; Negative #: 197)

Figure 37. Type I Sperm Spermiogenesis: The nucleus (N) continues to elongate and is now changed from a fine granular appearance of the chromatin to an electron dense appearance. (Sample Date: March, 1978; Photo Magnification: 4,464 X; Negative #: 10120) Marker = 1 μm.

Figure 38. Type I Sperm Spermiogenesis: The nucleus (N) begins to coil and forms a helix during the final sperm maturation stage. Note the coiled arrangement of the microtubules (MT) and the thin, diffuse layer of microtubules (arrow) near the nucleus. (Sample Date: March, 1978; Photo Magnification: 9,647 X; Negative #: 2517) Marker = 1 μm.

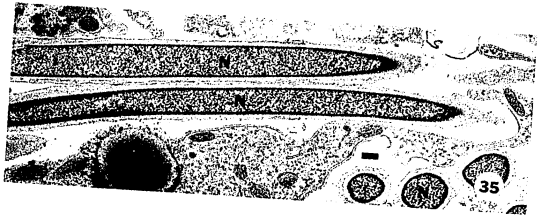
Figure 39. Type I Sperm Spermiogenesis: This enlargement of the previous figure shows the intricate arrangement of microtubules (arrow) that surround the nucleus. (Sample Date: March, 1978; Photo Magnification: 19,654 X; Negative #: 2518) Marker = 1 μm.

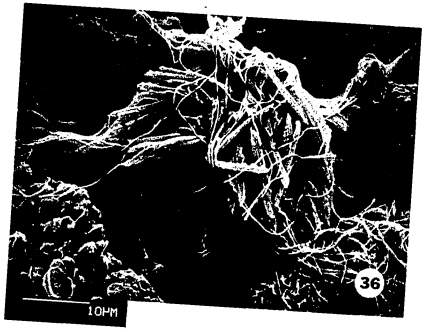
Figure 40. Type I Sperm Spermiogenesis: The microtubules (MT) do not appear to be in direct contact with the nucleus (N) as seen in this photo. (Sample Date: March, 1978; Photo Magnification: 19,654 X; Negative #: 2523) Marker = 1 μm.

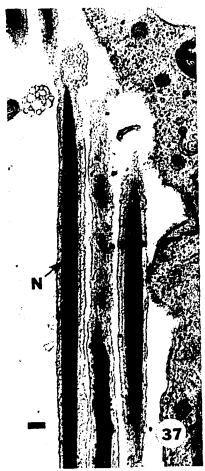
Figure 41. Type I Sperm Spermiogenesis: This transverse section suggests that the microtubules (MT) though coiled around the nucleus (N) do not come in contact with the nuclear membrane. (Sample Date: September, 1977; Photo Magnification: 19,654 X; Negative #: 2653) Marker = 1 μm.

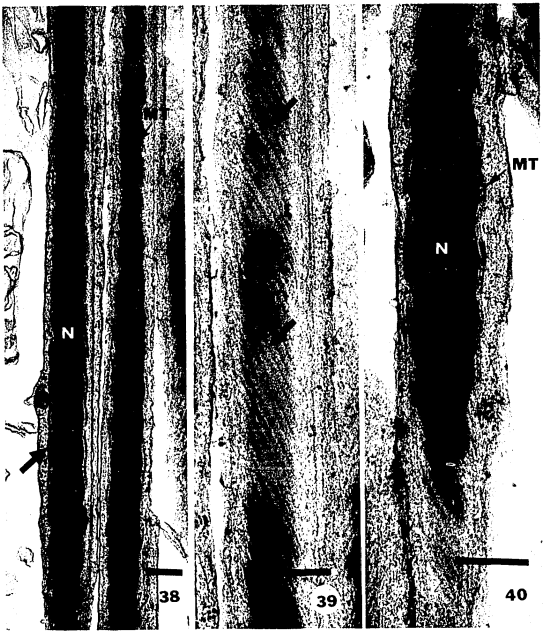
Figure 42. Type I Sperm Spermiogenesis: The neck region consists of the centriole (C), a single mitochondrion (M) located adjacent to the basal region of the nucleus (N). (Sample Date: September, 1977; Photo Magnification: 19,654 X; Negative #: 2650) Marker = 1 μm.

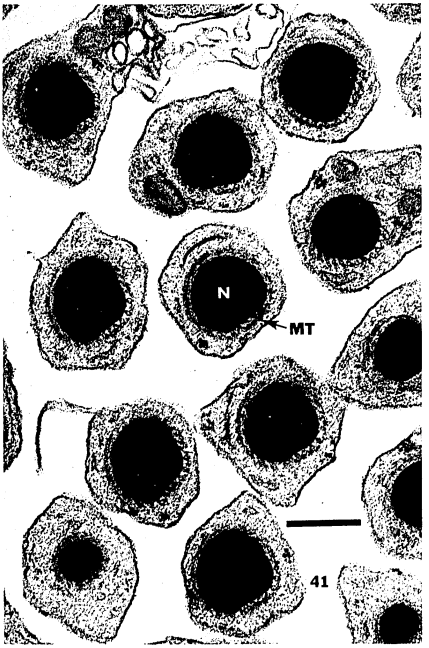
Figure 43. Type I Sperm Spermiogenesis: A transverse section through the neck region confirms a single mitochondrion (M). (Sample Date: September, 1977; Photo Magnification: 19,654 X; Negative #: 2655) Marker = 1 μm.

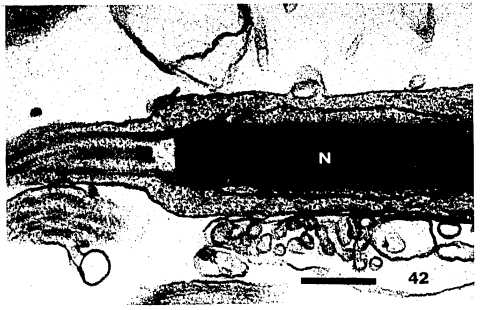


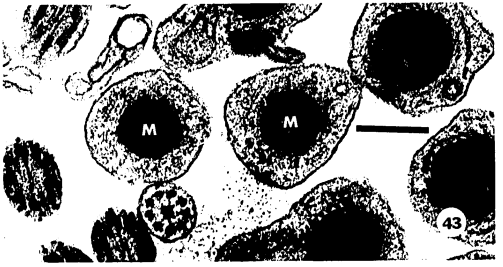












around the peripheral microtubules of the flagellum (Figure 44). These granules, approximately 20.0 nm in diameter, appear to be associated in some way with the outer microtubules. A long section (Figure 45) confirms the observation that these electron dense regions are granules and not another set of dense microtubules. An interesting association is made between the granules within the central nutritive mass (Figure 46) and those that are found within the mature midpiece (Figure 47). Both granules from the two regions are electron dense monoparticulate granules of about the same size. The central nutritive mass granule is approximately 18.3 nm in diameter and the midpiece granule is smaller at around 14.9 nm in diameter. The granules associated with the microtubules are around 20.0 nm in diameter. These granules are suggestive of glycogen.

The midpiece becomes heavily granulated and the ordered appearance of the granules as seen in Figure 47 changes to a disordered appearance of numerous granules between the midpiece membrane and the central core of microtubules. A degranulated region of the midpiece adjacent to the neck (Figure 48) is common. This region seems to vary from one sperm to the next. The width of the midpiece at maturation is around .800 μm in diameter. The overall length of the midpiece is around 30 μm.

The tail region is approximately 10 μm long (Figure 49). It is short and resembles the flagellum described in Figure 28 (p. 92). The tail is approximately .270 μm in diameter.

Figure 44. Type I Sperm Spermiogenesis: Electron dense granules probably glycogen (GL) are observed in close proximity to the peripheral microtubules (MT) within the midpiece. (Sample Date: June, 1976; Photo Magnification: 43,092 X; Negative #: 1211) Marker = 1 μm.

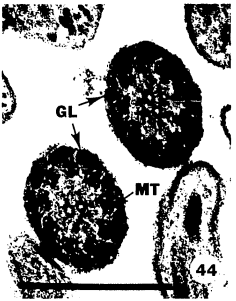
Figure 45. Type I Sperm Spermiogenesis: Electron dense granules probably glycogen (GL) are observed along the long axis of the midpiece. These granules appear to be associated with the microtubules (MT). (Sample Date: June, 1976; Photo Magnification: 43,092 X; Negative #: 1129) Marker = 1 μm.

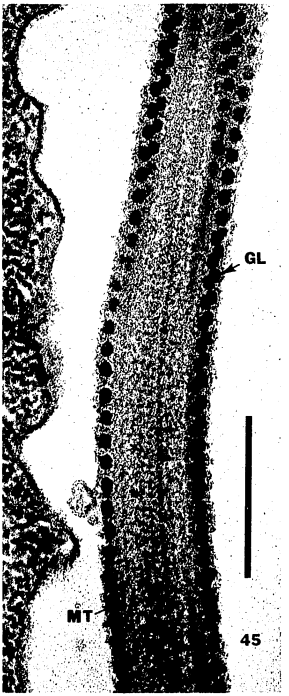
Figure 46. Type I Sperm Spermiogenesis: The electron dense granules (arrow) found within the central nutritive mass (CNM) are approximately the same size as those found in the midpiece. This suggests that the granules are glycogen. (Sample Date: March, 1978; Photo Magnification: 19,654 X; Negative #: 2508) Marker = 1 μm.

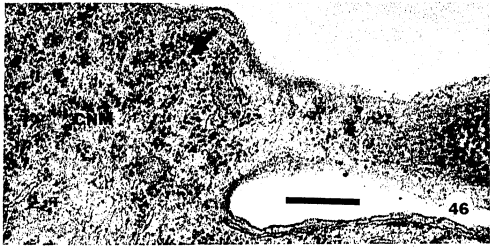
Figure 47. Type I Sperm Spermiogenesis: The midpiece is fully granulated with glycogen granules (arrow). This Type I sperm is almost fully matured at this point. (Sample Date: June, 1978; Photo Magnification: 14,742 X; Negative #: 1128) Marker = 1 μm.

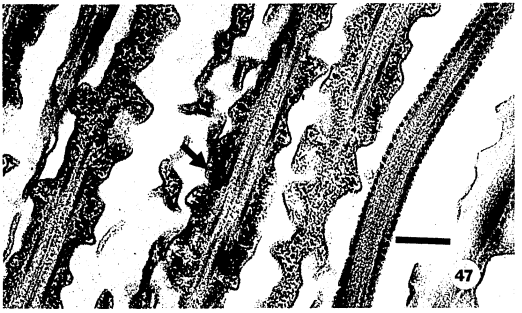
Figure 48. Type I Sperm Spermiogenesis: A short region of the midpiece near the neck (arrow) of the sperm is often without the numerous glycogen granules. (Sample Date: September, 1977; Photo Magnification: 9,647 X; Negative #: 2645) Marker = 1 μm.

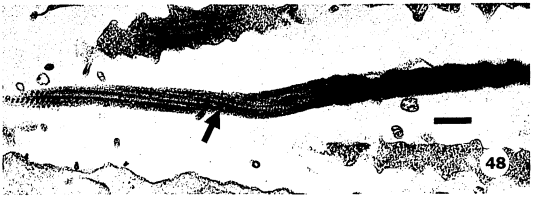
Figure 49. Type I Sperm Spermiogenesis: The mature Type I sperm is shown. (Sample Date: May, 1978; Photo Magnification: 2,500 X; Negative #: 48)

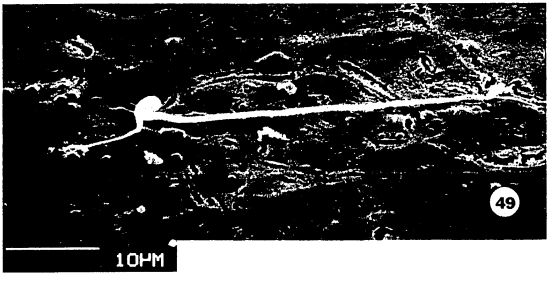












During the maturation process of the Type I sperm, certain cell products including membranes and organelles are cast off as debris into the sperm sac. This process is observed in Figure 42. Another observation of this process was seen in the head region toward the end of the nuclear maturation process. A membrane bound vesicle was observed in the basal areas of the head region (Figure 50). This vesicle appeared to surround cell debris and then release the captured contents outside of the maturing sperm (Figure 51).

The mature sperm, consisting of a head, neck, midpiece and tail region, is released from the central nutritive mass and stays within the sperm sac until such time that the sperm is deposited into the spermatheca. Figure 52 shows mature Type I sperm ready to be released from the central nutritive mass.

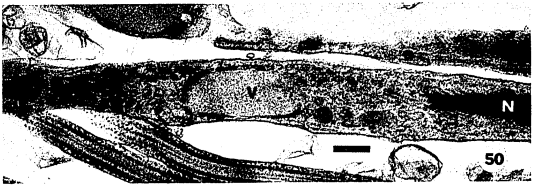
Spermiogenesis of Type II Sperm

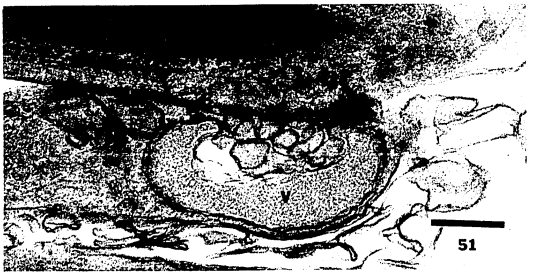
Spermiogenesis begins after the Type II morula has reached the 1024 nuclei stage. Mitochondria are observed to concentrate near the basal cytoplasmic region of the nucleus. This region begins to extend away from the nucleus and the beginning formation of the flagellum is seen (Figure 53). A distinct golgi is not observed at this time. As the cytoplasmic area continues to elongate, mitochondria are observed along the entire extension. These mitochondria appear to be centrally located along the length of the early developing flagella. At the end of this projection, the mitochondria are less centrally located. Of particular note is the shape of the

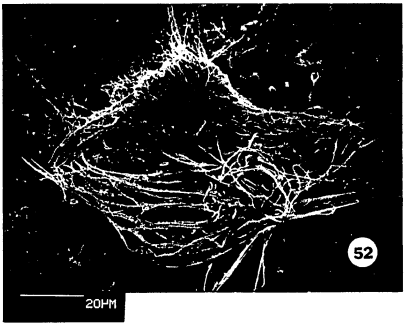
Figure 50. Type I Sperm Spermiogenesis: A membrane bound vesicle (V) appears to be in the process of getting unneeded cell components ready for release outside of the sperm. Nucleus (N), centriole (C). (Sample Date: March, 1978; Photo Magnification: 9,647 X; Negative #: 2519) Marker = 1 μm.

Figure 51. Type I Sperm Spermiogenesis: This membrane bound vesicle (V) near the neck region of the sperm appears to be releasing its contents of cell debris outside of the sperm. (Sample Date: March, 1978; Photo Magnification: 19,654 X; Negative #: 2524) Marker = 1 μm.

Figure 52. Type I and Type II Sperm Morula: Both Type I and Type II sperm are nearly matured but still attached to their respective morula. The Type I sperm are in the lower half of the photo and the Type II sperm are in the upper half of the photo. (Sample Date: October, 1978; Photo Magnification: 850 X; Negative #: 206)







mitochondria along the central portion of the flagellum. The mitochondria are elongated with the cristae running parallel with the long axis (Figure 54). As the differentiation of the flagellum continues, the mitochondria appear to dissolve into the cytoplasm as the central core of microtubules begin to appear (Figure 55). The mitochondria located at the distal regions of the flagellum are more rounded in appearance (Figures 53 and 54). At this point the nucleus has not begun to reduce and condense its nuclear contents. The nucleus is around 2 μm in diameter and contains the heterochromatin normally associated with these cells (Figure 53).

The flagellum is the first part of the sperm to become organized (Figure 56). The central core is composed of the typical 9:2 microtubule structure that is common throughout the animal kingdom (Figure 57). Often the peripherally located paired microtubules are connected to the central paired microtubules by a distinct spindle process (Figure 58). At the apical end of the central core of microtubules is the centriole (Figure 59). It should be pointed out that part of the flagellum will be associated with the middle piece that develops near the end of spermiogenesis.

As the flagellum continues to mature a distinct golgi complex is observed (Figure 60). Adjacent to or near the golgi is usually found one or two mitochondrion. At this point the number of mitochondria observed during the formation of the flagellum is no longer present except for one or two mitochondria adjacent to the golgi. Eventually a single mitochondrion will remain and will be

Figure 53. Type II Sperm Spermiogenesis; Mitochondria (M) are observed to concentrate near the basal cytoplasmic region of the nucleus (N). Note the beginning formation of the flagellum (F). (Sample Date: November, 1976; Photo Magnification: 4,320 X; Negative #: 2548) Marker = 1 μm.

Figure 54. Type II Sperm Spermiogenesis: Mitochondria (M) are elongated with the cristae running parallel along the long axis. The mitochondria are centrally located along the developing flagellum. (Sample Date: November, 1976; Photo Magnification: 9,647 X; Negative #: 2549) Marker = 1 μm.

Figure 55. Type II Sperm Spermiogenesis: As the differentiation of the flagellum continues the mitochondria (M) appear to dissolve into the cytoplasm as the central core of microtubules (MT) begin to appear. (Sample Date: November, 1976; Photo Magnification: 19,654 X; Negative #: 2553) Marker = 1 μm.

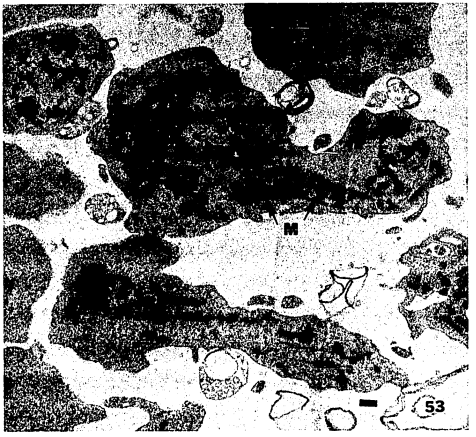
Figure 56. Type II Sperm Spermiogenesis: The flagellum matures rapidly in early sperm maturation and can be seen radiating away from the central morula. (Sample Date: October, 1978; Photo Magnification: 1,750 X; Negative #: 181)

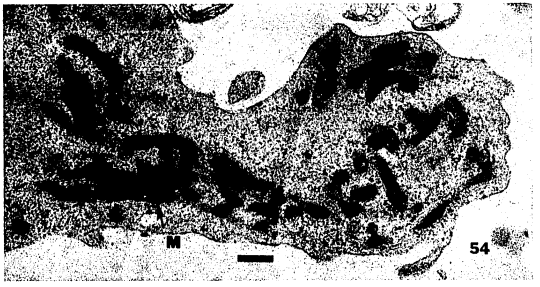
Figure 57. Type II Sperm Spermiogenesis: The flagellum (F) becomes well developed and as seen has the typical 9:2 microtubule structure (arrow). (Sample Date: March, 1978; Photo Magnification: 9,647 X; Negative #: 2462) Marker = 1 μm.

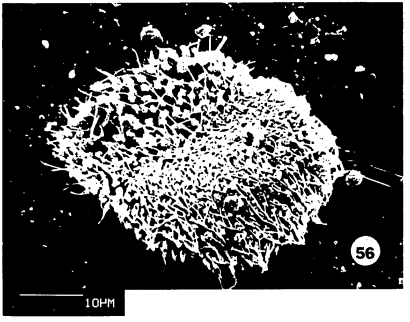
Figure 58. Type II Sperm Spermiogenesis: The 9:2 microtubules within the flagellum are often connected by a distinct spindle process (arrow). (Sample Date: September, 1977; Photo Magnification: 9,647 X; Negative #: 2668) Marker = 1 μm.

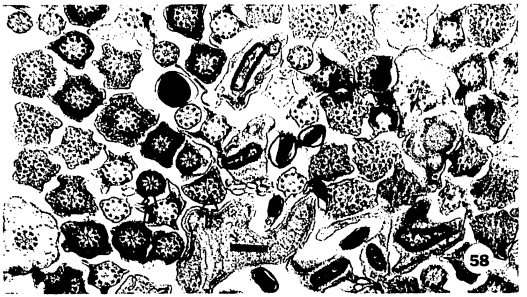
Figure 59. Type II Sperm Spermiogenesis: The centriole (C) is located at the apical end of the central core of microtubules (MT). The centriole is part of the neck region of the midpiece. (Sample Date: March, 1978; Photo Magnification: 4,464 X; Negative #: 10130) Marker = 1 μm.

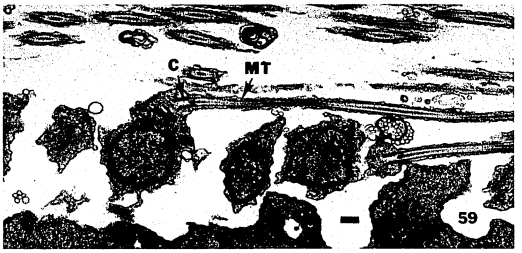
Figure 60. Type II Sperm Spermiogenesis: The golgi complex (G) becomes well organized as the flagellum matures. (Sample Date: May, 1978; Photo Magnification: 4,320 X; Negative #: 2600) Marker = 1 μm.

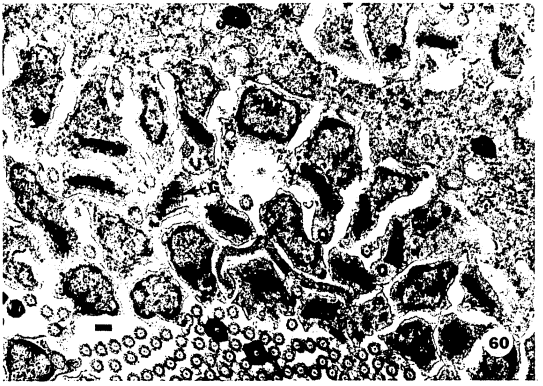












centrally located between the basal part of the nucleus and the centriole (Figure 61). This area is the neck region of the sperm and separates the head from its midpiece (Figure 61). Added support for a single mitochondrion in the neck region of the mature sperm is seen in Figure 62. This figure depicts a transverse section through the neck region with only one mitochondrion observable.

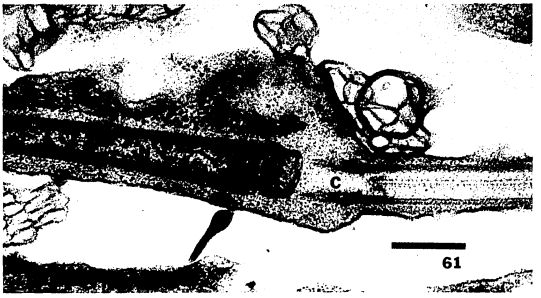
Throughout this early maturation process the nucleus has become smaller but still remains circular or spherical in shape. The original diameter of the nucleus, prior to the beginning of spermiogenesis, is around 2 μm. Subsequently, the nucleus begins to reduce and condense to a size of a little over 1 μm in diameter before the nucleus elongation process begins (Figure 59). The chromatin remains fairly diffuse throughout the nucleus through the initial reduction in overall size. Nuclear elongation is a complex process that appears to go through the stages of a manchette formation, continued condensation and reduction of the chromatin, and subsequent disappearance of the manchette.

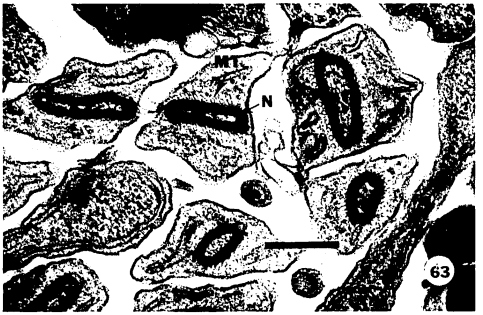
The formation of the manchette is indistinct for the Type II sperm. This is contrasted with a very distinct manchette in the Type I sperm (Figure 33, p. 96). Figure 63 shows an observable manchette in the Type II sperm. The microtubules making up the manchette measure approximately 22.5 nm in diameter. They are aligned equidistantly around the nucleus. The microtubules run on the long axis of the sperm. Whether or not the manchette is involved in the elongation process of the nucleus is not well under-

Figure 61. Type II Sperm Spermiogenesis: The neck region consists of a centriole (C), a single mitochondrion (M) located adjacent to the basal region of the nucleus (N). (Sample Date: September, 1977; Photo Magnification: 19,654 X; Negative #: 2639) Marker = 1 μm.

Figure 62. Type II Sperm Spermiogenesis: A transverse section through the neck region confirms a single mitochondrion (arrow). (Sample Date: May, 1978; Photo Magnification: 19,654 X; Negative #: 2633) Marker = 1 μm.

Figure 63. Type II Sperm Spermiogenesis: The manchette consists of microtubules (MT) around the nucleus (N). (Sample Date: September, 1977; Photo Magnification: 19,654 X; Negative #: 2669) Marker = 1 μm.





stood at this time. However, the association of the manchette with nuclear elongation is apparent.

The nucleus elongates to approximately 5 μm. At the apex, the nucleus is narrow and pointed (Figure 64). This apical region of the nucleus subtends the acrosome (Figure 64). The nucleus is approximately 87.0 nm in diameter. From this point the nucleus widens to approximately .320 μm about half way down the structure to the basal area adjoining the neck region. As seen in Figure 65, the nucleus also flattens with the long axis approximating .320 μm in diameter, and the short axis .100 μm. During the elongation phase the nucleus undergoes three distinct changes. The first as previously described is a diffuse arrangement of the chromatin throughout the nucleus (Figures 53, 57, 59, 60). The next change occurs as condensation of the chromatin into a granular arrangement throughout the elongating nucleus (Figure 61). The granulation appears to be coarse and not uniform in distribution within the nucleus. A partially electron dense matrix is seen in the background (Figure 61). A narrow electron dense outer layer of the nucleus is observed at this time (Figure 61). This outer region of the nucleus measures approximately 22.5 nm in width. In the final stages of sperm maturation, the nucleus becomes electron dense and therefore, uniformly dark throughout (Figures 64, 65).

The acrosome located at the tip of the nucleus is a narrow rodshaped structure measuring approximately .363 μm in length and 76.0 nm in diameter (Figure 64). The structure as seen in Figure 64 is less electron dense than the surrounding cytoplasm.

Figure 64. Type II Sperm Spermiogenesis: The head region of the nearly matured sperm consists of an electron dense nucleus (N) and a distinct acrosome (A). (Sample Date: March, 1978; Photo Magnification: 9,647 X; Negative #: 2456) Marker = 1 μm.

Figure 65. Type II Sperm Spermiogenesis: The midsection of the nucleus (N) as seen in transverse section is flattened and appears rod shaped. (Sample Date: May, 1978; Photo Magnification: 19,654 X; Negative #: 2587) Marker = 1 μm.

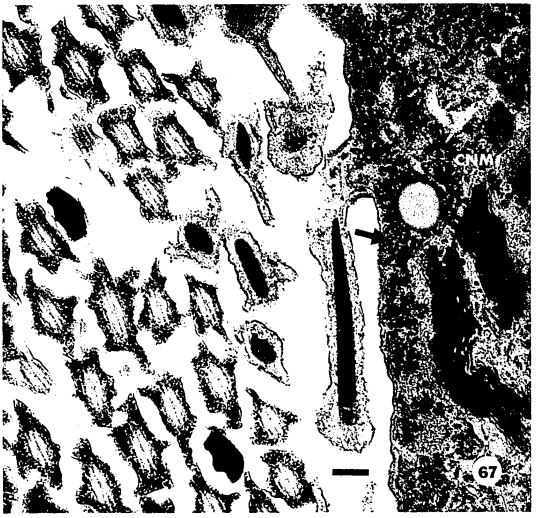
In summary, the acrosome, nucleus and surrounding cytoplasm compose the head region of the Type II sperm (Figure 64). The head region is appoximately 6 μm long and is approximately .350 μm wide at the widest point along this region.

The midpiece subtends the neck region and is approximately 65 μm long. This region is characterized by monoparticulate and clumped particulate electron dense granules located in the peripheral regions of the flagellum structure (Figure 66). These granules are not seen within the central microtubule core of the flagellum (Figure 67). The monoparticulate granules measure approximately 17.2 nm in diameter. The appearance of these granules was seen after the flagellum had developed and after the head region had reached an advanced maturation state—condensation and elongation of the nucleus with a developed manchette. At this stage of development the sperm is still attached to the central nutritive mass (Figure 67). Within the central nutritive mass are monoparticulate granules of approximately the same size as those found in the midpiece of the maturing sperm. The granules measure approximately 18.2 nm in diameter. Whether or not these granules are the same as those found in the midpiece has not been determined. However, an association of the two granules was observed. The granules are consistent with the size of glycogen granules.

The granulation of the midpiece is extensive (Figure 66). Of particular note is the fact that there appears to be a region between the neck and the midpiece that is without the granules (Figure 66).

Figure 66. Type II Sperm Spermiogenesis: Monoparticulate and clumped particulate electron dense granules are observed throughout the midpiece. These granules are probably glycogen (GL). (Sample Date: March, 1978; Photo Magnification: 9,647 X; Negative #: 2459) Marker = 1 μm.

Figure 67. Type II Sperm Spermiogenesis: Maturing sperm (arrow) are attached to the central nutritive mass (CNM). Note the electron dense granules within the central nutritive mass (arrow). These granules are approximately the same size as those observed in the transverse section of the midpiece located in the left portion of the photo. These granules are probably glycogen. (Sample Date: June, 1976; Photo Magnification: 9,431 X; Negative #: 1213) Marker = 1 μm.



This region without granules seems to vary from one sperm to another. The granules, as they become greater in numbers, seem to distend the outer membrane of the flagellum to a diameter of approximately .280 μm.

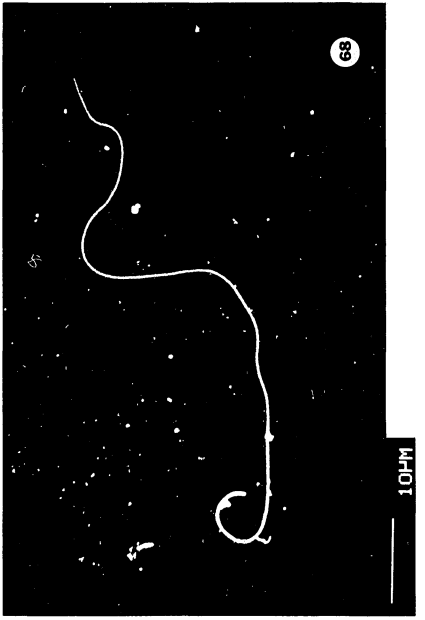
The tail region is short and is observed as the end piece of the mature sperm (Figure 68). The tail region is identical to the developing flagellum described in Figure 57 (p. 118). The tail is approximately .224 μm in diameter.

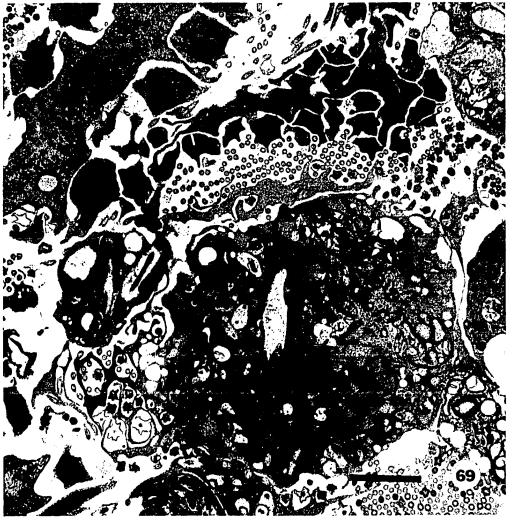
The maturation of the sperm takes place within the sperm sac with an individual sperm attached to the central nutritive mass. As spermiogenesis progresses, unneeded cytoplasm including membranes, organelles as well as unneeded nuclear materials is extruded into the sperm sac from the maturing sperm. Figures 59 (p. 119) and 61 (p. 122) show the release of unnecessary cell products into the sperm sac. These discarded cell products are absorbed by a phagocytic cell that is easily observed within the sperm sac (Figure 69). The properties of this cell and its origin are not understood at this time.

The mature sperm, consisting of head, neck, midpiece and tail regions, is released from the central nutritive mass and stays within the sperm sac until such time that the sperm is deposited into the spermatheca. Figure 52 (p. 113) shows mature Type II sperm ready to be released from the central nutritive mass.

Figure 68. Type II Sperm Spermiogenesis: The mature Type II sperm is shown. (Sample Date: June, 1978; Photo Magnification: 2,300 X; Negative #: 71)

Figure 69. Phagocytic Process: A phagocytic cell (PH) is commonly observed within the sperm sac. The cell appears to engulf discarded cell debris that arise during the maturation of the two sperm types. (Sample Date: June, 1976; Photo Magnification: 1,872 X; Negative #: 1196) Marker = 10 μm.





Development of the Sperm Sac, Sperm Ducts and the Penis Sheath

Initial findings suggest that the development of the male reproductive structures occurs from a glandular cell that appears to originate from the chloragogenous region (Figure 70). These cells are observed within the clitellar regions as an undifferentiated mass during the early development of the male structures (Figure 70). Such masses of undifferentiated cells are observed fairly commonly during the early part of the reproductive season. It appears that the sperm sacs are the first structures to develop in association with the release of the sperm morula from the inner regions of the cell mass (Figure 6, p. 55). The cell mass appears to differentiate into two regions: one region developing and releasing sperm morula and the other region serving to form the confines of the sperm sac. The mature sperm sac with developing sperm morulae is seen in Figure 5 (p. 52).

The sperm ducts appear to develop from the same undifferentiated cell mass that begins to appear as tubules along the clitellum (Figure 71). Differentiation of the ducts changes the appearance of the undifferentiated cells into the outer and inner layers of the ducts (Figure 72).

The penis formation occurs with a localization of undifferentiated cells taking the formidable penis sheath shape (Figure 73). Differentiation occurs over a period of time with growth of the calcareous penis structure in lengths (Figure 74) until it is mature (Figure 72).

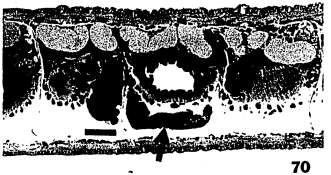
Figure 70. The mass of glandular cells (arrow) appear to originate near the chloragogen tissue. The primordial cells are first seen during the early development of the male genitalia. The cells are in segment 10. (Sample Date: November, 1976; Photo Magnification: 80 X; Stain: Toluidine Blue) Marker = 100 µm.

Figure 71. The sperm ducts appear to arise out of the same undifferentiated cell mass that begins to appear as tubules (arrow). (Sample Date: September, 1977; Photo Magnification: 80 X; Stain: Hematoxylin/Eosin) Marker = 100 µm.

Figure 72. Differentiation of the ducts changes the appearance of the undifferentiated cells into the outer and inner layers of the ducts (arrow). Penis sheath (PS), Male pore (MP). (Sample Date: June, 1976; Photo Magnification: 200 X; Stain: Hematoxylin/Eosin) Marker = 100 µm.

Figure 73. The penis sheath is formed initially by the primordial glandular cells that appear to secrete the chitinous substance that makes up the mature penis (arrow). Male pore (MP). (Sample Date: September, 1977; Photo Magnification: 200 X; Stain: Hematoxylin/Eosin) Marker = 100 µm.

Figure 74. The penis sheath continues to be formed from the secretions of the cells surrounding the structure (arrow). Spiral muscles (SM). (Sample Date: September, 1977; Photo Magnification: 200 X; Stain: Hematoxylin/Eosin) Marker = 100 µm.



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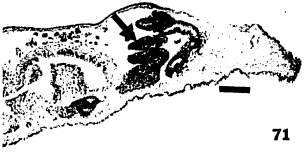

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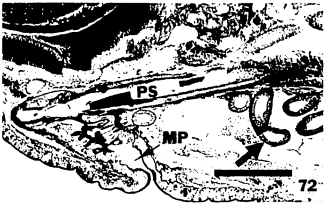

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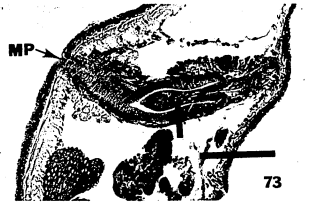
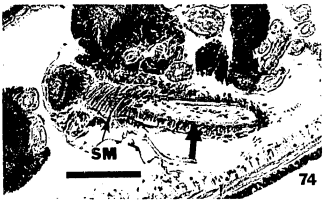




Sperm Transfer

Both Type I and Type II sperm appear to be transferred from one worm to another through an intimate contact. It appears that the penis erects and is positioned within the spermatheca for the sperm transfer. Erection of the penis was observed in Figure 75. Observations also suggest that the penis sheath may be left within the spermatheca at the completion of the sperm transfer.

Eggs, Nutritive Granules and Cocoons

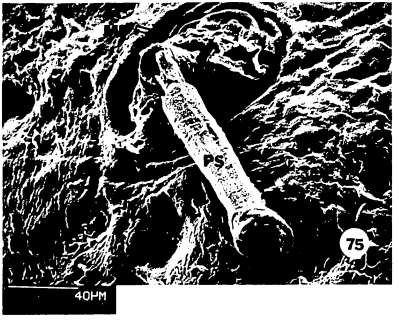
The entire process of fertilization, egg development, cocoon development does not appear to be understood fully. This area of research will require extensive study beyond the scope of the present work. However, certain findings should be presented since they contribute to current knowledge.

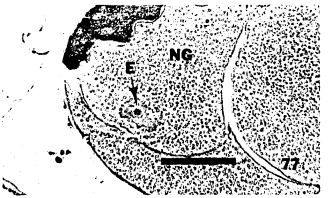
Nutritive granules made up the major portion of the egg sac (Figure 76). These granules are stained very dark with osmium suggesting a lipoprotein structure that would be consistent with a nutritive function for these granules. The granules are otherwise amorphous. The nutritive granules begin to appear in great numbers around the middle of the reproductive period (Figure 4, p. 46). Towards the end of the reproductive period eggs will begin to appear (Figure 77). The egg occupies a space within the egg sac surrounded by nutritive granules. In some unknown way, the egg with surrounding nutritive granules becomes organized into a single spherical mass

Figure 75. The penis sheath (PS) becomes erect and can be seen as it emerges through the male pore (MP). (Sample Date: April, 1978; Photo Magnification: 475 X; Negative #: 16)

Figure 76. The nutritive granules (NG) are found within the egg sac. These granules occupy a greater portion of the egg sac along several segments within the clitellum area. (Sample Date: April, 1978; Photo Magnification: 32 X; Stain: Toluidine Blue) Marker = 100 μm.

Figure 77. The egg (E) is surrounded by the nutritive granules (NG) within the egg sac. (Sample Date: May, 1978; Photo Magnification: 200 X; Stain: Hematoxylin/ Eosin) Marker = 100 μm.





(Figure 77). Whether or not fertilization of the egg has occurred is not known at this time. The spheroid becomes membrane bound which may suggest that fertilization of the egg has occurred. As many as six of these egg containing spheroids become organized and, in some unknown way, become organized into a cocoon (Figure 78). The cocoon is an eggshaped structure with two nipples on opposite ends of the structure (Figure 78). The nipples are plugged with what appears to be a glycoprotein material (Figure 79). Within the cocoon can be observed the development of individual worms (Figure 80). As the worms develop the nutritive granules are apparently consumed. Mature sperm or sperm products were not observed within the cocoon structure.

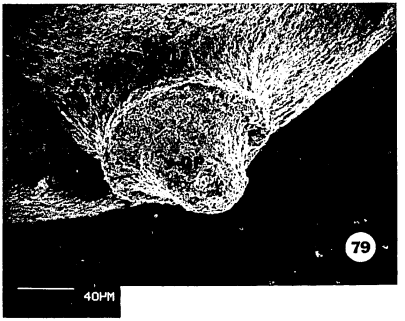
After the worms have matured, they will exit through one of the nipples. The glycoprotein plug has been dissolved allowing the young worms to exit.

Figure 78. The cocoon is an egg shaped structure with two nipples (NI) on either end of the structure. Within the cocoon are usually six spheroids containing a single egg surrounded by nutritive granules. (Sample Date: April, 1978; Photo Magnification: 32 X; Stain: Glutaraldehyde/ Osmium tetroxide) Marker = 100 μm.

Figure 79. The nipple (NI) of the cocoon is plugged with what appears to be a glycoprotein material (GP). (Sample Date: April, 1978; Photo Magnification: 375 X; Negative #: 3)

Figure 80. The young worms develop within the cocoon. Towards the end of the developmental period the plugs within the nipples are dissolved and the worms exit through the opening. (Sample Date: April, 1978; Photo Magnification: 32 X; Stain: Glutaraldehyde/ Osmium tetroxide) Marker = 100 μm.

78



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DISCUSSION

This study was designed with the following objectives:

1. To investigate the reproductive activity of *Limnodrilus hoffmeisteri* under field conditions by analyzing water chemistry and selected physical parameters as they may be related to observed morphological development of the reproductive organs.
2. To describe in detail the morphological changes that occur during spermatogenesis using observations made with light microscopy, transmission electron microscopy, and scanning electron microscopy.
3. To compare morphologically the two pathways of sperm development and to determine how the resulting sperm differ.

These objectives serve as the guidelines for the development of the discussion section. In general, the observations and findings of the present study accomplished the tasks set forth in the objectives. The accumulated evidence presented in the results section offers additional possibilities for an understanding of the reproductive process of *L. hoffmeisteri* as it is related to its environment.

The Reproductive Season for *Limnodrilus hoffmeisteri*

The observations that follow were made from monthly samples. This procedure appears to be one of the first to examine reproductive activity at a cellular level from samples taken from the environment. Kennedy (1966) measured sexual maturity by examining the thickened clitellum of *L. hoffmeisteri* and concluded that a single intense breeding activity in certain study areas occurred in early spring. The observation of the thickened clitellum would not allow the researcher to examine the development of the male and female organs which develop prior to the thickening of the clitellum.

The results suggest that the reproductive season for *L. hoffmeisteri* inhabiting the Little Calumet River (41° 34' 22" N, 87° 28' 30" W) occurs from early fall to late spring. This process begins with the development of the reproductive organs. Evidence indicates that the male structures are the first to develop. A glandular appearing tissue appears to form in the region of the chloragogen tissue within the clitellar segments (Segments 9-13). This tissue appears to be involved not only in the formation of the sperm sac but also in the release of sperm morulae that will mature within the sperm sac. Additionally, this primordial tissue appears to be involved in the development of the sperm ducts and ultimately the formation of the penis sheath through which the sperm is transferred to a spermatheca. The development of the male organs occurs within the early months of the reproductive season. Although the

development of the female organs was not studied in detail, a few observations suggest that these structures mature near the middle of the reproductive season with the appearance of the egg sac with nutritive granules. Egg development appears to occur near the end of the reproductive season. The end of the season is marked by the appearance of cocoons that contain developing worms. The end of the season is also marked by the decline in numbers of the sperm products. During this period of time one observes the major reproductive activity during the year. A low level of reproductive activity is observed during the rest of the year. This activity is marked by the appearance of sperm products as well as the appearance of nutritive granules. However, developing eggs were not observed during the period of low reproductive activity. Cocoons with developing eggs were not seen even though there is the suggestion that a low level of reproduction was occurring.

The evidence suggesting a single reproductive season for tubificid worms in temperate climates is found in a number of sources. Poddubnaya (1959) suggested a single reproductive peak from May to July as measured by cocoons. Dixon (1915) and Mehra (1926) observed sexual maturity in *Tubifex tubifex* from autumn months to the spring months. Kennedy (1966) reported that *L. udekemianus* would reach peak fecundity in late winter to early spring. All these findings suggested some variation in reproductive activity during these periods. However, the evidence for *L. hoffmeisteri* as reported in the Results section and the observations

of the above researchers indicate that major reproductive activity occurs between fall and spring.

It also appears that environmental factors may be involved in regulating this activity. But, the environmental factors that may regulate the reproductive process in *L. hoffmeisteri* are not defined. The chemical data as presented in the Results section did not appear to interfere with the developmental stages of the reproductive season. It is assumed that the chemical and physical phenomena that were observed do not interfere with reproductive activity for *L. hoffmeisteri*. Previous reports by Kennedy (1966), Grigelus (1961), Poddubnaya (1959), Timm (1962), and Matsumoto, Yamamoto (1966) have indicated that breeding activity ceases or is interrupted by decreasing or increasing temperatures. These reports indicate that reproductive activity is interrupted at temperatures below 8°C - 12°C. The present findings suggest that water temperature may have an influence at these lower temperatures but does not interrupt the entire process. The observations suggested that mitotic or meiotic activity of sperm morulae may be reduced when they are subjected to lowered water temperature. These observations were recorded during the December, January, and February collections. However, oxygen levels were at their lowest recorded levels during the same months, a factor that could also influence the ability of cells to divide since it is assumed that oxygen would be necessary for energy transport mechanisms. Further examination of the data indicates that during this same period the worms were

actively producing nutritive granules within the egg sac of the female organs. This may suggest that the worms were physiologically channeling energy into the production of the nutritive granules since the sperm morulae were already well developed. The influence of seasonally lower water temperature as well as lower oxygen levels on the reproductive activity within *L. hoffmeisteri* are not well understood from the information available from the present study. It is reasonable to assume that the influence may be minimal since these measured parameters did not appear to interrupt or stop the reproductive process.

In summary, the reproductive period for *L. hoffmeisteri* as observed in the Little Calumet River (41° 34' 22" N, 87° 28' 30" W) occurs from early fall through late spring. The period is marked by the development of the reproductive organs in the early fall months, followed by the development of sperm products within the sperm sac. The female organs appear to mature in middle to late winter with the appearance of nutritive granules followed by the developing eggs in early spring. This cycle is completed in early to late spring with the appearance of cocoons containing developing embryos. It is also apparent that a low level of reproductive activity occurs throughout the rest of the year. This is usually observed in worms with developed sperm sacs and sperm products, having matured penis and some developed egg sacs with nutritive granules. Eggs have not been observed during this low level of reproductive activity.

A Reexamination of Characteristics Used to Identify *Limnodrilus hoffmeisteri*

The morphologic characteristics used to identify *L. hoffmeisteri* were presented in the Review of Literature (p. 7) and hence will not be repeated here. Information regarding the stages of penis development of *L. hoffmeisteri* and possible mistaken identification with that of another species of *Limnodrilus* will be presented.

During the development of the reproductive organs of *L. hoffmeisteri*, it was shown that the penis undergoes different stages of development (Figures 73, 74, p. 139); that is, the penis gradually elongates with the deposition of the chitinous sheath by glandular appearing cells that take the form of the penis. The matured penis (Figure 72, p. 138) is characteristic of the worm and serves to identify the species. The formation of the penis was studied with 5 μm serially sectioned worms in order to observe the development of the sheath. It became clear that the sheath develops gradually with a cellular demarcation of the entire length of the mature penis sheath within the clitellar region. The penis sheath develops slowly and deposition of the chitinous material begins at the base of the penis structure, gradually elongates and achieves the appearance of the matured penis.

This developmental process appears to have led to an identification problem concerning two species of *Limnodrilus*: *L. hoffmeisteri* and *L. profundicola*. Brinkhurst (1965, 1971) in describing *L. profundicola* bases the distinguishing difference between the two worms

on the size of the penis sheath. *L. profundicola* is said to have a penis up to seven (7) times longer than broad, while *L. hoffmeisteri* has a penis up to 14 times longer than broad. Aside from this distinction the two worms are relatively the same in structure. Also both are said to live in a similar habitat.

Whole worms are usually used for identification since this saves time. Consequently, this process was used in the present investigation. A number of *L. profundicola* were identified in the early and late fall collections, the period marking the beginning of the reproductive period for *L. hoffmeisteri*. Subsequent collections failed to identify *L. profundicola*. All the worms were identified as *L. hoffmeisteri* or *Tubifex tubifex*. This information suggested that a closer examination of the earlier *L. profundicola* identifications should be made. Serially sectioned worms that would be identified as *L. profundicola* indicated that the early stages of penis development were occurring. This was marked by glandular-like cells that apparently are involved in laying down the chitinous material of the penis sheath. These cells would not be identified when examining whole worms through a microscope. This information suggests that *L. profundicola* is an immature *L. hoffmeisteri*. These conclusions are based on the fact that the penis sheath is the only major observable difference between the two worms and that this identification of *L. profundicola* was made only during the early months of the reproductive season for *L. hoffmeisteri* in which it has been shown that the reproductive organs are being formed.

Tubificidae Spermatogenesis, An Overview

A clear understanding of spermatogenesis within the tubificid worms has not been attained. The evidence presented in the Results section has answered many questions that have been alluded to in the past. These results and the interpretation of the results will, in the future, require a careful review by other investigators in order to ascertain if the present interpretations are correct since these interpretations are not generally supportive of the findings of most of the previous work that was examined (see Review of Literature, p. 8).

The Testicle or Testicular-like Tissue

Published accounts have described a testicle that is responsible for releasing the morulae into the sperm sacs (Brinkhurst, 1971). The sperm morulae were said to migrate upon release from this structure throughout the length of the sperm sac that often occupies segment 9 through 14 when fully developed. Questions as to how a single testicle could produce so many morulae were posed by Dixon as early as 1915. She noted that the tremendous numbers of sperm morulae coming from a single testicle would be quite a feat for such a small structure. However, she lacked the evidence to support the claim that the sperm morulae do not come from a single testicle but are formed and released from a glandular appearing group of cells that appear to originate in the region of the

chloragogen tissue. This study has presented evidence (Figure 6, p. 55) that shows the release of sperm morulae from a glandular appearing group of cells. These same cells have been observed to form the sperm sac boundaries and are associated with the formation of the male genitalia. The interpretation for the evidence gathered in the present study suggests that the testicle or testicular-like structure that is observed in mature worms is not actually involved in producing and releasing sperm morulae into the sperm sac. This process was accomplished during the early stages of gonadal development in the way described above. The remaining testicular-like tissue is probably a remnant of the overall process.

The Sequence of Spermatogenesis

The release of a morula containing a given number of cells from the testicular-like glandular cells is in agreement with previously published studies related to the clitellate oligochaetes. The number of cells in a released morula does vary from published accounts. Mehra (1926) reported that 6-10 cells would make up the morula while Dixon (1915) suggested that a single cell was released. Hatai (1900) and Bugnion and Popoff (1905) reported an 8 cell morula would be released. These findings are partially in agreement with the observations reported in the present study. The major difference is that two distinct types of sperm develop concurrently within the same sperm sac. Repeated observations of the sequence of events for the two sperm types suggest that a four (4) cell morula

and an eight (8) cell morula is released representing the Type I and Type II sperm respectively.

Additional clarifications are needed with respect to the developing morulae after they are released into the sperm sac. Two events have been reported to occur in succeeding developmental stages. Dixon (1915) believed that the development of the morula occurred with several nuclear divisions from the single cell that she reported was released into the sperm sac. Mehra (1926) opposed this viewpoint and suggested from his studies that the morula develop with succeeding cellular divisions. Careful examination of available data suggests that both events — cellular divisions and nuclear divisions — occur within the spermatogenesis sequence but not necessarily in the ways described by either Dixon (1915) or Mehra (1926).

The Type I sperm undergoes four (4) cellular divisions from the four (4) cell stage. The resulting 32 cells lose their distinct cell characteristics when the cell membrane disappears. At this point, there are 32 nuclei that will now divide. Two nuclear divisions follow, and the morula contains 128 nuclei located on the periphery of the morula sphere.

A similar process occurs for the Type II sperm. The first stage is marked by an eight (8) cell morula that undergoes three (3) cellular divisions. At this time the morula is composed of 64 cells. During this stage of development the 64 cells will transform to 64 nuclei within the morula sphere. Four (4) nuclear divisions follow

until the morula contains approximately 1024 nuclei located on the periphery of the morula structure. Correll (1969) suggested that the maximum number of nuclei was in excess of 500. Certainly, the nuclei in the last three developmental stages of the Type II sperm are difficult to count accurately. However, careful and repeated examination of the samples has indicated that there are indeed nearly 1024 nuclei that will undergo spermiogenesis.

Cellular and nuclear divisions are clearly observed in the spermatogenic sequence for *L. hoffmeisteri*.

The Beginning of Spermiogenesis

The last developmental stage prior to the beginning of spermiogenesis is the 128 nuclei stage for Type I sperm and the 1024 nuclei stage for the Type II sperm. An examination of the literature suggests that for clitellate oligochaetas the last developmental stage prior to the beginning of spermiogenesis is the 128 cell stage (Anderson, *et al*, 1967; Grassé, 1959; Ferraguti and Lanzavecchia, 1971). Certainly, the findings of Type I sperm are in agreement with these reports. Ferraguti and Lanzavecchia (1971) depicted a morula (=cytophorous) that contained more than the 128 nuclei for *Limnodrilus* and *Tubifex*. Although no counts of the developing sperm were presented in their report, it is assumed that these morulae are comparable to the Type II sperm 1024 nuclei stage and to the 500+ stage reported by Correll (1969).

Spermiogenesis

The process of spermiogenesis has been reported to go through three distinct stages within the animal kingdom (Franzen, 1956, 1974, 1977; Alfzelius, 1972; Fawcett, *et al*, 1971; Yasuzumi, 1974). These stages include (1) cell elongation, (2) reduction of both nucleus and cytoplasmic volume, and (3) the formation of distinct cell compartments within the mature sperm-head, midpiece, tail. The data from the present study agree with these findings. Both Type I and Type II sperm have been shown to follow this pattern of development. Since the findings are well documented in the Results section (p. 86), these developmental stages will not be described in this section.

Ferraguti and Lanzavecchia (1971) described spermiogenesis for *Limnodrilus udekemianus* and *Tubifex tubifex* with major emphasis on the manchette development and microtubules within the developing sperm. The findings of Ferraguti and Lanzavecchia (1971) are similar to those for the Type I sperm described for *L. hoffmeisteri*. A notable difference is observed during the nuclear condensation and elongation stage during which a set of dense microtubules (Figure 33, p. 96) is observed associated with the inner nuclear membrane. These dense microtubules appear to be associated with the microtubules that make up the manchette. This relationship is not currently understood. The dense microtubules become obscure as the nucleus continues to elongate and condense. The observations that the microtubules are associated with the twisting of the nucleus into a helix for the Type I sperm is similar to that reported by Ferraguti and Lanzavecchia (1971).

Perhaps, another question that remains regarding the maturation of the Type I sperm is the lack of an acrosome. Since an acrosome was not observed, this characteristic may be useful in distinguishing between the Type I and Type II sperm.

Aside from the association of the Type I sperm with the reported findings of Ferraguti and Lanzavecchia (1971), additional studies were not found that describe a similar process of spermiogenesis for the Type II sperm. It should be noted that the observations made by Correll (1969) in an unpublished master's thesis included a description of a single sperm type for *Peloscolex multisetosus*. A review of the results of the thesis suggest that the findings included two sperm types instead of one. This will be discussed in the next section.

Evidence of Dimorphism — Type I and Type II Sperm

Perhaps, the most important observation made from the present study is the development of two distinct sperm types within a single organism. The evidence for such a process is presented in the Results section and will not be repeated here except where certain points need clarification.

Previous reports have described aberrant, giant, oligopyrene, apyrene sperm types throughout the animal kingdom (Yasuzumi, 1974). In general, these sperm types have been described as nonfunctional and the results of, as an example, polyploidy. Published evidence

that suggests a dimorphic development of different sperm types within a single organism has not been yet found.

It should be noted that the controversy of sperm dimorphism has existed for some time (Dixon, 1915). The literature abounds with descriptions of aberrant sperm types within many groups of organisms (Yasuzumi, 1974). The clitellate oligochaetes are no exception and are reported to have aberrant sperm that probably are not functional in the reproductive process (Tuzet, 1946). Aberrant is used as a collective term for the many descriptive words given to these sperm. These descriptive words include, among others, giant, apyrene and oligopyrene.

Within the family Tubificidae, a number of reports exist that describe aberrant sperm within the sperm sac. Historically, Dixon (1915) appears to be the first to document the occurrence of two sperm types within *Tubifex rivulorum* (=*tubifex*). The two sperm were easily distinguished as one was larger than the other. Dixon tried without success to establish the developmental sequences of the two sperm. She suggested that the two sperm represented a true process of sperm dimorphism within the animal kingdom even though the developmental process of the two sperm was not established. Dixon did establish that the two sperm had distinct head, midpiece and tail regions.

Mehra (1926) dismissed Dixon's view of sperm dimorphism within the family Tubificidae. Mehra reported that the large sperm was apyrene or without a nucleus and did not have the three distinct

regions of a mature sperm-head, midpiece, and tail. This suggested that the sperm would be nonfunctional. It was also argued that the large sperm was not found in great numbers and probably represented some sort of degenerative process in sperm development.

Correll (1969) reported finding a giant sperm within the sperm sac of *Peloscolix multisetosus*. This sperm type was simply dismissed as a process of polyploidy and was not discussed.

According to the results of this study, Dixon (1915) was probably correct in suggesting that the two sperm types within *T. rivulorum* (=*tubifex*) represent sperm dimorphism. The evidence appears to indicate clearly that this is the situation for *L. hoffmeisteri*. The developmental stages of the two sperm types have been established for *L. hoffmeisteri* (Figure 7, p. 57). The data also suggest that the Type I and Type II morulae are released from a common primordial tissue within the developing sperm sac (Figure 6, p. 55). Ultrastructural examination of the two sperm types appear to establish clearly that each sperm type has a head, midpiece and tail region. This agrees with the findings of Dixon (1915) and disagrees with those of Mehra (1926). Within the head region of the large sperm or Type I sperm of *L. hoffmeisteri*, a nucleus is clearly seen (Figure 38, p. 102). From the evidence presented, it is concluded that the development of the two sperm types within the sperm sac of *L. hoffmeisteri* is a process of sperm dimorphism. Both sperm types appear to be functional since it is shown that each type matures with distinct head, midpiece and tail regions. These

findings suggest that a reevaluation of the existence of two sperm types within members of the family Tubificidae should be undertaken in order to understand better the reproductive processes within these organisms. Such investigations will require an indepth study following the same or similar methodology used in this study.

Type I Sperm and Type II Sperm — A Comparison

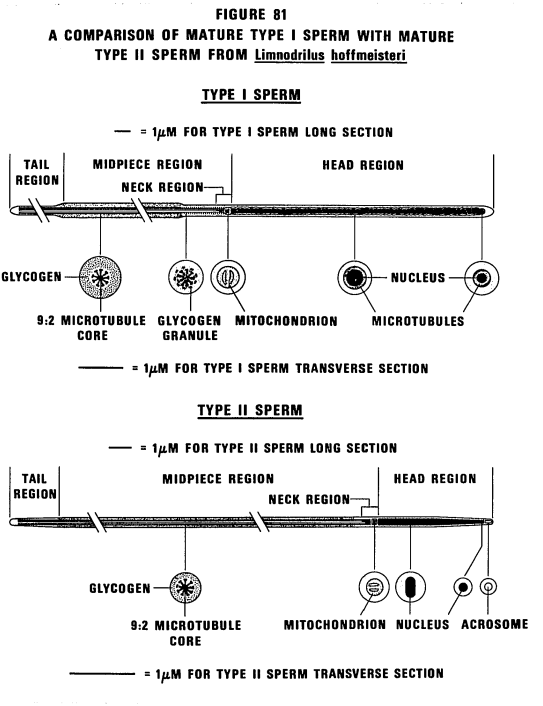
The terms Type I and Type II have no real functional relationship to the two sperm observed in *L. hoffmeisteri*. The designation of Type I is given to the sperm that has the fewer developmental stages prior to spermiogenesis. The Type II designation is simply given to the other sperm type.

The Type I and Type II sperm are compared here in order to better understand the similarities and differences between the two sperm. The Type I sperm undergoes six developmental stages prior to spermiogenesis while the Type II sperm undergoes eight developmental stages prior to spermiogenesis. Obvious differences are seen in the size and shape of the developing morulae (Figure 7, p. 57).

The sequences of spermiogenesis are similar for both sperm types. Both sperm types undergo cell elongation, reduction of both nucleus and cytoplasmic volume, and form distinct cell compartments within the sperm-head, midpiece and tail.

The mature Type I and Type II sperm are similar with the exception of the overall sizes and major differences within the head region.

FIGURE 81
A COMPARISON OF MATURE TYPE I SPERM WITH MATURE TYPE II SPERM FROM Limnodrilus hoffmeisteri



Head

The Type I sperm has a helically arranged nucleus (Figure 81) whereas the Type II sperm has a flattened elongated nucleus that is pointed at the apex (Figure 81). The size and shape of the nucleus is the difference that distinguishes between the Type I and Type II sperm within the head region. The Type I sperm does not appear to have a morphologically identifiable acrosome (Figure 81) whereas the Type II sperm does (Figure 81).

Midpiece

The midpiece for both sperm types consists of the neck as well as a region extending down the flagellum marked by an accumulation of glycogen granules that surround the central core of microtubules. The microtubules are arranged in the typical 9:2 pattern. In the neck region, both Type I and Type II sperm have a single mitochondrion that subtends the nucleus. Immediately below the mitochondrion is the centriole from which the central core of microtubules originates. A short distance from the centriole, glycogen granule accumulations are observed. This accumulation of glycogen is observed along the major portion of the flagellum and marks the midpiece area. The major difference between the Type I and Type II sperm is seen in the length and width of the midpiece (Figure 81).

Tail

The tail is short for both types of sperm. The tail region is marked by a lack of glycogen granules as seen in the midpiece (Figure 81). The central core of microtubules is arranged in the 9:2 pattern for both types of sperm. Within the tail region, the major difference between the two sperm is the length and width (Figure 81).

In summary, the Type I and Type II sperm are similar with respect to the three regions of the mature sperm. The major differences between the two are seen in the head region in which the nuclei are obviously different in size and shape. There appears to be a lack of an acrosome in the Type I sperm whereas the Type II sperm has a distinct acrosome. Other obvious differences are simply size relationships including length and width of the three regions of the mature sperm.

CONCLUSIONS

1. The reproductive season for *Limnodrilus hoffmeisteri* from the Little Calumet River, Hammond, Indiana (41° 34' 22" N, 87° 28' 30" W) occurs from early fall until late spring. The season begins with the development of the reproductive organs during the fall months and is completed in late spring with the appearance of cocoons.
2. A low level of reproductive activity, marked by the appearance of reproductive organs, sperm products and nutritive granules, was seen throughout the remaining months of the year. During these times eggs were not seen. Therefore, it is believed that the worms were not reproducing during these months.
3. The reproductive season for *L. hoffmeisteri* suggests that certain environmental factors may regulate the reproductive activity within a population of worms. However, these factors were not identified from the data collected. An analysis of chemical and physical data used in this study failed to suggest recognizable effects on the reproductive behavior. Possible exceptions were seasonally lower water temperature and oxygen levels. During this time period (December through February) mitotic (or meiotic) activity of sperm morulae was low. At the same time, formation of nutritive granules increased. Seasonally lower water temperature and oxygen levels did not interrupt the reproductive process once it was initiated.

4. The development of the penis sheath occurs gradually with elongation of the chitinous structure. Such a short penis sheath is the species characteristic for *L. profundicola*. The present evidence recognizes that penis sheath appears during the early months of the reproductive season for *L. hoffmeisteri*. It is concluded that the shortened penis sheath indicates an immature form of *L. hoffmeisteri* and that the use of the character to describe *L. profundicola* is incorrect.
5. A primordial grouping of cells that appears to originate from near the chloragogen tissue in the clitellum segments is responsible for differentiating into the male organs. These same cells were seen to develop into the sperm sac as well as release sperm morulae into the sperm sac. It is concluded that a single testis does not serve to release morulae into the sperm sac as had previously been thought.
6. The development of two distinct types of sperm within the same organism has been established. It is concluded that the process is an example of sperm dimorphism within the animal kingdom.
7. The early developmental stages of Type I and Type II sperm undergo cell divisions. The resulting cells make up the morulae structures. The final developmental stages are observed by nuclear divisions. The central portion of the morula is described as the central nutritive mass.
8. Spermiogenesis of both Type I and Type II sperm is marked by three stages: (1) cell elongation, (2) reduction of both

nucleus and cytoplasm, and (3) the formation of distinct cell compartments within the mature sperm-head, midpiece, and tail. These stages of spermiogenesis are in agreement with those observed among most representatives of the animal kingdom.

9. Morphological differences distinguish the two sperm. The Type I sperm is wider, has a distinct midpiece containing glycogen, and is marked by a long helically arranged nucleus within the head region. The Type II sperm is longer and narrower and has a midpiece that contains glycogen. The Type II sperm nucleus is short and flattened and subtends an acrosome at the apex of the head.

10. Both Type I and Type II sperm appear to be viable, based on the morphological observations of the sperm using light microscopy, transmission electron microscopy, and scanning electron microscopy.

11. The use of the techniques described, including histological procedures, periodic sampling within the environment that the organism inhabits, chemical analysis, measurement of physical parameters, are all necessary in order to understand better the life history of *L. hoffmeisteri*. The findings of this study suggest that further research will require the use of these techniques, as well as improved techniques, in order to examine life histories of other organisms that inhabit similar environments.

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