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Cytogenic Studies of a Chesapeake Bay Population of *Molgula Manhattensis* (Dekay) (Ascidiacea: Stolidobranchia)

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CYTOGENETIC STUDIES OF A CHESAPEAKE BAY POPULATION OF MOLGULA MANHATTENSIS (DEKAY) (ASCIDIACEA: STOLIDOBRANCHIA)

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INTRODUCTION

Tunicates are marine protochordates whose importance in the evolutionary heirarchy of vertebrates is uncertain. On the basis of past research into chordate chromosomes and DNA content (Atkin et al., 1965; Ohno and Atkin, 1966; Taylor, 1967), it has been postulated that a series of gene duplications, via individual gene multiplication and chromosome polyploidization, occurred while the vertebrates were still aquatic. With few exceptions, DNA content in natural groups (e.g., placental mammals, birds, etc.) is fairly constant (Atkin et al., 1965). One exception is the fishes in which the range of DNA values is 19 - 3,540% that of the mammals (Ohno and Atkin, 1966). This wide range of values lends credence to a theory of polyphyletic origin with the lowest value (19%) possibly reflecting the original vertebrate genome. Atkin and Ohno (1967) have since reported lower values in tunicates that may reflect the original chordate genome. Fourteen chromosome number reports and one DNA content estimate are known for over two thousand species of the class Ascidiacea. Also, relationships between oogenesis and chromosome (and DNA) duplication are not known in the tunicates. This paucity of information may contribute to the uncertainty of the tunicate's phylogenetic position.

In Molgula manhattensis, cytoplasmic inclusion, accessory cell development (Burren, personal communication), and yolk formation (Crampton, 1899) during oogenesis have been studied. Conflicting data concerning the life cycle of M. manhattensis, a cosmopolitan species,

have been reported by American and British scientists (Costello et al., 1957; Millar, 1952; Grave, 1933), but no oogenic studies have been published on Western Atlantic populations. No attempt has been made to relate DNA and chromosome replication to histological changes occurring in oogenesis. The purpose of this research is to study the visual changes of gonadal cells during gonadogenesis, to record variations in total gonadal DNA quantity, and to study the chromosomes. These data are to be compared with the literature of other tunicates, protochordates, and lower vertebrates. Continuities or discontinuities in either chromosomal complement or DNA quantity are of importance to the evolutionary geneticist in supporting or opposing the proposed scheme of chordate evolution.

LITERATURE REVIEW

Gonadogenic Studies

Water temperature is believed to be the principal factor for gonadal development in temperate zone tunicates (Goodbody, 1961; Orton, 1920; Millar, 1954). Although some tunicates spawn continuously (Dybern, 1965; Millar, 1952; 1971), it is believed that most temperate species have a spawning period which becomes more distinct with a greater difference between summer and winter temperatures. Millar (1958) suggested a relationship between commencement and extent of breeding and geographical distribution of species.

During a three year study of growth relationships versus water temperature in a Scandinavian population of Ciona intestinalis, Dybern

(1965) reported gonadal activity in early February at water temperatures between 0 to 4°C. He observed mature sex cells within gonoducts by April (4 - 8°C) and larvae by May (10 - 15°C). Peak population increase occurred when the temperature stabilized near 10°C. Ciona was found to have two maximum spawning periods, from May to June (6 - 11°C) and from August to September (12 - 16°C), based on larvae retrieved from plankton samples. After the fall spawning, the gonads were cleared of eggs and sperms by phagocytic cells.

Millar (1952) studied sexual reproduction in Scottish populations of Botryllus schosseri and Diplosoma listerianum. In the former, few gonads were visible in January to March and ovaries and testes were small. Rapid gonadal development was observed by March to April and some larvae were present by late March. From May to June, gonads were most evident in the population. By late September, the colonies had returned to their overwintering state. Sexual reproduction in D. listerianum followed the same pattern with sexual inactivity occurring from January to March and active gonadal development between March to April. A sharp rise in colonies containing eggs was observed in May. Developing embryos were seen in late May and gonadal inactivity had returned by mid-July.

Millar (1954) recorded sexual development in comparison with time and water temperature in a Scottish population of Dendrodoa grossularia. From January to March, the peripheral phagocytes removed unshed eggs and sperms from the previous gonadal cycle. New oocytes increased in size and by March (7°C) the gonads reached their fullest development

and gametes were liberated. After spawning, phagocytes reappeared and resorption of residual gametes proceeded rapidly. By August to September (11°C), the gonads matured again and spawning occurred when the water temperature dropped.

Low temperature limits to spawning were reported by Millar (1954). He also proposed that there may be upper temperature limits. Histological examination of July gonads showed large, yolky eggs that were not shed, but retained and phagocytized. Millar suggested that with temperatures over 15°C ovulation was progressively less and that above 20°C was totally suppressed. The fall spawning period was the result of reduced temperatures stimulating ovulation.

Dybern (1965) and Millar (1952, 1954) are summarized and compared with the results of the present study in Table 5.

Whittingham (1967) reported the effects of intensity and wave-length of light on the shedding of gametes by Ciona intestinalis and Molgula manhattensis. Both species were found to shed shortly after exposure to light, but shedding was markedly reduced with low light intensities. The excitatory wave-length was probably between 500 and 700 nm. Since the experiments were performed with sexually mature animals and with constant water temperature, no tests were made of possible environmental factors which could initiate gonadal development.

In histological studies of Molgula manhattensis, Crampton (1899) described oocyte growth in relationship to yolk origin and egg polarity. When the primary oocyte was first distinguishable from surrounding

follicles and interstitial cells, it measured 8 - 10 μm and contained a large, vesicular nucleus (3 μm), nucleolus, and scattered granular masses. A "nucleo-albumin" or albuminous yolk-matrix originated in the cytoplasm near the nucleus. As the yolk-matrix fragmented, granular elements scattered throughout the cytoplasm. Progressive vacuolation of the cytoplasm increased cell size although no chemical alteration of cell constituents was identified. Finally, the dispersed yolk-matrix granules enlarged to yolk-spheres.

With the exception of Crampton (1899) and Loyez (1909), the prevailing theory before 1927 was that yolk arose from the mitochondria. However, Harvey (1927) reported lipoids from test-cell granules and albuminous material from Golgi apparatus were "combined or mixed" with mitochondrial metabolites around and inside mitochondria to form yolk. The yolk nucleus (yolk-matrix) was not present in very young oocytes and Harvey suggested it was secreted by the Golgi apparatus early in oogenesis. As the cell grew, the yolk nucleus enlarged and from 5 - 12 (sometimes more) new yolk nuclei appeared. Volume of yolk nuclei increased until maximum cytoplasmic staining and then the nuclei fragmented. These fragments were dispersed in the cytoplasm before yolk formation had progressed very far.

Harvey (1927) reported the nucleus was clear with a large acidophilic nucleolus in very young oocytes before yolk formation. As the oocytes grew, the nucleus enlarged rapidly but slower than the cytoplasm and nucleolus. The nuclear membrane developed long lobes and the nucleolus vacuolated. As yolk formed, the nucleus assumed its

original shape. When yolk formation ended, the nucleolus fragmented into spherical droplets. The nuclear membrane disintegrated and these droplets were scattered throughout the cytoplasm. The nucleus reformed, smaller in diameter than previously. Meiosis began and proceeded to metaphase, where it remained during egg extrusion from the ovary and until sperm penetration.

Burren (personal communication) reported oocytes of M. manhattensis passed through two stages of development. Young oocytes were surrounded by a single follicle layer until the latter part of the first stage when an outer follicle of stromal cells was formed. In nuclei of very small oocytes, Feulgen - positive DNA was detectable as a thin layer around the nuclear membrane. Considerable synthesis of RNA, proteins, mitochondria, and multivesiculate bodies occurred during this stage of development. During the second stage, yolk, which was comprised of yolk platelets, free polysaccharide granules, and lipochondria, originated from the multivesiculate bodies.

Oocyte nuclei increased in diameter from 8 - 45 μm during ovarian development. Nucleoplasm contained proteins and a sulphydryl-rich nucleolus. The latter persisted until the oocyte matured and then became elliptical and disappeared.

DNA Content Studies

Estimates of cellular DNA content may help in placing chordate classes in the phylogenetic heirarchy. Since Atkin et al. (1965) postulated that a series of gene duplications occurred while the vertebrates were still aquatic, there is special interest in DNA

studies of aquatic chordate classes.

Atkin et al. (1965) measured diploid DNA content in many chordate classes by microspectrophotometry and compared these with the human value as 100% (Table 1). Placental mammals (90 - 100% DNA content) and Aves (44 - 59% DNA content) were uniform groups. The large difference between birds and placental mammals indicated that these two groups evolved from different reptilian lineages. Within class Reptilia, two discrete DNA groups were discernable, one (order Squamata) that readily associated with Aves and the other (orders Chelonia and Crocodylia) that readily associated with placental mammals.

Ohno and Atkin (1966) reported diverse values for cellular DNA content for Amphibia (Table 1). In agreement with this is the wide range of values reported for Pisces. Both Amphibia and Pisces values tend to support a polyphyletic origin for terrestrial vertebrates from aquatics. Ohno and Atkin believed that subclass Crossopterygii and amphibians belonged to the same lineage, but because of their diversed DNA values were probably not directly related to the main vertebrate lineages. Other Pisces orders values (subclass Neopterygii) were comparable with mammalian. Order Isospondyli readily associated with reptilian orders Chelonia and Crocodylia.

Smaller DNA values have been reported for lower chordates (Atkin and Ohno, 1967). A urochordate (Ciona intestinalis) had 6% and a cephalochordate (Amphioxus lanceolatus) had 17%. From this data, Atkin and Ohno suggested that the vertebrate's foundation genome contained very little DNA and in the early stages of evolution a series of gene

TABLE 1 Chromosome Number and DNA Content of Some Chordates
(Taken from Atkin et al., 1965; Atkin and Ohno, 1967; Ohno and Atkin,1966)

	2N	% DNA (human value 100%)
Subphylum Urochordata		
Ciona intestinalis	28	6
Subphylum Cephalochordata		
Amphioxus lanceolatus	--	17
Subphylum Vertebrata		
Class Cyclostomata		
Lampetra planeri	--	38
Eptatretus stoutii	48	78
Class Pisces		
Subclass Crossopterygii		
Order Dipnoi	38	3,540
Subclass Neopterygii		
Order Isospondyli	60±	80
Order Ostarophysi	102±	52
Order Percomorphi (2 sp.)	46-48, 60	31-35
Order Microcyprini	48	19-23
Order Heterosomata (2 sp.)	48	19-23
Class Amphibia		
Order Caudata	24-28	705-2,789
Order Salientia	-----	104
Class Reptilia		
Order Squamata (6 sp.)	30,36,46	60-67
Order Chelonia (2 sp.)	52,66	80-89
Order Crocodylia	42	80-89

TABLE 1 continued

	2N	% DNA (human value 100%)
Class Aves		
Order Columbiformes	80±	44-59
Order Galliformes	78±	44-59
Order Passeriformes	80±	44-59
Order Psittaciformes	58±	44-59
Class Mammalia		
Order Rodentia (1 sp.)	17/18	90
(2 sp.)	40,44	100
Order Perissodactyla	64	100
Order Carnivora	78	100
Order Primates	48	100

duplications occurred. This probably accounted for the wide range of DNA values found in primitive Chordata and Cyclostoma.

Chromosome Studies

Table 2 lists the known ascidian chromosome reports. Variation is obvious and some may have resulted from difficulties encountered by early workers with staining techniques and extremely small chromosomes. One possible indication that some variation may not be real is that all chromosome counts reported after 1954 are multiples of 7, 8, or 9 while those prior to this date vary, at times, from these base numbers.

Colombera (1969, 1970, 1971a, b, c) and Taylor (1967) reported extreme contraction of spermatocyte bivalents in several ascidian species. In oocyte bivalents, Colombera was able to detect chiasmata and centromeres and to classify chromosomes according to number and position of chiasmata. Oocyte bivalents of Ascidiella aspersa (Colombera, 1971a) were reported as two bodies, joined by one or two chromatin bridges, ranging in length from 2.5 to 4 μm. The bivalents were classified as having one or two terminal chiasmata, or two terminal and two subterminal chiasmata. Mitotic chromosomes from embryonic tissue of A. aspersa showed 9 pairs of metacentric "autosomes", ranging in length from 3 to 6 μm.

The 8 oocyte bivalents of Phallusia mammillata (Colombera, 1971c) were classified as without chiasmata, or with one terminalized chiasma, or with one terminalized and one unterminalized chiasma. The number and position of chiasmata in bivalents from different oocytes were similar. The 8 pairs of mitotic chromosomes from embryonic tissue

TABLE 2 Ascidian Chromosome Reports Arranged by Suborders

	2N	N	Reference
Order Pleuogona			
Suborder Stolidobranchia			
Botrylloides leachi	32	16	Colombera, 1970
Botryllus schlosseri	--	7 or 8	Milkman and Therrien, 1965
Dendrodoa (=Styelopsis) *grossulari*	4	2, 4, or 8	Julin, 1893
Pyura (=Styelopsis) *microsmus*	--	8	Zwillenberg and Zwillenberg, 1954
Styela (=Tethyum) *plicata*	32	16	Minouchi, 1936a
	32	16	Taylor, 1967
Order Enterogona			
Suborder Phelbobranchia			
Ascidia mentula	--	9	Boveri, 1890
	18	9	Colombera, 1970
Ascidiella aspersa	18	9	Colombera, 1971a
Ciona intestinalis	18	9	Boveri, 1890
	--	6	Morgan, 1942
	28	14	Taylor, 1967
Phallusia mammillata	13-16	8	Hill, 1895
	--	8	Minganti, 1956
	16	8	Colombera, 1971c
Order Enterogona			
Suborder Aplousobranchia			
Clavelina lepadiformis	18	9	Colombera, 1971b
Aplidium proliferum	--	14	Zwillenberg and Zwillenberg, 1954
Sidnyum elegans	--	9	Zwillenberg and Zwillenberg, 1954

TABLE 2 continued

	2N	N	Reference
Unknown Orders			
Distalpia occidentalis	--	12	Bancroft, 1899
Polystomum integerrimum	20	10	Minouchi, 1936b

were metacentric and ranged in length from 2.4 to 5.4 μm.

Clavelina lepadiformis (Colombera, 1971b) was similar to other ascidians that had been analyzed cytogenetically in that the chromosomes were metacentric and had separating daughter kinetochores, homologs exhibited somatic association, and sex-chromosomes were absent. The 9 oocyte bivalents were classified as having one unterminalized chiasma, or one random and two terminalized chiasmata, or two unterminalized random chiasmata, or one terminalized and one random chiasmata, or one terminalized random chiasma. The chiasmata were easily detectable but varied in number from one nucleus to another.

MATERIALS AND METHODS

Ten to 15 specimens between 10 and 16 mm in length were hand collected by careful severing of the animal's stalk with a knife. Collections were made every other week from January 5, 1972 through June 21, 1972 from the southern end of the Sailing Club's floating dock located in Willoughby Bay at the Naval Base, Norfolk, Virginia. Environmental data were recorded within 1 meter of the surface. Water temperature was measured with a standard centigrade thermometer at the collecting site. Salinity and pH were measured from water samples immediately upon returning to the lab, the former with a hydrometer and the latter with a Beckman Zeromatic pH meter.

Histological Examination

Upon returning to the laboratory, three tunicates of the same approximate size were washed in sea water and fixed overnight in

methanol-acetic acid solution (3:1). Molgula has two gonads, one above the heart and one within the gut loop. The gonad located above the heart was removed from each animal, dehydrated in an ethanol-water series following Gray (1964) and infiltrated with paraffin in a vacuum oven. The embedded gonads were placed in a freezer to harden, serially sectioned with a microtome at 10 µm, placed on slides, and hydrated in an ethanol-water series following Gray (1964).

The slides from one tunicate were rinsed in running water for approximately 5 minutes, stained for 5 minutes in Gray's Celestine Blue B, and rinsed again in running water. The sections were counter-stained in Gray's Double Contrast for 3 minutes, drained, blotted, differentiated in a series of absolute ethanol, absolute ethanol-xylene (1:1), xylene and mounted in permount (Gray, 1964).

Slides from two tunicates were stained in Delafield's haematoxylin. Hydration was the same as above. After staining for 10 minutes, the slides were rinsed in distilled water (5 minutes), differentiated in an acid-ethanol solution (0.1% HCl in 70% ethanol), and blued to proper color density in 0.1% sodium bicarbonate in tapwater. The slides were rinsed in running water for 5 minutes, counter-stained in eosin for 1 minute, drained, dipped sequentially in the following: 80% ethanol, 95% ethanol, absolute ethanol, absolute ethanol-xylene (1:1), xylene, and mounted in permount (Gray, 1964). Measurements were made with an A-O Phasestar microscope, using a Filar micrometer eyepiece. Egg counts were made at 430 diameter magnification.

Colorimetric Estimation of DNA

Purified nuclei of both gonads from one tunicate dissected upon returning to the laboratory were obtained by swelling cells with reticulocyte standard buffer (RSB) (0.01M NaCl, 0.01M Tris HCl pH 7.4, 0.0015M $MgCl_2$), then homogenized. Nuclei were resuspended in buffer (100mM NaCl, 20mM $MgCl_2$) to which a mixed detergent solution (2 parts 10% Tween 40:1 part 10% sodium deoxycholate) had been added (Penman, 1969). DNA was extracted from washed nuclei with 2.5N and 0.5N $HClO_4$ following Burton (1956). Samples of all extracts were analyzed immediately. The remainders of the extracts were stored in a Revco freezer at -40°C and were retested at the end of the study as duplicate samples.

Two ml of diphenylamine reagent were added to 1 ml of extract. This was covered and incubated for a minimum of 20 hours at 25 - 30°C. Using a Gilford Spectrophotometer 240, optical density (595 and 700 nm) was read against an incubated blank containing 0.5N $HClO_4$. The acid blank was tested against a distilled water blank before each run to determine amount of contaminant in the reagent (Shatkin, 1969). Before each series of samples were tested, cuvets were soaked overnight in 0.5% HCl, cleaned, polished, and calibrated with distilled water.

Standard solutions of salmon sperm DNA (Calbiochem), determined to be 97.2% pure by ultra violet spectral analysis and extinction coefficient (Felsenfeld, 1968) were prepared according to Shatkin (1969) with final concentrations corrected to 100%. Dilutions were measured from 65 to 5 µg at 5 µg intervals. Readings were also made

at 7, 4, 3, 2, and 1 µg. Standards were measured twice *in toto* and one or two selected dilutions were retested with each gonadal sample. The standard curve was drawn using linear regression (Fig. 1). Amount of DNA per ml of sample was determined from the standard curve. Total content was determined by multiplying DNA per ml by the amount of extract obtained, and dividing by the wet weight of each gonad (i.e., DNA / gonadal weight).

Chromosome Studies

The remaining "gut-loop" gonad of one tunicate from the histological portion of the study plus both gonads from a different tunicate were squashed in order to study chromosomes. The tunicates were fixed in methanol-acetic acid (3:1) upon returning to the laboratory and left overnight in the fixative. They were then stored in 70% ethanol until examined. The gonads were washed in distilled water, hydrolyzed in 1N HCl at 60°C for up to 10 minutes, washed in successive washes of distilled water and 55% acetic acid, stained in aceto-orcein for 10 to 15 minutes, and squashed. Excess stain was washed away with 55% acetic acid. The squashes were sealed with a paraffin-balsam mixture and stored in a refrigerator (Colombera, 1970; Taylor, 1967).

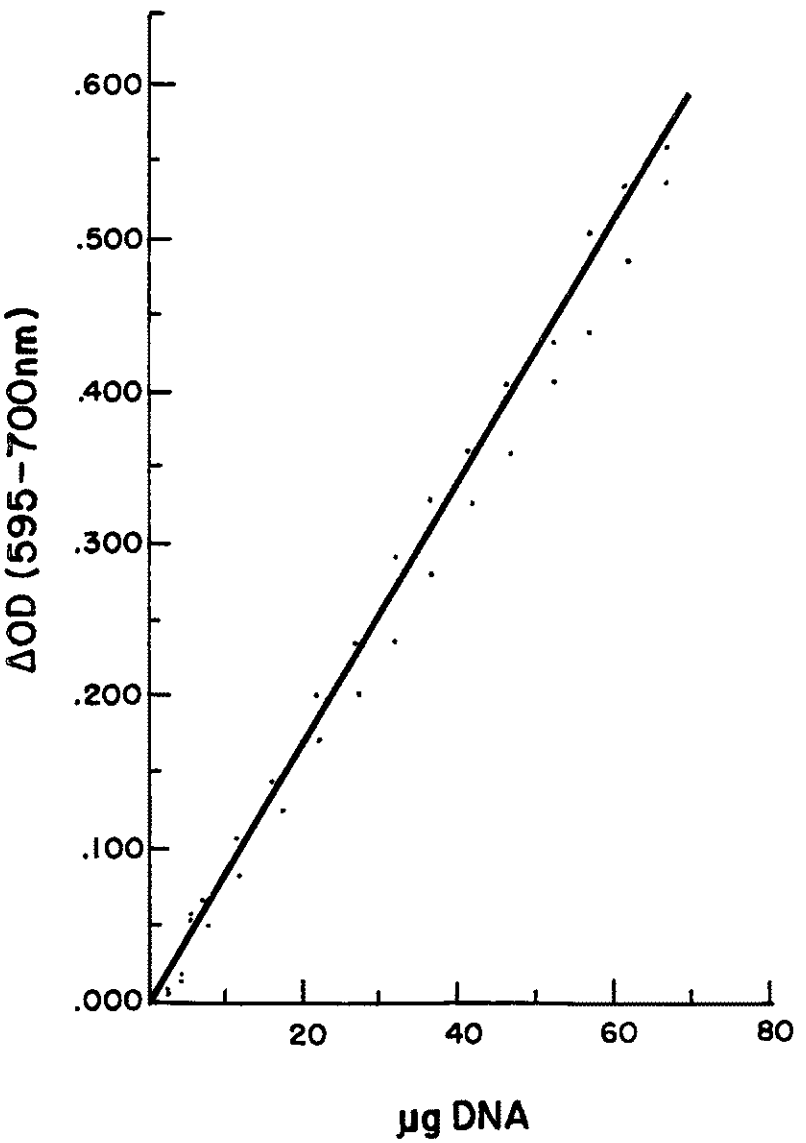
RESULTS

Gonadogenic Results

Oogonia (3 - 5 µm) were scarce and hard to distinguish during January to February, even though both gonads were clearly visible.

FIGURE 1

Linear Regression Curve of µg DNA Versus Δ Optical Density Used as Standard Curve for DNA Estimates



Many small oocytes (6 - 8 μm) were present and thickened chromatin strands were evident in some larger cells. Yolk-ladened eggs (80 - 106 μm), containing 12 - 18 yolk nuclei which encircled the nucleus, were prevalent (Fig. 2). In these eggs, the nucleus was unstained, large (46 - 58% of average cell diameter), and contained a nucleolus (31 - 67% of average nuclear diameter) which stained with both counterstains. Sperms were present in January but not in late February, suggesting possible phagocytosis occurred in the interim. The cytoplasm of some eggs stained a yellow-brown which may indicate the beginning of atrophication (Fig. 3). Little interstitial tissue was evident during this period. Water cavities made up much of the gonadal volume.

Small oocytes increased in size by addition of yolk from March to April. The nucleolus, which during the previous two months contained 1 to 3 vacuoles, became highly vacuolated. There was no discernable change in nucleolar or nuclear volume associated with increased vacuolation. By mid-April, some sperms were present and probable mature eggs (100 μm) were visible in the gonoducts at the time of collection. Suspected phagocytes were not evident. Free interstitial cells were prevalent in the outer gonadal membrane. Nuclear staining, cytoplasmic masses (12 - 18) again occurred in the large eggs. There was a rise in the number of follicles and interstitial cells, which coincided with an increase in gonadal volume. The gonadal membrane was thicker and more fibrous than previously. Water cavity volume decreased as the testicular and ovarian volumes increased. Metaphase chromosomes

FIGURE 2 Yolk Nuclei Encircling Nucleus
430X Magnification

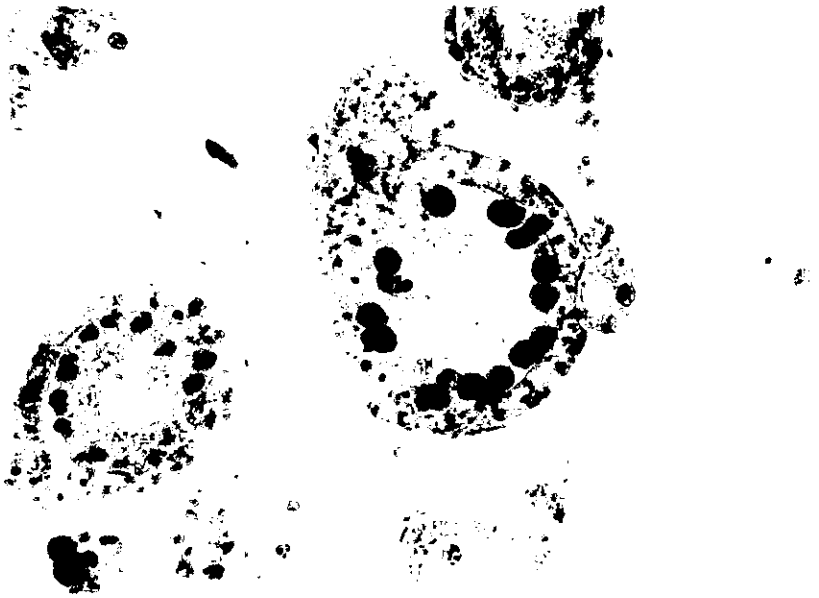
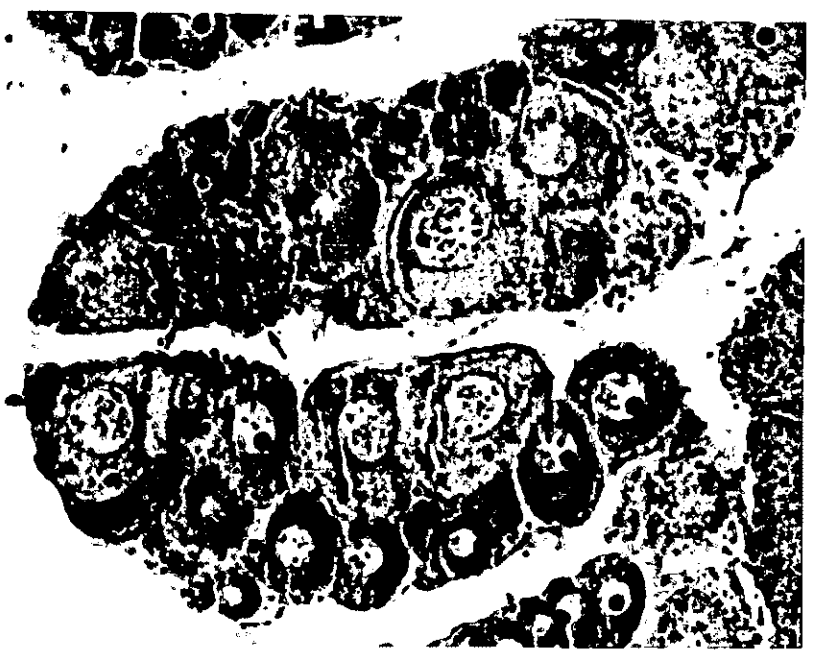


FIGURE 3 Atrophied Yolk-Ladened Eggs (Arrows)
430X Magnification



were observed during this period and will be described later.

Immature tunicates were observed in the study population in mid-May. Few sperms and eggs were evident during the remainder of May (Table 3). Indications of probable phagocytosis and atrophication of residual gametes were again evident. At this time, a change in the stainability of yolk-ladened eggs was noticed. Suspected phagocyte numbers subsided rapidly and by early June only a few were observed. Small oocytes (29 - 60 μm) with 1 to 3 vacuoles in their nucleolus, and some sperms, were prevalent at the end of this study, probably signifying the commencement of a new cycle. Total interstitial tissue decreased and gonads were not as large as those previously seen.

DNA Results

There was a wide range of DNA content per gonad during the collecting period (Table 4). The steep incline from March 29 to April 12 was accompanied by the first appearance of eggs within the oviduct (Fig. 4). A second increase was evident at the end of the sampling period (May 24 to June 21), indicative of the commencement of a new cycle.

During the sampling period, salinity and pH remained fairly stable, but temperature varied considerably (Fig. 5). Maximum temperature increase and the sharp incline in DNA content coincided closely (Fig. 4).

Chromosome Results

Molgula manhattensis was found to have very small chromosomes in spermatocytes. Meiotic metaphase counts varied and were inconclusive. However, the most frequently encountered number was 10 with a range

TABLE 3: Average Number of Oocytes in a 430X Field

Date			Average Numbers
January	5		54
	19		34
February	2		68
	16		61
March	1		64
	15		46
	29		77
April	12	(Eggs in gonoducts)	68
	26		27
May	5		18
	24	(Immatures)	39
June	7		60

TABLE 4: DNA Values for Molgula Manhattensis Gonads

Date		Gonad Measured	Gonad Weights ($\times 10^{+3}$ µg)	Total DNA (µg)	DNA/µg G. Weight ($\times 10^{-3}$ µg)
January	19	Heart	31.1	14.620	0.47
	"	Loop	19.6	10.417	0.53
February	2	Heart	24.7	28.373	1.14
	"	Loop	10.6	8.307	0.78
	16	Heart	48.3	5.283	0.10
	"	Loop	19.6	4.399	0.22
March	1	Heart	71.2	97.177	0.63
	"	Loop	33.4	10.200	0.30
	15	Heart	80.1	7.342	0.09
	"	Loop	15.4	2.125	0.13
	29	Heart	60.1	55.160	0.91
	"	Loop	22.6	28.827	1.27
April	12	Heart	59.9	203.433	3.39
	"	Loop	39.0	72.137	1.84
	26	Heart	78.5	239.504	3.05
	"	Loop	35.0	41.891	1.19
May	10	Heart	125.0	298.512	2.39
	"	Loop	188.4	138.054	0.73
	24	Heart	64.4	112.302	1.74
	"	Loop	41.6	32.663	0.78
June	7	Heart	81.9	122.630	1.48
	"	Loop	38.9	99.343	2.55
	21	Heart	18.6	66.409	3.57
	"	Loop	7.3	27.756	3.80

FIGURE 4 µg DNA per µg Gonadal Weight for Heart and Loop Gonads and Temperature (°C) for Each Sampling Date

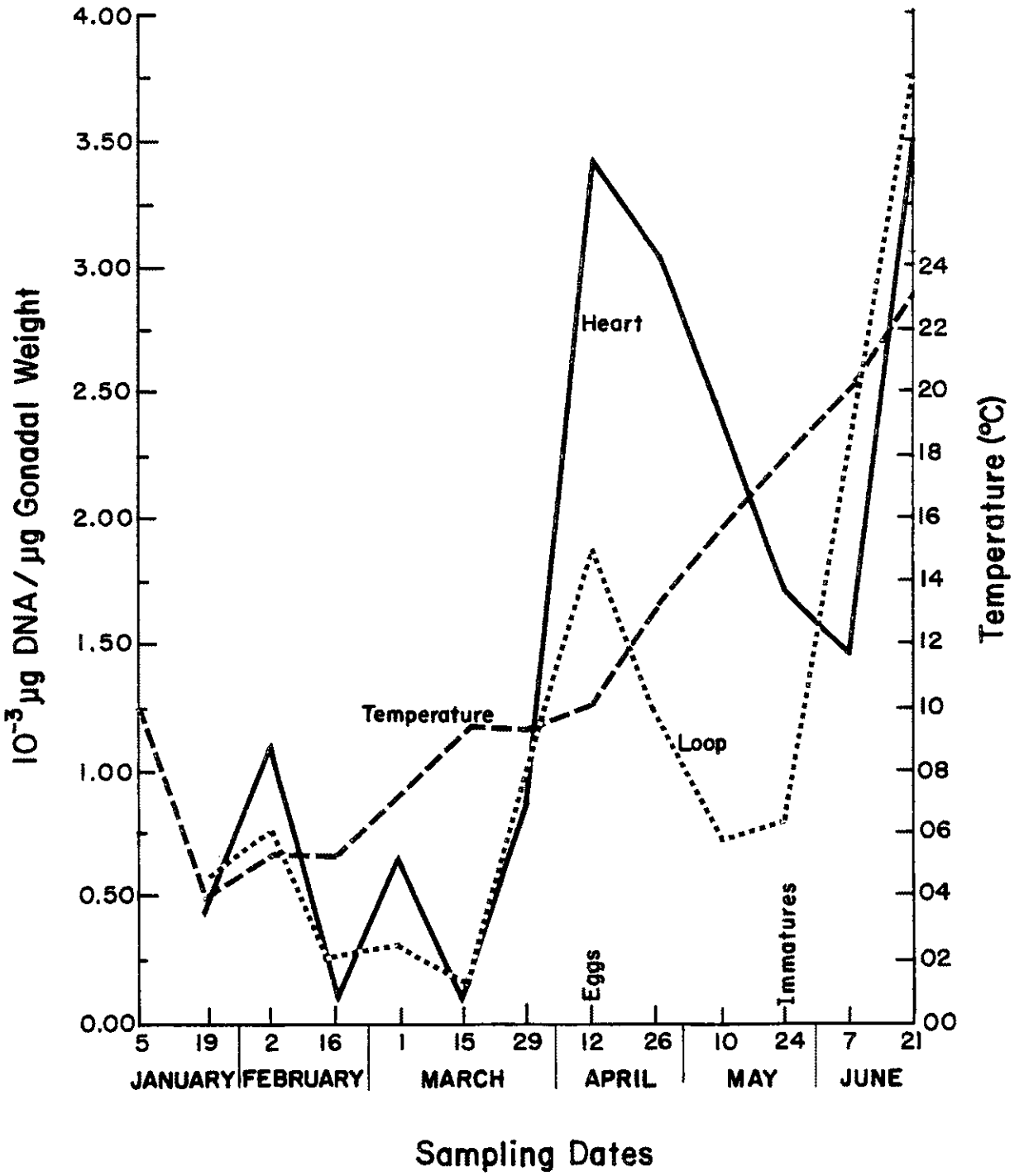
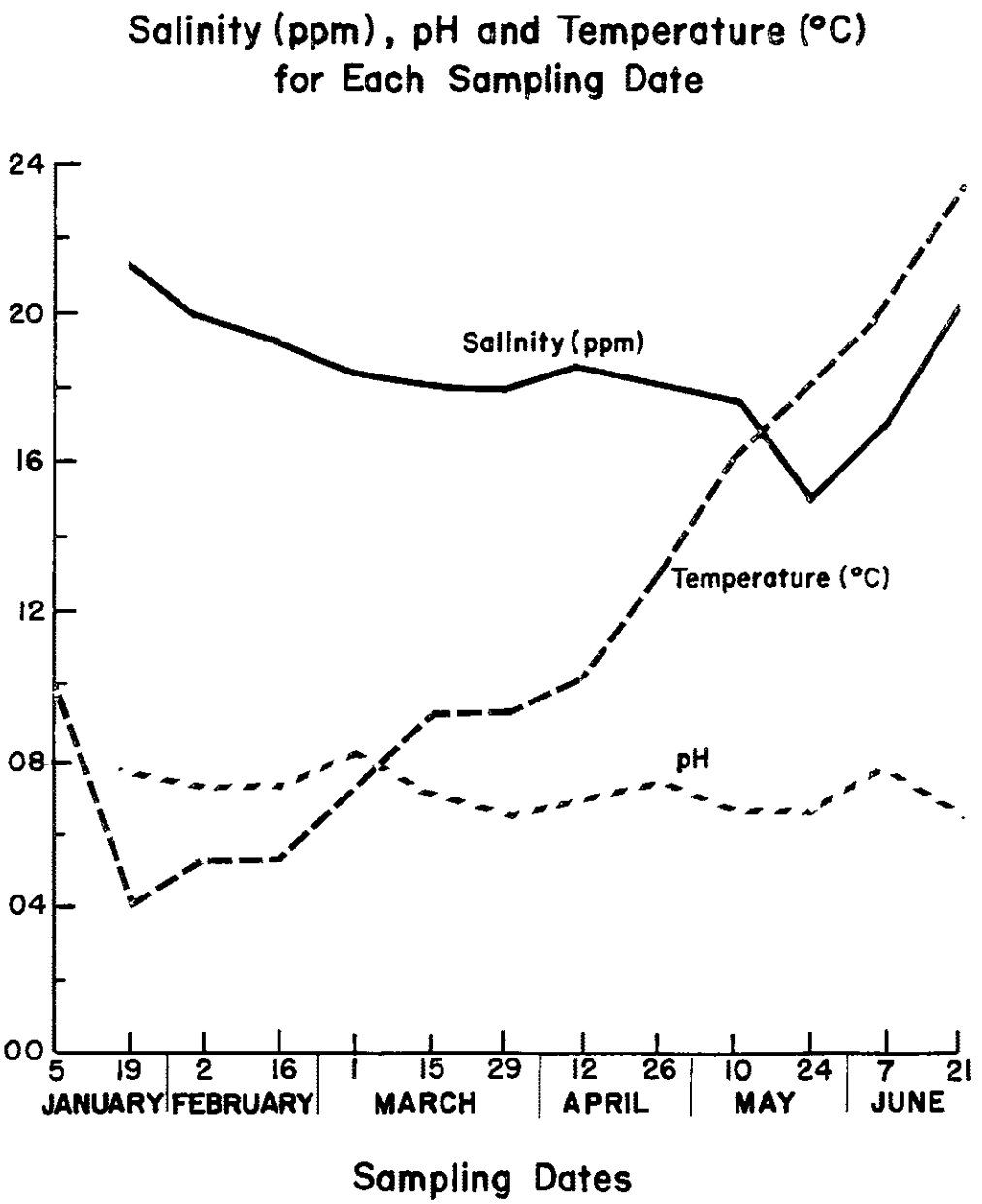


FIGURE 5


Salinity (ppm), pH and Temperature (°C)
for Each Sampling Date
24
20
16
12
08
04
00
Salinity (ppm)
Temperature (°C)
pH
5
19
2
16
1
15
29
12
26
10
24
7
21
JANUARY
FEBRUARY
MARCH
APRIL
MAY
JUNE
Sampling Dates

from 8 to 15. A typical cell is shown in Fig. 6. Individual bivalents were uniformly less than 1.0 μm; thus, no details of the karyotype could be discerned.

Ovarian chromosomes were not distinguishable from the surrounding cytoplasm which was found to stain positive with both aceto-orcein and Feulgen reagent.

DISCUSSION

Results of the present study (Table 5) were commensurate with previously published results (Orton, 1920; Dybern, 1965; Millar, 1952; 1954), with regard to water temperature as an essential factor controlling gonadal development. The pH and salinity had no apparent effect. However, Dybern (1965) found gonadal maturation in Scandinavian populations of Ciona intestinalis during February while Millar (1952, 1954) did not find significant development in Scottish populations of B. Schosseri, D. listerianum, and D. grossularia until March to April. Initiation of gonadal development in the current study was during January. Apparently, water temperature ascent initiated gonadal development, but the threshold temperature for stimulation is different for different species and probably for different geographical populations of the same species.

Although some tropical tunicates spawn continuously (Millar, 1971; Dybern, 1965), it was believed that temperate species had distinct spawning periods. Millar (1954) theorized that temperatures above 20°C (or below 11°C) suppressed ovulation, producing two spawning

FIGURE 6 Typical Spermatocyte Chromosomes
1000X Magnification

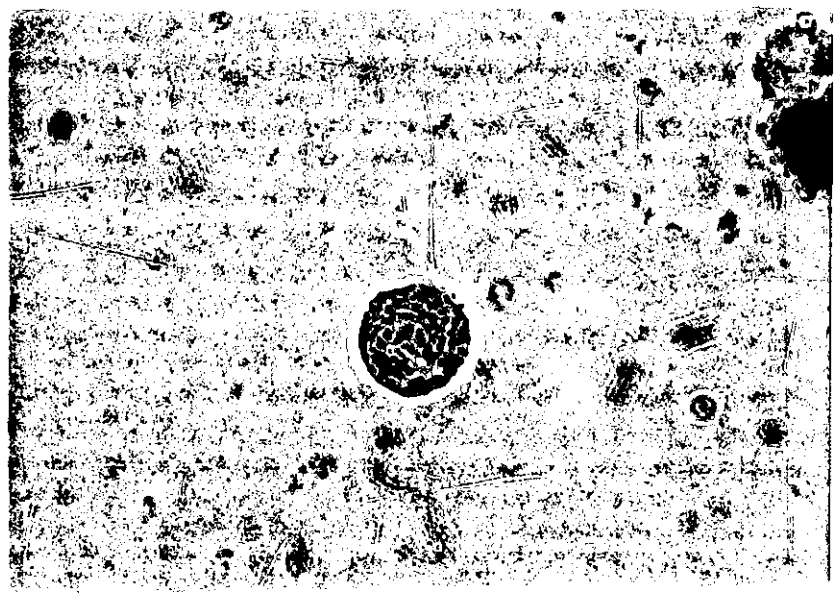


TABLE 5 Ascidian Gonadal Development Versus Water Temperature

	C. intestinalis (Dybern, 1965)	*B. schosseri* (Millar, 1952)	*D. listerianum* (Millar, 1952)	*D. grossularia* (Millar, 1954)	*M. manhattensis* (Crawford, 1973)
Gonadal Initiation	February 0-4°C	March	March	January - February 6°C	January 4-10°C
Rapid Gonadal Changes	----------	March-April 7°C	March-April	March-April 7°C	March 7-9°C
Sex Cells in Gonoducts	early April 4-8°C	----------	----------	March 7°C	mid-April 10°C
Larvae in Plankton	May 10-15°C	late March	May	May 8-9°C	mid-May 16-18°C
Spawning Peaks	May - June and August	May - June	May - June	May and August	May
Upper Limit to Spawning	above 20°C	----------	----------	above 20°C	above 20°C
Lower Limit to Spawning	below 12-10°C	----------	----------	below 11°C	----------

maxima limited to the summer. Applying Millar's theory to Dybern's (1965) plankton data, two peaks would be expected in the summer months. These occurred, indicating that Millar's theory was probably applicable to Ciona. Measurements of DNA quantity should remain constant if continuous spawning was evident in M. manhattensis, but results did not show this (Table 4, Fig. 4). Total gonadal DNA increased from January to April, dropping sharply after spawning. DNA peaking is possibly the result of suppressed egg release (water temperature 18 - 23°C) thus Molgula appears to adhere to Millar's theory. Corroborating evidence was found in histological examinations of late May to June gonads in which yolk-ladened eggs were apparently undergoing either phagocytosis or atrophication.

Crampton (1899) reported the diameter of oogonia of M. manhattensis as less than 8 µm. In the current study, they were found to be 3 - 5 µm. Although Crampton's descriptions were vague, the present data seems comparable to general details given for maturing oocytes. Harvey (1927) referred to 5 to 12 yolk nuclei circumventing the cell nucleus during early yolk formation in Ciona. These dark staining masses were probably the enlarged yolk-spheres described briefly by Crampton (1899) in M. manhattensis. They were also observed in the present study. Both papers reported granular elements (yolk nuclei) in small oocytes during early yolk formation, but the present data indicated they were in larger cells (80 - 106 µm) during late yolk formation.

Harvey (1927), but not Crampton (1899), reported increased

vacuolation of the nucleolus during oogenesis. No correlation between vacuole number and nucleolus diameter was reported for this occurrence and none was discerned in the current study even though the same phenomenon was observed. After yolk formation, the nucleolus fragmented in Ciona. Burren (personal communication) reported its disappearance after oocyte maturation in M. manhattensis, but the nucleolus remained intact throughout the present study.

Carlisle (1951) described phagocytosis of nearly ripe ova by surrounding follicle cells in Ciona intestinalis and Millar (1954) observed the same process in Dendrodoa grossularia testis and ovaries. Pérés (1952) reported phagocytosis of the testis of C. intestinalis. Millar (1954) believed phagocytes were derived from surrounding blood or connective tissue in testes. He observed them migrating into the central lobes, ingesting residual sperms, and then withdrawing to the periphery to eventually disappear. Neither phagocytosis nor atrophication had previously been reported in M. manhattensis. Evidence of both was observed during the present study. What appeared to be atrophing, yolk-ladened eggs (Fig. 3) were seen during periods of sexual inactivity (January and June). It was doubtful that atrophication occurred in June because of the time involved to bring the gonads to breeding conditions again. Mistaken atrophied eggs might have been follicle cells described by Millar (1954) after they phagocytized eggs.

Taylor (1967) reported spermatocyte chromosomes of Ciona intestinalis and Styela plicata as too small (probably less than 3 μm

by my estimate) for any description of the karyotype. Colombera (1969, 1970, 1971a, b, c) observed extreme contraction in spermatocyte bivalents of several species, but was able to make idiograms of oocyte chromosomes (range of length: 2.4 to 5.4 μm). Ovarian bivalents were obscured by the cytoplasmic staining in the present study. Grosch (1958) reported a similar occurrence in Molgula with Feulgen stain and demonstrated increased staining during oocyte maturation. Cytoplasmic deoxyribosides have been reported for two chordates and one echinoderm (Hϕff-Jorgensen and Zeuthen, 1952) and were probably responsible for the staining in Molgula. Small contracted spermatocyte chromosomes were inconclusive as they tended to clump together. Their size and numbers were comparable to Taylor (1967) and the genetic material possessed by Molgula manhattensis was probably similar to that of Ciona intestinalis (Atkin and Ohno, 1967).

The present results confirm the protochordate findings of Atkin and Ohno (1967) and Taylor (1967) in support of the proposed theory of polyphyletic evolution of terrestrial vertebrates from aquatics. The small amount of DNA in the gonads (maximum 3.8×10^{-3} μg DNA/μg gonadal weight) and the chromosome size indicated that cellular DNA quantity was quite small and probably close to 6% of the human value.

Obviously, many questions remain unanswered. Future cytogenetic studies should attempt to positively determine if an ecological trigger mechanism is present and identify it if possible. This could be accomplished by culturing Molgula under constant background conditions, but tunicates are difficult to culture. Work in culturing

technique alone is essential.

Techniques for chromosome analysis must be improved. Removal of yolk from oocytes to facilitate chromosome analysis as well as the development of methods for spreading the chromosome figures are both areas in need of technique improvement. The latter applies to testicular material also. In fact, the whole area of testicular development is virtually untouched and spermatogenesis has not been described. Fine structure studies of gonadal and associated tissue would be highly desirable.

SUMMARY

Visual changes of the nucleus, variations in total gonadal DNA quantity, and the chromosomes of Molgula manhattensis were studied. Results were compared with studies from other protochordate and lower chordate literature concerning a proposed scheme of chordate evolution. Environmental data (i.e., surface salinity, water temperature, and pH) were recorded during the study. Histological results showed Molgula gonadal development was similar to Ciona intestinalis with respect to increased vacuolation of the nucleolus and to development of the yolk nuclei. Evidence of atrophication and of phagocytosis of residual gametes was found. Corroborating data from colorimeteric estimations of gonadal DNA indicated two spawning peaks during the summer months. It was suggested that M. manhattensis egg release was suppressed by water temperatures above 20°C and that the fall spawning was a result of reduced temperatures that stimulated ovulation.

It was difficult to identify specific chromosomal structures, sizes, and numbers. When seen, they were small and clumped together. However, the size of the chromosomes and quantity of gonadal DNA were in apparent agreement with previous tunicate literature.

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