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The Dynamics of Oogenesis and the Annual Ovarian Cycle of *Stichopus californicus* (Echinodermata: Holothuroidea)

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Abstract. The major cytological stages of oogenesis in the holothurian *Stichopus californicus* are morphologically separated in distinct classes of tubules, the discrete anatomical units comprising the ovary. Primordial germ cells are in the connective tissue compartment of the gonad basis. Mitotic oogonia are in the smallest and most anterior set of primary ovarian tubules. Oocytes in the early prophase stages of meiosis I are in secondary tubules located more posteriorly. Diplotene oocytes occur in larger, more posterior secondary tubules. The most advanced oocytes are in the most posterior fecund tubules. *S. californicus* oocytes bear nuage, a subcellular marker common to many germ line cells. The striking axial polarization of these oocytes, evident as the egg axis, is indistinguishable from the apical basal axial polarization in epithelial germ line cells. These axes are congruent throughout the developmental history of the oocyte. I present a model for the annual cycle of the *S. californicus* ovary and assess its application to other holothurians. I infer function from the structure of oogenesis presented here; and contrast this information with what is known about oogenesis in the other echinoderm classes.

Introduction

The only detailed histological investigation of holothurian oogenesis is that of Théel (1901) who reported annual cycling of the gonadal tubules in the synallactid aspidochirote *Mesothuria intestinalis*. Because *M. intestinalis* is hermaphroditic, a fine structural study of oogenesis in a gonochoric holothurian will be useful. It would also be of interest because many comparative anatomists consider holothurian gonads primitive (Hyman, 1955; Smiley, 1988b). The simplicity of the holothurian ovary makes it a promising model for all echinoderm gonads. Such a study will also shed light on the development of polarization within the holothurian oocyte. These oocytes bear an apical protuberance, making them among the most visibly polarized in the animal kingdom (Smiley and Cloney, 1985). The protuberance is the site of polar body formation during meiosis, and marks the animal pole of the egg axis (Smiley and Cloney, 1985). Analysis of the development of epithelial cellular polarization in the early germ line cells is also of interest because of its likely importance in the development of embryonic pattern.

In this paper, I present a detailed fine structural analysis of oogenesis in the aspidochirote holothurian *Stichopus californicus*. I also report on an analysis of the annual cycle of this ovary, from resorption of the spent fecund tubules, through oogenesis, culminating in the inception of vitellogenesis in oocytes that will be spawned in the next reproductive season.

Materials and Methods

Stichopus californicus adults, collected by dredging or diving in nearby waters, were maintained in running seawater aquaria at the Friday Harbor Laboratories, University of Washington, from 1980 through 1985. Ovaries from more than 150 individual *S. californicus* were fixed in all months of the year except December. Dissection was done rapidly and all stages of fixation were done on ice using described methods (Smiley and Cloney, 1985).

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Tissues were dehydrated in acetone and embedded in epon. Semithin (1 μm) sections were examined from each of several blocks made from each ovary fixed. Thin sections from more than 25 individuals were examined in a Philips 301 electron microscope.

S. californicus juveniles were raised from eggs fertilized *in vitro*, as described previously (Smiley, 1986, 1988a). After settlement, fingerbowls containing juveniles were barely submerged in aquaria. This allowed suspended food to flow into the bowls, which were cleaned weekly by rinsing. Maintained in this manner, juvenile *S. californicus* grow well their first fall and winter. Juveniles were fixed in January and February following fertilizations in May, June, and July. *Stichopus californicus*, Clark's (1901) generic redesignation of *Holothuria californica* Stimpson (1857) is also referred to as *Parastichopus californicus* Deichmann (1937).

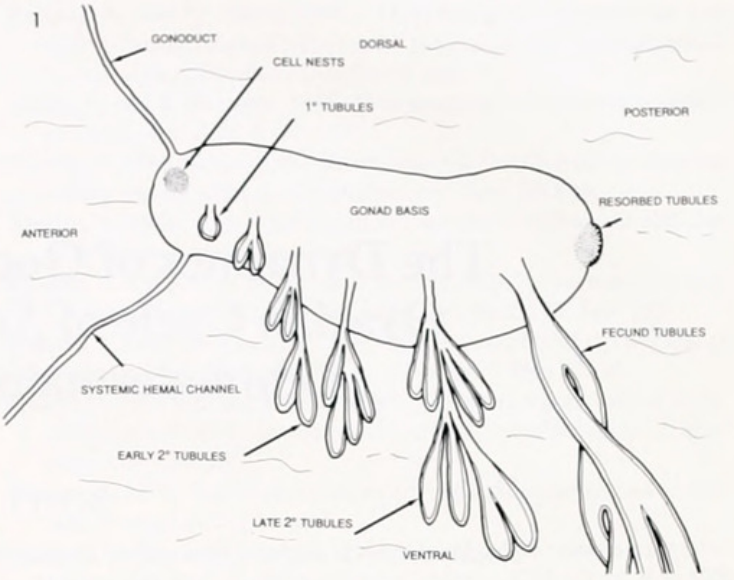

Figure 1. Diagrammatic depiction of a left lateral view of the *Stichopus californicus* ovary. This ovary is bilaterally symmetric about the dorsal mesentery.

Results

The results are organized into two parts. The first is a description of the anatomy and histology of the ovary, including the fecund tubules before and after spawning, and progressing anteriorly. This section gives the light microscopical level distinctions between classes of tubules. The second part covers the fine structural changes in the ovarian inner epithelium. This includes a detailed analysis of the dynamics of the somatic and germ line cells beginning with the cell nests of the gonad basis and culminating in vitellogenesis in the most posterior tubules.

Anatomy and histology of the ovary

Fecund tubules. The ovary of *Stichopus californicus* consists of three classes of tubules attached to a central *gonad basis* (Fig. 1): fecund, secondary, and primary tubules. While the three size classes are distinguished by a number of criteria, they represent stages in a continuum, in which the fecund tubules are the most posterior and the largest. These attach to the most posterior regions of the basis and bifurcate many times along their length. They contain vitellogenic oocytes in the months prior to spawning, and post vitellogenic oocytes in the weeks immediately before.

The fine structural organization of fecund ovarian tubules in *S. californicus* was recently reported (Smiley and Cloney, 1985). Tubules are the structural unit in holothurian gonads, and each consists of three tissues. Outermost is a *complex peritoneum* composed of peritoneal epithelial cells, nerves, and muscle cells. Innermost is the *inner epithelium* composed of oocytes and somatic inner epithelial cells of two types. Lying between the basal laminae of the peritoneum and the inner epithelium is the *connective tissue compartment* composed of fibers, the mesenchymal cells, and the genital hemal sinus. The genital hemal sinus is not lined by cells but is a lacuna within the connective tissue compartment. The oocyte basal lamina (oolamina) limits direct connection of the oocyte and the jelly space with the fluid in the genital hemal sinus. No outer sac of tissues surrounds holothurian gonads.

Spent tubules. Spawning evacuates post-vitellogenic oocytes from the fecund tubules (Smiley and Cloney, 1985). Just after spawning, spent tubules are a maximum of 4 cm long, and their pigmentation has decreased markedly. A week later, they have shrunken and become a rust color owing to phagocytosis (Fig. 9). This phagocytic action reduces the ovarian inner epithelium to few identifiable structures; the most obvious are the oocyte oolaminae (Fig. 9). The densely staining material in peritoneal cells and coelomocytes are birefringent residual bodies, products of phagocytic activity. A month after spawning, only pigmented plaques on the posterior of the gonad basis remain, testifying to the former presence of spent fecund tubules (Fig. 1). Since these tubules are lost, subsequent generations of oocytes must be recruited from other ovarian tubules.

Secondary tubules. The secondary tubules are just anterior to fecund tubules on the gonad basis (Fig. 1). They average 2.5 cm in length and 0.35 mm in diameter. They branch once or twice along their length, and each branched portion is slightly elongate. Their histology is similar to the fecund tubules, but the peritoneal epithelial cells are more columnar in secondary tubules (Fig. 2), and the genital hemal sinus is less extensive, giving the connective tissue compartment a more fibrous appearance. Fibroblasts, morula cells, and especially petal-

oid amoebocytes are more common in the connective tissue compartment of secondary tubules. Secondary tubules can be divided into two categories. Late secondary tubules are more posterior on the basis; early ones are more anterior.

In late secondary tubules, coincident with the onset of vitellogenesis in the fall, somatic inner epithelial cells form the inner wall of the ovary, and surround the oocytes (Fig. 10). Together, the inner epithelium and connective tissue compartment of these tubules form stubby longitudinal folds (Fig. 2) that run short distances along the length of the tubule. These develop into the long deep longitudinal folds in fecund tubules (Smiley and Cloney, 1985). Some inner epithelial cells form a follicle around the oocytes; these adhere to the oocyte surface, and a jelly space is not present. The lumen of late secondary tubules is much less occluded by oocytes than that of fecund tubules. Sexes can be reliably identified from late secondary tubules, after oocytes have formed germinal vesicles.

The peritoneum of early secondary tubules is thinner than in late secondary tubules, but is otherwise similar. The inner epithelium in early secondary tubules is simple in spite of its stratified appearance (Fig. 3). Here, longitudinal folds are reduced in size compared to late secondary tubules. The monociliated somatic cells are smaller than oocytes and have nuclei with denser peripheral heterochromatin. Even by electron microscopy, it is not possible to distinguish between oocytes and spermatocytes in early secondary tubules, or between oogonia and spermatogonia in primary tubules. The ovarian lumen of early secondary tubules is not seriously occluded by oocytes or longitudinal folds (Fig. 2) and is more expansive than in late secondary tubules. Within it is weakly staining hemal fluid containing morula cells and petaloid amoebocytes.

Primary tubules. The primary tubules, the smallest and most anterior on the gonad basis, are often difficult to discern even with magnification (Fig. 1). The most anterior are less than 2 mm in length, 0.25 mm in diameter, and do not bifurcate along their length. Those primary tubules closest to secondary tubules are slightly larger than those more anterior, and they may branch, but the tips of their branches are globose rather than elongate. The distinction between primary and secondary tubules is made on size, position, and cytology. There are no longitudinal folds of the inner epithelium and the connective tissue compartment in primary tubules (Fig. 4).

The histological organization of primary tubules is similar to the other tubules (Smiley and Cloney, 1985), but the connective tissue compartment is substantially smaller, particularly the genital hemal sinus. The lumen of primary tubules also contains hemal fluid which appears to be identical with that found in the genital hemal sinus of late secondary and fecund tubules. In spite of the adhering junctions between cells of the inner epithelium, fluids and mesenchymal cells within the lumen appear to mix freely with those in the genital hemal sinus. I conclude that the lumen of primary and early secondary tubules is in direct connection with, if not a part of, the ovarian connective tissue compartment.

Gonad basis. The gonad basis in *S. californicus* is a 5 to 7 mm long saddle-shaped thickening (Fig. 1) of the dorsal mesenteric connective tissue compartment (Smiley and Cloney, 1985; Cameron and Fankboner, 1986). It is totally enclosed by the dorsal mesenteric perivisceral peritonea (Fig. 6), which are structurally identical to, and continuous with, the investing peritonea of the ovarian tubules (Smiley and Cloney, 1985). The dorsal mesentery results from the lateral fusion of the left and right somatocoels during metamorphosis (Smiley, 1986), and the peritonea of the gonad basis and tubules have the same ontogeny.

The gonoduct inserts into the dorsal anterior aspect of the gonad basis and ascends within the connective tissue compartment of the dorsal mesentery to the gonopore located anteriorly in interambulacrum *CD* (Hyman, 1955). In all specimens sectioned (Fig. 8), the thinner wall of the gonoduct faces one side of the dorsal mesentery, and the long axis of its elliptical lumen is parallel with it. The duct is lined with a simple epithelium composed of columnar and exaggeratedly columnar monociliated cells. No *genital cord* (Théel, 1901; Haanen, 1914) is present adjacent to the gonoduct in *Stichopus californicus,* nor is there any genital rachis (Smiley, 1988b). Entering the basis at a ventral anterior aspect is a channel connecting the genital hemal sinus of the gonad with the dorsal hemal structures of the gut (Fig. 1).

The composition and structure of the connective tissue compartment in the gonad basis is complex (Figs. 6, 7). Within it, muscle cells run between the fibers of the connective tissue matrix. Lacunae are found in the connective tissue of the gonad basis, but based on evidence from serial semithin sections, neither the genital hemal sinus of late secondary and fecund tubules, nor the central lumina of primary and early secondary tubules are in direct connection with these lacunae. The gonad basis contains a reduced central lumen continuous with lumina from more advanced tubules (Fig. 6). Columnar somatic inner epithelial cells of the less advanced tubules partially occlude the opening near the point of their insertion onto the gonad basis. At the most anterior lateral aspect of the gonad basis, the connective tissue compartment contains small cell nests, about 50 μm in diameter (Fig. 7), similar to those described for *Holothuria parvula* (Kille, 1942). In *S. californicus,* each cell nest has a reduced central lumen isolated from the lumen of the gonad basis. The nests are separated from the connective tissue compartment by a basal lamina (Fig. 13).

Cytology of the inner epithelial cells

Primordial germ cells in the gonad basis. The cells comprising the cell nests are easily differentiated into two

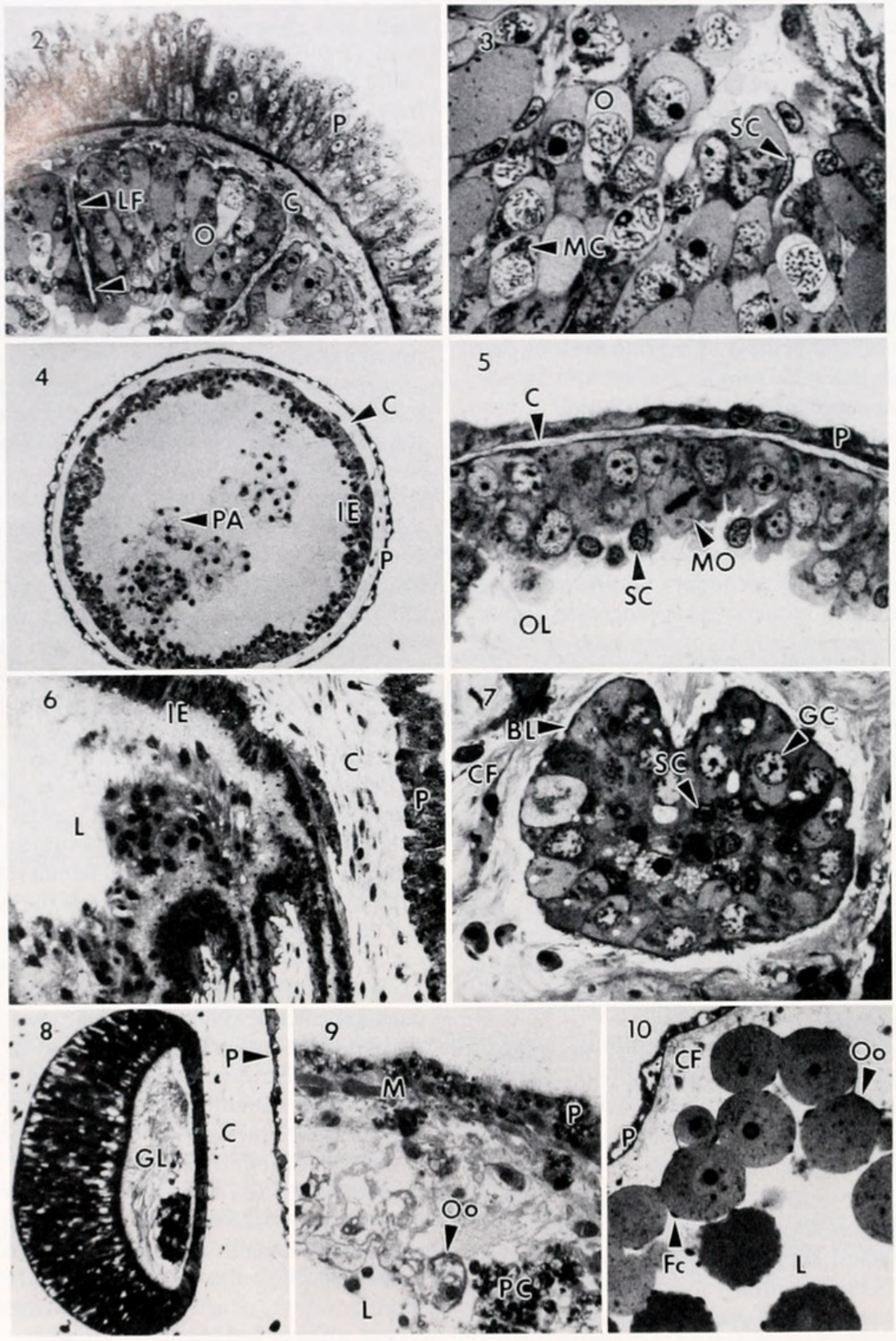

Figure 2. Light micrograph of a cross section of an early secondary tubule. C. connective tissue compartment, LF. incipient longitudinal folds, O. oocyte, P. peritoneum. The arrows demark a longitudinal fold. 480×.

Figure 3. Light micrograph of the inner epithelium in an early secondary tubule. MC. mitochondrial cloud in a spireme stage oocyte, O. oocyte, SC. inner epithelial somatic cell. 910×.

Figure 4. Light micrograph of a cross section of a primary tubule. C. connective tissue compartment, IE. inner epithelium, P. peritoneum, PA. petaloid amoebocytes within the lumen. 175×.

Figure 5. Light micrograph of details on the inner epithelium of a primary tubule. C. connective tissue compartment, MO. mitotic oogonium, OL. ovarian lumen, P. peritoneum, SC. somatic cell of the inner epithelium. 1050×.

types found in approximately equal abundance (Fig. 13). The smaller type, averaging about 6 μm in diameter, have more electron-dense cytoplasm, nuclei with distinct peripheral heterochromatin, prominent centrioles and a cilium; these are interpreted as somatic cells. The larger cells (Fig. 13), averaging about 10 μm in diameter, have more electron-lucent cytoplasm, nuclei with reduced peripheral heterochromatin, prominent centrioles (Fig. 18), and appear to bear a cilium. The cytoplasm in the larger cells (Fig. 13) contains aggregations of mitochondria and associated electron-dense bodies (Fig. 19), here called *type I nuage.* Nuage is germ line specific electron-dense granules, unbound by membranes, and has been described for germ line cells from other species (Eddy, 1975; Kessel, 1983). These criteria indicate that the larger cells (Figs. 7, 13) are *primordial germ cells* (*PGC*s), the progenitors of the mitotic gonial cells, themselves precursors of spermatocytes or oocytes. PGCs lack yolk, are bound to neighboring cells with adhering junctions (Fig. 19), and appear attached to the thickened basal lamina (Fig. 13).

Oogonial mitoses in primary tubules. The inner epithelium of primary tubules contains two distinct cell types, somatic cells and oogonia (Fig. 5). The smaller cells, with more basophilic cytoplasm and nuclei with more distinct peripheral heterochromatin are somatic cells, homologous with the smaller cells in cell nests within the gonad basis. These monociliated cells are not organized into a simple epithelium typical of late secondary and fecund tubules. Their jumbled arrangement makes the organization difficult to classify (Fig. 5). TEM reveals that all cells have adhering junctions attaching them to their neighbors, and the basal most cells rest on a basal lamina, therefore the inner cells of primary tubules form a true epithelium.

Within primary tubules, germ line cells of the inner epithelium are less electron dense, and their nuclei show substantially less peripheral heterochromatin than somatic cells. They average about 10 μm in diameter, lack yolk, and contain type I nuage. Like the somatic cells, these are bound to neighbors by adhering junctions, and appear attached to the thickened basal lamina. Germ line mitoses are frequently encountered, and are restricted to the primary tubules (Fig. 5) in *S. californicus.* Mitoses indicate that these germ line cells are oogonia, which correspond to PGCs in cell nests. The criteria supporting this conclusion include: (1) they are part of the inner epithelium of the tubules, (2) they are larger and are morphologically similar to other germ line cells, (3) their staining pattern is more like germ cells than somatic cells, and (4) because TEM reveals that they contain type I nuage.

The frequency of mitotic figures among the oogonia of primary tubules is higher than that found in any other ovarian tissues of *S. californicus.* The plane of the division spindles in oogonia is parallel to the inner epithelium, preventing daughter cells from being pushed into the tubule lumen at mitosis. Oogonia occur throughout the length of the primary tubule; indicating there is no discrete zone of mitotic proliferation in this tissue. Peritoneal epithelial cells in primary tubules also divide, although not as frequently as oogonia.

Prophase of meiosis I in early secondary tubules. In the inner epithelium of secondary tubules, the somatic cells cannot be distinguished as parietal or follicular. These cells are bound to other inner epithelial cells, somatic and germ line, by adhering junctions; or they may be closely applied to the surface of adjacent cells but showing no junctions. The somatic cells are smaller than germ line cells and have nuclei with peripheral heterochromatin.

The germ line cells here are quite small relative to those in late secondary tubules, and their chromosomes are in configurations characteristic of early prophase stages of meiosis (compare Fig. 21). These cells average about 15 μm in diameter, lack yolk, have adhering junctions with adjacent cells, contain nuage, and appear to be associated with a special basal lamina, the oolamina. Because of the small oocyte size, oolaminae in early secondary tubules extend over a greater area at the basal surface of the oocyte than in the fecund tubules (Smiley and Cloney, 1985), but it has the same staining properties and fine structure. The differences in relative size of the oolamina in secondary and fecund tubules are probably a result of oocyte growth.

An aggregation of densely staining particles lies adja-

Figure 6. Light micrograph of the gonad basis. C. connective tissue compartment, IE. inner epithelium, L. lumen, P. peritoneum. 375×.

Figure 7. Light micrograph of a cell nest within the connective tissue compartment of the gonad basis. BL. basal lamina limiting the nest, CF. connective tissue fibers, GC. germ line cell, SC. somatic cell. 1025×.

Figure 8. Light micrograph of the gonoduct. C. connective tissue compartment of the dorsal mesentery, GL. gonoduct lumen, P. peritoneum. Note the absence of a genital cord. 255×.

Figure 9. Light micrograph of a cross section of a resorbing spent tubule. M. muscle cells of the complex peritoneum, L. ovarian lumen, Oo. oolamina, P. peritoneum, PC. petaloid amoebocytes containing lipofuscin granules. 525×.

Figure 10. Light micrograph of a cross section of a late secondary tubule. CF. connective tissue fibers, Fc. follicular inner epithelial cells, L. ovarian lumen, Oo. oolamina, P. peritoneum. 250×.

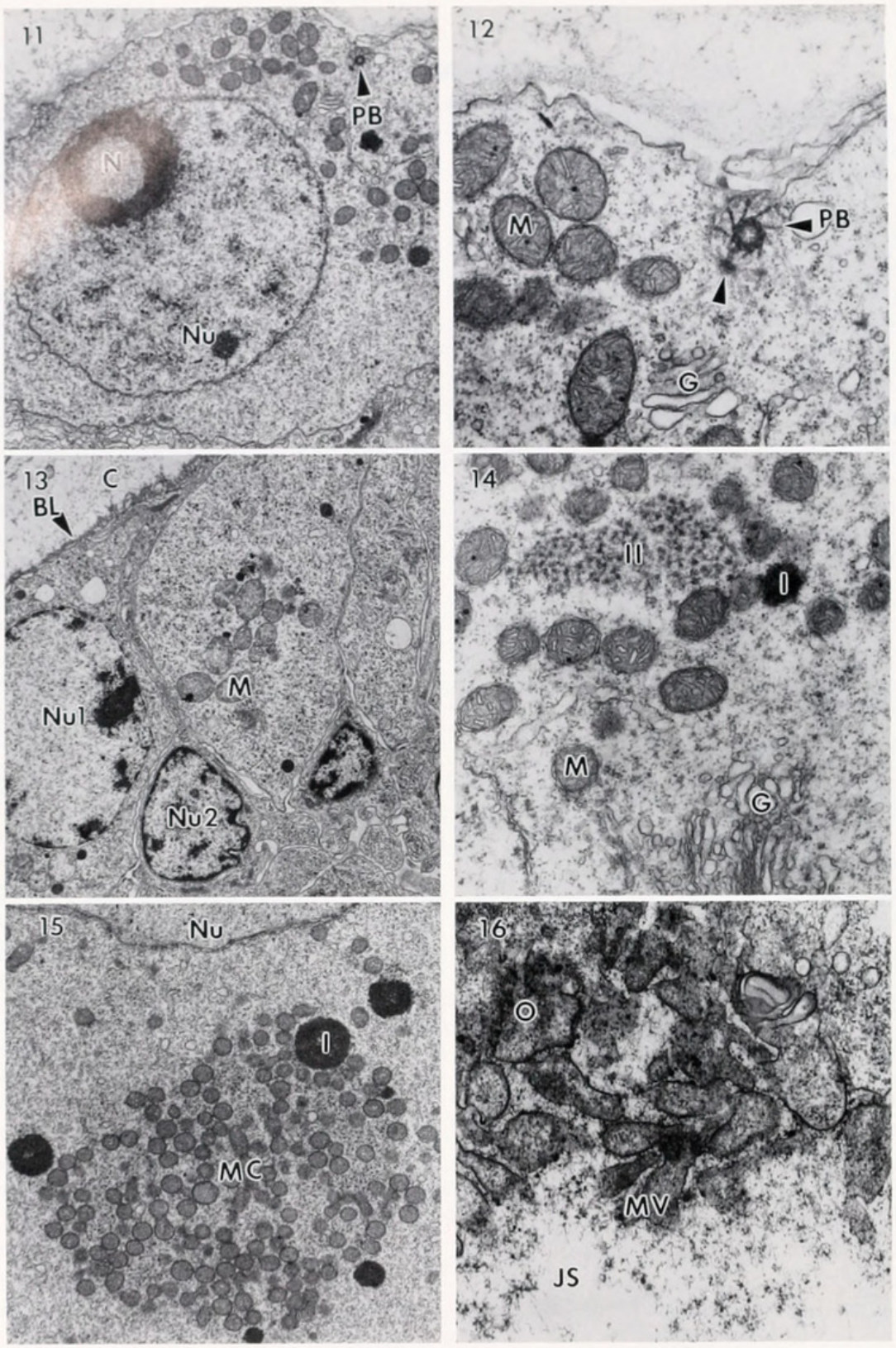

Figure 11. TEM of a germ line cell from a late primary tubule. N. nucleolus, Nu. nucleus, PB. pericentriolar body (satellite structures). 6980×.

Figure 12. TEM at higher magnification of the centriolar satellite structure in Figure 11. G. Golgi body, M. mitochondrion, PB. pericentriolar body (satellite structures). Arrowhead points to microtubules radiating from the centriole. 21,700×.

Figure 13. TEM of cell nest within the gonad basis. BL. basal lamina separating cell nest from, C. connective tissue compartment, M. mitochondria, Nu1. nucleus of a germ line cell, Nu2. nucleus of a somatic cell. 5770×.

Figure 14. TEM showing nuage in a spireme stage oocyte from an early secondary tubule. G. Golgi body, M. mitochondrion, I. type I nuage, II. type II nuage. 13,330×.

Figure 15. TEM showing mitochondrial cloud from a previtellogenic oocyte in a secondary tubule. MC. mitochondrial cloud, Nu. nucleus of oocyte, I. type I nuage. Type II nuage is located between the mitochondria of the cloud. 8200×.

Figure 16. TEM vegetal microvilli in an oocyte from a late secondary tubule just beginning vitellogenesis. Many of the particulate densities are precipitated lead stain, an artifact. JS. jelly space, MV. microvilli. O. oocyte. 20,000×.

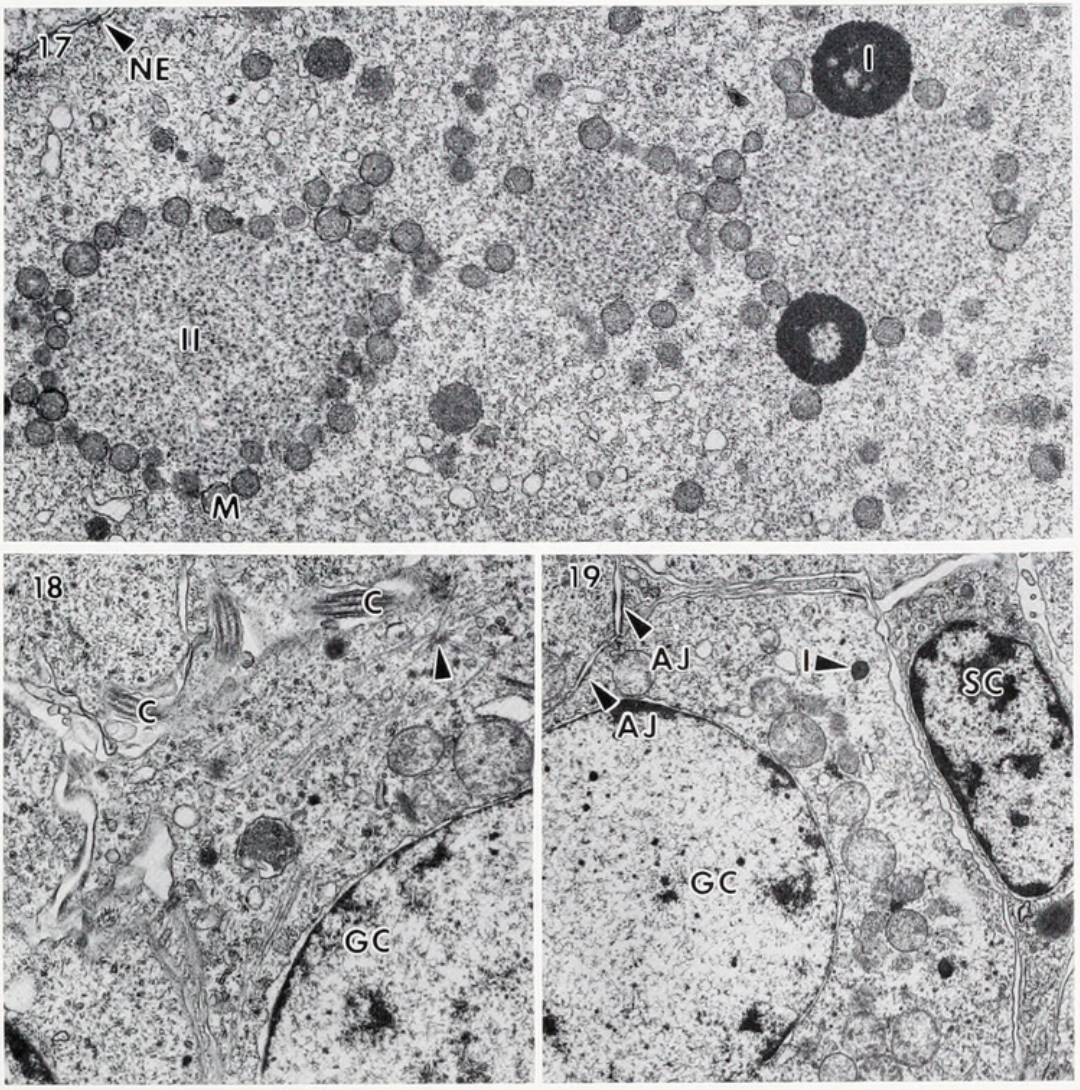

Figure 17. TEM showing unusual spherical aggregations of mitochondria. M. spherical aggregations of mitochondria, NE. nuclear envelope, I. type I nuage, II. type II nuage. 13,060×.

Figure 18. TEM of cilia found on somatic and germ line cells within the cell nests. C. cilium, GC. germ line cell nucleus. Arrowhead marks an apical centriole in this germ line cell. 11,150×.

Figure 19. TEM showing intercellular junctions between adjacent inner epithelial cells in the cell nests. AJ. adhering junctions, GC. germ line cell, SC. somatic cell, I. type I nuage. 8360×.

cent to the nucleus, in the vegetal region of prediplotene oocytes of the secondary tubules (Fig. 3). These are not yolk, but a cloud of mitochondria (Fig. 15). As oogenesis progresses these mitochondria become arranged in striking spherical aggregations which enclose granular electron-dense material (Figs. 14, 17), here called *type II nuage,* which is ultrastructurally indistinguishable from similar nuage found in other echinoderm oocytes (Millonig *et al.,* 1968; Eddy, 1975). Type I nuage (Fig. 14) is also present in these oocytes. Oocytes of secondary tubules have a centriole located in the peripheral cytoplasm (Figs. 11, 12). The centriole has a full compliment of satellite structures (Fig. 12) as would be expected in a cilium producing centriole, yet holothurian oocytes are not known to bear a cilium and none were found in these sections. The centriole and the mitochondrial cloud define the future egg axis, judged by their relationship to the oolamina. It is possible that the centriole acts to fashion the oocyte protuberance, which contains dense arrays of microtubules (Smiley and Cloney, 1985).

Diplotene oocytes in late secondary tubules. The term previtellogensis is used here to include all phases of oocyte development beginning with the production of oocytes in the mitotic proliferation of oogonia, and extending to the inception of active vitellogenesis. Cytologically, the earlier portions of oocyte previtellogenesis can be referred to as spireme stages (Wilson, 1925), describing the characteristic chromosome morphologies. The spireme stages of leptotene, pachytene, and zygotene are evidently accomplished rapidly in *S. californicus* since they are only rarely encountered in sections. The chromosomes decondense at diplotene; the nucleus enlarges and assumes an expanded germinal vesicle configuration, giving the oocyte its most recognizable morphol-

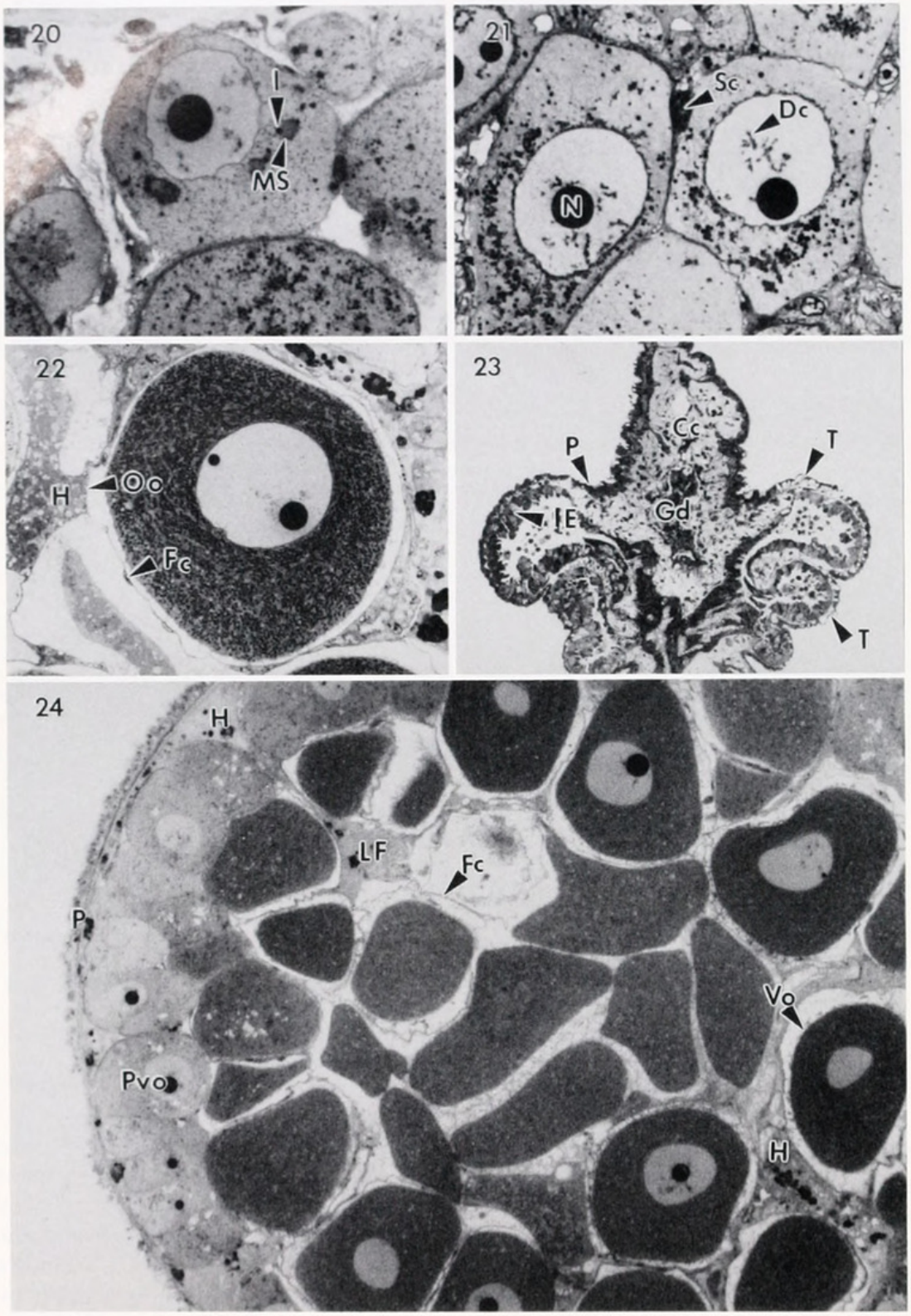

Figure 20. Light micrograph of previtellogenic oocytes in a late secondary tubule. MS. spherical aggregations of mitochondria (see Fig. 17). I. type I nuage. 1130×.

Figure 21. Light micrograph of diplotene configuration to chromosomes of secondary tubule oocytes. Dc. diplotene chromosomes, N. nucleolus, Sc. somatic cells. 940×.

Figure 22. Light micrograph of early vitellogenic oocyte in the late secondary or early fecund tubule. Fc. follicle cells, H. genital hemal sinus of the connective tissue compartment, Oo. oolamina. 375×.

Figure 23. Light micrograph of frontal section through the developing gonad of a juvenile *S. californicus*. CC. connective tissue compartment, Gd. gonoduct, IE. inner epithelium, P. peritoneum, T. tubules. 75×.

Figure 24. Light micrograph of a cross section of a vitellogenic tubule. Fc. follicle, H. genital hemal sinus, LF. longitudinal fold, P. peritoneum, Pvo. previtellogenic oocytes, Vo. vitellogenic oocytes. 180×.

ogy. The diplotene stage extends into the vitellogenic period, and is terminated when the chromosomes enter diakinesis. Diplotene has the longest duration of any of these stages in *S. californicus* (Fig. 20) and most oocytes in late secondary tubules have their chromosomes in this configuration.

The morphology of oocytes and somatic cells in secondary tubules changes greatly during early oocyte growth and differentiation. When tubules have advanced to where their oocytes are in the diplotene stage, somatic inner epithelial cells adhere to the surface of the oocyte (Fig. 10). During the transition from early to late secondary tubules, these somatic cells form junctional complexes between themselves, creating a true follicle around the oocyte, but it is only in late secondary tubules that the parietal inner epithelium is clearly identifiable (Fig. 24). When a follicle is present around secondary tubule oocytes, the jelly space is absent. Oocytes adhere to the connective tissue layer of the ovary (Fig. 10) by their oolamina in all but the most advanced secondary tubules (Smiley and Cloney, 1985), and attempts to manually dislodge oocytes with fine needles were unsuccessful. Oocytes here average about 40 μm in diameter, contain type I and II nuage, and for the most part lack yolk. The large diplotene germinal vesicle nucleus is probably active in synthesizing messages required for further development, but no specific information on this point is available.

Vitellogenesis in smaller fecund tubules. Stichopus californicus oocytes start to accumulate yolk late in the fall, beginning at about the end of October in the San Juan Archipelago population. The onset of vitellogenesis occurs only in late secondary tubules, and is heralded by a substantial increase in cellular debris found in the genital hemal sinus of these tubules (Fig. 22), during the resorptive period and torpor that follows spawning (Fankboner and Cameron, 1985; Cameron and Fankboner, 1986). Vitellogenesis initially proceeds at a leisurely pace, determined by the slow increase in dense basophilic granules in oocytes of animals fixed during November. By late November, there is a marked increase in the number of granules per oocyte section, indicating that the pace of vitellogenesis has increased. The pace continues to increase until mid January when it appears to level off. The accumulation of cellular debris within the genital hemal sinus is paralleled by elaboration of microvilli at the vegetal pole of the oocyte adjacent to the oolamina (Fig. 16).

TEM reveals that the majority of these granules are yolk platelets (Smiley and Cloney, 1985; Fig. 23), but some are mitochondria, and others type I nuage. During vitellogenesis the spherical aggregations of mitochondria that surrounded nuage in early secondary tubules break up. The mitochondria and nuage disperse within the ooplasm and are not localized to the perinuclear vegetal region. Vitellogenesis is detectable earlier in more centrally located oocytes (Fig. 24). Oocytes near the periphery of tubules are the last to show an increase in size and in accumulation of the dense basophilic yolk granules diagnostic of vitellogenesis. The average diameter of oocytes increases during the late fall and through winter and early spring in *S. californicus*.

The juvenile gonad. In juvenile *Stichopus californicus,* the gonad has started to develop by three to four months after metamorphosis (Fig. 23). The initial structures to form include an expanded central connective tissue component of the gonad basis, a developing gonoduct, and two to four pairs of tubules, similar to the conditions reported for *Holothuria parvula* (Kille, 1942). The tubules are small and unbranched. The most posterior tubules are slightly larger than those more anterior, but the germ line cells within their inner epithelia are all in the proliferative oogonial stage, as determined by the presence of mitotic figures in serially sectioned gonads. Juveniles are difficult to raise, and consequently are rare. I took serial 1 μm sections of the gonads from the four specimens I raised, and found no genital rachis (Hyman, 1955; Smiley, 1988b) present in any of these specimens.

Discussion

Structural aspects of oogenesis in Stichopus californicus

This data shows that the gametogenic holothurian ovary consists of three classes of ovarian tubules, which can be defined by their size, their location along the gonad basis, and the cytological stage of the germ line cells within them. These results are harmonious with and expand upon those of Théel (1901), Mitsukuri (1903), and Kille (1942) concerning the function of the smaller ovarian tubules anterior on the basis in other holothurians. However, my results are in conflict with the arguments of Delage and Hérouard (1903) who suggested that the function of the smaller tubules was to provide a fluid that augmented the spawn. The tubules represent a continuum between the smallest most anterior primary tubules, and the largest most posterior fecund tubules. This continuum is broken by the annual episodic cytological changes in the development of the oocytes. All primary tubules, whether larger or smaller, contain only oogonia. All secondary tubules, whether early or late, contain previtellogenic oocytes. The onset of vitellogenesis in secondary tubules during the fall is a convenient marker for determining the initial stage of the fecund tubules.

This description of the resorption of spent tubules, the progressive increase in size of the secondary tubules during autumn, and the localization of separate functions to particular classes of tubules supports the hypothesis that tubules are progressively recruited to a more posterior position along the flanks of the gonad basis as they develop. Direct testing of this hypothesis is not easy. Vital

dye markers disappear long before a year has passed, *S. californicus* eviscerates when tagged, and animals kept in aquaria for more than a few months shrink in size, and fail to develop oocytes properly (Smiley, pers. obs.). In the field, individuals range freely and readily change depth (Courtney, 1927). The most direct test of this hypothesis would be to mark mitotic oogonial cells with radioactive thymidine. A sizable number of animals would have to be tagged to insure significant recovery, and doses of the marker would have to be initially high to be detected reliably. Because the morphological evidence is so compelling, use of this much radioisotope is probably not warranted.

Oocyte polarity and attachment

Holothurian oocytes are among the most visibly polarized in the animal kingdom (Smiley and Cloney, 1985). In fully formed oocytes, this polarization is referred to as the egg axis, which passes from the oocyte protuberance at the animal pole, through the eccentric germinal vesicle, to the oolamina at the vegetal pole. This axial polarization develops gradually as oocytes increase in size and is continuously congruent with an epithelial cell polarization of the germ line cells that is evident from the time they are first identifiable in the cell nests. This epithelial character of the polarization is defined by the presence of a luminal apical surface, the apical centriole, junctional complexes with adjacent cells, and a basal lamina.

The centriole of *S. californicus* oocytes is apical (animal quadrant), and similar in position to those in oocytes of the asteroid *Pisaster ochraceus* (Schroeder and Otto, 1984; Schroeder, 1985). This is in contrast to reports in *Xenopus* oocytes of a basal centriole (in the vegetal quadrant) which is associated with the mitochondrial cloud (Al-Mukhtar and Webb, 1971; Coggins, 1973), a position not homologous to that in any other epithelial cell. I contend that these workers have mistakenly assigned a basal position to this centriole because they assumed a vegetal location for the aggregated mitochondria of the Balbiani body. But, the terms 'Balbiani body' and 'yolk nucleus' actually mean any basophilic zone near the germinal vesicle (Nørrevang, 1968), and neither aggregated mitochondria nor Golgi bodies are restricted to the vegetal quadrant or to the mitochondrial cloud according to other investigators (Heasman *et al.*, 1984; Wylie *et al.*, 1985).

Wilson (1925) suggested promorphological homology for the vegetal location of the oocyte centrioles through his comparison of oocytes with spermatozoa which have a centriole basal to the nucleus. However, his depiction of spermatozoa is inverted compared to their true promorphological architecture. The trailing flagellum is actually an apical cilium; and the centrioles of spermatozoa are apical to the nucleus. In other words, sperm swim backwards. Consequently, if the centriole is indeed vegetal in *Xenopus* oocytes, one would expect to identify centrioles in two different axial positions in premetamorphic tadpole gonocytes. Al-Mukhtar and Webb did not report this observation. Recent analyses using immunocytochemical methods did not identify a vegetally located centriole in *Xenopus* (Palacek *et al.*, 1985; Dent and Klymkowsky, 1988). Given these arguments, it seems prudent to reinvestigate the axial pattern in *Xenopus* prediplotene oocytes using these techniques.

In some previous descriptions of other holothurians (Inaba, 1930) and other echinoderms (Boveri, 1901), oocytes were often thought to be attached to the somatic ovary by their vegetal surfaces. Other investigators hold that oocytes are attached by the animal surface (Lindahl, 1932; Monné, 1946, Holland *et al.*, 1975). In *Stichopus californicus,* both views are correct depending on the stage of oogenesis. In early stages, oocytes are connected to the somatic ovary by the oolamina at their vegetal pole. In more advanced stages, the protuberance, an animal pole elaboration, connects oocytes to the cells of the somatic ovary. Sections through less fully developed ovaries might lead one to erroneously conclude that holothurian oocytes are always attached by their oolaminae. Information derived from thicker sections of poorly fixed and paraffin embedded specimens might also lead to erroneous conclusions.

Origin of the germ cells

If we accept type I nuage as a more critical marker of germ line cells than either alkaline phosphatase or dense RNA accumulation (Eddy, 1975), then PGCs can be reliably identified only with TEM or immunocytochemistry (Strome and Wood, 1982, 1983). Between the somatocoels in newly metamorphosed *S. californicus* there is set of mesenchymal cells which contain dense RNA accumulations characteristic of germ line cells, but these do not have unambiguous type I nuage as determined by TEM (Smiley, 1986). In the present study, I show that when *Stichopus californicus* is six months old, gonadogenesis has begun, and unambiguous PGCs are located in the connective tissue compartment of the dorsal mesentery. The location and timing of the appearance of these germ line cells is consistent with previous reports of the onset of holothurian gonadogenesis (Cuénot, 1948; Wootton, 1949). These results support the views of Nieuwkoop and Sutasurya (1981) who concluded that echinoderm germ cells first become visible some months after settlement. However, primordial germ cells have been described in newly metamorphosed echinoids (Houk and Hinegardner, 1980).

These arguments should not be interpreted to mean either that germ line cells of holothurians or other echinoderms show an irrevokable lineage restriction, or that

there is an inviolable restriction barring other cell types from becoming germ line cells. The results of Kille (1942), on posterior half regenerates of *Holothuria parvula* following binary fission, suggest that at least peritoneal epithelial cells can transform into germ line cells under some conditions. Théel (1901) described cell aggregations lying against the ascending gonoduct in the dorsal mesentery of adult *Mesothuria intestinalis,* as a genital cord, which contained cells that he interpreted to be PGCs. The histological description of the genital cord in *M. intestinalis* does not match that of the cell nests in the gonad basis of *S. californicus,* but the location and description of *M. intestinalis* PGCs themselves are quite similar to those of *S. californicus* reported here. Presumptive PGCs occur in cell nests lodged within the dorsal mesentery of the aspidochirote *Holothuria parvula* (Kille, 1942). Until PGCs have been described in holothurians from different taxa, we must assume that the differences in location and structure of the germ line bearing tissues reflect intraordinal variations.

Cytological aspects of oogenesis

Nuage. This is the first specific identification of nuage in a holothurian oocyte. Nuage is electron-dense material, lacking a unit membrane, which is found in the cytoplasm of germ line cells where it commonly occurs in two forms (Eddy, 1975, Kessel, 1983). Type I nuage is about 1 μm in diameter and is a granular electron-dense material (Fig. 17) forming rough spheres. Type II nuage consists of minute particulate electron-dense granules surrounded by a homogeneous matrix which is slightly more electron dense than ordinary cytoplasm and which excludes ribosomes (Figs. 14, 17). Both types of nuage are often associated with mitochondria, especially in female germ line cells (Fig. 17; and Millonig *et al.,* 1968). The relationship between nuage and the mitochondrial yolk cloud is likely to be a fundamental one judging from the association between these elements in the oocytes of species from numerous phyla (Eddy, 1975). To date there is no detailed explanation for this association.

In echinoderms, a number of different names have been applied to nuage including 'dense lumps' in oocytes of the crinoid *Nemaster rubignosa* (Holland, 1971) and probably the extruded nucleoli in oocytes of the holothurian *Thyone briareus* (Kessel, 1966). Type I and type II nuage have been reported in oocytes of adult echinoids (Millonig *et al.,* 1968). Houk and Hinegardner (1980) found structures similar to type I nuage, which they called goniosomes, in cells presumed to be PGCs in newly metamorphosed *Lytechinus pictus,* and types I and II nuage were found in oocytes of *Xenopus laevis* (Al-Mukhtar and Webb, 1971; Coggins, 1973). Available reports suggest that all animals probably have some form of germ line specific granule, whether it be called nuage (Eddy, 1975), polar granules as in *Drosophila melanogaster,* or P granules as in *Caenorhabditis elegans* (Wolf *et al.,* 1983). Nuage appears to be germ line specific, because somatic cells have not been shown to contain it (Eddy, 1975). Determination of the origin of germ line cells in other echinoderms may be facilitated by using nuage as a marker. Nuage is distinct from the *heavy* (dense) *bodies,* large granular inclusions surrounded by annulate lamellae often encountered in a variety of echinoderm oocytes (Afzelius, 1957; Eddy, 1975; Kessel, 1966; see Smiley and Cloney, 1985, Fig. 22).

Nuage is no longer aggregated in *S. californicus* oocytes after the onset of vitellogenesis. The spherical aggregations of mitochondria which surround type II nuage disperse, presumably carrying nuage along with them. A similar dispersal was noted in echinoids (Millonig *et al.,* 1968). If nuage is a germ line determinant, then its dispersal may be expected in those animals, such as echinoderms, where regulative development is the rule. If the nuage were not to disperse it would be absent from isolated blastomeres which then would lack the germ line determinant, a condition incompatible with numerous surgical studies on echinoderm embryos (Hörstadius, 1973). In support of this assessment is a recent report by Wylie *et al.* (1985), which suggests that the mitochondrial cloud of *Xenopus laevis* disperses throughout the vegetal ooplasm when the oocyte becomes fully formed. In this case mitochondria and nuage remain somewhat localized to the vegetal quadrant.

Mitochondrial cloud. This structure (Fig. 15) has been referred to as the yolk nucleus, the Balbiani body, and the Balbiani vitelline body in other oocytes (Nørrevang, 1968; Guraya, 1979), as well as the mitochondrial cloud (Heasman *et al.,* 1984; Wylie *et al.,* 1985). The definitions for these terms appear to be capricious; they refer to a number of structures having only a juxtanuclear position and basophilic staining characteristics in common (Nørrevang, 1968; Guraya, 1979). The term 'mitochondrial cloud' is at least accurately descriptive and is used here.

In *S. californicus,* the mitochondrial cloud is always found in the vegetal hemisphere of developing oocytes, close to the nuclear envelope. In sections through secondary tubules (Figs. 2, 3), adjacent oocytes appear to have their mitochondrial clouds in opposing orientation. But the complex topology of the inner epithelium shows that this is not the case (Smiley and Cloney, 1985). In these oocytes, as well as in those of *Xenopus laevis,* the vegetal mitochondrial cloud appears to be the site for mitochondrial proliferation (Heasman *et al.,* 1984), judging by the frequency with which dumbell-shaped mitochondria are encountered. However, the identity of the bio-

chemical pathways controlling this proliferation are not known. Among other echinoderms, a mitochondrial cloud is found in crinoid oocytes (Holland, 1976). The report that echinoid oocytes contain yolk nuclei (Verhey and Moyer, 1967) must be reinterpreted, because the micrographs these investigators present as documentation of their claim show only annulate lamellae (their Fig. 20).

Vitellogenesis. Intense vitellogenesis begins in the late secondary tubules after the autumnal resorption of the viscera in *Stichopus californicus.* At this time, the genital hemal sinus contains cellular debris and unidentifiable particulate matter, and the number of yolk granules in oocytes increases. Vitellogenesis begins in oocytes at the center of the *S. californicus* ovary, but an explanation for this is not readily apparent. It appears likely that oocyte growth constricts passage of hemal fluid through the genital hemal sinus toward the interior of the tubule (Smiley and Cloney, 1985). But rapid growth of central oocytes, occluding only the most central parts of the ovary, would still allow the peripheral oocytes access to an unrestricted hemal sinus.

Yolk proteins appear to be preferentially taken up at the vegetal pole of the *S. californicus* oocyte, based on fine structural information alone. Elongate microvilli are found at the vegetal surface of *S. californicus* oocytes during the post resorptive period of vitellogenesis (Fig. 16). Similar structures have been described at the presumptive vegetal pole of an asteroid oocyte (Beijnink *et al.,* 1984). Some elasipod holothurians have very large eggs, and uptake occurs over the entire egg surface (Eckelbarger, pers. comm.).

Recent studies of echinoid vitellogenesis indicate that cells within the echinoid ovary, either accessory cells or oocytes or both, may synthesize some of the yolk glycoproteins (Ozaki *et al.,* 1986; Shyu *et al.,* 1986). Cells explicitly homologous to the accessory cells do not occur either in any holothurian yet described (Smiley and Cloney, 1985) or in crinoids (Holland *et al.,* 1975). Consequently, these classes require a different mode of nutrient replenishment, which may be provided by the coelomocytes of the perivisceral coelomic fluid. The most abundant protein found in echinoid coelomic fluid shows immunocytochemical cross reactivity with the egg 23S yolk glycoprotein (Giga and Ikai, 1985a, b). Coelomocytes in the coelomic fluid may be the largest single source for all yolk glycoproteins, at least in *Dendraster excentricus* (Harrington and Ozaki, 1986), but ovary (Ozaki *et al.,* 1986) and gut (Shyu *et al.,* 1986) also produce yolk precursor proteins. Results from the experiments of Shyu *et al.* (1986), while documenting that the coelomic fluid contains large amounts of the yolk precursor proteins in *Strongylocentrotus purpuratus,* appear to contradict the conclusion that coelomocytes are a major source. Shyu *et al.* (1986) did not include a divalent cation chelator in their coelomocyte culture medium, and they only labeled for about one quarter the time that Harrington and Ozaki did. In the absence of 10–50 m*M* EDTA, echinoid petaloid amoebocytes undergo an irreversible clotting reaction (Otto *et al.,* 1979; Edds, 1980). This suggests that the failure of Shyu *et al.* to record radiolabeled amino acid incorporation into vitellogenin in the coelomocytes might be due to differences in the duration of labeling or to the clotting response. Future research into holothurian vitellogenesis should include an examination of coelomocytes to see if they are a rich source of vitellogenins. If they prove to be, then the absence of a discrete nutrient storage organ in this class might be explained, and the pathway of nutrient replenishment proposed by Smiley and Cloney (1985) supported.

The annual cycle of the S. californicus *ovary*

The results of this study demonstrate that holothurian oogenesis follows the same cytological course that has been described for a number of other animals in different phyla (Wilson, 1925). In all non parthenogenetic animals, the PGCs, proliferating oogonia, spireme stages of meiosis I prophase, diplotene, and diakinesis sequence is followed in exact order. Repetition of this order, coupled with the localization of these clearly interpretable cytological stages into discrete and linearly ordered structures within the *S. californicus* ovary, offers a simple explanation of the annual ovarian cycle in holothurians.

The cytology of the germ line cells of cell nests, primary and secondary tubules, and the complete resorption of spent fecund tubules support the idea that more anterior tubules are progressively recruited to a more posterior position on the gonad basis with the same timing as that shown by changes in the cytological stages in the oocyte nuclei. This suggestion was also made in other aspidochirotes by Mitsukuri (1903) for *Stichopus japonicus,* Kille (1942) for *Holothuria parvula,* and Deichmann (1948) for *Neostichopus grammatus,* and for the dendrochirote *Thyone briareus* by Kille (1939). This notion of progressive recruitment is attractive in *S. californicus* because it is a large animal that doubtless lives for many more than six years, and probably spawns in all but the first three (Fankboner and Cameron, 1985; Cameron and Fankboner, 1986). If progressive recruitment of new tubules did not occur in *S. californicus,* then after its sixth year the animal would be without spawn.

The Model

My results provide the information necessary to propose a model of how the annual cycle of the *S. californicus* ovary progresses. The annual cycle begins with the resorption of spent tubules in year **N,** and can be summarized as follows. Some of the nutrients derived from

phagocytosis of the spent tubules are taken up into the genital hemal sinus of the secondary tubules, as indicated in Figure 22. Concurrent with the increase of material in the genital hemal sinus, vitellogenesis begins in these oocytes. Therefore, from late fall to early spring, secondary tubules become the fecund tubules of year **N + 1** and are located at the posterior of the gonad basis. Immediately after resorption of the spent tubules, the genital hemal sinus and lumina of primary tubules and hemal lacunae in the connective tissue compartment also become occluded with debris and nutrients. The primary tubules, having completed the oogonial proliferative divisions, become the secondary tubules of year **N + 1** and are now located further back on the basis. During the fall, winter, and early spring, oocytes in these new secondary tubules (year **N + 1**) undergo the early prophase stages of meiosis I, culminating in diplotene. Nests of cells within the gonad basis emerge from the connective tissue compartment during the early fall. Surrounded by the perivisceral peritoneum, they become the primary tubules of year **N + 1** and are found in the anterior location characteristic of primary tubules.

General applicability of the model

Hyman (1955) suggested that resorption of spawned tubules would prove to be the rule among holothurians. If spent tubules are resorbed after spawning, it is likely that progressive recruitment of tubules also occurs, with anterior less advanced tubules moving to a more posterior location concomitant with advancement in the cytological development of the germ line cells. Progressive recruitment of ovarian tubules has been strongly suggested in several aspidochirotes; *Stichopus californicus* (this study), *Mesothuria intestinalis* (Théel, 1901), as well as in the dendrochirote *Thyone briareus* (Kille, 1939). However, there is too little comparative information on oogenesis in apodan, molpadian, dactylochirote, and elasipod holothurians to assess the applicability of this model to them. The best information supporting this model are the surgical experiments of Kille (1939) with the dendrochirote *Thyone briareus.* These showed that of all the ovarian tissues, the gonad basis alone can regenerate oocytes. When only fecund tubules were ablated, new oocyte-bearing tubules regenerated from the basis. When the entire gonad including the basis was removed, regeneration failed. Kille's (1942) study of the gonad in *Holothuria parvula,* an animal which also reproduces asexually through binary fission, provides additional support for this model.

The data and analysis presented in this paper, while considerably more precise in determining cell and tissue level changes, is limited by this precision, and cannot supplant population level surveys. Information derived by the gonad index method may be applicable in assessing this model's generality. From the data presented here, it is clear that these gonad index studies measure vitellogenesis and not the process of oogenesis. A number of species have been investigated in this way, including *Stichopus californicus* (Cameron and Fankboner, 1986), *Stichopus japonicus* (Tanaka, 1958), *Thelenota ananas* and *Microthele nobilis* (Conand, 1983), and the data they present are entirely consistent with the pattern described here (see Smiley *et al.,* for a more complete review). Consistency, not confirmation or refutation, is all that can be expected from such studies, because gonad index assessments only measure average changes in populations. Analysis of reproductive cycle data derived from gonad indices of populations of many different holothurians shows that most species have the general features predicted by this model. This includes marked diminishment in gonad index after spawning, the subsequent further reduction in index corresponding to phagocytosis of spent tubules, the measurable lowest size corresponding to the basis and more anterior tubules, and the gradual build up in index as vitellogenesis for the next season commences (Smiley *et al.,* 1988).

This model may not account for the gonad index data of male holothurians of any order, nor of small and hermaphroditic holothurians, such as *Cucumaria curata* (Rutherford, 1973), or *Rhabdomolgus ruber* (Menker, 1970). Because many Arctic, Antarctic, and deep sea species are thought to produce eggs continuously (Feral and Magniez, 1985; Tyler *et al.,* 1985) they may also pose problems for this model.

Comparisons with other echinoderm classes

The anatomical and histological simplicity of this holothurian ovary offers unique information that is applicable to understanding the dynamics of oogenesis in other echinoderm ovaries (Smiley, 1984, 1986, 1988b; Smiley and Cloney, 1985; Smiley *et al.,* 1988). It is difficult to study microscopic morphological changes among a synchronously developing population of oocytes in other echinoderms. Primordial germ cells, mitotic oogonia, and early meiotic prophase staged oocytes are considerably smaller than previtellogenic or vitellogenic oocytes; they can be quite difficult to locate within the inner epithelium. This difficulty is reflected in the limited reports on the earliest stages of oogenesis in other echinoderms (Holland *et al.,* 1975; Walker, 1982). These same structural problems have prevented biochemical analysis of oogenesis in any echinoderm. Given the identifiable and discrete localization of the major cytological stages in oogenesis in the holothurian ovary, such biochemical analyses may now be possible.

Unanswered questions

This study offers a number of important observations and analyses which require more detailed investigation. Among these are the possible signalling role of the resorptive phase and the onset of torpor in driving subsequent oogenesis. There is no clear description of the earliest stages of development in the PGCs of any holothurian, nor of their ultimate source. If cells other than those containing nuage are capable of transforming into PGCs (Kille, 1942), then the conditions under which this transformation can occur are important to know. This knowledge would allow a more detailed analysis of the mechanisms of coordinated control over the regulatory pathways for differentiation in an echinoderm, an elusive problem made more difficult by the extreme regulative development of these animals. The mechanisms of control over the proliferative divisions of the mitotic oogonia are not known, nor is the control of entry into the prophase stages of meiosis I or the biochemical details of these changes. Even if the onset of vitellogenesis is controlled by resorption of the viscera, a mechanism that could explain how the interior oocytes of late secondary tubules are directed to begin vitellogenesis prior to the peripheral oocytes is elusive. We have very little information on the relative contribution of the oocyte itself on yolk formation in any holothurian species. Finally, the reasons for the general resorption of the viscera of this holothurian each fall, even in those individuals that have not yet reached sexual maturity (Fankboner and Cameron, 1985), remain a mystery.

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