

## ULTRASTRUCTURAL ASPECTS OF SEXUAL REPRODUCTION IN THE RED TIDE DINOFLAGELLATE *GONYAULAX TAMARENSIS*<sup>1</sup>

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### ABSTRACT

The marine dinoflagellate *Gonyaulax tamarensis* Lebour is best known for its propensity to form blooms known as red tides in coastal waters worldwide. This paper examines the sexual cycle of this organism using light and electron microscopy. Sexual reproduction begins with contact between thecate gametes which subsequently shed their thecae to fuse along their pellicular layers. Nuclear fusion occurs well after cytoplasmic fusion and is characterized by several distinctive features: a highly vesiculate nucleoplasm without microtubules; nucleoli and V-shaped chromosomes about the nuclear envelope distal to the region of nuclear contact; and each chromosome possesses a longitudinal line, the central chromosomal axis. Fusion results in a planozygote with numerous cytoplasmic storage products and a slightly thickened layer beneath the pellicle. Subsequent loss of thecal plates and a thickening of the sub-pellicular layer results in a non-motile hypnozygote. A newly-formed hypnozygote possesses numerous minute papillae along its outer surface, formed by the up-folding of the accumulating wall layer. Maturation of the hypnozygote wall results in a smooth three-layered wall, the outermost layer of which is the pellicular layer. Hypnozygote germination produces a large quadriflagellate planomeiocyte with a single nucleus and thecal plates identical to vegetative cells. Two subsequent divisions, presumably meiotic, result in four cells morphologically identical to vegetative cells.

**Key index words:** dinoflagellates; life cycle; sexual reproduction; ultrastructure; zygotes

The earliest account of sexual reproduction in dinoflagellates was that of Joseph (1879) who described the fusion of swimming cells of *Peridinium stygium* Ehrenberg. Later, Pouchet (1883) described stages of gamete fusion and nuclear rotation in several species. Other early reports describing sexuality in dinoflagellates were regarded with much skepti-

cism until von Stosch (1964, 1965) documented the process in two species of *Ceratium*. Since that time sexual stages have been reported for over 20 species of dinoflagellates (Beam and Himes 1980, Pfister and Anderson 1987).

Sexuality is now regarded as important and common in the life cycles of dinoflagellates. Aside from the clear benefit of genetic recombination, sexuality is also beneficial in many cases because it results in a resting cyst which can aid in species dispersal, initiate blooms and aid in survival during adverse conditions. The importance of sexuality in the "red tide" dinoflagellate *Gonyaulax tamarensis* Lebour (synonyms *Protogonyaulax tamarensis* Taylor and *Alexandrium tamarense* Halim) is well established (Anderson and Wall 1978, Turpin et al. 1978). Sexual reproduction in this organism involves the formation of motile gametes which fuse to form a motile zygote (planozygote). The presumably diploid planozygotes swim about for several days before losing motility and becoming thick-walled resting cysts (hypnozygotes). The transition from planozygote to hypnozygote begins as the motile planozygote drops its flagella and settles from the water column. As is the case with other dinoflagellates, hypnozygote development occurs with the deposition of a thick, multi-layered wall formed internal to the thecal plates. Hypnozygotes are a dormant phase of the dinoflagellate life cycle. Under normal culture conditions, the dormancy period of hypnozygotes from different species is highly variable, lasting from as few as 12 h in *Peridinium gatunense* Nygaard (Pfister 1977), to several weeks in *Woloszynskia apiculata* von Stosch and *Gymnodinium pseudopalustre* Schiller (von Stosch 1973), to several months for *Gonyaulax tamarensis* (Anderson 1980). In those cases where a single germling emerges from the hypnozygote (as with *G. tamarensis*) prior to nuclear divisions, this cell is believed to still retain the diploid condition and is termed a planomeiocyte (von Stosch 1973). The planomeiocyte cell divides relatively rapidly so that within 24 h of excystment, the first division has produced two cells. Within another day four cells are present, each resembling a typical vegetative cell

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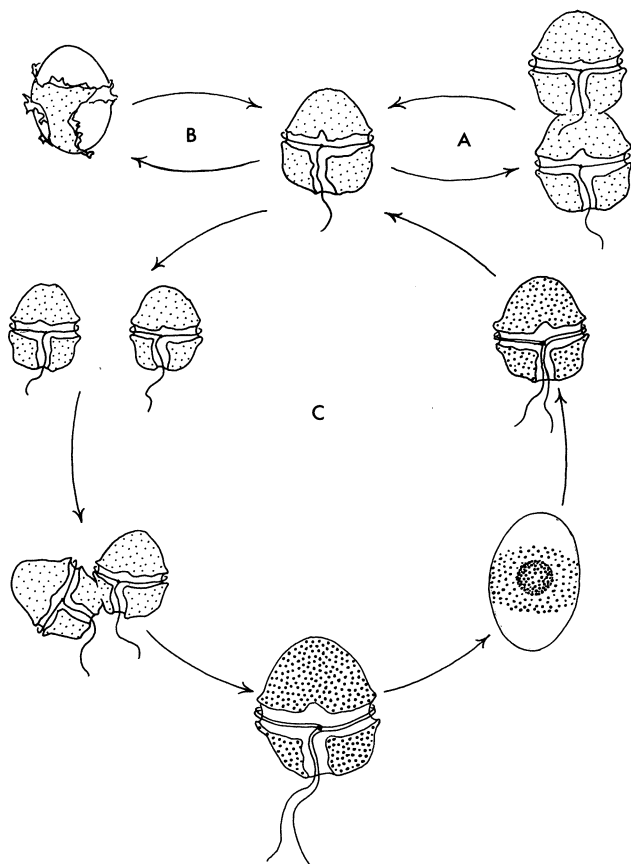


FIG. 1. Stages in the life cycle of *Gonyaulax tamarensis*. From the central vegetative cell three cycles radiate. Loop A depicts mitotic cell division, the most common form of cell reproduction in dinoflagellates. Loop B portrays temporary cyst formation; short term cysts which withstand temporarily adverse environments. Loop C shows the sexual life cycle in which motile gametes fuse to form a large quadriflagellate planozygote followed by a non-motile hypnozygote and finally the excysted motile planomeiocyte. Divisions of the planomeiocyte regenerates normal vegetative cells.

in size and shape (Pfiester 1976, 1977, Anderson and Wall 1978, Walker and Steidinger 1979, Sako et al. 1985).

Past studies of sexuality in dinoflagellates have generally been a combination of life history descriptions and induction experiments (von Stosch 1973, Pfiester 1975, 1976, 1977) or physiological investigations (Anderson et al. 1984, Binder and Anderson 1987). Relatively little is known of cell morphology and development during the stages of dinoflagellate sexuality, in part because of the difficulty in obtaining good fixations due to the thick impermeable cyst wall. Spector et al. (1981) described the process of gamete fusion at the ultrastructural level for the freshwater dinoflagellate *Peridinium cinctum* Lindemann, but information on the sexuality of marine dinoflagellates is limited. The life cycle of *G. tamarensis* has been largely worked out by light microscopy and field studies. In this paper we describe the entire sexual cycle in this

important dinoflagellate at the ultrastructural level and compare the results with those known from other dinoflagellates. This is the first paper to present the entire sexual life history of one dinoflagellate at the ultrastructural level.

#### MATERIALS AND METHODS

*Gonyaulax tamarensis* var. *excavata* was maintained at 20° C in K medium (Keller and Guillard 1985) without added silica under a 14:10 h L:D cycle. The clone (GTMP) was started from a single hypnozygote cyst isolated by D.M. Anderson from Mill Pond on Cape Cod, Massachusetts in 1978. Cells were cultured in 25 mL medium in Surfasil (Pierce Chem., Rockford, Illinois) treated 50 mL glass tubes. Procedures for preparing medium and glassware were as described in Anderson et al. (1984).

Sexual stages of *G. tamarensis* were induced by transferring log phase vegetative cells into nitrogen depleted medium (Anderson et al. 1984). Fusing cells were noted in the cultures from 5–7 days after transfer and were distinguished from dividing cells by the oblique angle of the paired cells' cingula during fusion (dividing cells have parallel oriented cingula). Planozygotes were characterized by their double set of flagella, larger size and darker pigmentation compared with vegetative cells. Hypnozygotes appeared at the bottom of culture tubes approximately 2 weeks after sexual induction. For the study of hypnozygote germination, cysts were collected by one of us (DMA) from bottom mud samples (4–5 cm deep) from Perch Pond, Cape Cod, Massachusetts. From this depth cysts are presumed to be several years old (Anderson, pers. comm.). Cysts were held in the dark at 20° C for approximately 1 month before germination studies. Individual cysts were pipetted from microscope well slides containing bottom mud and each was transferred to a separate well in a 96-well tissue culture plate filled with 130  $\mu$ L growth medium. The plate was incubated at 15° C under a 14:10 h L:D cycle using cool-white fluorescent lighting. After approximately 3 days motile germlings (planomeiocytes) were observed. Cells soon divided and were pipetted at the 1-, 2- and 4-cell stage into separate microcentrifuge tubes and fixed for electron microscopy.

Samples for transmission electron microscopy were fixed simultaneously with a solution of 3% buffered glutaraldehyde (phosphate buffer pH 6.8–7) and 2% buffered OsO<sub>4</sub> for 7 min at room temperature. Samples were rinsed twice in buffer, rinsed again in distilled water and dehydrated in a graded ethanol series (25, 50, 75, 95, 100, 100%) on ice for 5–10 min each. Samples were embedded in epoxy resin (Polybed 812, Polysciences, Warrington, Pennsylvania), spread into a thin film on a teflon-coated glass microscope slide and covered with a teflon-coated coverslip. After polymerization of the resin (60° C, 24 h), cells of interest were circled with a marker under a light microscope, cut out of the thin resin layer, and mounted on an epoxy block for ultramicrotomy. Serial sections were cut, post-stained with uranyl acetate and lead citrate, and observed on a Philips 300 or Siemens 1A transmission electron microscope. For scanning electron microscopy samples were fixed and dehydrated as above followed by critical point drying, sputter coating with gold-palladium and observations on an Hitachi SEM.

#### RESULTS

The complete life cycle of *G. tamarensis* as detailed from light microscopic studies (Dale 1977, Anderson and Wall 1978, Turpin et al. 1978, Anderson 1980) is depicted in Figure 1. Loop A depicts mitotic cell division, the most common form of cell reproduction in this and other dinoflagellates. Loop B depicts temporary cyst formation: short term cysts formed when vegetative cells encounter adverse en-

vironmental conditions. Loop C is the sexual cycle and the subject of this paper.

Sexuality begins with the appearance of fusing gametes in cultures within 4–5 days of inoculation into nitrogen-depleted medium. Fusing cells, generally observed toward the bottom of culture tubes, are distinctive because they tend to swim in rather tight circles. The isogametes are always thecate cells, somewhat smaller than vegetative cells (20–25  $\mu\text{m}$  vs. 30–35  $\mu\text{m}$ ) and less heavily pigmented. Fusion is typically initiated at the hypothecal region of each with one cell slightly higher than the other, although other orientations have been observed. Contact often occurs at a skewed angle so that the two cingula are at an oblique angle to each other (Fig. 2). Examination of early gamete fusion reveals a narrow cytoplasmic connection between the cells near their basal bodies (Fig. 4). The nuclei remain far apart during the early stages of fusion, and often the chromosomes of each of the two nuclei lie at right angles with respect to each other. As a result, the chromosomes of one nucleus appear in cross section whereas in the second nucleus they are seen in longitudinal section (Fig. 5). As fusion proceeds and the cytoplasmic connection widens, the nuclei migrate toward the region of fusion. As the nuclei approach each other, they realign so that their chromosomes are parallel to each other and their nucleoli are situated distal to the area of early nuclear fusion (Figs. 6, 8). When the gamete nuclei contact they are characterized by numerous cytoplasmic invaginations (Figs. 6, 8) making the separate nuclear boundaries difficult to distinguish. Unlike dividing nuclei of *G. tamarensis*, in which microtubules are present within mitotic channels (Fritz 1986) microtubules have not been observed at any time within the nuclear invaginations of fusing cells.

Initial cell contact and the start of fusion begins with fully thecate gametes. The actual process of cytoplasmic fusion, however, involves not the fusion of thecae, but instead the fusion of gamete pellicular layers (Fig. 7, inset). Some thecal plates are lost during the fusion process, although others may remain to surround the gametes until late in cytoplasmic fusion. Within the fusing nuclei the arrangement of chromosomes and nucleoli appears to be very precise. In serial sections of fusing cells, V-shaped chromosomes are found attached to the nuclear envelope only along the distal portion, away from the area of initial nuclear fusion (Figs. 8, 9). The free ends of the chromosomes, therefore, face the area of initial contact. The nucleoli, moreover, are positioned at the back of the fusing nuclei and are directly opposed to each other (Fig. 8).

Another feature common to the chromosomes of fusing cells is a line, which we designate the central chromosomal axis, running the entire length of each chromosome (Figs. 5, 9). Serial sections demonstrate that the axis is surrounded by a thin boundary of less dense material. The axis and surrounding halo

are apparent, within each chromosome, in only three successive serial sections (40–80 nm sections), the central axis itself is visible in only one section.

The completion of cytoplasmic and nuclear fusion results in the formation of a motile quadriflagellate planozygote. The planozygotes are characteristically larger than vegetative cells (40–50  $\mu\text{m}$  in length) and more densely pigmented. Planozygote chromosomes are often seen as pairs with the light microscope (Fig. 3). Two trailing flagella are characteristic of this stage (Fig. 10). Thecal plate arrangements appear to be very similar in planozygotes and vegetative cells, although planozygotes often have a more elongate epitheca (Fig. 11). The internal features of the planozygote, however, appear rather different from vegetative cells. The heavier pigmentation observed with the light microscope is due to a denser cytoplasm with less vacuolar space and an increase in lipid storage bodies and starch grains (Fig. 12). The planozygote amphipha is changed as well. The region just inside the pellicular layer is thickened with a dense material (Fig. 13, arrowhead) reminiscent of the material which makes up the temporary cyst wall (Fritz 1986). The planozygote remains motile for several days before transforming into a non-motile resting cyst.

The cellular changes involved in the transformation from a motile planozygote to a hypnozygote begin as the flagella and thecal plates are shed and the cell elongates (Fig. 14). The deposition layer beneath the pellicular layer, which began to thicken in the planozygote stage, increases in thickness and begins to fold up, forming minute projections (papillae) which appear on the outer cyst wall (Figs. 15, 16, inset). The young hypnozygote wall has a roughened appearance (in addition to the minute papillae), and it retains some of the remnant features of the motile cell. The cyst in Figure 16 retains an equatorial band which marks the former cingulum region and a depression where the flagella previously exited the theca.

Figures 17 and 18 are of the walls of two hypnozygotes from culture in different stages of development. Figure 17 shows a cell which possesses the roughened cyst wall characteristic of newly-formed hypnozygotes, whereas Figure 18 is of an older cell with a thicker, more smoothly-surfaced wall. Both cysts contain large amounts of starch and lipid. The lipid bodies in Figure 17 are arranged along the periphery of the cell just beneath the cyst wall. The wall at this stage consists of a single layer of material internal to the pellicular layer. In contrast, the cyst wall in Figure 18 is thicker and smoother and composed of two distinct layers beneath the electron dense pellicular layer (layer 1 in this micrograph). Layer 2 is a thickened band of less dense material which occasionally appears fibrous, and layer 3 is an inner amorphous band of material. Adjacent to the numerous lipid bodies located in the peripheral cy-

toplastm are numerous regularly spaced oval bodies (0.5–1.0  $\mu\text{m}$  diam) filled with many electron dense particles arranged in concentric rings (Fig. 17). In a single thin section through the cyst 8–12 of these bodies are found. The accumulation body, or red drop, commonly reported in light microscopic studies is visible in Figure 18. The large, centrally located nucleus contains typical banded chromosomes with numerous thin (15–25 nm wide) cables within the nucleoplasm (Fig. 19). The cables are located near the nucleolus and in serial sections appear to emanate from it. Several chromosomes within the hypnozygote nucleus lie very close together, but no physical connections between such chromosomes were observed.

For the study of mature hypnozygotes and excysted planomeiocytes, we used hypnozygotes obtained from field samples. The field collected cysts in Figures 20, 21 and 23 are estimated to be two years old. Scanning electron micrographs of these mature hypnozygotes reveal a smooth featureless external cyst wall (Fig. 20). The minute pellicular projections observed on cultured younger cysts are no longer present, but thin sections of mature hypnozygotes show some similarities with the younger cysts. The accumulation body is still present as well

as numerous starch grains and lipid bodies (Fig. 21). The mature cyst wall is, however, somewhat different (Fig. 23). The electron dense outer wall component (1) derived from the pellicular layer is still present, but the inner component (2) is thicker and now has a definite stratified appearance. Also, the amorphous inner wall region (3) is less evident in the mature cysts. In order to rule out the possibility that the surface papillae seen in the young cysts are culture artifacts, we examined laboratory cultured hypnozygotes approximately 1 year old. The surface morphology of these cysts as viewed with the scanning electron microscope (Fig. 22) shows a smooth featureless wall similar to that of the field cysts.

When mature hypnozygotes taken from the field and held in the dark at 20° C were placed in fresh growth medium at 15° C under a 14:10 h L:D regime, excystment occurred within 3–5 days. Upon excystment a single motile cell (planomeiocyte) emerged, leaving an empty cyst casing behind. The planomeiocytes are elongate cells somewhat rounded at both ends. Emergent planomeiocytes were fixed for electron microscopy within 24 h of excystment and by this time possessed a fully developed amphiesma with thecal plates (Figs. 24, 25). As in the previous stage, planomeiocytes possess numerous

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FIGS. 2–5. Cytoplasmic fusion in *Gonyaulax tamarensis*. FIGS. 2, 3. Light micrographs. FIG. 2. Fusing cells. Note two gametes' cingula positioned at oblique angles to each other (arrows). Scale bar = 25  $\mu\text{m}$ . FIG. 3. Nuclear fusion results in large nucleus with paired chromosomes (arrow). Scale bar = 10  $\mu\text{m}$ . FIGS. 4, 5. Electron micrographs of early fertilization. FIG. 4. Fusing gametes at region of early cytoplasmic fusion. Relative positions of the two basal bodies, as determined by serial sections, designated by X's. Scale bar = 2  $\mu\text{m}$ . FIG. 5. Fusing gametes with nuclei at right angles to each other. Scale bar = 4  $\mu\text{m}$ .

FIGS. 6–9. Nuclear fusion in *Gonyaulax tamarensis*. FIG. 6. As gamete nuclei begin to fuse their boundaries become difficult to distinguish and the central area of nuclear fusion becomes highly vesiculate. Scale bar = 4  $\mu\text{m}$ . FIG. 7. Gamete fusion involves the merging of pellicular layers. Thecae are shed late in fusion. Ecdysed plates shown in upper and lower region of micrograph. Scale bar = 2  $\mu\text{m}$ . Inset: Higher magnification of pellicle (arrow) region outlined. Scale bar = 0.5  $\mu\text{m}$ . FIG. 8. As gamete nuclei draw together, their nucleoli (n) remain distal to the region of fusion. Serial sections show that V-shaped chromosomes (arrowheads) are attached to distal region of nuclear envelope, their free ends pointing towards each other. Scale bar = 3  $\mu\text{m}$ . FIG. 9. Region of nuclear envelope distal to contact site. V-shaped chromosome attached to envelope. Central chromosome axis visible in portion of chromosome (arrowhead). Scale bar = 0.5  $\mu\text{m}$ .

FIGS. 10–15. Planozygotes and early hypnozygotes of *Gonyaulax tamarensis*. FIG. 10. Light micrograph of planozygote showing two trailing flagella (arrowheads). Scale bar = 20  $\mu\text{m}$ . FIG. 11. Scanning electron micrograph of planozygote. Thecal tabulation similar to vegetative cell, but epitheca appears slightly elongated. Scale bar = 5  $\mu\text{m}$ . FIG. 12. Transmission electron micrograph of planozygote. Cytoplasm more condensed than vegetative cell with many starch grains (s) and lipid bodies (l). Scale bar = 5  $\mu\text{m}$ . FIG. 13. Section through planozygote wall. Arrowhead denotes area just inside pellicular layer thickened with dense material. Scale bar = 1  $\mu\text{m}$ . FIG. 14. Light micrograph of newly-formed hypnozygotes. Scale bar = 25  $\mu\text{m}$ . FIG. 15. Transverse section through newly-formed hypnozygote showing folding of pellicular layer forming minute papillae on surface. Scale bar = 5  $\mu\text{m}$ .

FIGS. 16–19. Hypnozygotes of *Gonyaulax tamarensis* from culture. FIG. 16. Scanning electron micrograph of newly-formed hypnozygote. Scars of former cingulum (C) and location of flagellar insertion (arrow) still evident. Scale bar = 10  $\mu\text{m}$ . Inset: Higher magnification of cyst wall showing minute papillae. Scale bar = 1  $\mu\text{m}$ . FIG. 17. Section through wall area of newly-formed cyst. Wall composed of outer pellicular layer (arrowhead) and thick underlying layer of less density. Spherical body with concentric rings of electron dense particles lies beneath wall adjacent to lipid body (l). Scale bar = 0.5  $\mu\text{m}$ . FIG. 18. Newly-formed hypnozygote showing wall area with 3 distinct layers; the outer pellicular layer (1), a thickened less-dense middle layer (2) and an inner amorphous layer (3). Accumulation body (a) present in lower right. Scale bar = 0.5  $\mu\text{m}$ . FIG. 19. Nucleus of hypnozygote. Numerous nuclear microcables evident (arrowhead). Scale bar = 1  $\mu\text{m}$ .

FIGS. 20–23. Mature hypnozygotes of *Gonyaulax tamarensis*. FIG. 20. Scanning micrograph of approximately two year old cyst collected from field samples. Wall surface is smooth and featureless. Scale bar = 15  $\mu\text{m}$ . FIG. 21. Section through mature field cyst showing nucleus (N), starch grains (s) and the thickened cyst wall. Scale bar = 10  $\mu\text{m}$ . FIG. 22. Laboratory cultured hypnozygote approximately 1 year old. Wall surface smooth as on mature field cysts. Scale bar = 20  $\mu\text{m}$ . FIG. 23. Higher magnification of mature cyst wall, comprised of a thin pellicular layer (1), a thick stratified layer (2) and an inner amorphous layer (3) much reduced in thickness from the younger cysts (c.f. Fig. 18). Scale bar = 1  $\mu\text{m}$ .

