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Effects of Temperature Conditioning on Development and Germination of *Gonyaulax tamarensis* (Dinophyceae) Hypnozygotes

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EFFECTS OF TEMPERATURE CONDITIONING ON DEVELOPMENT AND GERMINATION OF *GONYAULAX TAMARENSIS* (DINOPHYCEAE) HYPNOZYGOTES[1]

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ABSTRACT

Plankton samples from a dense bloom of the toxic red tide dinoflagellate Gonyaulax tamarensis *Lebour were incubated in the laboratory to study the formation and development of thick-walled, overwintering cysts. Samples contained very few cysts, and fusing cells were seen only twice. However, thousands of elongate cysts were formed from large posteriorly biflagellated cells (presumed to be planozygotes) that comprised approximately 50% of the initial motile population. Development of these new cysts (hypnozygotes) was studied under different storage conditions. Each hypnozygote lost pigmentation and formed a thick cell wall during the first several days of storage, producing a starch-filled cell with a yellow accumulation body. Starch reserves decreased rapidly during storage at warm (22 C) temperatures and more slowly at 5 C (with no apparent effect from light, dark, or added nutrients). Excretion of mucilaginous material was also observed. Periodic germination experiments showed that 22 C temperatures led to rapid development of the hypnozygote, with germination possible 1 mo after formation, whereas 5 C storage retarded development, with the first revivals after nearly 4 mo. Excystment was initiated only after an applied stimulus—either a temperature increase or decrease depending on the previous storage temperature. Cysts formed during the late spring in temperate waters are thus capable of excystment several months later as fall temperatures decrease; those formed in the fall take longer to mature, but are viable after six months of overwintering. Alternating spring and fall blooms seeded by germinating populations of dormant cysts are thus possible, a finding consistent with recent field observations.*

Key index words: cyst; dinoflagellates; Gonyaulax; *hypnozygote; planozygote; red tide; resting spore,* Gonyaulax; *sexual reproduction,* Gonyaulax; *toxic blooms, dinoflagellate*

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Evidence is accumulating rapidly in support of the early hypothesis (11, 12, 16) that the germination of overwintering cysts of *Gonyaulax tamarensis* Lebour provides the "seed population" for toxic blooms of this dinoflagellate. Once the thick-walled, ovoid cysts (hereafter termed hypnozygotes) were identified and isolated from sediment samples (2, 3, 15), it was possible to study their geographic distribution and the details of excystment and bloom initiation (1, 2).

Despite this progress, little is known about *G. tamarensis* hypnozygote formation, maturation, and development. The link between sexual reproduction and hypnozygote formation has been well-documented for numerous freshwater dinoflagellates, with sexuality induced by manipulation of temperature, photoperiod, or nutrients in laboratory cultures (8–10, 13, 14). For example, nitrogen depletion induces gamete formation in *Peridinium cinctum* (O.F.M.) Ehrenberg. The small, naked, isogamous gametes then fuse and form motile zygotic cells (planozygotes) which remain active for 2 wk before they are transformed into thick-walled hypnozygotes (8).

Similar efforts with marine photosynthetic dinoflagellates have had limited success, probably due to the difficulty in providing laboratory conditions that adequately reproduce the physical and chemical properties of the marine environment. Variations in culture conditions often produced thin-walled, nonmotile cells (termed temporary, pellicle or ecdysal cysts (2, 3, 15), but sexual fusion was not documented until Turpin et al. (15) used nitrogen depletion techniques on cultures of a Pacific strain of *G. tamarensis.* They describe large "lumpy" planozygotes resulting from the plasmogamy, followed several weeks later by the formation of smooth-walled, oval hypnozygotes. Although the hypnozy-

gotes did not develop the characteristic cell contents of *G. tamarensis* cysts isolated from sediment samples, the authors attribute the differences to maturation processes occurring in the sediments. One of the objectives of this paper is to document the transition from planozygote to hypnozygote so as to fill this gap in our understanding.

The same culturing problems that have hindered the study of sexuality in marine dinoflagellates have precluded detailed examination of the maturation and development of newly-formed hypnozygotes. Culture studies of freshwater dinoflagellates suggest that age and previous temperature "conditioning" are critical factors in the excystment process (6, 7, 14). For marine species, the only data are those from sequential incubations of hypnozygotes of unknown age isolated from sediment samples "conditioned" at different ambient temperatures. Results show a variation of 1–4 mo in the period of obligate dormancy for *Gonyaulax* species (1, 4, 17). The second objective of this paper is to describe the effects of temperature, light, and nutrients on the dormancy of new *G. tamarensis* hypnozygotes. These were obtained through laboratory incubation of a natural population of the toxic dinoflagellate collected during a dense spring bloom.

Nomenclature. Those studying *G. tamarensis* are presently confronted with a confusing array of terms describing the two nonmotile, or encysted stages observed (2, 3, 15). In this paper, the term planozygote describes the large, longitudinally biflagellated motile cell that is transformed into a thick-walled, oval resting cyst (in like manner termed hypnozygote). Since the entire *G. tamarensis* life cycle has not been observed sequentially, this change is not fully warranted at this time, yet the accumulated evidence now seems sufficient to justify these sexual terms. Rationale for this choice is discussed later.

MATERIALS AND METHODS

In April 1978, a toxic bloom of *G. tamarensis* occurred in Perch Pond (Falmouth, Massachusetts). Details of the species identification and the sampling location are given by Anderson and Wall (2) and Anderson and Morel (1). Hypnozygotes used in this study were obtained from the bloom in two ways:

i) On 28 April, water samples and plankton net tows were collected and taken immediately to the laboratory. The *G. tamarensis* cell density was 3×10^5 cells$\cdot l^{-1}$ and the water temperature 12 C. After removal of the large zooplankton with 80 μm mesh, the samples were poured into separatory funnels and maintained at 15 C on a 14:10 LD cycle, 50 μEin$\cdot m^{-2} \cdot s^{-1}$ cool-white illumination. Those cells that lost their motility and settled were withdrawn and resuspended in another funnel to ensure that all collected cells were nonmotile. Essentially all of the settled cells were new hypnozygotes. After 2 wk of this collection procedure, separate aliquots of the new cysts were stored in the nutrient depleted sea water at 5 and 22 C, both with and without light. A fifth sample was spiked with nutrients (100, 10, 10 μM nitrate, phosphate and silicate, respectively) and placed at 22 C in the light.

ii) On 8 May a sediment sample from Perch Pond was collected using a plankton net towed across the sediment surface. After 1 wk of dark storage at 15 C, this sample was divided and stored in the dark at 5 and 22 C, in sea water from the original site. At the time of collection, the *G. tamarensis* cell density was <50 cells$\cdot l^{-1}$ and the water temperature 16 C.

For discussion purposes only, cysts collected from the plankton samples using separatory funnels will hereafter be called planktonic hypnozygotes whereas those from the sediment sample will be termed sediment hypnozygotes. This distinction refers only to the different collection procedures. The dates 3 and 6 May indicate the approximate dates of formation of sediment and planktonic hypnozygotes (±1 wk).

Germination of sediment hypnozygotes followed the methods of Anderson and Wall (2) whereby individual cells were micropipetted from a sonicated and size-fractionated sediment slurry and incubated in f/2 medium (5) at 15 C and 50 μEin$\cdot m^{-2} \cdot s^{-1}$ in sealed Palmer-Maloney nannoplankton slides. In most instances, planktonic hypnozygotes were isolated directly from the stored sample without sonication, but for comparative purposes, several samples were sonicated and processed as if sediment was present. After 1 wk of incubation, excystment success was determined (defined as the successful emergence of the protoplast from the cell wall). At least 50 hypnozygotes were incubated for each determination.

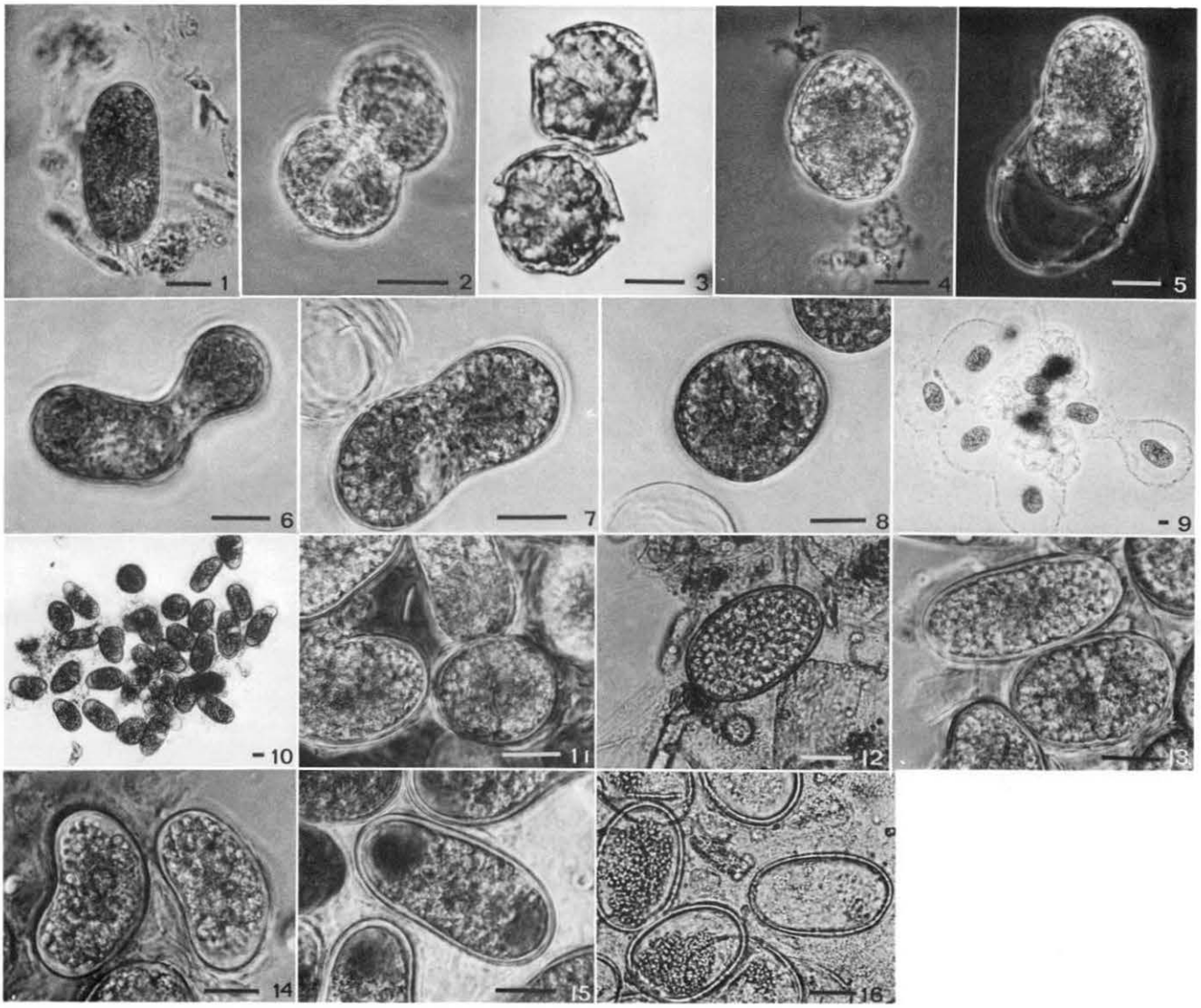
RESULTS

Hypnozygote formation. Plankton net tows examined shortly after collection contained only two starch-filled, deeply pigmented elongate cells that could subsequently be identified as new *G. tamarensis* hypnozygotes (Fig. 1). During this early observation, what appeared to be fusing cells were observed twice, but both pairs were inhibited by isolation and incubation in Palmer-Maloney slides. In both, the fusing cells were spherical and similar in size (25–30 μm diam; (Fig. 2). No further sexual pairing was observed, so additional verification was impossible.

Motile *G. tamarensis* cells in the natural populations were of two types. Approximately half were small cells (25–35 μm diam) nearly spherical in shape (Fig. 3). Others were slightly elongate and much larger (40 × 55 μm), with pigments and thecal patterns similar to normal vegetative cells (Fig. 4). Posterior biflagellation was observed in the large cells.

During laboratory incubation of the natural plankton populations, the larger cells lost their motility and settled to the bottom of the culture vessel. In most cases, the protoplast then emerged from the apical region of the theca, constricting somewhat during emergence so as to form a thin-walled, oval cell with normal vegetative pigmentation (Fig. 5). For unknown reasons, in some cases the protoplast remained within the theca and thus retained the more rounded shape of the large vegetative cell; while in other cases, ecdysis produced irregular, peanut-shaped hypnozygotes (Fig. 6).

Hypnozygote development. One day after initial loss of motility, the outer layer of the cell wall began to separate from the protoplast at either or both ends of the cyst (Fig. 7). Simultaneously, the pigments began to disappear from the periphery of the cell, leaving a clear band that gradually increased in size. After 3 days the pigments had accumulated into a yellow region at the center of the cell (Fig. 8) in a



FIGS. 1–16. Developmental stages of *Gonyaulax tamarensis* planozygotes and hypnozygotes. Scale = 15 μm. FIG. 1. Deeply-pigmented new hypnozygote from plankton tow. FIG. 2. Fusing gametes observed immediately after sample collection. FIG. 3. Normal vegetative cells after division. FIG. 4. Planozygote from plankton tow, posterior biflagellation not visible. FIG. 5. Hypnozygote emerging from theca of planozygote. FIG. 6. Peanut-shaped hypnozygote, still fully pigmented, 1 day after ecdysis. FIG. 7. One day old hypnozygote, pigment beginning to recede from periphery, outer cell wall separating from protoplast. FIG. 8. Rounded 2 day old hypnozygote; note pigment accumulating into yellow region in center. FIG. 9. Rings of mucilaginous material excreted by 2 day old hypnozygotes. FIG. 10. Small clump of hypnozygotes, 2 mo old. FIG. 11. Thick cell wall visible after 5 days storage in light at 5 C; cysts completely filled with starch. FIG. 12. Typical starch-filled hypnozygote isolated from sediment sample after spring bloom. FIG. 13. Starch-filled hypnozygotes after 3 mo of 5 C storage. FIG. 14. Slightly reniform hypnozygotes showing reduced starch and pale microgranular material becoming visible after 9 days of 22 C storage. FIG. 15. Fully developed hypnozygote after 1 mo of 22 C storage; microganular material with deep brown tint. FIG. 16. Dead hypnozygotes after 4 wk storage.

manner similar to that described by Pfiester (9). These accumulation bodies were somewhat obscured by the starch grains that filled the hypnozygotes completely at this stage of development.

When several of these planktonic hypnozygotes were placed in a Palmer-Maloney slide, a distinct ring of a mucilaginous substance formed around each cyst after 2 days (Fig. 9). No bacterial motion was evident within these rings although there was considerable activity throughout the culture slide. This mucilage was apparently responsible for the tendency of the new hypnozygotes to clump together, with some accumulations numbering in the thousands (Fig. 10).

Four or five days after loss of motility, the hypnozygote cell wall was noticeably thicker, even for those cells still enclosed by a theca (Fig. 11). The only visible internal contents were the yellow accumulation body and the starch grains that filled the cell.

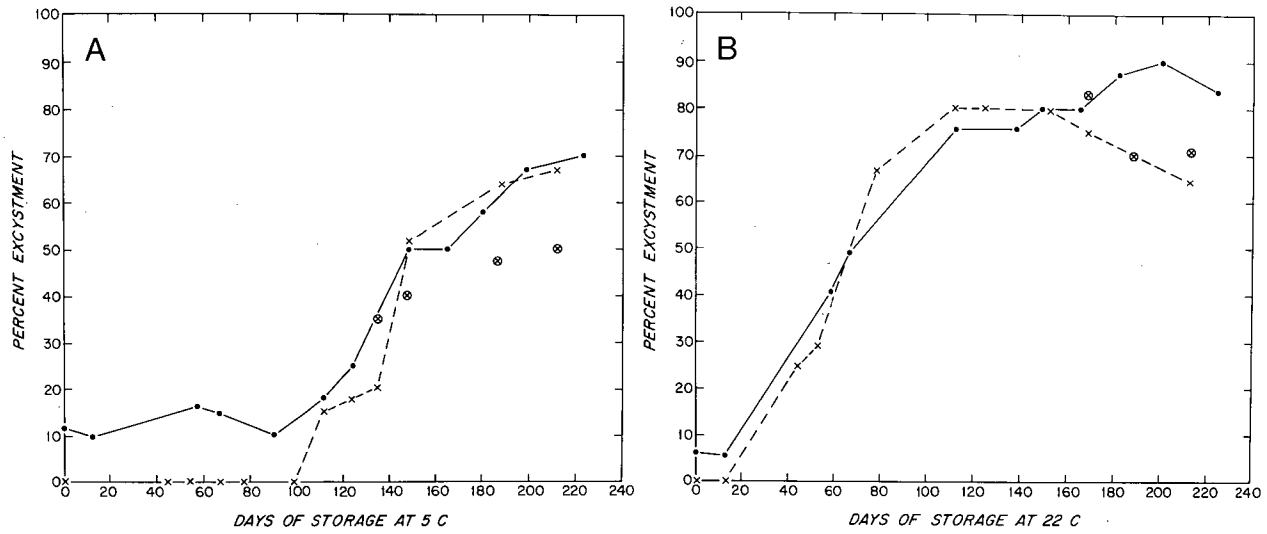


FIG. 17. Excystment success of new *G. tamarensis* hypnozygotes stored at 5 C (A) and 22 C (B), incubated at 15 C: (●——●) = hypnozygotes isolated from sediment sample collected on 8 May (isolation required mild sonication), just after the spring bloom and stored in the dark at 15 C for 1 wk prior to permanent storage (sediment hypnozygotes): (×——×) = hypnozygotes collected over 2 wk period during laboratory incubation of plankton samples from the spring bloom (planktonic hypnozygotes: isolation required no sonication): ⊗ = planktonic hypnozygotes sonicated prior to incubation.

Ninety percent of the hypnozygotes in a sediment sample collected on 8 May were identical in appearance to the starch-filled cysts described above (Fig. 12). The remaining 10% were mature hypnozygotes (2, 3, 15) with a narrow band of starch grains at the center of the cell, and microgranular material in Brownian motion at each end.

Separate samples of the planktonic hypnozygotes were stored at 5 and 22 C in both light and dark, and one 22 C sample was also spiked with nutrients. Periodic observations over several months indicated that only temperature affected development significantly (i.e., that neither light, dark, nor added nutrients affected the pattern of development determined by the storage temperature). Those hypnozygotes stored at 5 C changed very little, so that after 3 mo many cysts were still filled with starch (Fig. 13). In contrast, the starch content of hypnozygotes stored at 22 C began to decrease visibly after only 9 days of storage. As the starch grains began to disappear from the pole regions of the cyst, lightly colored microgranular material became visible (Fig. 14). After 1 mo of 22 C storage, most hypnozygotes had lost 50% of their internal starch and appeared identical to the mature hypnozygotes described previously (Fig. 15). In some, the accumulation body remained yellow while in others it developed a reddish hue. In most cases, the microgranular regions darkened to a brownish color.

Three weeks after formation, dead hypnozygotes began to appear in all of the stored planktonic samples. Initially only a small number of those observed were dead, but as time progressed, this fraction increased to, and in some cases exceeded, 50%. No mortality was observed for the hypnozygotes in the sediment samples stored under the same conditions as the planktonic samples. The first sign that a cell was no longer alive was the disappearance of the yellow accumulation body. Subsequently the internal starch became more granular in appearance, gradually receding to the center of the cell until only an empty (and seemingly unruptured), thick-walled cyst casing remained (Fig. 16).

Duration of dormancy. Planktonic and sediment hypnozygotes were stored in the dark at 5 and 22 C and then periodically incubated at 16 C to determine dormancy characteristics. The first excystment from planktonic samples stored at 5 C occurred after nearly 4 mo of storage, with 50% germination after 5 mo (Fig. 17A). In contrast, throughout the first 3 mo of storage, a low level of excystment (10–15%) was observed for sediment hypnozygotes stored at 5 C, after which germination increased at a rate similar to that of the planktonic samples. Also shown in Fig. 17A are the results obtained when planktonic hypnozygotes were sonicated prior to incubation, simulating the isolation procedure used with sediment samples. On three occasions, sonication decreased excystment success by 10–15%; once a 15% increase was observed.

Planktonic cysts stored at 22 C became viable significantly faster than those maintained at 5 C (Fig. 17B). The first excystment occurred after ca. 1 mo of storage, followed by a rapid increase to 80% success after 4 mo. Sediment cysts followed a similar trend with the exception that some excystment (6%) was observed during the first 2 wk of storage (Fig. 17B). When planktonic cysts from 22 C were sonicated, the results were within 5–8% of those for unsonicated samples.

DISCUSSION

Hypnozygote formation. A dense population of *G. tamarensis* collected during a spring bloom produced thousands of thick-walled, ovoid cysts during laboratory incubation. Two sizes of motile cells were present in the population, yet cysts identical to those isolated from sediment samples were formed only from the large posteriorly biflagellated cells (Figs. 4, 5). Although sexual fusion was observed only twice, the large cells are assumed to be planozygotes, the small cells gametes and/or vegetative cells, and the cysts hypnozygotes. Laboratory incubation probably inhibited further copulation, with the bulk of the fusion having occurred during a phase of the bloom prior to sample collection. These inferences are based on the following observations: i) Turpin et al. documented sexual fusion of *G. tamarensis* in culture, producing large planozygotes (15); ii) many of the large cells in the natural population described here had posterior biflagellation, a characteristic used by von Stosch to identify planozygotes of the freshwater dinoflagellates *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* Stosch at all levels of maturity (14); and iii) Anderson and Wall described posterior biflagellation for the emerging *G. tamarensis* cells excysting from sediment samples (2)—a characteristic again used by von Stosch as an indication that the germling cell is a planomeiocyte and the cyst a hypnozygote.

Details of the origin of these planozygotes are still uncertain. It is noteworthy that the only observed sexual fusion was between cells of similar size (Fig. 2), in contrast to the observations of Turpin et al. (15) who described the fusion of large "female" cells (50 μm diam) and small "male" cells (25–30 μm). This anisogamous fusion produced large "lumpy" planozygotes due to the very slow (2 wk) incorporation of the male gamete into the much larger female. The resulting hypnozygotes lacked certain characteristics of cysts from sediment samples, a difference attributed to maturation processes in the sediments. The planozygotes from the natural plankton sample described here were not irregularly shaped and the resulting hypnozygotes rapidly developed the thick wall and cell contents of those in the sediment samples. Perhaps a more reasonable explanation of the findings of Turpin et al. (15) would thus be that their populations were still adversely affected by some of the culturing artifacts that have made it so difficult to observe sexuality in marine dinoflagellates. In other words, they succeeded in inducing sexuality in a small fraction (<1%) of their population, but perhaps their observations were of gametes, planozygotes, and hypnozygotes still subject to a low level of culturing stress.

As discussed earlier, the development of the hypnozygote includes a thickening of the cell wall, rapidly reduced pigmentation leading to a yellow accumulation body, the excretion of a mucilaginous outer layer, and the gradual utilization of starch reserves. Germination of the hypnozygote produces a large elongate cell with posterior biflagellation as described by Anderson and Wall (2). (Von Stosch's description of *Gymnodinium pseudopalustre* includes two posterior flagella before and after the hypnozygote stage as well; 14).

Dormancy of hypnozygotes. Under the conditions of this study, the major factor affecting the length of the mandatory period of dormancy after hypnozygote formation was storage temperature. Other factors (light, dark, nutrients) did not alter basic results. (Note that the presence of bacteria in the stored samples makes the nutritional status of the unspiked sea water uncertain.) Hypnozygotes that either form in the fall or accumulate in deeper, colder waters will thus mature relatively slowly, presumably due to a slow rate of metabolism and the subsequent prolongation of the biochemical transformation needed to produce a viable cyst. This is best illustrated by excystment results for the planktonic cysts in Fig. 17A, and by the high starch content of three month old cysts stored at 5 C (Fig. 13). At this temperature, nearly four months of dormancy are necessary before any excystment occurs. Sediment hypnozygotes show the same sharp increase in germination after nearly four months, but a low level of excystment was also observed prior to this increase. One could argue that this early germination was a result of the sonification procedure used during isolation. However, it is more probable that some of the hypnozygotes in the original sediment sample had formed (and matured) the preceding year but had not excysted by the time they were collected and stored at 5 C. Subsequent incubations at 16 C initiated excystment. This is consistent with the observation that 90% of the newly-collected sediment cysts were starch-filled while 10% had the internal contents typical of mature cysts.

G. tamarensis hypnozygotes formed in temperate estuaries in the spring may not be exposed to winter temperatures for 6 mo or more. Under these warm water conditions, maturation proceeded rapidly with excystment possible one month after formation with 22 C conditioning. Here again, not all cysts developed equally fast, but after three months, over 80% were viable. This rapid development was associated with an equally fast reduction in the quantity of internal starch reserves.

The only comparable data on this required period of dormancy for marine *Gonyaulax* species are those relying on germination of cysts from sediment samples conditioned to natural temperatures. Wall and Dale reported that cysts of *G. digitalis* (Pouchet) Kofoid from the Woods Hole region had a 3 mo mandatory resting period during which excystment did not occur (17). Anderson and Morel found a 1 mo period of declining viability for hypnozygotes of *G. tamarensis* taken from the sediments of a Cape Cod salt pond during a spring bloom, after which excystment success increased gradually through the

summer (1). Dale et al. describe a 4 mo resting period for *G. tamarensis* (var. *excavata*) isolated from sediment samples from 90 m depths in the Gulf of Maine (4). In light of the results presented here, it seems clear that ambient "conditioning" temperature was the underlying reason for such differences. This study also shows that not all hypnozygotes stored at one temperature become viable at the same time—there can be a variation of 3–4 mo (Fig. 17A).

The development of hypnozygotes seems to be correlated with visible changes in cell contents, as older cells contain less starch and more visible microgranular material at each pole. This does not concur with the description by Dale et al. of maturing *G. tamarensis* hypnozygotes from 90 m depths in the Gulf of Maine (4), where the only reported differences over 4 mo were color changes of both the accumulation body and the microgranular regions. (Similar changes were observed during the present study, but not for all hypnozygotes.) Since Dale et al. (4) described a 4 mo mandatory resting period but did not report starch-filled hypnozygotes at the onset of their observations, it is possible that the environmental conditions occurring during the planozygote stage directly affect the quantity of internal reserves available to the new hypnozygote. The population from Perch Pond might then have stored more starch than the equivalent population from Monhegan Island in the Gulf of Maine. Alternatively it is possible that the deep water cysts originated in the warmer surface or estuarine waters where they had sufficient time to utilize some of the starch reserves before settling offshore.

It has been demonstrated that mature *G. tamarensis* hypnozygotes conditioned to cold (5 C) and warm (22 C) temperatures will excyst if the temperature increases or decreases respectively (1). This dual mechanism is consistent with the observed periods of required dormancy after hypnozygote formation (Fig. 17). Thus hypnozygotes formed during a bloom in the late spring, early summer are fully capable of excystment by the time the temperature begins dropping several months later, whereas those formed in the fall take longer to mature, but still have sufficient time given the approximate 6 mo interval between fall and spring. Alternating spring and fall blooms are thus possible in certain temperate waters, a bloom sequence that has in fact been observed in a Cape Cod (Massachusetts) salt pond (1). Rapid maturation due to warm water conditioning also explains the short period of reduced germination observed for *G. tamarensis* hypnozygotes in that pond following a spring bloom. However, additional field data from Anderson and Morel suggesting that cold temperatures shorten the mandatory resting period (1) are not in agreement with this study, perhaps because their fall sediment samples included both newly-formed and unexcysted, mature hypnozygotes, making interpretation of germination experiments uncertain.

Initiation of excystment. In this study, excystment of mature cold- or warm-conditioned hypnozygotes was initiated by a temperature increase or decrease respectively, confirming earlier laboratory and field observations (1). Cysts not exposed to a temperature change remained dormant at both 5 and 22 C (for over 10 mo at this writing). Since these two temperatures are similar to those found during winter and summer in the Cape Cod region, temperature change would appear to be a realistic prerequisite for excystment under natural conditions. It is not known, however, whether *G. tamarensis* hypnozygotes stored at a constant optimal growth temperature (e.g., 13 C) would still require such an external stimulus prior to excystment. Perhaps germination would proceed spontaneously after a suitable maturation period, as is the case with certain freshwater dinoflagellates where excystment occurs without changes in culture conditions (7, 10, 14). Von Stosch (14) and Huber and Nipkow (6, 7) indicate that cold storage retards the excystment of freshwater dinoflagellates and results in more complete and better synchronized germination upon incubation at higher temperatures, but this temperature pretreatment is not a prerequisite to germination.

Two comments are appropriate concerning the methodology used in this study. First, it is not known why a significant fraction of the planktonic cysts died during storage. Since no mortality was observed for the sediment cysts stored under identical conditions, the deaths must be linked to the formation and collection procedures. Perhaps the laboratory conditions somehow altered the metabolic transition from planozygote to hypnozygote for some cells. This seems unlikely though, since all new hypnozygotes looked alike until the first deaths occurred after three weeks, and those that did not die were normal in all respects. Another alternative is that the deaths resulted from the abnormal clumping together of hundreds of cells at the bottom of the separatory funnels or from the abnormally high hypnozygote density in the 250 ml storage jars. Excreted mucilage or other byproducts, fungal growth, or even a lack of water exchange could have affected them. The lack of bacteria within the rings of mucilage in Fig. 9 might be indicative of a toxic excretion product. Until a valid explanation is provided, high cyst concentrations and clumped accumulations should be avoided if this method of collection is used in the future.

The isolation of cysts from sediment samples is often greatly facilitated by sonication (which liberates individual cells by disaggregating the detrital material surrounding them). Since hypnozygotes are not visibly damaged by this procedure, its use presents no problems for certain qualitative studies. Other types of investigations could be affected, however, either through the organism's response to the ultrasound or to the thermal increase that is sometimes unavoidable with this instrument (1). Al-

though a detailed examination of this phenomenon was not within the scope of this study, some inferences are possible based on the germination results of hypnozygotes isolated with and without sonication. In general, Fig. 17 demonstrates excellent agreement between the two methods if, as discussed earlier, one accepts the existence of a small fraction of mature hypnozygotes in the original sediment sample. On a more detailed level, sonication of planktonic cysts at 22 C resulted in slight positive and negative differences from the excystment percentage of unsonicated samples, but these variations were clearly within the error of this germination method. At 5 C, the differences were larger (up to 15%) but both positive and negative as well. Thus, the preliminary indication is that at warm temperatures, proper use of sonication should have little effect on quantitative results, while at low temperatures, some scatter might be introduced. This conclusion is valid only with respect to the relatively large temperature change used here as a stimulus for excystment. Results from more precise experiments using small temperature changes (1) remain uncertain. It is hoped that either through the collection procedures described here or through advances in culturing methodologies, hypnozygote assemblages can be obtained for detailed physiological and biochemical studies without the possible uncertainties associated with sonication.

In summary, this study addresses an important gap in our understanding of the *G. tamarensis* life cycle. It describes the transition from planozygote to hypnozygote and demonstrates the major effect of temperature on the developmental process. Although it represents just one aspect of a complete sequential cycle, the evidence is consistent with previous observations, leaving little doubt that Turpin et al. (15) were justified in linking sexual fusion to the formation of the thick-walled, ovoid cyst that is so important in the natural ecology of *G. tamarensis*.

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