STUDIES ON THE DEVELOPMENT OF THE SEA URCHIN
STRONGYLOCENTROTUS DROEBACHIENSIS.
I. ECOLOGY AND NORMAL
DEVELOPMENT

R. E. STEPHENS

*Marine Biological Laboratory, Woods Hole, Massachusetts 02543, and the
Department of Biology, Brandeis University,
Waltham, Massachusetts 02154*

The sea urchin *Strongylocentrotus droebachiensis* is one of the most widely distributed of the echinoderms. Its occurrence is circumpolar, extending into the boreal regions of both the northern Atlantic and Pacific Oceans (Mortensen, 1943). This organism was the first echinoid to be described embryologically (Agassiz, 1864) but identity of later larval stages was a matter of dispute for some time (Mortensen, 1943). The true echinoplutei were finally described over a century after Agassiz's original study (Strathmann, 1971).

Ethel Browne Harvey, in her classic work "The American Arbacia and Other Sea Urchins" (Harvey, 1956), treats this species only briefly. Both Harvey (1956) and Boolootian (1966) describe a comparatively short breeding season for specimens obtained north of Cape Cod. The fertilized eggs typically develop with asynchrony, are large and yolky, and have heavy jelly coats. These points have caused *S. droebachiensis* to be regarded as a rather undesirable organism for the more sophisticated studies of cell division and early development. Some large-scale biochemical work has recently been carried out with gametes of *S. droebachiensis* (*e.g.*, Kolodny and Roslansky, 1966; Stephens, 1967; or Stephens, 1970) but the "more reliable" *S. purpuratus* or *Arbacia punctulata* dominate the literature of cellular and developmental biology.

Neither Agassiz's early account (1864) nor any later work describes the development of *S. droebachiensis* under controlled laboratory conditions. This report presents such sequences, relating development to natural environmental temperatures, and consequently suggests optimal conditions for fertilization and synchronous development. These methods and developmental schemes serve as the basis for further studies on mitotic spindle assembly and control, patterns of protein synthesis, and the mechanism of ciliogenesis (Stephens, 1972; in preparation).

MATERIALS AND METHODS

Experimental animals

Strongylocentrotus droebachiensis, 2″–4″ in diameter, was collected from various subtidal areas of Cape Cod Bay during the fall and winter of 1966–1971 and maintained either in running sea water of ambient ocean temperature or in closed aquaria at 4° C utilizing sub-sand filtration. The food supply for animals maintained in running sea water consisted of *Laminaria* while that for animals in the

closed system consisted of a mixed algal population growing on the walls of the tank; the latter animals were generally used within a week or two after collection. In the Cape Cod population, no differences in the degree of gonad development were noted between freshly-collected specimens and those maintained in the running sea water system. This might be expected since both sets of specimens experienced essentially the same seasonal temperature change and had available the same food supply. Ripe animals maintained in the closed system at 4° C remained in breeding condition for at least two months beyond the time of natural spawn-out, if fed an occasional piece of *Laminaria*. For comparison, urchins were also obtained from the Boothbay Harbor area of Maine through commercial sources and were generally used immediately. Urchins collected from Maine prior to the breeding season and maintained in the Woods Hole sea water system underwent gonad development coincident with those of the native Cape Cod population.

Gametes

Eggs were obtained by injection of 0.53 M KCl into the perivisceral cavity (Palmer, 1937; Costello, Davidson, Eggers, Fox, and Henley, 1957). For a typical 3″ diameter urchin, two injections of 2 ml each were made at diametrically-opposite points in the peristome. The urchin was placed atop a 100 ml beaker filled with filtered, ice-cold sea water, aboral side down, and the beaker nearly immersed in cold running sea water. Thirty minutes were usually sufficient to obtain the maximum number of eggs, even at 0° C. Alternatively, eggs may be shed in a refrigerator or cold room whose temperature does not exceed 8° C.

Sperm were obtained by removal of the testes when a male was detected by the above injection method. The testes were briefly rinsed in cold sea water, lightly blotted, and placed in a covered plastic petri dish on ice. "Dry sperm," exuded from the testis, was diluted 1:20 with cold sea water containing 10^{-4} M EDTA (Tyler, 1953) and used for fertilization within 5 minutes.

After shedding, the eggs were washed by decantation at least twice with 10 times their volume of cold filtered sea water. The eggs were either used immediately or else washed once more with cold Millepore-sterilized sea water containing 0.05% sulfadiazine (Tyler and Tyler, 1966), resuspended in fresh cold sulfadiazine-sea water, and kept on ice in a covered Stender dish. The eggs were adjusted to such a concentration that they formed a layer no more than two cells thick; the depth of the fluid in the dish was 1 centimeter. Under these conditions, 90–95% of the eggs were fertilizable for 1–2 days; thereafter fertilizability dropped off to about 10% in 7 days. Eggs fertilized during this 1–2 day period developed normally but not nearly as synchronously as those fertilized immediately after shedding.

Fertilization and development

One ml of 1:20 sperm suspension was typically mixed with 10 ml of eggs in 200 ml of sea water. The eggs were allowed to settle, the sea water was decanted, and the eggs were washed twice with filtered sea water. The egg suspension was partitioned into Stender dishes in such a manner that the eggs formed a layer no more than two cells thick, in fluid no more than 1 cm in depth.

The dishes were then maintained on ice, in the running sea water system, or in a temperature-controlled water bath. The fertilization, washing, and transfer operations were carried out at the same temperature as embryonic development.

Removal of the jelly coat

The developmental studies carried out in this report were all done with un-treated eggs, but often removal of the jelly coat (Tyler and Tyler, 1966) is some-times desirable in order to decrease the egg volume, to remove adhering bacteria, or for various biochemical isolation procedures. The eggs, in at least 10 times their volume of cold sea water, are rapidly adjusted to pH 5 (no less!) with dropwise addition of 0.1 HCl. The eggs are immediately spun down in a hand centrifuge and washed three times by centrifugation with cold filtered sea water. Exposure to acid conditions must be minimized in order to obtain maximum fertilization and optimum synchrony. When this procedure is carried out care-fully, there are no significant developmental differences between normal and de-jellied eggs.

Glassware

All glassware used was soaked for at least one hour in Alconox detergent, rinsed 10 times with tap water, 10 times with deionized distilled water, and allowed to dry protected from dust or vapors of fixatives. Containers for handling the eggs or embryos were pre-chilled to the temperature of the experiment. Sterile disposable plastic petri or tissue culture dishes (Fisher or Falcon) were found to be non-toxic for both fertilization and development, requiring no pre-washing.

Microscopy

Developing embryos were photographed through either Zeiss phase-contrast or Leitz polarization optics. Fields of embryos were photographed through a Wild M-5 stereomicroscope equipped with a phototube and Polaroid filters. Ciliary motility in later stages was arrested with osmium tetroxide vapor fixation. Flatten-ing of embryos was prevented through the use of 0.18 mm thick coverglass placed beneath the usual coverglass as a spacer. Kodak Panatomic-X film was used throughout and was developed in Kodak Microdol-X developer. Magnification calibration was obtained through photographs of a stage micrometer taken through the same optical system as the embryo.

RESULTS

Breeding season

Based upon observations made since the winter of 1966–67, the breeding sea-son of *S. droebachiensis* from Cape Cod Bay encompasses nearly four months. Ripe eggs can be obtained in mid-December from about $\frac{1}{10}$ of the females and by late January nearly half of the females are fertile. The period from mid-February until mid-April represents the maximum period of fertility with 99% fertilization in $\frac{9}{10}$ of the females; maximum egg volume and essentially 100% fertilization are found from mid-March until mid-April, after which a rapid spawn-out takes place.

During particularly cold winters, the fertility period extends into May. The males are ripe nearly two months earlier and remain ripe about a month later than the females; sperm can usually be obtained throughout the year in small amount. No attempt was made to relate gonad index (weight of gonad/weight of animal) to fertility for it was found that the gonad index in early January was not significantly different from that in mid-April, the former time representing gonads full of eggs but in the germinal vesicle stage.

Urchins obtained from Maine in 1964–65 and 1966–67 showed a much more limited season, with the beginning of the season taking place about one month later and spawn-out occurring coincident with or even two weeks earlier than in urchins from Cape Cod Bay. Figure 1 illustrates monthly mean water tempera-
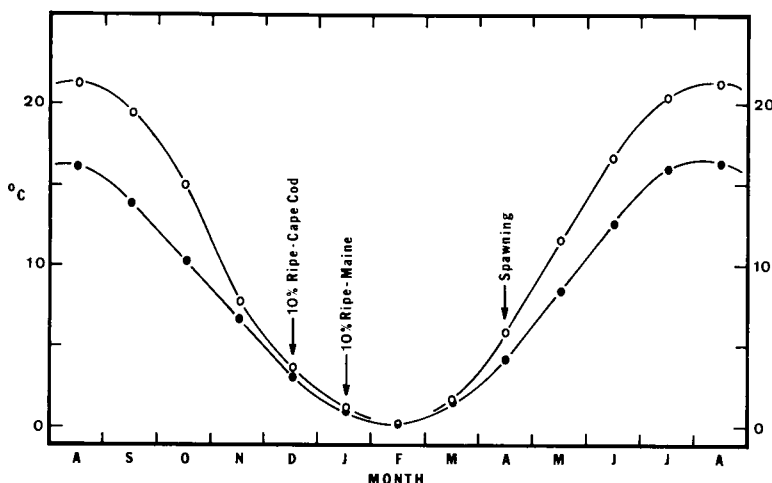

FIGURE 1. Mean monthly sea temperature for Boothbay Harbor, Maine (filled circles) and for Woods Hole, Massachusetts (open circles); from the data of Taylor, Bigelow, and Graham, 1957.

tures off Boothbay Harbor, Maine, and Woods Hole, Massachusetts; superimposed upon these temperature curves are data relating to the breeding season of *S. droebachiensis* from Maine and from Cape Cod Bay.

Some qualitative differences in egg properties have been noticed in animals collected from these two populations, but these differences are difficult to evaluate. Most batches of eggs from the northern *S. droebachiensis* are yellow-orange in color and are frequently mixed with large amounts of mucus. The urchins from Cape Cod Bay also produce some batches of eggs with this intense coloration but most are yellow or even pale-yellow to colorless; they are consistently free of mucus. Taken at the maximum of the breeding season, eggs from the northern population are somewhat less synchronous than those from Cape Cod Bay. Maine urchins wholly ripened in the running sea water system at Woods Hole show no such differences in color or synchrony, so such effects are probably environmental, most likely related to food supply.

Developmental sequence

Even though having twice the diameter and developing at temperatures 10–20° C lower, the fertilized egg of *S. droebachiensis* develops along a time scale proportional in all respects to that of the more commonly studied *Arbacia*. Table I lists events in the first division of *S. droebachiensis* at 0° C, 4° C, and 8° C. When temperature variation is held to within ± 0.2° C, synchrony at the first division is excellent. At 8° C, 90% of the cells cleave at ± 5 minutes of the time cited. At 0° C, the comparable range is ± 15 minutes. In both cases, this range represents an interval of about 6% of the total division time. With temperature fluctuations of 1–2° C, particularly at prophase, the degree of asynchrony is doubled or tripled. Figure 2 plots the log of the time of metaphase and cytokinesis *versus* temperature for both the first and second division of *S. droebachiensis*. Temperatures above 10° C cause gross asynchrony while those above 12° C arrest cell division irreversibly in at least 80% of the cells; 14–15° C is lethal.

TABLE I

Temperature dependence of first division events, time in minutes ($\pm 5\%$)

	0° C	4° C	8° C
Fertilization membrane complete	10	7	5
Sperm aster	80	50	30
Union of pronuclei	110	65	40
Hyaline layer complete	200	120	75
Nuclear membrane breakdown	350	210	130
Metaphase	405	250	150
Anaphase	430	260	160
Telophase	460	280	170
Cleavage	510	310	190

Fry (1936) has noted that, in *Arbacia,* metaphase always occurs at a time point that is 80–85% of the cleavage time regardless of temperature; R. E. Kane of the University of Hawaii (personal communication) has made similar observations with regard to the various temperate and tropical sea urchins that he has studied. It is obvious from Figure 2 that this rule holds true in the case of *S. droebachiensis* over its entire temperature range in both the first and second division.

The developmental stages through the four-armed echinopluteus (whereafter feeding is necessary) at 8° C are illustrated in Figure 3, demonstrating both the size and the relative clarity of the egg and embryo. Table II contains additional information about later events in development at 0° C, 4° C, and 8° C.

Regardless of temperature, problems occur at the hatching of the blastula. Growth in sea water from the laboratory system is quite normal up to this point, but after hatching the blastomeres disintegrate. Transfer to Millepore-sterilized sea water or sea water containing sulfadiazine just prior to hatching entirely prevents this disintegration. Tyler and Rothschild (1951) recommend penicillin for apparently the same reason. Other marine embryos (*e.g., Arbacia punctulata* or

Asterias forbesi) show no such sensitivity when grown in untreated sea water. Examination of embryo cultures after disintegration reveals a large population of *Pseudomonas* sp. and other unidentified bacteria. Apparently *S. droebachiensis* is particularly susceptible to bacterial action.

Discussion

Conditions influencing breeding

Harvey (1956) reports that *S. droebachiensis* from Salisbury Cove, Maine, had unripe eggs in January and February, showed maximum ripeness in March
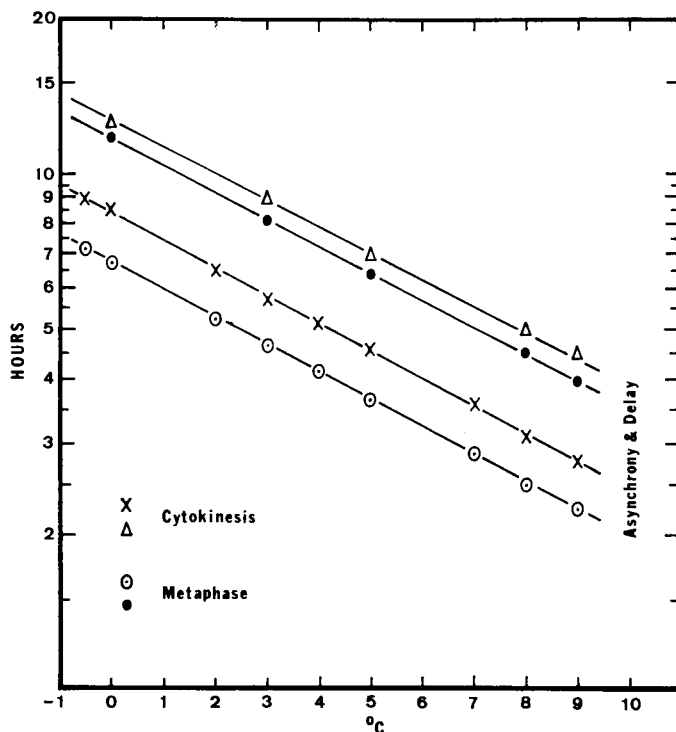
FIGURE 2. Semi-logarithmic plot of the mean time for metaphase and cytokinesis *versus* temperature for the first and second divisions.

and April, and was fully spent by mid-May. Boolootian (1966), on the other hand, cites February and March as the peak season, with some ripe gametes obtainable in January but spawn-out in March for animals collected from Lamoine, Maine. The animals employed in this report, obtained from Boothbay Harbor, Maine, correspond most closely with Harvey's data, while the Cape Cod Bay population appears to ripen coincidentally with the population cited by Boolootian but remains ripe much longer. Sverdrup, Johnson, and Flemming (1942) report that *S. droebachiensis* in Norway has a breeding season from December through April, but gives no location, quantitative fertility data, nor seasonal temperature variation.
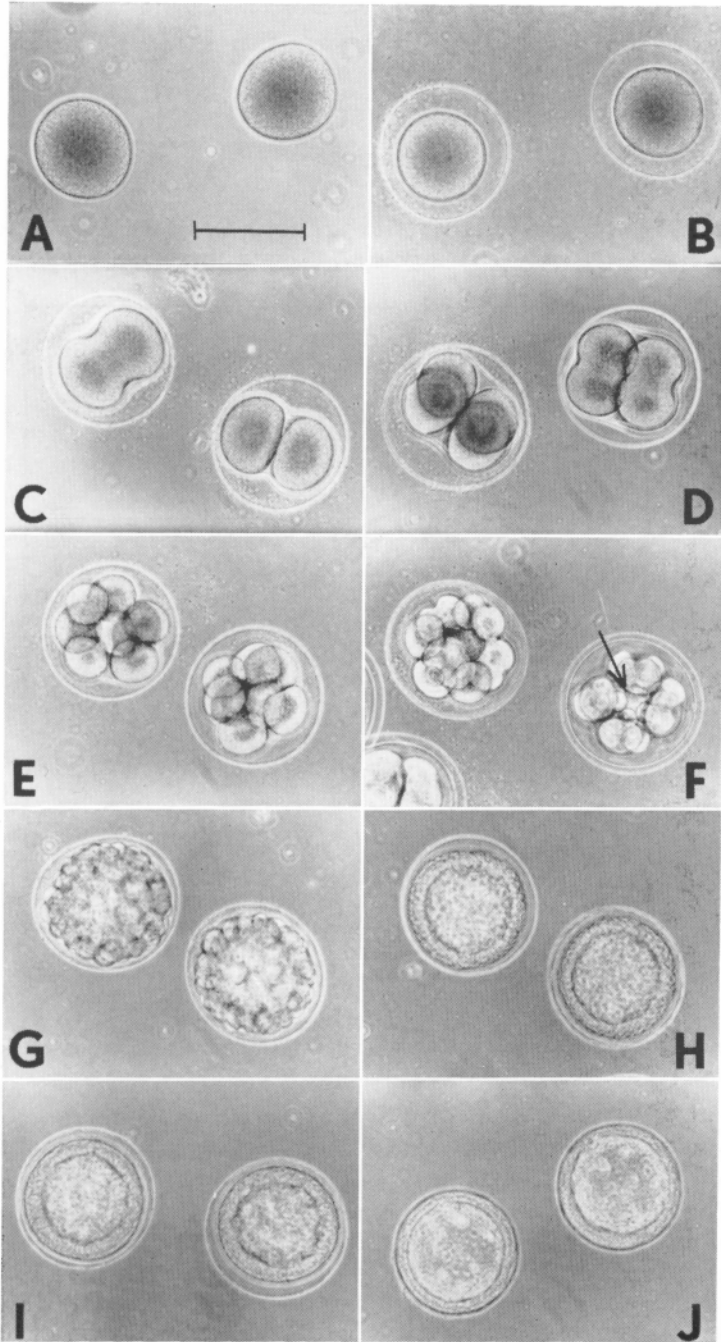
FIGURE 3. Developmental stages for fertilized eggs of *Strongylocentrotus droebachiensis* at 8° C; *A*—unfertilized egg (scale = 200 μ); *B*—5 minutes after fertilization; *C*—first division; 3 hr; *D*—second division, 5 hr; *E*—8-cell stage, 6½ hr; *F*—16-cell stage, 8½ hr (arrow: micromeres); *G*—early blastula, 15 hr; *H*—mid-blastula, 20 hr; *I*—cilia formation, beginning 24 hr; *J*—hatching, 30 hr; *K*—ciliated blastula, 32 hr (scale = 50 μ); *L*—early gastrula,
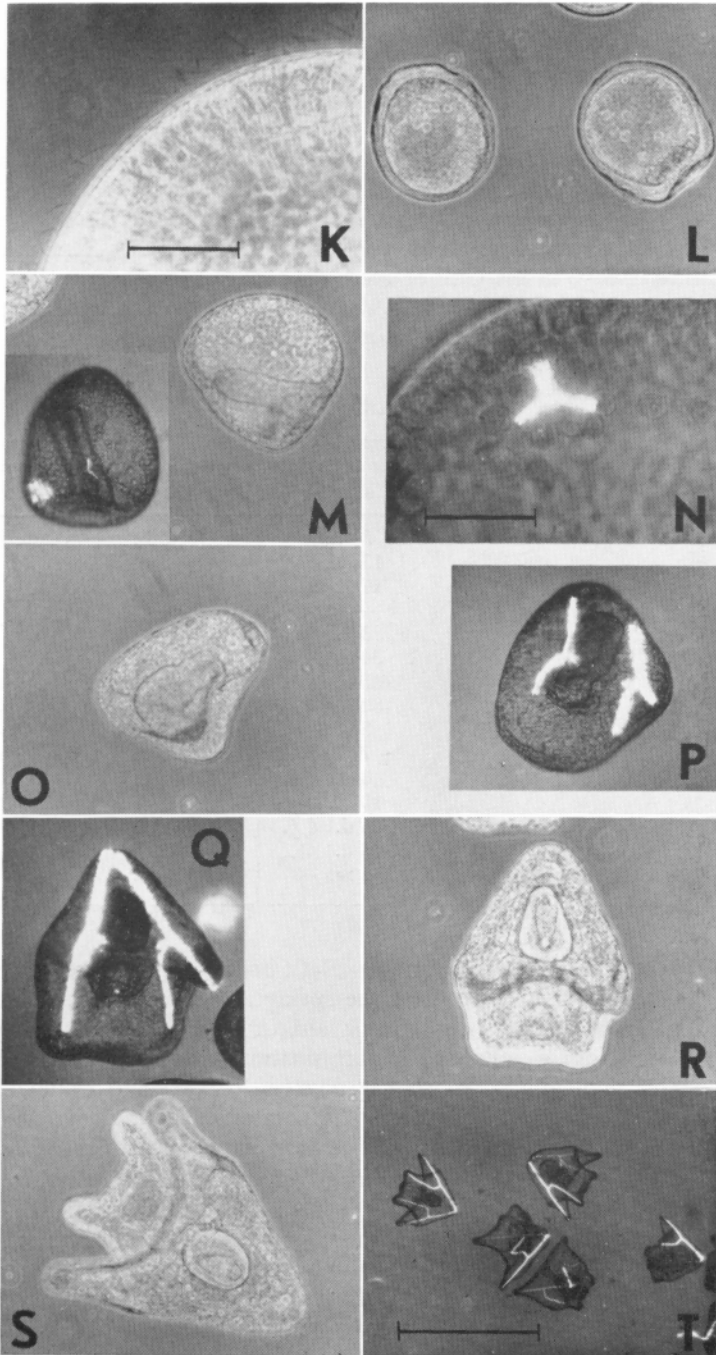
45 hr; *M*—late gastrula, spicule formation (inset: polarized light), 72 hr; *N*—tridentate spicule, polarized light, 72 hr (scale = 50 μ); *O*—prism stage, 96 hr; *P*—same as *O*, polarized light; *Q*—anal arm, polarized light, 5 days; *R*—same as *Q*, phase-contrast; *S*—oral arm, 7 days; *T*—pluteus, polarized light, 12 days (scale = 1 mm).

It is generally agreed that annual gonad development and spawning is fre-
quently correlated with seasonal temperature maxima or minima.   Boolootian
(1966) remarks that the rate of temperature change may initiate gonad develop-
ment while the reverse change may induce spawning, the gonad between these
extremes requiring stronger stimuli for spawning the further it is from the time
and temperature of natural spawning.   If one accepts this general idea and applies
it to *S. droebachiensis,* the lengthened breeding season of the Cape Cod Bay
population may be reasonably rationalized.   From Figure I it may be seen that
in the fall the shallow waters of Cape Cod drop rapidly in temperature while
the temperature change in the Gulf of Maine is moderated by its mass.   This rapid
drop-off in temperature, particularly in the Billingsgate Island area where most of
the Cape Cod Bay animals were obtained, should influence early gonad develop-

TABLE II

Temperature dependence of development $(\pm 10\%)$

	0° C	4° C	8° C
2 Cell	$8\frac{1}{2}$ h.	5 h.	3 h.
4 Cell	$13\frac{1}{2}$ h.	8 h.	5 h.
8 Cell	17 h.	$10\frac{1}{2}$ h.	$6\frac{1}{2}$ h.
12 Cell	—	—	$8\frac{1}{4}$ h.
16 Cell	23 h.	14 h.	$8\frac{1}{2}$ h.
32 Cell	30 h.	18 h.	11 h.
Cilia formation	65 h.	40 h.	24 h.
Hatching	80 h.	50 h.	30 h.
Invagination	5 d.	75 h.	45 h.
Mid-gastrula	7 d.	100 h.	60 h.
Spicule formation	8 d.	5 d.	72 h.
Prism stage	10 d.	$6\frac{1}{2}$ d.	96 h.
Anal arm	14 d.	8 d.	5 d.
Oral arm	18 d.	11 d.	7 d.
Maximum pluteus (without feeding)	1 m.	20 d.	12 d.

ment according to Boolootian's postulate.   Both bodies of water reach temperature
minima at the same time and warm in the spring at almost the same rates.   Peak
season and spawn-out thus should occur almost coincidently.   Yearly seasonal
variation would, of course, influence the termination of spawning.   The differences
in the two Maine populations reported by Harvey (1956) and Boolootian (1966)
may simply reflect cooling and warming differentials.   These arguments assume,
of course, that an adequate and comparable food supply exists in all cases.

Since the Cape Cod Bay population does differ somewhat in habitat, breeding
season, and egg appearance from its Maine counterpart, it might be mentioned
at this point that both populations are unquestionably of the same species.   Cri-
teria outlined by Swan (1962) for distinguishing various *Strongylocentrotidae*
indicate that the species clearly is *droebachiensis.*   Out of roughly 5000 indi-
viduals examined over the past eight years, only one specimen, obtained in 1966
by commercial dredging off the coast of Maine, was tentatively identified as *S.
pallidus.*   The concept of physiological race (see for example Loosanoff and

Nomejko, 1951) also does not apply in this case since both populations spawn at essentially the same temperature, in spite of widely different summer temperature maxima, and animals from Maine subject to Woods Hole water temperatures ripen coincidently with the natural Cape Cod population.

Ecological considerations

The inability of *S. droebachiensis* to develop normally above about 10° C even under the most controlled laboratory conditions would indicate that this temperature represents an upper limit for larval development. The metamorphosed urchin and the adult clearly are exposed to temperatures 5–10° C warmer during mid-summer, but the distribution of *S. droebachiensis* correlates well with a 10° C upper limitation, for it is found in seas where the temperature during the early spring months rarely exceeds 6–8° C (Mortensen, 1943). South of Cape Cod the urchin is still found, but generally at moderate depths in the cold bottom waters. Since the larvae survive temperatures below zero quite well, the northern limitation in distribution would be determined only by actual freezing of coastal waters, permitting circumpolar distribution. But it is apparent from the laboratory behavior that the planktonic larvae of *S. droebachiensis* would not survive well in the more temperature surface waters to the south of Cape Cod.

Strongylocentrotus found in Cape Cod Bay occurs chiefly at Billingsgate Island, off Barnstable Harbor, and on the northeast jetty of the Cape Cod Canal. The latter two populations have become quite depleted recently, with no obvious cause. Past history of these beds indicates quite a variability in size-class and population density from year to year (John Valois, personal communication), something that would not be predicted for an indigenous steady-state population. One simple explanation for this variability is that a major proportion of settling larvae originate from the more northerly regions and are carried south to Cape Cod Bay; yearly variation in prevailing winds plus local environmental conditions would thus determine the success of an immigrant population. Such larval dispersal is now considered to be an important factor in genetic exchange in shallow-water marine populations (*cf.* Scheltema, 1971).

The extreme susceptibility of the newly-hatched blastulae to bacterial action, whether at the upper or lower reaches of its viable temperature range, would imply that survival of larvae in waters of high organic content would be substantially reduced. The sea water intake at the Marine Biological Laboratory is adjacent to a sewer outfall; as discussed above, larvae survive only when the water is rendered sterile or when sulfadiazine is added to the sea water. A similar situation might be envisaged in nature where organic pollution of a bay or estuary not subject to appreciable tidal action may result in mass mortality of these larvae. Of course, many other factors besides simple bacterial action may affect larval development; Wilson and Armstrong (1961) cite "biological differences" between various samples of sterile sea water, detected through their influence upon the embryogenesis of *Echinus esculentus*. Whatever the specific cause, the disappearance of large beds of *S. droebachiensis* in the vicinity of population centers should not be unexpected.

Timetable of development

The whole developmental program, at least through the pluteus stage, behaves as a tape played at various speeds depending upon temperature. Such proportionate time-temperature relationships are really not too surprising taken in the context of an average activation (Q_{10}) seen in other studies of echinoderm early development. Tyler (1936, 1942) observed no temperature optimum for either respiration or development, but rather a logarithmic time-temperature relationship apparently related to an over-all Q_{10} much as noted here for *S. droebachiensis*. Hoadley and Brill (1937) studied the timing of the first three cleavages of *Arbacia punctulata* and *Chaetopterus pergamentaceus* (annelid) and found an inverse logarithmic relationship with temperature.

What is somewhat surprising is the temperature range, specifically its lower limit. Perfectly normal pluteus larvae can be produced from eggs fertilized and grown entirely at *minus* 1° C. Considering modern observations on the role of microtubules in mitotic spindle structure (Inoué and Sato, 1967) or in later differentiation (Tilney and Gibbins, 1969) and the use by these and other workers of hypothermic treatment to abolish microtubules and thus stop mitosis or primary mesenchyme migration, it is interesting—to say the least—to have normal development at or below 0° C. The existence of diverse fauna at either low temperatures, high hydrostatic pressures, or both (*cf.* Sanders and Hessler, 1969), conditions under which microtubules or other low temperature and high pressure-sensitive organelles should be non-existent, may point to the evolution of organisms whose structural proteins associate more strongly or whose control mechanisms differ from the more commonly studied temperate littoral or terrestrial forms. In fact, mitotic spindle assembly at low temperature in *S. droebachiensis* confirms both stronger association and temperature modulation of assembly of spindle proteins (Stephens, 1972).

The use of S. droebachiensis *as an embryological material*

When obtained in shallow subtidal areas, *S. droebachiensis* has a breeding season quite comparable to its more commonly studied west coast relative, *S. purpuratus*. Simple precautions in laboratory handling make it as reliable as any other sea urchin. Its large, comparatively clear egg and its prolonged development are unique advantages; these facts, coupled with the large volume of obtainable eggs and good synchrony, would suggest that *S. droebachiensis* could be a valuable experimental organism.

Difficulty in laboratory use of *S. droebachiensis* as an experimental material can be easily attributed to several separate causes, all of them "violations" of the urchin's normal ecology. Upon collection, the animals must not be subjected to temperatures appreciably higher than those of their habitat; as with other sea urchins, warming will induce shedding of gametes. The same considerations must be made for the gametes themselves; shedding below 10° C is essential for high fertilization and synchronous development. During embryogenesis the temperature must remain constant and below 10° C for normal development and synchrony. After hatching the blastulae are extraordinarily sensitive to bacterial action and precautions must be taken to assure near-sterile conditions. As with

most other embryos, overcrowding and lack of oxygen retard or arrest development; no more than 1 cm of sea water and cells one or two layers thick assure proper aeration through diffusion. Mechanical agitation or aeration of de-jellied eggs during the initial part of the first division (while the hyaline layer forms and the fertilization membrane hardens) generally results in cell clumping or even lysis. After the first cleavage, the cells are fairly insensitive to aeration or agitation in a reciprocating bath. All fertilization, washing, and transfer operations should be done with pre-chilled glassware to avoid temperature shock.

Choice of a pale yellow or near-colorless egg batch assures relative cell clarity. Slight compression (about 25%) is usually sufficient for observation of the mitotic apparatus, either in phase-contrast (by exclusion of granules) or in polarized light. A heat filter and temperature-controlled stage are essential if the cell is to be observed for any length of time. Reference to Figure 3 will show that most developmental events or relevant structural features are readily seen in *S. droebachiensis*.

SUMMARY

1. Methods for obtaining viable gametes and embryos of the arctic-boreal sea urchin *Strongylocentrotus droebachiensis* are presented and practical suggestions are made regarding the suitability of this material for embryological use.

2. The useful breeding season extends from early January to mid-April for animals obtained from shallow subtidal regions of Cape Cod Bay or beginning roughly a month later for animals collected from the Gulf of Maine. The "season" can be extended by at least two months by holding the ripe animals at 4° C.

3. The time course for the first division and for development to the four-armed echinopluteus are given for various temperatures. Development time follows an inverse log relationship with temperature over the range of $-1°$ C to 9° C.

4. The susceptibility of the eggs and embryos to temperatures in excess of 10° C and of the hatched blastulae to bacterial action are discussed in regard to laboratory experimentation and the natural distribution of the organism.

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