FERTILIZATION IN PECTINARIA (=CISTENIDES) GOULDII

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Pectinaria gouldii was selected for study because the animal was readily available and its egg is more suitable for phase-contrast microscopy that those of many other marine invertebrates, owing to the lack of interfering cytoplasmic structures such as refractile droplets or coarse granulations. The observations are published because, with the exception of Tweedell's (1962) report on cytoplasmic inclusions, only brief notes on *Pectinaria* eggs have appeared in the literature (Wilson, 1936; Costello *et al.*, 1957; Tweedell, 1959, 1960, 1961), and because fertilization in these eggs has several features of interest.

Methods

The animals were collected in the Woods Hole area during June and July, 1961, and maintained in the laboratory in fingerbowls containing sand and supplied with running sea water. Gametes were obtained by removing an individual to a separate dish and carefully breaking down the conical tube in which the animal lives, starting from the thin end. As a rule, shedding occurred during this process and it was mostly these gametes that were used in the studies to be described. More rigorous methods were often needed to provoke shedding, such as prodding or pinching the animal with forceps, but gametes thus obtained were in the main found to be unsatisfactory, most of the eggs being incompletely grown and the spermatozoa unlikely to leave their packets (Fig. 1) and develop free motility.

Eggs were examined by phase-contrast microscopy, both in the fresh state and after fixation and staining as whole-mounts. A solution of 10% glacial acetic acid in absolute alcohol was used for fixation; staining was effected with aceto-carmine (Schneider's). Some eggs in the fresh state were examined by fluorescence microscopy; they were treated with acridine orange solution (0.0005% in sea water) and ultraviolet radiation. Eggs after semination were also fixed in 10% formalin solution, embedded in paraffin, sectioned at 6 μ and stained with haematoxylin and eosin.

Observations

Primary oocyte

The *Pectinaria* egg took the form of a concavo-convex disc 50–60 μ in diameter and 25–30 μ in thickness. It was bounded by a closely applied transparent vitelline membrane about 2 μ thick. The cytoplasm contained numerous finely granular elements and was devoid of large lipoidal droplets.

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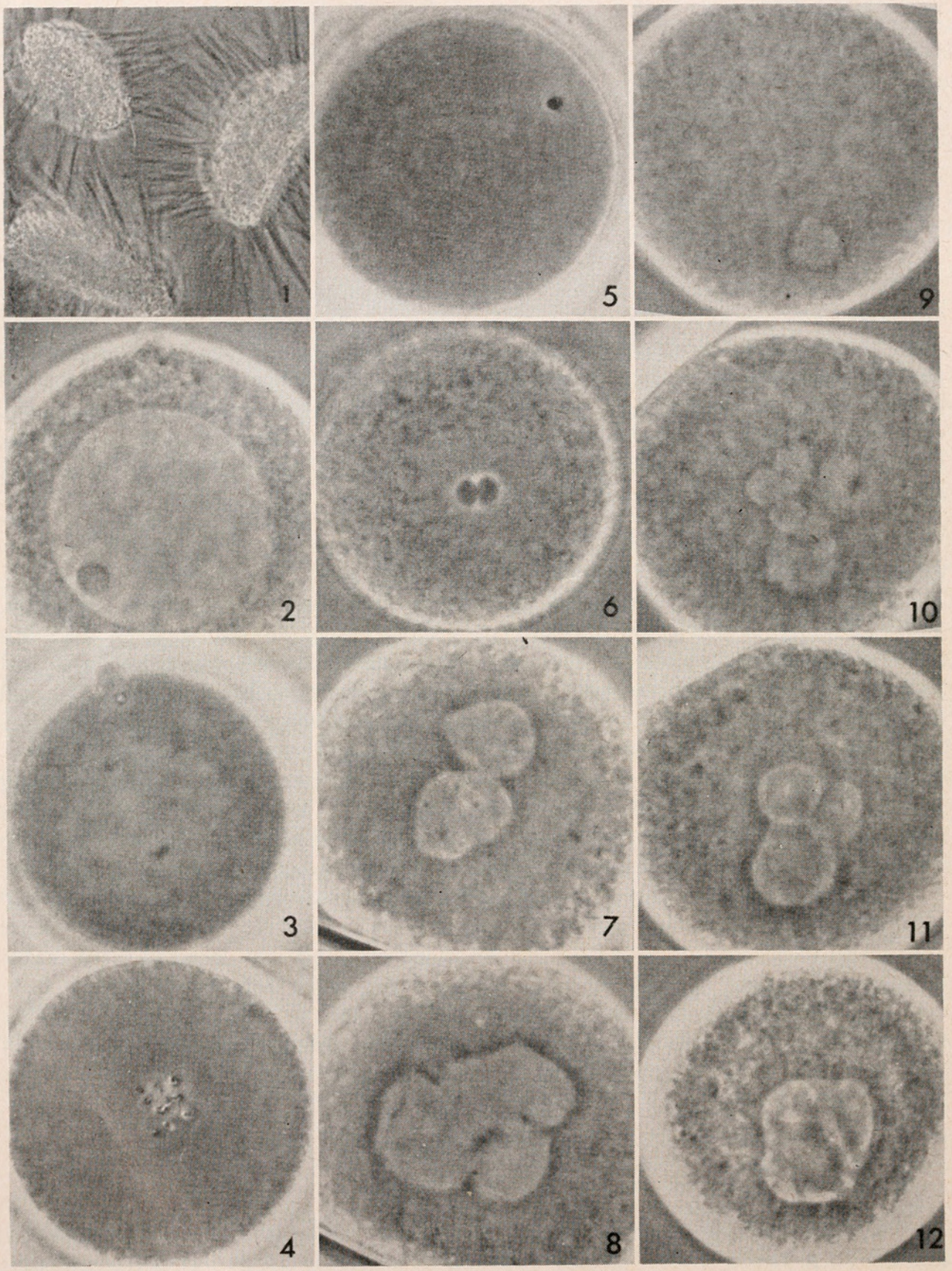

All photographs were taken by phase-contrast microscopy and are reproduced at × 1300.

FIGURE 1. Sperm packets. (Fresh material.)

FIGURE 2. Primary oocyte with intact germinal vesicle. A spermatozoon had recently entered the egg (at top). (Fresh material.)

The germinal vesicle (Fig. 2) was large relative to the size of the egg, its diameter being about half that of the vitellus. A single nucleolus was usually present, in addition to a number of scattered chromosomes. In the living egg, the chromosomes were only vaguely distinguished but they became more evident when they condensed just before breakdown of the germinal vesicle. In the condensed phase of the fixed egg, they could clearly be seen to have the forms characteristic of bivalents. The attempt was made to count the chromosomes in 26 such eggs; estimates ranged from 17 to 21, with a mean of 19.2, uncertainty being due particularly to the presence of several very small chromosomes.

Primary oocytes treated with acridine orange solution and ultraviolet radiation exhibited bright green fluorescence in a zone around the nucleolus and in numerous cytoplasmic granules, and bright red fluorescence in about an equal number of cytoplasmic granules. The nucleolus itself, the nucleoplasm and the hyaline cytoplasm did not have noticeable fluorescence.

Oocytes examined histologically displayed a moderate cytoplasmic basophilia, especially in the granular elements, and a strong basophilia in chromosomes and in the band of material surrounding the nucleolus.

Maturation

The egg was always shed as a primary oocyte; maturation changes began almost immediately, the proportion of eggs showing the changes varying between 10% and 90% in different individuals. The chromosomes condensed, though remaining scattered, the nuclear membrane became irregular in outline and disappeared, and the chromosomes aggregated in the center of the egg as a loose group which became the metaphase plate of the first polar spindle (Fig. 4). Further progress was seen only after sperm penetration; when this occurred, meiosis was resumed and the first and second polar bodies were successively separated. The polar bodies were always extruded into the middle of the concave side of the egg (Fig. 6). The first polar body had a tendency to divide partially or completely, and thus three polar bodies were occasionally seen. Eggs could be left in sea water for at least three hours after shedding without losing their capacity to proceed, on semination, with apparently normal fertilization.

FIGURE 3. A similar egg to that in Figure 2, showing germinal vesicle (with two or three bivalents in approximate focus), a sperm head in the cytoplasm and a residual fertilization cone. (Acetic alcohol and acetocarmine.)

FIGURE 4. Primary oocyte showing polar view of the first meiotic metaphase plate. (Acetic alcohol and acetocarmine.)

FIGURE 5. A similar egg to that in Figure 4, showing a recently penetrated sperm head which had lost its refractility and become stained. (Acetic alcohol and acetocarmine.)

FIGURE 6. Secondary oocyte; surface view showing polar bodies. (Fresh material.)

FIGURE 7. Ootid with male and female pronuclei approaching full development. (Fresh material.)

FIGURE 8. Ootid with two male pronuclei—which appear fused, but were in fact separated by their nuclear membranes—and smaller female pronucleus. (Fresh material.)

FIGURES 9–12. Successive stages of pronucleus development in an egg in which polar body emission failed and two female pronuclei were formed. Seemingly intact nuclear membranes still separated the pronuclei in Figure 12 which give the appearance of having fused. (Fresh material.)

Sperm penetration

The frequency with which sperm packets broke up to liberate free and actively motile spermatozoa differed much between different animals. With good material, complete break-up of the packets occurred within 5 or 10 minutes.

Spermatozoa were occasionally seen to become attached to the vitelline membrane and then pass gradually through this structure; actual entry into the vitellus was not witnessed, the spermatozoa under observation all being stopped apparently at its surface. Many instances were encountered in which a spermatozoon had just entered the egg cytoplasm, and generally the sperm head lay within a small fertilization cone. There was no evidence of elevation of a fertilization membrane. Penetration could apparently occur at any point on the egg surface except in the concave region. A little further progress could be watched until the sperm head became indistinguishable among the cytoplasmic granules. Subsequent stages were studied in fixed and stained eggs, and it was evident that the sperm head enlarged and lost its refractility as it advanced into the egg (Fig. 5). The course of events of entry into eggs before the breakdown of the germinal vesicle was broadly the same as that into eggs of later stages, except that in the former case rather larger fertilization cones were often formed and the sperm head remained refractile and did not enlarge (Figs. 2 and 3). Fertilization proceeded only in eggs penetrated after the germinal vesicle had started to break down. Allowing eggs to stand in sea water for three hours after shedding and before semination did not affect the frequency of sperm penetration.

Pronucleus development

During its early stages of development, the female pronucleus could clearly be seen to be made up of separate portions, karyomeres (Fig. 10), which later came together and fused to form a single nucleus. Evidence of septa could also be seen in the early male pronucleus (Fig. 9). Residual walls or strands tended to persist in both pronuclei at later stages, and seemed to be responsible for irregularities in the outlines of the pronuclei which often looked roughly polygonal (Figs. 7, 8 and 12). At full development, male and female pronuclei came into close apposition and sometimes gave the impression of having undergone fusion (Fig. 12); in no instance, however, could it be said with certainty that actual fusion had occurred, for there always seemed to be a thin membranous structure separating the two nuclei. The chromosome groups deriving from the male and female pronuclei in the prophase of the first cleavage division were regularly found to be quite separate.

Observations indicated that the male pronucleus was formed almost always before the female; it was possible for successful fertilization to be initiated by sperm penetration occurring as soon as the germinal vesicle had broken down, and thus the male pronucleus was formed during the early maturation changes in many eggs. Male pronucleus development then seemed to be suspended until female pronucleus development was under way, for intermediate and later stages of growth were regularly found to be synchronous in the two pronuclei.

In several trinuclear eggs that had two polar bodies (presumptive polyspermic eggs), two pronuclei were seen to be equal in size and both were larger than the

third (Fig. 8); in a trinuclear egg that had no polar body (in which it was surmised that two female pronuclei had developed from the egg chromosomes after anaphase separation), two pronuclei were smaller than the third (Figs. 9–12). The inference is that in this species the male pronucleus is larger than the female. The pronuclei contained several small nucleoli (Fig. 7).

Anomalies

The chief anomalies noted were: (1) polyspermy, (2) refertilization, (3) possible early gynogenesis, and (4) failure of polar-body formation. There were no instances of androgenesis or of spontaneous development beyond the first polar metaphase (rudimentary parthenogenesis).

Polyspermy. The occurrence of polyspermy was inferred, in early fertilization, from the presence of two or more sperm heads in the vitellus, or, at later stages, from the presence of three or more pronuclei in eggs having two (or three) polar bodies (Fig. 8). A total of 163 polyspermic eggs was recorded; one was tetraspermic, fourteen trispermic and the rest dispermic. The tetraspermic egg and all but one of the trispermic eggs had intact germinal vesicles. Some eggs observed in stages of the first cleavage division had multipolar spindles; in six, the spindle was tripolar, and in two tetrapolar. These eggs all had two polar bodies and were also assumed to have undergone polyspermic fertilization.

Refertilization. The term is used to denote the entry of a second (or third) spermatozoon into the vitellus much later than the first (or first two), so that the male elements are clearly in different stages of development. The distinction is drawn with polyspermic fertilization in which the male elements are in the same stage of development and apparently advance synchronously. Altogether, ten examples of refertilization were seen, in each of which there was a sperm head that had not undergone noticeable change. Four of these eggs had in addition late-stage male and female pronuclei, two showed an early male pronucleus with the egg chromosomes in the second polar prophase, two had early male pronuclei and egg chromosomes in second polar metaphase, one egg was in the metaphase of the first cleavage division and one egg was two-cell.

Gynogenesis. Possible examples of early gynogenesis were presented by three eggs; each had a well formed female pronucleus and two polar bodies, but the penetrating sperm heads had not appreciably changed.

Failure of polar-body formation. Two eggs were seen in the course of fertilization, both of which lacked polar bodies; they were kept under continuous observation while the pronuclei formed. In one, there were two presumptive female pronuclei and a larger male pronucleus (Figs. 9–12). In the other the sperm head changed into an early male pronucleus, and the egg chromosomes, having failed to undergo anaphase separation, became incorporated into a single female pronucleus, which was about the same size as the male pronucleus.

Time relations and incidence of features of fertilization

The time relations of fertilization were studied in four experiments, the results of which were reasonably consistent; details of the most elaborate experiment are

set out in Table I. From these sets of data, the times for fertilization stages were estimated to be as follows: Breakdown of the germinal vesicle became evident a few minutes after shedding and most eggs showed these changes by 10 minutes. The first polar division had advanced to metaphase in a few eggs by 20 minutes and most eggs had reached (or, if penetrated, had passed) this stage within the first hour. Sperm penetration began immediately after semination and virtually all the eggs that were destined to be penetrated contained spermatozoa by 10 minutes after semination. Most of the eggs that were penetrated and lacked the germinal vesicle exhibited first polar bodies and early male pronuclei between 20 and 25 minutes after semination. Second polar bodies and female pronuclei made their appearance within the next 10 minutes. The whole pronuclear phase of fertilization lasted about half an hour, condensation of chromosomes in the prophase of the first cleavage division being evident in most eggs between 50 and 60 minutes after semination. Mitosis seemed to pause in metaphase, and cytoplasmic cleavage was completed in most eggs 20 to 30 minutes later, namely about 80 minutes after semination.

The following inferences are drawn from the observations summarized in Table I. Some eggs fail to undergo any maturation changes, even after standing in sea water for an hour or more: the data show that the proportion of oocytes with intact germinal vesicles fell to 51% (23% + 20% + 8%) at 45 minutes after shedding and suffered only a small reduction thereafter. At each interval from 45 minutes onwards, it was found that roughly half the unchanged eggs had been penetrated by spermatozoa; evidently, sperm penetration neither provoked nor inhibited maturation. The frequency of sperm penetration into primary oocytes, whether before or during maturation, did not change significantly from that observed at 10 minutes after semination—this was despite the presence, for well over an hour, of many active spermatozoa in the medium. The proportion of eggs penetrated was somewhat higher in those with maturation changes (67%) than in those without (54%). On the other hand, the incidence of polyspermy was much higher in eggs with intact germinal vesicle (24% of penetrated eggs) than in eggs at later stages (4% of penetrated eggs).

Discussion

The limits of the breeding season of *Pectinaria* at Woods Hole have not yet been determined, but Costello *et al.* (1957) noted that ripe animals could be secured at least during August. The present investigation was carried out on animals collected in June and July, which may have been before the peak of the season. This would explain why many animals could not be induced to shed, why spermatozoa obtained from others often failed to become free-swimming, and why occasionally as few as 10% of oocytes underwent maturation. The relatively low rate of sperm penetration that occurred (around 60%) despite the presence of many surplus free-swimming spermatozoa may have been owing also to unripeness of animals providing the gametes.

According to Costello *et al.* (1957), polar bodies are separated about 29 minutes after semination, and the first cleavage occurs at about 54 minutes. The corresponding times recorded in the present series were 30–35 minutes and about

TABLE I

Stages of maturation and fertilization in eggs examined at various times after shedding and semination (at 18° C.)

Time after shedding (min.)		15	30	45	60	90	120	150	Eggs		
Time after semination (min.)		—	—	10	25	55	85	115	Total	Penetrated	Polyspermic
Primary oocyte	with germinal vesicle	175 (87)	140 (68)	54 (23)	53 (17)	59 (18)	63 (16)	83 (25)	992	365 (54*)	89 (24*)
	+ 1 sperm			47 (20)	44 (14)	65 (20)	57 (15)	63 (19)			
	+ > 1 sperm			20 (8)	14 (3)	18 (5)	25 (7)	12 (4)			
	late prophase	27 (13)	62 (30)	29 (12)	2 (0.5)	1 (0.3)	3 (1)	1 (0.3)	1015	617 (67*)	16 (4*)
	+ 1 sperm			17 (7)	5 (1.5)	5 (1.5)	1 (0.3)	0			
	+ > 1 sperm			2 (1)	0	1 (0.3)	0	0			
	1st metaphase		5 (2)	22 (9)	59 (18)	59 (18)	78 (20)	50 (15)			
	+ 1 sperm			44 (19)	37 (12)	1 (0.3)	2 (0.5)	3 (1)			
	+ > 1 sperm			2 (1)	1 (0.3)	0	1 (0.3)	1 (0.3)			
Secondary oocyte	+ 1 sperm				103 (32)	4 (1)	2 (0.5)	0			
	+ > 1 sperm				1 (0.3)	2 (0.5)	0	0			
Ootid	+ 1 sperm					114 (34)	48 (13)	8 (2)			
	+ > 1 sperm					2 (0.5)	2 (0.5)	1 (0.3)			
2-cell							101 (26)	99 (30)			
3-cell								7 (2)			
Total eggs		202	207	237	319	331	383	328	2007	—	—
Total penetrated eggs		—	—	132 (56)	205 (64)	212 (64)	239 (62)	194 (59)	—	982	—

Figures in parenthesis are percentages.

* Eggs recovered before semination were not included in the calculation of these percentages. Cleaved eggs were not included in the calculation of the incidence of polyspermy for the lower group (4%).

80 minutes. The authors just cited asserted also that pronuclear fusion took place at about 40 minutes; in the present series, the early prophase changes of the first cleavage division were observed between 50 and 60 minutes after semination. The implication is that fertilization was slower in the present series, probably owing to differences in water temperature (24° C. in the study of Costello *et al.*, 18° C. in the present series).

Two features having temporal association with the breakdown of the germinal vesicle are worthy of comment, and they may be attributable to the same underlying cause. These features are the large differences in the incidence of polyspermy before (24%) and after (4%) germinal vesicle disappearance, and the difference in the fate of penetrating sperm heads before and after the event. The difference in the incidence of polyspermy was the more striking since eggs with intact germinal vesicle showed a lower frequency of sperm penetration (54%) than did those undergoing maturation (67%). The deficiency in the former group presumably lay in the block to polyspermy, and the inference is that, when maturation begins, the nature of the reaction shown by the egg plasma membrane to sperm contact undergoes an important change. Normally, sperm penetration involves early fusion between egg and sperm plasma membranes (Colwin and Colwin, 1960; Szollosi and Ris, 1960; Friedmann, 1962), and it may well be that the block to polyspermy is provoked by such fusion. However, in oocytes before maturation, sperm entry may possibly occur in the way it was classically supposed to happen, namely, by a process resembling phagocytosis. With a phagocytic form of sperm absorption, the spermatozoon would be engulfed with its membrane intact and surrounded at a short distance by an envelope of egg plasma membrane. Early stages of what could be phagocytic engulfment of a sperm by a *Lytechinus* primary oocyte have been described by Franklin and Metz (1962). Under these circumstances, the sperm head would be shielded from cytoplasmic agents normally responsible for bringing about its metamorphosis into a male pronucleus. Thus, the high incidence of polyspermy and the persistence of unchanged sperm heads in the oocyte before breakdown of the germinal vesicle can both be explained by the assertion that the reactivity of the egg plasma membrane to sperm contact is not yet of the kind needed for normal fertilization.

The fluorescent colors displayed by oocytes treated with acridine orange and ultraviolet radiation conform in general to the descriptions given by Tweedell (1959, 1960, 1962). They are difficult to interpret. In fixed mammalian tissue subjected to the same treatment, yellow and red fluorescence was shown to be associated with the presence of DNA and RNA, respectively (Armstrong, 1956). Living mammalian eggs were found to have strongly green and red fluorescent structures; here the green color was regularly evident in DNA-containing elements but the distribution of the red color was consistent with an association, not with RNA, but with mononucleotides (Austin and Bishop, 1959). In *Pectinaria,* the red fluorescence could reasonably be ascribed to the presence of mononucleotides, but the cytoplasmic green fluorescence seems most unlikely to be indicative of DNA. The cytoplasmic DNA of *Paracentrotus* eggs is not demonstrable by histochemical methods, owing apparently to its low order of polymerization (Hoff-Jørgensen, 1954), and the acridine-orange-induced green fluorescence in the cytoplasm of *Arbacia* oocytes was not removed by DNAase (H. Esper, 1962, personal com-

munication). Possibly, green fluorescence in these marine eggs can be ascribed to component proteins having an appropriate degree of polymerization.

The work described in this report was done when the author, a member of the External Scientific Staff, Medical Research Council, London, was F. R. Lillie Memorial Fellow for 1961 at the Marine Biological Laboratory, Woods Hole, Massachusetts. The observations on induced fluorescence were made in collaboration with Dr. C. B. Metz who also provided the fluorescence microscope and other facilities required for this procedure.

Summary

1. The eggs shed by *Pectinaria gouldii* were in the stage of primary oocytes with intact germinal vesicles. Maturation began promptly, but proceeded only to the first metaphase, which some eggs reached in 20 minutes, and further progress depended upon sperm penetration.

2. Spermatozoa entered eggs rather more readily after than before breakdown of the germinal vesicle, and only those that entered after the event developed into pronuclei. It is suggested that spermatozoa entering eggs before maturation may be engulfed in a manner resembling phagocytosis, as distinct from membrane fusion. Polar bodies were extruded about 20–25 minutes and 30–35 minutes after semination at 18° C. Male pronuclei were evidently larger than the female. Early prophase of the first cleavage mitosis was seen between 50 and 60 minutes after semination, and cell division took place 20 to 30 minutes later at 18° C.

3. The incidence of polyspermy observed was 24% before and 4% after breakdown of the germinal vesicle. Eggs undergoing refertilization, possible early gynogenesis, and development after failure of polar-body formation were also seen.

4. Oocytes treated with acridine orange displayed green and red cytoplasmic granules, as well as a green zone around the nucleolus. The red fluorescence may have denoted the presence of mononucleotides; the green fluorescence was not considered specific to DNA.

LITERATURE CITED

Armstrong, J. A., 1956. Histochemical differentiation of nucleic acids by means of induced fluorescence. *Exp. Cell Research,* **11**: 640–643.

Austin, C. R., and M. W. H. Bishop, 1959. Differential fluorescence in living rat eggs treated with acridine orange. *Exp. Cell Research,* **17**: 35–43.

Colwin, A. L., and L. H. Colwin, 1960. Changes in the spermatozoon during fertilization in *Hydroides hexagonus* (Annelida). II. Incorporation with the egg. *J. Biophys. Biochem. Cytol.,* **10**: 255–274.

Costello, D. P., M. E. Davidson, A. Eggers, M. H. Fox and C. Henley, 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Marine Biological Laboratory, Woods Hole, Massachusetts.

Franklin, L. E., and C. B. Metz, 1962. Electron microscope study of sperm entry into sea urchin oocytes. *Biol. Bull.,* **123**: 473.

Friedmann, I., 1962. Cell membrane fusion and the fertilization mechanism in plants and animals. *Science,* **136**: 711–712.

HOFF-JØRGENSEN, E., 1954. Deoxynucleic acid in some gametes and embryos. *In:* Recent Developments in Cell Physiology, p. 79. Ed. by J. A. Kitching. Butterworths Scientific Publications, London.

SZOLLOSI, D. G., AND H. RIS, 1960. Observations on sperm penetration in the rat. *J. Biophys. Biochem. Cytol.*, **10**: 275–283.

TWEEDELL, K. S., 1959. Induced fluorescence in marine eggs. *Biol. Bull.*, **117**: 429–430.

TWEEDELL, K. S., 1960. Differential staining of cytoplasmic inclusions in eggs of *Pectinaria*. *Biol. Bull.*, **119**: 345.

TWEEDELL, K. S., 1961. Factors affecting germinal vesicle breakdown in *Pectinaria* (*Cistenides*) *gouldii*. *Biol. Bull.*, **121**: 412.

TWEEDELL, K. S., 1962. Cytological studies during germinal vesicle breakdown of *Pectinaria gouldii* with vital dyes, centrifugation and fluorescence microscopy. *Biol. Bull.*, **123**: 424–449.

WILSON, D. P., 1936. Notes on the early stages of two polychaetes, *Nephthys hombergi* Lamarck and *Pectinaria koreni* Malmgren. *J. Mar. Biol. Assoc.*, **21**: 305–310.