FERTILIZATION AND COMPLETION OF MEIOSIS IN SPAWNED EGGS OF THE AMERICAN OYSTER, *CRASSOSTREA VIRGINICA* GMELIN

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It is of fundamental importance to an understanding of the genetic system of the commercially valuable American oyster, *Crassostrea virginica* Gmelin, to know whether its meiosis is of the normal type found in the vast majority of metazoa or one of the various anomalous types. These anomalous forms have developed in particular groups in association with parthenogenesis or male haploidy, and even in some groups with a bisexual method of reproduction and diploidy in both sexes (WHITE, 1954). Protandry and hermaphroditism are common in the Mollusca (WHITE, 1954).

Irrespective, however, of whether they are normal or atypical, cytogenetic knowledge of meiosis and fertilization in the American oyster is indispensable to its genetic study and selective breeding. Such information is particularly important because eggs like those of this bivalve mollusk, which are fertilized externally and complete maturation after fertilization, are especially susceptible to wide variations of one or both maturation divisions (WILSON, 1953). Laboratory and hatchery rearing of oyster larvae depends on laboratory-stimulated development of the gonads of adult animals, proper maturation or meiosis of the eggs, and successful fertilization of normal eggs from induced spawnings (LOOSANOFF and DAVIS, 1963).

Early cytological studies of the chromosomes, cell division, and development of marine eggs (see WILSON, 1953, for review) did not include any critical studies of the Ostreidae. In more recent years marine animals have not been much considered in cytogenetic researches even though improvements in chromosome techniques (SHARMA and SHARMA, 1965) have removed many of the methodological obstacles to their cytological study.

An illustrated account is presented here of the completion of meiosis and fertilization in mature, spawned eggs of *C. virginica*; it is based on the study of thousands of eggs from numerous crosses of gametes of different

parents. Observations were supplemented and confirmed over a period of a year while we were preparing a karyotype of the American oyster chromosomes and in the course of other genetic studies (LONGWELL and STILES, 1967).

MATERIALS AND METHODS OF PREPARATION

All oysters were from wild *C. virginica* stock local to the vicinity of the harbor of New Haven, Connecticut. Eggs and the sperm used to fertilize them came from thermal-induced spawnings of adult male and female oysters conditioned in the laboratory for spawning (LOOSANOFF and DAVIS, 1963).

Oysters of the oviparous *Crassostrea* species are usually not hermaphroditic and functional eggs and sperm are only rarely found together. Age and environment, however, affect the frequency of hermaphroditism in these oysters. *C. virginica* is protandric and the degree of protandry varies greatly among juvenile, 1-year-old oysters in their first breeding season (GALTSOFF, 1964).

Fixation was in Carnoy's fluid (ethyl alcohol: glacial acetic acid, 3:1) for a few minutes to several days. Squashes were made in 1-2% orcein in 45-60% acetic or proprionic acid.

Before squashes were made, yolk was extracted from the eggs in a micro Soxhlet apparatus with a 1:1 mixture of methyl alcohol and chloroform. The usual extraction time approximated 2 hours (LONGWELL and STILES, 1968).

Eggs were examined and photographed with a 100X Zeiss Neofluar phase-contrast objective.

REGULAR MEIOSIS AND FERTILIZATION IN SPAWNED EGGS OF THE AMERICAN OYSTER

Normal, unfertilized, spawned eggs of *C. virginica* are either at Prometaphase or Metaphase I of meiosis. Occasionally a spawned egg is at Diakinesis (Fig. 1) or late Diplotene. Few unfertilized eggs proceed beyond Metaphase I.

Fig. 1. — Ten pairs of Diakinesis chromosomes of an unfertilized egg of *C. virginica.* ×2100.

Fig. 2. — Metaphase I bivalents of a *C. virginica* egg with the round, dense sperm nucleus at the right of the metaphase plate (at arrow). ×2000.

Fig. 3. — Late meiotic Anaphase I in a *C. virginica* egg showing the reduced number of chromosomes and the male pronucleus (at arrow). ×2250.

Fig. 4. — Portion of an egg of *C. virginica* showing a Metaphase II chromosome group, first polar body nucleus (to the right of the metaphase group), and chromosomes developing from the male pronucleus (group at lower left). ×2500.

Fig. 5. — The 10 highly contracted Metaphase II chromosomes of a *C. virginica* egg. (Two chromosomes are lying close together). ×2500.

Fig. 6. — The two polar body nuclei of a fertilized egg of *C. virginica.* One has a more diffuse and irregular outline than the other. ×2100.
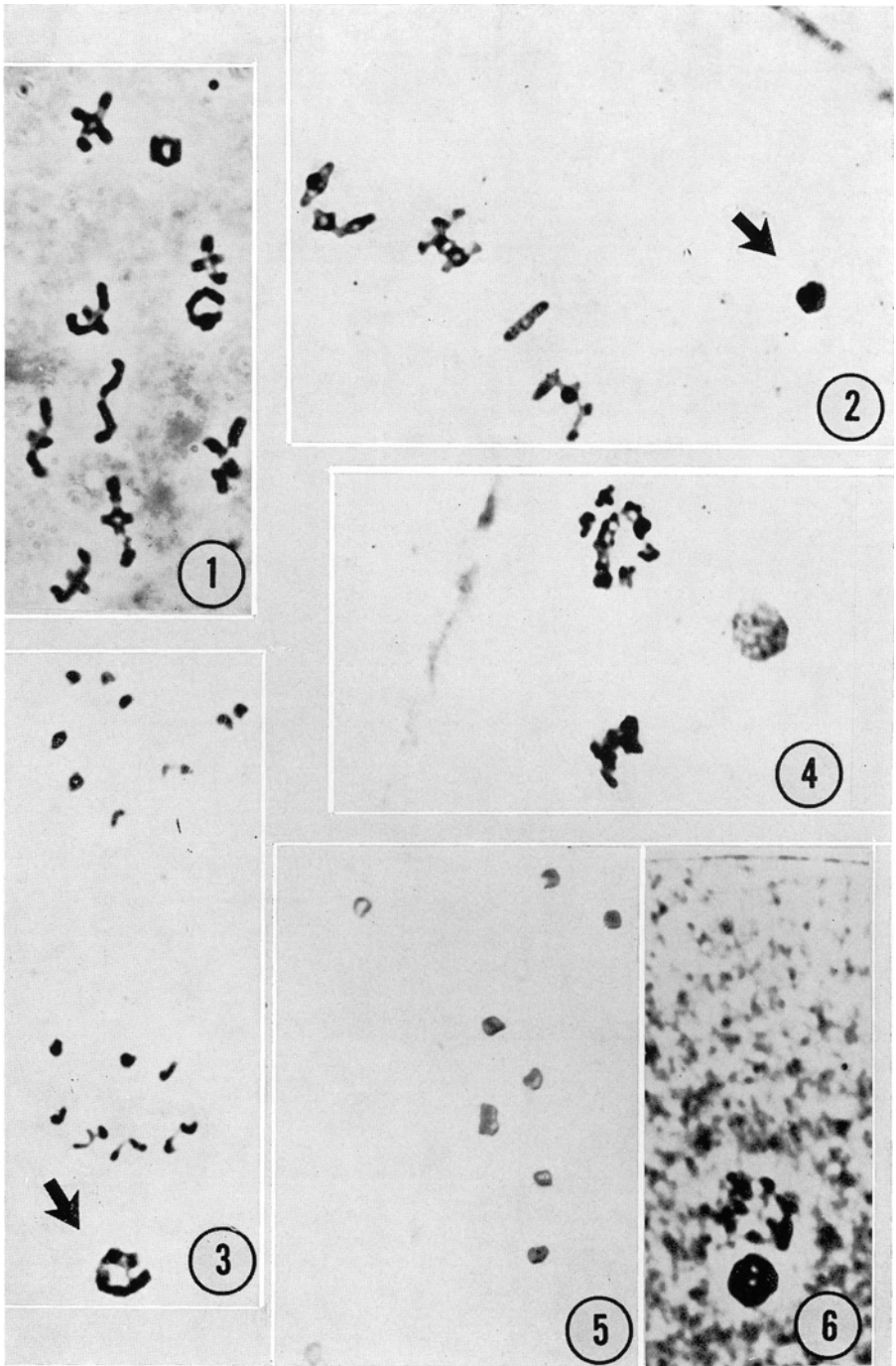
Immediately after fertilization the dense, compact sperm head is clearly recognizable in the egg cytoplasm. The size of this sperm head soon begins to increase. Figure 2 shows the 10 bivalents of the female aligned across the Metaphase I spindle and the swollen but still dense sperm nucleus lying to one side of the division group.

Once fertilized the eggs rapidly proceed to complete meiosis.

The first meiotic division is reductional. Each Anaphase I group has 10 chromosomes (Fig. 3).

About Telophase I of the female gamete the sperm nucleus has been transformed into the diffuse, vesicular male pronucleus, which soon takes the general appearance of a prophase (Fig. 3).

One of the Telophase I groups forms the first polar body nucleus (Fig. 4). The other Telophase I chromosomes pass directly into the equational plate of the second meiotic division with its 10 super-contracted chromosomes (Figs. 4 and 5).

The second, innermost polar body nucleus is formed from one of the Telophase II groups. Figure 6 illustrates the two polar body nuclei. These nuclei are extruded from the eggs and constricted off them with a small portion of cytoplasm.

During Division II of the female gamete, the male pronucleus gives rise to the 10 chromosomes of the male parent.

No prolonged female pronuclear stage was observed.

The chromosomes of the egg and sperm are combined in one group through their orientation on the first cleavage spindle. For some time before this stage and to the onset of the first cleavage, the prometaphase chromosome sets of the male and female gametes are readily distinguishable from one another by virtue of the differences in their position in the egg. Individual chromosomes of each group can be conveniently and easily studied at this stage. At this time the chromosomes of the female tend to be more elongated than the usually shorter, denser chromosomes of the male gamete (Fig. 7). Once they are fully oriented on the metaphase plate the chromosome set of each gamete is equally contracted. This stage is reached approximately 1 hour after the addition of the sperm suspension to the eggs.

As cleavage begins, the presence of two polar body nuclei and the

Fig. 7. — The haploid chromosome sets of the male and female gamete of *C. virginica* just before their coming together on the first cleavage spindle. The 10 more elongated chromosomes of one group are the female chromosomes; the 10, shorter, denser chromosomes are the group brought into the egg by the sperm. To one side of the photomicrograph are the two polar body nuclei (at arrow), an indication of normal meiosis. ×1600.

Fig. 8. — First cleavage anaphase in a *C. virginica* egg after successful completion of meiosis and fertilization (normal chromosome number and normal polar body nuclei — to the left of the egg). ×1600.
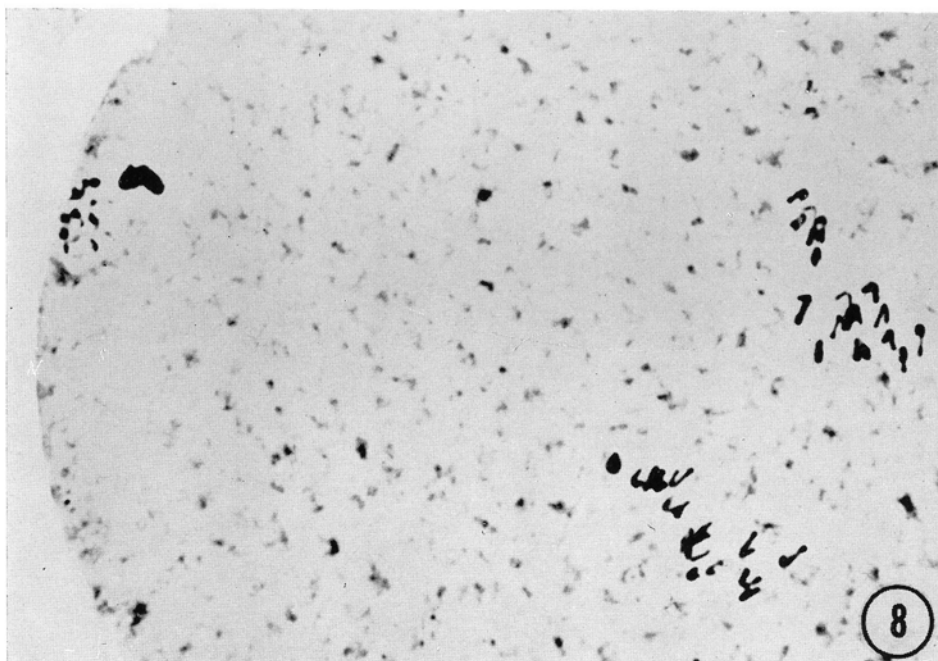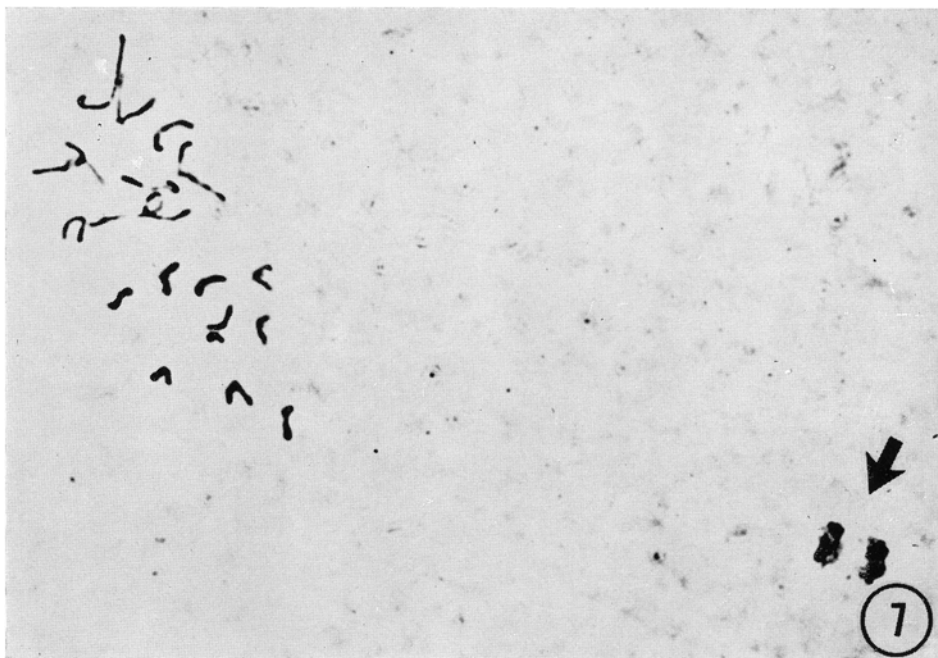
absence of any other nuclear group in the egg aside from the cleavage chromosomes indicate that meiosis and fertilization were at least superficially normal (Fig. 8). Figure 9 shows a somewhat later cleavage embryo with its two polar body nuclei.

The first, outermost polar body enters division usually at the 64-cell stage of the embryo. In abnormal eggs this nucleus tends to begin dividing earlier. This division has the appearance of a colchicine metaphase with much contracted easily studied chromosomes (10) and no organized spindle (Fig. 10). These polar body chromosomes have never been observed to enter anaphase, although embryos were studied cytologically until just before the emergence of the trochophore larvae at which time the polar bodies are lost.

Sometimes it seems to be the second, innermost polar body nucleus that enters this abortive mitosis. No eggs have ever been observed in which both polar body nuclei are in division.

Karyotype studies on early cleavage chromosomes of this oyster (LONGWELL and STILES, 1967) have shown them tto be about 2 to 3.5 microns long at colchicine metaphase. No unusual phenomena are associated with early cleavage.

Thus far, meiosis in male oysters has been only cursorily examined. It also seems to be of the normal type. The small size of the spermatocytes, however, impedes their analysis.

IRREGULAR MEIOSIS AND FERTILIZATION IN SPAWNED EGGS OF THE AMERICAN OYSTER

Various deviations from the usual sequence of these events of meiosis and fertilization were not infrequent in the material used in this study. Aneuploid, haploid, triploid, tetraploid, and chromosomally mosaic embryos are the result of such disturbances. They no doubt lead to poor embryo and abnormal larval development, and death in a significant number of cases.

Polyspermy can occur in *C. virginica* and more than one sperm nucleus can be resolved into chromosomes in a single egg. These extra nuclear groups can either remain *in situ* in the cytoplasm or several of them may fuse with the female group with the resultant formation of a multipolar spindle.

Fig. 9. — Early cleavage embryo of *C. virginica* showing its two polar body nuclei (at arrow). ×1200.

Fig. 10. — Metaphase of a polar body (at arrow) division in a *C. virginica* egg. Seven of the 10 chromosomes show at the level of focus at which this photomicrograph was taken. ×1600.
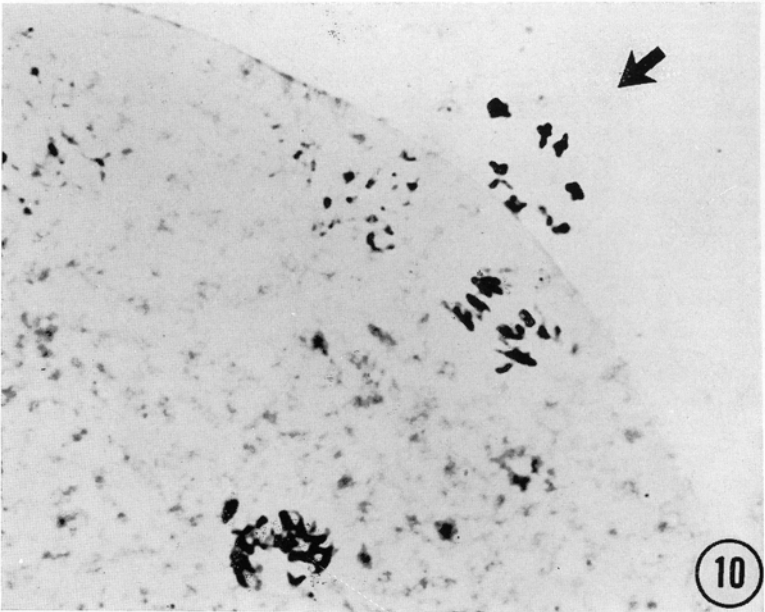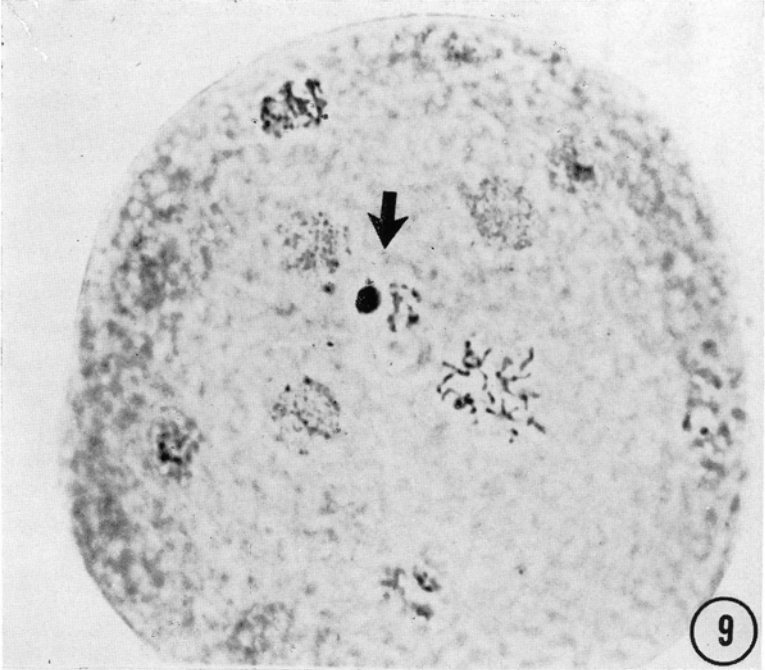
The incidence of parthenogenesis is low and variable in eggs of our local, wild oysters conditioned and spawned under laboratory conditions. A Metaphase I group in unfertilized eggs of the American oyster occasionally begins, after some time, to divide equationally. The two products of the female second meiotic division sometimes fuse in fertilized and unfertilized eggs alike. Some unfertilized eggs complete meiosis normally and then with only the female set of chromosomes commence to cleave.

COMPARISON OF MEIOSIS AND FERTILIZATION PHENOMENA AMONG MOLLUSCAN AND OTHER EGGS

As in the eggs of C. virginica, maturation of most other molluscan eggs begins spontaneously after spawning in sea water with the breakdown of the germinal vesicle (RAVEN, 1958). Like this bivalve mollusk, many other molluscan, as well as annelid and ascidian eggs, remain in metaphase until the sperm enters the egg or until the egg is stimulated to divide by artificial parthenogenesis (CONKLIN, 1917).

Meiosis, as it occurs in the American oyster, is of the typical type. It is uncertain just what is the cause or significance of the marked deviations from regular meiosis and fertilization sometimes observed.

As noted previously by GALTSOFF (1964), the time it takes for C. virginica eggs to be fertilized and to reach different stages of meiosis varies somewhat. This variation must depend on the maturity of the gametes, environmental factors, and genetic constitution of the eggs.

GALTSOFF (1964) earlier reported that polyspermy can occur in C. virginica eggs. Polyspermy was reported to be a common cause of abnormal development in the clam, Cumingia, also a bivalve mollusk (of the mud and sand dwelling Semelidae) when fertilization was delayed or when eggs received undue handling (MORGAN, 1910).

In more typical cases of female meiosis the first polar body divides while the second one is being formed, but exceptions are frequent. The second polar body rarely divides (CONKLIN, 1917). That the behavior of the two polar body nuclei in the American oyster should be rather unpredictable is not unexpected. In Loligo, a cephalopod mollusk, the first polar body divides more than once, whereas in other mollusks the polar body never divides (RAVEN, 1958).

EXPERIMENTAL GENETICS AND CELL BIOLOGY OF THE OYSTER EGG

The facility with which every later stage of female meiosis and fertilization can be followed in the oyster, is of much value in breeding

studies and further makes these molluscan eggs excellent material for experimental studies on genetics and cell biology.

Parthenogenesis, with and without polar bodies, has been induced in several bivalves japanese *Ostrea gigas*, the japanese oyster (INABA, 1936); in *Cumingia* (MORRIS, 1917); in the boring clams, *Barnea* (RAVEN, 1958); and in *Caecella*, also a lamellibranch (RAVEN, 1958). Sporadic or induced parthenogenesis in the American oyster might well be of much practical use in selective breeding of oysters, as it has been in the production of homozygous, diploid stocks of maize (CHASE, 1964).

Formation of polar body nuclei is of particular significance, then, as a means of estimating the frequency of natural parthenogenesis in different American oyster stocks, and as an indicator of the success of attempts at inducing parthenogenesis.

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SUMMARY

Meiosis and fertilization in spawned eggs of the American oyster, *Crassostrea virginica*, are of the normal type found in the large majority of the metazoa and higher plants. Various deviations from the regular sequence of these events were not infrequent, however, in oyster eggs examined in this study. Meiosis and fertilization can be cytogenetically followed critically in these eggs, which, consequently, offer many opportunities for experimental manipulation of the genetic material.