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CHORDATA (Vertebrata)

(TELEOSTEI)

Fundulus heteroclitus and *F. majalis*

LIVING MATERIAL:

The sexes of both species of Fundulus are quite easily identified and obtained. The mature *F. heteroclitus* female is pale olive in color and usually has no definite bars or spots, although young females may have indistinct, dark, transverse bars on the sides; the dorsal fin is non-pigmented. The adult male of this species is a dull, dark green color, with narrow, ill-defined transverse bars composed of silvery spots; the dorsal fin is black-pigmented, in a mottled pattern.

The pale olive *F. majalis* female has a pattern of heavy, black longitudinal stripes on the sides, and a non-pigmented dorsal fin. The sides of the somewhat darker male bear approximately 12 broad, dark transverse bars, and there is a striking black patch on the dorsal fin.

BREEDING SEASON:

Material is best and most abundant, as a rule, during the first three weeks of June, but small numbers of fertilizable eggs have been procured through July 15 at Woods Hole, Mass.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Fish are usually delivered by the M. B. L. Supply Department in mixed lots, but it is advisable to segregate the sexes, to prevent spawning. Males and females should be placed in separate aquaria until needed, and after they have been stripped, they should be removed to a discard tank. An adequate supply of running sea water is, of course, essential.

B. Procuring Gametes: Both eggs and sperm are obtained by "stripping": the fish is held firmly in one hand while gentle pressure is applied to its abdomen with the thumb and forefinger of the other hand. As these fingers are drawn towards the anus of the fish, the pressure forces out the gametes. If the fish is held in front of a strong light source during the stripping process, the eggs may be seen passing through the oviduct which runs along the anal fin.

C. Preparation of Cultures: Strip the eggs into a clean four-inch fingerbowl which has been moistened with filtered sea water. Strip the milt into a small amount of sea water, and mix the suspension with the eggs in ¼ inch of sea water. The eggs should be inseminated as soon as possible after they are obtained from the body of the female. After 30–45 minutes, change the sea water and leave the eggs in about a ¼- to ½-inch depth of sea water. Keep the fingerbowl covered with a glass plate to prevent evaporation; do not allow the eggs to clump or accumulate in one spot. The water should be changed at least twice daily.

D. Methods of Observation: To remove the sticky outer jelly layer, roll the eggs on a piece of filter paper or paper towel until the surface of the outer membrane is left smooth and clean. This same procedure should be followed daily for stock cultures, in order to prevent clumping of the eggs.

For experimental work, where it is essential to obtain development as nearly normal as possible, the eggs are usually examined uncovered in shallow depression slides; they may be manipulated with hair loops. For classroom study, when the eggs are to be observed over long periods of time and a specific orientation is desired, either of the following methods is suggested: (1) Place the eggs in sea water in special culture slides having a depression of 1.7 to 1.8 mm. (slightly less than the diameter of the eggs); it is then possible to roll the eggs to the desired position by moving the coverslip. (2) If these special slides are not available, the eggs may be placed in a drop of sea water on an ordinary glass slide and covered with a very thin, flexible sheet of mica; water is then withdrawn (using lens or filter paper) until capillary attraction causes a pressure on the egg, so that it can be rotated as in the previous method.

Recently, Trinkaus and Drake (1956) have described a method for the *in vitro* culture of Fundulus blastoderms isolated from the subjacent periblast and yolk mass.

E. Permanent Total Preparations: Fix the eggs in Stockard's solution (formalin, 5 parts; glacial acetic acid, 4 parts; glycerine, 6 parts; distilled water, 85 parts). This turns the protoplasm white but leaves the yolk transparent. The fixative may be used as a preservative, or the material can be transferred to 10% formalin after two days.

F. Preparation of Eggs for Sectioning: Eggs to be sectioned must be dechorionated before fixation, so that fluids can penetrate to the interior. (For details of this process, see the paper by Nicholas, 1927.) The following schedule for dehydration and embedding is useful.

1. Fix in Bouin's or Zenker's solution, 12–24 hours.
2. Dehydrate as usual through the alcohol series (up to and including 95% alcohol), leaving the eggs in each for one hour.
3. Absolute alcohol, two hours—use several changes.
4. Equal parts absolute alcohol and amyl acetate, two hours.
5. Amyl acetate, 24–48 hours.
6. Equal parts amyl acetate and paraffin, 12 hours (incubate at 30°).
7. Three changes of infiltrating paraffin (15 minutes in each); embed in 56–58° paraffin.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: Eggs stripped from a female fish into diluted sea water (70% fresh water, 30% sea water) retain the morphological characteristics of freshly-extruded eggs, including the yolk platelets, oil drops, membranes, etc. A micropyle is present, but it must be observed before removal of the chorionic jelly.

B. Fertilization and Cleavage: In order to follow all the pre-cleavage changes, it is important to (1) record the exact time of insemination, and (2) transfer the eggs *immediately* to a slide (see above) for observation. Polar bodies have not been described for Fundulus eggs, and it is not certain what stage the egg nucleus is in at the time of fertilization. No fertilization membrane is given off.

There is a gradual accumulation of the egg protoplasm at one pole of the egg, 25–35 minutes after fertilization, to form the blastodisc or germ-disc. A groove

on the surface of this blastodisc is the first indication of cleavage; it usually occurs two to three hours after fertilization. The cleavages continue for a considerable period without much change in the over-all form from that of the original blastodisc; this is called the period of the high blastula. Details of the process of cleavage are given by Oppenheimer (1937).

C. Time Table of Development: The following schedule is based on observations made at room temperatures which approximated 22–25° C. Times are recorded from insemination.

Stage	Time
Blastodisc formation	25–35 minutes
First cleavage	2–3 hours
Four-cell stage	2½–3½ hours
Eight-cell stage	4–5 hours
Sixteen-cell stage	4½–5½ hours
Early high blastula (Oppenheimer Stage 8)	10 hours
Late blastula (Oppenheimer Stage 9)	12 hours
Expanding blastula (Oppenheimer Stage 11)	17 hours
Early gastrula; embryonic shield (Oppenheimer Stage 12)	1 day
Middle gastrula; keel (Oppenheimer Stage 13)	2 days
Late gastrula; closure of blastopore (Oppenheimer Stages 14–15)	2½–3 days
Formation of brain and auditory capsules; 4–14 somites (Oppenheimer Stage 18)	3½ days
Heart-beat, embryonic circulation (Oppenheimer Stage 20)	4 days

D. Later Stages of Development: The periblast appears 16–24 hours after fertilization. The uncleaved protoplasm around the margin of the group of blastomeres is called the marginal periblast, while that beneath the blastodisc (visible only in sections) is the central periblast. At about this same time, the large, pinkish periblast nuclei may be visible. The nuclei of the marginal row of cells gradually become free of cell outlines, continue their divisions and migrate into the marginal periblast, converting it into a nucleated but non-cellular structure. Subsequent to the nucleation of the periblast, the blastoderm changes in form and size, and the embryo is now referred to as a blastula. Soon the margin of the blastodisc thickens (due both to a peripheral increase in cells and to a thinning of the central part of the disc), to form the germ-ring; this structure is best observed in eggs of *F. majalis*.

During the next few hours, the germ-ring grows completely over the surface of the yolk mass, so that the uncovered portion of the egg (the blastopore) is finally covered. This process of blastopore closure occurs after the first stages of formation of the embryonic axis. Under favorable conditions, it is sometimes possible to observe the beginning of gastrulation; a slight indentation appears at

the edge of the germ-ring, usually when the yolk is about one-fourth covered. Staining with neutral red (one or two drops of a 0.5% solution in a Syracuse dish of sea water) may make easier the identification of the germ-ring and periblast.

While the germ-ring is extending around the yolk, the embryonic axis is being established. The first indication of this process is a cellular thickening, the embryonic shield, resulting from a more active movement of cells in one region of the germ-ring. It is usually initiated when the blastoderm has covered from one-quarter to one-third the surface of the yolk. When the blastoderm has spread to cover approximately one-half the yolk, the embryonic shield has become a bluntly triangular area, extending from the margin of one portion of the germ-ring almost to the center of the blastoderm. The shield can best be identified in profile view. As the blastoderm spreads over the surface of the yolk, the embryo grows rapidly in length, and becomes segmented; this segmentation is confined to the mesoderm.

It is suggested that embryos be removed from the chorion for observation of the later developmental stages. Although this de-chorionation is rather difficult at early stages, it can readily be accomplished later, with the use of sharpened forceps or beading needles. Injury to the yolk sac should be avoided.

After hatching, the young fish may be studied in detail if they are anaesthetized with chloretone. The paper by Oppenheimer (1937) contains further details of developmental stages.

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CHORDATA (Vertebrata)

(TELEOSTEI)

Menidia sp.*

LIVING MATERIAL:

Two species (and probably several sub-species) of the common silversides are available in the Woods Hole, Mass., area. Menidia is characterized by the presence of a longitudinal silvery stripe, which runs along the flanks of the adult.

It is difficult to distinguish between *Menidia menidia* and *M. beryllina* solely on the basis of external morphology of the adults, although *M. beryllina* is somewhat smaller than *M. menidia* and has a colorless peritoneum, as opposed to the black peritoneum of *M. menidia.* The eggs of *M. beryllina* are somewhat smaller than those of *M. menidia,* and each has 8 to 14 attaching threads per egg, in contrast to the larger number (about 40) characteristic of the egg of *M. menidia.* All the attaching threads of the *M. menidia* egg are of approximately equal size, but Moulton (personal communication) reports that one thread of the egg of *M. beryllina* is notably larger (by a factor of two or three) than the remaining ones. Further details may be found in the book by Breder (1948).

During the breeding season, the females of both species are considerably plumper than the males, but other criteria for distinguishing between the sexes are somewhat unsatisfactory. Often, the females in a school of Menidia out-number the males (Kendall, 1901).

The adults were formerly very abundant in the Eel Pond, and some are still available there, as well as at other collecting sites (frequently in the same locales as Fundulus). Kendall (1901) reported that Menidia was common at that time about the wharves in Great Harbor, at Woods Hole.

BREEDING SEASON:

From mid-June to mid-July; the last two weeks in June are probably most favorable (Moulton, personal communication). Bumpus (1898) reported that eggs are also obtainable early in June.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The fish live well in aquaria supplied with running sea water, but it is important that they be transferred to such aquaria as soon as possible after collection.

B. Procuring Gametes: Eggs and sperm are obtained by stripping the fish (see the section on Fundulus, p. 224 of this manual). Some immature eggs (which lack the characteristic attachment threads) are usually obtained from the females; such eggs are pale in color and smaller than ripe eggs, and do not tend to cling together as do mature eggs.

*Much of the material on which this section is based was obtained from Dr. James M. Moulton, to whom we are most grateful.

C. Preparation of Cultures: Eggs may be inseminated by the same general methods described for Fundulus. The cultures are best kept on the sea water table; a temperature of 18 to 19° C. is apparently most favorable (Moulton, personal communication).

D. Methods of Observation: The attachment threads may be cut off close to the egg surface, using a sharp scalpel or razor blade. (See, also, the methods used for observation of Fundulus eggs.)

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg of *M. beryllina* is approximately 0.75 mm. in diameter (Breder, 1948), while that of *M. menidia* is somewhat larger and measures about 1.2 mm. in diameter (Nichols and Breder, 1927). The eggs of both species are clear and somewhat yellowish in color, and two to three oil droplets (which later coalesce into one) are present. The attachment threads arise from a very circumscribed area of the chorion, 180 degrees from the future site of origin of the blastodisc (Moulton, personal communication).

B. Fertilization and Cleavage: The sperm enters the egg through a micropyle. Polar bodies have not been observed in developing Menidia eggs (Moulton, personal communication), and the stage of the egg nucleus at the time of fertilization is not known. Formation of the germinal disc and cleavage are, in general, similar to the same processes in the Fundulus egg. During the course of development, the egg of Menidia becomes free within the chorion, so that the position of the attachment threads is no longer a criterion of the polar axis.

C. Time Table of Development: The development of Menidia eggs is slow; Moulton (personal communication) observed the following schedule, at a temperature of 18–19° C. The times are recorded from insemination.

Stage	Time
Germinal disc	By 40 minutes
First cleavage	60 minutes **
Eight to 32 cells	3 hours, 50 minutes
Beginning of expanding blastula	16 hours, 20 minutes
Early embryonic shield; germ-ring halfway around yolk	27 hours, 35 minutes
Beginning of gastrulation	36 hours
Yolk plug; optic vesicles to closed blastopore	39 hours, 35 minutes
Eyes formed; heart beating	6 days
Hatching	15 days

D. Later Stages of Development: The later development is like that of Fundulus, except that by four days, the eggs are clear and transparent, so that observation of the embryo is easier. The large oil droplets coalesce approximately 16 hours after insemination, to form a single drop. At six days, the eyes are well formed, the heart is beating and Kupffer's vesicle is clearly visible. The circulatory system in a 48-hour embryo is diagrammed by Clark and Moulton (1949). Shortly before hatching, the chorion becomes very soft and flabby.

** From the paper by Bumpus (1898); the temperature is not specified.

Figures of many stages in the development of *M. menidia* are available in the paper by Kuntz and Radcliffe (1917).

SPECIAL COMMENTS:

The localization of the egg attachment threads to a circumscribed area, together with the small number of oil droplets, facilitate study of this form, especially in early stages (Clark and Moulton, 1949). Thus, the eggs of Menidia have some advantages over those of Fundulus, for both study and experimentation.

In addition, the spawning season of Menidia is usually somewhat more prolonged than that of Fundulus.

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CHORDATA (Vertebrata)

(TELEOSTEI)

Tautogolabrus [=*Ctenolabrus*] *adspersus*

LIVING MATERIAL:

Pelagic eggs may be obtained from the scup (*Stenotomus chrysops*) and the mackerel (*Scomber scombrus*), but must be stripped and fertilized immediately, as the fish are taken from the traps. The cunner, Tautogolabrus, is more useful for the study of pelagic egg development, for it may be brought to the laboratory and stripped as needed. Fish of this genus are quite common.

Cunners should be caught on the same day they are to be used; females are ordinarily obtained only after 12 noon. The male has a somewhat brighter green color than the female, and can also be distinguished by its bright red cloacal lining.

BREEDING SEASON:

This is usually concurrent at Woods Hole, Mass., with the breeding season for Fundulus (June and, occasionally, part of July).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The sexes should be segregated and the animals maintained in large aquaria with adequate supplies of running sea water.

B. Procuring Gametes: Eggs are stripped into a four-inch fingerbowl containing a small amount of filtered sea water; milt is stripped into a large fingerbowl containing sufficient sea water to cover the bottom. It is almost essential to use several layers of cloth for holding the fish while they are being stripped, because they are extremely active and slimy, and have very sharp spines in the dorsal fin.

C. Preparation of Cultures: As soon as possible after stripping, the sperm suspension should be poured into the dish containing the eggs, and the time recorded. Let the mixture stand undisturbed for one-half minute, then add fresh sea water and decant into a graduate cylinder or an Erlenmeyer flask, adding sufficient sea water to bring the meniscus to near the top of the cylinder, or to the neck of the flask. Viable eggs will float to the top and collect at the edge of the meniscus. They should then be pipetted off and placed in covered four-inch fingerbowls containing ¼ inch filtered sea water. Store the dishes on the sea water table where they will keep cool; pelagic eggs of this type are very sensitive to such environmental factors as temperature and oxygen supply.

Only glass-clear eggs are suitable for study; if ova show the slightest opacity, they are either immature or dead. Similarly, the presence of bits of tissue adhering to eggs indicates that they are immature and should be discarded. To obtain later stages of development, not more than three to six embryos should be placed per four-inch fingerbowl; the sea water should be changed twice daily and dead (opaque) embryos removed immediately.

D. Methods of Observation: For observing the formation of polar bodies, the blastodisc and early cleavage, it is advantageous to place the microscope in a hori-

zontal position, so that the blastodisc may be studied in a profile view; it is difficult to observe the polar bodies by any other method.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg is approximately 0.8 to 1.0 mm. in diameter; it is, as noted above, transparent and contains no oil droplets.

B. Fertilization and Cleavage: The polar bodies, which appear as small, clear beads on the surface of the blastodisc, are given off 5 to 10 minutes after insemination. Cleavage is rapid, occurring about once every 20 minutes at temperatures of 16 to 18° C.; the nuclei are sometimes visible between divisions, as pinkish bodies.

C. Later Stages of Development: Because of the beautiful clarity of the egg, this form is very favorable for the study of later stages of teleost development, including the formation of the germ-ring, embryonic shield and Kupffer's vesicle. Development is rapid and hatching occurs in about four days. The details of embryogenesis are described by Newman (1915), Kuntz and Radcliffe (1917) and Breder (1948). Diagrams are available in the paper by Kuntz and Radcliffe (1917).

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CHORDATA (Vertebrata)

(TELEOSTEI)

Other Teleost Eggs of the Woods Hole, Mass., Region, Suitable for Experimental Use

I. *Cyprinodon variegatus* (broad killifish)

A. Breeding season: June through mid-July.
B. Developmental rate: Moderately rapid; hatches in 5–8 days.
C. Egg characteristics: Demersal; 1.3–1.5 mm. in diameter; spherical, slightly yellow; yolk almost transparent and colorless; fibrous, sticky coat; small oil droplets present; micropyle visible.
D. Special comments: Females with ripe eggs are rather difficult to obtain. The larvae live well in fingerbowls of sea water.
E. Pertinent references: Breder (1948); Newman (1907, 1915).

II. *Opsanus tau* (toadfish)

A. Breeding season: June and July.
B. Developmental rate: Very slow; hatches in 10–26 days.
C. Egg characteristics: Demersal; 5 mm. in diameter; large adhesive disc at center of vegetal pole, opposite micropyle; deep amber in color.
D. Special comments: Fairly readily obtained; the eggs are found attached inside submerged objects (tin cans, old boots, etc.), or they can be inseminated artificially (by allowing the eggs to flow from the opened ovary into a dish containing just enough water to cover them; fertilize after the eggs have attached to the dish).
E. Pertinent references: Clapp (1891, 1898, 1899); Sumner (1903); Wallace (1899).

III. *Scomber scombrus* (mackerel)

A. Breeding season: Mid-May to June and very early July.
B. Developmental rate: Rapid; usually hatches in 60 to 72 hours.
C. Egg characteristics: Pelagic; 1.2 mm. in diameter; faintly pink in color, transparent; one large oil globule; very sensitive to changes in temperature, 16° C. being optimum.
D. Special comments: The eggs are fairly readily obtained; females contain enormous numbers of eggs, but they must be stripped at the fish traps. Even at optimum temperatures, the mortality rate is high.
E. Pertinent references: Newman (1915, 1918); Russell (1939); Worley (1933).

IV. *Stenotomus chrysops* (scup)

A. Breeding season: Early June.
B. Developmental rate: Very rapid; hatches in 48 hours.

C. Egg characteristics: Pelagic; 0.8 mm. in diameter; colorless and very transparent; one large oil droplet.
D. Special comments: *Mature* females are rather difficult to obtain, but large numbers of eggs may be obtained from a single female. The fish must be stripped as soon as the eggs are ripe. Hatched embryos will live for a few days in fingerbowls of sea water.
E. Pertinent references: Breder (1948); Newman (1915).

V. *Strongylura marinus* (billfish)

A. Breeding season: June.
B. Developmental rate: Slow; the time of hatching has apparently not been recorded.
C. Egg characteristics: Demersal; 3 mm. in diameter; very clear and transparent; long tufts of adhesive threads.
D. Special comments: Not very common; probably it is best to strip and inseminate the eggs at the fish traps, although this can be done later, at the laboratory. The percentage of fertilized eggs is not very high, but the enormous numbers of eggs obtained from one female often assure a good supply of eggs. Embryos can be raised to the hatching stage.
E. Pertinent references: Breder (1948).

VI. *Syngnathus fuscus* (pipefish)

A. Breeding season: Mid-May through June or possibly early July. Males with young in their brood-pouches have been found in July and early August.
B. Developmental rate: Not known.
C. Egg characteristics: Pelagic; carried by male in a ventral brood-pouch; 0.75–0.85 mm. in diameter; nearly opaque; contains numerous orange oil droplets.
D. Special comments: Large numbers of eggs and larvae are found in the brood-pouches of the males—as many as 200. Eggs apparently cannot successfully be removed from the brood-pouch until the yolk sac is completed, but after that time, they will develop in fingerbowls of sea water.
E. Pertinent references: Agassiz and Whitman (1885); Cohn (1904); Huot (1902); Cunningham (1895).

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