

## Embryonic, larval, and post-larval development in the symbiotic clam *Codakia orbicularis* (Bivalvia: Lucinidae)

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**Abstract.** *Codakia orbicularis* is a large Caribbean lucinid clam with chemoautotrophic bacteria in its gill cells. Its development from spawning up to 2.5 mm shell-length juveniles is described using light, scanning, and transmission electron microscopy. Embryonic development, from large eggs with an abundant vitelline supply, takes place within individual glycoprotein capsules up to the veliger stage. After hatching, 48 hours after fertilization, swimming veligers develop to swimming-crawling pediveligers, then to benthic, crawling plantigrades in 16 days without developing an umbonate secondary larval shell. This first phase of metamorphosis is completed without any special environmental stimulation. However, without addition of a suitable substrate, a delay of metamorphosis occurs at the end of the plantigrade stage whereas, in planktotrophic bivalves, a developmental hiatus has been described at the end of the pediveliger stage. When a sterile sand fraction is added, plantigrades enter a second phase of metamorphosis, differentiate gill filaments, a siphonal septum, and a byssal gland, and secrete a fast-growing juvenile shell, which becomes umbonate. Metamorphosis is completed with the differentiation of the unique excurrent siphon in 2 mm shell-length juveniles. Such a developmental pattern is different from the planktotrophic and from the lecithotrophic developments already described for bivalves. *C. orbicularis* is a facultative planktotroph with a two-step metamorphosis including long planktonic and benthic stages without significant growth, contrasting with the rapid growth of the post-larval shell. The whole larval and post-larval development occurs without the presence of chemoautotrophic symbionts. Therefore, symbiosis is not necessary to achieve metamorphosis.

*Additional key words:* facultative planktotrophy, metamorphosis

*Codakia orbicularis* (LINNÉ 1758) is a large lucinid clam, up to 90 mm in shell-length, ranging from Florida through the Caribbean region to Brazil (Abbott 1974). Adult individuals, which live in shallow-water sea-grass beds, harbor sulfur-oxidizing chemoautotrophic bacteria in their gill cells (Berg & Alatalo 1984; Frenkiel & Mouëza 1995) like every species of the eight genera of the family Lucinidae so far examined (Reid 1990). Descriptions of larval development in members of the eulamellibranch superfamily Lucinacea are scarce. Two developmental patterns are known to date, one in a member of the family Thyasiridae, *Thyasira gouldi* (Blacknell & Ansell 1974) and the other in a tropical member of the family Lucinidae, *C. orbicularis* (Alatalo et al. 1984). Both descriptions are supported by observations on living and fixed larvae

using light microscopy. The first one was done before the discovery of sulfur-oxidizing bacterial symbiosis in Thyasiridae (Dando & Southward 1986) or in other marine invertebrates (Cavanaugh et al. 1981; Felbeck et al. 1981; Cavanaugh 1983). In the second one, Alatalo et al. (1984) postulated that larval development may be sustained by chemoautotrophic nutrition, but gave no information about the presence of bacteria during developmental stages.

To date, four species of bivalves known to host chemoautotrophic sulfur-oxidizing bacteria in adult gill-cells have been raised successfully (Blacknell & Ansell 1974; Alatalo et al. 1984; Gustafson & Reid 1986, 1988a; Gustafson & Lutz 1992), and interest in the relationships between hosts and their bacterial endosymbionts during developmental stages is increasing. Gill endosymbionts are vertically transmitted from parents to offspring in the protobranchs *Solemya reidi* (Cary 1994) and *S. velum* (Krueger et al. 1996), and

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environmentally transmitted to the new host generation in *C. orbicularis* (Gros et al. 1996). Embryonic and larval development of the members of the protobranch family Solemyidae, *S. reidi* (Gustafson & Reid 1986, 1988a,b), and *S. velum* (Gustafson & Lutz 1992), have been described using electron microscopy whereas information is lacking on embryonic stages, metamorphosis, and juvenile features of *C. orbicularis*.

Planktic larval development has been described in several bivalve species including mussels (Bayne 1976), oysters (Andrews 1979), clams, and scallops (Sastry 1979; Cragg & Crisp 1991). The most common sequence is external embryonic development resulting in a free-swimming ciliated trochophore, which, upon secretion of the first larval shell, prodissoconch I, becomes a planktonic, D-shaped veliger larva. In planktotrophic species, the free-swimming D-larva grows rapidly and becomes an umbonate veliger, or veliconcha, as its mantle secretes prodissoconch II.

The pediveliger is defined as a transitional stage due to the simultaneous presence of functional velum and foot, allowing for alternating swimming and crawling. Regression of the velum commits the larva to benthic life; the crawling postlarva develops functional gill filaments and settles as a crawling, early spat, called a plantigrade (Bayne 1976). The most critical step in metamorphosis appears to be the shift of ciliary feeding and oxygenation functions from velum to gills. In the development of some planktotrophic veligers, a hiatus takes place and a delay of metamorphosis may be observed when pediveligers do not receive appropriate cues for settlement (Bayne 1965, 1976; Hadfield 1978). During such a delay of metamorphosis, metamorphic competence is retained during a period determined by the nutritional status of the pediveligers; growth ceases, and mortality increases, probably due to the lack of ciliary cleansing mechanisms no longer performed by the velum and not yet assumed by the gills.

As soon as the gill filaments increase in size and number, plantigrades become resistant to previously deleterious bacteria and acquire specific behavior; oysters cement onto a suitable substrate (Cranfield 1973, 1974), mussels stick to it with byssus threads (Bayne 1976), whereas burrowing species crawl onto the sediment and acquire siphons (Quayle 1952; Ansell 1962; D'Asaro 1967). The crawling plantigrade appears as a short transitory benthic stage common to species which will develop various specific behaviors and complete metamorphosis. The onset of permanent benthic life is also contemporary to the secretion of the dissoconch, which exhibits specific shape and ornamentation. However, the development of gill filaments

appears as a prerequisite for metamorphosis, whereas secretion of the dissoconch appears as a result of successful metamorphosis.

According to Ockelmann (1965), lecithotrophic development described in several species, is correlated with a short pelagic life and with the absence, or poor development, of prodissoconch II, which distinguishes the shells of lecithotrophic larvae from those of late, planktotrophic larvae. Alatalo et al. (1984) observed that *C. orbicularis* had some features of lecithotrophic species but a long planktonic stage, and postulated that it could have a mixed nutrition, involving lecithotrophy, facultative planktotrophy, and chemoautotrophy. These authors took for granted that metamorphosis could proceed to completion in *C. orbicularis*, as in other species with a planktonic larval stage, as soon as the plantigrade early spat had acquired benthic behavior.

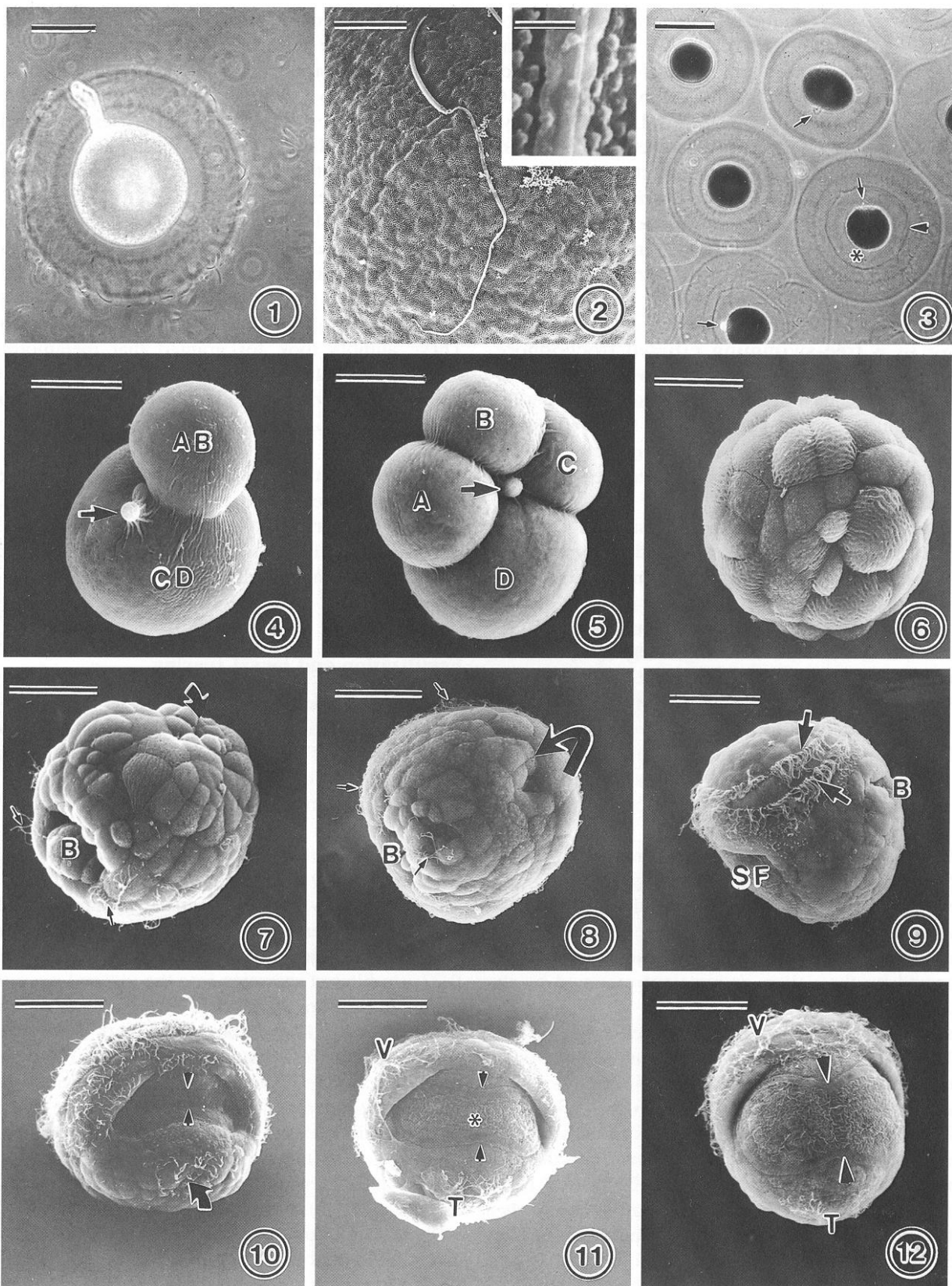
In the present paper, we describe, with scanning and transmission electron microscopy, the embryonic, larval, and post-larval development of *C. orbicularis*, obtained repeatedly up to the fully metamorphosed juveniles, 2.5 mm in shell-length.

## Methods

### Fertilization and larval rearing

Adult specimens of *Codakia orbicularis* were collected from shallow-water sea-grass beds off the island of Guadeloupe (French West Indies) during the period of gonad repletion (from May to September). All seawater used, from spawning to metamorphosis, was filtered through a 5- $\mu\text{m}$  Millipore cartridge filter, then UV treated (Protoco LM6-S86; 2  $\times$  80 Watts). Shells were cleaned by scrubbing, then rinsed in filtered seawater, and spawning was induced by injection of 0.3 ml of a 4 mM serotonin solution (5-hydroxytryptamine creatinine sulfate complex; Sigma Chemical Co.) in 0.22- $\mu\text{m}$  filtered seawater, into the visceral mass (Matsutani & Nomura 1982). The breeding clams were put in individual glass dishes filled with seawater and transferred to fresh seawater as soon as the emission of gametes started. Oocytes and sperms were mixed in a 1-liter cylinder, until the appearance of the first polar body. Fertilized eggs were washed on a nylon screen (100- $\mu\text{m}$  mesh) to eliminate excess sperms, then placed in 20-liter tanks at a density adjusted to 20,000 eggs liter<sup>-1</sup>. Cultures were maintained at 25–27° C without antibiotics and gently aerated with 0.22- $\mu\text{m}$  filtered air bubbling to avoid sedimentation of eggs during embryonic development.

After hatching, straight-hinge veliger larvae were collected on a nylon screen (100- $\mu\text{m}$  mesh) and placed in 20-liter tanks at a density adjusted to 8,000 larvae



liter<sup>-1</sup>. They were treated with 30 mg liter<sup>-1</sup> of chloramphenicol and 60 mg liter<sup>-1</sup> of streptomycin for one day. During larval development, no air bubbling and no antibiotics were used; seawater was exchanged and 0.5 ppm of sodium hypochlorite (NaClO) added every day. Larvae were fed only from the second week of development, after each water change. Unicellular algal cultures were provided at an initial density of 60 cells  $\mu\text{l}^{-1}$  (*Nannochloropsis* sp.) and the diet was progressively increased to 150 cells  $\mu\text{l}^{-1}$  in mixed rations (*Nannochloropsis* sp., *Pavlova lutheri*, and *Isochrysis tahiti*).

Metamorphosis was induced by addition of sterile sand with a grain size smaller than 200  $\mu\text{m}$ . Thereafter, seawater was changed every other day, without NaClO added. Juveniles, from a mean shell-length of 500  $\mu\text{m}$ , were placed in trays containing sterile sand, and seawater exchange was limited to 10% once a week. The juveniles were distributed into two batches, both cultured in sterile sand, one with addition of 1  $\mu\text{M}$  sulfide ( $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ ), which is the estimated concentration in sea-grass beds (Fisher 1990), and the other without anything added.

Eggs, embryos, larvae, and juveniles were observed alive on a Leica Orthoplan fitted with a Vario Orthomat photomicroscope and prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

## SEM preparation

Embryos were sampled at 3-h intervals throughout embryonic development, fixed in a solution of 2.5% glutaraldehyde in seawater, rinsed in cacodylate buffer (0.1 M cacodylate buffer adjusted to pH 7.2 and 1,000 mOsM with 0.4 M NaCl), then dehydrated in an acetone series. Larvae and juveniles were put in 25 ml of seawater in a glass beaker and anesthetized by adding one drop of  $\beta$ -phenoxyethanol (Sigma) according to Waller (1981). One hour later, larvae and juveniles, unable to retract the velum and foot, were killed by addition of 1 ml of 25% glutaraldehyde. They were collected and fixed, for 1 h at room temperature, in 2.5% glutaraldehyde in cacodylate buffer. After a rinse, specimens were fixed for 45 min at room temperature in 1% osmium tetroxide in the same buffer, rinsed in distilled water, and dehydrated in an acetone series. After critical point drying using  $\text{CO}_2$  as transitional fluid and sputter coating with gold, they were observed in a Hitachi S-2500 SEM at 20 kV accelerating voltage.

## TEM preparation

Specimens were prefixed for one hour at 4° C in 2.5% glutaraldehyde in cacodylate buffer, 2 mM  $\text{CaCl}_2$  being added for better membrane preservation. After a brief rinse, they were fixed at room temperature for 45 min in 1% osmium tetroxide in the same buffer,

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**Figs. 1–12.** Unfertilized oocytes to the saddle-stage trochophore. Figs. 1 & 3, phase contrast; Figs. 2 & 4–12, SEM.

**Fig. 1.** Live oocyte with several sperms around the jelly coat. The clear germinal vesicle and oocyte stalk indicate that it is not fertilized. Scale bar, 100  $\mu\text{m}$ .

**Fig. 2.** Sperm with a long curved head lying on the surface of an oocyte. Scale bar, 12  $\mu\text{m}$ . Inset: higher magnification showing the undulating membrane along the flagellum. Scale bar, 0.3  $\mu\text{m}$ .

**Fig. 3.** Fertilized eggs. Arrows indicate the first polar body; star indicates the vitelline space limited by a conspicuous fertilization membrane (arrow head). Scale bar, 150  $\mu\text{m}$ .

**Fig. 4.** 2-cell embryo, 130 min after fertilization ( $T_0+130$  min), characterized by two unequal blastomeres (AB and CD). The first polar body (arrow) is near the cleavage plane. Scale bars, 30  $\mu\text{m}$  in Figs. 4–12.

**Fig. 5.** 4-cell embryo characterized by 3 equal blastomeres (A, B, C) and one larger (D), with the polar body between them (arrow).

**Fig. 6.** Non-ciliated morula at  $T_0+6$  h.

**Fig. 7.** First ciliated gastrula at  $T_0+15$  h. Blastopore (B); shell-field invagination (curved arrow); motile cilia (arrows).

**Fig. 8.** Gastrula at  $T_0+21$  h. The shell-field begins to evaginate (curved arrow). Blastopore (B); motile cilia (arrows).

**Fig. 9.** Early trochophore stage at  $T_0+24$  h. The prototroch consists of two bands of motile cilia (arrows). Blastopore (B); shell-field (SF).

**Fig. 10.** Dorsal view of an early trochophore ( $T_0+24$  h) showing the first organic shell material, which is limited to a narrow band (between arrow heads) within the shell-field recess. The first cilia of the telotroch are beginning to develop (curved arrow).

**Fig. 11.** Beginning of the saddle stage in a trochophore ( $T_0+24-27$  h). The organic shell material (asterisk) appears as a wrinkled layer (between arrow heads) within the shell-field recess. Telotroch (T); velum (V).

**Fig. 12.** Dorsal view of an older trochophore stage. The secreted shell has a saddle shape; the hinge line is delineated by arrow heads. Telotroch (T); velum (V).

rinsed in distilled water, postfixed with 2% aqueous uranyl acetate for one more hour (Hayat 1970; Silva et al. 1971), and decalcified overnight in 1% aqueous ascorbic acid at room temperature (Dietrich & Fontaine 1975), then dehydrated through an ascending series of ethanol and propylene oxide. After embedding in an Epon-Araldite resin mixture according to Mollenhauer (in Glauert 1975), semi-thin and thin sections were cut on a Leica Ultracut E ultramicrotome. Semi-thin sections, 0.5  $\mu\text{m}$  thick, were stained with 0.5% toluidine blue in 1% borax buffer. Thin sections, 80 nm thick, were collected on collodion-coated 100-mesh grids, contrasted 30 min in 2% uranyl acetate in distilled water and 10 min in 0.1% lead citrate before examination in a Hitachi H-8000 TEM at 100 kV accelerating voltage.

## Results

### Fertilization and embryonic development

Spawned eggs are large spherical cells, 92 to 108  $\mu\text{m}$  in diameter, with a large supply of vitelline platelets. They are surrounded by a thick jelly coat composed of an inner layer (up to 40  $\mu\text{m}$  wide) overlying the vitelline membrane and an outer layer (up to 80  $\mu\text{m}$  wide), distinguishable by phase contrast, so that the total diameter of the oocyte and jelly coat is about 350  $\mu\text{m}$  (Fig. 1). This jelly coat, already present around full-grown oocytes inside the ovary, is made up of glycoproteins and proteoglycans synthesized by the oocyte itself during vitellogenesis (Frenkiel, unpubl.). After spawning, it is swollen by hydration of its proteoglycan components. The oocyte stalk, which crosses the two layers of the jelly coat (Fig. 1) disappears only after fertilization. Sperms, approximately 66  $\mu\text{m}$  long (Fig. 2), have a long curved head and a peculiar tail characterized by a continuous undulating membrane along the flagellum (Mouëza & Frenkiel 1995). They move slowly with a pendular movement.

During fertilization, many sperms are found attached to the jelly coat but there is no evidence of polyspermy. The first polar body, opposite to the site of sperm entrance, is detected within 30 min after contact between oocyte and sperm ( $T_0+30$  min). Fertilized eggs are characterized by the appearance of a perivitelline space 35  $\mu\text{m}$  wide (Fig. 3) limited by the fertilization envelope, composed of the vitelline coat associated with some fibrous material expelled from the egg after fertilization (Frenkiel, unpubl.). Due to expansion of the perivitelline space during embryonic development, the egg capsule (which persists until the veliger stage) enlarges, to a mean of 490  $\mu\text{m}$  before hatching.

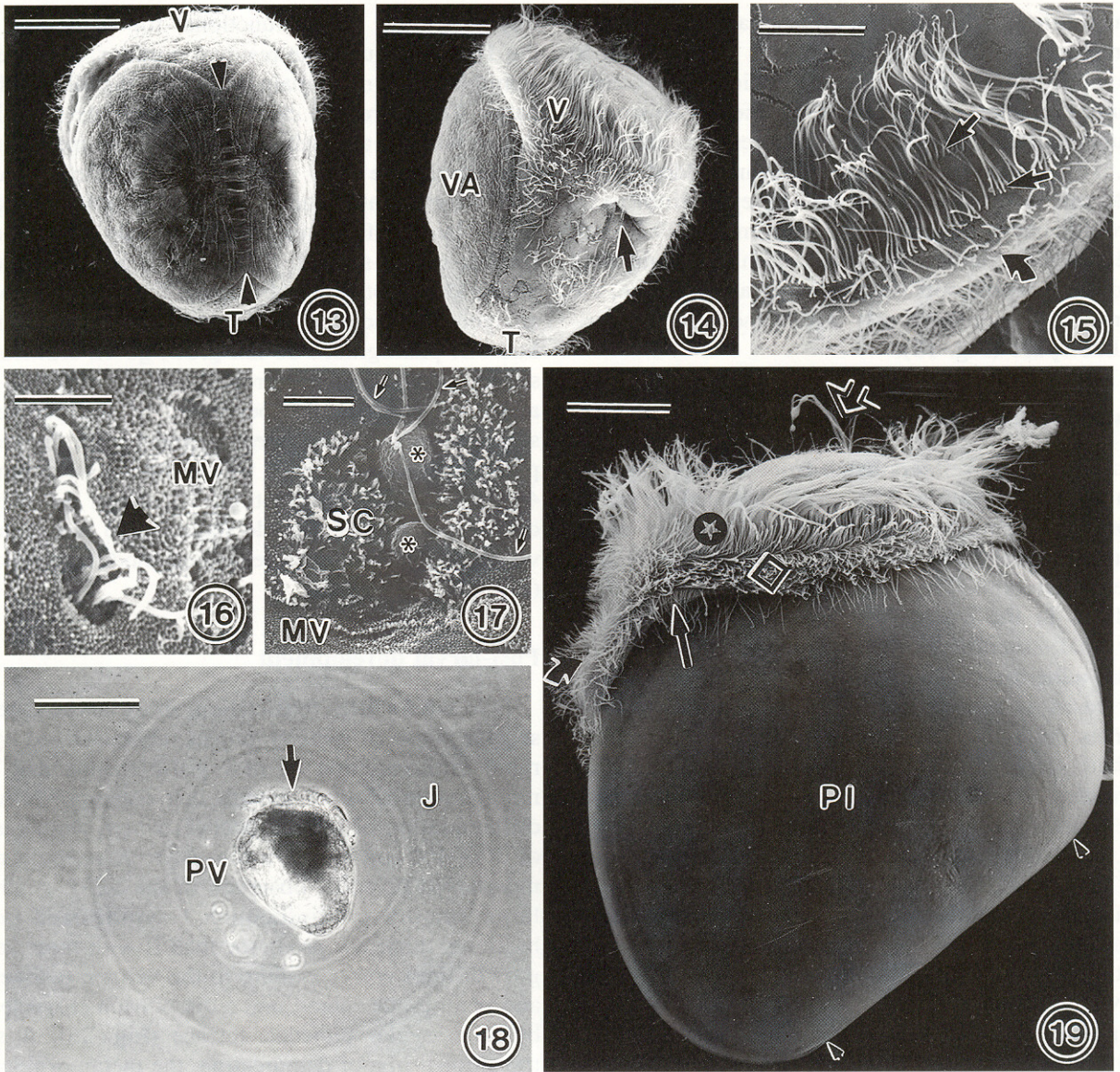
The first cleavage begins at  $T_0+130$  min and results

in two unequal blastomeres, AB and CD (Fig. 4), the polar body being in the cleavage plane; no polar lobe was observed. The second cleavage, at right angles to the first, produces 3 equal blastomeres (A, B, C) and a larger D blastomere (Fig. 5). Successive cleavages follow a spiral pattern and result in a non-ciliated morula at  $T_0+6$  h (Fig. 6), then in a non-ciliated blastula 3 h later. At  $T_0+12$  h, invagination of the blastopore at the vegetal pole indicates the early gastrula stage. Subsequent cell divisions lead to the development of a ciliated gastrula, which begins to rotate within the egg capsule at  $T_0+15$  h (Fig. 7). The blastopore is large, its circular margin marking the border between ectoderm and endoderm. At the same time, a shell-field invagination (as defined by Eyster & Morse 1984) appears dorsal to the blastopore (Fig. 7). During gastrulation, the blastopore is displaced anteriorly over the ventral side because of the growth of the dorsal region of the embryo.

At  $T_0+21$  h, the shell-field begins to evaginate (Fig. 8) while cilia distributed around the anterior region prefigure the future prototroch. Subsequent cell divisions lead to the early trochophore stage within 24 h (Fig. 9). At this stage, the prototroch is composed of two bands of motile cilia that divide the trochophore into two regions: a pretrochal area (anterior) and a post-trochal area (posterior); in the latter, the blastopore is on the ventral side and the shell-field is on the dorsal side. The first cilia of the telotroch appear in the posterodorsal region (Fig. 10). The shell-field evagination appears as a transverse pad (Fig. 11); it progressively grows and folds into right and left halves, which will expand over the body. In a dorsal view, the secreted shell material has the appearance of a saddle with a wrinkled surface hanging down on both sides of the larva; the bilateral symmetry becomes obvious and the hinge line well delineated (Fig. 12).

At  $T_0+27$  h, the saddle expanding over the body forms two valves, which compress the trochophore laterally (Fig. 13). A functional velum originating from the prototroch (Fig. 14) is composed of three discrete ciliary bands: (i) preoral cilia about 20  $\mu\text{m}$  long, distributed in two lines separated by a narrow space, 6–7  $\mu\text{m}$  wide; (ii) an adoral band composed of cilia about 5  $\mu\text{m}$  long, randomly distributed; and (iii) a postoral band that merges with the postoral tuft composed of short cilia (Figs. 14, 15). At the same time, the first cilia of the apical tuft appear in the central region of the velar disk (Fig. 16).

At  $T_0+39$  h, late trochophores develop to early veligers. The velar ciliary bands increase in density; the mouth is masked by cilia of the adoral and postoral bands; the telotroch overlaps the anal region. Between



**Figs. 13–19.** Late trochophore to hatching. Figs. 13–17 & 19, SEM. Fig. 18, phase contrast.

**Fig. 13.** Dorsal view of a trochophore stage at  $T_0+27$  h. The secreted shell expands over the body and forms two valves, which compress the trochophore laterally. The hinge line is clearly delineated (arrow heads). Telotroch (T); velum (V). Scale bar, 30  $\mu\text{m}$ .

**Fig. 14.** Trochophore stage at  $T_0+27$  h, ventral view. Mouth (arrow); telotroch (T); valve (VA); velum (V). Scale bar, 30  $\mu\text{m}$ .

**Fig. 15.** Higher magnification showing the outer preoral band consisting of a double row of long cilia (straight arrows) and the adoral band consisting of short cilia (curved arrow). Scale bar, 10  $\mu\text{m}$ .

**Fig. 16.** Early apical sense organ of a trochophore at  $T_0+27$  h. Microvilli (MV); ciliary tuft (arrow). Scale bar, 2  $\mu\text{m}$ .

**Fig. 17.** One of the two putative sense organs differentiated symmetrically on each side of the mouth-anus axis on a trochophore at  $T_0+39$  h, posterodorsal view. Microvilli (MV); short cilia (SC); smooth protuberance (stars); telotroch cilia (arrows). Scale bar, 2  $\mu\text{m}$ .

**Fig. 18.** Veliger enclosed in the jelly coat just before hatching. Jelly coat (J); perivitelline space (PV); velum (arrow). Scale bar, 120  $\mu\text{m}$ .

**Fig. 19.** D-larva just after hatching ( $T_0+48$  h). The straight hinge line is indicated by an arrow head. The velar crown is composed of a post-oral band (arrow); an adoral band (square); and an outer preoral band (star). Mouth (curved arrow); apical tuft (open arrow); prodorsoconch I (PI). Scale bar, 30  $\mu\text{m}$ .

the anal tuft and the postoral ciliary band, two organs, symmetrical with respect to the axis from mouth to anus, appear in the posteroventral region; they consist of numerous short cilia surrounding one or two smooth protuberances, the function of which remains unknown (Fig. 17). Some long sensory cilia are added to the apical tuft whereas the surrounding epithelium bears microvilli but is devoid of cilia. From  $T_0+39$  h until hatching, the calcified shell valves enlarge until they enclose the whole soft body. During all this time, the embryos rotate continuously within the enlarged perivitelline space (Fig. 18).

### Larval development

Straight-hinge veligers hatch from the egg capsules 48 h after fertilization. The newly hatched larvae of *C. orbicularis* range from 160 to 170  $\mu\text{m}$  in shell-length (Fig. 19). The first shell, prodissoconch I, is large and smooth. The velum retains the same ciliary bands as in the trochophore stage, but they acquire their final structure. In the preoral ciliary bands, 5 to 9 cilia adhering together from a short distance above their bases up to their tips compose cirri 25  $\mu\text{m}$  long, whereas the adoral and postoral bands are composed of short single cilia about 10  $\mu\text{m}$  long and randomly distributed (Fig. 20). The central region of the velar disk bears a principal tuft composed of long single cilia surrounded by some secondary tufts constituted by a few, short, thin cilia (Fig. 21).

During the first week of larval development, veliger larvae are nourished by abundant vitelline platelets, mostly located in the velum, dorsal region, and stomach wall, and need no additional food. At the end of that period, the stomach has developed two dorsal digestive diverticula and a ventral style sac. From then on, larvae were fed to compensate for the decrease in number of vitelline platelets; this feeding maintained the dark color of the digestive diverticula typical of well-nourished larvae. The pediveliger stage is initiated by the appearance of the pedal anlage posteroventral to the velum. About 12 days after fertilization, the foot (Fig. 22) has a ciliary tuft near the heel and an unciliated groove that runs along its ventral face and onto its tip, which is partly covered by a cap of cilia (Fig. 23), but is still not functional.

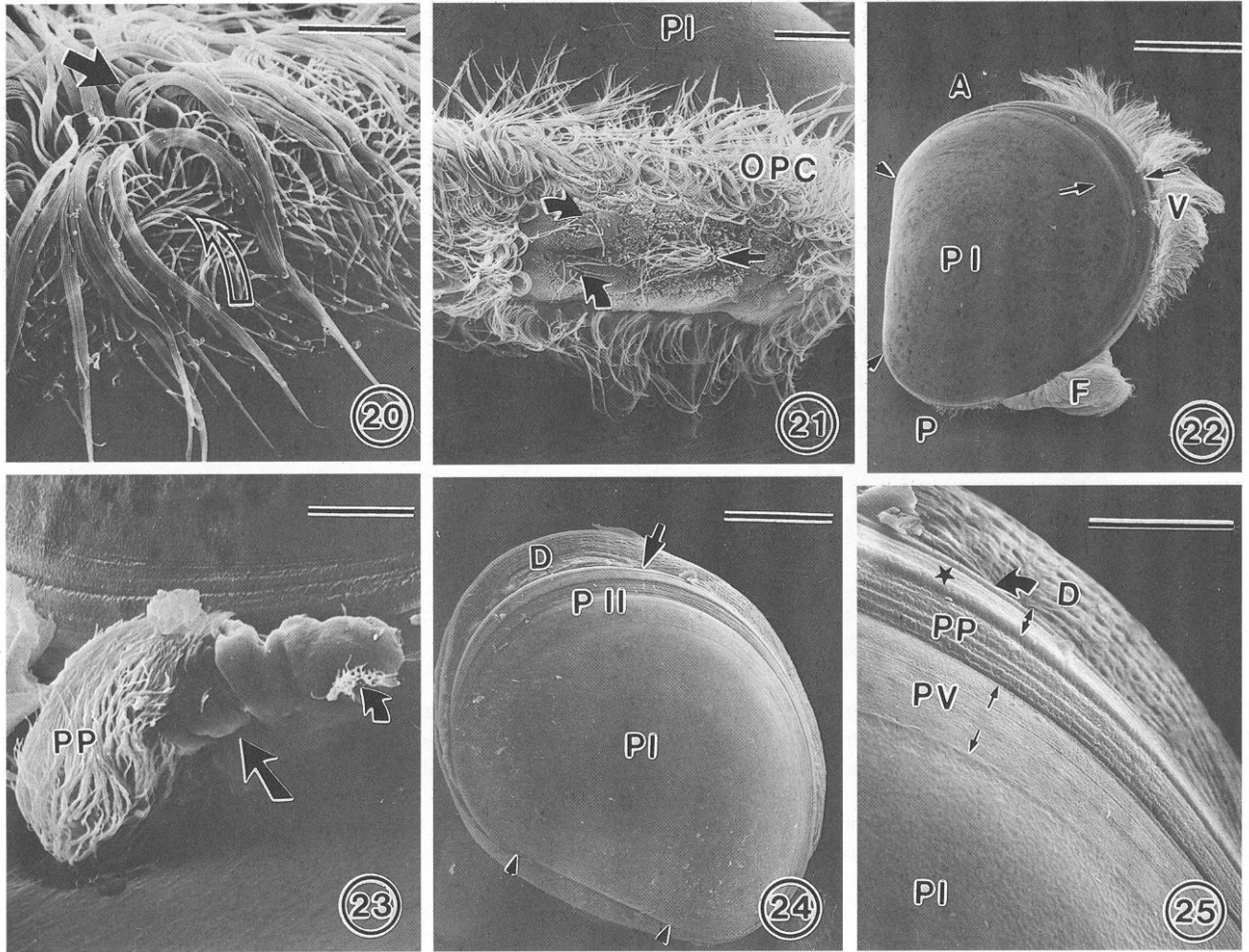
From the 15th day, a pair of statocysts can be detected at the base of the well-differentiated foot, which is protruded from the shell and explores the outside area. Ctenidial anlagen, originating from a ridge of the inner face of the mantle, appear on each side of the posterior region of the body. Thereafter, pediveligers swim with extended velum, and crawl on the bottom with extended foot. The velum decreases in size rap-

idly and is no longer functional for swimming after the 16th day, but some residual portions, identified on semithin sections, remain as part of the ciliated labial palps. Therefore, the crawling plantigrade stage is attained without any substrate added. The mean size of this plantigrade early spat is no more than 190  $\mu\text{m}$  in shell-length; thus, there is little growth during the whole larval life up to the plantigrade stage, and the shell is still D-shaped. If no sand is added, metamorphosis does not proceed further; the symmetrical gill anlagen become elongated but no other gill filaments bud off; plantigrades do not grow, go on crawling for several weeks, and die progressively. Even with sand added as early as the beginning of the pediveliger stage, there is a long latent period before the second phase of metamorphosis, whose earliest occurrence is 4–5 weeks after fertilization. Therefore, the minimal duration of the plantigrade stage is 2–3 weeks during which no anatomical or behavioral modifications occur (Fig. 30).

### Post-larval development

Five weeks after fertilization, most larvae enter the second phase of metamorphosis, characterized by the rapid differentiation of new gill-filaments and by a modification of shell growth that corresponds to the differentiation of the dissoconch, which attains a size of 300–400  $\mu\text{m}$  within a few days. A recently metamorphosed juvenile, observed in the SEM, exhibits a large smooth prodissoconch I synthesized before hatching, a narrow prodissoconch II with three sets of growth lines corresponding to the veliger, pediveliger, and plantigrade stages, and a dissoconch characterized by an irregular punctate surface (Fig. 25). A sharp transitional line of demarcation, the metamorphic line, is obvious at the prodissoconch II-dissoconch boundary (Figs. 24, 25). Juveniles up to 300  $\mu\text{m}$  shell-length still possess a straight hinge line with dorsal margins slightly roundish. The typical umbonate shape appears thereafter, but the adult ornamentation of the shell will not appear before several weeks. In the posteroventral region, gill filaments lengthen progressively from the symmetrical gill anlagen and a siphonal septum separates the inward and outward pallial currents in individuals of 400  $\mu\text{m}$  shell-length (Fig. 26). However, no siphon may be observed at this stage either in living individuals or in semi-thin sections. In the posterior part of the foot, the byssal gland, made up of large clear cells (Fig. 26), opens through a short canal onto the ventral groove which runs from the heel to the tip of the foot.

Further development is characterized by the increasing number and elongation of gill filaments, and by



**Figs. 20–25.** Larvae and early juveniles. SEM.

**Fig. 20.** Velum of a D-larva. Outer preoral cirri (black arrow); cilia of the adoral and post-oral bands (open curved arrow). Scale bar, 5  $\mu\text{m}$ .

**Fig. 21.** Velum, ventral view. Prodissoconch I (PI); outer preoral cilia (OPC); straight arrow indicates the principal tuft and curved arrows indicate the secondary tufts of the apical sense organ. Scale bar, 10  $\mu\text{m}$ .

**Fig. 22.** Late pediveliger. The hinge line is indicated by arrow heads. Prodissoconch II (between arrows) is composed of two sets of growth lines. Anterior (A); foot (F); posterior (P); prodissoconch I (PI); velum (V). Scale bar, 70  $\mu\text{m}$ .

**Fig. 23.** Foot of a pediveliger characterized by a ciliated tuft (curved arrow) near the heel and by an unciliated byssal groove (arrow) along its ventral face opening onto the tip of the foot, which is partially covered by a cap of cilia, the propodium (PP). Scale bar, 5  $\mu\text{m}$ .

**Fig. 24.** Early metamorphosed juvenile (5 weeks old). The umbone becomes roundish while the hinge line (delineated by arrow-heads) is still observable. Prodissoconch I (PI); prodissoconch II (PII); dissoconch (D); metamorphic line (arrow). Scale bar, 70  $\mu\text{m}$ .

**Fig. 25.** Higher magnification of the ventral margin of a shell valve in an early metamorphosed juvenile. Prodissoconch I (PI) is smooth with regular shallow pits; prodissoconch II has three sets of growth lines (between arrows) corresponding to veliger (PV), pediveliger (PP), and plantigrade (star). The metamorphic line (curved arrow) delineates the dissoconch (D) characterized by a punctate structure. Scale bar, 20  $\mu\text{m}$ .

the differentiation of the excurrent siphon, which begins to develop in 1.5 mm shell-length juveniles (Fig. 27) and is fully differentiated in 2 mm shell-length juveniles (Fig. 28). Six months after metamorphosis, juveniles measure 2 to 2.5 mm in shell-length, in the laboratory conditions. They possess well-developed

gills with about 50 filaments and a single retractable siphon; therefore, they are able to burrow. From 2 mm shell-length, radial lines crossing growth lines give to the dissoconch the cancellate aspect typical of the adult shell.

Metamorphosed juveniles are very active and are so





**Figs. 26–28.** Sections of juvenile clams. Light micrographs of semi-thin sections of the posterior region.

**Fig. 26.** 400  $\mu\text{m}$  juvenile. Posteroventral gill anlagen (curved arrows); digestive diverticula (D); gill (G) with 4 elongated filaments and the rudimentary first one (arrow heads); intestine (I); byssal gland (star) inside the foot (F); siphonal septum (straight arrow). Scale bar, 50  $\mu\text{m}$ .

**Fig. 27.** 1.7 mm juvenile. The excurrent siphon (S) begins to differentiate in the posteroventral region by elongation of the mantle edge (arrow heads). Digestive diverticula (D); heel of the foot (F); gill filaments (G); kidney (K); ligament (L). Scale bar, 125  $\mu\text{m}$ .

**Fig. 28.** 2.3 mm juvenile. The siphon (S) results from the elongation of the fused inner mantle folds (arrow) whereas the middle (M) and outer (O) folds are separated. Gill filaments (G); intestine (I); isthmus (IS); kidney (K). Scale bar, 150  $\mu\text{m}$ .

resistant to bacterial diseases that they need only one or two washes per week; however in the same batch, growth is variable and it is usual to observe juveniles of various sizes together with plantigrades that had not completed metamorphosis.

### Gill ultrastructure

Except for the most anterior filament, all the gill filaments in juveniles from 400  $\mu\text{m}$  to 2 mm shell-length are composed of a frontal ciliated zone and a lateral zone devoid of cilia (Fig. 29). Each gill filament consists of a simple epithelium which, at the level of the ciliated and intermediary zones, is in contact with a stiff connective tissue axis; in the lateral zone the connective tissue is occupied by blood lacunae separated from the epithelial cells by a basal lamina. The ciliated zone is composed of four main cell types: (i) frontal cells bearing short cilia without precise orientation; (ii) narrow prolaterofrontal cells bearing two rows of long cilia; (iii) large eulaterofrontal cells bearing a large curved cirrus; (iv) eulateral cells constitut-

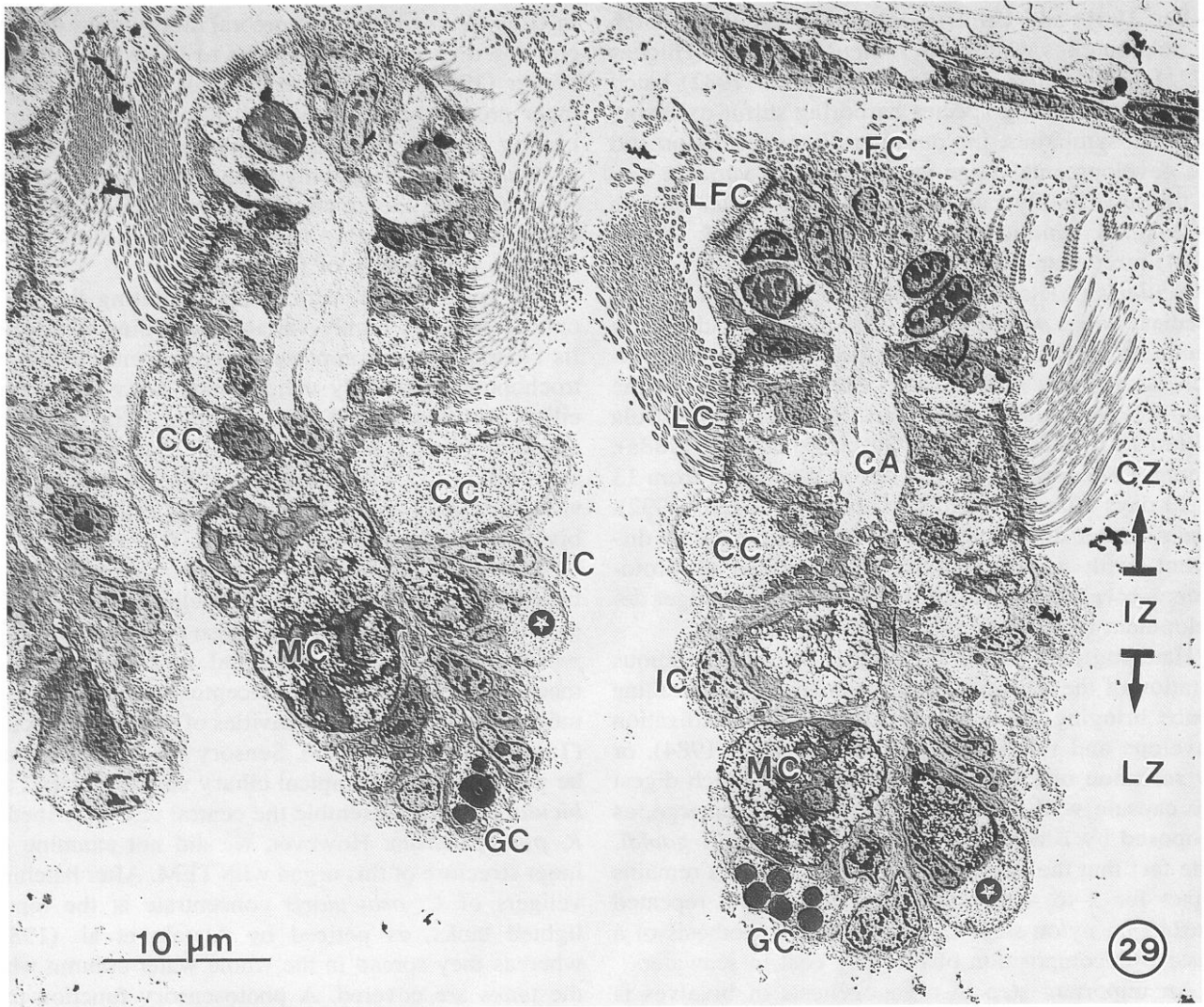
ing a functional cluster composed of three ciliated cell types. The lateral zone is composed of four cell types: (i) mucocytes typified by clear granules and numerous Golgi stacks; (ii) granule cells containing large membrane-bound osmiophilic inclusions; (iii) intercalary cells characterized by an elongated nucleus in an apical position, a narrow base, and a trumpet-shaped apical area covered by microvilli; (iv) undifferentiated cells with a basal nucleus and a cytoplasmic volume containing scarce organelles, mostly mitochondria.

No differentiated bacteriocytes with intracellular bacteria were detected in any gill filaments examined in juveniles cultivated in sterile sand either with or without additional sulfide. Moreover, no putative cryptic form of gill-endosymbiont was observed either in the gill filaments or in any larval tissues, which contain only large vitelline platelets.

### Discussion

#### Fertilization and embryonic development

Spawning induction was the first challenge to overcome in order to obtain the development of *Codakia*



**Fig. 29.** Gill filaments in a 1.5 mm shell-length juvenile clam. TEM. Frontal cells (FC), laterofrontal cells (LFC), and lateral cells (LC) are the main components of the ciliated zone (CZ), which has a collagen axis (CA). A clear cell (CC) represents the intermediary zone (IZ). Granule cells (GC), intercalary cells (IC), mucus cells (MC), and putative bacteriocytes without bacteria (star) are the main components of the lateral zone (LZ).

*orbicularis*. Alatalo et al. (1984) tried several techniques to trigger spawning; some successful spawning was obtained using  $H_2O_2$  as described by Morse et al. (1979); these eggs were not fertilized but the authors succeeded in fertilizing a batch of stripped eggs. Serotonin stimulation, first used by Matsutani & Nomura (1982) for the scallop *Patinopecten yessoensis*, induced reliable mass spawning from *C. orbicularis* during the whole breeding period (Frenkiel & Mouëza 1988). The sperm balls described by Alatalo et al. (1984), which were not observed with serotonin stimulation, appear to be immature sperms attached to their nurse cells.

SEM observations demonstrate that the polar bodies appear opposite to the site of sperm entry; however, it

is uncertain whether the fertilizing sperm can enter anywhere on the surface of the oocyte through the jelly coat or only through the oocyte stalk, which remains accessible after spawning and disappears after fertilization. In lucinid species, the jelly coat produced in addition to the vitelline coat by the oocyte contains glycoproteins as well as proteoglycans as described in *Lucina pectinata* (GMELIN 1791) (Frenkiel et al. 1997). It is swollen after spawning by hydration of its proteoglycan components but glycoproteins are likely to support recognition receptors for sperms.

A large jelly coat surrounding the egg has been reported in several species of bivalves; it may disappear at an early stage during cleavage (Creek 1960) or at various stages of development; *Pandora inaequalvis*

(Allen 1961) and *Venus striatula* (Ansell 1961) hatch as trochophores whereas *Scrobicularia plana* (Hughes 1971) and *Arctica islandica* (Lutz et al. 1982) hatch as veligers. Among species harboring sulfur-oxidizing gill endosymbionts, the duration of encapsulation and the developmental stage at hatching are variable too. In the superfamily Lucinacea, *Thyasira gouldi* hatches as a small benthic pediveliger (Blacknell & Ansell 1974) more than 50 days after fertilization whereas *C. orbicularis* (Alatalo et al. 1984) and the other lucinid species, *Linga pensylvanica* (LINNÉ 1758) and *Lucina pectinata* (Mouëza, unpubl.), hatch 48 h after fertilization, as veligers with a large vitelline supply. In the family Solemyidae, *Solemya reidi* hatches as a gastrula within 18 h of fertilization (Gustafson & Reid 1988a), whereas *Solemya velum* hatches as a juvenile from 13 to 23 days after fertilization (Gustafson & Lutz 1992). Their further developmental pattern is completely different, with a pericalymma larva typical of proto-branch bivalves instead of trochophore and veliger development typical of Eulamellibranchia.

Hatching may be effected either by the continuous rotation of the veliger within the enlarged perivitelline space bringing about the erosion of the fertilization envelope and soft jelly coat (Alatalo et al. 1984), or by secretion of enzymes from the larva, which digest the capsule wall and allow the veliger to emerge, as proposed by Blacknell & Ansell (1974) in *T. gouldi*. The fact that the capsule of undeveloped eggs remains intact for 3 to 4 days in seawater, despite repeated washes on nylon screen, rules out the hypothesis of a passive decomposition of the jelly coat in seawater.

An important step in organogenesis of bivalves is shell secretion. A shell gland has been described before gastrulation in *Ostrea edulis* (Horst 1883–1884 in Waller 1981). In other bivalve species, invagination of the shell-field may occur at various development stages. It has been described in early trochophores of *Spisula solidissima* by Eyster & Morse (1984). In *C. orbicularis*, invagination of the shell field begins during gastrulation, and the first secreted shell material appears in the early trochophore. The irregular wrinkles observed on the surface of the early shell (from 24 to 39 h after fertilization) may be either artifacts of preparation for SEM or the punctate-stellate pattern described in straight-hinge veliger larvae of *O. edulis* (Carriker & Palmer 1979; Waller 1981).

### Larval development

The velum is not such a uniform structure as it appears from observations of living bivalve veligers. Through SEM, Waller (1981) showed that the velar crown of *O. edulis* is composed of four ciliary bands.

In *C. orbicularis*, the two preoral ciliary rows are both composed of long cirri similar to those described by Waller (1981) in the outer preoral band whereas the inner preoral band, composed of short single cilia, is lacking in *C. orbicularis*. Therefore, the velum of *C. orbicularis* comprises only three ciliary bands, the outer preoral, the adoral, and the postoral bands; however, descriptions of bivalve veligers are too scarce to discuss the significance of this difference.

The apical organ, which appears during the trochophore stage, is highly variable according to species. Its central cirrus, composed of coalescent cilia in the trochophore and early veliger, may be replaced by a ciliary tuft composed of individual cilia in *Pecten maximus* (Cragg & Crisp 1991). It is represented by short cilia in *O. edulis* (Waller 1981) whereas it consists of a stiff apical tuft in several eulamellibranch bivalves (Tardy & Dongard 1993). It is considered to be a sense organ because of its non-motile cilia and because of the behavior of the veliger larvae. Ultrastructural study of the apical sense organ in *Ruditapes philippinarum* reveals a central unit interpreted as mechanoreceptor or chemoreceptor, and a peripheral unit composed of ciliated cavities of unknown function (Tardy & Dongard 1993). Sensory functions may also be postulated for the apical ciliary structures of *C. orbicularis*, which resemble the central unit described in *R. philippinarum*. However, we did not examine the inner structure of this organ with TEM. After hatching, veligers of *C. orbicularis* concentrate at the top of lighted tanks, as noticed by Alatalo et al. (1984), whereas they spread in the whole water column when the tanks are covered. A photosensory function may be postulated for the apical complex of the free-swimming veliger but remains to be demonstrated.

During the first week of larval development, larvae are nourished by vitelline platelets mostly located in the dorsal edge, velum, and stomach wall. Alatalo et al. (1984) supposed that plantigrades may be obtained without any algal food provided, owing to the large supply of vitelline platelets observed in veligers; however, the veligers are able to ingest algae shortly after hatching, as demonstrated by the stomach color. In planktotrophic larvae, a short lecithotrophic larval development is followed by an extended mixotrophic phase during the veliger and pediveliger stages (Lucas et al. 1986). The lecithotrophic larvae of *C. orbicularis* behave as facultative planktotrophs, ingesting food if it is available but developing similarly without any sign of starvation at least until the pediveliger stage. Such a nutritional schedule may improve the physiological condition, as demonstrated by Kempf & Hadfield (1985) for the lecithotrophic nudibranch mollusc *Phestilla sibogae*, in which fed larvae retained meta-

morphic competence longer and survived a delay of metamorphosis better than unfed ones.

Larvae of *C. orbicularis* have a prodissoconch II limited to a narrow fringe around the margin of the prodissoconch I, considered by Ockelmann (1965) as characteristic of the shells of lecithotrophic larvae with a short planktonic larval stage such as *P. inaequalvis* (Allen 1961) (Fig. 30). The developmental pattern of *C. orbicularis* is characterized by the large size of prodissoconch I at hatching followed by poor growth and by a planktonic life in the same range of duration as for planktotrophic veliger larvae such as *Chione cancellata* (D'Asaro 1967) or *Scrobicularia plana* (Frenkiel & Mouëza 1979) (Fig. 30). Therefore, the small size of prodissoconch II, which results in a smaller size at metamorphosis than for the planktotrophic larvae, is not due to a short planktotrophic phase, as in *P. inaequalvis* (Allen 1961) but rather to a low growth rate and a different life-history (Fig. 30). However, the larval shell of *C. orbicularis*, which is not umbonate before the postlarval growth of the dissoconch, appears more similar to the pandoracean larval shell described by Chanley & Castagna (1966) than to a typical planktotrophic veliger shell.

### Post-larval development

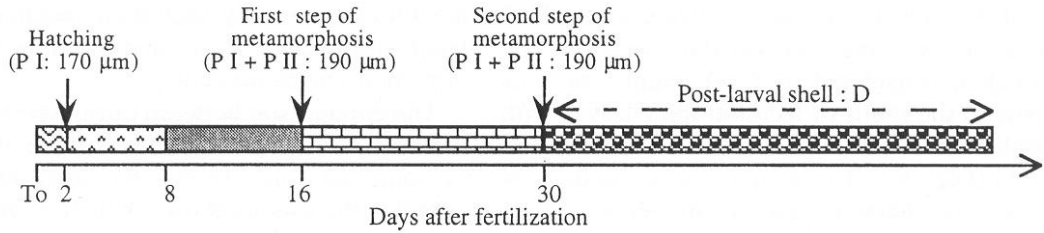
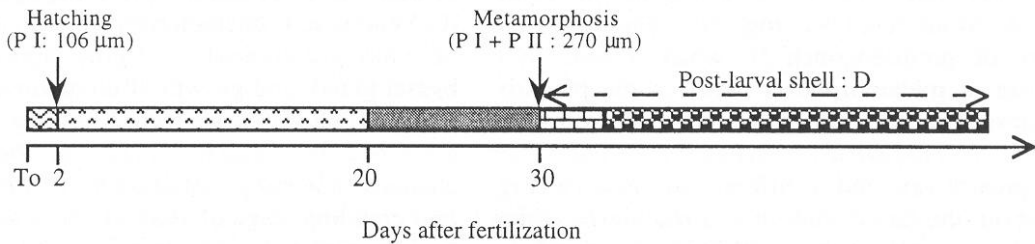
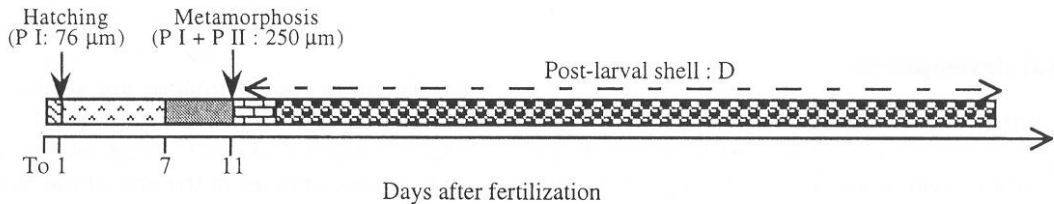
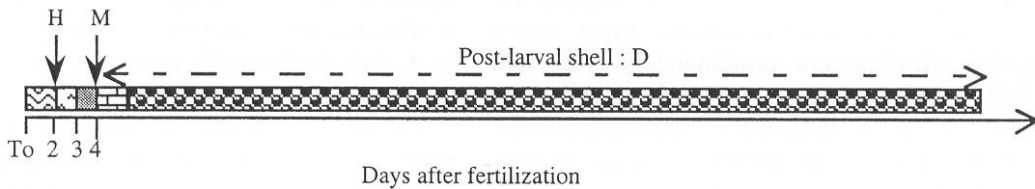
According to Alatalo et al. (1984), larvae complete metamorphosis, 16 days after fertilization, without added substrate. Our conclusions diverge from those of Alatalo et al. (1984) in several points, from that stage, which they illustrated very precisely (fig. 2F). This figure corresponds, in our observations, to an ~200- $\mu$ m plantigrade with only the first ctenidial anlagen composed of two roundish ciliated crypts, which do not become gill filaments spontaneously. If no substrate is added to the culture, these plantigrades die progressively without completing metamorphosis. Conversely, if a sterile sand fraction is added as substrate, the plantigrades enter a new developmental phase, characterized by elongation and multiplication of gill filaments, rapid growth of the newly set dissoconch, and complete metamorphosis.

Alatalo et al. (1984) noted that although no byssal gland is present, juvenile clams do not burrow. This is true up to the plantigrade stage but we observed a byssal gland on sections of juveniles ranging from 300  $\mu$ m to 2.5 mm in shell-length. Moreover, their finding of a rudimentary siphon developed by fusion of the mantle edges from the pediveliger stage (12 days after fertilization) was not confirmed in our study. In several batches of healthy larvae reared in nutritional conditions supporting complete metamorphosis, we did not observe either the siphon or the siphonal septum in

plantigrades, before the differentiation of the dissoconch. The rudiment of the unique lucinid siphon was not observed in any sectioned specimens with shell-length less than 1.5 mm and becomes functional in 2 mm shell-length juveniles.

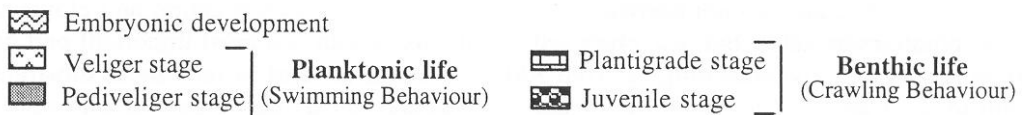
The comparison between our observations and those of Alatalo et al. (1984) demonstrates that the last developmental stage shown by these authors had not completed metamorphosis, which is characterized by the presence of several elongated gill filaments, a large byssus gland, and a functional excurrent siphon (Frenkiel & Mouëza 1979). The pattern of metamorphosis as described for various burrowing species of the family Veneridae is characterized by the simultaneous loss of velum, differentiation of gills, siphonal septum, and byssal gland, and growth of dissoconch (Quayle 1952; Ansell 1962; D'Asaro 1967). All these modifications described by Quayle (1952) as the most evident changes indicating metamorphosis are effective in the first crawling stage of venerid clams and metamorphosis is completed soon after the plantigrade stage. The excurrent siphon is elongated very early from the siphonal septum in *Chione cancellata* (D'Asaro 1967) as well as in *Anomalocardia brasiliensis* (Mouëza, unpubl.) and the incurrent one appears a little later without developmental hiatus. Larval growth and metamorphosis in the Tellinacea are similar to that of Veneridae, with a large umbonate prodissoconch II, but a developmental hiatus with an increased mortality occurs in these species at the end of the pediveliger stage. A delay of metamorphosis may occur in *S. plana*, at the pediveliger stage, but no further hiatus of metamorphosis occurs after the plantigrade stage. The elongation of siphons occurs later (Caddy 1969; Frenkiel & Mouëza 1984) and these siphons become functional in 1 mm shell-length juveniles well after the differentiation of gills and byssal gland.

The developmental pattern of *C. orbicularis* differs from those already described. Larval development and the first phase of metamorphosis take place with negligible growth up to the plantigrade stage, with or without sand added. There is no delay of metamorphosis at the end of the pediveliger stage but a developmental hiatus takes place at the end of the plantigrade stage. Multiplication and elongation of gill filaments, which is a most important part of metamorphosis, requires sand as settlement substrate. In our study, metamorphosis was triggered with sterile sand because the plantigrades were not resistant enough to bacterial infection to withstand the addition of crude sand. However, even if sand was added as early as the pediveliger stage, the rapid differentiation of numerous gill filaments and secretion of the dissoconch did not begin before the larvae were 4–5 weeks old. The de-

*Codakia orbicularis* (this study)*Scrobicularia plana* (Frenkiel & Mouëza 1979)*Chione cancellata* (D'Asaro 1967)*Pandora inaequalvis* (Allen 1961)

H : Hatching (P I : 150  $\mu\text{m}$ ) ; M : Metamorphosis (P I + P II : 175  $\mu\text{m}$ )

**Legends :** P I : prodissoconch I ; P II : prodissoconch II ; D : dissoconch.



**Fig. 30.** Comparison between developmental stages in *Codakia orbicularis* and in typical planktotrophic and lecithotrophic species. Planktotrophic species may have a longer (*Scrobicularia plana*) or a shorter (*Chione cancellata*) planktonic life. Classical lecithotrophic species (*Pandora inaequalvis*) have a much shorter planktonic life.

velopment of gill filaments appears as a necessary condition for juveniles to increase their resistance to bacterial diseases. The siphonal septum, constituted by the fusion of the inner folds of the mantle edge, develops later and the elongation of the single excurrent siphon is delayed until juveniles reach 2 mm. This siphon, typical of the Lucinacea (Allen 1958), is considered neotenic by Ansell (1962) as it retains the structure and retraction mode by inversion of the primary siphon of other burrowing bivalve species.

The developmental pattern of *C. orbicularis* is characterized by a small prodissoconch II similar to the pandoracean type and by a delay of dissoconch secretion to the second phase of metamorphosis. All the metamorphic events which occur simultaneously in the family Veneridae may be dissociated in other bivalves. To define which event indicates the beginning of post-larval life may help to clarify the developmental stages. If it is the resorption of the velum, the plantigrade is the first post-larval stage in Lucinidae as well as in other Eulamellibranchia. If it is the secretion of the dissoconch, the plantigrade is a larval stage in Lucinidae whereas it is postlarval in other Lamellibranchia. In fact, the most important event is a metamorphic transition, which is prerequisite for post-larval growth and development. A developmental hiatus occurs during this metamorphic transition, which takes place at the end of the pediveliger stage, in most lamellibranch species. In *C. orbicularis*, the crawling plantigrade, which has not overcome the developmental hiatus, cannot develop essential features necessary to the life of a burrowing species. Therefore, it is not a postlarval stage but it is no longer a larval stage and metamorphosis is dissociated into two phases, the first, usual one, at the end of the pediveliger stage, and the second, unusual one, at the end of the plantigrade stage.

### Ultrastructure of gill filaments in juveniles

The ciliated and intermediary zones of the gill filaments observed in juveniles cultured in sterile sand with or without additional sulfur appear identical to those described in wild juveniles (Frenkiel & Mouëza 1995). Conversely, the lateral zone remains short, without differentiated bacteriocytes; nevertheless, it is occupied by cell types similar to those in gill filaments of wild juveniles and adults, i.e., granule cells, intercalary cells, and mucus cells (Frenkiel & Mouëza 1995), but also by atypical undifferentiated cells which are likely to be putative bacteriocytes. No bacteria were observed in gill filaments examined from 300  $\mu\text{m}$  to 2.5 mm juveniles. Thus, morphogenesis of the gill filaments of *C. orbicularis* does not depend on the

presence of the sulfur-oxidizing bacterial gill-endosymbionts, and the hypothesis that larvae of *C. orbicularis* may obtain nutrition through intracellular chemoautotrophic bacteria, put forward by Alatalo et al. (1984), is questionable. The term "chemoautotrophic development" proposed by these authors does not apply to *C. orbicularis*, although it may apply to bivalve species in which sulfur-oxidizing symbiotic bacteria are vertically transmitted, such as *S. reidi* (Gustafson & Reid 1988b; Cary 1994), *S. velum* (Krueger et al. 1996), *Calyptogena magnifica*, *C. pacifica*, and *C. phaseoliformis* (Cary & Giovannoni 1993).

We obtained several batches of juveniles up to 2.5 mm in shell-length with numerous gill filaments and a well-differentiated excurrent siphon but without endosymbionts. These results demonstrate that (i) larval development takes place without the presence of sulfur-oxidizing bacterial endosymbionts; (ii) the association between *C. orbicularis* and its endosymbionts is not necessary for metamorphosis and post-larval development. However, no adult or juvenile specimens of *C. orbicularis* collected in the wild (Berg & Alatalo 1984; Frenkiel & Mouëza 1995) have been found without chemoautotrophic bacteria within their gill bacteriocytes, which implies that the association between this bivalve species and its chemoautotrophic bacteria occurs at some postmetamorphic developmental stage.

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