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The Spawning and Early Development of the Hawaiian Acorn Worm (Hemichordate), *Ptychodera flava*

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ABSTRACT—The spawning and early embryogenesis of the hemichordate, *Ptychodera flava*, in Hawaii are described in detail and illustrated with photographs of living material. Natural spawning in the evenings of early December was induced by a shift of seawater temperature from about 22°C to about 26°C. The fertilized egg divides equally and slowly at first, reaching 8 cells at about 5 hr after insemination at room temperature (20-24°C). Divisions then appear to become slightly unequal and by 9 hr the embryo has divided into about 100 cells. The blastocoel forms during cleavage as an irregular space that, when viewed from the side, tends to appear oblate and ultimately appear crescent-shaped as the vegetal plate thickens into the blastocoel. The archenteron forms at about 18 hr as a cleft beginning at the vegetal pole and extending into the vegetal plate. As development proceeds, the embryo expands and by 24 hr forms a typical deuterostome gastrula with an outer sphere of ectoderm and a inner tube of endoderm connected at the blastopore. An outpocketing of the gut appears at the tip of the archenteron over the next 4 hr to form the protocoel which will become the proboscis coelom. Approaching 40 hr the gut becomes asymmetric and over the next few hr contacts the ectoderm to form a mouth. Hatching occurs during this time at about 45 hr of development. Morphogenesis continues to produce an early tornaria larva by about 60 hr.

INTRODUCTION

We are interested in molecular developmental mechanisms that permitted and/or accelerated the evolution of chordates (urochordates, cephalochordates and vertebrates) (see reviews by Satoh and Jeffery, 1995; Satoh, 1995). Many extant invertebrate groups and several hypothetical forms have been considered as potential chordate ancestors, and evolutionary pathways from advanced invertebrates through primitive chordates to vertebrates have been a subject of vigorous discussion for more than a century (Haeckel, 1868; Garstang, 1928; Berrill, 1955; Jefferies, 1986). Recent morphological analyses (Schaeffer, 1987; Peterson, 1995) as well as molecular phylogenetic studies (Wada and Satoh, 1994; Turbeville *et al*., 1994) support the idea that echinoderms, hemichordates and chordates may share a common ancestor and form the monophyletic group of deuterostomes.

The embryonic development of echinoderms and primitive chordates (urochordates and cephalochordates) has been studied extensively at the molecular level (reviewed by Davidson, 1990; Satoh *et al*., 1996; Holland and Garcia-Fernàndez, 1996). In contrast, there has been no molecular investigations of hemichordate development. Recent advances in understanding the genetic program which specifies the chordate body plan have raised interest in the molecular phylogeny of the deuterostomes and require renewed study of the development of the minor deuterostome phyla (Krumlauf, 1994; Davidson *et al*., 1995). The relationship of the hemichordates and the echinoderms was recognized from their similar larval forms (e.g., Willmer, 1990; Nielsen, 1995). From the time of Bateson (1885), the hemichordates have been linked to the chordates because they share chordate-specific structures and organization that can be related to the chordate body plan (Nielsen, 1995; Peterson, 1995). This makes them an important focus of any study aimed at elucidating the molecular phylogeny and evolution of the genetic programs specifying the chordate body plan. The latest comprehensive review of the literature on the development of hemichordates was published by Hadfield (1975) more than 20 years ago. Because of difficulty in obtaining gametes and embryos of acorn worms in general, studies of molecular developmental biology of Enteropneusta have not progressed well. Here, as a first step to study the molecular mechanisms of hemichordate development, we report a conventional induction of natural spawning and the normal time course of early embryogen-

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esis of the Hawaiian acorn worm, *Ptychodera flava*. We present phase and differential interference contrast photographic images of the various stages of the living embryos.

MATERIALS AND METHODS

Adults of *Ptychodera flava* Eschscholtz [as *P.f. laysanica* Spengel (Edmondson, 1946)] were collected during December at the sand bar, Kaneohe Bay, Oahu, Hawaii, and maintained in glass aquaria with 3 to 4 cm of sand from their native habitat covering the bottom in the Kewalo Marine Laboratory of University of Hawaii. Neither sperm nor eggs that were active could be obtained from dissected animals, as described below. Therefore, gametes were obtained from animals which were naturally spawning in the laboratory and fertilization was carried out in plastic dishes. Animals generally spawned in the evening after being left undisturbed in the aquaria for several hr. As described below, we succeeded in the induction of natural spawning, and thus we observed spawning of more than 50 females and males, each. We observed embryogenesis of more than 30 batches of eggs. Eggs seemed to respond best to fertilization within 6 hr after shedding. Sperm can be stored at least one week in the refrigerator. The fertilized eggs were moved into glass beakers and washed with filtered seawater several times and allowed to develop at room temperature (20-24°C). Routine daily washing was made with several changes of fresh filtered seawater containing Streptomycin (50 mg/l). Photomicrographs of *P. flava* embryos and larvae were taken through a Zeiss Axiphot with 20X and 40X phase contrast and DIC (Differential Interference Contrast) objectives. The magnifications and other details are given in the explanation of figures.

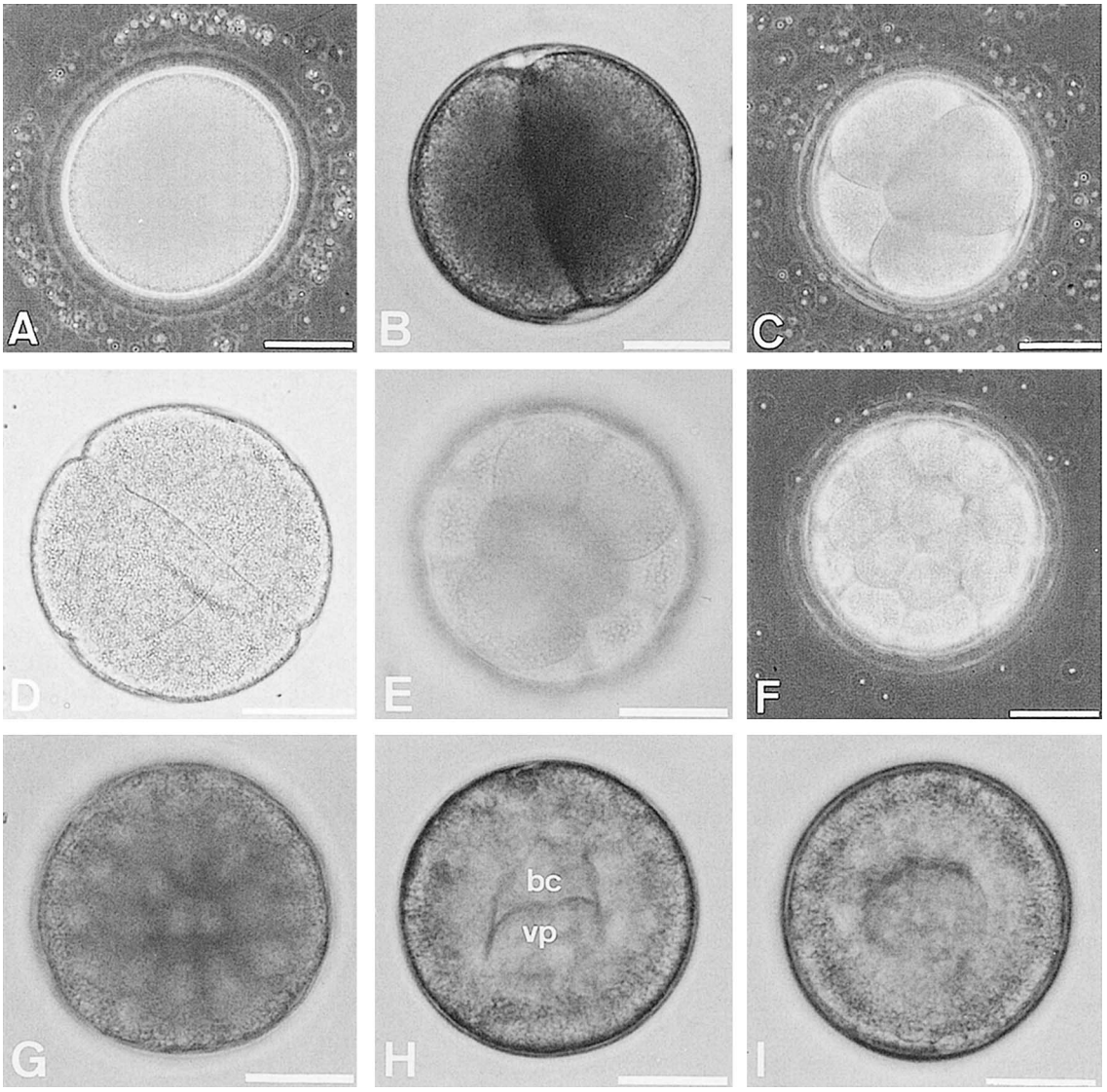

Fig. 1. Cleavage stages of *Ptychodera flava*. (**A**) A fertilized egg, 20 min after insemination. (**B**) The 2-cell stage, 3 hr after insemination. (**C**) The 4-cell stage, 4 hr after insemination. (**D**) The 8-cell stage, 5 hr after insemination. (**E**) The 16-cell stage, 6 hr after insemination. (**F**) The 32-cell stage, 7 hr after insemination. (**G**) About 100-cell satge embryo, 9 hr after insemination. (**H**) Blastula, 13 hr after insemination. The blastocoel (bc) and vegetal plate (vp) are evident. (**I**) Blastula, 13 hr after insemination. Phase contrast photographs. **D** is slightly compressed by a cover glass. Scale bar represents 50 μm.

RESULTS

Induction of natural spawning by a shift of seawater temperature

Numerous *P. flava* females and males can be collected in Hawaii (see Nishikawa, 1977 for *P. flava* in the coast of Japan). In late November and early December, the ovaries of ripe females have many oocytes with germinal vesicles. Artificial induction of oocyte maturation would be very convenient to collect large lots of embryos that may be used for molecular studies of development. We attempted to induce artificial oocyte maturation by various approaches.

Firstly, after dissecting out from the gonad, fully grown oocytes were kept undisturbed in seawater for several hr to overnight, in the hopes that, as is the case for some marine animals, oocyte maturation might occur spontaneously under this condition (e.g., Sakairi and Shirai, 1991). However, *P. flava* oocytes never matured in seawater.

Secondly, we treated oocytes with various reagents that have been shown to induce oocyte maturation in other marine animals. The reagents included calcium ionophore A23187 (Osanai and Kuraishi, 1988; Guerrier *et al*., 1993), NH_4Cl (Guerrier *et al*., 1986; Deguchi and Osanai, 1994), teophilin (Freeman and Ridgway, 1988), caffeine (Yamashita, 1988), 1-methy-adenine (Kanatani *et al*., 1969), and DTT (Maruyama, 1980). We treated oocytes with various concentrations of these reagents for several hr. However, the germinal vesicle never broke down. We also treated oocytes with various combinations of the reagents (A23187+NH_4Cl and A23187+ NH_4Cl +teophilin) with no avail.

Thirdly, we examined effects of seawater temperature shift. Colwin and Colwin (1962) reported that the shift of seawater temperature from about 27°C to 22°C could induce natural spawning of another hemichordate *Saccoglossus kowalevskii* (Agassiz). We adopted this method to *P. flava*. Natural spawning time of *P. flava*, both females and males, is around 6 pm at dusk. After several trials, we found that the following treatment resulted in the most frequency of spawning in both females and males. Namely, adults were kept for at least 6 hr in seawater at about 22°C, then around 5 pm the seawater was exchanged so that seawater temperature shifted to about 26°C. Animals were then kept in dark at this temperature. Thus, we were able to induce natural spawning and embryogenesis of more than 50 adults.

Fertilization, cleavage and blastula formation

The eggs of *P. flava* are about 120 μm in diameter, yellow and partially clear (Fig. 1). They are surrounded by a thick mucilaginous jelly coat with an underlying vitelline membrane that is slightly raised from the egg surface (Fig. 1A). Upon sperm attachment, a fertilization membrane is formed, which is tightly applied to the surface of the zygote (Fig. 1B). The tightness of the fertilization membrane is maintained until the time of hatching (Figs. 1 and 2).

The approximate time course of *P. flava* development is presented in Table 1. Depending on batches, the time from insemination to first polar body formation was very variable from 0.5 hr to 1.5 hr, although it was rather constant within a batch of eggs. Second polar body was formed about 40 min thereafter. As shown in Fig. 1B for first cleavage, the polar body is a landmark of the animal pole during early development. Cleavage is holoblastic, radial, and nearly equal. The first and second cleavages are meridional and equal (Fig. 1B, C). The first cleavage occurs at about 3±0.5 hr after insemination. Thereafter, cleavage occurs at about 1 hr intervals. In contrast to early embryos of other hemichordates including *Saccoglossus kowalevskii* (Colwin and Colwin, 1953) and *Saccoglossus horsti* Brambell et Goodhart (Burdon-Jones, 1952), our present observation demonstrated that the cleavage products of *P. flava* appear to adhere and the blastomeres remain apposed (Fig. 1B-D). For this reason, the zygote remained completely spherical and a cleavage pore which was reported in *S. kowalevskii* embryos (Colwin and Colwin, 1953) was hardly observed. The third cleavage is latitudinal and again equal (Fig. 1D) as in *Balanoglossus clavigerus* Delle Chiaje (Heider, 1909) and *Ptychodera bahamensis* Spengel (Payne, 1937). Just as described by Colwin and Colwin (1953) for fourth cleavage in *S. kowalevskii*, the 16-cell embryos of *P. flava* as shown in Fig. 1E are arranged as three tiers of cells: an upper animal tire of 8 cells and two 4-celled vegetal tiers with the most vegetal quartet being composed of the smaller cells.

As cleavage proceeds, shown at 7 hr (Fig. 1F), 9 hr (Fig. 1G) and 13 hr (Fig. 1H, I), all blastomeres become progressively smaller. The blastocoel is evident as an irregular space by the 16 cell stage at about 6 hr (Fig. 1E). Over the next 6 hr it expands into a more regular space that attains about 40% of the diameter of the embryo. During this period the blastocoel remains flattened such that it appears oblong (Fig. 1E, G) or round depending on the angle from which it is viewed. Ultimately the vegetal plate thickens and forms a mound in the blastocoel such that the blastocoel takes on a crescent shape when viewed from the side (Fig. 1H). The blastocoel in the same embryo appears round when viewed from the animal or vegetal pole (Fig. 1I).

Table 1. Time table of development of *Ptychodera flava* at 20-24°C

Developmental stages	Time (hr) after insemination
1st polar body formation	1
2nd polar body formation	1.6
2-Cell stage	3
4-Cell stage	4
8-Cell stage	5
16-Cell stage	6
32-Cell stage	7
About 100-cell stage	9
Early blastula	12
Late blastula	16
Early gastrula	18
Middle gastrula	20
Late gastrula	24
Hatching	44
Early tornaria larva	4 days

Gastrulation

The first indication of archenteron formation is the appearance of a cleft at the vegetal side, at about 18 hr after fertilization (Fig. 2B). This cleft rapidly extends to form the archenteron (Fig. 2C) which rounds up and becomes more tubular (Fig. 2D-F). At the same time the ectodermal cells become thinner and the ectoderm expands slightly (Fig. 2C-I).

The drastic changes during gastrulation in shape of the embryo observed in *S. kowalevskii* (Colwin and Colwin, 1953) were not observed in *P. flava*. From about 25 hr to 40 hr the primordial gut appears to be a closed sac attached to the ectoderm at the place of the closed blastopore (Fig. 2F, G). Later the anus appears to open at the point of blastopore closure. During gastrulation, external ciliation appears and by the time of blastopore closure, the embryo has begun to rotate within the enclosing membrane. At about the same time, the protocoel, which will form the proboscis coelom, begins to arise by an anterior swelling of the archenteron (Fig. 2F, G). This

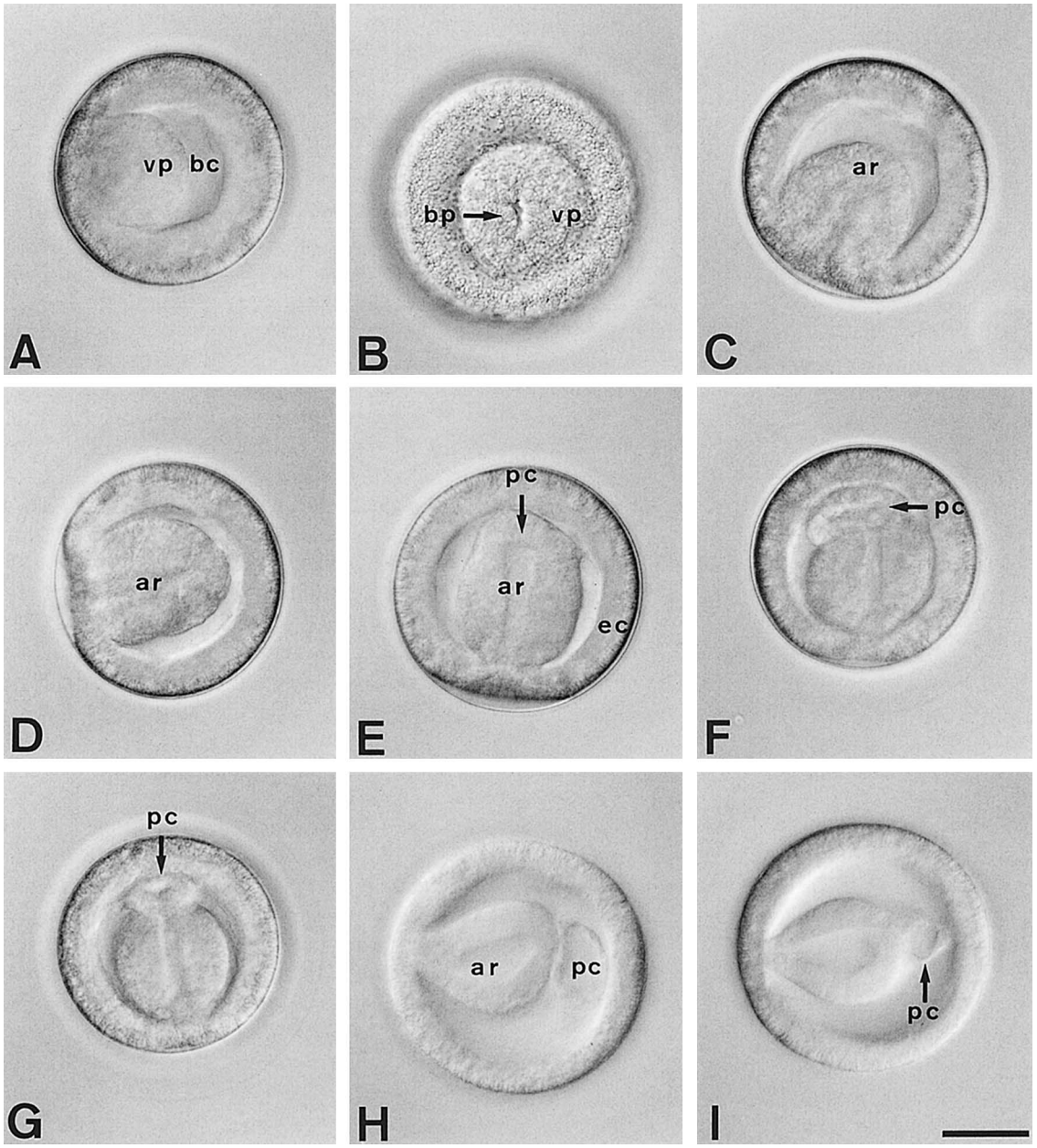

Fig. 2. Gastrulation of *Ptychodera flava*. (**A**) A late blastula showing blastocoel (bc) and vegetal plate (vp), 17 hr after fertilization. (**B**) An early gastrula viewed from the vegetal pole showing an early blastopore (bp) at the vegetal plate (vp), 18.8 hr after fertilization. (**C**) Side view of the early gastrula showing a profile of the archenteron (ar), 19.8 hr after fertilization. (**D**) Mid-gastrula, 20.3 hr after fertilization. (**E**) Late gastrula showing the early stages of formation of the protocoel (pc), 24 hr after fertilization. ec, ectoderm. (**F**) Lateral and (**G**) frontal view of late gastrula showing formation of protocoel (pc), 29.3 hr after fertilization. (**H**) Lateral and (**I**) frontal view of late gastrula at 39 hr after insemination, showing the pinching off of the protocoel (pc). DIC photographs. Scale bar represents 50 μm for all photographs.

lobe then gradually pinches off from the archenteron and becomes a separate cavity (Fig. 2H, I). The blastocoel cavity also begins to expand and the ectoderm thins dramatically, a process that continues as the tornaria larva develops.

After the protocoel separates from the archenteron at about 40 hr of development, an anterior prolongation establishes a thin connection to the apical plate ectoderm directly beneath the apical tuft (Figs. 3C and 4A). A second tubular evagination from the protocoel makes contact with the dorsal wall of the embryo opening to the outside to form the proboscis pore (Figs. 3C and 4A). An extension from one side of the tip of the archenteron bends ventrally and contacts the body wall between 40 and 45 hr of development.

Hatching of the embryo

The embryo hatches at about 45 hr of development (Fig. 3). One side of the embryo, usually the posterior side becomes slightly protruded (Fig. 3A). The embryo breaks the enclosed membrane at this side (Fig. 3B), and slips out of the membrane (Fig. 3C). The hatching process is completed within a few hr.

Formation of early tornaria larvae

After hatching the cilia soon grow longer at the anterior end of the embryo, forming an apical tuft (Fig. 4A-D). The ectoderm at the point of contact with the archenteron becomes thick, forms an oral plate (Fig. 3C) and the mouth eventually opens at this site (Fig. 4A). By this time, several mesenchyme cells appear in the blastocoel (Fig. 4A, B) and as development proceeds, the cells increase in number and show active locomotion with filopodia. Between 2 and 4 days of development the larva elongates in the anteroposterior axis and the gut differentiates into three sections, the esophagus, stomach and intestine (Fig. 4A-D).

By the time of 4 days a wide band of elongate cilia, longitudinal ciliary band is formed encircling the anterior part of the larva (Fig. 4E, G), and soon after the telotroch becomes noticeable encircling the anus of the larva (Fig. 4E, G, I). In addition, a pair of eye spots, visible only in the DIC photographs of later larva (e.g., Fig. 4F, G), appears near the apical tuft. Over the next 3 days the structures of the tornaria larva continue to expand and differentiate (Fig. 4F-I) and the larva becomes clear, transparent and beautiful. We could not detect development of the mesocoel, metacoel, and stomochord (buccal diverticulum) during the period of our observations.

DISCUSSION

Induction of natural spawning by a shift of seawater temperature

P. flava adults are rather abundant on the sandy coast of the Oahu Island, Hawaii. Therefore, it is not difficult to obtain the materials. However, their spawning season is restricted to late November or December (Hadfield, 1975). Ripe animals may shed gametes by responding to the seasonal decrease of seawater temperature which occurs at this time of year. As shown in this study, natural spawning is easily induced by a shift of seawater temperature of gravid adults brought into the laboratory. This is very useful for future studies of *P. flava* development.

We tested various methods to induce oocyte maturation without success. Studies to devise methods to induce oocyte maturation and sperm activation artificially will remain a major goal of our work.

Embryogenesis of *P. flava*

The descriptions of blastula formation and gastrulation which we presented here are slightly different from previous explicit descriptions of these events in enteropneust hemichordates. When compared to the major descriptions of embryogenesis of hemichordate species (Bateson, 1885; Burdon-Jones, 1952; Colwin and Colwin, 1953), our results suggested moderate diversity of early development in these forms. It is well described that hemichordates exhibit two general modes of development, direct and indirect; the latter occurring through an extended pelagic planktotrophic tornaria larval stage. Most detailed descriptions of blastula formation and gastrulation, have come from the larger eggs of direct developing forms (Bateson, 1885; Burdon-Jones, 1952; Colwin and Colwin, 1953). Embryogenesis of early stages of *Ptychodera*

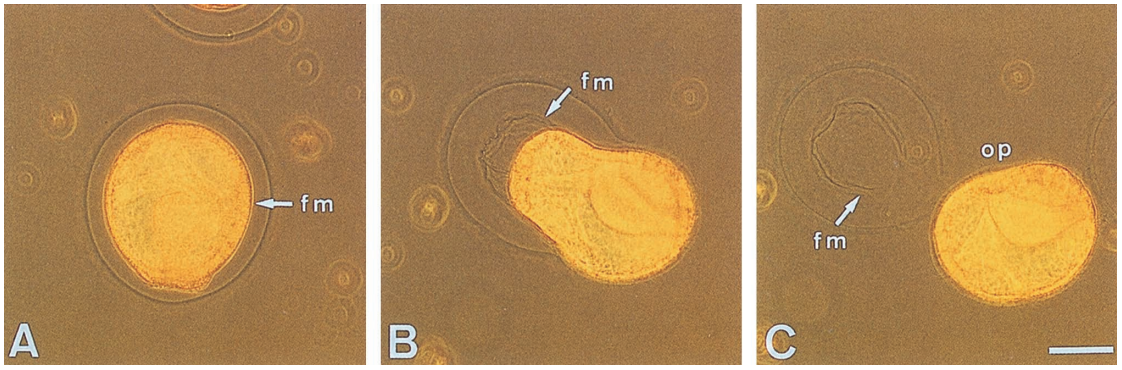

Fig. 3. Hatching of larvae. (**A**) Late gastrula in the fertilization membrane (fm), 44.2 hr after insemination. (**B**) Larva extruding from the fertilization membrane (fm), 44.3 hr after insemination. (**C**) Hatched larva and empty fertilization membrane (fm) remained, 44.5 hr after insemination. op, oral plate. Phase contrast photograph. Scale bar represents 50 μm for all photographs.

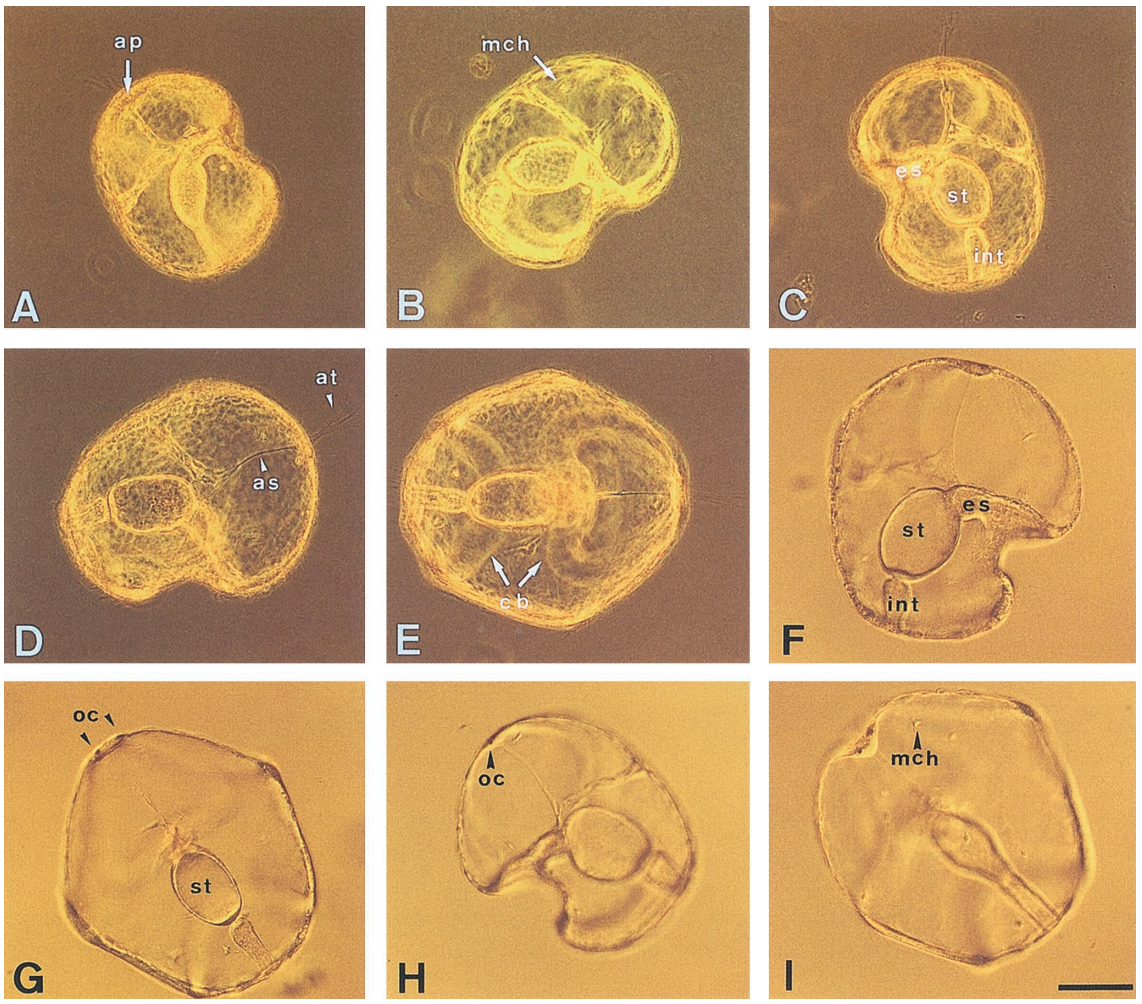

Fig. 4. Formation of tornaria larvae. (**A**) Larva at 63 hr after insemination viewed from right side. (**B**) A 3-day-old tornaria, right side view and (**C**) left side view. (**D**) A 4-day-old tornaria, left side view and (**E**) posterior view. (**F**) A 5-day-old tornaria, right side view and (**G**) posterior view. (**H**) A 7-day-old tornaria, left side view and (**I**) posterior view. ap, apical plate; as, apical strand; at, apical tuft; cb, ciliary bands; es, esophagus; int, intestine; mch, mesenchyme cells; oc, ocellus; st, stomach. **A**, **B**, **C**, **D** and **E** are phase contrast photographs while **F**, **G**, **H** and **I** are DIC photographs. Scale bar represents 50 μm for all photographs.

bahamensis, which represents an indirect developing form, has also been described (Payne, 1937). Here we note that in *P. flava*, instead of forming a large, hollow spherical blastula which is invaginated to form the gastrula, the blastocoel forms as a smallish disk shaped space in what appears to be a rather compact mass of cells. It never expands to more than 40% of the diameter of the egg and as the vegetal plate thickens it comes to resembles a crescent underlain by a mound of vegetal plate cells. Although the lack of an expanded blastocoel has not been explicitly noted previously, this conclusion is compatible with previous descriptions and the few illustrations presented in previous works on early development of *P. bahamensis* (Payne, 1937) and/or *P. flava* (Rao, 1954; Hadfield, 1975).

P. flava development is also distinct from that described for other hemichordates beginning from the first cleavage, when the first two blastomeres of *P. flava* appear to remain adhering such that the cleaved zygote maintains an essentially spherical profile (Fig. 1). This may be compared with *Balanoglossus clavigerus* (Bateson, 1885) or *Saccoglossus kowalevskii* (Colwin and Colwin, 1953), where the first blastomeres take the form of weakly attached ovate cells.

In *S. horsti* (Burdon-Jones, 1952), *S. kowalevskii* (Colwin and Colwin, 1953) and the Indian *P. flava* (Rao, 1954), third cleavage is slightly unequal so that in 8-celled embryos the cells of the animal tier are smaller than the vegetal four. As previously described by Hadfield (1975) for *P. flava* from Hawaii, the blastomeres of the 8-cell stage in the material we examined appeared quite equal. However, the blastomeres of the 16-cell stage do appear unequal as described and illustrated.

Previous explicit descriptions of hemichordate gastrulation do fit the classical image of gastrulation by invagination of a hollow ball (Bateson, 1885; Burdon-Jones, 1952; Colwin and Colwin, 1953). Because the blastocoel never becomes expanded in *P. flava*, the blastula never resembles a hollow ball and does not gastrulate according to this classical picture. In our observation of *P. flava* the archenteron appears

as a cleft in the thickened vegetal plate, apparently beginning externally at the vegetal pole and progressing toward the animal pole as it opens and the cells undergo morphogenesis into a well formed archenteron. These variation of early embryogenesis may be caused by adhesive properties of embryonic cells or may be the result of a restrictive fertilization membrane that holds the cells together such that the embryo remains compact.

As mentioned in the Introduction, recent cloning of developmentally important genes may be opening new possibilities to interpret the origin and evolution of chordates. Our particular interest is the genes that specify embryonic cells yielding notochord, since the notochord is one of the most prominent feature of chordates (Satoh and Jeffery, 1995). The *Brachyury* gene has been implicated as a master control gene of notochord differentiation. Isolation of a cDNA clone for a hemichordate homologue of *Brachyury* will be described elsewhere.

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