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The Development and Larval Form of an Echinothurioid Echinoid, *Asthenosoma ijimai*, Revisited

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Abstract. The modified development from cleavage to late larval form of the echinothurioid echinoid, *Asthenosoma ijimai,* was re-examined using light microscopy and scanning electron microscopy of whole and sectioned stages. Although an original study (Amemiya and Tsuchiya, 1979) reported direct development without evidence of a pluteus larva, we found that the unusual development can be interpreted as a topologically reflected, reduced pluteus, with vestigial larval arms and a greatly reduced larval skeleton. This developmental pattern produces the third and most reduced pluteus form known among the six echinoid lineages with modified development that have been studied thus far. Features such as an equal fourth cleavage, extrusion of yolk into the blastocoel, and the presence of large numbers of cells within the blastocoel are convergent with traits reported for other species with modified development. Coelom formation is clearly modified from that of species with feeding larval development, but notably the hydrocoel begins to develop podial buds prior to separation from the archenteron. Echinothurioid sea urchins are considered to be the most primitive living euechinoids, and in *A. ijimai* the timing of mesenchyme cell ingression and the formation of epineural folds were similar to these features in other euechinoids. Indentation of the juvenile oral surface relatively late in larval development raises the possibility that the amniotic invagination (vestibule), common in all other euechinoids, may be a trait incorporated into the development of echinoids at the time of origin of the echinothurioids. The structural comparisons reported here show a need for further detailed morphological studies of developmental modifications in other echinoid species.

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Introduction

Most sea urchins species (*ca.* 66%) develop through a feeding larval stage, the echinopluteus, for several to many weeks before metamorphosing into juvenile echinoids (see review by Emlet *et al.,* 1987). At least 14 times among living taxa, however, the feeding larval stage has been lost, and these species undergo a modified and abbreviated development before juvenile sea urchins are formed (Emlet, 1990; see also Strathmann, 1978; Raff, 1987). At present, about ten species from six of the lineages with modified development have been investigated, and some description of their embryonic and larval development is available. Descriptive and analytical research has been conducted on the following: two cidaroids (*Phyllacanthus imperialis,* Olson *et al.,* 1988; *P. parvispinus,* Mortensen, 1921; Parks *et al.,* 1989); two echinothurioids (*Asthenosoma ijimai,* Amemiya and Tsuchiya, 1979; *A. sp.,* Uehara and Amemiya, unpub. obs.); one temnopleuroid (*Holopneustes purpurescens,* V. Morris, Univ. Sydney, unpub.); one echinometrid (*Heliocidaris erythrogramma,* Mortensen, 1921; Williams and Anderson, 1975; Parks *et al.,* 1988); two clypeasteroids (*Peronella japonica,* Mortensen, 1921; Okazaki and Dan, 1954; Okazaki, 1975; *P. rubra,* Amemiya and Emlet, unpub. obs.); and two brooding spatangoids (*Abatus agassizi,* Larrain, 1973; *A. cordatus,* Schatt, 1985, 1988). Many of the other echinoid lineages with modified development occur in deeper seas or antarctic seas and are difficult to collect for study (*e.g.,* lineages of temnopleuroids, holasteroids and other lineages of cidaroids and spatangoids, *c.f.,* Mortensen, 1936; Fell, 1976).

The most recent studies have focused on species with very highly modified development, often referred to as direct development. These studies have examined heterochrony (changes in relative timing) of developmental events, modifications of cleavage patterns, the resultant cell lineages, and cell movements (*e.g.,* Parks *et al.,* 1988, 1989; Wray and Raff, 1989, 1990; Henry and Raff, 1990). Additional immunofluorescence studies have drawn inferences about gene expression from specific markers for gene products [*e.g.,* the monoclonal antibody B2C2 to mesenchyme-derived antigens or antibodies to serotonergic neurons (above citations, Bisgrove and Raff, 1989)]. Due to the extreme degree of developmental modifications, these studies usually emphasize how different the morphogenetic patterns are from those of species that develop through a pluteus larva (*e.g., Heliocidaris erythrogramma,* Wray and Raff, 1990). With the exception of the above mentioned studies on *Abatus cordatus, Heliocidaris erythrogramma,* and *Peronella japonica,* either no information or only limited information is available on the internal morphological aspects of development of species with modified development. Both the extreme modification, and a lack of detailed morphological information, limit our understanding of how these developmental modifications may have evolved.

This report—an extension of an earlier one (Amemiya and Tsuchiya, 1979)—describes selected features of morphogenesis in the echinothurioid echinoid *Asthenosoma ijimai,* from cleavage through late larval development. Comparisons are also made with unmodified pluteal development, as well as with the modified developmental patterns occurring in other species. The echinothurioids are a particularly important group to study: first, because all of them seem to have modified development (reviewed in Emlet *et al.,* 1987); and second, because they are considered to be the earliest living branch of the euechinoid lineage, and thus the second oldest lineage of echinoids after the cidaroids (Smith, 1984). Because the cidaroids and euechinoids differ in many developmental features (Emlet, 1988), we should inquire whether the development of the echinothurioids shows greater affinity with other euechinoids, or with the more primitive cidaroids. The observations presented here, together with the comparisons with other species, point up the need for additional morphological studies of developmental modifications in other echinoid species.

Materials and Methods

Adults and larvae

Adults of *Asthenosoma ijimai* Yoshiwara were collected at a depth of 20 m off Misaki Marine Biological Station in Sagami Bay, Japan. Adult specimens were dissected, and fully matured gametes were obtained. Eggs were washed twice in filtered seawater (0.22 μm) and fertilized by mixing with a small amount of undiluted sperm. Fertilized eggs were washed three times in filtered seawater, and cultured in unstirred, one-liter glass beakers at 20°C or at room temperature (25–28°C). The stages and times for sectioned material presented here are from cultures at room temperature. No food was added to the larval cultures. At various times after fertilization, living larvae were photographed under a dissecting microscope, and aliquots were fixed for examination by light or scanning electron microscopy (SEM).

Preparation of sectioned and stained material

Larvae were fixed for 1 h in seawater containing 4% or 10% formalin at room temperature and preserved in 70% EtOH. Preserved specimens were dehydrated through graded ethanol series and embedded in Spurr embedding media (Polysciences, Inc). Sections, 5–8 μm thick, were stained with Richardson's stain (1% Azure II in distilled water combined with 1% methylene blue in 1% sodium borate, Richardson *et al.,* 1960). The serial sections of larvae embedded in epoxy resin were traced with camera lucida and digitized so that 3-D images of the sections could be constructed (PC3D program, Jandel Scientific, Inc.).

Immunofluorescence and H33258 staining

Immunofluorescence staining with skeletogenic mesenchyme specific monoclonal antibody B2C2 was conducted according to the methods of Parks *et al.* (1988). Embryos, larvae, and juveniles were fixed for 50 min in seawater containing 4% formalin, washed in artificial seawater, dehydrated in a graded ethanol series, embedded in polyester wax (BDH, Ltd), and sectioned at a thickness of 5 μm. The rehydrated sections were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-TW20) and incubated with culture fluid containing the monoclonal antibody B2C2, diluted 1:20 in PBS-TW20, for 40 min at room temperature in a humidified chamber. The slides were washed in PBS-TW20, incubated with FITC-conjugated, goat anti-mouse, IgG antibodies (diluted 1:200 in PBS-TW20) for 40 min, and rinsed again. For detection of cell nuclei, some sections were incubated with H33258 (Hechst, Inc.) at a concentration of 0.5 μg/ml PBS for 10 min instead of, or after, treatment in primary and secondary antisera. Fluorescence was observed and photographed with a Nikon fluorescence microscope.

Scanning Electron Microscopy (SEM)

Specimens were fixed and preserved as indicated above, or they were fixed for 1 h in a mixture of 2% gluteraldehyde (Taab Lab.) and 1% osmium tetroxide (OsO_4, Taab Lab.)

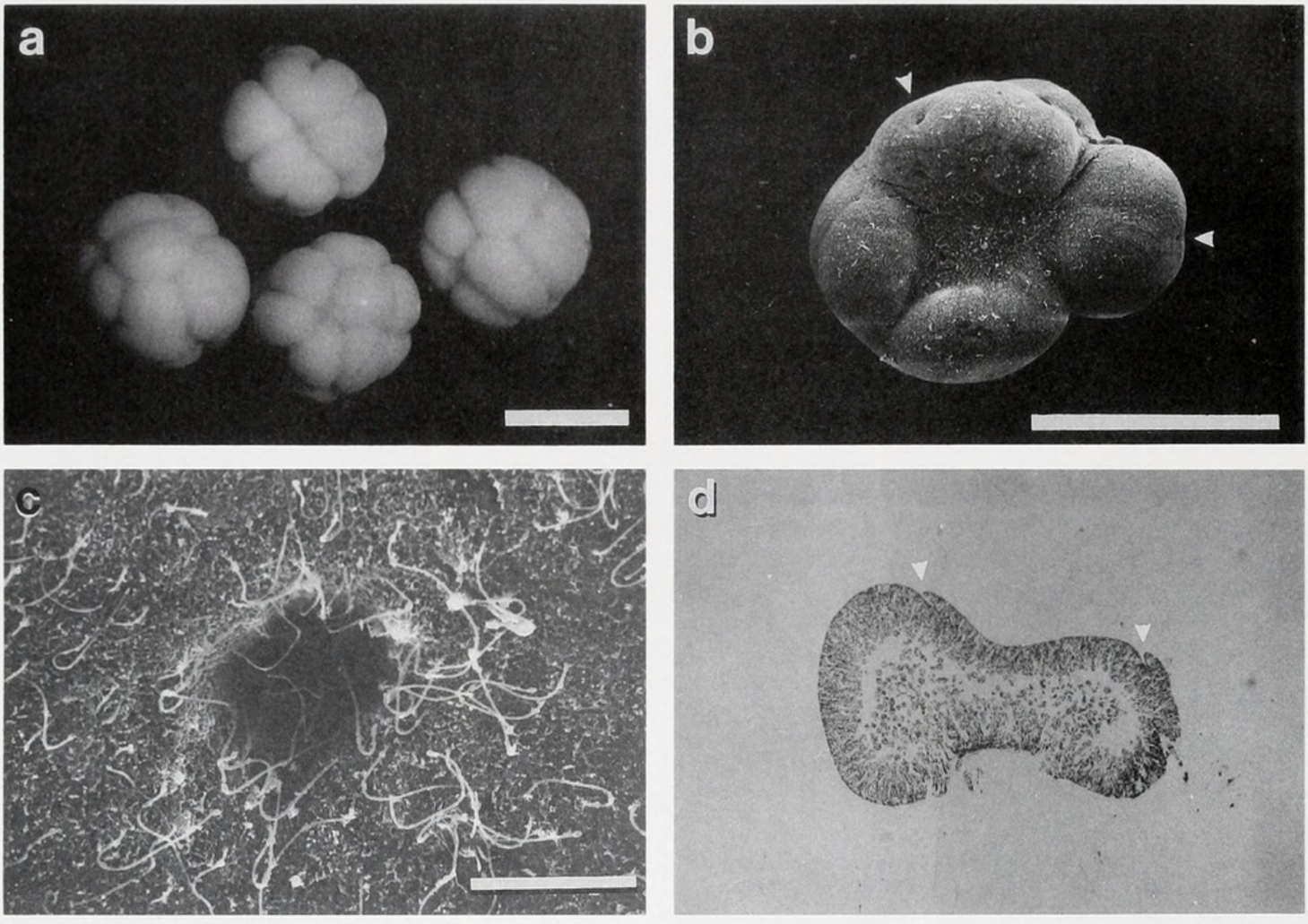

Figure 1. Early embryonic stages of *Asthenosoma ijimai*. a. Sixteen-cell stage embryos randomly oriented, show that all cells are approximately the same diameter after the fourth cleavage. Scale bar, 1 mm. b. SEM of a 21.5-h, lobate blastula. Arrowheads mark pits in ectoderm. Scale bar, 1 mm. c. Close-up SEM of ectodermal pit marked by right arrow in b. Scale bar, 25 μm. d. Section of a 21.5-h blastula shows yolky cytoplasm in the blastocoel and ectodermal pits (arrowheads). Same scale as b.

in 0.45 *M* sodium acetate buffer (pH 6.4) at room temperature (Harris and Shaw, 1984) and preserved in 70% EtOH at 4°C. The preserved specimens were dehydrated through a graded ethanol series, dried at the critical point (Hitachi HCP-1 drier) with liquid CO_2 as a transitional fluid, and sputter-coated with gold (Eiko IB-3 ion coater). Observations were made with a Hitachi HHS-2R SEM. To examine the inside of larvae with SEM, specimens were embedded in polyester wax and sectioned by microtome to expose a particular cross section. These specimens were incubated in absolute ethanol at 40°C for 12 h to remove wax (Armstrong and Parenti, 1973) and then subjected to critical point drying as described above.

Clearing larvae

Live larvae of *Asthenosoma* are opaque orange-yellow (Amemiya and Tsuchiya, 1979), and it was impossible to see internal structures in these or in fixed, preserved specimens. However, larvae could be rendered translucent by clearing with solutions of benzyl benzoate and benzyl alcohol mixed in ratios of 2:1, 1:1, or 1:2, depending on the desired refractive index. Fixed larvae were first dehydrated to 100% EtOH, then transferred into the clearing solution where the remaining EtOH was allowed to evaporate. Upon clearing, larval dimensions remained the same, and no osmotic effects were discerned. To search for calcareous deposits, cleared larvae were observed under crossed-polarized light.

Results

Observations on soft-tissue development

Eggs, cleavage, external aspects of larvae, and metamorphosis have been described by Amemiya and Tsuchiya (1979). The fourth cleavage of embryos of *Asthenosoma ijimai* was almost equal, giving rise to 16 blastomeres of similar size. Figure 1a shows that there was some

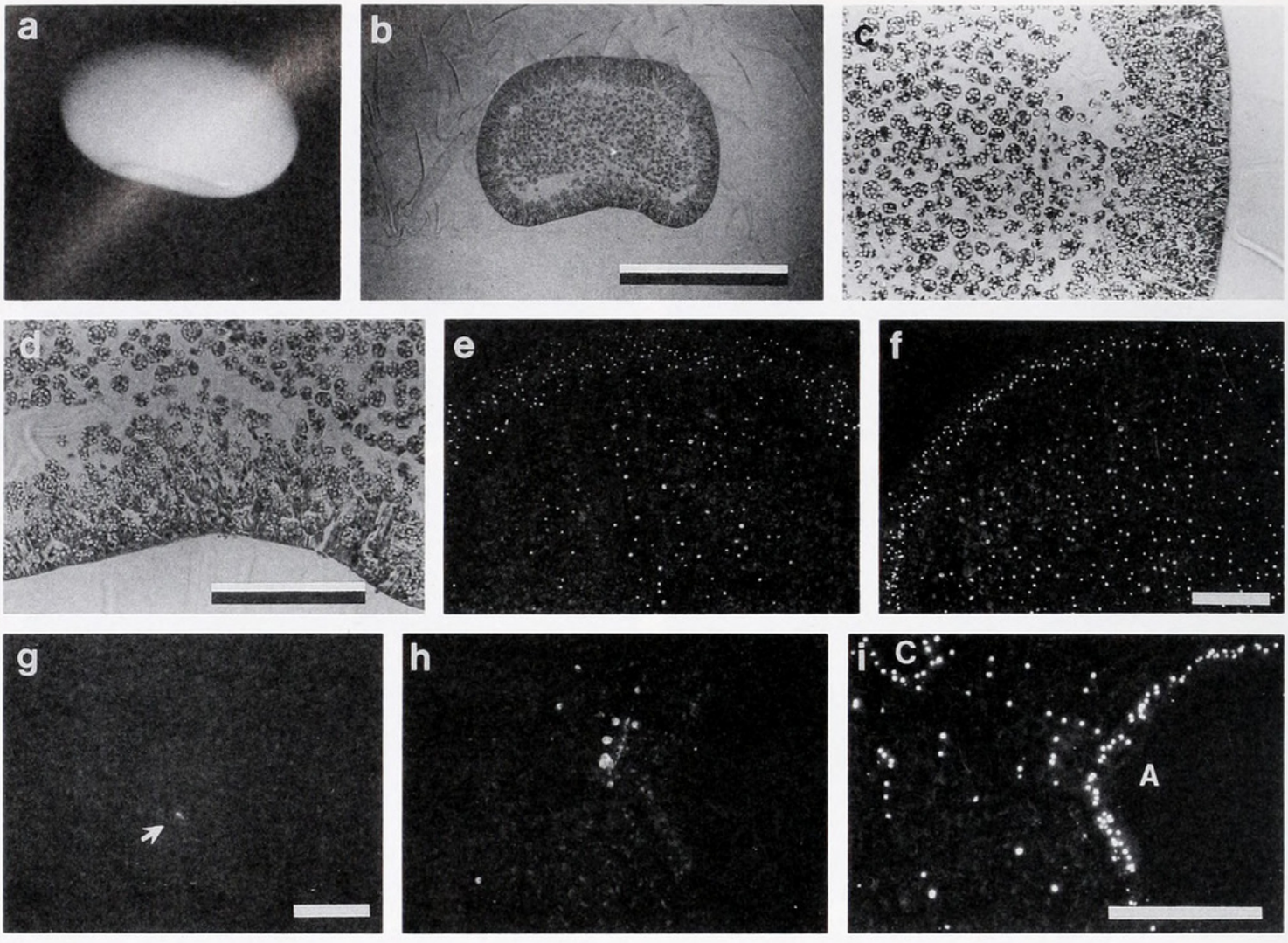

Figure 2. Later embryonic stages of *Asthenosoma ijimai.* All embryos and sections are oriented with the animal pole up. a. Light micrograph of a live, 25.5-h-old, early gastrula. Same scale as b. b. Section through animal-vegetal axis of flattened gastrula (25.5 h), showing blastocoel filled with yolky cytoplasm. Scale bar, 1 mm. c. Lateral wall of gastrula (25.5 h), shows yolky cytoplasm exocytosing from basal ends of ectodermal cells. Same scale as d. d. Vegetal wall of gastrula (25.5 h), showing exocytosis of cytoplasm and ingression of cells. Scale bar, 200 μm. e. Section of a 25.5-h gastrula shows fluorescently staining nuclei (H33258 fluorescent dye) of ectodermal and probable mesenchyme cells. Same scale as f. f. Section of 35-h gastrula, with fluorescently stained nuclei. Scale bar, 200 μm. g. Section of 51.5-h embryo, stained with B2C2 antibody, arrow shows first occurrence of expression of MSP-130 glycoprotein associated with blastocoelic cells. Scale bar, 50 μm. h, i. Section of an 88.5-h larva, doubly stained with B2C2 antibody (h) and H33258 fluorescent dye (i) shows not all blastocoelic cells express MSP-130. The concentrated clusters of nuclei in (i) are epithelia of the archenteron (A) and a coelomic compartment (C). Scale bar, 200 μm.

variation in size of the blastomeres in 16-cell embryos, but there was no evidence of micromeres at the vegetal pole. The absence of an unequal, fourth cleavage parallels the cleavage patterns of other echinoid species large yolky eggs and modified development (Williams and Anderson, 1975; Raff, 1987; Parks *et al.,* 1989).

Like other echinoderm species with yolky eggs, embryos of *Asthenosoma ijimai* formed wrinkled blastulae (Amemiya and Tsuchiya, 1979; Parks *et al.,* 1989). This stage was followed by egression and loss of wrinkles and led to a lobate blastula (Fig. 1b). At this stage, small, cylindrical pits were present on the external surface of the embryo and passed into the ectodermal layer (Fig. 1b, c, d). Serial sections showed that some of these pits terminated blindly within the ectoderm, while others passed through the ectoderm to another external opening. Several of these pits or passages coincided with large indentations in the embryo's surface and, therefore, may have been the remnants of the wrinkled indentations. Parks *et al.* (1989) reported pits in the yolky embryo of the cidaroid *Phyllacanthus parvispinus,* though they did not see any association of pits with egression tracks, where wrinkles diminished. Further work is necessary to determine whether the pits in the two species arise by similar mechanisms.

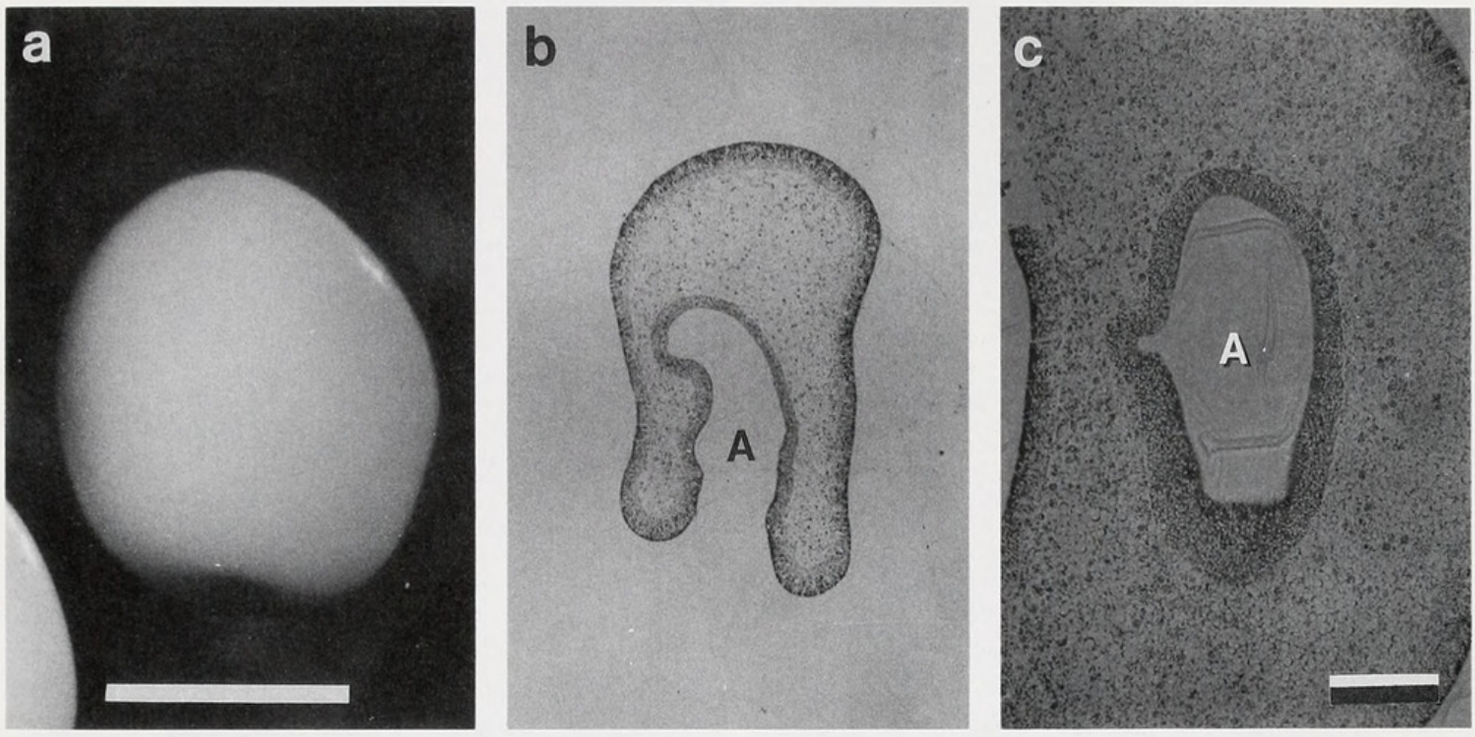

Figure 3. A 51.5-h larvae of *Asthenosoma ijimai,* all with anterior end up. a. Light micrograph of a live larva with dorsal swelling on the right. Scale bar, 1 mm. b. Medial sagittal section through late gastrula. The tip of archenteron (A) has curved toward the ventral surface. Same scale as a. c. Frontal section shows a small outpocketing on the left side of the archenteron (A) that extends dorsally in other sections. Scale bar, 200 μm.

Sections of this 21.5 h stage showed anucleate, yolky cytoplasm being released into the blastocoel from the basal ends of most ectodermal cells (Fig. 1d, see also Fig. 2c, d). Sections also revealed that one indented surface was extruding considerably more material than other surfaces into the blastocoel (Fig. 1d). The fluorescent stain, H33258, revealed considerable numbers of nuclei in the blastocoel, indicating mesenchyme-like cell ingression at the onset of gastrulation.

Gastrulation had begun by 25.5 h after fertilization, and embryos were compressed along the animal-vegetal axis, with a large indentation at the vegetal pole (Fig. 2a). At this stage, the blastocoel was filled with yolky cytoplasm (Fig. 2b, c, d). Counts of fluorescently stained nuclei showed a mean of 98 cells per 5-μm section (n = 3 sections, S.D. = 11) and were scattered among the yolky cytoplasm in the blastocoel (Fig. 2e). The number of staining nuclei, and thus the number of cells, increased to a mean of 375 per section (n = 3 sections, S.D. = 9.6) in the mid-gastrula stage at 35 h (Fig. 2f). The source of these additional blastocoelic cells is either ingression from the vegetal pole (Fig. 2d) or cell division. Fluorescent staining also showed that cell division in the ectoderm was continuing because the number of nuclei in the ectoderm increased between 25.5 and 35 h (Fig. 2e, f).

A positive reaction of B2C2 antibody with blastocoelic cells, indicating expression of MSP-130 glycoprotein, was found first at 51.5 h post fertilization (Fig. 2g). Later observations on mesenchyme cells associated with skeleton in larvae of *Asthenosoma ijimai* showed that these cells reacted with B2C2, suggesting that MSP-130 is expressed by the skeletogenic cells in this species just as in other echinoids. By 88.5 h post fertilization, after skeletogenesis had begun, sections labeled with both nuclear stain (H33258) and B2C2 antibody revealed that only a fraction of blastocoelic cells were skeletogenic (Fig. 2h, i).

In the present paper, the identities of the dorsal and ventral surfaces are reversed from those described in the initial paper on development of *Asthenosoma ijimai* (Amemiya and Tsuchiya, 1979). By 35 h post fertilization, the embryos elongated along the animal-vegetal axis, with the blastopore located off center, toward the ventral surface. At 51.5 h, the late gastrulae had swollen dorsal sides (Fig. 3a). Internally, the archenteron had grown over half the length of the embryo, and the apical (anterior) end curved toward the ventral side of the embryo (Fig. 3b). In addition to the large curved tip of the archenteron, another small outpocketing was forming on the left side of the archenteron and was growing dorsally (Fig. 3c).

Serial sections of stages at 51.5, 56.5, and 63 h post-fertilization showed progressive changes in the development of the archenteron and coelomic pouches (Fig. 4). When the archenteron reached its full length, one or two slender, epithelial projections on the left side and an-

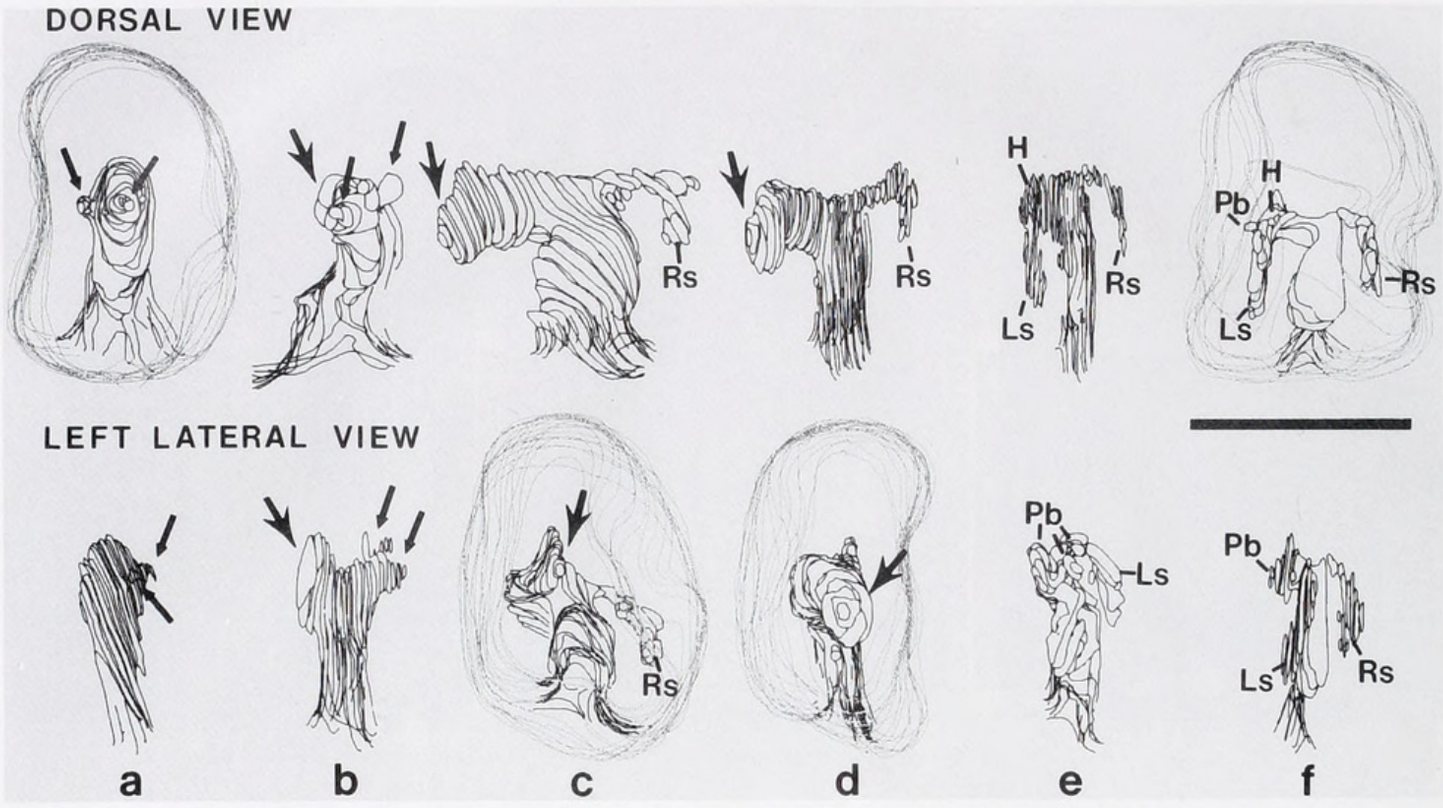

Figure 4. Three-dimensional reconstructions of the archenteron and coelomic pouches from serial sections of 51.5-, 56.5-, and 63-h larvae of *Asthenosoma ijimai*. In each column the same larva is shown in approximate dorsal view and approximate left lateral view. The animal pole, corresponding to the anterior end, is toward the top of the figure; the vegetal pole, corresponding to the posterior end, is toward the bottom of the figure. Line segments are tracings of the inner surface of the archenteron and coelomic pouches (black). The outer surface of the larval ectoderm (gray) is included in a, c, d and f for reference. Small arrows, left lateral and dorsal projections from archenteron; large arrow, tip of archenteron; Rs, right somatocoel; Ls, left somatocoel; H, hydrocoel; Pb, podial bud of hydrocoel. See text for explanation. a., b. 51.5 h. c., d. 56.5 h. e., f. 63 h. Scale bar, 1 mm.

terodorsal surface of the archenteron grew dorsally (small arrows, Fig. 4a, b). Of the three 51.5-h specimens that were serially sectioned, two showed both projections (Fig. 4a, b), and one showed a left lateral projection only (not figured). With further development, the much larger ventral tip of the archenteron bent and extended ventrally without contacting the blastocoel wall (large arrow, Fig. 4b, c). Between 51.5 and 56.5 h, the archenteron underwent torsion, twisting approximately 90° counterclockwise, when viewed from the animal pole. This twist reoriented the tip of the archenteron toward the left side of the larva, and the slender projections toward the right side of the larva (Fig. 4b, c). One of the two slender projections extended laterally and posteriorly (toward the vegetal end) to form the right somatocoel (Fig. 4c–f). Comparisons among serially sectioned larvae suggest that either of the slender projections could form the right somatocoel, and the other slender projection apparently did not continue to grow. Subsequent to 56.5 h, the tip of the archenteron grew a projection dorsally and posteriorly, which formed the left somatocoel (Fig. 4e, f). By 63 h, while still attached to the main body of the archenteron, the tip of the archenteron began to develop into the left hydrocoel with buds that became the coelomic lining of the five, primary podia (Fig. 4e, f; Fig. 5c, d).

Externally, changes between the 56.5 and 63 h stages produced four large and rounded lobes that grew into projections called "para-arms" by Amemiya and Tsuchiya (1979). One pair of these bilaterally symmetrical projections is located dorsally and laterally relative to the blastopore and projects posteriorly, away from the animal pole. The second pair is located on the dorsal surface just anterior to the other pair and also projects dorsally (Fig. 5a). The surfaces of the larvae were uniformly ciliated (Fig. 5b). No developing stages, even in the region of the para-arms, showed cilia collected into discrete rows such as found in the ciliated bands of pluteus larvae. No dorsal hydropore was present despite internal development of somatocoels and the left hydrocoel.

By 75.5 h post-fertilization, the five bulges of the primary podia were externally visible and arranged in a circle on the left lateral surface (Fig. 6a). Sections of this stage

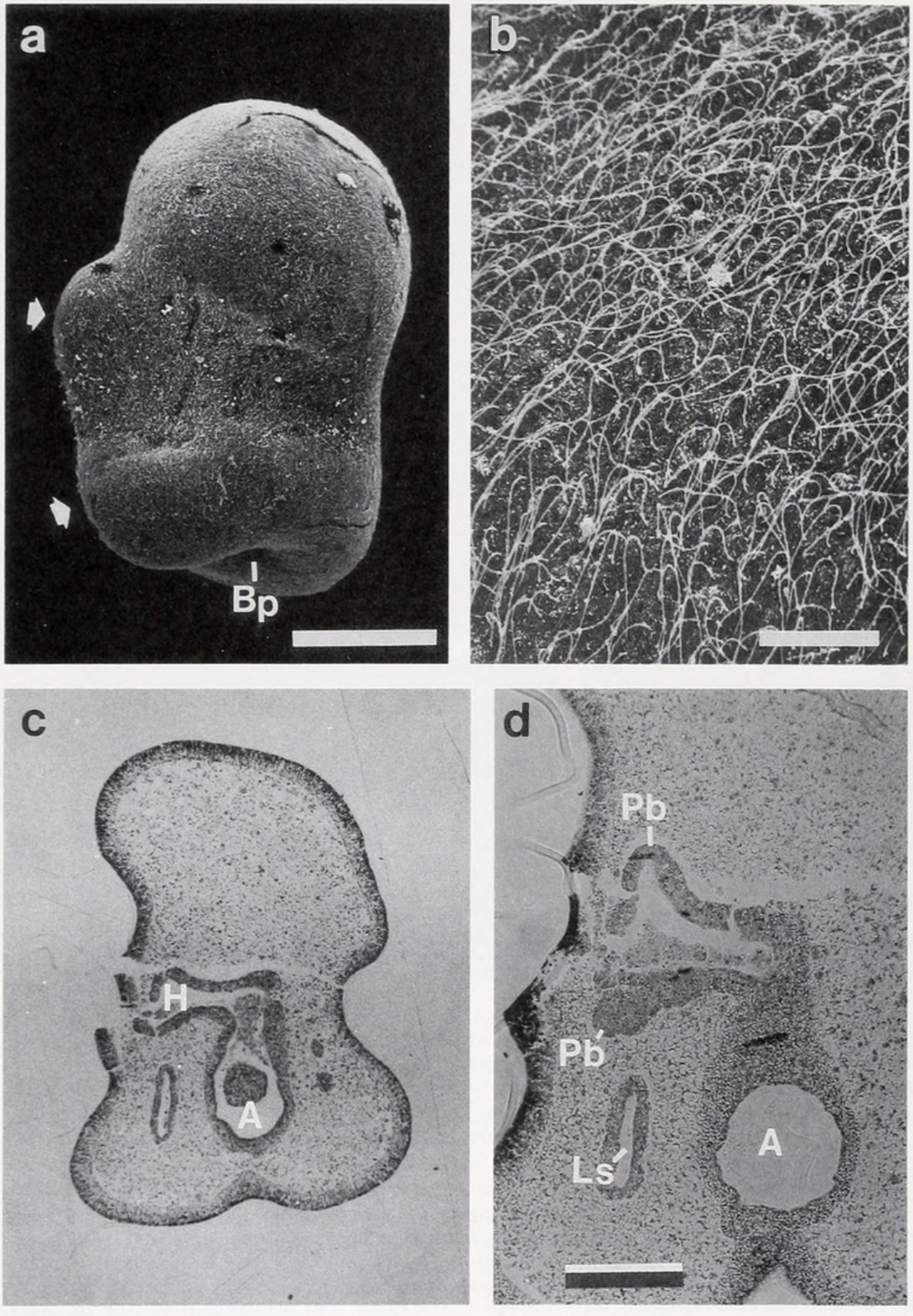

Figure 5. A 63-h larvae of *Asthenosoma ijimai,* all oriented with the anterior end toward the top of the figure. a. SEM of a whole larva, right ventral view (dorsal side is on the left) shows two right para-arms (arrows) and the blastopore (Bp). Scale bar, 0.5 mm b. Close-up of uniformly ciliated epidermis. Scale bar, 25 μm. c. Frontal section shows the leftward oriented archenteron with coelomic components of podia near its tip. The hydrocoel (H) is developing before being separated from the archenteron (A). (This larva was damaged during embedding, but a clear interpretation of sections was still possible.) Same scale as a. d. Higher magnification of a more dorsally located frontal section from same larva as c. Hydrocoelic components of podial buds (Pb) are present. Ls, left somatocoel. Scale bar, 200 μm.

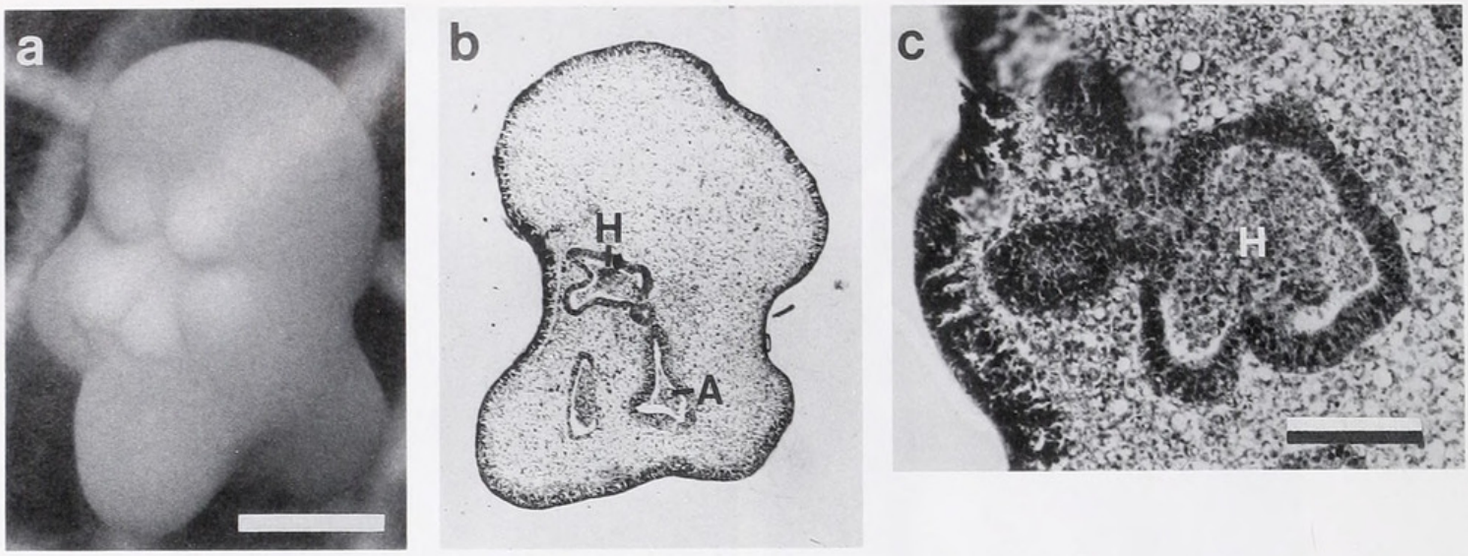

Figure 6. A 75.5-h larvae of *Asthenosoma ijimai*. a. Light micrograph of live specimen shows five primary podia just beginning to form on left side of larva. Scale bar, 0.5 mm. b. Frontal section with continued hydrocoelic (and podial) development. The hydrocoel (H) is almost completely separated from the archenteron (A). Same scale as a. c. Detail of hydrocoel (H) with parts of two podial extensions from a different section of the same larva as b. Scale bar, 100 μm.

showed the hydrocoelic compartments with thickened epithelia beneath the podial swelling of the ectoderm (Fig. 6c). Serial sections revealed that the connection between the hydrocoel and archenteron was greatly reduced in one larva (Fig. 6b) and completely severed in a second larva examined. All coelomic and archenteric cavities contained stained materials that appeared to be yolky cytoplasm and some cells (Fig. 6b, c).

By 101 h post fertilization, primary podia elongated to 0.2 mm length (Fig. 7b, c, d). Sections of the juvenile oral surface showed folds of ectodermal tissue lying between the five primary podia (Fig. 7c, d). These folds were evidently epineural folds that were growing over the juvenile oral surface to form the epineural sinus (von Ubisch, 1913; Hyman, 1955; Emlet, 1988). SEM observations of the external surface of the developing juvenile oral region confirm that these epineural folds were spreading toward the oral center (Fig. 7f–h).

Coincident with the lengthening of the primary podia and development of the epineural folds, the oral surface sank to become indented in the surface of the developing larva. This indentation was notable in live specimens viewed from the side at 101 h (Fig. 7b), as well as in sectioned material (Fig. 7c) and in specimens fixed for SEM (Fig. 7f). Though the developing juvenile oral surface was never deeply enclosed as occurs within the amniotic invagination (or vestibule) of the euechinoids, the oral surface was further sunken in living larvae nine days post-fertilization (Fig. 8a). Fourteen days after fertilization, the oral surface was no longer evidently sunken, and the larval para-arms and anterior yolky mass have moved away from the oral surface toward the aboral surface of the juvenile (Fig. 8b, c).

At 101 h post-fertilization, a hydropore was evident on the dorsal surface of the larva (Fig. 7a). The location of this pore was near the median side of the base of right anterior para-arm. Sections of 101-h-old larvae showed that the hydropore was joined to the hydrocoel via a canal lined by a thick epithelium (Fig. 7e). In sections of younger larvae (88.5 h), this hydroporic canal invaginated from the larval surface but was not yet joined to the coelomic cavities. Sections of 14-day larvae showed the hydropore connected to the hydrocoel by a stone canal (Fig. 8c, e). Also by this stage, epineural folds had joined to form an epineural sinus (Fig. 8d, e).

Observations on the calcitic skeleton

Larval stages at 58, 63, 75.5, 88.5, and 101 h after fertilization were cleared to look for calcareous skeletal spicules within developing embryos. No evidence of calcification was seen in 58- and 63-h specimens, even though the latter had begun to form the para-arms (Figs. 4f and 9a). The first evidence of calcification was found in 75.5-h specimens (Fig. 9b). In these, para-arms were well formed, and podial bulges had just begun to form. One calcareous plate-like ossicle was embedded in the base of each para-arm. In the more advanced 75.5-h specimen of the two observed, a fifth calcareous ossicle was present and located centrally between the four para-arms (Fig. 9b). In 88.5-h specimens, the five ossicles had grown into plates, and those in the para-arms had formed fenestrated rods that projected toward the distal ends of the para-

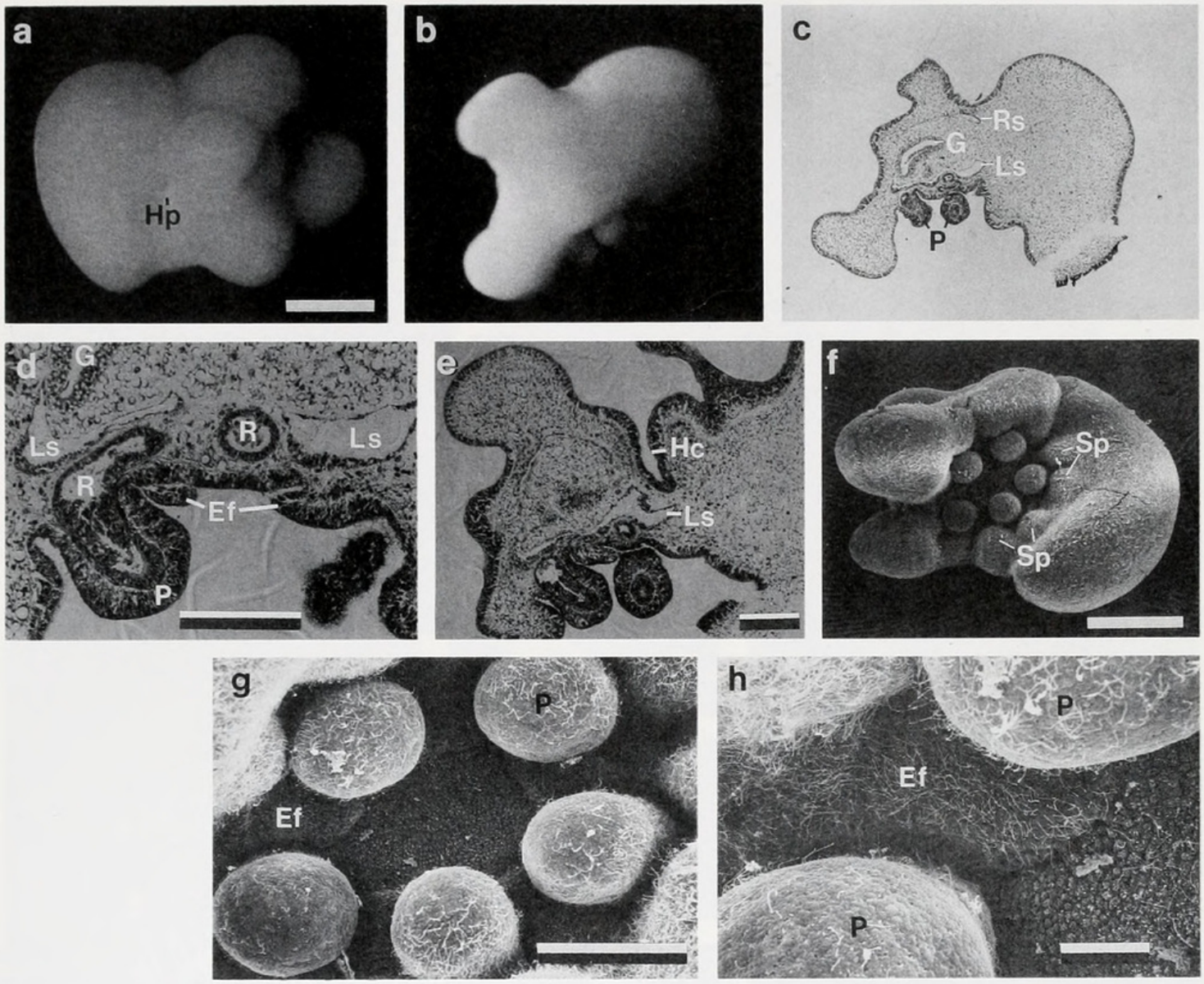

Figure 7. A 101-h larvae of *Asthenosoma ijimai*. a. Light micrograph of dorsal side of live specimen. Note the hydropore (Hp) and four para-arms (to right). The anterior end is to the left of figure. Scale bar, 0.5 mm. b. Light micrograph of ventral side of live specimen. The anterior end is to the right of figure. Same scale as a. c. Medial frontal section through larva shows developing internal structures and juvenile oral region. P, podia; Rs, right somatocoel; Ls, left somatocoel; G, remnant of archenteron and future gut. Same scale as a. d. Close-up of juvenile oral region, with podia (P), epineural folds (Ef), radial canals of water vascular system (R), and left somatocoel (Ls). Scale bar, 200 μm. e. Section at the level of the hydropore shows invaginated canal (Hc). In an adjacent section, the canal joins the hydrocoel. Scale bar, 200 μm. f. SEM of oral region of larva, shows five podia, and bulges for spines (Sp) sunken into the left larval surface. Scale bar, 0.5 mm. g. Close-up SEM of oral region showing inward movement of epineural folds (Ef) between podia (P). The infolding epidermis is strongly ciliated whereas the original floor of the oral region is sparsely ciliated. Scale bar, 200 μm. h. High magnification view of a single epineural fold (Ef) moving between two adjacent podia (P). Scale bar, 50 μm.

arms (Fig. 9c). These rods were particularly well developed in the two right para-arms and had just begun to form in the two left para-arms. The centrally located plate showed no evidence of an attached rod. Each of the calcified skeletal plates, with or without rods attached, behaved optically like a single crystal when rotated through polarized light (Fig. 9d–f). This observation confirmed the structural appearance that plates with attached rods were a single skeletal unit. Also in the 88.5-h larvae, several other calcification centers had formed and ossicles were growing (Fig. 9c).

Calcification in 101-h larvae was even more developed (Fig. 9g). These larvae had well-developed podial buds (Fig. 7b) and, on one specimen, the buds for spines were developing on the circumference of the juvenile oral surface (see Fig. 7f). As with earlier stages, fenestrated rods

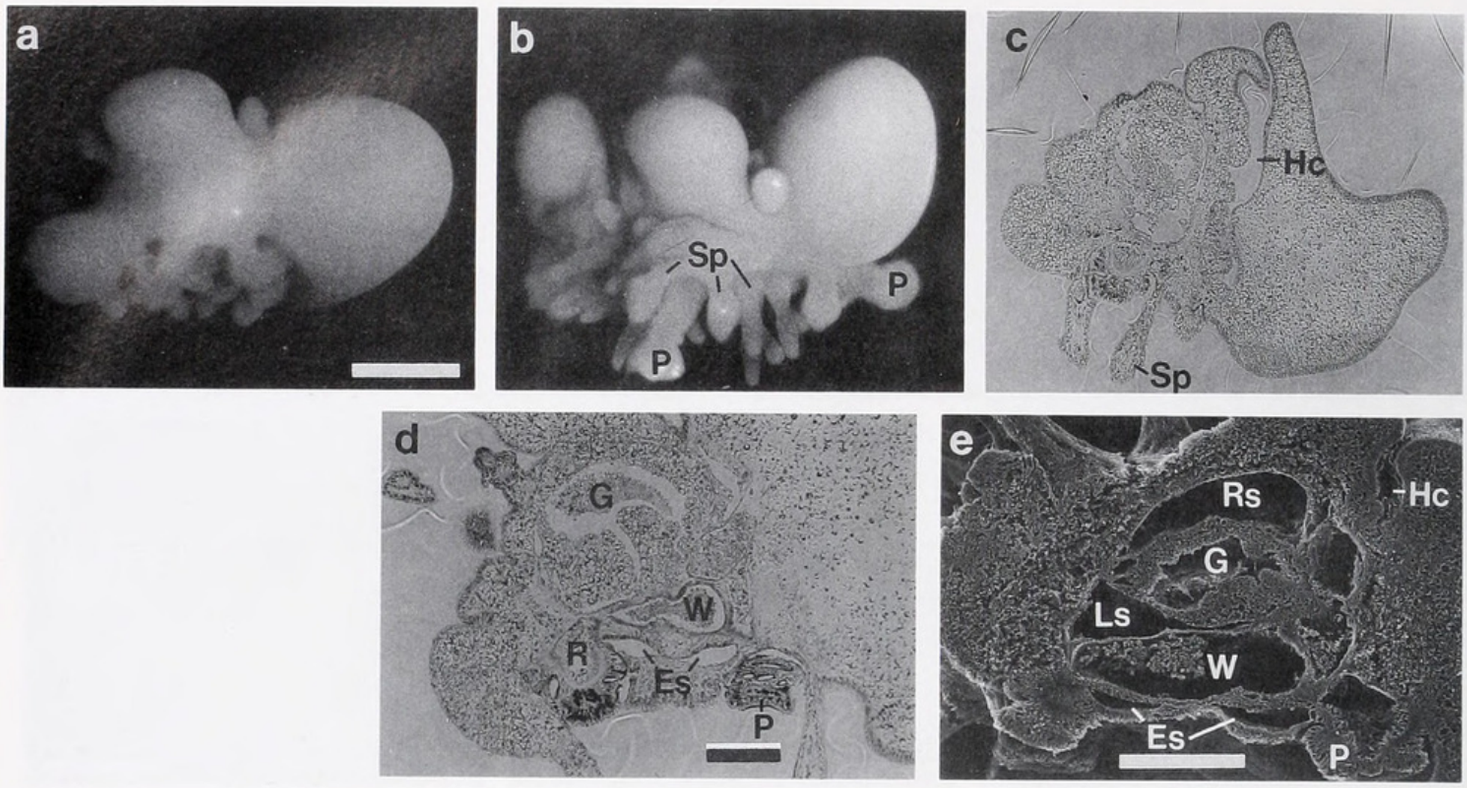

Figure 8. Later stages of larval development of *Asthenosoma ijimai*. For all specimens, the anterior end is to the right of figure. a. Ventral side of live specimen nine days after fertilization. Scale bar, 0.5 mm. b. Ventral side of live specimen 14 days after fertilization. The larval para-arms and anterior yolky mass have been contorted toward the juvenile aboral surface. P, podia; Sp, spines. Same scale as a. c. Fourteen-day post fertilization, approximate frontal section at the level of the hydropore and hydroporic canal (Hc). Same scale as a. d. Close-up of juvenile oral region showing epineural sinuses (Es), gut (G), water vascular system (W), radial canal (R), and podia (P). Compare with e. Scale bar, 200 μm. e. SEM of partially sectioned specimen showing similar structures as seen in d. Hc, hydroporic canal; Rs, right somatocoel (aboral part of body cavity); Ls, left somatocoel (oral part of body cavity). Scale bar, 200 μm.

were associated with plates in the para-arms and not with other ossicles. In one larva, each of the spine buds contained a growing spicule. In this same larva, the two ossicles of the left para-arms and three additional ossicles formed a circle beneath the juvenile oral surface that represented the five ocular plates of the adult skeleton.

Discussion

Larval structure of Asthenosoma ijimai

Our re-examination of the larval development of *Asthenosoma ijimai* has demonstrated several morphological features that were not reported in the initial study of this species. Amemiya and Tsuchiya (1979) reported that the early post-gastrula of *A. ijimai* resembled an early bipinnaria and not a prism larva. That study also reported the appearance of para-arms later in development and distinguished these projections from pluteus larval arms, because the former apparently lacked larval spicules and apparently arose from different regions of the larva. On this basis Amemiya and Tsuchiya concluded that, during development, *A. ijimai* passes from the gastrula stage to metamorphosis without showing any evidence of a pluteus larval form. They also concluded that the development of *A. ijimai* represents a second example of direct development (*sensu* Hyman, 1955) for an echinoid, the first being that of *Heliocidaris erythrogramma* (development originally described by Mortensen, 1921, but also by Williams and Anderson, 1975). Amemiya and Tsuchiya (1979) identified the surface on which para-arms arose in embryos of *Asthenosoma* as the ventral surface because of its resemblance to the ventral (oral) surface of early bipinnaria larvae of asteroids. Amemiya and Tsuchiya (1979) also incorrectly stated that the five primary podia were on the ventral surface, although Amemiya (1980) reported that primary podia arise lateral to the ventral surface. In the present study, the surface on which the para-arms arose has been identified as the dorsal surface based on observations of internal structures and on comparison with the primitive pluteus morphology. In this new orientation, the primary podia form on the left side of the larva.

A number of newly observed structures and their positions lead us to reinterpret the larval development of

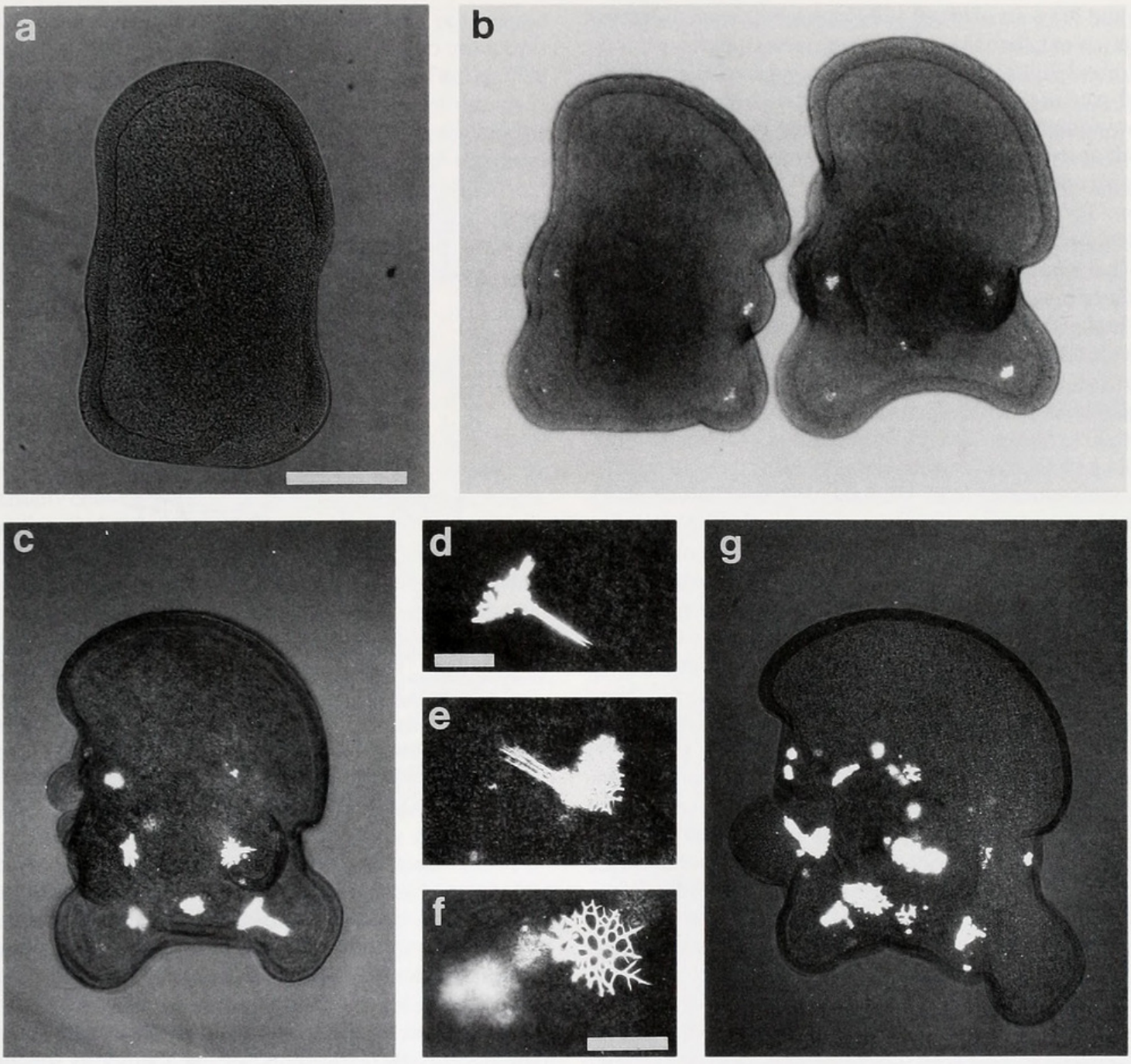

Figure 9. Skeletal development in various, cleared stages of larvae of *Asthenosoma ijimai*. All larvae are viewed from the dorsal side in partially polarized light. a. A 63-h larva shows no evidence of calcareous skeletal elements. Scale bar, 0.5 mm. b. Two 75.5-h specimens show the very first signs of skeletal development. One calcareous element is associated with each para-arm. The specimen on the right was an additional calcareous element centrally located between the para-arms. Same scale as a. c. A 88.5-h larva with continued skeletal development. Each calcareous element associated with a para-arm has formed a plate-like ossicle and shows substantial or initial formation of a rod attached to the plate. Other calcification centers have also begun. Same scale as a. d. Close-up of plate-like ossicle and rod from right posterior para-arm of a 88.5-h larva. Scale bar, 100 μm. e. Another plate-like ossicle and rod from a 101-h larva. Same scale as d. f. Central plate-like ossicle without an associated rod from a 101-h larva. Scale bar, 100 μm. g. A 101-h larva with many calcification sites. Same scale as a.

Asthenosoma ijimai as that of a highly modified pluteus larva. The two pair of bilaterally symmetrical para-arms arising from posterior and dorsal parts of embryo, each one containing a calcareous, fenestrated skeletal element, appear to be vestigial larval arms. We reject an alternative interpretation that the fenestrated rods are juvenile spines, because the spines form in association with plates that are separate elements (Gordon, 1926a, b). Because the skeletal elements are fenestrated, we interpret the para-arms as reduced post-oral and postero-dorsal arms (the

1st and 3rd pairs of arms) of a pluteus. Fenestrated skeletal rods are only known for these arm pairs in pluteus larvae (Mortensen, 1921; Emlet, 1982). In typical plutei, the second pair of arms to form is the anterolateral pair that always contains simple calcareous rods (Mortensen, 1921). Each anterolateral rod is an outgrowth from the pair of spicules that also form the postoral rods and body skeleton. The postoral rods are so reduced in *A. ijimai* that anterolateral rods are absent.

There are also several differences in the early formation of fenestrated spicules in a pluteus and those in *A. ijimai*. (1) In the pluteus, a fenestrated rod grows from a triradiate spicule (Okazaki, 1975) and later elaborates a plate at its proximal base (Emlet, 1985, and unpub. obs.). In contrast, skeletal elements in *A. ijimai* form proximal, reticulate plate-like ossicles that later form reduced, fenestrated rods. (2) In a pluteus, calcareous rods extend and consequently the arms elongate (*e.g.*, Okazaki, 1975); in larvae of *A. ijimai*, para-arms are already present before spicules elongate. In actuality, formation of arm buds in the absence of spicules can still occur in plutei (Yasumasu *et al.*, 1985; Emlet, pers. obs.) indicating that the epidermis of the arm regions is apparently distinct prior to its association with spicules. This last observation is consistent with the formation of arm buds in *A. ijimai*.

Additional support for the identification of the para-arms as homologues of the first and third arm pairs of a pluteus larva comes from the following evaluation of arm position. Rather than being directed anteriorly (in the direction of swimming) as they are for a pluteus larva, arms and their associated skeletal elements are reflected dorsally and posteriorly at the surface of the very large yolky larva (Fig. 10). In echinoids with plutei, the gastrula forms a prism larva when rods of the first pair of larval spicules lengthen into postoral, body, and anterolateral rods and deform the ectoderm (Horstadius, 1939; Okazaki, 1975). The prism's ventral surface (defined by the association of the archenteron tip with that surface) flattens to become the pluteus oral surface; the prism's dorsal surface (opposite the ventral surface) distends to become the aboral surface, terminating at the posterior end of the pluteus. During the prism stage, the ciliated band forms and serves as a landmark dividing oral and aboral ectoderm (*c.f.*, Davidson, 1986). The postoral arms grow anteriorly from the positions lateral to the blastopore. Late in the four-armed stage, a second pair of triradiate spicules appears at dorsolateral edges of aboral surface near the ciliated band (see Fig. 10), and these form the (usually) fenestrated posterodorsal rods (*e.g.*, Mortensen, 1921; Okazaki, 1975). The postoral and posterodorsal arms thus extend the ciliated band anteriorly and are located at the edge of the concave oral and convex aboral ectoderm. If the posterior end of the pluteus were not convex, and if the aboral surface lay in one plane, the positions of the postoral and

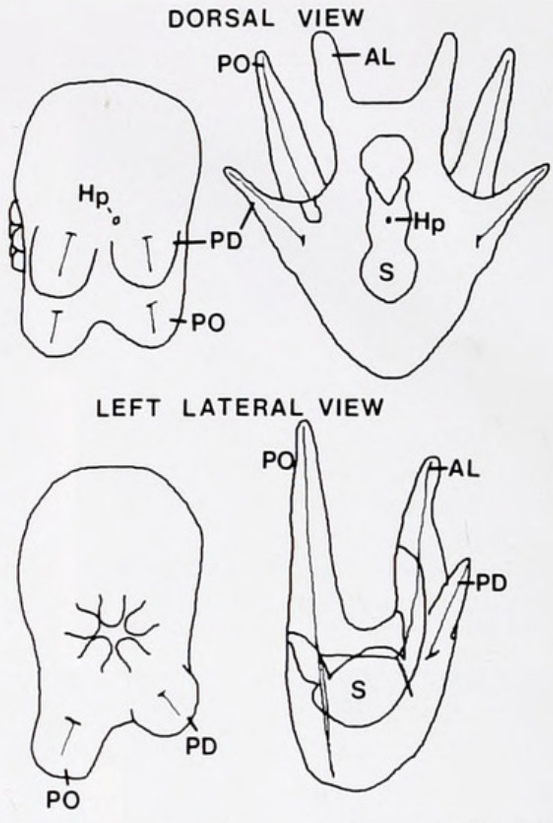

Figure 10. Schematic of a larva of *Asthenosoma ijimai* and a pluteus (*Strongylocentrotus franciscanus*) viewed from dorsal and left lateral orientations. In *A. ijimai* the para-arms are reflected posteriorly and contain reduced skeletal elements. These bilaterally symmetric arms and spicules are in positions that can be considered homologous with the postoral (PO) and posterodorsal arms (PD) of the pluteus. The anterolateral arms and rods (AL) have been lost in *A. ijimai*. Hp, hydropore; S, stomach.

posterodorsal arms of a pluteus would conform with the para-arms of *Asthenosoma ijimai* (see Fig. 10). This description is consistent with the hypothesis of homology between the identified arms and skeletal elements in plutei and larvae of *A. ijimai*.

The position of another newly observed structure, the hydropore, is also consistent with and supports this interpretation of vestiges of pluteus larval development. In both pluteus larvae and those of *Asthenosoma ijimai*, the hydropore opens medially to, and anterior of, the bases of the posterodorsal arms (Figs. 10, 7a). A clear difference is, however, that the hydropore opens just after coelom formation in pluteus development and it opens only after advanced coelomic development in *A. ijimai*.

If the boundary between oral and aboral ectoderm has remained associated with the epidermal regions of the arms, this reinterpretation of the larval form of *Asthenosoma ijimai* implies that the large, rounded, anterior end of the larva is covered by oral ectoderm and that aboral ectoderm may be restricted to that region associated with the para-arms. For *A. ijimai*, there may be a reversal in the relative area (and shape) of the oral ectoderm and aboral ectoderm compared to that in plutei (Fig. 10). It

may be possible to test this hypothesis with cell lineage studies or with immunocytochemical probes to transcripts of the CyIII actin gene or the Spec gene, which are specific to aboral ectoderm in plutei of *Strongylocentrotus purpuratus* (Cox *et al.,* 1986; Davidson, 1986). Enlargement of the oral ectoderm and reduction of aboral ectoderm has been demonstrated in cell lineage studies of *Heliocidaris erythrogramma* (Wray and Raff, 1990). Further work will be required to determine whether this apparent similarity represents a new case of parallelism in echinoid developmental patterns.

Comparisons between pluteus development and modified development

Even though larvae of *Asthenosoma ijimai* retain several reduced pluteus structures, several other features are partially convergent with other echinoid species that have modified development. Developmental comparisons among species that form plutei, *A. ijimai,* and other species with modified development allows inferences about morphogenetic changes that may occur during evolution from pluteus development to highly modified (*e.g.,* direct) development.

An equal fourth cleavage, documented here for *Asthenosoma ijimai,* is a common feature of species with highly modified development and is correlated with the production of a large number of mesenchyme cells (Raff, 1987; Parks *et al.,* 1989). Raff (1987) suggested that the large number of mesenchyme cells is a requirement for acceleration of development of the adult rudiment. For *A. ijimai,* only a fraction of the large number of blastocoelic cells become skeletogenic and only after a delay relative to species with feeding larvae. A large number of mesenchyme cells is also produced from the relatively large micromeres of an unequal fourth cleavage by embryos of *Peronella japonica,* and some of these cells also produce larval skeletal rods (Okazaki and Dan, 1954; Okazaki, 1975). These comparisons suggest that the loss of the expression of larval skeleton is independent of, and follows amplification of, the cell lineage that putatively produces adult skeleton.

The growth and behavior of the archenteron and coeloms of *Asthenosoma ijimai* appears to be intermediate between that of species with pluteus development and that of the other species with modified development. In species with feeding larvae, the archenteron grows into the blastocoel, reaching approximately $^4/_5$ of the distance toward the animal pole prior to bending toward, and attaching to, the blastocoel wall where the larval mouth forms. In most species for which modified development has been described, the archenteron invaginates less than half way into the blastocoel: *Peronella japonica* (Mortensen, 1921; Okazaki, 1975); *Heliocidaris erythrogramma* (Williams and Anderson, 1975; Wray and Raff, 1989); *Phyllacanthus parvispinus* (Parks *et al.,* 1989). In *Asthenosoma ijimai,* the archenteron invaginated as much as $^2/_3$ of the way into the blastocoel. Unlike what has been reported for other species with modified development, in *A. ijimai* the tip of the archenteron curved toward the ventral surface of the blastocoel and subsequently underwent torsion to the left side of the larva.

Enterocoely, where left and right coelomic pouches are budded off completely from the anterior end of the archenteron, is the only means of coelom formation known in pluteus development (*e.g.,* Okazaki, 1975). Both of these pouches divide again to form anterior axocoelic and posterior somatocoelic sacs. The left axocoel subsequently grows a canal to form the dorsal hydropore, and another extension of this sac forms the left hydrocoel (Hyman, 1955). Development of bilateral coelomic pouches followed by posterior growth and formation of somatocoels also occurs in *Asthenosoma ijimai,* yet in a distinctive way: two outpocketings from the archenteron form the left and right somatocoels, and precocious hydrocoelic lobes grow from the tip of the archenteron (Figs. 4; 5c, d). No obvious axocoelic sacs and no hydroporic canal are formed during this sequence. Later, a hydropore does form and joins with the water vascular system. In other species with modified development, the coelomic pouches are usually produced in pairs at the tip of the archenteron, with one sac substantially larger than the other (*e.g.,* Williams and Anderson, 1975; Wray and Raff, 1989). Several species with highly modified development are reported to form additional coelomic sacs by shizocoely from aggregated mesenchyme cells (*e.g.,* Williams and Anderson, 1975; Schatt, 1985). These patterns suggest that a transition from enterocoely to a combination of enterocoely and shizocoely may take place only after considerable modification of development has already occurred. For species with modified development in general, detailed descriptions of how coelomic pouches give rise to different coelomic sacs are currently lacking, and additional studies are needed.

The orientation of the adult oral-aboral axis relative to the plane of bilateral symmetry of the pluteus is conserved in several species with modified development, but is apparently lost in others. Loss of symmetry is not related to the degree of loss of pluteus features. Evidence for retention of pluteus larval symmetry and its relation to juvenile rudiment formation has already been presented for *Asthenosoma ijimai.* In *Phyllacanthus imperialis,* with two pairs of larval arms, a reduced preoral region, and no larval mouth, the juvenile oral surface forms on the left side of the larval body (Olson *et al.,* 1988). A reduced bilateral symmetry is also present in *Heliocidaris erythrogramma,* which has one coelomic pouch (the left one) larger than the other (Williams and Anderson, 1975) and

a bilaterally symmetric (larval) serotonergic ganglion (Bisgrove and Raff, 1989). In *H. erythrogramma,* the coincident arrangement of these two sources of bilaterality provides evidence for conservation of the adult oral-aboral axis (Bisgrove and Raff, 1989). Departures from conservation of relative positions of larval and adult axes occur for three other species. *Peronella japonica* and *P. rubra* (with very similar development) have bilaterally symmetric larvae, but the juvenile rudiment forms centrally, with the juvenile oral surface directed anteriorly and dorsally (Okazaki, 1975; Amemiya and Emlet, unpub. obs.). Loss of the primitive larval-adult arrangement of axes may be related to the retention of one pair of larval arms (postorals) and the loss of the more dorsal pair. Retention of only one well-developed pair of larval arms may force the rudiment to develop on the dorsal side, whereas in the absence or reduction of both pairs (*e.g., H. erythrogramma, A. ijimai*), or retention of both pairs (*P. imperialis*), the juvenile oral surface would not be shifted. This mechanistic hypothesis does not apply to *Phyllacanthus parvispinus* which lacks a larval skeleton and has bilobed, asymmetric coeloms which do not coincide with the orientation of the serotonergic neurons (Park *et al.,* 1989). Further morphological studies of the formation and growth of the archenteron and coeloms of both *P. japonica* and *P. parvispinus* are needed to determine how primitive larval and adult oral aboral axes have been rearranged.

Euechinoid characters in echinothurioid development

Early ingression of cells from the blastular wall in *Asthenosoma ijimai* (Fig. 1d) is comparable to primary mesenchyme ingression, which occurs prior to gastrulation in other euechinoids and is different from the later ingression known among the cidaroids (Schroeder, 1981).

Beginning with the first appearance of podia and continuing until the adult skeleton is well-developed (Fig. 8b, c), the juvenile oral region of *Asthenosoma ijimai* sinks into the surface of the larva (Figs. 7b, c, f; 8a). Within this indentation, both podia and oral spines grow. Though there is no early invagination and enclosure of the juvenile oral surface that could be clearly identified as an amniotic invagination, the strong indentation may be a morphogenetic process equivalent to vestibule formation. Other species with modified development either have or lack an amniotic invagination, consistent with their phylogenetic position as cidaroids or euechinoids (see Parks *et al.,* 1989), and this sunken condition is, therefore, not simply due to the yolkiness of the larva. A comparison of our figures with those of the cidaroid *Phyllacanthus parvispinus,* which lacks an amniotic invagination, shows that the oral surface of *A. ijimai* is considerably more indented than that of the cidaroid (Parks *et al.,* 1989, fig. 3f). Our observations raise the possibility that a partial, possibly primitive, form of an amniotic invagination may be present in echinothurioids.

Parks *et al.* (1989) also examined sectioned material of *A. ijimai* and concluded that an amniotic invagination was absent. These authors hypothesized that an amniotic invagination arose in the euechinoid lineage after the echinothurioid branch. They hypothesized further that, because the two most primitive lineages of echinoids, the cidaroids and echinothurioids, lacked an amniotic invagination, the absence of this character was primitive for echinoids. Our observations suggest that their first phylogenetic hypothesis may not be accurate, but our findings are consistent with the hypothesis that the amniotic invagination is a derived character in euechinoids. Based on comparisons of the fate of larval epidermis among echinoderm classes, Emlet (1988) suggested the same hypothesis that the primitive condition for echinoids is the absence of an amniotic invagination.

The formation of epineural sinuses in *Asthenosoma ijimai* matches very closely the original descriptions of the same process occurring within the amniotic invagination of other euechinoids [compare Fig. 7f–h with original text-figs. e–h of von Ubisch, 1913 (text-figs. f, g reprinted in Hyman, 1955, p. 497)]. By contrast, epineural sinus formation in this echinothurioid differs from that described for the cidaroid, *Eucidaris thouarsi* (Emlet, 1988). In *E. thouarsi,* epineural folds were present, but not clearly evident when observed by SEM. Sections of *E. thouarsi* showed epineural folds closely adhered to the developing juvenile oral surface on the left side of the larva, whereas in euechinoids, the epineural folds were not so closely adhered. Emlet (1988) hypothesized that the pattern in *E. thouarsi* might reflect a different mechanism of epineural fold movement (from that in euechinoids) but might also result from the open condition of the surface upon which this process occurs in *E. thouarsi.* The largely open nature of the oral surface in *A. ijimai,* and the distinctly euechinoid appearance of its epineural folds, suggest that the open condition of the epineural folds in *E. thouarsi* was not a cause for their appearance. This observation dismisses Emlet's (1988) hypothesized explanation for convergence of the epineural folds of *E. thouarsi* and the ophiuroid, *Ophiopholis aculeata* (Olsen, 1942) but leaves standing the hypothesis that cidaroids and ophiuroids have similar means of epineural sinus formation (Emlet, 1988).

Conclusions

In this re-examination of the larval morphogenesis of *Asthenosoma ijimai,* evidence has been presented to show that *A. ijimai* has retained previously unrecognized, reduced pluteus characters. As such, this larval form is the most reduced pluteus yet described, being considerably

more modified than larvae of *Phyllacanthus imperialis* (Olson *et al.*, 1988) and *Peronella japonica*. This contribution brings to three the number of lineages with modified development and with pluteus characters that are retained to varying degrees. In contrast, four lineages (*Phyllacanthus parvispinus, Heliocidaris erythrogramma,* a temnopleuroid, and *Abatus* species) have lost most, if not all, primitive larval characters. (The genus *Phyllacanthus* stands alone as being represented in both groups, but it is not known whether non-feeding development has evolved independently for the two species: *P. imperialis* and *P. parvispinus*.) Comparative experimental and cell lineage information about species with reduced larval features must now be collected if we are to determine 1) whether there is a common, possibly convergent, theme of developmental changes, or 2) whether modifications to cleavage and cell lineage fates are additional changes occurring after the loss of feeding and the reduction of the pluteus form. The detailed description presented here adds to the growing collection of comparative data on modified development; but it also indicates that the basic morphological changes occurring in other species with modified development—including two that are already well studied, *Heliocidaris erythrogramma* and *Peronella japonica*—ought to be re-examined.

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