Left-right positioning of the adult rudiment in sea urchin larvae is directed by the right side

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SUMMARY

Indirect-developing sea urchins eventually form an adult rudiment on the left side through differential left-right development in the late larval stages. Components of the adult rudiment, such as the hydropore canal, the hydrocoel and the primary vestibule, all develop on the left side alone, and are the initial morphological traits that exhibit left-right differences. Although it has previously been shown that partial embryos dissected in cleavage stages correctly determine the normal left-right placement of the adult rudiment, the timing and the mechanism that determine left-right polarity during normal development remain unknown. In order to determine these, we have carried out a series of regional operations in two indirect-developing sea urchin species. We excised all or a part of tissue on the left or right side of the embryos during the early gastrula stage and the two-armed pluteus stage, and examined the left-right position of the adult rudiment, and of its components. Excisions of tissues on the left side of the embryos, regardless of stage, resulted in formation of a left adult rudiment, as in normal development. By contrast,

excisions on the right side of the embryos resulted in three different types of impairment in the left-right placement of the adult rudiment in a stage-dependent manner. Generally, when the adult rudiment was definitively formed only on the right side of the larvae, no trace of basic development of the components of the adult rudiment was found on the left side, indicating that a right adult rudiment results from reversal of the initial left-right polarity but not from a later inhibitory effect on the development of an adult rudiment. Thus, we suggest that determination of the left-right placement of the adult rudiment depends on a process, which is directed by the right side, of polarity establishment during the gastrula and the prism stages; however, but commitment of the cell fate to initiate formation of the adult rudiment occurs later than the two-armed pluteus stage.

Key words: Sea urchin, Adult rudiment, Left-right asymmetry, Half embryo, Handedness

INTRODUCTION

Indirect-developing sea urchins pass through planktotrophic larval stages during which they form an adult rudiment, and then metamorphose into benthic juveniles (Okazaki, 1975). The structures of the larva are arranged on the basis of animal-vegetal (AV), oral-aboral (OA) and left-right (LR) axes (Hörstadius, 1973; Davison et al., 1998): the archenteron elongates along the AV axis during the early gastrula stage, then begins to bend along the OA axis towards the oral ectoderm to form a larval mouth, and the tip of the archenteron subsequently buds out the bilateral coelomic sacs along the LR axis. The morphology of the larva at the beginning of the larval stage is basically LR symmetric. At later stages, however, the larva undergoes differential LR development to form the adult rudiment on the left side of the body (Czihak, 1965; Okazaki, 1975).

The adult rudiment comprises the left coelomic sac and the vestibular ectoderm, which are initially located remote from each other within the left side of the larva. During the initial

stage of development of the adult rudiment, the hydropore canal and the hydrocoel develop in the left coelomic sac, and a small epithelial invagination begins to develop on the left side of the oral ectoderm as the primary vestibule (Runnström, 1917; Gustafson and Wolpert, 1963; Czihak, 1965; Hörstadius, 1973; Pehrson and Cohen, 1986). The corresponding parts on the right side of the larva do not develop these organs, so that the hydropore canal, the hydrocoel and the vestibule are left-side-specific traits in normal development. Although the exact timing of development of these organs differs between different species, these traits characterize the earliest LR asymmetry in the morphology of the larva generally among indirect-developing species (Okazaki, 1975; Raff, 1987).

Previous studies of indirect-developing sea urchins have shown that the polarity for differential LR development can be re-established in half embryos dissected meridionally to the AV axis. The first and second cleavages are meridional to the AV axis, and the halves separated along these planes in early development up to the blastula stage have been shown to develop into the pluteus (Hörstadius and Wolsky, 1936;

Hörstadius, 1973), and further into sexually mature sea urchins (Marcus, 1979; Cameron et al., 1996). The ability to re-establish LR polarity in the meridional twins of *Lytechinus variegatus* was studied by McCain and McClay (McCain and McClay, 1994). Their study demonstrated that the meridional halves of the cleavage stage were able to coordinate the polarity between the OA and the LR axes to re-establish the normal LR development of the hydropore canal. The meridional dissection was performed as late as the mesenchyme blastula stage. Although 22% of the meridional halves showed abnormalities, such as a right pore canal or bilateral pore canals, many of the mesenchyme blastula halves (78%) re-established normal LR polarity. The study indicated that the meridional halves re-establish normal LR polarity during cleavage, and the ability of the halves to re-establish normal LR polarity was not completely lost as late as the mesenchyme blastula stage (McCain and McClay, 1994).

Development of the adult rudiment is accelerated in a direct-developing sea urchin, *Heliocidaris erythrogramma,* whose vestibule develops soon after the gastrula stage (Williams and Anderson, 1975; Wray and Raff, 1990). Although the orientation of the first cleavage to the LR axis is variable in some sea urchin species (Kominami, 1988; Henry et al., 1992; Ruffins and Ettensohn, 1996; Summers et al., 1996), the first cleavage invariably occurs in the mid-sagittal plane in *H. erythrogramma* (Wray and Raff, 1990; Henry and Raff, 1990; Emlet 1995). The developmental potential of the left and right halves to re-establish the LR polarity for the vestibule was investigated in *H. erythrogramma* (Henry and Raff, 1990; Henry and Raff, 1994), by taking advantage of the first cleavage, which predicts the future left and right sides of the larvae. Both the left and right halves directed the formation of a vestibule throughout the early blastula stage, although the regulatory ability declined in the left halves as development proceeded. Normal LR polarity was demonstrated to be retained in the left halves, whereas the polarity was sometimes reversed in the right halves (Henry and Raff, 1990; Henry and Raff, 1994). The potential of the left and the right halves to re-establish LR polarity was also investigated in two starfish species (Hörstadius, 1928; Hörstadius, 1973). The LR development of the hydrocoel in a direct developer, *Asterina gibbosa*, and of the hydopore canal in an indirect developer, *Astropecten aranciacus* remained normal in the left halves dissected at the gastrula stage, but was impaired in an unpredictable manner in the right halves, with the hydrocoel or the hydropore canal being formed on the left, on the right, on both the left and the right, or on neither side (Hörstadius, 1928; Hörstadius, 1973). Thus, the results of the dissection experiments for *H. erythrogramma* and for the two starfish species all indicated that the normal LR polarity was fixed in the left halves, whereas the LR polarity was unstable in the right halves.

There have been no experiments to examine the potential of indirect-developing sea urchins to re-establish the LR polarity in half embryos that were clearly identified as having originated from the left or the right half of the embryo. The finding that the meridional halves of indirect developing species re-established normal LR polarity indicates that determination of LR polarity is mediated by cell interactions (McCain and McClay, 1994), but it is not known when or which cell interactions determine the normal LR position of the adult rudiment during development. As a first step in investigating the process that determines the LR placement of the adult rudiment in spatial and temporal contexts, we investigated whether excisions on the left and right side of the embryos have a different effect on LR placement of the adult rudiment in two indirect-developing sea urchins, *Hemicentrotus pulcherrimus* and *Scaphechinus mirabilis*. We excised whole or part of the tissue on the left or the right side of embryos during the stages that ranged from the early gastrula stage to the two-armed pluteus stage. The operated larvae were cultured and examined for the LR position of the adult rudiment, and of components of the adult rudiment that develop morphological LR differences in the larva, such as the hydropore canal, the hydrocoel and the primary vestibule. In contrast to the previous results found in the direct-developing sea urchin *H. erythrogramma* and the two starfish species, the indirect-developing sea urchins examined in the present study showed abnormal LR patterning in the left halves but not in the right halves. Furthermore, regional excisions in the mid-late gastrula stage revealed that a part of right side tissue was indispensable for the formation of an adult rudiment on the left side of the larva, whereas that on the left side was not. Thus, the left-right differential effects of excisions in the present study indicate that LR polarity in formation of the adult rudiment is directed by the right side, in larvae of the indirect-developing sea urchins.

MATERIALS AND METHODS

Treatment and culture of embryos

The sea urchins, *Hemicentrotus pulcherrimus*, were collected around the Miura and Bousou peninsulas on the Pacific coast and off Tsushima Island in the Japan Sea. The sand dollars, *Scaphechinus mirabilis,* were collected around the coasts of Iwate and Aichi Prefectures on the Pacific Ocean, in Mutsu Bay in Aomori Prefecture and off islands in the Setouchi district. To obtain gametes, a small volume of 10 mM acetylcholine chloride solution dissolved in filtered sea water (FSW) was injected into body cavity of the animals. Eggs were fertilized with diluted sperm in FSW, washed several times and cultured in a Petri dish filled with FSW. The culture temperature for early development through the two-armed pluteus stage was within the range of 12-18°C for *H. pulcherrimus* and 16-20°C for *S. mirabilis,* and allowed normal development of both species. At later stages, after the late two-armed pluteus, the larvae were cultured in FSW containing diatoms *Chaetoceros gracilis* as food and cultured according to the method described previously (Amemiya, 1996; Aihara and Amemiya, 2000). Culture temperature was 12°C for *H. pulcherrimus* and 18°C for *S. mirabils*. The larvae of *H. pulcherrimus* and *S. mirabilis* were examined under an optical microscope every 5 days and 3 days, respectively. After every examination, the sea water was changed, and a new suspension of diatoms was added. The larvae were cultured throughout the larval feeding stage until they underwent metamorphosis, a period of about 50 days for *H. pulcherrimus* and 30 days for *S. mirabilis.*

Microsurgery

Microsurgical operations were performed under a dissecting microscope (WILD MZ8: Leica) with a glass needle produced by manually pulling over a tiny gas flame. After the operation, the larvae were examined through an optical microscope (OPTIPHOTO or BIOPHOTO: Nikon) to confirm that the excision had been made as planned.

Vital staining

Vital staining was performed with 1% Nile Blue Sulfate (Schmid Gmbh) in a solution containing 10% agarose. A glass capillary whose opening was about the same diameter of an egg was filled with the dye and used to apply it to the embryos. The stained embryos were immediately transferred to a new dish and operated on. The position of the dye marks in the operated larvae was checked with an optical microscope.

Measurements of arm length

The length of the larval arms was measured with a micrometer under an optical microscope with a ×20 or ×10 power objective.

Statistics

The statistical significance of consistent inversion (in the right-EME cut) was tested against the null hypothesis that the probability of forming the adult rudiment on the left side or the right side of an individual was equal. A formation of a left adult rudiment and a right adult rudiment in the outcomes were scored as 1 point for the left side and for the right side, respectively. Outcomes such as bilateral adult rudiments or no adult rudiment on either side were given a score of 0.5 point each on the left and right side. Significance was tested by cumulating one tail of binomial distributions (Sokal and Rohlf, 1995).

The significance of differences of the mean and the variations in the length of arms among different groups of the left and right halves was tested by analysis of variance (ANOVA) for a single classification (Sokal and Rohlf, 1995). An F_s value of less than 1 means that variations between groups are smaller than the variation within each group, indicating that the difference between groups is not significant. Probability was tested only when F_s was more than 1.

RESULTS

Embryo dissection along the plane of bilateral symmetry in the early gastrula stage

The position of the plane of bilateral symmetry (midplane) during the cleavage of *H. pulcherrimus* and *S. mirabilis* is not predictable, because the orientation of the first cleavage plane to the midplane of the embryo varies within each species (Kominami, 1988) (T. Minokawa and S. A., unpublished). It first become possible to identify the left and the right sides of the embryos at the early gastrula stage based on bilateral clusters of cell aggregates of primary mesenchyme cells (PMCs) (Okazaki, 1975; Hardin et al., 1992). The earliest dissections in the present study were performed during two different stages: the earliest gastrula stage, immediately before buckling of the vegetal plate to generate the primary invagination of the archenteron; and the early gastrula stage, as soon as the buckling of the archenteron began.

First, we investigated whether dissection of the early gastrulae along the midplane evoked reversal of the OA polarity, as reversal of the OA polarity had previously been observed in embryos dissected meridionally during cleavage (Hörstadius and Wolsky, 1936; McCain and McClay, 1994) and in the blastula stage (Hörstadius and Wolsky, 1936). A medium concentration of Nile Blue Sulfate was used to stain the embryos, because it had been demonstrated to have no biasing effect on the polarity of the OA or the LR axis (Lindhal, 1932; Hörstadius, 1973; Henry et al., 1992; Henry and Raff, 1994), as confirmed by the following findings. The left or right side of an embryo was stained with Nile Blue Sulfate at the earliest stage of gastrulation. The embryo was immediately bisected along the midplane (Fig. 1A), and the location of the staining
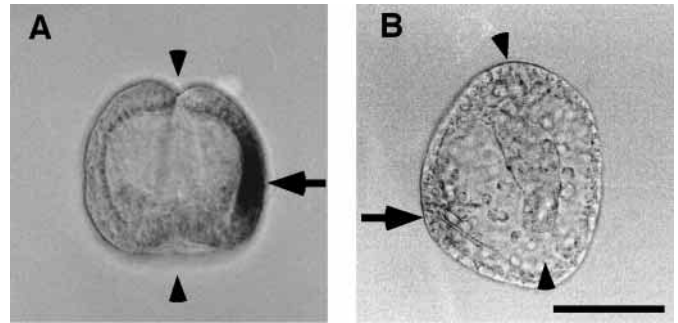

Fig. 1. Vital dye staining of the embryos. The embryos are viewed from the aboral (dorsal) side, with the animal side at the top. The left and right sides of the embryos correspond to those of the viewer. (A) An early gastrula *H. pulcherrimus* embryo stained with Nile Blue dye on the right side (arrow). The pressure of the glass needle has slightly indented the embryo along the midplane (arrowheads), through which the embryo is about to be dissected. (B) An *H. pulcherrimus* larva derived from the left half of an embryo that had been stained on the left side and dissected along the midplane in the early gastrula stage. The archenteron of the half larva is indicated by arrowheads. The staining is on the left side of the half larva (arrow). Scale bar: 100 μm.

in the each half was investigated in the two-armed pluteus stage. However, as no staining was ever detected in the opposite half, the left halves were examined after staining the left side, and the right halves were examined after staining the right side. In the 30 left halves examined, staining was always found on the left side (*n*=24/30, Fig. 1B), the only exceptions being some specimens in which the staining was so weak that its exact position could not be identified (*n*=6/30). Similarly, right staining was found exclusively on the right side (*n*=27/31) of right-half larvae, and the only exceptions were specimens in which no staining could be identified (*n*=4/31). These results indicate that left and right halves obtained by midplane dissection at the earliest gastrula stage do not reverse the polarity of the OA axis. Therefore, the left side of the left halves was always derived from the left side of the unseparated whole embryos, and the right side of the right halves from the right side.

We next examined the LR position of the adult rudiment in the half larvae. The results of the dissections of the earliest gastrula and the early gastrula were similar, and are shown in Table 1. The right halves generally developed the adult rudiment on the left side (Fig. 2A). A few right halves formed the adult rudiment on the right side, but only at a much lower rate. In the left half larvae, on the other hand, the proportions of larvae with a right adult rudiment (Fig. 2B) and with a left adult rudiment (Fig. 2C) were about equal. The results indicated that the dissection during the earliest gastrula and the early gastrula stage had different effects on the left and right halves in regard to the LR position of the adult rudiment. The LR position of the rudiment was essentially normal in the right halves, whereas there was a high rate of reversal of the LR position in the left halves. Because the earliest gastrula and the early gastrula stages were close in time, and because the effect of dissection on the LR position of the adult rudiment were basically the same at both stages, the results at both stages have been combined and the stage of the dissections will subsequently be referred to as the early gastrula stage.

Fig. 2. LR placement of the adult rudiment in left and right half of *S. mirabilis* larvae dissected in the early or mid-late gastrula stage. The LR axis of a larva coincides with that of a gastrula embryo. The axial relationship of the animal-vegetal and the oral-aboral embryonic axes is as follows: the larval oral (anterior) side corresponds to the embryonic oral side, while the larval-ventral and the larval-dorsal sides correspond to the vegetalmost and the animal-side portions, respectively, of the embryonic aboral side. The larvae are viewed from the dorsal side, with the oral side at the top. The left and right sides of the larvae correspond to those of the viewer. (A) A larva corresponding to the six-armed pluteus derived from the right half dissected in the early gastrula stage and developing an adult rudiment on the left side (arrow). (B) A larva corresponding to the six-armed pluteus derived from the left half dissected in the early gastrula stage and containing a developing hydrocoel (white arrow) on the right side. Evidence of the vestibule (black arrows) is also visible on the right side of the larva. (C) A larva corresponding to the six-armed pluteus derived from the left half dissected in the early gastrula stage, with a vestibule (black arrow) and a hydrocoel (white arrows) developing on the left side. (D) A larva corresponding to the six-armed pluteus derived from the left half dissected in the mid-late gastrula stage and developing an adult rudiment on the right side (large arrow). The left anterolateral arm (small arrow with c) and skeletal rod of the right anterolateral arm (small arrow with d) and the left (small arrow with a) and right (small arrow with e) postdorsal arms are growing in the half larva. No postoral arm is growing on the cut side of the larva, but the left postoral arm (small arrow with b) can be seen to be growing in the larva. Scale bars: 100 μm.
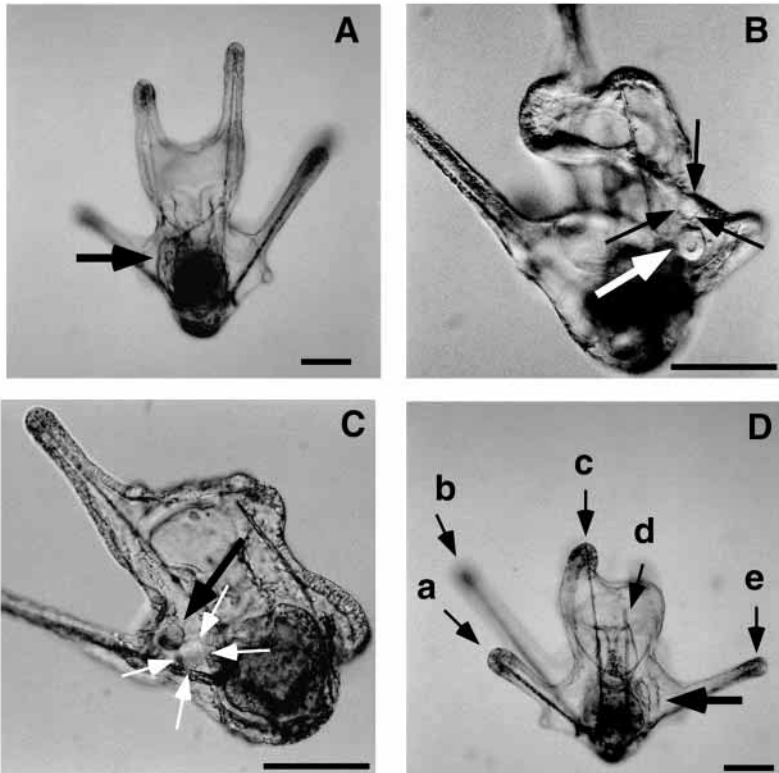
Dissection of the embryos in the midplane in the mid- to late-gastrula stage

LR placement of the adult rudiment in the larvae

The LR placement of the adult rudiment was investigated in half larvae dissected along the midplane in the mid- to late-gastrula (mid-late gastrula) stage, during which the length of the archenteron ranged from half of its full length to the full length (Table 2A). Most of the right halves of *S. mirabilis* (82%) and *H. plucherrimus* (100%) formed a left adult rudiment, and LR defects were observed in only three *S. mirabilis* larvae, two of which had no adult rudiment and the other of which had bilateral adult rudiments. The results indicate that normal LR placement can be re-established in most of right halves of both species. By contrast, the majority of the left halves of *H. plucherrimus* (75%) and of *S. mirabilis* (83%) formed a right adult rudiment (Fig. 2D), indicating a high rate of reversal of LR placement. The percentages of reversal of LR placement of the adult rudiments in the left half

Table 1. Left-right placement of the adult rudiment in half larvae dissected in the early gastrula stages

	Number operated	Number survived	Left-right placement			
			L	L and R	R	None
(A) Left half						
Earliest gastrula						
S. mirabilis	13	8	4	0	4	0
Early gastrula						
S. mirabilis	21	18	8	0	10	0
H. pulcherrimus	18	12	5	0	7	0
(B) Right half						
Earliest gastrula						
S. mirabilis	14	9	8	0	1	0
Early gastrula						
S. mirabilis	18	12	12	0	0	0
H. pulcherrimus	18	12	11	0	1	0

Table 2. Left-right placement of the adult rudiment and of the hydropore canal in half larvae dissected in the mid to late gastrula stage

(A) Left-right placement of the adult rudiment in the half larvae

	Number operated	Number survived	Left-right placement			
			L	L and R	R	None
Left half						
S. mirabilis	21	12	1	0	10	1
H. pulcherrimus	13	8	1	0	6	1
Right half						
S. mirabilis	21	17	14	1	0	2
H. pulcherrimus	13	11	11	0	0	0

(B) Left-right placement of the hydropore canal assessed in the early four-armed stage in the half larvae of *H. pulcherrimus*

	Total	Left-right placement			
		L	L and R	R	Not identified
Left half	29	0	3	22	4
Right half	22	19	0	0	3

larvae were higher than would be expected if LR placement were random ($P<0.15$ and $P<0.02$ for *H. pulcherrimus* and *S. mirabilis*, respectively, exact probability), confirming that placement of the adult rudiment was biased to the right side. Taken together, these results indicate that the right halves of both *H. pulcherrimus* and *S. mirabilis* generally form a left adult rudiment after dissection in the mid-late gastrula stage. The left halves dissected in this stage showed a strong tendency to reverse LR polarity in regard to formation of the adult rudiment.

Severe defects of LR patterning were not linked to severe defects in the larval arms

In the two-armed pluteus stage, the dissected halves had a smaller body cavity and shorter arms on the operated side than the unoperated side. The body cavity on the cut side of almost all the operated larvae grew to be almost the same size as on the other side during subsequent development, but the postoral arm on the cut side generally did not regenerate (Fig. 2D), and the length of the anterolateral arm on the cut side (arrow with d in Fig. 2D) frequently remained shorter than on the unoperated side in the late larval stage (arrow with c in Fig. 2D). The shorter arms on the cut side indicate that the dissection impaired other aspects of larval development besides LR patterning. In previous experiments in which the gastrula embryos of the starfish *Asterina gibbosa* were dissected, the defects in the LR pattering were confined to the right halves, and the survival rate of the right halves was lower than that of the left halves, indicating that the defective LR patterning was linked to defects in other aspects of development (Hörstadius, 1928; Hörstadius, 1973). Moreover, as the variation in arm length in the present study suggested that the dissections conferred different degrees of damage on different individuals, even though every embryo was cut in approximately the midplane, we investigated whether the defects of LR patterning were linked to severe impairment of arm growth. The following analysis is based on investigation of whether a consistent difference in arm length was found either between the left and right halves, or between larvae having a left adult rudiment and larvae having a right adult rudiment.

We first examined the unoperated side of the left and the right halves of *S. mirabilis* dissected during the early to late gastrula stage for differences in arm length by measuring the length of postoral-arm-rod plus body-rod (po-length), and the length of the anterolateral-arm-rod plus body-rod (al-length) (see Fig. 3A for the arm notation). The results showed no differences in po- or al-length between the left and right halves at the two-armed pluteus stage ($n=22$, Fs=0.0017 and Fs=1.33 for po- and al-length, respectively, single classification ANOVA for two samples, see Materials and Methods), or in po-length ($n=20$, Fs=0.073), al-length ($n=19$, Fs=0.13), or in length of the posterodorsal rod (pd-length) ($n=17$, Fs=0.59) between the left and right halves at the six-armed stage. These results indicate that midplane dissection has no differential effect on arm growth on the unoperated side in left and right halves dissected during the gastrula stage. The same test was performed on larvae dissected in the two-armed pluteus stage, and no significant differences were found between the left and right halves (data not shown).

We next examined arm length on the cut side as an indicator of the degree of direct damage by the operation. Because the size of the larvae differed between individuals, the ratios between arm length on the cut side and the al-length on the unoperated side were calculated. The postoral arm was usually did not regenerate on the cut side in the half larvae, and it was not assessed. The relative al-length (Fig. 3B, left) and pd-length (Fig. 3B, middle) of *S. mirabilis* larvae measured at the six-armed stage was calculated for three groups: the group with a right half and a left rudiment (□ or ■), the group with a left half and a right adult rudiment (◇ or ◆), and the group with a left half and a left adult rudiment (△ or ▲). Each dot indicates the arm length of one individual. As indicated by the scattering of dots at different levels along the vertical axis, the relative length of both the al- and pd-arms varied between different individuals within each group. The range of variations in both al-arm and pd-arm lengths in the three groups substantially overlapped, and the absence of any significant differences between the arm length for the three groups indicated that the operation inflicted similar damage on the growth of the arms on the cut side in *S. mirabilis* (Fs=2.30, 38 degrees of freedom, $P\gg0.5$ for pd-length, Fs=0.213, 37 degrees of freedom for al-length, ANOVA with three samples). No significant differences were found between the relative al-length of larvae with a left adult rudiment (+) and larvae with a right adult rudiment (×) ($n=9$, Fs=0.99) among *H. pulcherrimius* left halves dissected at the early gastrula stage (Fig. 3B, right).

To summarize, arm length on the cut side and on the unoperated side did not significantly differ either between the left and right halves, or between larvae with a left adult rudiment and larvae with a right adult rudiment in the left halves. These results indicated that even though LR patterning was defective in the left halves alone, there was no difference in damage to arm growth between the left and the right halves. Reversal of the LR position of the adult rudiment was not linked with the severe damage evaluated on the basis of arm length.

LR positioning of the hydropore canal in *H. pulcherrimus* half larvae

We next investigated the LR position of the hydropore canal in the left and the right halves of *H. pulcherrimus* as the earliest morphological LR marker in this species. The hydropore canal is a tubular organ that develops from the left coelomic sac and connects the coelomic cavity of the adult rudiment to the dorsal opening. It normally develops only on the left side. In several species of sea urchins, including *H. pulcherrimus*, the left hydropore canal forms during the late two-armed or early four-armed pluteus stage (Gustafson and Wolpert, 1963; Pehrson and Cohen, 1986; McCain and McClay, 1994), and in other species, including *S. mirabilis*, it develops later, during the six-armed stage. Although formation of the hydropore canal usually results in eventual development of the adult rudiment, degeneration of the pore canal sometimes occurs under experimental conditions. For example, two types of abnormal LR patterning of the hydropore canal and adult rudiment have been reported for the meridional halves of *Lytechinus variegatus*: a right pore canal and bilateral pore canals (McCain and McClay, 1994), and in one of the larvae with bilateral pore canals in that study the left canal degenerated and the adult rudiment developed on the right side alone. In the present study, the majority of the left halves dissected in the mid-late

gastrula stage formed a right adult rudiment, and no larvae with bilateral adult rudiments were found (Table 2A). The present results raise two possibilities: (1) that the hydropore canals develop on both sides in (some of) the left halves but the left one degenerates subsequently; or (2) that the hydropore canal develops on only the right side of the left halves. To determine which actually occurs, we examined the LR position of the hydropore canal in the half larvae of *H. pulcherrimus* dissected in the mid-late gastrula stage (Table 2B, Fig. 4).

When the LR position of the hydropore canal was assessed in 29 left halves in the early four-armed pluteus stage, 22 were found to have developed a right pore canal (Fig. 4A,B), three had developed bilateral pore canals and four had no identifiable pore canal on either side. The later development of eight of the 22 four-armed plutei with a right pore canal was observed, and all eight eventually developed a right adult rudiment. These findings showed that the pore canal did not develop on the left side in the majority of the left halves (Fig. 4A). Two of the three four-armed plutei with bilateral pore canals survived; one developed a right adult rudiment and the other developed a left adult rudiment. This finding showed that degeneration of the pore canal occurred in the minority of the left halves. The later development of the four left halves with no identifiable pore canal at the four-armed stage was also examined.

Three of them survived; two developed a right adult rudiment and one developed bilateral adult rudiments. This finding shows that the absence of a pore canal at the four-armed stage does not necessarily mean no formation of the adult rudiment on that side, but it does imply retarded development of the pore canal.

The position of the pore canal in the right halves was examined next. Of the 22 right halves examined in the four-

Fig. 3. Comparison of the arm length of larvae derived from the left and the right halves of embryos dissected in the gastrula stage. (A) This larva corresponds to the six-armed pluteus derived from the left half and shows the parts of the larvae that were measured. The length of the anterolateral-arm-rod plus body-rod (al-length), postoral-arm-rod plus body rod (po-length), and the postdorsal-arm-rod (pd-length) were measured. (B) Differences in the arm length of the left and the right half larvae. The al-length on the unoperated side represents the length of the larva along the anteroposterior axis in the late larval stage. The ratio of the length of the arms on the cut side to the anteroposterior length of one larva is plotted, and the differences in arm length on the cut side between populations are shown. The plots for both the al- and the pd-lengths on the cut side of *S. mirabilis* show similar distributions in the group with right halves and a left rudiment, the group with left halves and a left rudiment, and the group with left halves and a right rudiment. The plots also show similar distributions for the al-length on the cut side of the *H. pulcherrimus* larvae in the group with left halves and a left rudiment, and the group with left halves and a right rudiment.

Comparison of arm length in half larvae

A   Measure of arm rods
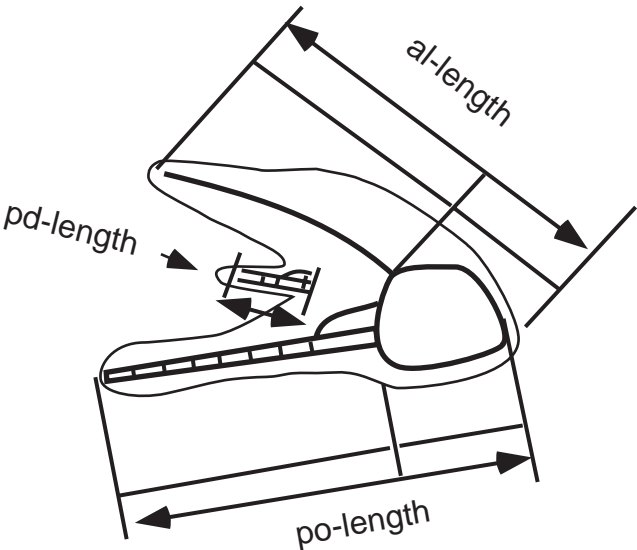
B   Length of the arms on the cut side of the larvae dissected in the midplane in the gastrula stage
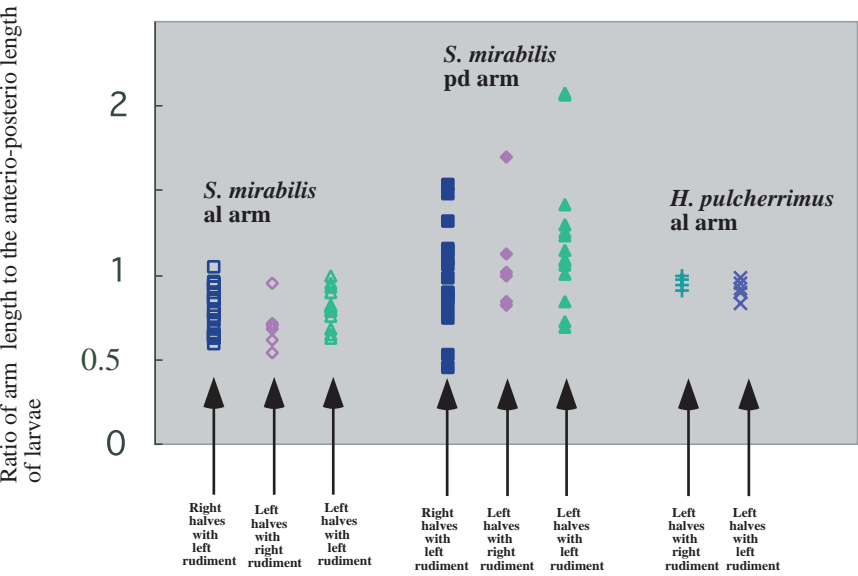
Fig. 4. LR positioning of the hydropore canal in the left half *H. pulcherrimus* larvae dissected in the mid-late gastrula stage. Two left lateral views of a four-armed pluteus, with the oral side (anterior side) to the left of the figures are shown. (A) A larva with the focal plane on the left coelomic sac (arrow). No hydrocoel has developed from the left coelomic sac. (B) The same larva as shown in A, with the focal plane on the right coelomic sac. A hydrocoel (arrow) is seen developing from the right coelomic sac. Scale bar: 100 μm.
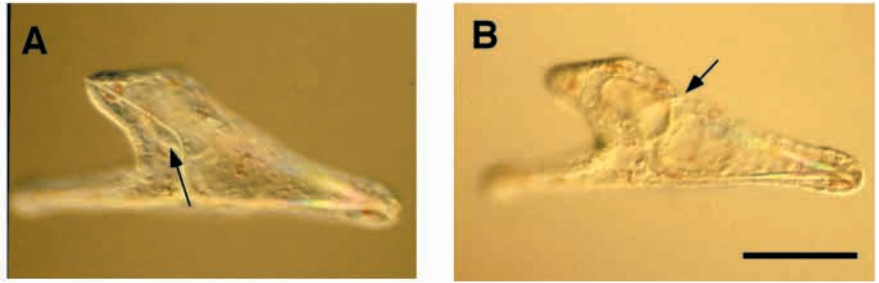
armed stage, 19 had a left pore canal and the other three had no identifiable pore canal. All three larvae with no pore canal were confirmed to eventually develop a left adult rudiment during subsequent development, indicating that the right halves consistently develop a left pore canal.

To summarize, the pore canal formed only on the right side in the majority of the left halves, indicating that development of the pore canal was generally not initiated on the left side of the left halves. In the right halves, the pore canal generally formed on the left side. In both the left and right halves, the LR position of the hydropore canal at the four-armed stage generally coincided with the LR placement of the adult rudiment, although the pore canal degenerated in a minority of the left half larvae.

Regional operations in the mid- to late-gastrula stage

Excision of tissue containing a portion of the archenteron

The results of the midplane dissection in the early gastrula and in the mid-late gastrula stage indicated frequent occurrence of reversal of LR polarity during formation of the adult rudiment in the left halves, but that reversal seldom occurred in the right halves. One possible interpretation of the different outcomes is that removal of tissue on the right side and left side has different effects on LR positioning during formation of the adult rudiment. Next, we examined the effect of regional excisions on LR placement of the adult rudiment (Fig. 5). The regional excisions were performed in the mid-late gastrula stage to take advantage of the fact that the percentage of polarity reversals in the left halves was highest at this stage so that the left and the right halves would be most distinctly different with regard to LR placement of the adult rudiment.

First, the tissue in the right- or the left-animal side of the embryos was dissected out (Fig. 5A,B). In this operation, the entire mass of tissue containing the archenteron was excised in the midplane on the animal side, but only a part of the lateral tissues was excised on the vegetal side so that tissue adjacent to the midplane was left in the embryo. The operation recapitulated the midplane dissection in the animal side, while only a smaller region was excised on the vegetal side. This operation was designated a right- or left-EME cut, as the ectodermal, mesodermal and endodermal (EME) tissue was removed on the animal side. The operated embryos developed into pluteus

larvae that had a smaller body cavity on the cut side (Fig. 5C). The unoperated side appeared morphologically normal. A coelomic sac formed on the cut side, the same as observed in previous studies, in which the archenteron was ablated in the late gastrula stage (Czihak, 1965; McClay and Logan, 1996). The coelomic sac on the cut side of the larvae in the present study was smaller than on the unoperated side.

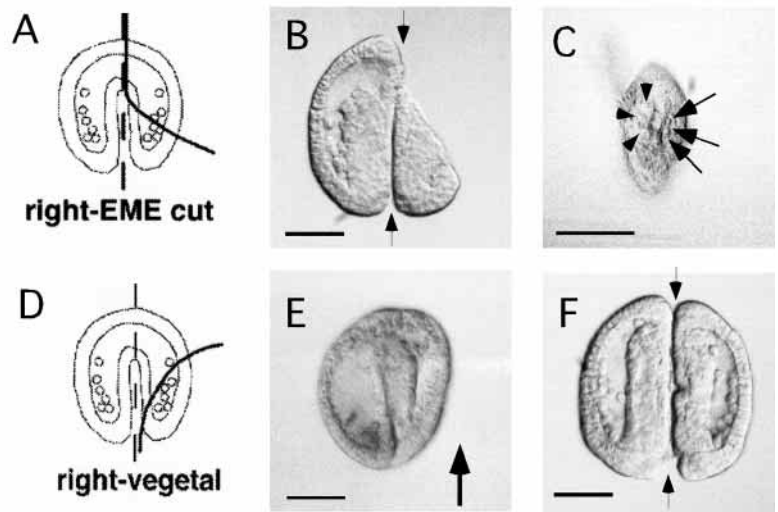The right EME cut resulted in reversal of the LR placement

Fig. 5. Excision of the tissue lateral to the midline, including a part of archenteron and ecto-mesodermal tissue. The embryos are viewed from the aboral side, with the animal side at the top, and the larvae are viewed from the dorsal side, with the oral side at the top. The left and right sides of the embryos correspond to those of the viewer. (A,B) A schematic drawing (A) and a bright field image (B) of an embryo subjected to the right EME cut. (A) The mid-sagittal plane of the embryo is represented by a broken line and the cut plane by a broad line. (B) An *S. mirabilis* embryo after the right EME cut. The right animal side of the embryo has been removed. The embryo was first marked with the glass needle in the mid-sagittal plane (arrows) to confirm that the cut region has not invaded the left side of the embryo. The upper part of the archenteron in the right side area was removed. The region corresponding to the ablated archenteron on the left side remains in the embryo. (C) A fixed specimen of two-armed pluteus larvae of *S. mirabilis* after the right EME cut in the gastrula stage. At this stage, the operated right side of the larva is smaller than the intact left side. The right coelomic sac (arrows) has formed on the right side of the foregut. The right coelomic sac is smaller than the left coelomic sac (arrowheads). (D,E). A schematic drawing (D) and a bright field image (E) of an embryo in which the right vegetal side has been removed. (D) The mid-sagittal plane of the embryo is represented by the broken line, and the cut plane by the broad line. (E) An *H. pulcherrimus* embryo whose right vegetal side (arrow) has been removed. (F) An *S. mirabilis* embryo subjected to the sham operation. The embryo is almost completely divided in the mid-sagittal plane (arrows). Scale bars: 50 μm.

of the adult rudiment in most of the operated larvae (Table 3A, Fig. 6A). Among the *H. pulcherrimus* specimens, seven out of the nine larvae had a right adult rudiment, and only two had a left adult rudiment. Among the *S. mirabilis* specimens, 12 out of the 17 larvae that survived formed a right adult rudiment, four had no adult rudiment on either side, and only one had a left adult rudiment. Statistical analysis confirmed that the right-EME cut, rather than randomizing the direction of adult rudiment formation, consistently biased it toward the right side (*H. pulcherrimus* $P<0.09$; *S. mirabilis*, $P<0.007$, exact probability). The results indicate that the LR placement of the adult rudiment was basically reversed in the larvae subjected to the right EME cut.

In previous studies, the vestibule degenerated in larvae whose left coelomic sac had been ablated (Czihak, 1965), and in larvae exposed to Ca^+- and Mg^{2+}-free sea water (Runnström, 1925), indicating that degeneration can occur even after the primary vestibule has formed. When we examined normal *S. mirabilis* larvae for the LR position of the primary vestibule, one of the earliest traits that indicates morphological LR differences in this species, the primary vestibule developed on the left side of the oral ectoderm in the early six-armed pluteus stage and was subsequently associated with the left coelomic sac (Fig. 6B). We then investigated whether the invagination of the vestibule was initiated on the left side of the larvae subjected to the right EME cut, and found that 83% of the operated larvae had a vestibule on the right side alone (Table 3B, Fig. 6C). This finding indicates that vestibular invagination of the left ectoderm generally did not occur in the larvae subjected to the right EME cut, even though the left side was not operated upon (Fig. 5A,B).

Degeneration of a right primary vestibule was found in a minority of the larvae. Of the 15 larvae that had a right vestibule, two did not develop a hydrocoel on either side of the coelomic sac, and the vestibule ultimately degenerated (Table 3B, the fourth column). These results indicated that the vestibule degenerated in only a minority of the larvae, and that formation of the primary vestibule generally led to formation of the adult rudiment, although not always. The LR placement of the hydrocoel was also examined. Formation of the hydrocoel was followed by formation of the adult rudiment in every specimen, and thus the LR placement of the hydrocoel coincided with that of the adult rudiment. The finding that the LR placement of the vestibule and the hydrocoel was generally reversed by the right EME cut was confirmed in *H. pulcherrimus* as well (data not shown). We also confirmed that the left half of both *H. pulcherrimus* and *S. mirabilis* dissected in the mid-late gastrula stage generally did not initiate formation of either the left vestibule or the left hydrocoel (data not shown).

The left-EME cut was performed in *H. pulcherrimus* embryos (Table 3A), and the adult rudiment was formed on the normal left side of all 10 larvae that survived. The results of the EME cut indicate that the effect of the left- and the right-animal side excisions of the embryo on the LR placement of the adult rudiment is basically the same as the effect of excision of the whole left side and the whole right side in the midplane dissection, respectively.

We then examined whether the excision of the right vegetal

Fig. 6. LR placement of the adult rudiment in the late pluteus *H. pulcherrimus* larvae from which part of the tissue lateral to the midplane, including the archenteron and the ecto-mesodermal tissues, has been removed in the mid-late gastrula stage. The larvae are viewed from the dorsal side, with the oral side at the top. The left and right sides of the larvae correspond to those of the viewer. (A) An eight-armed pluteus larva of *H. pulcherrimus* subjected to the right EME cut in the gastrula stage, as shown in Fig. 5A,B. The adult rudiment has formed on the right side of the larval body (arrow). (B) A control *S. mirabilis* larva in the early stage of adult rudiment formation. A vestibule (large arrow) and a hydrocoel (small arrow) have formed on the left side of the larva. There are no marks for vestibule formation in the ectoderm (large arrowhead) and for hydrocoel formation in the right coelomic sac (small arrowhead) on the right side of the larva. (C) An *S. mirabilis* pluteus larva in the early stage of adult rudiment formation that was subjected to the right EME cut in the gastrula stage. A vestibule (black arrow) and a hydrocoel (white arrow) have formed on the right side of the larva. Note that there are no marks for vestibule formation in the ectoderm (black arrowhead) or for hydrocoel formation in the left coelomic sac (white arrowhead) on the left side of the larva. (D) A six-armed pluteus *H. pulcherrimus* larva from which the tissue on the right vegetal side has been removed in the mid-late gastrula stage as shown in Fig. 5D,E. A vestibule (large arrow) and a hydrocoel (small arrow) have formed on the left side of the larval body. The right postoral arm did not regenerate and is absent, whereas the left postoral arm (arrowhead) is intact. Scale bars: 100 μm.
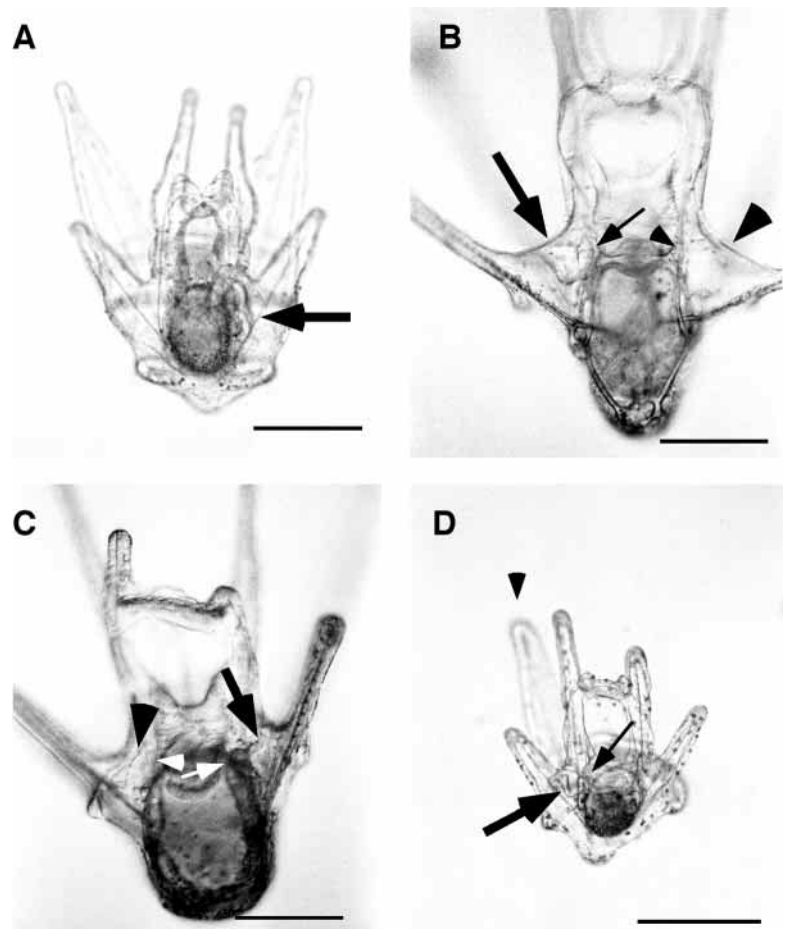
Table 3. Left-right placement of the adult rudiment and of the vestible in larvae in which portion of tissue including a part of archenteron has been removed during mid to late gastrula stage

(A) Left-right placement of the adult rudiment in the opearted larvae

	Number operated	Number survived	Left-right placement			
			L	L and R	R	None
Right EME cut (Fig. 5A,B)						
S. mirabilis	25	17	1	0	12	4
H. pulcherrimus	14	9	2	0	7	0
Left EME cut						
H. pulcherrimus	16	0	10	0	0	0
Right vegetal cut (Fig. 5D,E)						
S. mirabilis	17	11	11	0	0	0
H. pulcherrimus	24	16	16	0	0	0
Sham operation (Fig. 5F)						
S. mirabilis	19	15	15	0	0	0
H. pulcherrimus	17	13	13	0	0	0

(B) Left-right placement for the vestibular invagination and the adult rudiment in S. mirabilis larvae subjected to the right EME cut

	Total	Left-right placement of vestibule			
		L	L and R	R	None
Left-right position of adult rudiment	18	2	0	15	1
L	1	1	0	0	0
L and R	0	0	0	0	0
R	11	0	0	11	0
None	3	0	0	2	1
ND*	3	1	0	2	0

*Number of larvae that formed the vestibule but died before forming the adult rudiment.

Table 4. Left-right placement of the adult rudiment in the larvae of which part of the tissue lateral to the archenteron has been removed (EM cut) during mid to late gastrula stage

	Number operated	Number survived	Left-right placement			
			L	L and R	R	None
Right EM cut						
S. mirabilis	22	18	12	0	6	0
H. pulcherrimus	15	14	11	0	3	0
Left EM cut						
S. mirabilis	26	18	18	0	0	0
H. pulcherrimus	15	14	14	0	0	0

Excision of tissues beside the midplane region

The series of EME cut experiments revealed that removal of the right animal side was effective in disrupting the LR position of the adult rudiment, whereas the excisions that avoided this region had no effect on the LR position of the adult rudiment. We next wondered whether removal of only the epithelium on the right animal side, avoiding the archenteron, would affect LR placement (Fig. 7A,B). However, in reality it was impossible to leave the archenteron completely intact in the mid-late gastrula stage, because filopodial tracts from part of the secondary mesenchyme cells (SMCs) attached the inner wall of the ectodermal epithelium (Hardin and McClay, 1990). Besides, among four types of the SMCs, part of pigment and blastocoelar cells migrated out of the archenteron at the early gastrula stage (Okazaki, 1975; Ruffins and Ettensohn, 1996; Kominami, 2000), and as a result the cells that had already left the archenteron were removed from embryos by this cut. We refer to this operation the EM cut, as the ectodermal and mesodermal (EM) tissue was excised from the embryo. The EM cut did not directly damage the endoderm or the SMCs that evaginate from the archenteron in a later stage, such as the coelomic sac and pharyngeal muscle cells, because these cells do not leave the archenteron until the late prism stage (Gustafson and Wolpert, 1963; Okazaki, 1975; Ishimoda-Takagi, 1984; Ettensohn and Ruffins, 1993). The morphology of the tip of the archenteron at the two-armed stage appeared normal in the EM-cut larvae (Fig. 7C), whereas the coelomic sacs of the EME-cut larvae were clearly smaller on the cut side (Fig. 5C).

The right EM cut resulted in reversal of the LR position of the adult rudiment in 33% of the *S. mirabilis* larvae and 21% of the *H. pulcherrimus* larvae (Table 4), and the rest of the larvae formed a left adult rudiment. The percentage of reversal of the LR position of the rudiment was substantially lower than after the right EME cut. The hydropore canal, the hydrocoel and the primary vestibule did not develop on the left side in larvae with a right adult rudiment after the right EM cut (data not shown). The left EM cut had no effect on the normal position of the adult rudiment, indicating that the EM cut was effective in perturbing the LR patterning only on the right side. No other LR defects, such as bilateral adult rudiments or the absence of an adult rudiment were found among larvae subjected to the EM cut.

Dissection of *H. pulcherrimus* larvae in the midplane in the prism and the two-armed pluteus stages

In experiments performed in the mid-late gastrula stage, most

side affects LR placement of the adult rudiment. The tissue on the right vegetal side including the archenteron, the ectodermal epithelium and the mesenchyme cells was excised (Fig. 5D,E). All *H. pulcherrimus* and *S. mirabilis* larvae formed the adult rudiment on the left side after this operation (Table 3A, Fig. 6D), indicating that excision of the right vegetal side of the embryo had no effect on the normal LR placement of the adult rudiment. The effect of the sham operation along the midline of the embryos was examined next (Fig. 5F). The sham operation consisted of cutting the embryos almost completely in half along the midplane by stopping the cut slightly before the left and right halves were completely separated. The only connection between the left and right halves was the extracellular matrix (hyaline layer) in the plane of dissection. The embryos eventually developed into apparently normal plutei, and all formed a left adult rudiment (Table 3A). These findings indicate that the normal LR placement of the adult rudiment was not disrupted by the sham operation.

The above results of the regional operations indicated that removal of the right animal side generally resulted in reversal of the LR position of the primary vestibule and the adult rudiment, and that removal of the left animal side or the right vegetal side had no effect on the normal LR placement of the adult rudiment.

Fig. 7. Excision of ecto-mesodermal tissue lateral to the midplane, avoiding the archenteron (EM cut). (A,B) A schematic drawing (A) of ablations of ecto-mesodermal tissue lateral to the archenteron (right EM cut) and a bright field image (B) of an embryo subjected to the right EM cut. The embryos are viewed from the aboral side, with the animal side at the top. (A) The mid-sagittal plane of the embryo is represented by a broken line, and the cut plane by a broad line. (B) An *S. mirabilis* embryo subjected to the right EM cut. The right animal region lateral to the archenteron was removed from the embryo. The epithelium of the embryo has adhered at the cut plane so that the cut end is closed. The archenteron was not severely damaged and is almost intact. (C) An oral side view of a two-armed pluteus *S. mirabilis* larva subjected to the right EM cut. The animal side is towards the top, and thus the left and the right sides of the larva are on the right and the left, respectively, of the viewer. The morphology of the coelom of the right coelomic sac (arrow with r) on the cut side appears normal, the same as the left coelomic sac (arrow with l). Scale bars: 50 μm in B and 100 μm in C.

of both the left-half larvae and larvae subjected to the right EME cut formed a right adult rudiment, and components of the adult rudiment, such as the hydropore canal, the hydrocoel and the vestibule, did not develop on the left side of these larvae with a right adult rudiment. As the adult rudiment normally forms on the left side, we expected that the left halves dissected at later stages to form a left adult rudiment. To determine when the dissection resulted in formation of a left adult rudiment in the left halves, we dissected *H. pulcherrimus* embryos in the mid-sagittal plane in the prism stage and the early two-armed pluteus stage (Table 5, Fig. 8).

The left halves obtained by dissection in both the stages developed a left hydropore at the same time as the controls. The right side of the larvae was smaller at the two-armed stage, and there was no clear coelomic sac of the right side. A right coelomic sac regenerated by the four-armed pluteus stage. By the six-armed stage, the body cavity and the coelomic sac on the right side of the left halves had grown to about the same size as on the left side. All of the surviving eight left halves dissected in the prism stage and all of the surviving nine left halves dissected in the early two-armed pluteus stage formed an adult rudiment on both the left and the right sides (Fig. 8A). These results indicate that left halves dissected either in the prism stage or in the pluteus stage generally form an adult rudiment on both the left and right sides. The LR position of the adult rudiment in the right halves was examined after dissection in the early two-armed pluteus stage, and all of the nine right halves were found to have formed a left adult rudiment (Fig. 8B). This finding indicates that the right halves retain an ability to direct formation of a left adult rudiment in the early two-armed pluteus stage. The results of dissection in the late two-armed pluteus stage were basically the same for both the left and right halves (data not shown), as in the early two-armed pluteus stage.

Table 5. Left-right placement of the adult rudiment in half larvae of *H. pulcherrimus* dissected in the prism stage and the two-armed pluteus stage

	Number operated	Number survived	Left-right placement			
			L	L and R	R	None
Left half						
Prism	11	8	0	8	0	0
Pluteus	16	9	0	9	0	0
Right half						
Pluteus	16	9	9	0	0	0

To summarize, removal of all of the tissue on the right side did not affect the formation of the left adult rudiment from at the prism stage of *H. pulcherrimus* onwards. In addition, removal of the whole right side in the prism stage and two-armed pluteus stage elicited formation of the adult rudiment on the right side. The fact that the left halves did not fail to form an adult rudiment on the left side suggests that a critical change involved in the process of formation of the adult rudiment occurs on the left side of the embryo during the period between the gastrula stage and the prism stage. In addition, removal of the whole left side in the two-armed pluteus stage was shown not to affect formation of an adult rudiment on this side in larvae of *H. pulcherrimus*.

DISCUSSION

Mechanism of determination of the LR placement of the adult rudiment in indirect-developing sea urchins

Previous studies in which the sea urchin embryos were exposed to abnormal concentrations of sea water (MacBride, 1918; Ohshima, 1922) or sea water containing abnormal ion ingredients (Runnström, 1925; Hörstadius, 1973) showed that
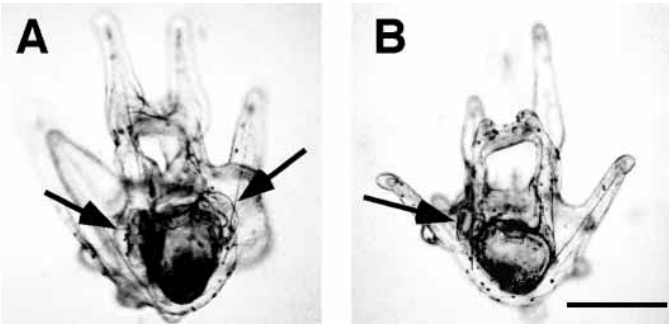
Fig. 8. LR placement of the adult rudiment in the left and right half *H. pulcherrimus* larvae dissected in the two-armed pluteus stage. The larvae are viewed from the dorsal side, with the oral side (anterior side) at the top. The left and right sides of the larvae correspond to those of the viewer. (A) A larva corresponding to the six-armed pluteus derived from the left half dissected in the two-armed pluteus stage, with an adult rudiment on both the left and the right sides (arrows). (B) A larva corresponding to the six-armed pluteus derived from the right half dissected in the two-armed pluteus stage, with an adult rudiment on the left side (arrow). Scale bar: 100 μm.

LR placement of the adult rudiment could be disrupted only in a small fraction of the larvae exposed. Pronase treatment effectively impaired the LR placement of the adult rudiment among the survivors, but significantly reduced the survival rate of the embryos (Marcus, 1981), and as a result generally less than 10% of the embryos treated exhibited abnormal LR placement. These previous studies showed that the larvae continued to retain a strong preference for establishing normal LR polarity under toxic conditions that impair development. As a whole embryo was exposed and a variety of defects other than those of the LR patterning were induced by the exposure, processes that determine LR polarity could not be identified. Czihak (Czihak, 1965) performed targeted destruction of the left coelomic sac by UV irradiation, leaving the presumptive vestibule region intact. The vestibule invagination occurred at the proper time on the left side of the larvae, indicating that the larvae had retained their ability to establish normal LR polarity with respect to formation of the vestibule (Czihak, 1965). Meridional halves dissected during cleavage have also been shown to re-establish the normal LR polarity (McCain and McClay, 1994; Cameron et al., 1996). Thus, previous studies conducted on indirect-developing sea urchins have shown a strong tendency of the larvae to establish normal LR polarity under variety of experimental conditions. The fact that meridional halves can re-establish normal LR polarity suggested that the processes that establish LR polarity during the normal development of indirect-developing sea urchins involve cell interactions (McCain and McClay, 1994). However, neither the stage profile nor the spatial profile for the cell interaction that determine LR polarity during normal development has been identified.

Regional dissection of sea urchin embryos and larvae at different stages between the early gastrula and the two-armed pluteus in this study revealed that the LR placement of the adult rudiment in larvae was basically impaired only in the left halves and not in the right halves. The present findings and previous reports suggest that the three mechanisms described below are involved in determination of LR placement of the adult rudiment.

First, the present findings suggest that in the mid- to late-gastrula (mid-late gastrula) stage the left side of the embryo is not yet specified to autonomously form the adult rudiment. The left halves dissected in the mid-late gastrula stage and the larvae subjected to the right EME cut generally failed to develop any components of the adult rudiment, such as the hydropore canal, the hydrocoel or the primary vestibule, on their left side (Fig. 4A, Fig. 6C, Table 2, Table 3B). The left side of the left halves and the right EME cut-larvae was derived from the left side of the unseparated whole embryo, as demonstrated by the vital staining experiments for the left and right halves (Fig. 1). Because the vestibule region, which is located between the anterolateral and postoral arms (Czihak, 1965; Okazaki, 1975) (Fig. 6B), is remote from the midplane, it is reasonable to conclude that the tissue adjacent to the vestibule was not displaced by the operation and shifted into the cut side of the operated larvae. Thus, the position of the vestibule region in relation to the adjacent tissue may not have been significantly changed by the operation. Indeed, the fact that many (50%) of the left halves dissected in the early gastrula stage formed a left adult rudiment indicates that the presumptive vestibule region was able to differentiate a

LR placement of the adult rudiment in half larvae

Development of left half larvae   **Development of right half larvae**
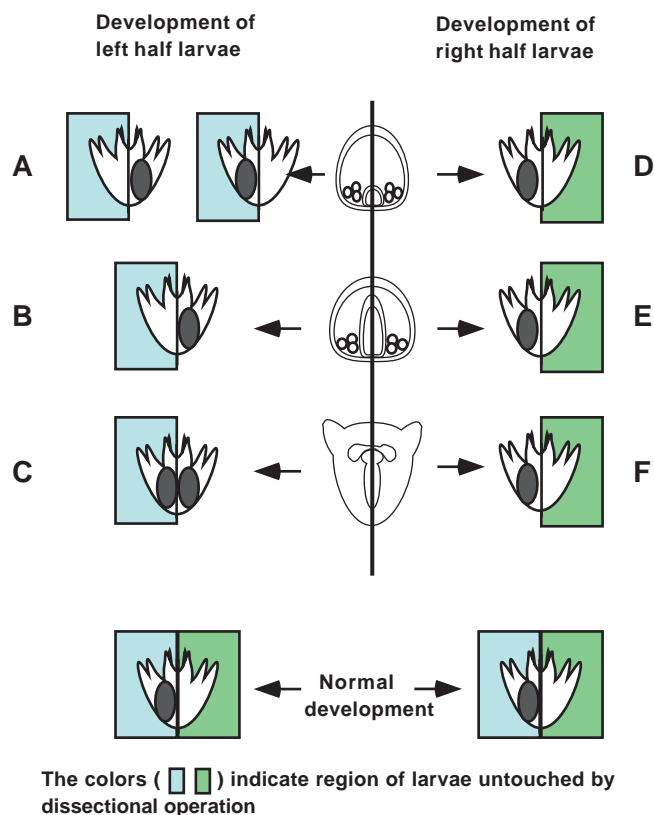
Fig. 9. A schematic representation of the LR placement of the adult rudiment in the left and the right half larvae subjected to dissection at three different stages during the period from the early gastrula stage to the two-armed pluteus stage. The left side of the left halves (colored blue in A-C) and the right side of the right halves (colored green in D-F) have been untouched by the dissecting operation. The LR placement of the adult rudiment in the left halves showed distinct patterns according to the stage at which they were dissected. Larvae with a left adult rudiment and larvae with a right adult rudiment were found in about equal proportions after dissection in the early gastrula stage (A). Almost all of the left half larvae formed a right adult rudiment after dissection in the mid-late gastrula stage (B), and the left half larvae formed a left- and a right-adult rudiment after dissection in the prism-pluteus stage (C). Normal LR placement of the adult rudiment was always re-established in the right halves dissected in the early gastrula stage (D), the mid-late gastrula (E) and in the prism-pluteus stage (F).

vestibule (Fig. 2C). Therefore, the finding that the primary vestibule invagination did not occur cannot be attributed to a direct effect of the operation. The fact that an excision far from the vestibule region altered the fate of this region suggests that the initial cues that determine the formation of the vestibule in intact whole embryos originate either in the midplane region or the right side. The fact that formation of the vestibule is a prerequisite for the formation of the adult rudiment suggests that formation of the adult rudiment cannot be initiated autonomously by the left side.

Second, the findings suggest that normal LR polarity for the direction of formation of the adult rudiment is directed by the right side. It has been shown previously that meridional halves dissected during cleavage stages are capable of coordinating

LR polarity with the polarity of the oral-aboral (OA) axis to develop a left hydrocoel (McCain and McClay, 1994). The present study showed that the establishment of LR polarity was disrupted in the left halves dissected during the period between the early gastrula and the two-armed pluteus stages (Fig. 9A-C), whereas normal LR polarity was correctly re-established in the right halves (Fig. 9D-F). This finding strongly suggests that LR polarity is coordinated with OA polarity by the right side during normal development, and therefore that the right side controls the process of polarity establishment in left side tissue. The hypothetical effect of the right side on the left side appears to proceed during the period between the mid-late gastrula stage and the prism stage, because the left halves dissected in the prism stage and the pluteus stage never failed to develop the adult rudiment on their left side (Fig. 8A, Fig. 9C, Table 5), indicating that contact with the right side of the intact whole embryos before the prism stage was sufficient to initiate the formation of the left adult rudiment and that no further influence from the normal right side was needed. This suggests that some induction from the right side to the left side that takes place between the mid-late gastrula and the prism stages is involved as one of the mechanisms that establishes normal LR polarity in the direction of formation of the adult rudiment.

Third, the findings suggest that proximate cues to initiate or not to initiate the morphogenesis of the adult rudiment are still not triggered in the two-armed pluteus stage. In the present study, the left and right half larvae of *H. pulcherrimus* dissected in the two-armed pluteus stage formed an adult rudiment on the cut side (Fig. 8A,B, Fig. 9C,F, Table 5). When an excision biased toward the right side so that it included outer portion of the right coelomic sac and the potential right vestibule region was conducted in the two-armed pluteus stage, it induced formation of an extra adult rudiment on the right side in more than 50% of the operated larvae, even though both the ectoderm along the midplane region and the digestive tract were left intact (Aihara and Amemiya, 2000). These results that excisions in two-armed pluteus larvae induced formation of the adult rudiment indicate that tissue in the vicinity of the midplane and tissue on the right side of the normal larvae retain an ability to convert the cell fate so that the tissues develop into the adult rudiment. In addition, during the assessment of LR positioning of hydropore canal in the present study, the two left halves of *H. pulcherrimus* that had bilateral hydropore canals eventually formed only one adult rudiment on the left or the right side, indicating that the LR placement of the hydopore canal does not always coincide with the eventual LR placement of the adult rudiment. Discordance between the eventual LR placement of the adult rudiment and the pore canal, the vestibule, and the hydrocoel has been reported by several predecessors (Runnström, 1917; Ohshima, 1922; Hörstadius, 1973; McCain and McClay, 1994). The discordance between the LR placement of the early traits and the eventual LR placement of the adult rudiment suggests that processes that determine whether or not to initiate the formation of the adult rudiment operate in the late pluteus stages before the morphological LR asymmetry is definitively established.

Taken together, the above findings suggest that the left side is not committed to form the adult rudiment autonomously in the mid-late gastrula stage, and that some induction from the right side to the left side that takes place between the mid-late gastrula stage and the prism-pluteus stages is involved in the

determination of normal LR polarity in the direction of formation of the adult rudiment. These findings suggest that one of crucial changes during the process of determining the LR placement of the adult rudiment occurs between the gastrula stage and the prism stage. This period coincides with the period when some evidence of LR asymmetry first arises at the tip of the archenteron, as previously shown by asymmetric expression of transcripts such as the *snail*-related gene (Hardin, 1995), β-catenin (Miller and McClay, 1997a), and cadherins (Miller and McClay, 1997b), or by asymmetric allocation of descendants of small micromeres into the coelomic sacs (Pehrson and Cohen, 1986; Ettensohn and Ruffins, 1993) (M. Aihara and S. A., unpublished). It has also been suggested previously and here in this study that the definitive decision to turn on or turn off morphogenesis of the adult rudiment is not yet complete in the two-armed pluteus stage.

Modes of re-establishing LR polarity in the left and right halves of different animal species

Previous studies of separated or conjoined half embryos of echinoderms (Hörstadius, 1928; Hörstadius, 1973; Henry and Raff, 1990; Henry and Raff, 1994) and vertebrates (Spemann and Falkenberg, 1919; Levin et al., 1996; Nascone and Mercola, 1997) showed that LR patterning defects occurred in the right halves but not in the left halves. LR polarity was either randomly re-established among different individuals (Henry and Raff, 1994) or was randomized among different organs within the same individual (Hörstadius, 1928; Hörstadius, 1973; Spemann and Falkenberg, 1919; Levin et al., 1996; Nascone and Mercola, 1997), and thus there was no consistency in the type of LR patterning defects that occurred in the right halves.

In comparison with the above results in previous studies, the results obtained for the dissected left and right halves of indirect-developing sea urchin larvae in this study showed unique features. First, LR patterning defects were generally found only in the left halves, not in the right halves (Fig. 9), providing the first examples of LR patterning defects being elicited in left halves but not in right halves. Besides the two species investigated in this study, we confirmed that LR defects in five other indirect-developing sea urchins belonging to three different orders are also confined to occurring only in the left halves after dissection in the gastrula stage (M. A. and S. A., unpublished), suggesting that the confinement of LR defects to the left halves is common to indirect-developing sea urchins. A second unique feature of our findings was uniformity in the manner of occurrence of LR defects between different individuals that was observed in the two stages: (1) LR polarity in the placement of the adult rudiment was reversed in almost all of the left halves dissected in the mid-late gastrula stage (Table 2), indicating that the reversal of polarity in the larvae was not random, which was further confirmed by the right EME cut in the same stage (Table 3); and (2) the left halves of *H. pulcherrimus* larvae dissected in the prism stage and pluteus stage consistently formed bilateral adult rudiments (Table 5). The consistent occurrence of one specific type of LR patterning defect being induced in every individual has never been found for LR halves in other animal species. Thus, the unique features found for indirect developing sea urchins in regard to bias in the occurrence of defects between the LR halves and to

uniformity in the occurrence of defects provided evidence that the confinement of defects to the right halves or the randomness in the occurrence of defects, which have been generally found in the previous studies (Spemann and Falkenberg, 1919; Wilhelmi, 1921; Hörstadius, 1928; Hörstadius, 1973; Henry and Raff, 1990; Henry and Raff, 1994; Nascone and Mercola, 1997; Brown and Wolpert, 1990; Levin, 1997; Ramsdell and Yost, 1998), are not equally conserved among animal species.

Despite the differences described above, the mode of occurrence of LR defects in all of the previous and the present experiments to produce left and right halves, including both separated and conjoined twins, is universal in that the left and right halves have shown different susceptibility, with LR defects consistently biased to occur in only one of the halves. The finding that patterning in only one side of the halves sustained the damage of the disruptions of LR interaction suggests that cell fate determination of the LR patterning of only one side is influenced by the opposite side in the normal whole embryos. Thus, these different animals appear to share a common process in which determination of cell fate on the left or right side is influenced by the other side as a general framework for the mechanism that establishes LR patterning.

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