

Sharon B. Minsuk · Mary E. Andrews · Rudolf A. Raff

## From larval bodies to adult body plans: patterning the development of the presumptive adult ectoderm in the sea urchin larva

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**Abstract** Echinoderms are unique among bilaterians for their derived, nonbilateral adult body plan. Their radial symmetry emerges from the bilateral larval body plan by the establishment of a new axis, the adult oral–aboral axis, involving local mesoderm–ectoderm interactions. We examine the mechanisms underlying this transition in the direct-developing sea urchin *Heliocidaris erythrogramma*. Adult ectoderm arises from vestibular ectoderm in the left vegetal quadrant. Inductive signals from the left coelom are required for adult ectodermal development but not for initial vestibule formation. We surgically removed gastrula archenteron, making whole-ectoderm explants, left-, right-, and animal-half ectoderm explants, and recombinants of these explants with left coelom. Vestibule formation was analyzed morphologically and with radioactive in situ hybridization with *HeET-1*, an ectodermal marker. Whole ectodermal explants in the absence of coelom developed vestibules on the left side or ventrally but not on the right side, indicating that left–right polarity is ectoderm autonomous by the gastrula stage. However, right-half ectodermal explants robustly formed vestibules that went on to form adult structures when recombined with the left coelom, indicating that the right side retains vestibule-forming potential that is normally suppressed by signals from the left-side ectoderm. Animal-half explants formed vestibules only about half the time, demonstrating that animal–vegetal axis determination occurs earlier. However, when combined with the left coe-

lom, animal-half ectoderm always formed a vestibule, indicating that the left coelom can induce vestibule formation. This suggests that although coelomic signals are not required for vestibule formation, they may play a role in coordinating the coelom–vestibule interaction that establishes the adult oral–aboral axis.

**Keywords** Echinoderms · Symmetry · Axial patterning · Vestibule

### Introduction

Studies of the larval-to-adult transition in echinoderms can help illuminate two related and fundamental problems in evolution and development. First, how do modified body plans evolve, especially when the modifications involve the radical reorganization of body axes? Such reorganization may underlie several major events in animal evolution, including the origin of Bilateria (Finnerty et al. 2004) and dorsoventral inversion during chordate evolution (Gerhart 2000), as well as the origin of pentaradial symmetry in echinoderms (Popodi and Raff 2001). Second, how have biphasic (indirect developing) life cycles evolved from monophasic (direct developing) ancestors? Such transitions have been important in bilaterian evolution, but the nature and identity of the ancestors have been controversial, and the mechanisms by which these transitions have occurred are poorly understood (Peterson et al. 2000; Sly et al. 2003).

Bilateral symmetry arose early in the evolution of animal phyla and is a fundamental and widely conserved feature of the largest of the three major animal subgroups, the Bilateria. The echinoderms have abandoned this body plan for a new one with no apparent relation to the old. Interestingly, the new, pentaradial body plan is present only in the adults; the larvae from which they develop are bilaterally symmetric.

The adult echinoderm body plan is not formed by a direct transformation of the larval body plan—such as occurs gradually in metamorphosing vertebrates or hemimetabolous insects, or even as in holometabolous insects such as *Drosophila*, in which the transformation is more abrupt. In

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S. B. Minsuk · M. E. Andrews · R. A. Raff  
Department of Biology and Indiana Molecular Biology Institute,  
Indiana University,  
Bloomington, IN, 47405, USA

Present address:

S. B. Minsuk (✉)  
Konrad Lorenz Institute for Evolution and Cognition Research,  
Adolf Lorenz Gasse 2,  
3422 Altenberg, Austria  
e-mail: sharon.minsuk@kli.ac.at  
Tel.: +43-2242-3239013  
Fax: +43-2242-323904

these and most other Bilateria, there is a one-to-one correspondence between the adult and larval body axes, and in segmented animals even between the adult and larval segments. In contrast, the echinoderm adult and larval body plans share no such correspondence (MacBride 1903; Hyman 1955; Okazaki 1975). The three orthogonal larval axes are replaced by a single oral–aboral (OA) axis around which the pentaradial adult is organized. (The larval dorsal–ventral or DV axis is often referred to as the oral–aboral axis, but this larval OA axis is not related to the adult one, and we will refer to it exclusively as the DV axis to avoid confusion.) The future adult mesoderm arises from small sets of “set-aside” cells (Davidson et al. 1995; Peterson et al. 2000; Sly et al. 2003) that develop into a series of larger coelomic cavities and interact with the ectoderm to form the adult body. Sea urchin coeloms develop asymmetrically, with three compartments on the left side and only two on the right; two of those on the left (the hydrocoel and the left somatocoel) combine with the left-side ectoderm to give rise to the pentamerous adult structures. In euechinoid sea urchins, the presumptive adult ectoderm is also a distinct set-aside tissue, which invaginates to form a pouch called the vestibule. This comes into contact with the hydrocoel, and where they meet they establish the future adult (the adult rudiment) centered on the oral pole of the adult OA axis, around which the five individual rays develop. This axis thus lies roughly along the preexisting larval left–right (LR) axis, and depends upon the formation of mesodermal and ectodermal precursors along that axis (McCain and McClay 1994; Summers et al. 1996; Henry 1998). However, the adult OA axis does not arise from a direct transformation of the larval LR axis, but arises *de novo* as a result of local interactions (Minsuk and Raff 2002, and in preparation). Although the OA axis arises along the larval LR axis in sea urchins, it is not equivalent to the larval LR axis in all echinoderms. It has shifted its orientation in the larva multiple times during echinoderm evolution. Thus, the OA axis lies along the DV axis in ophiuroids (brittle stars), and along the animal–vegetal (AV) axis in crinoids (sea lilies and feather stars), holothuroids (sea cucumbers), and the unique asteroid (starfish) *Pteraster tessellatus* (Hyman 1955; Smiley 1986; Hendler 1991; Holland 1991; Smiley et al. 1991; McEdward 1992; Janies and McEdward 1993). Among those with an AV-oriented adult axis, adult oral corresponds to the vegetal pole of crinoid embryos, but to the animal pole of holothuroids and *P. tessellatus*.

What all echinoderms have in common is the biphasic life cycle and the coelomic mesodermal set-aside tissues that define the adult axis and pentamerous body plan. In contrast, the sister group of the echinoderms, the hemichordates, shares the biphasic developmental mode and homologous coeloms (Peterson et al. 1997, 1999a; Cameron et al. 2000), but not the body plan transformation. Hemichordates retain bilateral symmetry throughout their lives, transitioning gradually from larval to adult morphology without any axial reorganization (Hyman 1959; Hadfield 1975; Peterson et al. 1999a; Urata and Yamaguchi 2004). Echinoderm life cycle and body plan evolution can thus be broken down into two steps: the origin of the biphasic mode with coelomic set-

aside cells in a bilaterally symmetric common ancestor of echinoderms and hemichordates and the subsequent reorganization(s) of the adult axes in the echinoderm lineage.

The formation of the mesodermal set-aside tissues and their interaction with ectoderm are key to understanding these events, but little is known about their regulation—how the asymmetries arise, how the ectodermal and mesodermal components are brought together at the right time and place, or how pentamery is established. These questions have been inaccessible largely due to the difficulty of studying them in the common echinoderm model species—indirect-developing sea urchins in which the rudiment develops only after a long period of larval feeding and growth. Numerous sea urchin species have functionally abandoned this stage of the life cycle, concomitantly losing, to varying degrees, the larval structures required for swimming and feeding, and shifting the developmental emphasis to early and fast rudiment formation (Raff 1996; Wray 1996). Although commonly referred to as “direct developers,” these species generally still have a bilaterally symmetric larval developmental stage (Hyman 1955; Emler 1995), albeit highly modified, followed by a metamorphosis into a pentaradial adult. This has allowed us to address the regulation of this transition in the direct developer *Heliocidaris erythrogramma*. Vestibule and coelomic development have been modified in this species, but they retain their LR asymmetry, and converge on the formation of a conserved rudiment and a typical adult (Williams and Anderson 1975; Haag and Raff 1998; Ferkowicz and Raff 2001). In addition, the much larger size of these embryos greatly facilitates microsurgical approaches (Minsuk and Raff 2002).

The vestibular ectoderm gives rise to adult structures such as the tube foot ectoderm and the central nervous system. As we showed previously (Minsuk and Raff 2002), the development of these structures requires inductive signals from the left coelom, but the initial formation of the vestibule itself takes place in the absence of coelomic mesoderm after the surgical removal of the mid-gastrula stage archenteron (from which coelomic mesoderm is derived), and is therefore ectoderm autonomous by that stage. Likewise, molecular subdivision of the vestibule (restriction of the ectodermal marker *HeARS* to the vestibule roof) requires the coelomic signal, whereas the earlier establishment of vestibule identity (down-regulation of the marker *HeET-1*, restricting it to the larval ectoderm) occurs autonomously in the absence of coelom. Here we extend this analysis to ask how the location of the vestibular ectoderm is determined. We find that the position of the vestibule on the larval left side is independent of mesodermal signaling. However, all ectoderm has the potential to differentiate as vestibule. Its restriction to a region in the left vegetal quadrant is brought about by a combination of early patterning along the AV axis, and inhibitory planar signals in the ectoderm along the LR axis that suppress vestibule development on the right side. Furthermore, although left coelom is not required for vestibule development, it can nonetheless induce vestibule development in animal pole ectoderm where

it would not otherwise occur, suggesting a role for coelomic signaling in regulating normal vestibule development.

## Materials and methods

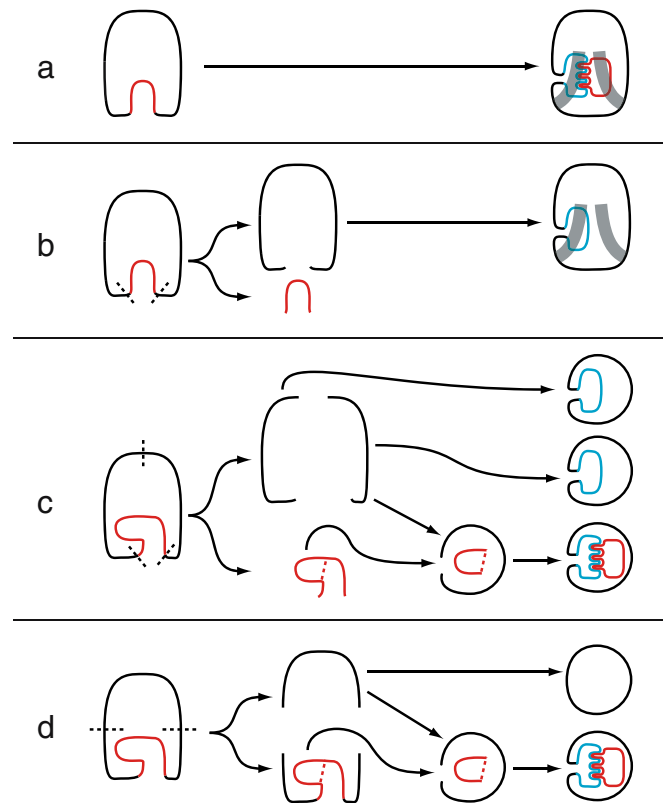
### Embryo culturing and staging

Gametes of *Heliocidaris erythrogramma* were obtained from adults injected with 0.55 M KCl. Embryos were cultured at 19–25°C, in 0.45- $\mu$ m Millipore-filtered sea water (FSW). We defined early, mid-, and late gastrula stages based on the depth of archenteron invagination, the length–diameter ratio of the embryo, the degree of left-coelom development, and internal characteristics detectable only during surgery. In lateral or DV profile, *early gastrulae* have an animal–vegetal length approximately equal to their diameter, and they have only a shallow archenteron; when surgically removed, the archenteron does not hold its shape but relaxes to a flat epithelium. *Mid-gastrulae* begin to elongate, so that the embryo length becomes noticeably greater than the diameter. The mid-gastrula archenteron forms a distinct cylindrical tube visible through the blastopore, and when surgically removed, it retains its shape. Left coelom formation begins toward the end of this stage, but is not externally visible; it can sometimes be detected during surgery as a symmetrical or asymmetrical swelling at the distal archenteron tip. No morphological LR asymmetry is present in the ectoderm, however. *Late gastrulae* have a narrower, constricted blastopore and a distinct left coelom, which is visible externally through the transparent ectoderm (see below). The ectoderm remains symmetrical. The left coelom establishes direct physical contact with the ectoderm, and adhesion between them increases. We consider the end of the late gastrula stage to be the point when this adhesion becomes too great to allow removal of the archenteron/coelom. This marks the beginning of the early larval stage, during which the vestibule and ciliated band appear. Timing of these stages varies with temperature and between spawnings. In these experiments, the mid-gastrula stage began at 13–15 h postfertilization. Late gastrula began at 17–19 h and ended around 20–21 h.

### Microsurgery

*Isolated ectodermal explants* were made as described in Minsuk and Raff (2002). Briefly, mid-gastrulae were soaked in  $\text{Ca}^{2+}$ -free seawater, and the archenteron was removed by cutting around the base of the blastopore with an eyebrow-knife. The remaining ectoderm was then cultured in FSW (Fig. 1b).

*Left- and right-half ectoderm pairs* were made at the late gastrula stage by cutting the ectoderm along the plane of bilateral symmetry before removing the archenteron, as follows. Younger gastrulae are filled with lipid droplets visible through the transparent ectoderm over the entire embryo surface (Williams and Anderson 1975; Henry et al. 1991). The first indication of axial orientation arises when a large gap in the field of droplets appears on the left side of



**Fig. 1** Schematic diagram of surgical procedures and results. *Dashed lines* indicate surgical cuts. Undifferentiated gastrula ectoderm and (in the last column) differentiated larval ectoderm are drawn in *black*; differentiated vestibular ectoderm is *blue*. Archenteron and left coelom are drawn in *red*. *Thick gray bars* in **a** and **b** show the relative position of ciliated band on the embryo surface. **a** In normal larvae, two spurs of the ciliated band flank the dorsal midline, thereby identifying the DV and LR axes. The vestibule develops on the left. The vestibule and left coelom interact to form adult structures (three tube feet shown). **b** Archenterons were removed from mid-gastrulae and discarded. The remaining ectodermal explants usually developed normal ciliated bands, allowing identification of axes. Vestibule development was heavily biased to the left side. **c** Left and right ectodermal halves were separated from the archenteron and coelom of late gastrulae. Cultured alone, both ectodermal regions developed vestibules. (Distinct larval axes could not be identified, so vestibule position was not scored.) Right-half ectoderm recombined with isolated left coelom developed rudiments. **d** Animal-half ectoderm from mid- to late gastrulae, cultured alone, frequently failed to produce vestibular ectoderm. When recombined with left coelom, vestibules and rudiments always developed

the embryo, representing the exclusion of droplets where the left coelom contacts the ectoderm (Minsuk and Raff 2005). Once the left side was identified, the ectoderm was bisected left from right, keeping the lipid gap midway between the dorsal and ventral incisions. This does not result strictly in bisection along the symmetry plane, because normal vestibule is slightly closer to the ventral than to the dorsal midline. However, it results in a right-half explant composed of the tissue most distant from any vestibular influence. Each half was then individually separated from the archenteron and coelom (Fig. 1c) and cultured in its own well of a 24-well culture dish. We thereby kept track of the

identity of each half (left or right), as well as their original pairing.

*Animal-half ectoderm* was prepared at the mid- to late gastrula stage by cutting the ectoderm along the circumference halfway between the blastopore and the animal pole (Fig. 1d). The archenteron and coelom remained entirely within the discarded vegetal half.

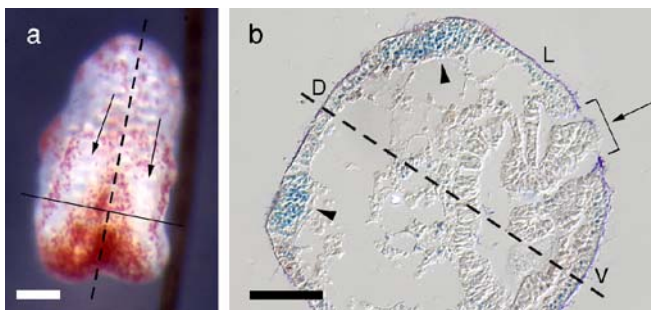
*Ectoderm–coelom recombinants* were made by cutting the left coelom away from the archenteron (Minsuk and Raff 2002) and inserting it into a right-half or animal-half ectoderm, which healed around it (Fig. 1c and d).

## Scoring

*Vestibule location in whole-ectoderm explants* Whole-ectoderm explants with distinct DV and LR axes were scored for the location of the vestibule relative to these axes. The dorsal and ventral midlines were first identified according to the pattern of ciliated band development (Emlet 1995; Byrne et al. 2001); paired projections of the ciliated band toward the animal pole identify dorsal (Figs. 1a,b and 2). Left and right meridians were then defined as those meridians halfway between the dorsal and ventral midlines, i.e., the middle of the left and right sides, where the larval surface is intersected by a frontal plane bisecting the DV axis. Vestibules were then scored by visual inspection as left, right, or ventral, according to their proximity to the ventral midline or to the left or right meridians. (Vestibules are

never located dorsally because the ciliated bands occupy that position by definition). Vestibules approximately midway between left and ventral were scored as left ventral.

*Presence or absence of vestibule in half-ectoderm explants* Each half-ectoderm was scored for the presence or absence of vestibule. Vestibular ectoderm is visually distinct before invagination and constriction of the vestibule opening (Minsuk and Raff 2002, 2005); when possible, its development was observed and noted in live explants as it occurred. Vestibules were not visible after completion of invagination, so all explants were fixed in 2% paraformaldehyde in FSW at 29 h or later, embedded in Paraplast, and sectioned at 5 or 10  $\mu\text{m}$ . Some explants were stained with toluidine blue and eosin and scored morphologically. Compared with the more free-form larval (nonvestibular) ectoderm invaginations that occur as a result of imperfect healing after surgery, true vestibules had characteristic features by which they could be distinguished: consistently large size, frequently arc- or bowl-shaped profiles, small constricted openings, and, frequently, differences in histological staining properties (see Fig. 3). To supplement the scoring of ambiguous cases and to validate our morphological scoring criteria, we performed radioactive *in situ* hybridization on the remaining explants with a probe to *HeET-1*, which labels larval ectoderm (see Fig. 4), as previously described (Angerer and Angerer 1991; Haag and Raff 1998).

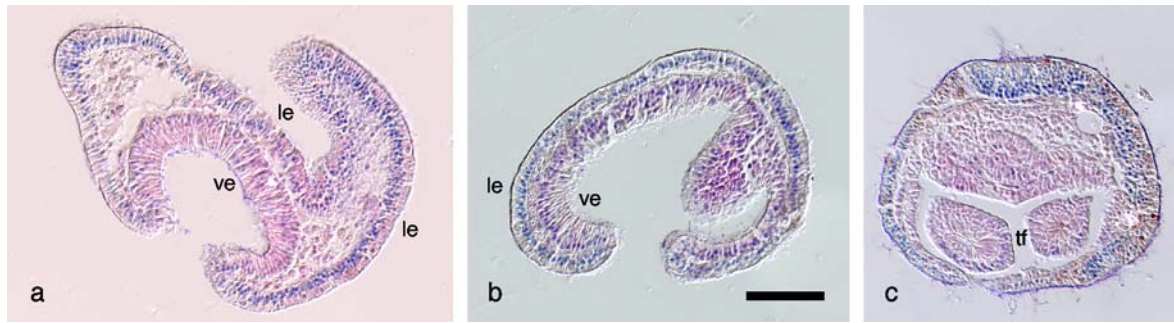


**Fig. 2** **a** Bilateral symmetry in an ecto-larva at 43 h. *Dorsal view*, vegetal at bottom. The ciliated band itself is not visible in the photograph, but its location is revealed by the pigmentation pattern. The wide red region at the vegetal end is flanked laterally by two thin red stripes, and the ciliated band follows the paired unpigmented gaps between the central pigmented region and the lateral stripes (arrows). At the vegetal end of the larva, these two ends of the ciliated band loop around the left and right (not visible in this view) to meet on the ventral side. *Thin solid line* indicates the plane of section used in **b**. *Dashed line* indicates the dorsal midline. **b** Ecto-larva at 47 h. *Paraffin section*, nuclei stained with toluidine blue. *Transverse section*, viewed from the animal side (i.e., from above the plane marked in **a**); the two regions of darkly stained, thickened ectoderm (arrowheads) are the paired dorsal spurs of the ciliated band. *Dashed line* therefore indicates the plane of larval symmetry, intersecting the ectoderm at the dorsal and ventral midlines (D, V). The left side of the larva (L) is to the upper right. The vestibule is the invaginated cavity; the vestibule opening can be seen slightly ventral to the midpoint of the left side (arrow). (The apparent double opening is the result of a wrinkle in the vestibular ectoderm protruding toward the opening. Such wrinkles frequently occur after surgical intervention.) Scale bars: **a** 100  $\mu\text{m}$ ; **b** 50  $\mu\text{m}$

## Results

In the absence of coelomic mesoderm, the vestibule forms with normal polarity

We previously showed that after removal of the archenteron from mid-gastrulae, before the appearance of the morphologically asymmetrical coelomic mesoderm, the remaining ectoderm can heal and develop in culture, becoming an “ecto-larva” lacking most endodermal and coelomic structures but developing normal larval ectodermal features such as ciliated bands, as well as vestibular ectoderm (Minsuk and Raff 2002). The vestibule often invaginates normally to form an internal cavity with a small opening; in other cases it evaginates. We revisited this experiment to determine where the vestibule in ecto-larvae is positioned. At the time of surgery, no morphological indicators of the LR or DV axes are present in the ectoderm. In normal larvae, these axes later become discernible by reference to the ciliated band (Emlet 1995; Byrne et al. 2001), which is bilaterally symmetric with distinct dorsal projections (Figs. 1a,b and 2a). Relative to these axes, the vestibule opening is located on the left side of the embryo, slightly closer to the ventral than to the dorsal midline, and just above the vegetal part of the ciliated band. We scored the location of the vestibule opening (or of the evaginated vestibular ectoderm) in ecto-larvae relative to the axes indicated by the ciliated band. Fig. 2b illustrates the relationships between the ciliated bands, vestibule opening, and DV and LR axes as viewed in



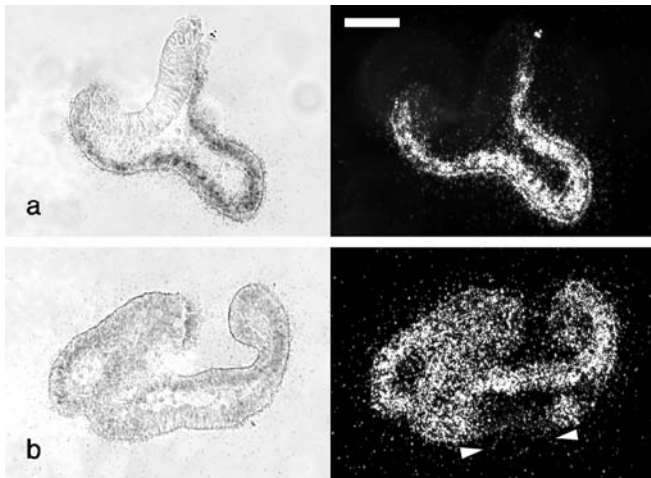
**Fig. 3** **a** Right-half ectodermal explant, 29.5 h, showing a well-developed vestibule (*ve*, lower left), as well as an extra fold of larval ectoderm (*le*, upper right) lacking the distinct morphology of vestibules. Note the difference in staining properties of the two, which was helpful in scoring other more morphologically ambiguous cases.

**b** Left-half ectodermal explant, 31 h, also containing a vestibule. **c** Recombinant of right-half ectoderm with left coelom, 53 h. Tube foot development shows ability of the vestibule to respond to coelomic signals and undergo normal morphogenesis. *tf* tube feet. Bar: 50  $\mu$ m

transverse section. (Actual scoring was done in intact ecto-larvae, live or fixed.)

We made and scored ecto-larvae from ten separate spawnings. Of a total of 70 ecto-larvae, 20 could not be scored for vestibule position: 4 of these died before ciliated band development, 1 became radialized (an occasional defect even of intact embryos under laboratory conditions; Minsuk and Raff 2005), and 15 developed a convoluted larval morphology as an artifact of poor healing, preventing the meaningful assignment of axes. The remaining 50 ecto-

larvae developed normal larval morphology (Fig. 2a) and were scored for vestibule position (see “Materials and methods”). Position along the AV axis was consistently normal, just above the vegetal ciliated band. Although many of the vestibules developed near the ventral midline, the majority developed laterally and these were found almost exclusively on the left side (Table 1, Fig. 1b). Since normal vestibules are located slightly ventral to the midpoint of the left side, specimens scored “left” and “left ventral” are both quite close to the normal location. This bias shows that isolated mid-gastrula ectoderm in the absence of coelom can establish LR asymmetry with robustly correct polarity, although we have not ruled out a role for coelom in regulating the precise DV or mediolateral position of the vestibule.



**Fig. 4** In situ hybridization with radioactive *HeET-1* probe. Bright field, left; dark field, right. Bright silver grains in the dark field images indicate signal. **a** Right-half ectoderm, 31 h, with vestibule. *HeET-1* is expressed in the larval ectoderm but not in the thicker vestibular ectoderm in the early stages of invagination. **b** Left-half ectoderm, 30 h. This explant underwent abnormal morphogenesis and illustrates the use of *HeET-1* to validate our morphological scoring. When we observed this explant during life, we noted that it had apparent vestibular ectoderm (based on pigmentation pattern and other optical properties) that failed to invaginate and apparent ordinary larval ectoderm on the other side that did invaginate. *HeET-1* is expressed throughout the invagination but is down-regulated on the opposite side of the explant (between arrowheads), confirming both that we can correctly identify vestibular ectoderm regardless of its morphogenesis and that we can distinguish nonvestibular invaginations from true vestibules. Bar: 50  $\mu$ m

In the absence of left-half ectoderm, the right-half ectoderm regulates and generates a vestibule

To investigate the potential for vestibule development in the right-side ectoderm, we dissected right- and left-half ectodermal explants from 21 late gastrulae (before the appearance of any asymmetry in the ectoderm) in four different spawnings, and cultured each explant separately until at least 29 h postfertilization. Unlike intact ectoderm, half-ectoderm explants could not be scored for vestibule position, because overall larval morphology formed abnormally after healing; the larval axes were not distinct enough to provide the appropriate landmarks. Therefore, we simply scored each explant for the presence or absence of vestibule by visual inspection during life, followed either by histological examination of fixed and sectioned larvae (Fig. 3a,b), or by in situ hybridization with *HeET-1* (Fig. 4), an ectodermal marker that distinguishes between larval and

**Table 1** Vestibule location scores in 50 ecto-larvae

Left	17 (34%)
Left ventral	9 (18%)
Ventral	23 (46%)
Right ventral	0 (0%)
Right	1 (2%)

vestibular ectoderm (see “Materials and methods”). *HeET-1* is expressed in all ectoderm before vestibule formation and is then down-regulated in the vestibule as it forms, becoming restricted to larval ectoderm and thereby forming a negative image of the vestibule (Haag and Raff 1998; Minsuk and Raff 2002). In addition to the vestibule, folds of larval ectoderm were frequently present, but these could be distinguished from vestibule by the above procedures (see “Materials and methods”; Figs. 3 and 4).

We scored all 21 pairs except for two left halves (one died before vestibule formation, and one was damaged before scoring). Vestibule was present in 18 of the 19 left halves (95%) and 16 of the 21 right halves (76%), demonstrating that even as late as the advanced gastrula stage containing a well-developed left coelom (and only a few hours before morphological vestibule development), the right half of the ectoderm retains substantial vestibule-forming potential (Table 2, Fig. 1c).

Half-ectoderm explants, like intact ecto-larvae (Minsuk and Raff 2002), did not develop rudiments; they lack the required inductive signal from the left coelom. Nine recombinants were made by transplanting a left coelom into a right-half ectodermal explant. Eight of these developed vestibules and subsequently tube feet (Figs. 1c and 3c), demonstrating that the vestibules produced by right-half ectoderm are able to respond to coelomic induction and undergo rudiment morphogenesis normally.

In isolated animal-half ectoderm, robust vestibule formation does not occur autonomously, but can be induced by left coelom

Once the vestibule has formed, further development of adult structures requires inductive signals from the left coelom (Minsuk and Raff 2002). However, we wished to know whether the initial formation of vestibule might also involve such signals. To test for the presence of vestibule-inducing signals, we required a tissue that, in the absence of coelom, does not produce vestibule autonomously. In normal embryos, presumptive vestibule arises from the animal blastomeres, but by the end of gastrulation lies mostly in the vegetal half of the ectoderm (Wray and Raff 1990, 1991; Haag and Raff 1998; Ferkowicz and Raff 2001). To deter-

**Table 2** Presence/absence of vestibule in paired left- and right-half ectodermal explants

19 Pairs:	
Present in both halves	13
Present in left half only	5
Present in right half only	1
Absent in both halves	0
2 Unpaired right halves (left halves not scored):	
Present	2
Absent	0
Left halves with vestibule	18/19 (95%)
Right halves with vestibule	16/21 (76%)



**Fig. 5** **a** Animal-half ectodermal explant, 51.5 h. No vestibule is present. (The small fold in the ectoderm is unambiguous larval ectoderm; compare Fig. 3a.) **b** Recombinant of animal-half ectoderm and left coelom, 49 h, with vestibule and tube feet (*tf*). Bar: 50  $\mu$ m

mine whether animal-half gastrula ectoderm has the desired properties, we made 53 animal-half ectodermal explants from seven different spawnings and scored for the presence of vestibule as above (Figs. 1d and 5a, Table 3). Vestibule formed in 20 explants (38%), and ambiguous cavities that we were unable to identify with certainty were formed in another 7 (13%). Therefore, vestibules formed in at most 51% of animal-half explants, demonstrating that although animal-half ectoderm does have vestibule-forming potential, it has substantially less than is present in left- or right-half ectoderm.

To test whether left coelom can induce vestibule formation in this nonvestibular tissue, recombinants were made by transplanting a left coelom into an animal-half ectodermal explant. We found that 100% of the recombinants (21 from three different spawnings) produced vestibules, unlike the animal-half ectoderm alone (Figs. 1d and 5b, Table 3). Tube feet were present in all cases. Therefore, the presence

**Table 3** Presence/absence of vestibule in animal-half ectodermal explants, with and without implanted left coelom

	Explants alone	Recombinants
Present	20 (38%)	21 (100%)
Ambiguous	7 (13%)	0
Absent	26 (49%)	0
Total	53 (100%)	21 (100%)

of left coelom induced robust vestibule formation that in many of these explants would not have otherwise occurred.

## Discussion

Isolated ectoderm contains sufficient information to specify the location of vestibule on the left side of the larva

The isolated intact ectoderm of *H. erythrogramma* develops normal larval ectodermal features including the bilaterally symmetric ciliated band (Minsuk and Raff 2002). The DV and LR axes are specified during early cleavage stages in indirect developers (McCain and McClay 1994; Summers et al. 1996; Henry 1998). They are specified even earlier, before first cleavage, in *H. erythrogramma* (Henry and Raff 1990; Henry et al. 1990), but are not fully committed until at least gastrulation (Henry and Raff 1994; Minsuk and Raff 2005) and are not evident morphologically until the formation of the left coelom, followed by the vestibule. Does the developing mesoderm impose asymmetry on the ectoderm, or does asymmetry arise independently in the two tissues? By scoring the location of the vestibule in ecto-larvae we have tested for a mesodermal role.

The development of a normal ciliated band after the removal of the archenteron indicates that the axial system as a whole remains intact. It is against this axial system and the ciliated band that we measured the location of the vestibules that formed in the ectodermal explants. More than half formed on the left, and most of the remainder formed near the ventral midline. This pronounced left-side bias shows that the ectoderm retains not only the larval bilateral symmetry, but also the L/R polarity information required for establishing the vestibule on the correct side. Formation of the vestibule represents a transition in axial organization, initiating adult ectodermal development.

The frequent formation of vestibules near the ventral midline could imply that the coelom is involved in establishing ectodermal LR asymmetry, and that in the absence of coelom, the ectoderm may remain bilaterally symmetric. However, because the ecto-larvae that did establish asymmetry formed their vestibules overwhelmingly on the left side and thus were correctly polarized, coelom cannot be involved in establishing polarity (which side is which). It seems unlikely that an ectoderm unable to reliably establish its own asymmetry could nonetheless autonomously determine the polarity. Therefore, we suggest that the formation of some vestibules near the ventral midline is better explained by a role for coelom in regulating DV position (see below) than by a role in regulating LR asymmetry. Alternatively, this variation may simply have been an artifact of surgery.

We do not know when the ability to establish LR asymmetry becomes autonomous to the ectoderm. Coelomic precursors in the archenteron or vegetal plate may send directional signals before the coelom develops. Unfortunately, ecto-larvae isolated at or before the early gastrula

stage fail to heal properly, and die. Therefore, other methods must be used to address this question.

Inhibitory signals within the ectoderm repress vestibule development on the right side

Previously, we showed that isolated whole ectoderm retains full vestibule-forming potential (Minsuk and Raff 2002). Here we have shown that left and right halves isolated at the late gastrula stage have the same potential. This indicates that vestibule development on the right is normally suppressed by a signal from the left, occurring after the late gastrula stage. Separation of the ectoderm into right and left halves interrupts this signal, allowing the right side to regulate and form vestibule robustly.

Henry and Raff (1994) reported similar results but concluded that the signal operates earlier, the right half being largely determined before gastrulation. We now have a better understanding of vestibular morphology (Minsuk and Raff 2005), and the present methods allow greater certainty in identifying the left and right halves as well as in isolating the ectoderm from coelomic signals. We find that full vestibule-forming potential persists in isolated right-side ectoderm at least until the late gastrula stage, immediately before the initial thickening of the vestibular ectoderm.

In two indirect-developing species, Aihara and Amemiya (2001) similarly found that right-half embryos could produce rudiments. However, in contrast to results in *H. erythrogramma* (Henry and Raff 1994), they found situs inversus in left halves, but not in right halves, and concluded that the right half controls LR axial polarity. Thus, there may be signals traveling in both directions along the axis.

Unlike the right halves, the animal halves suffered a substantial reduction in vestibule-forming potential, indicating that fates along the AV axis have been largely determined by the mid-gastrula stage (see also Henry and Raff 1990). The AV axis of sea urchins is specified maternally, and fates along the axis are determined progressively in response to vegetal signals (Brandhorst and Klein 2002; Angerer and Angerer 2003; Kauffman and Raff 2003). That we found any vestibule-forming potential at all in animal halves may indicate that AV patterning is not yet complete in the mid-gastrula, or more likely, that the presumptive vestibule extends more than halfway toward the animal pole, so that small amounts of it were inadvertently included in the animal explants.

### A role for coelomic signaling

We previously showed that development of central nervous system and tube foot ectoderm in the vestibule requires inductive signaling from the coelom (Minsuk and Raff 2002), but the ectoderm autonomy of vestibule specification makes it difficult to test for a possible role of coelomic signals in this earlier event. The reduced developmental

potential in animal-half ectoderm has allowed us to test for such signals, and we find that coelom does induce vestibule in that tissue. This does not prove that it induces vestibule at the normal location, but it does demonstrate that a signal is present, and at the right time (before vestibule development), and that at least one region of larval ectoderm can respond to that signal by differentiating as competent vestibular ectoderm instead. This suggests that although the left coelom is not required either for the initiation of normal vestibule development or for its restriction to the left side of the ectoderm, coelomic signaling may play a complementary role, fine-tuning the size or location of the vestibule. The variability in vestibule position that we saw in our whole-ectoderm explants (Table 1) would be consistent with such a role, although we have not ruled out surgical disruption as the cause of that variability. Subsequent morphogenetic and signaling interactions (Minsuk and Raff 2002, and in preparation) require that the vestibule and coelom be properly oriented to one another, and this could be facilitated by vertical signals, i.e., signals from one epithelial layer to another (Poznanski et al. 1997). Certain observations in indirect-developing sea urchins agree with this possibility. In these urchins, vestibule develops in two phases. First, it forms an initial invagination, and, subsequently, it orients toward and extends to meet the hydrocoel. Early studies (reviewed by Hörstadius 1973) suggested that the second phase, but not the first, was influenced by the presence or absence of hydrocoel.

If the coelomic signal to which our animal-half ectoderm responded does indeed have a role in normal patterning, it would be distinct from the role previously shown, the induction of further development in an existing vestibule to form advanced rudiment. The coelom would be involved in two different events in two different domains: first, regulation of vestibule formation as a whole, and then, the induction of adult structures in a more restricted subdomain, the vestibule floor (Minsuk and Raff 2002).

### Body plans and life history

The echinoderm–hemichordate common ancestor probably resembled extant hemichordates, which have a biphasic life cycle, a bilaterally symmetric larva, and a system of small set-aside coeloms that give rise to the mesoderm of a bilaterally symmetric adult, without axial transformation (Peterson et al. 2000; Nakano et al. 2003; Sly et al. 2003). The adult ectoderm of hemichordates, unlike the mesoderm, is formed from the larval ectoderm as a whole, without a special ectodermal set-aside compartment, as the initially small coeloms expand during the late larval stages to fill the adult body (Hyman 1959; Hadfield 1975; Peterson et al. 1999a; Urata and Yamaguchi 2004). The larval and adult development of euechinoid sea urchins, including *H. erythrogramma*, differs from that of hemichordates in two major aspects: the compartmentalization of adult development into a distinct adult rudiment, anatomically isolated from the rest of the larva; and the de novo formation of an adult OA axis and pentaradial symmetry within that rudiment. These process-

es require new mechanisms of control: the separation of ectoderm into distinct larval and adult compartments; the asymmetric patterning of the presumptive adult tissues, both mesodermal and ectodermal, as well as their precise mutual coordination; and the establishment of the adult axes and body plan.

Both the degree of compartmentalization and the details of axial transformation vary among the echinoderm classes, indicating a complex mosaic evolution. The histolyzation of the larval ectoderm and its replacement by the vestibular ectoderm at metamorphosis to give rise to the adult ectoderm is a special feature of euechinoids. Cidaroid sea urchins lack a vestibule and produce the adult rudiment exposed on the exterior left side of the larva; most of the larval ectoderm is retained and becomes the adult ectoderm (Emler 1988), without any set-aside contribution. The same is true in asteroids (Hyman 1955; Chia and Walker 1991). In holothuroids, ophiuroids, and crinoids (Hyman 1955; Smiley 1986; Hendler 1991; Holland 1991; Smiley et al. 1991), the larval ectoderm is largely retained, but a vestibular invagination provides the oral part of the adult ectoderm, in a process quite analogous (although probably not homologous) to that in euechinoids. Thus, ectodermal compartmentalization appears to have arisen several times, convergently and to different degrees. In all cases, however, regardless of the presence or absence of a vestibule, the interaction of the hydrocoel with the overlying ectoderm produces a similar response, in which the ectoderm provides the covering for pentaradial extensions of the hydrocoel, producing organs associated with the water vascular system (the tentacles or buccal podia in holothuroids; tube feet in the other classes). Thus, it seems likely that the mesoderm–ectoderm signaling events discussed here and elsewhere (Minsuk and Raff 2002) could predate the evolution of compartmentalized vestibular ectoderm seen in euechinoid sea urchins. Such compartmentalization would require new regulatory pathways to delineate the ectodermal compartment and coordinate its relationship to the mesoderm, as suggested by a recent comparison of hemichordate and sea urchin patterns of *Brachyury* gene expression (Peterson et al. 1999a,b). This transcription factor, involved in mesoderm specification in chordates, is expressed in all three coeloms of the hemichordate *Ptychodera flava* but nowhere in the ectoderm. In the sea urchin *Strongylocentrotus purpuratus*, its mesodermal expression has become restricted to the middle coeloms alone (on the left side, to the hydrocoel), but interestingly it is also expressed in the overlying vestibular ectoderm. The coincident coelomic restriction and vestibular recruitment suggests coordinated modifications in both the ectoderm and the mesoderm, associated with the evolution of a specialized set-aside ectodermal region and its localized interaction with mesoderm. The pathway may be a cause of, or a response to, the patterning events we discuss here. Studies in other echinoderms would clarify the role of *Brachyury* in the evolution of their adult development and metamorphosis.

The pentaradial adult body plan and its de novo emergence from the bilateral larva is common to all extant echinoderms, but the manner of its emergence varies. The



several coelomic compartments vary somewhat independently in their development among the echinoderm classes, but we can take the hydrocoel as the primary indicator of adult axis formation. Even so, the variation is complex, as the axis in some taxa moves during its development, making the comparison between the classes dependent on developmental stage. The hydrocoel usually arises on the left, but only in echinoids and asteroids does it remain in place, forming the OA axis on the larval left side (Hyman 1955; Chia and Walker 1991; Pearse and Cameron 1991). In ophiuroids and holothuroids, it curves into a ring around the larval esophagus, and interacts with the ectoderm around the larval mouth to form the adult mouth in the same location (Hyman 1955; Smiley 1986; Hendler 1991; Smiley et al. 1991). In the ophiuroids this results in the alignment of adult oral with larval ventral (Hyman 1955; Hendler 1991), but in the holothuroids the rudiment then migrates to the animal pole (Hyman 1955; Smiley 1986; Smiley et al. 1991). Thus, in holothuroids the hydrocoel forms along one larval axis (LR), it interacts with presumptive adult oral ectoderm along a second (DV), and finally the definitive adult OA axis aligns along yet a third (AV). In stalkless crinoids (feather stars), the hydrocoel and vestibule do not arise on the left side at all, but are located ventrally from their earliest development (Hyman 1955; Holland 1991). (The process may be slightly different in stalked crinoids, or sea lilies; Nakano et al. 2003.) The rudiment then rotates within the larva to align with the AV axis as in holothuroids, but in this case forming the definitive adult mouth at the vegetal pole (Hyman 1955; Holland 1991). In the unique asteroid *P. tessellatus*, the hydrocoel arises in a ring around the archenteron, which in the case of this completely direct developer lacking any larval mouth, is always oriented toward the animal pole, where the adult mouth forms (McEdward 1992; Janies and McEdward 1993). It is universally accepted that the adult OA axes of all echinoderms are homologous; this axis has therefore wandered relative to the larval bilateral axes during echinoderm evolution. The common tendency to treat the adult OA axis of sea urchins as synonymous with larval LR is therefore an oversimplification belied by its evolutionary history.

The evolution of larval ectodermal compartmentalization, and of larval-to-adult axial reorganization, could be causally linked. The former could have resulted in a more modular organization, with more localized control and de novo patterning of the adult axes, making them less dependent on global larval axial patterning information. Such a release from constraints may have made possible both the modified origins of the presumptive adult tissues and the positional shifts of the adult axes, thus giving rise to the differences in adult axis formation among the echinoderm classes (Hyman 1955; Smiley 1986; Hendler 1991; Holland 1991; Smiley et al. 1991; Nakano et al. 2003). Ongoing effects of a high degree of modularity may underlie more recent reorganizations of axis formation at lower taxonomic levels, including the species level (McEdward 1992; Janies and McEdward 1993; Kerr and Kim 1999; Minsuk and Raff 2005). The evolution of compartmentalization and the

ability of OA axis formation necessitate mechanisms for controlling the patterning of presumptive adult mesoderm and ectoderm and coordinating their combined development. We have begun to map out the regulation of vestibular ectoderm formation and vestibule-coelom interaction in sea urchins here and elsewhere (Minsuk and Raff 2002, 2005 and in preparation). Further studies of this critical developmental transition in sea urchins and the other echinoderm classes, as well as of coelom-ectoderm interactions in hemichordates, will be important in understanding deuterostome body plan and life history evolution.

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