Evolution of Developmental Control Mechanisms

Juvenile skeletogenesis in anciently diverged sea urchin clades

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Abstract

Mechanistic understanding of evolutionary divergence in animal body plans derives from analysis of those developmental processes that, in forms descendant from a common ancestor, are responsible for their morphological differences. The last common ancestor of the two extant subclasses of sea urchins, i.e., euechinoids and cidaroids, existed well before the Permian/Triassic extinction (252 mya). Subsequent evolutionary divergence of these clades offers in principle a rare opportunity to solve the developmental regulatory events underlying a defined evolutionary divergence process. Thus (i) there is an excellent and fairly dense (if yet incompletely analyzed) fossil record; (ii) cladistically confined features of the skeletal structures of modern euechinoid and cidaroid sea urchins are preserved in fossils of ancestral forms; (iii) euechinoids and cidaroids are among current laboratory model systems in molecular developmental biology (here Strongylocentrotus purpuratus [Sp] and Eucidaris tribuloides [Et]); (iv) skeletogenic specification in sea urchins is uncommonly well understood at the causal level of interactions of regulatory genes with one another, and with known skeletogenic effector genes, providing a ready arsenal of available molecular tools. Here we focus on differences in test and perignathic girdle skeletal morphology that distinguish all modern euechinoid from all modern cidaroid sea urchins. We demonstrate distinct canonical test and girdle morphologies in juveniles of both species by use of SEM and X-ray microtomography. Among the sharply distinct morphological features of these clades are the internal skeletal structures of the perignathic girdle to which attach homologous muscles utilized for retraction and protraction of Aristotles’ lantern and its teeth. We demonstrate that these structures develop de novo between one and four weeks after metamorphosis. In order to study the underlying developmental processes, a method of section whole mount in situ hybridization was adapted. This method displays current gene expression in the developing test and perignathic skeletal elements of both Sp and Et juveniles. Active, specific expression of the sm37 biomineralization gene in these muscle attachment structures accompanies morphogenetic development of these clade-specific features in juveniles of both species. Skeletogenesis at these clade-specific muscle attachment structures displays molecular earmarks of the well understood embryonic skeletogenic GRN: thus the upstream regulatory gene akl and the gene encoding the vegfr signaling receptor are both expressed at the sites where they are formed. This work opens the way to analysis of the alternative spatial specification processes that were installed at the evolutionary divergence of the two extant subclasses of sea urchins.

Introduction

This is our initial report on an evolutionary research project, the specific objective of which is to determine the developmental programs that underlie divergent morphogenetic processes distinguishing cidaroid and euechinoid sea urchins. We are interested in characters that can be tracked in the fossil record, so that paleontological evidence can be used to establish the polarity, and the plesiomorphy vs. novelty, of characters in each lineage. Fortunately, the growing fossil record is providing high-resolution evidence of skeletal structures in Paleozoic and Triassic sea urchin clades that is directly relevant to the emergence of the modern euechinoid and cidaroid subclasses. To attain our ultimate goals it would be necessary to gain experimental access to the developmental processes by which distinct euechinoid vs. cidaroid skeletal morphologies arise in their respective adult body plans. We have discovered such processes taking place in juveniles in the weeks immediately following metamorphosis. However, specific developmental mechanisms have rarely if ever been
studied at the molecular level in juvenile sea urchins, and an initial suite of methodological problems had first to be overcome. Our objectives in this work were (i) to identify divergent aspects of juvenile skeletogenesis that are specifically canonical to either of the two echinoid subclasses, using as laboratory sea urchin models the cidaroid *Eucidaris tribuloides* and the euechinoid *Strongylocentrotus purpuratus*; (ii) to characterize the morphogenesis of these features and determine when this occurs; (iii) to learn how to visualize gene expression in the relevant spatial phases of juvenile skeletogenesis; (iv) to obtain initial evidence that might relate these processes to the well known mechanisms of skeletogenesis in echinoid embryogenesis.

Going back in deep time, the fossil record shows that organization of the radial test endoskeletons of the various clades of Paleozoic echinoids varied enormously in respect to the absolute and relative numbers of columns of ambulacral vs. interambulacral plates (ambulacral plates are those containing perforations through which tube feet extend) (Kier, 1965). In contrast, the pentaradial tests of all modern echinoids, i.e., including both cidaroid and all regular euechinoid sea urchins, display a constant alternation of two ambulacral columns with two interambulacral columns of test plates. Many shared characters identify the Mississippian to Permian echinoid stem group *Archaeocidaris* as the closest known common ancestor of cidaroids and euechinoids (Kroh and Smith, 2010), though *Archaeocidaris* displays two columns of ambulacral plates alternating with four columns of interambulacral plates. The first well preserved forms of cidaroid and euechinoid lineages, known from the Permian and Triassic respectively (Kier, 1977; Smith and Hollingworth, 1990), display the crown group feature of two columns of ambulacral plates offset with two columns of interambulacral plates. However, the detailed evolutionary steps intervening between *Archaeocidaris* and the earliest crown group Mesozoic cidaroids and euechinoids remain obscure, and, as we report elsewhere, additional paleontological evidence is now leading to significant revision of current scenarios. It is clear (contrary to the conventional description of cidaroids as “primitive”) that both modern echinoid subclasses retain some plesiomorphic characters, such as their large spines and their tubercular support structures, plus a number of features relating to their coronal plating (Smith, 2005), and it is these features which lead to the conclusion that the last common ancestor was derived from the *Archaeocidaris* stem lineage. However, the paleontological record is likely missing intermediates between the *Archaeocidaris* stem lineage and crown group euechinoids and cidaroids. Each subclass also presents features that are derived with respect to the *Archaeocidaris* common ancestor as well as plesiomorphic characters shared with *Archaeocidaris*. Two prominent derived features of the endoskeleton distinguish cidaroid from euechinoid sea urchins. The first of these is the organization of their ambulacral test plates. The second is the entirely distinct morphology of the bony protrusions from the radial perignathic girdle which serve as attachment anchors for the powerful paired muscles that retract Aristotle’s lantern and the five teeth suspended within from the extruded position (Wilkie et al., 1998; Kroh and Smith, 2010). These skeletal features are illustrated below. Lantern and dental morphology, and the presence or absence of buccal notches, provide an additional sets of distinguishing characters (Smith and Hollingworth, 1990), but we have not addressed these more difficult features as they are less frequently preserved paleontologically and more difficult to study developmentally.

Results and discussion

**Morphological differences in the skeletal structures of cidaroid vs. euechinoid adult body plans**

In modern echinoids the endoskeletal test plates develop essentially in the following manner. The dorsal-most or apical plates, that is, the 10 plates surrounding the anus, including the five that contain the gonopores, and the other five (ocular) plates are present in very young metamorphosed juveniles. Formation of these plates is initiated in larval life, prior to metamorphosis. In young juveniles, circular rings consisting of horizontal rows of the body wall test plates, ambulacral and interambulacral, are delaminated downward from a generative zone immediately surrounding the apical plates. This process continues in juveniles for the first few weeks after metamorphosis, until the adult number of plate rows is produced (e.g., 14), such that the most adoral plate rows (furthest down) are developmentally the oldest, and the most adapical, adjacent to the apical plates, are the youngest. Thus, as rows are added, the form of the juvenile gradually changes from an almost flat pancake-like structure containing only a very few lateral plate rows to a globular one. Again in contrast to Paleozoic forms, the growth of the animal in post-juvenile life occurs by continuing accretion of biominal to the periphery of pre-existing plates, rather than by continuing formation of numerous additional plates (Smith, 2005).

A phylogenetically distributed endoskeletal character sharply distinguishes the ambulacral test plates of cidaroid and euechinoid sea urchins. This is that cidaroid plates each bear a single pair of pores and the initial plate boundaries are also the final plate boundaries, while in euechinoids, the initially formed plates (consisting of a primary plate and numerous demiplates) progressively fuse, so that in the aggregate the resulting compound plates contain many pore pairs (Kroh and Smith, 2010). In the *Archaeocidaris* stem group, ambulacral plates are exclusively simple, and thus the simple plating in cidaroids, as opposed to compound plating, is the plesiomorphic character. The comparison is shown graphically and photographically for *S. purpuratus* and *E. tribuloides* in Fig. 1. The mechanism of fusion involves overgrowth of the tuberclear biominal mounds onto adjacent demiplates. In our observations of test formation the earliest plate fusions could be observed only towards the end of the several week period we studied. Thus plate fusion is a relatively later event in body wall test formation, following delamination of all the plate rows and development of the initial sets of spines, tube feet and other external organs. Though a valuable subclass diagnostic, the progressive nature and relatively late process of ambulacral plate fusion did not recommend itself as a likely target for developmental investigation. Furthermore, plate fusion is a character that is present in one clade, the euechinoids, and entirely absent in the other, the cidaroids, rather than a character that develops differently in the two clades, but which, since it exists in both, might lend itself to differential developmental comparison.

A second, and for us more exciting distinction, is in the five pairs of muscle attachment structures of the perignathic girdle (Wilkie et al., 1998). These muscles mobilize the jaw of the sea urchin, which comprise the pentaradial Aristotle’s lantern structure in which the teeth are mounted. The muscle attachment mechanism motivates the physical deployment of the teeth, which can be extruded during feeding. Euechinoid sea urchins produce 10 erect structures known as auricles which are located exactly on the interior edges of the perignathic ambulacral plates, extending upward into the interior of the animal (Fig. 2A–C). In some clades, such as the *Strongylocentrotidae*, the two auricles present in each ambulacral area merge above the ambulacral plates forming an inverted “V”. In contrast, cidaroid sea urchins develop for this purpose five pairs of broader double-pointed protrusions known as apophyses, which grow out of the inner edges of the interambulacral adoral test plates (Fig. 2D–F). Though as we see in the following the functions of auricle and apophysis are similar, in that they both anchor the retractor muscles, these structures are strikingly different in form. In addition, they develop 180° out of phase with one another spatially, as they are ambulacral in the
euechinoid but interambulacral in the cidaroid. Furthermore, neither auricles nor apophyses are plesiomorphic, since Archeocidaridari
ds lacks both types of perignathic girdle structure and the protractor and retractor muscles apparently just attached to the base of the adoral plates surrounding the peristome.

Morphogenesis of the auricles or apophyses in very young post-
metamorphosis sea urchins

X-ray microtomography of juveniles only 3 and 4 weeks old indicated that the internal 5-fold radial structures of Aristotle’s
tlantern have already formed, as can be seen for both species in the images of Supplemental
Fig. 1A and B. Here Fig. S1A1 and S1B1 are focused externally on the apical plates, which can be seen particularly clearly in the 4-week old Euclidaris juveniles. Of main importance here are the images in Fig. S1A2 and S1B2, which provide horizontal computational sections about 3/4ths of the way
down toward the oral surface, at exactly the level where the test
ends at the peristomial opening. On this circular edge auricles or
apophyses will form, and the importance of these images is that they show that this developmental process has already begun.

Thus Fig. S1A2 displays the five pairs of retractor muscles of the juvenile Strongylocentrotus, connecting to the perignathic girdle exactly
at the five ambulacral radii, as can be seen by their radial correspondence with the podia pores. Fig. S1B2 specifically displays the pairs of nascent apophyses in E. tribuloides, the inter-
ambulacral protrusions extending inward from the test rim in the
image and connecting to the retractor muscles. The morphological evidence in these images suggests that auricle and apophysis development must have begun at even earlier stages than these 3–4 weeks old juveniles.

The alternative positional phases of the nascent ambulacral
auricles of S. purpuratus vs. the nascent interambulacral apo-
physes of E. tribuloides are clearly visualized in the SEM images of the juvenile tests in Fig. 3A and B. Side views of the whole test of a 3-week Strongylocentrotus juvenile reveal its flattened shape, due
to the early stage of the process of test row formation, as only 4–5 rows of body wall plates have yet formed (Fig. 3A1 and A2). Yet even in these few rows the ambulacral plates are clearly evident by their tube foot pores, which are lacking in the adjacent interambulacral columns. Ambulacral and interambulacral plates can similarly be seen in the dissected test fragment in Fig. 3A3. Turned over, to enable visualization of the internal surface of the same fragment, three nascent auricles can clearly be seen at the positions of the S. purpuratus ambulacral plates (Fig. 3A4).

Parallel displays of E. tribuloides tests, from juveniles of similar weeks post-metamorphosis, provide equivalent information (Fig. 3B).

Here can clearly be seen two nascent apophyses, located exactly opposite the two interambulacral plate columns visualized in this test fragment (Fig. 3B4; see legend).

In order to determine exactly how and when these clade-
specific muscle attachment structures develop, we carried out a
timed series of SEM observations on sectors of juvenile test from both species. Here we focused on the terminations of the body
wall plate columns at the nascent perignathal girdles at the oral extremes of the test. As shown clearly in Fig. 4A, the development of the ambulacral auricles of S. purpuratus juveniles initiates just
after one week following metamorphosis, and by four weeks the conical forms of these trabecular skeletal projections is well
established. Fig. 4B shows that the interambulacral apophyses of E. tribuloides appear even more precociously, as the initial apo-
physal projections can be seen even in one-week old juveniles. This difference may reflect only the higher temperature at which E. tribuloides lives, i.e., 24 °C rather than 15 °C. An interesting develop-
mental insight suggested by Fig. 4 is that the specific regions where the auricles will develop in S. purpuratus are positioned by
two specific features of the ambulacral plates: they are located exactly between the distal boundaries of the ambulacral plates and
the oral most podia pore in this plate. In E. tribuloides the
apophyses are again located at the distal plate boundaries, here of the interambulacral plates, and again adjacent to adoral most
pores, but here the pores are across the boundary in the adjacent
ambulacral plate, and apophysis growth begins next to the 2 aboral most podia pores. One interpretation is that cells at the edges of the skeletal matrix, in the relevant pores and at the plate boundaries, are sources of the signals used to specify the locations of these skeletal projections. In addition to the developmental sequences, Fig. 4 thus provides exact information on the differences distinguishing euechinoids from cidaroids in the initial developmental location of auricles vs. apophyses.

Visualization of juvenile skeletogenesis in situ by transcriptional expression of the sm37 biomineralization gene

In order to demonstrate the processes rather than the products of skeletogenesis, we adapted procedures for section in situ hybridization earlier used for studies of skeletogenesis in advanced S. purpuratus larvae (Gao and Davidson, 2008). The procedure (see Methods) permitted spatial localization of signal in high-resolution morphological maps of whole mounts and serial sections of S. purpuratus and E. tribuloides juveniles, shortly after metamorphosis. The sm37 probe used for this series of observations represents a well-characterized biomineralization gene that is actively expressed in embryonic spicule development, (Lee et al., 1999; Livingston et al., 2006). Successive focal plane image series from whole mount hybridization of a 3-week old S. purpuratus juvenile are shown in Fig. S2; and compete horizontal and parasagittal section series stained for sm37 expression in 3-week old E. tribuloides juveniles can be seen in Figs. S3 and S4 respectively. Developmental expression of the sm37 gene can be seen in many regions where skeletogenesis is taking place in the close-up views of Fig. 5. The whole mount in situ in Fig. 5A1 shows labeling in nascent spines, pedicellariae, structures of Aristotle's lantern (out of focus), and in focus, within the growing apical plates. A consistent observation was that in growing plates sm37 transcripts are localized in small spots. The explanation is that, as seen in the scanning EMs of Fig. 3, the stereom biomineral of the test plates has a reticular form in which the cavities are probably the location of cell bodies where this mRNA accumulates (though the skeletogenic tissue is syncytial); thus growth by accretion will
occur throughout the external surfaces. Observations confirm that the dimensions of the mRNA “dots” are the same as those of the pits in the stereom. Another view of this process is afforded in the high magnification in situ hybridization image of a test wall section shown in Fig. 5A2. Here the blue represents smt37 mRNA stain, closely interdigitated in a complementary manner with the gray stereom biomineral, as visualized in polarized light.

Similarly, Fig. 5B displays the multiple body parts where skeletogenic functions are active at this stage in *E. tribuloides*. For orientation, the horizontal plane of the in situ hybridization
reproduced at high magnification in Fig. 5B3 is mapped on microtomographic reconstructions in Fig. 5B1 and B2. Fig. 5B3 reveals intense sm37 transcription on either external surface of the test plates, indicating plate growth by accretion, and also at pore boundaries, where the tube feet are protruding through the ambulacral wall. Interestingly, dense staining is observed as well in the five teeth, which were not known previously to express this same biomineralization gene. The parasagittal section in Fig. 5C3, which is similarly oriented three-dimensionally in Fig. 5C1 and C2, displays in addition an internal locus of skeletogenesis, the pyramidal walls of Aristotle’s lantern.

Additional sections shown in Supplemental information (Figs. S5 and S6) provide further details on the course and mode of juvenile test growth. In Fig. S5(A–C) we see horizontal sections of an eight-week post-metamorphosis Strongylocentrotus juvenile. By this later stage the in situ hybridization signal clearly marks the junctions between plates, indicating continued growth by lateral accretion, just as predicted (Smith, 2005). The vertical, or apical to oral section in Fig. S5D shows a thin layer of active tissue on the still growing apical plates, but much more dense sm37 activity further down in the younger plates of the body wall, as can be seen particularly well on the left side of the specimen. Fig. S6

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**Fig. 4.** Developmental series displaying initial stages of morphogenesis of preignathic muscle attachment structures in *S. purpuratus* and *E. tribuloides*. SEM observations of preignathic girdle growth were obtained from dissected tests of sea urchin juveniles collected weekly, immediately after metamorphosis. Specimens are oriented aboral side upwards, oral side downwards. (A) *Strongylocentrotus purpuratus*. (A1–A4) Inside views of dissected sectors of tests from juveniles aged 1–4 weeks, each consisting of 6–8 columns of plates. (A1’–A4’) Higher magnification of the ambulacral region within the rectangles indicated in (A1–A4). Primordial auricles arise on the oral ends of the ambulacral plates and occupy the narrow space between the 1st podial pore (white asterisks) and the interambulacral/ambulacral boundary (red dashed line). (A1’–A4’). Higher magnification views of the auricles in (A1’–A4’) tracking the sequential trabecular growth of the auricle which progresses from a dendritic structure at weeks 1 and 2, to a fenestrated block at week 3, to the conical structure seen at week 4. (B) *Euclidaris tribuloides*. (B1–B4) Inside views of dissected test sectors from juveniles aged from 1 to 4 weeks, each of which consists of 4 columns of plates. One column of ambulacral plates always appears on each side of the two interambulacral plate columns after dissection, a result of the susceptible breakpoint along the boundary between the two ambulacral columns. The growth of apophyses in *E. tribuloides* is more conspicuous than that of the auricles in *S. purpuratus* of the same ages, and their V-shaped topology is visible from the beginning. (B1’ and B2’). Higher magnification of the interambulacral regions indicated by the rectangles in (B1 and B2). Primordial apophyses arise at the edges of the interambulacral plates, next to the 1st two podia pores in the adjacent ambulacral plate (white asterisks). Plate surfaces are more rough and imbricated than in *Strongylocentrotus*, though the interambulacral/ambulacral boundaries (red dashed lines) are still discernible under higher magnification. (B1”–B2”) Higher magnification views of the apophyses in (B1’) and (B2’) respectively, showing the curved apices and trabeculae. Scale bar 50 μm in A1–A4 and B1–B4.
displays further sections through the test of a three-week old *Eucidaris* juvenile. In this younger animal *sm37* expression is seen to extend throughout the depth of the plates, heaviest in more oral locations as indicated in the parasagittal section (Fig. S6C). It is clear from these observations that the skeletogenic program in which *sm37* expression participates is deployed in spatially specific ways in numerous external structures of these juvenile sea urchins. It could be said with some justification that the particular mechanism that controls the spatial deployment of this particular program is indeed that which mediates all the major features of the external morphology of these animals, as well as controlling their rapid juvenile growth.

Skeletogenic regulatory gene expression in the auricular and apophyseal muscle attachment structures of *Strongylocentrotus* and *Eucidaris* juveniles

Our specific aim has been to gain an experimental handle on the developmental biology of the clade-specific perignathic muscle attachment structures, so as to provide exactly as possible an indication of just when, where and how their different morphogenesis is executed. From the foregoing we learn exactly when and where development of the auricles and apophyses is initiated. The observation in Figs. 6 and 7 show respectively that, just as implied by the appearance and growth of these trabecular structures (Fig. 4), their appearance de novo in juveniles is indeed a skeletogenic process; and also that it involves key signaling and regulatory gene expressions that perform known roles in the skeletogenic GRN of sea urchin embryos.

As summarized above, in euechinoids the 10 ambulacral auricular projections are the attachment sites for the 10 retractor muscles (Ziegler et al., 2012), while in cidaroids the same function is executed by the five double flanges of the interambulacral apophyses. The retractor muscles extend from the movable lantern jaws to the immovable perignathic girdle anchors on the edge of the test (Wilkie et al., 1998). In situ section hybridizations with *sm37* in young juveniles, both *S. purpuratus* (Fig. 6A) and *E. tribuloides* (Fig. 6B), when viewed at high magnification, show unequivocally that the skeletal structures which form the immediate anchors for the retractor muscles are in process of development morphogenesis. The horizontal section in Fig. 6A1 is cut at a level that just catches the tips of four of the auricular flanges as they extend upward from the oral test rim (cf. Fig. 2). These junctional complexes are shown at high magnification in Fig. 6A2–A4. In each case the resolution is sufficient for visualization of the bundles of longitudinal muscles constituting the retractor. The muscles terminate at the auricular skeletomuscular junction, apparently a specialized, thin, non-biominalized zone, and immediately beneath this zone active *sm37* expression occurs.

**Fig. 5.** Juvenile skeletogenesis visualized by expression of *sm37* skeletogenesis gene. (A) *Strongylocentrotus purpuratus*. (A1) Whole mount viewed externally after staining, focus plane on apical plates in order to demonstrate apical plate boundaries and synthetic activity in internal stroma, denoted by stained dots. (A2) High magnification image of nascent test from juvenile after in situ hybridization for *sm37*; the syncytial stroma is stained, while the stereom biomineral is unstained. (B) *Eucidaris tribuloides*. (B1) Tomographic reconstruction of adult *Eucidaris tribuloides*, indicating orientation of horizontal sections in B2 and B3. (B2) Tomographic coronal or horizontal section of adult *Eucidaris tribuloides* for anatomical reference, to indicate positions of lantern and of ambulacral pores with respect to the test plating. (B3) In situ hybridization of 3 weeks post-metamorphosis juvenile showing staining for *sm37*. Red letters: (a) test wall, (b) tooth, (c) radiole (spine), (d) interpyramidial muscle, (e) podia (tube foot), (f) pore pair, (g) hemipyramid, and (h) pharynx. Note that test plates (a) and teeth (b) stain intensely. (C) *Eucidaris tribuloides*. (C1) Tomographic reconstruction of adult *Eucidaris tribuloides* indicating orientation of vertical sections in C2 and C3. (C2) µ-CT through lateral section of adult *Eucidaris tribuloides* showing position of lantern with respect to test plating. (C3) In situ hybridization of three-week post-metamorphosis juvenile showing staining for *sm37*. Red letters: (a) test wall; (b) tooth; (c) radiole (spine); (d) interpyramidial muscle; (e) podia (tube foot); (f) pore pair; (g) hemipyramid; (h) pharynx; (k) peristome. Spines, test plates and also skeletal elements of Aristotle’s lantern (hemipyramid) all stain for *sm37* expression.
Throughout the developing auricular skeletal element. In Fig. 6A5 the vertical sections display both the retractor muscles and elements of the perignathic auricles to which they anchor; again these skeletal anchor points are sites of active sm37 expression. Dramatic sm37 expression can be seen at the apophyses of juvenile E. tribuloides as well in the vertical sections of Fig. 6B1–B4. The beautiful high resolution transverse image in Fig. 6B5 shows how closely applied to the muscle termini are the thin zones of the cellular framework of the auricles. Red letters: (a) apophyses; (b) retractor muscles. Note that the retractor muscles attach to the apophyses; (c) hemipyramid; (d) tooth.

Conclusions

In this work we demonstrate experimental access to a specific developmental process of skeletogenesis, one which gives rise to cladistically diverged features of the adult body plans of echinoids. These anciently diverged features are the distinctly positioned and distinctly structured skeletal retractor muscle anchors of the perignathic girdle. Here we show that these morphologically specific projections from the oral test plates are undergoing active developmental skeletogenesis during the first weeks after metamorphosis in young juveniles of both S. purpuratus and E. tribuloides. We can now confront directly developmental programs that diverged well before the Permian/Triassic extinction, as the echinoid clades separated, and in the process, we have opened the way technically to experimental exploration of juvenile skeletogenesis. The value of this exploration is that it is conceived in direct relation to ongoing paleontological research being carried out in parallel that will elucidate more exactly the evolutionary pathway to the divergence: that is, we can study the same perignathic skeletal structure through the lenses of both deep time change, and the genetic regulatory program for development. The evolutionary conundrum that infuses this work is both obvious and perplexing. It is obvious that in order to comprehend the genomic changes that account for evolutionary alterations in body plan features, it is necessary to track their cause in the encoded developmental regulatory processes that generate these features (Britten and Davidson, 1971; Davidson and Erwin, 2006; Peter and Davidson, 2011). But in practice it is often perplexing to
find accessible examples where such enquiries might be truly satisfying, which means that the project has to be constrained by a requirement for rigorous cladistic logic, and also that it has actually to be directly relevant to the general problem of body plan evolution. What such constraints boil down to is that the characters under study must be derived, with respect to a plan evolution. What such constraints boil down to is that the characters under study must be derived, with respect to a plan evolution.

Methods

Culturing Et and Sp juveniles

Et adults were collected from San Diego, California, and Et adults were purchased from KP Aquatics, LLC (Key Largo, Florida). Embryos and larvae were handled and cultured as described elsewhere (Schroeder, 1981; Leahy, 1986). Both species usually became competent for metamorphosis about 4 weeks after fertilization. Using the aforementioned methods, induction of Sp larvae to metamorphose is very well established and robust in our experiments. The induction of Et metamorphosis is less well understood (Emlet, 1988). We found the following protocol to be best for inducing Et larvae to metamorphose:
1. One week prior to metamorphosis, a petri dish is placed inside the Et adult aquarium in order to allow microbes to collect on the plate’s surface.
2. Collect larvae and transfer to a flask.
3. Vigorously shake the flask manually for 10 min for turbulence treatment (Gaylord et al., 2013).
4. Add KCl to a final concentration of 75 mM, mount the flask on a shaker running at moderate speed, and expose the larvae to KCl for 1 h.
5. Collect and culture larvae on the petri dish prepared above, and leave them for 2–3 days.
6. Collect the unsettled larvae, culture them with abundant Rhodomonas lens for 3 days, and repeat steps above.

The newly metamorphosed juveniles were transferred to a petri dish coated with diatoms and raised to as late as 8 weeks after metamorphosis. Juveniles were collected at different stages and treated for the following μCT, SEM and WMISH analysis.

X-ray microtomography

X-ray microtomography is a non-invasive way to study sea urchin juvenile development internally and externally. Sample preparation and scanning is quick and convenient. The method is becoming more and more promising as the latest technology now approaches a resolution ≤ 350 nm.

Juveniles were prepared in one of two ways for the μCT analyses. The purpose of the first method was to scan whole specimens, including both their soft tissues and skeletal elements. For this, juveniles were first fixed in glutaraldehyde and then suspended in a sealed micropipette tip for scanning. The focus of this method was to scan skeletal elements only, and for this, juveniles were briefly treated with sodium hypochlorite, suspended in ethanol, air-dried at 4 °C, and mounted on Styrofoam for scanning.

The μCT scanning was undertaken at the Saban Research Institute at the Children’s Hospital Los Angeles using a Bruker SkyScan 1172 tomography system equipped with 100 kV Hama-matsu X-Ray source. Scanning parameters were 40 kV source voltage, 161 mA source current, 6 W power, no filter, 3425 ms exposure time, 3600 angular steps over 360° with 5 averaged images per rotation position, 4000 × 2672 pixel detector size, and about 21 h scan time. Image reconstruction was accomplished using the software NRecon GPU Cluster (Bruker SkyScan, Bruker Scientific Instruments, Germany). The datasets were visualized using the software VGStudio 2.2 (Volume Graphics GmbH, Germany).

The voxel resolution of our μCT datasets was ~1 μm, and for structures above this level of resolution, such as the plates, the spines and the teeth as a whole they are clearly discernible. For structures below this level of resolution, however, such as the trabeculae and pores in the stereom and boundaries of individual plates they are too small to be resolved as discrete entities, a limitation to the use of current laboratory X-ray μCT systems in the study of sea urchin juvenile skeletogenesis.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to study the structures visible only at a higher resolution than that achievable with μCT. Tests from juveniles at different stages of post-metamorphic development were extracted after etching with sodium hypochlorite and sodium hydroxide. A specimen’s peri- gnathic girdle was only observable after the extracted tests were delicately disarticulated under a dissection microscope. Tests to be scanned were air-dried, coated with Pt at 8 nm thickness and observed with a ZEISS 1550VP Field Emission SEM (Caltech GPS Division Analytical Facility, Pasadena, CA). Most images were taken using the following parameters: SE2 detector, 10 kV accelerating voltage, 30 μm aperture, and 5–8 mm WD.

Whole mount in situ hybridization

The WMISH procedure for sea urchin embryos and larvae is a robust, well-established protocol. Specific changes were made to optimize its use for juvenile sea urchins. Juveniles from both species were fixed at different developmental stages with 2% paraformaldehyde and 2% formaldehyde in MOPS buffer for 1 h at room temperature followed by overnight incubation at 4 °C. Fixed juveniles were washed five times with MOPS buffer and stored indefinitely in 70% ethanol at −20 °C until needed. Rehydration was accomplished before hybridization with three washes of 15 min each in TBST buffer. Hybridization was conducted at 60 °C for 3 days in a solution consisting of 50% deionized formamide, 5 × SSC, 1 × Denhardt’s, 1 mg/ml BSA, 1 mg/ml yeast tRNA, 50 μg/ml Heparin, 0.1% Tween-20 and 1 ng/μl riboprobes. Riboprobes containing digoxigenin-UTP were synthesized by conventional methods; for the specific sm37 sequences used as probes in this study see the supplemental data (Fig. S8). Post-hybridization washes occurred as follows: Juveniles were washed in fresh hybridization buffer two times for 1 h each under hybridization conditions and subsequently washed in 2 ×, 0.2 ×, 0.1 × SSCT buffer sequentially for 20 min each. Juveniles were then washed three times in MABT buffer at R/T, then blocked with 10 mg/ml BSA in MABT buffer for 20 min at R/T, and then with 10X goat serum plus 1 mg/ml BSA at 37 °C for 30 min in MABT buffer. Incubation with a 1/1000 dilution of the alkaline phosphatase conjugated Fab fragments (Roche Biosciences) was performed overnight at 4 °C. The antibody was removed with six washes in MABT buffer over an interval greater than 6 h. After two washes in alkaline phosphatase buffer for a total of an hour, the staining was developed by conventional methods with NBT/BCIP. The staining reaction was stopped by dilution in MABT buffer, and juveniles were subsequently stored in a 1:1 mixture of 100% glycerol and MABT for downstream applications. Stained juveniles were observed in whole mount display using an inverted microscope, and the whole staining pattern was recorded at different focus depth along the aboral–oral axis. For sectioning, juveniles were oriented horizontally or vertically to their aboral–oral axis and embedded in Fluka Durcupan Water-Soluble Embedding Medium and sectioned using a Leica Ultracut UCT microtome in serial 4 μm Durcupan sections.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.01.017.
References


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Supplementary data: Fig. S1. Micro-ct scans of Juvenile Strongylocentrotus purpuratus and Eucidaris tribuloides. (A) Three-week post-metamorphosis Strongylocentrotus purpuratus juvenile. (A1) View of apical end. (A2) Computational section through test showing lantern. (B) Four-week post-metamorphosis Eucidaris tribuloides juvenile. (B1) View of apical end. (B2) Computational section through test showing lantern. Fig. S2. Whole mount in situ hybridization staining for sm37 in three-week post-metamorphosis Strongylocentrotus purpuratus juvenile. Plane of focus shifts through juvenile from adapical end (A) to adoral end (I). Fig. S3. Complete sequential horizontal sections of whole mount in situ hybridization staining for sm37 in three-week post-metamorphosis Eucidaris tribuloides juvenile. Sections ordered from below adoral end (A) to apical end (AS). Fig. S4. Complete sequential vertical sections of whole mount in situ hybridization staining for sm37 in a three-week post-metamorphosis Eucidaris tribuloides juvenile. Sections ordered along the axis vertical to the aboral–oral axis. Fig. S5. Staining for sm37 in eight-week post-metamorphosis Strongylocentrotus purpuratus juvenile. (A–C) Horizontal sections. (D) Lateral section. Red letters: (a) test wall; (b) radiole (spine); (c) hemipyramid; (d) tooth, (e) interpyrimidal muscle; (f) podia (tube foot); (g) ampullae. Fig. S6. Staining for sm37 in three-week post-metamorphosis Eucidaris tribuloides juvenile. (A and B) Horizontal sections. (C) Lateral section. Red letters: (a) test wall; (b) radiole (spine); c, podia (tube foot); d, interpyrimidal muscle e; hemipyramid. Fig. S7. SpSm37, EtSm37, EtAlx1 and EtVegfR sequences used as probes in this study.