Cleavage and gastrulation in the shrimp *Penaeus (Litopenaeus) vannamei* (Malacostraca, Decapoda, Dendrobranchiata)

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Abstract

While most malacostracan crustaceans develop through superficial cleavage, in the Amphipoda, Euphausiacea, and Dendrobranchiata (Decapoda) cleavage is complete. Euphausiaceans and dendrobranchiate shrimp share a similar early cleavage pattern, early cleavage arrest and ingestion of mesendoderm progenitor cells, a ring of crown cells (prospective naupliar mesoderm) around the blastopore, and hatching as a nauplius larva. Yet recent phylogenies do not support a close relationship between Euphausiacea and Decapoda. In addition, some variation is reported in the timing of mesendoderm cell arrest and number of crown cells for a number of dendrobranchiates. To determine the representative pattern of development in the Dendrobranchiata, embryos of the Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei* were stained with Sytox Green to label chromosomes and nuclei and examined with confocal microscopy. The early cleavage pattern, mesendoblast arrest and subsequent ingestion at the 32-cell stage, presence of 8 initial crown cells, and fates of the mesendoblasts are the same for *P. vannamei* (family Peneaeidae) and *Sicyonia ingentis* (family Sicyoniidae). The lineage of the primordial endoderm cells differs from that reported for *P. kerathurus*. These characters were discussed in the context of the evolution of development in the Dendrobranchiata and in comparison to the Euphausiacea.

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Keywords: Cleavage; Gastrulation; Shrimp; Dendrobranchiata; *Litopenaeus vannamei*

1. Introduction

Malacostracan crustaceans develop by a wide variety of cleavage and gastrulation modes to a diverse number of larval forms and adult body plans (Anderson, 1973; Scholtz, 1997; Scholtz, 2000; Gerberding and Patel, 2004). Cleavage can be total, as in the primitive *Anaspides* (Syncarida), krill (Euphausiacea), penaeoidean shrimp (Decapoda, Dendrobranchiata), and Amphipoda, or superficial, as in most of the remaining malacostracan clades (reviewed by Gerberding and Patel, 2004). In groups which undergo total cleavage, gastrulation can occur by ingestion into a hollow blastula, as in *Anaspides* (Hickman, 1937), by ingestion of mesendoderm and invagination of naupliar mesoderm, as in penaeoidean shrimp and krill (Hertzler and Clark, 1992; Alwes and Scholtz, 2004), or by variants of ingestion and invagination in amphipods (Gerberding et al., 2002; Scholtz and Wolff, 2002; Wolff and Scholtz, 2002).

Comparisons between the Dendrobranchiata and Euphausiacea are interesting since they share adult morphological features and develop to a free-swimming nauplius larva, while other malacostracans pass through an ‘egg-nauplius’ or undergo direct development (Scholtz, 2000). Dendrobranchiate shrimp and krill also share a similar pattern of total cleavage and mode of gastrulation (Taube, 1909, 1915; Zilch, 1978, 1979; Hertzler and Clark, 1992; Alwes and Scholtz, 2004). However, recent studies based on an extensive number of characters from morphology, anatomy, and embryology (Richter and Scholtz, 2001) and 28S rDNA (Jarman et al., 2000) place the Euphausiacea closer to malacostracan clades other than decapods.

A recent study examined cleavage and gastrulation in the euphausiacean *Meganycitiphanes norvegica* by DNA staining and fluorescence microscopy (Alwes and Scholtz, 2004). This study confirms and extends the similarities between euphausiacean and dendrobranchiate cleavage and gastrulation, which include: (1) a similar arrangement...
of 4-cell stage blastomeres; (2) an invariant cleavage pattern, including mirror-image patterns and two interlocking semicircles of cells through the early cleavages; (3) the cleavage arrest at the 32-cell-stage and subsequent ingestion of two cells Xd and Xv, at the vegetal pole, which identify the dorsal–ventral axis, and may give rise to endoderm and germ line (Taube, 1909, 1915) or mesendoderm and germ line (Zilch, 1978, 1979; Hertzler and Clark, 1992; Hertzler, 2002); and (4) the radial division of cleavage-retarded crown cells (Kranzzellen) in a ring around Xd and Xv, which later forms the naupliar mesoderm. Differences between euphausiaceans and dendranchiates include the cell lineage of the crown cells and position of the dorsal–ventral axis (Alwes and Scholtz, 2004).

The classification of penaeoidean shrimp is controversial at present, centered on the question of whether six subgenera of *Penaeus senso lato* are natural groups that should be raised to the generic level (Pérez Farfante and Kensley, 1997; Baldwin et al., 1998; Maggioni et al., 2001; Lavery et al., 2004). A recent study of 26/28 of the recognized species of genus *Penaeus s.l.*, based on the mitochondrial 16S and COI genes, found support for the monophyly of some subgenera, e.g. *Litopenaeus* and *Farfantepenaeus*, but not for others, e.g. *Penaeus s.s.* and *Marsupenaeus* (Lavery et al., 2004). The first comprehensive phylogeny of *Penaeus* provides an opportunity to ask questions about the evolution of development in the Dendrobranchiata. Embryological characters can be mapped onto the phylogeny to produce hypotheses about homology versus convergent evolution and the direction of evolutionary change (Raff, 1996).

While the details of dendrobranchiate development are best known for the ridgeback prawn *Sicyonia ingentis*, incomplete data are available for other species (Table 1), including the sergestoidean shrimp *Lucifer* (Brooks, 1882), the kuruma shrimp *Penaeus* (*Marsupenaeus*) *japonicus* (Hudinaga, 1942; Kajishima, 1951), the caramote shrimp *Penaeus* (*Melicertus*) *kerathurus* (= *P. trisulcatus*) (Heldt, 1938; Zilch, 1978, 1979), the Green tiger shrimp *Penaeus semisulcatus* (Kungvankij et al., 1980), and the Indian white shrimp *Penaeus* (*Fenneropenaeus*) *indicus* (Morelli and Aquacop, 2003). Two embryological characters can be compared from the literature: the stage at which mesendoderm cell arrest occurs, which is 64-cells for *P. japonicus* and 16-cells for *P. kerathurus*, and the initial number of crown cells, which is 8 for both *P. japonicus* and *P. kerathurus* (Kajishima, 1951; Zilch, 1979). In addition, there are claims of embryos with ‘64-cells’ and ‘128-cells’ for *P. kerathurus* and *P. semisulcatus* (Heldt, 1938; Kungvankij et al., 1980), which do not account for the arrest of the mesendoderm cells. It was therefore of interest to examine the pattern of cleavage and gastrulation in detail in another representative of the Dendrobranchiata, the Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei*. This species is the most commonly farmed shrimp species in the western hemisphere and thus represents an important agricultural model. A comparison with *S. ingentis* showed an identical pattern of cleavage, time of mesendoderm cell arrest, and number of initial crown cells formed. In addition, development of the mesendodermal derivatives in *P. vannamei* was identical to that described for *S. ingentis*. The primordial endoderm cell derived from the ventral mesendoblast Xv in both *P. vannamei* and *S. ingentis*; in contrast, the primordial endoderm cell is reported to derive from the dorsal mesendoblast Xd in *P. kerathurus*.

### Table 1

Partial classification of relevant malacostracan crustaceans, with references referred to in the text

<table>
<thead>
<tr>
<th>Class Malacostraca</th>
<th>Order Euphausiacea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superorder Eucarida</td>
<td><em>Euphausia sp.</em> (Taube, 1909, 1915)</td>
</tr>
<tr>
<td>Order Euphausiacea</td>
<td><em>Meganyctiphanes norvegica</em> (Alwes and Schultz, 2004)</td>
</tr>
<tr>
<td>Family Penaeidae</td>
<td></td>
</tr>
<tr>
<td><em>Penaeus (Fenneropenaeus)</em></td>
<td></td>
</tr>
<tr>
<td><em>indicus</em> (Morelli, 2003)</td>
<td></td>
</tr>
<tr>
<td><em>P. (Litopenaeus)</em></td>
<td></td>
</tr>
<tr>
<td><em>vannami</em> (present study)</td>
<td></td>
</tr>
<tr>
<td><em>P. (Marsupenaeus)</em></td>
<td></td>
</tr>
<tr>
<td><em>japonicus</em> (Kajishima, 1951)</td>
<td></td>
</tr>
<tr>
<td><em>P. (Melicertus)</em></td>
<td></td>
</tr>
<tr>
<td><em>kerathurus</em> (Zilch, 1978, 1979)</td>
<td></td>
</tr>
<tr>
<td>Family Sicyoniidae</td>
<td></td>
</tr>
<tr>
<td><em>Sicyonia ingentis</em></td>
<td></td>
</tr>
<tr>
<td>(Hertzler and Clark, 1992; Hertzler, 2002)</td>
<td></td>
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<tr>
<td>Family Solenoceridae</td>
<td></td>
</tr>
<tr>
<td><em>Solenocera koelbeli</em></td>
<td></td>
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<tr>
<td>(Lavery et al., 2004)</td>
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</tr>
<tr>
<td>Superfamily Segestoidae</td>
<td></td>
</tr>
<tr>
<td>Family Luciferidae</td>
<td></td>
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<tr>
<td><em>Lucifer sp.</em> (Brooks, 1882)</td>
<td></td>
</tr>
<tr>
<td>Suborder Pleocyemata</td>
<td></td>
</tr>
</tbody>
</table>

After Martin and Davis (2001) and Pérez Farfante and Kensley (1997). Note that recent studies have questioned the close relationship of the Euphausiacea to the Decapoda (Jarman et al., 2000; Richter and Schultz, 2001).

2. Material and methods

2.1. Broodstock and spawning

*Penaeus vannamei* broodstock for this study were maintained at the Hawaii Oahu Suisan, Inc. hatchery in Kahuku, Hawaii in March 2002, as described in Wyban and Sweeney (1991). A 12-ft diameter recirculating maturation tank, containing about 35 females and 45 males, was placed on a reverse photoperiod so that ‘sunset’ occurred at 6 a.m. to induce mating. Mated females were sourced 2 h later and placed in a bucket with 5-L of seawater with gentle aeration at 27 °C, and monitored for spawning activity. Animals were allowed to spawn for 1 min then were removed from the bucket, which was then swirled periodically to keep the oocytes in suspension while the jelly was extruded. The
culture was maintained at 27 °C during the sampling period, through 8 h post-spawning (ps). Samples were taken every 15 min, passed through a 500 μm nylon mesh to remove the jelly, briefly pelleted with a hand centrifuge, and fixed and stored in 90% methanol-50 mM EGTA, pH 8.0.

2.3. Cell nomenclature

The cell nomenclature in Fig. 1 is based on that proposed for the dendrobranchiate shrimp 

Sicyonia ingentis (Hertzler and Clark, 1992; Hertzler, 2002) and the euphasiacean

Meganyctiphanes norvegica (Alwes and Scholtz, 2004). The four capital letters A, B, C, and D are used for the 4-cell blastomeres, with the D cell containing the vegetal pole and giving rise to the mesendoderm cells.

3. Results

3.1. Fertilization and zygote

Oocytes are spawned arrested at meiosis I and sperm immediately bind and enter. Meiosis is resumed with the formation of two polar bodies, one outside and one inside a hatching envelope (not shown). No attempt was made to follow the location of the polar bodies during cleavage, to determine the relation of the polar axis to the first cleavage plane. Development proceeds within the hatching envelope, from which the nauplius larvae hatch within 12 h. While newly-spawned oocytes are oblongate, the fertilized egg becomes perfectly spherical, with a diameter of 230 μm and a uniform distribution of yolk granules (Fig. 2(A)). Sperm nuclei were present in oocytes by 12.5 min ps, and pronuclear migration occurred from 15–25 min ps (data not shown), in a similar fashion as in 

S. ingentis (Hertzler and Clark, 1993).

3.2. First division and 2-cell stage, second division and 4-cell stage

The first cleavage occurred at about 40 min ps at 27 °C. The cleavage pattern of 
P. vannamei was similar to that described previously for 

S. ingentis (Hertzler and Clark, 1992). A revised nomenclature (Fig. 1) was used to label the early blastomeres, based on Hertzler and Clark (1992) and a recent study on the krill 

M. norvegica (Alwes and Scholtz, 2004). First cleavage was complete, resulting in a 2-cell stage of approximately equally sized blastomeres, AB and CD (Fig. 2(C) and (D)). If the relation of these cells to the polar axis is the same as that determined for 

S. ingentis, the CD blastomere contains the vegetal pole (Hertzler and Clark, 1992).

The second division planes formed synchronously at a 45° angle to each other, resulting in a 4-cell stage with blastomeres oriented in a close packing arrangement (Fig. 2(E)–(H)). All four blastomeres appeared to be the same size in intact embryos, so that no asymmetries could be detected at the 4-cell stage. The cell identities and sister cell relationships in Fig. 2 were inferred by tracing spindle orientations back from the 32-cell stage, and from live embryo lineage tracing in 

S. ingentis (Hertzler and Clark, 1992; Hertzler et al., 1994). Blastomeres A and C contacted each other at a cross-furrow running in the mid-sagittal plane on the dorsal side (Fig. 2(F)), while blastomeres B and D touched in a transverse furrow on the ventral side (Fig. 2(H)). A and C thus became left and right territories while B and D were anterior and posterior, respectively.

3.3. Third division and 8-cell stage

The third division, from 4- to 8-cells, was now orthogonal to the preceding one, with A and C spindles and B and D spindles orienting end to end. As in 

S. ingentis and 

M. norvegica, this resulted in two interlocking semicircles of cells, AC and BD. A divided into the dorsal cell A1 and ventral A2, while C divided into the dorsal cell C1 and ventral C2 (Fig. 2(J) and (L)). A1 and C1 thus met at the AC cross-furrow, while A2 and C2 lay at the ends of the AC band. From left ventral to right ventral, the cells in the AC band consisted of A1p, A1, C1, and C2p. B divided into the dorsal B1 and ventral B2, while D divided into the dorsal D1 and ventral D2 (Fig. 2(J) and (L)). B1 and D1 met at the BD cross-furrow, while B2 and D2 lay at the ends of the BD band. From the dorsal, anterior to the dorsal, posterior, the cells in the BD band consisted of B1, B1p, D1, and D1p.

3.4. Fourth division and 16-cell stage

The fourth division was synchronous, with spindles in the AC and BD bands in a row of four side-by-side spindles, yielding a 16-cell stage (Fig. 2(J) and (L)). The AC band divided in the anterior–posterior direction, with A1 forming A1a and A1p, A2 forming A2a and A2p, C1 forming C1a and C1p and A2 forming A2a and A2p (Fig. 2(N)). The BD band divided left and right, with B1 forming B1a and B1l, B2 forming B2a and B2l, D1 forming D1a and D1l, and D2 forming D1a and D1l (Fig. 2(N) and (P)).

3.5. Fifth division and 32-cell stage

In the synchronous fifth division, the spindles of the AC
and BD bands formed two rows of four cells with spindles oriented end-to-end, resulting in a 32-cell stage embryo (Fig. 2(M)–(P), Fig. 3(A), (D) and (G)). The AC and BD bands divided dorsal–ventrally. A IIa formed AIIad and AIIav, AIIp formed AIIpd and AIIpv, AIa formed AIad and AIav, and AIp formed AIpd and AIpv (Fig. 3(B), (C), (E) and (F)). C IIa formed CIIad and CIIav, CIIp formed CIIpd and CIIpv, CIa formed CIad and CIav, and CIp formed CIpd and CIpv.

Fig. 1. Cell lineage of *Penaeus vannamei*, showing the relative timing of cleavage divisions in min ps at 27 °C (A) and naupliar mesoderm and mesendoderm detail (B–E). (A) The overall lineage is color coded as to germ layer, with ectoderm in blue, mesoderm in red, endoderm in yellow, and germ line in purple. The nomenclature refers to the most commonly observed cleavage pattern, where DIIr = X, the progenitor of the two mesendoderm cells. The AB blastomere contains the animal pole, while the CD blastomere contains the vegetal pole. A and C make cross-furrow contact, as do B and D. The subscript I refers to the cell adjacent to the cross-furrow (e.g. A1), while the subscript II refers to the cell away from the cross furrow (e.g. A2). Additional designations are based on relative axial positions, either anterior–posterior (a, p), dorsal–ventral (d, v) or left–right (l, r). Lineage of the ectoderm is shown through 7 cell divisions. The lineage and nomenclature of the 9 crown cells K1–K9 are shown through two additional divisions. The derivatives of the mesendoderm are: the endodermal yolk cells, primordial endoderm cell E, mesoteloblast M, and germ cell G. (B) 122-cell stage, vegetal view showing replicated crown cells. (C) Dorsal (Xa) and ventral (Xv) mesendoblasts, with the rest of the embryo omitted for clarity. (D) Result of first (anterior–posterior) mesendoderm division, posterior view. (E) Result of second mesendoderm division, posterior view. (F) Result of third mesendoderm division, posterior view. (G) Result of fourth mesendoderm division, posterior view.
and C\textsuperscript{p}v (Fig. 3(B), (C), (E) and (F)). B\textsubscript{H}r formed B\textsubscript{H}ad and B\textsubscript{H}av, B\textsubscript{H}l formed B\textsubscript{H}pd and B\textsubscript{H}pv, B\textsubscript{a} formed B\textsubscript{ad} and B\textsubscript{av}, and B\textsubscript{p} formed B\textsubscript{pd} and B\textsubscript{pv} (Fig. 3(E) and (F)). D\textsubscript{H}r formed D\textsubscript{H}ad and D\textsubscript{H}av, D\textsubscript{H}l formed D\textsubscript{H}pd and D\textsubscript{H}pv, D\textsubscript{a} formed D\textsubscript{ad} and D\textsubscript{av}, and D\textsubscript{p} formed D\textsubscript{pd} and D\textsubscript{pv} (Fig. 3(B) and (C)).

### 3.6. Sixth division and 62-cell stage

The sixth division was synchronous in all but two 32-cell stage blastomeres. The two mesendoblasts arrested their cell division, while the remaining blastomeres continued dividing (Fig. 3(A)–(C), (G)–(I)). The two mesendoblasts defined
the dorsal–ventral axis, with \( X_d \) dorsal and \( X_v \) ventral. As in \( S. \) ingentis, two pairs of mirror image patterns were observed at the 32-cell stage. In the DII pattern, the mesendoblasts derived from either D IIlo or D IIr (e.g. in Fig. 3(A)–(C)), while in the DI pattern, the mesendoblasts derived from either DIlo or DIr (e.g. in Fig. 3(G)–(I)). The frequencies of these patterns differed from \( S. \) ingentis, however, with the DIIr pattern (designated Ddr in Hertzler and Clark, 1992) much more common than the DIl (Ddl), DIIl (Ddl) and DIl (Dvl) patterns. 25 embryos were optically sectioned and reconstructed for 3-D analysis. The DIIr pattern accounted for 64% of the embryos (Table 2). Therefore, this pattern was used as the reference cell lineage in Fig. 1 and designated as cleavage type I. The mesendoblasts \( X_d \) and \( X_v \) ingressed into the blastocoel during the 62-cell stage, to become surrounded by the rest of the embryo and exposed only at the blastopore at the posterior end (Fig. 4(A)–(C)).

Fig. 3. 32-cell stage \( P. \) vannamei, stained with Sytox Green for chromosomes and nuclei. Half-embryo projection of optical sections (A, D, G), with corresponding drawings showing cleavage planes (B, E, H) and labeled cells (C, F, I). Cleavage type I (DIIr \( \equiv X \)) is shown in posterior view (A, B, C) and same embryo in anterior view (D, E, F). Cleavage type II (DIl \( \equiv X \)) is shown in posterior view (G, H, I). Scale bars=60 \( \mu \)m. Time = 150 min.
Four C cell derivatives and five D cell derivatives adjacent to the mesendoblasts divided so that the posterior descendants remained in contact with the mesendoblasts (Fig. 4(A)–(C)). In embryos where the mesendoblasts derived from D_{1r}, the C derivatives C_{Ipdp} (K1), C_{ipv} (K2), C_{Ipd} (K3), and C_{ipv} (K4) adjacent to the mesendoblasts were delayed in their cell cycles relative to the ectoderm cells, as were the D derivatives adjacent to the mesendoderm cells, D_{rdr} (K5), D_{rdl} (K6), D_{ldr} (K7), D_{rlr} (K8) and D_{ldr} (K9). These cells were designated crown cells, homologous to the 9 cells found in S. ingentis and 8 cells found in euphausiaceans (Taube, 1909; Alwes and Scholtz, 2004).

The division of the crown cells was delayed relative to that of the ectoderm cells, and asynchronous relative to one another. Embryos in various stages of crown cell division were observed at 195 min ps (Fig. 4(A), (G), (I)–(K)). The dorsal–ventral orientation of the embryo could be determined by the position of the AC band of ectoderm cells and by the pattern of crown cells surrounding the mesendoblasts. Five crown cells made contact with X_d, while 6 crown cells made contact with X_v. After their division, 18 crown cells were present in two concentric rings (Fig. 4(L)). In later stage embryos, the crown cells gave rise to the naupliar mesoderm, as described in other penaeidean shrimp (Zilch, 1979; Hertzler and Clark, 1992).

### 3.7. Seventh and eight cleavages to 122- and 244-cell stages, first mesendoblast division

The two mesendoderm cells remained in interphase, while the remaining cells went through the 7th cleavage division to form a 122-cell embryo (Fig. 4(K) and (L)). By 210 min ps, the ectoderm was in mitosis of the 8th cleavage, with the crown cells again lagging behind the ectoderm cells (data not shown). The timing of cell divisions in the ectoderm was not followed further. The outer ring of crown cells entered mitosis several minutes before the inner ring of crown cells (Fig. 5(A)). The mesoderm cells, which had remained in interphase for the preceding three cell cycles, underwent their first division after arrest (corresponding to their 6th cycle) at 240 min ps (Fig. 5(B) and (C)). X_d and X_v both divided in an anterior–posterior direction, resulting in X_{d,a}, X_{d,p}, X_{v,a}, and X_{v,p}.

### 3.8. Second mesendoblast division

At 270 min ps, the four mesendoblast derivatives entered mitosis again (their 7th cycle), with the anterior cells X_{d,a} and X_{v,a} dividing left–right to form X_{a,l}, X_{a,r}, X_{a,l}, and X_{a,r} (Figs. 5(D) and (G); 6(A) and (B)). The posterior cells X_{a,p} and X_{p} divided in an oblique dorsal–ventral direction (Fig. 5(E) and (H)). X_{a,p} formed X_{dpd} at the dorsal anterior and X_{d,dp} at the ventral posterior, while X_{p} formed X_{pa} = M at the ventral anterior and X_{pp} = G at the dorsal posterior. The naupliar mesoderm, descended from the crown cells, continued to proliferate around the mesendodermal pyramid (Fig. 6(E)–(H)), and further posterior in a layer beneath the ectoderm (Fig. 5(F) and (I)). The resulting 8 mesendodermal derivatives were oriented as described for P. kerathurus as a ‘mesendodermal pyramid’ (Zilch, 1979).

In the 285 min ps embryo, the four endodermal yolk cells X_{dpd}, X_{dar}, X_{dal} (Fig. 6(A) and (B)) and X_{dpv} (Fig. 6(C) and (D)) were present dorsally. These are thought to give rise to the midgut-coeca digestive glands (Zilch, 1979). X_{dar} and X_{dal} lie below the yolk endoderm and are slightly larger than the yolk endoderm cells (Fig. 6(A) and (B)). These are the primordial endoderm cells Er and El, which undergo two subsequent anterior–posterior divisions by 7 h ps and later form the midgut epithelium (Zilch, 1979). The largest cell at the ventral anterior is the primordial mesoteloblast M (Fig. 6(A) and (B)). This stem cell undergoes teloblastic cell divisions to form the post-naupliar mesoderm (Zilch, 1979; Hertzler, 2002). Its sister cell G is thought to be the primordial germ cell (Fig. 6(C) and (D); Zilch, 1979; Hertzler, 2002).

### 3.9. Divisions and arrest of endoplasts and mesoteloblast

By 315 min ps, the first indications of segmentation are evident in the ectoderm of embryos oriented on their dorsal or ventral sides (Fig. 6(E)–(L)). The primordial mesoteloblast M remains undivided as the largest cell in the embryo, reliably marking the ventral side (Fig. 6(E) and (I)). The primordial germ cell G is also undivided, and positioned as a ‘cap’ at the ventral posterior of the mesendodermal pyramid (Fig. 6(F) and (J)). The endodermal derivatives X_{al}, X_{ar} (Fig. 6(G) and (K)), and X_{dpd} (Fig. 6(H) and (L)) divided slightly before El, Er (Fig. 6(F) and (J)) and X_{dpv} (Fig. 6(G) and (K)). El and Er divided to form the smaller cells el1 and er1 at the anterior and the larger cells El1 and Er1 at the posterior (Fig. 7(B) and (F)). The naupliar mesoderm continued to proliferate, lying underneath the ectoderm (Fig. 6(E)–(L)). By 330 min ps, the primordial mesoteloblast divided in an anterior–posterior direction, leaving a smaller daughter m1 at the anterior and a larger daughter M1 at the posterior (Fig. 7(A), (E), (I) and (M)). The primordial germ cell remained undivided with a large nucleus, posterior to M (Fig. 7(A), (E), (I) and (M)). The yolk endoderm cells and primordial endoderm cells El and Er were present in more dorsal sections (Fig. 7(B)–(D), (F)–(H)). By 375 min ps, El1 and Er1 again divided anterior–posterior to form the smaller cells el2 and er2 at the anterior and larger cells El2 and Er2 at the posterior (Fig. 7(J), (N),

### Table 2

Frequencies of cleavage patterns in *P. vannamei* embryos

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<tr>
<th>Progenitor cell at 8-cell stage</th>
<th>D_{tr}</th>
<th>D_{tl}</th>
<th>D_{fr}</th>
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<tr>
<td>Number</td>
<td>16</td>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Percent</td>
<td>64</td>
<td>28</td>
<td>4</td>
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By 390 min ps, the mesoteloblast M1 divided to form m2 at the anterior and M2 at the posterior (Fig. 7(K), (O), (Q) and (R)). At 420 min ps (7 h ps) the stem cells M2, G, El2, and Er2 were located in the mandibular segment. The endoblasts El2 and Er2 shifted from their original left–right relationship to a more anterior–posterior one (Fig. 7(S) and (T)), as has been observed for P. kerathurus (Zilch, 1979) and S. ingentis (Hertzler, 2002). This is a second indication of left–right asymmetry, although it is not clear whether El2 or Er2 is the cell that is shifted to the posterior.

4. Discussion

4.1. Homologous patterns of early cleavage pattern and mesendoblast arrest in dendrobranchiate shrimp

Comparing P. vannamei (family Penaeidae) and S. ingentis (family Sicyoniidae) confirms that a shared, invariant, early cleavage pattern occurs in dendrobranchiate shrimp. Homologous patterns of early cleavage and mesendoblast arrest and subsequent development occur in
the Dendrobranchiata, but the stage at mesendoderm arrest varies by genus (summarized in Fig. 8). The 4-cell stage is composed of blastomeres in a close-packed arrangement, with B and D forming a transverse cross furrow and A and C forming a sagittal cross furrow. The next cleavages are tangential, with each subsequent division orthogonal to the preceding one, which forms two semicircles of interlocking cells. Evidence of this pattern exists as well for *P. kerathurus* and *Parapenaeus longirostris* (Heldt, 1938, Figs. 35 and 38). Cleavage is complete and synchronous until the 32-cell stage in *S. ingentis* and *P. vannamei*, at which time the two mesendoblasts arrest for the next three cell cycles of the rest of the embryo, producing first a 62-cell then a 122-cell stage. In *P. indicus* (family Penaeidae), the mesendoblasts also arrest at the 32-cell stage (Morelli and Aquacop, 2003). This contrasts with reports for *P. kerathurus* and *P. japonicus* (both family Penaeidae), where the mesendoderm cells arrest at the 16- and 64-cell stage, respectively (Zilch, 1978, 1979; Kajishima, 1951). This consequently would produce embryos of 30- and 58-,
section with ventral mesoderm cell m1, mesoteloblast M1, and primordial germ cell G. (J, N) More dorsal section from same embryo as I, M with division of El1 and Er1. (K, O) Ventral section with division of mesoteloblast M1. (L, P) More dorsal section from same embryo as K, O showing position of El2, Er2, and G. (Q, R) Ventral section with positions of ventral mesoderm cell m2, mesoteloblast M2, and G. (S, T) More dorsal section from same embryo as Q, R showing shifted positions of El2 and Er2.
and 114-cells in *P. kerathurus*, and a 126-cell stage for *P. japonicus*. Reports of dendrobranchiate embryos with 64-cells in the older literature should be viewed with caution until confirmed by detailed analysis.

Mapping the stage at mesendoblast arrest onto a dendrobranchiate phylogeny (Fig. 8) suggests that arrest at the 32-cell stage is the basal condition, since the outgroup species to the Penaeidae, *S. ingentis* (family Sicyoniidae), shares this character with two members of the family Penaeidae. Furthermore, the Euphausiacea, an outgroup to the Dendrobranchiata, also have an arrest at 32-cells of apparently homologous cells. Two clades of the genus *Penaeus* s.l. have been recently identified, based on mitochondrial DNA sequences: *Melicertus* + *Marsupenaeus* and *Penaeus* s.s. + *Fenneropenaeus* + *Farfantepenaeus* + *Litopenaeus* (Lavery et al., 2004). Mesendoderm cell arrest at a stage other than 32-cells (either 16- or 64-cells) is consistent with the grouping of *P. (Melicertus) kerathurus* and *P. (Marsupenaeus) japonicus* in the *Melicertus* clade (Baldwin et al., 1998; Lavery et al., 2004) and may be an autapomorphy of this group.

What molecular mechanism might account for the different timing of mesendoderm cell arrest? It seems likely that the mesendoderm fate and cell division pattern is specified by cytoplasmic determinants at the vegetal pole, including cell cycle regulators (Hertzler et al., 1994). Perhaps larger or smaller areas of localized cell cycle determinants may be present, so that the threshold concentration for their effect is reached earlier in *P. kerathurus* and later in *P. japonicus*. Alternatively, the different timing of arrest of the mesendoderm cells may be due to a differential timing of translation of cell cycle regulators. One candidate for an intrinsic cell cycle regulator is the Retinoblastoma (Rb) protein. Maternal Rb mRNA could be localized at the vegetal pole then translated into protein in the mesendoblasts to cause their arrest in G1. The resumption of mitosis in the mesendoblasts might require the inactivation of Rb, perhaps by signaling through the hedgehog pathway as has been shown in the *Drosophila* eye (Duman-Scheel et al., 2002). Mesendoblast arrest and resumption of cell division in dendrobranchiate shrimp offers a simple model to study the regulation of the cell cycle during development.

Mirror-image cleavage patterns were observed in *P. vannamei*, as for *S. ingentis*, but the frequency of these patterns differed between the two. In *S. ingentis*, the DII (Dd) pattern occurred in about 3/4 of the observed cases, while the DI (Dv) pattern occurred about 1/4 of the time (Hertzler and Clark, 1992). In *P. vannamei*, the DII pattern occurred in 92% of the embryos examined, while the DI pattern occurred in 8%. Of the DII embryos, in *S. ingentis* about 1/2 were of the DII (Ddl) and 1/2 were DIIr (Ddr), while in *P. vannamei*, 64% of the embryos were of the DIIr type. Thus in *P. vannamei* there is a bias towards the DII (and perhaps the DIIr) pattern of cleavage, so the cell lineage in Fig. 1 is based on this pattern. The mechanism of this bias is a matter of speculation, but the observations may indicate that one pattern of genetic ‘handedness’ is favored over the other, as has been documented for some gastropod mollusk. The isolation and characterization of these determinants is underway in *Lymnaea* (Harada et al., 2004). Similar genes may cause the mirror-image patterns observed in *P. vannamei*, but whether the observed patterns occur in a dominant-recessive maternal effect is beyond the scope of this study.

### 4.2. Homologous patterns of crown cell division and fate in dendrobranchiate shrimp

In both *S. ingentis* and *P. vannamei*, the crown cells (prospective naupliar mesoderm) form a ring of nine division-retarded cells around the ingressed mesendoderm cells. The lineage and division of the crown was examined in more detail in the present study. Their lineage was inferred from several lines of evidence, including (1) their position within the semicircular bands of cells identified in previous cleavage stages, and (2) lineage tracing studies performed in live *S. ingentis* embryos (Hertzler and Clark, 1992; Hertzler et al., 1994). In *S. ingentis*, 4-cell injections of lineage tracer revealed that

![Fig. 8. Partial phylogeny of the Dendrobranchiata (modified from Baldwin et al., 1998; Lavery et al., 2004), mapping embryological characters onto the tree. Representative species from each clade within the family Penaeoidea are shown, with representatives of family Sicyoniidae and Order Euphausiacea shown as outgroups. Three embryological character states are shown adjacent to the tree: the stage at mesendoderm cell arrest (#X), the number of initial crown cells (#C), and the identity of the primordial endoderm cell (E). Hypothesized transitions in embryological characters are: (1) change from 8 to 9 crown cells, (2) mesendoderm cell arrest from 32-cell stage to 16 cells, (3) change from 9 to 8 crown cells, and (4) mesendoderm cell arrest from 16 cells to 64 cells.](image-url)
the naupliar mesoderm derived from the C and the D blastomeres. As inferred in this study, the crown cells K1, K2, K3, and K4 derive from the C blastomere, with K1 the dorsal-most cell in the ring, while K5, K6, K7, K8, and K9 derive from the D blastomere, with K5 and K6 being the only sister pair, at the ventral side. In P. japonicus and P. kerathurus, 8 crown cells are present (Kajishima, 1951; Zilch, 1979). The number of crown cells that form correlates with the time at which the mesendoderm cells arrest and ingress inside the embryo; species were the mesendoderm cells arrest at 32-cells have 9 crown cells, while species where the mesendoderm cells arrest at 16- or 64-cells have 8 crown cells. Embryos with nine initial crown cells may be the basal character state for the Dendrobranchiata, since the outgroup Sicyonia has this condition.

In P. vannamei, the crown cells were observed to divide late than the ectoderm, but asynchronously with each other. In both P. vannamei and S. ingentis, the first crown cell division is oriented radially into the blastopore. Subsequent divisions occur along the anterior–posterior axis to build up a layer of mesoderm basal to the overlying ectoderm. By following their development over sequential stages, it is clear that the crown cell derivatives are naupliar mesoderm, as opposed to the posterior teloblastic mesoderm that originates from the primordial mesoteloblast.

4.3. Homologous fates of the mesendoderm in dendrobranchiate shrimp?

As first described by Zilch (1978, 1979) for P. kerathurus, the mesendoderm divides in an invariant pattern and is hypothesized to produce yolk endoderm cells, the primordial endoblast, the primordial mesoteloblast, and the primordial germ cell. These cell types were identified by morphological and ontogenetic criteria. The endodermal yolk cells continue a regular series of cell divisions and later become vacuolated in the course of their differentiation to the epithelium of the digestive gland. Their nuclei become highly condensed in S. ingentis (Hertzler, 2002). The primordial endoblast is retarded in division relative to the yolk endoderm and divides to form the midgut epithelium; the primordial mesoteloblast is the largest side at the ventral side, and undergoes teloblastic divisions to eventually form an arc of cells on the ventral side. The primordial germ cell has ‘an extremely large nucleus with clearly visible chromosomes’ (Zilch, 1979). These fate designations, while likely to be accurate, should be considered provisional until they can be confirmed by molecular markers. With this caveat, homologous cells to P. kerathurus were found in P. vannamei by position relative to other cells and timing and orientation of cell division. The primordial endoderm cell was X₄a in both S. ingentis (Hertzler, 2002) and P. vannamei. In contrast, in P. kerathurus the primordial endoderm cell is proposed to be a dorsal mesendoblast derivative, equivalent to X₄pd (Zilch, 1979).

4.4. Comparison to Euphausiacea

A number of authors have noted the similarities of early cleavage and gastrulation in dendrobranchiate and euphausiacean shrimps (Taube, 1909; Shiino, 1957; Zilch, 1979; Hertzler and Clark, 1992; Alwes and Scholtz, 2004). A recent study of the euphausiacean M. norvegica has revealed the similarity of early cleavage in detail, including the interlocking bands of cells and arrest at 32-cells of two presumed mesendoderm cells (Alwes and Scholtz, 2004). Furthermore, a similar pattern of crown cells (presumptive naupliar mesoderm) forms in both dendrobranchiates and euphausiaceans. Based on their distinctiveness and complexity, these patterns are hypothesized to be homologous (Alwes and Scholtz, 2004). Alwes and Scholtz were careful not to assign fates to the presumed mesendoblasts and naupliar mesoderm cells in M. norvegica, but it seems reasonable to use them as a working hypothesis until more detailed lineage tracing studies become available in both taxa. It will be of particular interest to follow the fates of the mesendoderm in Euphausia, one is reported to generate endoderm, while the other forms endoderm and the germ cells (Taube, 1909, 1915; Shiino, 1957). This corresponds with dendrobranchiates, where the dorsal mesendoblast gives rise to endoderm only (endodermal yolk cells), while the ventral mesendoderm gives rise to the endoblasts, primordial germ cell, and mesoteloblast (Zilch, 1978; Hertzler, 2002; present study). The fates of the mesendoblasts may therefore be homologous as well, providing further embryological evidence for a sister relationship between the Euphausiacea and the Dendrobranchiata. In contrast, some recent studies have suggested that Euphausiaceans are the sister group to the Peracarida/Pancarida (Jarman et al., 2000; Richter and Scholtz, 2001). The resolution of this conflict must await further embryological and molecular evidence.

4.5. Conclusions on the evolution of development in the Dendrobranchiata

An embryo with 9 crown cells was likely the condition for the last common ancestor of the Penaeidae and Sicyoniidae. The number of crown cells is predicted to be nine for P. indicus, based on the pattern that holds for species that have mesendoderm arrest at 32-cells. In contrast, in the Euphausiacea, 8 crown cells form, so a transition from 8 to 9 crown cells must have occurred after the divergence of the Dendrobranchiata and Euphausiacea from their last common ancestor (Fig. 8, step 1). The number of crown cells has reverted to 8 in the clade containing P. japonicus and P. kerathurus, which indicates that this character may be linked to a change in the stage at mesendoderm cell arrest (Fig. 8, step 3.)

The observation that both S. ingentis and P. vannamei share the embryological character of mesendoderm cell
arrest at 32-cells suggests that this state was also shared by the common ancestor of the Sicyoniidae and Penaeidae. The stage at mesendoderm cell arrest (32-cells) is also known for *P. indicus*, further supporting 32-cells as the ancestral condition. Finally, in the Euphausiacea, the presumptive mesendoderm cells also arrest at 32-cells, suggesting that this was the condition for the last common ancestor of the Decapoda and the Euphausiacea. In the clad containing *P. japonicus* and *P. kerathurus*, at least two transitions have occurred for this character, from mesendoderm cell arrest at 32-cells to arrest at either 16- or 64-cells. Given that *P. kerathurus* is the most basal member of this clad (Lavery et al., 2004), the first transition was likely from 32-to 16-cells (Fig. 8, step 2), then from 16- to 64-cells (Fig. 8, step 4).

Finally, the lineage of the primordial endoderm cell as Xₐa is shared in *S. ingentis* and *P. vannamei*, while the lineage of this cell is X_dp in *P. kerathurus*. If the cell division pattern can be taken as a reliable indicator of the endoblasts, then this indicates that Xₐa was also the primordial endoderm cell in the last common ancestor of the Sicyoniidae and the Penaeidae. With the data from *P. vannamei*, it is more likely that X_dp was incorrectly identified as the primordial endoblast in *P. kerathurus*, and that Xₐa is likely to be the correct identity. An early marker for endoderm would be very useful for a more definitive identification of the endoderm and solution to these conflicting results.

In summary, the cell lineage of *P. vannamei* provides a reference for an economically important member of the Dendrobranchiata, as well as comparative data for studies of the evolution of development in this group and the Euphausiacea. It will be of interest to obtain additional data for other members of the Penaeidae, as well as for the other families of the Dendrobranchiata to test the hypotheses suggested in Fig. 8.

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