Cleavage and gastrulation of the dendrobranchiate shrimp *Penaeus monodon* (Crustacea, Malacostraca, Decapoda)

Caterina Biffis a, Frederike Alwes a, b, Gerhard Scholtz a, *

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**A B S T R A C T**

The cleavage pattern of the black tiger shrimp *Penaeus monodon* was analyzed from the first division until gastrulation. Observations were based on microscopy combined with the use of fluorescent dyes, histological techniques, and computer based three-dimensional reconstructions. Early cleavage is holoblastic and follows a stereotypic pattern, which largely corresponds to what is known from other dendrobranchiate decapods. However, for the first time in this group, we report the presence of an intracellular structure throughout early development. This intracellular body (icb) marks the lineage of one of the two enlarged and division-delayed mesendoderm cells that initiate gastrulation. The identity of the icb as a natural marker and putative determinant of the germ line and its implications on the establishment of the body axes are discussed. The icb as a landmark reveals that the same stereotypic cell division pattern can lead to different fates of individual cells. Hence, the results of this study permit an additional approach to study the relation between cell lineage pattern and the identity of cell lineages.

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1. Introduction

The Crustacea not only show the greatest variety of early developmental modes at all levels, but are also the arthropod group in which the most examples of holoblastic or total cleavage types are found (e.g. Weygoldt, 1960; Siewing, 1969; Fioroni, 1970; Anderson, 1973; Scholtz, 2000, 2004; Scholtz et al., 2009a,b). Some of these cleavage patterns have been shown to be highly stereotypic with predictable positions, sizes, and directions and timing of divisions of individual blastomeres (e.g. Kühn, 1913; Fuchs, 1914; von Baldass, 1937, 1941; Bregazzi, 1973; Scholtz and Wolff, 2002; Alwes and Scholtz, 2004). The occurrence of two chiral variants, or mirror images, has been described for dendo-branchiate cleavages (*Sicyonia ingentis*: Hertzler and Clark, 1992; Hertzler et al., 1994). Moreover, in addition to the mirror images, a varying position of the two mesendoderm cells within the D derivatives in the dendrobranchiate *Sicyonia ingentis* indicates two additional clonal types, the so-called ‘ventral type’ and ‘dorsal type’ (Hertzler and Clark, 1992, Hertzler et al., 1994). A similar variation of the early cleavage pattern has not been observed in other crustaceans.

Here we describe the early cleavage pattern and gastrulation of *Penaeus monodon* (Fabricius 1798), the black tiger prawn of Australian waters, by means of histology and fluorescence microscopy using CLSM and three-dimensional (3D) image visualization software. We found an overall pattern, which is in many aspects similar to that of the other Dendrobranchiata studied so far (e.g. Zilch, 1979; Hertzler and Clark, 1992; Hertzler, 2005). In addition, we detected an intracellular structure with a specific relation to the progeny of the D quadrant that most likely represents a natural marker for the germ line. With this marker as a reference point we...
were able to discriminate a number of different developmental types with respect to the fate of individual cells.

With our results, we contribute to the question of the ancestral pattern and the evolution of dendrobranchiate early development. Furthermore, our data tentatively suggest that the germ line in *Penaeus monodon* might be determined at an early stage and that the germ line in turn might influence the fate of other cells. This suggests that the same initial stereotyped cell division pattern can lead to different cell fates.

2. Materials and methods

2.1. Collecting specimens

Specimens of the black tiger shrimp *Penaeus monodon* were collected by G. Scholtz at the Australian Institute of Marine Science (AIMS) in Townsville (Australia) in July/August 2005. Adult females were kept together with males in large seawater tanks at room temperature. Females that were apparently ready for spawning were isolated in smaller tanks equipped with a light barrier system that measured swimming activity. The increased frequency of the movement of spawning animals led to an alarm sound. The eggs were fixed in phosphate buffered 4% formalin (4% formalin, 1× PBS; 1.86 mM NaH2PO4, 8.41 mM Na2HPO4, 175.0 mM NaCl, pH 7.4) in defined intervals (around every 20 min) monitoring the cells at the cross-furrow as I, and the cells at the end of each ring as II. The descendants of these cells are marked by adding \( a \) or \( p \) for anterior and posterior, \( v \) or \( d \) for ventral and dorsal, and \( r \) or \( f \) for right and left (Fig. 1). The two sister cells which arrest their division after the fifth division cycle are designated with the capital letters \( X \) and the respective points \( v \) (ventral) and \( d \) (dorsal). The capital letter \( K \) followed by a number (1–9) is used to identify each of the nine ‘crown cells’ ('Kranzellen') at the time they are recognizable in their characteristic pattern surrounding the division arrested cells \( Xd \) and \( XV \) during gastrulation (e.g. see Fig. 1).

M1 and M2 designate the two mirror image cleavage patterns, which represent two observed chiral variants of the same pattern at each cell stage. T1 and T2, i.e. cleavage type I and type 2, indicate the two alternative patterns which are recognized at the 8-cell stage. As cell division proceeds, the number of alternative patterns increases and at the 32-cell stage the cleavage types are labeled by adding the letter ‘a’ or ‘b’ (e.g. T1a) (compare Table 1 with Fig. 9 in the discussion). However, it must be stated that not all of these patterns were observed in the later stages. This might be due to the limited numbers of embryos investigated for each stage.

2.2. Nuclear staining and 3D reconstruction

For fluorescent-light microscopic investigations, eggs were stained with the DNA-selective fluorescent dye Hoechst (bis-benzimide, H33258; Molecular Probes) and with Sytox Green nucleic acid stain (5–7020; Molecular Probes). The eggs were washed in PBS and were then incubated in Hoechst-solution (0.9 µg Hoechst in 1 ml) for 10 min. Unbound dye was removed by washing in PBS for several times: 2 × 10 min, 6 × 30 min.

The staining procedure with Sytox Green (Molecular Probes) follows Hertzler and Clark (1992). After washing in 1× TBS (Tris buffered saline; 150 mM NaCl; 10 mM Tris; pH 7.5) (2 × 5 min, 4 × 30 min), the eggs where incubated for 3 h with Sytox Green solution (1 µM Sytox Green 5 µM; 2000 µl TBS) and then rinsed again in TBS (2 × 10 min; 4 × 30 min) and mounted in DABCO-glycerol (2.5 mg/ml DABCO [1.4-diazobicyclo[2.2.2] octane] in 90% glycerol-PBS).

The eggs were observed with an epifluorescence microscope (Zeiss Axioscope II) and relevant stages were scanned with a confocal microscope (Leica DM IRE2). The image stacks produced by the confocal microscope were analyzed by the 3D visualization software Imaris 4.2.05 (Bitplane AG, Zürich). Among the various display modes and image processing tools of Imaris 4.2.05 mainly the program modules of the surpass mode were applied for the documentation: the rendering of the 3D objects which represent the single blastomeres based on manually defined contours of the 2D images in the program module ‘Contour Surface’. The positions and the mitotic state of nuclei were visualized by manually adding spots as virtual 3D objects in the program module ‘Spots Measurement’.

2.3. Histology

Some Sytox Green-stained eggs were additionally chosen for histological investigations. Eggs were dehydrated in a graded ethanol series (5–10% steps) up to 98% ethanol and transferred to a methacrylate embedding medium (Technovit, Kulzer) following the product instructions. Eggs were oriented in relevant positions under a fluorescence microscope (Zeiss Axioscope II). Serial semithin sections (1–2 µm) were produced using a Zeiss micrometre (HM 355). Sections were dried on slides at 90 °C and stained with 0.1% Toluidine-blue following standard protocols. Details of histological sections were digitized by an AxioCam HRC (Zeiss) connected to an Axioskop II (Zeiss).

2.4. Nomenclature

The nomenclature is based on that proposed for the dendrobranchiate shrimp *Penaeus* (Lithopenaeus) *vannameti* (Hertzler, 2005), which agrees with the nomenclature used for the cell lineage of the euphausiacean *Meganicypridina norvegica* (Alwes and Scholtz, 2004).

At the 2-cell stage the blastomeres are designated with capital letters, the smaller cell \( AB \) and the larger cell \( CD \). At the 4-cell stage \( AB \) produces the daughter cells \( A \) and \( B \), and \( CD \) divides into \( C \) and \( D \). The pointers \( I \) and \( II \) are added after the next division to mark the cells at the cross-furrow as \( I \), and the cells at the end of each ring as \( II \). The descendants of these cells are marked by adding \( a \), \( p \) or \( v \) for anterior and posterior, \( d \) for ventral and dorsal, and \( r \) for right and left (Fig. 1). The two sister cells which arrest their division after the fifth division cycle are designated with the capital letters \( X \) and the respective pointers \( v \) (ventral) and \( d \) (dorsal). The capital letter \( K \) followed by a number (1–9) is used to identify each of the nine ‘crown cells’ ('Kranzellen') at the time they are recognizable in their characteristic pattern surrounding the division arrested cells \( Xd \) and \( XV \) during gastrulation (e.g. see Fig. 1).

M1 and M2 designate the two mirror image cleavage patterns, which represent two observed chiral variants of the same pattern at each cell stage. T1 and T2, i.e. cleavage type 1 and type 2, indicate the two alternative patterns which are recognized at the 8-cell stage. As cell division proceeds, the number of alternative patterns increases and at the 32-cell stage the cleavage types are labeled by adding the letter ‘a’ or ‘b’ (e.g. T1a) (compare Table 1 with Fig. 9 in the discussion). However, it must be stated that not all of these patterns were observed in the later stages. This might be due to the limited numbers of embryos investigated for each stage.

3. Results

The fertilized eggs of the black tiger shrimp *Penaeus monodon* are spherical, with a diameter of about 200 µm. They are surrounded by two transparent envelopes that tightly adhere to the egg. The perivitelline space is almost imperceptible under a common stereo microscope. In Hoechst-stained eggs the perivitelline space appears as a brightly stained unspecific background (Fig. 2A), whereas in Sytox Green-stained eggs this area remains unstained.

Polar bodies are evident in all the stages analyzed here, but their position does neither show any relation to the first cleavage plane nor any indication to the orientation of the body axes of the future embryo.

The eggs are isolecithal, with yolk distributed in dense granules (Fig. 2B). Close to the nuclei the perinuclear cytoplasm (Hofplasma) is connected to the thin layer of periplasm by reticoplasm threads, forming a 3D mesh-work through the yolk. While mitosis occurs and chromosomes arrange in metaphase, the perinuclear cytoplasm elongates following the direction of the spindle (Fig. 2B). An inclusion object, which differs in shape, size, and position from the nuclei and the polar bodies, can be detected after the 2-cell stage to the 122-cell stage. Under fluorescence in Sytox Green-stained eggs, but not in Hoechst-stained eggs, this body appears as a bright light ovoid shape and is embedded within the
Fig. 1. Cell lineage nomenclature in the early embryonic stages of *Penaeus monodon*. Schematic overview of the nomenclature used for the cell lineage of *Penaeus monodon* from the first cleavage to the sixth cleavage (62-cell stage). The scheme provides also an overview on the origin of the two mesendoderm cells (Xv and Xd) and of the crown cells (K1–K9) in the cleavage type M1, T1b (at the right side). The colors are assigned according to the cell lineage of the corresponding blastomeres at the 4-cell stage.
periplasm of one cell (Fig. 3A). Histological sectioning confirms this position as a dense Toluidine-blue stained body within the periplasm. The cell membrane shows a depression in this area (Fig. 3B).

To simplify further description, this body is denoted as icb, i.e. intracellular body. The icb labels the D quadrant and always one of the D derivative cells. From the 5th cleavage on, the icb is found in one of the two D derivative cells, which arrest their division and indicate the future site of the blastopore during gastrulation. Therefore, the icb is suitable as a morphological marker of the vegetal pole. Furthermore, starting with the 4-cell stage, the icb is observed in an asymmetric position shifted towards the blastomere C and its derivatives.

3.1. From the 2-cell stage to the 4-cell stage: a close non-planar packing of the blastomeres

At the 2-cell stage, blastomeres are slightly unequal in size. The larger blastomere is designated as CD, the smaller as AB. CD contains the icb opposite to the furrow plane shifted to one side of the blastomere (Fig. 4). The division from the 2- to the 4-cell stage is synchronous and slightly unequal. The spindle axes of the two dividing blastomeres are obliquely oriented to each other. The resulting four blastomeres are arranged in a non-planar packing: AB generates A and B, while CD produces C and D. Two additional planes of contact are created other than the division furrows. They lie at right angle to each other and form the cross-furrows (Figs. 5C,D). These cross-furrows are situated between two blastomeres which have no sister cell relationship. Hence, blastomere C is in contact with A, and D contacts B at the opposite side (Figs. 5A,B). The cell D is larger than the other blastomeres and contains the icb shifted towards the sister cell C (Figs. 5B–D). The pattern of the four blastomeres is observed in two chiral variants, the mirror images designated M1 and M2.

3.2. From the 4-cell stage to the 16-cell stage: two interlocking cell bands

At the transition from the 4-cell stage to the 8-cell stage, the mitotic spindles of A and C point end to end to each other as well as the spindles of B and D, with those of A and C perpendicular to those of B and D. The third cleavage is synchronous: A generates A1 and A2, C generates C1 and C2; B divides into B1 and B2 and D produces D1 and D2. Therefore, at the 8-cell stage the blastomeres form two bands of four cells each, which are interlocked as two horseshoe-like sheets. The AC-band is composed of A1, A2, C1, and C2 and interlocks with the BD-band formed by B1, B2, D1, and D2 (Figs. 5E, F). A1 and C1 are each situated at the end of the AC-band (Figs. 5E,F,H), while B1 and D1 each bound the BD-band at the opposite side (Figs. 5E, F,G). A1 and C1 meet at the AC cross-furrow (Fig. 5G), while B1 and D1 meet at the BD cross-furrow (Fig. 5H).

Beginning with the 8-cell stage a small blastocoel forms (Fig. 6).

The division of D is slightly unequal and the somewhat larger cell extends deeper into the blastocoel and includes the icb. The icb (Figs. 5E,G,H) is always shifted towards the C descendant blastomeres, which adjoin the icb containing cell on either the left or the right side. Its position therefore facilitates the identification of two mirror images: to the right side in M1 (Figs. 5E–H) or to the left side in M2. Among the 12 embryos investigated in detail, ten show the icb in D1 located at the end of the BD-band, while two embryos show the icb in blastomere D2 within the BD-band. The location of the icb in either D1 or D2 characterizes two different cleavage types called T1 and T2.

![Fig. 2. General structure of the blastomeres of embryos at different stages in P. monodon. A. Hoechst-stained egg at the 16-cell stage. Confocal image stack (Imaris, section mode). The perivitelline space (ps) is stained unspecifically. The polar body (pb) is located at the periphery of the perivitelline space. The nuclei (n) in prophase are quite round. They are surrounded by the perinuclear cytoplasm (pen). The cell membranes (m) are not stained and they appear as a thin dark web around cells. B. Semi-thin section of a Toluidine-blue stained egg at the 2-cell stage. The yolk is uniformly distributed in droplets and it is surrounded by the periplasm (pep). The perinuclear cytoplasm (pen) surrounds chromosomes (cr) which are going to arrange in metaphase during mitosis. The section is parallel to the plane of the spindles (sp1) of one blastomere (AB) and orthogonal to the other (sp2 of CD).](image-url)
were present. The remaining four embryos, which show the descendants of DI, show the intracellular body (icb) in one of the two blastomeres. cr: chromosomes in the heterochromatic nucleus; pen: perinuclear cytoplasm. Toluidine-blue stained semi-thin section at the 4-cell stage showing the intracellular body (icb). The membrane is depressed in the region which bounds the intracellular body (icb) while the surrounding area is poor of yolk.

The fourth division is synchronous and perpendicular to the previous one. The spindles of the four blastomeres in each band are oriented side by side. The nuclei are off-center, located in apical position within the cells, which show a conical shape. The resulting two in DIl and two in DIr. Hence, as in T1, the two mirror images M1 and M2 can be detected in T2 (see Fig. 9).

3.3. From the 16-cell stage to the 32-cell stage: two D derivatives are delayed in their division

During the fifth cleavage, the 16 blastomeres are arranged in two bands of eight cells each with the spindles oriented end-by-end. The fifth cleavage planes are perpendicular to those of the previous division. The result is an extension of the two interlocking cell bands into a 32-cell stage with the AC- and BD-bands arranged in two rows of eight cells each. AAv, AAd, AApv, and AApd, CAv, Cad, CApv, and CApd are located at the two ends of the AC-band; AAv, AAd, AApv, and AApd, CAv, Cad, CApv, and CApd are located at the AC cross-furrow. The BD-band is formed by BBrv, BBrd, BBrv, and BBrd, BBrd, BBrv, and BBrd locking the band with DBrv and DBlv, respectively. The BD-band is bounded by BBrv, BBrd, BBrv, and BBrd at the one end and by DBrv, DBrd, DBlv, and DBld at the other end (compare Figs. 7A–E and 9).

The fifth division is not synchronous: one of the four D derivatives shows a consistent delay in its division. Its position within the cleavage pattern varies as well as the location of the icb, which has always been found in the delayed D blastomere. As a consequence, different cleavage types are observed in different embryos at the...
32-cell stage. \textbf{T1} is the cleavage type in which either cell \textbf{Df} (Figs. 7A,B) or \textbf{Dil} (Figs. 7C,D) is delayed. We distinguish between \textbf{T1a} and \textbf{T1b} with respect to the position of the \textbf{icb} either in \textbf{Dfrd}, \textbf{Dfld} or in \textbf{Dfrv}, \textbf{Dflv}. The location of the \textbf{icb} in \textbf{Dfrd} and \textbf{Dfrv} represents the mirror image \textbf{M1}, and in \textbf{Dfld} and \textbf{Dflv} the mirror image \textbf{M2}.

Among the 15 embryos analyzed, eight were of \textbf{T1b}, two as \textbf{M1} and six as \textbf{M2}. The occurrence of \textbf{T1a} was observed in one embryo in the mirror image \textbf{M2}. \textbf{T2} is the cleavage type in which either \textbf{Dfr} or \textbf{Dfl} is delayed. \textbf{T2a} and \textbf{T2b} are the two cleavage types, which show the \textbf{icb} located in the blastomeres \textbf{Dfrd}, \textbf{Dfld} or \textbf{Dfrv}, \textbf{Dflv}, representing

**Fig. 5.** 4–8–16 cell stages. Views of different Sytox Green-stained eggs at the 4-cell stage (A–D), at the 8-cell stage (E–H), and at the 16-cell stage (I–K). Each stage is represented by different aspects of the same egg. Confocal image stacks processed by the software Imaris: section mode (in A–B; E–F); 3D reconstruction of the blastomeres, Imaris: surpass view: contour surfaces, spots measurements (in C–D; G–H; J–K). In A–B, E–F and I the lines indicate the sister cell relationships. In C–D, G–H, J and K the volume of each blastomere is represented by a transparent surface which allows the observation of the position of each nucleus inside represented by colored spheres. The \textbf{icb} and the polar body (pb) are pointed out by white arrows and are represented by light blue spheres smaller than the nucleus spheres. The \textbf{icb} is always shifted towards the cells derived from \textbf{C}. In D, H and K the volume objects are rotated of 180° in the respect of the volume objects in C, G and J. Colors are assigned according to the cell lineage of the corresponding blastomeres. A. View of the top surface of the image stack of the confocal scanned egg at the 4-cell stage. A and C on focus at the top surface of the egg are not directly sister cells and create an additional plane of contact (the cross-furrow) other than the division furrow. B. View of the bottom surface of the image stack of the same egg at the 4-cell stage. C–D. 3D reconstruction of the 4 blastomeres. The cross-furrows (cf white broken line) between the blastomeres A, C and B, D are shown in C and D, respectively. E. View of the top surface of the image stack of the confocal scanned egg at the 8-cell stage. View of one end of the \textbf{AC}-band (green broken line) with \textbf{C1} and \textbf{C2} on focus at the top surface of the egg. The \textbf{BD}-band is viewed by the red and blue broken line interlocking the \textbf{AC}-band. \textbf{Df} contacts \textbf{Bf} within the band. \textbf{Dh} and \textbf{Dg} define the two ends of the \textbf{BD}-band. The \textbf{icb} (white broken line) is located in \textbf{Dh} shifted towards the derivative \textbf{C} cells. F. View of the bottom surface of the image stack of the same egg at the 8-cell stage. View of one end of the \textbf{AC}-band (yellow broken line) with \textbf{Aa} and \textbf{Ab} on focus at the bottom surface of the egg. \textbf{Bh} is on focus at the end of the \textbf{BD}-band (red and blue broken line). G–H. 3D reconstruction of the 8-cell stage. In G, \textbf{Ab} contacts \textbf{C1} within the \textbf{AC}-band while \textbf{Dh} and \textbf{Dg} are at the two ends of the \textbf{BD}-band. In H, \textbf{Bf} contacts \textbf{Bf} within the \textbf{BD}-band while \textbf{C1} and \textbf{C2} are at the two ends of the \textbf{AC}-band, respectively. The two bands are interlocked as two horseshoe-like sheets. I. View of the top surface of the image stack of the confocal scanned egg at the 16-cell stage. View of a part of the \textbf{BD}-band (red and blue broken line). The sister cells \textbf{Br} and \textbf{Bl} contact \textbf{Dr} and \textbf{Dl} within the \textbf{BD}-band (on focus at the top surface of the egg). The sister cells \textbf{Dr} and \textbf{Dl} are located at one end of the \textbf{BD}-band. \textbf{C1a} and \textbf{C1p} (in focus on the left) define one end of the \textbf{AC}-band (green and yellow broken line). The \textbf{icb} (white broken line) is located in \textbf{Dfr} shifted towards the derivative \textbf{C} cells. J–K. 3D reconstruction of the stage with 16 blastomeres. In J, the sister cells \textbf{C1} and \textbf{C2} contact \textbf{Aa} and \textbf{Ab} within the \textbf{AC}-band while the sister cells \textbf{Bf}, \textbf{Br} and \textbf{Df}, \textbf{Dl} are at the two ends of the \textbf{BD}-band. In K, the sister cells \textbf{Br} and \textbf{Bl} contact \textbf{Dr} and \textbf{Dl} within the \textbf{BD}-band while the sister cells \textbf{C1a}, \textbf{C1p} and \textbf{Aa}, \textbf{Ab} are at the two ends of the \textbf{AC}-band.
M1 or M2. T2a was observed in six embryos, three as M1 and three as M2 (see e.g. Fig. 7E). There was no T2b among the 32-cell embryos studied, since we did not observe the icb occurring in Ddrv or Dplv. In summary, the delay in the division within the D quadrant is thus observed in all four possible positions of the 16-cell stage. This delay also designates the region of the vegetal pole marking the area where gastrulation takes place. After the division of the delayed D blastomere, no matter which one it is, the resulting sister cells are called Xv and Xd.

The cells Xv and Xd, are enlarging and going to arrest their division for the next three cycles of cleavage remaining in interphase, while the other blastomeres continue dividing. These enlarged two cells correspond to the cells that have been described as the two mesendoderm cells in the penaeids *Penaeus kerathurus* (Zilch, 1978) and *Penaeus vannamei* (Hertzler, 2005) and in Sicigonia ingentis (Hertzler and Clark, 1992).

3.4. From the 32-cell stage to the 62-cell stage: the arrested division of two D derivatives and the pattern of the ‘crown cells’

During the sixth cleavage the embryo consists transiently of 60 blastomeres with a cleavage direction perpendicular to the previous one, and with the two putative mesendoderm cells Xv and Xd arrested in division (Figs. 7F,G). Xv and Xd still keep contact to the surface of the egg, but they assume an elongated shape filling the blastocoel. Their enlarged nuclei are located in an apical position and are arrested in interphase (Fig. 7F). The blastomeres at the animal pole are advanced in their division compared to those at the vegetal pole, thus showing an increasing retardation along the animal-vegetal axis. As a consequence the cleavage pattern of the two interlocking cell bands is still preserved at the animal pole (Fig. 7H), whereas at the vegetal pole it is distorted by the presence of Xv and Xd (Fig. 7G).

With the 62-cell stage the two interlocking bands are broadened to four rows of eight cells each, except for the site of the two division arrested cells Xv and Xd. The cross-furrow at the vegetal pole is deviated: four of the eight C derivative cells and five D derivative cells remain adjacent to Xv and Xd. Xv and Xd are eventually surrounded by nine cells, which are designated as crown cells. The crown cells are arranged in a stereotyped pattern, which occurs in the two mirror images mentioned above. At one end the four C derivative cells, which contact Xd and Xv are Cppdp (K1), Cvpvp (K2), Cppdp (K3), and Cvpvp (K4) in both the variants (see e.g. Fig. 7G), which means in M1 they are arranged clockwise and in M2 they surround Xv and Xd anticlockwise. At the other end, the lineage of the five D derivative blastomeres forming the crown cells is different in the two mirror images. In M1 they correspond clockwise to the two sister cells Drrdr (K5) and Drrdl (K6) and to Dllrd (K7), Dllrv (K8), Dlldr (K9). While in M2 they correspond anticlockwise to the two sister cells Dlrdl (K5) and Dlldr (K6) and to Dllrd (K7), Dllrv (K8), Dllrd (K9). In all 62-cell stage embryos examined we detected the icb in the large blastomere Xv, which is the descendent of the division-delayed blastomeres Ddur or Duil of the 32-cell stage (Figs. 7F,G). Accordingly, only the cleavage type T1b with its two mirror images M1 and M2 was found.

3.5. From the 62-cell stage to the 122-cell stage: the beginning of gastrulation

At the 62-cell stage, Xv and Xd are still arrested at the interphase, while the other cells continue to divide. Xv and Xd enlarge, elongate, and extend deeper into the interior of the embryo (Fig. 7.I). The nine crown cells follow this movement by arranging around Xv and Xd before starting the next division. Therewith, an opening is formed at the vegetal pole which establishes the position of the blastopore (Figs. 7J–M). The icb is still visible within Xv and its position is towards the opening of the blastopore (Figs. 7J–M). During the seventh cleavage the gradient of the cell division delay along the animal-vegetal axis becomes more distinct. While at the animal pole the cells have already completed their division, the crown cells at the vegetal pole and the cells surrounding them are delayed in their division. Thus, a transient stage of 113 cells follows. At the animal pole the spindles are oriented perpendicularly to those of the preceding cleavage and the pattern of the two interlocking cell bands is still preserved. At the vegetal pole the crown cells divide and the orientation of their spindles is turned towards the blastopore so that they show a radial arrangement around Xv and Xd (Figs. 7J,L). Their further divisions remain radially oriented and do not follow the rule of perpendicularity.

At this stage, the pattern of the crown cells is preserved and becomes more evident due to the expression of a gradient in their division occurring in two chiral variants. K4 and K5 extend deeper upon Xv and Xd and touch each other. Their spindles are oriented end-by-end along the mediasagittal axis (Fig. 7I). The remaining seven crown cells are arranged around the future blastopore, clockwise in M1 and anticlockwise in M2 (compare Figs. 7I, K with L–M). They all keep contact with Xv and Xd. K4 and K5 are delayed in their mitosis compared to the other K cells which are in the same stage of division. This delay is accompanied by the delayed division.
of the two sister cells in the outer ring which surrounds the crown cells (Figs. 7L,M). In M1 it corresponds to the delay of Dlvdv as sister cell of K9 and of Dlvld as sister cell of K0 (Fig. 7M); while, in M2 the delay concerns Dlvdrr as sister cell of K9 and Dlvrv as sister cell of K9 (Fig. 7K). The subsequent divisions of the crown cells show also a radial orientation towards the blastopore. The crown cells and their derivatives move into the blastopore and form a tier of the “archenteron” wall, posterior to the mesendoderm cell, forming the advanced gastrula (Fig. 8). As has been suggested for other penaeoidean shrimps (Zilch, 1978; Hertzler and Clark, 1992), the crown cells presumably give rise to the naupliar mesoderm (Hertzler, 2005). At the animal pole the mitoses proceed at the usual pace and under the usual spatial rules, thus preserving the two interlocking cell bands. The two mesendoderm cells show their enlarged nuclei and they do not participate in the next division. Then the shape of the mesendoderm cells appears flattened against the basal wall of the ectoderm and the icb is no longer visible (Fig. 8).

4. Discussion

4.1. The early cleavage of Penaeus monodon reveals an invariant cell division pattern that is homologous to that of other dendrobranchiates

The present study describes the early cleavage of the dendrobranchiate shrimp Penaeus monodon based on fixed stages stained with fluorescent dyes combined with laser scanning microscopy and 3D visualization software. The results show that the cell divisions follow an invariant pattern in terms of: (1) the formation of the cross-furrow between two cells which are not sister cells at the 4-cell stage; (2) the arrangement of the blastomeres in two interlocking semicircles and cell bands during the subsequent divisions; (3) the arrest in division of two D derivative blastomeres that initiate gastrulation at the vegetal pole; and (4) the formation of a ring of crown cells around the gastrulation center. These aspects of the early cleavage pattern correspond to what is known from other dendrobranchiates that have been investigated in this respect (e.g. Zilch, 1978; Hertzler and Clark, 1992; Hertzler, 2005).

The formation of the two cross-furrows between two non-sister cells at the 4-cell stage is a pattern, which has been described in all investigated dendrobranchiates, as well as the arrangement of the blastomeres in two interlocking semicircles and cell bands in the subsequent divisions. As already reviewed by Hertzler (2005), the pattern differs among the dendrobranchiates species in the stage at which the delay of the two mesendoderm cells occurs and in the number of the crown cells. In Penaeus monodon this delay occurs at the 32-cell stage which is the same stage as in the two penaeoideans Penaeus indicus (Morelli and Aquacop, 2003) and Penaeus vannamei (Hertzler, 2005), in the sicyonid Sicyonia ingents (Hertzler and Clark, 1992) and in the sergestoidean shrimp Lucifer spec. (Brooks, 1882). Different situations are described in Penaeus japonicus, in which the two large mesendoderm cells divide in division at the 62-cell stage (Kajishima, 1951), and in Penaeus kerathurus, where they are reported to arrest division in the 16-cell stage (Zilch, 1979). We found nine crown cells in Penaeus monodon and this corresponds to the number observed in Sicyonia ingents (Hertzler and Clark, 1992) and in Penaeus vannamei (Hertzler, 2005), while in Penaeus japonicus and in Penaeus kerathurus eight...
crown cells have been reported (Kajishima, 1951; Zilch, 1979). According to Hertzler (2005), the differences in Penaeus japonicus and Penaeus kerathurus concerning the cell stage, at which the mesendoderm cells are, delayed and arrested in their divisions or the number of crown cells, must be considered to be derived within the dendrobranchiates.

With the present study on Penaeus monodon we contribute to all four aspects of dendrobranchiate development mentioned above. Our results indicate that Penaeus monodon corresponds to what has been suggested by Hertzler (2005) to be the ground pattern for Dendrobranchiata.

The radial orientation of the mitotic spindles of the crown cells in Penaeus monodon corresponds to what has been described for the nine crown cells in Sicyonia ingentis (Hertzler and Clark, 1992) and Penaeus vannamei (Hertzler, 2005) at their first appearance (the 62-cell stage). A similar arrangement has also been reported for the eight crown cells of Penaeus japonicus (Kajishima, 1951) and Penaeus kerathurus (Zilch, 1979). In addition to the radial spindle orientation, we found for the first time a stereotypic division pattern during the mitoses of the crown cells within the Dendrobranchiata. In Penaeus monodon the cleavage pattern of the crown cells is maintained in a stereotypic arrangement in the next division (to the 122-cell stage), which is observed occurring in two mirror images. So far, no corresponding stereotypic crown cell division pattern has been described in detail in Dendrobranchiata.

4.2. The pattern of the crown cells: a comparison within malacostracans

Among other malacostracan groups with total cleavage such as, for instance, Amphipoda (Gerberding et al., 2002; Scholtz and Wolff, 2002; Wolff and Scholtz, 2002), Anaspidacea (Hickman, 1937), and Euphausiacea (Taube, 1909, 1915; Alwes and Scholtz, 2004), only the latter shows distinct correspondences to the cleavage pattern of the Dendrobranchiata (Alwes and Scholtz, 2004; Scholtz et al., 2009b). This concerns the relative size, position and division pattern of blastomeres, the two interlocking cell bands, and the delay in the division of the two large mesendoderm cells (see Alwes and Scholtz, 2004; Hertzler, 2005; Scholtz et al., 2009b). In addition, the arrangement of the crown cells is one of the shared characters between Euphausiacea and Dendrobranchiata (Taube, 1909; Kajishima, 1951; Zilch, 1978; Hertzler and Clark, 1992; Alwes and Scholtz, 2004; Hertzler, 2005). In both groups, the mitotic spindles of the crown cells are radially oriented at the vegetal pole and the division is delayed compared to other cells.

Nevertheless, due to the partially unresolved body axis relationships it must currently remain an open question, whether the stereotypic crown cell division pattern observed in Penaeus monodon and Meganyctiphanes norvegica is an additional similarity between the early development of Dendrobranchiata and Euphausiacea.

4.3. The icb and the establishment of the body axes with respect to the cell lineage

Concerning the cell lineage, the composition of the crown cells differs in comparison between Penaeus monodon and Meganyctiphanes norvegica. In Penaeus monodon they are composed of
four derivatives of C (K1, K2, K3, K4) and five derivatives of D (K5, K6, K7, K8, K9), while in Meganyctiphanes norvegica they are composed of four derivatives of A (K1, K2, K3, K4), one of C (K5) and three of D (K4, K5, K6). The composition differs also within these species, since the cell lineage of the D derivative cells in Penaeus monodon is different between the two observed mirror images (see results), as well as the cell lineage of K5 and K6 in Meganyctiphanes norvegica differs between the two observed mirror images (Alves and Scholtz, 2004). This variability in terms of cell lineage within the same pattern has been already described in Sicyonia ingentis (Hertzler and Clark, 1992) and more recently in Peneaus vannamai (Hertzler, 2005). The variants of different types besides the two mirror images are based on the positional variation of the two division-delayed mesendoderm cells and give rise to the ‘ventral type’ and the more frequent ‘dorsal type’ (Hertzler and Clark, 1992; Hertzler, 2005). The variants of different types besides the two mirror images are based on the positional variation of the two division-delayed mesendoderm cells and give rise to the ‘ventral type’ and the more frequent ‘dorsal type’ (Hertzler and Clark, 1992; Hertzler, 2005). In these two types the composition of the crown cells also differs, as the ‘ventral type’ also includes derivatives of B while in the ‘dorsal type’ they are composed only of derivatives of C and D. The inclusion of B derivatives (rather than C derivatives) in the composition of the crown cells comparable to the ‘ventral type’ has not been found in Meganyctiphanes norvegica.

In Penaeus monodon the observed occurrence of different cleavage types in the stages prior to the formation of the crown cells allows arguing for the existence of higher variability in the cleavage pattern of the crown cells. The occurrence of the types T2a and T2b in Penaeus monodon with the mesendoderm cells located within the BD-band leads to the expectation of a crown cell composition in which B derivatives are included as well (Fig. 9). However, this could not be confirmed by the present study and will need to be studied by other methods.

The icb in Penaeus monodon is found either in Xv or in Xd in different embryos. Therefore, it enables us to identify additional cleavage types to what has been described in earlier studies of dendrobranchiates (Hertzler and Clark, 1992; Hertzler, 2005). Another consequence is that the establishment of the dorsoventral axis, which is described previously by the division into the two mesendoderm cells, can be discussed from a different point of view. Assuming that the icb may be a cell specific determinant indicating the primordial germ cell (PGC) and thus giving rise to the germ line (see discussion below), two explanations for the establishment of the dorsoventral axis are possible: (1) the germ line can be derived from either the dorsal or the ventral mesendoderm cell in the different cleavage types of Penaeus monodon. This stands in contrast to previous studies on other dendrobranchiates, which report that the germ line precursor cell always originates from the ventral mesendoderm cells (Zilch, 1978, 1979; Hertzler, 2002, 2005). (2) The germ line in both cleavage variants in Penaeus monodon is always determined on either the ventral or the dorsal side. In this case, the icb would be the establishing factor of the dorsoventral axis and the remaining cells of the embryo would not be determined prior to the division of the two mesendoderm cells.

However, since we did not trace the fate of the embryos to a stage in which the ventral-dorsal orientation could be clearly shown, we are not able to say whether we have different dorsoventral orientations with respect to the cell lineage. Hence, this issue must remain unresolved at present. A solution to this problem lies in further studies applying suitable methods, such as cell lineage tracing, in order to trace the development up to stages with morphologically unambiguous axes.

Fig 9 summarizes the different cleavage types in relation to the position of the icb and the resulting cell fate of individual cells. It clearly demonstrates that the cleavage pattern, in terms of cells size, cell arrangements, and spindle direction, can be similar or even the same but the position of the icb within the D derivatives, and therefore, the fate of individual cells, such as the mesendoderm cells, is not fixed.

In a recent study applying blastomere isolations and cell recombination in Sicyonia ingentis embryos, Wang et al. (2008) have shown that the mesendoderm cells play a central role for the determination of the behavior and fate of adjacent cells. The mesendoderm cells influence the direction of the mitotic spindles of the crown cells and they are capable of inducing an oriented cell division to neighboring cells, irrespective of their cell lineage. Taken together with the presence of the icb in Penaeus monodon these results of Wang et al. (2008) suggest that the icb might indeed determine the cell fate of the surrounding cells, i.e. crown cells, and may therefore also determine the fate of other cells.

4.4. The icb as a putative natural marker of the germ line

Extavour and Akam (2003) and Extavour (2008) list a number of characteristics for the recognition of PGCs in metazoans, among them the occurrence of granular inclusions and the expression of germ cell specific genes, such as vasa. Granular inclusions as a general term for granular components in germ plasm have been found in many metazoans (reviewed in Extavour and Akam, 2003; Extavour, 2008). They are described as, e.g., P bodies, nuages, or mitochondrial clouds. Among the Crustaceae, we find many examples for an early segregation of dense granular material that is associated with oneblastome. One well-known example is the cladoceran Polyphemus pediculus (Kühn, 1913). The granular material has been suggested to originate from a transformed nurse cell remnant and it persists in one blastomere during the early cleavages. Eventually it is found in two division-delayed micromeres that give rise to the germ line (Kühn, 1913). In amphipod malacostracans, PGCs have been identified based on cell lineage tracing with in vivo markers (Gerberding et al., 2002; Wolff and Scholtz, 2002), and by ablation experiments (Extavour, 2005), but so far no icb or a corresponding structure has been described. Recent studies with the zinc-finger-containing gene vasa as a marker trace the germ line development in the amphipod Parhyale hawaiaensis (Extavour, 2005; Özhan-Kızıl et al., 2009) and the cladoceran Daphnia magna (Sagawa et al., 2005) and show in both species an early restriction of the germ line. These gene expression studies impressively confirm the identification of the germ lines based on natural markers as in cladocerans (Kühn, 1913) and cell lineage tracing with injected dyes as in amphipods (Gerberding et al., 2002; Wolff and Scholtz, 2002). Vasa-RNA is also found to be expressed in male and female gonads of Penaeus vannamai (Aflalo et al., 2007) and its specific expression in female late oogenesis suggests a possible contribution as a maternal factor for the determination of germ lineages, as has been suggested as early as 1913 by Kühn.

In Dendrobranchiata, the PGCs are suggested to derive from the ventral of the two large mesendoderm cells (Zilch, 1978, 1979; Hertzler, 2002, 2005). However, yet no unambiguous evidence for a PGC or an earlier precursor of the PGC has been found for this group. The icb detected by us in Penaeus monodon might be a good candidate for a natural germ line marker in a dendrobranchiate representative. It is a granular inclusion related to a specific cell lineage involved in gastrulation. Furthermore, the icb is not stained by Hoechst which is a sensitive DNA staining, but by Sytox Green which is a general staining for both nucleic acids (DNA and RNA). This indicates an accumulation of RNA to the icb structure. However, from this alone we cannot conclude a germ line specific role of the icb and whether this can be linked to any RNA mediated gene regulation of the germ line. The suggestion of the icb as a PGC marker and its specific function can be tested by future investigations applying molecular and in vivo cell lineage approaches. Furthermore, it remains to be seen whether corresponding features occur in other representatives of the Dendrobranchiata.
Fig. 9. Scheme of the cleavage types in *P. monodon*. Schematic representation of the different cleavage types of *P. monodon* from the 4-cell stage to the 62-cell stage in the two mirror images M1 (A) and M2 (B). Each stage is represented with the BD-band cut at the cross-furrow and the B and D blastomeres brought to the side of the AC furrow maintaining their location in the pattern. The AC-band is shown as disconnected from the BD-band at its two ends. Each blastomere is represented by a colored circle. Colors are assigned according to the cell lineage of the corresponding blastomeres. The sister cell relationships are represented by black lines which connect the two sister cells. At the 32- and 62-cell stage the two derivative D blastomeres which give rise to the two mesendoderm cells are colored in grey. The crown cells at the 62-cell stage are underlined by adding a grey circumference to the related circle. The icb is represented by a turquoise ovoid on the side of the corresponding D derivative blastomere. Please note that the pattern T1a in M1 has not been found in our samples and that not all possible cleavage types have been observed in all stages.
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