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Cleavage, gastrulation, and germ disc formation of the amphipod *Orchestia cavimana* (Crustacea, Malacostraca, Peracarida)

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Abstract

Investigations of amphipod embryonic development have a long tradition. However, many aspects of amphipod embryology are still controversial. These concern, among others, the nature of the cleavage, the origin of the germ disc, and the mode of gastrulation. On the other hand, amphipods show the same characteristic type of invariant cell division pattern in the germ band as other malacostracans. Since amphipods seem to undergo a stereotyped pattern of early cleavage they are highly interesting for our understanding of the evolution of arthropod development. In this paper, we describe the cleavage pattern of the amphipod crustacean *Orchestia cavimana* from the zygote to gastrulation and the formation of the germ disc using direct observation, scanning electron microscopy, histology, video recording, and lineage tracing with a vital dye. The early development follows the mode of a total, radial, unequal cleavage with a determinate stereotyped pattern. A small transient blastocoel is formed. The 8-cell stage is characterised by 4 micromeres and 4 macromeres. One quadrant is smaller than the others. There are two kinds of eggs that show a mirror handed image. The 16-cell stage is the last regular stage after which the blastomeres divide highly asynchronously. The germ disc is formed by the descendants of the macromeres and some micromere derivatives. The other micromeres constitute the extra-embryonic region. Migration of macromere descendants is involved in germ disc formation accompanied by the extrusion of the yolk. During this process some vitellophages are formed. The gastrulation *sensu stricto* is initiated by the micromere derivatives of the smallest quadrant at the anterior of the forming germ disc. A true blastopore occurs which involves an invagination and the immigration of cells. Our data help to correct erroneous interpretations of former students of amphipod development. We can show that many characters of amphipod embryonic development are apomorphic supporting amphipod monophyly. With the present investigation we contribute to a complete understanding of the embryonic cell lineage of amphipods from the egg to segment formation and organogenesis.

Contents

Introduction

The early development of arthropods exhibits a great variety of modes with respect to virtually all aspects at every level. We find all sorts of cleavage modes from total to superficial (Anderson 1973; Scholtz 1997), different ways of gastrulation (Weygoldt 1979), numerous modes of germ band for-

mation and segmentation processes (Scholtz 1997), and all kind of larval stages up to direct development (Anderson 1973). This diversity at the cellular, morphological and ecological levels is also reflected at the genetic level where early pathways are evolutionarily altered despite a similar resulting or subsequent pattern (Abzhanov & Kaufman 1999; Jockusch et al. 2000; Davis & Patel 2002). This high degree of variation can be found even within arthropod subgroups such as malacostracan crustaceans or hymenopteran insects (Grbic 2000; Scholtz 2000).

The present study describes the early cell lineage during cleavage, gastrulation and germ disc formation of an amphipod crustacean. Although investigations on amphipod embryology started as early as 1837 with Rathke's report, there are still many controversial views and open questions concerning the exact mode of amphipod embryonic development. Like all other malacostracans studied, amphipods undergo a distinct stereotyped pattern of cell divisions from the formation of the postnaupliar germ band up to segmentation, neurogenesis, limb bud formation, and mesodermal differentiation (Dohle & Scholtz 1988; Scholtz & Dohle 1996; Scholtz et al. 1994; Scholtz 1990, 2000; Gerberding & Scholtz 1999, 2001). However, most of the decapod and peracarid species studied with respect to their cell lineages in the germ band undergo a superficial cleavage that does not allow the tracing of early cell lineages (if at all present) up to germ disc formation and gastrulation. This is different in amphipods; there is some evidence from several studies that the early cleavage of amphipods shows a stereotyped pattern (e.g. van Beneden & Bessels 1869; Ulianin 1881; Langenbeck 1898; Heidecke 1904; Rappaport 1960; Bregazzi 1973; Meschenmoser 1987). However, in some respects these investigations led to contradictory results. For instance, La Valette St. George (1860), Ulianin (1881) and Mergault & Charniaux-Cotton (1973) claimed that the cleavage is not total but that the central yolk mass remains undivided whereas authors such as Langenbeck (1898) and Pereyaslawzewa (1888a) suggested a clear total cleavage mode. Several authors (e.g. Ulianin 1881; Bregazzi 1973; Mergault & Charniaux-Cotton 1973) stated that the germ disc originates from the micromeres of the 8-cell stage. In contrast, Langenbeck (1898) and Rappaport (1960) found that the macromeres of the 8-cell stage give rise to the germ disc cells. Furthermore, some authors suggested that the cleavage pattern of amphipods shows aspects of spiral cleavage (Sheader & Shia 1970) which has been denied by other researchers (Scholtz 1997; Wolff & Scholtz 2002).

In order to gain a better picture of early amphipod development we applied several techniques such as direct observation of living embryos, video recording, scanning electron microscopy (SEM), plastic embedding for histology, and the vital dye DiI as a marker to study cell lineage. In particular, we address the following questions: what type of cleavage occurs, total or superficial, spiral or radial? Where do the germ disc cells come from? When are the axes of the embryo determined? What kind of gastrulation is found? In a first paper, we traced the fate of individual cells of the 8- and 16-cell stages (Wolff & Scholtz 2002). Here we describe the cleavage pattern in detail, the formation of the germ disc, and the gastrulation process.

Our results confirm that the amphipod *Orchestia* shows total cleavage with a highly stereotyped cell lineage. The germ disc is formed by derivatives of both micromeres and macromeres with the macromeres giving rise to the largest part of the ectoderm and to mesendodermal parts of the embryo whereas the micromeres contribute partly to the mesendoderm and to the extra-embryonic ectoderm. The cleavage is not spiral. Gastrulation takes place at the anterior end of the germ disc.

The general pattern of early amphipod development is unique among malacostracans and crustaceans in general. It shows a number of apomorphic characters related to cleavage, gastrulation, germ band proliferation, and gene expression unifying the different amphipod taxa.

Material and methods

Maintaining of the animals and embryos

Specimens of the terrestrial amphipod species *Orchestia cavimana* were collected from beaches of the Tegeler See (Berlin) and from the river Weser

close to the town of Elsfleth where they live in and feed on detritus (Rudolph 1995). The animals were maintained in a terrarium at 18-20°C and fed with carrots and rolled oats. To receive eggs in relevant stages the animal in a so-called praecopula (the male carries the female around for a while before copulation) were caught and isolated. Usually after about 10-14 hours the animals separate and the female's ventral brood pouch (marsupium) contains eggs in an early stage of cleavage. Females with eggs in their marsupium where carefully dazed in mineral water containing CO_2. The eggs were washed out of the marsupium from anterior with a Pasteur pipette. The eggs were transferred to salt dishes containing a saline which mimicks the liquid in the marsupium (isopod saline : NaCl-frog saline = 1:2; [isopod saline: 12 g NaCl, 1,6 g KCl, 1,6 g $CaCl_2$, 1,6 g $MgCl_2$, 0,2 g $NaHCO_3$ to 1 l aqua dest.; NaCl-frog saline: 115 mM NaCl, 2,5 mM KCl, 2,15 mM $Na_2HPO_4*2H_2O$, 0,85 mM $NaH_2PO_4*H_2O$, 1,8 mM $CaCl_2*H_2O$]).

Live observation

For the observation of living eggs and embryos we used stereomicroscopes (Zeiss, Leica) equipped with a camera and a cold-light source. Broods that were transferred to saline in salt dishes were continuously observed (although the light had to be turned off from time to time to avoid damage of the embryos). The progress of development was documented by drawings and photographs.

Video recording

The eggs were transferred in saline (see above) into a specially designed glass container (30 ml) with a bottom made of a cover slip and pút on an inverted microscope (Zeiss Telaval 31). Fixation of the eggs was done with a rubber ring glued on the cover slip. The saline was oxygenated with an aquarium pump through a filter (0.2 ìm) and changed every second day. The light came from a cold light source. Permanent light destroys the eggs, so a switch was integrated in the set up which turned on the light for just one shot of the recording system (camera: Sony SSC-M370CE, analogous recorder: Panasonic). The frequency was one picture per minute. Later the recorded sequences were digitised. The video films are put on the homepage of Contributions to Zoology (http://www.uba.uva.nl/ctz).

Histology and scanning electron microscopy

For semi-thin sections the embryos were fixed for 30 min to 2 h in buffered 5% formaldehyde, Bouin's solution or a mixture of picric acid and sublimate. The egg envelopes were removed during fixation (if possible) to allow better penetration. After washing in distilled water and a dehydration in an ethanol series the embryos were transferred to the metacrylate embedding medium (Technovit) following standard protocols. Serial semi-thin sections (3µm) were produced with a Jung microtome. Toluidine-blue was used for staining. Some whole-mount preparations were done of eggs of various stages. In this case the fluorescent dye Bisbenzimide (Hoechst) was used to stain nuclei. The stained eggs were transferred into glycerol and covered with a cover slip. Micrographs were taken with a Zeiss Axiophot 1 equipped with a digital camera (Nikon D1).

The same solutions for fixation as for histology were used for scanning electron microscopy. After dehydration the embryos were transferred into acetone, dried at the critical point (Balzers Union) and sputter coated (Balzers Union). Observations were done with a Philips scanning electron microscope.

Injections

The eggs in the 8-cell stage were fixed on microscopic slides under small cover slips that were equipped with plasticine feet at the corners. The eggs could be brought in the right position by carefully shifting the cover slip. With soft pressure on the cover slip the eggs were fixed for the injection. All macromeres of the 8- cell stage were marked subsequently. To get suitable needles for the injection, pipettes (Hilsberg, diameter 1,0 mm, thickness 0,2 mm) were pulled (KOPF Puller 720). After

this, the tips had to be sharpened (Bachofer). The angle of the cutting edge varied between 20 and 30 degrees. The fluorescent marker was sucked into the injection-needle. DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanin perchlorate, Molecular Probes) was used as a vital marker. DiI is a lipophilic fluorescence-dye which binds to the cell membrane. This guarantees that the dye is exclusively restricted to the daughter cells. A further advantage is the kind of the application. The injection needle does not have to penetrate the membrane of the marked cell. It is enough to penetrate both embryonic covers and to put down a drop of DiI on the cell membrane. This drop diffuses over the hole cell surface. The cell marking was done with an inverse microscope equipped with a micromanipulator (Leica DMIRB). The opaque purple yolk makes the eggs non-transparent. Therefore, during DiI injection a cold light source was used to illuminate the eggs from the side. After the penetration of the two embryonic covers (chorion, vitelline membrane) a drop of DiI was set on the membrane of single cells. The diffusion was observed under blue fluorescence light. This wavelength causes only weak stimulation of DiI, and thus the dangers of dye fading and damage to the egg were avoided. After injection the eggs were put into a petri dish with saline (see above) and kept at 18-20°C in darkness. After a defined period of development the marked eggs were put on a slide and fixed with a cover slip. The observations were done with a fluorescence-microscope (Zeiss Axiophot1) using blue light or green light (strongest stimulation of DiI) and the results were documented with analogous and digital cameras (Zeiss C 80 (Agfa CT 100), Nikon D1). The analogous slides were digitalized with a scanner (EPSON Diascan) and tailored with Corel Draw to the desired size. The pictures were not retouched. If necessary, the embryos were dissected in buffered 4% formaldehyde-solution and embedded in DABCO-Glycerol (25 mg DABCO [1,4 diazabicyclol-2,2,2-octane, Merck] in 1ml PBS [8 g NaCl, 0,2 g KCl, 1,44 g Na_2HPO_4, 0,24 g KH_2PO_4, pH 7,2]to 9 ml Glycerol).

Nomenclature

The blastomeres of the 2-cell stage cannot be reasonably labelled because the fate of the individual cells is unclear. At the 4-cell stage distinct differences between the quadrants are recognisable (Fig. 1). Viewed from 'dorsal' (the future micromere region), the smallest blastomere is named **A** followed by **B**, **C**, and **D** in a clockwise sequence. The 8-cell stage shows a characteristic pattern with four micromeres and four macromeres. As in the 4-cell stage the descendants of the smallest quadrant are labelled **A** (macromere) and **a** (micromere) the other blastomeres are labelled accordingly in a clockwise sequence. Nomenclature for the 16-cell stage. Since the symmetry axis of the embryo runs through the **A/C** axis, the blastomeres are labelled according their position to the length axis of the embryo left (**l**) and right (**r**) and anterior (**a**) and posterior (**p**). After the 16-cell stage the cleavage products of equatorial cleavages are named **d** for blastomeres closer to the micromere pole and **v** for blastomeres closer to the macromere pole.

Results

The first four cleavages

The oval shaped fertilized egg is about 500 µm long and 300 µm in diameter. It is rich of purple coloured marbled yolk containing many droplets of lipid. Polar bodies or other differentiations cannot be seen, and therefore no polarity can be recognised (Fig. 1A). The nucleus lies eccentrically in a stellate mass of yolk-free protoplasm which has thin protrusions passing through the yolk forming a three-dimensional net. This plasmatic net is connected with a thin layer of periplasm surrounding the yolk.

The first cleavage at first becomes visible as a furrow on one side propagating around the egg. The cleavage is total and meridional, and with some variation the cleavage furrow is oriented obliquely to the long axis of the egg (Fig. 1B). The nuclei of the two daughter cells lie excentrically.

The second cleavage is again meridional. It leads to a first distinct differentiation of cells which allows to identify each cell with respect to its position and size. The two blastomeres divide asym-

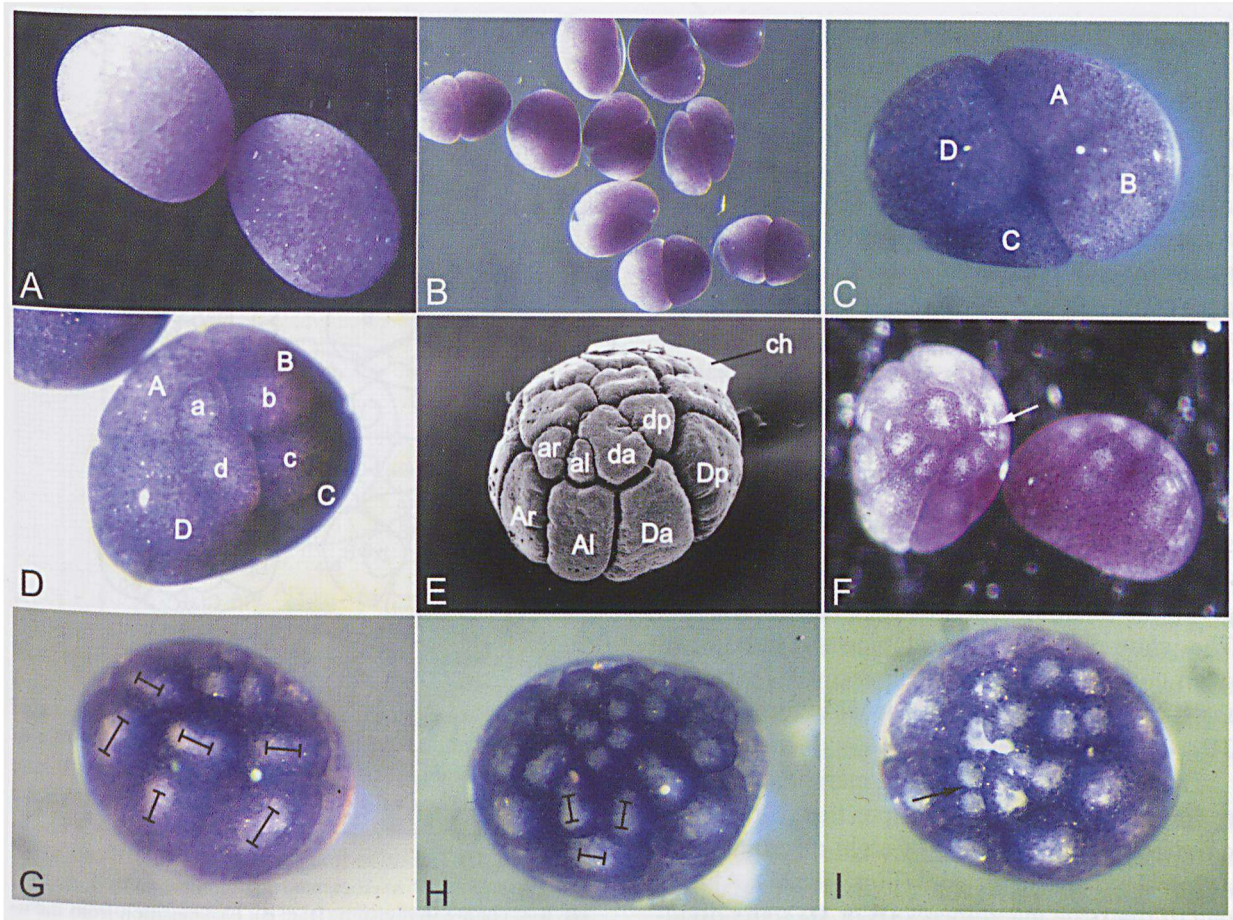


Fig.1. Cleavage in *Orchestia cavimana.* A) Two living uncleaved eggs with the characteristic marbled purple yolk. B) The 2-cell stage (living eggs). The cleavage plane shows a certain variability. C) The 4-cell stage (unstained, living egg). The two small blastomeres **A** and **C** and the two large blastomeres **B** and **D** are recognisable. **A** is the smallest blastomere. The two large blastomeres touch each other in the median region whereas the small blastomeres are separated. D) The 8-cell stage(unstained, living egg). A tier of 4 micromeres (**a-d**) lies on top of 4 macromeres (**A-D**). The cells show a radial arrangement. **A** is the smallest macromere, **a** the smallest micromere. E) SEM picture of a 16-cell stage. The blastomeres are radially arranged. The **A** quadrant is still the smallest. ch = chrion. F) Two living eggs starting the 5th cleavage. The white cytoplasm appears at the surface. The macromeres finished their division (right egg, lateral view) whereas the micromeres (left egg, "dorsal" view) are still in mitosis (elongated white cytoplasm). The cells **al** and **ar** have not yet started their 5th cleavage (arrow). G) The 6th cleavage of the macromeres, lateral view. From left to right: the derivatives of **Bp**, **Cl**, and **Cr** are seen. Sister cells are connected by a line which indicates the spindle direction. This pattern is typical for the derivatives of macromeres **B**, **C** and **D**. The derivatives of **A** show a somewhat different pattern (see Figs. 8A, B) (compare Figs. 4C, 5). H) The 7th cleavage of the macromeres. As in (G) the derivatives of **Bp**, **Cl**, and **Cr** can be seen (compare Fig. 5). I) The 5th cleavage of the micromeres ("dorsal" view). All micromeres underwent their division except **al**, **ar** (arrow), **ba**, and **da** (compare Fig. 4C).

metrically producing a larger and a smaller descendant each (Figs. 1C, 2A,B). They do not divide synchronously (Fig. 2A). The division planes are parallel. The resulting two larger descendants **B** and **D** meet at the top and the bottom of the egg, whereas the smaller blastomeres **A** and **C** have contact only in the centre of the egg (Figs. 1C, 2A,B, 3A). The cell **A** is the smallest blastomere (Figs. 1C, 2B). Again the relative size of the blastomeres and the cleavage planes show some variation. Therefore, the cell **A** is not always unambiguously identifiable.

The third division is the first equatorial cleavage. Each of the four blastomeres divides highly

unequally with a clearly radial spindle orientation. The divisions are not synchronous and show no predictable sequence. Only the smallest blastomere **A** always divides last. The result of the third cleavage are four micromeres labelled **a, b, c, d** and four macromeres **A, B, C, D** (Figs. 1D, 2C). The size relations of the quadrants at the four-cell stage are maintained during the third and subsequent cleavages (Figs. 1D, 2C). Since the **A** blastomere of the four-cell stage is the smallest, its descendants are also the smallest macromere **A** and the smallest micromere **a** of the eight-cell stage. During the 8-cell stage the colour of the micromeres becomes lighter than the one of the macromeres. The smallest blastomere **a** is most advanced with respect to this. This phenomenon is caused by migration of the nuclei and the cytoplasm towards the periphery of the micromeres (compare Figs. 1D and 1F). The white colour of the cytoplasm is due to calcium concrements (Meschenmoser 1987). During the 8-cell stage a small blastocoel is formed (Fig. 3B).

The fourth cleavage is again meridional leading to a 16-cell stage with an upper tier of eight micromeres and a lower tier of eight macromeres in a typical radial arrangement (Figs. 1E, 2F). Always one of the large macromeres (**B** or **D**) starts dividing. Its corresponding micromere mostly divides next. Then a macromere divides, followed by its sister micromere. Again, the sequence of divisions is not strictly determined. Only the smallest macromere **A** is always the last macromere to divide and the smallest micromere **a** is the last blastomere at all undergoing fourth cleavage (Figs. 2D-F). When it has finished its mitosis, the 16-cell stage is complete.

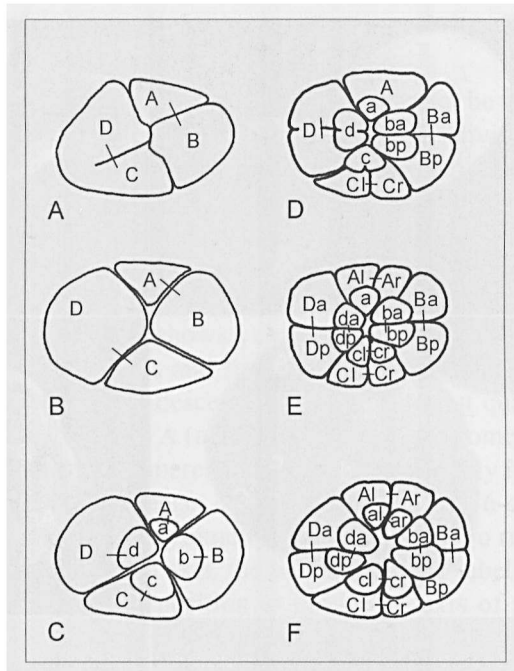


Fig. 2. Six stages of the same egg showing the sequence of early cleavages. A) Transition from the 2-cell stage to the 4-cell stage; meridional cleavage. The blastomeres **A** and **B**, and **C** and **D** are sister cells. The cleavage furrow runs from the centre to the margin of the egg. B) The 4-cell stage. C) The 8-cell stage. D) Transition from the 8-cell to the 16-cell stage. Except for **A, a** all micromeres and macromeres have divided (**B, b, C**) or are in division (**D, c, d**). E) Transition from the 8-cell to the 16-cell stage. All cells but micromere **a** have finished their divisions. F) The 16-cell stage. The open space between the micromeres allows a view into the blastocoel (see Fig. 3).

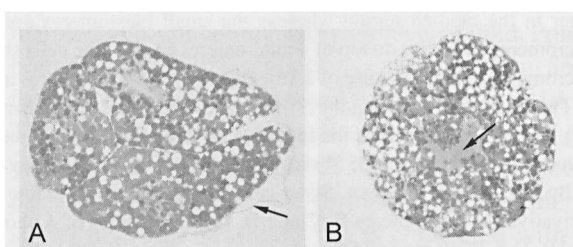


Fig. 3. Semi-thin sections through eggs during early cleavages. A) Horizontal section through the macromeres of an 8-cell stage. It is evident that all blastomeres are separated by cell membranes which is typical for total cleavage. The cytoplasm and the nuclei lie in the centre of each cell. The arrow marks the **A** quadrant. B) Transverse section of an egg after the 16-cell stage. The arrow points to the blastocoel.

The fifth cleavage and further

The second and third cleavage divisions leading to the 4- and 8-cell stages are already not synchronous. This tendency becomes much more distinct during the fourth cleavage. Nevertheless, a 16-cell stage still develops. However, this is the last "regular" stage. This is due to several factors:

(i) From the 16-cell stage on mitoses of the macromeres are always in advance as compared to those

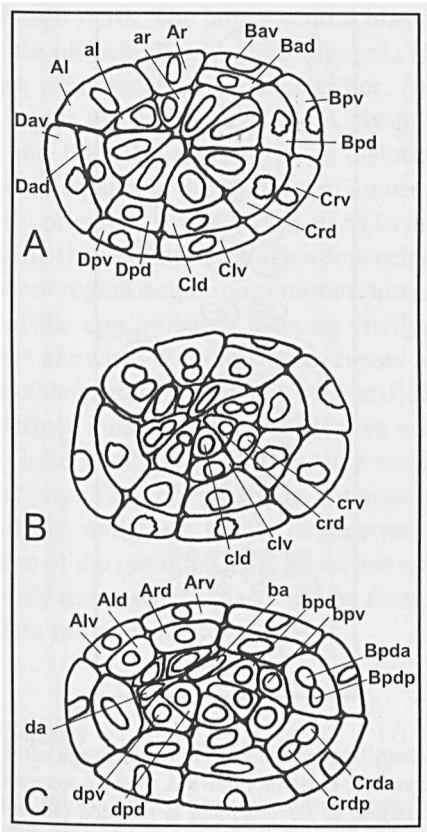


Fig. 4. Three stage of the same egg showing the sequences of the 5th and 6th cleavage of the macromeres and of the 5th cleavage of the micromeres. A) The eight micromeres of the 16-cell stage are still undivided. The elongated cytoplasm (except in **al** and **ar**), however, indicates the beginning of the next (5th) cleavage. The 5th cleavage of the macromeres is almost finished. Only the blastomeres **Al** and **Ar** have not finished their mitosis. B) A slightly advanced stage compared to that of (A). Most micromeres underwent the 5th cleavage. C) The beginning of the 6th cleavage in the macromeres. The upper macromere derivatives show a meridional cleavage whereas the lower derivatives undergo an equatorial cleavage (compare Figs. 1, 5). The 5th cleavage of the micromeres is still incomplete. The derivatives of **a** and the adjacent cells **ba** and **da** show a delayed development (see Fig. 1I).

of the micromeres – with increasing tendency (Figs. 1F-I, 4). During germ disc formation the macromeres are about three divisions ahead. (ii) The cells **Ar** and **Al**, the smallest macromeres, and **ar** and **al**, the smallest micromeres, and their adjacent cells show retarded mitoses further along in development (Fig. 4).

Once the 16-cell stage is complete, the macromeres are next to divide. Again a determined sequence is not to be noticed. Each cell can start this phase with the exception of the blastomeres **Ar** and **Al** which always divide last. The cleavage of the cells **Ba, Bp, Cl, Cr, Da, Dp** is equatorial oriented and unequal (Figs. 1F, 4). The lower positioned daughter cells **Bav, Bpv, Clv, Crv, Dav,** and **Dpv** are of longitudinal shape and relatively large, whereas the upper descendants **Bad, Bpd, Cld, Crd, Dad,** and **Dpd** are smaller, round, and lie around the equatorial area of the egg (Fig. 4). The spindle directions of the single divisions are not strictly determined. Their orientation depends on the space and the position of the cell, which are both due to earlier cleavages. In contrast to all the other macromeres, **Al** and **Ar** divide more or less equally (Fig. 4, see also Fig. 8A). The descendants **Alv** and **Ald** and **Arv** and **Ard** show mostly a rhomboid arrangement, which is due to the inclined spindle axes. Their nuclei and the white cytoplasm are now seen at the egg surface. This stands in contrast to the situation in the other macromeres, where the nuclei and the plasma reach the egg surface in somewhat later stages.

The mitoses of the fifth cleavage in the macromeres are mostly finished by the time the micromeres start to divide. Sometimes the small macromeres **Al** and **Ar** divide synchronously with the micromeres starting the fifth cleavage. As in the macromeres, a defined sequence of mitoses cannot be found in the micromeres. Often they divide almost synchronously, with the exception of the blastomeres **al, ar** and **ba, da** which show a retarded further development (Figs.1I, 4). As in the macromeres, the spindle orientations are more or less perpendicular to the one of the previous cleavage. The smallest micromeres, **al** and **ar**, undergo their mitoses last. The spindle axes of this division are perpendicular to the one of the previous mitosis. Furthermore, the spindles are slightly inclined towards the interior of the egg. Consequently, the daughter cells of **al** and **ar** are in a lower position somewhat sunken into the yolk. This is the beginning of the blastopore formation in the area of the gastrulation centre. The blastomeres adjacent to **al** and **ar**, the cells **ba** and **da**, show a retarded development in this stage. Their daughter cells also become involved early in the gastrulation process. However, these phenomena do not show an identical pattern

in all eggs. The sequence of these mitoses is apparently not fixed. During the sixth cleavage of the macromeres the retarded micromeres sink deeper into the yolk. Thereby they undergo a change of their shape. The nuclei and the yolk-free cytoplasm are now superficial and the yolk of each cell is shifted towards the centre of the egg (Figs. 1I, see also Figs. 7, 8). Thus, the cells assume a columnar appearance.

The sixth cleavage of the macromere region starts before the micromeres have finished their fifth cleavage. First the smaller cells **Bad**, **Bpd**, **Crd**, **Cld**, **Dpd**, and **Dad** divide almost synchronously into **Bada** and **Badp**, **Bpda** and **Bpdp**, **Crdr** and **Crdl**, **Cldr** and **Cldl**, **Dpda** and **Dpdp**, **Dada** and **Dadp** (Figs. 1G, 4C). All these mitoses are equal. The spindle axes are again perpendicular to those of the previous divisions. This means it is in principle a meridional cleavage. However, as in the last cleavages many deviations can be found caused by earlier positional changes of single blastomeres. These deviations of spindle direction can be up to 90°. During these processes there exist about 10 to 13 micromeres. The subsequent divisions of the larger macromere derivatives **Bav**, **Bpv**, **Crv**, **Clv**, **Dpv**, and **Dav** are again unequal and the spindle direction is vertical as in their last cleavage (equatorial cleavage) (Figs. 1G, 4C). The upper derivatives **Bavd**, **Bpvd**, **Crvd**, **Clvd**, **Dpvd**, and **Davd** are again roundly shaped and smaller than their lower sister cells **Bavv**, **Bpvv**, **Crvv**, **Clvv**, **Dpvv**, and **Davv** which are elongated (Fig. 1H). As in the previous cleavages, the descendants of the blastomeres **A** divide much later. Beside their retardation the mode of their mitoses is different. **Arv** and **Ard**, **Alv** and **Ald** divide equally again, and the arrangement of the daughter cells does not show the typical pattern that is found in the other macromere derivatives.

The sixth cleavage in the micromere area starts before the **A** group of macromeres has undergone its sixth cleavage. Again, no predictable pattern in the sequence can be seen (data not shown).

The seventh cleavage of macromere descendants follows the same mode as described for the sixth cleavage. The basal macromere derivatives divide unequally with an equatorial division plane. Thus they keep up their spindle orientation starting from the 16-cell stage onwards. This mode of division is best described as teloblastic with the large basal blastomeres showing the asymmetric unidirectional division sequence which is typical for teloblast stem cells. The smaller macromere derivatives, on the other hand, always alternate their division plane about 90° (Fig. 5). This mode is kept up to eighth cleavage. Beyond this stage an analysis is made impossible by the cell migration during formation of the germ disc. The mitoses of the seventh cleavage in the macromere area again begin in the smaller cells close to the micromere descendants (Fig. 1H). They are synchronous in a larger region. As before, all these events show some variations concerning the sequence, the spindle direction and the area where

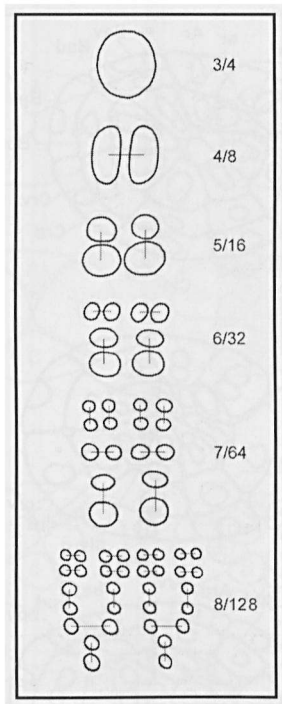


Fig. 5. Schematic representation of the cleavage pattern of the macromeres **B**,**C**, **D** lateral (view). **A** shows a somewhat different pattern and its development is retarded (see text). The ventralmost cell from the 5th cleavage on behaves like a stem cell with asymmetric divisions in dorsal direction. The dorsal cells follow the typical sequence of perpendicularly oriented cleavage planes. The left number indicates the cleavage stage, the right number represents the number of macromeres at that stage. The latter is idealised since the mitoses of the macromeres are not synchronous (in particular, those of **A**) but it gives an estimate of the cell numbers.

the cleavage starts. The larger ventral blastomeres lag a little bit behind with their mitoses. They do not show a predictable sequence either. As in all earlier stages the divisions,of the **A** group are retarded and the cleavage pattern is different. All mitoses are equal and the spindles are more or less vertically oriented. It was observed in living eggs that during the seventh cleavage a few cells of the macromere region begin to immigrate towards the centre of the egg probably forming vitellophages (data not shown). Whether these events show a reproducible pattern could not be clarified. Neither a defined number nor a specific area was identified. These vitellophages fill out the small blastocoel cavity. They obviously do not undergo further mitoses and their nuclei degenerate during formation of the germinal disc. In sections of these stages only traces of cytoplasm can be found in the yolk (data not shown).

Mirror images

There are two kinds of eggs showing a mirror symmetry. This can be recognised from the 4 to 8-cell stage on. For example, if one looks on the upper side of eggs at the 16-cell stage there is to be seen that the cells **ar, al**, **Ar,** and **Al** point in one case towards the left direction in the other towards the right (Fig. 6). The relation of these two types of eggs in every single brood is about 1/1. This phenomenon can be stated up to relatively late embryonic stages as is indicated by the inclination of the germ band in relation to the long axis of the egg.

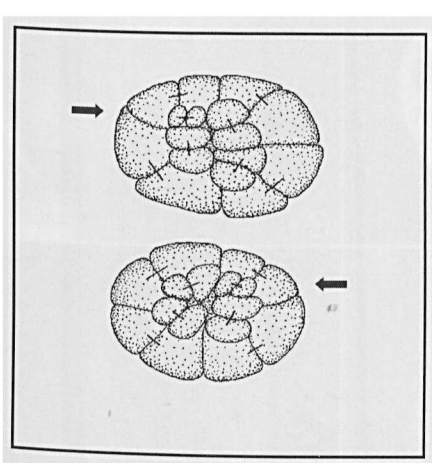


Fig. 6. Mirror images of eggs at the 16-cell stage. The two types of eggs with a left oriented **A** quadrant (arrow) (upper egg) and a right oriented **A** quadrant (arrow) (lower egg).

The formation of the germ disc

The cleavage divisions of the macromeres have the result that the macromere derivatives have the similar size and appearance as the micromere derivatives (Figs. 7A, 8B). Furthermore, a differentiation takes place mainly concerning the **A** region, the smallest quadrant. Both the macromeres **Al**, **Ar** as well as the micromeres **a, b, d** and their descendants, respectively differ from their neighbour cells with regard to their position, their morphology and the retardation and orientation of their mitoses. As described above, the nuclei together with the cytoplasm migrate relatively early (after the 16-cell stage) towards the egg surface whereas the yolk of these cells is transferred towards the egg centre. The derivatives of **A** are characterised by a round margin of the white cytoplasm which stands in contrast to the stellate or amoeboid shape of the cytoplasm in the other macromere descendants. Together with these changes of the cell shape a retardation of the divisions occurs. During the eighth cleavage division of the other macromeres they differentiate in a comparable way. The nuclei and the cytoplasm of the macromere derivatives migrate towards the egg periphery, they become superficial (Fig. 9). The external shape of the blastomeres changes from spherically to flat. This coincides with the separation of the yolk from the cytoplasm of each cell. The process of yolk extrusion leads to central yolk compartments which are surrounded by membranes. This phenomenon is comparable to what has been described for other crustaceans as so-called yolk pyramids (Dotterpyramiden, Fioroni 1987). Whether these membranes disappear in advanced stages is not clear. I some sections it looks as if, but this might be just an artefact caused by the fixation. As a result, the cells have the appearance of roundly white isles surrounded by the purple yolk (Figs. 7, 8). The cytoplasm loses its protrusions the edges are even. Figure 10 shows these events schematically. These morphological changes of the

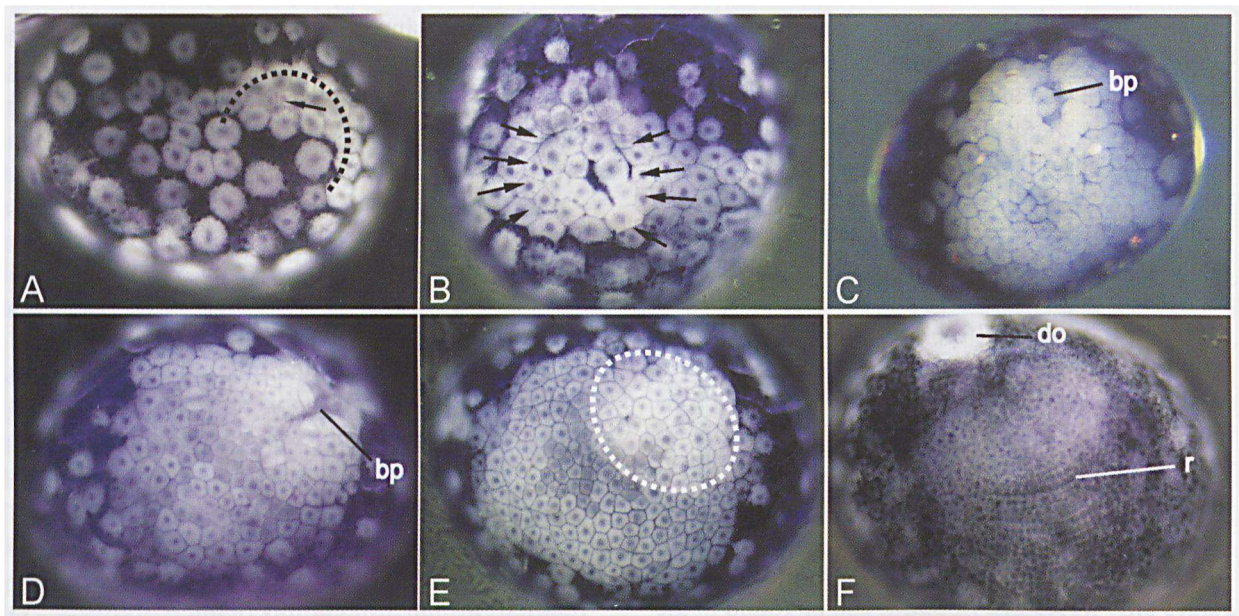
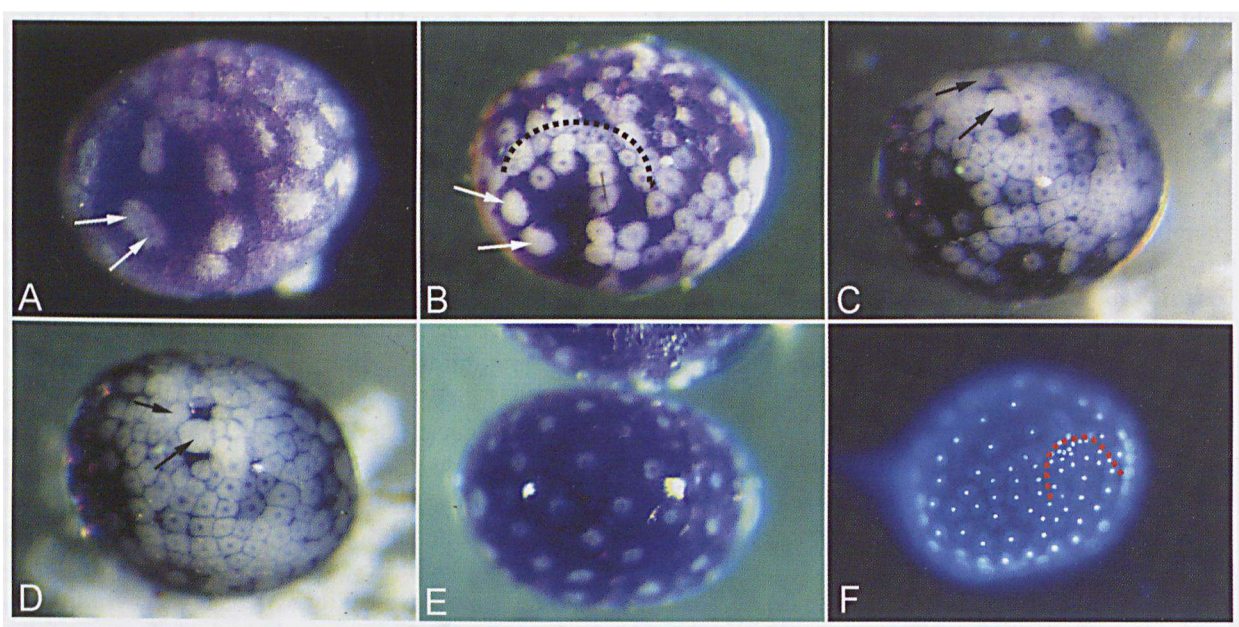


Fig. 7. Stages of the gastrulation. A) Formation of the gastrulation centre in the region of the retarded derivatives of micromeres **a**, **b** and **d** (arrow). These cells start to delaminate into the interior of the egg. The macromere derivatives migrate towards this area. This stage is characterised by a sickle shaped arrangement of the cells at the anterior margin of the gastrulation centre (dotted line). B) Beginning of the blastopore formation in the gastrulation centre. The derivatives of macromeres **B** and **D** start to overgrow immigrating and invaginating mesendoderm and germ cell precursors. The arrows indicate the migration of the macromere derivatives. C) A real blastopore (bp) has formed at the anterior of the forming germ disc. D) The blastopore (bp) is almost closed forming an elongated slit. E) Germ disc after the closure of the blastopore. The area of the mesendoderm appears lighter due to the two cell layers (dotted line). E) Beginning of the ectoderm row formation (r) at the boundary between the naupliar and post-naupliar regions. The dorsal organ (do) lies anterior to the germ disc.



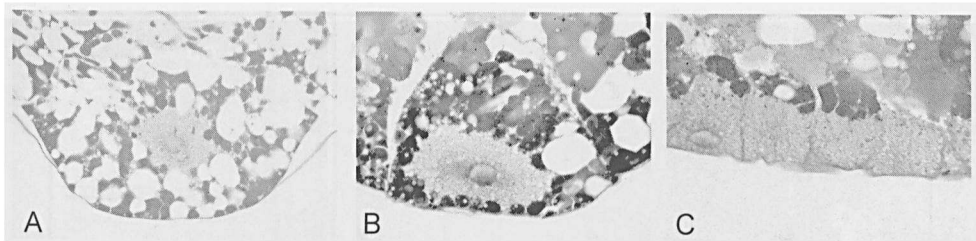


Fig. 9. Histological sections showing the change of cell shape during development. A) Blastomere at an early cleavage stage (about 16-cell stage). The cytoplasm and the nucleus are located in the centre of the cell surrounded by yolk. B) Blastomere at a stage of around 30 cells. The cell contains still a large amount of yolk but the cytoplasm and the nucleus have migrated to the egg surface. C) Cells of an early germ disc. Only some yolk droplets are found in some of the cells. The yolk lies in the centre of the egg forming compartments surrounded by membranes.

macromere derivatives occur first in the equatorial area surrounding the egg. Later the other macromere derivatives follow. Thereby all the macromere descendants begin to migrate together toward the initial gastrulation centre, thus forming a compact germ disc (Figs.7, 8, 10, 11). Correspondingly, the micromere region becomes extended. This way it covers the biggest part of the egg surface (Figs. 8, 10, 11). In contrast to the macromere derivatives, most of the micromere derivatives do not extrude all of their yolk (Figs. 8E, 11). Figure 11 shows macromere derivatives, which are differentiated as germ disc cells, in comparison with micromere derivatives, which are part of the extra-embryonic region ("extra-embryonic ectoderm" see Anderson 1973).

In summary, one can note three major events, which are undergone by the macromere derivatives, forming the germ disc: (i)The migration of the nuclei and the surrounding cytoplasm towards the egg periphery. (ii)The extrusion of the yolk. (iii)The migration towards the initial gastrulation centre.

With formation, the germ disc consists of about 120 – 130 cells. The extra-embryonic area includes about 30 blastomeres. These numbers correspond relatively good (even when the retarded blastomeres **A** and **a**, **b**, **d** are considered) with the number of cleavage divisions, which took place up to this time in the macromeres (8/128) and in the micromeres (6/32).

Gastrulation

In *Orchestia* gastrulation happens in three phases:

1. The immigration of several macromeres during the seventh cleavage probably forming vitellophages.
2. The sinking in of the micromeres in the **a** and adjacent regions forming the initial centre for gastrulation (Figs. 7, 8, 12).

←

Fig. 8. Migration and change of the shape of macromere descendants during germ disc formation (compare Figs. 9, 10). Panels A to D show the same egg at different developmental stages. The area of quadrant **A** is in the front. In panels C and D the egg is slightly rotated backwards. The cells **Arvd** and **Arvv** (arrows) serve as landmarks because in this particular embryo they showed an aberrant development (for some reasons they did not divide and they had problems with the extrusion of their yolk). A) The macromeres **B** to **D** undergo the 7th cleavage (see Fig. 5). **A** is in the 6th cleavage. The cell surface is still rounded and the cytoplasm is surrounded by yolk. B) Cell migration has started. The Macromeres **B** to **D** are at least in the 8th cleavage, **A** is in the 7th cleavage stage. The cells used as landmarks (arrows) have not divided. The cell surface is now flat and the yolk is internalised. The cells lie closer together. C) Migration and yolk extrusion have proceeded and the early germ disc is recognisable. The arrows point to the cells that serve as landmarks. Gastrulation has stated (see Figs. 7B, C). D) Advanced formation of the germ disc. The blastopore lies in front of the marked (arrows) cells at the upper left. Almost no yolk is visible between the cells. E) Micromere area of another egg at the same stage as that in D. Most micromere descendants keep their yolk and are evenly distributed at the prospective dorsal side of the embryo. They are not involved in embryo formation (extra-embryonic ectoderm). F) Embryo stained with fluorescent dye showing a similar stage as in C. The sickle shaped arrangement of cells at the margin of the gastrulation centre is marked (dotted line).

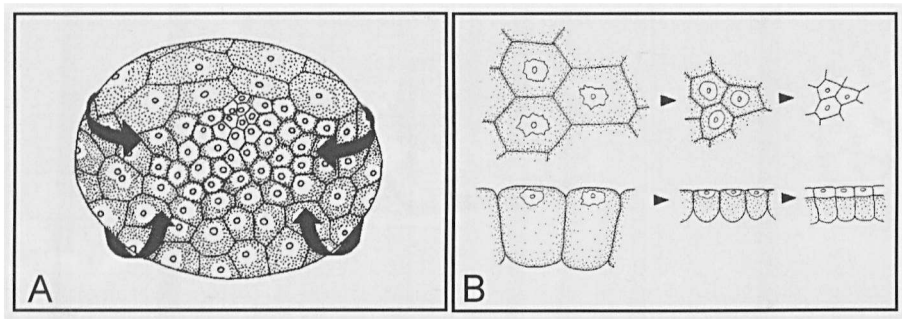


Fig. 10. Schematic representation of germ disc formation. A) shows the migration of macromere derivatives during germ disc formation (arrows). B) depicts the changes in cell morphology during migration and germ disc formation. Upper row surface view, lower row lateral view of cells. See text for explanation.

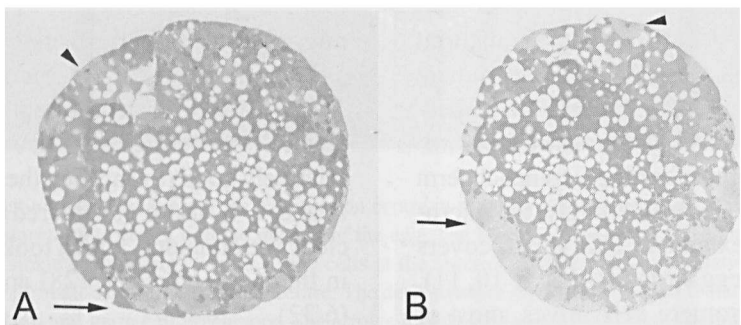


Fig. 11. Sagittal sections through eggs in different stages of germ disc formation. A) Early germ disc formation. The arrow points to the anterior end of the forming germ disc. The arrowhead marks a typical dorsal micromere derivative. B) Slightly advanced stage of an early germ disc. The arrow points to the anterior end of the forming germ disc. The macromere derivatives to the right show the appearance typical for migrating cells. At the dorsal side the micromere derivatives with their characteristic shape are visible (arrowhead).

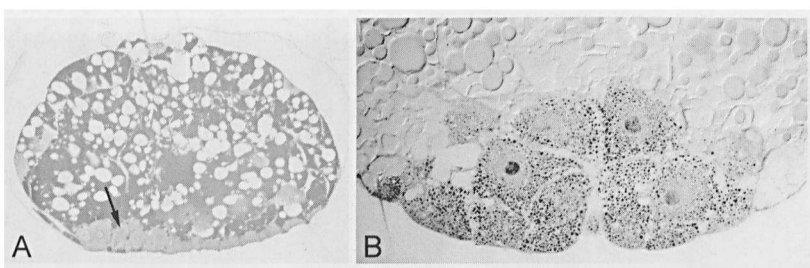


Fig. 12. Histological sections through the gastrulation centre. A) Sagittal section through an embryo at the onset of blastopore formation (anterior to the left). In the anterior area of the germ disc, there are several cell layers indicating the immigration of cells during early gastrulation. B) Transverse section through the blastopore in an advanced stage of gastrulation (compare Fig. 7).

3. The gastrulation *sensu stricto* by a combined invagination and immigration of anterior germ disc cells which gives rise to the main part of the mesendodermal mass (Figs. 7, 12).

Consequently, the two last phases are not strictly distinct. The last phase starts just when the germ disc consists of about 130 cells (Fig. 7). As mentioned above, the retarded micromere descendants lie at the anterior edge of the germ disc in a deeper position sunken into the yolk. From here a gastral groove or blastopore is forming which propagates in posterior direction (Fig. 7). It has about 1/3 of the length of the germ disc. The gastrulation is a combination of invagination and immigration of

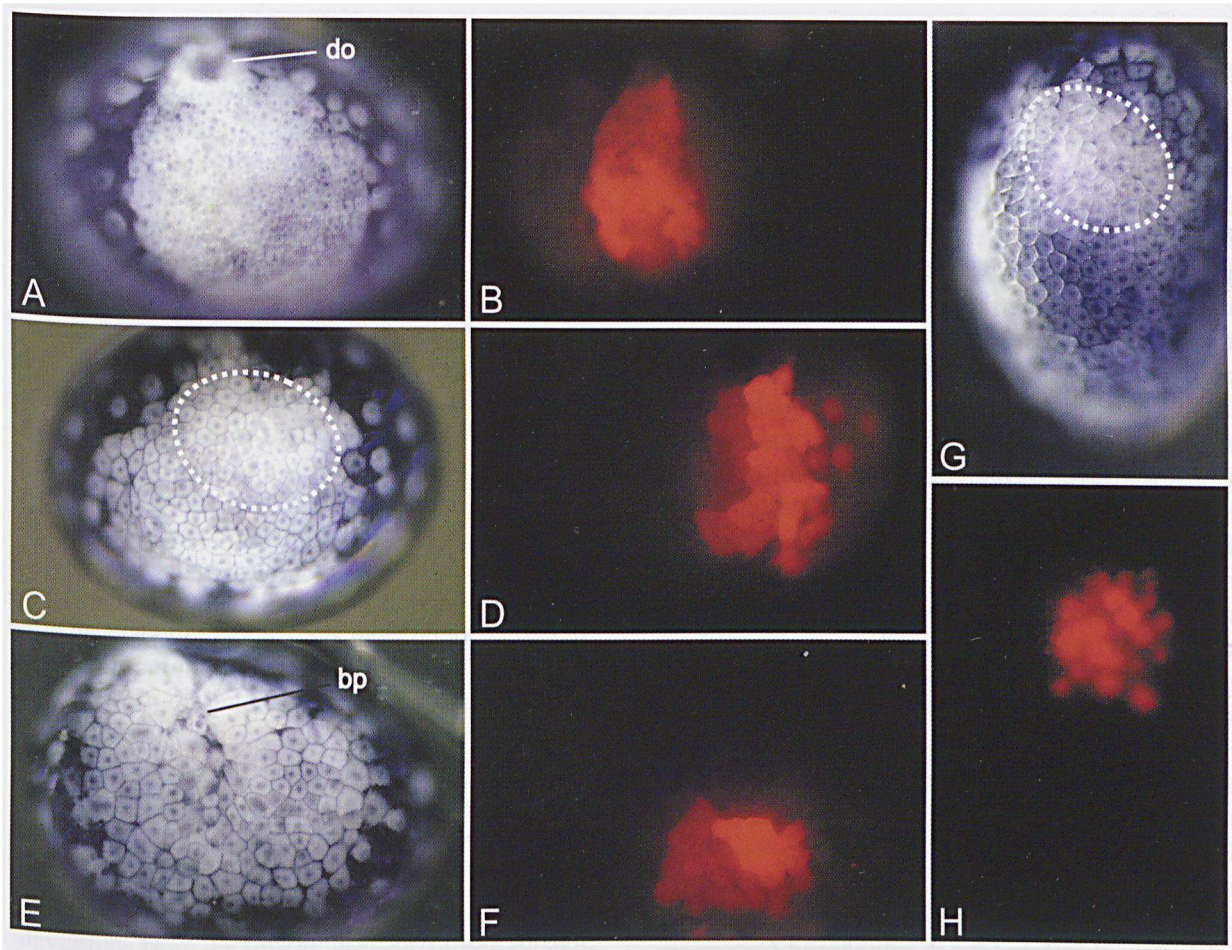


Fig. 13. Germ disc formation from macromere descendants (compare Wolff & Scholtz 2002). Tracing of the cell lineage using a vital dye. In every case one macromere of the 8-cell stage was labelled. A,B) Labelling of macromere **B**. A) shows the living egg with light from above. The dorsal organ (do) indicates anterior. B) shows the same egg under fluorescent light. The labelled cells (descendants of **B**) form the morphological right side of the germ disc. C,D) labelling of macromere **D**. C) depicts the living egg with light from above. Slightly earlier stage than in panel A. Anterior is up, the dorsal organ is not yet formed. The dotted line indicates the mesendodermal mass. D) shows the same egg under fluorescent light. The labelled cells (descendants of **D**) form the morphological left half of the germ disc. E,F) Labelling of macromere **C**. E) depicts the living egg during gastrulation with light from above. The blastopore (bp) marks the anterior end of the germ disc. F) shows the same egg under fluorescent light. Cells at the posterior margin of the germ disc (descendants of **C**) are labelled. G,H) Labelling of macromere **A**. G) depicts the living egg with light from above (same stage as panel C). The dotted line indicates the mesendodermal mass at the anterior of the embryo. H) shows the same egg under fluorescent light. Only cells of the mesendodermal mass (descendants of **A**) are labelled.

germ disc cells giving rise to the mesendodermal mass. The blastopore becomes closed by growing together of the two lateral edges in posteroanterior direction. After closure the germ disc shows a characteristic pear-like shape with a narrow anterior part (Fig. 7E). During gastrulation little mitotic activity can be seen within the germ disc. The cells of mesendodermal mass and the primordial germ cells are spread under the ectoderm layer (Figs. 7E, F). In late germ discs, this can be recognized by the brighter appearance of the anterior region due to the two layers of cells (Fig. 7E). The fate of the gastrulating blastomeres **A, a, ba**, and **da** has been described in another paper (Wolff & Scholtz 2002). The derivatives of **A** give rise to the mesoderm of the naupliar region and to anterior parts of the endodermal midgut glands, **a** forms the primordial germ cells, and the gastrulating descendants of **ba**

and **da** form the post-naupliar mesoderm and the posterior region of the endodermal midgut glands

After gastrulation has finished, some blastomeres (see Wolff & Scholtz 2002) in front of the germ disc begin to differentiate into cells forming the dorsal organ (Fig. 7F). For a detailed description of this structure see Meschenmoser (1996). The cell marking with DiI clearly reveals that the outer (ectodermal) layer of the germ disc is exclusively formed by derivatives of macromeres **B**, and **D**, and transiently of **C** (Fig. 13) (see Wolff & Scholtz 2002). The only part of the germ disc which is partly formed by micromere derivatives is the most anterior lower layer of the germ disc. Here lie the descendants of micromeres **a**, **ba** and **da** (not shown in this paper). Together with the derivatives of macromere **A** they form the mesendodermal mass and the primordial germ cells (see Fig. 13) (Wolff & Scholtz 2002). The complete germ disc is oval shaped and lies obliquely on the egg surface (Fig. 7). The mirror image germs are still discernible. The ectodermal cells of the germ disc start to become arranged in transverse rows during the formation of the dorsal organ or sometimes slightly later (Fig. 7F). In the mesoderm the mesoteloblasts are differentiated. The further development of the germ disc and germ band follows the same mode as described in detail for the amphipod *Gammarus pulex* (see Scholtz 1990; Scholtz et al. 1994; Wolff & Scholtz 2002).

Discussion

A secondary meroblastic radial cleavage despite yolky eggs

The present investigation reveals that the early embryonic development of the amphipod *Orchestia cavimana* follows the mode of a total, radial, unequal early cleavage. These results correspond, up to the 16-cell stage, to the descriptions and figures found in the papers of several authors who studied amphipod development (e.g. van Beneden & Bessels 1869; Rossiiskaya 1888, Pereyaslawzewa 1888a,b; Rossiiskaya-Koschewnikowa 1890, 1896; Wagner 1891; Langenbeck 1898; Heidecke 1904; Rappaport 1960; Bregazzi 1973; Mergault & Charniaux-Cotton 1973; Magniette & Ginsburger-Vogel 1982). However, some students of amphipod development came to the conclusion that the cleavage furrows are only superficial, leaving an undivided central yolk mass (La Valette St.George 1860; van Beneden & Bessels 1869; Ulianin 1881; Della Valle 1893; Mergault & Charniaux-Cotton 1973). Van Beneden & Bessels (1869) concluded that the eggs of all marine gammarid species cleave totally, and that, in contrast to this, the eggs of freshwater species (*Gammarus pulex* and *Gammarus poecilurus*) cleave superficially. This hypothesis is not correct. In her investigation, Pereyaslawzewa (1888a) shows clearly that *Gammarus poecilurus* undergoes a total separation of blastomeres and our own observations of the eggs of the freshwater species *Gammarus pulex* reveal the same total cleavage type as in *Orchestia* (unpub.). There is some evidence that the "superficial cleavage" in amphipods is an artefact due to the problems with fixation of the yolky eggs, and this may also be the reason for the opinion that the total cleavage changes to superficial right after the 16-cell stage (Heidecke 1904; Weygoldt 1958). We can clearly demonstrate by semithin sections and by the lineage tracing with DiI that the cleavage is entirely total until the yolk compartments are separated from the blastomeres during germ disc formation.

The later cleavage events, from the 16-cell stage on, are described in detail only by Langenbeck (1898) and in parts by van Beneden & Bessels (1869) who developed a schematic representation of later cleavage divisions. The problem of the interpretation of advanced cleavage stages is the variability between the eggs concerning the position and size of individual cells, the different division frequency of the blastomeres, the high degree of cell migration found in the macromeres, and the tendency of the macromere derivatives to decrease in size so that they become more and more difficult to distinguish from the micromeres and their progeny. A further complication is due to the mirror images of the germs (see below), which led investigators who did not notice this phenomenon to erroneous interpretations (e.g. Langenbeck 1898: Figs. 2 and 3). On the other hand, almost all cited authors report that from the 16-cell stage on the macromere divisions are in increasing advance compared to the micromeres and that the blastomeres of the small-

est quadrant (micro- and macromeres) show some retardation in their cleavage activity. Different views are presented by van Beneden & Bessels (1869) and Lalitha et al. (1989) who report synchronous divisions and by Mergault & Charniaux-Cotton (1973) and Bregazzi (1973) who write that the divisions of the micromeres are in advance – which is, at least for *Orchestia*, not correct. In principle, the descriptions given by Langenbeck (1898) correspond in several aspects very good to those found by us in *Orchestia*. Nevertheless, the above sketched difficulties of interpretation lead to some contradictions in her report. On page 308 she writes, "The subsequent cleavage planes (after the 8-cell stage) which divide the two larger macromeres are alternately meridional and equatorial, while the planes dividing **EF** (our blastomere **A**) and the micromeres are always meridional". The corresponding figures show different patterns resembling more the conditions we have found in *Orchestia*, i.e., the macromeres from the 16-cell stage on divide in principle as shown in Fig. 5. The schematic representation of advanced cleavage as presented by van Beneden & Bessels (1869) for several gammarid species also shows differences to the results given here with regard to the spindle direction of the upper macromere derivatives, where the authors presume deviations from the rule of perpendicularity, which we found only to occur in the lower large macromere derivatives giving rise to their smaller sister cells in only one direction. With regard to the mentioned difficulties of interpretation of advanced cleavage stages, we carefully presume that the mode found in *Orchestia* might be typical for amphipods in general. Although the differentiation of the **A** quadrant is recognisable in the figures of most amphipod embryologists the specific role of these cells forming an initial centre for germ disc formation and gastrulation has been only partially described by Langenbeck (1898) and by Rappaport (1960). The latter reports an ingression of cells in the **a** region after the 32-cell stage in *Marinogammarus*. This phenomenon does not correspond to the situation in *Orchestia*, where only a few cells lie in a lower position. In other gammarid species Rappaport (1960) could not find such ingression either. Perhaps, different species vary with this respect.

It is evident from our data on the radial pattern of early cleavages that the cleavage of *Orchestia* is not of the spiral-cleavage type; all blastomeres are arranged according to a modified radial cleavage. This conclusion is supported by the tracing of the lineage of individual cells at the 8- and 16-cell stages which shows remarkable differences between spiral cleavage and the cleavage mode of amphipods (Wolff & Scholtz 2002). Furthermore, it is clear that the cleavage of amphipods is highly derived among malacostracans and crustaceans in general. Again this is true for the cleavage pattern and the fate of single cells (this paper; Wolff & Scholtz 2002). This raises the question, what was the reason for the evolutionary shift from superficial cleavage towards total cleavage in amphipods with such an elaborated cleavage pattern. There is a problem. All amphipods studied possess relatively large yolky eggs. Accordingly, one would expect that the cleavage has to be of the superficial type. Moreover, amphipods are phylogenetically nested within the malacostracan taxon Peracarida which is characterised by brood care with a ventral brood pouch, direct development, yolky eggs, and superficial cleavage (Johnson et al. 2001; Richter & Scholtz 2001).

Evolutionary changes of cleavage from holoblastic to meroblastic or *vice versa* are often thought to be related to the size and the yolk content of the eggs. Famous examples for this are the transitions from holoblastic spiral cleavage towards discoidal cleavage in cephalopod molluscs or from holoblastic radial cleavage towards discoidal cleavage in amniote vertebrates. In both cases the change of cleavage type is correlated with an increase of egg size and yolk content. The opposite evolution can be seen within the isopod crustaceans which plesiomorphically show a superficial cleavage with yolky eggs and only some parasitic forms evolved early total cleavage correlated with small yolkless eggs (Strömberg 1971). However, Fioroni (1987: 173 f.) pointed out that there is no simple one to one correlation between egg size, yolk content and cleavage type – there are eggs of prosobranch gastropods which are much larger than those of some cephalopod representatives. Nevertheless, the prosobranch eggs follow the mode of spiral cleavage and those of cephalopods show a discoidal cleavage. This phenomenon can be interpreted as being due

to some sort of historical "burden" which must be considered in addition to mechanistic adaptive causes (Riedl 1975). Can this explanation be applied to the evolution of amphipod total cleavage? If so, we have to assume that the amphipod stem species possessed small eggs with only a small amount of yolk. This led to the evolution of total cleavage which was maintained (burden) even in the large yolky eggs we find in most amphipod species. Interestingly enough, the representatives of the Ingolfiellidea have about the smallest eggs among the amphipods. Ingolfiellidea are often considered as an early offshoot of the amphipod lineage. However, their cryptic lifestyle (meiofauna, stygobiont etc.) caused several apomorphic adaptive specialisations such as the loss of eyes. Nevertheless, perhaps we have to think of an amphipod stem species that was morphologically and with respect to size and lifestyle not so far away from the Recent ingolfiellid pattern. Unfortunately, nothing is known about ingolfiellid development (see below).

Mirror images

From the 8-cell stage on one can distinguish two types of eggs which are mirror images to each other. This is indicated by the position of the smallest quadrant, the **A** quadrant. The ratio between these mirror image germs is about 1:1. Mirror images are reported in the development of several crustacean species e.g. in other amphipods *Tryphoselis* (Bregazzi 1973), in several species of euphausiaceans (Taube 1909), in decapods (Hertzler & Clark 1992), in the cladocerans *Daphnia* and *Holopedium* (von Baldass 1937, 1941), and in the copepod *Cyclops* (Fuchs 1914). In all cases, the meaning is unclear and there is no obvious effect on adult morphology. Cell lineage tracing in *Orchestia* has shown that the **A** quadrant marks the anterior region of the embryo, **C** is at the posterior pole, and the **B** and **D** quadrants form the lateral parts (Wolff & Scholtz 2002, present study). This means that the asymmetry of the early embryonic stages in amphipods reflects the oblique orientation of the **A/C** axis with respect to the longitudinal axis of the egg. The embryo itself is symmetric. This stands in contrast to the phenomenon that a mirror image spiral cleavage leads to an inverted shell coiling in adult gastropods (van den Biggelaar 1991). About the causes for the mirror symmetrical eggs in crustaceans can only be speculated. One possible reason could be oogenesis in the paired ovaries indicating maternal axis determination through yolk distribution. Another possible cause could be the entry of sperm (see Hertzler & Clark 1992). At present we do not know how mirror symmetry is related to blastomere clonal relationships, i.e., whether the quadrants **A** and **B** are always sisters or whether **A** is the sister cell to **D** in eggs which are oriented to the right (see Figs. 2, 6). The high variability of the first cleavage plane makes it difficult to trace this but it also offers the possibility that there is always a clonal relationships between **A** and **B** and **C** and **D**.

Origin of the germ disc by migrations of macromere derivatives

In *Orchestia,* the early germ disc (before gastrulation) is mainly formed by macromere descendants. Only some immigrating cells in the retarded micromere area become part of the germ disc. About the half of the early micromere descendants remains extra-embryonically (the derivatives of **c**, **bp**, and **dp**) (see Wolff & Scholtz 2002). Whether all macromere derivatives are involved in early germ disc formation remains unclear. There are some hints that at the margin, and in particular, at the posterior region of the germ disc some macromere derivatives are not involved in embryo formation (Wolff & Scholtz 2002). Only Langenbeck (1898), Rappaport (1960) and Meschenmoser (1987) describe the role of the macromeres for germ disc formation in a corresponding way. Bergh (1894) and Weygoldt (1958) report a migration of blastomeres forming the germ disc but they do not say from which cells they arise. Rappaport (1960) confirms the function of macromeres forming the germ disc by destruction experiments in the 8-cell stage in *Marinogammarus*. When he destroyed the micromeres, yolk free cells, which are characteristic for the germ disc, differentiated in more advanced eggs. When the macromeres were punctuated, such cells were not seen. Nevertheless, most authors

suppose that the germ disc is given rise by micromere derivatives (van Beneden & Bessels 1869; Ulianin 1881; Heidecke 1904; Sheader & Chia 1970; Bregazzi 1973; Mergault & Charniaux-Cotton 1973; Magniette & Ginsburger-Vogel 1982). However, in most of these investigations the cleavages after the 16-cell stage are either traced incompletely or not at all. The early differentiation of the retarded micromeres and the size and appearance of the early micromere area may have led to the opinion that the micromeres form the germ disc. All this makes the conclusion evident that in amphipods in general the germ disc is mainly built up by macromere descendants. The formation of the germ disc is accompanied by dramatic migrations of the macromere derivatives towards the area of gastrulation. Furthermore, during migration the macromeres get rid of their yolk content by a mechanism which is still not completely understood. Rappaport (1960) describes the phenomenon as cell division without nuclear division. Our data are in agreement with this view.

Gastrulation in amphipods - the only "protostome" malacostracan crustaceans

Gastrulation in *Orchestia* is a highly complex process showing some more general features such as the subdivision into several phases (Weygoldt 1979; Fioroni 1987) that is shared with other arthropods and some features typical for amphipods. Gastrulation in *Orchestia* involves the immigration or delamination of single cells and an invagination. The first cells that participate in gastrulation are vitellophages which are presumably formed by blastomeres which immigrate during early cleavage. The gastrulation *sensu stricto* is started by the immigration of the derivatives of micromeres **a** after the 16-cell stage followed by neighbouring cells. As is shown by Wolff & Scholtz (2002) the descendants of **a** are the primordial germ cells whereas derivatives of micromeres **b** and **d** and of macromere **A** are responsible for the formation of the mesoderm and endoderm. The area of the blastopore is spatially related to the region of the formation of the stomodaeum and marks the anterior pole of the forming germ disc. The major part of the germ disc is formed by the macromere derivatives which aggregate by cell migration towards this region. Further cells immigrate but in addition invagination takes place in the same region forming a true blastopore. An anterior gastrulation has also been described in *Gammarus pulex* (Bergh 1894; Weygoldt 1958; Scholtz 1990), in *Parhyale hawaiensis* (Gerberding et al. 2002), and from the figures in several publications it can be deduced that this is the case for other amphipod species as well (e.g. Heidecke 1904; Pereyaslawzewa 1888b). Older reports about a mesoderm formation by immigrating cells spread all over the germ band (e.g. Ulianin 1881; Langenbeck 1898; Heidecke 1904) have been shown to be incorrect (Weygoldt 1958; Scholtz 1990). Sometimes a posterior gastrulation is reported (e.g. Langenbeck 1898; Margault & Charniaux-Cotton 1973; Lalitha et al. 1989). The figures and the description of Langenbeck (1898) are somewhat ambiguous and a reinvestigation of the gastrulation in *Microdeutopus* seems justified. In the case of *Orchestia gammarellus* the posterior blastopore is an erroneous observation because the authors oriented the germ disc upside down (Margault & Charnier-Cotton 1973: Planche 4) Similarly, Lalitha et al. (1989: Fig. 8) mistook the forming caudal furrow as blastopore. Interestingly, all other malacostracans show a blastopore or gastrulation centre in the posterior region of the forming germ disc with a close spatial relationship between the gastrulation area and the proctodaeum (e.g. Weldon 1892; McMurrich 1895; Taube 1909, 1915; Manton 1928, 1934; Hickman 1937; Scholl 1963; Dohle 1970, 1972; Zilch 1974, 1978, 1979; Scholtz 1984, 1992; Hertzler & Clark 1992; Hertzler 2002). In other words, amphipods are the only true "protostomes" among malacostracan crustaceans, whereas the other groups show a "deuterostome" type of development.

The embryonic development of amphipods shows many apomorphic characters

The monophyly of the taxon Amphipoda has never been seriously questioned. However, there are dramatic morphological differences between the representatives of the large amphipod taxa Gammaridea, Ingolfiellidea, Caprellidea, and Hyperiidea (Gruner

1993), and there is just one single apomorphic character of adults which occurs consistently in all amphipod subgroups – the basal fusion of the first thoracic appendage, the maxilliped (Richter & Scholtz 2001). In contrast to this there are several embryonic characters that exhibit a number of clear apomorphies for the Amphipoda, although at present the available data are still somewhat patchy. In particular, there are no embryological data at all for the Ingolfiellidea but this group is of great interest since the size of the eggs in the ovary in relation to the size of the oostegites (Siewing 1963) indicates that they do not carry the eggs in the brood pouch but rather deposit them in the environment (Gruner 1993).

The list of (potential) developmental apomorphies for Amphipoda is as follows:

1) The early cleavage pattern with the characteristic size relations, the arrangement of the blastomeres in the 4- to 16-cell stages and the increasing asynchrony after the 16-cell stage. A comparable pattern is not found in any other malacostracan or non-malacostracan crustacean group. This cleavage pattern has been shown for many representatives of the Gammaridea (van Beneden & Bessels 1869; Langenbeck 1898; Rappaport 1960 Bregazzi 1973; Gerberding et al. 2002; Wolff and Scholtz (2002); present study). Pereyaslawzewa (1888b) describes the same pattern for *Caprella ferox* (Caprellidea). For Hyperiidea there is only one short description without figures for *Parathemisto gaudichaudi* by Sheader (1977) but this description and the discussion suggest that the cleavage follows the same mode as in other amphipods.

2) Anterior gastrulation with the blastopore spatially related to the position of the stomodaeum. As mentioned above other malacostracans posses a gastrulation centre at the posterior, more closely related to the forming proctodaeum. This character has been demonstrated so far only for gammaridean species (Weygoldt 1958; Scholtz 1990; Gerberding et al. 2002; Wolff & Scholtz 2002; present study), but the figures in Pereyaslawzewa (1888b) indicate that the situation in caprellids is comparable.

3) A germ band with a growth zone lacking ectodermal teloblasts. All other malacostracans seem to possess ectoteloblasts (Scholtz 2000). Again, the Gammaridea are best studied with respect to this character (Bergh 1894; Langenbeck 1898; Dohle & Scholtz 1988; Scholtz 1990, 2000). However, Scholtz (1990) mentions that the same character occurs in *Hyperia galba* (Hyperiidea) and figures 28 and 41 in the article of Pereyaslawzewa (1888b) indicate that ectoteloblasts are also absent in Caprellidea.

4) The 90° spindle orientation to the midline of the cell c2 during the first differential cleavage of derivatives of each ectodermal row in the post-naupliar germ band of amphipods. Other malacostracans show an angle of the spindle direction of the corresponding cells of about 45° (Dohle 1976; Scholtz 1984; Dohle & Scholtz 1988). Our knowledge about this character in amphipods is currently restricted to several species of the Gammaridea (Scholtz 1990; Scholtz et al. 1994).

5) A delay of the expression of the segment polarity gene *engrailed* with respect to cell division in the ectodermal rows of the post-naupliar germ band. In amphipods the *engrailed* expression is switched on one cell cycle later than in decapods, mysids, and isopods (Scholtz et al. 1993; Patel 1994; Hejnol 2002). This phenomenon has been shown for several gammaridean species including *Orchestia cavimana* and *Gammarus pulex* (Scholtz et al. 1993; Scholtz & Dohle 1996).

Although not all characters are investigated for all amphipod subgroups, and although the monophyly of the amphipod taxa Gammaridea, Ingolfiellidea, Caprellidea, and Hyperiidea is not in all cases proven, not to speak about sister group relationships between these groups, this list is promising in establishing a set of powerful amphipod apomorphies. However, this list also demonstrates the incompleteness of our knowledge. For instance, investigations about the embryology of the aberrant group Ingolfiellidea are urgently needed.

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