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# ONTOGENETIC DEVELOPMENT OF *PORCELLIO SCABER*: STAGING BASED ON MICROSCOPIC ANATOMY

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ABSTRACT

Arthropods exhibit a vast variety of morphological forms. Developmental studies on diverse arthropod groups will help clarify how these forms evolved during arthropod evolution. The terrestrial isopod *Porcellio scaber* is a suitable novel animal model for developmental studies; it is well adapted to terrestrial habitats and these adaptations make the species interesting from the evolutionary perspective. *Porcellio scaber* is already extensively used in ecotoxicological and physiological studies and its biology is well known, but a comprehensive description of its ontogenetic development is still missing. Here, a staging system of *P. scaber* embryos and marsupial mancas based on morphological observations with light microscopy techniques is presented. An important adaptation of oniscids to terrestrial lifestyle is formation of specific structures in the digestive system, which enable effective water retention and good digestion of cellulose-rich plant material. Differentiation of the digestive system during *P. scaber* embryogenesis is described and the results show that digestive system is well developed already in late-stage embryos. The presented staging system should provide a useful basis for the expanding field of gene expression studies during arthropod embryogenesis and enable comparative developmental studies between arthropod groups.

KEY WORDS: gut morphology, light microscopy, ontogenetic development, *Porcellio scaber*, staging system

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## INTRODUCTION

Arthropods are by many measures the most successful animals on Earth as they have adapted to life in the sea, on land, and in the air, and have consequently evolved a large variety of morphological forms. The study of arthropod development helps to understand better how changes in developmental mechanisms have led to such diverse forms during arthropod evolution (Schram and Koenemann, 2004). Traditionally, studies of arthropod development employ embryos of *Drosophila* (Campos-Ortega, 1985), but the embryonic development of increasing numbers of arthropod species is being studied, especially at the level of gene expression.

Hughes and Kaufman (2000) proposed that any novel model organism for developmental studies should be carefully chosen and its phylogenetic position should be considered. To represent a larger taxon, a species with basal characteristics should be selected, or alternatively, a species with derived or unique mechanism of development could be chosen to study evolution of developmental mechanisms. Hughes and Kaufman (2000) suggested as one of these novel model organisms the common rough woodlouse, the crustacean *Porcellio scaber* (Latreille, 1804). Since crustaceans are a sister group of insects in arthropod evolution, studies of crustacean development can answer some of the pressing questions regarding the relationship between Insecta and Crustacea, and assist in gaining a better understanding of arthropod evolution (Browne et al., 2005).

Many characteristics make *P. scaber* an ideal model organism for developmental studies. The species is a representative of terrestrial isopods (Oniscidea), a unique group of crustaceans well adapted to land habitats. Highly derived characteristics, such as pseudotracheal lungs, marsupial development, meroblastic cleavage, and direct development, make *P. scaber* a suitable model for studies of evolution of adaptations to terrestrial life (Schmidt, 2008). Moreover, *P. scaber* is feasible to work on as individuals are easily bred in the laboratory, and females have large numbers of embryos throughout the year. Embryos can be kept in vitro in isopod saline, which markedly facilitates manipulation. The biology of *P. scaber* is well studied and much is known about its physiology, behaviour, life history, population biology, and evolutionary patterns (Alikhan, 1995; Warburg, 1987; Schmidt, 2008; Sfenthourakis et al., 2004). *Porcellio scaber* is used extensively as a bioindicator in ecotoxicological studies, mostly in the field of heavy metal contamination, due to its ability to accumulate and tolerate high amounts of metals (Hopkin, 1989; Hopkin et al., 1993; Drobne, 1997; Drobne and Hopkin, 1995; Drobne and Hopkin, 1994; Odendaal and Reinecke, 2007; Witzel, 1998, 2000).

A few papers on isopod embryonic development were published in late nineteenth and early twentieth centuries. McMurrich (1895) studied ontogenetic development of *P. scaber* in his paper on embryology of isopod crustaceans. Descriptions of embryogenesis of several other isopod species were also published, the most thorough being those of *Irona* (Nair, 1956), *Idotea* (Strömberg, 1965), and *Limnoria* (Strömberg, 1967). Although detailed, these descriptions do not provide an easy-to-use staging system necessary for contemporary studies, since they describe mainly the formation of separate tissues and organs with a focus on their functional interdependence and temporal successions and they lack general descriptions of the

appearance of the embryo in a particular stage of development.

Some individual events in *P. scaber* embryogenesis, such as leg-to-maxilliped transformation and germ-band formation have already been studied in great detail, and also few general staging systems have been published (Whitington et al., 1993; Brena et al., 2005) but a comprehensive staging system is still not available. In this paper, we present such a staging system with emphasis on characteristics that have not been covered in papers published so far. Our observations are based on morphological changes that can be observed visually in live specimens or by simple DAPI (4',6-diamidino-2-phenylindole) staining of whole-mount embryos with no need for sectioning. The staging system described here is focused on differentiation of the digestive system, namely the structure and function of the midgut glands, stomach, and hindgut.

This proposed staging system of *P. scaber* development is expected to provide a useful basis for the continually expanding field of gene expression analysis and to facilitate comparison of embryonic development between related crustacean species. As stressed by Brena et al. (2005), gene expression can be a dynamic process and use of convenient but overly simplistic staging diagrams could lead to erroneous conclusions. Short periods of specific gene expression could potentially not be detected, and therefore the diversity of genetic expression and a possible variety of functions could be overlooked. The staging system presented here should help eliminate such cases as it is quite extensive and divides the developmental period of *P. scaber* into considerably more stages than in published staging systems. A similar comprehensive staging system based on external morphology of live embryos and DAPI staining of another peracaridan crustacean, the amphipod *Parhyale hawaiensis* (Dana, 1853), was recently published (Browne et al., 2005), in which they emphasized the importance of investigations using non-model taxa for achieving a better understanding of arthropod evolution. As *P. scaber* is used as an appropriate model organism in various biological and toxicological studies, the proposed developmental staging system is intended to enable further comparative and embryological studies, both at functional and at molecular levels.

## Materials and Methods

### Experimental Animals

To study in detail the external morphology of *P. scaber* during embryogenesis, embryos and mancas at different stages of development were liberated from the brood pouch using entomologic forceps. Broods of 75 females were separately cultured in 6-well, tissue culture plates in artificial marsupial saline (Surbida and Wright, 2001) at 21°C ± 1°C.

### Staging Method

In vivo light microscopy and DAPI (4',6-diamidino-2-phenylindole) visualisation of nuclei in fixed embryos were used to create reference stages of *P. scaber* embryonic development. Staining with aqueous DAPI solution of whole mount embryos was performed as described by Browne et al. (2005). Chorions of individual embryos were removed and embryos were washed with methanol for 1 min and incubated overnight at 4°C in 50% (v:v) glycerol/1 x PBS containing 1 mg/ml DAPI. Live and DAPI-stained embryos were observed with bright field, fluorescence and differential interference contrast (DIC) microscopy techniques with a dissecting microscope Leica MZFLIII and with microscope Zeiss AxioImager Z.1.

## Results

The events of embryonic development of *P. scaber*, from released fertilized eggs to hatching of the embryos, last approximately 25 days at room temperature and daily light cycle. After hatching, embryos are termed marsupial manca larvae, or mancas, and they stay inside the brood pouch for up to ten days before they are released into the environment. We divided this ontogenetic development inside the marsupium into 20 stages, designated S (from S1 - fertilized egg to S20 - marsupial manca). Staging is based on percentages of development as the rates of development differ among individual embryos of the same brood and several embryos cease to develop at different stages. Staging based on absolute time of development would, therefore, not be appropriate.

The term early-stage embryo is used for embryos with no visible limb buds. Embryos are termed mid-staged after limb buds start developing and until the dorsoventral rotation of the embryo inside the vitelline membrane. Stages after this rotation until hatching represent late-stage embryos. Because postembryonic ontogenetic development of *P. scaber* is discussed elsewhere (Tomescu and Craciun, 1987), we describe marsupial mancas only briefly.

### S1 0-4% Development - Fertilized Eggs Released Into Brood Pouch

Eggs (Fig. 1a) are filled with small yolk granules and are irregularly shaped as a result of compression within the marsupium. Yolk colour ranges between shades of yellow, orange, and red, and is uniform within a single brood. No tissue or cells are visible on the egg surface. Due to opaqueness of the yolk, the intravitelline nuclei cannot be observed in live specimens. The only distinct membrane is a transparent, acellular, and sometimes slightly wrinkled chorion separated from the egg by a fluid filled space. The inner vitelline membrane is closely apposed to the egg surface.

### S2 8-24% Development - Early Stages of Embryo Development

After five intravitelline divisions, the resulting 32 nuclei reach the egg surface. Most nuclei aggregate into a one-cell thick germdisc, while a few nuclei cover the extra-embryonic egg surface. The germdisc grows by a continued proliferation of cells (Fig. 2a). Gastrulation begins after about 32 cells have aggregated into a germdisc. The main gastrulation movement is ingression, accompanied by invagination and delamination (Hejnol, 2002; Gerberding, 2004). The multilayered germdisc bulges slightly above the yolk surface (Fig. 1b) and the vitelline membrane starts to detach from the egg surface above the developing germdisc.

### S3 28% Development - Teloblasts Appear

Gastrulation is completed when a crescentic row of large teloblasts appears in front of the gastrulation centre

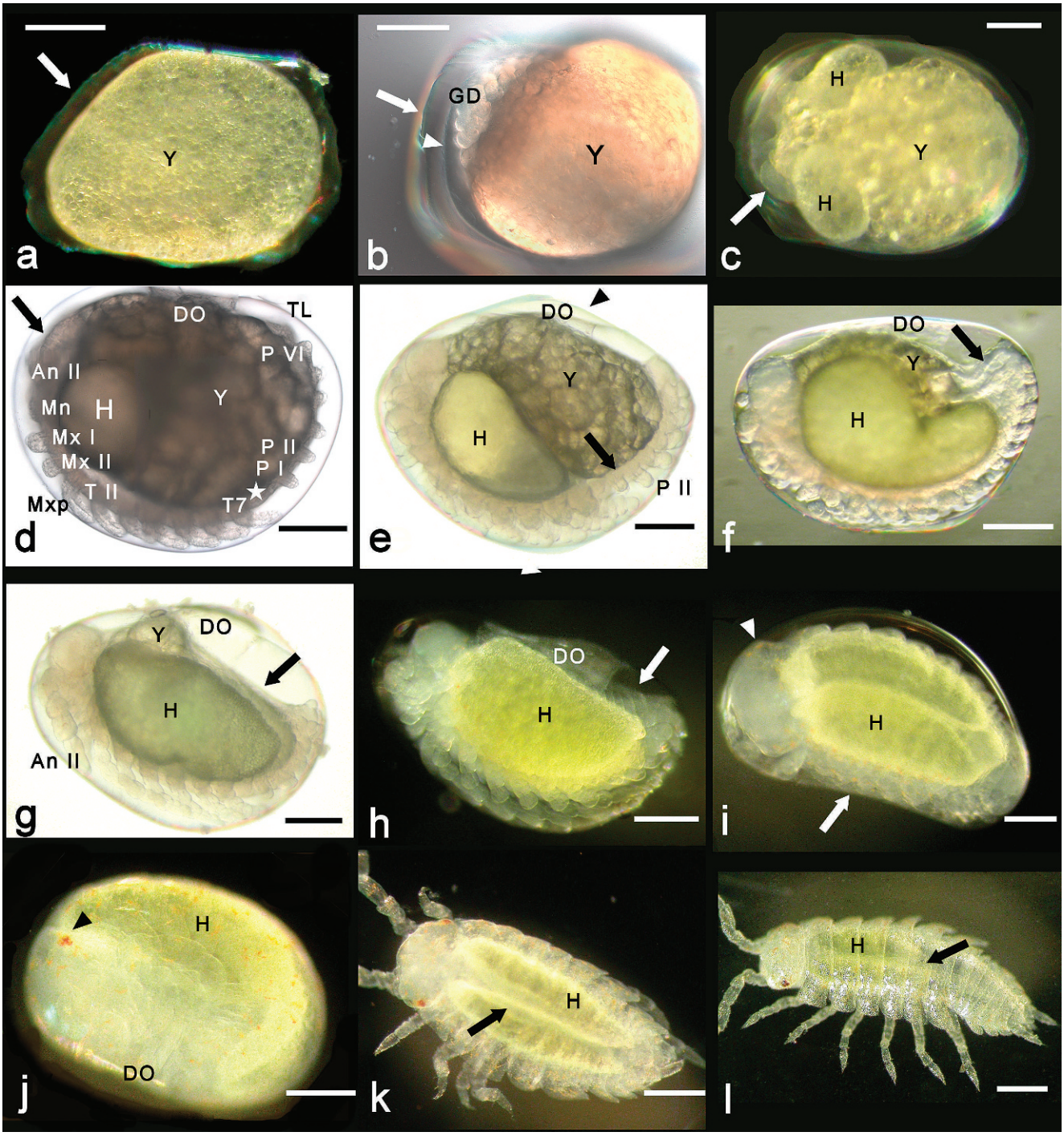


Fig. 1. Bright field and differential interference contrast (DIC) of *P. scaber* ontogenetic development. Embryos oriented with ventral side downwards and head to left. An II - antennae; DO - dorsal organ; GD - germdisc; H - hepatopancreatic glands primordia; Mn - mandible; Mx I - maxillula; Mx II - maxilla; P I - pleopod I; P II - pleopod II; P VI - uropod; II; Mxp - thoracopod I/maxilliped; T II - thoracopod II/pereiopod I; TL - telson; Y - unenclosed yolk;. Bar - 200 µm. a, Stage 1 (S1), fertilized egg filled with small yolk granules, no cells visible on the surface; arrow - chorion. b, Stage 2 (S2), germdisc; arrow - chorion; arrowhead - vitelline membrane, detached above the germdisc. c, Stage 8 (S8), disc-shaped hepatopancreatic gland primordia; unenclosed yolk aggregated into larger globules; arrow - prominent optical lobe. d, Stage 9 (S9), segmentation of pereiopods into distinct podomeres; no reduction of P I; arrow - optic lobe; star (*) - pereomere VI, which does not grow pereiopod buds prior to the juvenile phase. DIC. e, Stage 11 (S11), bean-shaped midgut glands; arrow - reduction of the pleopod I; arrowhead - chorion. f, Stage 13 (S13), 75% of the yolk enclosed by midgut glands; arrow - hindgut bent at 90° angle. g, Stage 14 (S14), a small amount of yolk unenclosed into the midgut glands; pigmentation of the body wall; arrow - hindgut straightened out. h, Stage 15 (S15), yolk completely enclosed into the midgut glands; prominent saddle-shaped dorsal organ; arrow - hemitergites fused into discernible tergites. i, Stage 16 (S16), dorsoventral rotation of the embryo inside the vitelline membrane; arrow - dorsal organ located ventrally; arrowhead - pigmented eye. j, Stage 18 (S18), embryo tightly rolled up inside the vitelline membrane, just prior to hatching; arrowhead - eye. k, early stage 20 (S20), recently hatched marsupial manca; hepatopancreatic glands extended into the pleon; arrow - dorsally located heart. l, late stage 20 (S20), marsupial manca just before the release from the marsupium; arrow - midgut glands notably reduced in size.

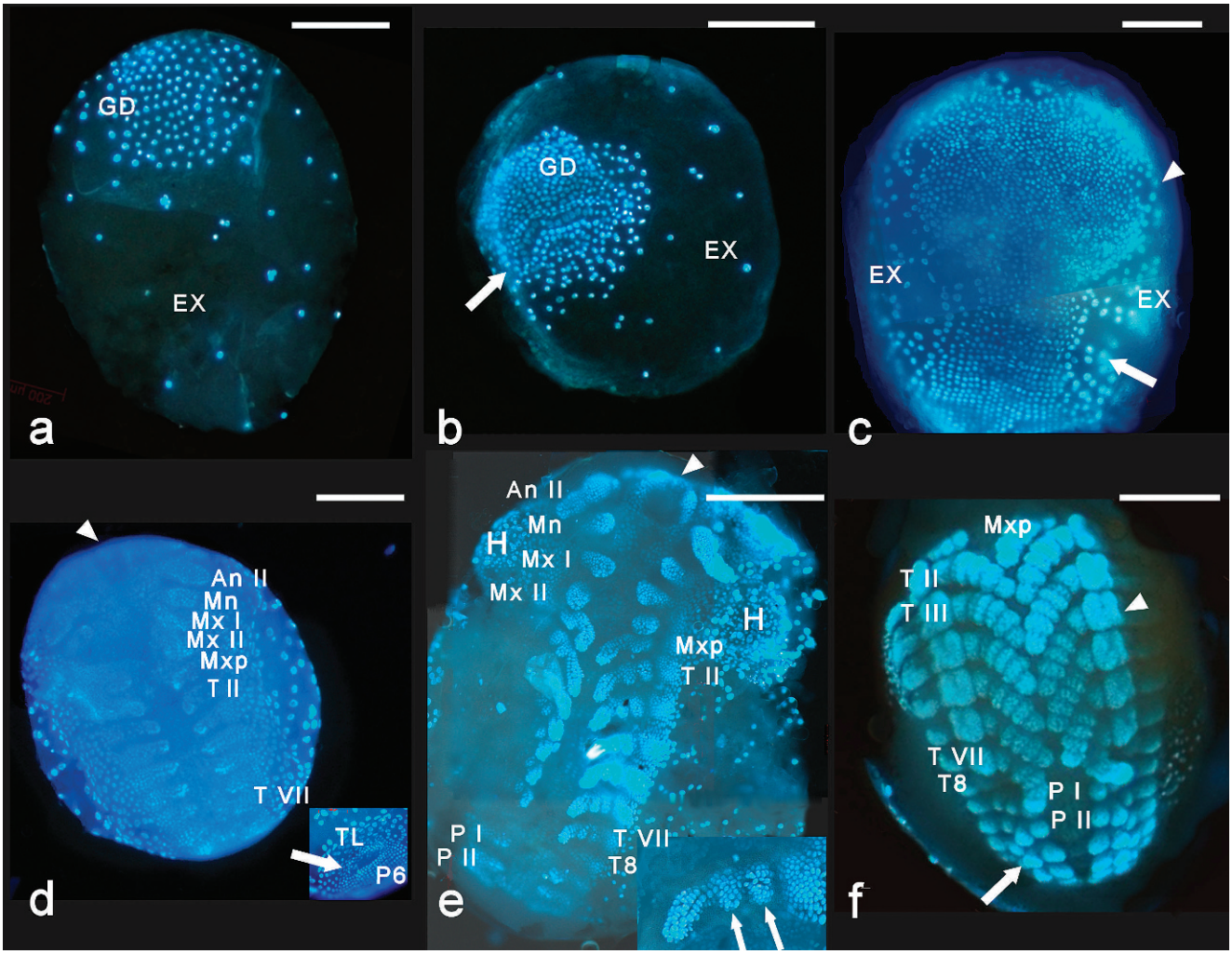


Fig. 2. DAPI images of early embryogenesis of *P. scaber*. An II - antennae; DO - dorsal organ; EX - extra-embryonic nuclei; GD - germdisc; H - hepatopancreatic glands primordia; Mn- mandible; Mx I - maxillula; Mx II - maxilla; P I - pleopod I; P II - pleopod II; P6 - pleomere 6; II; Mxp - thoracopod I/maxilliped; T II - thoracopod II/pereiopod I; T8 - thoracomere 8; TL - telson. Bar 200 µm. a, Stage 2 (S2), germdisc. Bar - 200 µm. b, Stage 3 (S3), arrow - a crescentic row of large ectoteloblasts. c, Stage 5 (S5), elongation of the germ band; arrow - post-naupliar germ band composed of regular transverse rows of cells; arrowhead - naupliar region with scattered cells. d, Stage 6 (S6), appearance of the limb buds; anterior thoracic limb buds more developed than posterior buds; arrowhead - stomodeum invaginated between the antennae; insert - proctodeal invagination (posterior is up). e Late stage 7 (S7), thoracic limbs elongated with two proximal outgrowths; insert - enlarged pereiopod I, arrows - a presumable exopod bud and a coxal plate on pereiopod I; arrowhead - stomodeal opening with thickened labrum on the anterior margin; f, Stage 9 (S9), legs segmented into seven podomeres; exopod buds no longer present; arrowhead - well developed coxal plates; arrow - bilobate pleopods.

(Fig. 2b). Mesendodermal cells can be discerned under the ectoderm and differentiated teloblasts start to bud off daughter cells anteriorly. The vitelline membrane is further detached above the developing embryo.

### S4 32% Development - Pear Shaped Embryo with Head Lobes

The germ band is notably larger than in S3 and appears pear-shaped, its wider part representing the developing head lobes. The post-naupliar part of the germ band (posterior to the mandibular segment) consists of several transverse cell rows. Large ectoteloblasts are discernible in the posterior part of post-naupliar region. The anterior part of the post-naupliar germ band, which will give rise to most of the segments of the maxillula, maxilla, and thoracopod I/ maxilliped, is of non-teloblastic origin.

### S5 36% Development - Elongation of Germ Band

The naupliar part of the germ band (anterior to the maxillulary segment), formed by irregularly patterned cells, is quadrifolium-shaped (Fig. 2c). The post-naupliar germ band elongates posteriorly and is composed of many characteristic transverse cell rows. Pleonic segments are not yet completely formed. No appendage formation is yet observable. Many extra-embryonic cells with large nuclei cover the prospective dorsal egg surface. The vitelline membrane is not completely detached.

### S6 40% Development - Limb Buds Appear, Invagination of Stomodeum and Proctodeum

Elongation of the germ band is completed and all pleonic segments are formed. The head is much wider than the rest of the embryo. Small appendage buds progress in an

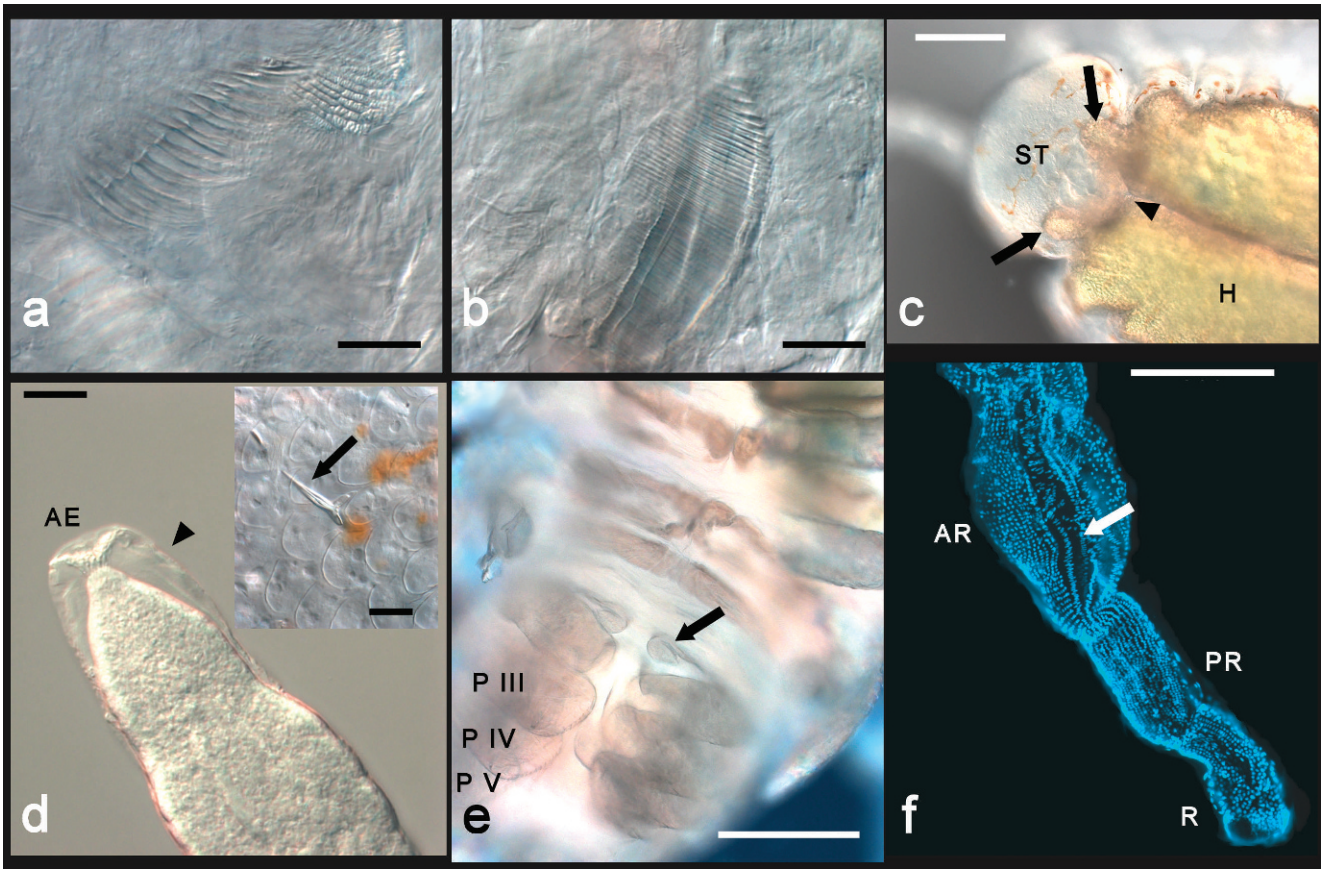


Fig. 3. Selected details of *P. scaber* ontogenetic development. AE - antennal aesthetascs; AR - anterior hindgut region; H - hepatopancreatic gland; MA - masticatory apparatus of the stomach; PF - primary filter of the stomach; PR - papillate hindgut region; P II - exopod of pleopod II; P III - pleopod III; P IV - pleopod IV; P V - pleopod V; R - rectum; SF - secondary filter of the stomach; ST - stomach; T8 - thoracomere 8. Bar: a, b, d - 20 µm; c, e, f - 200 µm. a, Stage 18 (S18), primary filter and masticatory apparatus of the stomach; differential interference contrast (DIC). b, Stage 18 (S18), the secondary filter of the stomach; DIC. c, Stage 15 (S15) the second pair of midgut diverticula - arrows; arrowhead - a narrow canal between the midgut lobes. d, Stage 16 (S16), apolysis of cuticle on appendage tips - arrowhead; insert – Stage 18 (S18), cuticular scales; arrow - sensory seta. DIC. e, Stage 20 (S20), reduced endopod of pleopod I - arrow; thoracomere 8 devoid of appendage buds. f, Stage 20 (S20), hindgut differentiated into anterior region and papillate region; arrow - well developed typhlosole in the anterior region. DAPI staining.

anterior to posterior direction (Fig. 2d). Antennal buds are the stoutest and longest. Mandibles, maxillulae, and maxillae are visible. Maxilliped buds and thoracopod II/pereiopod I buds are similar in shape. Pleopods are not yet visible. Invaginated stomodeum is discernable at the level of antennae. Hindgut invagination is located mid-ventrally between the pleomere 6 and the telson (Fig. 2d insert). Lateral embryonic tissue grows dorsalward and displaces dorsally located saddle-shaped sheet of extra-embryonic nuclei. This saddle-shaped sheet detaches from the egg from the periphery inward and forms the dorsal organ underlying the vitelline membrane. Yolk granules fuse into larger blocks.

### S7 44% Development - Dorsal Flexion, Biramous Pereiopods, and Visible Midgut Gland Primordia

The embryo is dorsally bent or C-shaped as it grows longer than the anterior-posterior egg axis. Two discoid midgut gland primordia appear dorsolaterally at the level of the maxillulae (Fig. 2e). The labrum is formed as a thickening on the anterior stomodeal margin. The future labium is seen as two medial thickenings (paragnaths) between the mandibles behind the stomodeal opening. Segmentation of the antennae is indicated. Mandibles, maxillulae, and maxillae are small, unsegmented, uniramous buds. The more anterior pereiopods are elongated, and two lateral lobes grow from their basal parts (Fig. 2e insert). The distal bud represents rudimentary exopod, while the proximal bud will subsequently expand into a coxal plate. The more posterior pereiopods are still uniramous and short. In late S7, all pereiopods (thoracopods II-VII) bear exopod buds, and the anterior pleopod buds appear.

### S8 48% Development - Bilobate Pleonic Limb Buds

The head is elongated and flattened with distinctive optical lobes (Fig. 1c). The longest appendages are caudally curved antennae. Exopod buds are no longer visible on the anterior pereiopods. Five bilobate pleopod buds and a pair of similarly shaped uropod buds are formed. The two discoid midgut gland primordia are prominent, the proctodeum has invaginated further, and the vitelline membrane is detached from the embryo.

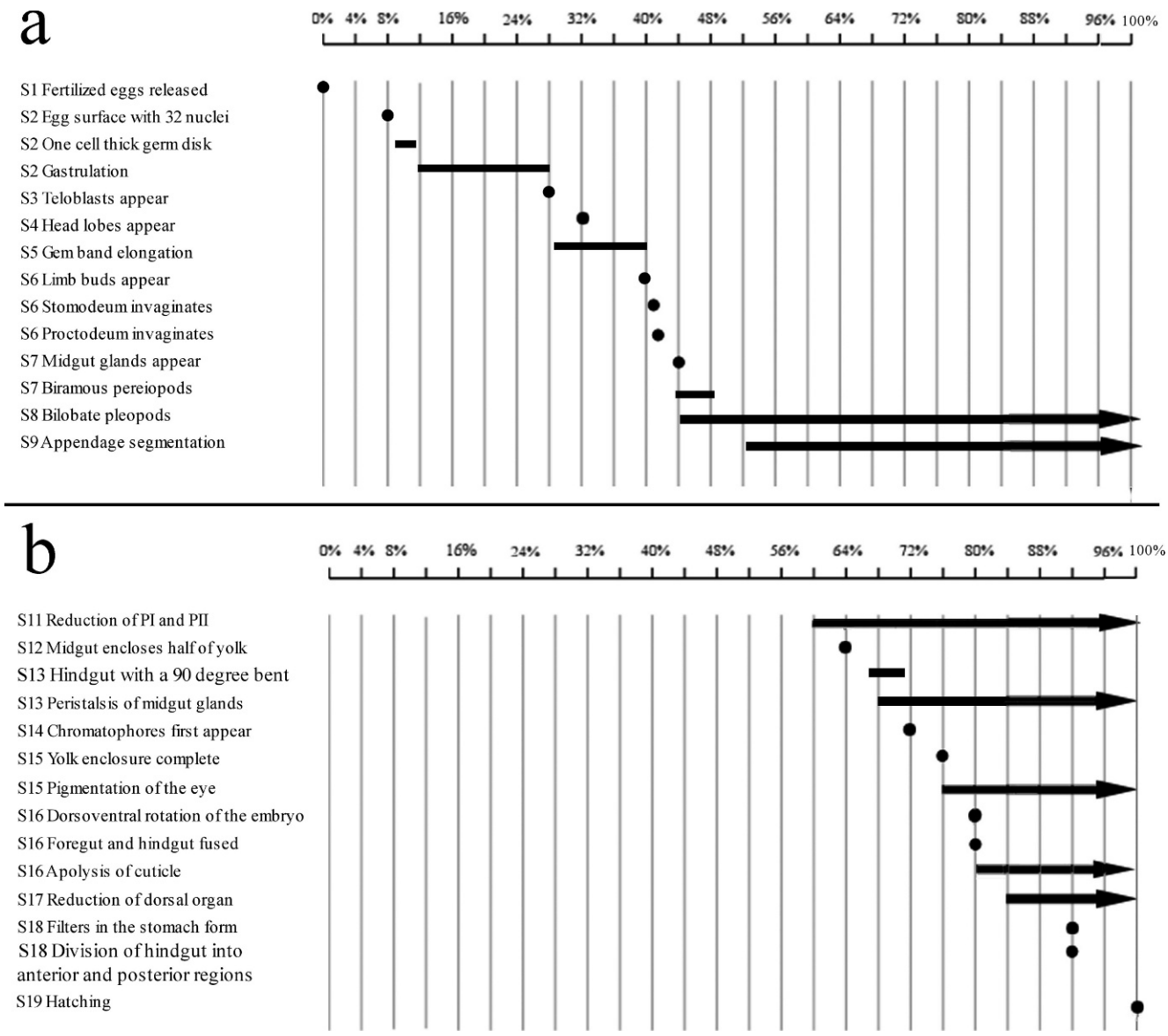


Fig. 4. *Porcellio scaber* embryogenesis 0-100%. Axis representing percentage of embryonic development runs from left to right along the top of panels a and b. Stage number and short descriptor run from top to bottom along the left side of panels a and b. a, Embryonic events and stages from stage S1 to stage S9. Events persisting past 100% of the development are marked by arrowheads at far right. b, Embryonic events and stages from stage S11 to stage S19.

### S9 52% Development - Segmented Appendages

Rounded optical lobes protrude from the head (Fig. 1d). Antennae have six segments and seven short podomeres can be distinguished in all thoracic appendages (Fig. 2f). The pereiopods have prominent square coxal plates. The maxillipeds are still shaped as pereiopods but lack the prominent coxal plates. The seventh pereiomere shows no indication of limb bud formation. Pleopod I is the largest and no sign of its later reduction is detectable. Hemi-tergites of the pereion and pleon with epimeres (the left and right parts of the tergites, which will fuse together at a later stage of development as the embryo closes dorsally over the remaining yolk) are visible. The saddle-shaped dorsal organ has not yet completely detached from the egg surface.

### S10 56% Development - Ventrally Fused Midgut Diverticula

Segmentation of short antennulae is noticeable, and segmentation of antennae and thoracic appendages is pronounced. The exopods of pleopods are larger than endopods. Pleopods VI, which will become caudally directed uropods, differ in shape from other pleopods and are slightly pointed. The two midgut gland lobes are fused ventrally at the midline and enclose approximately 20% of the yolk.

### S11 60% Development - Reduced Pleopods I and II

Midgut lobes extend caudally appearing bean-shaped (Fig. 1e). Exopods and endopods of pleopod I, and

endopod of pleopod II, are notably reduced. Pleopods III-V are elongated. The saddle-shaped dorsal organ is well developed. Chorion is still present.

### S12 64% Development - Expanded Midgut Lobes

The head is large and compact. Midgut lobes are extended and enclose approximately one half of the yolk. Each midgut diverticulum opens antero-medially into the unenclosed yolk. The hindgut extends cranially.

### S13 68% Development - Well-Developed Midgut Glands, Hindgut Bent at 90° Angle

The midgut glands enclose three quarters of the yolk (Fig. 1f). Peristaltic constrictions of the midgut glands pump the yolk inside the lobes. The hindgut is bent at a 90° angle and pushes forward to the unenclosed yolk in the shrinking yolk sac. The anterior hindgut wall is thinner than lateral walls.

### S14 72% Development - Pigmentation Visible

Scattered orange chromatophores appear on the body surface but eye pigmentation is not yet visible (Fig. 1g). Hemi-tergites of the pleon are fused into tergites. The dorsal organ is connected to the embryo only by a thin cord. The midgut glands occupy a large part of the embryo and only a small amount of yolk immediately behind the head remains unenclosed. The hindgut has almost reached the foregut.

### S15 76% Development - Yolk Enclosure Complete

The yolk is completely enclosed inside the midgut glands (Fig. 1h) and the two midgut lobes are connected anteriorly through a narrow canal. The second pair of midgut diverticula appears as two small protuberances (Fig. 3c). Peristalsis of the midgut glands is vigorous and the hindgut comes into contact with the foregut. All hemitergites are fused into distinct tergites. The first orange-red pigment appears in the retinal cells of the eye. The chorion has now disintegrated and the embryo is surrounded only by the vitelline membrane with an underlying prominent saddle-shaped dorsal organ.

### S16 80% Development - Rotation of the Embryo Within Vitelline Membrane, Eye Pigmentation Apparent

The embryo rotates in the dorsoventral axis within the vitelline membrane, and becomes ventrally bent (Fig. 1i). As a result, the uropods and telson point in an antero-ventral direction and the dorsal organ faces the ventral part of the embryo. During rotation, the cord connecting the dorsal organ with the embryo is severed. Chromatophores form irregular networks, and the eyes are notably pigmented. Apolysis, i.e., detachment of cuticle from the underlying epithelium, starts on appendage tips (Fig. 3d). Aesthetascs grow on antennae and legs have claw-shaped dactyli. The foregut and hindgut are fused.

### S17 84% Development - Reduction of Dorsal Organ

Apolysis of cuticle is advanced. Mandibular incisor and molar processes acquire their characteristic shape. The uropods and telson grow apical hairs. The saddle-shaped dorsal organ facing the ventral side of the embryo is much reduced in size.

### S18 88-96% Development - Formation of Stomach Filters and Differentiation of Anterior and Posterior Hindgut Regions

Due to water uptake, embryos swell inside the vitelline membrane (Fig. 1j). The antennal distal segment bears long aesthetascs. Characteristic setae and/or spines form on appendages and distinctive cuticular scales are observed (Fig. 3d, insert). The cuticular masticatory apparatus and primary and secondary filters of the stomach are formed (Fig. 3a, b). The midgut glands open into the stomach. The second pair of midgut diverticula extends caudally. The hindgut is divided into anterior and papillate regions. Formation of the typhlosole on the dorsal wall of the anterior region is indicated. A pulsating tubular heart is developed dorsally above the hindgut.

### S19 100% Development - Hatching

Embryos hatch from the vitelline membrane due to swelling and active movement (Fig. 1k). The midgut glands shorten as the yolk inside the lumen is consumed. The eyes are dark brown. Mandibular incisors and molars are sclerotized. Exopods of pleopods II-V grow setal tufts on the distal margin. The endopod and exopod of pleopod I and the endopod of pleopod II are visible as minute buds (Fig. 3e).

### S20 Postembryonic Development - Further Consumption of Yolk in Glands, Marsupial Moulting

The yolk inside the glands is further reduced and the pigmentation is darker. Cuticular scales have a rough surface. Some mancas have moulted for the first time. The anterior hindgut region is dilated and the typhlosole is pronounced (Fig. 3f). At the end of S20 the midgut glands are considerably shortened (Fig. 1l) and the second midgut diverticula extend to the pereomere II. Mancas are ready to be released from the marsupium.

## Discussion

Recent reports on *P. scaber* embryogenesis mostly rely on the staging system described by Whitington et al. (1993), but because this study dealt with neural development in embryos, the earlier developmental stages and marsupial mancas are not described. Our observations (Fig. 4) on mid- or late-stage embryos are generally in good agreement with Whitington's descriptions, although some important differences should be mentioned. We confirmed that dorsoventral rotation of the embryo inside the vitelline membrane occurs at 80% of the development. Yolk enclosure, however, is usually not complete by 70% of the development as described in Whitington, but only by

76% of the development. We observe first pigmented chromatophores already prior to the dorsoventral rotation, at 72% of the development, while Whitington reports that pigmentation only becomes visible at 80%. The eyes start showing pigmentation as early as 76% and not as late as 90% of development. Whitington also describes extension of the hindgut corresponding to 60% of development which corresponds to 75% yolk enclosure. Our results show that hindgut starts extending even before 50% of the yolk is enclosed inside the midgut glands. We also observe earlier appearance of first limb buds, which are already quite prominent at 45% of the development, when Whitington first mentions them.

The staging system of Whitington does not describe embryonic stages prior to 45% of developmental period. However, an important study of germ band formation and elongation in *P. scaber* is presented by Hejnol and co-workers (2002, 2006), who provide a detailed description of cell division patterns in the ectoderm of the post-naupliar germ band in *P. scaber*. The naupliar segments of *P. scaber* are formed, as in all arthropods, by scattered cell divisions without any obvious pattern, whereas the post-naupliar germ band consists of cells that divide in a stereotypic pattern, typical for all malacostracan crustaceans (Hejnol et al., 2006; Dohle et al., 2004; Scholtz and Dohle, 1996; Scholtz, 1997). In *P. scaber*, the posterior part of the post-naupliar germ-band is formed by ectoteloblasts, as in most malacostracans, except the amphipods. The anterior part of the post-naupliar germ band in *P. scaber*, however, is formed by two cell rows, which are of non-ectoteloblastic origin. These cell rows give rise to most of the segment of maxillula up to the anterior part of the first thoracic segment. We observe that the crescentic row of large ectoteloblasts anterior to the gastrulation centre forms at 28% of the development. These ectoteloblasts divide during subsequent germ band elongation to produce transverse rows of ectoteloblastic progeny, easily seen in DAPI stained embryos. When development reaches 40%, all body segments are formed and differential cell divisions in each segment generate minute limb buds in the anterior to posterior direction. All appendage buds are present before 50% of development.

Full or 100% of the developmental period is usually defined as hatching from the embryonic membrane, which is caused by vigorous skeletal muscle movements that become noticeable few days before hatching. Muscles of the heart also become functional at that time as the heart starts beating regularly.

Marsupial manca larvae of *P. scaber* stay inside the marsupium for up to ten days before they are released into the environment. We only briefly describe the development of marsupial mancas as the main visible characteristics are yolk consumption with consecutive shortening of the midgut glands, and development of elaborate cuticular differentiations, such as hairs, setae, spines, and scales. Development of post-marsupial mancas has been described elsewhere (Tomescu and Craciun, 1987; Brum and Araujo, 2007).

A simple staging of *P. scaber* embryonic development has also been described by Brena et al. (2005) where they observe an interesting phenomenon, a ''Lazarus developmental feature'' (Minelli, 2003). They noticed that pleopod I and the endopod of pleopod II first start developing normally during early limb bud formation but later regress to only minuscule rudiments. In our staging system, we describe this early development and subsequent reduction of pleopod I and the exopod of pleopod II, which differ somewhat from the observations by Brena because they, for instance, observe limb primordia already at 10% of the development. Contrary to the pleomere 1 and 2, the thoracomere 7, which develops normally, does not grow a limb bud until after the first post-marsupial moult (Tomescu and Craciun, 1987).

A similar inhibition of limb bud formation during embryogenesis is found in Asellota, where in females the first pleopods are lacking completely, and in males they are only present as small, uniramous structures, a unique innovation within the Isopoda (Wilson, 1987, 1989). Pleomere 1 does not develop limb buds during early limb bud formation in the marine asellote *Munnopsurus atlanticus* (Bonnier, 1896) (Elizalde and Sorbe, 1992) and in *Asellus* (Needham, 1938). Only later, during the marsupial manca stage, small pleopod I buds appear in males. Pleopods II, however, appear as small buds already during early embryogenesis, but they remain small relative to pleopods III-V (Elizalde and Sorbe, 1992; Needham, 1938).

In their study on germ band elongation and early limb formation in *P. scaber*, Hejnol and Scholtz (2004) report that exopod formation in pereiopods of *P. scaber* is prevented during limb formation and therefore pereiopods develop as uniramous buds. We, however, observed the appearance of transitory rudiments of the exopods on all pereiopods during early limb development in stage S7. The observed outgrowths are presumably exopod lobes, which is corroborated by their lateral appearance, similarly to exopods in crustaceans with biramous limbs (Pabst and Scholtz, 2009). These exopod buds, however, completely disappear by stage S9 and could therefore easily be overlooked. Similar transient exopod buds in other isopods have also been described by McMurrich (1895) and Nair (1956).

## Development of the Digestive System in Embryos of *P. scaber*

Terrestrial isopods, or Oniscidea, have evolved specific structural and physiological adaptations to terrestrial habitats, unique among crustaceans, which makes oniscids suitable model organisms for studies of evolution of such adaptations. Some prominent adaptations concern their digestive system, which supports effective digestion of cellulose-rich leaf litter and active water conservation.

Studying differentiation of the digestive system during oniscid development should help clarify how specific adaptations to terrestrial life evolved in crustaceans. A comprehensive histological study on origin and fate of the digestive system elements in *Porcellio laevis* (Latreille, 1804) embryos was presented by Goodrich (1939) and by Štrus et al. (2008) who described the ultrastructure of the digestive system of late-stage embryos, marsupial mancas, and adults in *Porcellio scaber*. In the present work, we

focus on sequential appearance of specific characteristics during digestive system formation. The stomodeum first becomes noticeable in mid-stage embryos at 40% of the development, after all the segments of post-naupliar germ band are formed and the appendage buds appear, and it begins to invaginate at the level of the developing antennulae and antennae. Posteriorly the invagination is flanked by the paragnaths, which are located medial to the mandibular buds. This position of the paragnaths supports claims of Wolff and Scholtz (2006) that they are not appendages but rather sternal outgrowths of the mandible segment. Only later in the development does the stomodeal opening shift posteriorly, so that the oral opening is positioned at the level of the mandibles in adults.

Shortly after the stomodeum becomes visible, the proctodeum starts invaginating mid-ventrally at the level between the developing pleomere 6 and the telson, which, in adults, are fused into a pleotelson (Knopf et al., 2006). The proctodeum then grows towards the elongating foregut. At the time of stomodeal and proctodeal invagination a prominent change in yolk composition occurs. Yolk granules start to fuse into larger blocks, a phenomenon first observed in *Porcellio* by McMurrich (1895), and also described for other malacostracans and *Daphnia*, where this fusion commences similarly at the time of the first limb bud formation (Garcia-Guerrero and Hendrickx, 2004; Kotov and Boikova, 2001). The stomodeum and the proctodeum fuse just prior to the dorsoventral rotation of the embryo within the vitelline membrane.

In their reviews of structure and function of the digestive system of terrestrial isopods, Hassal and Jennings (1975), Hames and Hopkin (1989), and Štrus et al. (1995) stress the importance of the typhlosole canal and the partition of the hindgut into anterior and papillate regions as an adaptation to terrestrial lifestyle. Such a structure of the hindgut enables effective water retention, a prerequisite for survival in dry conditions (Hames and Hopkin, 1989). We observed a subdivision of the hindgut into an anterior and papillate region, as well as a differentiation of a typhlosole in the dorsal wall of the anterior hindgut chamber, in late-stage embryos still enveloped in the vitelline membrane. The typhlosole is first noticeable only as two rows of laterally elongated cells with markedly elongate nuclei. In marsupial mancas, the two rows expand into two prominent dorsal ridges protruding from the dorsal wall. Thick circular muscles separate the papillate region from the short rectum, which functions to produce the flattened faecal pellets (Hames and Hopkin, 1989).

A significant adaptation of terrestrial isopods to a cellulose-rich diet occurs with a stout and complex foregut equipped with a well-developed masticatory apparatus, which is absent from primitive amphibious species *Ligia* and *Ligidium* (Štrus et al., 1995). Rows of compact teeth forming the abrasive plates of the masticatory apparatus can be distinguished in late-stage embryos at around 90% of development. Other prominent cuticular structures of the proventriculus are primary and secondary filters, which are also already well developed before hatching of the late-stage embryos from the vitelline membrane.

An important question concerning evolution of oniscids involves the formation and fate of the midgut in the definitive gut (Štrus et al., 2008). In amphibious species, a ring of endodermal midgut cells can be found at the junction of the ectodermal foregut and hindgut, but in terrestrial oniscids, a true midgut is absent from the digestive tract and can only be found in the annexes of the digestive canal and the hepatic diverticula (Štrus et al., 1995). In late-stage embryos, no endodermal cells can be found in the digestive tract (Štrus at al., 2008). The question remains, whether a transitory midgut appears in the developing digestive tube prior to the fusion of the foregut and the hindgut. Also, molecular mechanisms underlying gut formation should be investigated as it has been shown that evolutionarily conserved mechanisms guide endoderm formation in arthropods (Nakagoshi, 2005). To address these questions, we propose examining embryos in stages S14 and S15 just prior to the dorsoventral rotation of the embryo when the foregut and the hindgut are not yet fused.

In adult *P. scaber*, the only endodermal parts of the digestive system are the midgut or hepatopancreatic glands. Our results show that midgut glands primordia appear at 45% of the development. The muscle investment of the midgut glands becomes functional before 70% of the developmental period, demonstrated by the peristaltic contractions of the midgut diverticula.

We further describe the outgrowths of the second pair of midgut diverticula in late-stage embryos; interestingly they remain short even at the time of the release of mancas from the marsupium, although all four hepatopancreatic diverticula are of the same length in adults.

According to our observations, the digestive system of *P. scaber* is morphologically well differentiated by the end of the embryonic period, indicating that the digestive system is elaborated for feeding well before mancas are released from the brood pouch. In some oniscid species, marsupial mancas feed on their underdeveloped siblings (Warburg, 1994). Although no such intramarsupial cannibalism was directly observed in our study, the well-developed digestive system of late-stage embryos and the asynchronous development of a single brood indicate marsupial mancas of *P. scaber* could potentially exhibit cannibalism.

A prominent feature of a mid-stage embryo is a saddle-shaped dorsal organ underlying the vitelline membrane. Our observations are in agreement with Goodrich (1939) who first described dorsal organ formation during *P. scaber* embryogenesis as formation of an ''amnion,'' or a sheet of secondary extra-embryonic nuclei. In mid-stage embryos at 40% development, cells from the proliferating lateral edges of the embryo start to grow dorsally and displace the saddle-shaped sheet of extra-embryonic cells. This sheet gradually separates from the underlying yolk from the periphery inward to form the dorsal organ underlying the vitelline membrane. Just before the dorsoventral rotation of the embryo inside the vitelline membrane, the embryo is only connected with the dorsal organ by a thin cellular cord. During dorsoventral rotation this cord is severed and in late-stage embryos the dorsal

organ steadily degenerates. Similar observations have been described for *Irona* (Nair, 1956), and for *Oniscus asellus* (Linnaeus, 1758) (Meschenmoser, 1996). Meschenmoser conducted a comparative study on peracarid dorsal organs and their formation and proposed nutritional and osmoregulatory role of the dorsal organ, which still remains to be confirmed.

## Conclusions

The period of early-stage embryos is characterized by segmentation, gastrulation, and germ band elongation. These key events are easily observed by simple DAPI staining; but in live, unstained embryos they can be quite inconspicuous, and the developing germ band is seen only as a whitish sheet of cells.

The mid-stage embryos can be recognized mainly by the growth and differentiation of the digestive system, which is clearly visible through the transparent envelopes. Two midgut gland primordia become visible shortly after all limb buds appear and enclose the yolk as they grow. The midgut glands have enclosed all the yolk by the time of the dorsoventral rotation of the embryo inside the vitelline membrane, which marks the transition into the late embryonic period. Also by this time the digestive tube is completely formed by the fusion of the stomodeum and the proctodeum. The late-stage embryo begins to move inside the embryonic envelope and beating of the heart can be observed. Body and eye pigmentation become apparent and specific cuticular differentiations, such as setae, spines, and scales, form on the body surface. The cuticular masticatory apparatus and filters of the stomach and typhlosole in the hindgut anterior region also develop in late-stage embryos.

The period of the marsupial manca is characterized by the formation of elaborate cuticular structures and rapid yolk consumption. Approximately 10 days after hatching, marsupial mancas leave the marsupium, and with successive moults the post marsupial mancas develop.

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