Andreas Wanninger *Editor*

Evolutionary Developmental Biology of Invertebrates

Vol. 3 Ecdysozoa I: Non-Tetraconata

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Cover illustration: Stripe-like expression of the homeobox gene *engrailed* in the spider *Parasteatoda tepidariorum*. See Chapter 5 for details.

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Preface

The evolution of life on Earth has fascinated mankind for many centuries. Accordingly, research into reconstructing the mechanisms that have led to the vast morphological diversity of extant and fossil organisms and their evolution from a common ancestor has a long and vivid history. Thereby, the era spanning the nineteenth and early twentieth century marked a particularly groundbreaking period for evolutionary biology, when leading naturalists and embryologists of the time such as Karl Ernst von Baer (1792–1876), Charles Darwin (1809–1882), Ernst Haeckel (1834–1919), and Berthold Hatschek (1854–1941) realized that comparing ontogenetic processes between species offers a unique window into their evolutionary history. This revelation lay the foundation for a research field today commonly known as Evolutionary Developmental Biology, or, briefly, EvoDevo.

While for many of today's EvoDevo scientists the principle motivation for studying animal development is still in reconstructing evolutionary scenarios, the analytical means of data generation have radically changed over the centuries. The past two decades in particular have seen dramatic innovations with the routine establishment of powerful research techniques using micromorphological and molecular tools, thus enabling investigation of animal development on a broad, comparative level. At the same time, methods were developed to specifically assess gene function using reverse genetics, and at least some of these techniques are likely to be established for a growing number of so-called emerging model systems in the not too distant future. With this pool of diverse methods at hand, the amount of comparative data on invertebrate development has skyrocketed in the past years, making it increasingly difficult for the individual scientist to keep track of what is known and what remains unknown for the various animal groups, thereby also impeding teaching of state-of-the-art Evolutionary Developmental Biology. Thus, it appears that the time is right to summarize our knowledge on invertebrate development, both from the classical literature and from ongoing scientific work, in a treatise devoted to EvoDevo.

Evolutionary Developmental Biology of Invertebrates aims at providing an overview as broad as possible. The authors, all renowned experts in the field, have put particular effort into presenting the current state of knowledge as comprehensively as possible, carefully weighing conciseness against level of detail. For issues not covered in depth here, the reader may consult additional textbooks, review articles, or web-based resources,

particularly on well-established model systems such as *Caenorhabditis elegans* (www.wormbase.org) or *Drosophila melanogaster* (www.flybase.org).

Evolutionary Developmental Biology of Invertebrates is designed such that each chapter can stand alone, and most chapters are dedicated to one phylum or phylum-like taxonomic unit. The main exceptions are the hexapods and the crustaceans. Due to the vast amount of data available, these groups are treated in their own volume each (Volume 4 and Volume 5, respectively), which differ in their conceptual setups from the other four volumes. In addition to the taxon-based parts, chapters on embryos in the fossil record, homology in the age of genomics, and the relevance of EvoDevo for reconstructing evolutionary and phylogenetic scenarios are included in Volume 1 in order to provide the reader with broader perspectives of modern-day EvoDevo. A chapter showcasing developmental mechanisms during regeneration is part of Volume 2.

Evolutionary Developmental Biology of Invertebrates aims at scientists that are interested in a broad comparative view of what is known in the field but is also directed toward the advanced student with a particular interest in EvoDevo research. While it may not come in classical textbook style, it is my hope that this work, or parts of it, finds its way into the classrooms where Evolutionary Developmental Biology is taught today. Bullet points at the end of each chapter highlight open scientific questions and may help to inspire future research into various areas of Comparative Evolutionary Developmental Biology.

I am deeply grateful to all the contributing authors that made *Evolutionary Developmental Biology of Invertebrates* possible by sharing their knowledge on animal ontogeny and its underlying mechanisms. I warmly thank Marion Hüffel for invaluable editorial assistance from the earliest stages of this project until its publication and Brigitte Baldrian for the chapter vignette artwork. The publisher, Springer, is thanked for allowing a maximum of freedom during planning and implementation of this project and the University of Vienna for providing me with a scientific home to pursue my work on small, little-known creatures.

This is the first of three volumes dedicated to animals that molt during their life cycle, the Ecdysozoa. It covers all non-hexapods and non-crustaceans, i.e., the Cycloneuralia, Tardigrada, Onychophora, Chelicerata, and Myriapoda. While the Nematoda and all other phyla are treated in their own chapters, the remaining cycloneuralians are presented jointly due to the paucity of available developmental data for their individual subclades.

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January 2015

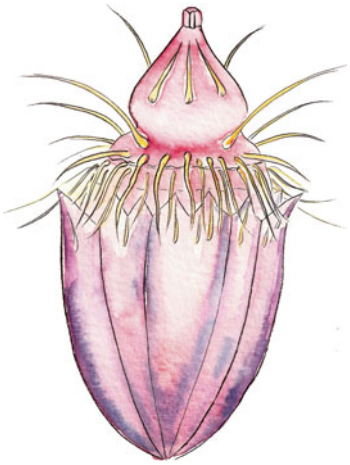
Andreas Wanninger

Contents

Cycloneuralia

1

Andreas Hejnol



Although commonly considered a subtaxon of Cycloneuralia, the Nematoda are covered separately in the following chapter of this volume.

Chapter vignette artwork by Brigitte Baldrian.

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INTRODUCTION

The current view is that Cycloneuralia together with the Panarthropoda form the Ecdysozoa. Cycloneuralia comprises the sister taxa Nematoida (Nematomorpha + Nematoda; development of the latter is treated separately in Chapter 2) and Scalidophora (Priapulida, Kinorhyncha, Loricifera) (Fig. 1.1). The taxon Cycloneuralia has been erected based on morphological data by Ahlrichs (1995), and the main defining character is a circumpharyngeal brain that forms an equally thick ring around the foregut. The taxon Scalidophora is characterized by scalids on its introvert. The Cycloneuralia were proposed before the results of the seminal work of Aguinaldo et al. (1997) who found the first molecular evidence for the Ecdysozoa. Since then, molecular phylogenies have consistently supported the Ecdysozoa but largely fail to provide a solid support for the Cycloneuralia. One problem is that most studies do not include the Loricifera in their analyses – and those who do only receive low support for their placement at any branch within Ecdysozoa (Park et al. 2006; Sørensen et al. 2008). Another issue is that in most phylogenomic studies, tardigrades group together with nematodes (Philippe et al. 2005; Hejnol et al. 2009; Borner et al. 2014), which is likely an artifact that could only be eliminated in some approaches (Dunn et al. 2008; Campbell et al. 2011). However, studies with increased taxon sampling should help to resolve the ambiguous results in the molecular phylogenies in the near future (Dunn et al. 2014).

Cycloneuralia have no ciliated epidermis and the "larvae" likewise lack cilia. Most cycloneuralian species are interstitial and thus tiny (less than 0.5 mm). Some groups, such as priapulids, nematodes, and nematomorphs, have species that are more than a centimeter in size. Most cycloneuralians have a "terminal" mouth that is located at the anterior-most tip of the animal. Some nematodes have shifted the mouth to the ventral or dorsal side of the body (Fitch and Sudhaus 2002). Interestingly, the mouth starts to be formed in the priapulid *Priapulus caudatus* on the ventral side (Martín-

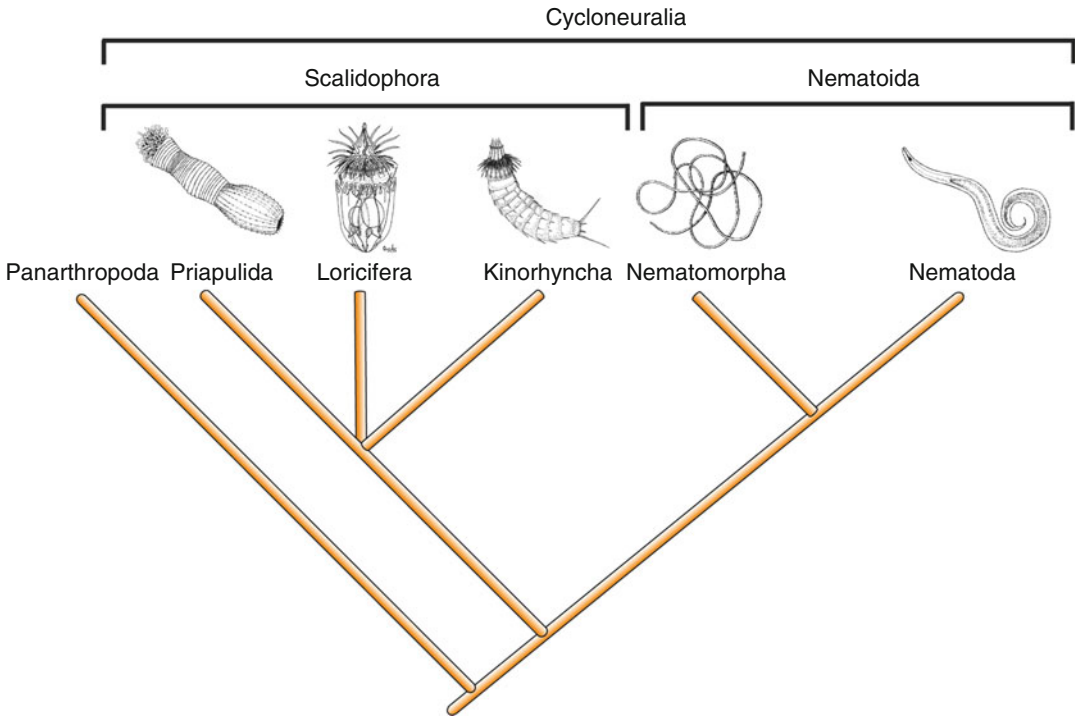

Fig. 1.1 Cycloneuralian phylogeny (Based on Dunn et al. (2008) and Ahlrichs (1995))

Durán et al. 2012; 2015), and in nematodes the mouth is also not formed at the anterior tip of the embryo. The Cycloneuralia are also characterized by a more or less elaborated structure, the so-called introvert. The mouth can be extruded and invaginated, and the animals use this for locomotion and/or feeding.

Scalidophoran taxa have scalids on their introvert. The cuticle is mainly made of chitin but contains also other materials such as collagen. Chitin as a component of the cuticle is likely a plesiomorphy since also arthropods possess a chitinous cuticle. To grow in size or to metamorphose, cycloneuralians need to molt.

Cycloneuralian "larvae" are miniature copies of the adults and have all organ systems present or show at least anlagen of them. Larval stages differ slightly between the molts, and different lineages show a different number of larval stages. Most cycloneuralian groups are free-living and interstitial (Kinorhyncha, Loricifera, some Priapulida), but some have evolved into parasitic species (Nematoda) or are exclusively parasitic (Nematomorpha). The broad diversity of cleavage patterns and developmental modes make it difficult to reconstruct the developmental aspects of the last common cycloneuralian ancestor. However, it seems likely that a radial cleavage with deuterostomic gastrulation was part of the cycloneuralian ground pattern.

PRIAPULIDA

Priapulids – also called penis worms – are sausage-shaped, marine worms that live burrowing in the mud or are interstitial (Schmidt-Rhaesa 2013). There are only 19 described recent species, but these animals have been abundant during the Cambrian and most likely had an important ecological role (Vannier et al. 2010; Boyle et al. 2014). Priapulids are free-spawning and of separate sex. The embryogenesis of priapulids has been described in the two larger species *Halicryptus spinulosus* (Zhinkin and Korsakova 1953) and *Priapulus caudatus* (Zhinkin 1949; Lang 1954; Wennberg et al. 2008; Martín-Durán et al. 2012). No studies have been conducted on interstitial species so far.

Cleavage

Cleavage in Priapulida is total and equal and follows a radial pattern that seems to be stereotypic (Zhinkin 1949; Lang 1954; Wennberg et al. 2008). The most detailed work has been conducted on the species *Priapulus caudatus* (Wennberg et al. 2008). The first cell division of the approximately 60 µm large embryo produces two equally sized blastomeres (AB and CD) (Wennberg et al. 2008). The cleavage is meridional to the animal-vegetal axis. After the next round of cell divisions – which are also meridional – the embryo consists of four blastomeres: A, B, C, and D. The eight-cell stage is achieved by another round of cell division and contains four blastomeres and a vegetal quartet of slightly larger blastomeres (Wennberg et al. 2008). All blastomeres from the eight-cell stage divide equatorially and equally. The 16-cell stage embryo is composed of four rows of four blastomeres each, of which the vegetal blastomeres are slightly larger than the animal blastomeres. During the next round of cell divisions, the directions of mitoses differ between the blastomeres. The vegetal-most and the animal-most quartets of blastomeres divide along the meridional axis, while the two median quartets divide in equatorial direction. The 32-cell stage is composed of an animal and a vegetal hemisphere of each two animal-vegetal quartets and a median ring of eight blastomeres. In the transition of the 32-cell to the 64-cell stage, the blastomeres follow a similar pattern as in the cell division round before. Again, the two animal-most or vegetal-most quartets divide equatorially, while all other more median rings of blastomeres divide meridionally. The blastomere rings of the 64-cell stage are composed of the following number of blastomeres from animal to vegetal: 4-4-8-16-16-8-4-4. After, the 64-cell stage gastrulation begins.

Gastrulation

Gastrulation has been studied in detail in the species *Priapulus caudatus* (Wennberg et al. 2008; Martín-Durán et al. 2012; Martín-Durán and Hejnol 2014; 2015). Gastrulation starts with the immigration of the vegetal-most blastomeres of

the embryo (Wennberg et al. 2008; Martín-Durán et al. 2012). These comprise the endodermal and mesodermal precursors and likely also the germ line. One day after the cells have immigrated, F-actin is prominent at the contact zone of the median cells (Martín-Durán et al. 2012; Martín-Durán and Hejnol 2015). This is the cell border that will form the future gut lumen. Twenty-four hours later, an indentation is formed slightly vegetal from the equator of the embryo. This indentation will form the border of the introvert (Martín-Durán et al. 2012). At the same time, the stomodeum forms at the animal-vegetal region of the embryo. This is also the first timepoint when the radial symmetry of the embryo is broken and the embryo becomes bilaterally symmetric (Martín-Durán and Hejnol 2015). The mouth forms separate from the blastopore at the animal pole and the blastopore becomes the anus – accordingly, the development of *Priapulus caudatus* is deuterostomic (Martín-Durán et al. 2012; Martín-Durán and Hejnol 2014; 2015).

Organogenesis

The musculature becomes visible 6 days after fertilization, and the lumen of the digestive tract is present, but without cavity (Fig. 1.2A). The anlage of the introvert is internalized and this stage is named "introvertula" (Martín-Durán et al. 2012; Martín-Durán and Hejnol 2015). The nervous tissue likely separates from the ectoderm at about the same time of development. All major organ systems are developed at least as anlage in the first larval stage that hatches from the egg (Fig. 1.2).

Postembryonic Development

Priapulids have several larval stages. Investigations of the hatching larval stages have been conducted on *Halicryptus spinulosus* (Janssen et al. 2009) and *Priapulus caudatus* (Wennberg et al. 2009). The hatching larvae are pear shaped and have a functional introvert. No lorica is present in this first larval stage (Fig. 1.3A). The larva hatches with the developed digestive tract but with the mouth and anal opening covered by the cuticle (Wennberg et al. 2009). This is also true for the hatching larva of *Halicryptus spinulosus*, which also lacks a lorica but is slightly more motile than the hatching larva of *P. caudatus* (Janssen et al. 2009). Two weeks after hatching, the larva undergoes the first ecdysis and becomes the first lorica larva (Wennberg et al. 2009). This larval stage is the first to possess a lorica and is about twice the size of the hatching larva (approx. 170 µm). The mouth is still covered with cuticle, which also means that the first larval stages still use the nutrients of the yolk and do not actively feed (Wennberg et al. 2009). The next larval stage of *Priapulus caudatus* is called the second lorica larva (Wennberg et al. 2009). This is the first larval stage that has a definitive mouth opening that is armored with pharyngeal teeth. The study of Wennberg et al. (2009) clearly shows that all previous studies on priapulid larvae have exclusively described later stages – despite the fact that some authors assumed these were "hatching larvae" or were described as earliest stages (Lang 1954; Higgins et al. 1993; Adrianov and Malakhov 1996). This demonstrates that it is necessary to obtain data from animals cultured in the laboratory and that studies that depend on dead or collected material from the wild can only provide a very limited picture of priapulid development. This is also true for other cycloneuralian taxa such as Loricifera.

Molecular Approaches

Molecular studies on priapulids are in their infancies. The most accessible species are *Priapulus caudatus* and *Halicryptus spinulosus*. The Hox gene complement has been studied with degenerate PCR (de Rosa et al. 1999), and the mitochondrial genome has been analyzed (Webster et al. 2006). Both sets of data indicate a rather slow rate of evolution on the molecular level. The Hox gene complement of priapulids seems to include all paralog groups, although the orthology assignments are only based on parts of the homeodomain

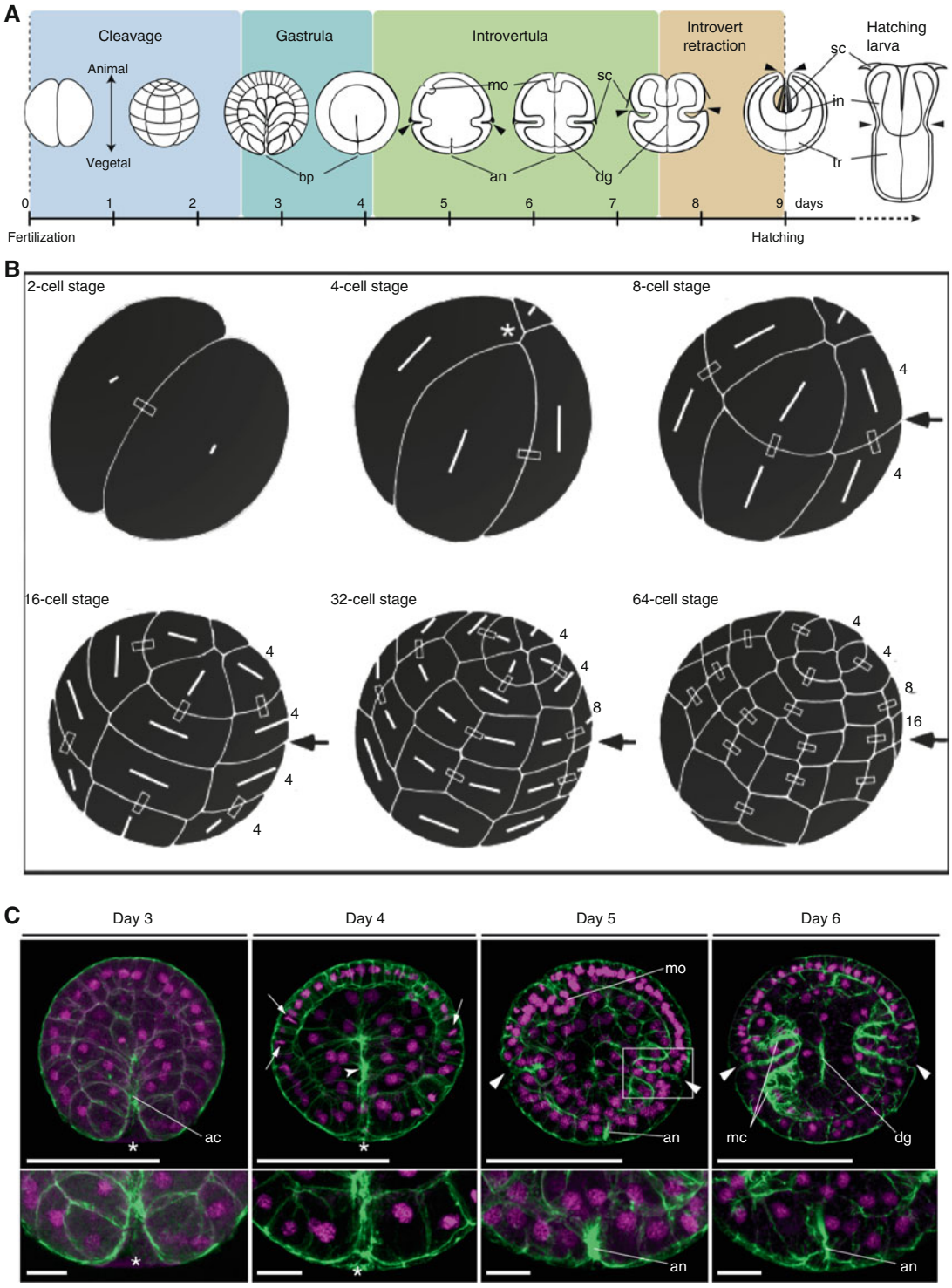

Fig. 1.2 Development of *Priapulus caudatus*. (**A**) Time course of development until hatching at approximately 10 °C (After Martin-Duran et al. 2012). (**B**) Cleavage pattern (From Wennberg et al. 2008). (**C**) Gastrulation (After Martin-Duran et al. 2012). *Bp* blastopore, *mo* mouth, *an* anus, *dg* gut, *sc* scalids, *in* introvert, *ac* archenteron, *arrowheads* introvert-trunk border, *asterisk* blastopore

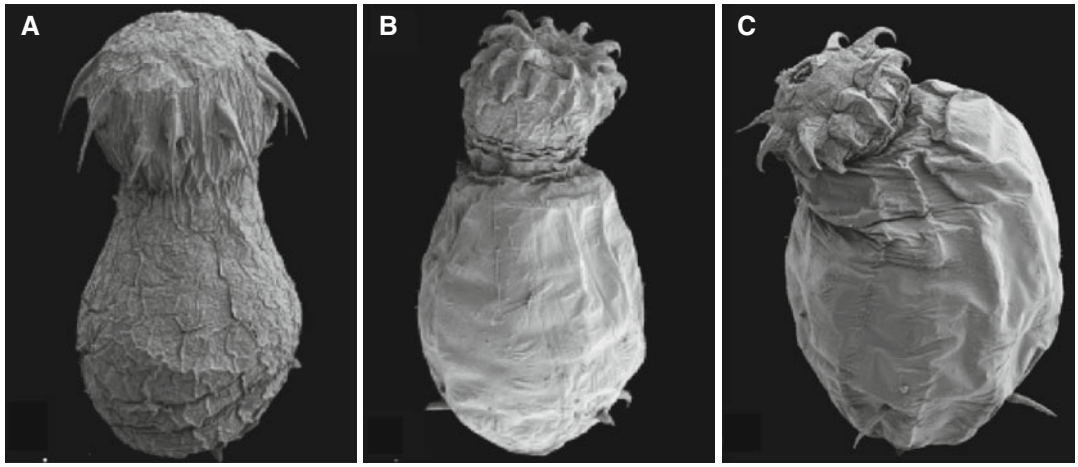

Fig. 1.3 Early larvae of *Priapulus caudatus*. (**A**) Hatching larva. (**B**) First lorica larva. (**C**) Second lorica larva

(de Rosa et al. 1999). Sequencing the whole genome of a priapulid species will provide more insights into clustering and complement in the future.

Whole mount in situ hybridizations have been conducted on developmental stages using blastopore, mouth, and anus marker genes and could confirm the deuterostomic mode of development in *Priapulus caudatus* (Martín-Durán et al. 2012). The gene expression patterns are largely similar to that of *Drosophila. Brachyury* is expressed only in the hindgut and not in the mouth, *FoxA* is expressed all along the alimentary canal, while *goosecoid* is expressed in the foregut and *caudal* is expressed in the hindgut (Martín-Durán et al. 2012; Martín-Durán and Hejnol 2015).

KINORHYNCHA

Kinorhynchs – also called mud dragons – are exclusively marine and interstitial invertebrates. There are close to 200 species described. Kinorhynchs possess a peculiar segmented body of 11 trunk segments, also called zonites, to avoid unwanted speculations about their homology to arthropod segments. The cuticle of the body is often covered with spines. Kinorhynch locomotion is driven by the protrusible introvert, and the diet of these worms is likely detritus and bacteria (Neuhaus 2013).

Embryogenesis

The embryology of kinorhynchs (*Echinoderes kozloffi*) had not been studied until recently (Kozloff 2007). Kozloff's (2007) pioneering work uses only light microscopy but provides some insights into their cleavage pattern (Fig. 1.4). During egg deposition the embryos get covered with detritus and are hard to detect. This makes embryological studies on kinorhynchs difficult. The egg size can differ between females of one species – likely due to different sizes of the females. Smallest *E. kozloffi* eggs found are 54 μm in diameter but may be also of ovoid shape with a length of 72 μm. The duration of the cell cycle during the first cleavages is about 1 h (Kozloff 2007). The juvenile worm hatches after 9 days at around 9° Celsius. The first cleavages are described as equal but can be slightly asynchronous (Kozloff 2007). After the 16-cell stage, the embryo seems to internalize some cells (Fig. 1.4E). How this occurs remains unclear. However, the embryo is compact and does not seem to possess a blastocoel. Two days after the first cleavage, the embryo does not show any landmarks that would indicate an orientation of the differentiation of cells (Fig. 1.4E). After 5 days, the embryo seems to extend and bend inside the egg shell (Fig. 1.4F) – 6 days after first cleavage, the internal tissues are visible (Fig. 1.4G). In summary, *Echinoderes kozloffi*

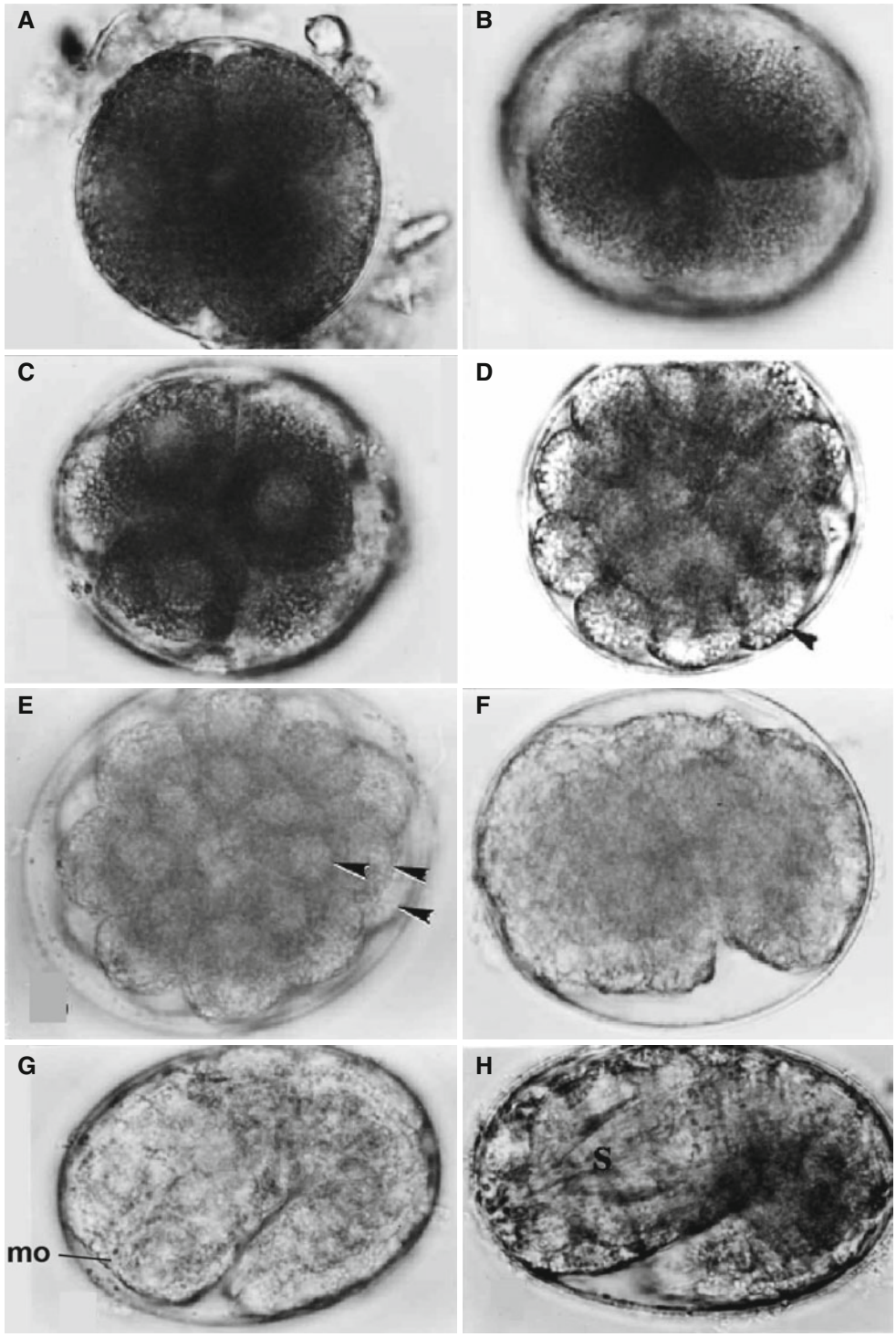

Fig. 1.4 Development of *Echinoderes kozloffi* (Kinorhyncha) (From Kozloff 2007). (**A**) Two-cell stage. (**B**) Four-cell stage. (**C**) Eight-cell stage. (**D**) Pre-gastrula stage. (**E**) Later stage, likely with internalized cells (*arrowheads*). (**F**) Embryo that has begun to extend along the anterior-posterior axis. (**G**) Bended worm, mouth opening (*mo*) to the *left*. (**H**) Prehatching stage, cells are differentiated with scalids (*s*) visible

seems to have radial cleavage, and the description of Kozloff that the blastomeres get organized into "layers" may indicate a more organized cleavage pattern – more similar to priapulids rather than to the highly variable cleavage program of nematomorphs (see below). Gastrulation remains unclear and thus also the relation of the site of cell internalization to the future openings of the digestive tract.

Postembryonic Development

Postembryonic development was first described in detail by Kozloff (1972) and revised previous studies by Nyholm (1947), who claimed that a three-segmented larva hatches from the egg. In fact, the juvenile kinorhynch hatches with 9 segments (or zonites) and gradually increases the number during 5–6 juvenile molts to 13 (Neuhaus 1993, 1995; Sørensen et al. 2010). No details concerning the changes in the internal organs or organogenesis altogether during the different juvenile stages are known.

LORICIFERA

Loriciferans were first described in 1983 by Kristensen (1983). Since then, a growing number of species (>30) have been described from this taxon. The animals are exclusively marine and interstitial and are found in shallow waters as well as in the deep sea. Recently, an anoxic species was described from the Mediterranean Sea (Danovaro et al. 2010). The taxon is characterized by a "lorica," a cuticular and thickened structure that protects the animal and is also found in the larva.

Embryogenesis

There are no descriptions of loriciferan development. Some authors have observed a developmental stage inside the fixed material (Heiner and Kristensen 2009), which is not sufficient to draw conclusions on their early development.

Larval Stages

The life cycle of loriciferans has been reconstructed solely based on fixed material, and different samples have been assigned to the same species (Heiner Bang-Bertelsen et al. 2013). So far, no description of a living loriciferan larva has been published. Thus, the data for the life cycle of Loricifera has to be interpreted with care before actual living stages are observed and described (see also Priapulida section). Nanoloricidae appear to have the simplest of the reconstructed life cycles (Fig. 1.5). Part of the life cycle is the Higgins larva that is similar to the adult and molts several times before the sexually mature adult emerges (Heiner Bang-Bertelsen et al. 2013). At first glance, the larvae and adult loriciferans look similar to priapulid lorica larvae. However, this similarity seems to be only superficial given the high number of cells the loriciferans possess, which is, for example, reflected by their complex musculature (Neves et al. 2013).

NEMATOMORPHA

Nematomorphs – or horsehair worms – are parasitic and have a life cycle that includes changes in the hosts. The reproductive phase is during a free-living stage. About 350 species have been described.

Life Cycle

Nematomorphs are parasites that first infest an intermediate host and subsequently the main host (Fig. 1.6). A review about the life cycles is given in Hanelt et al. (2005). Figure 1.6 shows

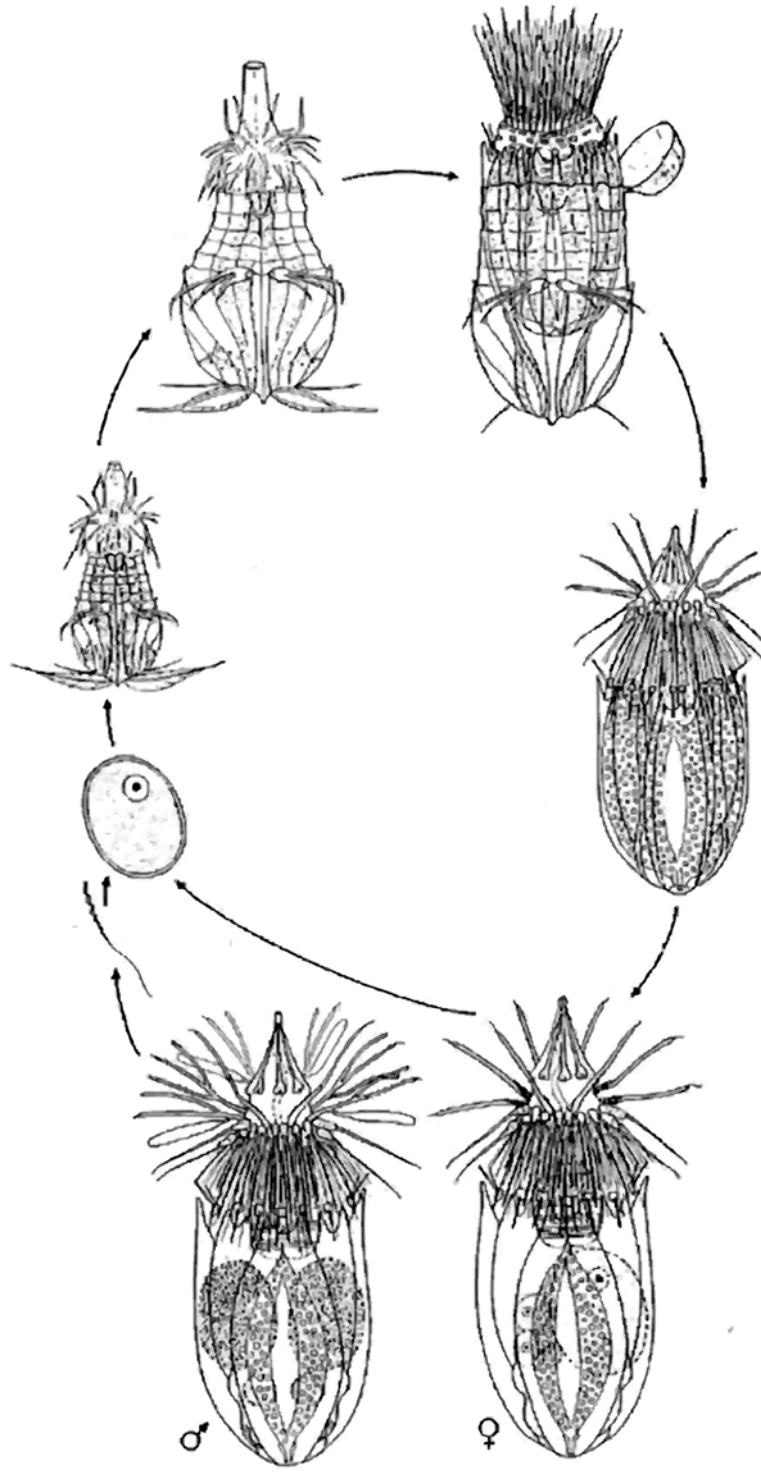

Fig. 1.5 Life cycle of *Nanaloricus mysticus* (Loricifera) (From Kristensen 1991) The adults of separate sex produce gametes. After fertilization the egg develops into the "Higgins larva." After several molts the larva undergoes a final molt ("metamorphosis") to an adult. The adult continues molting until sexual maturity (courtesy of RM Kristensen, copyright 2015, all rights reserved)

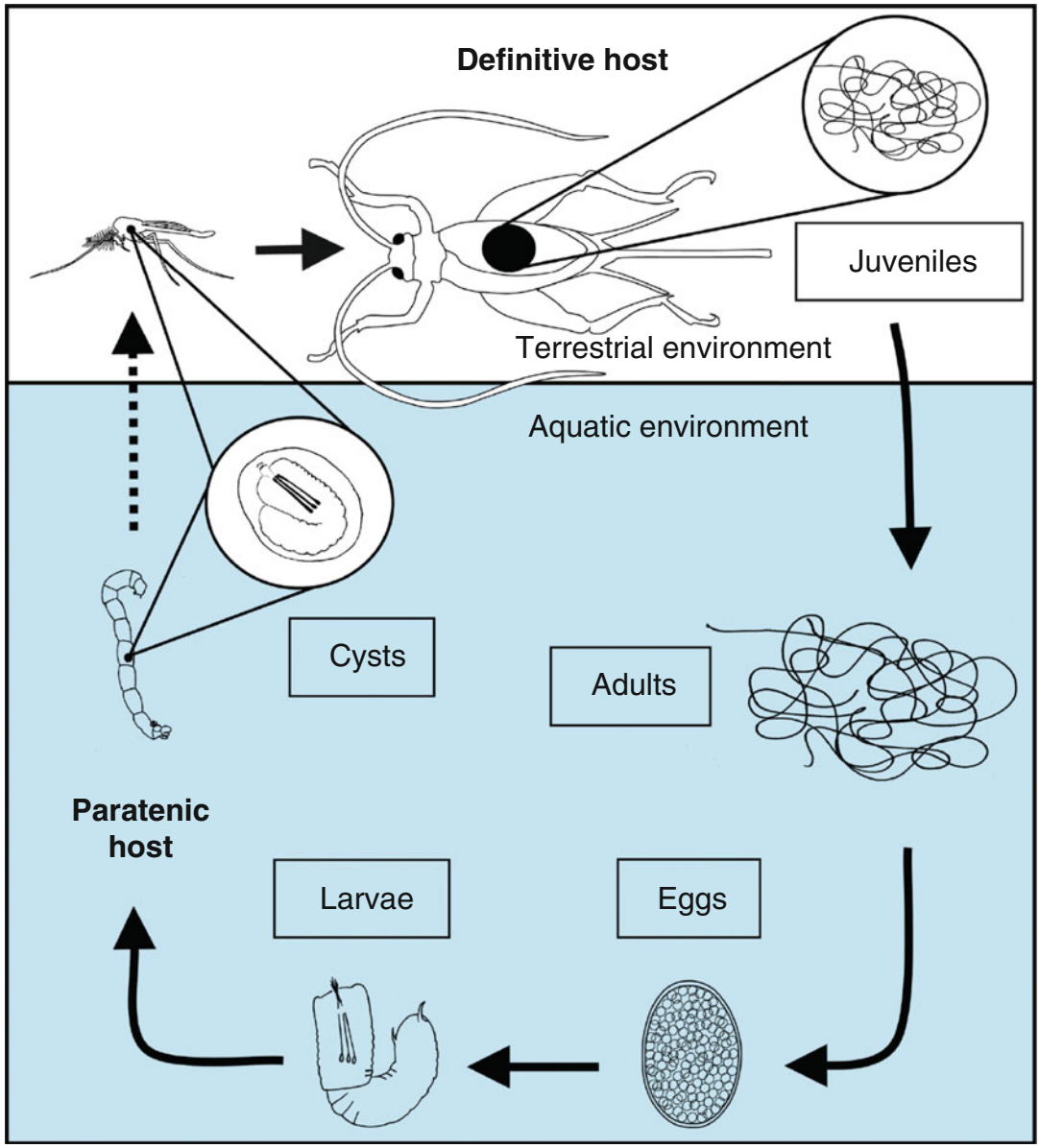

Fig. 1.6 Typical gordiid life cycle (From Hanelt et al. 2012) Gordiids have a simple life cycle that has one aquatic paratenic host and a definitive terrestrial host. The adults grow to sexual maturity in the definitive host before they are released into the water. The adults of separate sex copulate and lay fertilized eggs. These eggs develop into larvae, which either penetrate the epidermis or enter the host through the mouth. Inside the host the larvae encapsulate in cysts. These cysts remain in the host through metamorphosis. Terrestrial predators take up the cysts, and inside the host the cysts release the worm which then develops into an adult

a typical gordiid life cycle. The worms accumulate often in bulks ("gordian knot") in rivers and ponds, where they copulate and males transfer sperm to the females. After fertilization, the adult females deposit millions of eggs in egg strings and die afterward. The nematomorph larva hatches and infests the paratenic host – limnic or marine arthropods – either through the digestive tract or by penetrating the skin. In the intermediate host, the larva encysts. These cysts persist

in the intermediate host until they are eaten by terrestrial arthropods. These are the definitive hosts in which the cyst releases the larva, which then develops into an adult. Nematomorphs are able to change the behavior of the terrestrial host in that it – contrary to common behavior – seeks for aquatic environments. There, the adults crawl out of the body of the arthropod and enter the aquatic realm – the cycle is closed.

Cleavage

The cleavage pattern has been studied in different species, and all reports agree that it is total and equal (Tratiakow 1901; Montgomery 1904; Meyer 1913; Mühldorf 1914; Inoue 1958; Malakhov and Spiridonov 1984). Only the two-cell stage does not show variation (Fig. 1.7), while all following cleavage stages are highly variable (Montgomery 1904; Mühldorf 1914; Malakhov and Spiridonov 1984). The early development is thus highly regulative and does not follow a cleavage program that allows the tracing of individual blastomeres. The four-cell stage can have the blastomeres in a row or in a tetrahedral arrangement, and individual blastomeres can be delayed in their division so that three-cell and five-cell stages are common (Meyer 1913; Inoue 1958). The irregular cleavage pattern results in a 16-cell stage that has a large blastocoel (Fig. 1.7D).

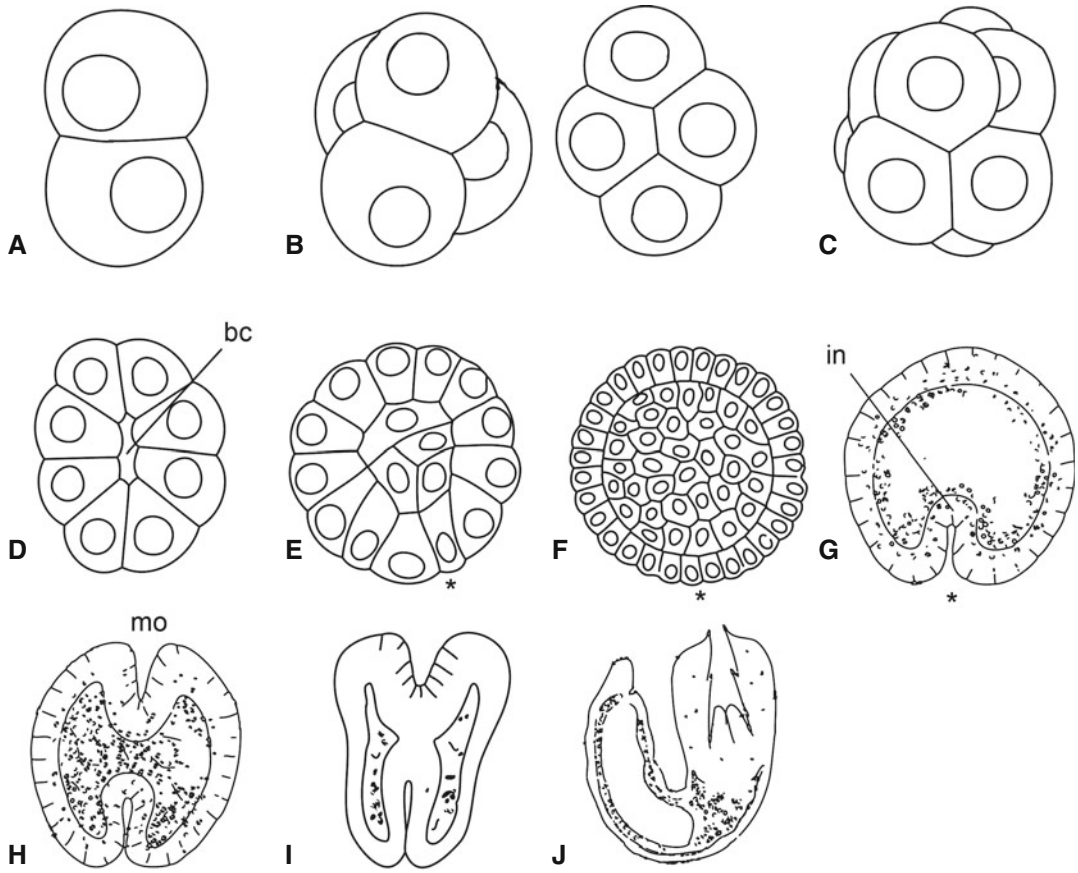

Fig. 1.7 Development of the nematomorph *Gordius* sp. (After Malakhov and Spiridonov 1984). (**A**) Two-cell stage. (**B**) Variable arrangements of the blastomeres at the four-cell stage. (**C**) Eight-cell stage. (**D**) Optical section through a 16-cell stage showing the blastocoel (*bc*). (**E**) Gastrula with inner cell mass. (**F**) Later gastrula. (**G**) Formation of the pseudointestine (*in*) at the vegetal pole, asterisk indicates the site of gastrulation (blastopore) according to the authors. (**H**) Mouth (*mo*) and proboscis formation. (**I**) Digestive tract formation. (**J**) Prehatching larva

Gastrulation

Gastrulation in all nematomorph species investigated so far has been reported as a unipolar ingression of cells (Tratiakow 1901; Montgomery 1904; Meyer 1913; Mühldorf 1914; Inoue 1958; Malakhov and Spiridonov 1984). Meyer (1913) and Montgomery (1904) reported that at first two blastomeres immigrate, which then divide, resulting in a blastocoel that is filled with blastomeres. After this immigration the ectoderm of the pseudointestine forms at the blastopore at the posterior end of the embryo (Montgomery 1904; Meyer 1913; Malakhov and Spiridonov 1984) (Fig. 1.7F, G). All authors agree that the blastopore corresponds to the site of hindgut formation, which means that nematomorphs are deuterostomic. The proboscis is formed later at the opposite – anterior – end of the embryo (Fig. 1.7H).

Organogenesis

The pseudointestine is the first internal organ that forms (Montgomery 1904; Meyer 1913; Malakhov and Spiridonov 1984). It remains unclear if this pseudointestine will also form the intestine of the juvenile or adult. The opening of the intestine will likely form the cloacal opening of the juvenile and adult. Mesodermal cells are the remaining blastomeres that do not contribute to the gut formation and will form the musculature and the gonads of the larva (Fig. 1.7H–J). Malakhov and Spiridonov (1984) describe the anlage of the ventral nerve cord as a row of individual blastomeres. More detailed information regarding organogenesis of the nematomorph larva is lacking.

OPEN QUESTIONS

- Description of cleavage, gastrulation, and organogenesis in Loricifera and Kinorhyncha
- Fate map and cell lineage of Priapulida and Nematomorpha
- Molecular mechanisms of basic developmental processes including developmental gene expression in all groups

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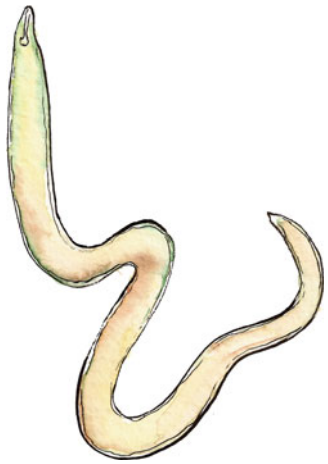
Nematoda

2

Ralf J. Sommer



Although commonly considered a subtaxon of Cycloneuralia, the Nematoda are covered separately in this chapter.

Chapter vignette artwork by Brigitte Baldrian.

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INTRODUCTION

Nematoda (roundworms) are mostly small animals in the range of only millimeters. While they are hard to see without a microscope, nematodes represent the largest animal phylum with an estimated number in the range of one to ten million species (Lambshead 1993). Nematodes are characterized by three general features. Besides species richness, these are numerical abundance and ecological omnipresence because they usually occur in high numbers and they are found in most ecosystems. For example, in some soil samples, nematodes can occur in excess of one million individuals per square meter (Floyd et al. 2002). The highest diversity of nematodes is found in marine environments and in terrestrial settings, often in association with arthropods or other invertebrates. Some nematodes are important parasites of plants, livestock, and humans. In the last 15 years, molecular phylogenetics has resulted in a comprehensive understanding of the relationships among nematodes that can serve as the basis for evolutionary considerations (van Megen et al. 2009). For example, molecular phylogenetics convincingly showed that parasitism has evolved at least seven times independently in nematodes, involving both plant and animal parasitism (Fig. 2.1; Blaxter et al. 1998). By now, many parasitic nematodes have their genome sequenced (Fig. 2.1), representing a promising starting point to understand associated biological processes (for a review, see Sommer and Streit 2011).

One particular nematode species, *Caenorhabditis elegans*, serves as an important model organism for both basic and applied research and plays a pivotal role in the elucidation of basic principles of biology and biomedical research. Not surprisingly, therefore, the knowledge available from *C. elegans* also served as a starting point for studies in evolutionary biology and EvoDevo. The success of *C. elegans* as a model results from the easiness of its culture in the laboratory (Brenner 1974). With a life cycle of only 3 days (20 °C), *Escherichia coli* as food source, and self-fertilization as the typical mode of reproduction, *C. elegans* can be cultured indefinitely in large numbers.

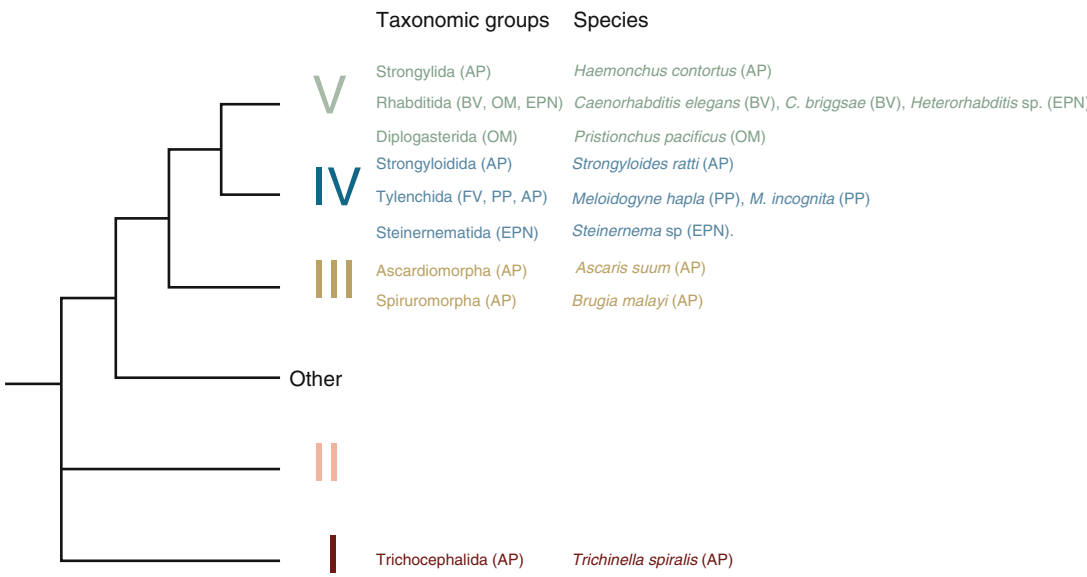

Fig. 2.1 Phylogenetic relationship of nematodes and their associations. *Roman numerals* indicate the distinction of five clades according to Blaxter et al. (1998). Species with a published genome sequence are indicated in *bold*. *AP* animal parasite, *BV* bacteriovore, *EPN* entomopathogenic nematode, *FV* fungivore, *OM* omnivore, *PP* plant parasite (Redrawn from Sommer and Streit (2011).)

C. elegans has a typical nematode life cycle. It undergoes embryonic development within an egg shell, and postembryonic development consists of four stages, called juvenile stages (J1–J4), separated by molts (Fig. 2.2; Wood 1988). In nematode evolution, particularly the evolution of parasitic forms, this general life cycle has been modified in numerous ways. As nematode life cycles are described in numerous textbooks, the following just gives a very brief summary of the different ecologies and life cycles of nematodes.

Saprobiotic, bacterial-feeding nematodes are common in the family Rhabditidae to which *C. elegans* belongs, but also in the Diplogastridae, including another nematode model, *Pristionchus pacificus*. Under favorable conditions, the development of *C. elegans* takes as little as 3 days, and the adult reproductive life span is also in the order of several days. In contrast, under unfavorable conditions, these worms form a non-feeding but motile alternative J3, called a dauer juvenile, which will be discussed below in greater detail (Fig. 2.2). In the Rhabditidae and Diplogastridae families, an androdioecious mating system has evolved multiple times independently (Denver et al. 2011). Hermaphrodites can mate with males but can also self-fertilize. In both families, the best-studied representatives, i.e., *C. elegans* and *P. pacificus*, are hermaphroditic.

However, other ecologies and life cycles have evolved repeatedly. The genera *Steinernema* and *Heterorhabditis* are two phylogenetically unrelated groups of entomopathogenic nematodes (Fig. 2.1; Gaugler 2002). These nematodes form

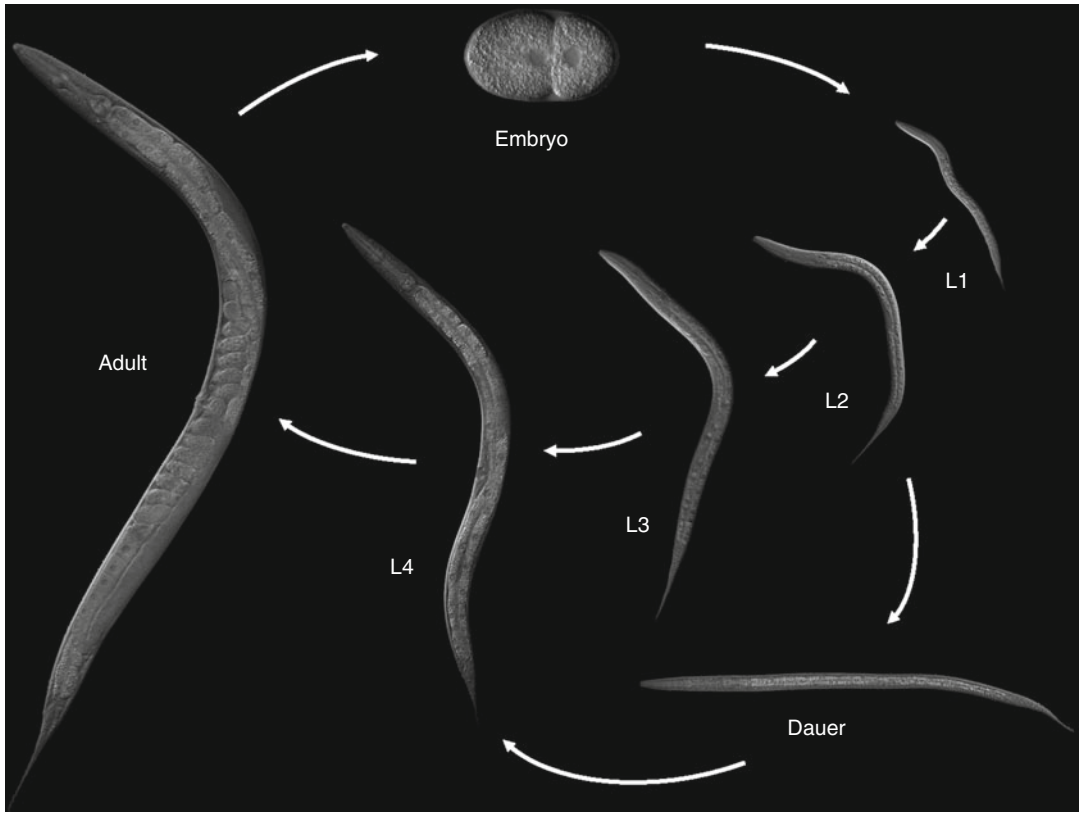

Fig. 2.2 The basic nematode life cycle exemplified with *C. elegans*. *C. elegans* has a simple life cycle that can be completed in 3 days in the laboratory. The self-fertilizing hermaphrodite lays eggs which hatch into an *L1* larva. Four larval stages are separated by molt. Larvae grow primarily by the enormous increase in the reproductive organ, the gonad. Adults can lay more than 200 eggs and can live for several weeks. When conditions become harsh, animals will redirect their development and form an arrested dauer larva, an alternative L3 stage (© Ralf J. Sommer 2015. All Rights Reserved)

infective larvae that carry a species individual of symbiotic bacteria, *Xenorhabdus* spp. in the case of *Steinernema* spp. and *Photorhabdus* spp. in that of *Heterorhabditis* spp. Worms invade insect larvae and release the bacterium, which reproduces very rapidly and kills the insect by the secretion of a number of toxins providing the food for nematodes to grow on the insect carcass. Other nematodes are gastrointestinal parasites of vertebrates, i.e., *Haemonchus contortus* living in the abomasum of sheep and goats, *Ascaris suum* in the small intestine of pigs, and members of the genus *Strongyloides* in the small intestine of mammals. In contrast to these cases, *Trichinella spiralis* is an intracellular parasite. Finally, nematodes are equally successful parasites of plants, with members of the genus *Meloidogyne* spp. being among the most important agricultural pests.

C. elegans and nematode anatomy, gene sequences and expression patterns have been extensively studied in the last two decades, providing detailed insight into the comparative biology of these organisms. Detailed ONLINE platforms (see paragraph below) have been established that summarize and review these topics in form of "living," regularly updated, chapters. Therefore, the reader is referred to these contributions for a comprehensive summary and state-of-the-art description of expression patterns and gene sequence similarities. Instead, the following text provides an overview on EvoDevo studies in free-living nematodes with the aim to highlight important conceptual findings, such as developmental systems drift. Also, the author wants to highlight the need for integrative research programs, which in nematodes can be very fruitful by combining laboratory studies with fieldwork. Throughout the text, review articles focusing on specific aspects of nematode EvoDevo are mentioned in the respective paragraphs. For example, phylogeny, genomics, and the evolution of reproductive systems are not covered here, and the reader is referred to other recent review articles (Denver et al. 2011; Sommer and Streit 2011; Schierenberg and Sommer 2014).

NEMATODE ONLINE PLATFORMS: WORMBASE, WORMBOOK, AND WORMATLAS

C. elegans is one of the most important model organisms in the modern life sciences and provided detailed mechanistic insight into many fields of biology, including developmental biology and neurobiology. Building on the cellular understanding of embryonic and postembryonic development with the formation of 959 cells in the adult hermaphrodite, generations of scientists have studied many developmental, cellular, and physiological processes in great detail. Fortunately, *C. elegans* has not only been at the forefront of basic research but has also launched several ONLINE platforms for the transparent and open access-driven distribution of knowledge (Table 2.1) that can serve as road model for other research communities. The three most important ONLINE platforms are discussed below.

WormBase (www.wormbase.org) represents a searchable database for all aspects of worm biology. WormBase covers the anatomy and cellular composition of *C. elegans* and contains detailed information on all genes. Gene data include gene classes, ontology, and potentially related human disease genes. Similarly, WormBase covers mutant phenotypes including those obtained by reverse genetic tools, such as RNA interference and the "Million Mutation Project." The latter represents a mutagenesis project, in which a large number of viable mutants have been generated and all resulting lines have been whole genome sequenced and can be distributed upon request. In WormBase, the pages on individual genes also cover the expression patterns, sequence similarity to other nematodes and other model organisms, information about transgenic strains, and links to all related papers. WormBase is regularly updated, and at the time of writing (August 2014) is in version WS243.

WormBook (www.wormbook.org) represents an ONLINE review of *C. elegans* biology that is regularly updated. WormBook covers all areas of worm biology, including development, genetics,

Table 2.1 ONLINE platforms for *Caenorhabditis elegans* and nematode biology

1.	WormBase	www.wormbase.org	Genetic, biochemical and molecular information, expression patterns, sequence information, complete reference list, link to researchers	*C. elegans*, other nematodes, other model organisms
2.	WormBook	www.wormbook.org	Worm (*C. elegans*) biology, methodology, evolution	*C. elegans* and other nematodes
3.	WormAtlas	www.wormatlas.org	*C. elegans* anatomy with focus on electron microscopy	*C. elegans*

cell biology, and biochemistry. It contains a WormMethods section and covers other nematodes, making it an important tool for evolutionary biologists.

WormAtlas (www.wormatlas.org) represents a database featuring the behavioral and structural anatomy of *C. elegans*, making use of the unique serial reconstruction of the worm body by transmission electron microscopy. It contains handbooks for the hermaphrodite, the male, and the dauer stage and as resources provides worm images, a slidable worm, and worm wiring diagrams. Together, these three comprehensive ONLINE platforms make *C. elegans* and worm biology available for scientists in all research fields.

Finally, the *C. elegans* Genetics Center (CGC) distributes all type of reagents necessary to research throughout the world. All of the above-mentioned platforms as well as CGC are funded through the National Institute of Health (NIH) in the United States of America.

NEMATODE EVODEVO

Building on the detailed knowledge about embryonic and postembryonic processes in *C. elegans*, nematode EvoDevo studies cover a variety of developmental patterns and processes. For embryonic developmental processes, the reader is referred to a recent review describing in detail many aspects of early and late embryogenesis throughout the Nematoda (Schierenberg and Sommer 2014). In the following, a brief overview about the evolution of selected embryonic and postembryonic processes is provided. These studies have focused largely on members of the Rhabditidae and Diplogastridae family because nematodes in these groups can often be cultured in the lab similar to *C. elegans*. Also, in a selected group of nematodes, a functional toolkit was developed that provides mechanistic insight into the evolution of developmental processes.

Sex Determination

Sex in nematodes is most often genetically determined and involves sex chromosomes. Diploid species with sex chromosomes have females with an XX karyotype and males with an XY or an XO karyotype. *C. elegans* and its relatives have XX/XO karyotypes, and intense studies over the last two decades provided a detailed account of the genetics and the molecular biology of sex determination in *C. elegans* (Fig. 2.3; Zarkover 2006). Specifically, in *C. elegans*, the ratio of X chromosomes to autosomes controls a complex signaling pathway of negative-acting factors, and XO animals activate a master sex determination switch gene *xol-1*. In contrast, XX animals suppress *xol-1* activity, resulting in the activation of the zinc finger transcription factor TRA-1, thereby suppressing male development in hermaphrodites. Upstream of *tra-1* is a series of regulators consisting of *her-1*, *tra-2*, and three *fem* genes, with the FEM protein ubiquitin ligase complex eventually targeting TRA-1 for degradation (Fig. 2.3). In hermaphrodites, the absence of HER-1 results in TRA-2 inhibiting the ubiquitin ligase complex, and TRA-1 is finally active to repress male development.

Detailed comparative studies in different *Caenorhabditis* species revealed that sex determination appears to evolve rapidly (Haag et al.

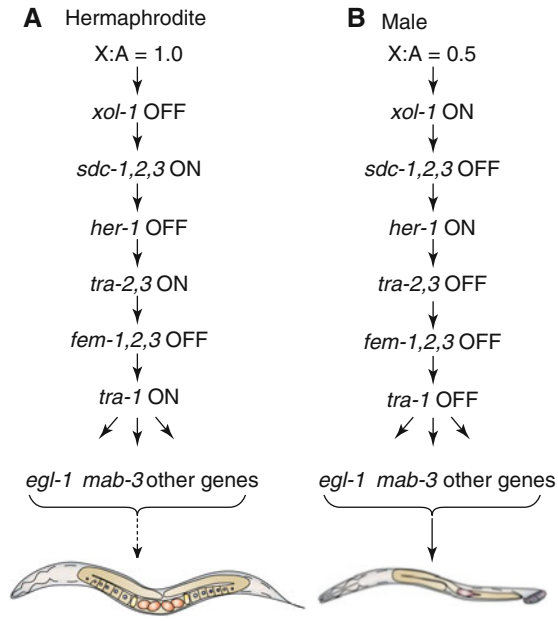

Fig. 2.3 Somatic sex determination in *C. elegans*. Genetic model for sex determination in hermaphrodites (**A**) and males (**B**). A series of negative regulatory interactions triggered by the X:A ratio (i.e., the ratio between sex chromosomes and autosomes) results in high TRA-1 activity in hermaphrodites and low TRA-1 activity in males. TRA-1 regulates transcription of various sex-specific genes, such as *egg-laying defective 1* (*egl-1*) and *male abnormal 3* (*mab-3*). Mutations in *C. elegans* sex determination genes result in distinct phenotypes: *tra* transformer of XX animals into males, *fem* feminization of XX and XO animals, *her* hermaphrodization of XO animals (Redrawn from Sommer and Bumbarger WIRE 2012.)

2002). These studies also suggest that the more downstream players in the gene cascade are more conserved. For example, TRA-1 is highly conserved in evolution although the sex determination machinery is evolving rapidly. Interestingly, the regulation of TRA-1 seems to be highly species specific. In *C. elegans*, mutations in the genes *fem-2* and *fem-3* result in females that lack the short period of spermatogenesis typical for hermaphrodites. In contrast, these genes are dispensable for hermaphrodite development in the close relative *C. briggsae*, indicating that *C. elegans* and *C. briggsae* acquired their hermaphroditic mode of reproduction independently from male/female ancestors (Hill et al. 2006).

The gonochoristic *C. remanei* is a close relative of *C. briggsae*, representing the ancestral mode of reproduction. Generally, it is assumed that the evolution of self-fertilizing hermaphroditism, as observed in nematodes, is a late event in evolution and cannot be reverted, resulting in an evolutionary dead end. In the case of *C. remanei* and *C. briggsae*, it was shown that mutations in just two genes are sufficient to allow the transition from females to hermaphrodites (Baldi et al. 2009). Specifically, lowering the activity of *tra-2* by gene knockdown via RNAi generated animals capable of making spermatids, but they remained dysfunctional unless a second mutation in the sperm activation gene *swm-1* was introduced.

Comparative Cell Lineage Analysis

Cell lineage analysis was the most important approach besides genetics that made *C. elegans* a unique model in developmental genetics. Due to its transparency and the small number of cells, the complete postembryonic cell lineage of *C. elegans* has been determined (Sulston and Horvitz 1977; Kimble and Hirsh 1979). The postembryonic lineage was found to be invariant resulting in a final number of 959 cells for adult hermaphrodites and 1,031 cells in males.

The comparison between the complete postembryonic cell lineages of *C. elegans* and the phylogenetically distant nematode *Panagrellus redivivus* revealed high similarities but also important differences that can be used to identify categories of lineage transformations (Fig. 2.4; Sternberg and Horvitz 1981, 1982; Sommer et al. 1994). Basically, five types of cell lineage transformations can be distinguished: (1) fate switch of a cell to a fate normally associated with another cell, (2) polarity reversal in the lineage generated by a blast cell, (3) alteration in the number of rounds of cell divisions, (4) changes in the relative timing of divisions, and (5) altered segregation of developmental potential, such that a fate normally associated with a specific cell instead becomes associated with its sister. Besides these general features, the postembryonic cell lineage of *C. elegans* and comparisons between different nematodes provided a foundation for many experimental and functional investigations of postembryonic processes. In the following, a few landmark results from nematode EvoDevo studies are provided.

Gonad Development

In general, the structure of nematode gonads varies substantially between the two sexes and across species, with male gonads always being monodelphic (one armed), whereas female/hermaphrodite gonads can be didelphic (two armed) or monodelphic (Fig. 2.5; Chitwood and Chitwood 1977). In *C. elegans* hermaphrodites, the two gonadal arms develop nearly symmetrically from two somatic precursor cells, Z1 and Z4, which form the anterior and posterior arm, respectively (Kimble and Hirsh 1979). Z1 and Z4

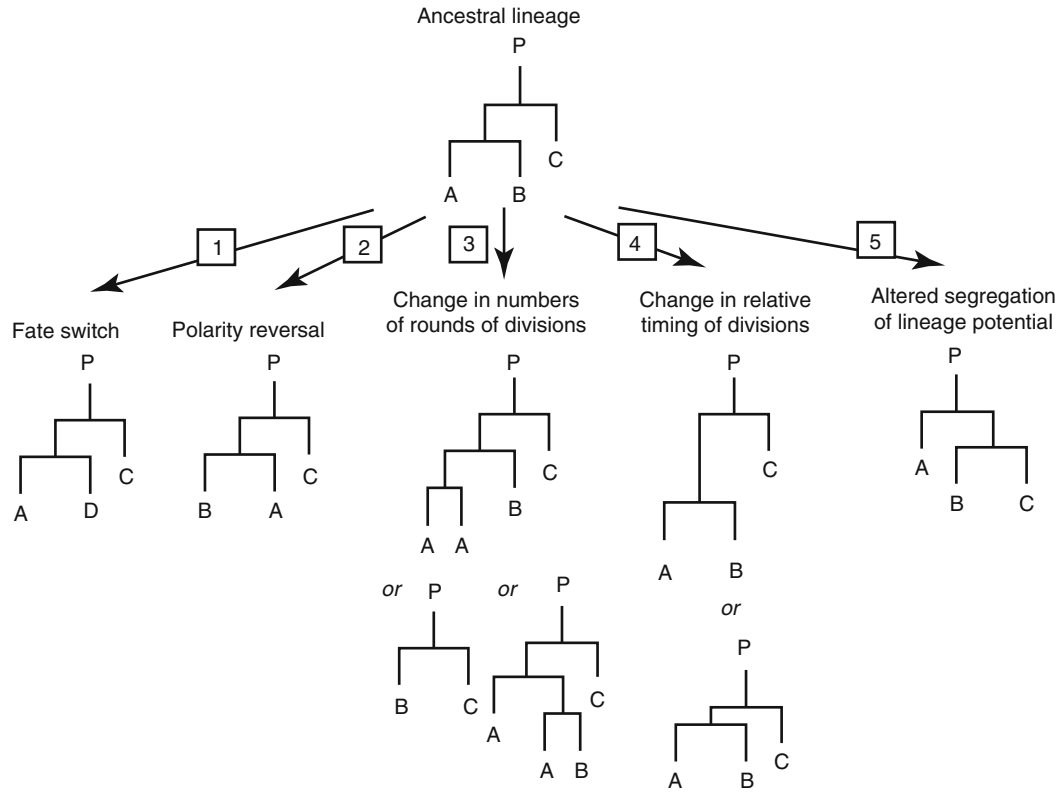

Fig. 2.4 Cell lineage transformations. Five possible kinds of changes in cell lineage pattern found in nematodes. *P*, precursor cell; *A–D*, different cell types (From Fitch and Thomas (1997), after Sommer et al. (1994). © Ralf J. Sommer 2015. All Rights Reserved)

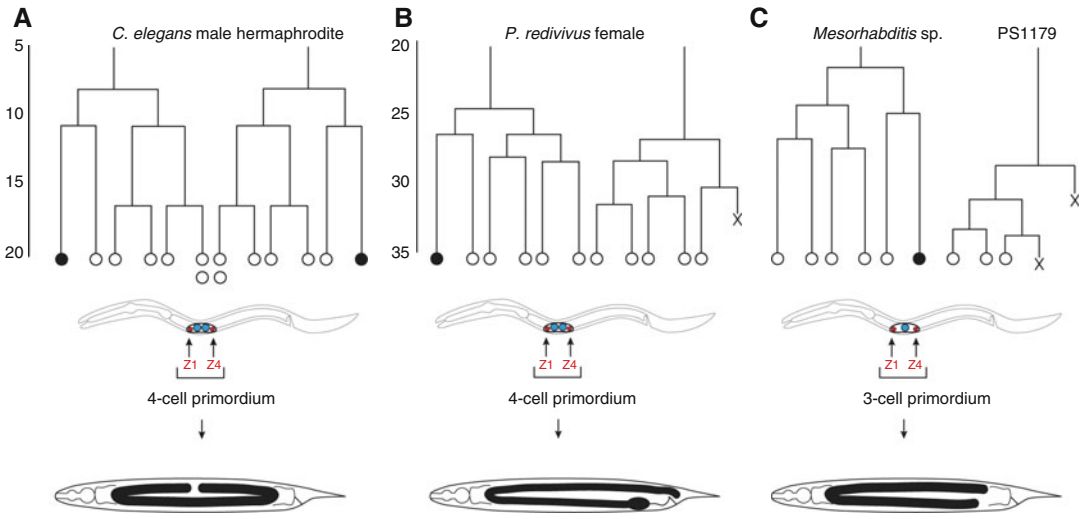

Fig. 2.5 The evolution of gonad development and schematic representation of nematode gonads and their evolutionary modifications. Cell linage and gonad development of (**A**) *C. elegans* hermaphrodites, (**B**) *Panagrellus redivivus* females, and (**C**) *Mesorhabditis* sp. 1,179 females. Lineages: *line length* represents the relative timing of divisions. Terminal Xs at the *lineage base* represent cell deaths; *black circles* represent the distal tip cell; *white circles* represent all other fates. Thus, the fate of these particular cells is sex specific. *Mesorhabditis* starts with a three-cell primordium as a single germ line precursor is present; all others start with a four-cell primordium with Z1 and Z4 as precursor of the somatic gonad (*red circles*) and Z2 and Z3 as precursor of the germ line (*blue circles*). Lower images show final anatomy of the gonad. *C. elegans* hermaphrodites have a didelphic gonad, others a monodelphic gonad (Redrawn from Sommer and Bumbarger, WIRE 2012. © Ralf J. Sommer 2015. All Rights Reserved)

give rise to a distal tip cell (dtc; Z1.aa and Z4.pp), which promote growth and shape of the gonadal arm (Fig. 2.5).

Panagrellus redivivus was the first species with a monodelphic female gonad to be studied in greater detail (Sternberg and Horvitz 1981). Cell lineage analysis revealed that gonad monodelphy resulted from the programmed cell death of the posterior dtc Z4.pp, revealing an astonishing simple mechanism: the programmed cell death of a single cell has a major influence on the overall anatomy of the nematode – a monodelphic vs. didelphic gonadal structure (Fig. 2.5). Within the family Rhabditidae, monodelphy has evolved repeatedly, and in these cases, the first asymmetry between the anterior and posterior part of the gonad can be seen already at the time of Z1 and Z4 division (Fig. 2.5; Felix and Sternberg 1996). Thus, already the descriptive analysis of cell lineage patterns in nematodes can explain major morphological differences among species.

Comparative Gene Expression Studies

The development of powerful genomic and transcriptomic tools in recent years also helped advancing the understanding of comparative embryogenesis in nematodes, allowing for the first time a link between morphological stages of development and the underlying molecular activities (Levin et al. 2012). While comparative cell lineage studies focused largely on the early embryo, transcriptomic tools allow later stages to be analyzed in similar detail. Levin and coworkers (2012) defined the expression profile of *C. elegans* at a genome-wide level, distinguishing ten developmental stages from the four-cell embryo to the hatching L1 larva. Specifically, 6.790 *C. elegans* genes were clustered in 20 distinct developmental profiles based on their dynamic expression patterns. Comparing different stages revealed two drastic transitions in gene expression profiles associated with gastrulation (stage 3 of Levin et al. 2012) and ventral closure

(stage 7). Functional characterization indicated that gastrulation shows intestine-enriched gene expression, whereas ventral closure around stage 7 shows the expression of marker genes known to be involved in the specification and differentiation of neurons and muscles (Levin et al. 2012). Thus, a transcriptomic approach provides new inroads into the analysis of neurogenesis and myogenesis, which are complicated to study by cell lineage analysis given their late occurrence in the *C. elegans* embryo.

The same authors have added to their analysis of *C. elegans* four additional *Caenorhabditis* species, which differ in their length of embryogenesis. Interestingly, the analysis and the comparison with *C. remanei*, *C. briggsae*, *C. brenneri*, and *C. japonica* revealed qualitatively similar relationships in expression profiles to those found in *C. elegans*. These similarities were independent of timing, which differed up to 20 % between the five *Caenorhabditis* species. Together, these studies revealed the existence of two conserved "developmental milestones," characterized by expression dynamics and the activation of key developmental regulators. Also, this study indicates that the use of transcriptomics in comparative cell lineage analysis and EvoDevo is a powerful tool, which in the future can hopefully be applied to postembryonic processes.

Conservation of Developmental Control Genes: A Slightly Different Perspective

EvoDevo research over the last two decades has resulted in the truism that developmental control genes are highly conserved throughout evolution. However, in nematodes, or at least *C. elegans*, many of these stories are slightly different. For example, *C. elegans* does not contain a typical Hox cluster. While six Hox-type genes have been found, only four of them are in a cluster-like region, and only one, *ceh-13*, the labial-like Hox gene of *C. elegans*, is involved in and is essential for embryogenesis (Aboobaker and Blaxter 2003). The other three classical Hox genes, *lin-39*, *mab-5*, and *egl-5*, play important roles in postembryonic development, where they guide cell fate decisions in different body regions. Most of their defined roles are as transcription factors acting downstream of signaling pathways such as EGF/RAS and Wnt signaling. For example, vulva development, to be discussed below, requires double input of *lin-39* at different levels of the genetic hierarchy (Sternberg 2005). The functions of Hox genes in nematode development evolve rapidly, as reduction-of-function and loss-of-function mutants in homologous Hox genes in *C. elegans* and *P. pacificus* resulted in completely different phenotypes (Sommer 2008).

For other important developmental regulators, the story told in nematodes is even more different. For example, many studies have shown the transcription factors *groucho* and *hairy*, originally identified by genetic studies in flies, to be highly conserved in animal evolution (Rebeiz et al. 2005). However, there are no *groucho* and *hairy* genes *in C. elegans*, indicating that complex body plans can develop without this pair of genes. In contrast, genetic and molecular studies in *P. pacificus* revealed the existence of a *groucho* and *hairy* module with functions related to insects and vertebrates (Schlager et al. 2006). Thus, there can be major differences in the genetic composition of species within one phylum without these differences being associated with drastic changes of the body plan, as *P. pacificus* and *C. elegans* both look like typical free-living nematodes. The following paragraph will describe in greater detail one example of nematode EvoDevo and the conceptual conclusion of "developmental systems drift," the notion that homologous structures can be specified by non-homologous molecular mechanisms.

NEMATODE VULVA DEVELOPMENT AND DEVELOPMENTAL SYSTEMS DRIFT

Comparative Vulva Development

The vulva is the egg-laying organ and mating structure of nematode females and hermaphrodites, and vulva formation in *C. elegans* represents one of the best-studied developmental

processes in animal development (Fig. 2.6; for a review, see Sternberg 2005). This precondition makes vulva development a unique paradigm and reference system for comparative EvoDevo studies with a particular emphasis on the underlying mechanisms. The vulva derives from the ventral epidermis in all nematodes studied to date, with the ventral epidermis consisting of 12 epidermal blast cells, called P1.p to P12.p from anterior to posterior (Fig. 2.6). Six of these 12 cells are set aside early in development, and a subset of 3 cells will later be induced to form the vulva.

Specifically, the six ventral epidermal cells P(3–8).p do not fuse with the hypodermis during early *C. elegans* larval development, like their anterior and posterior counterparts P(1,2,9–11).p (Fig. 2.6). They are named vulval precursor cells (VPCs) because they all have the potential to form part of the vulva. However, under unperturbed conditions, only three of these six VPCs, P(5–7).p, form vulval tissue due to an inductive signal from the gonadal anchor cell (AC; Fig. 2.6). P(5–7).p adopt a 2°-1°-2° fate pattern and form the vulva, whereas the three remaining VPCs, P(3,4,8).p, adopt an epidermal, so-called 3° fate (Fig. 2.6; 1°, 2°,3° denotes a fate hierarchy; ablation experiments indicate that cells compete for a higher ranked fate). P5.p and P7.p have a 2° fate and produce seven progeny each, which form the anterior and posterior part of the vulva. In contrast, P6.p acquires the 1° fate and generates eight progeny forming the central part of this organ. Together, six VPCs adopt one of three alternative fates, resulting in a stereotypical 3°-3°-2°-1°-2°-3° pattern.

In *C. elegans*, vulva formation is induced by a signal from the anchor cell (AC), and the specification of vulva cell fate in *C. elegans* requires a complex network of signaling processes. The AC secretes an epidermal growth factor (EGF)-type ligand that is transmitted in the VPCs by RAS, a central member of various signal transduction pathways. A Notch-type lateral signaling process acts downstream or in parallel to EGF/RAS signaling to specify vulval fates. Once P6.p has been specified as the 1° cell by the AC-derived signal, it signals its two neighbors P5.p and P7.p to adopt a 2° fate via Notch signaling (Sternberg 2005).

Comparative studies on vulva development were initiated in the 1990s (Sommer et al. 1994). These studies revealed that the basic pattern of vulva formation is highly conserved in nematode evolution with P(5–7).p forming the vulva in most representatives studied to date, and the 2°-1°-2° pattern is found to be a basic principle of vulva formation (Kiontke et al. 2007). Surprisingly, however, there is substantial variation with respect to vulva induction. While *C. elegans* requires the AC for vulva induction, some nematodes which form their vulva in the posterior body region rely on cell-autonomous specification processes (Sommer and Sternberg 1994). Again other species require a continuous inductive signal, e.g., *P. pacificus* (Sigrist and Sommer 1999). For a detailed overview, the reader is referred to another recent review (Schierenberg and Sommer 2014). The following concentrates on molecular studies in one particular nematode, *P. pacificus* (see also boxed text), and the observed general phenomenon of developmental systems drift.

The Development of a Satellite Model System: *Pristionchus pacificus* vs. *Caenorhabditis elegans*

Caenorhabditis elegans is one of the most important model systems in modern biology (http://www.wormbook.org). As a point for detailed mechanistic and functional comparisons, *Pristionchus pacificus* was developed as a satellite model system. Since its description as a novel species in 1996, a functional toolkit has been generated in this species. *P. pacificus* is a member of the Diplogastridae family and thus only distantly related to the other nematode model system, *Caenorhabditis elegans*, which belongs to the Rhabditidae (Fig. 2.1). While *P. pacificus* and *C. elegans* differ in many developmental characteristics, they share many technical features, such as a short generation time, simple laboratory culture, self-fertilization as a mode of reproduction, and spontaneous male formation in laboratory cultures. In general,

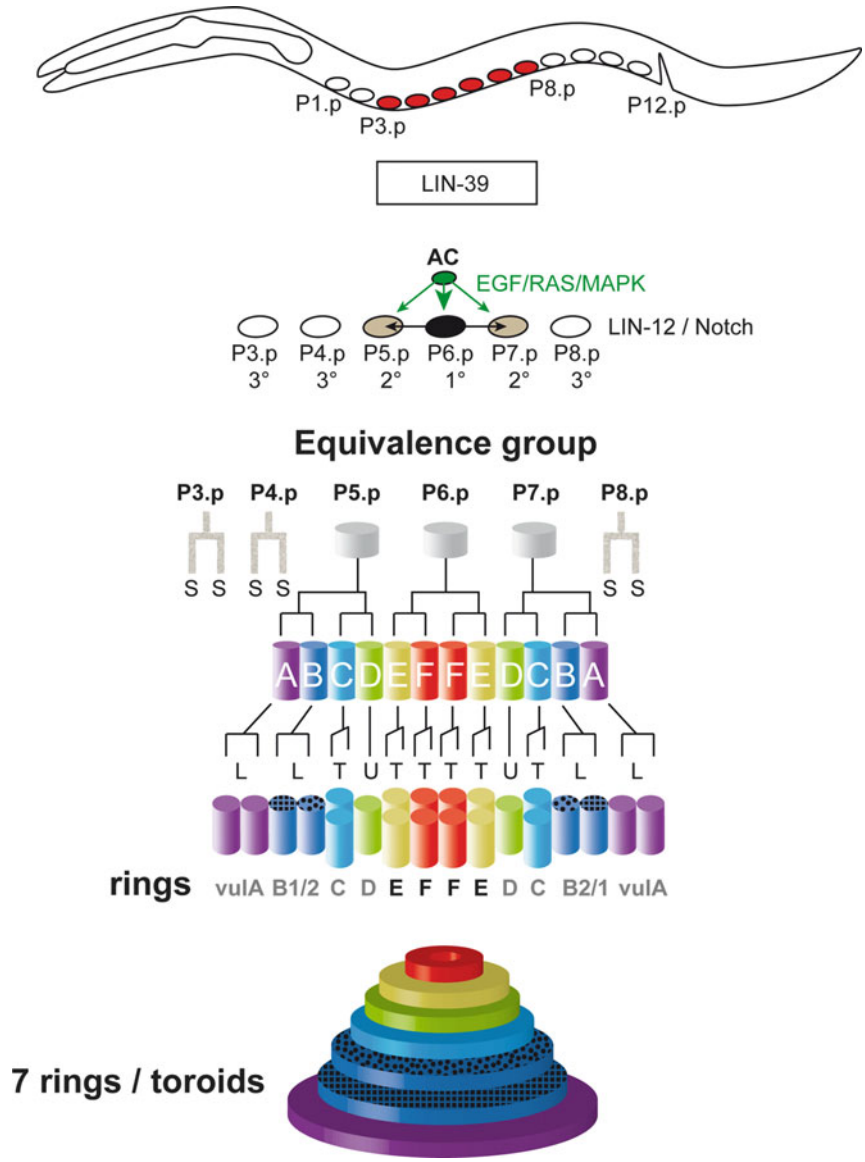

Fig. 2.6 Vulva development in *C. elegans*. *LIN-39* first determines the vulva equivalence group from P(3–8).p and acts as transcription factor downstream of EGF/RAS signaling. The midbody Pn.p cells P(3–8).p are set aside for vulva formation. Other Pn.p cells fuse in the L1 stage. P(3–8).p can adopt one of three alternative fates. P6.p adopts the inner vulval fate (1°, *black oval*), P(5,7).p the outer vulval fate (2°, *gray ovals*), and P(3,4,8).p a non-vulval fate (3°, *white ovals*). This spatial pattern of cell fates relies on an induction of vulval fates by the anchor cell (AC, *green oval*) of the gonad through EGF/Ras/Map kinase signaling (*green arrows*) and lateral signaling between P6.p and its neighbors through a Delta-Notch pathway (*black arrows*), which inhibits the 1° fate and activates the 2° fate in P(5,7).p. P(5–7).p forms vulval tissue by dividing three times and generating a total of 22 progeny. These 22 progeny partially fuse in later development and form a total of seven rings, often called toroids, as indicated in the bottom part of the figure. These rings form the scaffold-like structure of the vulva, which connects the uterus to the outside environment. Each fate corresponds to a specific cell division pattern that is executed in the late L3 stage, with characteristic orientations of the third round of division: *T* transverse division (*left-right*), *L* longitudinal (anterior-posterior division), *U* undivided. In the L4 stage, the symmetric cells of the P5.p and P7.p lineages, and of the two daughters of P6.p, migrate toward each other, fuse, and form seven superimposed syncytial rings around a vulval invagination. The two sisters of the B granddaughter form two rings, vulB1 and vulB2; the progeny of all other granddaughters form a single ring (Modified and redrawn with permission from Kiontke et al. (2007).)

hermaphrodites are modified females that produce sperm during a short period of larval development to become mature adult females. Hermaphrodites will use their self-sperm for fertilization. However, they can mate with males, the latter of which can easily be obtained and maintained under laboratory conditions as a result of meiotic nondisjunction of the X chromosome. These reproductive features of *P. pacificus* simplify a number of forward and reverse genetic tools, which facilitate mechanistic studies similar to *C. elegans*, *Drosophila* or *Arabidopsis*. In addition to forward and reverse genetics, DNA-mediated transformation is available in *P. pacificus*, providing an important tool for the manipulation of the organism under laboratory conditions.

Developmental Systems Drift

The theory of developmental systems drift was introduced by True and Haag in 2001 and provides a concept to explain the discrepancy between *macroscopic diversity* as seen in animals, plants, and fungi and *microscopic uniformity*, describing the general notion that animal life relies on a small number of signaling pathways that are conserved throughout the animal kingdom to regulate development in diverse organisms. True and Haag argued that the development of conserved morphological structures could involve large-scale modifications in their regulatory mechanisms and that developmental specification mechanisms might evolve rapidly and independent of the morphological structures they are specifying (True and Haag 2001). One example is the rapid evolution of sex determination in animals (see above).

The comparison of the molecular mechanisms involved in vulva formation in *C. elegans* and *P. pacificus* represents a second example for developmental systems drift. Vulva development in *P. pacificus* involves a set of evolutionary conservations and modifications when compared to *C. elegans*. First, the vulva is a homologous organ because it is formed from homologous precursor cells in both species. In contrast, vulva induction in *P. pacificus* is a continuous process that requires multiple cells and extends over more than 10 h of larval development (Sigrist and Sommer 1999). Detailed genetic and molecular studies revealed that *P. pacificus* vulva induction relies on Wnt signaling rather than EGF signaling (Tian et al. 2008; Wang and Sommer 2011).

There are two astonishing aspects of the different regulatory input to vulva induction. First, the role of EGF vs. Wnt signaling in *C. elegans* and *P. pacificus* evolved in the absence of changes in the genomic composition of these two nematodes. Both species contain largely similar genes in their genome that encode for ligands, receptors, cytoplasmic regulators, and transcription factors of all essential signaling pathways (EGF, Wnt, FGF pathways), and all these genes are 1:1 orthologs. Thus, changes in vulva induction do not depend on gene duplications and/or losses. Second, the change between *C. elegans* and *P. pacificus* occurred without major changes in gene expression, as all studied EGF and Wnt pathway genes in *P. pacificus* show expression patterns similar to *C. elegans* (Tian et al. 2008; Wang and Sommer 2011). Thus, changes in vulva regulation do also not depend on expression pattern changes. Rather, the functional specificity of individual genes and their encoded proteins changed during the course of evolution. One prime example is the LIN-17 protein, which represents a Frizzled-type receptor in nematode Wnt signaling. Genetic studies indicate that *Cel-lin-17* functions as an agonist that transmits Wnt-ligand signaling information (Sawa and Korswagen 2013). In contrast, *Ppa-lin-17* acts as an antagonist of Wnt signaling, indicating that the readout and regulatory linkage of proteins in regulatory networks can change substantially during the course of evolution (Wang and Sommer 2011). These findings result in the major conceptual conclusion that homologous structures formed by homologous cells can nonetheless be specified by completely different and unrelated molecular mechanisms, an extreme and prime example of developmental system drift.

INTEGRATIVE EVOLUTIONARY BIOLOGY

Research in EvoDevo as described above for nematodes tries to identify general principles involved in the generation of biological diversity. The idea to compare the development of different organisms in an evolutionary and phylogenetic context basically goes back to Darwin's principle of modification. However, the observed phenomenon of developmental systems drift, which is emerging as a general principle in EvoDevo if studies are performed in a mechanistic and causative framework, indicates the limits of a pure EvoDevo approach. Consequently, several authors have argued for more integrative approaches to tackle the evolution of function and form by considering all those research areas involved in the regulation and evolution of phenotypes (Gerhart and Kirschner 1997; Schlichting and Pigliucci 1998; West-Eberhard 2003; Sommer 2009). This results from the general notion that at least three research areas are involved in the study of the generation of form and diversity.

First, developmental biology and EvoDevo try to elucidate how morphological structures are formed throughout the ontogeny of the individual and how genetic and molecular alterations result in phenotypic evolution. This monograph represents a testimony of the success of EvoDevo in this research paradigm. However, additional research disciplines not covered in EvoDevo are also important for the understanding of biological diversity. A second research area is ecology, which contributes to the understanding of diversity by indicating how the environment influences development, resulting in evolutionary change ("EcoEvoDevo"). An ecological perspective on developmental processes is crucial to understand the generation of novelty over evolutionary timescales (Gilbert and Epel 2009). Third, population genetics describes how modifications and novelty arise as "natural variation" in populations (Lynch 2007). A population genetic perspective is thus a prerequisite for obtaining a comprehensive understanding of phenotypic change. It has been argued previously that a comprehensive understanding of the evolutionary forces that generate biological diversity requires integrative approaches that bring developmental biology and EvoDevo closer to evolutionary theory (Sommer 2009). Such integrative studies, when performed in the same organism to study the same patterns and processes, can be integrated into a comprehensive framework.

Developmental systems drift as observed between nematode species in sex determination and vulva development immediately results in the idea that distinct molecular mechanisms are recruited in response to the adaptation to different environments. Unfortunately, little is known about the exact ecology of many model systems in developmental and molecular biology. This was initially also true for the nematode model organisms. Recent years have seen major changes, resulting in the beginning of the description of the environment in which *C. elegans* and other nematodes can be found. *C. elegans* and other rhabditids but also *P. pacificus* can be found in soil samples, and interestingly, the strains used as "wild-type," *C. elegans* N2 and *P. pacificus* PS312, were indeed isolated from soil (Brenner 1974; Sommer et al. 1996).

However, work during the last decade has provided more detailed insight into the ecology of both species. *C. elegans* can most reliably be found in rotten fruits, often rotten apples (Felix and Duveau 2012). It is most reasonable to assume that *C. elegans* arrives on the apple fruit with a vector, probably insects, although details about the propagation are currently unknown. For nematode dispersal, it is long known that many species contain a specific dispersal stage, the arrested dauer larva (for details, see below). The association of nematode dauer larvae with insects has been called "phoresy" and represents a behavior also known from other invertebrates as a dispersal strategy (Poulin 2007).

The ecological association of *P. pacificus* had also been unknown for a long time. Detailed studies over the last decade, however, have provided ample of evidence for *Pristionchus* nematodes to be associated with scarab beetles (Fig. 2.7; Herrmann et al. 2006, 2010). While most *Pristionchus* species have a nearly species-specific association with scarab beetles, *P. pacificus* is found in association with different

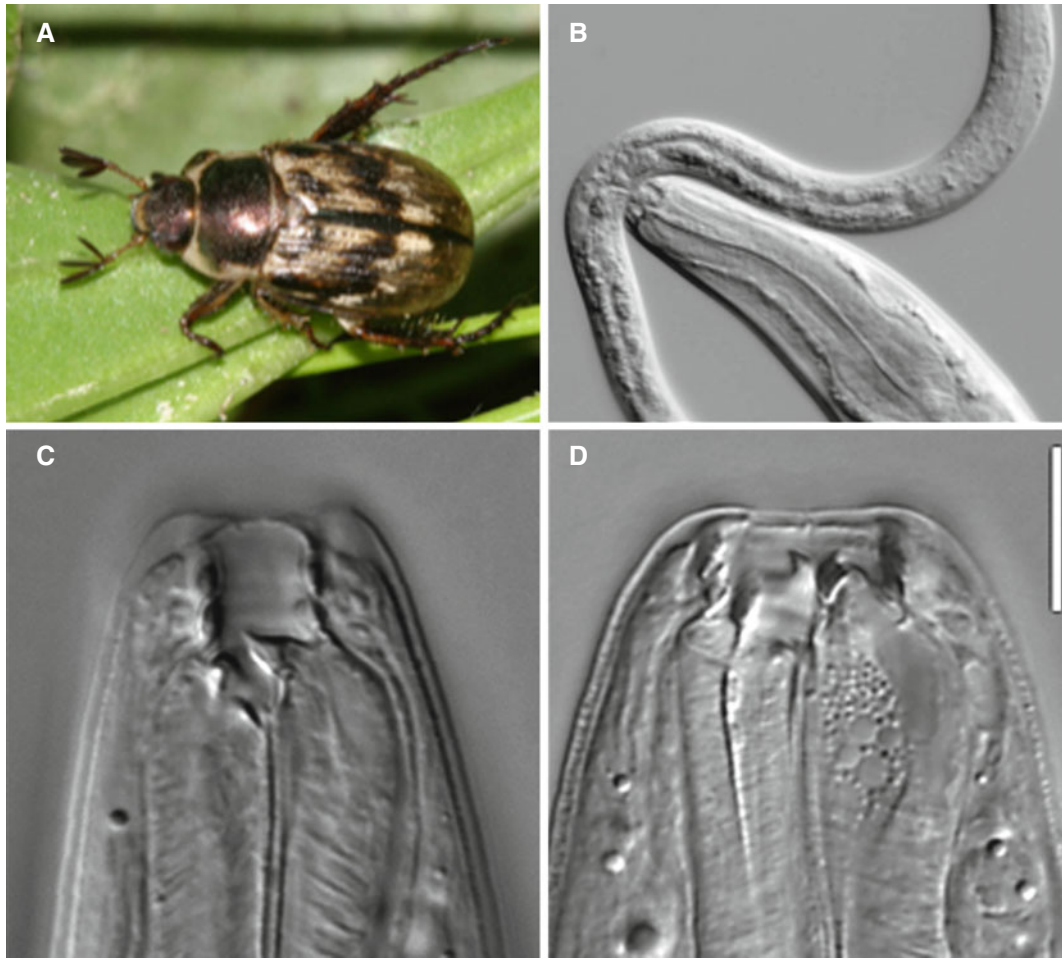

Fig. 2.7 Development of ecologically relevant traits. *P. pacificus* is found in tight association with scarab beetles, such as *Exomala orientalis* (**A**). It is an omnivorous feeder that can predate on nematode prey (**B**). For this to be achieved, *Pristionchus* and related nematode have evolved novel morphological structures in forms of teeth-like denticles. The mouth form of *P. pacificus* is dimorphic and can be either eurystomatous (**C**) or stenostomatous (**D**). The mouth dimorphism represents an example of phenotypic plasticity. Scale bar equals 10 μm (© Ralf J. Sommer 2015. All Rights Reserved)

scarabs in different parts of the world. *Pristionchus* nematodes rest on the beetle in the dauer stage and can use their insect vectors also for dispersal (Weller et al. 2010). In addition to this phoretic behavior, however, *P. pacificus* often remains on the beetle and waits for its natural death to feed on the developing microbes on the beetle carcass. Such associations have been called "necromeny" and are often considered to represent an additional step beyond phoresy toward the evolution of parasitism (Poulin 2007).

The inroads into the ecology of *P. pacificus* and related organisms have resulted in new research avenues. The following two paragraphs of this chapter will summarize recent findings of the development and function of dauer larvae and the evolution and development of feeding structures that have evolved in the context of the nematode-beetle ecosystem. These studies indicate the importance of considering EvoDevo questions in the context of ecology and, ultimately, population genetics.

THE DEVELOPMENT AND EVOLUTION OF DAUER LARVAE

Optimal growth conditions in the laboratory are unlike the harsh conditions that nematodes are exposed to in their natural environment. To survive adverse conditions, some groups of nematodes have evolved an effective survival strategy; a specialized alternative larval stage that is resistant to various environmental stresses called "dauer larvae" (Fig. 2.2). Dauer larvae show morphological, physiological, and behavioral adaptations that are not observed in other stages. This includes a closed mouth, a remodeled pharynx, and a thicker cuticle. Usually, dauers are formed as an alternative to the third larval stage, representing an example of phenotypic plasticity. The facultative nature of dauer formation enables a boom-and-bust lifestyle of nematodes: worms reproduce as much as possible while food is available but form arrested dauer larvae after food depletion. Besides survival, dauer larvae are specifically adapted for dispersal, enhancing the chances of finding a new food source (see above). It should be noted, however, that the exact ratio of direct vs. indirect development of *C. elegans*, *P. pacificus*, or other free-living nematodes is currently unknown. These uncertainties represent important shortcomings, as knowledge on the total number of nematode generations per year would be important for the understanding of divergence times between species.

In *C. elegans*, dauer formation is regulated by at least three environmental cues, i.e., starvation, high temperature, and high population density (Hu 2005). Among the many aspects of dauer biology, the genetic mechanisms regulating the entry into the dauer stage are most extensively studied, mainly using *C. elegans* (Hu 2005). In short, the decision to enter direct or indirect development is controlled by multiple signaling pathways, including insulin and TGF-ß signaling, which converge at the regulation of a hormone and the nuclear hormone receptor DAF-12. The transcription factor DAF-16 acts in parallel to DAF-12. Mutations in the *daf-12* and *daf-16* genes result in similar phenotypes deficient in dauer formation (Kenyon 2010).

Upstream of these signaling pathways act a number of small molecules to sense environmental cues, in particular population density. High population density triggers dauer formation and was recently shown to depend on a class of glycosides called ascarosides (for a review, see Ludewig and Schroeder 2013). Ascarosides contain a dideoxyhexose, ascarylose, as the sugar moiety, and they act as pheromones in the regulation of aggregation, mating, and the control of dauer development. However, little is known about the factors involved in linking ascaroside signaling to the signaling processes involved in dauer regulation.

EvoDevo studies on nematode dauer development have also focused on *P. pacificus*. As in *C. elegans*, dauer larvae are formed in response to starvation and high population density, involving a pheromone. The elucidation of the chemical nature of these small molecules resulted in the surprising finding that this nematode produces small molecules of a different composition and a much higher complexity than *C. elegans* (Bose et al. 2012). While still in their infancy, these studies might indicate that nematodes in general are very diverse in their secondary metabolite production, an idea that would correlate with the enormous genomic diversity seen in comparative nematode genome projects (for a review, see Sommer and Streit 2011).

The dauer larval stage as survival and dispersal stage is of tremendous importance for nematode ecology and evolution. Evolutionary theory would predict that the ecological properties of dauer larvae are under strong selection, resulting in natural variation for various dauer traits. Recent studies have started to investigate natural variation for dauer development by comparing multiple isolates of *P. pacificus* from around the world. Indeed, experimental studies of 16 *P. pacificus* strains showed that all strains produced a dauer pheromone (Mayer and Sommer 2011). Surprisingly, however, 13 of these 16 pheromones induce the highest rate of dauer formation in individuals of other genotypes, rather than of their own genotype. This cross-preference might point toward neutral evolutionary processes or

might be a sign of intraspecific competition, a previously unconsidered aspect of dauer formation (Mayer and Sommer 2011).

Indeed, more recent studies showed that the small molecule profiles of six sympatric and allopatric *P. pacificus* strains differ substantially from each other (Bose et al. 2014). Also, these strains differed in their dauer formation response to individual small molecules, and there was limited correlation between small-molecule production and sensing in individual strains. Finally, intraspecific competition was directly observed in a specifically designed competition assay between three sympatric strains from La Réunion Island and two allopatric strains from California and Ohio (Bose et al. 2014). Such studies begin to add novel, previously unconsidered aspects to our understanding of nematode dauer formation and its ecological relevance. Competitive interactions are part of evolutionary arm races that result in novelty and are predicted in an environment such as the scarab beetle ecosystem that *P. pacificus* lives in. *P. pacificus* dauer larvae of different haplotypes are often found on the same beetle individual, which might indicate that intraspecific competition is of relevance in the wild (Morgan et al. 2012).

THE EVOLUTION OF NOVELTY

EvoDevo research focuses primarily on two objectives: first, finding developmental regulators conserved during the course of evolution and, second, determining the changes resulting in the modification of development and ultimately morphology and form. These objectives are fully in line with Darwin's principle of common ancestry and "modification," resulting in the diversity of life seen all over the planet. However, besides modification, the evolution of novelty represents a second important objective necessary to understand how the diversity of form known today has been acquired. The evolution of novelty is often attributed to key innovations, which are defined as phenotypic traits that allow the subsequent radiation and success of a taxonomic unit (West-Eberhard 2003). One prime example of a key innovation is the neural crest, which resulted in the radiation of the vertebrates (Hall 1999).

Some authors have argued that phenotypic plasticity plays a crucial role in the generation of morphological novelty, a hypothesis that has been put forward under the term "facilitator of phenotypic evolution" (West-Eberhard 2003). The shared genetic control of dauer formation in free-living nematodes and infective juveniles in parasitic nematodes has been discussed as an example supporting this hypothesis (Poulin 2007; Sommer and Ogawa 2011). The following provides another example of phenotypic plasticity in form of a mouth dimorphism, which is unique for the genus *Pristionchus* and some related genera of the Diplogastridae family (Kanzaki et al. 2012).

P. pacificus worms are omnivorous feeders. With their necromenic lifestyle, they can find bacteria, protozoa, fungi, and other nematodes on beetle carcasses (Bento et al. 2010). *P. pacificus* is equipped with versatile teeth-like denticles in its mouth, and it can assume two forms, the so-called eurystomatous (EU) and the stenostomatous (ST) form (Fig. 2.7). EU worms are distinguished by a bigger clawlike dorsal left denticle and an extra right ventral denticle not present in ST animals, in addition to quantitative differences in the shape of the buccal cavity in EU worms (Fig. 2.7). The mouth of an individual nematode is irreversibly determined and executed during larval development. Interestingly, selection line experiments of ST and EU worms over several generations have indicated that the development of the mouth form is phenotypically plastic, representing another example of phenotypic plasticity in nematodes (Bento et al. 2010).

Several environmental perturbations strongly influence the mouth-form decision, with starvation showing one of the strongest effects (Bento et al. 2010). Given that starvation also regulates dauer formation in *P. pacificus* and *C. elegans*, genetic experiments have shown that dauer and mouth-form formation are regulated in part by similar molecular mechanisms. Specifically, the mouth-form decision is controlled by the nuclear hormone receptor *Ppa-daf-12*, and application of one of its steroid hormone ligands, dafachronic

acid (DA), also influences the mouth-form ratio (Bento et al. 2010). Thus, endocrine signaling module DAF-12/DA has been independently co-opted to the mouth-form regulatory network.

In contrast, other studies showed that mouth-form regulation relies on a number of novel regulators. First, unbiased chemical studies using NMR and mass spectrometry indicated a number of complex and novel small molecules to be involved in the regulation of the mouth form. For example, the dimeric ascaroside derivative *dasc#1* specifically regulates mouth form but not dauer development in *P. pacificus* (Bose et al. 2012). More recent genetic studies indicated that a novel sulfatase gene is part of a developmental switch mechanism that regulates the mouth-form decision in *P. pacificus*. Mutations in the *eud-1*/sulfatase result in the absence of the Eu mouth form, whereas overexpression fixes this form (Ragsdale et al. 2013). Surprisingly, it was shown that natural variation in mouth-form frequencies among natural isolates of *P. pacificus* involved expression differences of *eud-1*. Thus, microevolutionary differences can be attributed to a gene originally identified in a genetic screen. Using the possibility to form hybrids between *P. pacificus* and its gonochoristic sister species *P. exspectatus* provided further evidence for a role of the *eud-1* sulfatase also in the macroevolution of the mouth dimorphism (Ragsdale et al. 2013). The role of *eud-1* as a developmental switch brings long-standing research of phenotypic plasticity into the realm of molecular biology. In particular, the confirmation of the prediction of developmental switches (West-Eberhard 2003) indicates how the combination of laboratory studies and fieldwork (population genetics and the result isolation of wild strains) can result in new insight, helping to better integrate knowledge in evolutionary biology.

OUTLOOK

Nematode EvoDevo is an attractive research area given the easiness with which multiple nematodes can be studied in the laboratory. Not surprisingly, therefore, work on nematodes has contributed to the important conceptual finding of developmental systems drift, in particular with detailed studies on sex determination and vulva development.

Given some of the attractive technical features of nematodes, this group of animals might also play an important role for the next conceptual challenges in EvoDevo and, more generally, evolutionary biology. Specifically, the author of this chapter wants to highlight that detailed integrative case studies in a diversity of organisms, including insects, cnidarians, and nematodes and covering diverse approaches involving genetics, molecular biology, ecology, and population genetics, are necessary to obtain a broad and comprehensive picture about evolution and its underlying mechanisms and causes. Therefore, the most pressing open questions in nematode EvoDevo relate to the analysis of ecologically relevant traits. This involves a different perspective of research. One example that can highlight these novel needs and which has been outlined above is nematode dauer development. For a long time, researchers have focused purely on the developmental and genetic aspects of dauer formation. However, dauer development also represents an example of phenotypic plasticity, providing an important link to ecology and evolution. Any finding made for the regulation of dauer development has to be considered in the context of these disciplines. A population genetic (natural variation) perspective of dauer development can provide important new insights and questions, which will ultimately redirect research into new avenues (Mayer and Sommer 2011; Bose et al. 2014). Thus, modern EvoDevo research needs broad perspectives and open-minded researchers because development and organisms are linked to ecology and evolution ("EcoEvoDevo"). The time is ripe.

OPEN QUESTIONS

How Questions

- Development of reverse genetic tools for gene knockouts, i.e., TALEN and CRISPR
- Molecular phylogenic framework for EvoDevo organisms

- Case studies I: The evolution of sex determination and the mode of reproduction
- Case studies II: The evolution of the nervous system in the context of the organism's ecology
- Case studies III: The evolution of embryogenesis in the context of the organism's ecology

Why Questions

- Isolation of strains for a natural variation (population genetic) perspective
- Knowledge on the environment of EvoDevo model organisms
- Analysis of adaptive and nonadaptive forces acting on EvoDevo traits (i.e., sex determination, mode of reproduction, gonad development)
- The molecular mechanisms of developmental systems drift

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Tardigrada

3

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Chapter vignette artwork by Brigitte Baldrian.

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INTRODUCTION

Tardigrades, also known as "water bears," are microscopic invertebrates usually not exceeding 1 mm in length (Fig. 3.1A, B; Dewel et al. 1993; Kinchin 1994; Nielsen 2012). They are found in a variety of marine, limnic, and limno-terrestrial habitats, such as benthos, soil, and moss cushions. Many species can form environmentally resistant cysts, or tuns, that allow them to survive desiccation, freezing, high radiation levels, and other environmental extremes (Jönsson 2001; Møbjerg et al. 2011). Some species have even survived an exposure to outer space (Persson et al. 2011; Rebecchi et al. 2011). However, the mechanisms that enable them to withstand these extreme conditions remain poorly understood (Ramløv and Westh 2001; Hengherr et al. 2008; Schokraie et al. 2011; Halberg et al. 2013).

The Tardigrada consists of over 1,000 described species, divided into two major subgroups: the Eutardigrada and Heterotardigrada (Fig. 3.2; Guidetti and Bertolani 2005; Nichols et al. 2006; Degma and Guidetti 2007; Guidetti

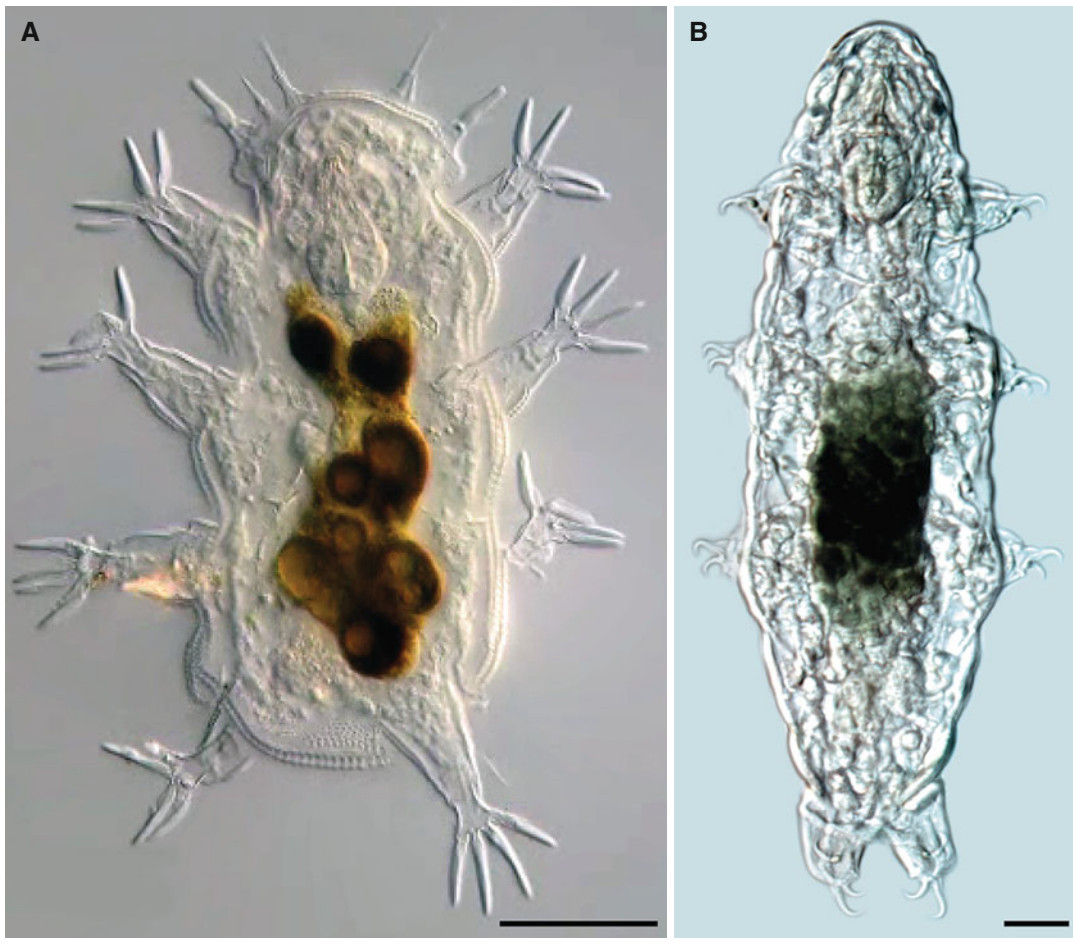

Fig. 3.1 Representatives of the two major tardigrade subgroups. (**A**) *Orzeliscus belopus*, a marine heterotardigrade, in dorsal view. (**B**) *Hypsibius dujardini*, a limno-terrestrial eutardigrade, in dorsal view (From Mayer et al. (2013b), creative common license of BMC). Scale bars: 30 µm

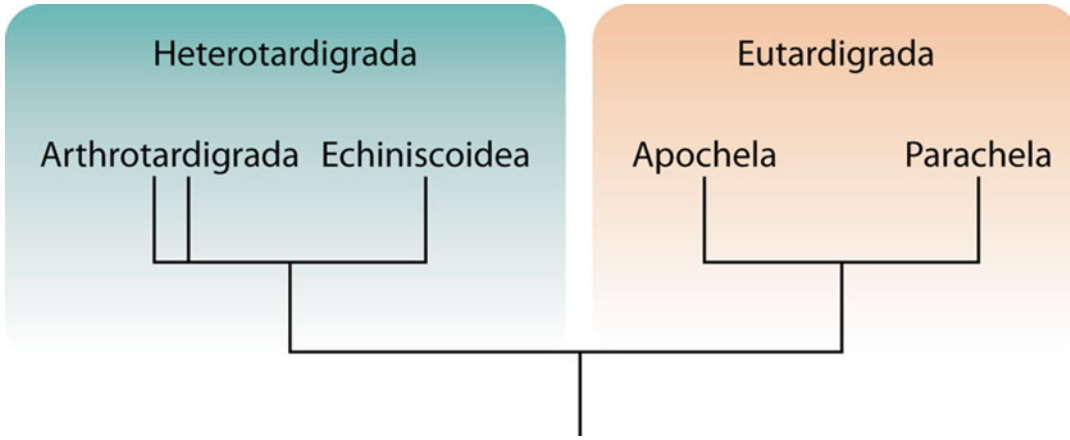

Fig. 3.2 Phylogeny of Tardigrada. Topology according to Guil and Giribet (2011). The *double line* for Arthrotardigrada indicates the putative non-monophyly of this group

and Bertolani 2011; Degma et al. 2014; Bertolani et al. 2014). Representatives of Eutardigrada are characterized by a body up to 1 mm in length with a smooth, unornamented cuticle (Fig. 3.1B). They are further split into two subgroups, Parachela and Apochela, both of which are likely monophyletic (Fig. 3.2; Guil and Giribet 2011). Species of Heterotardigrada typically do not exceed 500 µm in length (Fig. 3.1A) and exhibit a highly diverse cuticle and individual claws (not doubled as in eutardigrades). Heterotardigrades are also subdivided into two groups (Fig. 3.2). The Arthrotardigrada includes the vast majority of known marine species, while the Echiniscoidea consists of the armored, limno-terrestrial species and a few marine species (Nelson 2002; Nelson et al. 2009). The Arthrotardigrada is considered to be polyphyletic, as the Echiniscoidea is nested within it (Guil and Giribet 2011). Despite their morphological diversity, marine tardigrades are relatively understudied and represent only about 15 % of all described species, likely owing to their small size, low abundance, and difficulties with collection.

A third putative tardigrade subgroup, the Mesotardigrada, consists of a single species, *Thermozodium esakii*, described from a Japanese hot spring (Rahm 1937). This species has been reported to have an intermediate morphology between eutardigrades and heterotardigrades, possessing features that are characteristic of both groups. However, the type material has since been lost and the type locality destroyed by an earthquake. Since no additional specimens have been found, this taxon is considered dubious and is not accepted by most authorities (Nelson 2002). In addition, a fossil has been described from the Middle Cambrian that is undoubtedly a tardigrade, although it cannot be assigned to any extant taxon (Müller et al. 1995). This fossil, likely a member of the tardigrade stem group, strikingly resembles extant tardigrades, except that it possesses only three pairs of legs instead of four. Nevertheless, it indicates that tardigrades have changed relatively little in hundreds of millions of years.

The body of the extant eutardigrades and heterotardigrades is typically barrel-shaped and comprises a head and four trunk segments, each with a pair of unjointed legs, or lobopods (Dewel et al. 1993; Nielsen 2012). The entire body, including any sense organs and locomotory appendages, is enclosed within a chitinous cuticle, which is molted periodically throughout the entire life cycle (Walz 1982; Greven and Peters 1986). The morphology of the cuticle can vary greatly between species, and its various plates and elaborations are often some of the most striking features of a tardigrade (Greven 1972; McKirdy et al. 1976; Grimaldi de Zio et al. 1982;

Kristensen 1987; Guidetti et al. 2000). Sclerotized claws or adhesive organs, which are also cuticular, are attached to each of the eight legs in most species (Schuster et al. 1980; Grimaldi de Zio et al. 1987; Kristensen and Higgins 1989). Marine tardigrades usually have 4 individual claws mounted on digits, while terrestrial tardigrades have either 2 double claws or up to 12 individual claws attached directly to the foot. Tardigrade claws are extremely morphologically diverse and, as a result, present a powerful taxonomic tool at all levels within the entire group (Pilato 1982; Renaud-Mornant 1982; Bertolani and Kristensen 1987). All cuticular structures, including the claws and mouthparts, are also expelled during molting and subsequently reformed.

The mouth is positioned terminally or ventrally and leads into a buccal tube, which is associated with a complex buccopharyngeal apparatus that is unique to the Tardigrada (Schuster et al. 1980; Eibye-Jacobsen 2001a; Guidetti et al. 2012, 2013). The mouth leads into the buccal tube, which then follows into a muscular, pharyngeal bulb (Dewel and Clark 1973a; Walz 1973; Eibye-Jacobsen 2001b). The buccal tube is flanked laterally by a pair of sharp stylets responsible for piercing the food item, the contents of which are then ingested through the mouth and buccal tube via the sucking action of the pharynx (Marcus 1929; Ramazzotti and Maucci 1983). The secretion mechanism of the stylets and stylet supports and the organization of their associated musculature suggest that these structures are modified limbs (Halberg et al. 2009; Nielsen 2012; Ou et al. 2012). The pharyngeal bulb is followed by a short esophagus, a simple midgut, and a short, ectodermal hindgut (Dewel and Clark 1973a, b, c; Greven 1976; Dewel and Dewel 1979; Dewel et al. 1993; Rost-Roszkowska and Poprawa 2008; Nielsen 2013). Tardigrades generally feed on bacteria, algae, plant matter, and other microorganisms, while a few predatory species feed on nematodes and rotifers (Marcus 1927; Doncaster and Hooper 1961; Kinchin 1994; Hohberg and Traunspurger 2009; Schill et al. 2011).

Malpighian tubules are the main organs of the excretory system, which has been examined exclusively in eutardigrades (Dewel and Dewel 1979; Węglarska 1980). Three Malpighian tubules are present in most eutardigrades – two lateral and one dorsal – that open into the gut, between the midgut and hindgut. These structures likely serve a dual function, as they are also responsible for osmoregulation (Greven 2007). This is especially evident in the marine eutardigrade, *Halobiotus crispae*, where the Malpighian tubules are exceptionally large (Kristensen 1982). No such structures have been reported from heterotardigrades.

The central nervous system of tardigrades consists of a dorsal ganglionic brain and four trunk ganglia, which are interconnected by somata-free connectives (Doyère 1840; Greeff 1865; Marcus 1929; Zantke et al. 2008; Persson et al. 2012, 2014; Mayer et al. 2013a, b; Schulze and Schmidt-Rhaesa 2013; Schulze et al. 2014). Of the four trunk ganglia, the first three are similar in size, while the fourth is slightly smaller. A central fiber mass connects the two hemiganglia, which are fused along the midline. Additional, extra-ganglionic commissures link the connectives anterior to the second, third, and fourth trunk ganglia (Doyère 1840; Greeff 1865; Plate 1889; Mayer et al. 2013a; Schulze and Schmidt-Rhaesa 2013). In each leg-bearing segment, a pair of leg nerves arises from its respective trunk ganglion. The anterior of the two leg nerves is associated with a peripheral ganglion within the leg (Mayer et al. 2013a).

Despite the recent progress in tardigrade neuroanatomy, ample controversy remains regarding several other aspects of the nervous system. For example, the presence of a subpharyngeal/subesophageal ganglion remains dubious (Zantke et al. 2008; Persson et al. 2012, 2014; Mayer et al. 2013a, b; Schulze and Schmidt-Rhaesa 2013; Schulze et al. 2014), and the segmental composition of the tardigrade brain is still uncertain. It is unclear whether the brain comprises one or several segments or whether it is a non-segmental structure (Dewel et al. 1999; Zantke et al. 2008;

Persson et al. 2012, 2014; Mayer et al. 2013b; Schulze and Schmidt-Rhaesa 2013; Schulze et al. 2014). Recent findings on the stomatogastric nervous system, however, suggest that the tardigrade brain comprises only one segment, which is homologous to the protocerebrum of arthropods and onychophorans (Mayer et al. 2013b).

Tardigrade sensory structures are most evident as filamentous cirri (also called "cephalic appendages") on the head of marine tardigrades. A full set of cephalic sense organs consists of up to 13 structures: paired internal, external, and lateral cirri; up to 3 pairs of clavae (cephalic papillae); and an unpaired, dorsal median cirrus (Kristensen 1981; Wiederhöft and Greven 1999; Dewel and Eibye-Jacobsen 2006). Every sense organ is constructed in a similar fashion and resembles the sensory setae of arthropods (Kristensen 1981). Most eutardigrades lack such sense organs entirely but have sensory fields on the head and around the mouth cone (Walz 1978; Kristensen 1981).

Few dedicated studies regarding the eyes have been performed to date (Greven 2007). Pigmented, cuplike eyes – generally black, brown, or red – are found in most eutardigrades and echiniscoideans (Marcus 1929). These eyes are present inside the lateral lobes of the brain rather than being adjacent to the body wall (Greven 2007). Such eyes are absent in arthrotardigrades, although they may simply have escaped detection due to a lack of shading pigment or, alternatively, these animals have other types of photoreceptors (Kristensen 1978).

The body musculature is organized into many strap-shaped muscles that can be grouped as ventral, dorsal, and lateral (Marchioro et al. 2013). Most dorsal and ventral muscles are longitudinal and span the length of the body. Leg muscles generally span the entire length of the leg. The number of fibers in each of the first three pairs of legs is relatively similar, while that of the posterior-most leg pair is reduced (Schmidt-Rhaesa and Kulessa 2007; Halberg et al. 2009; Marchioro et al. 2013). Eutardigrades have a higher number of muscle fibers than do heterotardigrades, but each fiber tends to be thinner. Heterotardigrade muscles are also cross-striated, while those of eutardigrades are obliquely striated, except for those associated with the buccopharyngeal apparatus, which are cross-striated in all tardigrade species (Schmidt-Rhaesa and Kulessa 2007; Halberg et al. 2009; Schulze and Schmidt-Rhaesa 2011; Marchioro et al. 2013).

The reproductive system consists of a single large gonad dorsal to the midgut (Dewel et al. 1993). All marine tardigrades are dioecious (separate male and female sexes), where a pair of sperm ducts is present in males and a pair of prominent seminal receptacles is often visible in females (Kristensen and Higgins 1984, 1989; Hansen et al. 2012). The male gonopore is tube-shaped, whereas the female gonopore is surrounded radially by six or seven conspicuous cells (Dewel and Dewel 1997). The gonopore of both sexes is positioned anterior to the anus. In eutardigrades, both male and female reproductive tracts open into the rectum, forming a true cloaca (Dewel and Dewel 1979; Kinchin 1994). Dioecious species may exhibit minor sexual dimorphism, evident in the gonopore, total body size (males are often smaller than females), and, in some species, the primary clavae (Renaud-Mornant and Deroux 1976). Freshwater species may be dioecious or parthenogenetic, while hermaphroditism is restricted to limno-terrestrial species, which represent all three reproductive modes (Bertolani 2001).

Tardigrades are perplexing in terms of evolution because their exact phylogenetic position has not yet been resolved (Jenner and Scholtz 2005; Dunn et al. 2008; Meusemann et al. 2010; Rota-Stabelli et al. 2010; Campbell et al. 2011; Rehm et al. 2011). They are widely accepted as panarthropods, being closely related to arthropods and onychophorans (velvet worms), but their precise relationship to one of these animal groups remains controversial (Rota-Stabelli et al. 2010; Campbell et al. 2011; Nielsen 2012; Mayer et al. 2013a). All panarthropods share several features, including a dorsal brain, ventral nerve

cords, segmented body, and paired locomotory appendages (Nielsen 2012; Persson et al. 2012, 2014; Mayer et al. 2013a). Additionally, molecular data based on 18S rRNA and mitochondrial sequence data as well as microRNA sequences support onychophoran/arthropod rather than cycloneuralian affinity of tardigrades (Garey et al. 1996, 1999; Giribet et al. 1996; Garey 2001; Ryu et al. 2007; Rota-Stabelli et al. 2010; Campbell et al. 2011). Because of their key phylogenetic position, tardigrades play an important role for understanding the evolution of arthropods.

EARLY DEVELOPMENT

Tardigrade eggs can be deposited either freely into the environment or into the old exuvium after ecdysis (Kinchin 1994). Those that are laid freely often have elaborate and diverse processes projecting from the egg shell that are of high taxonomic value (Bertolani and Rebecchi 1993). The function of these elaborations is not known, but they have been postulated to help maintain position in the substrate, avoid predation, or slow dehydration (Kinchin 1994). On the other hand, eggs that are left in the shed exuvium are usually smooth. Ultrastructural studies of egg capsules have been done only in three tardigrade species – all eutardigrades – but all eggs examined to date have a double egg shell consisting of an inner vitelline envelope and an outer, multilayered chorion (Węglarska 1982; Poprawa 2005, 2010). The chorion is secreted first by both the oocyte and the gonad wall, before secretion of the vitelline envelope by the oocyte (Poprawa 2010). Development time varies greatly between species and also depends on environmental factors such as temperature. For example, the development time of *Hypsibius dujardini* is approximately 4 days (Gabriel et al. 2007), while that of *Halobiotus crispae* is 2 weeks (Eibye-Jacobsen 1996/97).

Hypsibius dujardini: An Emerging Model for Embryonic Development

The eutardigrade *Hypsibius dujardini* (Doyère, 1840) has recently been emerging as a model organism for evolutionary developmental biology (Fig. 3.1B). This species, typically not exceeding 750 µm, is commonly found in freshwater habitats of most European countries, where it feeds on unicellular algae (Marcus 1929). *Hypsibius dujardini* shows a number of features that make it amenable to developmental studies, such as a compact genome and short generation and development times (Gabriel et al. 2007). Additionally, *Hypsibius dujardini* is primarily parthenogenetic (although males have been reported), making it ideal for continuous culturing (Gabriel et al. 2007). In fact, one strain in particular that was collected from a benthic pond sample in Great Britain in 1987 has been in culture ever since (Gabriel et al. 2007). Cultured females usually deposit two to ten smooth eggs (diameter 50–75 µm) into the shed exuvium after ecdysis and the transparent eggs and embryos allow for live imaging during development (Figs. 3.3A–H and 3.6A, B; Gabriel et al. 2007; Tenlen et al. 2013). A number of protocols for embryos of *Hypsibius dujardini* have already been established, including those for cytochemical and immunolabeling experiments as well as for disruption of gene function using parental RNA interference (Figs. 3.4A–C and 3.5A, B; Gabriel and Goldstein 2007; Tenlen et al. 2013). In recognition of its value as an arthropod outgroup, *Hypsibius dujardini* has been selected for genome sequencing as part of the Ecdysozoan Sequencing Project (http://www.genome.gov/10002154).

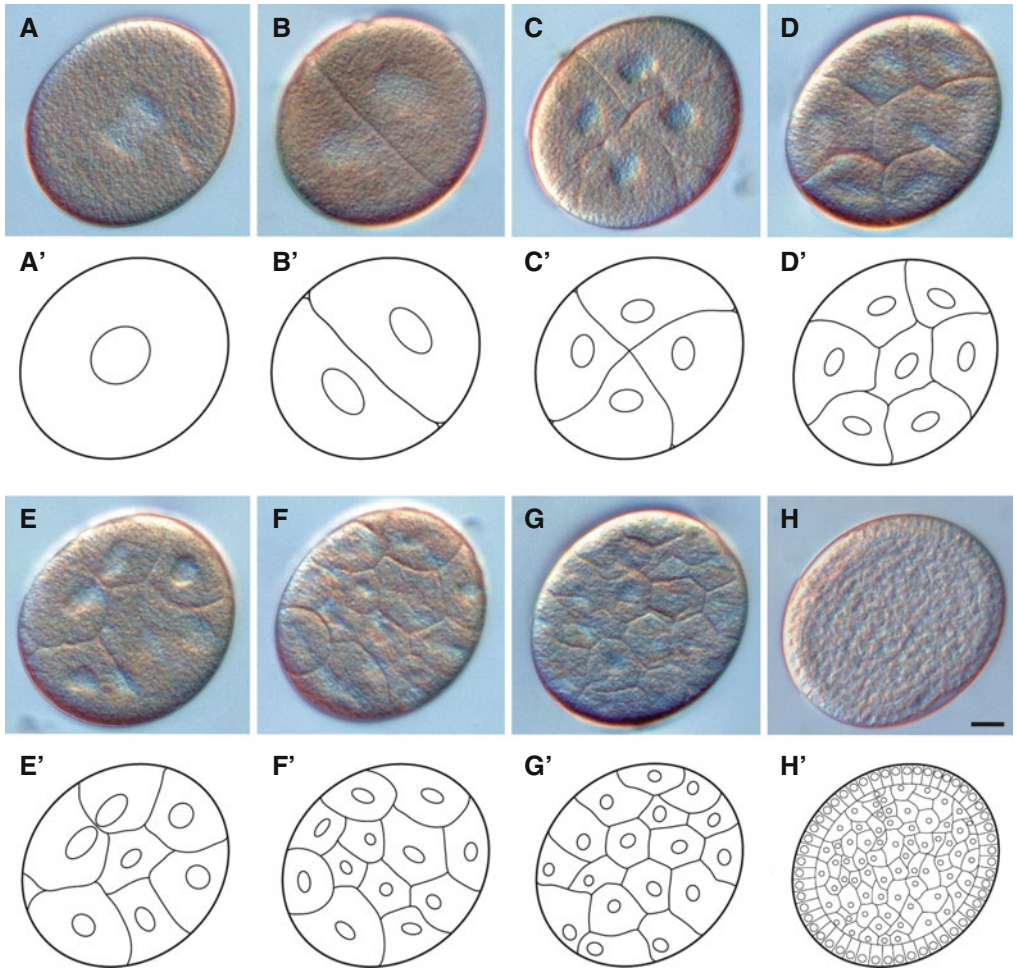

Fig. 3.3 Cleavage in *Hypsibius dujardini*. Light micrographs (**A**–**H**) and their corresponding schematic drawings (**A′**–**H′**). Whole embryos except in **H′**, which represents only a subset of the embryo. (**A**, **A′**) One-cell stage. (**B**, **B′**) Two-cell stage. (**C**, **C′**) Four-cell stage. (**D**, **D′**) Eight-cell stage. (**E**, **E′**) Eight-cell stage. The nuclei of two adjacent cells have migrated toward each other indicating the future ventral side of the embryo. (**F**, **F′**) 16-cell stage. (**G**, **G′**) ~32-cell stage. (**H**, **H′**) Late gastrula, ~500 cells. Scale bar in **H** for all images: 10 μm

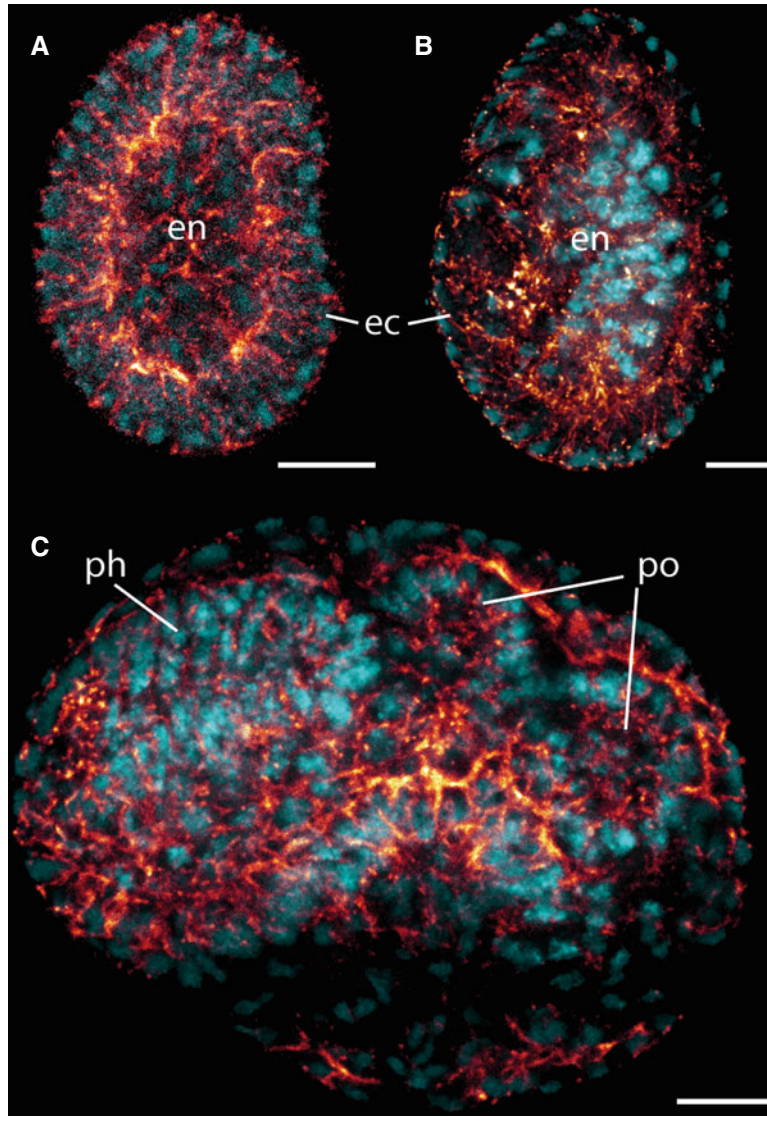

Fig. 3.4 Early developmental stages of *Hypsibius dujardini* stained with a fluorescent marker for filamentous actin (phalloidin-rhodamine, *glow* scale) combined with a nuclear marker (SYBR® Green, *cyan*). Confocal micrographs, maximum projections. (**A**) Gastrula. An epithelium is formed by migration of cells into the center of the embryo and subsequent closure of the resulting gap by epiboly. (**B**) Gastrula. Shortly after the epithelium formation is completed, the embryo starts to elongate, resulting in a flexed shape. (**C**) Later developmental stage, after gastrulation and elongation of the embryo. Segmental mesodermal pouches are seen in the posterior half of the embryo. The outline of the developing pharynx is already visible in the anterior part of the embryo. Abbreviations: *ec* ectoderm, *en* endoderm, *ph* pharynx, *po* mesodermal pouches. Scale bars: 10 µm

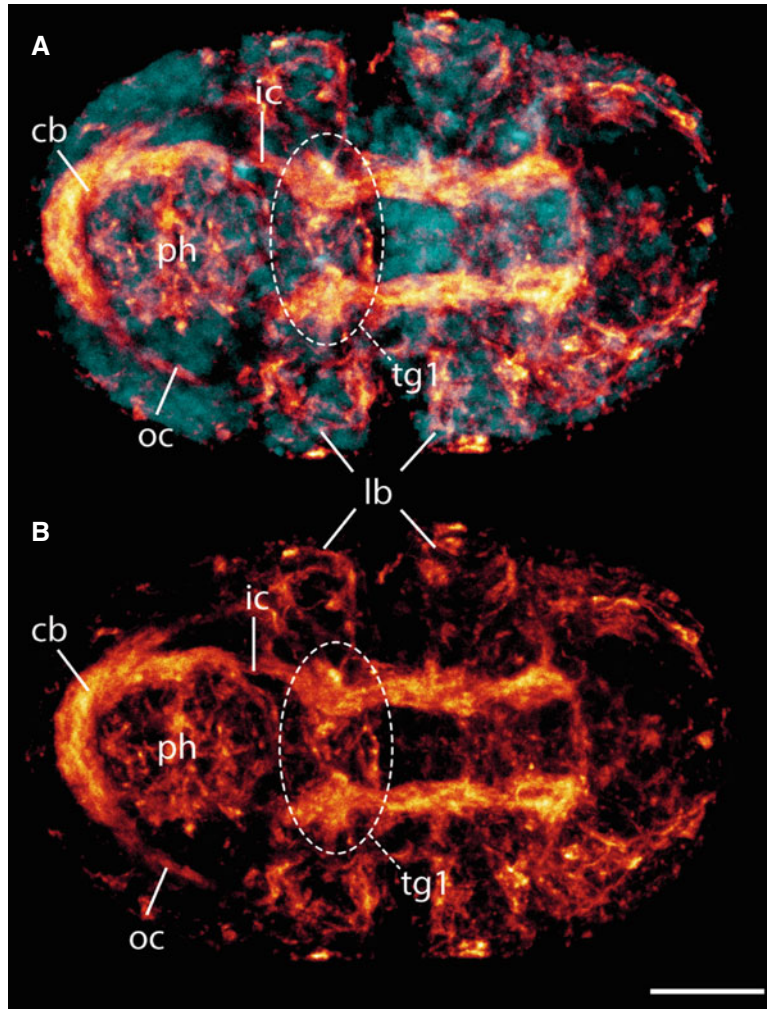

Fig. 3.5 Developing nervous system in an embryo of *Hypsibius dujardini* stained with phalloidin-rhodamine (*glow* scale) and SYBR® Green (*cyan*). Ventral view, anterior is *left*. Confocal micrographs, maximum projections. (**A**) Combined phalloidin-rhodamine and SYBR® Green labeling. (**B**) Separated phalloidin-rhodamine channel of the same embryo. Abbreviations: *cb* anlage of the central brain neuropil, *ic* inner connective, *lb* limb buds, *oc* outer connective, *ph* pharynx, *tg1* first trunk ganglion. Scale bar: 10 µm

Very few tardigrade developmental studies have been done in the past, and many of these were carried out over 80 years ago (von Erlanger 1895; von Wenck 1914; Marcus 1929). Cleavage in tardigrades is generally accepted as total, with evenly distributed yolk granules (=isolecithal) (Fig. 3.3A–H; Marcus 1929; Eibye-Jacobsen 1996/97; Hejnol and Schnabel 2005; Gabriel et al. 2007; Scholtz and Wolff 2013). However, conflicting reports regarding the cleavage pattern and potential cell fates have been published in recent years. Laser ablations of early blastomeres in *Thulinius stephaniae* (=*Thulinia stephaniae*; see Bertolani 2003) provide evidence for indeterminate cleavage (Hejnol and Schnabel 2005). Embryos in which one cell of the two-cell stage and one or two cells of the four-cell stage were ablated eventually hatched into normal juveniles.

In some cases, development took longer or the juveniles were smaller, but they still were able to form all tissues and structures (Hejnol and Schnabel 2005). Additionally, all ablated embryos still formed two germ cells, indicating that the germ line is not predetermined, but arises sometime later in development. *Thulinius stephaniae* therefore represents the highest regulatory potential described to date for a protostome (Hejnol and Schnabel 2005).

In contrast to the indeterminate cleavage observed in *Thulinius stephaniae*, Gabriel et al. (2007) reported a stereotyped cleavage pattern in *Hypsibius dujardini*. The authors consider it unlikely that the reported differences in cleavage patterns are due to discrepancies in the identification of individual cells, attributing them instead to real variation between tardigrade species. Despite laser ablation not being used in *Hypsibius dujardini*, the authors suggest that differing results are not unusual in other animal groups (e.g., Félix and Barrière 2005). For example, even closely related arthropod lineages often show wildly different cleavage patterns (Hejnol and Schnabel 2006; Scholtz and Wolff 2013). In either case, since total cleavage is found at least in some representatives of both Onychophora and Arthropoda (von Kennel 1885; Anderson and Manton 1972; Scholtz and Wolff 2013), this type of cleavage was most likely present in the last common ancestor of Panarthropoda.

Results concerning the synchrony of early cleavages have also been inconsistent. According to von Erlanger (1895) and Hejnol and Schnabel (2005), cleavage is synchronous for at least the first three divisions (Fig. 3.3A–C), while von Wenck (1914), Marcus (1929), and Eibye-Jacobsen (1996/97) found asynchronous cell divisions throughout embryonic development. In *Hypsibius dujardini*, two of the cells of the four-cell stage divide asymmetrically (Gabriel et al. 2007). The smaller of the daughter cells have a delayed cell cycle relative to all other cells until the ~60-cell stage, at which point one or both cells ingress. The blastula appears to be a solid ball of cells with no obvious blastocoel, i.e., a sterroblastula (Figs. 3.3H and 3.4A).

The mechanism of dorsoventrtal axis formation in tardigrades is not yet known but is most likely similar to that in onychophorans, crustaceans, and insects (Dearden and Akam 2001; Treffkorn and Mayer 2013), as there is no evidence of a cumulus or cumulus-like structure in the early tardigrade embryo. This is in contrast to the chelicerate and myriapod embryos, where the cumulus initiates the breakdown of radial symmetry (Sakuma and Machida 2002; McGregor et al. 2008). In *Hypsibius dujardini*, Gabriel et al. (2007) were able to recognize a stereotyped pattern of nuclear migrations on the ventral side of the animal, allowing for the identification of the dorsoventral axis as early as the four-cell stage. Similarly, following nuclear migrations during the elongation stage (shortly before the appearance of the mesodermal pouches) allows for the orientation of the anterior-posterior axis.

On the other hand, Hejnol and Schnabel (2005) were unable to identify the dorsoventral axis in early embryos of *Thulinius stephaniae*. The authors could not reliably follow the progeny of each cell across different embryos due to equal size and identical morphology of the blastomeres and variable spindle positions (Hejnol and Schnabel 2005). Unfortunately, polar bodies are not useful for egg orientation either (von Erlanger 1895; von Wenck 1914; Marcus 1929; Eibye-Jacobsen 1996/97). As a result, axes could not be determined in early embryos of this species, especially since there were no cell migrations before gastrulation. Both axes can only be identified at the initiation of gastrulation based on the position of the blastopore.

Gastrulation (Fig. 3.4B) is initiated via the first directed cell migrations that are detectable in the embryo (Hejnol and Schnabel 2005; Gabriel et al. 2007). In *Hypsibius dujardini*, the first cells to move are the progeny of the asymmetrically dividing cells, which migrate through the blastopore (Gabriel et al. 2007). Ectodermal cells then seal off this opening by epiboly (thinning and spreading of the ectodermal cell layer), forming an epithelium. The process is more complex in *Thulinius stephaniae*, where two distant openings

are present rather than one (Hejnol and Schnabel 2005). The primordial germ cells are the first to migrate and do so through the blastopore in the anterior region of the embryo. They are followed by the mesodermal and endodermal precursors, respectively. Meanwhile, the ectodermal precursors move in through the second, posterior opening but shortly resurface, plugging the pore. After gastrulation, a new pore arises at this position that will form the hindgut, while the anterior pore becomes the mouth (Hejnol and Schnabel 2005).

Primordial germ cells are likely specified as early as the two-cell stage in *Thulinius stephaniae* (Hejnol and Schnabel 2005). After migrating through the blastopore during gastrulation, the primordial germ cells temporarily linger at the ventral side of the developing midgut before migrating dorsally and posteriorly to the final position of the future gonad, above the gut. Similarly, von Wenck (1914) and Marcus (1929) described "early germinal cells" ("Urkeimzellen") in the ventral archenteron (embryonic gut). Eibye-Jacobsen (1996/97) did not find such cells in *Halobiotus crispae*, but attributes their perceived absence to a possible artifact of her specific protocol.

Mesoderm development historically has been a highly contentious issue in tardigrade development and remains to be resolved (von Erlanger 1895; von Wenck 1914; Marcus 1929; Eibye-Jacobsen 1996/97; Hejnol and Schnabel 2005; Gabriel et al. 2007). Initially, it was thought that the mesoderm originates from the archenteron; endomesodermal pouches pinch off from the archenteron and form four pairs of segmental sacs (=somites) that later differentiate into various mesodermal organs (von Erlanger 1895; Marcus 1929). Gabriel and Goldstein (2007) support this hypothesis by offering evidence of birefringent granules in the pouch cells, noting that similar granules are specific to the endodermal (=gut) cells in the nematode *Caenorhabditis elegans* (Siddiqui and Babu 1980). These cells are also believed to exhibit alkaline phosphatase activity, which is indicative of larval gut cells, at least in brachiopods (Freeman 2003). However, the data provided by Gabriel and Goldstein (2007) do not provide irrefutable evidence that the birefringent granules and alkaline phosphatase activity are indeed localized in cells of the mesodermal pouches and not in the developing gut cells.

While the presence of mesodermal pouches is widely accepted (Fig. 3.4C), a clear picture of their origin and fate has yet to emerge. Eibye-Jacobsen (1996/97) could not clarify the situation in *Halobiotus crispae*, but Hejnol and Schnabel (2005) provide evidence against mesoderm formation via outpocketing of the archenteron in *Thulinius stephaniae*. Instead, they contend that precursors of every germ layer are already present at the beginning of gastrulation. The mesodermal precursors proliferate as they migrate along the inside of the outer ectodermal layer, forming mesodermal bands that then separate into the four pairs of somites. It is uncertain whether the pouches are solid or hollow and whether or not their cavities, if present, are lined by true epithelia like the somites of onychophorans and arthropods (Anderson 1973; Mayer et al. 2004, 2005). Eibye-Jacobsen (1996/97) reported the presence of cavities within each pouch in *Halobiotus crispae*, but Hejnol and Schnabel (2005) described solid balls of cells in *Thulinius stephaniae*, although this could possibly be due to the compression of the embryo. In any case, mesoderm originating from the archenteron is a characteristic feature of deuterostomes, with a few exceptions (e.g., chaetognaths; Nielsen 2012). This casts further doubt on the idea that this mechanism also exists in the tardigrade embryo. As it stands, the origin of the mesoderm in tardigrades remains an open issue and requires more research to clarify.

The buccopharyngeal apparatus, pharynx, and esophagus are likely derived from ectoderm, as they arise from the region surrounding the stomodeum and contain cuticular elements (Marcus 1929; Dewel and Clark 1973a, b, c; Eibye-Jacobsen 1996/97, 1997; Gabriel et al. 2007). The earliest developing structure of the digestive tract is the pharynx; its outline is first evident

around the point when the mesodermal pouches appear (Fig. 3.4C; Eibye-Jacobsen 1997). The midgut is formed shortly thereafter (Gabriel et al. 2007). The stylets, buccal tube, and other cuticular structures of the buccopharyngeal apparatus are formed only when secretion of the cuticle begins, after neurogenesis. In contrast to the stylets and stylet supports, which are secreted by the salivary glands, the cuticular lining of the pharynx is most likely secreted by the pharyngeal cells (Marcus 1929; Dewel and Clark 1973b; Guidetti et al. 2012).

Little information is available on neurogenesis and neural development in tardigrades. Marcus (1929) described the nervous system as arising from a single, continuous anlage stretching from slightly anterior of the stomodeum to the proctodeum. He suggests that this anlage is first present around the stage when the mesodermal pouches are still attached to the midgut and gives rise to all structures of the nervous system simultaneously, including the brain. Eibye-Jacobsen (1996/97) supports his assertion that all nervous structures arise simultaneously but argues that this occurs later in development. However, more recent studies have provided no evidence for a continuous, unitary anlage of the nervous system in either *Thulinius stephaniae* or *Hypsibius dujardini* (Hejnol and Schnabel 2005; Gabriel et al. 2007). The four trunk ganglia instead arise from four neural progenitor cells; each progenitor cell, initially located in the ventral ectoderm, immigrates and gives rise to the corresponding trunk ganglion (Hejnol and Schnabel 2005). The brain of *Thulinius stephaniae* is also formed by neural progenitor cells, although their number is unknown (Hejnol and Schnabel 2005). Notably, the cerebral progenitors immigrate prior to those forming the trunk ganglia, suggesting that development of the tardigrade brain is initiated before that of the trunk ganglia. A putative anlage of the subpharyngeal/subesophageal ganglion was not detected by Hejnol and Schnabel (2005) in embryos of *Thulinius stephaniae*, nor is it seen in embryos of *Hypsibius dujardini* (Fig. 3.5A, B).

LATE DEVELOPMENT

When a tardigrade is ready to emerge from the egg (Fig. 3.6A), hatching is accomplished via a combination of swelling of the body due to water intake and stylet or hind leg action to pierce the egg membrane (Ramazzotti and Maucci 1983). The newly emerged juvenile is approximately three times the size of the egg (cf. Fig. 3.6A, B) and is immediately self-sufficient (Hallas 1972). In contrast to many arthropods, tardigrades do not display a true larva in the sense that juveniles do not undergo a radical metamorphosis, possess unique structures that are absent from the adults, or occupy different habitats. In fact, eutardigrade hatchlings are simply miniature versions of the adults (Bertolani et al. 1984).

Contrary to eutardigrades, heterotardigrades do exhibit minor postembryonic development primarily involving the cuticle and cuticular structures. This process can be separated into three stages (Bertolani et al. 1984). The first instar juveniles often have a reduced number of digits relative to the adult (usually two fewer) and lack an anus and gonopore. Despite the absence of a functional anus, the first instar is still a feeding stage with defecation occurring into the old cuticle during the first molt. The remaining claws and anus are generated at the beginning of the second stage, which usually, but not necessarily, corresponds to the first molt. At this point, the gonopore is still absent or, at most, severely underdeveloped. The third and final stage begins with the development of the gonopore and represents the complete adult morphology. The majority of the life cycle is spent in this stage (Bertolani et al. 1984).

A number of exceptions have been observed for various species, but they all essentially follow the same pattern. For example, the morphology and number of the cuticular projections of many echiniscids vary between juveniles and adults. In general, adults have more projections than juveniles, or especially in species where the number remains constant, e.g., *Cornechiniscus ceratophorus*, the projections are longer in adults (Maucci 1972; Ramazzotti and Maucci 1983). At least four juvenile stages have been observed in *Batillipes noerrevangi*, in which a

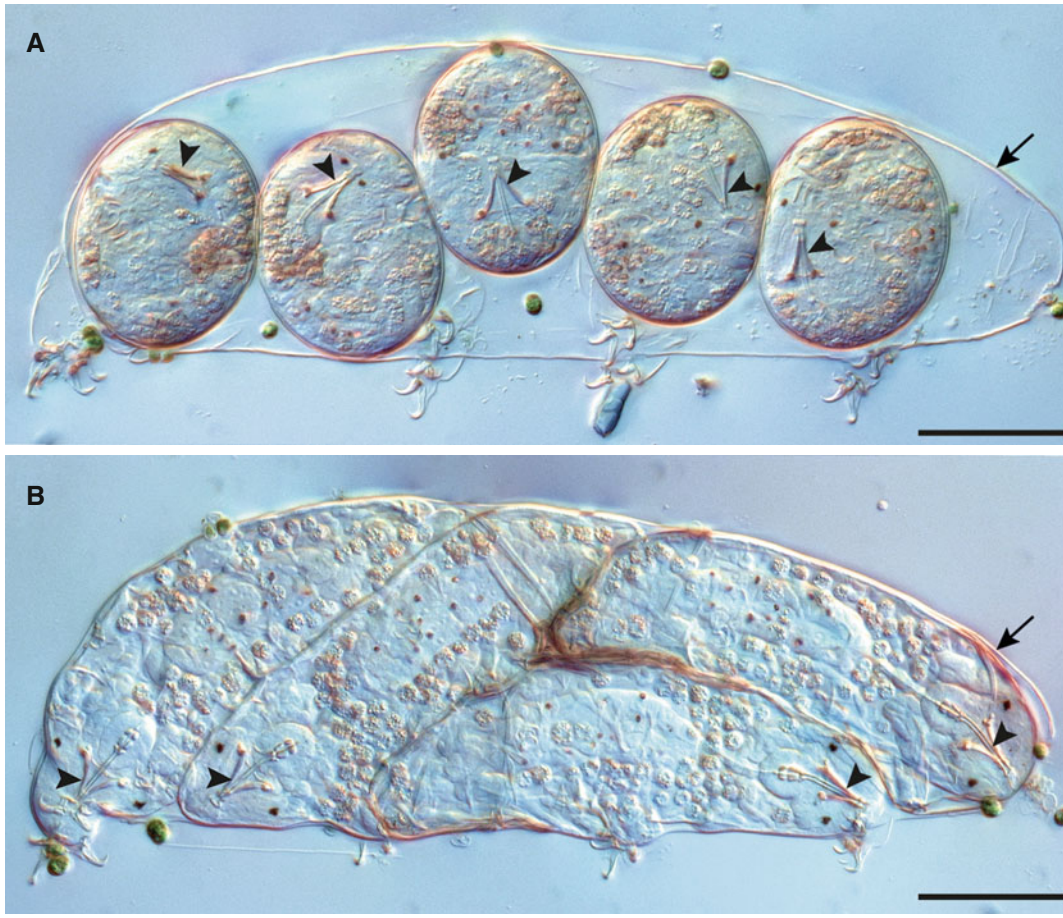

Fig. 3.6 Late developmental stages and hatchlings of *Hypsibius dujardini*. Light micrographs. (**A**) Fully developed embryos in the exuvium of the mother prior to hatching. (**B**) Hatchlings in the exuvium of the mother. *Arrowheads* indicate the buccopharyngeal apparatuses of the embryos and hatchlings. *Arrow* points to the shed exuvium of the mother. Scale bars: 50 μm

progressive increase in digits occurs from the first to the fourth pair of legs (Kristensen 1978). On the other hand, juvenile stages appear to be completely absent in *Tetrakentron synaptae*, possibly as an adaptation to the ectoparasitic lifestyle in this species (Kristensen 1980). Species-specific structures also often develop further in adults, for example, the cuticular expansions in species of *Florarctus* and *Actinarctus* (Grimaldi de Zio et al. 1980). The echiniscid *Mopsechiniscus imberbis* appears to be the sole exception to this pattern; the juvenile exhibits cuticular processes that are lost in the adult (Ramazzotti and Maucci 1983).

Since neither tardigrades nor onychophorans have a true larval stage, the last common ancestor of Panarthropoda most likely had a monophasic life cycle with direct development.

GENE EXPRESSION

To date, no mRNA expression studies have been carried out on tardigrades. The only study on the expression of developmental genes in the tardigrade embryo used cross-reactive antibodies against the Engrailed and Pax3/7 proteins to localize their expression during development in

Hypsibius dujardini (Gabriel and Goldstein 2007). In this species, the Engrailed protein is localized in rows of nuclei in the dorsal and lateral ectoderm at the posterior boundary of each developing segment (Gabriel and Goldstein 2007). This pattern resembles the segmental expression pattern of *engrailed* in various arthropods (Tautz 2004; Carroll et al. 2005; Damen 2007), suggesting an involvement of *engrailed* in the formation of segmental boundaries in tardigrades.

In contrast to Engrailed, the Pax3/7 protein initially does not show any segmental pattern of expression; it is instead first expressed ubiquitously in the posterior half of the embryo (Gabriel and Goldstein 2007). Later in development, however, when the morphological segments have become evident, Pax3/7 is detected in a segmentally iterated pattern in four bilaterally symmetric groups of ectodermal cells along the ventral midline as well as in a large domain in the head region of the embryo. These groups seem to correspond in position with the developing trunk ganglia and the brain (cf. Mayer et al. 2013a, b), suggesting a role of Pax3/7 in neural patterning (Gabriel and Goldstein 2007). Since *pax3/7* and its protein product are not expressed in a pair rule pattern in embryos of tardigrades or onychophorans (Gabriel and Goldstein 2007; Janssen and Budd 2013), the pair rule function of this gene might have evolved within the arthropods. However, functional gene analyses would be required to confirm this hypothesis.

Recently, Tenlen et al. (2013) developed a protocol that seems promising for the disruption of gene function in embryos of *Hypsibius dujardini*. They used double-stranded, RNA-mediated, parental RNA interference (RNAi), which mostly resulted in distinct and reproducible phenotypes. While the function of five out of six selected genes was disrupted successfully, no effect was seen with respect to the anterior Hox gene *Deformed*. Nevertheless, the study of Tenlen et al. (2013) shows that parental RNAi might be an effective method to dissect gene function in the tardigrade embryo, which will be helpful for understanding the evolution of developmental mechanisms in panarthropods.

OPEN ISSUES FOR FURTHER DEVELOPMENTAL STUDIES

- Type of cleavage: determinate versus indeterminate
- Origin and fate of mesodermal pouches/somites
- Expression patterns of key developmental genes, including axis-determining, segmentation, and Hox genes
- Neurogenesis and neural development
- Development of the gonad
- Embryology of heterotardigrades

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Onychophora

4

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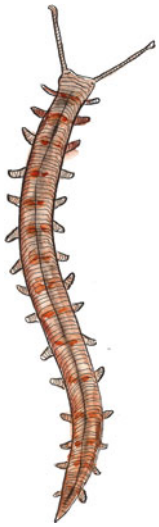

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INTRODUCTION

Onychophorans, or "velvet worms" (Fig. 4.1), are multi-legged, terrestrial invertebrates that inhabit decaying logs, soil, and leaf litter of tropical and temperate forests on landmasses that have resulted from the breakup of Gondwana (Fig. 4.2A; Brinck 1957; Ruhberg 1985; Allwood et al. 2010; Ruhberg and Mayer 2013; Murienne et al. 2014). The approximately 200 described species are classified into two major subgroups, the Peripatidae and Peripatopsidae, which might have diverged over 350 million years ago (Fig. 4.2A, B; Mayer and Oliveira 2011, 2013; Oliveira et al. 2012a; Murienne et al. 2014). The anatomy of onychophorans has changed little since the Early Cambrian, as they resemble fossil lobopodians – putative stem-group representatives of Panarthropoda (Onychophora + Tardigrada + Arthropoda) (Bergström and Hou 2001; Maas et al. 2007; Haug et al. 2012; Ou et al. 2012).

The onychophoran body consists of a head, a worm-shaped trunk, and an anal cone, which comprises a true, limbless segment (Mayer et al. 2005). The trunk is composed of 13–43 segments, each bearing a pair of unjointed limbs called lobopods that are equipped with a pair of sclerotized claws (Oliveira and Mayer 2013). The limbs of the head have been modified into three pairs of specialized appendages: the antennae, jaws, and slime papillae (Storch and Ruhberg 1993; Mayer et al. 2010a; Ruhberg and Mayer 2013). The slime papillae eject a sticky secretion, which is used for prey capture and defense (Manton and Heatley 1937; Ghiselin 1985; Read

Fig. 4.1 Selected species of Peripatopsidae from Australia. From *left* to *right*: *Euperipatoides rowelli*, *Phallocephale tallagandensis*, *Ooperipatellus insignis*, *Ooperipatus hispidus*, *Tasmanipatus barretti*, and *Tasmanipatus anophthalmus*. Images not to scale (length of photographed specimens ranges from 20 to 60 mm)

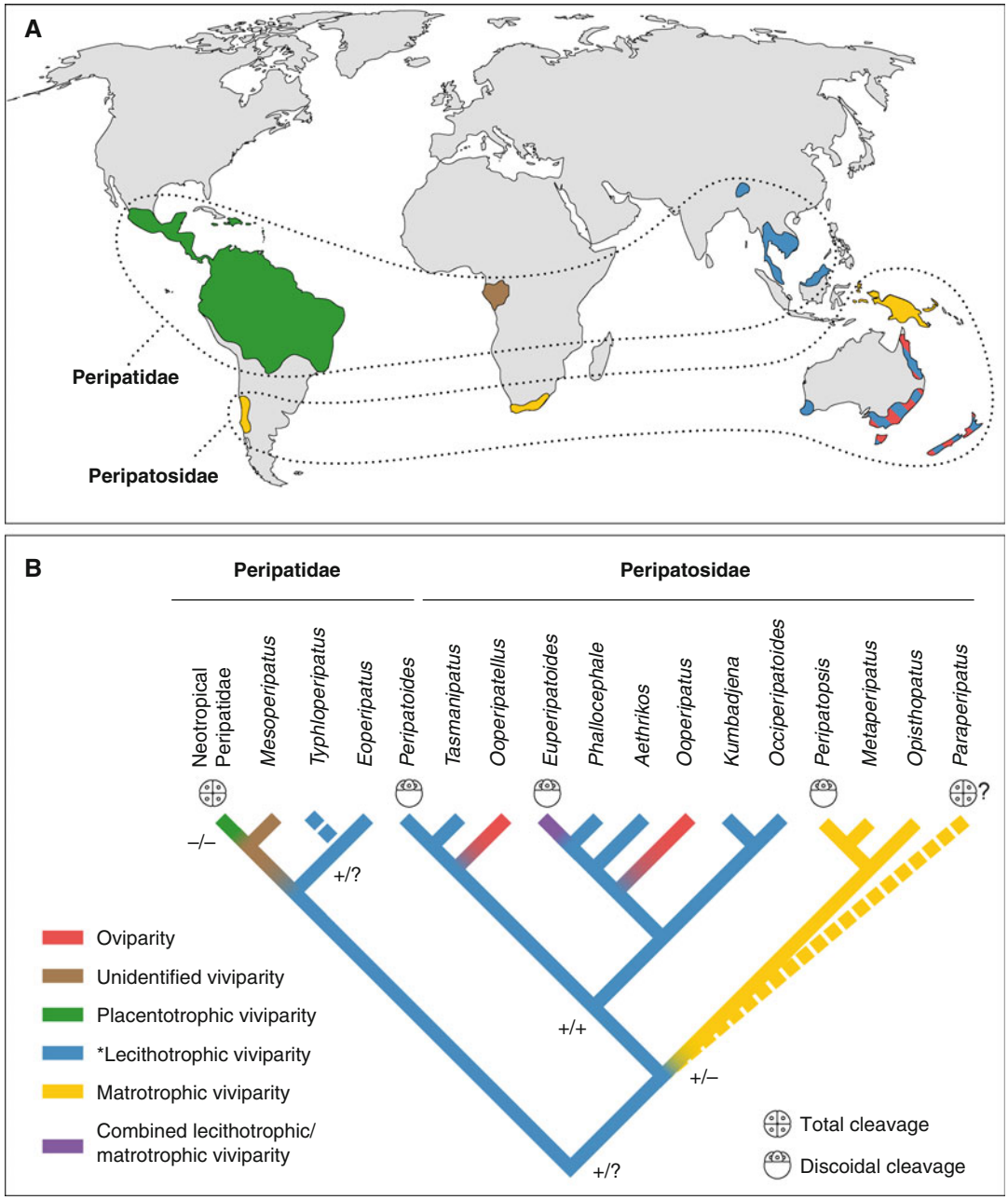

Fig. 4.2 Distribution of different types of nourishment supply to the embryo, embryonic envelopes, and cleavage types among onychophoran taxa. (**A**) Map of nourishment supply type by world region (*color coding* as defined in **B**). Note that the mode of viviparity in representatives of *Mesoperipatus* from tropical Africa remains unidentified (see Bouvier 1905) (distribution map modified from Oliveira et al. 2012a). (**B**) Mapping of nourishment supply type on a phylogenetic tree of Onychophora according to Murienne et al. (2014); note that many taxa were excluded from this study. *Dashed* branches indicate putative position of key taxa missing in the analysis of Murienne et al. (2014). *This mode might in fact be combined lecithotrophic/matrotrophic viviparity, which has been shown to exist in *Euperipatoides rowelli* (see Sunnucks et al. 2000). "+" and "–" at each node indicate, respectively, the presence/absence of the vitelline envelope (*left*) and the chorion (*right*); *question marks* indicate missing data. Cleavage type in *Paraperipatus* is uncertain (*question mark*)

and Hughes 1987; Storch and Ruhberg 1993; Baer and Mayer 2012). After the prey (e.g., crickets, woodlice) has been entangled and immobilized by slime threads, its cuticle is punctured using the jaws and digestive saliva is injected into the prey's body. The liquefied contents are then ingested using a suctorial pharynx (Manton and Heatley 1937; Baer and Mayer 2012; Mayer et al. 2013a; Nielsen 2013).

Onychophorans have a ventral mouth, which is surrounded by several lip papillae that are innervated by the brain (Ou et al. 2012; Martin and Mayer 2014). The oral cavity harbors the jaws and an unpaired tongue (Mayer et al. 2010a; Oliveira and Mayer 2013). The mouth leads into a muscular pharynx, which is succeeded by a tube-like esophagus, a thick-walled midgut, a short hindgut, and a terminal anus (Storch et al. 1988; Storch and Ruhberg 1993; Mayer et al. 2013a). Instead of Malpighian tubules, which are lacking in onychophorans, segmental nephridia serve as excretory (=renal) organs, most of which open to the exterior near the basis of each leg (Gabe 1957; Storch et al. 1978; Mayer and Koch 2005; Mayer 2006a). In some segments, however, the nephridia have been modified into the gonoducts of the genital tract, the labyrinth organs, and the genital, anal, and salivary glands (Ruhberg and Storch 1978; Storch et al. 1978, 1979; Lavallard and Campiglia 1988; Mayer and Koch 2005).

The male crural glands, which are thought to produce pheromones (Barclay et al. 2000a), also open to the exterior at the basis of each leg either in several trunk segments (in peripatopsids and representatives of *Peripatus*) or in only two pregenital segments (in most peripatids) (Reid 1996; Oliveira et al. 2012b, 2013a). The slime glands and the male anterior genital glands, the latter present only in some peripatopsids, might be derivatives of the crural glands of the corresponding body segments (Ruhberg and Storch 1977; Baer and Mayer 2012). The reproductive tract of males comprises paired testes, seminal vesicles, spermioducts, and an unpaired ductus ejaculatorius (Storch and Ruhberg 1990; Storch et al. 1995). The reproductive tract of females consists of an ovary; paired oviducts, which may or may not be associated with seminal receptacles and ovarian funnels; paired uteri; and a common duct leading into the vagina (Reid 1996; Herzberg et al. 1980; Brockmann et al. 1999, 2001; Walker et al. 2006; Mayer and Tait 2009). The genital opening of both sexes is situated either between the last (in peripatopsids) or penultimate leg pair (in peripatids). The females of oviparous species usually possess an ovipositor (Reid 1996).

The respiratory system of onychophorans consists of numerous unbranched tracheae, which open to the exterior via specialized atria (Storch and Ruhberg 1993; Hilken 1998; Oliveira et al. 2012b, 2013a; Ruhberg and Mayer 2013). The tracheae supply oxygen to most internal organs, including the reproductive, digestive, nervous, and muscular systems (Storch and Ruhberg 1993; Mayer and Tait 2009; Baer and Mayer 2012; Oliveira et al. 2013b). The musculature of the body wall consists of three layers: an outer circular, a diagonal, and an inner longitudinal muscle layer (Birket-Smith 1974; Hoyle and Williams 1980). The longitudinal layer is organized into two dorsal, two lateral, and three ventral bundles. An additional layer of transverse musculature bridges the ventral and dorsolateral body walls. While none of these layers alone show a segmental arrangement, the muscles associated with limbs are clearly segmental (Birket-Smith 1974; Hoyle and Williams 1980; Oliveira and Mayer 2013; Oliveira et al. 2013b).

In contrast to tardigrades and most arthropods, the onychophoran nervous system lacks metameric ganglia (Mayer and Whitington 2009a; Whitington and Mayer 2011; Mayer et al. 2013b). The two widely separated nerve cords, linked together by numerous median commissures, instead have a medullary organization, with neuronal somata distributed along the entire length of each nerve cord (Mayer and Harzsch 2007, 2008). Additional ring commissures interconnect the nerve cords with the two dorsolateral nerves and the dorsal heart nerve in an orthogonal fashion (Mayer and Harzsch 2008; Mayer and Whitington 2009a; Whitington and Mayer 2011). Only the leg nerves and the nephridial nerves show a segmental arrangement.

The onychophoran brain is a bilobed, ganglionic structure, which consists of the proto- and deutocerebrum (Holmgren 1916; Hanström 1928; Mayer et al. 2010a, 2013a; Martin and Mayer 2014; Mayer 2015). The protocerebrum innervates

the antennae and the eyes and the deutocerebrum supplies the jaws, whereas the anterior region of the ventral nerve cords that innervate the slime papillae is altogether separate from the brain (Mayer et al. 2010a). The eyes of velvet worms are simple structures that contain rhabdomeric photoreceptors and might be homologous to the median ocelli of arthropods (Dakin 1921; Eakin and Westfall 1965; Mayer 2006b). The eyes are unlikely to have high visual resolution and serve only for monochromatic vision (Hering et al. 2012 ; Beckmann et al. 2015).

As typical representatives of ecdysozoans (=molting animals), onychophorans must periodically molt their cuticle, a process most likely mediated by ecdysteroid hormones (Manton 1938a; Holliday 1942; Hoffmann 1997). Within the Ecdysozoa, onychophorans are united with tardigrades (water bears) and arthropods (spiders, centipedes, crustaceans, insects, and allies) in the clade Panarthropoda (Whitington and Mayer 2011; Giribet and Edgecombe 2012; Nielsen 2012; Mayer et al. 2013b). However, the phylogenetic relationship of these three major panarthropod groups is still controversial (Rota-Stabelli et al. 2010; Campbell et al. 2011; Mayer et al. 2013a, b). Because of its key phylogenetic position, Onychophora represents an important outgroup for understanding the evolution of arthropods, one of the most abundant and diverse animal groups on Earth (Zhang 2011, 2013).

EARLY DEVELOPMENT

Embryonic Nutrition

Despite high diversity in the modes of sperm transfer among onychophorans, ranging from dermal insemination (Manton 1938b; Mayer 2007) to the use of specialized head structures in males (Reid 1996; Tait and Norman 2001), all species studied to date exhibit internal fertilization. However, the types of nourishment supply to the embryo vary considerably (Table 4.1). For example, while some species of Australasian Peripatopsidae are oviparous (Fig. 4.3A), placentotrophic viviparity occurs in the neotropical Peripatidae (Fig. 4.2A, B; Kennel 1885, 1888; Dendy 1892; Ruhberg 1985; Brockmann et al. 1997; Norman and Tait 2008; Mayer et al. 2010b). Embryos of the latter are attached to the uterus wall via a hollow stalk, which is a differentiation of the dorsal extraembryonic tissue in the neck region of the embryo (Kennel 1885, 1888; Walker and Campiglia 1988, 1990). However, nourishment from the mother is not transferred via the stalk but rather via the embryonic surface (Walker and Campiglia 1990). On the other hand, the remaining onychophoran species are typically classified as "ovoviviparous," but as pointed out by Reid (1996), this classification might be an oversimplification because it does not take into account the entire diversity of embryonic nutrition in these taxa (Table 4.1). For example, the females of the peripatopsid *Euperipatoides rowelli* from Australia produce yolky eggs, but the embryo receives additional nourishment from the mother during development (Sunnucks et al. 2000). Hence, this species exhibits a combination of lecithotrophic and matrotrophic viviparity, a "mixed" mode that might also be characteristic of other lecithotrophic viviparous species (Tutt et al. 2002). In contrast, the females of *Metaperipatus inae* from Chile, *Peripatopsis sedgwicki* from South Africa, and *Paraperipatus* spp. from Indonesia and Papua New Guinea produce nearly yolkless eggs, and their embryos develop large "trophic vesicles" or "trophic organs" that might serve for the uptake of nourishment from the mother (Fig. 4.3B, C; Willey 1898; Bouvier 1905; Pflugfelder 1948; Manton 1949; Hofmann 1988; Mayer 2007). Accordingly, this mode is classified as non-placentotrophic, matrotrophic viviparity (Table 4.1).

Mapping these modes on the onychophoran phylogeny (Murienne et al. 2014) suggests that the last common ancestor of Onychophora exhibited either lecithotrophic viviparity or a combination of lecithotrophic/matrotrophic viviparity (Fig. 4.2B). Since no details are available on the embryology of *Mesoperipatus tholloni* from tropical Africa, placentotrophic viviparity might have evolved either in the neotropical Peripatidae or in the last common ancestor of the tropical African and neotropical Peripatidae. On the other hand, oviparity might have arisen independently at least twice within Peripatopsidae (Fig. 4.2B). Interestingly, at least some embryonic development of oviparous species occurs before egg

Table 4.1 Modes of reproduction and nourishment supply to the embryo in Onychophora (see Fig. 4.2A, B for summary)

Mode	Description	Geographical occurrence	Representative taxa	Notes
Oviparity	Yolky eggs with vitelline envelope and thick, sculptured chorion	Australia, New Zealand	Peripatopsidae: *Ooperipatus*, *Ooperipatellus*, others (see Reid 1996)	Embryos may be at advanced stages prior to deposition (Brockmann et al. 1997)
Lecithotrophic viviparity ([a]ovoviviparity)	Yolky eggs retained in uteri; no trophic interaction between embryo and mother. Vitelline envelope and chorion persist until birth; hatching and birth occur simultaneously	Southeast Asia, Australia, New Zealand	Peripatidae: *Eoperipatus*, *Typhloperipatus*	Whether this mode occurs independently or only in combination with matrotrophic viviparity is unknown
			Peripatopsidae: *Peripatoides*, *Tasmanipatus*, *Phallocephale*, others	
Matrotrophic viviparity ([a]ovoviviparity)	Eggs retained in uteri. Little or no yolk present; nourishment supplied by mother. Placental structures absent; vitelline envelope persists until birth; hatching and birth occur simultaneously; chorion absent	South Africa, Chile, Indonesia, Papua New Guinea	Peripatopsidae: *Peripatopsis*, *Opisthopatus*, *Metaperipatus*, *Paropisthopatus*, *Paraperipatus*	
Combined lecithotrophic/matrotrophic viviparity ([a]ovoviviparity)	Eggs retained in uteri. Yolk present; additional nutrition provided by mother. Vitelline envelope and chorion persist until birth; hatching and birth occur simultaneously	Australia	Peripatopsidae: *Euperipatoides rowelli*	
Placentotrophic viviparity	Small, yolkless eggs; live birth. Nourishment supplied exclusively by mother via placental structures; egg envelopes absent	Neotropics	All neotropical Peripatidae	This mode may also occur in *Mesoperipatus tholloni* from tropical Africa but has not been confirmed

[a]The term "ovoviviparity" has been applied to various species irrespective of the nature and number of embryonic envelopes and whether or not there is trophic interaction between embryo and mother. Due to this ambiguity, it is replaced herein by the more precise terms "lecithotrophic viviparity," "matrotrophic viviparity," and "combined lecithotrophic/matrotrophic viviparity"

deposition (Brockmann et al. 1997; Norman and Tait 2008), indicating that this mode has originated by heterochronic evolution from lecithotrophic or combined lecithotrophic/matrotrophic viviparity.

Embryonic Envelopes

The mature ovarian oocytes of onychophorans are initially surrounded by an extracellular coat: the so-called primary egg membrane (Fig. 4.4A, B; Evans 1901; Herzberg et al. 1980; Huebner and Lococo 1994; Brockmann et al. 2001; Mayer and Tait 2009). This primary egg membrane is not retained in the embryo but is thought to disappear after fertilization (Sedgwick 1888a; Manton 1949). The physical stability of the developing embryo is instead provided by specialized envelopes, the occurrence of which differs among onychophoran subgroups depending on the mode of nourishment supply during development (Fig. 4.2B, Table 4.1).

The embryos of the lecithotrophic viviparous species of Peripatopsidae from Australia and New Zealand are enclosed by two envelopes: the thin vitelline envelope and the chorion (Sheldon 1887, 1889a, b; Tutt et al. 2002; Walker and Tait 2004; Mayer and Whitington 2009a; Eriksson and Tait 2012). These envelopes persist until birth so that hatching and birth occur simultaneously. At least in the Australian peripatopsid *Euperipatoides rowelli*,

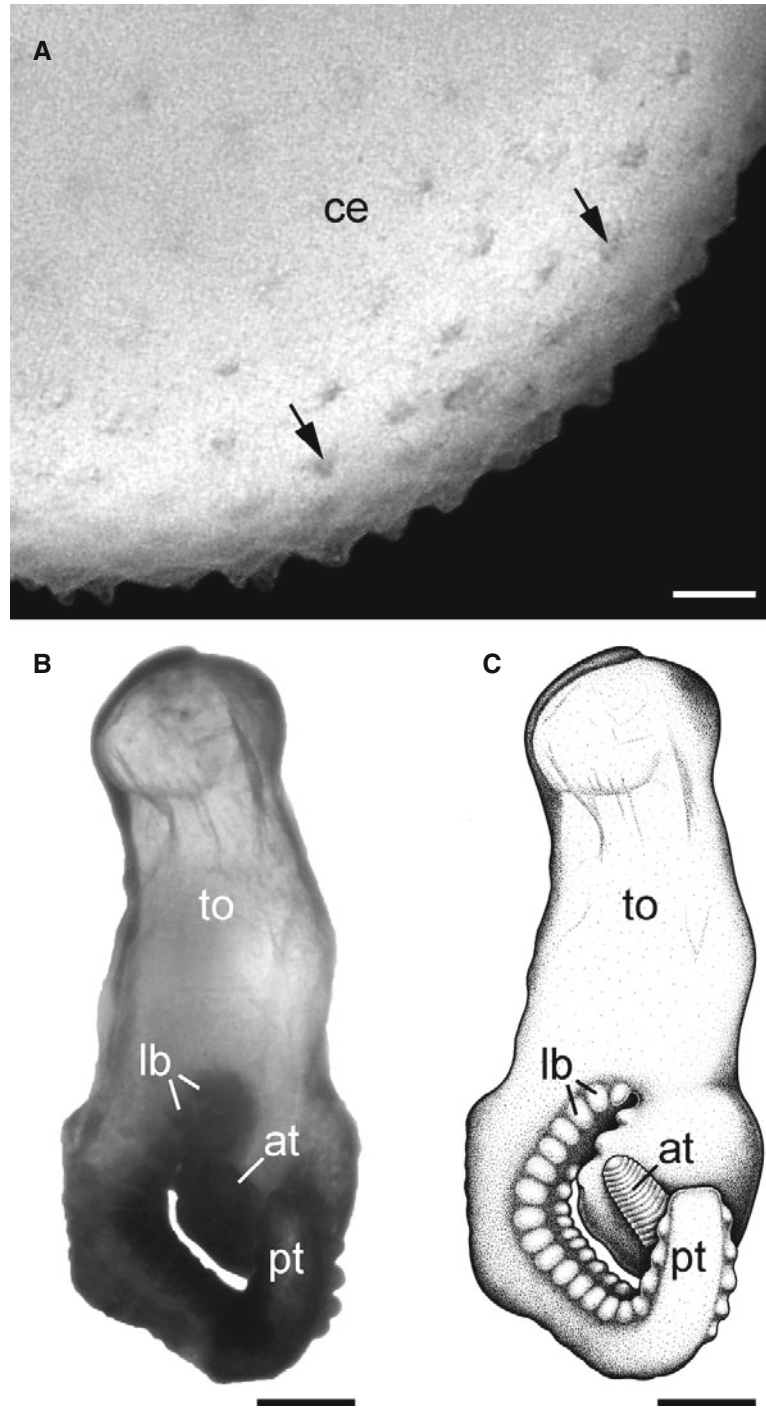

Fig. 4.3 Oviparity and matrotrophic viviparity in species of Peripatopsidae. Light micrographs (**A**, **B**) and line drawing (**C**). (**A**) Detail of the egg of the oviparous species *Ooperipatus hispidus* from Australia. Note the sculptured chorion (*arrows*). (**B**) Embryo of the matrotrophic viviparous species *Metaperipatus inae* from Chile. Light micrograph; note the prominent trophic organ (*to*). (**C**) Ink drawing of the same embryo (by Peter Adam). *Abbreviations*: *at* antenna, *ce* chorion (envelope), *lb* limb bud, *pt* posterior body region, *to* trophic organ. Scale bars: 100 µm (**A**) and 250 µm (**B**, **C**)

both the vitelline envelope and the chorion must be permeable to the nourishment supplied by the mother because the dry weight of the embryos of this species increases by about 10 % over the course of development (Sunnucks et al. 2000). The vitelline envelope and the chorion are also present in the oviparous species of Peripatopsidae from Australia and New Zealand with yolky eggs, but the chorion is thicker and sculptured (Fig. 4.3A; Dendy 1902; Brockmann

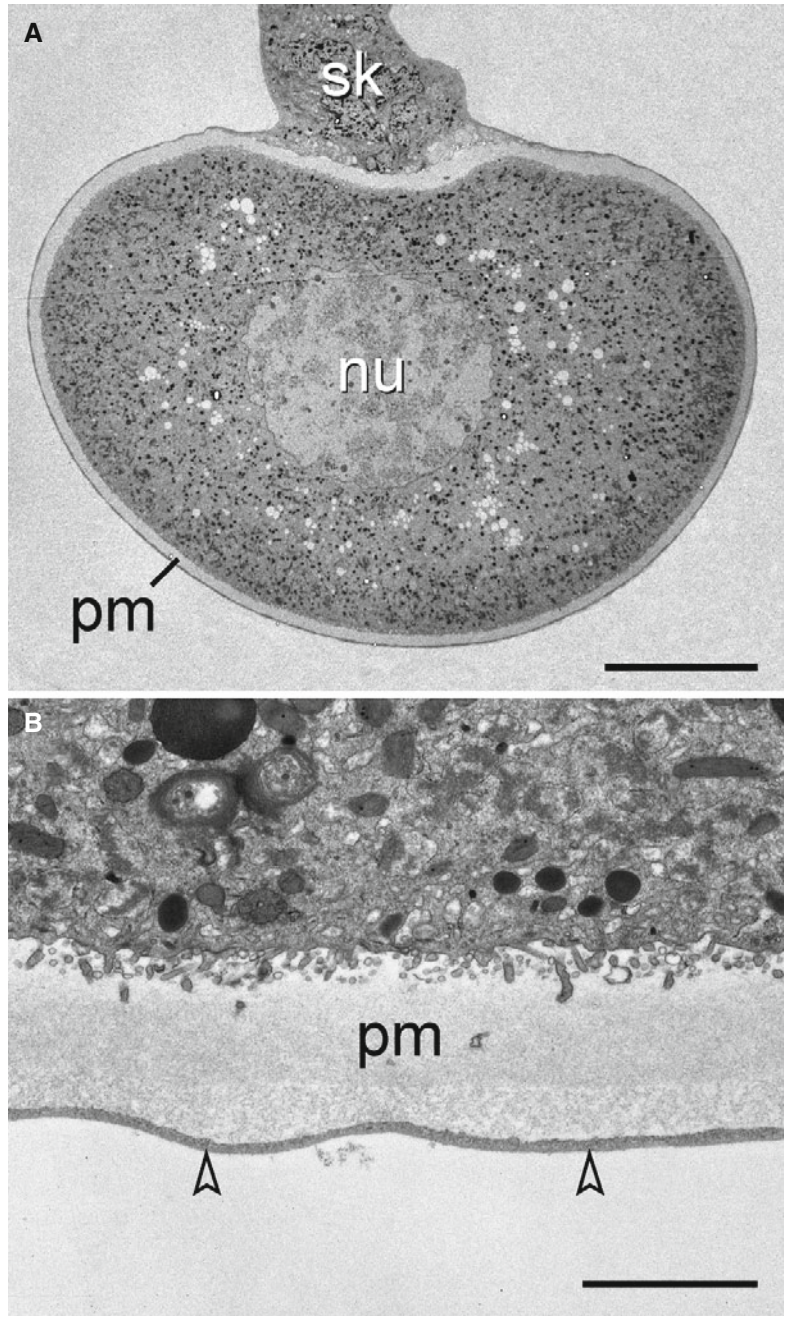

Fig. 4.4 Cross section of mature ovarian oocytes in matrotrophic viviparous species of Peripatopsidae. Transmission electron micrographs. (**A**) Overview of an oocyte in *Peripatopsis balfouri*. Note the stalk, which connects the oocyte to the ovary, and the central position of the nucleus. (**B**) Detail of the primary egg membrane of an oocyte in *Opisthopatus roseus*. *Arrowheads* indicate the basal lamina (=extracellular matrix). *Abbreviations*: *nu* nucleus, *pm* primary egg membrane, *sk* stalk. Scale bars: 20 µm (**A**) and 2 µm (**B**)

et al. 1997, 2001; Norman and Tait 2004, 2008). Embryos may already be at advanced developmental stages at the time of egg deposition, with juveniles hatching 7–17 months[1] thereafter (Dendy 1902; Brockmann et al. 1997). Unfortunately, the process of hatching has not been documented in detail.

In contrast to the oviparous and lecithotrophic viviparous species, only one envelope is found in representatives of the matrotrophic viviparous taxa of Peripatopsidae, including *Metaperipatus*, *Peripatopsis*, *Opisthopatus*, and *Paraperipatus* (Fig. 4.2B; Sedgwick 1888a; Willey 1898; Pflugfelder 1948; Manton 1949; Walker 1992,

[1] Juveniles of the oviparous species *Ooperipatus hispidus* kept in culture at 17 °C hatched only 4 months after egg deposition (unpublished data).

1995; Mayer et al. 2005). This envelope most likely represents the vitelline envelope, while the chorion is lacking. A vitelline envelope also seems to surround the yolky embryos in the lecithotrophic viviparous species of the Southeast Asian Peripatidae, including *Eoperipatus* and *Typhloperipatus* (Evans 1901; Kemp 1914). However, it is unclear whether there is an additional embryonic envelope in these species, which would correspond to the chorion (Fig. 4.2B). While no corresponding data are available for the peripatid *Mesoperipatus tholloni* from tropical Africa, the embryos of the neotropical Peripatidae generally lack embryonic envelopes and develop a placenta instead (Kennel 1885, 1888; Sclater 1888; Anderson and Manton 1972; Walker and Campiglia 1990; Campiglia and Walker 1995; Mayer and Whitington 2009b). Brockmann et al. (1999) reported an electron-dense, flimsy extracellular coat around the early embryo in *Epiperipatus biolleyi*, but the nature of this coat is unclear.

The distribution of embryonic envelopes among different onychophoran subgroups suggests that the embryo of the last common ancestor of Onychophora was enclosed at least by a vitelline envelope (Fig. 4.2B). Whether or not a chorion was present, or whether it evolved in the Australian and New Zealand species of Peripatopsidae, is unknown.

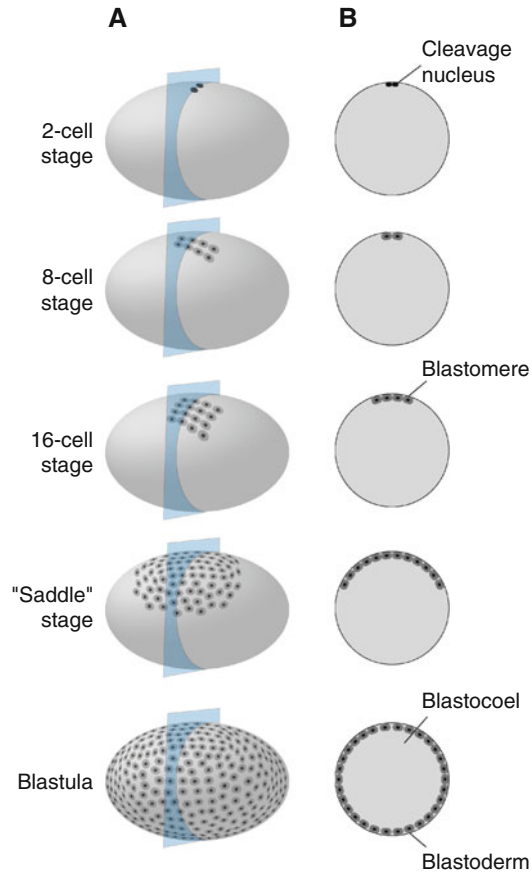

Fig. 4.5 Simplified diagrams of the discoidal meroblastic cleavage stages in Onychophora (reconstructed after Sheldon 1887, Manton 1949, and Eriksson and Tait 2012). (**A**) External view of cleavage stages. (**B**) Cleavage stages in cross section (sectioning plane represented in *blue* in **A**)

Cleavage Patterns

Cleavage patterns differ among the onychophoran subgroups. A closer look at the original descriptions reveals two major types: (i) discoidal meroblastic cleavage and (ii) holoblastic (=total) cleavage (Fig. 4.2B; see Gilbert 2013 for the definition of cleavage types). Superficial (=intralecithal) cleavage had been assumed to occur in the lecithotrophic viviparous species, such as *Peripatoides novaezealandiae* and *Euperipatoides kanangrensis* (Korschelt and Heider 1899; Anderson 1966, 1973; Eriksson and Tait 2012), but the described pattern is in fact typical discoidal rather than superficial cleavage (see critical discussion by Scholtz and Wolff 2013). In both species, one or two initial energids (=nuclei with associated cytoplasm) occur at the surface of the egg, whereas no nuclei are seen within the yolk mass up to the blastoderm stage (Fig. 4.5A; Sheldon 1887, 1888; Eriksson and Tait 2012). What Anderson (1973) regarded as "intralecithal cleavage nuclei" in *Peripatoides novaezealandiae* are in fact, according to Sheldon (1887), "protoplasmic masses" devoid of nuclei. Thus, the cleavage type found in the lecithotrophic viviparous species of Onychophora clearly differs from the superficial cleavage of arthropods, which is characterized by the intralecithal position of the early cleavage nuclei (e.g., Ho et al. 1997; Gilbert 2013; Scholtz and Wolff 2013.

As cleavage proceeds in the lecithotrophic viviparous onychophoran species, the two peripheral nuclei and their progeny divide synchro-

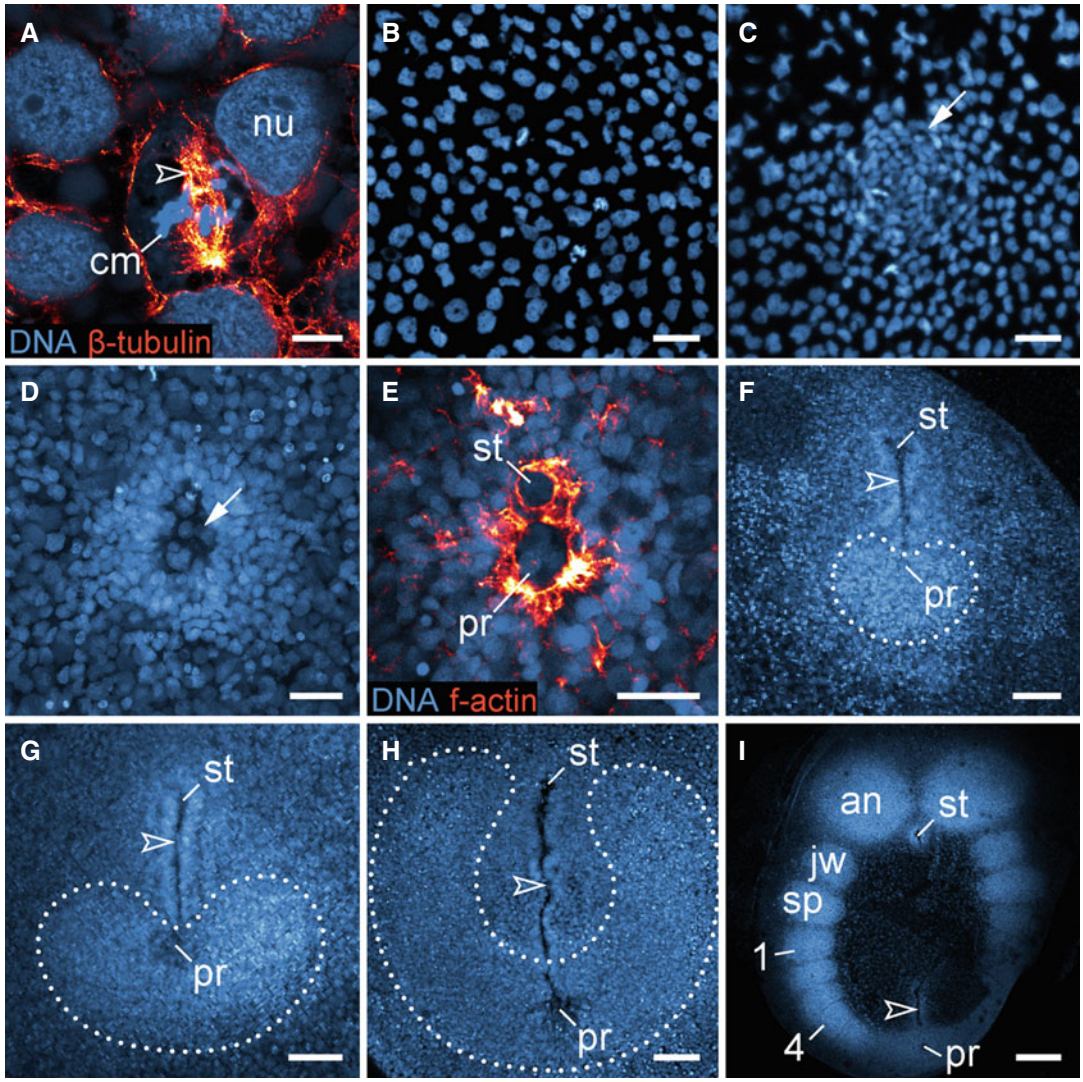

Fig. 4.6 Early development in *Euperipatoides rowelli* (Peripatopsidae). Confocal laser scanning micrographs of successive embryonic stages labeled with DNA marker bisbenzimide (*light blue* in **A**–**I**). Embryos in **A** and **E** were double labeled for β-tubulin and f-actin (phalloidin-rhodamine), respectively (*glow* scale). (**A**) Dividing cell in the blastoderm with a spindle apparatus (*arrowhead*). Note the asynchronous division compared to surrounding cells. (**B**) Blastoderm of a blastula. (**C**) Early gastrula. Note the concentration of nuclei/cells in the area of the future blastopore (*arrow*). (**D**, **E**) Gastrula. Note that the early blastopore (*arrow* in **D**) encloses the separate openings of the stomodeum and proctodeum (*glow* scale in **E**). (**F**–**H**) Germband formation. The germband (*dotted line*) arises posterior to the proctodeum (**F**) and elongates anteriorly (**G**) until it reaches the level of the stomodeum (**H**). During this process, the stomodeum and the proctodeum separate from each other, forming the blastoporal slit (*arrowhead*). (**I**) Early stage II embryo (staging according to Walker and Tait 2004). Note that four leg-bearing segments (*numbered*) have already been established, while a small remnant of the blastoporal slit still remains (*arrowhead*). *Abbreviations*: *an* antennal segment, *cm* chromosomes, *jw* jaw segment, *nu* nucleus, *pr* proctodeum, *sp* slime papilla segment, *st* stomodeum. Scale bars: 10 μm (**A**), 50 μm (**B**–**E**), 100 μm (**F**–**H**), and 250 μm (**I**)

nously up to the 32-cell stage, resulting in a regular arrangement of nuclei on one side of the egg (Fig. 4.5A, B; Eriksson and Tait 2012). From the 32-cell stage onward, cellularization (=membrane formation) is evident and cell division becomes asynchronous (Figs. 4.5A and 4.6A; Eriksson and Tait 2012; note that cellularization occurs at an earlier stage in *Peripatopsis* spp.

according to Manton 1949). Further cell division leads to a peripheral plate of cells, which spreads around the central yolk mass, giving rise to the blastoderm (Fig. 4.5A, B). Beginning at the 64-cell stage, yolk granules of the central yolk mass aggregate into separate, rounded compartments (Fig. 4.5A; Sheldon 1887; Eriksson and Tait 2012). Later in development, some of these yolk compartments have been reported to contain nuclei, the origin of which is unknown (Sheldon 1887; Eriksson and Tait 2012).

A strikingly similar cleavage pattern has been described from the matrotrophic viviparous species of *Peripatopsis* with yolkless eggs (Fig. 4.2B; Manton 1949). In these species, the initial energids are also located at the surface of the egg and their cleavages give rise to a "saddle" of blastomeres (Fig. 4.5B; Sedgwick 1885, 1886; Manton 1949). During the early cleavage stages, the cytoplasm of the egg breaks up into a number of non-nucleated spheres (="pseudoblastomeres" sensu Manton 1949). These spheres resemble the yolk compartments of the lecithotrophic viviparous species, although they are devoid of yolk and degenerate early in development (Manton 1949). From the "saddle" stage onward, Manton (1949) described three different modes of development in the four *Peripatopsis* species studied. While cell division within the "saddle" leads to a continuous blastoderm in *Peripatopsis moseleyi* and *Peripatopsis sedgwicki*, marginal cells migrate inside the embryo in *Peripatopsis balfouri*, after which the "saddle" spreads to form the blastoderm. The most divergent mode of development has been described from *Peripatopsis capensis*, in which the immigrated cells from the margins of the "saddle" give rise to an inner vacuolated epithelium around a central cavity (Sedgwick 1886; Manton 1949). This description requires confirmation, as it suggests that the embryo of *Peripatopsis capensis* has no real blastula stage but rather develops directly from the "saddle" stage into a gastrula. Despite these differences and the lack of yolk in the *Peripatopsis* species, their early cleavage pattern can be best classified as discoidal cleavage.

In contrast to non-placentotrophic species, cleavage is total and equal in the placentotrophic viviparous species of Peripatidae with very small, yolkless eggs (Fig. 4.2B; Kennel 1885; Sclater 1888, 1889; Anderson and Manton 1972). Early cleavage generates a morula, which becomes attached to the uterine wall (Kennel 1885; Sclater 1888, 1889; Anderson and Manton 1972; Walker and Campiglia 1990; Campiglia and Walker 1995). Further cell division results in a coeloblastula, with blastoderm surrounding a central blastocoel. Specialized cells of the blastula then give rise to a hollow stalk and the embryonic placenta. The maternal part of the placenta is generated by specialized areas of the uterine wall, consisting of several extracellular and cellular layers, including a syncytium (Anderson and Manton 1972; Walker and Campiglia 1990; Campiglia and Walker 1995). The stalk and the embryonic placenta are considered to be derivatives of the dorsal extra-embryonic tissue (Anderson 1966, 1973; Anderson and Manton 1972).

Putative total cleavage has also been reported from *Paraperipatus amboinensis* – a matrotrophic viviparous species of Peripatopsidae from Indonesia (Pflugfelder 1948). However, since embryos earlier than the 48-cell stage were not analyzed, this claim is doubtful and requires confirmation. The same holds true for *Paraperipatus novaebritanniae*, in which no early cleavage stages were analyzed (Willey 1898). Since embryogenesis of *Paraperipatus* species is strikingly similar to that of *Peripatopsis* and *Opisthopatus* species (Willey 1898; Manton 1949; Pflugfelder 1968; Walker 1995; Mayer et al. 2005), one would expect that cleavage patterns are similar in all matrotrophic viviparous species with yolkless eggs (Fig. 4.2B). Hence, it seems likely that species of *Paraperipatus* exhibit discoidal meroblastic rather than total equal cleavage.

Overall, only discoidal and total cleavage patterns have been confirmed in onychophorans (Fig. 4.2B), whereas the account of superficial cleavage seems speculative. It is also highly likely that the last common ancestor of Onychophora had indeterminate cleavage, as the anterior-posterior axis of the embryo is not predetermined in the egg (Eriksson and Tait 2012; Scholtz and Wolff 2013). Since neither onychophorans nor tardigrades (Chapter 3) exhibit superficial cleavage, it most likely evolved in arthropods – probably several times independently in different lineages (reviewed by Scholtz and Wolff 2013).

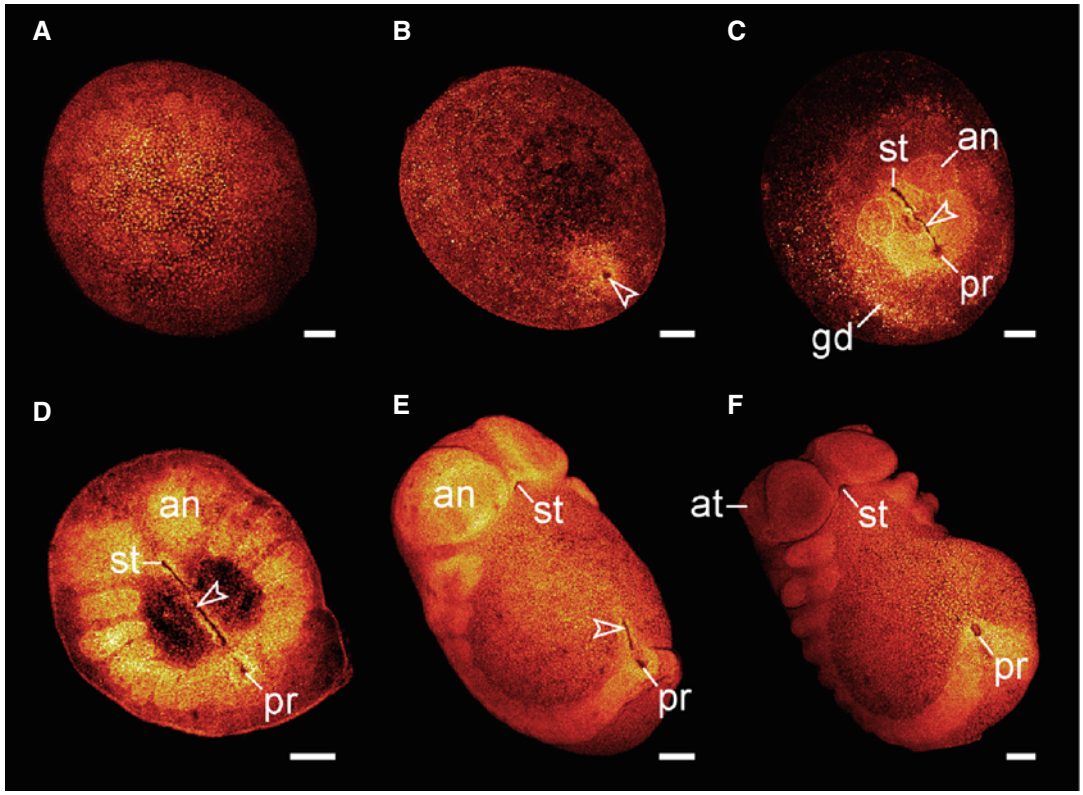

Fig. 4.7 Embryogenesis in *Euperipatoides rowelli* (Peripatopsidae). Confocal laser scanning micrographs of whole-mount embryos labeled with DNA marker bisbenzimide (*glow* scale). (**A**) Blastula. (**B**) Early gastrula. Note the developing blastopore (*arrowhead*). (**C**) Elongating germband-stage embryo. Note the slit (*arrowhead*) formed by the separation of the stomodeum from the proctodeum and the establishment of the first anterior-most somites. (**D**) Dissected germ disc of late elongating germband-stage embryo. Note the blastoporal slit between the stomodeum and the proctodeum (*arrowhead*). (**E**) Early stage II embryo (staging according to Walker and Tait 2004). The stomodeum has separated from the proctodeum, which remains associated with a small remnant of the blastoporal slit (*arrowhead*). (**F**) Late stage II embryo. The blastoporal slit has closed completely at this stage. *Abbreviations*: *an* antennal segment, *at* antenna, *gd* germ disc, *pr* proctodeum, *st* stomodeum. Scale bars: 200 μm

Gastrulation

The described modes of gastrulation differ even between closely related onychophoran species (Kennel 1885; Sedgwick 1885, 1886; Pflugfelder 1948; Manton 1949; Anderson 1966, 1973; Eriksson and Tait 2012). In many non-placentotrophic viviparous species, a germ disc arises prior to gastrulation (Figs. 4.6B, C and 4.7A–D; Manton 1949; Mayer and Whitington 2009b; Mayer et al. 2010b; Eriksson and Tait 2012). The germ disc comprises a condensed plate of cells, which shows an increased number of cell divisions relative to the surrounding blastoderm (Mayer et al. 2010b). The germ disc develops into the embryo, whereas the remaining blastoderm persists as dorsal extra-embryonic tissue (Figs. 4.6A–I and 4.7A–F).

In the onychophoran *Euperipatoides rowelli*, the germ disc initially occupies only a small area of the embryo, and an invagination in the center of the germ disc gives rise to a pit, i.e., the blastopore (Figs. 4.6D and 4.7B; Mayer and Whitington 2009b; Mayer et al. 2010b). The thickened walls of the blastopore suggest that cells begin to migrate from this region inside the embryo to form the endoderm and, later on, the mesoderm. A similar origin of these germ layers has been described from other non-placentotrophic onychophoran species (Evans 1901; Pflugfelder

1948; Manton 1949; Eriksson and Tait 2012), although the opening of the early blastopore was not reported in every case. Two separate openings occur within the blastoporal pit shortly after its formation: the stomodeum and the proctodeum (Fig. 4.6E). The anterior-posterior body axis is already established at this stage.

The mechanism of dorsoventral axis formation in onychophorans has not been studied in detail, but there is no evidence of a cumulus or cumulus-like structure in the early onychophoran embryo (Figs. 4.6, 4.7, 4.8, and 4.9; Mayer and Whitington 2009b; Treffkorn and Mayer 2013). In the chelicerate embryo, the cumulus is a conspicuous group of mesenchymal cells that expresses *decapentaplegic* (*dpp*) and initiates the breakdown of radial symmetry of the germ disc (Holm 1952; Akiyama-Oda and Oda 2003, 2006; McGregor et al. 2008. A cumulus-like structure has also been described from myriapods (Sakuma and Machida 2002, 2004), but there is no evidence of such a structure in crustaceans, insects, or tardigrades (Dearden and Akam 2001; Hejnol and Schnabel 2005; Gabriel et al. 2007; Treffkorn and Mayer 2013; Chapter 3).

After the anterior-posterior axis has been established in embryos of the onychophoran *Euperipatoides rowelli*, the germ disc increases in size, while the stomodeum and proctodeum move apart, thus giving rise to a longitudinal slit – the blastoporal slit – between the two openings (Figs. 4.6F–H and 4.7C, D). There is some controversy surrounding the origin of the endoderm. While Eriksson and Tait (2012) suggested that cells continuously immigrate from the entire bastoporal area in *Euperipatoides kanangrensis* – including the tissues surrounding the blastoporal slit, stomodeum, and proctodeum – Manton (1949) showed that immigration occurs only in the region posterior to the blastoporal slit in the *Peripatopsis* species. As a result, it is currently unclear whether this difference is due to real interspecific variation in the endodermal origin or to the limitations of the histological methods used.

During further development of non-placentotrophic onychophorans, the blastoporal slit is segregated from the stomodeum and proctodeum and subsequently closes by amphistomy (=lateral closure of the blastoporal lips) in an anterior-to-posterior progression (Figs. 4.6H, I and 4.7D–F; Mayer et al. 2010b; Eriksson and Tait 2012). A small remnant of the slit persists for some time anterior to the proctodeum (Figs. 4.6I and 4.7E). This slit has been interpreted erroneously as the putative proctodeum by some authors (Korschelt and Heider 1899; Manton 1949; Anderson 1973), but a more complete series of developmental stages revealed that the slit disappears completely during development, whereas the actual proctodeum, located posterior to this slit, persists throughout embryogenesis (Fig. 4.7C–F; Mayer et al. 2010b). Furthermore, the identity of the proctodeum has been confirmed by demonstrating the expression of a *wingless*/*Wnt1* homolog around its opening (Eriksson and Tait 2012), which resembles the situation in other bilaterians (Nulsen and Nagy 1999; Seaver and Kaneshige 2006; Holland et al. 2000).

The matrotrophic viviparous species *Peripatopsis capensis* shows a deviating pattern of gastrulation from other onychophoran species, as there seems to be no true blastula stage (Sedgwick 1885, 1886; Manton 1949). At the onset of gastrulation, cells immigrate from the margins of the saddle of blastomeres to give rise to the vacuolated endoderm (Manton 1949). The ectoderm then gradually grows around the endoderm and almost completely encloses it, so that only a small opening – the blastopore – persists (Sedgwick 1885, 1886). According to Sedgwick (1886), this opening then gives rise to the blastoporal slit, whereas Manton (1949) claimed that it closes completely and reopens again later to form the slit. Despite these differences in early embryogenesis, further development of *Peripatopsis capensis* resembles that of other *Peripatopsis* species (Manton 1949).

In contrast to the non-placentotrophic peripatopsid species, no germ disc formation is evident in the placentotrophic viviparous species of the neotropical Peripatidae (Figs. 4.8A–I and 4.9A–G; Sclater 1887, 1889; Kennel 1885; Anderson and Manton 1972; Mayer et al. 2010b). In embryos of these species, the zygote develops into a compact early cleavage embryo, i.e., the morula (Figs. 4.8A and 4.10A; Brockmann et al. 1999; Anderson and Manton 1972; Mayer and

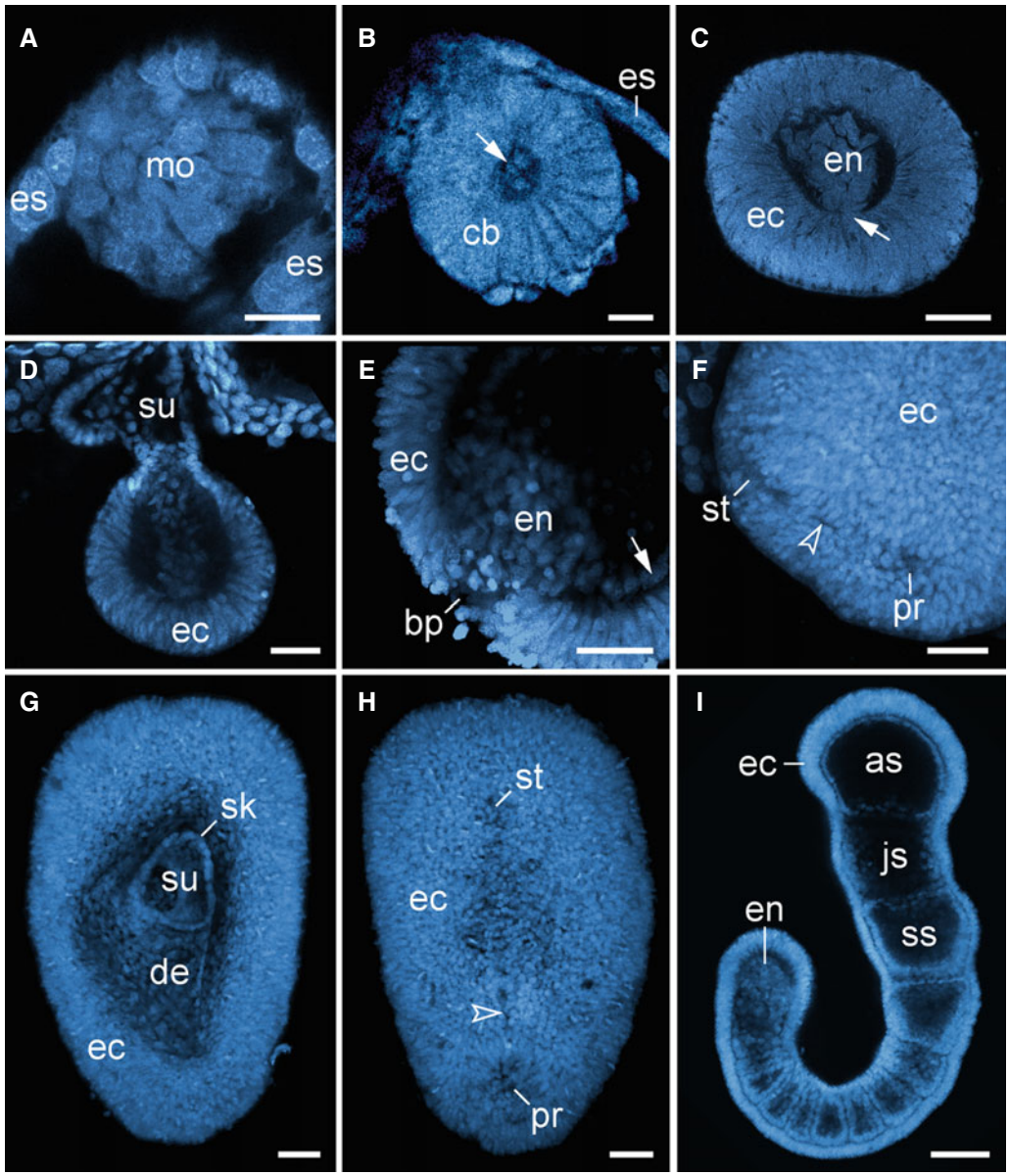

Fig. 4.8 Early development in neotropical placentotrophic viviparous species of Peripatidae. Confocal laser scanning micrographs of successive embryonic stages in *Epiperipatus biolleyi* (**A**–**H**) and *Principapillatus hitoyensis* (**I**) labeled with DNA marker bisbenzimide. (**A**) Morula. (**B**) Coeloblastula. *Arrow* points to the blastocoel. (**C**) Early gastrula. Note the site of endomesodermal cell migration (*arrow*). (**D**) Late gastrula. Note that the embryo remains connected to the maternal uterus via a hollow stalk. (**E**) Detail of the blastoporal area in a late gastrula embryo showing the continuous immigration of endomesodermal cells, which give rise to the endoderm (*arrow*) and mesoderm later in development. (**F**) Elongating embryo. Note that the stomodeum and the proctodeum move apart from each other, forming a blastoporal slit (*arrowhead*). (**G**, **H**) Elongating embryo at a later developmental stage in dorsal (**G**) and ventral perspective (**H**). Note the remnant of the blastoporal slit anterior to the proctodeum (*arrowhead*), similar to that observed in *Euperipatoides rowelli* (cf. Fig. 4.6I). (**I**) Embryo of early coil stage (staging according to Walker and Campiglia 1988). Note the coelomic cavities occupying most of the inner volume of the embryo, while endomesodermal cells continuously migrate in the posterior body region. *Abbreviations*: *as* antennal somite, *bp* blastopore, *cb* coeloblastula, *de* dorsal extra-embryonic tissue, *ec* ectoderm, *en* endomesoderm, *es* embryonic sac, *js* jaw somite, *mo* morula, *pr* proctodeum, *sk* stalk, *ss* slime papilla somite, *st* stomodeum, *su* stalk lumen. Scale bars: 20 μm (**A**, **B**), 50 μm (**C**–**H**), and 100 μm (**I**)

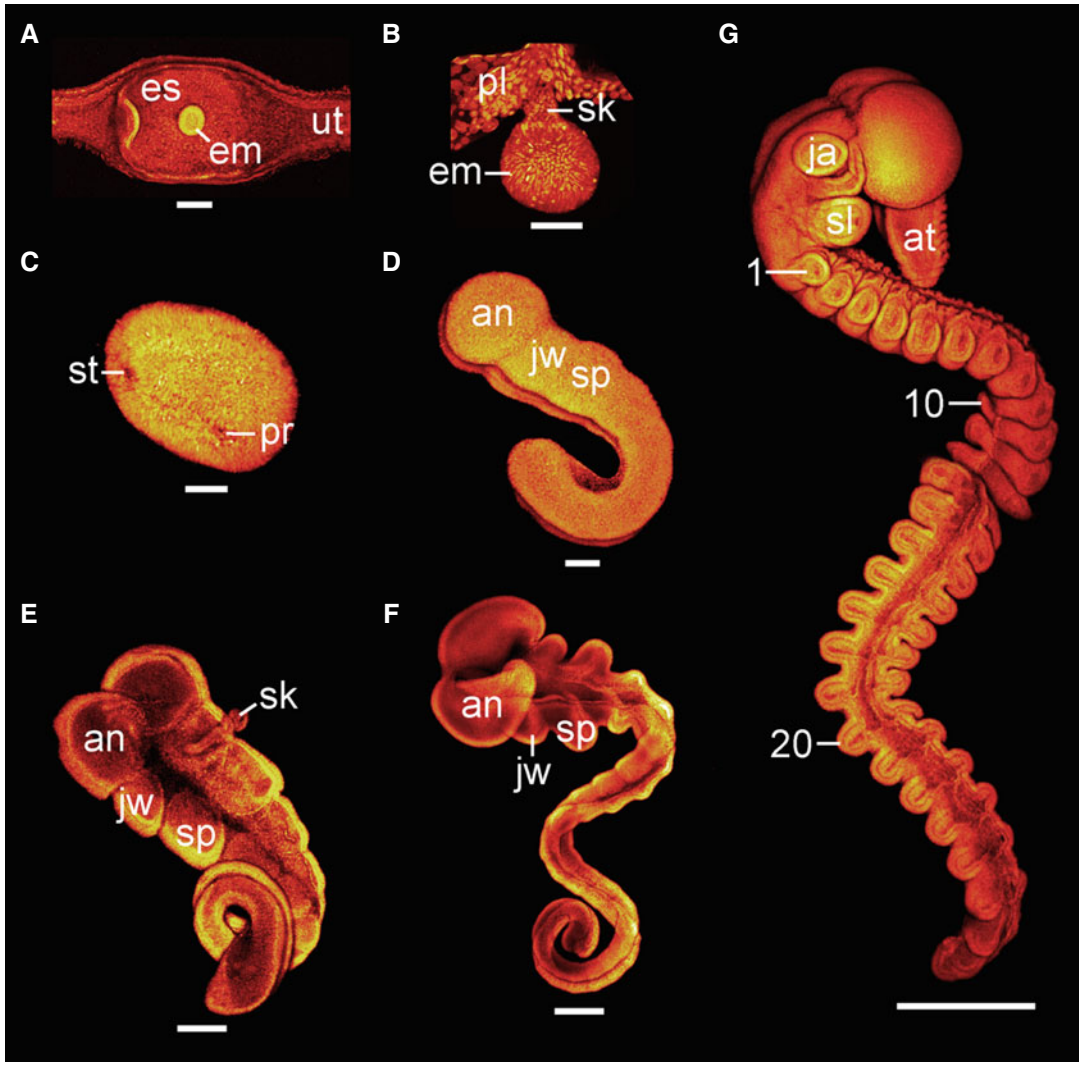

Fig. 4.9 Embryogenesis in placentotrophic viviparous species of Peripatidae. Confocal laser scanning micrographs of whole-mount embryos of *Epiperipatus biolleyi* (**A**–**C**, **E**, **F**) and *Principapillatus hitoyensis* (**D**, **G**) labeled with DNA marker bisbenzimide (*glow* scale). (**A**) Implanted early embryo (morula or early gastrula). (**B**) Gastrula. Note the stalk connecting the embryo to the maternal placenta. (**C**) Elongating embryo. The stomodeum is already separated from the proctodeum. (**D**–**F**) Embryos of successive developmental coil stages (staging according to Walker and Campiglia 1988). Note the stalk attached to the dorsal region of the embryo in (**E**). (**G**) Flexed-stage embryo. Developing leg-bearing segments are indicated by *numbers*. *Abbreviations*: *an* antennal segment, *at* antenna, *em* embryo, *es* embryonic sac, *ja* jaw, *jw* jaw segment, *pl* placenta, *pr* proctodeum, *sk* stalk, *sl* slime papilla, *sp* slime papilla segment, *st* stomodeum, *ut* uterus. Scale bars: 200 µm (**A**, **C**, **E**–**G**) and 100 µm (**B**, **D**)

Whitington 2009b). After becoming attached to the wall of the embryonic sac (specialized area of the uterus), the morula hollows out and gives rise to a spherical coeloblastula (Figs. 4.8B, 4.9A, and 4.10B). As the blastula increases in size via cell divisions, the blastocoel becomes more prominent. Specialized cells of the blastula, located at the attachment site to the uterine wall, then give rise to a hollow stalk, resulting in a bulb-shaped embryo (Figs. 4.8D, 4.9B, and 4.10B–D; Kennel 1885; Sclater 1887; Anderson and Manton 1972). The stalk cells later form a pseudostratified layer of the embryonic part of the placenta. At the onset of gastrulation, cells

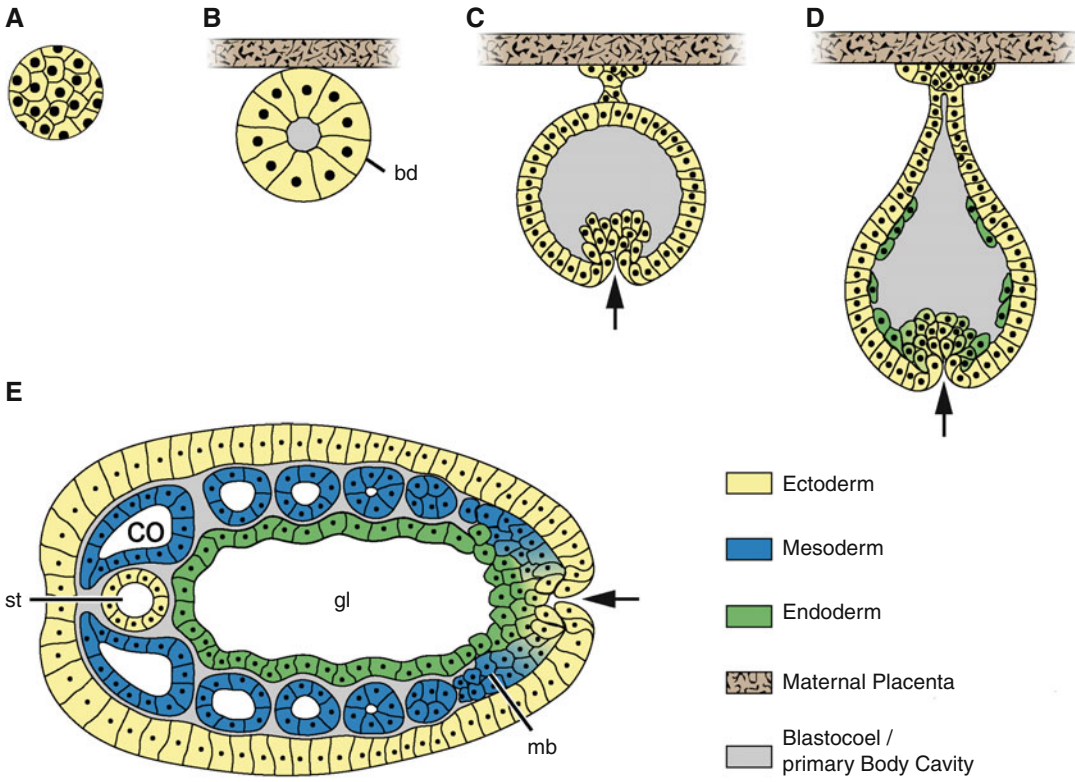

Fig. 4.10 Simplified diagrams of the development in placentotrophic viviparous peripatids (reconstructed after Kennel 1888, Walker and Campiglia 1988, 1990 and own data, cf. Fig. 4.11A, B). (**A**) Morula. Cleavage is total and equal in these species. (**B**) Coeloblastula. (**C**) Early gastrula. Note the endomesodermal cells starting to immigrate in the blastopore region (*arrow*). (**D**) Late gastrula. The immigrated endomesodermal cells spread around the blastocoel to form the endoderm. *Arrow* points to the blastopore region. (**E**) Horizontal section of an elongating embryo. The endomesodermal cells, which continue to immigrate in the proctodeal region (*arrow*), give rise to both endoderm and mesoderm. Note that the blastocoel persists as confluent spaces (=primary body cavity) between the ecto-, endo-, and mesoderm (contrary to Koch et al. 2014). *Abbreviations*: *bd* blastoderm, *co* coelomic cavity, *gl* gut lumen, *mb* mesodermal germband, *st* stomodeum

begin to immigrate in the blastoporal area at the opposite pole of the embryo (Figs. 4.8C, E and 4.10C, D; Kennel 1885). These cells form a compact mass that later spreads out around the blastocoel to form a single-layered endoderm (Fig. 4.10D, E).

While the blastopore persists as the proctodeum in the elongating embryo, the origin of the stomodeum is ambiguous in the neotropical Peripatidae. Kennel (1885) assumes an independent origin of the stomodeum and proctodeum, but these openings might also originate simultaneously in the blastoporal area and then move apart, as described herein from embryos of the lecithotrophic/matrotrophic viviparous species *Euperipatoides rowelli* (Figs. 4.6D–I and 4.7B–F). A common origin of these two openings in the neotropical Peripatidae is supported by the occurrence of a blastoporal slit between the stomodeum and proctodeum (Fig. 4.8F, H), again resembling the situation in the lecithotrophic viviparous species (cf. Figs. 4.6F–I and 4.7C–E). However, this slit disappears early in development, as it is not recognizable in the segmenting embryo thereafter. Further embryonic development of the neotropical Peripatidae, including growth and differentiation of the mesodermal bands and organogenesis, resembles that of the non-placentotrophic species, except that the coelomic cavities occupy most of the inner volume of the embryo (Figs. 4.8I and 4.11A–D; Kennel 1885, 1888; Anderson and Manton 1972;

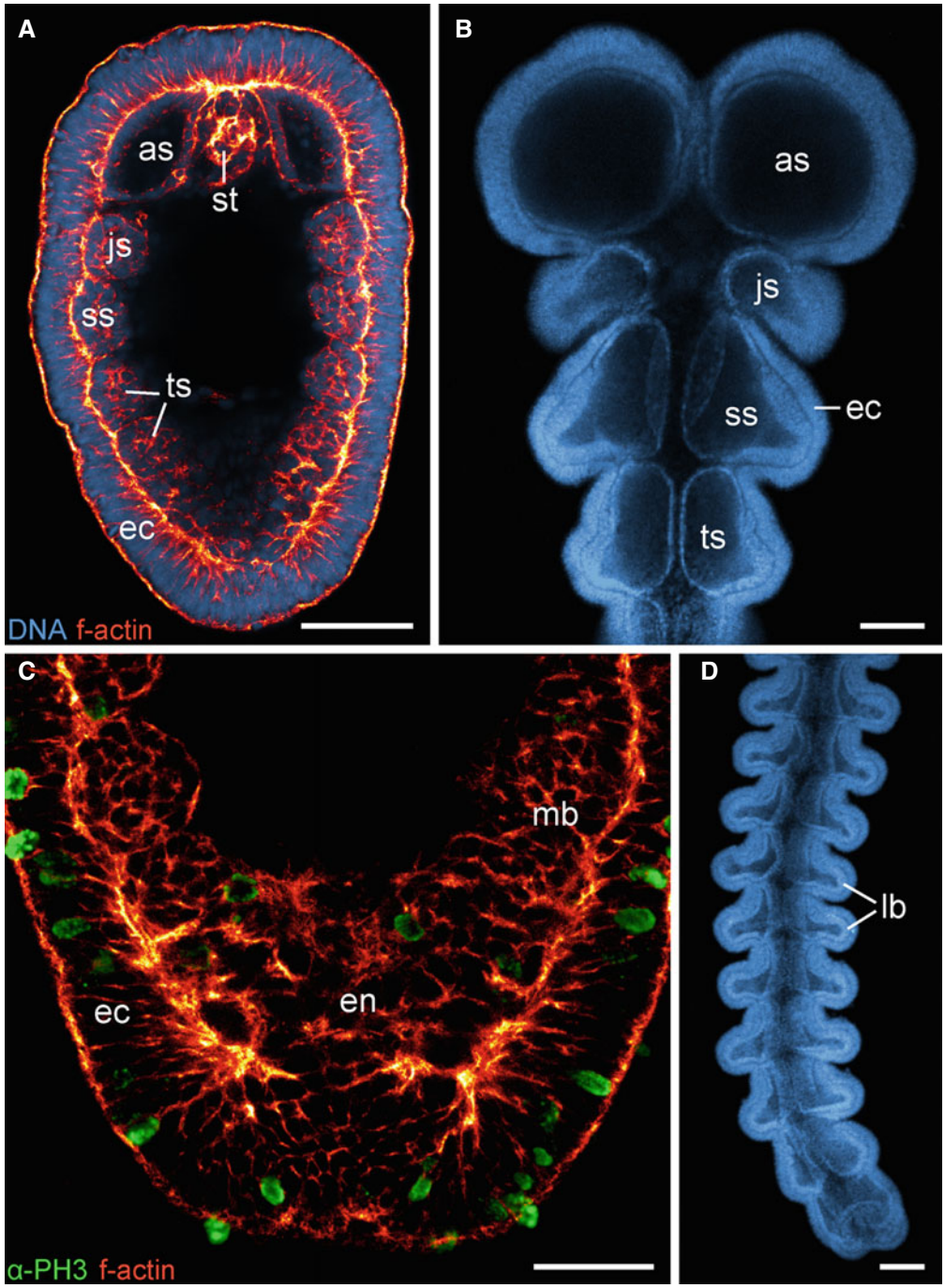

Fig. 4.11 Mesoderm formation and coelomogenesis in *Epiperipatus biolleyi* (Peripatidae). Confocal laser scanning micrographs. Anterior is up in all images. (**A**, **B**). Elongating embryo stage triple labeled with DNA marker bisbenzimide (*blue*), phalloidin-rhodamine (f-actin; *glow* scale) and anti-phospho-histone H3 antibody (α-PH3; *green*). Note the anterior-to-posterior development of coelomic cavities. Also note that α-PH3 immunolabeling reveals no posterior proliferation zone (**B**). (**C**, **D**) Anterior (**C**) and posterior body region (**D**) of a flexed-stage embryo labeled with DNA marker bisbenzimide. Note the large, segmental coelomic cavities. *Abbreviations*: *as* antennal somite, *ec* ectoderm, *en* endomesoderm, *js* jaw somite, *lb* limb bud, *mb* mesodermal band, *ss* slime papilla somite, *st* stomodeum, *ts* developing trunk somite. Scale bars: 100 μm (**A**, **C**, **D**) and 50 μm (**B**)

Bartolomaeus and Ruhberg 1999; Mayer et al. 2004; Mayer 2006a). Thus, despite the reduction of yolk and ventral extra-embryonic tissue and the presence of an embryonic stalk and placenta, early development of the neotropical Peripatidae is actually more similar to the lecithotrophic and matrotrophic viviparous species of Peripatopsidae than previously recognized (Anderson 1966, 1973).

Organogenesis

Mesoderm Formation and Coelomogenesis

Similar to the endoderm, the mesoderm of the onychophoran embryo originates from the endomesodermal tissue surrounding the proctodeum (Fig. 4.11A, B; Kennel 1885; Sedgwick 1886, 1887; Evans 1901; Manton 1949). The endomesoderm gives rise to a pair of ventrolateral mesodermal bands, which grow anteriorly between the developing midgut epithelium and the overlying ectoderm (Figs. 4.10E and 4.11A, B; Sedgwick 1887; Kennel 1888; Evans 1901; Manton 1949). As the bands grow, they subdivide into solid, metameric blocks of cells that hollow out by schizocoely in an anterior-to-posterior progression (Fig. 4.11A–D; Sedgwick 1887; Kennel 1888; Evans 1901; Manton 1949). These hollow spaces are the embryonic coelomic cavities (=somites), which are lined by true epithelia (Bartolomaeus and Ruhberg 1999; Mayer et al. 2004, 2005; Mayer 2006a).

Thus, like many arthropods, onychophorans show typical short germband development, since their body segments also arise in an anterior-to-posterior progression (Figs. 4.6, 4.7, 4.8, 4.9, 4.10, and 4.11; Kennel 1885, 1888; Sedgwick 1887, 1888b; Evans 1901; Manton 1949; Walker and Campiglia 1988; Campiglia and Walker 1995). The two reports (Walker 1992, 1995) of putative long germband development in *Opisthopatus cinctipes* are doubtful, as the thin linings of the dorsal coelomic compartments may have been overlooked in this species (see discussion by Mayer et al. 2005). Despite short germband development, labeling with specific cell division markers revealed no posterior proliferation or growth zone in Onychophora (Mayer et al. 2010b). The dividing cells are instead scattered along the body rather than being concentrated at the posterior end (Fig. 4.11B). Therefore, this body region should not be regarded as a proliferation zone like in annelids, but rather as a segment addition or segmentation zone (Mayer et al. 2010b).

Mixocoely: Fusion of Coelomic and Primary Body Cavities

The first cells of the developing mesoderm and endoderm grow into the blastocoel, forcing it into narrow, confluent spaces between the three germ layers (Figs. 4.10E and 4.12A). These spaces comprise the primary body cavity (gray in Figs. 4.10E and 4.12A–H) – lined by extracellular matrix – and are initially separate from the coelomic cavities that are enclosed by true epithelia, i.e., cells that are linked by apical junctions and rest on a basal lamina (Fig. 4.12K; Mayer et al. 2004, 2005; Mayer 2006a). Ultrastructural studies on embryos of *Epiperipatus biolleyi* revealed that the primary body cavity subsequently fuses with coelomic cavities by a process called mixocoely (Mayer et al. 2004; Mayer 2006a). While some portions of the coelomic walls retain their epithelial organization, such as in the anlagen of nephridia and their derivatives, their remaining portions are transformed into mesenchymal tissue that gives rise to the heart and musculature. Thus, the ultrastructural data from embryos of *Epiperipatus biolleyi* confirm the results of Kennel (1885, 1888) on the coelomic fate in other neotropical species, although his observations were heavily criticized (Sedgwick 1887; Evans 1901).

In contrast to the neotropical peripatids, Sedgwick (1887, 1888a, b) described a different coelomic fate in the peripatopsid *Peripatopsis capensis* (Fig. 4.12E–H). In this species, like in *Opisthopatus roseus* – another matrotrophic viviparous peripatopsid from South Africa – each unitary coelomic cavity is subdivided into a ventral and a dorsal compartment (Fig. 4.12E–J; Sedgwick 1887, 1888a, b; Mayer et al. 2005). While the ventral compartment develops into the nephridial anlage, the dorsal compartment is, according to Sedgwick (1888a, p. 81), “reduced

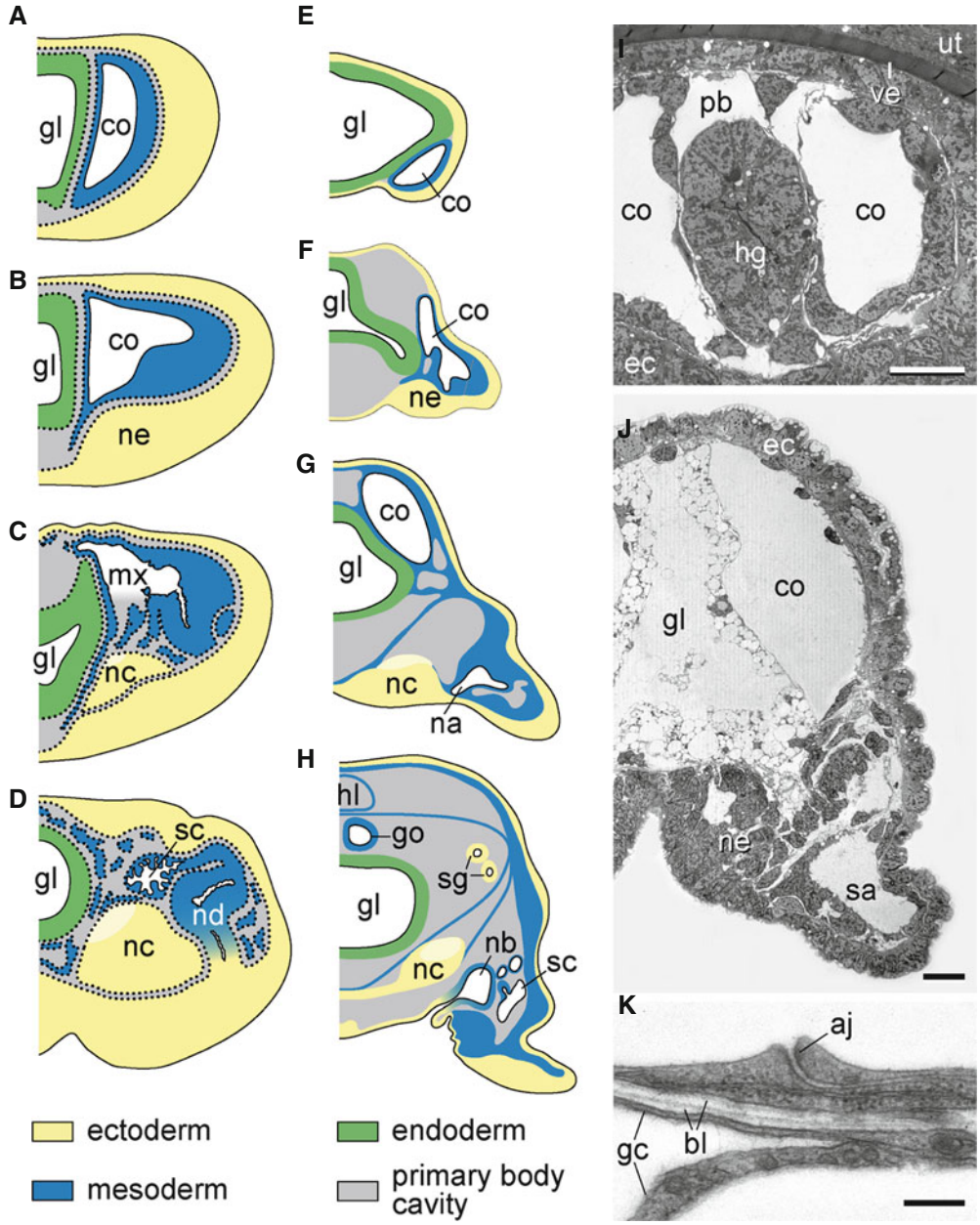

Fig. 4.12 Nephridiogenesis in Onychophora. Simplified diagrams of successive developmental stages (**A**–**H**) and transmission electron micrographs (**I**–**K**). (**A**–**D**) Nephridiogenesis in the peripatid species *Epiperipatus biolleyi* (reconstructed after Mayer et al. 2004; Mayer 2006a). *Dotted lines* indicate the basal laminae of the ecto-, endo-, and mesodermal cell layers (reconstructed based on the ultrastructural data). Note the fusion of the primary body cavity with the coelomic cavity, forming a mixocoel. (**E**–**H**) Nephridiogenesis in the peripatopsid *Peripatopsis capensis* (reconstructed after Sedgwick 1888a). The coelomic cavity is divided into a dorsal and a ventral compartment. (**I**, **J**) Cross sections of embryos of *Opisthopatus roseus* from South Africa, which show a similar situation to that in *Peripatopsis capensis*. Note the separation of the coelomic cavity into a dorsal and a ventral compartment (in **J**). (**K**) Detail of the coelomic lining of the dorsal coelomic compartment in (**J**). Note that the coelomic lining cells are linked by an apical junction and rest on a basal lamina (=extracellular matrix), characterizing the coelomic wall as a true epithelium. *Abbreviations*: *aj* apical junction, *bl* basal lamina, *co* coelomic cavity, *ec* ectoderm, *gc* gut cell, *gl* gut lumen, *go* gonad, *hg*, hindgut, *hl* heart lumen, *mx* mixocoel, *na* nephridial anlage, *nb* nephridial bladder, *nc* nerve cord, *nd* nephridioduct, *ne* neuroectoderm, *pb* primary body cavity, *sa* anlage of the salivary gland, *sc* nephridial sacculus, *sg* slime gland, *ve* vitelline envelope, *ut* uterus. Scale bars: 10 µm (**I**, **J**) and 200 nm (**K**)

in size … and finally vanishes." Likewise, Evans (1901, pp. 59–60) stated that various coelomic compartments become obliterated by the "coming together" of their walls in the Southeast Asian peripatid *Eoperipatus weldoni*. However, neither Sedgwick (1887, 1888a, b) nor Evans (1901) provide details on what actually happens to the epithelial linings of the coelomic compartments that are obliterated. It therefore seems likely that at least the "vanishing" coelomic compartments fuse with the primary body cavity, while their walls are transformed into mesenchymal tissue, similar to the process of mixocoely described from the neotropical peripatids (Kennel 1885, 1888; Mayer et al. 2004; Mayer 2006a). Since mixocoely (sensu Mayer 2006a) also occurs in various arthropods (e.g., Anderson 1973; Weygoldt 1986; Nielsen 2012; Koch et al. 2014 contra Bartolomaeus et al. 2009), this process was most likely present in the last common ancestor of Onychophora and Arthropoda.

Ontogenetic Fate of Coelomic Linings: Formation of Nephridia, Gonad, Heart, and Musculature

During onychophoran development, the epithelial linings of transitory coelomic cavities give rise to all mesodermal organs and tissues, including the heart, musculature, and nephridia (=renal organs) and their derivatives (Kennel 1885, 1888; Sedgwick 1887, 1888a, b; Evans 1901; Mayer et al. 2004, 2005; Mayer and Koch 2005; Mayer 2006a). In embryos of the South African peripatopsids, in which the coelomic cavity is subdivided into a dorsal and ventral compartment, the walls of the ventral compartment form the nephridium (Fig. 4.12E–H; Sedgwick 1887, 1888a, b; Mayer et al. 2005). More specifically, the lateral portion of this compartment develops into the sacculus, whereas the median portion gives rise to the nephridioduct (Fig. 4.12G, H; Sedgwick 1888a). During further development, the nephridial lumen opens to the exterior via a short, ectodermal duct. The mesodermal portion of the nephridioduct then elongates and forms several specialized regions, while the cells lining the sacculus differentiate into podocytes that are involved in ultrafiltration in the adult (Storch et al. 1978). A similar pattern of nephridial development occurs in the Australian peripatopsids *Euperipatoides rowelli* (Fig. 4.13A–E) and *Cephalofovea clandestina* (see Koch et al. 2014), although it is unclear whether the initial coelom is subdivided into a dorsal and ventral compartment, like in the South African peripatopsids.

In contrast, a subdivision of coelomic cavities does not take place prior to nephridiogenesis in embryos of the neotropical peripatids (Kennel 1888; Mayer 2006a). In these species, the nephridial anlage instead arises from, and subsequently buds off, the ventrolateral portion of a single coelomic compartment (Fig. 4.12A–D; Mayer et al. 2004; Mayer 2006a). Further development of nephridia is similar in all onychophoran species studied and results in a typical double-looped organization of the nephridioduct (Fig. 4.13E; Mayer 2006a; Mayer and Whitington 2009a). This loop is also evident in the modified nephridia (=labyrinth organs) of the fourth and fifth leg-bearing segments, although the function of these organs is unknown (Fig. 4.13F).

Euperipatoides rowelli: An Emerging Model for Developmental Studies

The peripatopsid *Euperipatoides rowelli* Reid, 1996 (leftmost specimen in Fig. 4.1) has recently been emerging as a model for evolutionary developmental biology (Table 4.2). Females of *Euperipatoides rowelli* typically do not exceed 6 cm in length, whereas males are usually smaller (Reid 1996; Curach and Sunnucks 1999; Sunnucks et al. 2000). Specimens of both sexes can be maintained in the laboratory for several years at 17–18 °C in plastic jars filled with a 2–3 cm layer of peat covered with damp paper towels to retain moisture (Baer and Mayer 2012). The species covers a wide geographic range and can be collected easily from decaying logs within and around national parks throughout Tallaganda (New South Wales, Australia), where it is highly abundant (Reid 1996; Curach and Sunnucks 1999; Barclay et al. 2000a, b; Sunnucks et al. 2000; Bull et al. 2013). In the last two decades, *E. rowelli* has become the most studied onychophoran species (Blaxter and Sunnucks 2011),

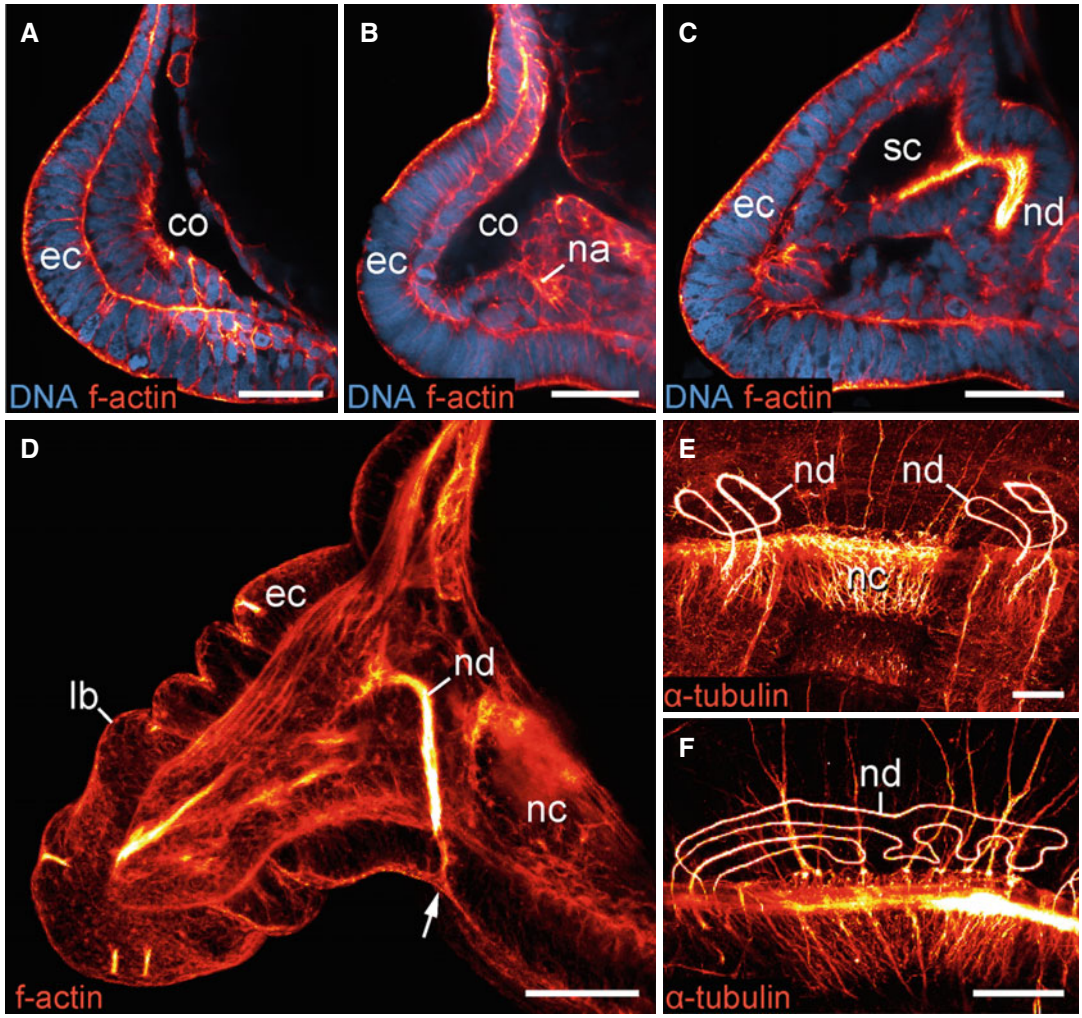

Fig. 4.13 Nephridiogenesis in the peripatopsid *Euperipatoides rowelli*. Confocal laser scanning micrographs. (**A**–**C**) Cross section of developing limbs in embryos of successive developmental stages double labeled with DNA marker bisbenzimide (*blue*) and phalloidin-rhodamine (f-actin; *glow* scale). Note that the lateral portion of the coelomic cavity gives rise to the nephridial sacculus, whereas the median portion forms the nephridioduct. The strong signal in the lumen of the nephridial duct (in **C**) results from numerous microvilli, which contain f-actin. (**D**) Cross section of a limb in a late developmental stage embryo labeled with phalloidin-rhodamine (f-actin; *glow* scale). Arrow points to the ectodermal duct, through which the nephridium opens externally at the basis of the limb. (**E**, **F**) The double-looped organization of fully developed nephridioducts. Late embryos labeled with phalloidin-rhodamine (f-actin; *glow* scale). Anterior is left. Note that the double-looped organization of the duct is also present in the modified nephridia (=labyrinth organs) of the fourth and fifth leg-bearing segments (in **F**). *Abbreviations*: *co* coelomic cavity, *ec* ectoderm, *lb* limb bud, *na* nephridial anlage, *nc* nerve cord, *nd* nephridioduct, *sc* anlage of the nephridial sacculus. Scale bars: 50 μm (**A**–**E**) and 100 μm (**F**)

the biology, anatomy, development, physiology, reproduction, phylogeny, and population genetics of which have been extensively analyzed (Table 4.2). Furthermore, the genome of *Euperipatoides rowelli* is currently being sequenced (http://www.hgsc.bcm.tmc.edu/content/i5k-velvet-worm), which will provide additional resources for working with this emerging "model" onychophoran species.

Table 4.2 Studies involving the onychophoran *Euperipatoides rowelli*

Primary focus	References
Biology, distribution, taxonomy, and conservation	Scott and Rowell (1991), Reid (1996), Curach and Sunnucks (1999), Barclay et al. (2000a, b), Clarke and Spier-Ashcroft (2001), Sunnucks and Tait (2001), Reinhard and Rowell (2005), and Blaxter and Sunnucks (2011)
Anatomy	Reid (1996), Strausfeld et al. (2006a, b), Walker et al. (2006), Mayer and Tait (2009), Baer and Mayer (2012), Mayer et al. (2013a, b), Oliveira and Mayer (2013), Martin and Mayer (2014), Mayer et al. (2014, 2015a), Mayer (2015)
Development	Walker and Tait (2004), Mayer and Whitington (2009a, b), Mayer et al. (2010a, b), Whitington and Mayer (2011), Ou et al. (2012), Treffkorn and Mayer (2013), Oliveira et al. (2013b), Martin and Mayer (2014), Franke and Mayer (2014), and Franke et al. (2015)
Reproduction	Curach and Sunnucks (1999), Sunnucks et al. (2000), Walker and Tait (2004), and Walker et al. (2006)
Population genetics	Sunnucks and Wilson (1999), Bull et al. (2013), and Bull and Sunnucks (2014)
Phylogenetic position	Tait et al. (1995), Reid (1996), Gleeson et al. (1998), Allwood et al. (2010), and Murienne et al. (2014)
Physiology	Woodman et al. (2007)
Other aspects	Haritos et al. (2010) and Baer et al. (2014) – study of slime and slime protein profiling; Hering et al. (2012) – opsin repertoire; Rowell et al. (1995) and Jeffery et al. (2012) – karyotype and genome size; Murdock et al. (2014) – decay of velvet worms; Beckmann et al. (2015) – spectral sensitivity; Mayer et al. (2015b) – pigment-dispersing factor neuropeptides

In the head region, the anlagen of nephridia persist in adult stages only in the slime papilla segment, in which they develop into the salivary glands (Kennel 1888; Sedgwick 1888a; Evans 1901; Mayer and Koch 2005). Each salivary gland anlage gives rise to a large, proximal, caecal part and a distal, glandular duct, the latter being associated with a sacculus (Storch et al. 1979). At the end of embryogenesis, the paired salivary gland anlagen open via a common duct into the definitive mouth cavity. Transient nephridial anlagen also occur in the antennal (=first) and jaw (=second) segments (Sedgwick 1887; Kennel 1888; Mayer and Koch 2005; Mayer et al. 2005). However, while the nephridial anlagen arise in a typical ventral position in the jaw segment, those of the antennal segment occur dorsally above each eye (cf. Fig. 4.19A, B; Kennel 1888; Mayer and Koch 2005; Mayer et al. 2005). This unusual position suggests that the onychophoran antennae are modified limbs of the anterior-most body segment that have been relocated antero-dorsally but still recapitulate a pair of transient nephridia at their bases (Mayer and Koch 2005). These transient nephridia, however, disappear before the formation of the photoreceptors.

The anlagen of nephridia in the posterior body region of onychophorans show multiple fates. In the post-genital segments, they are either rudimentary or give rise to accessory genital glands, whereas they form a pair of gonoducts in the genital segment (review Mayer and Koch 2005). The presumptive gonoducts subsequently become associated with the gonad, which arises from the dorsal coelomic walls of several posterior segments, including the genital segment (Sedgwick 1887; Kennel 1888; Evans 1901). The primordial germ cells, which arise earlier in development (Evans 1901; Manton 1949), become associated with the visceral wall of each coelomic cavity (Fig. 4.14A, B). They retain this position in the adult gonad, the lumen of which is a derivative of the embryonic coelomic cavities, and develop into the spermatogonia of the testes and the oogonia of the ovaries (Ruhberg and Storch 1976; Herzberg et al. 1980; Storch and Ruhberg 1990; Huebner and Lococo 1994; Storch et al. 1995; Brockmann et al. 1999, 2001; Mayer and Tait 2009). Irrespective of the ovarian type (exogenous versus endogenous), the ovarian anlage initially shows a sterile dorsal and a germinal ventral portion, the latter containing the presumptive oogonia

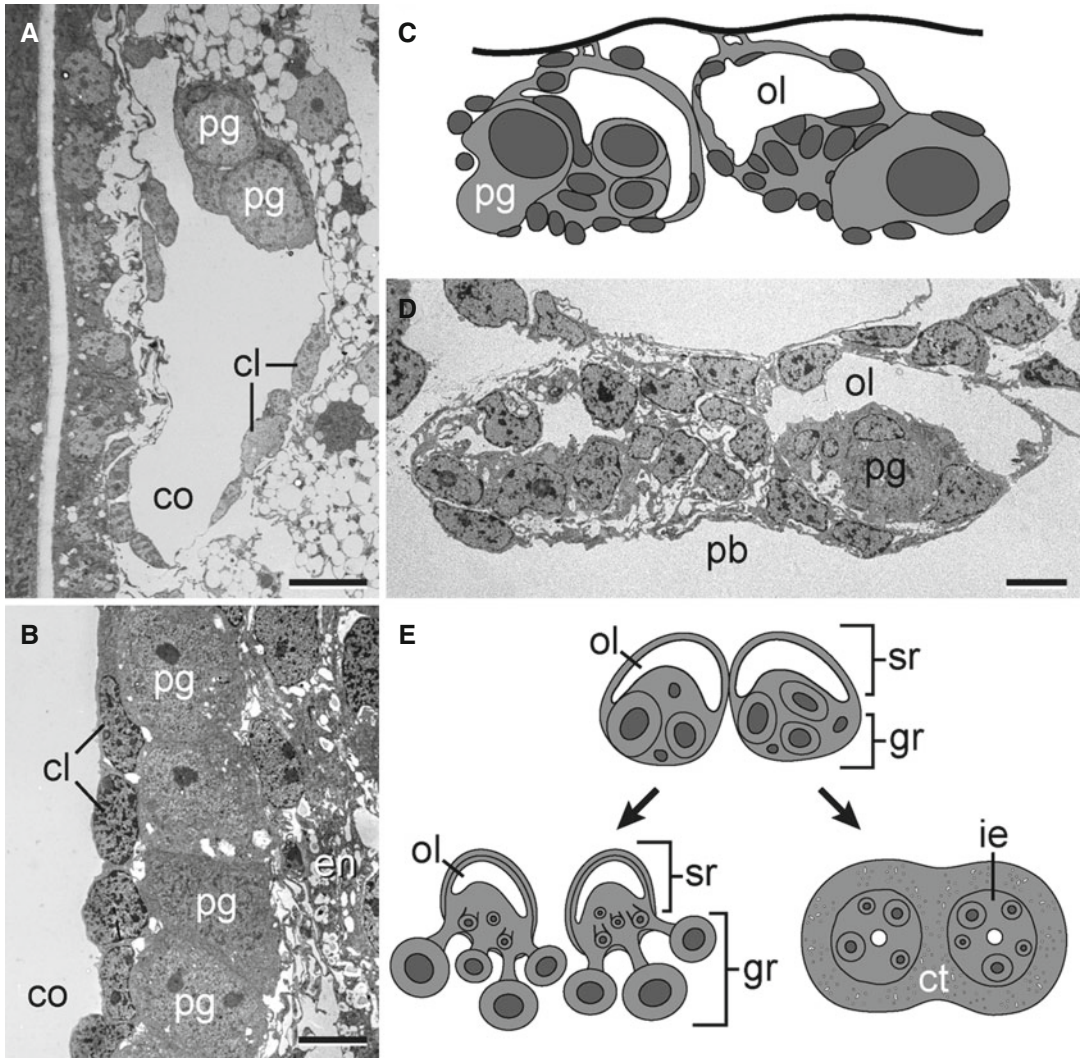

Fig. 4.14 Development of the onychophoran ovary. Transmission electron micrographs (**A**, **B**, **D**) and simplified diagrams (**C**, **E**). (**A**, **B**) Cross sections through the ovary anlage in an early embryonic stage of *Opisthopatus roseus* (Peripatopsidae) and *Epiperipatus biolleyi* (Peripatidae), respectively. The primordial germ cells are associated with the visceral coelomic wall in both species. (**C**) The anlage of an exogenous ovary in *Peripatopsis capensis* (redrawn from Sedgwick 1887). (**D**) Cross section through the anlage of the endogenous ovary in *Epiperipatus biolleyi*. Note the similar structure to the anlage of an exogenous ovary (represented in **B**). (**E**) Diagram of development of the ovarian anlage into different ovarian types. Note that the sterile dorsal portion and the germinal ventral portion of the ovarian anlage are retained in exogenous ovary (*left diagram*) but are modified in the endogenous ovary (*right diagram*) (diagrams of the adult condition modified after Mayer and Tait 2009). *Abbreviations*: *cl* coelomic lining cells, *co* coelomic cavity, *ct* connective tissue, *en* endoderm, *gr* germinal ovarian portion, *ie* inner (germinal) epithelium, *ol* ovarian lumen, *pb* primary body cavity, *pg* primordial germ cell, *sr* sterile ovarian portion. Scale bars: 10 µm (**A**) and 5 µm (**B**, **D**)

(Fig. 4.14C–E). This organization is retained in the exogenous and pseudoendogenous ovaries but modified in the endogenous ovary during embryogenesis (Fig. 4.14E; Mayer and Tait 2009).

Similar to the gonad, the onychophoran heart is also a derivative of dorsal coelomic walls (Sedgwick 1887, 1888a, b; Kennel 1888; Evans 1901). However, the coelomic linings do not directly contribute to the cardiac wall. Instead, individual cells of these linings first immigrate into the space above the midgut, which comprises the primary body cavity (Fig. 4.15A–C). These

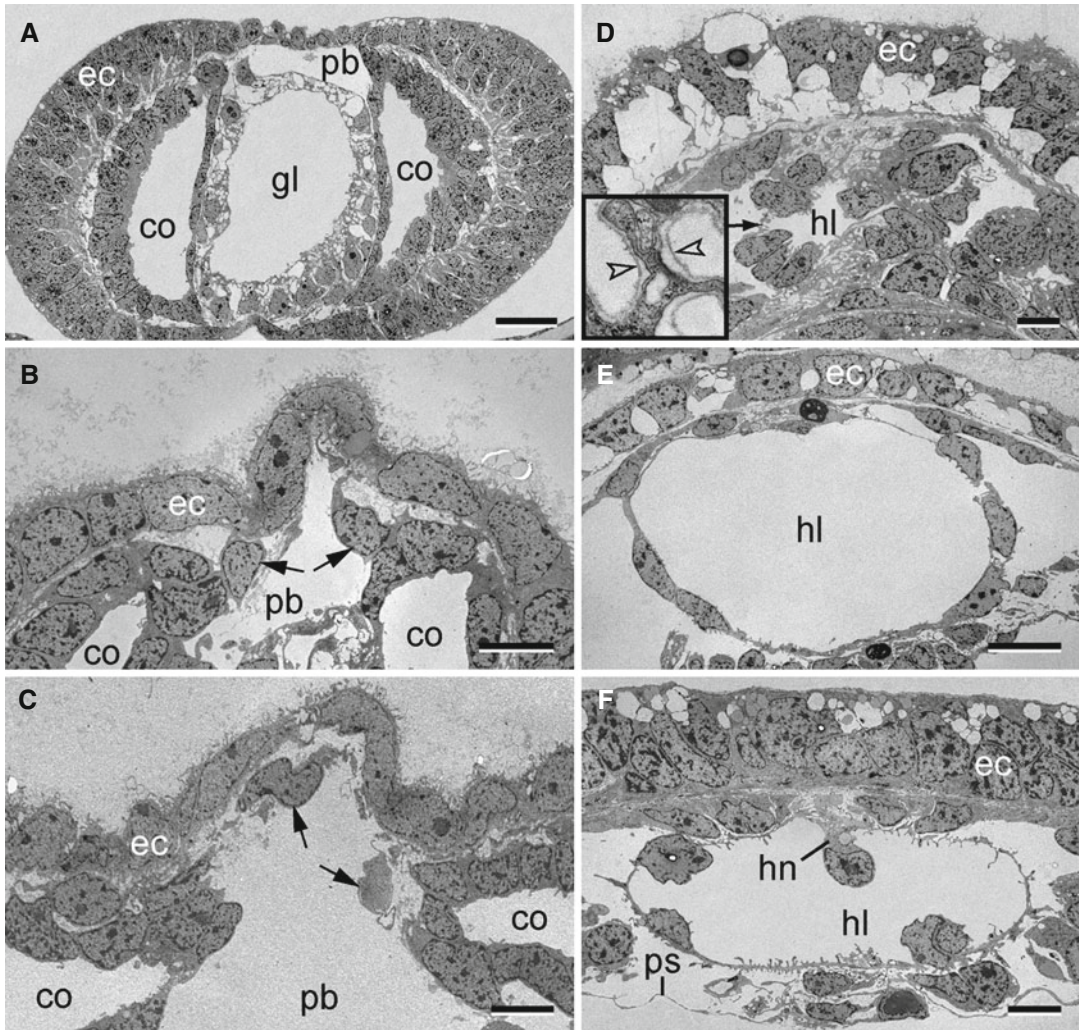

Fig. 4.15 Heart development in *Epiperipatus biolleyi* (Peripatidae). Transmission electron micrographs of successive developmental stages (cross sections). (**A**–**C**) Immigration of cells from coelomic linings (*arrows*) into the primary body cavity during early development. (**D**, **E**) Formation of a hollow tube – the future heart – by immigrated, mesenchymal cells. *Arrowheads* (*inset* in **D**) indicate the extracellular matrix on either side of the heart wall. (**F**) The complete heart with its associated dorsal nerve. *Abbreviations*: *co* coelomic cavity, *ec* ectoderm, *gl* gut lumen, *hl* heart lumen, *hn* heart nerve, *pb* primary body cavity, *ps* developing pericardial septum. Scale bars: 20 µm (**A**), 5 µm (**B**–**D**, **F**), and 10 µm (**E**)

immigrated, mesenchymal cells then form a hollow tube – the future heart – that develops muscle filaments and becomes associated with a dorsal nerve, i.e., the heart nerve (Figs. 4.15D–F and 4.16H; Nylund et al. 1988; Mayer and Harzsch 2008; Mayer and Whitington 2009a). A similar mode of heart development from apolar mesenchymal cells was described recently from arthropods, although the origin of these cells remains unclear (Koch et al. 2014).

The musculature of the onychophoran body arises from the remaining mesenchymal cells that had originated from the former coelomic walls (Kennel 1888; Sedgwick 1888a; Mayer et al. 2005; Mayer 2006a). While the initial muscle fibers of the body wall do not show any segmental arrangement, those within the limbs are arranged in a segmental fashion from the onset of myogenesis (Figs. 4.13D and 4.16A–I). The connective tissue, which contains muscle fibers

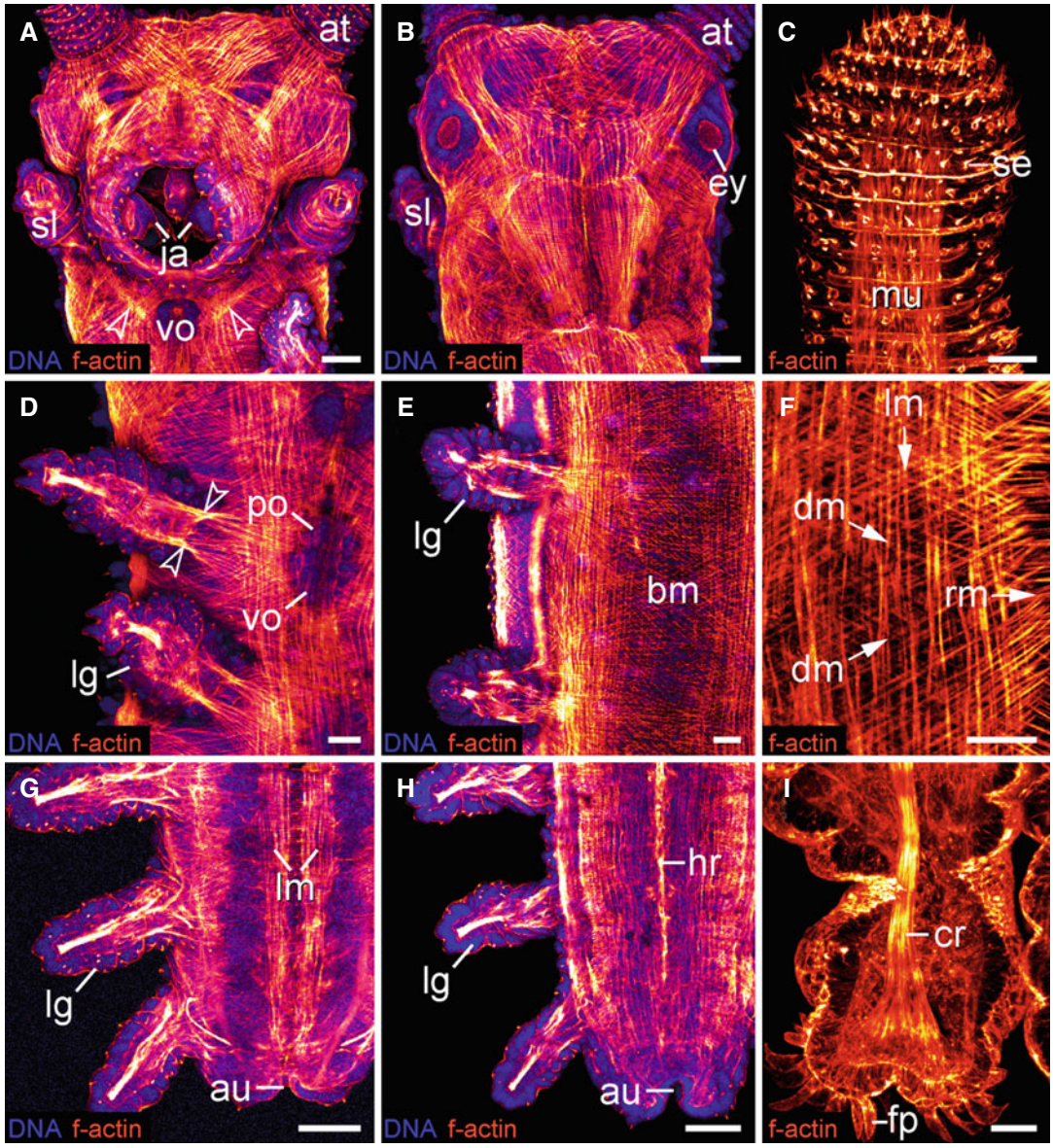

Fig. 4.16 The onychophoran body musculature. Confocal laser scanning micrographs of late-stage embryos of the peripatopsid *Euperipatoides rowelli* (**A**–**E**, **G**, **H**) and the peripatid *Epiperipatus biolleyi* (**F**, **I**). Embryos labeled with phalloidin-rhodamine (f-actin; *glow* scale in **A**–**I**) and double labeled with DNA marker bisbenzimide (*blue* in **A**, **B**, **D**, **E**, **G**, **H**). Anterior is up (in **A**–**H**), lateral is *right* (in **F**), and proximal is up (in **I**). (**A**) Head in ventral view. *Arrowheads* indicate the segmental, posterior depressor muscles of the slime papillae. (**B**) Head in dorsal view. (**C**) Detail of the antennal tip and its associated, modified musculature. (**D**) Ventral view of the trunk region. Note the association of the segmental, paired leg depressor muscles (*arrowheads*) with the median anlage of the ventral and preventral organs. (**E**) Dorsal view of the trunk region. (**F**) Detail of the ventral body wall musculature. The diagonal muscles consist of two layers and the longitudinal and ring muscles each consist of one layer. Orientations of muscle fibers indicated by *arrows*. (**G**) Posterior body region in ventral view. (**H**) Posterior body region in dorsal view. Note the developing musculature of the heart wall. (**I**) Detail of the muscles associated with the claws (=claw retractor). The foot projections are unique to placentotrophic species (e.g., Oliveira et al. 2012b). *Abbreviations*: *at* antenna, *au* anus, *bm* musculature of the body wall, *cr* claw retractor muscle, *dm* diagonal musculature, *ey* eye, *fp* foot projection, *hr* developing heart, *ja* jaw, *lg* leg, *lm* longitudinal musculature, *mu* musculature, *rm* ring musculature, *se* antennal sensilla, *sl* slime papilla, *po* preventral organ, *vo* ventral organ. Scale bars: 100 µm (**A**, **B**, **G**, **H**), 50 µm (**C**–**E**), and 20 µm (**F**, **I**)

and envelops most internal organs – including nerve cords, midgut, and genital tract – develops from a network of mesenchymal cells derived from disintegrating coelomic linings (Kennel 1888; Sedgwick 1888a; Mayer 2006a). Later in development, the muscle fibers of the body wall show the typical organization into the three muscle layers (Fig. 4.16F; cf. Hoyle and Williams 1980).

Within each developing limb, the future claw retractor muscle attaches to the distal epidermis of the foot (Figs. 4.13D and 4.16I; Oliveira and Mayer 2013), whereas the anterior and posterior leg depressor muscles become associated with specialized attachment sites, i.e., the ventral and preventral organs (Fig. 4.16D; Oliveira et al. 2013b). This arrangement of the ventral depressor muscles is also evident in the slime papilla segment, despite the anterior relocation of its appendages (Fig. 4.16A). Compared to the remaining limbs, the musculature of the antennae (Fig. 4.16C) and jaws (Oliveira and Mayer 2013) shows a modified organization, which might be due to the specialized function of these appendages.

Neurogenesis

At the onset of neurogenesis in Onychophora, neural precursors segregate as single cells from the neuroectoderm and move internally by a process called ingression (sensu Meyer and Seaver 2009). This process begins with single cells becoming bottle-shaped and subsequently detaching from the apical surface of the ectoderm (Fig. 4.17A, B; Whitington 2007; Mayer and Whitington 2009b; Whitington and Mayer 2011). These immigrated neural precursors form an internal layer of cells that show an increased number of mitotic divisions and express an *achaete-scute* homolog, which confirms their neural identity (Mayer and Whitington 2009b; Eriksson and Stollewerk 2010a, b; Whitington and Mayer 2011). The presumptive nerve cord, which arises directly basal to this layer, subsequently delaminates from the ectoderm and gives rise to a dorsal neuropil (Figs. 4.12A–H and 4.17C, D; Eriksson et al. 2003; Mayer and Whitington 2009b; Whitington and Mayer 2011). Within the neuropil, axonogenesis occurs in a cascade-like fashion from anterior to posterior along the body so that no segmental arrangement of pioneering neurons or their axons is evident (Fig. 4.18A, B; Mayer and Whitington 2009a; Whitington and Mayer 2011). The first axons appear in the antennal segment, followed by the jaw, slime papilla, and leg-bearing segments, respectively (Mayer and Whitington 2009a). Newly formed axons grow anteriorly and fasciculate with the anterior axons. After the longitudinal nerve cords have been established, the ring and median commissures as well as the leg nerves form, all of which are accompanied by glial cells (Fig. 4.18B–D; Mayer and Whitington 2009a; Oliveira et al. 2013b). During further development, the dorsomedian heart nerve and the two dorsolateral longitudinal nerves are formed by axons originating from the presumptive ring commissures, giving rise to an orthogonal organization of the nervous system (Mayer and Harzsch 2008; Mayer and Whitington 2009a).

Thus, in contrast to arthropods, neural development of onychophorans shows only little segmentation, as there is no indication of segmental ganglia or their rudiments (Mayer and Whitington 2009a; Whitington and Mayer 2011). The "metameric units" regarded as "ganglion anlagen" by Eriksson and Stollewerk (2010b) are in fact the anlagen of the ventral and preventral organs, which are clearly separated from the nervous system by several muscle layers (Fig. 4.18D; Mayer and Whitington 2009a) and serve as attachment sites for segmental limb depressor muscles (Fig. 4.16D; Oliveira et al. 2013b). Interestingly, gene expression data revealed that the homologs of *Notch* and *Delta* are expressed in each anlage of the ventral and preventral organs in embryos of *Euperipatoides rowelli* (Oliveira et al. 2013b). Similar double-paired domains of *Notch* and *Delta* are also seen in embryos of the closely related species *Euperipatoides kanangrensis*

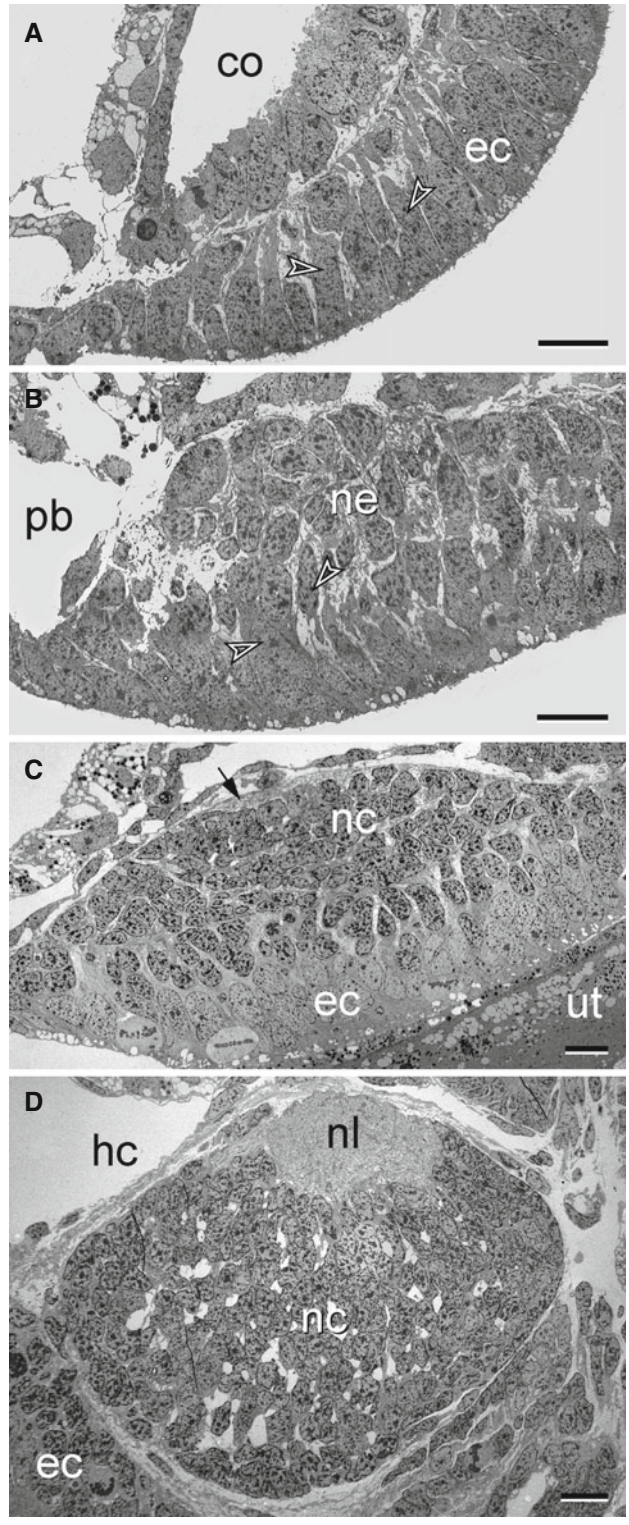

Fig. 4.17 Neurogenesis in the peripatid *Epiperipatus biolleyi*. Transmission electron micrographs of successive embryonic stages (cross sections). (**A**, **B**) Immigration of cells from the ectoderm to form the presumptive nerve cord. Note the elongated shape of detaching cells (*arrowheads*). (**C**, **D**) Establishment of the nerve cord neuropil. The nerve cord neuropil (*arrow* in **C**) originates when the nerve cord is already delaminated and increases in size as development proceeds. *Abbreviations*: *co* coelomic cavity, *ec* ectoderm, *hc* hemocoel, *nc* nerve cord, *ne* neuroectoderm, *nl* nerve cord neuropil, *pb* primary body cavity, *ut* uterus wall. Scale bars: 10 μm

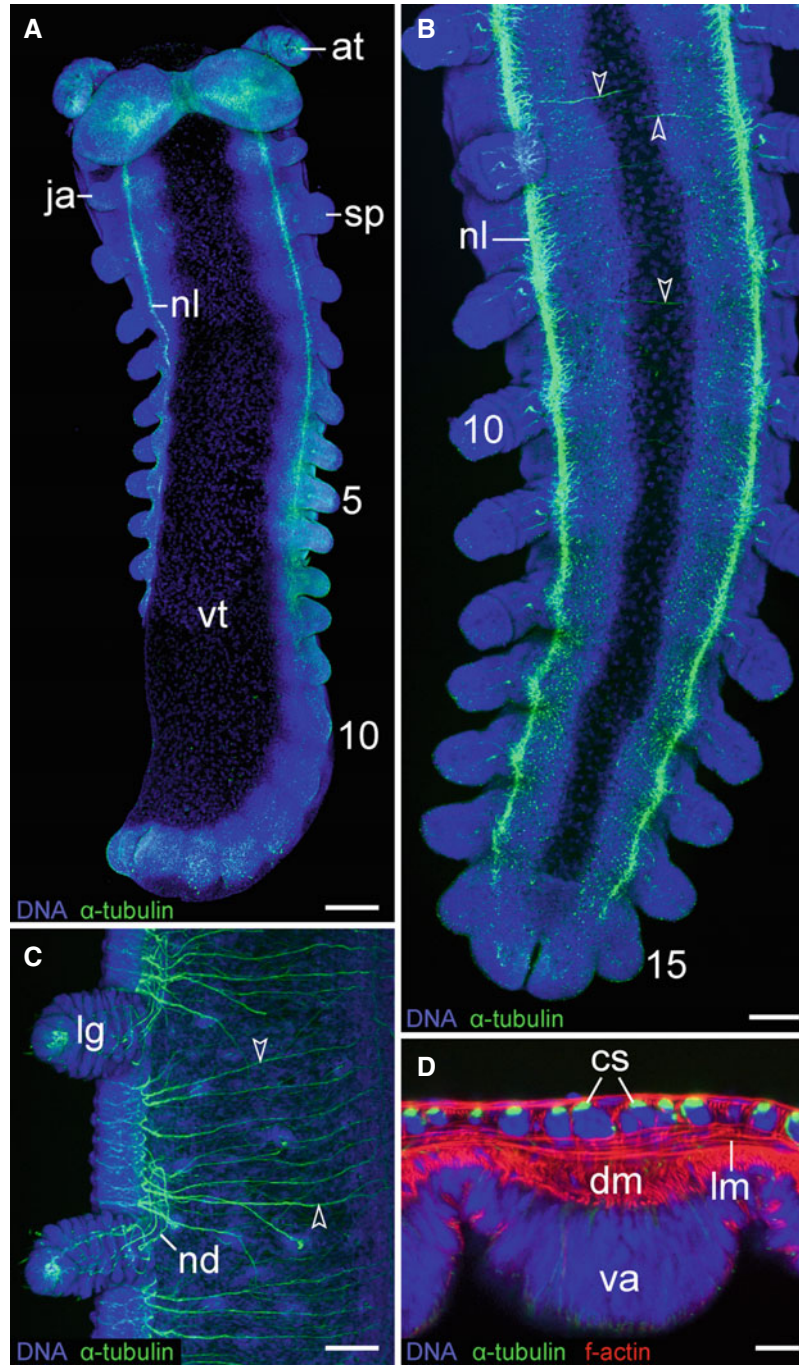

Fig. 4.18 Neural development in *Euperipatoides rowelli* (Peripatopsidae). Confocal laser scanning micrographs of late embryonic stages double labeled with DNA marker bisbenzimide (*blue* in **A**–**D**) and anti-acetylated α-tubulin (*green* in **A**–**D**) and triple labeled with phalloidin-rhodamine (f-actin; *red* in **D**). (**A**–**C**) Anterior-posterior development of the nerve cord. Anterior is up. The commissures (*arrowheads*) start developing after the nerve cord neuropils have been established. *Number* indicates the position of the corresponding leg-bearing segment. (**D**) Spatial relationship between commissures and the anlage of the ventral and preventral organs. Anterior is left. These structures remain separated from each other by three layers of musculature. *Abbreviations*: *at* antenna, *cs* median commissures, *dm* diagonal and ring musculature, *ja* jaw, *lg* leg, *lm* longitudinal musculature, *nd* nephridioduct, *nl* nerve cord neuropil, *sp* slime papilla, *va* anlage of the ventral and preventral organs, *vt* ventral extra-embryonic tissue. Scale bars: 250 µm (**A**), 100 µm (**B**, **C**), and 25 µm (**D**)

(Eriksson and Stollewerk 2010a, b), suggesting that these genes specify regions of the ectoderm that give rise to the ventral and preventral organs rather than being involved in onychophoran neurogenesis (Oliveira et al. 2013b contra Eriksson and Stollewerk 2010a, b).

Retrograde fills of segmental nerves in the head region revealed that the onychophoran brain consists of two segmental regions corresponding to the proto- and deutocerebrum of arthropods, whereas the major brain neuropils arise from a single commissure of the anterior-most (=anten-

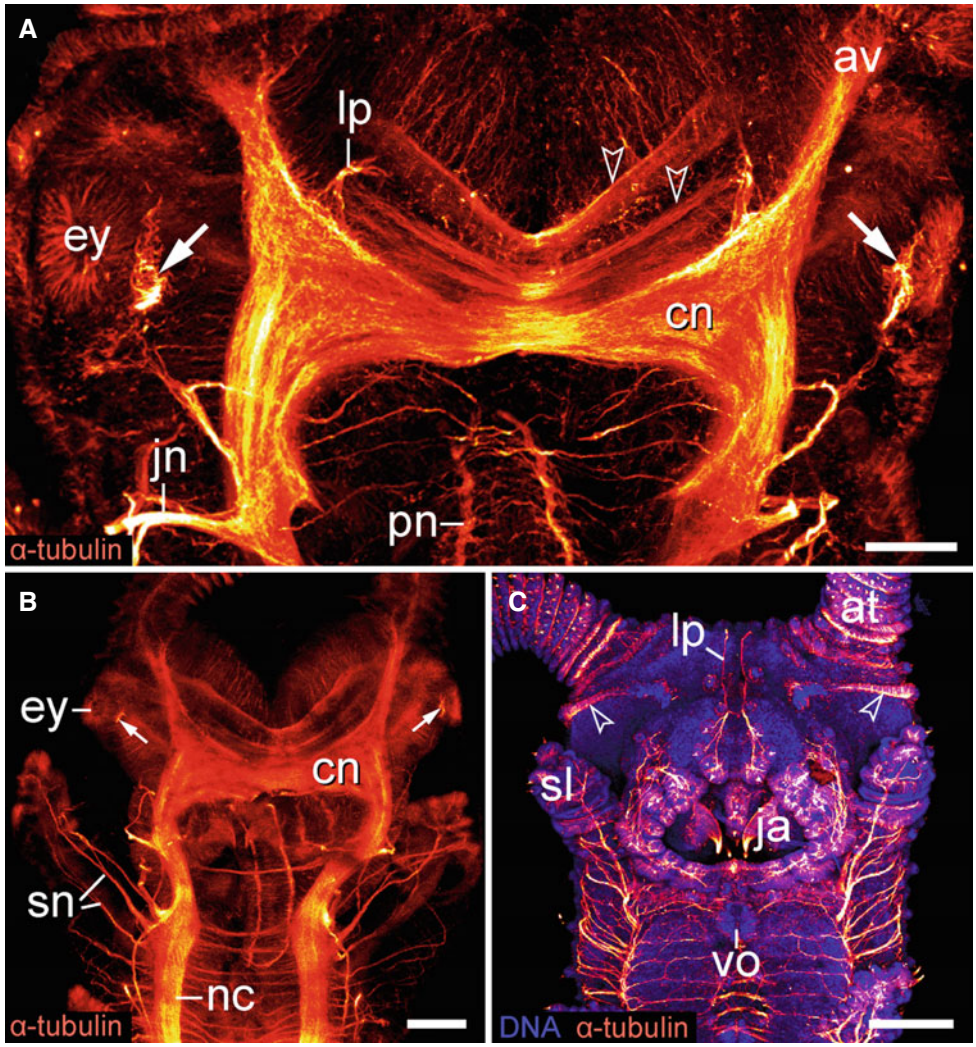

Fig. 4.19 Structure of the brain and associated structures in Onychophora. Confocal laser scanning micrographs of late embryos in the peripatid *Epiperipatus biolleyi* (**A**, **B**) and the peripatopsid *Euperipatoides rowelli* (**C**). Embryos labeled with anti-acetylated α-tubulin (*glow* scale in **A**–**C**) and double labeled with DNA marker bisbenzimide (*blue* in **C**). Anterior is up in all images. *Arrows* (in **A** and **B**) indicate labeled cilia of the rudimentary nephridia of the antennal segment. (**A**) Detail of the developing protocerebral brain neuropils and major tracts in dorsal view. The central brain neuropil arises from a single transverse commissure in the antennal segment, while two additional neuropils (*arrowheads*) arise anteriorly. (**B**) Dorsal view of the developing nervous system in the head region. Note that the slime papillae nerves are not associated with the protocerebrum, but rather with the nerve cords. (**C**) Ventral view of the nervous system of the head region. Note the complex innervation of the lip papillae around the definitive mouth opening. *Arrowheads* indicate developing frontal organs. *Abbreviations*: *at* antenna, *av* antennal nerve, *cn* central brain neuropil, *ey* eye, *ja* jaw, *jn* jaw nerve, *lp* anterior lip papillae nerve, *nc* nerve cord, *pn* pharyngeal loop nerve, *sl* slime papilla, *sn* slime papilla nerve, *vo* ventral organ. Scale bars: 50 μm (**A**), 100 μm (**B**), and 200 μm (**C**)

nal) segment (Mayer et al. 2010a; Whitington and Mayer 2011). This suggests that the three neuropils previously considered as the proto-, deuto-, and tritocerebrum (Strausfeld et al. 2006a) do not arise from three different segments but rather belong to the protocerebrum (Fig. 4.19A, B; Mayer et al. 2010a). The onychophoran brain has also been interpreted as a modified circumoral/circumpharyngeal ring, similar to the ring-shaped brain of cycloneura-

lians (Eriksson and Budd 2000). However, neither developmental nor neuroanatomical data support this view, as they show that onychophorans possess a typical ganglionic brain that, in contrast to the collar-shaped brain of cycloneuralians, is not subdivided into an anterior and posterior ring of perikarya separated by a ring-like neuropil (Ahlrichs 1995; Nielsen 2012; Martin and Mayer 2014).

Development of the Head and Cephalic Structures

Although there are only two segmental brain regions, the onychophoran head is composed of three cephalic segments, each equipped with a pair of modified limbs: the antennae, jaws, and slime papillae (Fig. 4.20A–D; Mayer et al. 2010a). Contrary to the suggestion of Frase and Richter (2013), the anterior-most region of the head clearly comprises a true segment, as it bears a pair of modified limbs (i.e., the antennae), transient nephridia, and embryonic coelomic cavities – structures that are characteristic of typical body segments in onychophorans (Figs. 4.11A, C, 4.19A, C, 4.20A–F, and 4.21A; Mayer and Koch 2005; Mayer 2006a; Mayer et al. 2010a). Moreover, neither anatomical nor developmental studies have provided evidence for a "presegmental acron" (Scholtz and Edgecombe 2006), suggesting that this structure does not exist in Onychophora (e.g., Kennel 1885, 1888; Sedgwick 1887, 1888a, b; Evans 1901; Eriksson et al. 2003, 2009; Mayer and Koch 2005; Mayer 2006a; Mayer et al. 2010a).

The three cephalic segments are clearly distinguishable in the early embryo but are rearranged later in development (Figs. 4.9D–G, 4.18A, and 4.20A–F; Kennel 1885, 1888; Walker and Tait 2004; Ou et al. 2012; Martin and Mayer 2014). While the antennae retain their frontolateral position, the jaws are subsequently incorporated into the definitive mouth cavity and the slime papillae move anteriorly to end up on either side of the head (Fig. 4.20A–F). The slime glands, which are regarded as derivatives of the crural glands (Storch and Ruhberg 1993), arise as ectodermal invaginations from distal portions of the slime papillae (Fig. 4.20A–D). The jaws become heavily sclerotized and their musculature, which is associated with a pair of long apodemes, becomes more prominent. These apodemes develop as ectodermal invaginations from the posterior region of the definitive mouth cavity and persist as hollow structures in the adult (Hewitt 1905; Oliveira and Mayer 2013).

The definitive mouth opening is surrounded by several lip papillae that originate from the three anterior-most body segments and are primarily innervated by the brain (Figs. 4.19C and 4.20A–D; Martin and Mayer 2014). The peculiar ontogeny and innervation pattern of the onychophoran mouth suggest that it is not homologous to the mouths of tardigrades or arthropods (Ou et al. 2012; Martin and Mayer 2014). In contrast, the embryonic stomodeum might well be homologous among these animal groups (Ou et al. 2012). The suctorial pharynx of onychophorans arises as an ectodermal invagination from the walls of the stomodeum at the border between the antennal and jaw segments (Ou et al. 2012). As is evident in cross sections of the embryonic heads, the pharyngeal lumen subsequently transforms from a slit-like to the triradiate shape found in adults (Fig. 4.21A–C; Mayer et al. 2005, 2013a; Nielsen 2013).

The hypocerebral organs develop as a pair of invaginations from the ventral ectoderm of the antennal segment (Fig. 4.20A). During embryogenesis, their anlagen lose their connection to the epidermis and become associated with the ventral surface of the brain (Sedgwick 1887; Kennel 1888; Evans 1901; Eriksson et al. 2003; Mayer et al. 2005; Mayer and Whitington 2009b; Oliveira et al. 2013b). The hypocerebral organs might serve a neurosecretory function in adults and are considered to be homologous to the *corpora allata* of insects (Sanchez 1958; Eriksson et al. 2005, 2013 contra Pflugfelder 1948: p. 479, who considered them as analogous structures). The frontal organs (Bouvier 1905; Oliveira et al. 2012b) also develop in the antennal segment as a pair of ventrolateral ectodermal ridges (Martin and Mayer 2014). These structures are initially associated with the anlagen of the hypocerebral organs, from which they subsequently separate, but their function remains unknown (Figs. 4.19C and 4.20A–D).

The onychophoran eyes develop as a pair of ectodermal invaginations from the dorsolateral

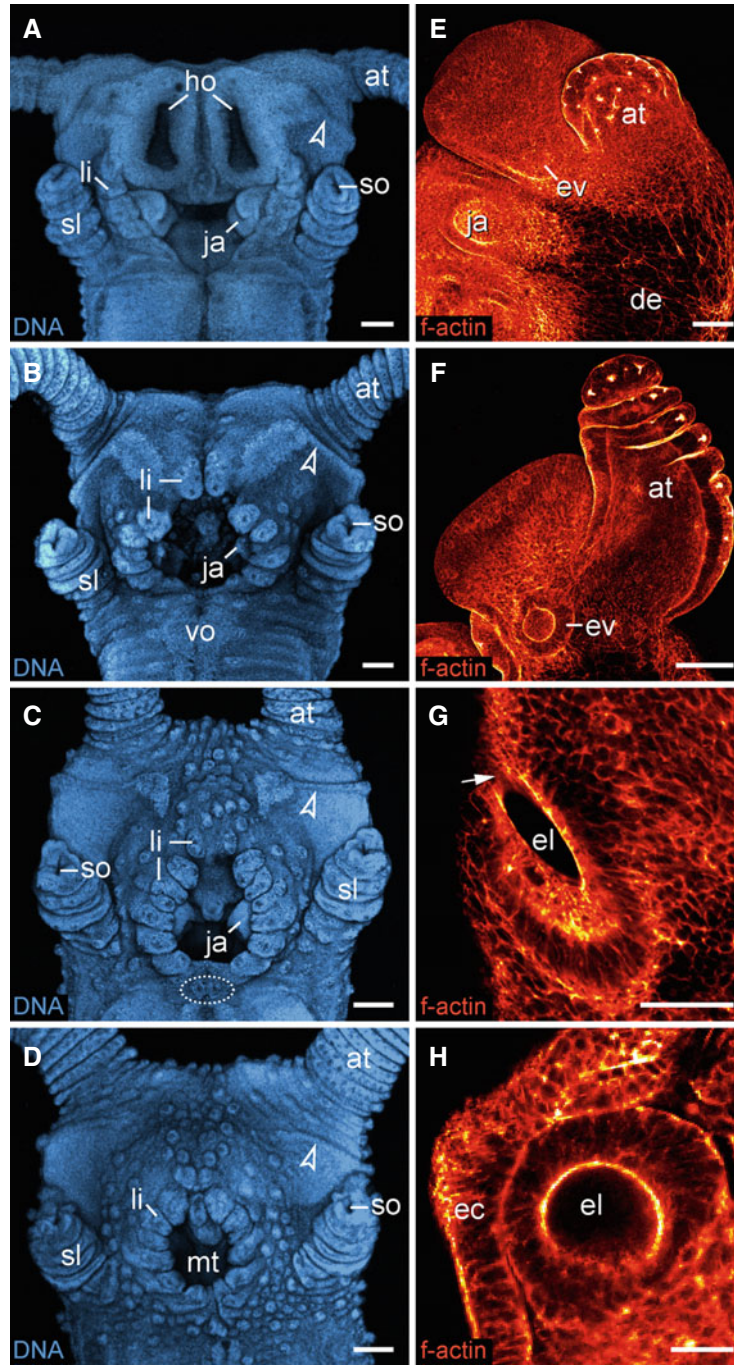

Fig. 4.20 Development of head and cephalic structures in Australian species of Peripatopsidae. Confocal laser scanning micrographs of successive developmental stages labeled with DNA marker bisbenzimide (**A**–**D**) and phalloidin-rhodamine (f-actin; **E**–**H**). *Tasmanipatus anophthalmus* (**A**), *Tasmanipatus barretti* (**B**), and *Euperipatoides rowelli* (**C**–**H**). Anterior is up in all images. (**A**–**D**) Development of the mouth and frontal organs. While the anterior-most pair of oral lips migrates ventrally from the frontal region of the antennal segment to form the definitive mouth, the posterior-most lip papillae (*dotted line* in **C**) originate from the third head segment (slime papilla segment). The jaws are gradually incorporated into the mouth cavity. Note the connection between the frontal organs (*arrowheads*) and hypocerebral organs (in **A**), which disappears in late developmental stages (**B**–**D**). (**E**–**H**) Eye development (overviews in **E** and **F**; details in **G** and **H**). The lumen of the eye vesicle initially opens to the exterior (*arrow* in **G**) and its closure occurs late in development. *Abbreviations*: *at* antenna, *de* dorsal extra-embryonic tissue, *ec* ectoderm, *el* lumen of the eye vesicle, *ev* eye vesicle, *ho* hypocerebral organ, *ja* jaw, *li* lip papillae, *mt* mouth, *sl* slime papilla, *so* slime papilla opening, *vo* anlage of the ventral organ of the slime papilla segment. Scale bars: 100 µm (**A**–**F**), 50 µm (**G**), and 30 µm (**H**)

regions of the antennal segment, near the antennal bases (Fig. 4.20E–H; Kennel 1888; Sedgwick 1888a; Walker and Tait 2004; Mayer 2006b; Eriksson et al. 2013). Each eye vesicle buds off from the ectoderm and its proximal cells develop into the retina, which contains the photoreceptors and shading pigment granules, whereas the distal cells give rise to the sub-cornea (Mayer 2006b). The cavity of the eye vesicle is subsequently filled with the vitreous body, secreted by the surrounding cells, and a cuticular cornea is produced by the overlying epidermal cell layer.

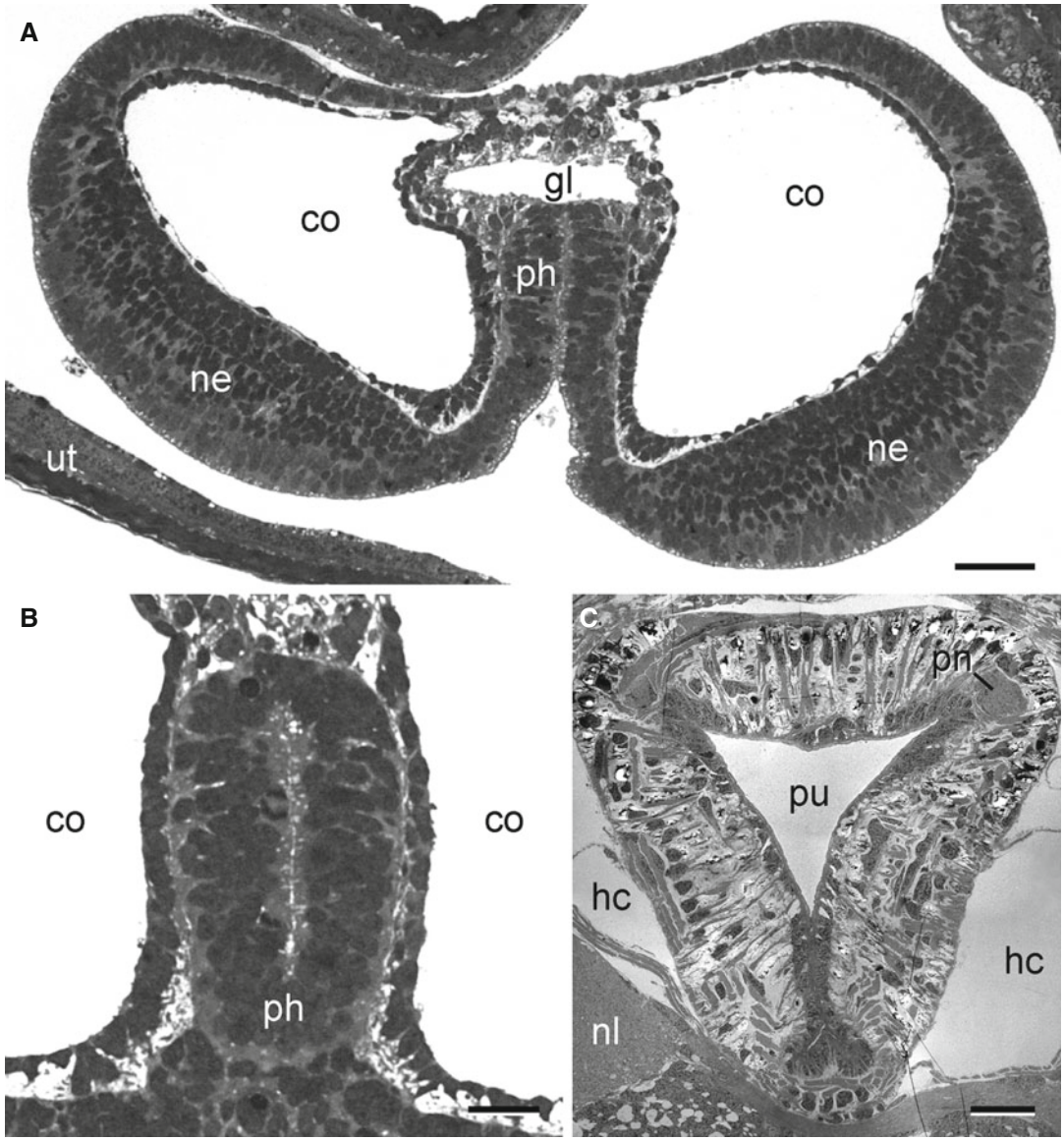

Fig. 4.21 Development of the pharynx. Light micrographs of semithin sections (**A**, **B**) and transmission electron micrograph (**C**) of the head in different developmental stages of the peripatid *Epiperipatus biolleyi*. Dorsal is up in all images. (**A**) Invagination of the ventral ectoderm to form the pharyngeal walls. (**B**) Cross section of an early embryonic pharynx with a slit-like lumen. (**C**) Cross section of the pharynx at an advanced developmental stage. The pharyngeal lumen has developed into a triradiate rather than slit-like shape. *Abbreviations*: *co* coelomic cavity, *gl* gut lumen, *hc* hemocoel, *ne* neuroectoderm, *nl* neuropil of the nerve cord, *ph* pharynx, *pn* pharyngeal loop nerve, *pu* pharyngeal lumen, *ut* uterus. Scale bars: 50 µm (**A**), 20 µm (**B**), and 30 µm (**C**)

LATE DEVELOPMENT

In contrast to many arthropods, onychophorans show no larvae or metamorphosis. Females of viviparous species give birth to live young, whereas oviparous females lay eggs, from which fully developed juveniles hatch after several months of development (Dendy 1892; Brockmann et al. 1997). The newborns and hatchlings have a full number of segments and resemble the adults

morphologically but are smaller and exhibit a weaker pigmentation of the integument. The tracheae of onychophorans apparently develop postembryonically. For example, in *Paraperipatus amboinensis*, they have been reported to arise from specialized epidermal cells that migrate internally and form the tracheal tubules (Pflugfelder 1955, 1962). The number of these tracheal tubules increases after each molt and the atria arise later (Pflugfelder 1955). Since neither onychophorans nor tardigrades have true larval stages, the last common ancestor of Panarthropoda most likely showed a monophasic life cycle with direct development.

GENE EXPRESSION

Segmentation Genes

In segmented animals, the maternal, gap, pair rule, and segment polarity genes control the subdivision of the anterior-posterior body axis into repeated units during development (review Sanson 2001). While currently no expression data on the maternal and gap genes exist for Onychophora, recent studies provided insights into the expression patterns of a number of pair rule and segment polarity genes in the onychophoran *Euperipatoides kanangrensis* (Eriksson et al. 2009; Janssen and Budd 2013). These studies revealed that among the pair rule genes studied, including *even skipped* (*eve*), *runt* (*run*), *hairy*/*Hes* (*h*/*Hes*), *Hes2*, *Hes3*, *odd-skipped* (*odd*), *odd-paired* (*opa*), and *sloppy-paired* (*slp*), only *eve* might play a role in the segmentation process, as it is the only gene that is expressed in the segment addition zone at the posterior end of the onychophoran embryo (Janssen and Budd 2013). Interestingly, the orthologs of *opa* and *slp* are expressed in the interpedal regions of the early embryo, i.e., between the developing limbs, indicating that these genes might be involved in the control or repression of limb development (Janssen and Budd 2013). However, functional analyses are required to further assess this hypothesis.

In contrast to many arthropods, there is only one ortholog of *pax3*/*7* (=*pairberry* according to Janssen and Budd 2013) in Onychophora, which is expressed in a segment polarity pattern along the body (Fig. 4.22A). This suggests that *pax3*/*7* had a role as a segment polarity rather than pair rule gene in the last common ancestor of Onychophora and Arthropoda, and this function has been retained in the onychophoran embryo. The onychophoran orthologs of the segment polarity genes *engrailed* (*en*), *cubitus interruptus* (*ci*), *wingless* (*wg*/*Wnt1*), *hedgehog* (*hh*), *Notum*, and *patched* (*ptc*) are also expressed in repeated sets along the body (Fig. 4.22C–F; Eriksson et al. 2009; Janssen and Budd 2013). While *ci* is expressed anteriorly and *en* and *hh* posteriorly with respect to each developing limb, the expression of *wg* appears anterior to each *engrailed* stripe in the ventral ectoderm and as an additional spot-like domain on the tip of each developing limb (Eriksson et al. 2009; Janssen and Budd 2013). Similar to *wg*, stripes of *Notum* emerge in the middle of each trunk segment and in the developing limb buds (Janssen and Budd 2013). These spatial expression patterns of the segment polarity genes seem to be conserved in onychophorans and arthropods, suggesting that the segment polarity gene network already existed in their last common ancestor (Janssen and Budd 2013).

The ortholog of *ptc* deviates from this conserved pattern. In arthropods, where this gene acts as a Hedgehog-binding receptor, it is downregulated by the *en*-expressing cells, resulting in two stripes located both anterior and posterior to the *hh*/*en* domains (Farzana and Brown 2008; Janssen et al. 2008; Hidalgo and Ingham 1990). In onychophorans, however, it is expressed only anterior, not posterior, to the *hh*/*en* domains (Janssen and Budd 2013).

The Wnt genes are also involved in the segmentation process of the onychophoran body. Orthologs of only 11 of the 13 known Wnt genes have been identified in the embryonic transcriptome of the onychophoran *Euperipatoides kanangrensis*, the missing ones being *Wnt3* and *Wnt8* (Hogvall et al. 2014). Most of these, including *Wnt1*, *Wnt4*, *Wnt5*, *Wnt6*, *Wnt9*, *Wnt11*,

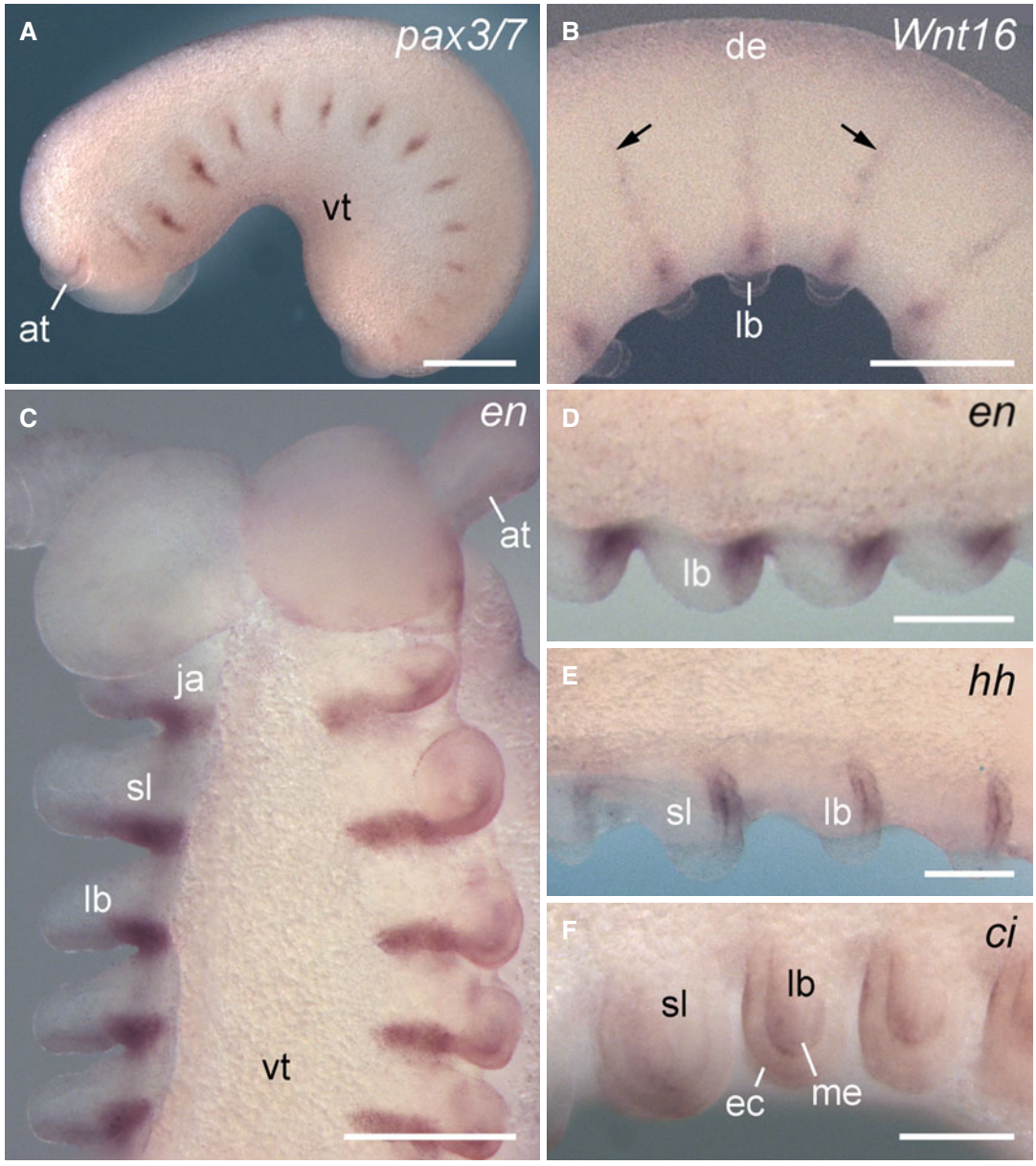

Fig. 4.22 Expression of segmentation genes in the peripatopsid *Euperipatoides rowelli*. Light micrographs. Anterior is left in **B** and **D**–**F**. (**A**) Expression of *pax3/7* in an embryo in lateral view. Note that this gene is expressed in all developing segments, corresponding to a segment polarity pattern. (**B**) Expression of *Wnt16* in the embryonic trunk in lateral view. Note that the expression extends to the dorsolateral integument (*arrows*). (**C**, **D**) Expression of *engrailed* (*en*) in the anterior (*ventral view* in **C**) and posterior (*dorsal view* in **D**) body regions. (**E**) Expression of *hedgehog* (*hh*) in the anterior body region in lateral view. Note that *hh* is expressed in a similar posterior pattern as *en* in the ectoderm and mesoderm, but the stripes are thinner (see also Franke and Mayer 2014). (**F**) Expression of *cubitus interruptus* (*ci*) in the anterior body region in lateral view. Note that this gene is expressed anteriorly with respect to each developing limb in both the ectoderm and mesoderm. *Abbreviations*: *at* antenna, *de* dorsal extra-embryonic tissue, *ec* ectoderm, *ja* jaw, *lb* limb bud, *me* mesoderm, *sl* slime papilla, *vt* ventral extra-embryonic tissue. Scale bars: 500 μm (**A**–**C**) and 200 μm (**D**–**F**)

and *Wnt16*, are expressed in a segment polarity gene-like fashion. Hence, these genes might play a role in the regionalization of individual segments in Onychophora (Hogvall et al. 2014). Notably, the segmental domains of *Wnt16* extend the furthest dorsally, suggesting that this gene is involved in the segmentation of the lateral and dorsal integument (Fig. 4.22B). For example, it might regulate the morphogenesis of segmental sets of dorsal plicae, the segmental number of which is conserved in different onychophoran clades (Oliveira et al. 2014a). On the other hand, the genes *Wnt2*, *Wnt4*, and *Wnt5* might play a role in specifying the identity of the antennal, jaw, and slime papilla segments, which would correspond to a gap- or Hox-like function. Moreover, several Wnt genes are clearly expressed in the segment addition zone of the onychophoran embryo, suggesting that they are involved in the segmentation process (Hogvall et al. 2014). Thus, the Wnt orthologs in onychophorans are much more functionally versatile than their counterparts in the arthropod embryo.

Hox Genes

The ancestral arthropod Hox cluster contained ten Hox genes, two of which (*Hox3* and *fushi tarazu*) have lost their homeotic function and acquired new roles in some arthropod groups (Carroll et al. 2005). Onychophorans possess the same set of ten Hox genes and all of them seem to have retained the ancestral homeotic pattern (Fig. 4.23; Grenier et al. 1997; Eriksson et al. 2010; Janssen et al. 2014). Each Hox gene is expressed from an anterior border, which varies among Hox genes, to the posterior limit of the animal. For example, the anterior expression borders of the Hox genes *labial* (*lab*), *proboscipedia*

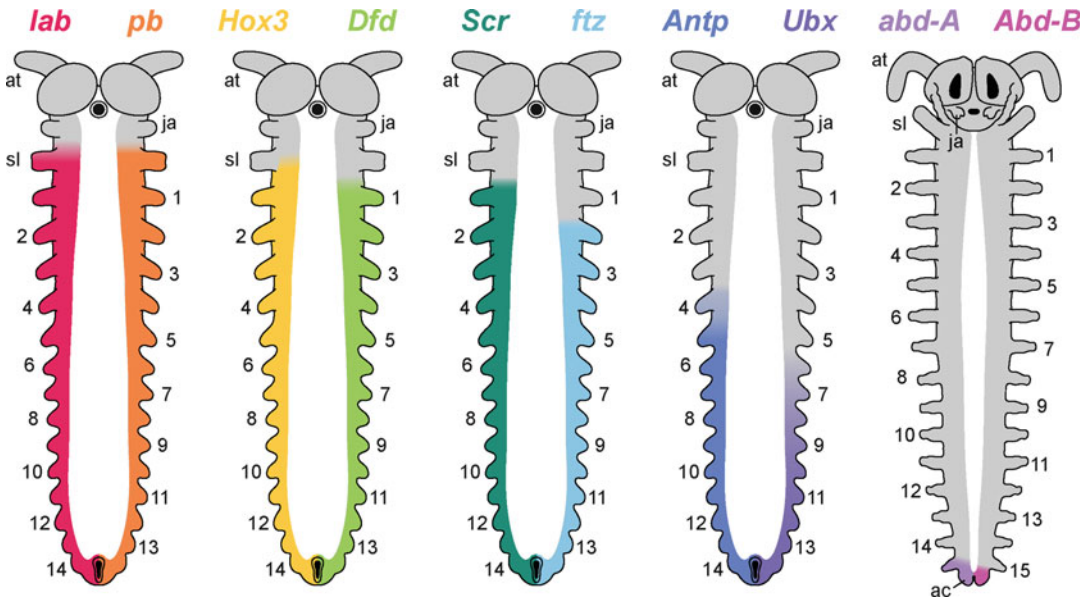

Fig. 4.23 Simplified diagrams showing the expression patterns of the ten Hox genes in the peripatopsid *Euperipatoides kanangrensis* (reconstructed after Janssen et al. 2014). The developing legs are numbered along the body. The expression of most Hox genes is illustrated in stage IV embryos, while that of *abd-A* and *Abd-B* is shown in a stage V embryo, in which all trunk segments have been established (staging according to Walker and Tait 2004). Note that the anterior expression border of *Dfd* was incorrectly identified as lying between the jaw-bearing segment and the first leg-bearing segment by Janssen et al. (2014). *Abbreviations*: *at* antenna, *ac* anal cone, *ja* jaw, *sl* slime papilla

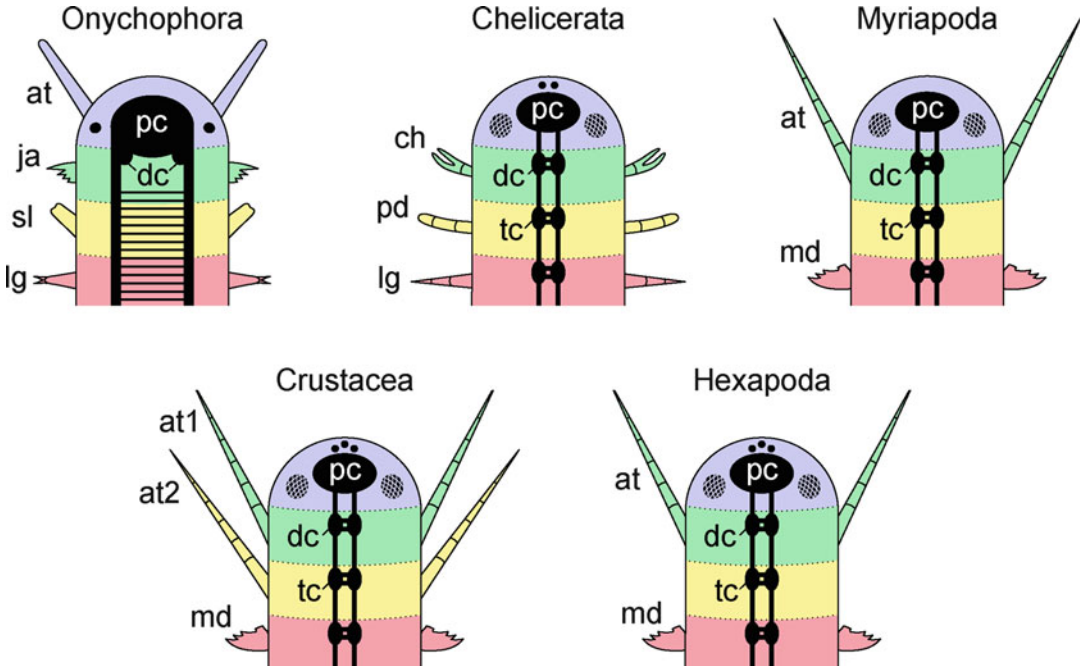

Fig. 4.24 Alignment of head segments among different panarthropod groups (simplified diagram modified from Mayer et al. 2010a, 2013a). *Color coding* indicates putative segment homology. *Abbreviations*: *at* antenna, *at1* first antenna, *at2* second antenna, *ch* chelicera, *dc* deutocerebrum, *ja* jaw, *lg* leg, *sl* slime papilla, *md* mandible, *pc* protocerebrum, *pd* pedipalp, *tc*, tritocerebrum

(*pb*), *Hox3*, and *Deformed* (*Dfd*) are highly conserved among arthropods and have been used to align the anterior segments between onychophorans and arthropods (Eriksson et al. 2010; Janssen et al. 2014). In the onychophoran *Euperipatoides kanangrensis*, the anterior expression borders of *lab* and *pb* are located at the anterior of the slime papilla segment, while the anterior border of *Dfd* expression appears between the slime papilla segment and the first leg-bearing segment (Fig. 4.23; note that the slime papilla segment was incorrectly interpreted as the jaw segment in Janssen et al. 2014). Based on the conserved anterior expression borders of these genes, the anterior segments of onychophorans and arthropods can be homologized as follows: (i) the jaw segment corresponds to the antennal/cheliceral segment; (ii) the slime papilla segment corresponds to the intercalary/pedipalp segment; and (iii) the first leg-bearing segment corresponds to the mandibular/first leg-bearing segment (Fig. 4.24).

According to Janssen et al. (2014), the anterior expression borders of *Hox3*, *Sex combs reduced* (*Scr*), and *fushi tarazu* (*ftz*) are located in the posterior half of the slime papilla and the first and second leg-bearing segments, respectively. However, the data provided are unclear and the expression of *Hox3* remains uncertain. While *Scr* and *ftz* are expressed in the entire limbs of the corresponding segments, no expression of *Hox3* is seen in the developing slime papilla (cf. Fig. 4.3 in Janssen et al. 2014). This contrasts with the findings of Eriksson et al. (2010), who detected *Hox3* expression in the entire slime papilla in at least some embryos (cf. Fig. 4.1E, F therein). Moreover, while Eriksson et al. (2010) found that the expression of *Hox3* disappears completely from the slime papilla segment at stage IV, Janssen et al. (2014) suggest that it persists in the ventral ectoderm of this segment throughout this stage.

The Hox-like expression pattern of *ftz* in *Euperipatoides kanangrensis* (Fig. 4.23) contra-

dicts the previous hypotheses of its involvement in nervous system development and segmentation in hexapods, some crustaceans, and myriapods (Hughes and Kaufman 2002a, b; Mouchel-Vielh et al. 2002; Damen 2002; Janssen et al. 2014). Thus, the last common ancestor of Onychophora and Arthropoda most likely exhibited the homeotic function of *ftz*. Interestingly, the anterior expression borders of *Scr*, *ftz*, and *Antennapedia* (*Antp*) move anteriorly during onychophoran development. Since this shift has not been reported from arthropods, it might be either an ancestral feature of Panarthropoda that has been lost in the arthropod lineage or a derived feature of Onychophora (Janssen et al. 2014).

The anterior expression border of *Antp* in *Euperipatoides kanangrensis* is located at the anterior of the fourth leg-bearing segment, i.e., the seventh body segment (Fig. 4.23). Although the expression of *Antp* is considerably weaker in this segment than in the subsequent segments, it corresponds closely to the anterior border of *Antp* expression in the seventh body segment of chelicerates and myriapods (Hughes and Kaufman 2002a; Janssen and Damen 2006; Schwager et al. 2007). In contrast, this border lies in the sixth body segment in crustaceans and hexapods (reviewed by Hughes and Kaufman 2002b), suggesting that the anterior shift of the *Antp* expression domain is a derived feature of Pancrustacea rather than Mandibulata, contrary to the assertion of Janssen et al. (2014).

The posterior Hox gene *Ultrabithorax* (*Ubx*) is the only Hox gene in *Euperipatoides kanangrensis* that does not show a distinct anterior expression border. Instead, it is expressed in an anterior-to-posterior gradient, beginning with the sixth leg-bearing segment (Janssen et al. 2014). The expression of *abdominal-A* (*abd-A*) is restricted to the genital (=last leg-bearing) segment and the anal cone (=posterior-most, limbless segment), which is consistent with a previous localization of the Ubx/abd-A proteins using a cross-reactive antibody in embryos of the onychophoran *Acanthocara kaputensis* (see Grenier et al. 1997). The posterior Hox gene *Abdominal-B* (*Abd-B*) is expressed in the mesoderm of the anal cone as well as the ectoderm surrounding the proctodeum in embryos of *Euperipatoides kanangrensis* (Fig. 4.23). Thus, contrary to its role in specifying the genital structure in some arthropods and vertebrates (Damen and Tautz 1999), *Abd-B* most likely specifies the identity of the anal segment in Onychophora.

Anterior Patterning Genes (Excluding Hox Genes)

As in many other bilaterians, the anterior-most body region of the onychophoran embryo is patterned by typical anterior/head patterning genes, including *six3*, *orthodenticle* (*otd/otx*), *pax6*, and *collier* (*col*) (Steinmetz et al. 2010; Janssen et al. 2011; Eriksson et al. 2013). In embryos of *Euperipatoides kanangrensis*, *six3* is expressed in the anterior-most region of the antennal segment, whereas *otd/otx* is expressed in the posterior region. Furthermore, *otd/otx* is also expressed in the neuroectoderm and eye anlagen of the antennal segment as well as in the anterior part of the stomodeum, which lies between the antennal and jaw segments (note that *optix* in Steinmetz et al. 2010 corresponds to *otd/otx* in Eriksson et al. 2013). In addition to *otd/otx*, the onychophoran eye anlage expresses a *pax6* homolog, which is considered to be a master control gene for eye development in various bilaterians (Gehring and Ikeo 1999; Kozmik 2008; Eriksson et al. 2013). Moreover, *pax6* expression is present in the invaginating anlagen of the hypocerebral organs in onychophorans, thus supporting the hypothesis of a neurosecretory/glandular function of these structures (Eriksson et al. 2005, 2013).

The expression pattern of *collier* (*col*) appears rather complex in the antennal segment of the early onychophoran embryo, as it also includes the anlagen of the brain and possibly the mesoderm later in development (Janssen et al. 2011). Its expression in the anterior rim of the head lobes as well as in the developing brain might be a conserved feature of onychophorans and all arthropods, whereas the involvement of this gene in specifying the intercalary segment of insects

and myriapods has been interpreted as a putative synapomorphy of these two arthropod groups (Janssen et al. 2011).

Interestingly, the dorsal patterning gene *decapentaplegic* (*dpp*) is also expressed in the early mesodermal somites of the antennal segment in the onychophoran *Euperipatoides rowelli* (Treffkorn and Mayer 2013). While this domain disappears during further development, additional domains arise in the dorsal mesoderm of the developing cephalic appendages, i.e., antennae, jaws, and slime papillae, as well as in the lateral ectoderm associated with the developing lip papillae surrounding the mouth (Treffkorn and Mayer 2013).

Limb Patterning Genes

In arthropods, proximo-distal limb patterning is regulated by the complementary activity of the signaling molecules Decapentaplegic (DPP) and Wingless (WG), which activate the genes responsible for this patterning (Prpic et al. 2003). Although the interactions of the DPP and WG proteins have not yet been established in onychophorans, mRNA expression patterns of the corresponding genes *dpp* and *wg* indicate a similar function of these genes in onychophorans and arthropods (Eriksson et al. 2009; Treffkorn and Mayer 2013). During the outgrowth of the limbs, *wg/Wnt1* is expressed in a spot-like domain at the distal tip of the limb as well as in a medial stripe in the ventral ectoderm. The expression domains are separated from each other by a region at the basis of each developing limb that lacks *wg* expression (Eriksson et al. 2009). Likewise, the genes *Wnt6*, *Wnt9*, *Wnt10*, and *Wnt11* are also expressed at the tip of each limb, whereas the expression of *Wnt5* and *WntA* occurs as a median ring in the slime papillae and walking limbs and that of *Wnt16* as a posterior stripe in each developing limb (Hogvall et al. 2014). These expression data suggest that Wnt genes are involved in proximo-distal growth rather than dorsoventral patterning of the onychophoran limbs.

The study of *dpp* expression in *Euperipatoides rowelli* embryos revealed that this gene is expressed in the dorsal portion of each developing limb (Treffkorn and Mayer 2013), which is in line with a conserved role of *dpp* in limb development among arthropods (Sanchez-Salazar et al. 1996; Jockusch et al. 2000; Niwa et al. 2000; Prpic et al. 2003; Prpic 2004; Angelini and Kaufman 2005. However, while *dpp* expression occurs in the dorsal mesoderm of the developing limb in onychophorans, it is expressed in the ectoderm in all arthropods studied thus far. Therefore, three different scenarios on the evolution of the *dpp* expression pattern have been proposed (Treffkorn and Mayer 2013). According to the first scenario, *dpp* was expressed in the mesoderm of the developing limb and the expression has shifted to the ectoderm in the arthropod lineage. The second scenario assumes that *dpp* was expressed in the ectoderm in the last common ancestor of Onychophora and Arthropoda and was shifted to the mesoderm in the onychophoran lineage. The third scenario proposes a *dpp* expression pattern in both mesoderm and ectoderm in the last common ancestor of Panarthropoda that was reduced in the ectoderm in onychophorans and in the mesoderm of arthropods.

In arthropods, the leg gap genes *homothorax* (*hth*), *extradenticle* (*exd*), *dachshund* (*dac*), and *Distal-less* (*Dll*) are responsible for the regionalization and segmentation of limbs (Prpic et al. 2003; Janssen et al. 2010). Expression studies of these genes in the onychophoran *Euperipatoides kanangrensis* revealed a similar mechanism of limb regionalization, even though the onychophoran limbs resemble non-segmented lobopodia (Janssen et al. 2010). This suggests that the mechanism of leg regionalization is conserved in Panarthropoda but has evolved for a function other than leg segmentation (Janssen et al. 2010). However, the overall expression patterns and dynamics of these genes show a higher degree of similarity of onychophorans to crustaceans and hexapods than to myriapods and chelicerates (Janssen et al. 2010). For example, the gene *exd* is expressed along the entire proximo-distal axis of the onychophoran leg. This is consistent with the *exd* expression pattern in crustaceans and hexapods but differs

from the expression in myriapods and chelicerates, where *exd* is expressed exclusively in the proximal portion of the developing limb (Gonzalez-Crespo and Morata 1996; Prpic and Tautz 2003; Prpic et al. 2003; Prpic and Telford 2008; Janssen et al. 2010). Similarly, *hth* expression is restricted to the proximal portion of legs in onychophorans, hexapods, and crustaceans, while its expression extends into the distal leg portion in myriapods and chelicerates (Prpic and Tautz 2003; Prpic et al. 2003; Prpic and Telford 2008; Janssen et al. 2010). The *dac* expression domain lies within the *Dll* domain in onychophorans, hexapods, and crustaceans but is first expressed in non-overlapping domains in myriapods and chelicerates. However, *dac* is not expressed in the onychophoran antennae (Janssen et al. 2010). It has been proposed that the distal limb patterning gene *aristaless* (*al*) instead adopted the leg gap function of *dac* in the onychophoran antennae and slime papillae, where it shows a typical leg gap gene-like expression pattern (Oliveira et al. 2014b). The *Dll* expression domain in the distal portion of the developing limb seems to be relatively conserved in arthropods and onychophorans. This indicates that the onychophoran and hexapod/crustacean state of leg gap gene expression is plesiomorphic, whereas the myriapod/chelicerate state is derived, suggesting a closer relationship of myriapods to chelicerates and, thus, supporting the Myriochelata hypothesis (cf. Mayer and Whitington 2009b).

In contrast to the limb regionalization mechanism by leg gap genes, the investigation of the distal limb patterning genes *clawless* (*cll*), *aristaless* (*al*), *rotund* (*rn*), *zinc finger homeodomain 2* (*zfh2*), *spineless* (*ss*), and *Lim1* revealed a higher divergence in their expression patterns between onychophorans and arthropods (Oliveira et al. 2014b). While *cll*, *al*, *Lim1*, and *ss* show at least some conserved features in onychophorans and arthropods, the expression patterns of *zfh2* and *rn* are considerably divergent. Furthermore, the expression patterns of these two genes differ between the various types of onychophoran appendages. Compared to insects, the expression patterns appear to be most similar in the walking limbs of onychophorans. Unfortunately, due to the lack of comparative data from non-insect arthropods, no conclusion can be drawn about whether or not the distal limb patterning mechanisms are conserved among all arthropods and onychophorans (Oliveira et al. 2014b).

OPEN QUESTIONS

- Mouth/anus formation in different onychophoran species
- Embryology of oviparous species and matrotrophic viviparous species of Peripatopsidae from Chile and Indonesia
- Number and nature of embryonic envelopes and cleavage type in Southeast Asian and tropical African Peripatidae
- Cleavage pattern and gastrula formation in *Peripatopsis capensis* from South Africa
- Coelomic fate in lecithotrophic viviparous peripatopsids
- Functional genomics in well-studied species, such as *Euperipatoides rowelli*

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Chelicerata

5

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INTRODUCTION

Chelicerate Phylogenetics

Chelicerata is a subphylum of arthropods that includes terrestrial as well as marine animals. Both the fossil record and molecular data place the origin of the chelicerates over 500 million years ago in the Cambrian (e.g., see Dunlop 2010; Rota-Stabelli et al. 2013). It has been shown that the chelicerates are a monophyletic group, and although they have previously been grouped together with the myriapods as Myriochelata, it is generally accepted that chelicerates represent the sister group of Mandibulata (pancrustaceans and myriapods) (Friedrich and Tautz 1995; Cook et al. 2001; Giribet et al. 2001; Hwang et al. 2001; Pisani et al. 2004; Dunn et al. 2008; Meusemann et al. 2010; Regier et al. 2010; Rota-Stabelli et al. 2011).

The chelicerates constitute two sister groups, the euchelicerates (Weygoldt and Paulus 1979) and the pycnogonids (sea spiders) (Fig. 5.1), which are united morphologically by the anterior-most pair of chelate appendages: the cheliceres of the former and the chelifores of the latter (reviewed by Dunlop and Arango 2005; Edgecombe 2010). This conclusion is supported by both neuroanatomy and Hox gene expression (Jager et al. 2006; Manuel et al. 2006; Brenneis et al. 2008).

There are more than 100,000 described species of chelicerates (Dunlop 2010) that can be subdivided into 14 recognised orders (Fig. 5.1;

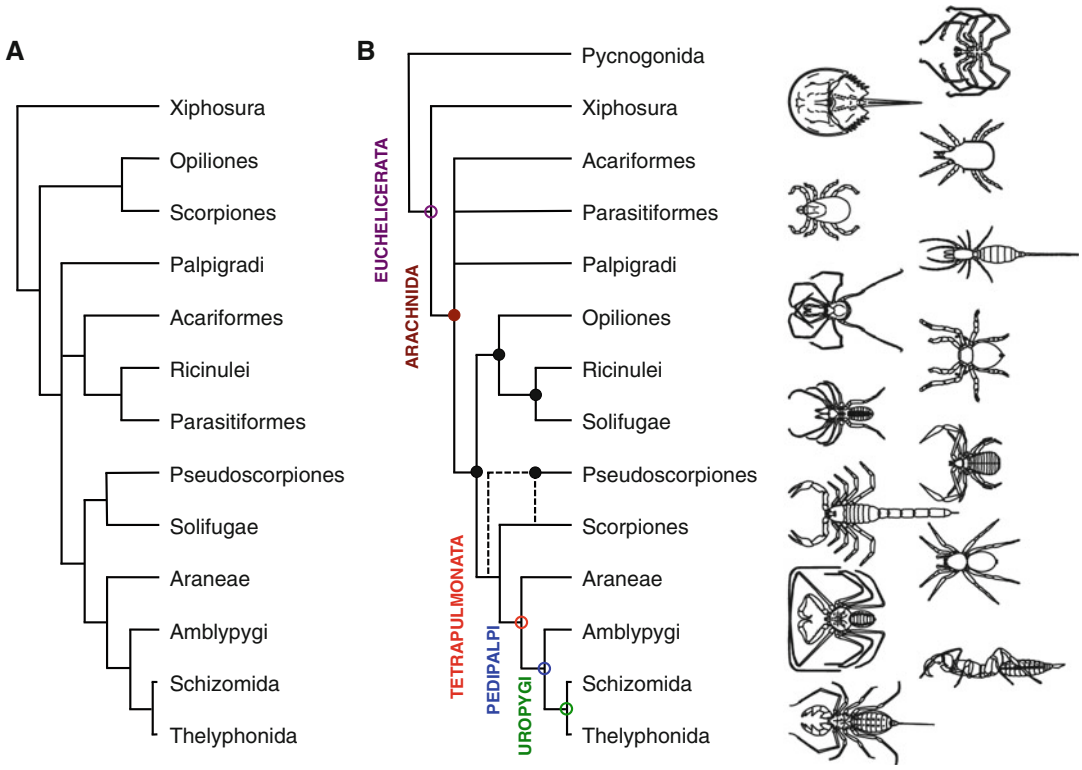

Fig. 5.1 Chelicerate phylogenies. (**A**) Phylogeny based on analysis of morphological characters by Shultz (2007). Note that pycnogonids were not included in this study. (**B**) Phylogeny based on the phylogenomic approach of Sharma et al. (2014a) using transcriptomic and genomic data. *Filled circles* indicate nodes that were supported only by subsets of the slowest-evolving loci used by Sharma et al. (2014a). The *broken lines* indicate two alternative relationships of Pseudoscorpiones to Scorpiones suggested by Sharma et al. (2014a) (© Prashant P. Sharma, 2015. All Rights Reserved)

reviewed by Dunlop 2010). The monophyly of the euchelicerates is very well supported by both molecular and morphological data (Fig. 5.1; Weygoldt and Paulus 1979; Dunlop 2010). While the consensus is that Arachnida (all terrestrial chelicerates) is also monophyletic, mainly based on morphological data (Wheeler and Hayashi 1998; Shultz 2007; Dunlop 2010; but see Giribet et al. 2002), molecular sequence data infrequently recover the monophyly of arachnids (Regier et al. 2010; Börner et al. 2014; Sharma et al. 2014a). A recent phylogenomic study by Sharma et al. (2014a), which used extensive molecular data, including transcriptomes and whole genomes, recovered a nested position of Xiphosura (horseshoe crabs) within arachnids, due to the placement of Pseudoscorpiones, Parasitiformes, and Acariformes. Indeed, the position of the Xiphosura was also found to be inconsistent with the monophyly of the arachnids by Roeding et al. (2009), Meusemann et al. (2010), and Börner et al. (2014). However, upon analysing only a subset of the most slowly evolving genes, Sharma et al. recovered maximal phylogenetic support for arachnid monophyly, suggesting that arachnid non-monophyly is attributable to systematic bias resulting from accelerated rates of evolution in certain "problematic" chelicerate orders (Fig. 5.1; Sharma et al. 2014a).

Among arachnids, the clades Tetrapulmonata, Pedipalpi, and Uropygi are strongly and consistently supported by both morphological and molecular data (Fig. 5.1; Wheeler and Hayashi 1998; Giribet et al. 2002; Shultz 2007; Dunlop 2010; Edgecombe 2010; Regier et al. 2010; Börner et al. 2014; Sharma et al. 2014a). However, the precise phylogenetic relationships of other arachnid orders have been much debated (Dunlop 2010). For example, there are conflicting views on whether Acari (Acariformes [mites] + Parasitiformes [ticks]) is monophyletic, although the most recent evidence supports the view that it is paraphyletic (Dunlop and Arango 2005; Pepato et al. 2010; Sharma et al. 2014a). The position of Opiliones (harvestmen) in the chelicerate tree has also proven to be enigmatic, but recent molecular data suggest that harvestmen form a group with Ricinulei (hooded tick spiders) and Solifugae (camel spiders) (Fig. 5.1B; Sharma et al. 2014a), although this relationship has not emerged from any previous studies (Fig. 5.1A; Wheeler and Hayashi 1998; Giribet et al. 2002; Shultz 2007; Dunlop 2010; Regier et al. 2010).

Resolving chelicerate and arachnid relationships is critical to our understanding of key evolutionary transitions, including many important open questions in evolutionary developmental biology. In this respect the continual expansion of chelicerate genomic resources holds great promise for resolving outstanding issues in the phylogeny of these animals, a necessary framework to explore their evolution and development.

Chelicerate Genome Biology

As with other organisms, the development of new sequencing technologies has allowed transcriptome and whole-genome sequencing of chelicerates that build on classical studies, mainly among spiders, of genome size and cytogenetics (Tsurusaki and Cokendolpher 1990; Chen 1999; Gregory and Shorthouse 2003).

The first chelicerate genome to be published was that of the two-spotted spider mite, *Tetranychus urticae* (Grbic et al. 2011). This was soon followed by the scorpion, *Mesobuthus martensii* (Cao et al. 2013), and two spiders (the social velvet spider, *Stegodyphus mimosarum*, and the Brazilian white-knee tarantula, *Acanthoscurria geniculata*) (Sanggaard et al. 2014) and the Atlantic horseshoe crab *Limulus polyphemus* (Nossa et al. 2014). In addition, the genome of the tick *Ixodes scapularis* has also been sequenced (www.vectorbase.org). Together, these genome sequencing projects corroborate the great variation in genome size among chelicerates and show that there are large differences in the predicted numbers of genes among these animals (Table 5.1). These genomes are only the tip of the iceberg, with several other chelicerate genomes likely to be available soon through initiatives such as i5K (http://www.arthropodgenomes.org/wiki/i5K).

Table 5.1 Chelicerate genome sizes

Order	Species	Genome size (Mb)	Predicted gene number	Reference
Xiphosura	*Limulus polyphemus*	2,740	>34,000	Nossa et al. (2014)
Acariformes	*Tetranychus urticae*	90	18,414	Grbic et al. (2011)
Parasitiformes	*Ixodes scapularis*	2,100	24,925	www.vectorbase.org
Scorpiones	*Mesobuthus martensii*	1,323	32,016	Cao et al. (2013)
Araneae	*Acanthoscurria geniculata*	6,500	73,821[a]	Sanggaard et al. (2014)
Araneae	*Stegodyphus mimosarum*	2,550	27,235	Sanggaard et al. (2014)
Araneae	*Parasteatoda tepidariorum*	1,200	up to 40,000	Posnien et al. (2014)

[a]For *Acanthoscurria geniculate* this is the predicted number of transcripts rather than genes

As well as whole-genome sequencing, there is already a large and growing number of transcriptome projects in various chelicerates to describe the general expression profiles of genes or to decipher tissue- or stage-specific expression (e.g., Croucher et al. 2013; Clarke et al. 2014; Posnien et al. 2014). Transcriptomics can tell us much about the gene content and expression profiles of the genomes of chelicerates, even for species for which the whole genome has not yet been sequenced.

Genomic sequencing of chelicerates has already provided considerable insights into the evolution of many important genes and gene families, from developmental genes to silk and venom genes. Intriguingly, it appears that there have been at least one and perhaps two whole-genome duplications in a horseshoe crab (Nossa et al. 2014). Even excluding the horseshoe crab, chelicerate genomes exhibit marked variability in genome size and content, with miniaturised genomes associated with gene loss in mites (*Tetranychus urticae*; Grbic et al. 2011) and genomes bearing among the largest known numbers of genes in arthropods (Table 5.1). Pinpointing gene family expansion and/or whole-genome duplication events has immediate downstream implications for understanding both the evolution of genomic architecture and gene regulatory networks in these animals.

The rapidly emerging genomic resources for chelicerates therefore represent new and exciting opportunities for the analysis of genome biology, gene expression, gene function, and gene regulatory evolution in existing chelicerate models and have great potential to empower investigation of evolutionary developmental biology in more enigmatic, understudied chelicerate lineages with interesting embryological and morphological features.

The Chelicerate Orders and Evolutionary Developmental Biology

The embryology of chelicerates has been studied for over 150 years (see below). Although some chelicerate models have made an important contribution to understanding animal evolution and development over the past 20 years, others remain very much understudied, as highlighted previously by Harvey (2002). Below, a short overview of the biology of each chelicerate order is given, together with a brief summary of their contribution and/or potential contribution to the field of evolution and development.

Pycnogonida (Sea Spiders)

Sea spiders are marine chelicerates that can live deep in the ocean and feed on sponges, cnidarians, and mollusks (Cobb 2010; Barreto and Avise 2011). These animals are characterised by their narrow cephalosoma, which carries the four sets of their appendages: the chelifores, palps, ovigers, and walking legs (typically four pairs; up to six pairs occur in a few lineages) (Figs. 5.1 and 5.2; Cobb 2010). The ovigers of pycnogonids

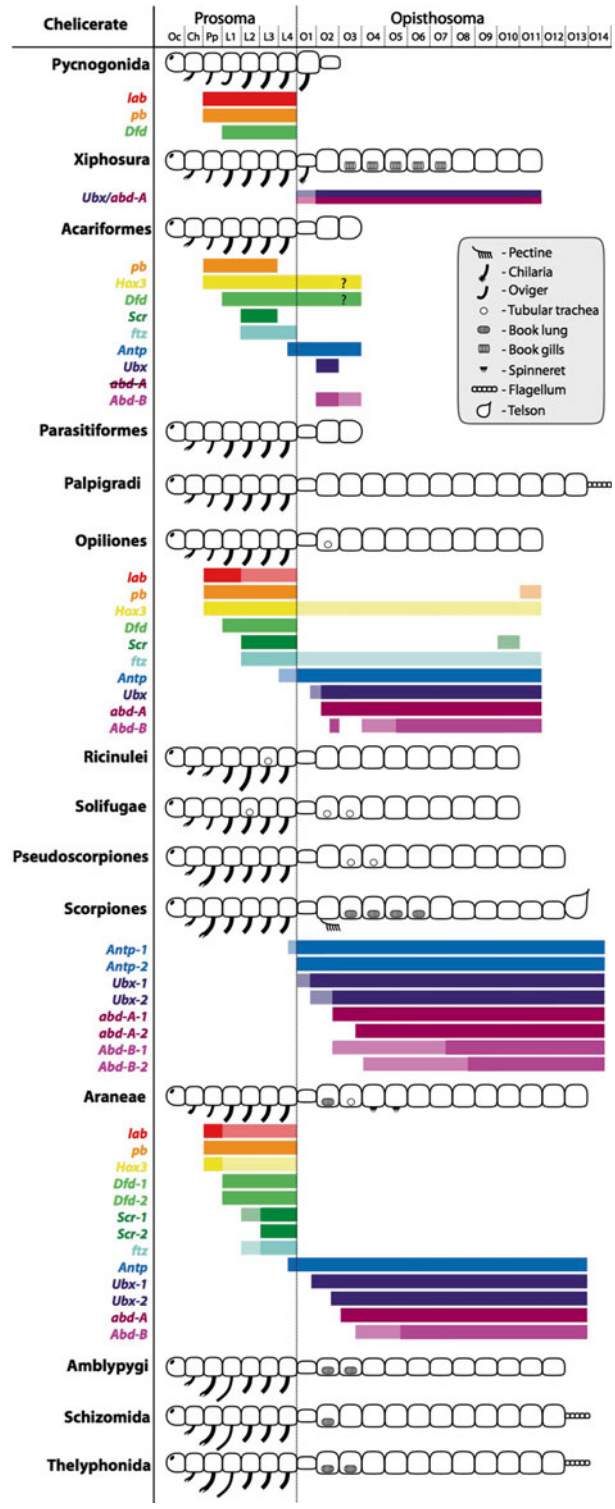

Fig. 5.2 Body plans and known Hox gene expression in chelicerate orders. The number of prosomal segments is conserved in Euchelicerata; variation in segment number of Pycnogonida is not shown. The eucheliceratе prosoma consists of six appendage-bearing segments: a pair of cheliceres, a pair of pedipalps, and four pairs of walking legs. There is considerable innovation in the function of these limbs, such as the long-range tactile first legs of Amblypygi or the muscular pedipalps of Scorpiones. In contrast to the prosoma, opisthosomal segment number is variable both between and within orders. The respiratory organs that are found in chelicerates differ in morphology as well as number and position. For instance, Xiphosura has book gills rather than the book lungs that are found in many other chelicerates. A tracheal respiratory system occurs in several arachnid orders as well as in derived spiders. The position of these is variable and can occur within the prosoma and/or opisthosoma (simplified to show typical positions of spiracles in Acariformes and Parasitiformes). Palpigrade opisthosomal "sacs" are of dubitable homology and are not shown here. Appendages shown that are specific to certain orders include pectines (scorpions), ovigers (pycnogonids), and chilaria (horseshoe crabs). In conjunction with morphological studies, expression of Hox genes in chelicerates has been characterised in Pycnogonida (Jager et al. 2006), Xiphosura (Popadic and Nagy 2001), Acariformes (Telford and Thomas 1998b; Barnett and Thomas 2013a), Opiliones (Sharma et al. 2012b), Scorpiones (Sharma et al. 2014b), and Araneae (Damen et al. 1998; Damen and Tautz 1999; Schwager et al. 2007). The variable anterior expression boundaries of posterior Hox genes are strongly consistent with involvement in patterning opisthosomal segment identity. Note that *abd-A* has been lost in mites. The conserved expression domains of *lab*, *pb*, and *Dfd* in the prosoma of Pycnogonida has contributed significantly to understanding segmental homology of arthropod head segments. In both Araneae and Scorpiones, Hox paralogs have been observed to have both spatial and temporal expression differences. *Oc* ocular segment, *Ch* cheliceres, *Pp* pedipalps, *L1–L4* walking legs, *O1*–O14 opisthosomal segments (© Alistar P. McGregor 2015. All Rights Reserved)

are unique to this group and in many species are typically used by males to carry masses of eggs deposited by females. Interestingly, given the narrow cephalosoma (anterior tagma) of sea spiders and the loss of the opisthosoma ("abdomen" or posterior tagma), the gonads and other organs are found in their appendages (Cobb 2010). However, the fossil record of pycnogonids, which extends to the Cambrian (Waloszek and Dunlop 2002), includes an extinct lineage with a long, completely segmented, limbless posterior region, indicating that stem pycnogonids once bore an opisthosoma (Bergström et al. 1980).

Since sea spiders are widely regarded as the sister group to the euchelicerates (Fig. 5.1; see above), knowledge of their development has great potential to inform our understanding of chelicerate evolution and development more generally. To date, many studies of sea spiders have had a phylogenetic focus, informed by characterisation of their neuroanatomy and Hox gene expression (see above; Arango 2002; Jager et al. 2006; Manuel et al. 2006; Arango and Wheeler 2007; Brenneis et al. 2008). Classical descriptions of pycnogonid development are rare and incomplete (Brenneis et al. 2011a, b). However, modern methodological approaches have recently been applied to describe the embryonic and post-embryonic development of sea spiders and to generate staging systems for *Pseudopallene* sp. and *Pycnogonum litorale* (Vilpoux and Waloszek 2003; Ungerer and Scholtz 2009; Machner and Scholtz 2010; Brenneis et al. 2011a, b). This work can serve as a platform for further studies of gene expression and possibly gene function in these animals and to help resolve questions regarding the evolution and development of chelicerates.

Xiphosura (Horseshoe Crabs)

Horseshoe crabs are the largest existing euchelicerates, although there are only four extant species (Obst et al. 2012). These chelicerates are marine and feed on other invertebrates and algae on the bed of shallow coastal waters (Ruppert et al. 2004).

Horseshoe crabs have a distinctive carapace that covers the dorsum of the cephalothorax and is joined by a hinge to the dorsal exoskeleton that covers the abdomen (Fig. 5.1). Posterior to the three-segmented cheliceres, horseshoe crabs have a sexually dimorphic pair of pedipalps and four pairs of walking legs (Fig. 5.2). Whereas in female horseshoe crabs the pedipalp is nearly identical to a walking leg, the pedipalps of mature males are modified to form terminally swollen, non-chelate "claspers" used to grasp females during mating. The last pair of walking legs, which is used for pushing on the substrate, is argued to be biramous because it exhibits a putative exopod called a flabellum that is sensory (Fig. 5.2; Snodgrass 1938). This putative homology is supported by the discovery of fossil synziphosurines with *bona fide* exopods on the pedipalps and all walking leg segments, suggesting that the flabellum is a vestige of the posterior-most exopod pair (Briggs et al. 2012). Other sensory organs include two ocelli on the carapace and two lateral compound eyes. Horseshoe crabs respire through five pairs of book gills located on abdominal segments three to seven (Fig. 5.2).

Female horseshoe crabs can lay thousands of eggs on beaches at high tide that are then fertilised by the males and covered in sand. Upon hatching the larvae then enter the sea. This has allowed researchers access to the embryos of these animals and their development has been described in some detail, as well as studied through embryonic manipulations (Kingsley 1892; Kishinouye 1893; Iwanoff 1933; Itow and Sekiguchi 1979, 1980; Sekiguchi et al. 1982; Itow 1990, 2005; Itow et al. 1991). Furthermore, there is a growing number of studies that have examined gene or protein expression during horseshoe crab embryogenesis and other aspects of development that have provided some valuable insights into evolutionary developmental biology (Popadic and Nagy 2001; Damen et al. 2002; Mittmann 2002; Blackburn et al. 2008). In addition, the recent sequencing of the genome of *Limulus polyphemus* (Table 5.1; Nossa et al. 2014) is anticipated to fuel further studies of gene expression in this species.

Scorpiones (Scorpions)

Scorpions are found in a range of habitats, from deserts to tropical rainforests. There are nearly

2,000 described species of scorpions. These chelicerates exhibit a familiar body plan that includes the characteristic pincers of the pedipalpal segment and the aculeus, or stinger, that harbours a barb coupled to a pair of venom-producing glands at the posterior end of the segmented metasoma (post-abdomen or tail) (Fig. 5.2; Beccaloni 2009). These structures are used in combination by these predators for defence and subduing their prey.

The cephalothorax of scorpions has a pair of median eyes and a variable number of lateral eyes (one to five pairs; Gromov 1998; Yang et al. 2013). Scorpions respire through four pairs of book lungs found on abdominal segments three to six (Fig. 5.2; Hjelle 1990), which correspond to embryonic abdominal segments four to seven (the first opisthosomal segment disappears during development). The second abdominal segment (or third embryonic abdominal segment) bears a pair of sensory pectines that is involved in chemoreception and detecting the substrate (Fig. 5.2; Hjelle 1990).

Scorpions are viviparous with embryogenesis taking place inside the females, which subsequently give birth to juveniles. Two distinct modes of development occur in scorpions. Apoikogenic development is characterised by large yolky eggs, surrounded by extra-embryonic membranes, and development occurs in the oviduct. In katoikogenic development, the eggs bear little or no yolk, and the embryos are nourished through connections of the ovariuterus that facilitate trophic exchange from the female's hepatopancreas (Hjelle 1990; Lourenço 2000); development occurs in modified, blind outgrowths of the ovariuterus. Development in either case can be prolonged, with a gestation period lasting 2–18 months in various species.

Despite the ensuing issue with access to embryos, protein and mRNA expressions have been studied during embryogenesis in species such as *Smeringurus mesaensis*, *Euscorpius flavicaudis*, and *Centruroides sculpturatus* (Table 5.2; Popadic and Nagy 2001; Simonnet et al. 2004, 2006; Sharma et al. 2014b, c). This means that it is possible to study the development of several aspects of scorpion morphology to provide new evolutionary insights due to the probable phylogenetic placement of these chelicerates as sister group to Tetrapulmonata (Regier et al. 2010; Sharma et al. 2014a). This includes the developmental patterning of the arachnid book lungs in spiders and scorpions, the serial homology of opisthosomal appendage types, and the sub- or neofunctionalisation of paralogous genes in both spider and scorpion genomes (Schwager et al. 2007; Cao et al. 2013; Sharma et al. 2014b).

Opiliones (Harvestmen)

Harvestmen live in a wide variety of temperate and tropical habitats worldwide, and they can be predators, scavengers, or even herbivores. More than 6,500 species of harvestmen have already been described, and there are estimated to be 10,000 extant species (Machado et al. 2007). These chelicerates are readily recognisable from the four pairs of elongated walking appendages of most species and are hence commonly known as "daddy longlegs" in some parts of the world. The long pedipalps of some harvestmen resemble legs, but in the suborder Laniatores, the pedipalps are raptorial and used to seize prey (Shultz and Pinto-da-Rocha 2007). The second pair of legs is usually longer than the other three pairs in phalangid (i.e., non-Cyphophthalmi) harvestmen, whereas the first pair is generally the longest in the primitive suborder Cyphophthalmi; the longest pair of legs is tactile and/or chemoreceptive throughout the order (Willemart et al. 2009).

Harvestmen respire through tracheal tubes with the spiracles (openings) located on the second opisthosomal segment (Fig. 5.2). These chelicerates do not synthesise silk or venom, but have evolved repugnatorial glands, which secrete acrid compounds including phenols (Raspotnig et al. 2012). The cephalothorax of phalangid harvestmen bears a pair of median eyes, but lacks lateral eyes. By contrast, cyphophthalmid harvestmen bear a single pair of eyes on the sides of the cephalothorax that are believed to be homologous to lateral eyes (Garwood et al. 2014).

Like some mites, male and female Phalangida have a penis and ovipositor, respectively, on the ventral cephalothorax, and thus, fertilisation is internal in these chelicerates; the plesiomorphic

Table 5.2 Expression of key developmental genes in chelicerates

Gene name	Species	Expression domain	Reference
Orthologs of *Drosophila* segmentation cascade genes			
engrailed	*Cupiennius salei*	Expression in segmental stripes at the anterior border of each parasegment (posterior border of each segment), six prosomal stripes, and 12 opisthosomal stripes; additional ocular spots in the pre-cheliceral lobe and expression anterior to the labrum	Damen et al. (1998), Damen (2002)
	Parasteatoda tepidariorum	Segmental stripe expression appears in nonsequential order in prosomal segments; subsequently, stripes in opisthosomal segments are sequentially added	Schwager et al. (2009)
	Phalangium opilio	Segmental stripes at the posterior border of each segment, seven opisthosomal stripes	Sharma et al. (2012b)
	Archegozetes longisetosus	Segmental stripes at the posterior border of each segment, cheliceral, pedipalpal and L1–L3 appear first, then O1, then L4 and lastly O2	Telford and Thomas (1998a), Barnett and Thomas (2012)
	Tetranychus urticae	Expression in pedipalp and four walking leg segments and two opisthosomal segments	Grbic et al. (2011)
runt	*Cupiennius salei*	Early: anteriorly moving stripes in the SAZ Late: neuroectoderm, head, and legs	Damen et al. (2000)
	Tetranychus urticae	Five pairs of ventral ring-shaped expression domains that later form stripes, segmental expression in the nervous system, later in the head lobes, legs	Dearden et al. (2002)
hedgehog	*Parasteatoda tepidariorum*	Early: expression at the rim of the germ disc that then forms a posteriorly moving stripe that will eventually split into three stripes in the pre-cheliceral region, the cheliceral and pedipalpal segment Late: segmental stripes (all segments, including a stripe in cheliceral lobe), SAZ	Pechmann et al. (2009), Akiyama-Oda and Oda (2010), Kanayama et al. (2011)
	Euscorpius flavicaudis	Posterior borders of segments, stripes in the SAZ before segment formation, labrum	Simonnet et al. (2004)
	Archegozetes longisetosus	Stripes at the posterior borders of segments, Ch, Pp, L1–L3 form first, then O1, then L4, and lastly O2. Expression is also found in stomodeum and proctodeum	Barnett and Thomas (2012)
fushi tarazu	*Cupiennius salei*	Groups of cells in the ventral neural ectoderm extending from the posterior portion of the first walking leg to the border between the fourth walking leg and the first opisthosomal segment, ring-shaped expression domains in the distal tips of the developing 3rd and 4th walking leg and weakly in the 2nd walking leg	Damen et al. (2005)
	Phalangium opilio	Distal tips of 3rd and 4th walking legs, ventral ectoderm expression expanding from the 2nd walking leg to the SAZ, later extending only to O4	Sharma et al. (2012b)

Pax group III	*Tetranychus urticae*	*Pax 3/7*: Initially three ventral stripes are expressed (Pp, L2, L4), a fourth stripe emerges between the two most anterior stripes (L1), and a fifth stripe (L3) appears between the two most posterior stripes, one small opisthosomal stripe Pax 3/7 protein expression: Segmental stripes in Ch, Pp, L1–L4, O1, O2. Pre-cheliceral CNS	Dearden et al. (2002), Davis et al. (2005)
	Cupiennius salei	*pairberry 1–3*: Dynamic SAZ expression, segmental expression in prosomal and opisthosomal segments Pax 3/7 protein expression: Ventral segmental stripes at the posterior segmental borders. O1 stripe is extended dorsally. Pre-cheliceral CNS, segmental neural cell clusters	Schoppmeier and Damen (2005), Damen et al. (2005), Davis et al. (2005)
	Limulus polyphemus	Pax 3/7 protein expression: Expressed as concentric rings from the posterior that form into stripes. Segmental neural cell clusters	Davis et al. (2005)
patched	*Parasteatoda tepidariorum*	Early: circular expression domain in the blastopore area, which expands to ubiquitous expression in the entire germ disc epithelium; followed by a reduction of expression to the rim of the germ disc Late: expression in the SAZ and in a stripe at the anterior border of the germband	Akiyama-Oda and Oda (2010), Barnett and Thomas (2013a)
	Archegozetes longisetosus	Early: expression commences as double stripes in prosomal segments corresponding to L1, L2, chelicerae, and pedipalps and in a broad expression domain in the growth zone Late: pronounced expression of a medial stripe in prosomal segments (excluding L3), followed by expression in a broad stripe in O1 and subsequently O3 and O4, which also eventually split into double stripes, in the two latter segments	
hairy	*Cupiennius salei*	Dynamic stripe formation in the SAZ before morphologically visible segmentation	Damen et al. (2000)
	Parasteatoda tepidariorum	Ring at the rim of the germ disc that will form a broad anterior stripe, which will later form stripes in the Ch and Pp segments. Circular expression in the centre of the germ disc that will clear from the centre to form a broad stripe that will later split into stripes in L2–L4. Opisthosomal stripes are sequentially added from the SAZ	Pechmann et al. (2009)
Head patterning genes			
orthodenticle	*Parasteatoda tepidariorum*	Early: ring around edge of germ disc, migrating wave of expression in the future head region Late: pre-cheliceral lobes and ventral midline	Akiyama-Oda and Oda (2003), Pechmann et al. (2009)
	Tegenaria saeva	Stripe in pre-cheliceral lobes, anterior to limb buds on O2–O5	Simonnet et al. (2006)
	Euscorpius flavicaudis	Early: pre-cheliceral stripe Late: pre-cheliceral lobes, ventral midline, lateral expression anterior to opisthosomal limb buds, and lateral dots in all metasomal segments	Simonnet et al. (2006)
	Archegozetes longisetosus	Pre-cheliceral lobes, ventral midline, possibly in labrum	Telford and Thomas (1998)
	Phalangium opilio	Eye fields, pre-cheliceral lobes, ventral midline, labrum	Garwood et al. (2014)

(continued)

Table 5.2 (continued)

Gene name	Species	Expression domain	Reference
empty spiracles	*Tegenaria saeva*	Posterior pre-cheliceral region, segmental patches neuroectoderm, walking legs, lateral stripes in O2–O5	Simonnet et al. (2006)
	Euscorpius flavicaudis	Posterior pre-cheliceral region, lateral segmental stripes in all segments, prosomal appendages	Simonnet et al. (2006)
	Phalangium opilio	Posterior pre-cheliceral region, lateral segmental stripes in all segments, prosomal appendages	Garwood et al. (2014)
cap-n-collar	*Phalangium opilio*	Weak ubiquitous expression	Sharma et al. (2014c)
	Centruroides sculpturatus	Weak ubiquitous expression	Sharma et al. (2014c)
Pax6	*Limulus polyphemus*	Head lobes and developing brain, paired clusters of cells in the ventral neuroectoderm and developing nervous system	Blackburn et al. (2008)
	Phalangium opilio	Head lobes, paired stripes in all prosomal and opisthosomal segments	Garwood et al. (2014)

Expression patterns of genes characterised in at least one chelicerate other than spiders, with the exception of Hox gene expression patterns and leg patterning genes, which are treated in Figs. 5.2 and 5.8, respectively

condition of fertilisation by spermatophores (indirect sperm transfer) occurs in Cyphophthalmi (Karaman 2005). Fertilised eggs are deposited singly or in batches that can number into the hundreds (Juberthie 1964). This means that some species of harvestmen can readily provide large numbers of embryos that can be collected at different stages to study the embryogenesis of these animals (Moritz 1957; Juberthie 1964; Muñoz-Cuevas 1971; Gnaspini and Lerche 2010). Indeed, RNA in situ hybridisation to visualise gene expression patterns (e.g., see Table 5.2) and RNAi to characterise gene function have already been established in *Phalangium opilio*. This has facilitated studying the regulation of development in this species compared to other animals, including analysis of Hox and leg gap genes (Fig. 5.2; Sharma et al. 2012a, b, 2013, 2014c; Garwood et al. 2014).

Solifugae (Camel Spiders)

Solifuges or camel spiders predominantly inhabit arid environments where they mainly predate on other arthropods, taking advantage of their speed and large powerful cheliceres (Punzo 1998). Anatomically, these arachnids are distinguished from others by their malleoli (sometimes called racquet organs). These are fan-shaped chemoreceptory organs that detect changes in the substrate, analogously to the pectines of scorpions (Brownell and Farley 1974). Being apulmonate arachnids, camel spiders lack book lungs, but have among the most densely branching tracheal system for respiration among arachnids (Fig. 5.2; Lighton and Fielden 1996). Although recent work has been carried out on the functional morphology of these arachnids (van der Meijden et al. 2012), camel spiders represent a rather understudied order of chelicerates, and there is a dearth of EvoDevo studies on the group. Although solifuges are difficult to collect and produce only one brood per year, culturing camel spiders in the laboratory is possible, albeit challenging, and females can lay clutches of up to 200 embryos (Punzo 1998). Therefore, there is potential that gene expression and gene function could be studied in camel spiders. The development of the malleoli and the genetic basis for lateral eye loss in many species of solifuges are opportune targets for evolutionary developmental study, particularly with reference to phalangid harvestmen, which also lack lateral eyes (Garwood et al. 2014).

Pseudoscorpiones (False or Book Scorpions)

There are over 3,200 species of pseudoscorpions, which occupy a wide range of habitats worldwide (Harvey 2011). These chelicerates prey on other invertebrates or are scavengers, and some have even adopted a commensal or phoretic (hitchhiking) strategy, living on and being dispersed by mammals, birds, and larger arthropods (Weygoldt 1970; Harvey 2002, 2011).

Pseudoscorpions have long modified pedipalps that terminate with chelae like scorpions, but they are distinguished from the latter in lacking the characteristic tail and stinger of scorpions, as well as median ocelli and pectines (Figs. 5.1 and 5.2). Like camel spiders and harvestmen, pseudoscorpions also lack book lungs and instead use spiracles and a tracheal system for respiration (Fig. 5.2; Weygoldt 1970; Lighton and Joos 2002; Harvey 2011). Like other chelicerates, most notably spiders, pseudoscorpions can also make silk, which is produced from prosomal glands and used for a variety of purposes, including sperm transfer and burrowing (Weygoldt 1970; Harvey 2011). Members of the suborder Iocheirata also synthesise venoms (Weygoldt 1970; Harvey 1992).

A multilocus phylogeny, which remains rare for several minor arachnid orders (Harvey 2002), has been proposed for pseudoscorpions and indicates that it is likely that venom only evolved once within this group and independently of scorpion and spider venom (Murienne et al. 2008). Although the morphology of these animals has been characterised in some detail for taxonomic purposes and aspects of their courtship behaviour described, these chelicerates have only recently been studied in the context of EvoDevo research (Jędrzejowska et al. 2013). Elucidating the genetic mechanism whereby chelate pedipalps are patterned in pseudoscorpions and scorpions may shed much needed light on how these groups are related (Fig. 5.1).

Acariformes (Mites)

To date nearly 50,000 species of mites have been described, although it is thought that there could be over a million species, and they therefore represent the most diverse group of chelicerates. These miniaturised arachnids can be free-living or parasitic and live in a wide range of habitats including aquatic environments (Beccaloni 2009).

The cephalothorax and truncated abdomen of mites are fused, but the body is divided into two autapomorphic tagmata—the anterior gnathosoma and the posterior idiosoma (Fig. 5.2)—although these can be covered by a single carapace in some species. The cheliceres and pedipalps of mites can vary in morphology between species and they have four pairs of walking legs as adults (Fig. 5.2). Most mites respire using a tracheal system and have up to four pairs of anteriorly positioned spiracles (Fig. 5.2). Sperm transfer is indirect in most mites with the males producing a spermatophore manipulated with their appendages. Females usually lay their eggs in soil or humus from which the larvae hatch up to 6 weeks later (Ruppert et al. 2004).

Research on mites has provided several important insights into evolutionary developmental biology (see below) through studying gene expression and gene function in species such as *Tetranychus urticae* and *Archegozetes longisetosus* (Table 5.2; Telford and Thomas 1998a, b; Dearden et al. 2000, 2002, 2003; Grbic et al. 2007; Khila and Grbic 2007; Barnett and Thomas 2012, 2013a). Furthermore, the genome of *T. urticae* has also been sequenced (Table 5.1; Grbic et al. 2011), which greatly complements the other tools and resources available for this species.

Parasitiformes (Ticks)

Ticks are highly speciose parasitic chelicerates that live on a range of hosts, including humans and domestic animals (Beccaloni 2009). The body plan of ticks is similar to that of mites (Fig. 5.2), although these two chelicerate lineages may not form a clade (the traditionally defined Acari; Fig. 5.1). The biology of ticks is highly relevant to health-related and agricultural interests, and the genome of *Ixodes scapularis* has thus been sequenced (Table 5.1). This has allowed comparisons of the sequences of important developmental genes to be made between this tick and other metazoans (e.g., Janssen et al. 2010). Furthermore, embryonic development has been described for *Rhipicephalus* (*Boophilus*) *microplus*, which involved using antibody stainings (Santos et al. 2013b). However, gene expression and function during tick development has not been studied to the best of our knowledge, although reports of the successful application of parental RNAi (e.g., la Fuente et al. 2007) might change this in the future.

Ricinulei (Hooded Tick Spiders)

Ricinulei represent a small (3 genera and only about 60 recognised species) and understudied order of chelicerates (Fig. 5.1; Harvey 2002; Botero-Trujillo 2014). These animals are small arachnids that live in leaf litter and caves, and most species lack eyes, although some species have basic lateral eyes (Beccaloni 2009).

Ricinulei exhibit two tagmata and also respire via a tracheal system (Fig. 5.2). Ricinulei are distinguished by a cucullus or cuticular hood that can be used to cover the cheliceres and mouthparts (Beccaloni 2009). The second pair of walking legs is longer than the others and is also sensory (Beccaloni 2009). The third walking legs of male Ricinulei are used for sperm transfer and exhibit species-specific modifications like the pedipalps of spiders (Legg 1977; Harvey 2002). Although several aspects of the morphology of Ricinulei have recently been described in great detail (Talarico et al. 2006, 2008a, b, 2011), there are no embryological or EvoDevo studies of these animals of which we are aware. Opportune targets for study of EvoDevo in this group include the differentiation of the sexually dimorphic third leg pair in males. In addition, a potential shared mechanism for the inhibition of L4 limb bud growth in first instars of Ricinulei, mites, and ticks may shed light on the phylogenetic affinities of the "acaromorph orders" (Fig. 5.1; Shultz 2007).

Palpigradi (Microwhip Scorpions)

There are approximately 80 species of microwhip scorpions (Fig. 5.1; Harvey 2002). These arachnids are widespread in tropical and subtropical regions and live in caves and damp soils (e.g., Smrz et al. 2013 and references therein).

Microwhip scorpions are very small (at most 3 mm in length), are eyeless, and exhibit a segmented flagellum at the end of their abdomen (Fig. 5.2; Beccaloni 2009). It was recently found that the species *Eukoenenia spelaea* feeds on cyanobacteria in caves, although very little else is known about the natural history of microwhip scorpions (Smrz et al. 2013). To the best of our knowledge, these rather enigmatic chelicerates have not been the subject of any embryological research. Microwhip scorpions only lay a few (one to three) embryos at a given time, and ovules of different developmental stages have been observed within the opisthosoma (Condé 1996).

Amblypygi (Whip Spiders)

Whip spiders are mostly found in tropical rainforests, and there are many cave-dwelling species. Only about 150 species of whip spiders have been described (Harvey 2003), and what is known of their biology has been previously reviewed in detail by Weygoldt (2000).

Whip spiders are similar in appearance to spiders, but are somewhat flattened in comparison. Furthermore, the cheliceres of whip spiders do not produce venom and they use modified pedipalps to capture prey (Fig. 5.2). These chelicerates can also be distinguished by their first pair of walking legs, which is elongated and tactile, and therefore considered to be antenniform (Weygoldt 2000). Whip spiders also have two sets of opisthosomal book lungs, but they do not have any other appendages on this tagma (Fig. 5.2) and they lack the ability to make silk. Like scorpions and thelyphonids (see below), parental care in this order consists of a female carrying hatchlings on her back until they reach a certain developmental stage and disperse. Unlike scorpions, only in amplypygids, thelyphonids, and pseudoscorpions do females carry eggs on the underside of the opisthosoma until hatching.

The embryology and morphology of whip spiders has been described in detail, although very little contemporary EvoDevo research has been carried out on these animals (Weygoldt 2000). However, such research would offer an interesting comparison to spiders due to the phylogenetic proximity of these two orders. The regulation of the development of the large, raptorial pedipalps and the elongate, antenniform first walking legs—in contrast to their shorter counterparts in spiders—constitute promising areas of future study (Weygoldt 2000; Harvey 2002).

Thelyphonida (Whip Scorpions)

There are over 100 described species of whip scorpions (Harvey 2002). These predators live in tropical climates and employ their enlarged raptorial pedipalps to grab prey (Fig. 5.2; Ruppert et al. 2004). To a lesser degree than in whip spiders, the first pair of legs of whip scorpions is elongated and tactile (Fig. 5.2). Whip scorpions also have a segmented opisthosoma that ends in an annulated flagellum, superficially resembling scorpions and conferring their common name (Ruppert et al. 2004). The abdomen of whip scorpions also carries two pairs of book lungs (Fig. 5.2) and two anal glands that are used to repel predators by spraying them with a mixture of acetic acid, caprylic acid, and other substances (hence, these animals are sometimes referred to as vinegaroons) (Eisner et al. 1961; Haupt and Müller 2004).

To date, whip scorpions have not been the subject of EvoDevo research. Like in Amblypygi, the embryos are carried in an external sac by the females, meaning that embryos of different developmental stages can be collected for analysis of gene expression and gene function (Ruppert et al. 2004). The miniaturisation of particular opisthosomal sternites in thelyphonids is of particular interest from the perspective of segmentation (Shultz 2007).

Schizomida (Short-Tailed Whip Scorpions, Microwhip Scorpions)

Short-tailed whip scorpions are close relatives of whip scorpions (Figs. 5.1 and 5.2) that live in tropical leaf litter (Santos et al. 2013a). Harvey (2002) estimated that there are over 500 extant species worldwide. Short-tailed whip scorpions resemble miniaturized whip scorpions. However, they are much smaller and have only one pair of book lungs (Fig. 5.2; Ruppert et al. 2004). The flagellum of short-tailed whip scorpions is also shorter than that of whip scorpions and confers their common name (Fig. 5.2). Interestingly, the flagellum is sexually dimorphic and is used during courtship, and it has been suggested that this structure may be involved

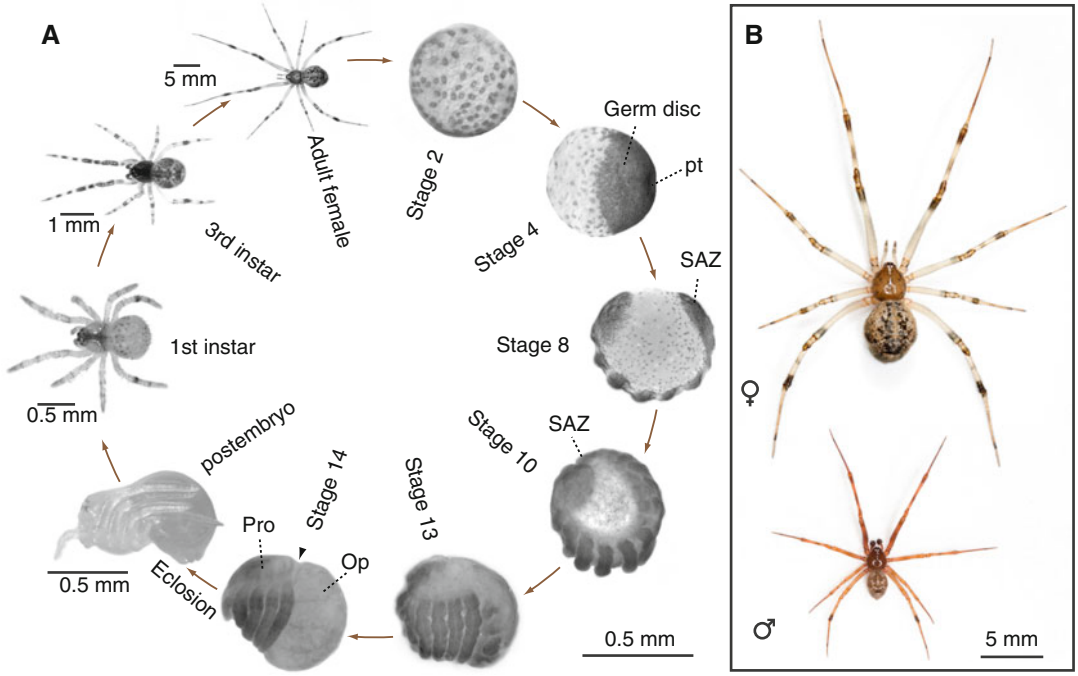

Fig. 5.3 The development and morphology of the spider *Parasteatoda tepidariorum.* (**A**) Stages of embryonic and postembryonic development: stage 2, cellularisation is complete, blastoderm formation; stage 4, germ disc including the primary thickening (*pt*) in the centre; stage 8, early germband with the segment addition zone (*SAZ*); stage 10, elongated germband with limbs; stage 13, end of inversion; stage 14, with distinct prosoma (*Pro*) and opisthosoma (*Op*) with constriction between them (*arrowhead*); postembryo; 1st instar, which exits the cocoon; 3rd instar, a free-foraging instar stage; female adult. Staging after Mittmann and Wolff (2012). In all images anterior is to the left. Scale bar is given with respect to the stage 2–14 embryos. (**B**) Adult female (*top*) and male (*bottom*). Anterior is to the top (Figure slightly modified and reproduced with permission from Hilbrant et al. (2012))

in species recognition (Harvey 2002). Like whip scorpions, the evolution and development of short-tailed whip scorpions is understudied, but the parallel evolution of a single pair of book lungs in schizomids and derived spiders from the ancestral condition of two pairs in Tetrapulmonata would be very interesting to explore further.

Araneae (Spiders)

Spiders have been intensively studied and they are the best-understood chelicerates in terms of their general biology, physiology, behaviour, development, and evolution (Fig. 5.1; Foelix 2010). Spiders are a speciose order of arachnids (over 40,000 described species) that exhibit a wide range of physiological and morphological adaptations, including silk and venom production, and morphological diversity of such appendages as the cheliceres and pedipalps (Foelix 2010).

Spiders have a prosoma and an opisthosoma with the former bearing the cheliceres, pedipalps, and four pairs of walking legs and the latter housing structures including the respiratory organs, genitalia, and spinnerets (Fig. 5.2). The group is distinguished from all other chelicerates in bearing spinnerets, modified appendages that constitute the web-spinning apparatus of spiders. The spinnerets and the webs of spiders have been argued to constitute key innovations that enabled considerable diversification in this group.

Spiders have also constituted the main model chelicerates used to address questions in evolutionary developmental biology. In particular, two Entelegynae, the central American wandering spider *Cupiennius salei* and the common house spider *Parasteatoda tepidariorum* (formerly *Achaearanea tepidariorum*; Fig. 5.3 and see boxed text), have provided great insights into

chelicerate, arthropod, and metazoan evolution and development (McGregor et al. 2008a; Hilbrant et al. 2012). More recently, the Haplogynae *Pholcus phalangioides* has been employed as a satellite model to provide a comparative perspective in spider EvoDevo within Araneomorphae (Pechmann et al. 2011), and there has also been one comparative gene expression study in a mygalomorph (Pechmann and Prpic 2009). The contribution of studies of gene expression (e.g., see Table 5.2) and gene function in spiders to our understanding of evolution and development is discussed in detail below.

The Common House Spider *Parasteatoda tepidariorum* as a Model for Evolutionary Developmental Biology

The common house spider, *Parasteatoda tepidariorum* (Koch 1841), native to South America, is synanthropic and presently distributed worldwide. *P. tepidariorum* hides in cobwebs in secluded areas. Due to the phylogenetic significance of chelicerates in arthropod phylogeny and the operational flexibility of this species, *P. tepidariorum* has become a powerful model organism in the field of evolutionary developmental biology. Females lay up to 400 embryos in silken egg sacs (cocoons) about every 5 days all year around under laboratory conditions. Due to the short fertilisation process, which takes about three minutes, embryos develop synchronously within one cocoon, which is particularly advantageous for developmental studies.

In embryos the first nuclear divisions take place in the centre of the spherical egg and cellularise when the cells start to migrate towards the periphery after about five divisions. Later, cells divide and aggregate to deploy the blastoderm at one hemisphere, where the blastopore forms in the centre upon gastrulation and invagination processes occur. After blastopore closure, the cumulus, an aggregation of mesenchymal cells in the centre of the germ disc, migrates underneath the ectodermal cell layer towards the periphery. This process specifies the DV axis and initiates the transformation from a germ disc to a germband (Fig. 5.3). The sequential addition of opisthosomal segments from the posterior segment addition zone follows, and the nervous system and appendages begin to form along the AP axis. At late stages of embryonic development, inversion processes occur where the embryo encloses the yolk and internal organs like the heart, digestive tract, and brain develop.

The whole developmental process until hatching lasts approximately 8 days and another 12 weeks for the spiderlings to develop to adulthood, including five molts for males and up to seven molts for females at 25 °C (see Fig. 5.3). Embryos of all embryonic stages can be fixed and used for in situ hybridisation and antibody staining to study mRNA and protein expression, respectively. Furthermore, gene function can be studied in *P. tepidariorum* with RNA interference: double-stranded RNA (dsRNA) injected into adult females results in several cocoons exhibiting a knockdown effect. Injecting a single cell of an embryo at the 16- or 32-cell stages with dsRNA generates clones of cells lacking gene function. The availability of transcriptomic sequences and, in the future, whole-genome sequence data will potentially allow genome-editing tools to be applied in *P. tepidariorum* to study the genetic regulation of the development of this spider in even greater detail.

In the following, a summary of the classic literature describing key aspects of the early and late development of the chelicerates is provided. Subsequently, studies that have focused on characterising gene expression and gene function in chelicerates are reviewed to highlight important insights into the evolution and development of these animals, other arthropods, and other metazoans.

EARLY DEVELOPMENT

The study of chelicerate embryos dates back to the very beginnings of invertebrate developmental biology. In 1824, Moritz Herold delivered what he claimed were the first studies of invertebrate development (Herold 1824)—and his first study subject were embryos of the European garden spider, *Araneus diadematus*. Most classical literature from the mid-late nineteenth century onwards has been extensively reviewed by Anderson (1973) and Yoshikura (1975), and to avoid duplicating these efforts, the reader may refer to their exhaustive listing of chelicerate embryological studies prior to 1975. In the following section, the focus is on describing key steps of chelicerate development that have been the focus of modern evolutionary developmental biology.

Cleavage

Most chelicerate eggs (with the exception of mites, ticks, and viviparous scorpions as well as sea spiders) are round or ovoid in shape, fairly large (0.5–3.5 mm), and rich in yolk. These types of eggs predominantly show superficial early cleavages (i.e., without cytokinesis/formation of membranes between the cleavage energids) that occur in the centre of the egg within the yolk (intralecithal) (Schimkewitsch 1887, 1898, 1906; Kingsley 1892; Iwanoff 1933; Moritz 1957; Juberthie 1964; Kondo 1969; Yoshikura 1969; Anderson 1973; Weygoldt 1975; Suzuki and Kondo 1995, 1994; Kimble et al. 2002; Kanayama et al. 2010).

The best-described examples of this cleavage mode are found in spiders, owing to the application of more sophisticated imaging techniques such as transmission electron microscopy (TEM) and, more recently, single-cell injection. In a close relative of *Parasteatoda tepidariorum*, *P. japonica*, the first four cleavages are synchronous and syncytial. The perinuclear cytoplasm is connected with the periplasm at the egg surface by thin strands that form along yolk columns, and the cell membrane invaginates from the surface, also along these yolk columns. At the 16-cell stage, cell membranes fuse and form the blastomeres, which then migrate to the embryo's surface (Suzuki and Kondo 1995, 1994). Kanayama et al. (2010) have confirmed these findings in *P. tepidariorum* by showing that fluorescent dyes injected into the surface periplasm at the 16-cell stage do not diffuse into neighbouring areas and subsequently will only be found in daughter cells of the injected cell. It has been argued that this type of superficial cleavage might be the ancestral cleavage mode in Chelicerata and that the cases of total cleavage seen in some mites, some ticks, pseudoscorpions, and viviparous scorpions are possibly derived and linked to the production of smaller, less yolky eggs (Anderson 1973; Wolff and Scholtz 2013).

In the case of mites and ticks, Laumann et al. (2010b) have argued that classical studies of their embryos might have wrongly attested to these chelicerates possessing superficial cleavage due to the techniques used to examine the embryos. Laumann et al. (2010b) base this judgment on the re-examination of the cleavage mode of *Archegozetes longisetosus* by traditional light microscopy techniques that failed to detect the total cleavage mode of this oribatid mite, which the authors previously had determined using TEM (Laumann et al. 2010a, b). The authors then conclude that since no modern studies in either ticks (Fagotto et al. 1988) or mites (Dearden et al. 2002; Walzl et al. 2004; Laumann et al. 2010a, b) have confirmed superficial cleavage, the ancestral cleavage mode within ticks as well as mites must have been total (Laumann et al. 2010a).

Most pycnogonids display total and equal cleavages that are irregular. This cleavage mode is therefore thought to be the ground pattern in pycnogonids (Ungerer and Scholtz 2009). However, there are certain groups of pycnogonids with larger, more yolk-rich eggs that display unequal, yet still total cleavages (Ungerer and Scholtz 2009). In some of these pycnogonids (*Callipallene* and *Propallene*) even the first cleavage is unequal, which is suggestive of an early cell fate determination that would make

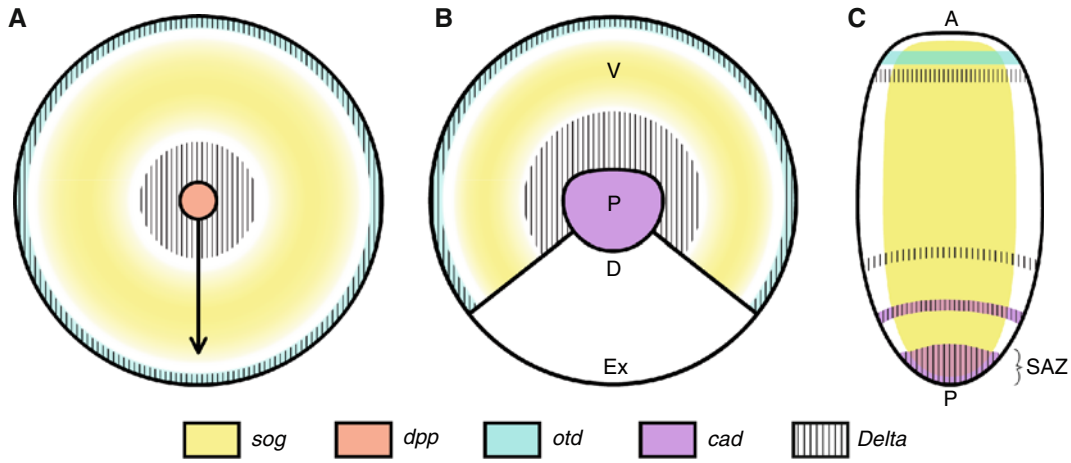

Fig. 5.4 Axis specification and formation of segments in spiders. (**A**) During early development blastomeres collect at one pole of the embryo to form a germ disc. At the centre of this is *decapentaplegic* (*dpp*) expression (*red*) from the mesenchymal cells of the cumulus. Surrounding the *dpp* expression is a circular domain of *Delta* (*Dl*) (hatched), then *short gastrulation* (*sog*) (*yellow*), with a co-expressed domain of *orthodenticle* (*otd*) (*light blue*), and a weak *Dl* signal around the periphery of the germ disc. As the *dpp*-expressing cumulus migrates, the radial symmetry is broken. (**B**) *dpp* expression then disappears when the dorsal field (*D*) starts to form. This dorsal region extends around the periphery of the germ disc forming the extra-embryonic (*Ex*) and dorsal tissues with the *sog* domain forming the ventral tissue (*V*). (**C**) Expression of *otd* and *Dl* in the periphery of the germ disc is later localised to the anterior prosomal region of the germband, with the opened central ring of *Dl* (*hatched*) moving to the approximate area where the prosoma/opisthosoma boundary develops. As the dorsal field opens up, the centre of the germ disc loses *Dl* expression and begins to express *caudal* (*cad*) in the forming caudal lobe (**B**). As the germband elongates, dynamic expression of *Dl* and *cad* in the segment addition zone (*SAZ*) buds off stripes associated with nascent opisthosomal segments (**C**). The exact spatial relationship of these genes' expression and which segments they form are still unclear. In (**B**, **C**), *A* anterior and *P* posterior ()

these embryos the only example of chelicerates showing determinate cleavage. Other chelicerates are not thought to specify cell lines early in development. However, cell lineage studies have so far only been attempted in spiders (Holm 1952; Kanayama et al. 2010) and a mite (Dearden et al. 2002).

Germ Rudiment Formation and Axis Formation

The Cumulus

The cumulus is a mesenchymal cell cluster that, in spiders, migrates from the centre of the germ disc to the rim of the germ disc and thereby breaks the radial symmetry of the embryo, establishing its dorsoventral (DV) axis (Fig. 5.4A). The cumulus has recently been shown to express *decapentaplegic* (*dpp*), and it is thought that Dpp protein is then received by germ disc epithelial cells and thereby represses ventralising *short gastrulation* (*sog*) expression (see below; Akiyama-Oda and Oda 2003, 2006).

The nomenclature of the cumulus has been confusing in classical chelicerate literature, since both the blastopore and the distinct, migrating cell group that originates from the blastopore form white, slightly elevated "cumulus-like" structures (from Latin, *cumulus*, meaning "heap" or "pile"). Therefore, classical literature has to be carefully judged for mislabeling the blastopore as a true cumulus. The cumulus' function as an organiser was first determined by Holm (1952) through cauterising and transplantation of cumulus material in embryos of the spider *Agelena labyrinthica*. Similar experiments as well as interspecific grafts have been performed on horseshoe crab embryos (Itow and Sekiguchi 1979; Itow 1990; Itow et al. 1991). Curiously, grafts of horseshoe crab "centre cells" from the blastopore region (but before actual cumulus

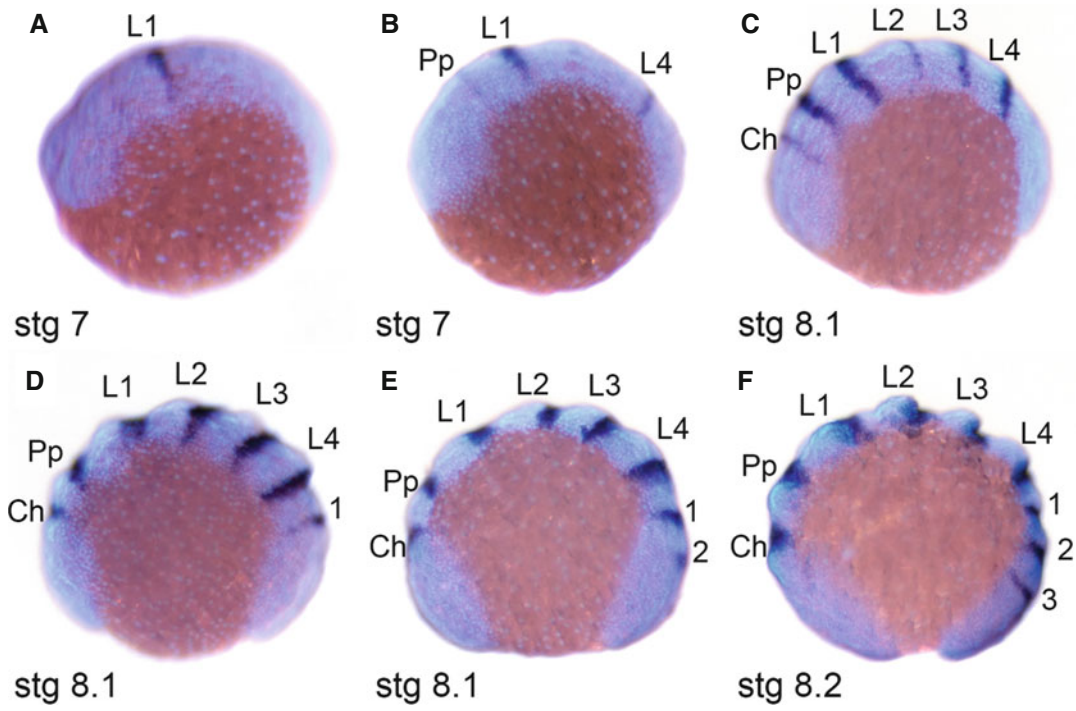

Fig. 5.5 Segmentation in spiders. Stripe appearance of the segmentation marker gene *engrailed* (*en*) in the spider *Parasteatoda tepidariorum*. The prosomal *en* stripes form nearly simultaneously, in a stereotyped order. (**A**) The first *en* stripe forms in L1. (**B**) Soon thereafter, stripes appear in L4 and the pedipalpal segment. (**C**) Subsequently stripes develop in L2, L3, and the cheliceral segment. (**D–F**) *en* stripes in the opisthosoma appear in a strict anterior to posterior order; only the first 3 of eventually 12 opisthosomal stripes are shown here. *Ch* cheliceral segment, *Pp* pedipalpal segment, *L* walking leg segments, 1, 2 and 3 opisthosomal segments (Figure slightly modified and reprinted from *Current Biology*, Schwager et al. (2009), with permission from Elsevier)

formation) are also capable of inducing a second embryonic axis in embryos of the frog *Xenopus laevis* (Itow 2005). However, Itow et al. claim that the posterior cumulus (i.e., the structure most likely homologous to the spider cumulus) has no effect on axis formation in the horseshoe crab (Itow 1990; Itow et al. 1991).

Migrating cumuli have also been noted in Amblypygi (Weygoldt 1975), Opiliones (Holm 1947; Juberthie 1964), possibly in a solifuge (Heymons 1904; Holm 1947) and most recently in a tick (Santos et al. 2013b). The cumulus has therefore been suggested as belonging to the ground pattern in Chelicerata (Hilbrant et al. 2012). However, the tick cumulus seems not to express Dpp, but instead, it appears to receive Dpp (Santos et al. 2013a, b). Accordingly, more evidence, especially molecular data, is required from chelicerate orders in which cumuli have not been described so far, to address the origin of the cumulus (with reference to the *dpp*-expressing structure observed in spiders) and perhaps ultimately to define this structure with respect to form, migration, developmental function, and gene interactions.

Segmentation

Most chelicerate embryos are of the short germ type, where a number of anterior segments is patterned by subdivision of the initial germ anlage and posterior segments are added sequentially from a posterior segment addition zone (SAZ). The initial germ anlage commonly forms all prosomal segments (pre-cheliceral lobe, cheliceral, pedipalpal, and the four walking leg segments—Pl, Ch, Pp, L1–L4), and a differing number of opisthosomal segments are added sequentially (Figs. 5.2, 5.3, and 5.5). This generalised form of

segmentation is found in spiders, harvestmen (Juberthie 1964), whip scorpions (Anderson 1973), Amblypygi (Weygoldt 1975), ticks (Anderson 1973; Santos et al. 2013b), and pseudoscorpions (Yoshikura 1975).

However, horseshoe crabs, scorpions and pycnogonids form only pre-cheliceral lobes and cheliceral and pedipalpal segments as well as a SAZ from their embryonic primordium, while walking leg segments and opisthosomal segments are then sequentially added from the SAZ (Anderson 1973; Itow and Sekiguchi 1980; Farley 2001; Brenneis et al. 2011b).

It is interesting to note that the anterior segments do not appear simultaneously, but instead are formed in a specific order that varies between groups. Where the timing of segment appearance has been observed (such as in spiders, Amblypygi and Xiphosura), the first segment to appear and the first segmental border to be established is usually the L1 segment or the Pp/L1 border (Anderson 1973; Weygoldt 1975; Itow and Sekiguchi 1980), and the last segment to be defined is most commonly the cheliceral segment. For example, in the spider *Parasteatoda tepidariorum*, *engrailed* (*en*) stripes appear first in L1, then Pp and L4 stripes emerge, then L2 and L3, and lastly Ch (Fig. 5.5; Schwager et al. 2009).

In contrast to the other chelicerates, the L4 segment of mites, ticks, and Ricinulei also derives from the SAZ. In case of the mite *Archegozetes longisetosus*, the remaining segments do not appear in sequential order from the SAZ, but, as evidenced by appearance of *en* and *hedgehog* (*hh*) stripes, first O1 is segmented, then L4, and finally O2 (Barnett and Thomas 2012).

As stated above, in almost all other chelicerates, opisthosomal segments are added sequentially from a SAZ. Despite the recent advances in our understanding of the genetic pathways involved in segmentation in spiders (McGregor et al. 2009; Hilbrant et al. 2012), we still lack insight into how exactly the SAZ of spiders and other chelicerates is organised, especially as cell division patterns have not been studied in detail, nor have cell movements been characterised. Generally, about 12 opisthosomal segments are formed from the SAZ. The first of these is later reduced to form the pedicel, linking the prosoma and opisthosoma in spiders and their close relatives. In other orders, O1 becomes greatly diminished (e.g., Opiliones) or almost completely removed (e.g., scorpions) in the course of embryonic development. In a few groups, such as opisthothele (non-mesothele) spiders, ticks, and mites, external opisthosomal segmentation is lost after embryogenesis (Anderson 1973; Yoshikura 1975).

Mites can also display a severe reduction in the number of opisthosomal segments: while most chelicerates develop around 12 embryonic opisthosomal segments (Fig. 5.2; Yoshikura 1975), in embryos of both *Tetranychus urticae* and *Archegozetes longisetosus*, only two *en* stripes are formed in the opisthosomal region (Grbic et al. 2011; Barnett and Thomas 2012). Ticks can show reduced opisthosomal segment numbers, but segments are clearly visible in embryos (Anderson 1973; Santos et al. 2013a, b). In addition, derived groups of mites (e.g., the gall mite family Eriophyidae) are even more segmentally aberrant, bearing only two legs and a worm-like body as adults.

Two groups that diverge from the general chelicerate segmentation pattern are (1) the katoikogenic scorpions, in which the mesosoma (the first eight embryonic segments of the opisthosoma in scorpions) is precociously segmented, with each segment bearing a pair of dorsolateral protrusions that supplement exchange surfaces with the mother, whereas the prosoma is segmented much later, and (2) the pycnogonids, which form a free-swimming larva that usually only possesses the cheliforal and two larval appendages (Vilpoux and Waloszek 2003; Machner and Scholtz 2010; Brenneis et al. 2013).

Development of the Nervous System

While the development of the nervous system in most Chelicerata has been described in classical literature (Anderson 1973), recent advances in imaging techniques as well as the use of molecular markers have allowed a more detailed look at chelicerate neurogenesis. However, these detailed

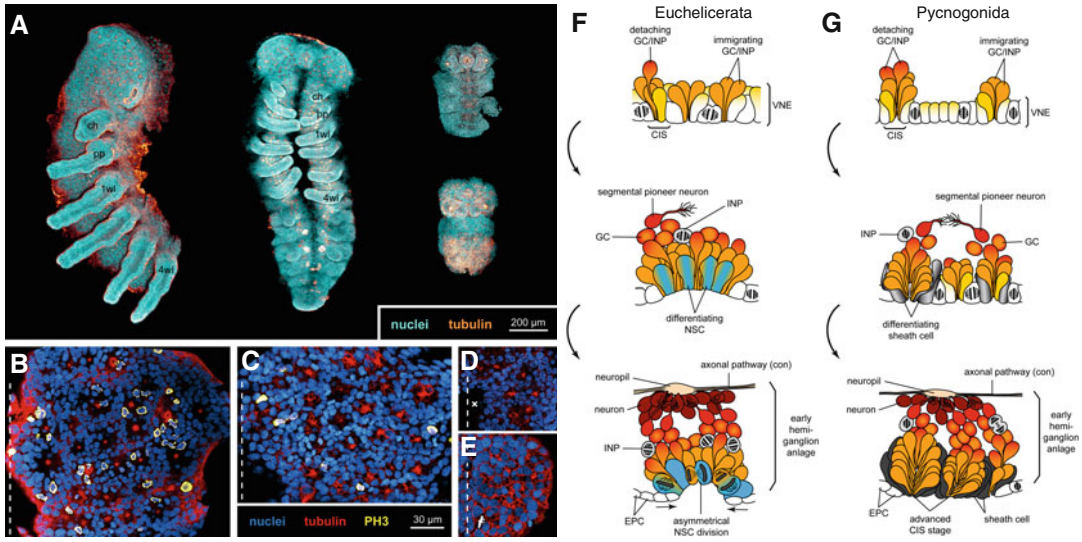

Fig. 5.6 Neurogenesis in euchelicerates and pycnogonids. (**A**) Confocal images of germbands of spiders and pycnogonids stained for tubulin and counterstained with a nuclear dye to illustrate cell internalisation sites (*CIS*) that are organised in a grid-like pattern in each hemisegment. From left to right: *Cupiennius salei* (left half of prosoma only), *Parasteatoda tepidariorum*, *Callipallene* sp. (*top right*), and *Pseudopallene* sp. (*bottom right*). (**B**–**E**) Apical horizontal sections of the ventral neuroectoderm (*VNE*) of a walking leg segment of (**B**) *C. salei* and (**C**) *P. tepidariorum*. Mitotic cells (stained by Phosphorylated Histone 3 antibody) are not found near CIS but are scattered throughout the hemi-neuromere. In the two pycnogonids (**D**) *Callipallene* and (**E**) *Pseudopallene*, the hemi-neuromeres are much smaller than found in the spiders. (**F**, **G**) Schematic sagittal sections through single hemi-neuromeres. Colours indicate different neuronal precursor (*NP*) cell types. In Eucelicerata (**G**, based mainly on *C. salei*), CIS form sequentially in the VNE, which shows unordered, mostly tangential cell divisions. Immature ganglion cells (*GCs*) delaminate and start differentiating basally to the CIS. CIS can also form cell-rich units, enclosed by glial-like sheath cells. Close to the forming neuropil, scattered symmetrically dividing intermediate neural precursors (*INPs*) can be found. Apically, the epidermis overgrows the hemi-neuromeres. In Pycnogonida (**F**, based mainly on *Pseudopallene* sp.), only few CIS and unordered, tangential cell divisions are found in the VNE. Basally, single GCs and INPs detach from the VNE and form a loose layer, in which neurons start differentiating. Apically, a central invagination forms and continues to deepen. Neural stem cells (*NSCs*), large, spindle-shaped cells, become discernible and start dividing tangentially or slightly obliquely, forming smaller daughter cells that invaginate independently. INPs also divide sub-apically. Epidermis overgrows the invagination site. *ch* cheliceral/cheliforal segment, *EPC* epidermis cell, *pp* pedipalpal segment, *wl* walking leg segment (Figure slightly modified and reproduced with permission from Brenneis et al. (2013))

studies of neurogenesis have only been performed on spiders, a horseshoe crab, and some pycnogonids.

Neurogenesis in spiders and horseshoe crabs takes place in the central ventral ectoderm. In each hemisegment about 30 groups of bottle-shaped neuronal precursors are specified and form cell internalisation sites (previously termed invagination sites). These sites are organised in a grid-like pattern and subsequently simultaneously delaminate from the neuroectoderm when early neurogenesis is complete (Fig. 5.6; Stollewerk et al. 2001; Mittmann 2002; Stollewerk and Chipman 2006; Doeffinger et al. 2010). In contrast to insects (Vol. 5) and crustaceans (Vol. 4), no neuroblasts (neural stem cells) are involved in neurogenesis in these chelicerates. Due to these differences with respect to Tetraconata and marked similarities with Myriapoda, it has been suggested that this mode of neurogenesis might be ancestral within arthropods (reviewed by Stollewerk and Chipman 2006).

Conversely, it has recently been shown that neurogenesis in pycnogonids surprisingly does involve neural stem cells (Fig. 5.6; Brenneis et al. 2013). Like the euchelicerates, neurogenesis in pycnogonids is initiated by formation of

post-mitotic neuronal precursor groups that form cell internalisation sites. In a second step, in pycnogonids, however, larger neural stem cells with high mitotic activity differentiate from the precursor groups and form ganglion cells by asymmetric cell divisions (Fig. 5.6; Brenneis et al. 2013).

Whether the pycnogonid neural stem cells have evolved convergently or, alternatively, chelicerates and myriapods have lost this cell type will, according to Brenneis et al. (2013), require studies of the molecular mechanisms of neurogenesis in pycnogonids, as well as detailed reinvestigation of neurogenesis in other arthropod groups. It is interesting to note that some authors of classical literature have noted the existence of neural stem cells within a few chelicerates (Anderson 1973). Because the study of neurogenesis with modern techniques is limited to just two chelicerate orders, re-examination of neurogenetic processes with advanced techniques is imperative in non-spider arachnids.

LATE DEVELOPMENT

Inversion

In most chelicerates the germband forms on the surface of the round or ovoid yolky egg. Hence, at some point during the development of the chelicerate embryo, the yolk has to be transferred into the embryo proper, specifically into the opisthosoma, where it will later be ingested by the hatchlings via the midgut. This problem is solved in two distinctive ways among the different chelicerate orders. The embryo either simply grows around the yolk dorsally until dorsal closure commences, or it undergoes a process termed "inversion". This process is most pronounced in entelegyne spiders, less so in more basally branching groups such as mygalomorph and haplogyne spiders, and is almost absent in mesothele spiders (Yoshikura 1975).

During inversion, the germband splits in half along the ventral midline and forms the ventral sulcus, which is only covered by a single layer of cells. The two halves, still connected at least at the anterior and posterior ends, move dorsally around the yolk, widening the ventral sulcus, until their dorsal sides converge at the dorsal midline. Only after dorsal closure do the two halves of the germband reconnect ventrally for ventral closure. During this process, most of the yolk is transferred into the opisthosoma (Anderson 1973).

The amount by which the ventral sulcus widens differs across spiders, and as mentioned before, is less pronounced in more basally branching groups. However, other chelicerates display inversion processes similar to spiders, most notably in Thelyphonida, Amblypygi (Weygoldt 1975), and possibly Solifugae, and others such as Opiliones and some mites show only a very slight widening of the ventral sulcus during the dorsal closure process (Anderson 1973). The process of inversion inherently has consequences for the behaviour of the midline and also for neurogenesis in spiders (Linne et al. 2012).

Development of Germ Cells

Characterisation of the developmental origin of germ cells of chelicerates is limited to classical, mainly histological, studies of only a few groups (spiders, scorpions, mites, ticks, harvestmen, and solifuges) (Anderson 1973).

In these studies, germ cells have mostly been reported to originate from the mesoderm later in embryogenesis, for example, at the posterior end of the germband in ticks (Aeschlimann 1958) or in spiders, where germ cells appear as segmental clusters close to the coelomic pouches in the opisthosoma (Kautzsch 1909; Strand 1906). In the early embryos of some spiders, harvestmen and solifuges primordial germ cells (PGCs) have also been described to originate in or near the blastopore (Faussek 1891; Brauer 1894; Heymons 1904; Montgomery 1909). Recently however, by assaying the mRNA and protein expression of two molecular germ cell markers, *piwi and vasa*, Schwager et al. (2014) did not find any evidence of germ cells near the blastopore in early spider embryos. Instead, in *Parasteatoda*, PGCs arise as segmental clusters in opisthosomal segments O2–O6.

Interestingly, none of the previous studies that found PGCs in or near the blastopore in harvestmen, scorpions, and solifuges were able to trace these cells to the gonads at later stages (Faussek 1889, 1891; Brauer 1894). Therefore, to determine the germ cell origin in these three groups, it will be essential to re-examine their embryos using molecular tools where possible.

In the only other modern study of chelicerate germ cells, in the spider mite *Tetranychus urticae*, the germ cell marker gene *vasa* has been used to identify a group of dispersed cells deep in the yolk as PGCs that later are thought to migrate towards the posterior of the embryo to form a cluster of germ cells near the prosomal/opisthosomal boundary (Dearden et al. 2003). This mode of germ cell specification from non-blastodermal cells does not match any of the modes described for the other chelicerates. Indeed, since *vasa* has also been found to be expressed in numerous other tissues, including stem cell-like cells, the cells described in *T. urticae* might not actually be PGCs. Examining PGC specification in the spider mite using more germ cell markers might help to shed further light on this issue (Schwager et al. 2014).

Development of Respiratory Organs

Among chelicerates, three main types of respiratory organs can be found: book gills, book lungs, and tracheae (Fig. 5.2). Book lungs and tracheae appear alone or in combination across the chelicerate orders (Fig. 5.2). Some miniaturised species (e.g., microwhip scorpions and some mites) lack specialised respiratory organs entirely, with gas exchange occurring through the cuticle (Ax 2000; Zhang 2003; Foelix 2010). Similarly, respiration in pycnogonids occurs through direct diffusion.

Book Gills and Book Lungs

The book gills of Xiphosura are thought to represent the most ancestral respiratory organ among euchelicerates, but their relationship to scorpion and spider book lungs is not well understood. Recent phylogenomic efforts suggest a single origin of the arachnid book lung, consistent with the anatomy of these organs in spiders, amblypygids, uropygids, and scorpions (Scholtz and Kamenz 2006; Regier et al. 2010; Sharma et al. 2014b). In *Limulus polyphemus*, book gill development commences with the formation of bilateral ridges on the opisthosoma, the primordia of the genital operculum, and the branchial appendage, which will later become the gill-bearing segment (Yamasaki et al. 1988). The genital operculum and the first branchial appendage further develop into a large lateral and a small medial lobe on the ventral side of the opisthosoma (Farley 2010). Trabeculae then become apparent on these opisthosomal segments, which will later function as space holders in the haemolymph channels of the book gills (Kingsley 1892). The surface of the operculum and the branchial appendage form small pores and invaginations, which may facilitate gas exchange. Cross sections of the operculum and branchial appendage have revealed that trabeculae bridge the lumen of these lobes and seem to be connected with the invaginations on the surface of these appendages (Farley 2010). At the stage of the swimming and burrowing first instar, the first branchial segment appears as a broad but thin appendage, which carries four gill lamellae. The book gills are therefore surface outgrowths of the first branchial appendage. The lamellar structures of the book gills provide the surface for gas exchange between water and haemolymph in horseshoe crabs. The invaginations at the surface of the operculum and the branchial segment are connected to the gill lamellae through the trabeculae (Farley 2010).

Arachnopulmonata (scorpions + tetrapulmonates) exhibit variable numbers of paired book lungs (Fig. 5.2). The "primitive" spiders (mesotheles, mygalomorphs, and most paleocribellates) exhibit two pairs of book lungs, but in labidognathous spiders (i.e., derived araneomorphs), the posterior pair has been modified into tubular tracheae (Kästner 1929; Yoshikura 1975). Scorpions exhibit four pairs of book lungs, while whip scorpions and whip spiders have two pairs, and microwhip scorpions only have one pair (Fig. 5.2; Levi 1967).

The development of scorpion and spider book lungs is uniform and first becomes apparent as an

ectodermal invagination at the posterior margin of an opisthosomal segment (Laurie 1890; Purcell 1909; Farley 2008). This invagination then increases in size, forming a pulmonary sac, while the limb bud itself ingresses into the ectoderm of the segment (Farley 2011). The anterior wall of the pulmonary sac develops projecting lamellae, which extend into the pulmonary sac (Anderson 1973; Farley 2010, 2011).

The fully developed book lungs in scorpions and spiders open as stigmata on the ventral side of the opisthosoma into the atrium, which enlarges into a cuticle-lined cavity (Kamenz et al. 2005). Cuticular invaginations filled with haemolymph, interspersed by air pockets, extend horizontally from the lung sinus opposite the atrium into the cavity. The name "book lungs" is derived from the stacked structure of the lamellae, where the oxygenation of the haemolymph occurs (Reisinger et al. 1991; Kamenz et al. 2005; Foelix 2010).

Tracheae

A tracheal respiratory system is found in mites, ticks, pseudoscorpions, camel spiders, harvestmen, hooded tick spiders, and, in conjunction with one pair of book lungs, most araneomorph spiders (Fig. 5.2). Tracheae can vary in structure and are either tubular (camel spiders, harvestmen, and some spiders) or sieve tracheae (pseudoscorpions, hooded tick spiders, some spiders) (Kamenz et al. 2005; Foelix 2010). The latter are composed of a bundle of tubes, which look like a perforated membrane in cross section, hence the name. It has been proposed that the sieve tracheae are derived from lung lamellae (Foelix 2010; Nentwig 2013).

In spiders the tubular tracheae are located on the third opisthosomal segment, behind the anterior pair of book lungs, and are visible as stigmata (openings), in close vicinity to the spinnerets (Fig. 5.2). Generally, a stigma leads into an atrium whence two lateral and two median tubes arise. The lateral tubes are connected to the second pair of book lungs and the median tubes originate from muscular insertions, which become hollow and function as breathing organs (Foelix 2010). Tracheae in spiders exhibit open ends, which are in direct contact with haemolymph that transports the oxygen to the organs. The localisation and expansion of the tubular tracheae, however, is not as uniform as for book lungs and can vary significantly between species ranging from a restriction to the opisthosoma to extensive branching up to the prosoma (Foelix 2010). Within spiders, tubular tracheae are regarded as more derived than book lungs, as they are not found in basally branching spiders or non-spider tetrapulmonates, which employ only book lungs (Höfer et al. 2000; Foelix 2010). The simultaneous knockdown of multiple posterior Hox genes results in homeotic transformation of book lungs (and possibly the tubular tracheae as well) to leg-like outgrowths in the spider *Parasteatoda tepidariorum*, corroborating the serial homology of paired respiratory organs and prosomal appendages in a tetrapulmonate arachnid (Khadjeh et al. 2012). The relationship between the tubular tracheae of spiders and those of apulmonate arachnids is not understood in the context of developmental genetics.

THE GENETIC REGULATION OF CHELICERATE DEVELOPMENT

Axis Formation

In chelicerates, the regulation of the formation of the anterior-posterior (AP) and dorsoventral (DV) axes are best understood in the spider *Parasteatoda tepidariorum*. During the formation of the germ disc in this spider (Fig. 5.3; see boxed text), the cumulus develops as a cluster of mesenchymal cells under the main epithelial disc (Fig. 5.4A). Gene expression and functional analyses of orthologous genes that pattern the body axes of other arthropods have highlighted the importance of the cumulus as a key signalling centre for embryonic organisation in the spider (see above; Oda and Akiyama-Oda 2008).

During the initial formation of the germ disc, Hh signalling plays a crucial role in coordinating the cumulus and controlling its movement (Akiyama-Oda and Oda 2010). Hh ligands from around the rim of the germ disc are received by

patched (*ptc*) and *smoothened* (*smo*). It has been suggested that Hh forms a positional value gradient and thereby high levels promote the presumptive anterior, while low levels at the centre of the disc designate the posterior region where the cumulus forms (Fig. 5.4A; Akiyama-Oda and Oda 2010). The movement of the cumulus to the periphery also relies on Hh signalling because parental RNAi against *ptc* and *smo* can perturb cumulus migration (Fig. 5.4A; Akiyama-Oda and Oda 2010).

As mentioned above, the migration of the cumulus from the centre to the periphery of the germ disc breaks the radial symmetry and forms the DV axis (Fig. 5.4; Akiyama-Oda and Oda 2003). While the basal mesenchymal cells of the cumulus migrate under the germ disc, they express *dpp*, which activates the phosphorylation of mothers against dpp (pMad) in the epithelium, possibly via cytonemes (Fig. 5.4A; Akiyama-Oda and Oda 2003). When the *dpp* expression reaches the rim of the germ disc, it represses part of the circular expression domain of *sog* (Fig. 5.4B; Akiyama-Oda and Oda 2006). This event is concomitant with the opening of the dorsal field and the loss of *dpp* expression as the cumulus disappears (Fig. 5.4B). The expression of *sog* retracts ventrally between the anterior expression of *orthodenticle* (*otd*) and *caudal* (*cad*) expression in the caudal lobe (Fig. 5.4B, C; Akiyama-Oda and Oda 2003; Pechmann et al. 2009). *sog* expression progressively narrows to the ectoderm of the ventral midline, surrounded by pMad in the dorsal region (Fig. 5.4C; Akiyama-Oda and Oda 2006).

Segmentation

Formation of the Caudal Lobe and Posterior Segmentation

Studying the genetic regulation of segmentation in chelicerates, especially spiders, has provided key insights into the evolution of segment formation among arthropods and even other metazoans with segmented bodies (Damen 2007; McGregor et al. 2008a, 2009; Oda and Akiyama-Oda 2008; Hilbrant et al. 2012). Before the appearance of segments, the DV and AP axes are defined, as well as the first regulatory steps that specify the germ layers (see above). The genetic regulation of these processes, again, has been most fully characterised in *Parasteatoda tepidariorum*. During early embryogenesis in this spider, the Delta-Notch pathway is involved in allocating cells to the ectoderm, mesoderm, and endoderm as well as specifying the caudal lobe that gives rise to the SAZ, from which subsequently the posterior segments are generated (Oda et al. 2007).

Concurrent with the formation of the cumulus, the centre of the germ disc begins to express *Delta* (*Dl*) (Fig. 5.4). Cells that express *forkhead* and *twist* (*twi*) near these *Dl*-expressing cells internalise beneath the epithelia and become endoderm and mesoderm cells, respectively (Oda et al. 2007). Subsequently, expression of *Dl* and *twi* clears from the centre of the germ disc and *cad* is expressed in the caudal lobe (Fig. 5.4B; Oda et al. 2007). Furthermore, these dynamic changes in gene expression that specify the caudal lobe and subsequently the SAZ all require *Wnt8* (McGregor et al. 2008b).

During the formation of the germband from the germ disc (Figs. 5.3 and 5.4), the posterior domain of *Dl* expression forms a stripe. Expression of *Dl* then reappears in the SAZ and subsequently dynamic stripes of *Dl* expression in the SAZ are associated with the formation of nascent segments from this tissue. Previously, it was also shown that such stripes of *Dl* expression in the SAZ are required for segmentation in *Cupiennius salei*, another spider (Stollewerk et al. 2003). Since *Dl* is also necessary for segmentation in the cockroach *Periplaneta americana* (Pueyo et al. 2008), this suggests that Delta-Notch, Wnt, and Cad organiser was used ancestrally for segmentation at least in arthropods and was subsequently lost in some lineages (McGregor et al. 2009; Wilson et al. 2010; Kainz et al. 2011; Chesebro et al. 2013). This work has also contributed to the debate about the evolution of segmentation in metazoans more generally (Couso 2009; Chipman 2010).

After the initial cues from Delta-Notch and Wnt have activated segmentation from the

posterior SAZ, it has been shown in both *Cupiennius salei* and *Parasteatoda tepidariorum* that the orthologs of the pair rule genes are then differentially activated across the AP axis. In *P. tepidariorum*, *Wnt8* may help to regulate the transcription of the primary pair rule gene *hairy* in the SAZ (McGregor et al. 2008b). In *C. salei*, dynamic stripes of *even skipped* and *runt-1* progress from the SAZ during the formation of nascent posterior segments (Damen et al. 2005). The secondary pair rule gene *pairberry-3* also exhibits dynamic expression in the SAZ but forms stable stripes in nascent segments (Damen et al. 2005). However, the other secondary pair rule genes, *odd-skipped-related-1*, *odd-paired* (*opa*), and *sloppy paired*, are not expressed in the SAZ but are observed in stripes anterior to this structure in the nascent segments (Damen et al. 2005). The primary pair rule genes therefore appear to initially define segments from the SAZ and then the secondary pair rule gene orthologs maintain segment positioning. Subsequently, the parasegmental boundaries are defined by *Wnt* and *en* expression (Damen 2002), which is now known to be a conserved feature of arthropod segmentation (Vols. 4, 5; Damen 2007).

Prosomal Segmentation

It has been shown in spiders that the mechanism and underlying genetic regulation of prosomal segmentation differ from that described above for the opisthosomal segments. In the presumptive prosoma, segmentation is achieved by subdividing a pre-existing field of cells into segments, and *engrailed* stripes do not appear sequentially in this region (see above and Fig. 5.5). This prosomal segmentation mechanism is similar to *Drosophila melanogaster* segmentation. Indeed, in *Parasteatoda tepidariorum* this process requires the ortholog of the *D. melanogaster* gap gene *hunchback*, and knockdown of this gene in *P. tepidariorum* also produces a gap gene phenotype with multiple missing adjacent segments (Schwager et al. 2009). Interestingly, in both *P. tepidariorum* and the haplogyne spider *Pholcus phalangioides*, *Distal-less* (*Dll*), a gene normally known for its involvement in appendage patterning (see below), is expressed in the presumptive prosoma (Pechmann et al. 2011). Even more surprisingly, *Dll* is required for formation of prosomal segments because inhibition of *Dll* expression in *P. tepidariorum* results in a gap-like phenotype (Pechmann et al. 2011).

In *Parasteatoda tepidariorum* embryos, the most anterior prosomal region, however, yet again uses a different segmentation mechanism that Kanayama et al. (2011) have termed "split-type segmentation". Here, first a wave of *otd* expression, in conjunction with a travelling wave of *hh* expression, is thought to specify the head segments (Pechmann et al. 2009; Kanayama et al. 2011). Then, the *hh* stripe splits to generate the cheliceral and pedipalpal segments, which also involves convergent extension movements and depends on an autoregulatory signalling network of *otd*, *hh*, *opa,* and *cubitus interruptus* (*ci*) (Kanayama et al. 2011).

Hox Genes and the Regulation of Segment Identity in Chelicerates

Hox genes are responsible for specifying segmental identity along the AP axis in bilaterian animals (reviewed in Carroll et al. 2005). In chelicerates, the evolution of particular Hox genes is correlated with differences among chelicerate body plans and compared to other arthropods. Generally, the spatial expression patterns of the prosomal Hox genes are well conserved, whereas those that are expressed in the opisthosoma are more divergent (Figs. 5.2 and 5.7; Abzhanov and Kaufman 1999; Schoppmeier and Damen 2001; Khila and Grbic 2007; Pechmann et al. 2011). This may correlate with the evolutionary conservation of the prosoma compared to the more variable opisthosoma.

In all chelicerate lineages studied to date (apart from mites), as well as mandibulate arthropods (Chapter 6; Vols. 4, 5) and Onychophora (Chapter 4), at least ten Hox genes have been identified (Fig. 5.2; Janssen and Damen 2006; Sharma et al. 2012a, 2013, 2014b; Barnett and Thomas 2013a; Janssen et al. 2014), which suggests that this was the ancestral number of Hox

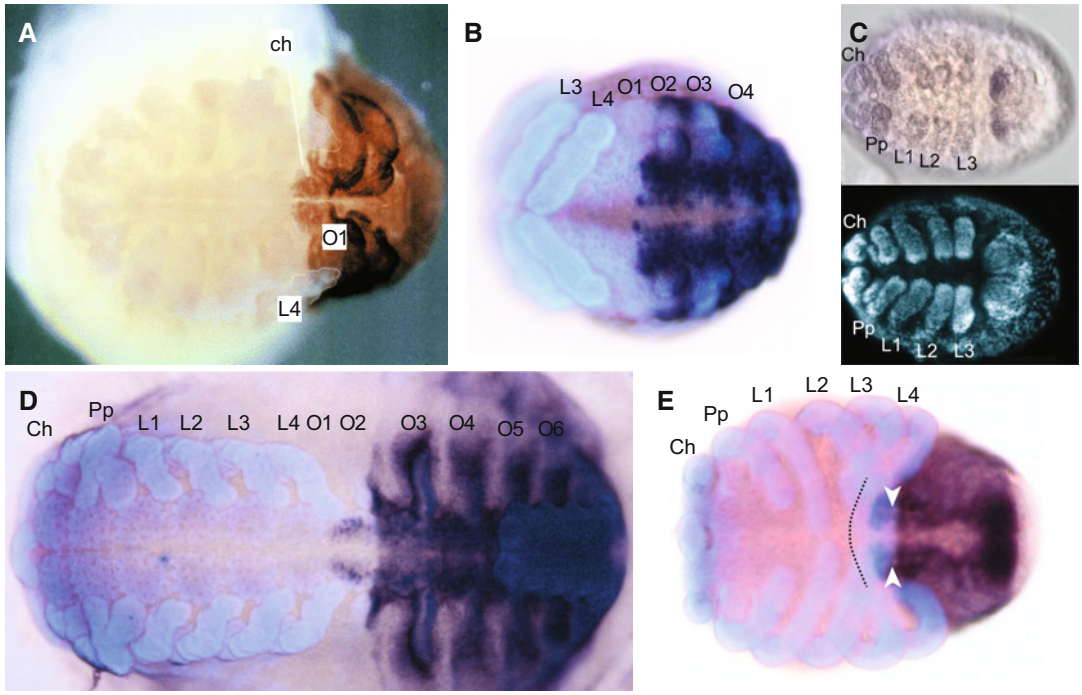

Fig. 5.7 Expression of *Ubx* in chelicerate embryos. (**A**) In the horseshoe crab *Limulus polyphemus*, Ubx/abd-A antibody staining is initially observed in O2 and all segments more posterior. In the later stage shown, it extends more anteriorly into the medial portion of O1 (the chilarial segment) (Slightly modified and reproduced from Popadic and Nagy (2001) with permission from John Wiley and Sons). (**B**) Expression of *Ubx-1* in the spider *Parasteatoda tepidariorum* extends ventrally into the posterior half of O1; otherwise, *Ubx-1* is expressed in O2 and all more posterior segments. (**C**) *Ubx* expression in the mite *Archegozetes longisetosus* (*top*, brightfield image; *bottom*, nuclear staining) is only found in O2 (Image slightly modified reproduced with permission of the authors of Barnett and Thomas (2013a)). (**D**) *Ubx-2* expression in the scorpion *Centruroides sculpturatus* is found in the ventral part of O2 and all segments more posterior. (**E**) In the harvestman *Phalangium opilio*, *Ubx* is expressed in O2 (*arrowheads* indicate the genital pores on O2, *dotted line* demarcates the prosomal/opisthosomal boundary) and all segments posterior to it. All embryos are oriented with anterior to the left. Embryos in (**B**, **D**, **E**) have also been stained with a nuclear dye. *ch* chilaria, *Ch* cheliceral segment, *Pp* pedipalpal segment, *L* walking leg segments, *O* opisthosomal segments

genes in arthropods. However, in *Cupiennius salei*, *proboscipedia*, *Deformed*, *Sex combs reduced*, and *Ultrabithorax* (*Ubx*) have all been found to be duplicated (Damen et al. 1998; Schwager et al. 2007). Furthermore, the paralogs have different spatiotemporal expression patterns, which suggests that there could have been significant duplication and divergence of Hox genes during the evolution of chelicerate body plans (Figs. 5.2; Schwager et al. 2007). Similarly, 19 Hox genes have been reported in the scorpion *Centruroides sculpturatus*, with two copies of each gene except for *Hox3* (Sharma et al. 2014b). Furthermore, different spatiotemporal gene expression patterns were observed for all four paralogous pairs of the opisthosomal Hox genes (*Antennapedia* (*Antp*), *Ubx*, *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*)) (Figs. 5.2 and 5.7; Sharma et al. 2014b). Intriguingly, shifts in anterior boundaries of opisthosomal Hox group paralogs are tightly correlated with shifts in segmental identity in the scorpion mesosoma and metasoma, consistent with the involvement of the paralogs in canonical Hox patterning (Sharma et al. 2014b).

Evolutionary changes to the Hox cluster are also found in the mite *Tetranychus urticae* (Grbic et al. 2011). This species has lost *abd-A* from its

genome (Grbic et al. 2011), which appears to be correlated with drastic reduction of the opisthosoma to only two segments (Fig. 5.2). This possible role of *abd-A* in defining opisthosomal segment number may also be consistent with the finding of a highly divergent *abd-A* in the sea spider and the reduction in size of this tagma in these animals (Manuel et al. 2006).

The expression domains of *Ubx*, *abd-A*, and *Abd-B* have also been found to be important in determining the identity of opisthosomal segments among chelicerates (Fig. 5.2; Damen and Tautz 1999; Popadic and Nagy 2001; Sharma et al. 2012b, 2014c; Barnett and Thomas 2013a). The different anterior expression domains of these Hox genes in harvestmen, scorpions, and spiders are correlated with the position of different segment types, such as book lungs, spinnerets, and the posterior-most undifferentiated segments (Fig. 5.2; Sharma et al. 2012a, 2014b). It therefore appears that the evolution of Hox gene expression is a likely mechanism for the diversification of chelicerates via the modification of posterior segment identity, a hypothesis that is beginning to be tested with functional tools in spiders (Khadjeh et al. 2012).

In addition, analysis of Hox gene expression has played a major role in solving the question of the evolution of arthropod head segments and their associated appendages (Telford and Thomas 1998a; Budd 2002; Maxmen et al. 2005; Scholtz and Edgecombe 2006; Brenneis et al. 2008; Damen 2010). Cheliceres and pedipalps (Fig. 5.2) were thought to be analogous to the intercalary and mandible segments in insects, respectively, due to their supposed innervation from particular regions of the ganglia. It has been further postulated that the segment in chelicerates that is analogous to the first antennal segment in myriapods, crustaceans, and insects has been lost during the course of evolution (Weygoldt 1985; Bitsch and Bitsch 2007). However, studies of Hox gene expression suggest that the segments bearing the cheliceres (and chelifores of pycnogoids) and pedipalps are homologous to the first antennal and intercalary (or second antennal) segments of mandibulates, respectively. Independent corroboration of this hypothesis is provided by the segmental organisation of the tripartite arthropod brain; both the first antennal segment and chelicer al (or cheliforal) segment are innervated by the deutocerebral ganglia (Telford and Thomas 1998a; Jager et al. 2006; Brenneis et al. 2008).

Appendage Development

The prosoma of euchelicerates comprises an evolutionarily conserved tagma, as inferred from segmental distribution of appendage types (Fig. 5.2). In other arthropod subphyla, genes including *Dll*, *homothorax* (*hth*), *extradenticle* (*exd*), and *dachshund* (*dac*) are required for appendage development, and it has been shown that these genes are also necessary for appendage development in chelicerates (Fig. 5.8; Prpic et al. 2001, 2003; Prpic and Damen 2004; Pechmann and Prpic 2009; Barnett and Thomas 2013b; Sharma et al. 2013).

The development of all the appendages requires *Dll*; knockdown of the expression of this gene inhibits outgrowth from limb primordia in, for example, spiders, mites, and harvestmen (Schoppmeier and Damen 2001; Khila and Grbic 2007; Pechmann et al. 2011; Sharma et al. 2013).

During the evolution of cheliceres, it appears that there has been a shift from primitive three-segmented cheliceres in orders like harvestmen, horseshoe crabs, and pycnogonids to the more derived two-segmented cheliceres of lineages like spiders (Sharma et al. 2012a, 2013; Barnett and Thomas 2013a; Brenneis et al. 2013; Brenneis and Scholtz 2014). Interestingly, an expression domain of *dac* in the proximal region of the harvestman *Phalangium opilio* is not found in arachnids that have cheliceres composed of two segments (Fig. 5.8; Sharma et al. 2012a), suggesting a role for this gene in the transition from three- to two-segmented cheliceres. Consistent with this hypothesis, knockdown of the expression of *dac* in *P. opilio* indicates that this gene is required for the development of the proximal cheliceral segment (Sharma et al. 2013). Further corroborating this mechanism, the proximal-most part of the cheliceres of the mite *Archegozetes longisetosus* transiently expresses

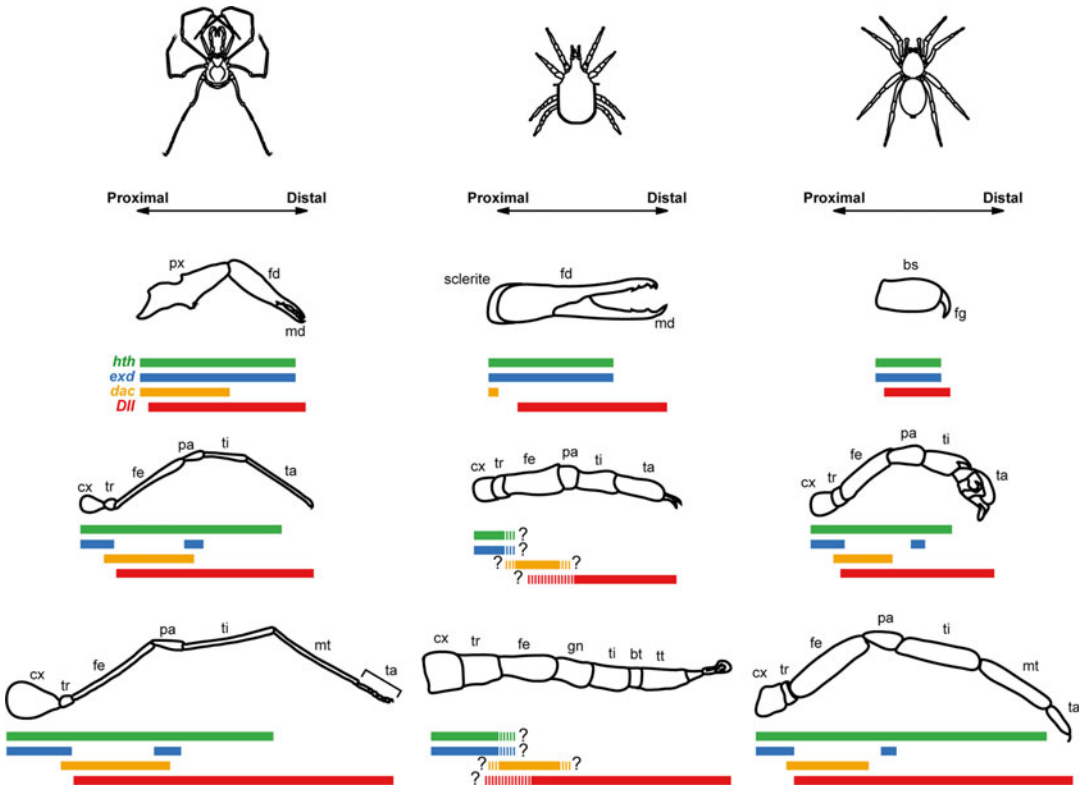

Fig. 5.8 Gene expression during appendage development. Comparative expression patterns of leg gap genes in three chelicerates. From *left to right*: Opiliones, Acariformes, and Araneae. Appendage types from *top* to *bottom* are chelicera, pedipalp, and walking leg. *Coloured bars* indicate expression domains of *homothorax* (*green*), *extradenticle* (*blue*), *dachshund* (*orange*), and *Distal-less* (*red*). Hashed bars in Acariformes indicate uncertainty of expression boundaries with respect to podomeres. *2nd* secondary article, *bs* basis, *bt* basitarsus, *cx* coxa, *fe* femur, *fg* fang, *gn* genu, *ma* mobile article, *mt* metatarsus, *pa* patella, *px* proximal segment, *ta* tarsus, *ti* tibia, *tr* trochanter, *tt* telotarsus (© Prashant P. Sharma, 2015. All Rights Reserved)

dac (Fig. 5.8; Barnett and Thomas 2013b). Accordingly, adults of many Acariformes form a sclerite in this region whose segmental nature had been debated, and *dac* expression in mite embryos suggests that this sclerite is a vestige of the fully formed proximal segment of groups like Opiliones and Xiphosura (Sharma et al. 2012a, 2013; Barnett and Thomas 2013b).

A separate aspect of the appendages that is evolutionarily labile and functionally significant to feeding in chelicerates is the gnathobases (endites), a separate ramus of the chelicerate appendage (Boxshall 2004). A variable number of gnathobases occurs across Chelicerata, and these structures have played a key role in morphological phylogenetic hypotheses of the group (Shultz 2007). For example, outgrowths of a single appendage pair, the pedipalpal gnathobases, form the "maxilla" of spiders (not homologous to the maxillae of mandibulates). Gnathobases of the same appendage pair form part of the subcapitulum of mites and ticks and putatively unite these as "Acari". In groups like Opiliones and scorpions, additional gnathobases occur on the walking leg segments; these fuse to form the preoral chamber, a structure that has putatively united harvestmen and scorpions in morphological phylogenies (Shultz 1990, 2007). In spiders (both araneomorphs and mygalomorphs), mites, and harvestmen, all outgrown gnathobases strongly express *Dll*, and knockdown of *Dll* expression results in the loss of these structures, together

with the distal telopod segments (Schoppmeier and Damen 2001; Khila and Grbic 2007; Sharma et al. 2013). These data suggest a common, but unknown, developmental patterning mechanism underlying morphogenesis of feeding structures derived from gnathobasis outgrowths.

Neurogenesis

Research on gene expression during neurogenesis in chelicerates has made an important contribution to our understanding of the evolution and development of arthropods more generally (Stollewerk and Chipman 2006). In arthropods the *achaete-scute* complex is important in the early stages of neurogenesis. It has been shown that the spider homolog *ASH1* has a similar function during the formation of neural precursor cells to that of crustaceans and insects (Doeffinger et al. 2010). High levels of *ASH1* expression induce the later invagination of regions to form the optic lobes, mushroom bodies, and arcuate body (Doeffinger et al. 2010).

Furthermore, Delta-Notch signalling determines neuronal precursor number through lateral inhibition across arthropod lineages (Stollewerk 2002), and the genes that are responsible for patterning neural fates are also somewhat conserved. For example, *en* (Doeffinger et al. 2010) and *gooseberry* (Jarvis et al. 2012) are involved in organising the neuroectoderm in chelicerates and mandibulates. Interestingly, alterations in Hox expression in the developing nervous system are also correlated with changes in Hox expression across the AP axis. These changes in both neurology and segment morphology due to Hox genes may help appendages to evolve sensory functionality (Jarvis et al. 2012).

Although some genes have conserved roles during neurogenesis, the function of others has diverged. For example, in the spiders *Cupiennius salei* and *Parasteatoda tepidariorum*, Netrins have been shown to have elements of conserved function in commissural axon guidance in the ventral midline with respect to insects and crustaceans (Linne and Stollewerk 2011). However, in *C. salei*, Netrins may also contribute to the correct differentiation of the axonal scaffold through maintaining short-range adhesive interactions between sheath cells and neural precursor cells (Linne and Stollewerk 2011).

Another gene that has diverged in function is *single-minded* (*sim*). In crustaceans and insects, *sim* functions as an important regulator of ventral midline development (Nambu et al. 1990, 1991; Vargas-Vila et al. 2010). This is in contrast to chelicerates, where *sim* is expressed in the median region of the ventral neuroectoderm and is not required for ventral midline development (Linne et al. 2012). It has been hypothesised that the midline precursors seen in crustaceans and insects evolved from an ancestral median area of ventral neuroectoderm. The modification of *sim* expression from the median to the midline tissue could be responsible for this change (Linne et al. 2012).

Therefore, while considerable progress has been made on understanding the evolution and regulation of neurogenesis in chelicerates, it is clear that further insights into the evolution of neurogenesis will be gained through investigation of gene expression and function in non-arachnid chelicerates like pycnogonids and horseshoe crabs (Brenneis et al. 2013; Brenneis and Scholtz 2014).

FUTURE RESEARCH FOCI FOR CHELICERATE EVODEVO

Many important questions in evolutionary biology can be uniquely addressed through evolutionary developmental study of Chelicerata, both via comparisons within chelicerates and between Chelicerata and other metazoans. Key processes that can only be deciphered through studies of chelicerates include the genetic basis for the synthesis of diverse and potent venoms (e.g., scorpion and spider venoms), the diversification of silk genes, and the evolution of terrestrialisation.

Newly sequenced genomes of non-developmental models have provided much needed insights as to genomic architecture and gene family diversification in notable chelicerate

groups (Table 5.1). Developmental studies using established chelicerate models, such as the spider *Parasteatoda tepidariorum*, can be expanded by availability of genomic resources (Posnien et al. 2014). This is complemented by the rapid dissemination of developmental transcriptomes and modern developmental techniques for satellite models such as the harvestman *Phalangium opilio* (Sharma et al. 2012a, 2013) and the scorpion *Centruroides sculpturatus* (Sharma et al. 2014b, c), coupled with refined understanding of phylogenetic relationships (Regier et al. 2010; Sharma et al. 2014a).

However, establishing laboratory cultures in concert with further development of gene expression and functional techniques in exemplars of other chelicerate orders would be insightful for a number of questions. For example, understanding the evolution and development of the specialised appendages (e.g., chelate pedipalps of pseudoscorpions and scorpions; antenniform legs of whip scorpions and whip spiders; sexually dimorphic appendages for sperm transfer in spiders and Ricinulei) could have a great impact on our understanding of origins of morphological novelties and diversity in arthropod appendages.

In the following, two examples of important evolutionary processes whose investigation requires the study of chelicerates are highlighted.

Terrestrialisation

Numerous selective pressures are proposed to have driven the ancestrally aquatic arthropods to adapt to terrestrial habitats (Little 2009). Modern phylogenomic assessments of arthropod relationships indicate multiple terrestrialisation events in the arthropod tree of life, particularly in Mandibulata (e.g., Hexapoda, Myriapoda, some lineages of malacostracan crustaceans). In Chelicerata, the earliest records of marine lineages are Cambrian fossil Pycnogonida, whereas horseshoe crabs and other extinct marine orders (Eurypterida and Chasmataspidida) were present by the Ordovician (Dunlop 2010). Nearly all arachnid orders are present in the fossil record by the Carboniferous (Petrunkevitch 1955; Selden et al. 1991; Dunlop 2010).

A scenario for chelicerate terrestrialisation is contentious. Some researchers have supported a single terrestrialisation event in the ancestor of a monophyletic Arachnida, based on morphology and/or the inferred improbability of terrestrialisation events (Scholtz and Kamenz 2006; Shultz 2007). Others have proposed an independent colonisation of land by scorpions, based on the interpretation of a marine (or at least aquatic) habitat of Palaeozoic scorpion fossils (Jeram 1997; Dunlop and Braddy 2001). At the core of the dispute is marked character conflict within both morphological and molecular phylogenetic datasets and the ensuing elusiveness of a robust chelicerate tree of life (Shultz 2007; Regier et al. 2010). However, there is now strong support for a single origin of the arachnid book lung due to the phylogenetic placement of scorpions as sister group to tetrapulmonates (Sharma et al. 2014a). Separately, the inference of multiple terrestrialisation events in mandibulate arthropods and concomitantly, of morphological convergence driven by terrestrial habitat (e.g., independent origins of tubular tracheae and Malpighian tubules in insects and myriapods), is now robustly supported by phylogenomic analyses. These discoveries discredit an argument for a single terrestrialisation event in the arachnid ancestor grounded on the assumption that terrestrialisation (and ensuing convergence in arthropods) is a historically rare or improbable event (reviewed by Shultz 2007; Sharma et al. 2014a).

Morphological and developmental comparison of book gills in Xiphosura and book lungs in Tetrapulmonata underlie the widespread view that book lungs developed from book gills via internalisation (Lankester 1881; Purcell 1909; Kamenz et al. 2005; Scholtz and Kamenz 2006; Farley 2010). The serial homology of the two appendage types is compelling (but see Dunlop 1997), but has yet not been demonstrated in the context of developmental genetics. Intriguingly, one previous study has suggested that book gills, both respiratory organ types of derived spiders (book lungs and tubular tracheae), as well as spider spinnerets and insect wings were all serial

homologs of crustacean gills, inasmuch as all of these originated from epipods (Damen et al. 2002). This argument, first made in support of a serial homology of insect wings and crustacean gills, was based on the differential expression of *pdm/nubbin* and *apterous* (*ap*); a solid expression domain of both genes is observed in the epipods of a fruit fly and a crustacean (wings and gills, respectively), whereas one or more rings of weak expression are observed in the distal endopods (legs) of the corresponding appendages (Averof and Cohen 1997). The similarity of expression patterns was the basis of the homology statement. Subsequently, Damen et al. (2002) showed that strong expression of *pdm/nub* and *ap* is observed in the book gills of *Limulus polyphemus*, as well as in the respiratory organs and spinnerets of the spider *Cupiennius salei*.

However, the inference that the respiratory organs of spiders originated as epipods is inconsistent with the recent functional work of Khadjeh et al. (2012), which demonstrated homeotic transformation of the book lungs to walking leg-like limb buds upon Hox gene knockdown, suggesting that book lungs (and possibly tubular tracheae) are derived from endopods. While no functional work has been conducted on spinneret development, the spinnerets of many basally branching spiders are also directly comparable to chelicerate endopods (e.g., walking legs) in that they can be segmented and leg-like in adults, and express all leg gap genes embryonically (Pechmann and Prpic 2009). One possible explanation is that *pdm/nub* is not a reliable and/or conserved marker for distinguishing endopods and epipods in chelicerates. Indeed, Damen et al. (2002) observed stronger expression of *pdm/nub* throughout the developing legs (endopods) of *Cupiennius salei* than had been observed in insect or crustacean legs, which questions the utility of this marker for discerning appendage rami in arachnids based on strength of expression level alone. While expression of one of the two spider *ap* paralogs seems to be consistent with the position of vestigial epipods (*ap-1* is expressed dorsally to the walking legs in later stages of *Cupiennius salei*), the fossil record of chelicerates reveals that biramous chelicerates bore exopods in this part of the body, not epipods (Boxshall 2004; Briggs et al. 2012). Together with documented homoplasy of certain genes' expression patterns (Janssen et al. 2011; Sharma et al. 2014c), these results indicate that the exact serial homology between the respiratory organs and prosomal appendages of chelicerates is not sufficiently clear at present.

Beyond these studies, essentially nothing is known about the genetic patterning of the book gills and book lungs, the development of chelicerate tubular tracheae, or the relationship between the tracheae of apulmonate arachnids and derived spiders. Therefore, two key experiments must be conducted towards understanding the evolution of respiratory systems in Chelicerata with existing EvoDevo resources. First, a double knockdown of the Hox genes *abd-A* and *abd-B* must be conducted in a spider and in an apulmonate arachnid (e.g., *Phalangium opilio*) to test the serial homology of the respiratory organs and the prosomal endopods (i.e., legs) of these groups, with the prediction that both respiratory organ types of these arachnids should be homeotically transformed to legs if they are serially homologous to prosomal endopods and to each other. Second, the function of *pdm/nub* and *ap* must be characterised in the spider, to assess the alternative hypothesis of an epipodal origin of respiratory organs and spinnerets. If this hypothesised homology statement was true (*sensu* Damen et al. 2002), then knockdown of *pdm/nub* should severely affect the development of the book lungs, tubular tracheae, and spinnerets, but only the segmentation of the prosomal appendages. This result would support the proposed homology to epipods, given that loss-of-function mutations of *pdm/nub* in *Drosophila melanogaster* result in loss of wing structures (Ng et al. 1995).

Evolution of the Spider Spinning Apparatus and Silk

Two minor orders of chelicerates produce silk, namely, some mites and pseudoscorpions, which utilise silks for tasks such as dispersal, protecting

eggs, and lining burrows (Beccaloni 2009). However, the most familiar silk-producing chelicerates are of course the spiders. Spiders produce diverse types of silk, which has greatly contributed to their successful adaption to different environments (Brunetta and Craig 2010). Spiders use silk to make cocoons to encase eggs and to build different types of webs (e.g., tube-, orb-, or wheel-shaped webs) as hiding places, to capture prey, and even as support for their respiration under water, as in the case of air bells of aquatic spiders (Brunetta and Craig 2010; Foelix 2010).

In the course of adapting to different environments, spiders have evolved morphological differences in their spinning apparatus and a great diversity in silk proteins within and between species (Marples 1967; Gatesy et al. 2001; Challis et al. 2006). The silk-producing organ of all spiders consists of the internal silk glands and the exterior spinnerets, but varies in number and composition between species.

Spider silk consists of fibrous proteins, which is stored in the silk glands in its liquid form and becomes solid through shearing upon excretion (Craig 1997). For various purposes, spiders can produce silks with distinct characteristics from different types of silk glands, which differ in morphology and function (Brunetta and Craig 2010). The simplest silk glands can be found in Orthognatha, whereas at least four different gland types occur in Ctenidae and up to eight distinctive types are present in Orbiculariae (Peters 1955; Mullen 1969; Palmer et al. 1982).

In most spiders, the spinnerets are located at the posterior end of the ventral side of the opisthosoma and consist of a varying number of spinneret pairs with various spatial arrangements (Marples 1967; Shultz 1987). Mesothelae exhibit four pairs of spinnerets, which is considered the "primitive" state. The more derived Orthognatha bear two to three pairs and some labidognathous spiders have two pairs of spinnerets, but additionally exhibit a specialised spinning structure, the cribellum (Shultz 1987). The spinnerets are covered with hairlike structures, the spigots, which are openings to the ducts that connect with the silk glands in the abdomen (Marples 1967).

Both the complexity of the spinning apparatus and the diverse composition of silks prompt questions regarding the evolutionary origins of the morphological and molecular apparatus underlying web spinning, with the corollary of the basis for spider web diversity. Different scenarios for the evolution of spigots and silk glands in spiders have been proposed. Some have argued that the silk glands evolved from a secretory organ, the coxal gland, on a modified leg segment and that the spigots derived from simple hair structures (Bristowe 1932; Butt and Taylor 1991). Another hypothesis proposes that spigots are modified sensory hairs, rather than simple hairs (Palmer 1991). Independently, it has been suggested that silk glands developed from epidermal invagination events, comparable to the male genital glands (epiandrous glands) (Palmer 1991; Craig 1997). Hypotheses grounded in such morphological studies are anticipated to be greatly informed by the advent of molecular and developmental genetic approaches. At present, comparatively little is understood about the genetic basis for spinneret and spigot development, whereas recent and redoubled efforts are shedding light on the characterisation of spider silk genes (Hayashi and Lewis 1998, 2000; Hayashi et al. 1999; Ayoub et al. 2007, 2013; Garb et al. 2010; Clarke et al. 2014; Sanggaard et al. 2014).

To elucidate the evolutionary rise of spinneret, silk gland, and silk protein diversity, these efforts should be complemented by comparative morphological, phylogenetic, and developmental studies, in tandem with comparative genetic and biochemical analysis of silk proteins. Such an integrative and cross-disciplinary pursuit is anticipated to inform understanding of spider diversification, as well as key innovations in evolution, more broadly.

In addition to the examples of terrestrialisation and silk production outlined above, there are several important open questions that can be addressed by future studies of chelicerates in comparison to those of other metazoans to provide new insights into evolutionary developmental biology. Some of these open questions are highlighted below, but this list is by no means exhaustive.

OPEN QUESTIONS

- How are book lungs, book gills, and tracheae patterned in the different chelicerate orders?
- What is the genetic basis for appendage diversity across Chelicerata and how is each appendage type specified?
- What is the genetic basis for sexual dimorphism in Chelicerata, and is this mechanism homologous to its mandibulate equivalent?
- How does the visual system develop, and what is the developmental genetic relationship between faceted eyes (Xiphosura only), lateral eyes (most arachnids), and median ocelli (all Chelicerata)?
- How is the development of the digestive system regulated in chelicerates?
- When during their development do chelicerates other than spiders and mites specify germ cells and which molecular mechanisms do they employ?
- How is the formation of the SAZ regulated and how are new segments generated from this tissue?
- Besides *hb* and *Dll*, which other factors are required for segmentation of the prosoma?

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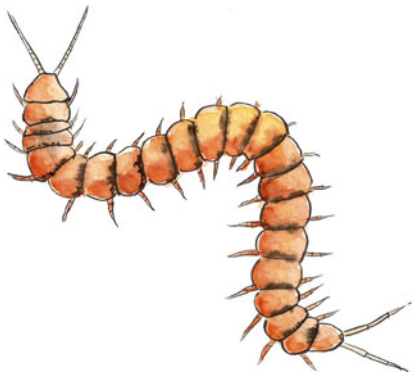
Myriapoda

6

Carlo Brena



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INTRODUCTION

Arthropods are composed of four major living groups, Chelicerata (Chapter 5), Myriapoda (herein), Hexapoda (Vol. 5), and the probably paraphyletic crustaceans (Vol. 4; see, e.g., Edgecombe 2010; Regier et al. 2010; Giribet and Edgecombe 2013). All recent molecular – but also morphological – phylogenies seem to show a strong support for a Hexapoda + crustacean clade (Tetraconata or Pancrustacea), with chelicerates never appearing as their sister group. The position of the myriapods is still not very strongly supported, and in the recent past some molecular phylogenies have grouped myriapods with chelicerates (e.g., Kusche and Burmester 2001; Mallatt et al. 2004; Pisani et al. 2004). Nevertheless, larger data sets and recognition of problematic issues like long branch attraction show a much stronger support for Mandibulata, where myriapods are sister group of the Pancrustacea. The support for this clade derives from molecular data (e.g., Kusche et al. 2003; Rota-Stabelli and Telford 2008; Regier et al. 2010; Rota-Stabelli et al. 2011, 2013; Rehm et al. 2014), development and gene expression data (e.g., Harzsch et al. 2007; Sharma et al. 2014), and morphological data (e.g., Harzsch 2004; Harzsch et al. 2005; Müller et al. 2007; Sombke et al. 2012; see also Giribet and Edgecombe 2013 for a general review).

According to the Mandibulata hypothesis, myriapods are particularly useful as an outgroup to polarise the character evolution – developmental or not – within Pancrustacea due to their basal position within Arthropoda and are thus pivotal for resolving any evolutionary scenario within this highly successful phylum (see, e.g., Saadaoui et al. 2011; Brites et al. 2013 but also discussion in Brenneis et al. 2013).

For this kind of evolutionary analysis, myriapods are not only particularly important due to their phylogenetic position but also because they have retained a rather primitive body plan, likely more ancestor-like than many other arthropods. Indeed, Myriapoda is the single major clade with no differentiation of the trunk in major components (tagmata, as, e.g., thorax and abdomen of insects). In particular, contrary to most arthropods, myriapods are characterised by a relatively uniform (homonomous) series of trunk segments, not significantly fused or diversified during development and still evident on the adult exoskeleton.

Indeed, in almost no other arthropod, the representation of reiterated morphological units along the anterior-posterior (AP) axis of the animal is as clear as in myriapods, which makes them particularly interesting for EvoDevo studies, as segmentation is a key character of several animal phyla, particularly the arthropods but also the annelids.

The apparent primitiveness of myriapods is marked also by the strong conservation of their body plan, which, within each of their four classes, does not exhibit the same level of diversification shown by most arthropod clades (see Fig. 6.1). This is also true across geological time since the very first time they appeared on land. Indeed, although the marine ancestor of myriapods is still unknown (but see Budd et al. 2001 for an upper Cambrian marine arthropod with some significant myriapodan feature), myriapods are among the very first Metazoa that appeared on land in the middle Silurian (millipedes, and late Silurian, centipedes) (Shear and Edgecombe 2010). Indeed, given the ancestral nature of their body plan, using data about their development may be the best choice to speculate about development modality in extinct groups like the trilobites (Ortega-Hernández and Brena 2012).

This morphological primitiveness is also mirrored at the genomic level, as the genome of the centipede *Strigamia maritima*, the only sequenced myriapod genome to date, shows several ancestor-like characters and conservation not seen in any other arthropod genomes (Chipman et al. 2014). For example, as far as developmental genes are concerned, the Hox cluster retains linkage to one ortholog of *evx/even skipped*, as it does in some chordates and cnidarians.

Independently from their position within the arthropod tree, the myriapods are clearly a well-supported monophyletic clade (Edgecombe

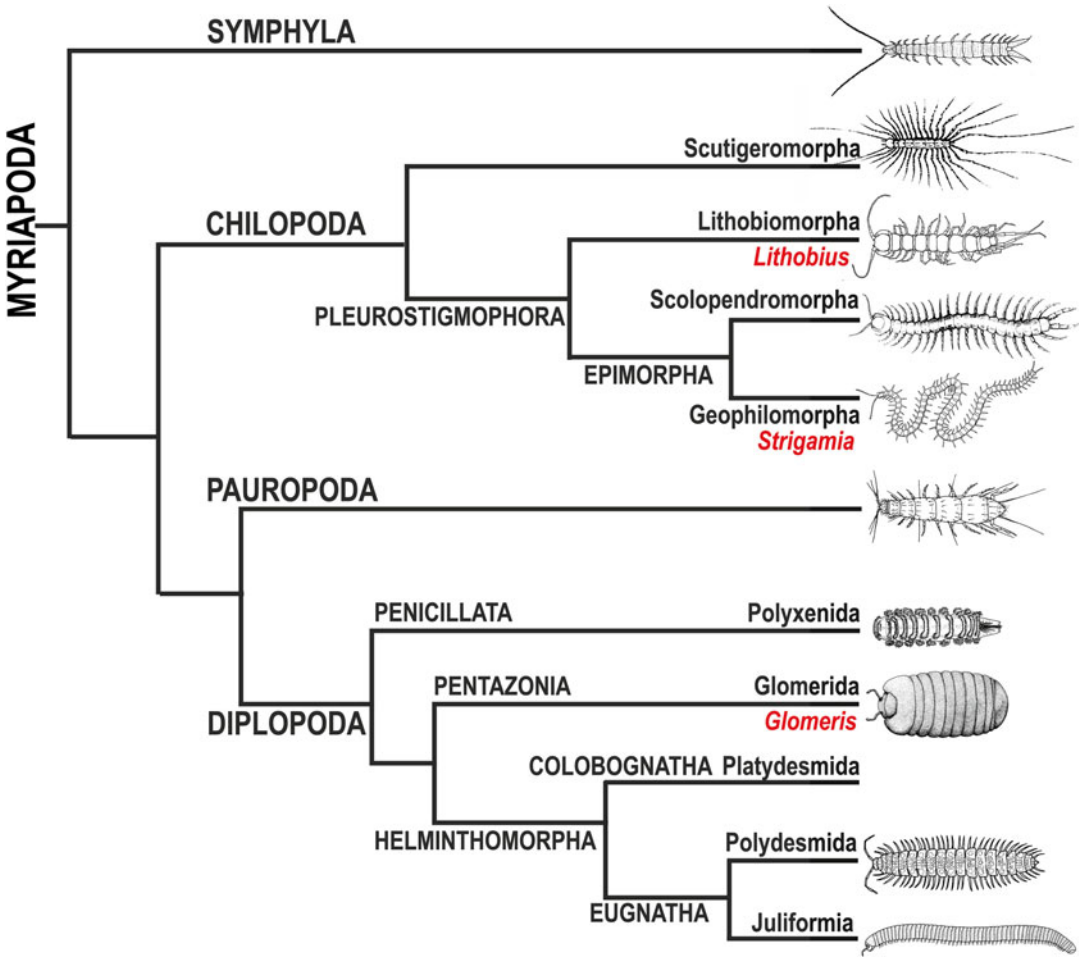


Fig. 6.1 Myriapod phylogeny. Simplified myriapod phylogenetic tree showing the main clades and in particular the ones which include species whose development has been studied. Reported in red are the three genera for which molecular developmental data have been published. While the internal phylogenies as shown here are well supported, the interrelationship between the main four groups is not yet fully resolved (see main text for discussion); the reported one is according to Rehm et al. (2014). Drawings are not to scale: see main text for sizes of different groups (Drawings modified after Newport (1845), Hopkin and Read (1992), Janssen et al. (2006))

2010; Giribet and Edgecombe 2013; Rehm et al. 2014). Myriapods are composed of four major clades: the millipedes (Diplopoda), the centipedes (Chilopoda), the pauropods (Pauropoda), and the symphylans (Symphyla), respectively, composed of 7,753, 3,100, 835, and 197 known species (Fig. 6.1; Zhang 2011). They have single antennae, mandibles, and uniramous legs. They are all terrestrial and, in correlation with this, have tracheae and Malpighian tubules.

Millipedes are slowly moving detritivores, using the mouthparts composed by the mandibles and a gnathochilarium to feed mostly on decaying vegetation (Hopkin and Read 1992). They rely for defence on production of toxins and/or on a strong, often calcified, exoskeleton, which they tend to roll in a planospiral coil, in the case of the longest ones, or in a ball, in the case of the pill millipedes, to protect the more vulnerable ventral side. Animals vary from 2 mm to 30 cm in size, although the Paleozoic 2-m long *Arthropleura* was very likely the most massive land arthropod that ever existed (Shear and Edgecombe 2010).

The head, typically convex, has a pair of antennae with seven to eight articles and, in most cases, simple eyes formed by lateral patches of ocelli. The first few dorsal cuticular plates (the tergites), including the very first one (the collum), correspond to single trunk metameres (see discussion below in section "Segmentation"), but in the following trunk, each dorsal cuticular plate (diplotergite) corresponds to two segments, represented by two pairs of legs, spiracles, ganglia, and ostia; this diplosegmentation is the most prominent feature of the clade. They have a maximum of 375 pairs of legs but may have as little as 11 of them (Enghoff et al. 1993).

Although the 16 orders appear to be monophyletic, the internal phylogeny of diplopods is not yet fully resolved (see, e.g., Shear and Edgecombe 2010; Brewer et al. 2012). Nevertheless, there seems to be a consensus at higher levels, corresponding to the simplified tree represented in Fig. 6.1 (see, e.g., Sierwald and Bond 2007; Shear and Edgecombe 2010; Blanke and Wesener 2014). These major clades somehow represent – or at least include – the four major (eco)morphologies of millipedes: the tiny bristly millipedes (Penicillata), the compact pill millipedes (Pentazonia), the worm-like flat millipedes (Colobognatha), and the elongated Eugnatha which includes the flat-backed Polydesmida and the archetypal, tubular millipedes, the Juliformia.

Pauropods are poorly known, tiny (0.5–1.9 mm) soft-bodied animals living in the forest litter, mostly feeding on fungi or decomposing plants (Fig. 6.1). As diplopods, they are dignathan, with a pair of mandibles and maxillae. The last head segment (the collum, not homologous to the millipede collum) is limbless, mostly developed ventrally. They have peculiar six-articled antennae branching into three flagella. They usually have a trunk with nine leg-bearing segments and a final limbless segment, and, except the first, segments with a tergal shield are alternated by segments without (see discussion below in section "Segmentation") (Tiegs 1947a, b).

Symphylans are small (1–8 mm), fast-moving inhabitants of moist soil and leaf litter, omnivorous but mostly feeding on roots or rotten vegetation (Fig. 6.1). They have three pairs of mouth parts as the centipedes but with the second maxillae fused to form a labium. The head has a pair of moniliform elongated antennae and, uniquely among terrestrial arthropods, the only two spiracles openings. All symphylans have 14 trunk segments, with legs only on the first 12 and spinnerets and trichobothria on the 13th and 14th segments, respectively, but they have a variable supernumerary number of tergites or scuta (15–24) (see discussion below in section "Segmentation"). Neither symphylans nor pauropods have eyes, and according to Shear and Edgecombe (2010), no reliable internal phylogenies have been proposed for these two groups.

Diplopods, pauropods, and symphylans are all progoneate, with the genital opening in a ventral position on the anterior trunk (respectively behind the second leg pair, between the third leg pair, and between the fourth leg pairs).

Centipedes are the only predatory myriapods, thanks to their main apomorphy, the forcipules, the first trunk limbs modified in venom injecting, pincer-like appendages; they can be used also to capture small vertebrates and as a means of defence (Lewis 1981; Edgecombe and Giribet 2007; Minelli 2011). They are trignathan, with mandibles and two pairs of separated maxillae. The internal phylogeny is rather well supported from both morphology and molecular data (Edgecombe and Giribet 2007; Shear and Edgecombe 2010), and from the more basal to the more derived, the four main orders (Craterostigmomorpha are represented only by a single species from Tasmania and New Zealand) represent four well-defined ecomorphotypes, somehow defining a continuum, from the long-legged, very fast house centipede (Scutigeromorpha) to the slower rock-crawling garden centipede (Lithobiomorpha), to the longer, more sinuous Scolopendromorpha, and to the extremely elongated leaf litter- and earth-dwelling Geophilomorpha (Fig. 6.1).

Ranging in length from around 1 cm to 30 cm in the larger tropical *Scolopendra*, centipedes have from 15 to 191 leg-bearing segments (value above 23 are almost exclusive of the geophilomorphs), and this number is always odd, notwith-

standing the high variability, even intraspecific, of this number that characterises most of the species with more than 27 leg-bearing segments (Minelli et al. 2000).

The phylogenetic relationship between myriapod classes has been a matter of controversy, and every combination has been proposed. Morphological data have traditionally supported the relationship with Chilopoda as sister group of Progoneata and Symphyla sister group of Dignatha (Pauropoda + Diplopoda) (Edgecombe and Giribet 2007; Shear and Edgecombe 2010). Up to now molecular data had lower support for alternative trees, but recent papers seem to show a stronger support for a basal position of symphylans (Miyazawa et al. 2014; Rehm et al. 2014), as shown in Fig. 6.1, and even a lower support for a sister group relationship between diplopods and chilopods (Miyazawa et al. 2014).

Diplopods, in particular, have an important ecological role in recycling plant debris, and both of them and the centipedes can be used as bioindicators (da Silva Souza et al. 2013; Gerlach et al. 2013). The economical impact of myriapods is rather limited, if not in specific environments, where symphylans like *Scutigerella immaculata* can be a pest, in particular in greenhouses.

The main myriapod impact may rely in the future in the great pharmacological potential of the apparently very specific centipede venom enzymes which have myotoxic, cardiotoxic, and neurotoxic activities; valuable chemical interest may arise also from the quinones and cyanide used by millipedes to repel predators (Undheim and King 2011; Haddad Jr et al. 2012; Liu et al. 2012).

EARLY DEVELOPMENT

Myriapods are animals in general difficult to handle and to keep in culture, and basic developmental techniques used in other arthropods, like, for example, live observations, egg injections, or cell marking, have proven to be extremely limited in the few available species. Most of what we know about myriapod descriptive development is based on rather old literature and on a very limited number of papers: the reader should be rather cautious in both taking for proven facts that are reported therein, in particular considering the technical limits of the time, and generalising to all classes what was shown for few single species.

All myriapod eggs are spherical, protected by a single membrane, the chorion, which tends to be rather permeable. The humidity of the egg is maintained thanks to enclosed brood chambers and maternal care (scolopendromorph and geophilomorph centipedes) and to the deposition deep in the soil (e.g., the millipede *Ommatoiulus*) or thanks to a mud cover or pellet including the egg (e.g., the centipede *Lithobius* and the millipede *Glomeris*) (Brena, personal observations). The eggs of centipedes are in general around 1–2 mm but can be larger, in particular in large tropical *Scolopendra*, but tend to be smaller in diplopods, from around 0.7 to 1.3 mm (these are actually the extremes of the centipede *Strigamia maritima*; Brena and Akam 2012). The symphylan *Hanseniella* has a 0.37 mm egg, and *Pauropus* has the smallest egg with 0.11 mm (Anderson 1973).

Cleavage and Blastoderm Formation

In myriapods the first subdivision of the zygote (cleavage of the egg) can be either total (or holoblastic), involving the whole egg, or intralecithal (or superficial, a kind of meroblastic cleavage), where the nuclear division is not followed by cytokinesis, but the cleavage products are represented by energids, nuclei surrounded by cytoplasm immersed in the yolk; this system, for example, is typical of most insects and chelicerates (Fig. 6.2; see also Chapter 5; Vol. 5 and Scholtz and Wolff 2013 for a general discussion on the terminology). Both systems are present in diplopods, where cleavage patterns can vary even within the same order (see below).

Cleavage is total in the two described species of pauropods and symphylans (Tiegs 1940, 1947b) and in a number of diplopod species belonging to Polyxenida, Glomerida, Polydesmida, and Juliformia (Fig. 6.2; Metschnikoff 1874; Lignau

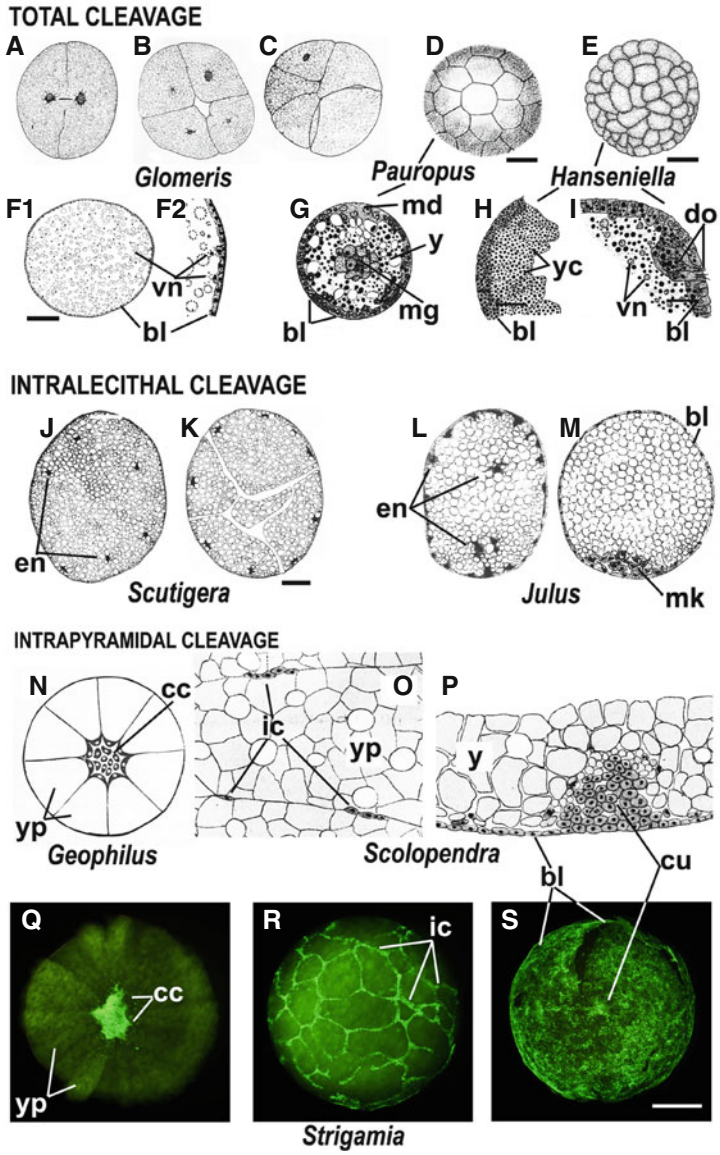


Fig. 6.2 Cleavage and blastoderm formation. Main cleavage modes: total cleavage (**A**–**E**) as exemplified by the millipede *Glomeris marginata* (**A**–**C**), the pauropod *Pauropus silvaticus* (**D**), and the symphylan *Hanseniella agilis* (**E**) and intralecithal cleavage (**J**–**L**), as exemplified by the centipede *Scutigera coleoptrata* (**J**, **K**) and the millipede *Julus terrestris* (L), with the intrapyramidal cleavage specific of more derived centipedes as *Scolopendra cingulata* (O), *Geophilus* (N), and *Strigamia maritima* (**Q**, **R**), where cleavage cells (cc) divide at the centre of the egg before migrating as intercalating cells (ic) to the periphery along the interpyramidal spaces. See main text for details. All cleavage modes lead to the formation of a blastoderm, initially uniform (F, *Glomeris* and H, *Hanseniella*) later to be differentiated (G, *Pauropus*, I, *Hanseniella*), often beginning with a first ingression of a cluster of cells (M, *Julus* and cumulus in P, *Scolopendra*, and S, *Strigamia*). (Reported stages are (**A**) two blastomeres, (**B**) 4 blastomeres, (**C**) 8 blastomeres, (**D**) about 40 cells, (**E**) 100 cells, (**F**, **H**) early blastoderm (**H**: 120 cells), (**G**, **I**) differentiated blastoderm, (**J**) 60 cells, (**K**) 150 cells with yolk pyramids, (**L**) early and (**M**) late stages of blastoderm formation, (**Q**) stage 1.2, (**R**) stage 1.4, (**S**) stage 2.1.) **F2** is a magnified section of **F1**. bl blastoderm, do dorsal organ, cc cleavage cells, cu cumulus, en energids ("segmentation masses" in Heathcote 1886), ic intercalary cells, md membrana dorsalis, mg midgut rudiment, mk mesodermal keel, vn vitellophage nuclei, y yolk, yc yolk cells, yp yolk pyramids. Scale bars: (**D**) 30 µm, (**E**) 100 µm, (**F1**) 200 µm, (**J**, **K**) approx. 300 µm; all based on egg size as reported in the original publication; (**Q**–**S**) 300 µm (**A**–**C**, **F** modified after Dohle (1964); (**D**, **E**, **G**–**I** after Tiegs (1940, 1947a), in part as modified by Anderson (1973); **J**, **K** after Knoll (1974); **L**, **M** after Heathcote (1886); **N** after Sograff (1883); **O**, **P** after Heymons (1901))

1911; Dohle 1964). Here, the first division splits the egg into two equal blastomeres (Fig. 6.2A). Starting from the second, perpendicular to the first, all following divisions are irregular, eventually giving rise to pyramidal yolky blastomeres radially arranged around a small, central blastocoel, while the spherical outline of the egg is maintained. The nuclei of these blastomeres migrate then towards the periphery of the egg. After a certain amount of mitoses, the cells undergo radial division, which eventually, through a slightly different process in the different clades, will produce a superficial uniform and monolayered blastoderm. In *Glomeris*, cell boundaries disappear to produce two populations of energids, one peripheral and one central. The marginal ones will then cut off as cuboidal blastoderm cells, leaving a central nucleate yolk mass (Fig. 6.2F; Dohle 1964). In the symphylan *Hanseniella*, a similar condition is reached without the blastoderm ever losing its cellular condition (Tiegs 1940). On the contrary, in *Pauropus*, the radial division leaves a central anucleate yolk portion of the original radial pyramidal blastomeres, which will eventually fuse to produce an anucleate yolk mass, with only two central yolky cells (Tiegs 1947a).

An intralecithal cleavage has been described in several species of diplopods belonging to the Juliformia and Polydesmida (Heathcote 1886; Cholodkowski 1895; Pflugfelder 1932) and the more basal scutigeromorph centipedes (Knoll 1974), where dividing energids are scattered within the yolk mass and cellularise just before or at the surface when they form the blastoderm (Fig. 6.2J–L).

With the exclusion of scutigeromorphs, the remaining centipedes (the Pleurostigmophora) show a peculiar system of cleavage, apparently a modification of an intralecithal cleavage (but see below) (Fig. 6.2N–O, Q–R). In these centipedes the yolk mass on fixed material appears to be subdivided into very irregular wedges departing from the centre of the egg or close to it. They are traditionally described as "yolk pyramids", although they are considered different from the "yolk pyramids" (total cleavage blastomeres) common to many arthropods, progoneate myriapods included. See Scholtz and Wolff (2013) for a general discussion on the terminology. They have been described in lithobiomorphs (Hertzel 1985), scolopendromorphs (Heymons 1901), and geophilomorphs (Sograff 1883; Brena and Akam 2012), and they are associated with a large number of energids/cells concentrated at the centre of the egg. In the geophilomorph *Strigamia maritima*, Brena and Akam (2012) have shown that these pyramids appear to reach the centre of the egg from the very early stages. It is not yet known whether they are delimited by a cellular membrane, but at least each pyramid is delimited by actin filaments and appears to be associated with the first cell bodies at their internal apex (Brena and Akam 2012) (Heymons (1901) himself interpreted them in *Scolopendra* as of a cellular nature). These data are consistent with the possibility that these chilopod yolk pyramids might indeed be actual blastomeres and that cleavage in *Strigamia* could be in fact at the beginning total. It is definitely clear in *Strigamia* that cellularisation, at least to a great extent, starts early and a population of yolk-free cells is quickly generated by subsequent divisions at the centre of the egg, clearly separated, by now, from the yolk pyramids (Fig. 6.2Q). By the time they reach the number of few thousands, these cells migrate centrifugally towards the surface, moving along the spaces between yolk pyramids. There is no apparent asymmetry in this migration, with the path conditioned by the irregularity of the yolk pyramids, as it is well marked by the irregular polygonal pattern of cells when they first appear at the surface of the yolk (Fig. 6.2R; Sograff 1883; Brena and Akam 2012). Shortly afterwards, cells spread over the surface to form a uniform blastoderm comprising 20–30,000 cells. Some cells interspersed in the yolk, possibly to be interpreted as yolk cells/vitellophages, may be the result of either incomplete migration or delamination from the surface. This whole cleavage process up to the appearance of the cells to the surface is exceptionally long in *Strigamia*, taking up to 46 % of developmental time, much more than the usual 10–20 %, typical, for example, of insects (Brena and Akam 2012).

This peculiar kind of cleavage/cell migration, which could be called "intrapyramidal cleavage", is unique among arthropods and is consistent with what was shown in the other studied Pleurostigmophora (Fig. 6.2N–O; Sograff 1883;

Heymons 1901; Hertzel 1985) and could well be an apomorphy of the group, although maybe with different levels of cellularisation of the migrating cells/energids. It remains an open question whether this peculiar cleavage system is an intralecithal-derived one, as that typology in the scutigeromorph outgroup may suggest, or whether it could be a total cleavage-derived one, a possibility left open by Brena and Akam (2012). Either hypothesis could phylogenetically be sound, considering the scattered distribution of total and intralecithal cleavage systems among diplopods, or the fact that both systems can be present in the same clades, Juliformia and Polydesmida. This implies that it might be relatively easy in evolution to move from one system to the other but, at the same time, makes the myriapod ancestral cleavage system uncertain.

In the progoneate myriapods, the blastoderm secretes a thin and highly resistant blastoderm cuticle under the chorion (Metschnikoff 1874; Tiegs 1940, 1947a; Dohle 1964). On the contrary, there is no such cuticle in chilopods (Anderson 1973) (this has been checked in *Strigamia* by Brena and Akam 2012), although in *Lithobius* there is apparently a temporary cuticle, which disappears during development (Hertzel 1983).

Blastoderm Differentiation and Gastrulation

A monolayered uniform blastoderm, covering the whole egg and, apparently, morphologically fully symmetric, is a common stage in all myriapods. In *Strigamia* this stage has been shown to be very brief (Brena and Akam 2012).

The first sign of differentiation of the blastoderm in progoneates is the thickening and condensation of cells on a portion of the blastoderm, associated with the assumption of columnar form and the proliferation of local cells ("ventral plate" in *Julus*) (Heathcote 1886; Tiegs 1940, 1947a; Dohle 1964). This is in fact the earliest delineation of a germinal area. Its appearance is soon associated with the first stages of gastrulation (in the strict sense of formation of germ layers), hence with the appearance of the first mesodermal cells. In symphylans and pauropods, mesodermal cells, delaminating from the blastoderm, uniformly underlie the central part of the germinal area, but in larger myriapods with many more cells, the internalised cells represent only a small cluster of the cell population, although the relative extension of the differentiated blastoderm and internalised plug of cells may change. In the chilopods *Scutigera* (Knoll 1974), *Scolopendra* (Heymons 1901), and *Strigamia* (Brena and Akam 2012), apparently only a well-defined and small cluster of cells, representing eventually the posterior pole of the animal, constitutes the very first differentiation of the blastoderm (Figs. 6.2P, S and 6.3G); in *Scolopendra dalmatica* (contrary to what happens in *S. cingulata*), this plug of cells appears on the surface of the egg even before the blastoderm itself (Heymons 1901). In *Julus* this cluster of mesodermal cells has been described as "mesodermal keel" (Fig. 6.2M; Heathcote 1886), but in the diplopod *Glomeris* (Fig. 6.3A; Dohle 1964) and the chilopods *Scutigera* (Knoll 1974) and *Scolopendra* (Heymons 1901), the whole plug of condensed cells recognisable from the surface has been called "Keimstelle", "cumulus primitivus", or simply cumulus, for the similarity with the cumulus primitivus of spiders (where this very same structure has been called also "primitive plate", "anterior cumulus", and "primary thickening" (Chaw et al. 2007). It is important to note that the myriapod cumulus (and spiders' "cumulus primitivus") is different from the "cumulus posterior" of spiders (cf. Chapter 5), a small cluster of internalised mesenchyme cells which migrates from this thickening towards the periphery of the germinal disc, thereby defining the major axis of the embryo, but disappears as the germband forms (Akiyama-Oda and Oda 2003, 2006). Unfortunately, this secondary element has been often referred to simply as the "cumulus", particularly in recent papers (e.g., Akiyama-Oda and Oda 2003, 2006; McGregor et al. 2008), but at the moment there is no reason to infer homology between this cumulus (posterior) of spiders and the cumulus of myriapods.

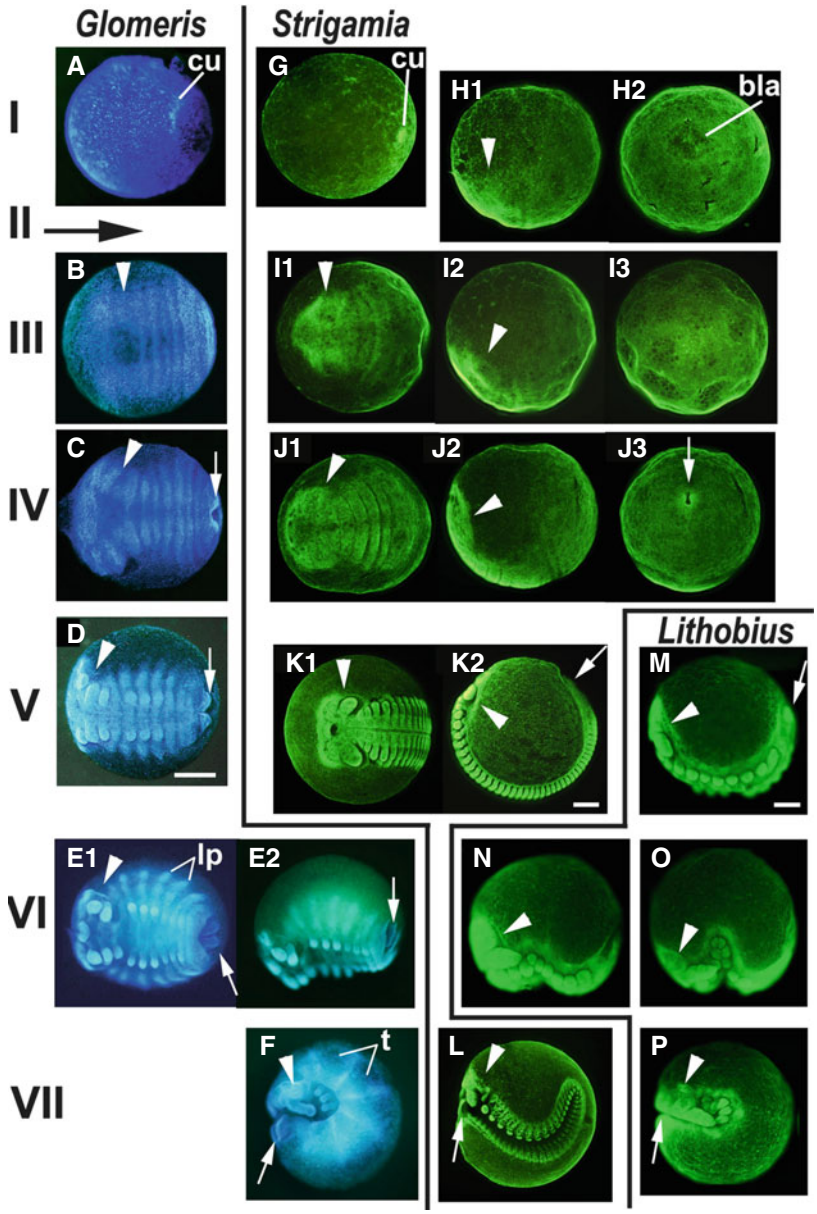


Fig. 6.3 Main phases of embryonic development in millipedes and centipedes beginning with blastoderm differentiation in the millipede *Glomeris* (**A**–**D**: *G. marginata*; **E**, **F**: *G. pustulata*) and the centipedes *Strigamia maritima* (**G**–**L**) and *Lithobius atkinsoni* (**M**–**P**). Embryos are aligned from top to bottom according to major developmental stages, only partially corresponding between the different species and independently from the specific staging system. See main text for details. (I) Early blastoderm differentiation (in A the regio germinalis is already partly formed). (II) Cephalic condensation (preceding the following stages only in *Strigamia*). (III) Delineation of the first segments. (IV) Early germband condensation/extension. (V) Later germband condensation/extension. (VI) Early ventral flexure. (VII) Early (slightly later in *Glomeris*) post-flexure embryos. Panels with the same letter but with sequential numbers are the same embryo viewed from different angles; *Glomeris* (except **E2** and **F**) and *Strigamia* left column, ventral view; *Glomeris* in **E2** and **F**, *Strigamia* central column and *Lithobius*, lateral view; *Strigamia* right column, posterior view. In all embryos, except the ones in posterior view, the anterior/head is to the left. The morphology of the embryos is highlighted by fluorescent nuclear staining (DAPI for *Glomeris*, SYBR Green for *Strigamia*, and Sytox Green for *Lithobius*). bla blastopore, cu cumulus, lp lateral plates, t forming tergites, arrowhead cephalic area/head, arrow proctodeum primordium/anus. Scale bars: 200 µm (**A** modified after Janssen (2012); **B**–**D** modified after Janssen and Damen (2006))

Additionally, a multilayered "cellular mass" has been described in *Scolopocryptops rubiginosus*, where it has been compared in behaviour with the cumulus posterior of spiders, thanks to its backward migration away from proctodeum before its disappearance (Sakuma and Machida 2002, 2003), but in this case the cellular mass is associated with later stages, after the formation of the germband, and there is again no evidence of a possible relation with the cumulus posterior in spiders, as remarked by the same authors (Sakuma and Machida 2003). This kind of late posterior distinct cellular mass was not observed in the geophilomorph *Strigamia* (Brena and Akam 2012; C. Brena, unpublished data).

It is possible that this initial cluster of cells may have an important role in very early blastoderm differentiation and, possibly, in breaking the radial symmetry of the embryo (see below). Certainly it has a precise function in starting the process of ingression of cells to form the internal germ layers, initialising gastrulation. Mesoderm, and later endoderm, formation is not fully understood in myriapods, in particular because published models are based strictly on cell morphology on serial sections and not on cell markers (e.g., Heymons 1901). Classical literature on all myriapods (but also some *Strigamia* preliminary work on expression of the mesoderm determinant gene *twist*; C. Brena and J. Green, unpublished data) seems to show that mesoderm forms both from cell ingression at a specific site (cumulus) at the posterior of the forming germinal area and as diffuse delamination of cells from the whole germinal area of the blastoderm (Heathcote 1886; Pflugfelder 1932; Dohle 1964). In *Julus*, *Hanseniella*, and *Scolopendra*, the mesoderm appears to be arranged in two parallel longitudinal bands under the ventrolateral surface of the embryo (Heymons interpreted them as deriving by cell migration from the cumulus) (Heathcote 1886; Heymons 1901; Tiegs 1940). In *Glomeris* and *Strigamia*, there are no indications of these bands (in *Strigamia*, neither at the gene expression level).

Given this scattered mesoderm ingression, in general a specific blastopore, i.e., a more well-indentified larger area of germ layer ingression, has not been recognised in myriapods (in *Scutigera* Knoll (1974) refers to the whole cumulus area also as a "blastoporus region"). The only exception to date is *Strigamia*, where it has been interpreted as a blastopore a large sub-circular area which remains monolayered and which is at the centre of an extending multilayered part of the blastoderm (Fig. 6.3H2; Brena and Akam 2012). Expression of genes associated with blastopore formation confirms this interpretation (Brena and Green, unpublished). Continuity of morphology and of gene expression in a series of fixed embryos suggests that the blastopore forms adjacent to or incorporates the early cell cluster.

All this initial cell condensation and proliferation and early phase of gastrulation in all myriapods result in a differentiation of the original blastoderm into two main regions. In the diplopods *Glomeris* and *Polyxenus*, the more differentiated ventral area, partly multilayered, is called "regio germinalis" and the undifferentiated dorsal area "regio dorsalis" (Dohle 1964, 1974). The latter in *Julus* has been called "dorsal ectoderm" (Heathcote 1886) and in symphylans, pauropods, and *Scolopendra*, "membrana dorsalis" (see, e.g., Figs. 6.2G and 6.4A, G; Heymons 1901; Tiegs 1940, 1947a).

Particularly in more recent literature, these two differentiated broad areas of the original blastoderm are commonly referred to as "germ disc" (or, before gastrulation, "blastodisc") and "extraembryonic ectoderm" (Anderson 1973), in analogy with the insects. In fact, myriapods do not have extraembryonic epithelial membranes like the insect amnion and serosa, discharged at hatching, and it is not clear how much of the dorsal undifferentiated cells are just co-opted later during dorsal closure (see below). In fact, the limits of the germinal region in these early stages, and more specifically of its multilayered component, are very smooth and not necessarily precisely identifiable (see, e.g., Fig. 6.3H1 and Brena and Akam 2012). In *Strigamia*, for example, several early patterning genes are indeed associated with the whole ectoderm, lacking to mark any clear differentiation between the two broad regions (see section "Gene expression").

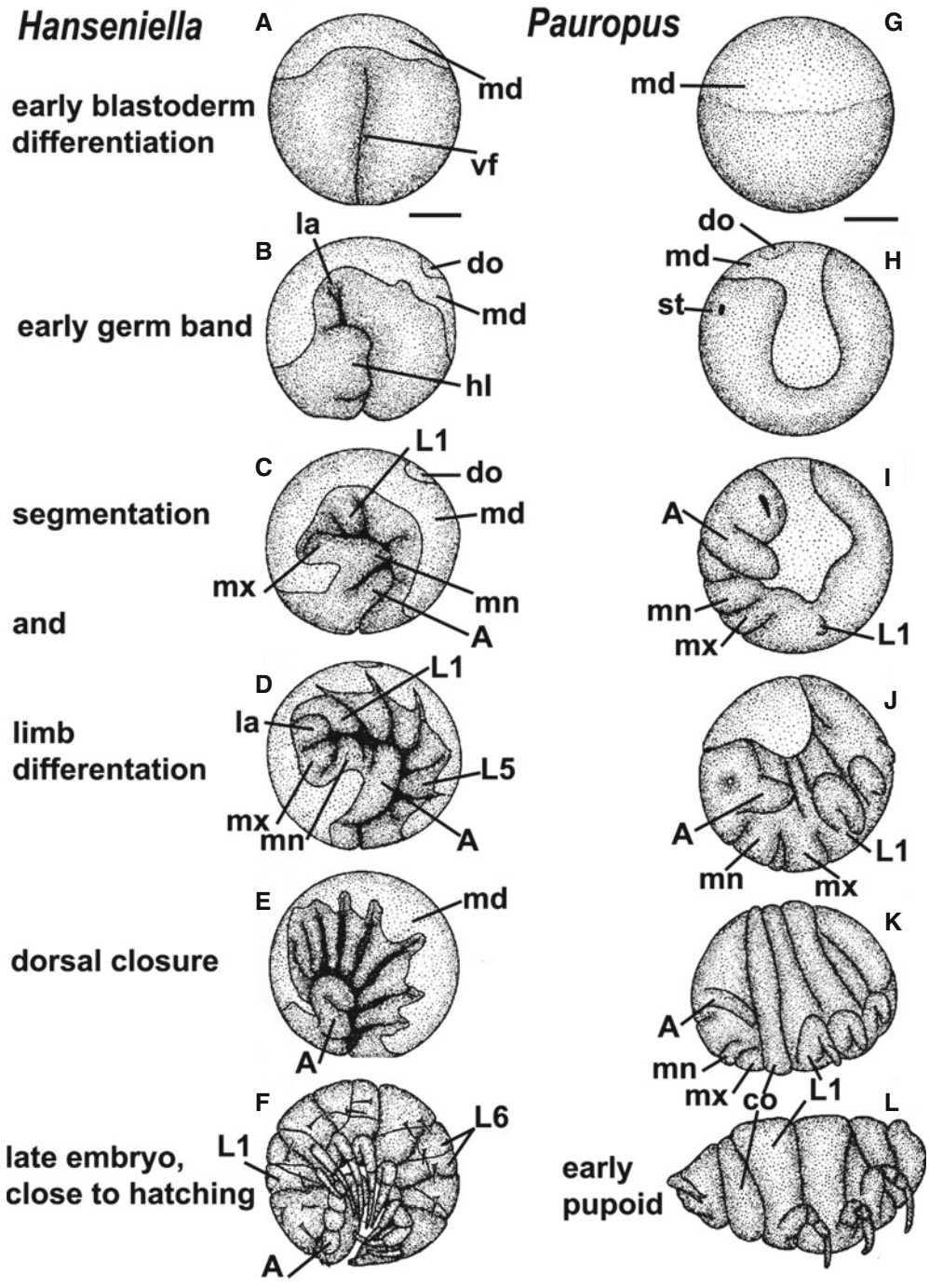


Fig. 6.4 Main phases of embryonic development in symphylans and pauropods. Schematic drawings of the main phases of embryonic development beginning with blastoderm differentiation in the symphylan *Hanseniella agilis* (**A**–**F**) and the pauropod *Pauropus silvaticus* (**G**–**L**). Embryos are aligned from top to bottom according to major developmental stages, as indicated in the figure. All specimens in lateral view with anterior/head to the left. A antenna, co collum, do dorsal organ, hl head lobe, L leg/leg segment, la labium, md membrana dorsalis, mn mandible, mx maxilla, vf incipient ventral flexure. Scale bars: *Hanseniella* 100 µm, *Pauropus* 30 µm (Modified after Tiegs (1940, 1947a), partly as modified by Anderson (1973))

Dorsal Organ

A distinct and relevant structure characterises the apical region of the membrana dorsalis of symphylans and pauropods, where a group of cells (20–30 in *Hanseniella*) enlarge and differentiate (in *Hanseniella* even before the germband has begun to form) into a secreting structure, the dorsal organ (Figs. 6.2I and 6.4B, H; Tiegs 1940, 1947a). In *Hanseniella* it secretes remarkable filamentous threads into the space between the chorion and the blastodermic cuticle, but these extraembryonic filaments are lacking in *Pauropus*. This structure is not present in diplopods and chilopods, but a remarkably similar organ is present in embryos of primitive hexapods (Collembola and Campodea) (Tiegs 1942a, b), although the phylogenetic distance would exclude any homology.

Seifert (1960) on *Polyxenus* and Dohle (1964) on *Glomeris* have revealed a group of cells which become deeper than the rest of the membrana dorsalis ectodermal cells, projecting downward into the yolk mass and whose fate is unknown.

Cephalic Condensation and Germband Formation

In the centipede *Strigamia*, the first morphological sign of the formation of an AP axis is a localised, ventral protrusion of the multilayered blastoderm at its anterior margin, which gives rise to the cephalic region (Fig. 6.3H1; Brena and Akam 2012). In *Glomeris* and other myriapods, the cephalic area condenses later, and thickening of germinal tissue is more uniform along the whole AP axis (Fig. 6.3B–D). In most Myriapoda there appears to be a general contraction of the germinal area and a latero-medial tissue convergence in the process that gives rise to the germband, a key developmental trait common to all panarthropods, hence considered their "phylotypic stage" (see discussion in Scholtz and Wolff 2013). This is very clear in *Glomeris* (Fig. 6.3B–D; Dohle 1964, 1974; Janssen et al. 2004; Janssen 2012) but even more so in *Pauropus* (Fig. 6.4G–I; Tiegs 1947a) and *Strigamia* (Fig. 6.3; Brena and Akam 2012), where previously the germinal area is particularly extended, covering two third of the egg. The germband takes shape not only through condensation of the widely extended germinal region but also through extension in the two opposite directions along the AP axis (see below). In symphylans and pauropods, the germband is not relegated to a portion of the surface of the egg as in most myriapods but occupies most of the egg (Fig. 6.4), being in symphylans fully bent from the beginning (see below as well as Fig. 6.4A and Tiegs 1940, 1947a).

Segmentation and Elongation

As the germband takes shape, the first segments appear as transverse bands delimited by furrows, the result of the thickening of ectodermal cells and the underlying re-arrangement of the mesoderm (Fig. 6.3B, H–I). In the centipede *Strigamia*, the first proper morphological segmental unit as a full transverse band is the mandibular one, and all the more posterior ones will follow it one by one, strictly sequentially along the AP axis (Fig. 6.3H–K). Anterior to the mandibular segment, only the antennal lobes (later limb buds) are recognisable, and no "anterior head segment" (see below) is defined by transverse furrows (Figs. 6.3 and 6.5; Brena and Akam 2012). In *Lithobius* the first morphological segment defined by a furrow is the mandibular one as well, while the antennae are already formed (Hertzel 1984). There are no precise sequential data for other chilopods. A similar sequence starting with the antennal lobes characterises pauropods (Tiegs 1947a) and maybe symphylans as well (Tiegs 1940), although in these miniaturised myriapods, segments appear more like large bulges associated with the early developed limb buds from the very beginning (Fig. 6.4). In the millipede *Julus*, the first appearance of segmentation on the surface is a furrow just posterior to the stomodeum, hence probably corresponding to the anterior margin of the mandibular segment as in chilopods, but details about the sequential appearances of the following segments are unknown (Fig. 6.6A–B; Heathcote

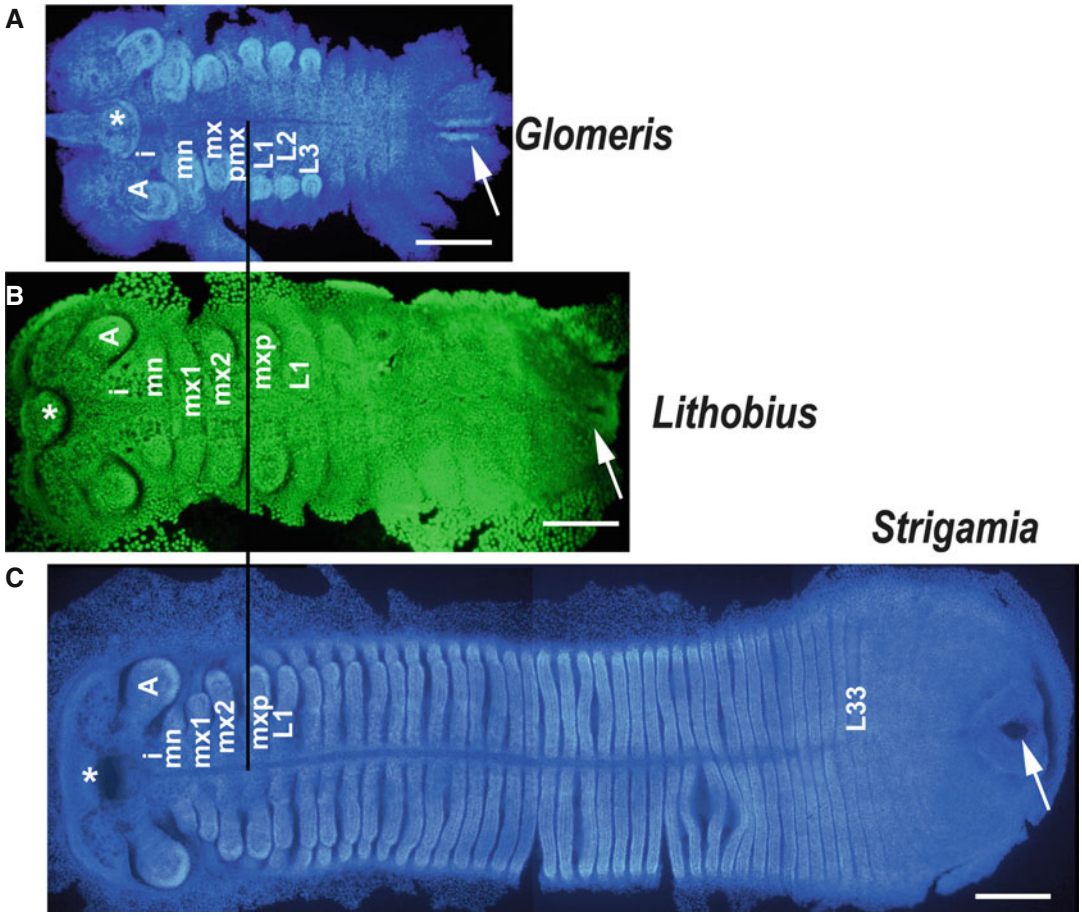


Fig. 6.5 The germband in centipedes and millipedes. Mid-late germbands of the millipede *Glomeris pustulata* (**A**) and the centipedes *Lithobius atkinsoni* (**B**) and *Strigamia maritima* (**C**), all to scale and aligned according to a line (black bar) defining the border between proper head and trunk; due to the differential development of the different parts, it is impossible to have a meaningful temporal alignment between the different species, but at least by stages here reported, there is no clear further elongation of the germband. Note that in *Glomeris* the dorsal tissue, which should be on the side of the germband (and is partially present on the mandibular segment), has been lost during dissection (this specimen is intermediate in development to the embryos in panel D and E of Fig. 6.3). The morphology of the embryos is highlighted by fluorescent nuclear staining (DAPI for *Glomeris* and *Strigamia* and Sytox Green for *Lithobius*). A antenna, L leg-bearing segment, i intercalary segment, mn mandible, mx maxilla (mx1, as first maxilla, in centipedes), mx2 second maxilla, mxp maxillipede, pmx postmaxillary segment, asterisk labrum, arrow proctodeum/anus. Scale bar: 200 μm

1886). All the first morphological segments seem to appear more or less simultaneously in the diplopods *Polydesmus* (Lignau 1911) and *Glomeris* (Fig. 6.3B; Dohle 1974; Janssen et al. 2004), deriving directly from the tissue of the initial regio germinalis; in *Glomeris*, when these first segments are well-defined on the surface of the germband, they include head and the first three (leg-bearing) trunk segments.

With maybe the exception of pauropods where the forming embryo tends to occupy most of the egg across all stages, as more segments are added at the posterior, there is a general tendency in myriapods for the germband to converge from the sides and, at the same time, for the most anterior segments to shift forward and condense to occupy a smaller fraction of the germband (Fig. 6.3). There is furthermore a slight elonga-

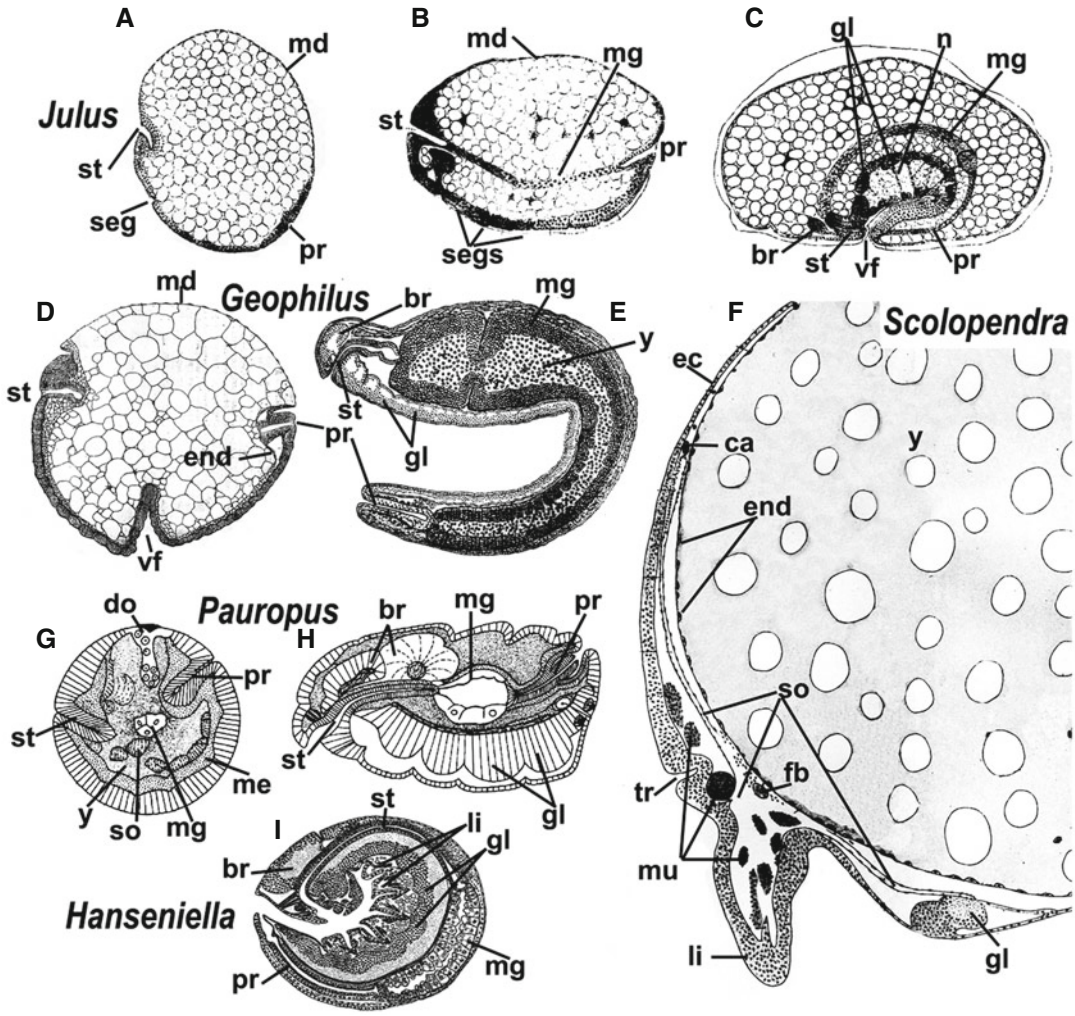


Fig. 6.6 Endoderm formation and organogenesis. Drawings, partly schematic, of sections of eggs/embryos at different stages of development of the millipede *Julus terrestris* (**A**–**C**), the centipedes *Geophilus ferrugineus* (**D**, **E**) and *Scolopendra cingulata* (**F**), the pauropod *Pauropus silvaticus* (**G**, **H**), and the symphylan *Hanseniella agilis* (**I**) to show different stages of formation of the gut, from early ingression/formation of stomodeum and proctodeum (**A**, **D**, **G**) to completion of the alimentary canal (**B**) and differentiation of nervous and muscular systems (**C**, **E**, **F**, **H**, **I**). All depictions are sagittal sections, except (**F**) which is a transversal section (**A**, **G** early germband stage; **B** mid-germband stage; **D** early ventral flexure; **C**, **F**, **I** after ventral flexure; **E**, **H** almost, or very early, postembryonic stages (probably proembryoid III, according to Brena 2014, and pupoid, respectively). br brain, ca cardioblast, ec ectoderm, end endoderm, do dorsal organ, fb fat body, gl ganglia, li limb, n nerve, md membrana dorsalis, me mesoderm, mg midgut (also midgut rudiment), mu muscles, pr proctodeum (or primordium of), seg segment (segs: segments), so somite/coelomic sac, st stomodeum (or primordium of), tr tracheal pit, vf ventral flexure, y yolk. (**A**–**C** modified after Heathcote (1886); **D**–**E** after Sograff (1883); **F** after Heymons (1901); **G**–**H** after Tiegs (1947a), in part as modified by Anderson (1973); **I** after Tiegs (1940))

tion of the germband, at least as measured from the stomodeum to the proctodeum (see below). This is rather limited in most myriapods, where only few segments are produced embryonically (anamorphic development) (e.g., in millipede *Glomeris*; see Dohle 1974; Janssen et al. 2004, or in the centipedes *Scutigera* (Knoll 1974) and *Lithobius*; see Hertzel 1984; Brena, unpublished

data). On the contrary, this elongation is evident in Epimorpha centipedes (Scolopendromorpha and Geophilomorpha), particularly so in geophilomorphs, the only myriapods producing a very high number of segments embryonically (see below section on Late development) (Fig. 6.5). In *Strigamia,* time-lapse movies have shown that the anterior margin of the head condensation moves forward over the underlying yolk, while the posterior thickening, originally associated with the blastopore, moves in the opposite direction, approaching the posterior margin of what is now becoming a shrinking posterior disc (Brena and Akam 2012).

The segmentation rate is only known for the centipede *Strigamia*. In this species, there are four phases of segment addition, each with a different pace (which is a constant one segment every 3.2 h at 13 °C, in the main phase, during stage 4) (Brena and Akam 2012).

Differentiation of Segments and Late Germband Extension and Dorsal Closure

In *Strigamia*, where segments are formed one by one from the very first segment, there is, consequently, a perfect developmental gradient along the AP axis. In *Glomeris* this gradient is only clearly evident for post regio germinalis segments (from the fourth trunk segment) (Fig. 6.5; Dohle 1964; Janssen et al. 2004; Brena and Akam 2012).

The first morphological differentiation which appears on segments is the limb bud, represented by an outgrowth, detaching more or less at two third of the medio-lateral extension of the segment. This, in a way, marks the differentiation, along this axis, between the ventral, the lateral, and the dorsal tissues. The ventral tissue is medial to the limb and is later involved in forming neural tissue, the lateral tissue is around and includes the limb, and the dorsal tissue is just external, i.e., lateral, to it. As the latter develops and extends further from the ventrolateral tissue, it may grow as to form a distinct patch of tissue, called in diplopod "lateral plate" by Dohle (1964), although it actually represents dorsal tissue (Fig. 6.3D–E; see also Fig. 6.11A). It may have a more or less developed mesodermal component depending on the stage and on the species: for example, at germband stages, mesoderm is present in this dorsal extension in the millipedes *Glomeris* and *Ommatoiulus* (Dohle 1974) but not in the centipede *Strigamia* (at least according to the mesodermal marker gene *twist*; C. Brena, unpublished data). Later in development this lateral extension representing dorsal tissue will start to differentiate its ectodermal component as dorsal tergite (a left and a right hemitergite per segment) (Fig. 6.3F). Eventually, in late embryonic stages and after ventral flexure (see below), the two hemitergites will fuse dorsally along the dorsal midline to form a single tergite, enclosing the whole egg ("dorsal closure"), a process delayed in centipedes (Fig. 6.3L, P; Dohle 1964; Ortega-Hernández and Brena 2012). During dorsal closure the fate of the dorsal membrane cells is unknown, although they might be just co-opted to contribute to the differentiated dorsal ectoderm.

Gnathal limb buds are specifically differentiated in the posterior head segments (see below), with chilopods differentiating also the first trunk segment as maxillipeds (Fig. 6.5). Both millipedes and pauropods lack the second maxillae on the last head segment, but only in millipedes the maxillae fuse to form the gnathochilarium, later in development (Tiegs 1947a; Dohle 1964), while in the symphylan *Hanseniella*, the two second maxillae fuse to form the labium (Tiegs 1940).

As the limb buds take shape, the underlining segmental mesodermal unit, partly included in the bud itself and traditionally referred to as "somite", may separate to create a coelomic cavity (Fig. 6.6F). These coelomic sacs are present, with different degree of development, in *Scolopendra* (Heymons 1901), *Lithobius* (Hertzel 1984), *Scutigera* (Knoll 1974), *Glomeris* (Dohle 1964), *Ommatoiulus* (Dohle 1974), and *Hanseniella* (Tiegs 1940). In geophilomorph centipedes, they appear to be present in germband stages only in the most anterior segment (C. Brena, unpublished data) and are barely developed at all in the miniaturised pauropods (Tiegs 1947a).

Ventral Flexure

In most myriapods, the late germband stretches medio-laterally and then flexes into the yolk near its midpoint, eventually bringing the ventral surfaces of the anterior and posterior halves of the germband into opposition (Fig. 6.3E, F, L–P; see also Fig. 6.6C, D). This dramatic and relative quick (at least in *Strigamia*, see time lapse in Brena and Akam 2012) is called dorsoventral – or simply ventral – flexure. It characterises stage 6 in both *Strigamia* (Brena and Akam 2012), *Scolopendra* (Whitington et al. 1991), and *Glomeris* (Dohle 1964).

Although in *Strigamia* the final phase of segmentation, interrupted during this movement, will retrieve later (Brena and Akam 2012), in *Glomeris* all eight embryonic leg-bearing segments (LBS) – present eventually at hatching – are already patterned (Janssen et al. 2004); this condition may characterise most anamorphic myriapods, as apparently is the case for *Lithobius* (with seven to eight LBS formed, Fig. 6.3M–O) and *Scutigera* (six to seven LBS present; Knoll 1974).

The ventral flexure appears extremely early in the symphylan *Hanseniella* (Fig. 6.4A; Tiegs 1940) and in the basal diplopods *Polyxenus* (Dohle 1964), where it even precedes germband formation/segmentation, appearing as a large transversal deep furrow. It is altogether absent in *Pauropus* (Tiegs 1947a).

In *Strigamia* (Brena and Akam 2012) and *Lithobius* (e.g., Hughes and Kaufman 2002a), the lateral spreading of the germband, which is associated with and precedes the ventral flexure, causes the interposition of an undifferentiated ectoderm between the medio-ventral neuroectoderm and the dorsolateral tissue. In *Scolopendra*, on the contrary, most of the membrane is interposed medially, splitting the neuroectoderm in a left and a right longitudinal band. This interposed epithelium has been called membrana ventralis (Heymons 1901) or even ventral extraembryonic ectoderm (Whitington et al. 1991) (but see discussion above). The interposition of a membrane is rather limited or absent in diplopods, and, given the precocious or absent flexure, altogether missing in symphylans and pauropods.

The neuroectoderm is medially split in spiders as well (Chapter 5; Stollewerk et al. 2001), where a similar flexure is called "inversion" (see Anderson 1973). This last movement characterises also some basal hexapods (and it is still called "ventral flexure", e.g., in the dipluran *Campodea*; Tiegs 1942b), although, given the phylogenetic position, it is difficult at this stage to infer any possible homology. In insects it has differentiated in a much more complex movement, formally defined there as blastokinesis, which involves a full sinking into the yolk of the embryo and the formation of the extraembryonic membranes (serosa and amnion; see Anderson 1973).

Proctodeum/Stomodeum: Endoderm and Gut Formation

At the posterior pole of the forming germband, a localised ectodermal cell population starts to invaginate to give rise to the proctodeum (Figs. 6.3C, J3 and 6.6A, D, G). It is clearly associated with the nucleus of early mesoderm formation as it appears just posterior to the cumulus in the diplopod *Glomeris* (Dohle 1964) and in close proximity with the blastopore in *Strigamia*. Indeed, in the latter the expression of several genes shows a clear continuity between the disappearance of the reducing blastopore and the appearance of the proctodeum (C. Brena, unpublished data; see below).

A similar medial invagination, eventually giving rise to the stomodeum, happens independently at the anterior of the germband, although this originally derives in millipedes and centipedes from a larger area, more or less between the ocular and the antennal segments (Figs. 6.3B, C, J1 and 6.6A, D, G). The proctodeum tends to appear earlier than the stomodeum, but the precise timing of their appearance is only known in a few species. In *Strigamia* the proctodeum appears at stage 3, at the appearance of the first segments, while the stomodeum takes proper shape only by early mid-segmentation (stage 4), while in *Lithobius* the stomodeal large area is

well formed already before trunk segmentation (Hertzel 1984; C. Brena, unpublished data). In the diplopod *Glomeris*, the proctodeum appears at stage 0.5, at the appearance of the first segments (with *engrailed* stripes up to T3, see below), while the stomodeum appears at stage 2 as mandibular and antennal stripes start to form (Janssen et al. 2004). According to Heathcote (1886), in *Julus* the proctodeum appears after the stomodeum, at the time of appearance of the first segments (Fig. 6.6A).

Subsequently, proctodeum and stomodeum invaginations extend further into the yolk mass and towards each other, as endodermal cells start to give rise to the midgut, ultimately connecting the two ectodermal invaginations to form a complete gut (Fig. 6.6).

Even more than for the mesoderm, to date the endoderm origin is still a matter of speculation based on cell morphology on serial sections. In *Scolopendra*, endoderm cells apparently derive from the blastoderm, either diffusely from the ventral surface according to Heymons (1901) or, according to Dawydoff (1956), from a localised small posterior ventral population of blastodermal cells in proximity of mesodermal proliferative cells. Endodermal cells will then spread around and will consequently enclose the yolk mass without entering it. On the contrary, in *Scutigera*, according to Knoll (1974), the endoderm derives entirely from the yolk in a rather complicated way through an initial aggregation in "nests". Whatever the origin, in centipedes endodermal cells give rise eventually to a midgut epithelium enclosing the yolk (Fig.6.6D–F) (Sograff 1883; Heymons 1901).

In diplopods, on the contrary, although Heathcote (1886) derived the midgut also from a central core of yolk cells, all other authors (Metschnikoff 1874; Cholodkowski 1895; Lignau 1911; Pflugfelder 1932; Dohle 1964) agree in deriving the midgut from the germband itself, as a strand of cells which runs through the centre of the yolk mass, derived (at least in *Glomeris*; Dohle 1964) from a localised ventral area of the blastoderm, immediately in front of the proctodeum (Fig. 6.6B).

In symphylans and pauropods, endodermal cells derive from radial division during cleavage (Tiegs 1940, 1947a). In symphylans they derive from the more internal of the nuclei scattered in the yolk mass, while in pauropods they originate from the two distinct cells cut off at the centre of the yolk mass during early cleavage (Figs. 6.2G and 6.6G). As in diplopods, these cells eventually give rise to a midgut tube joining the hindgut and foregut, surrounded by nucleated yolk, by now transformed in fat bodies (cells) (Fig. 6.6H, I).

At the conjunction between the proctodeum and posterior midgut, the proctodeum invaginates further forward bilaterally, producing two lateral long branches, the Malpighian tubules (see, e.g., Brena et al. 2006).

In *Julus* (Heathcote 1886), the midgut develops well before ventral flexure (Fig. 6.6B–C), and the same could hold true for *Glomeris* as at the time of the ventral flexure, the gut is complete (C. Brena, unpublished data). It is well developed rather early in pauropods and symphylans as well (Tiegs 1940, 1947a). In basal centipedes the timing of midgut completion is unknown, but in Epimorpha it is clear that the full formation of the gut is delayed even to postembryonic time (Fig. 6.6E). This is clearly associated with a general delay of embryonic development into postembryogenesis (Brena 2014).

Organogenesis

Small invaginations on the lateral ectoderm give rise to the tracheal pits and then to the tracheae in centipedes and millipedes (Fig. 6.6F; see in particular Heathcote 1886; Heymons 1901; Dohle 1964; Brena and Akam 2012; Ortega-Hernández and Brena 2012). Symphylans and pauropods lack trunk tracheal invaginations. A specific differentiation of the ectoderm characterises its ventromedial portion, where cluster of cells during germband stages ingress to generate the nervous system. This has been the focus of recent molecular developmental studies on neurogenesis (see below).

In *Scolopendra*, the mesoderm gives rise to a more or less developed coelomic cavity which

extends along most of the space between the endoderm covering the yolk and the ectoderm. Cells from its external (somatic) wall proliferate and differentiate to generate the longitudinal muscles and the musculature of the appendages, while cells from the visceral walls, lining the endoderm, form the midgut musculature (Fig. 6.6F; Heymons 1901). Cardioblast cells at the dorsal junction of somatic and visceral mesoderm give rise to the heart once the left and right segmental portions meet dorsally (Heymons 1901).

Description of mesoderm and endoderm differentiation and the resulting organogenesis has been the focus only of old studies based on serial sections. Comparison with modern molecular markers to confirm that the interpretation of those data is correct, is missing. For an extended description of those aspects of organogenesis, see, in particular, the detailed accounts for centipedes (*Scolopendra*, Heymons 1901 and *Scutigera*, Knoll 1974), millipedes (*Glomeris*, Dohle 1964 and *Platyrrachus*, Pflugfelder 1932), symphylans, and pauropods (Tiegs 1940, 1947a).

LATE DEVELOPMENT

All myriapods have direct postembryonic development, and they reach adult conditions through smooth changes, moult by moult. Indeed in many of them, as in several other arthropods (see, e.g., Minelli and Fusco 2013), there is an actual continuity between embryonic and postembryonic development, with the newly hatched animal persisting in the last embryonic cuticle within the open chorion for a while (this is particularly evident in *Strigamia*; Brena 2014). These are somehow delayed embryonic stages outside the chorion and are generally called embryoid or pupoid stages. They are typical of Juliformia and polydesmid millipedes, where a pupoid stadium is present, for example, in *Orthomorpha* (Dohle 1964), *Ommatoiulus* (Dohle 1974), or *Julus moreletti* (Metschnikoff 1874) (Fig. 6.7E). In Epimorpha centipedes where the first postembryonic instars are looked after by the mother for quite some time, this is even more clear, in particular in geophilomorphs where in *Strigamia* five embryoid stages separated by moults have been identified (proembryoid I–III, peripatus, and foetus) (Fig. 6.7G, H; Brena 2014). Peculiar is the case of pauropods where the transition between embryonic and postembryonic phase in *Pauropus* is represented by a "pupa" (two in *Gravieripus*; see Minelli et al. 2006), a capsule enclosing the animal, with specific long setae and barely marked signs of the limbs (the included pupoid is shown in Fig. 6.4L; see also Tiegs 1947a).

Although the general morphology of the animal is not really affected, in most myriapods, the major change in postembryonic development is the addition of segments (anamorphic segmentation). This appears to be the ancestral condition, and indeed only the derived Epimorpha centipedes form all their segments embryonically, at least almost. Indeed, with a condition that might be common to all of them, *Strigamia* in fact hatches with all prospective leg-bearing segments, but not with all legs (missing from the last segment) and without the genital segments. Not only these segments are normally added "anamorphically", but some specimens, at least in culturing conditions, may delay the morphological appearance even of the last leg-bearing segment to postembryonic time, somehow weakening the divide between anamorphic and epimorphic development (Brena 2014).

Much more extensively than in *Strigamia*, in general in myriapods most of the time a juvenile has segments with different degree of development: some with fully formed and functional legs, some with limb buds, and some are limbless altogether. This should be taken into account when comparing the number of segments of young instars in different species. Unfortunately, these details are only known for few species: the first instar of *Lithobius* has seven functional leg pairs + one limb bud + one barely developed segment (Hughes and Kaufman 2002b; C. Brena, unpublished data); in *Scutigera* it has five functional leg pairs but internally two additional well-developed limb buds (Fig. 6.7F; Knoll 1974).

There are three kinds of anamorphosis: euanamorphosis, when segments are added at each moult continuously for the whole life of the animal, teloanamorphosis when a species-specific

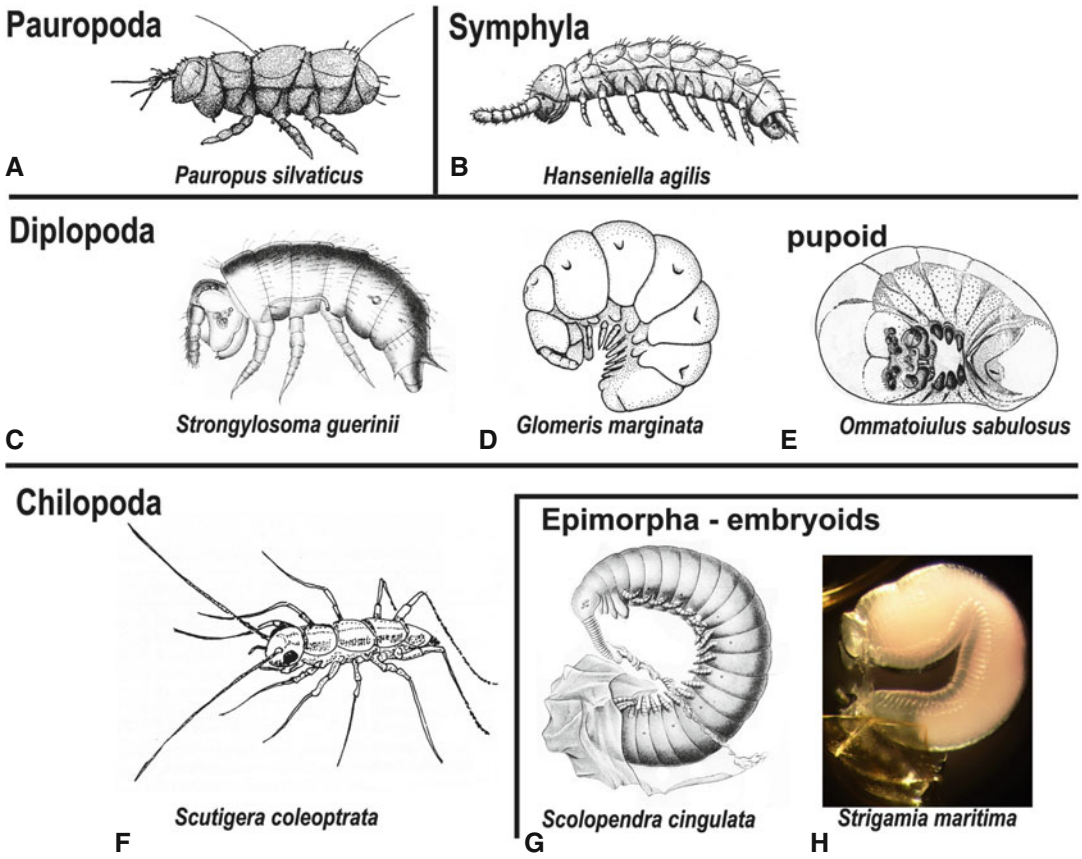


Fig. 6.7 First postembryonic instar across myriapods. Main typologies of first postembryonic stages ranging from the free-living anamorphic types like the ones of pauropods (**A**), symphylans (**B**), some polydesmid (**C**), and glomerid (**D**) millipedes and scutigeromorph centipedes (**F**) to the anamorphic pupoid of some juliform millipedes (**E**) to the epimorphic embryoid of epimorphic centipedes (**G**, **H**). Note that in pauropods an enclosed, inert "pupa" precedes the first instar shown in (**A**), and that what is shown in (**D**) is a very late embryonic stage, ready to hatch. (**A** modified after Tiegs (1947b); **B** after Tiegs (1945); **C** after Metschnikoff (1874); **D** after Dohle (1964); **E** after Dohle (1974); **F** after Knoll (1974); **G** after Heymons (1901))

number of segments is reached as the animal stops to moult, and hemianamorphosis, when addition of segments characterises only the first phase of juvenile development (Enghoff et al. 1993). All three kinds are present in diplopods, while the hemianamorphosis is typical of pauropods, symphylans, and scutigeromorph and lithobiomorph centipedes. In all these three classes, the anamorphic stages are, somehow misleading, traditionally called a "larva" (see Minelli and Fusco 2013 for a general discussion on the use, and abuse, of the term).

Among symphylans, *Hanseniella* first instar (usually called a prelarva, with non-functional mouthparts; Minelli and Fusco 2013) has seven leg-bearing segments and one apodous segment (Fig. 6.7B), while in *Scutigerella* it has 6 + 1 segments (Tiegs 1945). *Pauropus* first free instar has three leg-bearing segments and two apodous segments (Fig. 6.7A; Tiegs 1947b). In millipedes, where described, the first instar has the first three legs functional or well formed, while the remaining segments may have a different level of development. While *Polyxenus* has only five body segments (Schömann 1956), several species of polydesmids and juliforms have all up to the VI tergite but in general no limb bud except the first three (Figs. 6.7C, E; Metschnikoff 1874;

Heathcote 1886; Dohle 1964). *Glomeris*, on the contrary, is more developed and, besides the three functional leg pairs, has five limb buds and possibly nine total trunk segments, considering that the VII tergite is fully formed (Fig. 6.7D; Dohle 1964; Janssen et al. 2004; C. Brena, unpublished data). Even more developed is the first free larva of the platydesmid *Dolistenus savii* which, in addition to the first 3 segments with 3 functional leg pairs, has 20 diplosegments, each with 2 pairs of small legs (the last one has only small buds) (Silvestri 1950).

Besides the addition of segments in anamorphic species, the postembryonic development of myriapods is characterised by some minor continuous morphological changes, like the differentiation of the forcipules in centipedes (Dugon et al. 2012) or the sequential and stereotypic addition of setae in *Strigamia* (Horneland and Meidell 2009) or the ocelli in millipedes (Enghoff et al. 1993; Dhaenens and Van den Spiegel 2006). In particular Harzsch et al. (2007) have shown that the myriapod mechanism of eye (postembryonic) growth represents the ancestral euarthropod mode of visual system formation, similar to what found in trilobites and basal chelicerates.

GENE EXPRESSION

All modern EvoDevo analyses on myriapods are basically based on two European species (the millipede *Glomeris marginata* and the centipede *Strigamia maritima* – see boxed text) with some additional information on the centipede *Lithobius* (both the American *L. atkinsoni* and the European *L. forficatus*). Although these species are particularly prone to gene expression analysis on the germband, thanks to its extension on the surface of the egg, to date no functional technique has been proven successful. Pluriannual generation times and limits in culturing exclude any possible genetic hereditary studies.

We have just started to unravel myriapod development in terms of modern analysis of gene expression, and some of the concepts reported here are partially prone to change as more data become available.

Emerging Model Systems for Myriapod EvoDevo

Glomeris marginata

Glomeris marginata is the typical European pill millipede, 1.5–2 cm long, with a total of 17 pairs of legs in males and 19 in females, living on leaf litter, with a life cycle of few years. Specimens of this species can be found in relative high density, and once collected in spring and summer, it is easy to keep them in culture and have the females laying eggs for a while. These are singularly encapsulated in a mud shell and left at the surface, making them relatively easy to collect. *Glomeris marginata* development is the best described among millipedes, and its embryos are among the easiest to handle. It develops more precociously than other common European millipedes, like the julids, allowing developmental and molecular analysis of a higher array of segments and of more differentiated structures. All gene expression data published to date on millipedes are basically based only on this species, but functional molecular studies have as of yet failed to work. The genome of *Glomeris marginata* has not been sequenced yet.

Strigamia maritima

Strigamia maritima is a small 3–5 cm (around 1 mm in diameter) centipede with a variable number of leg-bearing segments, between 43 and 53. It has a 3-year life cycle and lives on the seashore on shingle beaches all along North European Atlantic coasts. It is the only known geophilomorph centipede (i.e., the centipedes with a high number of segments) that lives in very high density, also during parental care. This, in turn, allows for collection on site of a large number of eggs, although such a condition has been found to date only in Brora, northern Scotland, and off Galloway, eastern Ireland. The collected eggs (5–30 per female) can be grown in the lab, and their development can be delayed for a while by

lowering the culturing temperature. This process allows for experimental work on live eggs for not much more than 2 months per year. The adults can be easily kept in the lab, but they do not lay eggs.

Geophilomorphs have two characters which make them better-suited than other centipedes for evolutionary and developmental studies: they have variability of segment number and develop all their segments during embryonic time, a combination of characters essentially unique within all myriapods. Additionally, geophilomorphs have a small genome size, which made *Strigamia maritima* the selected species to be the first myriapod to be sequenced. The high quality of both sequencing and annotation should make this species a precious source of comparative genetic information. *Strigamia* is the only centipede for which a modern, detailed description of development is available, and most of the gene expression data published to date on centipedes are based on this species, although functional molecular studies have as of yet failed to work.

Germ Line

Primordial germ cells (PGCs) have been described in classical literature as appearing late in development from coelomic pouches of the mesoderm in millipedes, symphylans, and pauropods (Tiegs 1940, 1947a; Dohle 1964). Although the mechanism of PGC specification is unknown, it has now been shown through the expression of germ line genes *vasa* and *nanos* that PGS are specified much earlier than previously thought (e.g., as reported in Nieuwkoop and Sutasurya 1981; Extavour and Akam 2003), at least in the centipede *Strigamia* (Green and Akam 2014). Differentially expression of *vasa* and *nanos* shows that germ cells might be already determined at the cleavage stage, among cells at the centre of the egg. For sure PGCs are specified at least at the blastoderm stage where they accumulate at the blastopore, confirming somehow Heymons (1901) identification of a group of cells at the posterior end of the embryonic rudiment. PGCs then internalise through the closing blastopore and will eventually reach later in development the embryonic gonads after a long-range migration.

Blastoderm Early Signals: Blastopore, Proctodeum, and Stomodeum Determination

The blastoderm in myriapods shows a high level of cellularisation and, with the exception of the small symphylans and pauropods, is composed of a large number of cells (up to 20–30,000 in *Strigamia*) covering a large yolk-rich egg. This condition does not allow easy movement of morphogens or transcription factors as in the case of the well-known *Drosophila* syncytial blastoderm (Vol. 5, Chapter 1) and makes it difficult to imagine maternal determinants put in place during oogenesis to define the main egg axis (e.g., as *Drosophila* bicoid; McGregor 2005). This holds true in particular in the case of geophilomorph centipedes where a large population of cells are well cellularised at the centre of the egg before radially migrating to the surface. In *Strigamia*, *vasa* and *nanos2* transcripts and Vasa protein are present in developing oocytes (Green and Akam 2014), and transcripts of the early patterning gene *hunchback* (*hb*) (see below) was detected in *Glomeris* oocytes (Janssen et al. 2011a), but it is unknown whether these transcripts expressed on the blastoderm are still maternal (*orthodenticle* (*otd/otx*), see below, is clearly not; Janssen et al. 2011a).

The first differentiation of the blastoderm is represented by a multilayered cluster of cells, the cumulus. In *Strigamia* the cumulus appears to express specifically some important morphogens, like *decapentaplegic* (*dpp*) (Fig. 6.8A), which is important in animal embryos in defining major embryonic axes and promoting germband development. *dpp* expression later extends to the

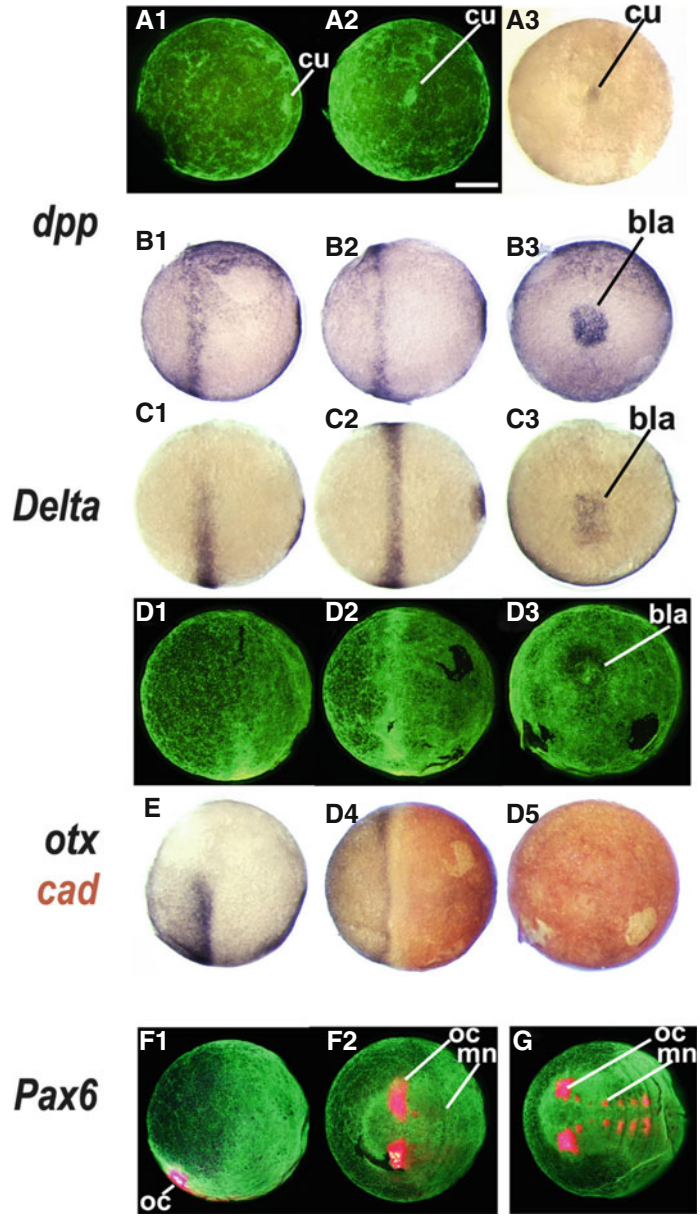


Fig. 6.8 Early blastoderm patterning. Early blastoderm molecular patterning in the centipede *Strigamia maritima*. *dpp* expression is associated with the cumulus (cu) at the beginning of the blastoderm stage (**A**) and is later expressed (**B**), like *Delta* (**C**), at higher level in correspondence of the blastopore (bla) and as a ring roughly delimiting the anterior head, expressing *otx*, while the posterior cap is dominated by *cad* (**D**, **E**). *Pax6* is at the beginning strongly expressed in the ocular lobes (oc) (**F**) but is then expressed in the neurogenic patches in every formed segment (**G**). (**B**–**E**) are eggs of similar, late blastoderm stage, while the embryo in (**F**) is at the beginning of segmentation and in (**G**) at the beginning of trunk segmentation. Panels with the same letter but with sequential numbers are the same embryo viewed from different angles (e.g., **A1** and **A2**) or under different illumination (e.g., **A2** and **A3**, respectively, fluorescent to show the morphology with the nuclear staining SYBR Green, and normal light to show the expression pattern). Left column: lateral view (ventral side on the bottom); middle columns and (**G**) ventral view; right column (except **G**); and **A2**, posterior view. In panels in lateral and ventral view, anterior is to the left. (**F**, **G**) false-coloured *Pax6* staining has been overlayed on the embryo viewed in fluorescent light. mn mandibular segment. Scale bar: 300 μm

whole blastopore (Fig. 6.8B). It is not known how the initial radial symmetry is broken in *Strigamia* or in any other myriapod, but certainly, this early expression of *dpp* is different from the expression of *dpp* in spiders, where it is strictly associated with the (secondary) cumulus posterior, an internalised cluster of mesenchymal cells which in its migration from the cumulus primitivus is pivotal in axial pattern formation (Chapter 5; Akiyama-Oda and Oda 2003, 2006).

In *Strigamia*, at – or just before – the appearance of the blastopore, the low-density cells defining it start to express the gene *brachyury* (*bra*), a mesodermal marker associated with the process of gastrulation (Green and Akam 2014). Later, as some of the *bra*-expressing cells inter-

nalise to form the mesoderm, the blastopore shrinks and *bra* is reduced to an arc of expression around its anterior margin. As in the anterior of the germband, the first morphological segments appear (see below), cells from this *bra-expressing* patch, by now also expressing *forkhead* (*fkh*, a marker of anterior and posterior gut primordia), start to invaginate to give rise to the primordium of the proctodeum. *fkh* is indeed expressed at the same time in the medial anterior cephalic area of low cell density that will give rise to the stomodeum (J. Green, unpublished data). The posterior part of the blastopore remains open and does not close until later in development.

Both hindgut and the reduced posterior blastopore are included in the *dpp* expressing patch (originally associated with the large blastopore), and the developmental series of embryos showing expression of different genes in that population of cells, leaves open the possibility that the hindgut derives indeed from the anterior margin of the blastopore (C. Brena, unpublished data).

As the blastopore closes completely at the posterior pole, the proctodeum primordium starts to be defined by a protruding ring. This ridge may still represent a site on mesodermal cells ingression, as, by now, it strongly expresses the mesodermal determinant *twist* (C. Brena, unpublished data). In both *Strigamia* and *Glomeris*, the anterior area of the proctodeum ridge, in particular its bilateral patches, must have a crucial developmental role considering that a large part of morphogens, signalling molecules, and transcription factors are expressed there at high level throughout germband stages. For example, this is true for the genes of the Wnt family (Janssen et al. 2004, 2010; Hayden and Arthur 2014; Janssen and Posnien 2014), whose signallings have been recognised as important for setting the posterior pole of early embryos in all animals (reviewed in Petersen and Reddien 2009).

Other factors may be involved in determining the anterior pole in an independent way. Indeed, the possibility of the appearance/formation on the surface of two different cumuli might be at the origin of the duplicated germbands with a single cephalic region (*Duplicitas posterior*, with different classes of duplication/fusion), found in *Glomeris* (Dohle 1964; Janssen 2013) and *Strigamia* (C. Brena, unpublished data).

Early Blastoderm Patterning

Data about gene expression patterns for very early blastoderm stages are practically absent in *Glomeris* and extremely limited in *Strigamia*. In *Strigamia* early patterning gene expression (see below) is already associated with the multilayered blastoderm (MLB) (stage 2.2; Brena and Akam 2012) and appears to follow its initial expansion, although to date the relation between the gene expression domain and the MLB is difficult to trace and in general not precisely known. Indeed, there seems to be some degree of independence at this stage between ectodermal gene expression and the extension of the underling internal layer. Nevertheless, in *Strigamia*, early gene expression appears to be stabilised when the MLB reaches its maximum expansion, at two thirds of the egg. Early pattering genes are clearly expressed all over the blastoderm (Fig. 6.8B–E). The anterior part of the egg diffusely expresses genes such as *otd/otx* (anteriorly overlapping *six3*), a conserved anterior determinant across arthropods, with other less anterior head determinants like *buttonhead/SP5* expressed in a more or less full ring around the egg, defining the posterior limit of the anterior cap (Steinmetz et al. 2010; Hunnekuhl 2013). Abutting it, the posterior egg expresses at high level the gene *caudal* (*cad*), a conserved posterior determinant involved in axis elongation across arthropods and bilaterians in general (Figs. 6.8D and 6.9D).

These two regions clearly represent a major subdivision of the body along the AP axis. The anterior cap corresponds to the tissue of the anterior head (ocular, antennal, and intercalary "segments"; procephalon sensu Snodgrass 1935),

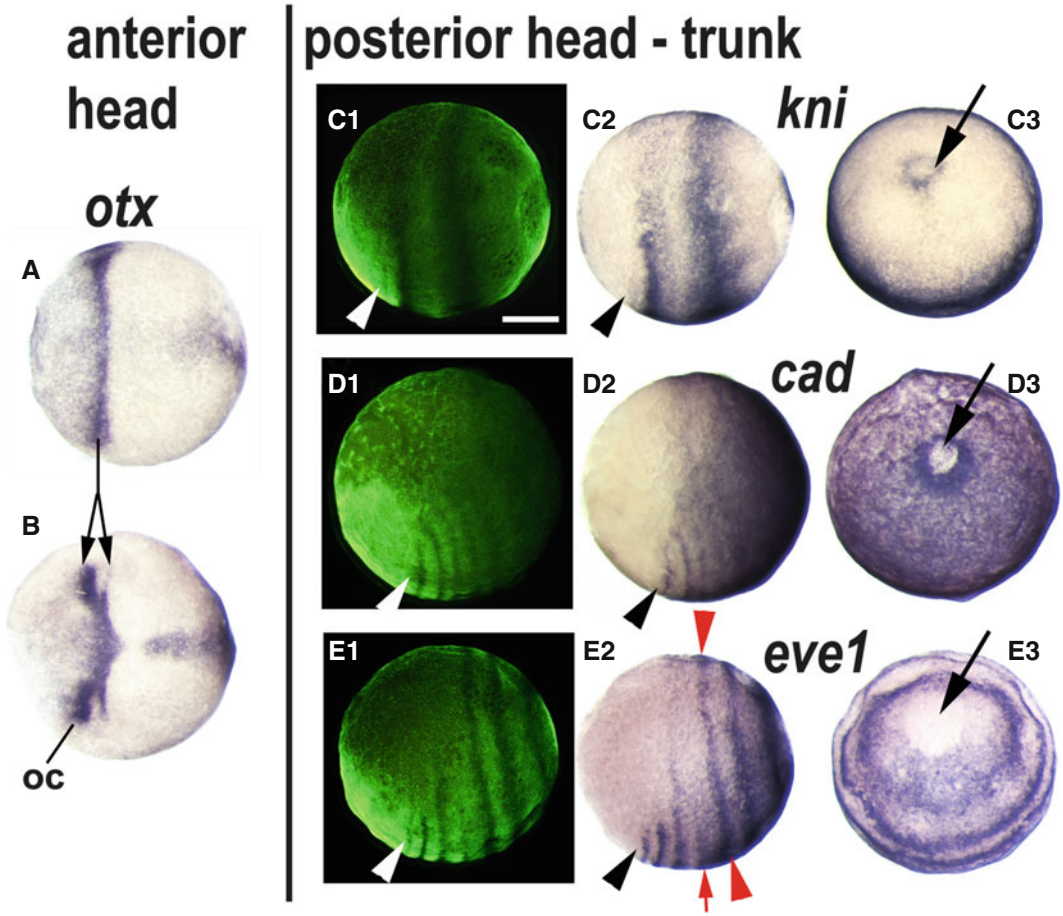


Fig. 6.9 Onset of periodic patterning – anterior head vs. posterior head and trunk. Early periodic patterning is shown in the centipede *Strigamia maritima*. Anterior head patterning appears to be the result of splitting of a broader domain associated with head extension, as shown by the gene *otx* in mid-late blastoderm stages (**A**, **B**). Posterior egg periodic patterning is associated with expression of broad dynamic stripes of *kni* (**C**), downregulation in segmental stripes of *cad* at its anterior border (**D**), and dynamic expression of pair-rule genes like *eve1* (**E**), where double periodicity rings (red arrowheads) expanding out of the proctodeum (black arrow) are later intercalated by single-segment periodicity stripes in the ventrally forming germband (red arrow). Panels with the same letter but with sequential numbers represent the same embryo viewed under different illumination (in a fluorescent light to show the morphology as highlighted by SYBR Green nuclear staining and in normal light to show pattern of gene expression) or from different angles (1 and 2 lateral view, 3 posterior view). (**A**, **B**) are in ventral view; in panels with lateral and ventral view, anterior is to the left. Embryos in **C**–**E** are roughly of the same age, after the appearance of the first morphological segment, the mandibular segment (black or white arrowhead). Embryo in (**A**) is the same as embryo in Fig. 6.8E. Black arrow marks the position of the forming proctodeum. oc ocular lobes. Scale bar: 300 μm

with its specific patterning system which has deep-rooted elements of homology across the Bilateria (see below). The posterior cap corresponds to all the remaining segments, starting from the mandibular one, i.e., gnathal segments (gnathocephalon) + trunk segments, characterised by elements of conservation at least common to all arthropods (see below).

In *Strigamia* the ring anterior to *cad* expression, more or less overlapping the border between anterior and posterior hemispheres, appears to be a key developmental boundary, where, at least in

their first appearance, several key developmental gene/markers are specifically expressed or expressed at higher levels. Among them are morphogens like *decapentaplegic* (*dpp*) but also signalling molecules like *Delta* or transcription factors like *twist* (*twi*) (Fig. 6.8B, C; Brena and Akam 2013; Hunnekuhl 2013; C. Brena, unpublished data). At the beginning, these genes mostly overlap, but a crucial later extension of the cephalic tissue along the AP axis induces a separation of parts of those domains (Fig. 6.9A, B) (see below).

Later ventral condensation of the regio germinalis, associated with forward extension of the cephalic area, affects the original pattering, breaking the original ring of expression and reducing it to a ventral stripe. A very similar breakage of an almost complete ring of expression of *otd/otx* characterises early spider development (Chapter 5; Akiyama-Oda and Oda 2003), although in the spider the ring is at the limit of a more reduced blastodisc, and the system associated with the breakage of this symmetry has not been identified in myriapods.

In myriapods the molecular system breaking the uniformity of the blastoderm is unknown. Gap genes are pivotal in differentiating the blastoderm in *Drosophila* along the AP axis, although their function in patterning the entire axis appears to be specific to long germband insects (see Jaeger 2011). Indeed, their most conserved – and thus probably ancestral – role is in head patterning and neurogenesis, as data from myriapods would confirm. Anterior gap genes involving both the anterior hemisphere and the area across the boundary seem to be rather conserved in both diplopods and chilopods (Janssen et al. 2011a; Hunnekuhl 2013; see below), but "trunk gap genes" appear in general to have a different role, possibly related to neurogenesis. In *Glomeris*, at the beginning *hb* appears to be expressed anteriorly, leaving open the possibility for a conserved function as a gap gene, but in *Strigamia* it is expressed diffusely on the whole germinal area, to be later segmentally patterned, as in *Glomeris*, and eventually restricted in later stage to the neuroectoderm (Janssen et al. 2011a). In *Strigamia*, *Krüppel*, although associated early with the posterior hemisphere, is then diffusely expressed, but at higher levels on the neuroectoderm. This diffuse mid-late expression characterises *Glomeris* as well (Chipman and Stollewerk 2006; Janssen et al. 2011a; C. Brena, unpublished data). In *Strigamia*, *tailless* is almost only associated with the ocular lobes (C. Brena, unpublished data). Of the genes analysed, the only trunk "gap" gene with a strong expression clearly preceding any segmentation gene is *knirps* (*kni*) in *Strigamia*. In early stages it is strongly expressed all over the posterior cap but is then dynamically downregulated in bands of more than one segment (Fig. 6.9C). It is then completely downregulated in all territory posterior to the second leg-bearing segment and upregulated again uniformly on the posterior unsegmented region on the final phase of segmentation (see below and Brena and Akam 2013; C. Brena, unpublished data).

Head Patterning

The head in myriapods is classically recognised as composed of six segments, as in all Mandibulata. From anterior to posterior, these segments are the ocular, antennal (corresponding to the cheliceral segment in chelicerates), intercalary (the latter called traditionally premandibular in diplopods and corresponding to the pedipalpal segment in chelicerates and to the second antennal segment in crustaceans), mandibular (first leg-bearing segment in chelicerates), first maxillary, and second maxillary segments (limbless "postmaxillary" in dignatha, becoming the collum in pauropods; see discussion below) and corresponding to the labial segment in symphylans and insects (Figs. 6.4 and 6.5). Whether all of them are "proper" segments or not, or whether there are even more than six, has been a matter of debate for a long time ("the arthropod head problem"; e.g., Scholtz and Edgecombe 2006). In fact even the nature of the head itself, or more in general its limits, may depend on specific uses. On the one hand, the head may include the forcipular segment, i.e. the first trunk segment of chilopods,

and traditionally includes the collum of diplopods, although developmental studies show that the collum of diplopods is just the tergite of the first trunk segment (Dohle 1974). On the other hand, in the EvoDevo field, the term "head" is actually often used to indicate only the anterior head, i.e., the procephalon composed of the first three segments. A firm distinction of this kind is rather appropriate as the procephalon appears to depend on a molecular patterning machine largely different from the rest of the body (gnathocephalon + trunk; although there is a rather clear overlap between the two systems, see below and, e.g., Peel 2004). This is rather clear in myriapods themselves, both from the first molecular patterning (see above) and from the following segmentation system (see below and Fig. 6.9). This, in turn, is reflected on how morphological segments appear: for example, in *Strigamia* on its forming flat germband, only – and all – post-intercalary segments appear as complete transversal bands delimited by furrows.

The patterning and early morphological diversification of the procephalon is more complex, reflecting also the fact that most of its diversification is in fact neural territories specification, and genes (as some "gap genes") patterning this region are involved in patterning also the neuroectoderm of the rest of the body. The anterior head is in fact constituted by the major anterior neural components, the brain and the major cephalic sensory structures (eyes – where present – and antennae), which probably trace back their origin and homologies outside the arthropods. The so-called ocular, antennal, and intercalary segments do correspond in fact to the protocerebrum, the deuterocerebrum, and the tritocerebrum subdivision of the brain. The protocerebrum in particular is a rather complex structure with a lateral ocular region and a medial central region, whose segmental or ancestral nature has been a matter of discussion for a long time (Urbach and Technau 2003; Scholtz and Edgecombe 2006; Posnien et al. 2010; Hannibal and Patel 2013).

In *Strigamia*, even at the morphological level, the way the anterior head is formed is complex and different from the rest of the body, and the subdivision in segments (see below) appears to be a direct result of these processes. In this species, analysis of the expression domains of different early patterning genes like *six3* and *otx* (Steinmetz et al. 2010; Hunnekuhl 2013; C. Brena, unpublished data) and morphology shows that the head is the result, partly, of condensation of the original anterior blastoderm cap but also of forward protrusion of the ventral portion of the posterior cap, although the real components of these complex cellular and expression dynamics are far from clear. This makes also rather difficult any attempt of precise alignment of early expression patterns between *Strigamia* and *Glomeris*.

Nevertheless, *Glomeris* and *Strigamia* show a clear conservation, in particular in their relative AP order, of the *Drosophila* so-called head gap genes: *otd/otx*, *empty spiracles* (*ems*), and *buttonhead* (*btd*)/*SP5* (Figs. 6.8D, E and 6.9A, B; Janssen et al. 2011a; Hunnekuhl 2013). In *Drosophila* these genes show larger areas of overlap, with *otd/otx*, for example, extended to include the antennal segment (see Cohen and Jürgens 1991), while restricted to the ocular segment in myriapods (not considering the later medial line expression; Fig. 6.9B, see also Fig. 6.14F). On the other side, there is apparently variability even within myriapods, with *btd* covering the whole anterior in *Glomeris* (Janssen et al. 2011a) and *SP5* originally just a ring in *Strigamia* (Hunnekuhl 2013).

Additionally, at least in *Strigamia* where early dynamics have been looked at in detail, the initial pattern is affected by the splitting/expansion of the original *SP5* ring of expression, with *ems* appearing between *otd* and *sp5only* later after condensation of the head (Hunnekuhl 2013). In general, although *Strigamia btd* expression is more similar to *Drosophila*, the expression pattern of these genes, and the associated dynamics, is more similar to the beetle *Tribolium*. This fact on the one hand may further support the arthro-

pod ancestral mode for the *otd-ems-btd* pattern and, on the other hand, weaken their role as proper gap genes as that has been dismissed functionally in *Tribolium* (Schinko et al. 2008; Hunnekuhl 2013). Indeed, the conservation of these anterior head determinants may go well beyond the arthropod ancestor, as some anterior expressing genes like *otx* and *six3* appear to be involved in defining this very ancestral region in a wide range of metazoans (Rubenstein et al. 1998; Lowe et al. 2003; Steinmetz et al. 2010; Hunnekuhl 2013; Sinigaglia et al. 2013).

General conservation of expression appears also at the level of secondary head patterning genes. *crocodile* (*croc*, a *Forkhead* gene) and *cap-n-collar* (*cnc*) are both expressed in association with the stomodeum as in other arthropods (Mohler 1993; Economou and Telford 2009; Janssen et al. 2011a; Hunnekuhl 2013). Additionally, *cnc* has a second domain of expression in the mandibular segment, where, in association with the Hox gene *deformed* (*Dfd*), it is required for its proper development in both *Tribolium* and *Drosophila* (see discussion in Sharma et al. 2014). This two-domain pattern is conserved in *Strigamia* (Hunnekuhl 2013) and *Glomeris* (Janssen et al. 2011a), as in crustaceans and insects, but it is expressed all along the body in chelicerates, resulting in a further support for the Mandibulata clade (Sharma et al. 2014).

Collier (*col*) is essential for *Drosophila* intercalary segment development, and it is early expressed in this segment in *Glomeris, Lithobius* (Janssen et al. 2011c), and *Strigamia* (Hunnekuhl 2013) in addition to insects (Schaeper et al. 2010) but is absent from the homologous segment in malacostracan crustaceans and in the spider (Schaeper et al. 2010). Possible scenarios based on the correlation between *col* and the lack of limbs in the intercalary segment have been discussed by Janssen et al. (2011b). However, the lack of a homeotic phenotype after removal of *col* in *Drosophila* (Crozatier et al. 1996, 1999; Schaeper et al. 2010) and the shifting of expression covering part of the antennal segment in *Strigamia* (Hunnekuhl 2013) (detailed alignment in *Lithobius* is missing; Janssen et al. 2011c) would rule out this hypothesis. In fact, in *Drosophila* there is strong evidence that *col* has a crucial function in head segmentation, where it acts on an intermediate level between head gap genes and segment polarity genes, taking input from both the trunk and the head patterning system (Crozatier et al. 1999) and *col* may have the same function in myriapods as discussed at length by (Hunnekuhl 2013).

Ocular Lobes Patterning

Glomeris, as most myriapods, has simple eyes, by no mean as complex as the eyes of insects; *Strigamia*, as all soil-dwelling geophilomorph, has no eye at all. Nevertheless, the ocular region of both myriapods seems to be characterised by the unique combination of transcription factors that induce ocular region differentiation in *Drosophila* (Noveen et al. 2000; Urbach and Technau 2003; Janssen et al. 2011a; Hunnekuhl 2013).

Two *Pax6* genes (critical for eye determination in *Drosophila*) are expressed in the ocular lobes in myriapods – in *Glomeris, Pax6.1* and *Pax6.2* (similar to *Drosophila eyeless* (*ey*) and *twin of eyeless* (*toy*), respectively; Prpic 2005) and in *Strigamia, Pax6A* and *Pax6B* (with a very similar expression pattern) – although they are also expressed in the neuroectoderm of every segment along the body (Fig. 6.8F, G; Hunnekuhl 2013). Other *Drosophila* eye development network genes which are expressed in myriapods in the optic lobes are *sloppy-pair* (*slp*) and *otx* (see also Figs. 6.9B and 6.14F; Steinmetz et al. 2010; Janssen et al. 2011a; Hunnekuhl 2013), *dpp* and *hedgehog* (*HH*) (Prpic 2004; Janssen 2012; Hunnekuhl 2013; C. Brena, unpublished data), *dachshund*, and *homothorax* (Prpic and Tautz 2003) and several *Wnt* genes (Janssen et al. 2004, 2010; Hayden and Arthur 2014; Janssen and Posnien 2014).

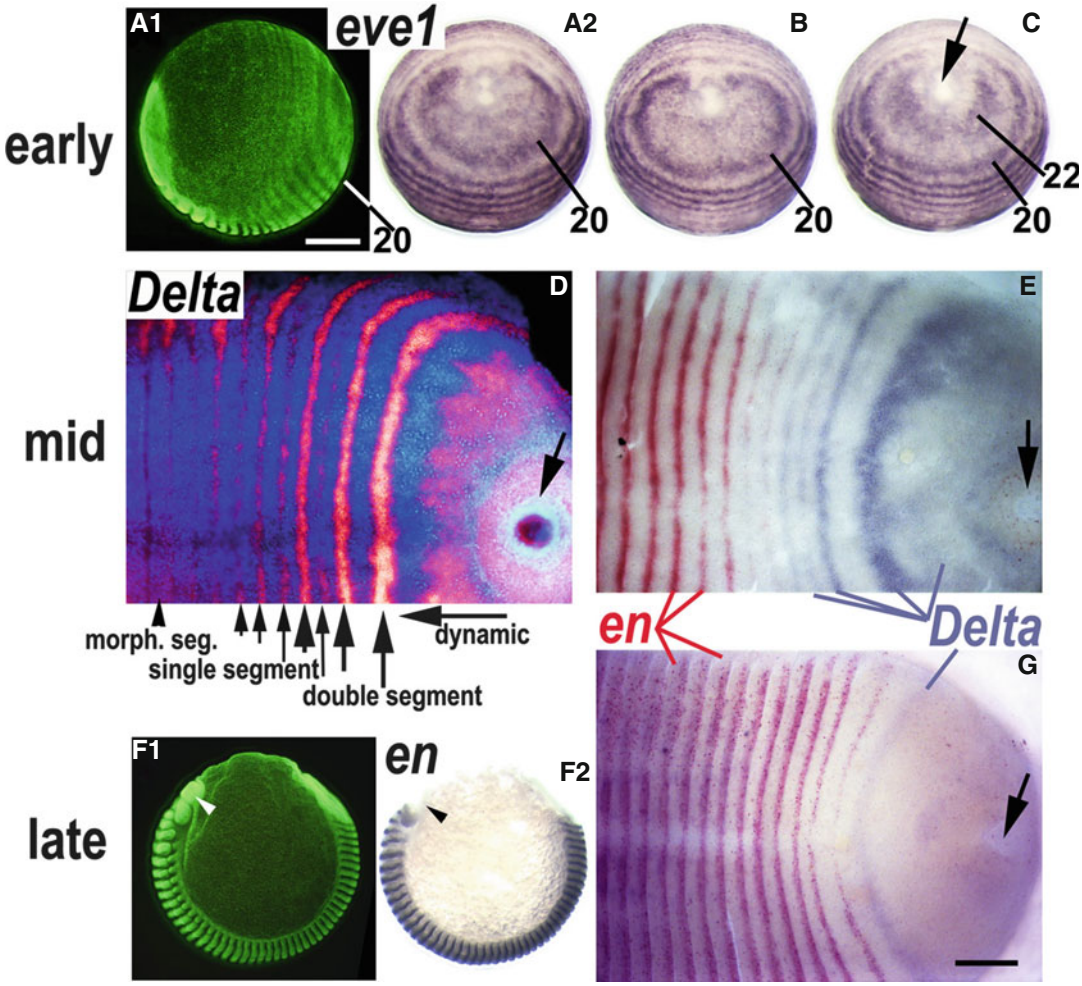


Fig. 6.10 Segmentation clock and late segmentation in *Strigamia*. In the centipede *Strigamia maritima*, in early mid-segmentation, periodic patterning appears as oscillatory periproctodeal expression involving pair-rule genes like *eve1* (**A**–**C**) and vertebrate-like segmentation clock genes like *Delta* (**D**–**E**) to be stabilised in double segment bands, intercalated later by single-segment periodicity stripes. Morphological segmentation is preceded by the segment polarity gene *en* (**E**–**G**). In late segmentation, as the single-segment front approaches the proctodeum (**E**), dynamic expression ceases, and single segments are produced sequentially one by one, with *Delta* expression reduced to a single stripe (**G**). Panels (**A2**, **B**, **C**) show three temporally ordered embryos showing a single cycle of dynamic expansion of the *eve1* band which will eventually correspond to the 20th leg-bearing segment. **A1**-**2** and **F1**-**2** are the same embryo viewed under different illumination (in 1 fluorescent light to show the morphology as highlighted by SYBR Green nuclear staining and in 2 normal light to show pattern of gene expression). In all panels, anterior is to the left. In (**D**), false-coloured *Delta* staining has been overlayed on the germband viewed in fluorescent light to show the morphology (DAPI nuclear staining). Arrows mark the proctodeum; arrowhead in **F** marks the ocular lobes, not expressing *en* in *Strigamia*. Scale bars: (**A**–**C**, **F**) 300 μm, (**D**, **E**, **G**) 100 μm

This conservation in gene expression between the eyeless *Strigamia*, *Glomeris* with simple eyes, and *Drosophila* with a complex eye would imply that probably this molecular signature is more associated with the early differentiation of this protocerebral area, presumably involved in the formation of such structures as the mushroom bodies, than with the differentiation of the eyes themselves (as discussed at length by Hunnekuhl 2013). To what extent the lack of expression of the segment polarity gene *engrailed* in the *Strigamia* ocular segment (Fig. 6.10F; Kettle

et al. 2003; Chipman et al. 2004a), on the contrary normally expressed in *Lithobius* (Hughes and Kaufman 2002b) and *Glomeris* (Janssen et al. 2004), is unknown.

Segmentation

Segmentation in animals can represent both a form and a process. In the first case, particularly evident in myriapods also in the adult trunk, "segmentation" is often used to indicate mere reiteration of morphological units along a given axis (usually the AP axis), irrespective of their mode of formation. In the second meaning, segmentation indicates the modality through which a periodic pattern arises on a uniform or broadly differentiated field of cells (either a whole blastoderm or a localised posterior growth zone), often through the differential expression of some "segmentation genes". In the following, it will be referred to this second meaning of the word segmentation.

When it comes to segmental patterning, two major problems arise when interpreting data based only on gene expression patterns. On the one hand, even genes which are bona fide homologous across clades may play different roles in different animals (an increased problem when a given gene belongs to a large family, like, e.g., the Wnt family, see below, where one-to-one homology is even more difficult to trace). On the other hand, several genes may be expressed in a reiterated pattern just because they are downstream to other proper segmentation genes and/or because they are associated with the morphology of the germband, a pronounced issue in organisms with extremely repetitive structures, limb buds included, such as myriapods (for a general discussion on segments and segmentation, see Hannibal and Patel 2013).

We still lack a general model explaining the appearance of segments in myriapods, in particular considering that data from the two main models (see boxed text) have been produced only in the last few years. Nevertheless, these data appear to show a uniformity of basic principles which appears to hold true for all myriapods beyond the substantial differences in segmentation of the two models.

Onset of Periodic Patterning: Pair-Rule Genes and Segmentation Clock

Two models explain the generation of segments in arthropods, and in both cases, the appearance of periodicity is associated with the expression of the pair-rule genes, the first "segmentation genes". In the best described model, the fruit fly *Drosophila*, segmental patterning is achieved simultaneously along the body axis by the combinatorial activation of pair-rule genes by overlapping broadly expressed "gap" genes (see Vol. 5, Chapter 1). In contrast, in most other arthropods, as in vertebrates, where the model has been analysed in much more detail (see, e.g., Oates et al. 2012), segmentation is a temporal sequential process associated with germband elongation, where a complex circuit of genes ("segmentation clock" genes in vertebrates, such as the Delta-Notch pathway genes), oscillating in their expression in an undifferentiated field of cells, transfer a periodicity in time into a periodicity in space. Involvement of vertebrate segmentation clock genes has been documented in different arthropods (Stollewerk et al. 2003; Chipman et al. 2004b; Schoppmeier and Damen 2005; Chipman and Akam 2008; Pueyo et al. 2008; Chesebro et al. 2012), but a cyclic wave of gene expression has recently been documented unambiguously only in the flour beetle *Tribolium* for the pair-rule genes *odd-skipped* (Sarrazin et al. 2012) and *even skipped* (El-Sherif et al. 2012).

A detailed analysis of the dynamics of gene expression from the earliest appearance of the gene across the whole embryonic development has shown that a similar segmentation clock associated with waves of expression operates in the centipede *Strigamia* as well (Brena and Akam 2013), where it has been shown to characterise all segments starting from the mandibular one, i.e., all segments deriving from the *cad*-expressing hemisphere of the blastoderm (Fig. 6.9). The mandibular segment is the most anterior segment expressing the pair-rule gene *even skipped 1* (*eve1*), which has a dynamic of expression in *Strigamia* strikingly similar to that found in

Tribolium (El-Sherif et al. 2012). From a broad posterior domain, *eve1* is downregulated in concentric rings covering the whole blastoderm and centred on the blastopore/proctodeum (Fig. 6.9E; Brena and Akam 2013). These rings appear one by one as waves propagating from the posterior pole (periproctodeal area), and they extend forward even after been fixed in their cellular expression, following the general forward convergent movement of the blastoderm/germband (Fig. 6.10A–C). These early rings appear at double segment periodicity and are subsequently intercalated by stripes defining single segment, within the ventral area corresponding to the forming germband (Fig. 6.9E; Brena and Akam 2013). A very interesting character is the apparent independence, at least in *Strigamia*, of this concentric early patterning process from the germband formation and axis elongation, two processes in general considered intrinsically interconnected (Brena and Akam 2013). The gene *Delta*, part of the Notch signalling pathway involved in vertebrate segmentation, has a very similar expression pattern (Fig. 6.10D; Brena and Akam 2013).

The possible involvement of a the Notch pathway in arthropod segmentation has been suggested for opisthosomal segment formation in spiders (Stollewerk et al. 2003; Schoppmeier and Damen 2005) and for abdominal segment addition in hemimetabolous insects (Pueyo et al. 2008; Mito et al. 2011; Chesebro et al. 2012), although those data are open to alternative interpretations (Oda et al. 2007; Kainz et al. 2011).

Also *Notch* in *Strigamia* (Chipman and Akam 2008; C. Brena, unpublished data) and *Delta* in *Lithobius* (Kadner and Stollewerk 2004) appear to show an early posterior variability of expression. More evident is the irregular expression in the periproctodeal area of *eve* in *Lithobius* (Hughes and Kaufman 2002b) and of many other pair-rule genes in both *Strigamia* (Chipman et al. 2004b; Chipman and Akam 2008; Green and Akam 2013; C. Brena, unpublished data) and *Glomeris* (Janssen et al. 2011b, 2012), where these genes resolve later in rings covering the whole egg, although details of their full dynamics are unknown. The variable early expression of all these genes is consistent with a cyclical wave model, implying that a segmentation clock, by no means necessarily homologous with the vertebrate one, may be ancestral in myriapods but also in arthropods, although some genes or some gene interactions may have changed through evolutionary time.

All genes homologous to *Drosophila* pair-rule genes have in general a conserved expression in both *Strigamia* and *Glomeris* (Janssen et al. 2011b, 2012; Green and Akam 2013). They are expressed in stripes preceding morphological segmentation, hence consistent with their possible role in segment patterning. Conserved appears also the hierarchical organisation characterising these genes in *Tribolium, Drosophila*, and the spider *Cupiennius salei* as well (Ingham 1988; Ingham and Gergen 1988; Damen et al. 2000; Schroeder et al. 2004; Choe et al. 2006). Indeed, they are clearly distinguished in early expressing primary pair-rule genes and secondary pair-rule genes. The primary pair-rule genes (*eve*, *runt*, *odd-skipped*, and *hairy*, with more than one homologous of each of these in *Strigamia*) show dynamic and then concentric rings of expression. The secondary pair-rule genes (*Pax3/7-2* (*pairberry* in *Glomeris*) and *sloppy-paired* (*slp*), with *odd-paired* expressed with a segmental pattern only in *Glomeris*), are expressed only slightly ahead of the segment polarity genes, as static stripes. Notwithstanding the general appearance, the relative expression of a number of the primary pair-rule genes is divergent between myriapods and insects (e.g., the relative expression domain of the gene *runt* is divergent between flies and beetles and between millipedes and centipedes; Choe and Brown 2009; Janssen et al. 2011b; Green and Akam 2013).

This early dynamic patterning process of the primary pair-rule genes has been shown in *Strigamia* to be specifically ectodermal (Green and Akam 2013), somehow confirming what was shown in insects and crustaceans, i.e., that segmentation is an intrinsic property of the ectoderm, with metameric structure of the mesoderm

induced by the ectoderm (Hannibal et al. 2012 and discussion in Scholtz and Wolff 2013).

Contrary to *Strigamia*, in the other myriapods, the pair-rule genes (or any other early segmental patterning gene) are expressed from the beginning at single-segment periodicity (Hughes and Kaufman 2002b; Janssen et al. 2011b, 2012). Indeed, notwithstanding the name, purely indicative of the homology of these genes with the *Drosophila* ones, no pair-rule expression has been reported for segmentation genes in the few studied short germband insects and crustaceans and in general in spiders if we exclude some anterior splitting behaviour (Grbic et al. 1996; Davis and Patel 2003; Copf et al. 2003; Damen et al. 2005; Liu and Kaufman 2005). There is one report of pair-rule patterning for a *paired* (*Pax3/7*) homolog in the prosoma of a mite (Dearden et al. 2002), and a similar behaviour is shown by *pairberry-1* in *Glomeris*, where strong and weak bands of expression are alternated (Janssen et al. 2012). In *Glomeris*, also the split of some single initial bands of different pair-rule genes covering initially two segments is considered by the authors indicative of a pair-rule nature (Janssen et al. 2012). A possible pair-rule involvement in dorsal patterning in millipedes is discussed below.

These scattered data suggest that a proper deployment of an initial pair-rule patterning would have independently evolved only in higher insects and in geophilomorph centipedes like *Strigamia*, where a process of secondary intercalation of segments might have been functional for the evolution of the much higher number of segments typical of this clade. In fact, in *Strigamia* itself not all segments are pre-patterned according to a double segment periodicity, but the last ten leg-bearing segments or so are patterned individually. This is associated with a broad change in the regulatory regime where oscillatory gene expression is suppressed and at least one other transcription factor not previously active during the main phase of segmentation, *knirps*, is upregulated (Fig. 6.10E, G; Brena and Akam 2013).

These results suggest that a conserved gene network, mainly involving pair-rule genes, may be rather conserved across arthropods as it is conserved along *Strigamia* developmental stages but that the mechanisms generating the initial periodicity may differ according to the clade and/or to the cell population environment, exactly as the cell population environment changes during *Strigamia* development. In this sense, the involvement of the Notch-Delta pathway may be just a means of coupling cell oscillations during segmentation within a large field of cells (see Oates et al. 2012), as it is the case in early-mid *Strigamia* embryonic stages. This cell coordination would not be necessary anymore in a more restricted cellular environment as in late *Strigamia* stages or in *Tribolium* embryogenesis, where indeed functional studies show that *Delta* does not oscillate and is not require for segmentation (Aranda et al. 2008; see also discussion in Valentin and Oates 2013).

Delineation of Segments: Segment Polarity Genes

In *Drosophila*, after pair-rule genes have set up a periodic pattern, some of their downstream target genes, "segment polarity" genes like *engrailed* (*en*) and *wingless* (*wg*), are crucial for setting up the polarity of individual segments (Vol. 5, Chapter 1). In *Drosophila,* these initial morphological segments are parasegments – later in development to be shifted by half a segment – and their boundaries are maintained by the mutual interaction of *wg/ci* (*cubitus interruptus*) expressing cells and *en/hh* expressing cells.

Although in myriapods the first appearing furrows are properly segmental (Hughes and Kaufman 2002b; Chipman et al. 2004a) and not parasegmental as in *Drosophila* (Martinez-Arias and Lawrence 1985), in *Lithobius* (Hughes and Kaufman 2002b), *Strigamia* (Chipman et al. 2004a; Hayden and Arthur 2014), and *Glomeris*, the expression patterns of *en* and *wg* and in *Glomeris* also of *hh* and *ci* (Janssen et al. 2004) are very similar to *Drosophila* and other arthropods (e.g., Patel et al. 1989; Martinez-Arias 1993; Nagy and Carroll 1994; Niwa et al. 2000; Damen 2002). Transcripts of *en* and *hh* colocalise to posterior cells of each segment, whereas *wg* and *ci* are expressed in anteriorly adjacent cells, demonstrating the conservation of the

parasegment boundary across arthropods (see below for peculiarity of dorsal patterning in diplopods).

A similar pattern of segment definition may be involved also in postembryonic segmentation, as *en* is expressed also in these stages in the centipede *Lithobius peregrinus,* although spatial information based on in situ hybridisation is missing (Bortolin et al. 2011).

Also the relative expression of the pair rule genes *slp* and *paired* with respect to *wg* and *en* at the parasegmental boundary, appears conserved between myriapods and insects, suggesting that functional interactions between these genes might be an ancient feature of arthropod segment patterning (Janssen et al. 2011b; Green and Akam 2013). On the contrary, the role of *eve* in setting the *en* border appears to have diverged within myriapods, with *eve* overlapping *en* expression in *Strigamia* as in insects, but barely so in *Lithobius* and not at all in *Glomeris* (Hughes and Kaufman 2002b; Janssen et al. 2011b; Green and Akam 2013).

Not only *wg* may be involved in segment patterning but also other genes of the large *Wnt* family. Myriapods have a rather ancestral array of *Wnt* genes, having 11 out of the 12 ancestral arthropod *Wnt* (Janssen et al. 2010). *wg/Wnt1*, *Wnt2*, *Wnt 4-7*, *Wnt9*, *Wnt11*, and *WntA* are present in both studied myriapods, but *Wnt8* is present only in *Glomeris* and *Wnt10* only in *Strigamia* (Janssen et al. 2004, 2010; Hayden and Arthur 2014; Janssen and Posnien 2014). They almost all show a reiterated expression along the AP axis, mostly related to what may be mesodermal somites – although published works do not mention mesodermal expression.

The genes *Wnt5*, *Wnt6,* and *Wnt10* in *Strigamia* according to Hayden and Arthur (2014) as well as the genes *Wnt5*, *Wnt6*, and *Wnt16* in *Glomeris* according to Janssen et al. (2010) and Janssen and Posnien (2014) appear to have comparable expression with *wg*, in the shape of transversal stripes mostly in the middle of each segment. They briefly anticipate the appearance of the morphological segment, suggesting a possible role in segment formation. Some of them may have a specific role in dorsal segmental patterning in millipedes (see below). Nevertheless, it is rather difficult to interpret the roles of *Wnt* genes solely from their expression pattern, given that these genes encode glycoprotein ligands involved in Wnt signalling, a process that regulates a wide range of developmental processes from cell proliferation and migration to segmentation and axis elongation. In fact, several of them have a strong expression associated with the proctodeum ring, a highly active area for many patterning genes (see above).

Anterior Head Segmentation Through Splitting

In myriapods the procephalon, determined initially by a specific combination of early expressing patterning genes (see above), may follow its own specific modality of segmentation. In *Strigamia* several segmental genes (like *en* and *Delta*; C. Brena, unpublished data) or genes expressed with a segmental pattern (like *twi* and *dpp*; C. Brena, unpublished data) appear later in this area and show a clear expansion and splitting (or intercalating) behaviour which appears associated with the forward extension of the cephalic tissue (as marked also by the expression of early patterning genes like *otx*, Fig. 6.9A, B), involving the whole region anterior to (and partly including) the mandibular segment. For example, it is clear that (contrary to what was suggested in Chipman et al. 2004a) the first *en* stripes centred on the intercalary segment also give rise to the mandibular and the antennal segments (C. Brena, unpublished data). A similar pattern is shown by *hh*, where an early single band of expression gives rise to the three segmental stripes, ocular, antennal, and intercalary (Hunnekuhl 2013). This is particularly interesting because a similar *hh* splitting pattern has been described also in *Glomeris* where the slipping involving only the antennal and ocular segments may depend on the minor expansion of the head tissue in this species (Janssen 2012). Although the splitting order may change, this form of *hh* anterior segmentation is probably ancestral to arthropods as it has been described also in spiders (Pechmann et al. 2009; Kanayama et al. 2011) and *Drosophila* (Lee et al. 1992). Whether this splitting is the result only of germband expansion or whether it may involve

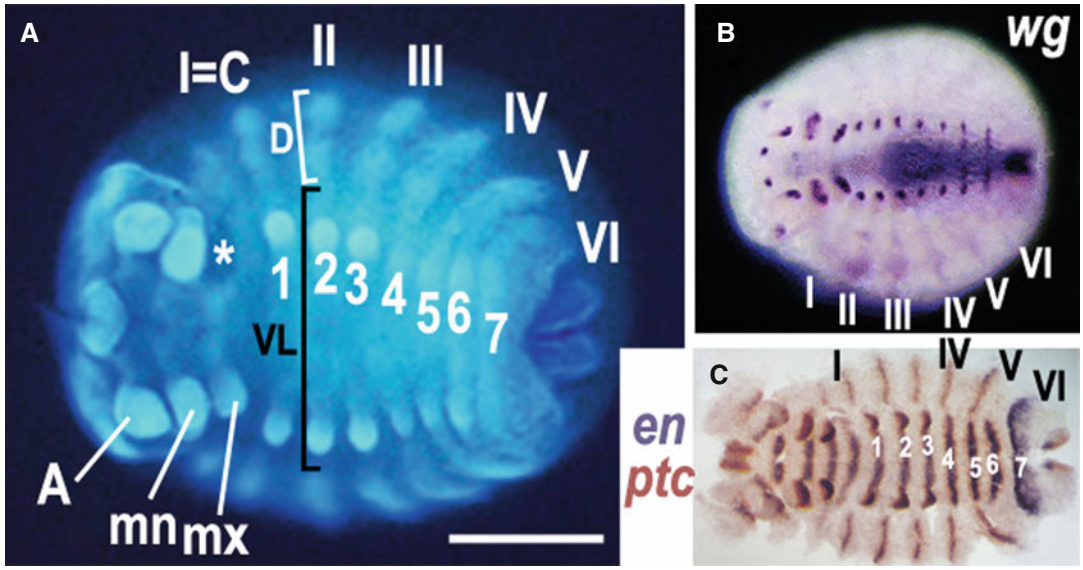


Fig. 6.11 Dorsoventral differentiation in millipedes. As for all millipedes, *Glomeris* shows a differentiation between segmentation in the ventrolateral (VL) and the dorsal (D) tissues (marked on the second trunk segment in (**A**) by a black bracket and a white bracket, respectively), both at the morphological (**A**) and gene expression level as indicated, for example, by the segment polarity genes *wg* (**B**) and *en* and *ptc* (**C**). A single "lateral plate" (I–IV), soon to give rise to a tergite, corresponds to each of the first four trunk segments, although they are forward shifted in relation to the ventral tissue during development (partly evident already at the stage shown here), while only one lateral plate, developing later into the first dip-lotergite (V), corresponds to the fifth and sixth ventrolateral leg-bearing segments, in correlation with the lack of dorsal expression of trunk stripe 6 of *en* and *ptc* (panel **C**). The first trunk tergite will eventually form the collum (**C**). All embryos are roughly of a similar age; (**A**) *G. pustulata* (DAPI fluorescent nuclear staining to show the morphology) and (**B**, **C**) *G. marginata*. All panels are in ventral view, anterior to the left. A antenna, mn mandible, mx maxilla – note that the maxilla left bud (asterisk) was accidentally lost during dissection; 1–7 indicate trunk, leg-bearing segments. Scale bar in (**A**) 200 µm. (**B**, **C** Modified after Janssen et al. (2008))

spreading of wave of expression as suggested by Kanayama et al. (2011) or by both processes is unknown.

Dorsoventral Differentiation

With the exclusion of centipedes, all other myriapods show some level of non-correspondence between the dorsal and the ventral subdivision of the exoskeleton. This complexity in identifying "segments" has been the source of endless disputes since the nineteenth century among zoologists. This has particularly been the case for diplopods, where there is a distinction between the first few segments (haplosegments, traditionally considered three, but see below), where tergites correspond to single leg pairs, and most of the trunk segments which are diplosegments, with a single tergite per pair of segments (see Janssen et al. 2006) for a general discussion on the issue).

To explain this lack of strict correspondence between tergites and ventral structures, a possible independent segmentation system for the ventral and dorsal side of the animal, was proposed, based on an apparent differentiation of some segmentation genes between the ventral and the dorsal anlagen of the first laid-down segments (basically the haplosegments) (Janssen et al. 2004, 2006, 2008, 2011b; Janssen and Posnien 2014). Among the key reasons for this hypothesis are the dislocated expression of segmentation genes (e.g., *en* is not at the posterior margin but in the middle) on the lateral plates (considered "dorsal segments" by Janssen et al. 2004; see above) and the apparent lack therein of proper *wg* expression in form of a stripe overlapping *en* expression

(see, e.g., Fig. 6.11B, C). In that sense, Janssen and Posnien (2014) hypothesise that *Wnt4* may be a substitute of *wg* for dorsal segmentation.

Unfortunately, the proper nature of the "dorsal segments" is not clear, and the hypothesis of dorsoventral decoupling of segmentation does not take into account the possibility that this apparent middle expression of *en* or *hh* on the lateral plates may just reflect a possible forward shifting of the ectoderm expressing *en* or *hh* on top of the dorsal mesodermal units, part of a migration and bending process that affects all the lateral (hence also dorsal) sides of the segments as they extend laterally and bend forward during the initial part of the process that will lead to the dorsoventral flexure (Figs. 6.3D–F and 6.11A). This later expansion could be also responsible for the (apparent) lack of medio-lateral continuity between the ventral and dorsal expression in the first four or so trunk segments of some genes like *en* (Fig. 6.11C). In fact, at trunk segment 5, where the bending/displacement of the dorsal side is very limited, there is a full dorsoventral continuity of *en* (or any other gene; Fig. 6.11C). Most importantly, there is in general a dorsoventral continuity – in fact all around the egg extending also in the undifferentiated regio dorsalis – of the pair-rule genes, the genes probably involved in determining the initial periodic – hence segmental – pattern (Janssen et al. 2011b, 2012). These genes do bend later as to match the dorsal position of segment polarity genes like *en* which, even if upregulated independently from the ventral side, will be in the expected position to be a normal downstream target of pair-rule genes. Indeed, the conserved function of polarising the posterior of the (dorsal ectodermal) segment is conserved, as *en* (and all other gene expressed there) later on in development marks the posterior boundary of the tergites (e.g., Janssen et al. 2004).

Resolving this issue in the diplopods will require identification of germinal layers and the degree of tissue migration. It will also require understanding of which are the limits between dorsal and ventrolateral tissues, in particular given that, apparently, published data show that no segmentation gene is really specific for the whole ventrolateral tissue as opposed to dorsal tissue, *wg*, or other *Wnt* genes included (compare Fig. 6.11A with 6.11B).

According to the published gene expression patterns, what really could appear to distinguish the dorsal from ventral side of the millipede embryo is the lack of complete dorsal extension of pair-rule and segment polarity gene stripes on trunk segment 6 and 8, exactly in correspondence with the first diplotergites (e.g., Fig. 6.11C and *eve* and *en* in Fig. 6.3B in Janssen et al. 11). In this sense, if this pattern is in particular conserved postembryonically for the following segments, there would be a rather straight correspondence involving a specifically dorsal pair-rule segmentation system at the origin of the diplosegments of millipedes. In other words, the diplotergites would be the outcome of an undivided dorsal tissue, in contrast with Janssen's (2011) suggestion that diplosegments in *Glomeris* are the result of fusion of the dorsal tissue of two adjacent segments, based on the pattern of later expressed genes like the myogenic gene *nautilus*.

All these published data on *Glomeris* – and in particular their reinterpretation – leave open the possibility that in fact all the complex mismatch between dorsal and ventral metameric structures in diplopods may be simply the result of two developmental processes affecting on one side the haplosegments and on the other the diplosegments. Indeed, the morphology and *en* expression pattern across all stages in Janssen et al. (2004) shows that the first segments up to the fifth are patterned uniformly singularly and should be considered from this point of view as the real haplosegments. Only subsequent movements shift the dorsal portion of the trunk segments forward, in such a way that the tergite of the first trunk segment becomes the collum, and the fifth tergite covers at the end of embryogenesis (almost) both the fourth and the fifth pair of legs. A similar relative dorsoventral tissue shifting might be indeed common in the development of other arthropods, such as the trilobites (Ortega-Hernández and Brena 2012). All following segments would be simply patterned coaxially but with a pair-rule system affecting only the dorsal, i.e., tergite, portion of the segmental units to produce diplosegments.

The apparent double segmentation of pauropods is of a completely different nature: contrary to what is commonly believed, the supposed "diplotergites" of *Pauropus* are just alternated single-segment tergites (starting from the second trunk segment) which, during postembryonic development, extend over each intercalated following segment, characterised by a reduced tergal wall and without a tergal shield (see, e.g., Fig. 6.7A and Tiegs 1947a). A similar simple developmental explanation shows that also in symphylans segments arise dorsoventrally uniformly at single-segment periodicity (Fig. 6.4E) but quite soon during development additional grooves subdivide the dorsal portion of some segments (Fig. 6.4F) giving rise to more than one scute per segment (Fig. 6.7B). These scutes will increasingly overlap only during postembryonic development, to the extent that in the adult it is difficult to recognise the correspondence between scutes and legs (Tiegs 1940).

Variability of Segment Number in Geophilomorphs

Myriapod species characterised by a large number of segments like helminthomorph millipedes and geophilomorph centipedes are characterised by extended interspecific variability in the number of segments, a variability which is often also intraspecific and which in geophilomorphs is most of the time also intersexual, with females having a modal value higher by two segments. Only geophilomorphs produce all their leg-bearing segments embryonically (but see Brena 2014), implying that their actual segment number is determined by the genetic and developmental conditions and not simply by the age of the animal as in many helminthomorph millipedes. Geophilomorphs are in fact a good model for evolutionary studies on segmentation, having a trait, the number of segments, variable, hence potentially subject to natural selection. Indeed, this trait would appear to have a hereditary component (Vedel et al. 2009) but also a clear environmental component, where temperature during developmental time is directly proportional to the final number of segments produced (Vedel et al. 2008); this explains the inverse relationship towards latitudes on natural population (Kettle and Arthur 2000). Interesting enough, temperature affects the final number of segments before the morphological segmentation starts or very early in the process, implying that the number of segments is influenced by the initial conditions of the embryo (e.g., its number of cells) more than by the segmentation process itself (Vedel et al. 2010). The genetic component associated with sex operates very early as well, with females reaching their higher number of segments well before the whole segmentation process is finished (Brena et al. 2013). This implies a genetic component on the way the segmentation process is set off, more than an effect on the duration of the process, or an association with the formation of the terminal genital segments.

Notwithstanding the high degree of variability in segment number, geophilomorphs, as all centipedes, have only even numbers of trunk segments, including the forcipular segment. The initial patterning of segments in pairs seems to explain very clearly this very strong developmental constraint (Chipman et al. 2004b). The discovery that the final phase of segmentation is at single-segment periodicity (see above) requires a more complex evolutionary scenario. The variable generation of segments by pair-rule patterning would have been imposed on an invariant underlying body plan, the one basically characterising all other centipedes where indeed segments, at least according to what we know from *Lithobius* (Hughes and Kaufman 2002b), are produced singularly (Brena and Akam 2013).

Hox Genes

Hox genes encode homeodomain-containing transcription factors that, by interacting with a large number of downstream targets (Pavlopoulos and Akam 2011), modulate many aspects of segment differentiation and development, ultimately playing a key role in specifying regional identity across the AP axis and, consequently, in the evolution of animal diversity (Akam 1998). Studied myriapods retain the whole arthropod ancestral complement of ten Hox genes: *labial* (*lb*), *proboscipedia* (*pb*), *Hox3*, *Deformed* (*Dfd*),

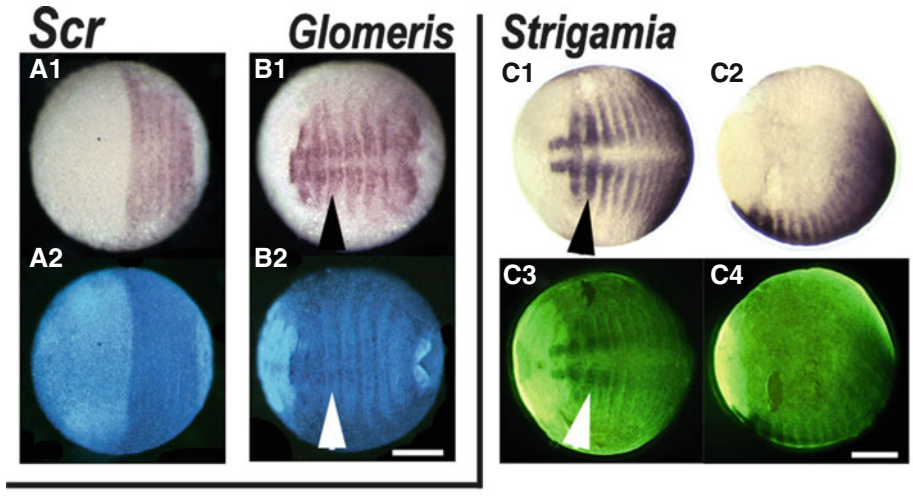
Sex combs reduced (*Scr*), *Antennapedia* (*Antp*), *fushi tarazu* (*ftz*), *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*). Except for the two *Hox3* genes, in *Strigamia* they are organised in an intact, well-ordered Hox cluster containing one ortholog of each of them (Chipman et al. 2014).

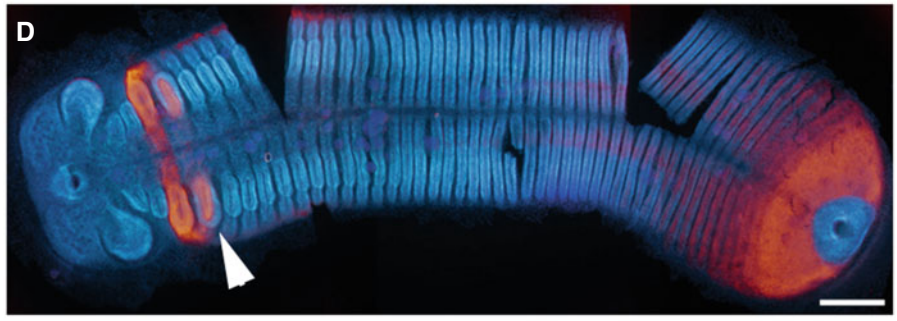
The expression pattern of the ten Hox genes has been studied in the millipede *Glomeris* (Brena et al. 2005; Janssen and Damen 2006) and in the centipedes *Lithobius* (Hughes and Kaufman 2002a) and *Strigamia* (Brena et al. 2006; C. Brena, unpublished data), and the Ubx/abd-A protein distribution has been shown in the scolopendromorph centipede *Ethmostigmus rubripes* (Grenier et al. 1997). Among these species there is a general conservation of the expression pattern, in fact, even in contrast with the different segmental specialisation that characterises millipedes and centipedes (with part of the peculiarities reported for *Lithobius* – Hughes and Kaufman 2002a – probably attributable to less clear in situ hybridisation data).

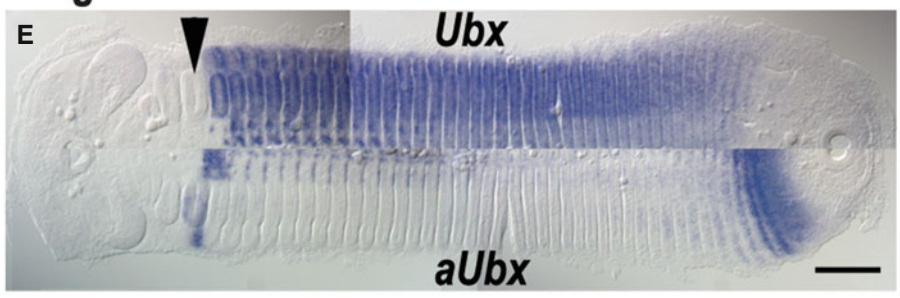
More than in any other arthropod, in myriapods the Hox gene expression domains overlap extensively, although the positioning of the anterior margins along the AP axis – and presumably their transcription – follows the canonical order in the cluster (collinearity); *Hox3a* and *Hox3b* in *Strigamia*, absent from the cluster in the genome, have a peculiar and distinct pattern restricted to the early stages and to the undifferentiated posterior pole around the proctodeum.

Contrary to the posterior margin, the anterior border is always well defined, but not only the boundaries of expression are slightly different along the medio-lateral axis (often with a more or less anteriorly extended expression on the medial neurogenic region), but they may also change during development (see, e.g., in *Strigamia Scr* in Fig. 6.12A–D and *antp*, *Ubx*, and *abd-A*; Brena et al. 2006), making somehow arbitrary the

Fig. 6.12 Hox genes. (**A**–**D**) Expression pattern of *Scr* is shown as an example of Hox gene expression, with its variability across stages (**A**–**D**) and between species, the millipede *Glomeris marginata* (**A**, **B**) and the centipede *Strigamia maritima* (**C**, **D**). The domain of *Scr* changes from a more uniform expression in late blastoderm stages (**A**) to segmental differentiation in mid stages, in particular between medio-ventral neuroectoderm and latero-dorsal tissues (**B**, **C**), to downregulation of expression in *Strigamia* in more mature (central) segments, with the exclusion of the two segments at the anterior border of expression (**D**). Note how at this stage, *Scr* is expressed in the whole ectoderm in the second maxilla segment while only in the mesoderm in the maxilliped (arrowhead). Although the general expression may be similar in similar stages (**B**, **C**), the precise anterior border of expression varies in the two species, with *Scr* being expressed at this stage only in the neuroectoderm of the first maxilla in *Strigamia*. In (**A**–**C**) the same embryos are viewed on top in normal light to show pattern of gene expression and below under fluorescent light to show the morphology as highlighted by DAPI (in A2, B2, and D) and SYBR Green (in C3-4) nuclear staining; B1–2 and C1, C3 ventral view; C2 same embryo as in C1 but in lateral view. In (**D**) false-coloured *Scr* staining has been overlayed on the germband viewed in fluorescent light to show the morphology (DAPI nuclear staining). (**E**) *Ubx* and antisense *Ubx* (*aUbx*) expression in *Strigamia* in a composite view of two mid-late germbands of the same stage aligned by inspection of DAPI-stained images and juxtaposed along the midline (Nomarski optics). *aUbx* expression precedes temporally *Ubx* at the posterior of the germband and has a more anterior expression domain including the maxilliped segment, and the two have a complimentary expression where the two domains correspond to each other. (**D**, **E**) are flat-mounted preparation. In all panels, anterior is to the left; arrowhead marks the first trunk segment (first leg-bearing segment in *Glomeris* and maxilliped in *Strigamia*). Scale bars (**A**, **B**) 200 μm; (**C**) 300 μm; (**D**, **E**) 200 μm. (**F**) Schematic representation of simplified Hox gene expression domains along the AP axis of myriapods, based on the millipede *Glomeris*, on the centipede *Strigamia* and, partly, on the centipede *Lithobius*. The actual expression domains may slightly vary between stages, tissues, and species (see above *Scr* expression and discussion in the main text). The anterior expression domain of *Hox3* and the anterior margin of expression of *Scr* and *abd-A* as reported here are specific to *Glomeris*. Diplop diplopods, Chilop chilopods. Anterior to posterior segments: oc ocular, A antennal, i intercalary, pmd premandibular, mn mandibular, mx maxillary (mx1 as first maxilla in centipedes), mx2 second maxillary, mxp maxillipedal, pmx postmaxillary, L leg bearing, pa periproctodeal area, av anal valves/proctodeum. Numbers of leg-bearing segments (arrows) with a uniform Hox expression vary between species. (**A**, **B** Modified after Janssen et al. (2006))







F Diplop / Chilop	oc	A	pmd / i	mn	mx / mx1	pmx / mx2	L1 / mxp	L2 / L1	L3 / L2	L4 / L3	→	pa	av
lab			■	■									
pb			■	■	■								
Hox3			■									■	
Dfd				■	■	■	■	■	■	■	■	■	
Scr					■	■	■	■	■	■	■	■	
ftz						■	■	■	■	■	■	■	
Antp							■	■	■	■	■	■	
aUbx							■	■	■	■	■	■	
Ubx								■	■	■	■	■	
abd-A									■	■	■	■	
Abd-B												■	■

positioning of the anterior margin of a bar on a diagram of expression (Fig. 6.12F).

Hox genes are in general expressed early within a large area of the blastoderm, well before the onset of overt segment formation; they are excluded from the first two head segments and are mostly within the hemisphere expressing *cad* (see above), known to be a regulator of Hox genes in vertebrates (Subramanian et al. 1995). The first three genes, *lab*, *pb*, and *Hox3* (only *Hox3b* and, only temporarily, in *Strigamia*, in addition to the posterior expression), appear as stripes defining the intercalary segment, otherwise not marked by any morphology in myriapods, testifying the conserved patterning of the tritocerebrum.

With the exception of *Abd-B*, relegated to an expression around and inside the invaginating proctodeum, all the following genes are expressed in myriapods across the whole trunk, with *Dfd*, *scr*, and *ftz* fading their expression in the middle of the AP axis as segments mature, except, for *ftz*, in the neuroectoderm.

In the posterior region, morphologically uniform, accumulation of Hox gene transcripts is in general strongly modulated by the maturing segment pattern (Fig. 6.12D) in *Strigamia* even at the initial double segment periodicity (see *Antp* in Brena et al. 2006), suggesting regulatory interactions at multiple levels of the segment patterning machinery. In this sense there is no reason to consider *ftz* more involved in segmental patterning than any other Hox gene as suggested by Hughes and Kaufman (2002a) based on the derived specific function that this gene has as a pair-rule gene in *Drosophila*.

In general, although the anterior margin of expression may slightly change between species and not fit with the margin of a given gene, in myriapods Hox genes follow the Hox code like all other arthropods, in such a way that every (anterior) differentiated segment has a specific combination of Hox genes, and all the uniform trunk segments express the same genes. Although this is rather strict in *Strigamia*, in *Glomeris*, surprisingly, the first three leg-bearing segments, morphologically uniform, show a differentiation of Hox gene expression, with, in particular, the first trunk segment matching the expression of the corresponding, differentiated, forcipular segment of *Strigamia* (Fig. 6.12F). This means, additionally, that the specific Hox code of this first trunk segment of *Strigamia* alone cannot explain the differentiation of maxillipeds in centipedes, as it has been well documented for other arthropods.

A peculiarity of *Ubx* expression and regulation, with some similarity with *Drosophila*, is the transcription from the opposite DNA strand of an antisense *Ubx*, overlapping the homeobox exon and expressed in an axially restricted pattern comparable to, but distinct from, those of the *Ubx*-coding transcripts (Fig. 6.12E). The expression pattern of *Ubx* sense and antisense transcripts is strikingly complementary, suggesting the possibility of antisense regulation of *Ubx* expression through a form of transcriptional interference (see Janssen and Budd 2010 for discussion on possible models of transcriptional regulation). This character must be ancestral in myriapods since it is present in *Strigamia*, *Lithobius*, and *Glomeris*, where this transcript shows the same pattern (Brena et al. 2006; Janssen and Budd 2010).

Limb Specification

In general, myriapods show a strong conservation of the *Drosophila*-like genetic cascade which is at the core of early limb differentiation. Early limb determinants are the morphogens *dpp* and *Wnt*, which are involved in determining the early gradients activating downstream limb genes: *dpp* is expressed in all limb buds in *Glomeris* (Prpic 2004) and *Lithobius* and *Strigamia* (C. Brena, unpublished data), and several *Wnt* genes are there expressed as well in *Glomeris* (Prpic 2004; Janssen and Posnien 2014) and *Strigamia* (Hayden and Arthur 2014), where they could operate in a redundant and/or in a combinatorial way, as they do in other arthropods (see discussion in Janssen and Posnien 2014). In particular, some of them, like *Wnt5* in *Glomeris*, may be involved in limb dorso-(ventral) patterning, as are probably the *dpp* and the *optomotor blind* genes, in a conserved way as in *Drosophila*, while the role

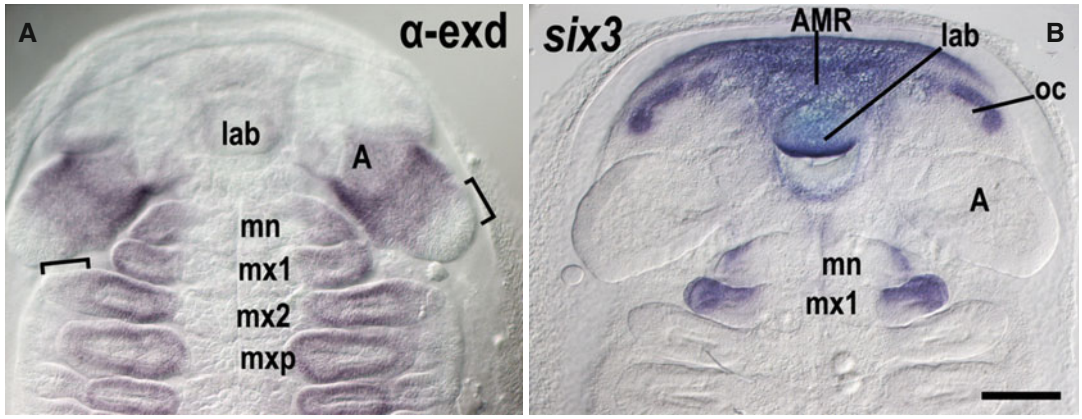


Fig. 6.13 Limb and labrum specification. Extradenticle protein domain (**A**), as shown by antibody (α-exd) and *six3* gene expression (**B**) in the head of a mid-late germband of the centipede *Strigamia maritima*. exd is excluded from the tip of most limb buds as marked by brackets on antenna (A) and second maxilla (mx2) with the exception of the mandible (mn) and the first maxilla (mx1). *six3* has a specific expression associated with the anterior median region (AMR), the anterior ocular lobes (oc), and the labrum (lab); in these late stages, it is also specifically expressed in part of the mandible and the first maxilla. mxp maxilliped. Flat mounted preparation viewed under Nomarski optics; anterior is to the top. Scale bar: 100 μm

of *H15* in ventral patterning is not as strongly supported by its expression (Prpic 2004; Prpic et al. 2005; Janssen and Posnien 2014).

Although there are some minor differences compared to *Drosophila*, the expression of *Distal-less* (*dll*), *dachshund* (*dac*), *extradenticle* (*exd*), and *homothorax* (*hth*) in *Glomeris* (Prpic and Tautz 2003) and *exd* in *Strigamia* (Fig. 6.13A) is compatible with a conserved role in providing positional identity along the proximo-distal axis of the limb, with *exd* and *hth*, *dac*, and *dll*, respectively, defining a proximal, a medial, and a distal identity. In *Glomeris* this pattern of expression is common in general to all legs and antennae limb buds but is differentiated in the mandibles and maxillae where *dll* is expressed uniformly only early (later only in a small domain, possibly in correlation with forming sensory organs), and *dac*, expressed in general as a ring outside the *dll* domain, is expressed in central spots, demonstrating the gnathobasic nature of these limbs (Prpic and Tautz 2003). This character has been verified for the mandible with a dll antibody in *Glomeris* (Scholtz et al. 1998) and in the polydesmid millipede *Oxidus gracilis* (Popadić et al. 1998) and for both mandibles and maxillae with an exd antibody in *Strigamia* (Fig. 6.13A), revealing a condition somehow common and possibly homologous to all other Mandibulata, where *dll* is either expressed only in association with mandibular palps (in crustaceans) or never expressed (in insects) (see Edgecombe et al. 2003). Note that in *Glomeris*, *dll* is transiently expressed also in the limbless intercalary and postmaxillary segment (Prpic and Tautz 2003) and that *H15* is not expressed ventrally in the maxillae, mandibles, and antennae (Prpic et al. 2005). Other genes like *six3* may have a specific expression in mandibles and first maxillae (Fig. 6.13B).

Labrum Specification

In myriapods, *dpp*, *dll*, and several *Wnt* genes are also expressed in the labrum, and indeed its possible homology with other appendages has been a matter of controversy for a long time (see, e.g., discussion in Posnien et al. 2009). This hypothesis has been in part supported by the fact that the labrum anlage is bilobed in *Glomeris* (Dohle 1964) as it is in several other arthropods, representing, in this hypothesis, the possible ancestral pair of limb buds of the ocular segment, fused later in evolution to form a single medial structure. Indeed, some early pat-

terning genes do show an initial bilateral expression in two spots, such as *dpp* in *Lithobius* (C. Brena, unpublished data.) and in *Glomeris* (Prpic 2004) and several *Wnt* genes in *Glomeris* (Janssen and Posnien 2014). Nevertheless, in *Strigamia*, where the labrum is always undivided from early stages onwards, *dpp* and *wg* never appear to have a bilateral expression (Hunnekuhl 2013; C. Brena, unpublished data) although for the other Wnt genes, the early expression is unclear (Hayden and Arthur 2014). On the other hand, Dll/*dll* expression is undivided both in *Glomeris* (Scholtz et al. 1998; Prpic and Tautz 2003) and *Oxidus* (Popadić et al. 1998), although its expression is shown only for mid-germband stages.

Regardless of the bilateral expression, the labrum primordium in *Strigamia* derives from a specifically patterned region, the anterior medial region (AMR), with its characteristic gene expression profile, including gene markers such as *six3, nk2.1, rx, hbn, and FoxQ2* (Fig. 6.13B; Hunnekuhl and Akam 2014). The expression profile of this region is in good part conserved between *Strigamia, Drosophila, Tribolium,* and *Oncopeltus* (see discussion in Hunnekuhl and Akam 2014) but is specific and distinct from the other appendages, which means that homologies between the labrum and the proper limbs are at least questionable.

Neurogenesis

Several proneural genes are known to be necessary for neural differentiation in *Drosophila*, and some homologous genes are expressed in myriapod neuroectoderm cells as they differentiate into neural precursors. The neurogenic genes *achaete scute (Ash)* (Dove and Stollewerk 2003; Kadner and Stollewerk 2004), *daughterless (da), Atonal (At), SoxB1, and snail (sna)* (Pioro and Stollewerk 2006) as well as *muscle segment homeobox (msh)* (Döffinger and Stollewerk 2010) are expressed in the neuroectoderm of the millipede *Glomeris* and part of them also in *Archispirostreptus* and the centipede *Lithobius*. Their expression shows that components of the genetic network involved in specification of neural precursors are rather conserved across the arthropods, although some changes have appeared during evolution. For example, the gene *At*, unlike its *Drosophila* homolog, is expressed in *Glomeris* in the cephalic appendages in a similar way as *dll* (Prpic and Tautz 2003); hence it is presumably associated with external sensory organs differentiation (Pioro and Stollewerk 2006).

Other conserved genes are associated with neural differentiation. The neurogenic genes *Notch* and *Delta*, which restrict the proportion of cells that adopt a neural fate at a certain time, are detected in both millipedes and centipedes in most neuroectodermal cells but accumulate at higher concentration in the invaginating neural precursor (Fig. 6.14B, C1, C2) (Dove and Stollewerk 2003; Kadner and Stollewerk 2004; Chipman and Stollewerk 2006). In *Strigamia*, *Delta* transcripts appear to accumulate at higher levels in single cells within the invagination groups. Although this apparent concentration may be due to the invaginating processes of the surrounding cells (Stollewerk and Chipman 2006), protein staining seems to confirm this single-cell higher expression (Fig. 6.14C1, C2).

Additionally, in *Strigamia*, the early patterning genes *Krüppel*, *hunchback* (Fig. 6.14A) (Chipman and Stollewerk 2006), and *engrailed* (Stollewerk and Chipman 2006) are expressed (or expressed at higher level) in subsets of neural precursors, possibly conferring them temporal identity, as they do in *Drosophila* (Isshiki et al. 2001).

In accordance with general timing of development, neurogenesis occurs simultaneously in the head and first trunk segments in *Glomeris* and *Lithobius* (Dove and Stollewerk 2003; Kadner and Stollewerk 2004; Pioro and Stollewerk 2006), as in the prosoma of spiders (Stollewerk et al. 2001). On the contrary, it is not synchronised, and there is an anterior-posterior gradient in neurogenesis in the millipede *Archispirostreptus* (Pioro and Stollewerk 2006), in the trunk segment of *Lithobius* (Kadner and Stollewerk 2004) and in all segments of *Strigamia* (Chipman and Stollewerk 2006; Stollewerk and Chipman 2006).

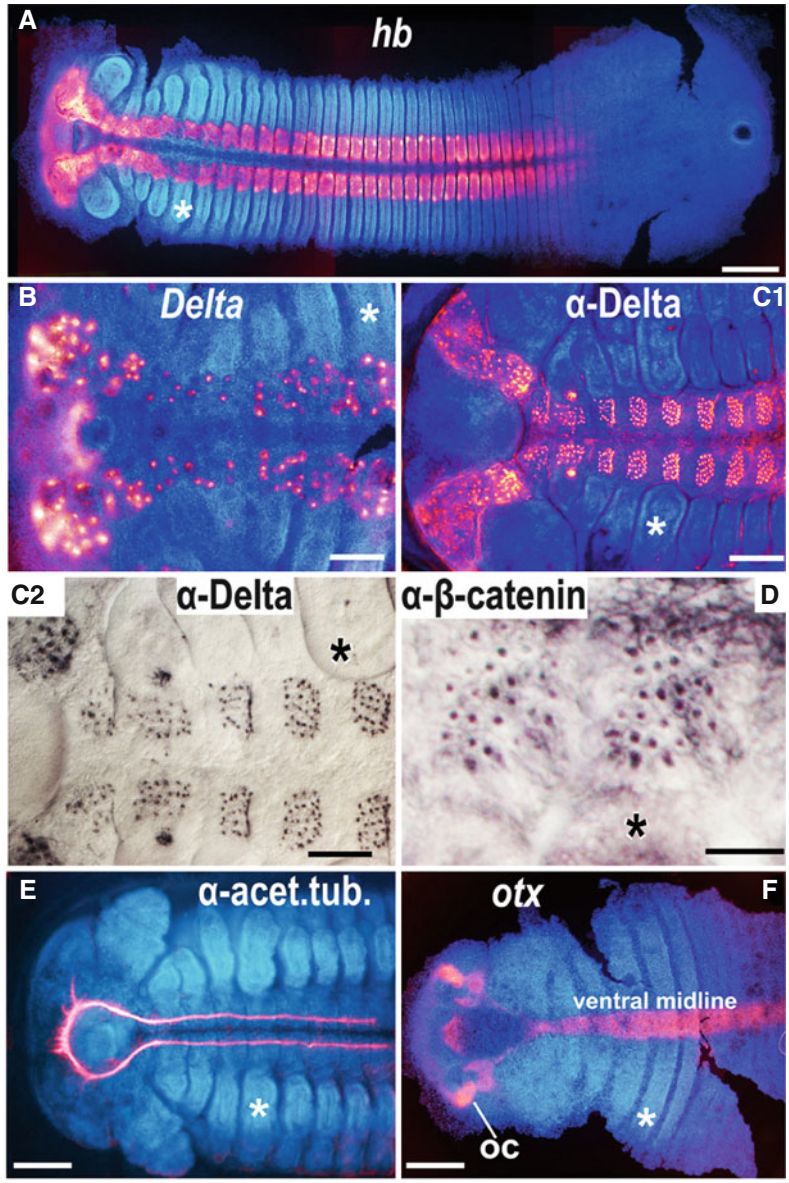


Fig. 6.14 Neurogenesis. (**A**–**D**) Molecular differentiation of the ventral neuroectoderm in the germband of the centipede *Strigamia maritima*, as shown by the expression of *hb* at mid-segmentation stage (**A**) and by the expression in specific invaginating neural precursor cells of the gene *Delta* in an early germband (**B**) and of the protein Delta in a late germband (C1 and C2) (Delta antibody, α-Delta, was specifically raised against *Strigamia* Delta). Invaginating neural precursor cells show also a specific presence of nuclear β-catenin (**D**). (**E**) In late *Strigamia* germband stages, in a centipede-specific way, a group of cells within the anterior medial region (AMR) give rise to pioneering axonal tracts progressively extending backwards, as shown by antibody against the neural marker acetylated tubulin (α-acet.tub.). (**F**) In addition to its anterior domain (oc ocular lobes), *otx* is expressed from early germband stages along the ventral midline, which is associated with neural development. In (**A**, **B**, **C1**, **E**, **F**) false coloured staining has been overlayed on the germband viewed in fluorescent light to show the morphology (DAPI nuclear staining); **C2** is a high magnification of **C1** viewed with normal light under Nomarski optics. All panels are flat-mounted preparations; anterior is to the left; asterisks mark the maxilliped segment. Scale bars: (**A**, **F**) 200 μm; B, C1, (**E**) 100 μm; (**C2**) 50 μm; (**D**) 20 μm

In contrast to insects and malacostracan crustaceans, where single stem cells from the ventral ectoderm (the neuroblasts) give rise to neural cells through a budding process, in myriapods and chelicerates all ventral neuroectodermal cells give rise to neural cells (Brenneis et al. 2013). These cells are distinct from the surrounding cells by the presence of nuclear β-catenin, a marker of the Wnt/β-catenin signalling pathway which is involved in neurogenesis (see, e.g., Zhang et al. 2011; Demilly et al. 2013). In general, these cells are post-mitotic immature neurons/glial cells that immigrate into the embryo as group of neural precursor ("cell internalisation sites" sensu Brenneis et al. 2013), composed of five to nine subapical, flask-shaped cells. They have been indentified in millipedes (Dove and Stollewerk 2003; Pioro and Stollewerk 2006), centipedes (Kadner and Stollewerk 2004; Chipman and Stollewerk 2006; Stollewerk and Chipman 2006), and symphylans (Mayer and Whitington 2009; Whitington and Mayer 2011). In all arthropod species investigated, independently of the different modes of neurogenesis and the minor differences in proneural gene expression, there are around 30 of these transient groups of neural precursors or neuroblast per hemisegment, arranged in seven transverse rows (see, e.g., Fig. 6.14C1, C2 and discussion in Döffinger and Stollewerk 2010). This condition supports the hypothesis that this stereotyped pattern of neuroblasts or neural precursors has been present in the last common ancestor of arthropods and is required for the generation of the highly conserved spatial pattern of the axonal scaffold (Döffinger and Stollewerk 2010).

Although proper neuroblasts are considered to be an apomorphy of the Tetraconata, in contrast to studied euchelicerate representatives, where mitosis occurs scattered in the neuroectoderm, in myriapods cell proliferation appears to be associated with the cell internalisation sites, indicating that some kind of neural stem cells may be involved in neurogenesis (Dove and Stollewerk 2003; Kadner and Stollewerk 2004; Whitington and Mayer 2011). Additionally, in *Strigamia* each cell internalisation site includes a larger cell, with a stronger neurogenic transcription profile. Both aspects could represent a first specialisation step "towards" tetraconate neuroblasts, in an evolutionary context where the diffuse post-mitotic neural precursor differentiation appears as a symplesiomorphy of chelicerates and myriapods (Brenneis et al. 2013).

Later in development in myriapods, the whole hemisegmental neuroectoderm invaginates and is then overgrown by the epidermis, giving rise to apical cell regions called "ganglionic pits" or "ventral organs", which will eventually differentiate as early hemiganglion anlagen, as described in classical developmental studies in scolopendromorph centipedes (Heymons 1901), scutigeromorph centipedes (Knoll 1974), symphylans (Tiegs 1940), and pauropods (Tiegs 1947a) (see also Fig. 6.6). Intriguingly, these histological studies reported cell divisions among those cells – especially as shown for pauropods by Tiegs (1947a) – which appear to be very similar in Pycnogonida (Brenneis et al. 2013). Nevertheless, a proper description of distribution of cell division among neural precursors in myriapods is still missing, although this would be crucial to understand neurogenesis evolution among arthropods, as discussed at length by Brenneis et al. (2013).

Cell internalising sites, as described above, are located throughout the whole neuroectoderm including the whole head with the ocular region, with the exception of the anterior medial region (see, e.g., Fig. 6.14C1; Hunnekuhl 2013; Hunnekuhl and Akam 2014; C. Brena, unpublished data). Later in development, from this region in *Strigamia*, some medio-ventral cells, arranged as a crescent anterior to the labrum, express the apical organ markers *collier (col), pro-hormone convertase 2 (phc2), and orthopedia (otp)* and differentiate into neural cells, later sinking beneath the epidermis. These cells then give rise to pioneering axonal tracts that will progressively extend with a long range backwards, a primary axonal scaffold of the central nervous system (Fig. 6.14E; Hunnekuhl 2013; Hunnekuhl and Akam 2014). This is a rather peculiar

axonogenesis described only for centipedes – as it has been described also in the scolopendromorph centipede *Ethmostigmus rubripes* (Whitington et al. 1991). Axonogenesis by segmental neurons begins later in development.

Before giving rise to the primary anterior axons, these cells at the centre of the anterior median region show a neurosecretory transcription profile typical of the apical organ of some marine spiralian larvae, e.g., the polychaete annelid *Platynereis dumerilii* (Vol. 2, Chapter 9), including nested domains of *FoxQ2* and *six3* (see Fig. 6.13B). They also express markers of vertebrate hypothalamic neurons, including *otp, neurophysin (vtn), and ventral anterior homeobox 1 (vax1)* (Hunnekuhl and Akam 2014).

In both *Strigamia* and *Glomeris*, also the midline cells, expressing *otx* (Fig. 6.14F) and *single minded (sim)*, a key factor for their determination, have been involved in neural development. A comparison of their expression pattern has shown that unpaired midline precursors evolved from the bilateral median domain of the ventral neuroectoderm in the last common ancestor of Mandibulata (Linne et al. 2012).

OPEN QUESTIONS

- What is the real nature and evolutionary origin of the yolk pyramids of higher centipedes? Does the migration of cells through the intrapyramidal spaces condition the distribution of genetic signal on the blastoderm?
- How is the apparent symmetry of the blastoderm broken and are there any similarities/homologies with spiders?
- How is gastrulation accomplished and mesoderm and endoderm genetically determined and subsequently formed?
- What is the relationship between the multilayered part of the blastoderm and the early genetic patterning of the egg?
- How do cell size, movement, and proliferation affect early patterning and the segmentation process? How are they involved in determining the final number of segments in geophilomorphs or the larger differences in segment formation between those centipedes and all the anamorphic myriapods?
- How does the cell population environment affect the deployment of a dynamic or a static segmentation process?
- What is the morphogenetic condition for pair-rule gene activity? To what extent is it present outside geophilomorph centipedes? Is it implicated in segment formation in *Scolopendropsis duplicata*, a scolopendromorph centipede with the number of segments almost duplicated?
- Is indeed pair-rule patterning involved in dorsal segmentation in millipedes? What are the real nature and the real limits of dorsal tissue in diplopods?
- What are the similarities between symphylans and the larger myriapods and could they be established as a more proper model system?
- How are neuromuscular and other major organ systems formed in the various myriapod subgroups?

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Index

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