Comparative development of spiralian larvae

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Scientific environment

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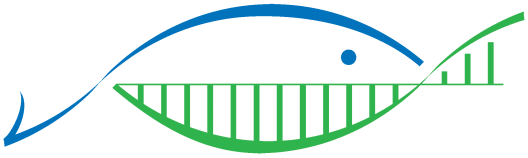

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Abstract

Bryozoans and brachiopods are sessile, mostly marine animals, that use an elegant crown of tentacles for filter-feeding. They are related to molluscs, segmented worms and other animals in a outstandingly diverse group of invertebrates named Spiralia. Most spiralians show a conserved pattern of embryonic development—spiral cleavage—but bryozoans and brachiopods deviate from their relatives. To better understand the developmental diversity and evolution of spiralian development, I examine bryozoan and brachiopod embryogenesis and larval morphology in comparison to other spiralians.

Some bryozoans develop through a unique stereotypic cleavage with biradial symmetry, and lack spiral cleavage. Here I describe the first detailed cell lineage of the bryozoan *Membranipora membranacea* by tracing the fate of embryonic blastomeres from the egg until the larval stage. I further investigate the molecular patterning of the larvae by analysing the expression of conserved developmental genes. Our data reveals several similarities between the fate map and gene expression of *M. membranacea* and the typical spiral-cleaving embryos, despite the loss of the spiral symmetry. The cell lineage resemblance might be a direct modification of the spiral cleavage pattern, or alternatively, be an evolutionary convergence that reflects a conserved underlying molecular patterning of the embryos.

Adult brachiopods do not have a segmented body, but their larvae have body boundaries that resemble annelid segments. To test whether genes involved in the patterning of segment boundaries also pattern brachiopod larval boundaries, I characterize the expression of the segment polarity genes *engrailed*, *wnt1* and *hedgehog* during the development of the brachiopods *T. transversa* and *N. anomala*. I found that *engrailed* is the only gene consistently demarcating the embryonic head/trunk boundary in the larvae of both species. Surprisingly, the gene expression profile at this brachiopod boundary is more similar to a boundary in the vertebrate brain than to segment boundaries. Our data suggests that the ancestral expression of *engrailed* was nonsegmental in the trunk ectoderm, and might have been independently recruited to the segment boundaries of annelids and arthropods.

This work provides basic embryological information, combining cell lineage tracing with morphological and molecular data for two understudied spiralian taxa, bryozoans and brachiopods. These comparative data bring insights to the evolution of two major morphological traits, spiral cleavage and segmentation, and to the evolution of the great diversity of spiralian larval forms.

List of publications

Papers included in the thesis

The following manuscripts are part of this thesis and will be referenced in the text as Paper I and Paper II.

Paper I: Vellutini BC, Martín-Durán JM, Hejnol A. **Evolutionary implications of the cell lineage and molecular patterning in the bryozoan *Membranipora membranacea***. Manuscript in preparation.

Paper II: Vellutini BC, Hejnol A. **Segment polarity genes in brachiopods support an ancestral nonsegmental role of *engrailed* for bilaterians**. Manuscript in preparation.

Additional of papers

During my doctoral training I have contributed to the publications below.

1. Martín-Durán JM, Vellutini BC, Hejnol A. **Evolution and development of the adelphophagic, intracapsular Schmidt's larva of the nemertean *Lineus ruber***. Evodevo 2015, 6:28.

2. Cannon JC, Vellutini BC, Smith III J, Ronquist F, Jondelius U, Hejnol A. **Xenacoelomorpha is the sister group to Nephrozoa**. Nature 2016 (accepted).

Contents

1 Introduction

1.1 Endless larval forms most beautiful

1.1.1 What a larva is

The Latin word *lārva* means *evil spirit*, *ghost* or *mask*[1]. In the 17th century, the naturalist Carolus Linnaeus was the first to employ the word larva to describe a stage in the life of an animal in which its adult form is still hidden or masked (Linnaeus, 1767, p. 534). An exemplar case of this new biological meaning is the maggot—the larval stage of a fly—whose wormy form and life style truly differs from its flying adult stage.

Not all larvae, however, are masked forms. The larval body of some marine snails[2], for example, is very similar to its adult body, except for the dazzling presence of a ciliated velum, used by the larva to swim and gather food (Collier, 1997). In more general terms, larval stages are considered to be a modification of embryonic development usually characterized by a morphology and habitat that are disparate from the adult stage (Hall and Wake, 1999). Because embryonic development can change in a multitude of ways, as evidenced by the great diversity of larval forms in nature (see below), there is no precise definition of *larva* (Hickman, 1999, Strathmann, 1993). Thus in practice, what a larva is, is defined case by case according to the organism and to one's research background.

The majority of animals on this planet have a complex life cycle with one or more larval stages. Collectively, marine invertebrates represent a great part of the observed larval diversity. Molluscs have the *veliger*, a shelled larva with the ciliated velum mentioned above; echinoderms have the *pluteus*, a spaceship-like larva with eight food-capturing arms, and the *brachiolaria*, a free-swimming larva driven by body-length dancing arms; bryozoans have the *cyphonautes*, a paper-thin triangular larva that sails over kelp blades; crustaceans have the *zoea*, an armored larva that swims as if using a jet pack; nemerteans have the *pilidium*, a larva with lobes and lappets in the form of a deerstalker cap... and this list goes on. The diversity of larval forms is astonishing (Figure 1.1).

Most of these charismatic larval figures were discovered in the 19th century by the naturalist founders of comparative embryology (Hall and Wake, 1999). At the time, the ideas of Karl Ernst von Baer and Ernst Haeckel had great influence on the understanding of embryonic development (Guralnick, 2002, Hall, 2000). Ontogeny was seen

[1] American Heritage® Dictionary of the English Language, Fifth Edition. (2011). Accessed November 13 2015 at `https://ahdictionary.com/word/search.html?q=larva`

[2] Michael Sars, one of the Norwegian biologists giving the name to the Sars Centre, was among the first to describe the development of molluscs from a swimming larva (Sars, 1837, Young, 1990).

as the unfolding of an immutable process that represents the evolutionary history of an organism—an idea known as recapitulation or Haeckel's biogenetic law: "ontogeny is a rapid and shortened recapitulation of phylogeny." (Gould, 1977, Haeckel, 1866).

These influential ideas were directly challenged by the mere existence of larvae. Or more generally, challenged by the existence of differentiated developmental stages that are, at the same time, functionally adapted to their environment and morphologically diverse. Such impressive variety of larval forms instigated questions about the relationship between the embryonic development of an individual (ontogeny) and the evolutionary history of a lineage (phylogeny).

Do larvae represent ancestral adult forms? How many times have larvae evolved? Are larval structures homologous or independently evolved? Soon, there was an urge to rationalize the diversity of larval forms into an evolutionary context.

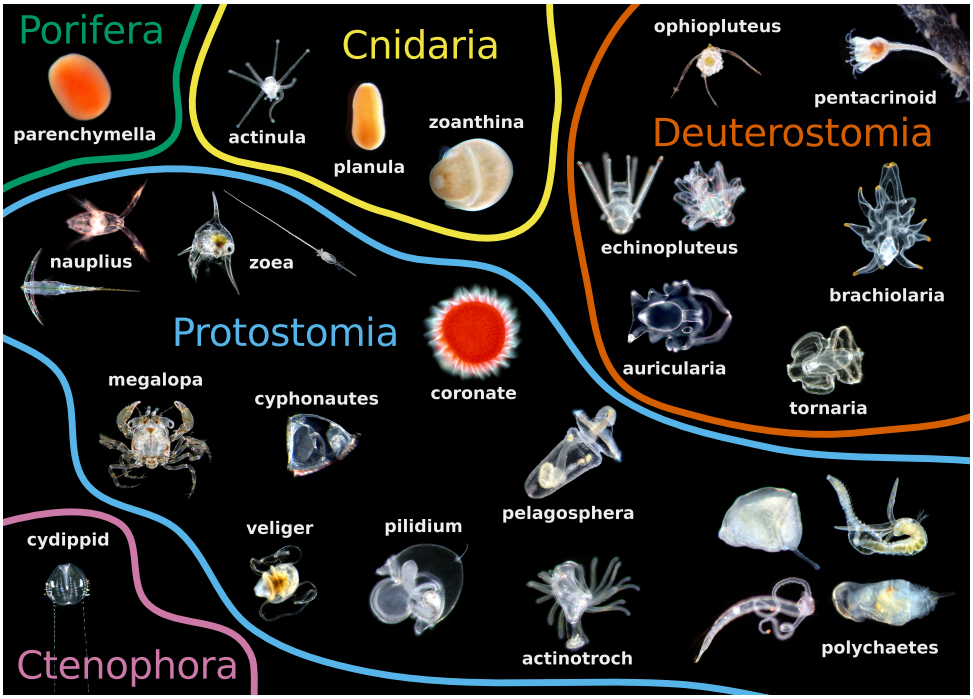

Figure 1.1: Sample of the diversity of metazoan larval forms. Larvae are not to scale. Photos from the *Cifonauta* marine biology image database (Migotto and Vellutini, 2011).

1.1.2 Larvae as the epitome of evolution

Francis M. Balfour set the pace on discussions about the evolutionary importance of larvae by addressing many of the fundamental questions regarding larval evolution (Balfour, 1874, 1880, 1881). He wondered about the ancestry of larvae. Can larvae reveal the ancestral forms of metazoans? He indicated tests to the predictions of recapitulation. Can we find a larva that corresponds to the adult of a related group? He asked whether larvae changed during evolution. How often do larval organs evolve? And

what might be the underlying mechanisms for the evolution of development. What guides the maintenance or atrophy of larval organs in adult stages? (Hall and Wake, 1999).

Perhaps, the greatest conceptual advance initiated by Balfour is that larvae are subject to variation and natural selection in the same manner as the adult stage (Balfour, 1874, 1881). In other words, he articulated the realization that evolution can occur at any developmental stage. However, if not all embryonic features represent ancestors (or ancestral traits), the foundation of the recapitulation theory is compromised. The evolutionary debate caused by larvae influenced a more informed way to make extrapolations from ontogeny to phylogeny (Hall, 2000, Hall and Wake, 1999). It was no coincidence that one of the most vehement opponents of Haeckel's recapitulation theory was a larvae affectionate, the biologist Walter Garstang who boldly concluded that "ontogeny does not recapitulate phylogeny, it creates it" (Garstang, 1922).

Present-day research shows that larval traits are evolutionary labile, and often correlate to ecological, developmental and other life-history factors (Strathmann and Eernisse, 1994). Evidence from diverse taxa, including gastropods (Collin, 2004), sea urchins (Raff and Byrne, 2006), ascidians (Jeffery and Swalla, 1992), sea stars (Byrne, 2006, Hart et al., 1997), nemerteans (Maslakova and Hiebert, 2014) and polyclad flatworms (Rawlinson, 2014), indicates that larval forms were modified, gained or lost in different lineages independently, and that the observed similarities are likely the result of convergent evolution.

These observations undermine scenarios about animal evolution that require the homology of larval characters (Jägersten, 1972, Nielsen, 1998, 2001, 2009, Peterson and Cameron, 1997) and are more consonant with the multiple independent evolution of metazoan larvae from a direct-developing ancestor (Page, 2009, Raff, 2008, Sly et al., 2003, Wray, 1995). Yet, the homology of larval characters such as the apical organ (e.g., Hunnekuhl and Akam, 2014, Marlow et al., 2014) or ciliated bands (e.g., Henry et al., 2007, Rouse, 1999) continues to be a central and lively discussed topic. For all the reasons above, larvae are a scandalous epitome of evolution, and the diversity of larval body patterns in marine invertebrates continue to provide a rich framework for evolutionary studies.

In this dissertation I examine the development of two unique larval forms, the cyphonautes larva of bryozoans and the unnamed nonfeeding larvae of brachiopods, in the context of two eye-catching animal traits, spiral cleavage (a conserved pattern of embryonic development) and segmentation (the partitioning of the body into repeated parts).

1.2 Spiral cleavage, an oblique matter

By the end of the 19th century, a series of biologists had dedicated themselves to following and discovering the fate of individual cells of an embryo during ontogeny. These works, known as cell lineage studies[3], were critical to disambiguate the relationship

[3]Also nicknamed *cellular bookkepping*, as recalled by E.G. Conklin: "...I followed individual cells through the development, followed them until many people laughed about it; called it cellular bookkeeping." (Bonner and Bell, 1984, p. 81).

between ontogeny and phylogeny, directly challenging the idea of recapitulation (Guralnick, 2002, Maienschein, 1978).

The detailed work of the cell lineage biologists Edmund B. Wilson, Edwin G. Conklin, Frank R. Lillie and others, revealed something remarkable. After carefully tracing the embryonic cells of different organisms, they discovered that animals such as molluscs, annelids, nemerteans and polyclad flatworms, whose adult stages are so different, actually share a similar embryogenesis[4] (Child, 1900, Conklin, 1897, Heath, 1899, Lillie, 1895, Mead, 1897, Wilson, 1892). Their embryos show the same cleavage pattern, in which cell divisions occur with the mitotic spindles oblique to the animal/vegetal axis, switching direction (clockwise and counterclockwise) at each division cycle (Costello and Henley, 1976, Hejnol, 2010, Henry and Martindale, 1999, Lambert, 2010). A quartet of vegetal macromeres sequentially gives rise to animal micromeres, and the resulting symmetry of these cleaving blastomeres, when viewed from the animal pole, was described as spiral. This developmental pattern thus became known as *spiral cleavage* (Wilson, 1892) (Figure 1.2).

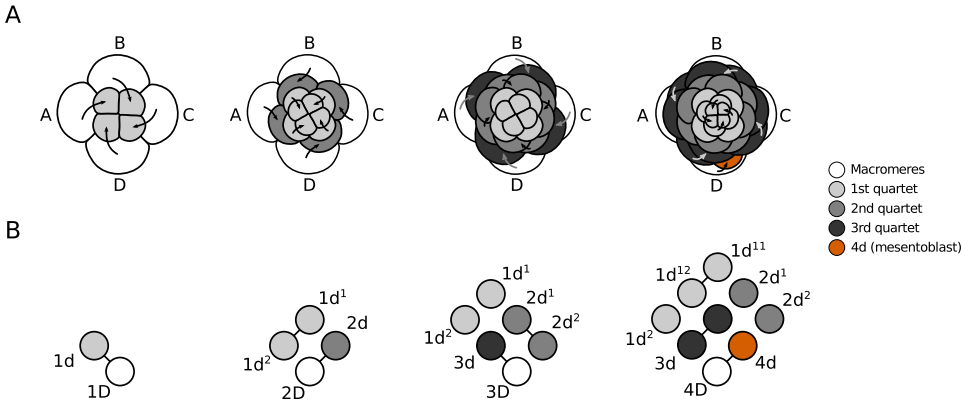

Figure 1.2: The spiral cleavage pattern. (A) Animal pole view of a generalized spiral-cleaving embryo. Arrows indicate the direction of cell divisions. Developmental sequence based on (Conklin, 1897). (B) Schematic diagram of cell divisions in the D quadrant in a lateral view (top: animal pole, bottom: vegetal pole). Cells are named with the standard spiral cleavage notation (Child, 1900, Conklin, 1897, Wilson, 1892). Representation based on Lambert (2010).

Because the cell divisions are stereotypic, individual blastomeres can be followed and compared between spiral-cleaving taxa in a fairly consistent manner. The ability to compare blastomere fates at this unprecedented cellular-resolution uncovered a surprising similarity in the fate maps of spiral-cleaving embryos (=annelids, molluscs, nemerteans and polyclad flatworms). The iconic example being the 4d mesentoblast, a well-conserved mesoderm precursor (Lambert, 2008). Overall, despite having the oblique cell divisions as an idiosyncrasy, spiral cleavage is understood today as a complex of developmental characters (Costello and Henley, 1976, Hejnol, 2010, Henry and Martindale, 1999, Lambert, 2010).

[4] "What a wonderful parallel is this between animals so unlike in their end stages! How can such resemblances be explained?" (Conklin, 1897, p. 195).

The empirical findings of cell lineage studies raised several important evolutionary questions regarding the evolution of development and the establishment of homologies (Guralnick, 2002). What are the underlying causes behind embryonic cleavage patterns—mechanical forces acting on the embryo or inherited historical factors? Are the events of early development necessary to build the adult characters? Is there an embryological criterion for homology? The ideas progressively moved towards a more evolutionary view of development, where ontogeny is not "a brief and rapid recapitulation of phylogeny" but an inherited product of evolution and subject to modification (Guralnick, 2002).

Even though most cell lineage biologists initially denied the systematic value of embryonic cleavage patterns, mainly in opposition to recapitulation (Guralnick, 2002), it was difficult to argue against the striking similarity between spiral-cleaving embryos, and dismiss their potential kinship[5]. Schleip (1929) was the first to propose a group to contain the animals displaying spiral cleavage—the Spiralia.

Recent metazoan-wide phylogenetic analyses corroborate the kinship between spiral-cleaving taxa, in a major protostome clade that is sister to the Ecdysozoa (e.g., insects) (Dunn et al., 2014). The latest works in protostome phylogenomics (Laumer et al., 2015, Struck et al., 2014) suggest that Spiralia (=Lophotrochozoa in some cases, see Hejnol (2010)) contains not only the typical spiral-cleaving groups, but several other taxa. Some spiralians (=animals that belong to the clade Spiralia) do not show any clear trace of spiral cleavage, such as bryozoans, brachiopods, gastrotrichs and rotifers, while others do exhibit spiral-like characters, such as gnathostomulids (Riedl, 1969), phoronids (Pennerstorfer and Scholtz, 2012) and entoprocts (Marcus, 1939, Merkel et al., 2012) (Paper I, Figure 1). What can we say about the evolution of these disparate cleavage patterns?

The spiral arrangement of embryonic blastomeres is present in the three main clades of Spiralia (Gnathifera, Lophotrochozoa and Rouphozoa), suggesting that this character is ancestral at least to the Lophotrochozoa-Rouphozoa clade (Paper I, Figure 1). This implies the spiral cleavage pattern was lost during the evolution of gastrotrichs, brachiopods, bryozoans and maybe rotifers. How did these groups lose spiral cleavage? Which aspects of a typical spiral-cleaving embryo did they lose, in addition to the spiral arrangement of the blastomeres? Are there any remnants of spiral cleavage?

The comparison between clades that have lost spiral symmetry, like bryozoans and brachiopods, and typical spiral-cleaving clades such as annelids and molluscs, can identify the traits that were lost, or are still shared, among these groups. This comparative approach can reveal novel insights about the evolution of spiral cleavage itself, and give rise to a broader perspective of the evolutionary mechanisms underlying spiralian development.

1.3 Segmentation, a question of boundaries

Annelids, arthropods and vertebrates show a remarkable morphological diversity (Chipman, 2010). Beneath this multiplicity of shapes and forms lies a common pattern

[5] "...if these minute and long-continued resemblances are of no systematic worth, and are merely the result of extrinsic causes, as is implied, then there are no resemblances between either embryos or adults that may not be so explained." (Conklin, 1897, p. 195).

of body organization—a trunk divided into repeated parts (Figure 1.3). This pattern and the developmental process that generates it are known as *segmentation* (Minelli and Fusco, 2004). While the vertebrate trunk is divided into somites[6] (a portion of the mesoderm), the body of annelids and arthropods is divided into intricate repeated compartments spanning the ectoderm and mesoderm—the segments (Scholtz, 2002a). The morphological similarity between these body segments previously was taken as support for a kinship between Annelida and Arthropoda, in a group called Articulata (Scholtz, 2002a, Seaver, 2003). In this scenario, segmentation would have evolved only once in the protostomes and once in the deuterostomes (Davis and Patel, 1999, Peel and Akam, 2003, Seaver, 2003).

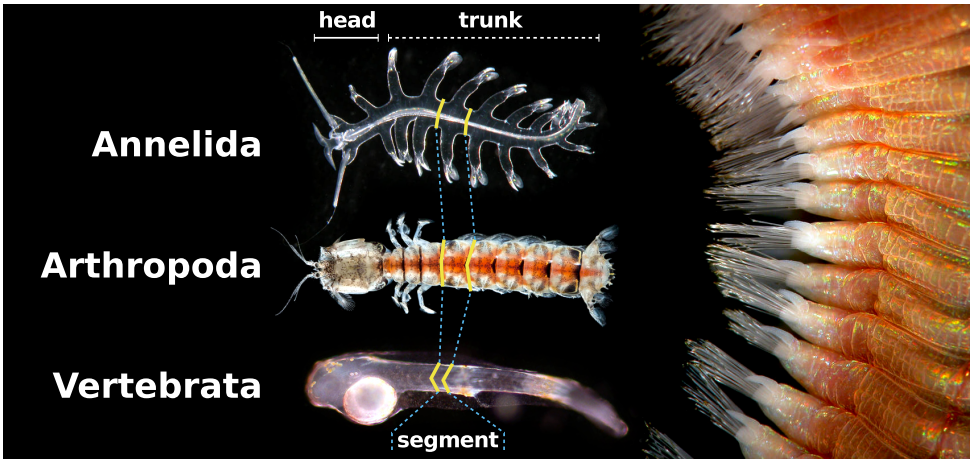

Figure 1.3: Taxa with a segmented trunk. Annelida: the holoplanktonik polychaete *Tomopteris* sp., Arthropoda: a mantis shrimp (Stomatopoda), Vertebrata: a Teleostei fish larva. Yellow lines mark the anterior and posterior boundary of one segment. Image on the right is a closeup of the ectodermal segmentation of the fire worm *Eurythoe complanata*. Images not to scale. Photos by Alvaro E. Migotto (Migotto and Vellutini, 2011).

Analyses arising from the area of molecular phylogenetics have disputed the monophyly of Articulata, suggesting that annelids and arthropods occupy different branches of protostomes, the Lophotrochozoa (=Spiralia) and Ecdysozoa, respectively (Aguinaldo et al., 1997, Eernisse, 1998). This phylogenetic hypothesis indicates that annelids and arthropods are more closely related to groups without body segmentation than to each other (Seaver, 2003); a topology that favors the independent evolution of annelid and arthropod body segmentation, in addition to the independent evolution of the different segmented tissues of vertebrates (Graham et al., 2014). Subsequent phylogenetic studies continue to corroborate the distant relationship between annelids, arthropods and vertebrates (Dunn et al., 2014, 2008, Edgecombe et al., 2011, Hejnol et al., 2009), reinforcing the homoplasy of their body segmentation (Paper II, Figure 1).

Remarkably, the molecular mechanisms of body segmentation in arthropods and vertebrates show a number of striking similarities (Damen, 2007, Davis and Patel, 1999,

[6]In addition to the somites, vertebrates also show segmentation in the rhombomeres and in the pharyngeal archs; segmented structures that likely evolved independently in the deuterostome lineage (Graham et al., 2014).

Kimmel, 1996, Patel, 2003, Peel and Akam, 2003, Seaver, 2003, Tautz, 2004). These molecular similarities were taken as evidence to support the homology of bilaterian segmentation (De Robertis, 1997, 2008, Dray et al., 2010, Kimmel, 1996), despite the opposing data from phylogenetics. To reconcile this apparent conflict between developmental and phylogenetic data, we must apply a comprehensive evolutionary approach to the problem.

The concept of segmentation is often used in a typological—and not evolutionary—manner (Budd, 2001). The result is a taxonomic bias, where the evolution of segmentation is regarded from the point of view of the groups considered to be segmented, i.e., annelids, arthropods and vertebrates (Budd, 2001). As a matter of fact, there is no conceptual basis to restrict segmentation to these three groups, because the repetition of parts along the body axis (Budd, 2001, Hannibal and Patel, 2013, Minelli and Fusco, 2004) also occurs in varying degrees in other bilaterians—usually considered to be pseudosegmented or unsegmented (Budd, 2001, Minelli and Fusco, 2004, Scholtz, 2002a, Willmer, 1990).

Another aspect to be considered is that segmentation—as much as spiral cleavage—is a complex of characters that ought to be individually compared between taxa (Scholtz, 2010). Breaking down segmentation into comparable traits (Scholtz, 2010), such as seriated nerve chords, segmented mesoderm or ectodermal boundaries, should provide a better overview of their evolutionary history.

Nevertheless, the sole comparison of traits between distantly related groups can still be misleading for understanding the evolution of a character (e.g., trunk segmentation), because the ancestral conditions of closer taxa are unknown. Since developmental mechanisms can be coopted to nonhomologous structures (Shubin et al., 2009), the phylogenetic context of a character is essential to distinguish homology from convergence. A recurrent proposal to better understand the evolution of segmentation is to expand taxonomic sampling (Arthur et al., 1999, Budd, 2001, Couso, 2009, Davis and Patel, 1999, Minelli and Fusco, 2004, Patel, 2003, Peel and Akam, 2003, Seaver, 2003, Tautz, 2004). Thus, examining segmentation traits in a wider range of taxa, including those without obvious segmented features, might help us to grasp the evolution of the developmental mechanisms that form repeated body parts in bilaterians.

1.4 Bryozoans and brachiopods

Bryozoans, brachiopods and phoronids are sessile coelomate animals that possess an anterior crown of ciliated tentacles—the lophophore (Ruppert et al., 2004). This distinct feeding apparatus and similar body morphologies were long recognized as evidence of their close affinities, and the group became known as the Lophophorata after Hyman (1959c). Because of their deuterostome-like embryological features (the presence of radial cleavage, enterocely and deuterostomy), the phylogenetic position of the lophophorates remained uncertain, and they were often considered within the Deuterostomia (Nielsen, 2001). Finally, the first molecular phylogenies placed them within the Protostomia with more confidence (Halanych et al., 1995). However, the monophyly and the exact relationships between the lophophorates and other protostomes is yet to be solved (Dunn et al., 2014), as some recent works find them paraphyletic (Dunn et al., 2008, Edgecombe et al., 2011, Hejnol et al., 2009) while others suggest the monophyly

of Lophophorata (Laumer et al., 2015, Nesnidal et al., 2013).

As detailed below, some bryozoans show a highly stereotypic cleavage pattern but without oblique cell divisions, that is an interesting comparison to the spiral cleavage pattern. Likewise, brachiopods can be informative to understand the evolution of segmentation mechanisms because their larval stages show putative segmented structures with a variety of ectodermal and mesodermal boundaries, that can be compared to segment boundaries.

1.4.1 Bryozoa (=Ectoprocta)

Bryozoans are common colonial animals that live attached to firm substrates, mostly in marine environments (Ruppert et al., 2004). Colonies are flat or arborescent and composed of diminutive individual functional units called zooids (Figure 1.4). The body of a typical feeding zooid consists of a tentacular crown (lophophore), a trunk with an u-shaped gut (the polypide), and a body wall that secretes the exoeskeleton case (the cystid). Each colony is formed by a single founding zooid (the ancestrula), derived from a metamorphosed planktonic larval stage.

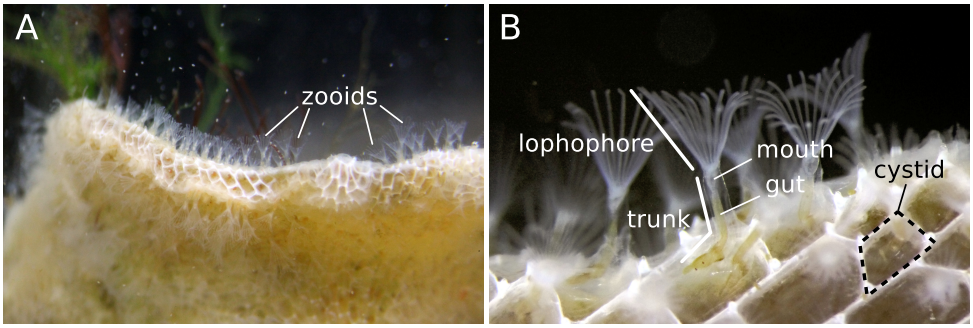

Figure 1.4: Colony and zooids of the bryozoan *Membranipora membranacea*. (A) Ripe colony on a kelp blade releasing eggs (white dots). Several zooids are everted with visible lophophores. (B) Closeup of everted zooids.

Bryozoans are divided in the monophyletic groups Phylactolaemata, Stenolaemata and Gymnolaemata (Waeschenbach et al., 2012). The three clades have fairly distinct developmental patterns regarding reproduction (e.g., brooding), early development (e.g., cleavage and gastrulation) and larval stages. The description below is based on the extensive reviews of bryozoan development by Hyman (1959b), Ström (1977), Zimmer and Woollacott (1977), Reed (1991) and Zimmer (1997).

Developmental diversity of bryozoans

Phylactolaemata are freshwater bryozoans that brood their embryos in invaginations of the zooid body wall (Hyman, 1959b, Ström, 1977, Zimmer, 1997). Holoblastic and irregular cleavage forms a blastula stage (Hyman, 1959b, Reed, 1991) that becomes bilayered by unipolar proliferation (Zimmer and Woollacott, 1977). After a placenta-like structure encircles the embryo, one to four polypide buds are formed at the central portion (Hyman, 1959b, Reed, 1991, Zimmer, 1997) and the remainder of the embryo

becomes ciliated (Reed, 1991, Zimmer, 1997). Upon release, the larva (an outer ciliated surface with polypides inside) swims for a short period and then undergoes metamorphosis, exposing the zooids (Hyman, 1959b, Zimmer, 1997). This larval stage—a swimming juvenile, in fact—shows no correspondent structures to the larvae of other bryozoan groups.

Stenolaemata bryozoans display polyembryony, where a single bilayered primary embryo develops from irregular cleavage stages and originates several secondary embryos (Ström, 1977, Zimmer, 1997). Secondary embryos differentiate into spherical larvae with a ciliated surface and two epidermal invaginations, one at the apical and one at the vegetal pole (Reed, 1991, Zimmer, 1997). The latter is the internal sac, an adhesive epithelium common to gymnolaemate larvae that is everted during metamorphosis and originates the cystid portion of the zooid (Zimmer, 1997). The larva—lacking muscles, coeloms, mesenchymal cells and nerves—settles and metamorphoses a few minutes after being released (Zimmer, 1997).

Gymnolaemata shows a characteristic cleavage pattern well-conserved within the group, in spite of the diversity of late larval stages (Zimmer and Woollacott, 1977). As summarized by Reed (1991) and Zimmer (1997), gymnolaemate cleavage is radial and holoblastic forming four apical and four vegetal blastomeres at the 8-cell stage. The fourth division results in a biradial embryo with a 4-by-2 array of cells in each pole. While the eight apical cells divide equatorially forming an additional row, the divisions on the vegetal side result in four inner cells surrounded by twelve outer vegetal cells. Gastrulation occurs at the sixth cleavage by invagination or by delamination of the inner vegetal blastomeres followed by epiboly of the vegetal plate (Hyman, 1959b, Reed, 1991, Zimmer and Woollacott, 1977).

Gymnolaemate larval forms: cyphonautes and coronate

Larvae of gymnolaemate bryozoans are classified into two types based on gross morphology, the shelled larva (cyphonautes) and the coronate larva (Zimmer and Woollacott, 1977) (Figure 1.5). The cyphonautes larva is triangular-shaped with a laterally compressed body, bilateral chitinous shells, a deeply invaginated oral field forming an internal cavity (vestibule) and a functional gut (planktotrophic) (Hyman, 1959b, Reed, 1991, Stricker et al., 1988a,b, Zimmer, 1997, Zimmer and Woollacott, 1977). The coronate larva is mostly spherical with long ciliated cells covering the surface (corona), but lacks a shell and a functional gut (Reed, 1991, Zimmer, 1997, Zimmer and Woollacott, 1977). There are at least five recognizable morphotypes of coronate larvae (Zimmer and Woollacott, 1977).

Despite the great morphological variability, the cyphonautes and the coronate larva have similar and likely homologous structures (Zimmer and Woollacott, 1977). A ciliary band (corona) divides the larval body into an aboral field, which contains the apical disc, the aboral epithelium and shell valves, and an oral field with the pyriform organ (ciliated glandular field of uncertain function), the internal sac (also shared with stenolaemate larvae), mouth and anus and vestibule (Reed, 1991, Zimmer and Woollacott, 1977). Even though the ancestral larval type for gymnolaemates could not be determined by a maximum parsimony analysis (Waeschenbach et al., 2012), these numerous shared structures, further similarities in muscles and neuronal connections, and the presence of gut rudiments in coronate larvae suggest the ancestral gymnolaemate

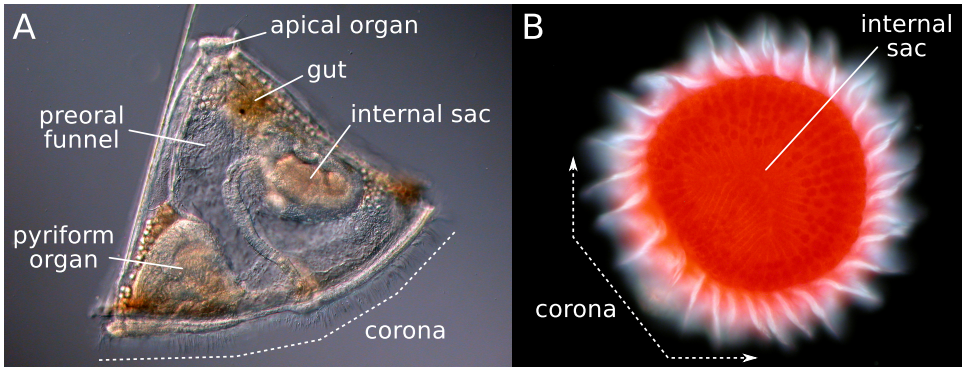

Figure 1.5: Gymnolaemate larvae. (A) Cyphonautes larva collected from plankton tow. (B) Coronate larva of *Watersipora subtorquata*. Photos by Alvaro E. Migotto (Migotto and Vellutini, 2011).

had at least a feeding larva (Zimmer and Woollacott, 1977). This idea does not imply that the cyphonautes larva directly represents the ancestral morphology, as has been suggested by Nielsen (1971). However, because the cyphonautes is found in different gymnolaemate clades, and is the only known planktotrophic larva of bryozoans, most authors consent that the cyphonautes morphology might be close to the ancestral form (Strathmann, 1978, Zimmer and Woollacott, 1977).

Embryonic origin of larval tissues and unanswered developmental questions

The organization of the gymnolaemate larval body (with a ciliated band between aboral/oral fields) can be traced back to the early embryo. Early embryological studies conducted by Barrois (1877), Prouho (1892), Calvet (1900), Pace (1906), Marcus (1938) and Corrêa (1948) provided the foundation for the fates of the bryozoan blastomeres. In general, the animal-most blastomeres of the 32-cell embryo originate the apical disc and aboral epithelium of the larva; the animal micromeres at the equator of the embryo form the corona; the twelve outer vegetal cells constitute the vestibule epithelium, the oral ectoderm, the pyriform organ and the internal sac; and the four inner vegetal blastomeres give rise to the endoderm and mesoderm (reviewed in Hyman, 1959b, Reed, 1991, Zimmer, 1997). Our understanding about the embryology of Gymnolaemata, however, is far from complete and several questions remain unsolved.

For example, the relation between the embryonic animal/vegetal axis and the body axes of the larvae remains unclear (Nielsen, 2005). Moreover, the fate of the blastopore, which closes in some species, is unsettled, and the protostomy of bryozoans is still an open matter (Gruhl, 2009, Marcus, 1938, Prouho, 1892, Zimmer, 1997). Finally, the fate of internalized cells has not been traced (Zimmer, 1997) and the source of mesoderm remains an especially contentious topic (Gruhl, 2009). Primary works observed mesodermal cells potentially derived from endodermal blastomeres, but failed to identify their cellular origin (Barrois, 1877, Calvet, 1900, Corrêa, 1948, d'Hondt, 1983, Pace, 1906, Prouho, 1892). Recent ultrastructural data suggest a different origin for the mesoderm, by the delamination of one ectodermal cell during gastrulation (Gruhl, 2009). Therefore, it is not yet demonstrated that the source of bryozoan mesoderm is endodermal, ectodermal or both.

Embryonic cell fates have only been systematically followed until the 64-cell stage (Corrêa, 1948, Pace, 1906) and, as of today, there is no detailed cell lineage of a bryozoan larva. For this reason, despite having a general overview of the cellular fates, the actual contribution of each blastomere to the larval structures remains unknown, and awaits a description with a higher level of cellular and temporal resolution.

Relevance of bryozoans to the evolution of spiral cleavage

As mentioned in the Section 1.2, bryozoans likely lost embryonic spiral symmetry, and thus are a valuable group to investigate the evolution of developmental patterns within Spiralia. However, there is more to it. The stereotypic cleavage of gymnolaemate bryozoans is suitable for reconstructing cell lineages, permitting a precise, cellular-scale comparison to animals with spiral-cleaving embryos. Moreover, the topology of the cell lineage of bryozoans constructed by Nielsen (2001) (based on Corrêa (1948)), suggests the fate map is comparable to spiral cleavage. Can one lose the spiral arrangement while maintaining the conserved cellular fates? Are there traces of spiral cleavage in bryozoan development? Or is the stereotypic bryozoan cleavage independently derived from spiral cleavage?

The comparison between bryozoans and other spiralians can not only reveal the evolutionary history of bryozoan development, but also bring a light to the evolution of spiral cleavage itself. In addition, gymnolaemate bryozoans have a planktonic larval stage with structures common to other spiralian larvae, such as an apical organ and a ciliated band. Thus, a multilevel comparison that includes the embryonic origin and fate of larval structures between bryozoans and other spiralians is a great basis to investigate the evolution and homology of spiralian larvae.

To approach these questions, I investigate the development of the gymnolaemate bryozoan *Membranipora membranacea* (Linnaeus, 1767), a species with the typical stereotypic cleavage pattern that gives rise to a planktotrophic cyphonautes larva.

Collection of *M. membranacea* in Bergen, Norway

Colonies of *M. membranacea* are commonly found at the sea shore near Bergen, Norway. The species occupies kelp blades growing off boat docks and can be easily collected by hand. We find reasonably large colonies (>10 cm) with ripe gametes between May and September in the Hjellestadosen bay. Collected kelp pieces with *M. membranacea* colonies are maintained in flowing tanks and remain viable for developmental studies for a week. A single colony can produce a vast amount of eggs per spawning. See Paper I for the detailed spawning procedures.

1.4.2 Brachiopoda

Brachiopods are benthic marine organisms possessing a shell with dorsal and ventral halves (Ruppert et al., 2004). The body enclosed in the bivalved shell consists of a large lophophore used for suspension feeding, a gut within a coelomic cavity and mantle epithelia with gonads extending internally on the shell walls. Individuals are attached to the substrate directly by the ventral half or by a muscular pedicle.

Brachiopoda has at least three lineages, the rhynchonelliforms as sister group to linguliforms and craniiforms (Bitner and Cohen, 2013) (Figure 1.6). The monophyly of

the group has been challenged since the suggestion that phoronids might branch within brachiopod lineages (Cohen, 2000, 2013, Cohen and Weydmann, 2005). This result, however, is not supported by broader molecular phylogenies, which place phoronids as sister group of Bryozoa (=Ectoprocta) (Dunn et al., 2008, Laumer et al., 2015, Nesnidal et al., 2013, Sperling et al., 2011). Independent of the position of phoronids, brachiopods share a fairly conserved early embryonic development and each of the three branches cited above has a characteristic larval form (Figure 1.7). Below, I summarize the embryology of brachiopods based on Hyman (1959a), Long and Stricker (1991) and Zimmer (1997).

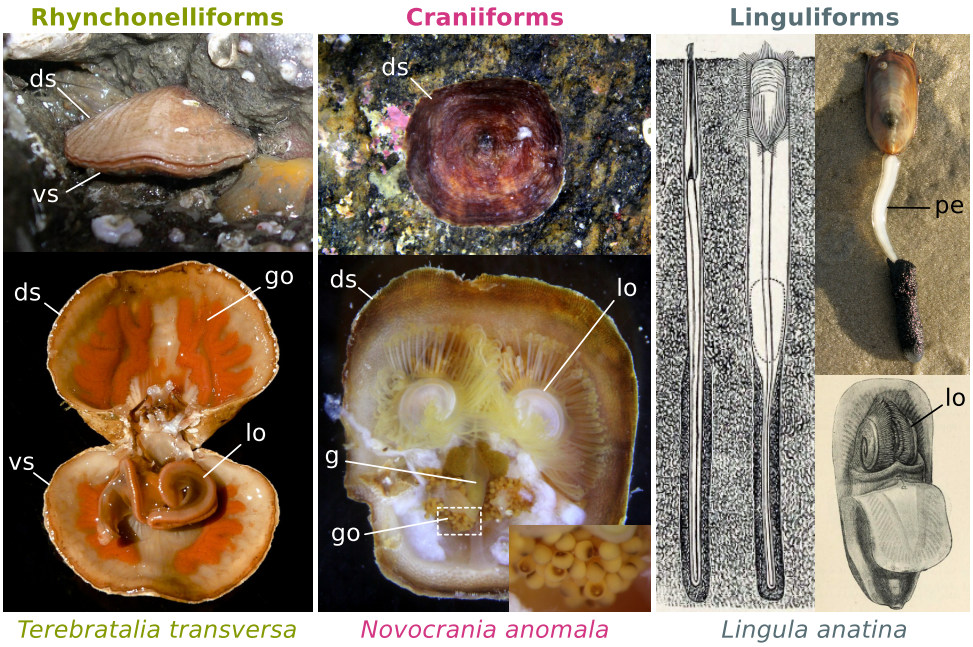

Figure 1.6: The lineages of Brachiopoda. Rhynchonelliforms: Live specimen of *Terebratalia transversa* (top, photo by Mary J. Adams) and internal anatomy of a ripe female individual (bottom, photo by Andreas Hejnol). Craniiforms: Live specimen of *Novocrania anomala* (top) and internal anatomy of a ripe female individual (bottom). Dashed area marks the gonadal region with mature eggs (inset). Linguliforms: Ilustration of the burrowing habit of *Lingula anatina* (left, British Museum). A live individual removed from the sand (top right, photo by Mark A. Wilson) and a dissected animal exhibiting the lophophore (bottom right, British Museum). ds: dorsal shell valve, vs: ventral shell valve, go: gonadal tissue, lo: lophophore, g: gut, pe: pedicle.

Development and larval diversity

Eggs are brooded or released into the sea and fertilized embryos undergo holoblastic equal cleavage with radial symmetry (Hyman, 1959a, Zimmer, 1997). A coeloblastula is formed and the embryo gastrulates by invagination of the vegetal plate (Hyman, 1959a, Zimmer, 1997). Formation of the mesoderm and coelomic sacs is variable between species (Hyman, 1959a). Mesoderm can be formed by a pair of cell masses proliferating next to the archenteron which later hollow out, by paired lateral pouches

branching off the archenteron, by a single anterior sac separated in two by the growth of the archenteron, by the evagination of a single posterior sac which later subdivides into an anterior and a posterior pair of coelomic pouches, and finally, by the progressive subdivision of paired posterior lateral pouches (reviewed by Hyman, 1959a). The external body wall (ectoderm) of all brachiopod embryos differentiates an anterior portion known as the *apical lobe* that forms the adult lophophore, and a posterior portion defined as the *mantle lobe*, that secretes the shell (Zimmer, 1997).

Linguliforms form a planktotrophic shelled larva with an apical lobe surrounded by a bivalved mantle lobe (Hyman, 1959a, Long and Stricker, 1991, Paine, 1963, Yatsu, 1902). Ciliation on the tentacle rudiments is responsible for locomotion and feeding; this larval stage lasts about a month (Zimmer, 1997). The morphology of the larva is close to that of the adults, and metamorphosis consists simply of the protrusion of an attachment pedicle after settlement (Long and Stricker, 1991, Zimmer, 1997).

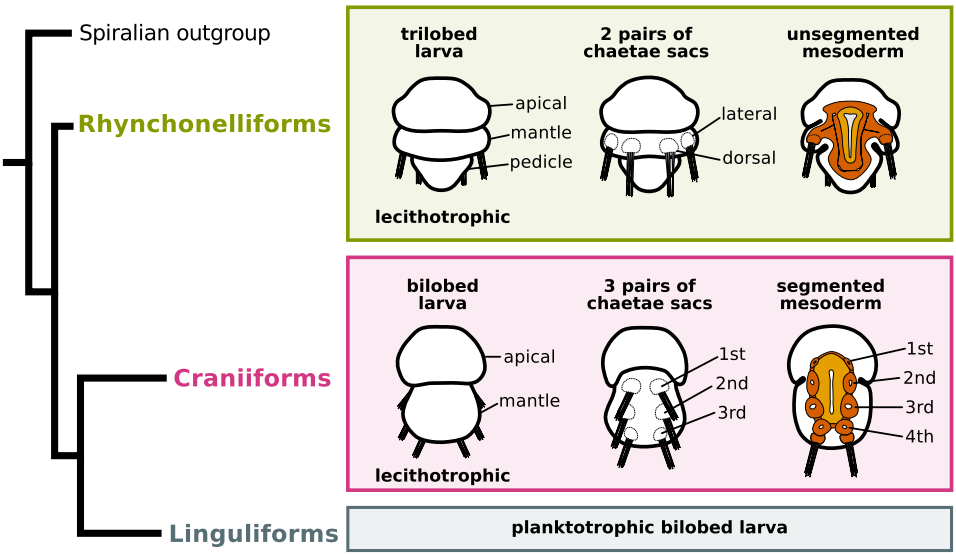

Figure 1.7: General morphology of lecithotrophic brachiopod larvae. Illustrations represent a ventral view (left), a dorsal view (center) and an internal view (right), depicting the mesoderm (red) and endoderm (yellow).

In contrast, craniiforms have a lecithotrophic larva with a short planktonic period, first described by Nielsen (1991). The body of the larva is divided into two lobes, an apical lobe with extensive ciliation and an elongated mantle lobe separated by a deep ectodermal furrow that demarcates the apical/mantle boundary (Nielsen, 1991). The mantle lobe contains three serially arranged pairs of chaetal sacs on the dorsal side (Freeman, 2000, Nielsen, 1991). Mesoderm morphology is unique among larval brachiopods, it consists of four pairs of serially arranged coelomic sacs, the three posterior being associated to the chaetal sacs (Freeman, 2000, Nielsen, 1991). During settlement, the larva is attached to the substrate by the posterior-most tip, and metamorphoses with the ventral surface down while the dorsal mantle secretes the dorsal valve of the shell (Altenburger et al., 2013).

Finally, rhynchonelliforms also have a short-lived lecithotrophic larva, but instead

of two lobes there is a differentiated posterior lobe, known as the *pedicle lobe* (Conklin, 1902, Long and Stricker, 1991, Morse, 1873a, Zimmer, 1997). Thus, in contrast to the bilobed larva of the craniiforms, the larva is divided into three portions separated by two transverse frontiers, the apical/mantle and the mantle/pedicle boundaries. The apical lobe is morphologically similar to that of craniiforms, but the mantle has only a lateral and a posterior pair of chaetal sacs (Long and Stricker, 1991, Zimmer, 1997). Larval mesoderm can be divided into an anterior and a posterior pair of sacs or be unsegmented depending on the species (Hyman, 1959a). The mantle lobe grows over the pedicle lobe during larval development, but at the time of metamorphosis the mantle lobe is reversed covering the entire apical lobe after the settlement of the larva (Franzén, 1969, Stricker and Reed, 1985a,b).

Relevance of brachiopod larvae to the evolution of segmentation

Overall, the larval stages of brachiopods show an exquisite diversity of morphological boundaries along the body, including partitions in the ectoderm and in the mesoderm. Such morphology and its evolutionary relevance has been widely discussed among embryologists since the earliest descriptions of brachiopod larvae.

Schmidt (1854) provided the first depiction of a brachiopod embryo, highlighting the division of the body into two unequal halves along the anteroposterior axis, separated by a deep constriction of the epidermis. Further observations revealed the planktotrophic larva of linguliforms (Müller, 1860, 1861), as well as other lecithotrophic larvae, with bodies divided externally into either three (Kowalevski, 1883, Morse, 1873a) or four (Lacaze-Duthiers, 1861) lobes along the anteroposterior axis.

The intriguing segmented appearance of these brachiopod larvae led some authors to suggest that brachiopods—at the time still considered to be "molluscs"—had closer affinities to the annelids (Agassiz, 1875, Kowalevski, 1883, Morse, 1870, 1873b). However, the implied idea that brachiopod larval lobes are homologous to annelid segments was strongly contested (Balfour, 1880, Dall, 1871, Shipley, 1883, Verrill, 1874). Lobes in larval brachiopods are not formed by a posterior growth zone, and despite being divided by deep ectodermal furrows, the mesoderm is not partitioned, as it is in the "true" segments of annelids (Balfour, 1880, Conklin, 1902, Shipley, 1883). Conklin (1902) gave the last word on the matter, concluding that the superficial appearance of segmentation in brachiopods is merely due to the mantle lobe being formed in the middle of the larval body, shaping an anterior and a posterior lobe in the lecithotrophic larvae (Conklin, 1902).

At the time, and for the following several decades, the larval stages of craniiforms remained unknown. It was only after the first descriptions of a craniiform larva (Freeman, 2000, Nielsen, 1991), and the discovery that the larval mesoderm is segmented into repeated coelomic sacs, that the idea that brachiopods might have had a segmented ancestor was revived (Balavoine and Adoutte, 2003, Temereva and Malakhov, 2011).

As pointed out above, annelid segments and brachiopod larval lobes are very different. Nevertheless, both are demarcated by distinct transverse ectodermal boundaries along the anteroposterior axis. How do brachiopod larval boundaries compare to annelid segment boundaries in terms of developmental mechanisms? Are these boundaries established by similar molecular pathways?

The comparison between brachiopods and annelids can reveal if the mechanisms

establishing segment boundaries are exclusive of the typical segmented groups, or if these developmental mechanisms also occur in other boundaries. Therefore, analysing the expression of "segmentation genes" in the putative segmented structures of brachiopod larvae might clarify the evolutionary context of these genes and their relation to the development of repeated structures.

To approach this question, I investigate the development of two brachiopod species with differing larval forms—the rhynchonelliform *Terebratalia transversa* (Sowerby, 1846) with a trilobed larvae with unsegmented mesoderm (Flammer, 1963, Long, 1964, Long and Stricker, 1991), and the craniiform *Novocrania anomala* (Müller, 1776) with a bilobed larva and serially arranged coelomic sacs (Freeman, 2000, Nielsen, 1991).

Collection of *N. anomala* in Bergen, Norway and *T. transversa* in Friday Harbor, USA

Ripe adult individuals of *N. anomala* can be collected by dredging rocky ocean floor (around 60m depth) in the Raunefjorden near Bergen, Norway during September and October. We bring the rocks with settled individuals to the laboratory, dissect the gonads to obtain gametes and fertilize after 24h. Fertilization is not completely synchronous and the success rate seems to depend on the maturation of the eggs. The other brachiopod, *T. transversa*, occurs in rocky ocean floor in Friday Harbor, USA and ripe adults can be collected by dredging in January. Maintenance and spawning methods are the same as for *N. anomala*, but in contrast, each *T. transversa* female can render vast amounts of embryos. For the detailed spawning procedures see Paper II.

2 Aims of the study

The goal of my doctorate work is to better understand the evolution of spiralian larval forms. For this, I analyze two groups—bryozoans and brachiopods—whose embryonic development deviates from well-known spiralian clades like annelids or molluscs. I examine several developmental aspects such as the fate of blastomeres, early embryonic patterning, the ontogeny of larval structures and the expression of molecular markers, to provide the basis for a solid comparison with other spiralians. Within a modern phylogenetic context, these comparative data can expose details about the evolution of embryonic and larval characters in the Spiralia.

My study is divided into two self-contained projects concerning different but equally relevant evolutionary questions, the evolution of spiral cleavage (Paper I) and the evolution of segmentation (Paper II). Each has a set of specific objectives that are described below.

Cell lineage of the bryozoan *Membranipora membranacea* (Paper I)

Their unique stereotypic cleavage pattern and larval morphology make the gymnolaemate bryozoans a pertinent comparative group to study the evolution of developmental patterns in Spiralia. I address the open questions about the development of bryozoans by studying *M. membranacea*, a species with a cyphonautes larva. My objectives are to:

- Elucidate the origin of larval structures (i.e., apical organ, ciliated band, shell, mouth, anus and gut) by tracing the embryonic cell lineage.
- Uncover the origin of the mesoderm.
- Clarify the relation between the embryonic animal/vegetal axis and the body axes of the cyphonautes larva.
- Characterize the activity of the MAPK pathway in bryozoan development.
- Better comprehend the body patterning of the cyphonautes larva by describing the gene expression of several developmental markers.
- Establish the evolutionary hypotheses about bryozoan development by comparing the cell lineage and gene expression to other spiralians.

Expression of segment polarity genes in larval brachiopods (Paper II)

Larval brachiopods display putative segmented characters such as transverse ectodermal boundaries and mesodermal partitions. In this project I test whether genes that pattern arthropod segment boundaries also correlate with the development of brachiopod larval boundaries. I analyze and compare gene expression during the development

of two species with different morphologies, *T. transversa* and *N. anomala* with the following objectives:

- Identify if the putative segmented boundaries in larval brachiopods are indeed repeated structures by conducting a detailed morphological analysis.
- Test if the molecular patterning of brachiopod larval boundaries is similar to the patterning of segment boundaries by characterizing the expression of the arthropod segment polarity genes *engrailed* (*en*), *wnt1* and *hedgehog* (*hh*).
- Establish the ground pattern for the expression of these genes in Brachiopoda by comparing species from two different lineages and larval morphologies.
- Better comprehend the evolution of segmentation mechanisms and their evolutionary significance by providing a closer phylogenetic comparison for the role of segment polarity genes.

3 Summary of the findings

3.1 Cell lineage of the bryozoan *Membranipora membranacea* (Paper I)

To uncover the embryonic origin of bryozoan larval structures, I describe a comprehensive cell lineage of the gymnolaemate *M. membranacea* using 4D microscopy. Additionally, I characterize the expression of several conserved developmental markers to further understand the body patterning of the cyphonautes larva by integrating cell lineage and gene expression data.

An embryo organized in quadrants with animal octets and vegetal twelve-tets

The *M. membranacea* embryo develops through the stereotypical cleavage pattern common in gymnolaemate bryozoans (Paper I, Figure 3). After the first two equal meridional cell divisions, the embryo undergoes an equatorial cleavage, forming four animal and four vegetal blastomeres. On the animal pole, the first quartet of animal blastomeres divides forming an octet (four inner and four outer cells). These cells divide synchronously in subsequent divisions and form the ectodermal structures from the apical organ to the ciliated band. The four vegetal blastomeres at the 8-cell stage divide as quartets forming twelve surrounding daughter cells at the 40-cell stage. While the four central blastomeres originate the gut and mesoderm, the remaining vegetal cells divide synchronously as a twelve-tet, and give rise to the vegetal ectoderm between the blastopore and the ciliate band.

Overall, each quadrant derived from the 4-cell stage contributes equally to the larval body, with corresponding blastomeres from the same octet or twelve-tet having similar fates. Further details are described below using a nomenclature adapted from spiral cleavage to accommodate the characteristics of bryozoan development, and yet serve as a comparative point to other spiralians (see Methods in Paper I). The most important information is that I named the quadrant that gives rise to the posterior region of *M. membranacea* as the D quadrant, to allow a comparison to spiral-cleaving embryos.

The nomenclature is mapped to the morphology of the embryos (Paper I, Figure 3 and 6) and to the cell lineage (Paper I, Figure S2). These figures might be used as a reference throughout the next sections.

Anteroposterior axis of the larva is orthogonal to the embryonic animal/vegetal axis

Live recordings from cleavage to larva allowed me to solve the relation between the embryonic and larval body axes. The animal/vegetal axis of the embryo does not correspond to the anteroposterior, but rather, to the apical/basal (or dorsoventral) axis of the larva. The anteroposterior axis of the larva is orthogonal to the embryonic animal/vegetal axis, running through the B–D quadrants (Paper I, Figure 2I and 3).

The gene *nk2.1* is involved in the patterning of the neural plate in vertebrates (Shimamura et al., 1995) and is expressed in anterior and ventral territories including the apical/neural plate and anterior endoderm (Lowe et al., 2003, Marlow et al., 2014, Takacs et al., 2004, Venkatesh et al., 1999). In *M. membranacea nk2.1* is only expressed in the B quadrant cells of the vegetal ectoderm, a region forming the oral ectoderm of the larva (Paper I, Figure 9 and 12). Expression of *nk2.1* suggests the aboral epithelium of the B quadrant has no ventral identity, providing additional support for an orthogonal anteroposterior axis of the larva.

Onset of bilateral symmetry at the 28-cell stage

During early cleavage, *M. membranacea* embryos are perfectly biradial with symmetric quadrants dividing synchronously (Paper I, Figure 4). In a live recording, the break in this symmetry can only be identified after the 48-cell stage by a delay in the division of two D quadrant cells, the animal blastomere $1d_o^{11}$ (Paper I, Figure 4) and the vegetal blastomere 3D. Thus, the bilateral symmetry of the embryo must be already established at the 48-cell stage, suggesting the existence of molecular asymmetries in earlier stages.

Indeed, three out of the five genes detected during cleavage, are expressed asymmetrically between the 32- and 40-cell stage (Paper I, Figure 9 and 10). The gene *gata456* is detected in the 3D blastomere (Paper I, Figure 11P), *nanos* in the cells $2d^L$ and 3d (Paper I, Figure 9) and *foxa* in all cells from the first twelve-tet except for $2d^R$ and 3d (Paper I, Figure 9 and 11C). The uneven localization of transcripts unambiguously delineates the left/right axis of *M. membranacea* slightly before the morphological evidence above. In fact, additional evidence from the activity of the MAPK pathway (see below) pushes the establishment of the bilateral symmetry further back, to the 28 cell stage.

Activated MAPK is only detected in the 3D blastomere

The molecular underpinnings of axis determination in spiral cleavage remain obscure, but the MAPK pathway has been implicated as the putative underlying signaling in molluscs (Henry and Perry, 2007, Koop et al., 2007, Lambert and Nagy, 2001, 2003). In the bryozoan *M. membranacea*, MAPK is activated at the 28-cell stage in a single vegetal blastomere—the 3D (Paper I, Figure 8). No other cells contain detectable levels of the activated form of MAPK, before or after this stage. The exclusive activation of MAPK in the 3D cell of the bryozoan is strikingly similar to the pattern found in equal-cleaving molluscs (Koop et al., 2007, Lambert and Nagy, 2003).

B quadrant does not contribute to the apical organ

Progeny of the first quartet of animal micromeres (1a–1d) originates the apical organ, the aboral epithelium and the ciliated band (corona) of *M. membranacea* cyphonautes larva (Paper I, Figure 5). The apical organ is derived from the apical-most cells $1a_i^1$, $1c_i^1$ and $1d_i^1$, but without any contribution of the blastomere 1b.

The genes expressed in the apical organ of *M. membranacea* are *dlx*, a gene involved in neurogenesis and proximodistal patterning (Panganiban and Rubenstein, 2002), and *six3/6* and *otx2*, transcription factors associated to anterior neural patterning (Marlow et al., 2014, Steinmetz et al., 2010). The expression of *dlx* correlates with the apical organ throughout development with transcripts detected in the first animal octet, the apical disc during gastrulation, and finally in the whole apical organ of the early larva (Paper I, Figure 9). Inner cells of the apical disc express *six3/6* during and after gastrulation (Paper I, Figure 9 and 11A), in a region occupied by serotonergic-positive cells of other cyphonautes larvae (Nielsen and Worsaae, 2010). During gastrulation, *otx2* is expressed in the apical region restricted to two anterior neuronal cells (Paper I, Figure 11B). Thus, *dlx* might participate in the early patterning of apical identities in *M. membranacea* while *six3/6* and *otx2* have more restricted domains consistent with a neural patterning role.

Corona and prototroch share some similarities in their embryonic origin

The embryonic origin of the prototroch—the primary ciliated band of several spiralian larvae—is conserved between annelids, molluscs and nemerteans, originating from $1a^1$–$1d^1$, $1a^2$–$1d^2$ and 2a–2d derivatives (Damen and Dictus, 1994, Henry et al., 2007, Maslakova et al., 2004a,b). The ciliated band of the cyphonautes larva—the corona—is derived from blastomeres that correspond in their position and lineage to the spiral cleavage $1q^1$ and $1q^2$ ($1q_i$ and $1q_e$) (Paper I, Figure S2). In contrast, the progeny of the second quartet does not seem to be part of the coronal cells in *M. membranacea*, like in other spiralians.

The expression of *otx* localizes to the prototroch of other spiralians, such as molluscs (Nederbragt et al., 2002) and annelids (Arendt et al., 2001, Marlow et al., 2014, Steinmetz et al., 2010). In the mollusc *Patella vulgata*, derivatives from the first and second quartet express *otx* (Nederbragt et al., 2002). Interestingly, the expression of *otx2* in the bryozoan *M. membranacea* occurs not only in the first quartet derivatives giving rise to the corona, but also in the second and third quartet blastomeres that form the twelve-tet at the vegetal ectoderm (Paper I, Figure 9 and 11B). The *otx2* domain encircles the bryozoan embryo in a pattern that closely resembles other spiralians, suggesting conserved molecular patterning between bryozoans and other spiral-cleaving embryos.

The blastopore forms the mouth of the cyphonautes larva

Our cell lineage and gene expression data indicate a protostomic development for *M. membranacea*, as suggested by Gruhl (2009) based on ultrastructural data. After gastrulation, the ectodermal cells bordering the anterior lip of the blastopore originate the anterior portion of oral ectoderm, and the inner endodermal cells close to the blastopore

form the anterior portion of the gut.

Further evidence of protostomy can be found in the expression of *nk2.1* and *foxa*. Expression of the anteroventral/foregut marker *nk2.1* borders the anterior lip of *M. membranacea* blastopore in the early gastrula, and later is expressed at the anterior portion of the preoral funnel, lining the larval mouth opening (Paper I, Figure 9). Expression of *foxa* is related to endoderm specification and commonly associated with the blastopore lip and foregut (Arenas-Mena, 2006, Boyle and Seaver, 2010, Oliveri et al., 2006). In the bryozoan, *foxa* transcripts also surround the blastopore during gastrulation (Paper I, Figure 9 and 11C). With the invagination of the vegetal ectoderm, the *foxa* domain is localized more anteriorly in the preoral funnel and around the larval mouth (Paper I, Figure 9 and 11D). Thus, independent evidence from gene expression, in addition to the lineage and ultrastructural data, support protostomy in *M. membranacea*.

Mesoderm is of endomesodermal origin

By tracking the fate of *M. membranacea* blastomeres with high temporal resolution, I found the cells 4a–4d, daughters of the four large vegetal blastomeres, are the first mesodermal blastomeres (Paper I, Figure 6). The anterior-lateral cells $4a^{A}$ and $4c^{A}$ originate the anterior muscles of the cyphonautes larva extending to the apical organ (Paper I, Figure 7). The progeny of $4b^{1}$ forms a distinct anterior stack of mesodermal cells of unknown function (Paper I, Figure 7). These cells express *foxf*, a transcription factor involved in mesoderm patterning and expressed mainly in visceral and anterior territories (Mazet et al., 2006, Passamaneck et al., 2015, Pérez Sánchez et al., 2002, Shimeld et al., 2010, Zaffran et al., 2001), supporting their mesodermal identity.

The fate of the 4d cell is unclear. It expresses *evx* (Paper I, Figure 11J and 11K), a gene also involved in the patterning of the posterior gut (de Rosa et al., 2005, Gorfinkiel et al., 1999, Thaëron et al., 2000), suggesting it might contribute to the larval hindgut. The transcription factor *foxc*, commonly expressed in anterior and posterior mesodermal domains (Häcker et al., 1995, Passamaneck et al., 2015, Shimeld et al., 2010), is expressed in ectodermal cells fated to the internal sac region of the bryozoan larva (Paper I, Figure 10). However, it is unclear if there is also mesodermal expression of *foxc* in the internal sac. Finally, a central regulator in mesoderm differentiation (Technau and Scholz, 2003), the transcription factor *twist*, is only transiently expressed in internalized cells during *M. membranacea* gastrulation (Paper I, Figure 10 and 11O), a pattern that differs from other spiralians (Dill et al., 2007, Nederbragt et al., 2002, Passamaneck et al., 2015, Perry et al., 2015, Pfeifer et al., 2013).

I did not observe the delamination of an anterior ectodermal cell as suggested by Gruhl (2009), but cannot discard the existence of ectodermally-derived cells contributing to the mesoderm of *M. membranacea*. Overall, our gene expression and lineage data suggests that the mesoderm of *M. membranacea*, unlike other typical spiral-cleaving taxa, is derived from multiple blastomeres of endomesodermal origin.

The progeny of 4A–4D forms the larval endoderm

M. membranacea endoderm is derived from the four large internalized blastomeres (4A–4D) (Paper I, Figure 6). This corroborates previous observations of bryozoan em-

bryogenesis and reveals another similarity to the development of spiral-cleaving embryos.

As shown above, the expression of the endomesodermal marker *gata456* (Patient and McGhee, 2002) occurs early in the bryozoan 3D blastomere (Paper I, Figure 10 and 11P). This gene continues to be expressed in the internalized blastomeres and the whole gut in later stages, suggesting the association of *gata456* with the endodermal development of *M. membranacea*, similar to other spiralians (Boyle and Seaver, 2010, Gillis et al., 2007, Passamaneck et al., 2015).

Posterior and germline genes are expressed in the internal sac

The region developing into the internal sac—the structure that forms the outer epidermal case of the zooid after metamorphosis—expresses three molecular markers in the late gastrula, the *bra* gene, related to blastopore, mesoderm and posterior/hindgut patterning (Technau, 2001); *foxc*, a gene expressed in the mesoderm; and *nanos*, a germline marker (Extavour and Akam, 2003, Juliano et al., 2010) (Paper I, Figure 9 and 10).

As reported above, *nanos* transcripts are restricted to a pair of posterior bilateral cells at the vegetal pole ectoderm (derived from $2d^{L}$ and 3d). These *nanos*-positive cells localize to the internal sac region in the late gastrula, but their actual fate is unclear. I could not distinguish if they become mesodermal. Since the internal sac is maintained through metamorphosis, it could be a potential region for blastemic tissues (i.e., the putative germ cells expressing *nanos*), in the cyphonautes larva of *M. membranacea*.

3.2 Expression of segment polarity genes in larval brachiopods (Paper II)

To better understand the role of typical "segmentation genes" in animal evolution, we analyzed the expression of the arthropod segment polarity genes *en*, *wnt1* and *hh* during the embryonic development of the brachiopods *T. transversa* and *N. anomala*. We directly compared the molecular profile of the brachiopod larval boundaries with previous data on the segment boundaries of annelids and arthropods.

Ectodermal boundaries of larval brachiopods are not repeated structures

Given the unsettled status of segmentation in brachiopod larvae, we initially asked if these ectodermal and mesodermal boundaries are indeed repeated structures along the body axis. We found that the two transverse ectodermal boundaries of the trilobed larvae of *T. transversa* do not share the same morphology and cannot be regarded as repeated structures. The anterior apical/mantle boundary is defined by an ectodermal furrow, while the posterior mantle/pedicle boundary is formed by a folding of the epithelia (Paper II, Figure 2–3). Both *T. transversa* and *N. anomala* share a similar morphological furrow at the apical/mantle boundary. The presence of an apical/mantle boundary in all taxa investigated so far, including the linguliform *Lingula anatina* (Yatsu, 1902), indicates the ancestral brachiopod had a larval body organized in an apical lobe forming

the lophophore, a mantle lobe forming the mantle of the adult shell, with an ectodermal furrow demarcating the boundary between the two.

Mesoderm of *N. anomala* is segmented into coelomic sacs

Hereby we provide an additional morphological description comparing the mesoderm of the two brachiopod species. The *T. transversa* larva does not show any sign of mesoderm subdivision, the tissue is unsegmented from anterior to posterior, simply expanding with the mantle lobe outgrowth (Paper II, Figure 3I–L). On the other hand, the mesoderm of *N. anomala* is progressively segmented into coelomic sacs, in tight association with the chaetal sacs on the dorsal surface (Paper II, Figure 3M–P). Even though the coelomic sacs are fused ventrally, the three posterior-most subdivisions of *N. anomala* mesoderm can be regarded morphologically as repeated structures.

Expression of *hedgehog* does not support a segment polarity role in brachiopods

Once we established a clear overview of the morphology of larval brachiopods, we tried to answer if genes involved in forming segment boundaries also pattern any of the brachiopod boundaries. Our null hypothesis was that *en* and *hh* would be coexpressed, with an adjacent non-overlapping stripe of *wnt1*, this being the expression pattern underlying the molecular signaling in arthropod segmentation.

At the apical/mantle boundary, we found that bilateral stripes of *en* expression precisely demarcate the posterior border of the ectodermal furrow (Paper II, Figure 4–5). This pattern is consistent between the two brachiopods, suggesting that it represents the ancestral condition for the expression of *en* at this developmental stage. While *en* expression precedes the morphological manifestation of the boundary, *wnt1* transcripts in *T. transversa* form a striped domain immediately anterior to the *en* domains, at the onset of the furrow formation (Paper II, Figure 4–5). Domains of *en* and *wnt1* at the apical/mantle boundary show tight correlation with furrow morphology and do not overlap (Paper II, Figure 5). This pattern is surprisingly similar to the parasegment boundaries of *Drosophila melanogaster* (Ingham and Martinez Arias, 1992) and the segment boundaries of the annelid *Platynereis dumerilii* (Prud'homme et al., 2003). However, in *N. anomala wnt1* is not expressed in the apical/mantle boundary (Paper II, Figure 4), suggesting a more labile evolutionary history for this ligand.

Because of this variability, we cannot assert if the correlation between the adjacent expression domains of *en* and *wnt1* and the apical/mantle boundary is ancestral, or not, for brachiopods. Hence, expression data from other species, specially *L. anatina*, will be crucial to solve this matter. Finally, we show the expression of *hh* is not related to any brachiopod ectodermal boundary, nor coexpressed with *en*; the *hh* transcripts are restricted to the endoderm of the larva (Paper II, Figure 4). The expression of *hh* does not support a typical segment polarity role—as known for arthropods—in the apical/mantle boundary of larval brachiopods (Paper II, Figure 9), despite the suggestive expression of *en* and *wnt1* in *T. transversa*.

Gene expression is not iterated through all mesodermal partitions

We further analyzed the expression of *en*, *wnt1* and *hh* in relation to the development of the segmented mesoderm of *N. anomala* and the unsegmented mesoderm of *T. transversa*. Expression of *en* in *N. anomala* localizes to the posterior border of the second and third coelomic sacs (Paper II, Figure 4). Interestingly, these mesodermal stripes appear in close contact to the ectodermal domains of *en*, established earlier in development (Paper II, Figure 4M–N). In *T. transversa* we see a similar correlation between the ectodermal expression of *en* and the mesodermal domains at the pedicle mesoderm (Paper II, Figure 4J). These patterns suggest the *en* ectodermal domains might induce the expression of *en* in the mesoderm of brachiopod embryos.

None of the other genes expressed in the mesoderm of *N. anomala* are iterated through all the four coelomic partitions. Transcripts of *pax2/5/8* were detected in a similar position as *en* (only in two coeloms), the genes *ptc* and *smo* are expressed in the anterior and posterior, respectively, and *gli* is expressed in the three posterior coeloms of *N. anomala*. Components of the Hedgehog pathway are expressed in a similar arrangement in the unsegmented mesoderm of *T. transversa*. Thus, even though the expression of some genes (e.g., *en*) suggest a role in the patterning of the coelomic sacs, none of the genes analyzed in this study clearly shows a repeated pattern of expression that matches the four mesodermal partitions of *N. anomala*.

Putative regulators of *en* expression demarcate the apical/mantle boundary

As described above, the only gene consistently expressed at the apical/mantle boundary in both larval brachiopods is *en*. However, the early bilateral patches of *en* do not encircle the whole embryo, suggesting there might be upstream factors positioning *en* expression and the apical/mantle boundary along the anteroposterior axis of brachiopod embryos. We have explored this possibility by examining the expression of genes that regulate *en* expression in other organisms. Published data on the pair rule genes, upstream factors in *D. melanogaster* segmentation cascade, showed no correlation to *en* expression or to the apical/mantle boundary. Thus, we investigated the expression of *pax6*, *pax2/5/8* and *fgf8/17/18*, genes known to regulate the expression of *en* in the axial patterning of the vertebrate brain (Araki and Nakamura, 1999, Matsunaga et al., 2000, Scholpp et al., 2003).

We found that *pax6* and *pax2/5/8* are expressed early in complementary patterns demarcating the anterior and posterior portions of the embryo at the radial gastrula stage (Paper II, Figure 6–7). The intersection between these two domains mark the position of the apical/mantle furrow, with *pax6* adjacent to the stripe of *en* expression (Paper II, Figure 7). Expression of *fgf8/17/18*, however, is mostly restricted to the developing chaetal sacs in both species. Thus, expression of *pax6* and *pax2/5/8* precedes the expression of *en* and extends through the whole embryo circumference. In addition, these patterns are consistent between the two brachiopods, suggesting these genes are good candidates for having a role in the differentiation between apical and mantle lobes and the establishment of the apical/mantle boundary. Our data on *T. transversa* embryos treated with pharmaceutical inhibitors provide additional support for an upstream role of *pax6*.

Over-activation of the Wnt pathway abolishes the anterior expression of *en* and *wnt1* but not of *pax6*

Because *wnt1* is expressed at the apical/mantle boundary of *T. transversa*, we tested whether the over-activation of the Wnt pathway affects the morphology and molecular profile of the boundary. Treatments with 1-azakenpaullone cause the posteriorization of the axial patterning, an anterior shift in the domains of gene expression and a failure to form the ectodermal furrow at the apical/mantle boundary (Paper II, Figure 8). This suggests that the proper placement of expression domains along the anteroposterior axis—likely dependent on Wnt signaling—is necessary for the formation of this ectodermal furrow. Embryos treated early in development show no traces of the wild type anterior domains of *en* or *wnt1*, but do have a *pax6* domain at the apical plate (Paper II, Figure 8). Embryos treated later have domains of *en*, *wnt1* and *pax6* at the anterior end, suggesting the period between mid-blastula and early gastrulation is crucial for the establishment of the anterior *en* and *wnt1* stripes in *T. transversa*. Thus, our experimental data supports our initial hypothesis that *pax6* might have an upstream role in the embryonic development of brachiopods.

Ancestral expression of *en* was nonsegmental

Since *en* was consistently associated with the apical/mantle boundary of brachiopods, we compared this pattern with all other bilaterians to recover the ground pattern of *en* expression in Bilateria. This comparative analysis reveals that the expression of *en* in the development of most bilaterians occurs as paired laterodorsal domains in the mid-body ectoderm (Paper II, Figure 10). These domains are usually associated with a great variety of epithelial boundaries later in development. Thus, the data suggests the ancestral expression of *en* at early developmental stages might have been a single pair of lateral domains in the trunk ectoderm.

4 Discussion and perspectives

I investigated the ontogeny of bryozoans and brachiopods in the context of two noteworthy features of animal development—spiral cleavage and body segmentation. My initial approach to the project can be summarized by two simple and rather naïve questions: *do bryozoans have spiral cleavage?* and *do brachiopods have segmentation?*

At a first read, the answers are a straightforward *no*. Most authors agree that bryozoans do not have spiral cleavage and brachiopods do not have segmentation (e.g., Couso, 2009, Hannibal and Patel, 2013, Seaver, 2003). However, it is also widely recognized that spiral cleavage and segmentation are not discrete characters, but a complex of morphological and molecular traits (Chipman, 2010, Hejnol, 2010, Minelli and Fusco, 2004, Scholtz, 2002a). Can any of these traits represent the essential qualities of spiral cleavage or segmentation? To answer this question we must delve into conceptual grounds.

The concept of segmentation is exemplar because it has been subject to extensive scrutiny over decades (Beklemishev, 1969, Budd, 2001, Chipman, 2010, Couso, 2009, Davis and Patel, 1999, Fusco, 2005, 2008, Graham et al., 2014, Hannibal and Patel, 2013, Minelli, 2009, Minelli and Fusco, 2004, Newman, 1993, Scholtz, 2002a, 2010, Seaver, 2003, Tautz, 2004). It is particularly difficult to reach a definition of *segmentation* that is objective and can encompass all the diversity of repeated structures of animals (Hannibal and Patel, 2013, Scholtz, 2002a). The concept has been historically tied to the general body morphology of annelids, arthropods and vertebrates, and these groups stand as types for what segmentation is (Budd, 2001). Considering segmentation as an "all-or-nothing" character prevents an evolutionary approach to the issue (Budd, 2001, Scholtz, 2010), a "conceptual trap" that can impair our understanding of the biological phenomenon behind the concept (Fusco, 2008). To overcome this issue, complex morphological characters need to be broken down into more objective and comparable traits and analyzed in a broader range of taxa (Budd, 2001, Scholtz, 2010).

Giving such perspective, what follows is an attempt to reveal insights about the evolution of spiralian development by comparing the individual traits of spiral cleavage and segmentation to the development of bryozoans and brachiopods, respectively.

4.1 The evolution of bryozoan development

The cleavage pattern of gymnolaemate bryozoan embryos is not found elsewhere in the metazoans, suggesting it is a derived feature of this group. Underlying this arrangement, however, I found that the embryo of *M. membranipora* is divided into quadrants where the four vegetal blastomeres sequentially give rise to cell quartets. As much as in the early stages of molluscs and annelids, the contributions of each quadrant are sym-

metrical and mostly equal. Thus, the most notable difference between gymnolaemate development and spiral cleavage lies in the complete absence of oblique cell divisions in bryozoans.

In *M. membranacea*, the blastomeres at the 4-cell stage have the same size and are not distinguishable from one another. Spiral-cleaving embryos where the first two cell divisions are equal and form blastomeres with the same size at the 4-cell stage are known as equal-cleaving (Freeman and Lundelius, 1992). The specification of the D quadrant in equal-cleaving molluscs occurs by inductive interactions between micromeres and one of the macromeres at the 32-cell stage (van den Biggelaar, 1977). My data suggest the D quadrant of *M. membranacea* might be specified at a corresponding stage (28-cell) when compared to molluscs. The specification of the D quadrant marks the establishment of the dorsoventral axis, which runs through the B–D blastomeres in both the bryozoan studied here and molluscs (van den Biggelaar, 1977). However, an important difference must be pointed out. While in molluscs, D quadrant specification establishes the dorsoventral axis, in *M. membranacea* the axis corresponds to the anteroposterior axis of the cyphonautes larva. Interestingly, in the bryozoan the D quadrant extends over the B quadrant (e.g., apical organ fates), suggesting an inclination in the axis that might be comparable to the 45 degree angle, relative to the animal/vegetal axis, that has been reported for the dorsoventral axis of spiral-cleaving embryos (Henry and Martindale, 1999).

To this extent, bryozoans and molluscs share some similarities in the timing of the specification of the D quadrant, but their larval axes do not show the same relative orientation to the embryonic animal/vegetal axis. Additional data on the determination of bryozoan axes, such as blastomere ablation or dissociation and a careful analysis of the macromere/micromere interactions, would certainly be worthwhile for a more in depth comparison with mollusc development.

In equal-cleaving molluscs, the identity of each quadrant is concealed until the centralization of the 3D macromere at the fifth cleavage (van den Biggelaar and Guerrier, 1979). At the equivalent cleavage cycle, I found the first indication of a break in the biradial symmetry of *M. membranacea* embryos, via the asymmetric activation of MAPK in the prospective 3D blastomere. The similarity of this bryozoan MAPK activity to equally-cleaving molluscs is striking. Perturbation experiments using an inhibitor of the MAPK pathway suggests that MAPK has a role in the dorsoventral specification of molluscs (Henry and Perry, 2007, Koop et al., 2007, Lambert and Nagy, 2001, 2003), but not in annelids (Amiel et al., 2013, Pfeifer et al., 2014). Similar experiments can reveal if the suggestive MAPK activity in the bryozoan has any correlation with the specification of the D quadrant. A comparative overview of MAPK activity in spiralians is lacking, and studies in other taxa such as phoronids, nemerteans, entoprocts and polyclads, might be especially informative to understand the role and evolution of MAPK signaling in spiralian development.

The larval mesoderm of *M. membranacea* originates from the fourth quartet of cells derived from the large vegetal blastomeres, as suggested by classical bryozoan embryology (Barrois, 1877, Calvet, 1900, Corrêa, 1948, d'Hondt, 1983, Pace, 1906, Prouho, 1892). I could not determine the fate of the 4d cell, but the progeny of 4a–4c forms mesodermal tissues of the cyphonautes larva. Multiple endomesodermal blastomeres originating mesoderm is not common in other spiral-cleaving embryos, in which only a single blastomere—the 4d—gives rise to mesoderm (Lambert, 2008). Although the

bryozoan 4d cell might originate mesoderm, the fact that others cells from the fourth quartet give rise to mesoderm is a significant difference to the spiral cleavage pattern.

The 4d cell is not the only source of mesoderm, and ventral micromeres, usually 3a and 3b, also contribute to the mesoderm, known as ectomesoderm (Boyer et al., 1996, Henry and Martindale, 1999, Lyons and Henry, 2014). Despite the suggestion that an ectodermal cell gives rise to larval muscles in *M. membranacea* (Gruhl, 2009), I did not find evidence of ectomesoderm in the cell lineage data. However, the bryozoan embryo is not fully transparent, complicating cell tracing in later stages, and thus I cannot disregard the possibility of ectodermal cells undergoing epithelial-mesenchymal transition in *M. membranacea*.

So far, the only putative candidates for ectomesoderm are the two vegetal ectodermal cells of the second and third quartet in the D quadrant expressing *nanos*, a gene required for germline development across Metazoa (Extavour and Akam, 2003, Juliano et al., 2010). *M. membranacea* pattern is not dissimilar from the expression of *nanos* in the mollusc *Haliotis asinina* (Kranz et al., 2010), but we did not detect *nanos* transcripts in the 4d cell as reported in *Ilyanassa obsoleta* (Rabinowitz et al., 2008). Nevertheless, if *nanos*-expressing cells in the bryozoan become mesodermal, it is a disparate origin of ectomesoderm when compared to other spiralians. Certainly, the origin of the germline in bryozoans is an interesting and largely unexplored topic. Germ cells have only been found after metamorphosis (Reed, 1991), and it is unclear if the germline differentiates during embryogenesis. Further gene expression studies following the fate of these cells through metamorphosis will certainly be revealing.

Overall, the fate map of the bryozoan shows a general resemblance to cellular fates in spiral-cleaving embryos. There is variation in some cell fates and in the mesoderm source, but the origin of the cyphonautes larval structures, such as the apical organ, corona and gut, is comparable to the origin of correspondent structures in the larvae of annelids, molluscs, nemerteans and polyclads. Did bryozoans lose the spiral symmetry while maintaining cellular fates or is the fate map similarity an evolutionary convergence?

Current protostome phylogenies (Laumer et al., 2015, Struck et al., 2014) indicate the spiral arrangement of embryonic blastomeres might be ancestral to Spiralia, and thus, have been lost in the bryozoan lineage. Within this framework, our data suggests that despite the modified orientation of mitotic spindles and cleavage pattern (e.g., octets and twelve-tets), gymnolaemate bryozoans kept a quadrant-based embryo with mostly similar blastomere fates. Some aspects of the differentiation of early blastomeres might have remained conserved, such as D quadrant specification and MAPK activity, but there was a late shift in the relation between the embryonic and larval axes, likely associated with the evolution of the unique morphology of the cyphonautes larva.

Nevertheless, it is uncertain if the stereotypic cleavage pattern of gymnolaemates represents the ancestral condition for the cleavage of bryozoans. The embryogenesis of phylactolaemates and stenolaemates is highly derived and the internal phylogeny of bryozoans is not yet sufficiently resolved (Waeschenbach et al., 2012). Thus, if the stereotypic cleavage pattern evolved *de novo* in gymnolaemate bryozoans, the above fate map similarities represent convergent traits. Therefore, the cell lineage coincidences might simply—but not less strikingly—reflect a conserved underlying axial patterning of spiralian embryos, with similar animal/vegetal identities that drive the blastomeres to correspondent fates.

4.2 The anterior boundary of larval brachiopods and the ancestral expression of *en*

The rich fossil record of brachiopods indicates that the evolutionary lineages of *T. transversa* (rhynchonelliforms) and *N. anomala* (craniiforms) diverged at least 500 million years ago (Bitner and Cohen, 2013) and that their respective larval forms might have evolved lecithotrophy independently, 300 million years apart (Freeman and Lundelius, 2005, 1999). Surprisingly, we found that most genes are consistently expressed between the two brachiopod larvae. Therefore, gene expression in brachiopod development is conserved to a certain extent, despite the long period of independent evolution and significant morphological differences between larvae.

In the mesodermal boundaries of *N. anomala*—the only clearly segmented structure of larval brachiopods—none of the candidate genes are expressed in an iterated pattern, repeated in the four pairs of coelomic sacs. Nevertheless, the mesodermal transcripts of *en* have an interesting spatial distribution, localized at the posterior portion of two coelomic sacs, resembling the somite expression of *en* in the more distantly related amphioxus (Holland et al., 1997) and onychophorans (Eriksson et al., 2009, Wedeen et al., 1997). This expression topology differs from the mesodermal patches found in annelids (Prud'homme et al., 2003, Seaver and Kaneshige, 2006). Clearly, further investigations are needed to elucidate the mesodermal role of *en* and the molecular framework involved in the partitioning of *N. anomala* mesoderm.

On the other hand, in the body wall of *T. transversa* lies the surprising adjacent stripes of *en* and *wnt1* demarcating the apical/mantle boundary of the larva. The pattern is striking because it closely resembles the expression in the parasegment boundaries of arthropods (Damen, 2007, Hughes and Kaufman, 2002, Ingham et al., 1988, Mellenthin et al., 2006, Nagy, 1994), and the expression in the segment boundaries of the annelid *P. dumerilii* (Prud'homme et al., 2003). The brachiopod data reveal that the abutting domains of *en* and *wnt1* demarcating a morphological ectodermal boundary is not exclusively associated with segment boundaries, but can occur in nonsegmental boundaries as well.

Despite this similarity, the expression of the Hedgehog pathway does not correlate with any ectodermal boundaries. These expression patterns do not support the involvement of a segment polarity signaling in the development of brachiopod larval lobe boundaries. In fact, in terms of gene expression topology, the apical/mantle boundary of brachiopods is more similar to a vertebrate brain boundary than to an annelid segment boundary.

Factors known to regulate *en* expression revealed two genes that correlate with the apical/mantle boundary, *pax6* and *pax2/5/8*. The spatial relation between the apical/-mantle furrow of brachiopods and the expression of *pax6*, *pax2/5/8* and *en*, is comparable to the gene activity defining the di/mesencephalon boundary in the brain of vertebrates (Araki and Nakamura, 1999, Matsunaga et al., 2000). A pattern also found in the neuroectoderm of hemichordates (Pani et al., 2012) and cephalochordates (Glardon et al., 1998, Kozmik et al., 1999), and that might be conserved within deuterostomes (Lowe et al., 2015). The consistent expression of *en*, *pax6* and *pax2/5/8* between brachiopods suggests that these genes might be related to the patterning of the apical and mantle lobe territories.

Given the prominent expression of *en* during brachiopod gastrulation, I examined the earliest developmental expression of *en* across bilaterians, to reconstruct its ancestral condition. The gene *en* is best known for its role in compartment boundaries in arthropod segmentation (Hidalgo, 1996, Kornberg, 1981). It has a pervasive role in neural differentiation, suggesting the ancestral function is related to neurogenesis (Gibert, 2002, Patel et al., 1989). Thus far, *en* has not been reported for ctenophores, sponges, placozoans or cnidarians, but it is widespread among bilaterians (Gibert, 2002), and possibly present in the last common ancestor of bilateral animals (Butts et al., 2008).

An extensive comparative survey, however, indicates a complementary hypothesis for the ancestral condition of *en* expression. The expression of *en* in the early stages of many bilaterians is similar, suggesting the ancestral embryonic expression of *en* was nonsegmental and near the region giving rise to the head/trunk boundary in adult stages. The expression in later stages is more variable and associated to a variety of developmental boundaries, including the segment boundaries of annelids and arthropods. Therefore, this comparative data suggests the deuterostome/protostome ancestor might have had *en* associated with the embryonic head/trunk boundary, and during evolution *en* was recruited multiple times for diverse developmental roles[1]. Additional gene expression studies in protostomes like gastrotrichs, rotifers, chaetognaths, nemerteans and priapulids are needed to test the hypothesis that *en* was originally related to axial patterning in ancestral bilaterians.

4.3 An evolutionary landscape for spiral cleavage and segmentation

This work provides basic embryological information for two understudied spiralian taxa, bryozoans and brachiopods. By combining cell lineage tracing with morphological and molecular data, I reveal the embryonic origin of the mesoderm, the fate of blastomeres and a peculiar pattern of MAPK activation in the development of the bryozoan *M. membranacea* (Paper I). I also tested if "segmentation genes" are expressed in the morphological boundaries of two species of brachiopods, *T. transversa* and *N. anomala*, and found that a nonsegmental larval boundary can be demarcated by the adjacent expression of *en* and *wnt1*, a pattern usually occurring in segment boundaries (Paper II).

[1]Harold Heath—a student of Conklin—was the first cell lineage biologist to try bridge the gap between early development and adult characters (Guralnick, 2002). And he did so by uniting the two main topics of this thesis, spiral cleavage and segmentation. To understand the origin of segmentation, he compared the embryonic origin of the body segmentation of annelids with the embryonic origin of the dorsal segmentation of chitons. He found that both segmented structures originate from the first somatoblast and are confined to the trunk region. Based on that he proposed a developmental hypothesis to explain the evolution of the annelid segmentation. Interestingly, he noted the two embryos have a groove separating the head/trunk boundary that occupy the same position in the embryo. Because the head/trunk boundary is a shared trait between the annelid and mollusc, while the trunk segmentation clearly differs between the two (despite having the same embryonic origin), Heath concludes the head must be an ancestral trait, and segmentation a derived character of each group. In Heath's own words: *"The 'head' therefore is phylogenetically the older portion of the Annelid while the greater portion of the trunk is comparatively a late formation. Metameric segmentation belongs to the trunk region and is therefore secondary and I should look upon the pro- and peri-stomium as one segment or better perhaps as the portion of the trochophore which has undergone no segmentation."* (Heath, 1899, p. 650). In a way, Heath's conclusion based on the cell lineage comparison parallels mine based on the expression of *en* for bilaterians.

Because bryozoans and brachiopods show divergent developmental traits (e.g., unique cleavage and larval body patterns), the data allows for an interesting comparative perspective, bringing insights into the evolution of spiralian development. Both the comparison between bryozoan development and spiral cleavage, and the comparison between the larval development of two brachiopod species, revealed that organisms shows a unique mixture of similarities and differences in their traits. In a sense, this is empirical evidence that evolutionarily independent lineages contain an assortment of ancestral and derived traits, and resemble their last common ancestor in varying degrees (Crisp and Cook, 2005).

Albeit not considered "to have" spiral cleavage or segmentation, bryozoans and brachiopods have developmental characters comparable to other spiralians. The similarities observed in this study incite evolutionary questions about the homology of these characters. For example, is the apical organ of the pilidium homologous to the apical organ of the cyphonautes? Is the corona of the cyphonautes homologous to the prototroch of a trochophore? Is the 3D blastomere of bryozoans homologous to the 3D macromere of molluscs? Is the apical/mantle boundary of brachiopods homologous to the head/trunk boundary of annelids? Is the embryonic head/trunk boundary homologous in all bilaterians?

A recurrent subject in this dissertation is that evolution can occur at any developmental stage without affecting previous or subsequent stages. Balfour (1874) recognized this aspect of biology in the diversity of larvae and argued that if homologous structures can have their embryonic origin changed, the lack of common origin does not imply in homoplasy[2]. The reasoning applies to the reverse case, the same embryonic origin does not imply the homology of two structures (Scholtz, 2002b, 2005). The evolutionary independence of developmental stages has a broad range of implications for the assignment of homologies between traits (Dohle, 1989, Scholtz, 2002b, 2005, Wagner and Misof, 1993).

In this context, the similar embryonic origin of the apical organ of the cyphonautes larva and the apical organ of the pilidium larva, cannot be a determinant criterion for establishing homology between these two structures. In the same manner, the fate of a cell might not be a good homology criterion because cells can be homologous at a specific stage, but give rise to divergent homoplasic structures. The embryonic head-/trunk boundary might be homologous among all bilaterians, but it does not imply that all the bilaterian heads are homologous. The evolutionary independence of developmental stages raises the intricate need to compare homologies by developmental stage (Scholtz, 2002b, 2005). However, there is a counterpart case. The corona of the cyphonautes is likely not homologous to the ciliated band of the pilidium, but the process of developing an anterior ciliated band might be homologous between the two larva (see Scholtz (2005) for the distinction between pattern and process homology).

These involved lucubrations only partially highlight the difficulty of identifying homologies. Recognizing evolutionarily independent morphological units (Scholtz, 2005, 2010) and identifying similarities is a crucial and necessary step to establish putative homologies (de Pinna, 1991). However, the "similarity or dissimilarity alone, no mat-

[2] "If we admit that organs can undergo changes, as to the primitive layer from which they are derived, important consequences must follow. It will, for instance, by no means be sufficient evidence of two organs not being homologous that they are not developed from the same layer. It renders the task of tracing out the homologies from development much more difficult..." (Balfour, 1874, p. 343).

ter how striking, do not support or refute homology propositions," and these putative homologies need to be tested against a phylogeny to determine if the similarities reflect the homology or homoplasy of a trait (de Pinna, 1991). Furthermore, other variables can affect the outcome of homology testing such as the robustness of a phylogeny and the extension of the taxonomic sampling (Abouheif et al., 1997).

Taking these aspects into account, perhaps a most-challenging animal group to reconstruct character evolution is the Spiralia. Spiralians are diverse—this incredible morphological variety complicates the identification of comparable traits for homology assessment. Spiralians are poorly known—even though there are well-studied planarians, molluscs and annelids, the developmental diversity of many spiralian lineages remain largely unexplored. Finally, spiralian relationships continue unsolved—despite the greater confidence of who are the spiralians, the affinities between the different lineages are still disputed.

Biologists may continue to fumble through evolutionary hypothesis for years to come, but the diversity and knowledge gaps within Spiralia are the very reasons that make these animals exhilarating to study. This doctorate work is but a little step towards a better understanding of the endless spectacle of spiralian larval forms and their enigmatic evolutionary histories.

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