Comparative analysis of acoel embryonic development

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Thesis for the degree of Philosophiae Doctor (PhD)
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All that you touch

You Change.

All that you Change

Changes you.

The only lasting truth

Is Change.

Octavia Butler

Scientific environment

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Author contributions

I hereby declare that I have written this thesis myself, with the contribution of other people in form of comments and formal corrections.

I also declare that I was involved in the design, in most of the experiments and in the analyses of the manuscripts I and II and I have written myself the manuscripts I and II. The contributions of the other authors to manuscripts I and II are as follow:

Dr Mark Q. Martindale and Dr Elaine C. Seaver (The Whitney Laboratory for Marine Bioscience, S. Augustine, Florida) performed the injections of fluorescent linage tracer on *Isodiametra pulchra* embryos and took the confocal images of the juveniles.

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Prof. Dr Andreas Hejnol (University of Bergen, Norway), as my supervisor, was involved in the design and the supervision of the projects and commented on the thesis and the manuscripts.

Manuscript III was written by Dr Jose M. Martín Durán (Queen Mary University of London). I contributed by cloning the Hox genes and performing the *in situ* hybridization on the embryos, juveniles and adults of the annelid *Dimorphilus gyrociliatus*. I have also read and commented on the manuscript draft.

Abstract

Acoels are bilaterally symmetric aquatic worms, that lack a through gut and have a single body opening to their digestive syncytium. Although they were initially placed within spiralians, because of their morphological affinities with turbellarians, acoels, together with nemertodermatids and *Xenoturbella* (Xenacoelomorpha), are now placed as sister group of all the remaining bilaterians (Nephrozoa = Protostomia + Deuterostomia), based on several molecular studies. This phylogenetic position gives acoels a critical role for our understanding of bilaterian evolution. Acoels show a stereotypic cleavage pattern, called duet cleavage, and also, they possess a regulative development, meaning that their embryos have the ability to adapt to perturbations of the normal development, such as the deletion of one or more blastomeres at different developmental stages. Although several studies described acoel cleavage pattern, a comprehensive study on their embryonic development, also uncovering the molecular patterning of developmental genes, is missing. Thus, to have a better understanding of the evolution of developmental traits, I analysed and compared the early embryogenesis of two acoels species, *Isodiametra pulchra* and *Convolutriloba macropyga*, focusing on the fate of the early blastomeres, on their ability to regulate (or not) cell ablations, and on the expression pattern of several developmental genes during the early cleavages. In the first part of this thesis, I provide a comprehensive study on the embryonic development of the acoel *I. pulchra.* The detailed fate map of the early blastomeres showed how they contribute to the germ layer derivatives and to the bilateral ground plan of these animals, while the immunostaining after the ablation of specific blastomeres showed the extent to which regulation occurs in these embryos. These data revealed similarities with the embryonic development of another acoel species *Neochildia fusca*, showing a general conservation of cell fates between the two acoel species, but also highlighted that the regulative potential is restricted to specific embryonic stages in the species *I. pulchra.* In the second part of the thesis, I analysed the spatial and temporal expression of 17 developmental genes, during the early development of the acoel *C. macropyga*. The expression of several ectodermal markers (*dlx, emx, gata1/2/3, otx, pax2/5/8, six3/6,* and *nk2.1*) in the micromere lineages revealed the ectodermal identity of the early micromeres, while the expression of the endomesodermal markers (*gata4/5/6*, *fox A* and *foxF*) in the macromere lineages revealed the endomesodermal identity of the early macromeres. The characterised molecular fates of the early blastomeres

are consistent with the acoel fate map and provide the first early expression analysis of regulatory genes in an acoel species. This thesis expands the knowledge on the embryonic development of acoels, combining classical embryological studies, such as the fate map analyses and the perturbation experiments, with modern molecular approaches, such as whole mount *in situ* hybridization (WMISH).

List of Publications

Papers included in the thesis:

The following papers are part of the thesis:

Paper I: Cetrangolo V., Martindale M.Q., Seaver E., Schnabel R., Hejnol A. **The fate map and regulative development analyses of the acoel *Isodiametra pulchra*. (**Manuscript in preparation)

Paper II: Cetrangolo V., Hejnol A. **Early blastomere identity revealed by gene expression in the acoel *Convolutriloba macropyga.*** (Manuscript in preparation)

During my training I contributed to the paper:

Martín-Durán J.M., Vellutini B.C., Marlétaz F., Cetrangolo V., Cvetesic N., Thiel D., Henriet S., Grau-Bové X., Carrillo-Baltodano A., Wenjia G., Kerbl A., Bekkouche N, Chourrout D., Gómez-Skarmeta J.L., Irimia M., Lenhardt B., Worsaae K., Hejnol A. **Conservative route to extreme genomic compaction in a miniature annelid.** (Manuscript submitted)

Contents

1. Introduction

1.1 The embryo and its development: how to form a new organism

If we look at all the diverse life forms in nature, we soon realise that there is a common feature shared by most of the organisms in the tree of life: the embryo. The embryo, from the Greek word ἔμβρυον (*embruon*) literally the "young one", is the unit by which a new organism starts to be formed and its creation is the way most life forms on Earth use to expand their populations and to transmit their genes to the next generations. After two individuals produce their gametes, haploid cells that carry a single copy of each chromosome, these cells will be combined together (fertilization) and generate a new cell, the zygote. This is the primordium of the embryo, in fact this single and new cell will go through a series of events and processes that will lead to the formation of a completely new individual, that carries both chromosomal sets of the parents. Therefore, the embryo represents the first step into the life of an organism. For this reason, the embryo and its development always represented a fascinating topic to study.

Over the years, scientists have dedicated their careers and lives to study the development of several animal embryos. Between the end of the 19th and the beginning of the 20th century, the studies of scientists such as Boveri, Driesch, Hertwig, and Loeb started to shed light on the fertilization process and on early embryonic development of the sea urchin (Ernst, 1997). About the same time, the embryologists Conklin, Lillie, Bresslau and Wilson followed individual cells during the development of ascidians, gastropods, acoels and annelids, revealing their specific fates during the ontogeny (Bresslau, 1909; Conklin, 1905b; Lillie, 1895; Wilson, 1892). In this way, they created the first cell lineage studies, tracking the cells to their ultimate fates. Meanwhile, Roux, Chabry, Spemann and Mangold, investigated the embryonic development of amphibians and ascidians (Chabry, 1888; Roux, 1895; Spemann and Mangold, 1924). The manipulation of embryos, which was then called experimental embryology,

allowed them to understand the response of the embryos to perturbations during the early development. With such manipulations, they began to study how the specification of blastomere fates occurs. Altogether, these studies built the foundations of the modern developmental biology. Then, the outbreak of developmental genetics (identification of genes that control the development) and molecular biology (interpretation of relationships and interactions between developmental genes), improved the study of embryological paths. Thanks to the synergy of these fields with the developmental biology, nowadays, we have great knowledge about the processes that constitute the embryonic development (Slack, 2013).

1.2 Early cleavage: the first steps of the embryo

After the fertilization of the two gametes, the first event that occurs is a series of mitotic divisions of the zygote. This initial phase, called cleavage, leads to the formation of multiple cells, the blastomeres, which in later stages are committed, differentiated and reorganised into distinct cell types (Wolpert et al., 2015). Each new blastomere needs to acquire a different fate to form tissues and organs, which will be placed according to the axes of the adult organism. Cell specification involves a change in the pattern of gene activity within a specific cell, that accordingly will lead to a variation in the protein composition of the cell (Davidson, 1990; Wolpert et al., 2015). Therefore, the cleavage phase, dividing the embryo in numerous blastomeres, creates different spatial territories which are then specified into different fates. Already Conklin, in the late 1800, observed that the cleavage pattern is linked with the fates of the blastomeres and proposed the terms *determinate* and *indeterminate* cleavage (Conklin, 1897). He defined those cleavage patterns that are constant in form and in which the cells invariably give rise to definite structures of the embryo or larva under normal developmental conditions, as *determinate*. On the other hand, he termed those cleavage patterns that are not constant in form, bearing no constant planes of localisation among embryos and in which the blastomeres are not predeterminate, as *indeterminate*. Nowadays, the cleavage pattern is usually classified as invariant or variable. An *invariant* cleavage pattern is defined by regular cleavage planes and equal positions of

blastomeres, which are constant in each embryo of a given species. The regular and specific position of the blastomeres, consequently produces predictable blastomere fates from embryo to embryo (Davidson, 1990). The invariant cleavage pattern is also known as stereotypic and it is observed in most bilaterian embryos. Thus, the determinate cleavage as defined by Conklin, refers to ontogenies with invariant cleavage pattern, in which individual blastomeres and their fates are identical among embryos. In contrast, a *variable* cleavage pattern is defined by irregular positions of the blastomeres, which are consequently not similar among embryos of a species. In this case, the cell fates are not predictable and the differentiated organs and tissues are generated by different lineages (Davidson, 1990). The variable cleavage pattern is mostly observed in vertebrates, especially in mammals. Thus, the indeterminate cleavage, proposed by Conklin, refers to the variable cleavage pattern.

1.3 Cell lineage and fate map analyses

During the late years of the 19th Century, among the pioneers of developmental biology, Wilson and Whitman created the term "cell lineage". Following the cells and their divisions throughout the development, they were able to trace out the fates of individual blastomeres (Whitman, 1878; Wilson, 1892). The initial cell lineage studies were performed by means of direct observation of the embryos, which were generally transparent and easy to access. One of the major advantages for cell linages analyses is the stereotypic and invariant cleavage pattern, which allows the identification of the cells (and consequently of fates) consistently in each embryo of a species. Also, the low number of cells allows the reconstruction of complete cell lineages, as seen in the complete cell lineage analyses of the nematode *C. elegans*, where individual worms are formed of 959 somatic cells (Sulston et al., 1983). In contrast, following the cells of opaque embryos, with a variable cleavage pattern, like fish or amphibians, is one of the main limitations to direct observation for the reconstruction of cell lineages. Almost a century after the first cell lineage studies, Vogt solved this problem by marking the surface of an amphibian blastula with a vital dye (i.e. non-toxic dye, Nile Red) (Vogt, 1929). With this technique, he was able to follow the cellular movements during the

gastrulation and created the first fate map of an embryo (Vogt, 1929). In fate map analyses, the marked cells are followed through morphogenetic movements and traced to their final fates without tracking specific cell divisions, then these fates are projected back into the progenitor cells (Klein and Moody, 2016). Nowadays, numerous techniques are available to follow the cells until their final fates and produce cell lineages and fate maps. Among them, one of the modern ways to reconstruct cell lineages is the four-dimensional - 4D - microscope system (Schnabel et al., 1997). In this system, a microscope is connected with a camera and a software, allowing the recording of the embryo at different focal planes, throughout the development. The recordings are then examined with the software which allows the analysis of cell divisions, cell positions and cell migration, producing the final cell lineage (Schnabel et al., 1997) . On the other hand, one of the most used technique to produce fate maps is the injection of vital tracers in individual blastomeres, creating the so-called *clonal* fate maps (Weisblat et al., 1978). In early fate map analyses with this technique, horseradish peroxidase enzyme - HRP - was injected into single blastomeres and the distribution of the tracer in the progeny of the injected cells was then observed in late embryonic stages (Weisblat et al., 1978). More recently, the introduction of fluorescent vital tracers allowed not only the identification of the blastomere fates (Schoenwolf and Sheard, 1990), but also the possibility to injected, simultaneously, multiple tracers into blastomeres, reconstructing the fate map of different blastomeres (Lyons et al., 2015).

Ever since then, cell lineage and fate map analyses were pursuit in many metazoan embryos, including ctenophores (Martindale and Henry, 1999), acoels (Bresslau, 1909), sea urchins (Cameron et al., 1987), amphibians (Moody, 1987a, b, 2000), fish (Kimmel and Warga, 1988), tunicates (Conklin, 1905b; Nishida and Satoh, 1983, 1985, 1989), annelids (Ackermann et al., 2005; Meyer and Seaver, 2010) , molluscs (Dictus and Damen, 1997; Hejnol et al., 2007), nematodes (Schnabel et al., 1997; Sulston et al., 1983), and arthropods (Gerberding et al., 2002; Hejnol et al., 2006), among others. The comparison of cell lineages among animals leads to the identification of common and likely ancestral embryological features such as cleavage patterns and cell fates of

specific blastomeres. For instance, the comparison of cell lineages and fate maps within Spiralia, a large and diverse group of bilaterians, led to the identification not only of a common cleavage pattern, i.e. the spiral cleavage pattern, but also of shared cell fates among blastomeres, possibly indicating a common evolutionary ancestor (Lambert, 2008, 2010). Therefore, lineage analyses allow the identification of cell fates in time and space and also, by revealing how these processes vary across animals, they contribute to a better understanding of the evolution of different embryonic development modalities.

1.4 Regulative potential and cell fate specification

At the time of the first cell lineage studies, questions arose regarding the way specification of cell fate occurs. Weismann proposed that the nucleus of the zygote contains some factors, which he called determinants, that were distributed differently in each new cell, producing different fates (Weismann, 1893). Therefore, according to Weismann's theory the fates of the cells were predetermined (Weismann, 1893). Although Weismann theory was purely theoretical, surely it paved the way to a new concept in developmental biology: the idea that determinants were inherited by the zygote and then differentially distributed in the embryonic cells. At that time, several embryologists, including Chabry, Conklin, and Hertwig, performed perturbation experiments with several embryos, in order to understand the developmental mechanisms that lead to the specification of cells and generally to the formation of a complete adult individual (Chabry, 1888; Conklin, 1905a; Hertwig, 1892). Among them, two embryologist, Roux and Driesch performed perturbation experiments on frog and sea urchin embryos, in the attempt to validate Weismann's theory. Roux ablated one blastomere at the 2-cell stage of a frog embryo and obtained a half-formed late embryo, claiming that the development was based on mosaic mechanism and that the cell fates are determined at each cleavage (Roux, 1895; Sander, 1991) In contrast, Driesch, by separating the two blastomeres at the 2-cell sage of the sea urchin, obtained two normal, but smaller, larvae (Driesch, 1892, 1909). He, therefore, defined the *regulative* development as the potential of an embryo to adapting to an interference, as

a removal of a part (Driesch, 1909). While Roux's experiment seemed to support Weismann's theory on the nuclear determinants, the experiment of Driesch seemed to reject it, opening new questions on how the cells are specified and the potential of the blastomeres is regulated. More perturbation experiments performed on ascidian embryo by Chabry and Conklin (Chabry, 1888; Conklin, 1905a) also showed opposite results to Driesch experiments. In his study, Conklin concluded that ascidians showed a *mosaic* development, since the embryos were missing the structures derived from the deleted cells (Conklin, 1905a). Thus, ascidians embryos were not able to adapt after the deletion or one or more parts. In these early times of developmental biology, it started the dichotomy between *regulative* versus *mosaic* development, and many embryos, mostly invertebrates, were initially identified as *mosaic* embryos, while the vertebrate embryos were identified as *regulative* (Conklin, 1905a; Wilson, 1892). Experimental embryology showed that the concepts of regulative and mosaic development are closely related to cell fate specification. In the regulative development, interactions between cells govern the cell fate specification, while in the mosaic development the fates are specified by maternally inherited factors (Lawrence and Levine, 2006). In this contest, in embryos that exhibit *regulative* development, the cells are *conditionally* specified through cellular interactions and also by the cellular environment that specific signalling molecules (morphogen gradients) create (Wolpert et al., 2015). Hence, in this case the specification of cells depends solely on the extracellular context and not on intrinsic cellular factors. When the cells are *conditionally* specified, because their specification depend on external signals, they retain a certain *plasticity*, i.e. the potential to differentiate into different fates, at least until a given time point during the embryonic development (Davidson, 1990). The conditional specification of cells was initially showed by Spemann and Mangold, in frog embryos. With a transplantation experiments in *Xenopus,* they showed that a group of cells, called organizer, can influence the specification of the neighbour cells, when moved into another cellular environment (Spemann and Mangold, 1924). Also, because of cellular interactions, Roux's deletion experiment on the 2-cell stage frog embryo was not correct. Although he ablated one cell, the blastomere was connected

with the non-deleted one and still producing signals, which finally led to the formations of a half-embryo. Already in 1892, Wilson discussed these results in his study, highlighting that in a normal development each blastomere is influenced by the others (Wilson, 1892). In contrast, in *mosaic* embryos, the cell fates are *autonomously* determined by maternally inherited morphogenetic determinants. In his studies, Conklin observed that the yellow pigment present in the ascidian zygote, was then distributed and localised into the cells, progenitors of the muscles. Although he did not believe that the yellow pigment determined the cell fate, he understood that as the yellow pigment was subsequently distributed only in the progenitor cells of the tail muscles, something similar was underlying the specification of these cells (Conklin, 1905a).

With the emergence of molecular approaches, embryologists are nowadays able to investigate the details of cell fate specification. Thanks to identification of the molecular markers, it become clear that the determinants are the gene products which can specify and determine the cells fates and, consequently, that no embryo is devoted to one or the other type of development (Lawrence and Levine, 2006). On the contrary, virtually all embryos display a combination of mosaic and regulative development, in which some cell linages are autonomously specified while other are conditionally specified by signalling molecules. In the abovementioned examples, the ascidian specific early blastomeres are determined *autonomously* by the maternal transcription factor *Macho-1* to differentiate into muscles, while the nervous system formation is controlled by Notch-Delta signalling through cell-cell interactions (Akanuma et al., 2002; Nishida and Sawada, 2001). On the other hand, sea urchin embryos, initially thought to be exclusively regulative embryos, possess also maternal determinants that determine the large micromeres at the vegetal pole. These cells are specified *autonomously* by the maternal determinants Disheveled and β-catenin to become endoderm and mesoderm and also they are able to produce signals to induce the specification of their neighbour cells (Croce and McClay, 2010). Similarly, in *Xenopus* embryos, the maternal factor VegT is essential for the specification of the primary germ

layer (Zhang et al., 1998). Therefore, since no embryo, taken as a *whole*, is completely mosaic or regulative, it seems correct to apply the mosaic or regulative potential to specific cell lineages or set of cell lineages, as suggested by Davidson (Davidson, 1990).

1.5 Developmental genes and the Evo-Devo era

The rise of molecular approaches applied into the embryology field not only showed that the genes and the signalling molecules are responsible for developmental mechanisms but also that many of the genes involved in developmental processes were present in different animal embryos, even distantly related as *Drosophila* and vertebrates (Gurdon, 1992). The comparison of the genes involved in embryonic development highlighted great molecular similarities among metazoan embryos. One example is the discover of the Hox genes, a group of transcription factors that are involved in the specification of structures along the anteroposterior axis. These genes are found in a vast diversity of embryos among metazoan (Carroll, 1995; Cook et al., 2004; Duboule, 2007; Ferrier, 2010). Similarly, several molecular pathways involved in developmental mechanisms are found across metazoan embryos. In particular, the BMP pathway which pattern the dorsoventral axis, the canonical Wnt pathway, also involved in the primary axis formation, and the Notch-Delta pathway, which promotes differential cell identities, are present in several metazoan species (Babonis and Martindale, 2017). This new approach to the comparative embryology highlighted, for the first time, the possibility to associate development and morphology, comparing them across distantly related species. Also, the emergence of new and powerful molecular techniques, such as whole mount *in situ* hybridization of the (WMISH), has given the possibility to follow the expression of the genes in embryos throughout the embryonic stages and opened new roads for the comparative embryology field (Koopman, 2001). Consequently, embryologists started to compare expression patterns and function of the developmental genes, to then study animal evolution. This gave rise to the new field of Evolutionary Developmental Biology (Evo-Devo), whose aim is to uncover the developmental mechanisms that govern the modification of

morphological inheritable characters, which are at the base of animal evolution (Hall, 2003). Over the years, the urge of uncover evolutionary changes that govern the developmental mechanisms led to the study of numerous animals. The first studies were mainly focus on distantly related species, which were already used in the lab and for which many molecular and genetic techniques were available, such as *Drosophila, C. elegans* and *Xenopus* (Gurdon, 1992). However, more animal species from across metazoans were selected and studied, expanding the knowledge on the evolution of developmental characters. As an example, the study of cnidarian and ctenophore gastrulation, from both the developmental and the molecular perspective, and the comparison with bilaterian embryos, revealed a change in the site of gastrulation and conservation of the genes involved in this process. This has an important impact on our understanding on the evolution of the developmental processes that led to the modification of body plan in bilaterian evolution (Martindale and Hejnol, 2009). Although nowadays we have a large knowledge on ontogenies, developmental genes and genomes of many metazoan embryos, there are still several species that remain scarcely studied, limiting a deep understanding on animal evolution. This highlights the need to expand the taxon sampling to groups with key phylogenetic positions, to increase our knowledge on animal evolution. In this regard, an interesting taxon to study is Acoela, as it holds a pivotal phylogenetic position.

1.6 Acoela

Acoels are small aquatic worms, abundant in the benthic aquatic environment, whose body size ranges from few micrometres to few millimetres in length (Fig.1) (Achatz et al., 2013; Haszprunar, 2016). They are bilaterally symmetric worms, that lack a through gut, having a single body opening to their digestive syncytium, homologous to the mouth of non-chordate bilaterians (Hejnol and Martindale, 2008a). Acoels also lack a coelom and the space between the digestive syncytium and the body wall is often filled with parenchymal cells and bodies of epidermal and gland cells (Smith and Tyler, 1985). Their nervous system shows a general centralization towards the anterior with a great variety of organizations from species to species. It mainly consists of a nerve net and commissures which can be organized in a ring, a barrel or a bilobate neuropil at the anterior end, with three to eight pairs of neurite bundles running along the anteroposterior axes and placed dorsal, ventral and laterally (Achatz and Martinez, 2012; Bery et al., 2010; Raikova et al., 2004; Reuter et al., 1998; Reuter et al., 2001; Semmler et al., 2010). At the anterior end, acoels typically possess the statocyst, a gravitational sensory organ composed of three cells: one lithocyte, bearing a statolith, and two parietal cells encapsulating the lithocyte (Bedini et al., 1973; Ehlers, 1991). A gland organ, called the frontal organ is also present at the most anterior end, and it is constituted by several mucus-secreting gland cells (Klauser et al., 1986; Smith and Tyler, 1986). Furthermore, some acoel species have eyes spots which can have a photoreceptor function (Lanfranchi, 1990; Yamasu, 1991). The musculature of acoels is composed of a grid of several types of muscles: longitudinal, circular, oblique, diagonal and U-shaped muscles run along the anteroposterior axis and also contribute to the copulatory organs (Chiodin et al., 2013; Hooge, 2001; Semmler et al., 2008; Tekle et al., 2007; Tyler and Rieger, 1999). While the multiciliate epidermis allows acoels to glide, the body-wall muscles net generates bending, shortening and lengthening movements (Tyler and Rieger, 1999). Furthermore, acoel possess the ability to regenerate their body after an injury or fission, an ability driven by special self-renewing cells, called neoblasts. These are pluripotent cells that govern the

regeneration process and also control the growth and the homeostasis of the body, being the only mitotically active cells of the body (De Mulder et al., 2009; Gehrke et al., 2019; Gehrke and Srivastava, 2016; Srivastava et al., 2014). The muscles can be also be involved in the regeneration process, helping the wound to close and providing positional information (Raz et al., 2017). Finally, acoels do not possess a circulatory system and excretory organs but they show an active excretion mode through their digestive-associated tissues (Andrikou et al., 2019).

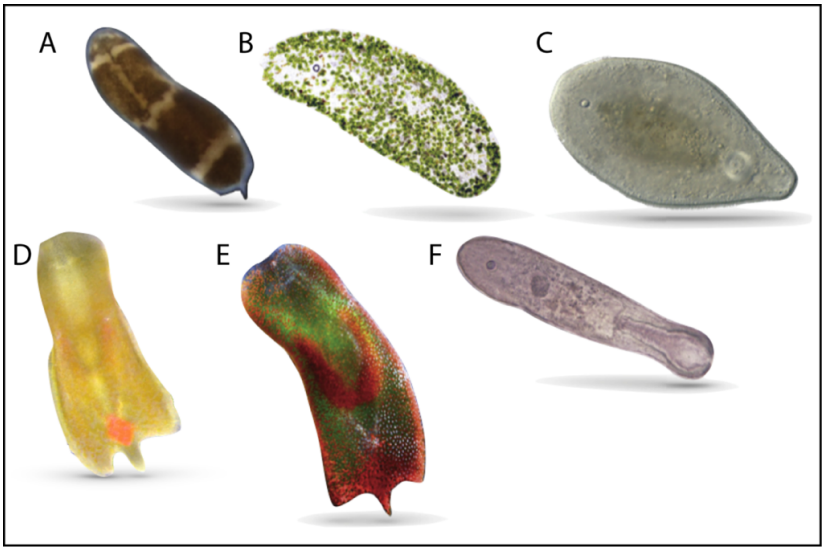

Figure 1.1 Acoels diversity A *Hofstenia miamia* **B** *Symsagittifera roscoffensis* **C** *Isodiametra pulchra* **D** *Convolutriloba macropyga* **E** *Convolutriloba longifissura* **F** *Diopisthoporus psammophilus***.** (Modified after Hejnol and Pang, 2016, Current Opinion in Genetics and Development)

1.6.1 Phylogenetic position of acoels

Because of their morphological affinities with Platyhelminthes, acoels and nemertodermatids (Acoelomorpha) were initially placed within the Platyhelminthes group, as part of "Turbellaria" (Ax, 1987; Ehlers, 1986). Morphological studies based on parsimony analyses started to consider the Platyhelminthes as a paraphyletic group and Acoelomorpha as early bilaterian offshoot (Haszprunar, 1996). Then, with the advent of molecular analyses, several studies started to support the phylogenetic position of Acoelomorpha as the early bilaterian lineage, sister group of all remaining Bilateria (Nephrozoa = Protostomia + Deuterostomia). The first studies supporting this position were based on partial or complete small subunit (SSU) ribosomal RNA analysis, on the *Hox* genes signatures and mitochondrial genome data, amongst others (Cook et al., 2004; Katayama et al., 1993; Mwinyi et al., 2010; Paps et al., 2009; Ruiz-Trillo et al., 2002; Ruiz-Trillo et al., 2004; Ruiz-Trillo et al., 1999; Telford et al., 2003). Then, the availability of more nucleotide sequence data allowed a broader analysis, supporting the position of Xenacoelomorpha (Acoelomoprha with the inclusion of *Xenoturbella*) as sister group of Nephrozoa (Dunn et al., 2014; Hejnol et al., 2009; Srivastava et al., 2014). Finally, a more recent analysis, done by Cannon and colleagues, reinforced this phylogenetic position, with a stronger support based on the analysis of more transcriptomes and using maximum likelihood and Bayesian inference methods (Cannon et al., 2016) (Fig. 1.2). In contrast with the position of Xenacoelomorpha as sister group to Nephrozoa, other studies initially placed *Xenoturbella* as sister group to Ambulacraria, within Deuterostomia (Bourlat et al., 2006; Bourlat et al., 2003). These first studies were based on analyses of mitochondrial genome, miRNA complements and amino-acids data sets. More recent studies expanded the data set with new data matrix including more amino-acids and more taxa selection, and placed Xenacoelomorpha (= *Xenoturbella* + Acoelomorpha) as sister group to Ambulacraria (Philippe et al., 2011; Philippe et al., 2019). Although this represents still an open question within the bilaterian phylogeny, the phylogenetic position of Xenacoelomorpha, as the sister group of the Nephrozoa, assigns to this group a critical role for understanding bilaterian evolution.

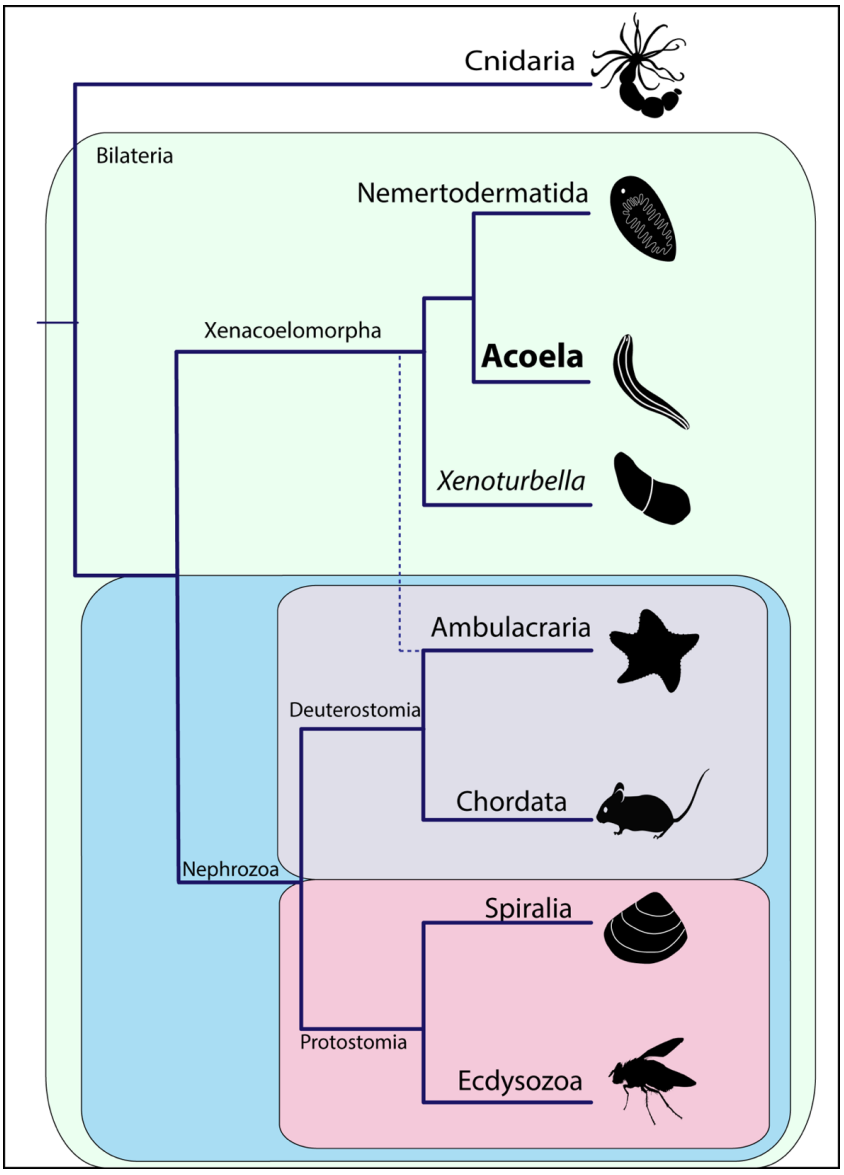

Figure 1.2 Phylogenetic position of Xenacoelomorpha (*Xenoturbella* + Acoela + Nemertodermatida) as sister group of Nephrozoa. In dashed line the position of Xenacoelomorpha as sister group of Ambulacraria, as hypothesize by Philippe et al. (2011, 2019).

1.6.2 Acoel development

Acoels are hermaphrodites and their gonads are not lined by a specific epithelium and are generally located on the lateral sides of the central digestive syncytium (Rieger et al., 1991). They reproduce sexually, with an internal fertilization by mutual sperm transfer and hyperdermal or hypodermal injection (Apelt, 1969; Bush, 1975). After fertilization, eggs are laid individually or in clusters through the mouth or the female gonopore (Rieger et al., 1991). All acoel species reproduce sexually, but some species also possess asexual reproduction through paratomy (preformation of organs before separation), architomy (formation of organs after the separations of the two individuals) and budding by which the daughter individual develops at the posterior end of the mother but with a reverse axis from the mother (Bartolomaeus and Balzer, 1997; Shannon and Achatz, 2007; Sikes and Bely, 2008, 2010).

Acoel embryos develop directly into juveniles, without the formation of a larval stage, and display a stereotypic and invariant cleavage pattern called duet cleavage. At each cell division, starting at the 4-cell stage, two large cells, the macromeres, give rise to two smaller cells called micromeres. The two newly formed micromeres and macromeres are called duet (Fig.1.3) (Boyer, 1971; Henry et al., 2000). This cleavage pattern is shared by all acoel species investigated so far, suggesting that it could be an ancestral feature of this group (Apelt, 1969; Bresslau, 1909; Gardiner, 1895; Georgévitch, 1899; Henry et al., 2000). In his studies on acoel embryonic development, Bresslau was the first to recognise a possible relationship between the cleavage pattern of acoels and the spiral cleavage pattern, thus connecting acoels with spiralians (Bresslau, 1909). Therefore, the nomenclature of the blastomeres initially followed the one for the Spiralia.

The first cleavage follows the animal/vegetal axis and divides the embryos meridionally in two equal blastomeres (A and B; Fig. 1.3A). In the second division, the mitotic spindles have an oblique 45° angle to the animal/vegetal axis and the two macromeres generate, with a counterclockwise movement, two smaller micromeres at

the animal pole the 4-cell stage. The first micromere duet 1a, 1b is localized towards the animal pole, while the first macromere duet 1A and 1B at the vegetal pole (Henry et al., 2000) (Fig.1.3B). At the third division the mitotic spindles are again slightly oblique and two new micromeres (second duet 2a, 2b) are generated from the macromeres, forming the 6-cell stage (Fig. 1.3C). The next cleavages divide the animal micromeres. The first micromere duet (1a and 1b) divides with the mitotic spindle oriented with a 90° angle and give rise to the micromeres, $1a^1$, $1a^2$ and $1b^1$,$1b^2$, forming the 8-cell stage (Fig. 1.3D). Then, in the division to the 10-cell stage, the second micromere duet (2a, 2b) divides, generating the micromeres $2a^1$, $2a^2$ and $2b^1$, $2b^2$ (Fig. 1.3E). At this point, the vegetal macromeres divide again, with their mitotic spindle perpendicular to the animal-vegetal axis and give rise to two new larger micromeres (third micromere duet 3a,3b) and two smaller macromeres (third macromere duet 3A, 3B; Fig 1.3F). Then at the 14-cell stage, gastrulation starts with the ingression of the third macromere duet (3A, 3B) into the embryo (Henry et al., 2000) (Fig. 1.3G). Meanwhile, the descendants of the first three micromeres duets continue to proliferate and form an outer layer that surrounds the inner macromeres and the ingression point (blastopore), at the vegetal pole (Henry et al., 2000; Ramachandra et al., 2002). After this proliferation, the embryo has about 200 cells and the formation of the tissues and organs begins. The outer layer of micromeres develop into ciliated epidermis, while the internalized macromeres form the muscles and the digestive syncytium (Ramachandra et al., 2002). Finally, the embryo develops into a juvenile, which hatches from the eggshell and has all the adult organs, except the gonads that will grow later.

Although several studies analysed the peculiar cleavage pattern of acoels, only two studies examined the developmental features of these embryos, by characterising the fate map of the early blastomeres and investigating the regulative potential of the embryo in the species *Neochildia fusca* (Boyer, 1971; Henry et al., 2000). Boyer in 1971 examined the regulative potential of *N. fusca* embryos by manual ablation of specific blastomeres. Deletions of one or two micromeres and one macromere at 4- 6- and 12-cell stages always result in normal juveniles, that display morphological traits

similar to the normal juvenile: presence of the mouth, the statocyst, pigmentation, body shape and muscular contraction. On the other side, deletions of two macromeres, at any of the mentioned stages, result in aberrant juveniles which generally display an outer layer of ciliated epidermal cells and a roundish body shape. The most significant results are the one after the deletion of one blastomere at 2-cell stage, since this is the classical experiment to determine the regulative capacity of an embryo. After this blastomere's deletion, a great percentage of normal juveniles develop. Therefore, altogether these data not only demonstrated that this acoel species has the ability to regulate blastomeres deletions, but also that cell-cell interactions are important for the normal embryonic development of this acoel species. Moreover, Henry and colleagues made the first fate map of an acoel embryo (Henry et al., 2000). Injecting a fluorescent lineage tracer into the first three micromere duets and in the second and third macromeres duet, they revealed the final fates of these early blastomeres. The first, second and third micromere duets give rise to all ectodermal derivatives, which include epidermal cells and nervous system, while all the endomesodermal derivatives, including the digestive syncytium, all the types of muscles and the statocyst, are generated from only two cells, the two macromeres 3A and 3B. This also pointed out that, in acoels, mesoderm come from only one source, namely the endomesoderm (Henry et al., 2000). Interestingly, injections into the first three micromeres duets also revealed the contribution of these micromeres to the body axes. Injections in the first duet micromere 1a and 1b label epidermal areas respectively on the dorsal and on the ventral side along the midline of the body, but, while the labelling in the dorsal side (1a) extend anterior-posteriorly, the labelled epidermal area on the ventral side (1b) localized only in the anterior half of the body. Then, injections in the second micromeres duet 2a or 2b mark epidermal areas respectively on the left and the right side, both extending along the anteroposterior and dorsoventral axes. Finally, injections in the third micromeres duet 3a or 3b mark ventral epidermal domains, posteriorly placed. With this first fate map of an acoel embryo, Henry and colleagues showed the identities of the early blastomeres corresponding to the different germ

layers, and also showed the axial distribution of the early blastomere progeny in the juvenile body.

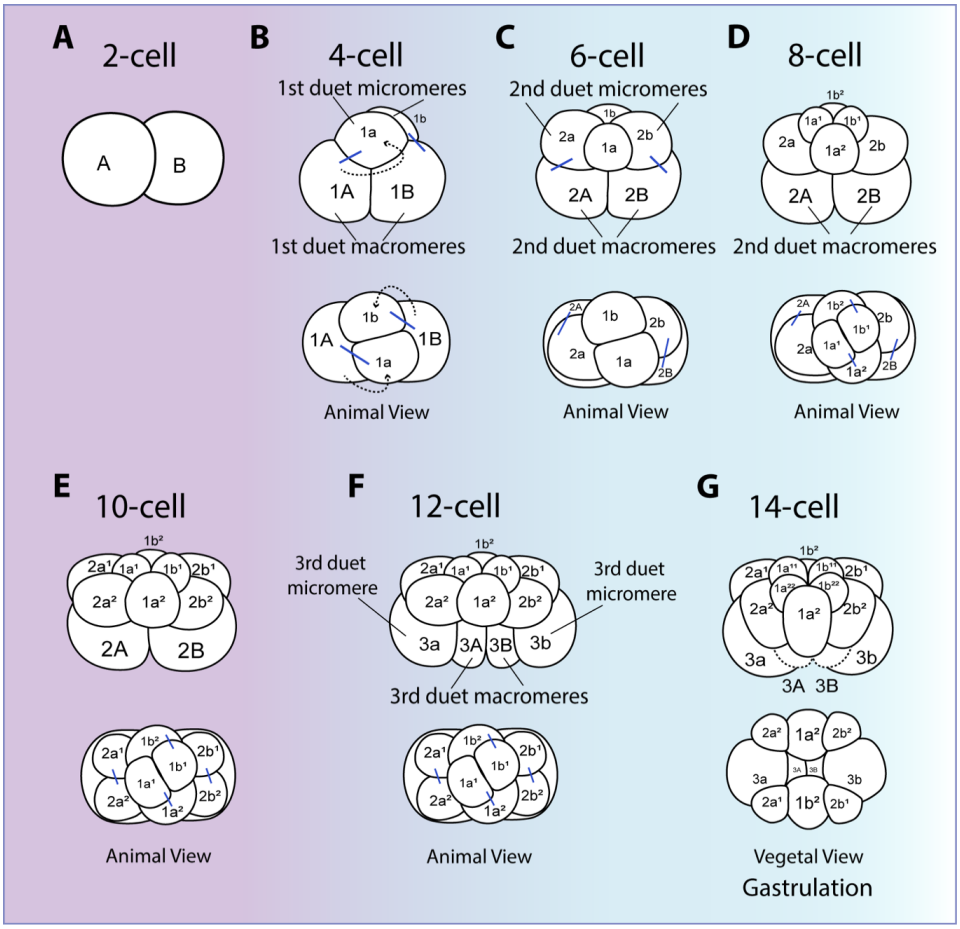

Figure 1.3 Acoel duet cleavage pattern and gastrulation. A 2-cell stage **B** 4-cell stage. Formation of the first duet micromeres and first duet of macromeres **C** 6-cell stage. Formation of the second duet of micromeres and second duet of macromere **D** 8-cell stage **E** 10-cell stage **F** 12-cell stage. Formation of the third duet of micromeres and third duet of macromeres **G** 14-cell stage. Gastrulation: ingression of the third duet of macromeres 3A, 3B.

1.6.3 *Isodiametra pulchra* and *Convolutriloba macropyga*

Acoela includes about 380 species divided in 9 large subclades, according to the last acoel phylogeny from Jondelius et al., 2011 (Jondelius et al., 2011). In the next paragraphs, I briefly introduce the two species examined in this thesis: *Isodiametra pulchra*, from the Isodiametridae and *Convolutriloba macropyga* from the Convolutidae.

Isodiametra pulchra (Smith and Bush, 1991)

The acoel *Isodiametra pulchra* is a small worm (1mm), from the meiofauna of the muddy, sandy beaches of the Atlantic coast of North America(Smith and Bush, 1991). *I. pulchra* has no pigmentation when observed at Differential Interference Contrast -DIC- but, because they feed on diatoms, often their central digestive syncytium has a greenish coloration (Fig. 1.4A). The epidermis is covered with cilia, which allow the animal to glide, and ciliated sensory receptors, while the muscle grid allows all the other types of movements (elongation, shortening) (Achatz et al., 2013). The mouth is located on the ventral side and it is aligned with the ventral funnel that this animal use to capture food. As part of the taxon Crucimusculata, *I. pulchra* possesses ventral crossover muscles beside the longitudinal, circular, oblique and diagonal muscles which form the muscles grid (Fig. 1.4D, D') (Jondelius et al., 2011; Ladurner and Rieger, 2000). A statocyst is typically present at the anterior end of the body in juveniles as well as in adults, and it is connected with muscles fibres and surrounded by neural commissures (Fig. 1.4A, C, C') (Achatz and Martinez, 2012). The nervous system is composed by commissures of neurons, condensed bilaterally around the statocyst. These commissures produce three main structures: a frontal nerve ring, an anterior commissure and a posterior commissure, which surround the statocyst. Also, four pairs of neurite bundles run along the anterior posterior axis on the dorsal, ventral, mid-ventral and lateral sides (Fig. 1.4C, C') (Achatz and Martinez, 2012). At the anterior tip of the body a gland organ is present, the frontal organ, which is composed by several mucus-secreting gland cells whose bodies lay just behind the statocyst

(Smith and Bush, 1991). Furthermore, the gonads are located on the lateral sides around the digestive syncytium (Fig. 1.4A, C, C'), same as with the neoblasts, which are self-renewing cells that allow the animal to regenerate (Chiodin et al., 2013; De Mulder et al., 2009). *I. pulchra* reproduces sexually, laying 1 or 2 embryos per animal, which directly develop into a juvenile. The embryonic development follows the acoel duet cleavage, and the muscles start to be formed at 50% of development, about 20-21 hours after egg-laying (Ladurner and Rieger, 2000). The first muscle fibres to appear are the primary circular fibres in the anterior part of the embryo, followed later by the primary longitudinal fibres, 23-24 hours after egg-laying. These establish the primary muscle grid which will be the template for the secondary muscle grid, that includes the oblique and diagonal muscles that cross the body (Ladurner and Rieger, 2000). The mouth is formed late during the development about 28-32 hours after egg-laying, at the centre and ventral side of the body (Ladurner and Rieger, 2000). Beside the description of the formation of muscles during the embryonic development, and a description of the juvenile nervous system (Achatz and Martinez, 2012), the embryonic development of this species remains scarcely investigated. Therefore, details about the development of *I. pulchra* are given in this thesis.

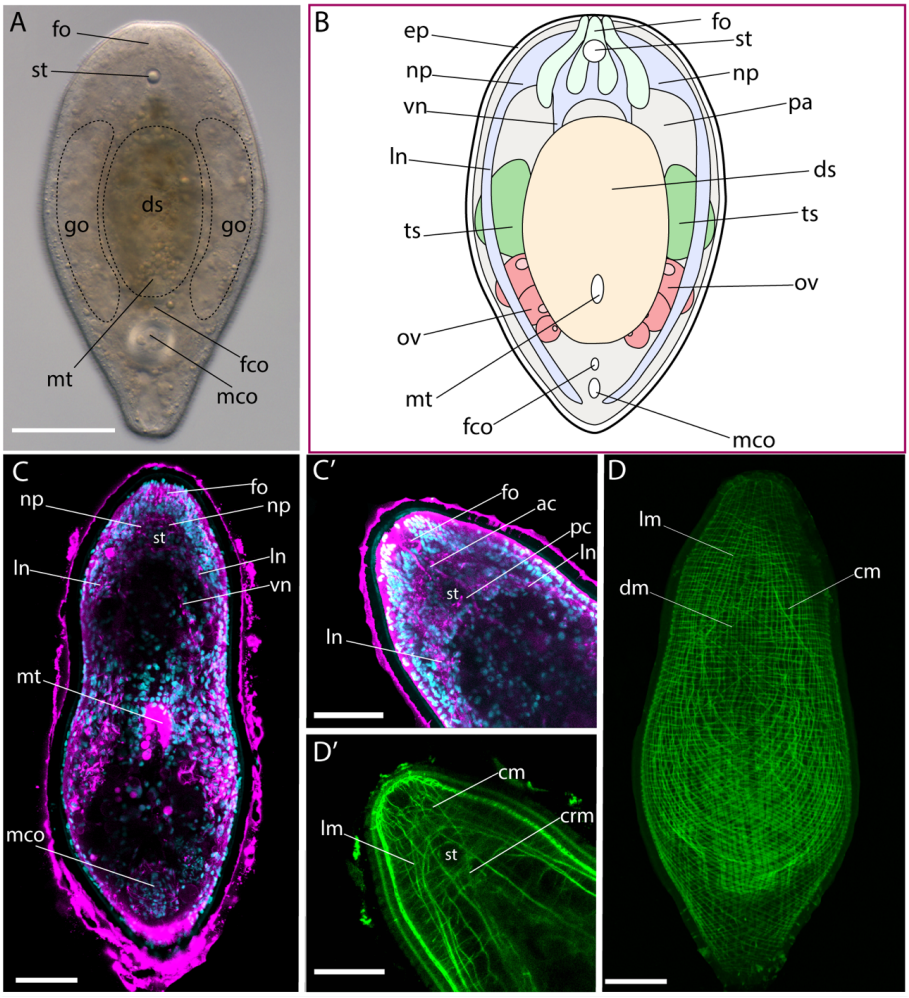

Figure 1.4 ***Isodiametra pulchra*****. A** Adult morphology under Differential Interference Contrast (DIC). Scale bar=100µm. **B** Scheme of the adult morphology. For simplicity dorsal neurite bundles are missing **C** CLSM (Confocal laser scanning microscopy) Z-projection of the nervous system, stained with Anti-tyrosinated tubulin (magenta) and DAPI marked the nuclei (cyan). **C'** Details of the nervous system **D** CLSM Z-projection of the musculature net. Muscles are stained with ND-Phallacidin (green). **D'** Details of the anterior part of the musculature net. Abbreviations: ac anterior commissure, cm circular muscles, crm cross muscles, dm diagonal muscles, ds digestive syncytium, ep epidermis, fco female copulatory organ, fo frontal organ, go gonads, lm longitudinal muscles, ln lateral neurite bundle, mco male copulatory organ, mt mouth, np neuropil, ov ovaries, pa parenchyma, pc posterior commissure, sc sensory cells, st statocyst, ts testis, vn ventral neurite bundle. C-D Scalebar=50 µm.

***Convolutriloba macropyga* (Shannon and Achatz, 2007)**

The species *Convolutriloba macropyga* was first discovered and described in 2007, and it was found in an aquarium with organisms collected in the Indo-Pacific (Shannon and Achatz, 2007). The size of *C. macropyga* individuals is between 2 and 10 mm and the animals have a green and orange coloration due to the symbiont zoochlorellae and due to scattered rhabdoid gland cells. In the aquarium, they feed on *Artemia sp.* and their mouth is located ventrally in line with their ventral funnel. The epidermis is completely ciliated, as in all other acoel species, allowing the gliding movements. However, differently from *I. pulchra*, the adult individual of this species does not possess a frontal organ and a statocyst at the anterior end of the body, but it bears two small eyespots (Fig. 1.5A, A'). The body-wall musculature is composed similarly to *I. pulchra*, of longitudinal, circular, oblique and diagonal muscles, which appear to be stronger on the ventral side, with inner longitudinal muscles and circular muscles surrounding the mouth (Shannon and Achatz, 2007). The nervous system is composed of two neuropil regions transversally connected by a commissure and from which two nerve cords originate and run frontally and laterally (Fig. 1A') (Shannon and Achatz, 2007). As other acoels the gonads are located laterally to the digestive syncytium, but the peculiarity of this species is the dual reproduction strategy. *C. macropyga* individuals can reproduce sexually, producing clusters of 50-150 embryos temporally synchronised, but they can also reproduce asexually budding off the new individuals from their posterior with a reverse inverted axis from the mother (Fig. 1.5B, C). Beside the description of the species and its placement into the internal phylogeny of acoels (Jondelius et al., 2011; Shannon and Achatz, 2007), there are no studies on the embryonic development of *C. macropyga*. For the purpose of this thesis though, *C. macropyga* is a lab culturable acoel with easily accessible embryos. This species, in fact, represents one of the most fecund acoels species: when it reproduces sexually, it lays embryos all year around and in clusters of many embryos. This make this species well suited for embryology and molecular studies.

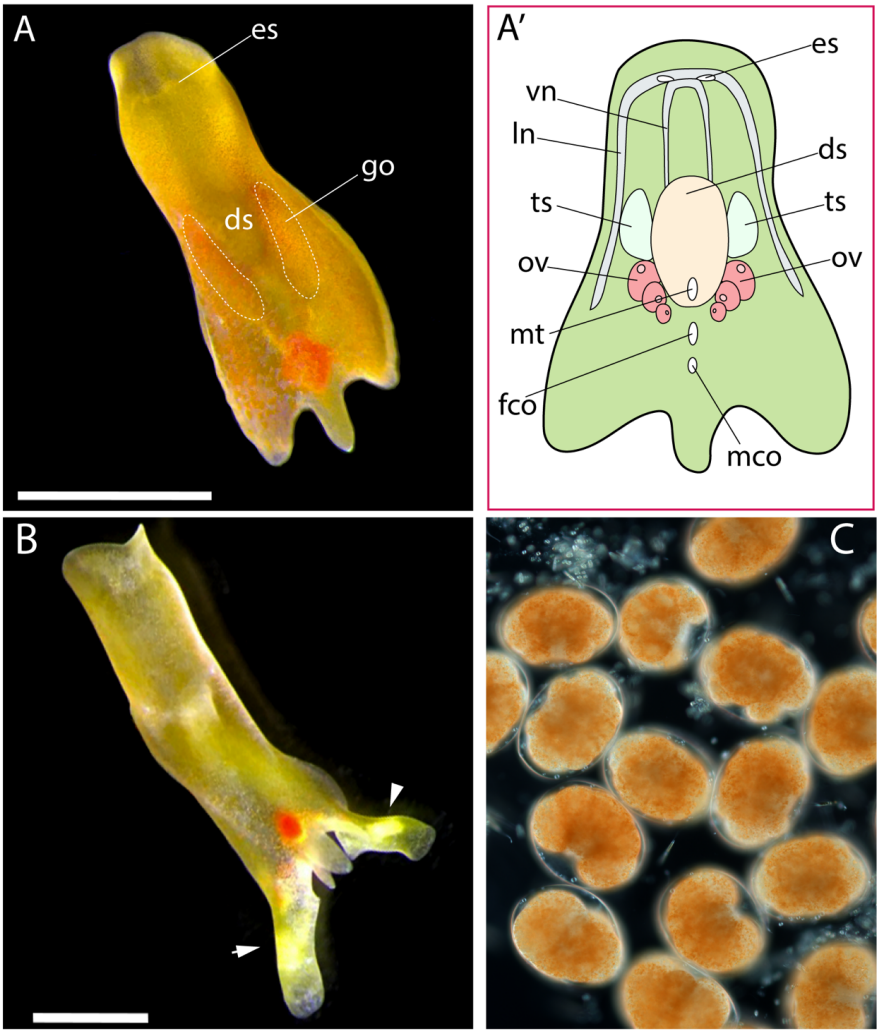

Figure 1.5 ***Convolutriloba macropyga*** **A** Adult individual of *C. macropyga* **A'** Scheme of *C. macropyga* adult morphology. **B** Adult specimen budding off two new individuals from the tail, indicated by the white arrowheads **C** Cluster of temporally synchronised embryos at gastrula stage. Abbreviations: ds digestive syncytium, es eye spot, fco female copulatory organ, go gonads, ln lateral neurite bundle, mt mouth, mco male copulatory organ, ov ovaries, ts testis, vn ventral neurite bundle. Scale bars 2mm.

2. Aim of the thesis

Reconstructing the evolution of molecular, morphological and developmental characters across animals is fundamental to evolutionary developmental biology (Balavoine and Adoutte, 2003; Hejnol and Martindale, 2008b; Hejnol et al., 2009). Species like *C. elegans, D. melanogaster, D. rerio*, and even *S. purpuratus* became models in developmental, molecular and cell biology due to the easiness of collecting and culturing them and the availability of genetic toolkits. Nevertheless, evolutionary conclusions, drawn on comparison among this handful of species, may result incomplete. Therefore, to have a better understanding of the evolution of developmental traits, it is important to expand the knowledge to animal taxa that are scarcely studied. In this context, acoels represent a key group to study developmental features because of their pivotal phylogenetic position, as sister group of Nephrozoa. Although the embryonic development of acoels was studied in several species, fate map analysis and a regulative development study are restricted to one species (Boyer, 1971; Henry et al., 2000). Also, the molecules underpinning the specification of cell fates during the embryonic development is poorly described (Hejnol and Martindale, 2008a, b, 2009; Ramachandra et al., 2002). Many assumptions on acoel embryonic development are based on few species studies (Apelt, 1969; Boyer, 1971; Hejnol and Martindale, 2008a, b, 2009; Henry et al., 2000; Ladurner and Rieger, 2000; Ramachandra et al., 2002). Therefore, the general purpose of this thesis is to expand the knowledge of the embryonic development of acoels. With this aim, I analysed and compared the early embryogenesis of two acoels species, *Isodiametra pulchra* and *Convolutriloba macropyga*, focusing on the fate of the early blastomeres, on their ability to regulate (or not) cell ablations, and on the expression pattern of several developmental genes during the early cleavages.

For this reason, this thesis is divided into two projects that elucidate different aspects of the early embryonic development with different approaches:

1- The fate map and regulative development analyses of the acoel *Isodiametra pulchra*. (**Paper I**)
2- Early blastomere identity revealed by gene expression in the acoel *Convolutriloba macropyga.* (**Paper II**)

3. Summary of the findings

3.1 Paper I: The fate map and regulative development analyses of the acoel *Isodiametra pulchra*.

To analyse the embryonic development of the acoel *Isodiametra pulchra,* I characterised the fate of the early blastomeres and their regulative potential. Firstly, I described the embryonic development of this species by differential interference contrast (DIC) microscopy; then, using injections of fluorescent lineage tracer dye in the early blastomeres, I analysed their fates. Finally, with a laser system, I deleted specific early blastomeres and I examined the morphology of the freshly hatched juveniles to characterise the regulative potential of these embryos.

Invariant and stereotypic cleavage pattern

Isodiametra pulchra embryos show a direct development, taking 42 hours to develop, after the egg is deposited, into a hatching juvenile (Fig. 2, Paper I). These embryos display the acoel-specific cleavage pattern called duet cleavage. Beginning at the 4-cell stage, a pair of larger cells, called macromeres, give rise to a new pair of smaller cells, called micromeres, at every new division round. This cleavage pattern is highly stereotyped and invariant from embryo to embryo, a characteristic that allows the identification of the blastomere fates among embryos. During the early cleavages, the micromeres orient themselves in a counterclockwise manner, with the mitotic spindle angle that varies among duets. In the first 3.5 hours after the eggs are laid, the cleavages generate the 2-, 4-, 6-, 8-, 10-, 12- and 14- cell stage embryos (Fig. 2A-G, Paper I). When the embryo reaches the 14-cell stage and comprises 12 micromeres and two macromeres, gastrulation starts. Gastrulation occurs with the ingression of the two macromeres 3A and 3B at the vegetal pole of the embryo (Fig. 2 G, G, Paper I). In the meantime, the micromeres continue to divide forming an outer layer that surround the internalised macromeres (Fig2. H-K, H'-I', Paper, I). After 24 hours, the embryo is a

ciliated ball that rotates inside the eggshell (Fig. 2L, Paper I). Finally, after 42 hours post egg-laying, the juvenile hatches from the eggshell (Fig. 3, Paper I).

Origin of ectoderm and its derivatives

The analysis of the juveniles developed after fluorescent dye injections in the first (1a,1b) second (2a,2b) and third (3a, 3b) micromere duets revealed the ectodermal fate of these micromeres. The injections in the first three micromere duets labelled epidermal cells, nervous system, gland cells and sensory cells, but the contribution of the duets to ectodermal derivatives varied from one duet to the other. Labelled epidermal areas distributed on the dorsal and ventral side were produced after ablations in the first micromere duet (1a, 1b; Figs. 4 and 9A, B Paper I), while labelled epidermal cells localised dorsoventrally on both lateral sides were produced from injections in the second micromere duet (2a, 2b; Figs. 5 and 9C, D, Paper I). Finally, posterior epidermal cells dorsoventrally distributed on the both later sides were labelled after ablations in the third micromere duet (3a, 3b; Figs. 6 and 9F, G, Paper I). Nervous system structures including neurons, the set of neurite bundles and the commissures around the statocyst were always labelled after injections in all the three micromere duets (Figs, 4, 5, 6 Paper I). Nevertheless, the injections highlighted that each duet gives rise to more ectodermal derivatives. The gland cells, composing the frontal organ, originate from the first micromere duet (1a,1b; Fig. 4 Paper I), while the rhabdoid gland cells, intermingled within the epidermal cilia, originate from the third micromere (3a, 3b; Fig. 6 Paper I). In addition, labelled sensory cells were always detected after the injections in the second and third micromeres duet (Figs. 5, 6 Paper I), indicating that these cells also come from the ectodermal lineage. Hence, ectoderm in *I. pulchra* includes epidermis and nervous system but also gland and sensory cells. In conclusion, these first three micromere duets give rise exclusively to ectodermal derivatives and never to endodermal or mesodermal cells.

Variability of the micromere descendant distribution

Although all the micromeres belonging to the first three duets possess an ectodermal identity, invariant from embryo to embryo, their descendants display a certain degree of variability. The first example of variability is the marked epidermal cells. Firstly, the number of labelled epidermal cells varied in a range between 30 and 60 among the juveniles belonging to different duets. Consequently, despite the labelled epidermal areas derived from a specific micromere duet were consistently located along the body axes, their boundaries changed from one juvenile to the other (Supplementary Fig. 1, Paper I). This indicates that the spatial arrangement of the epidermal cells, originated from one micromere, varies among juveniles, thus suggesting a degree of variability of the micromere descendants. Then, another example of variability is the distribution of the neurons derived from the second micromere duet (2a, 2b). After injections in the second micromere duet, the majority of neurons were labelled on the left or on the right side. Embryos injected into the micromere 2a displayed most of the marked neurons located on the left side (about the 90%), but few marked neurons (15%) were located on the right side. Conversely, embryos injected into the micromere 2b displayed most of the marked neurons located on the right side, but few labelled neurons located on the left side (Fig. 5, Paper I). This suggests a possible migration of the second duet micromere progeny across the midline of the juvenile body. Finally, the third example of variability is the ectodermal derivatives of the third micromere duet (3a, 3b). Although this micromere duet also give rise only to ectoderm, the labelled ectodermal derivatives coming from these micromeres varied among juveniles. Labelled sensory cells and rhabdoid gland cells were detected in all the examined juveniles, but just a small portion of juveniles displayed, together with the marked sensory cells, labelled epidermal cells posteriorly located (Fig. 6, and Supplementary Fig.1, Paper I). In conclusion, these observations indicate a variability in the spatial arrangement of the progeny of the early blastomeres in *I. pulchra*, in spite of the stereotypic cleavage pattern and invariant cell fates of these embryos.

Origin of endomesoderm

To characterise the fate of the macromeres, I analysed juveniles developed after injections in the macromeres belonging to the second duet 2A and 2B. In these juveniles, I observed labelling in all the types of muscles that constitute the muscular net (longitudinal, circular and diagonal fibres), indicating that the second duet of macromeres (2A and 2B) give rise to mesodermal derivatives (Figs. 7, Paper I). Because of the absence of gonads in the juveniles, the muscles were the only mesodermal derivatives that I could detect. Interestingly, another labelled structure after injections in the macromeres 2A and 2B was the statocyst (Fig. 7, Paper I). Not only the muscles around the statocyst but also the three cells that compose this structure (two parietal cells and one lithocyte) were marked in a portion of juveniles, probably indicating a mesodermal origin of this sensory organ. Finally, due to autofluorescence in the digestive syncytium in most of the examined juveniles, I could not distinguish any endodermal fate for the second macromeres duet. Altogether, these injections revealed the mesodermal (and possibly also the endodermal) fate of the second duet macromeres.

Deletion of early blastomeres revealed the regulative potential is restricted to specific cell stage.

To analyse the regulative capacity of *Isodiametra pulchra* embryos, I deleted one or more blastomeres in a single embryo at different stages, using a laser system. A summary of these deletions is presented in the Paper I (Tables 2 and 3 Paper I). The deletion of one blastomere at the 2-cell stage and of two macromeres at the 12-cell stage never produced normal juveniles (Fig. 8E-K and FF-HH, Paper I). These results indicate the at 2-cell and 12-cell stage, the embryo cannot compensate the loss of blastomeres. After the ablation of one micromere or one macromere at the 4-cell stage, instead, most of the juveniles looked similar to the control (Fig. 8L-AA, Paper I). Therefore, at 4-cell stage, the embryo seems to regulate and compensate for the loss of one micromere or macromere, although is not clear which other non-deleted

blastomere can compensate for the loss of the ablated one. In conclusion, these ablation experiments suggest that *I. pulchra* embryo possess a regulative ability which is temporally restricted to the 4-cell stage.

3.2 Paper II: Early blastomere identity revealed by gene expression in the acoel *Convolutriloba macropyga.*

C. macropyga embryos also display the invariant and stereotypic acoel-specific duet cleavage pattern. In this study, I analysed the spatial and temporal expression of evolutionary conserved developmental genes, during the embryogenesis of *C. macropyga.* For simplicity, I classified the embryonic development in four main stages (early cleavage, gastrula, post-gastrula and late stages) and then I examined the molecular patterning of ectodermal anterior markers (*dlx, emx, pax2/5/8, gata1/2/3, otx, six3/6,* and *nk2.1*) of posterior/hindgut markers (*bra* and *cdx*), of endodermal markers (*foxA, gata4/5/6a* and *gata4/5/6b*), and of mesodermal markers (*foxC, foxF, snail, pitx,* and *six1/2*) across the four main stages above mentioned.

Ectodermal and anterior markers characterise the ectodermal identity of the micromeres

The analyses of genes that are commonly used as ectodermal and anterior markers revealed the ectodermal identity of the micromeres belonging to the first (1a,1b), second (2a, 2b) and third (3a, 3b) duets. These genes can be categorised depending on the onset of their expression during the four main phases of *C. macropyga* embryonic development. The first group of genes (*dlx, emx* and *gata1/2/3*) was expressed at the early cleavage stages in the first, second and third micromere duets (1a, 1b; 2a, 2b; 3a, 3b). They continued to be expressed in the descendants of these micromeres, at the animal pole, throughout the remaining developmental phases (gastrula, post-gastrula and late stages; Fig. 3A-R, Paper II). The second group of genes (*pax2/5/8* and *otx*) began to be expressed at the post-gastrula, in the micromeres $1a^1$ and $1b^1$ (*pax2/5/8*) and $1a^2$ and $1b^2$ (*otx*) (Fig. 3V and BB, Paper II). Later, the expression is detected in the descendants of these micromeres (Fig. 3W-DD, Paper II). The third group of genes (*six3/6* and *nk2.1*) was detected only at the late stages in the presumptive neural descendants of the ectodermal micromeres, located at the animal pole of the embryos (Fig. 4A-L, Paper II). The expression of multiple genes in the micromere lineages

revealed the ectodermal fate of these blastomeres, while the different spatiotemporal expression of these ectodermal markers suggests a possible differential recruitment of these genes for the specification of the anterior and neuronal identities.

Posterior markers *bra* and *cdx* show differential expression profile

The expression of the posterior and hindgut marker *bra* was detected at early cleavage stages, in the two macromeres of the first duet (Fig. 4M-N, Paper II). However, at gastrula and post-gastrula stages, *bra* transcripts are detected in the micromeres $1a^2$ and $1b^2$ and their progeny, which surround the blastopore (Fig. 4O-R, Paper II). Then, in the late stages, *bra* is expressed in ectodermal precursors at the vegetal pole of the embryo (Fig. 4R, Paper II). The expression of *bra* suggests that in *C. macropyga* embryos this gene has a conserved role as a blastoporal and posterior marker. On the other hand, the posterior marker *cdx* was expressed only in the late stages, in a ring of presumptive mesodermal and neuronal cells. Nevertheless, in juveniles this gene is expressed along the anteroposterior axis in presumptive neuronal cells, suggesting that this gene might be involved in the specification of neuronal fates (Fig. 4X and Suppl. Fig.1 Paper II).

Endodermal identity of the macromeres is characterised by the expression of *foxA* and *gata4/5/6*.

The endodermal markers *foxA* and *gata4/5/6* were both detected in the macromeres but at different stages during the embryonic development of *C. macropyga.* The gene *foxA* was detected at post-gastrula, presumably in the daughter cells of the macromeres 3A and 3B, which according to the fate map of other acoels species are the endomesodermal precursors (Fig. 5D, Paper II) [(Henry et al., 2000) and the previous paragraph on *I. pulchra*]. The expression is retained in the progeny of these macromeres in the next stages, and in the late stages it expanded in an internal endodermal domain along the animal/vegetal axis, probably corresponding to the precursors of digestive syncytium (Fig. 5E, F, Paper II). Two copies of the gene *gata4/5/6* are found in the transcriptome of *C. macropyga*. *Gata4/5/6a* expression

started at the early cleavages in the second duet of macromeres (2A, 2B) (Fig. 5H, Paper II). The expression continued at the late stages in two endodermal and bilateral regions, presumptive progeny of the internalised macromeres (Fig. 5I-L, Paper II). G*ata4/5/6b*, the second copy, was instead expressed only at the late stages, in two presumptive endodermal regions at the animal pole of the embryo. (Fig. 5R, Paper II). These data revealed the endodermal fate of the macromeres, but the different expression time of the endodermal markers might suggest a possible sequential specification time.

Mesodermal markers are expressed after gastrulation

The expression of the mesodermal markers (*foxC, foxF, snail, pitx,* and *six1/2*) was not detected during the early cleavages. Only the gene *foxF* started to be expressed at post-gastrula stages, in the presumptive progeny the macromeres 3A and 3B (Fig. 6, Paper II). The internal mesodermal domain extended along the animal/vegetal axis and it seems to be adjacent or even to overlap with the expression domain of the endodermal gene *foxA,* while in late stages the expression is seen in mesodermal cells scattered at the vegetal pole (Fig. 6E-F, Paper II). The expression of this gene in the internalised macromere descendants not only indicates the mesodermal fate of these cells but also suggests that mesodermal domains are possibly adjacent to the endodermal domains expressing *foxA*. The reaming mesodermal markers (*snail, pitx,* and *six1/2*) were, instead, expressed only at the late stages in two bilateral domains, presumptive mesodermal precursors. Altogether, these data revealed the mesodermal fate of the macromeres, but they also suggest a possible late differentiation of endoderm and mesoderm.

4. Discussion

In order to expand the knowledge of acoel embryonic development, I analysed the ontogeny of two acoels species, *Isodiametra pulchra* and *Convolutriloba macropyga*. The leading aim of this thesis was to uncover the conservation of embryological features among acoel species, revealing blastomere identities through fate mapping and gene expression of conserved developmental genes. In the manuscripts (Paper I and Paper II) I discussed the specific results obtained in those studies, while in the following paragraphs I will discuss how the data obtained from these two species and works are related to each other and contribute to a broader understanding of the acoel embryonic development, framed in an evolutionary context.

4.1 Acoel blastomere fate comparison

All acoel species investigated so far share at least one common developmental character, namely the duet cleavage (Apelt, 1969; Boyer, 1971; Bresslau, 1909; Gardiner, 1895; Henry et al., 2000). Whilst an attempt of reconstructing the cell lineage has been made in the past (Bresslau, 1909; Gardiner, 1895), the complete fate map of the early blastomeres was done only twenty years ago on the species *Neochildia fusca* (Henry et al., 2000). In this study, they highlighted not only the fates of the blastomeres but also the bilaterally symmetrical contribution of the duets to the juvenile body. Although the fate map of *Isodiametra pulchra* highlighted more details on the contribution of the early duets to ectodermal and endomesodermal derivatives (Paper I), I found a general comparable pattern between *I. pulchra* and *N. fusca* embryos, in which the first three micromeres duets (1a, 1b, 2a, 2b and 3a, 3b) give rise to ectodermal derivatives, while the endomesodermal fates are produced by only two macromeres (2A, 2B). Also, the first micromere duet contributes to the dorsal (1a) and ventral (1b) epidermal cells, while the second micromere duet contributes to the left (2a) and right (2b) epidermal domains, setting the plane of bilateral symmetry, similar to the *N. fusca* duet contribution (Henry et al., 2000). Comparing the gene expression pattern of early embryos of *C. macropyga* with the fate map of *I. pulchra* and *N. fusca,*

I found a similar conservation of blastomere fates also in the embryos of *C. macropyga.* All the analysed ectodermal markers (*dlx, emx, gata1/2/3, pax2/5/8, otx, nk2.1,* and *six3/6*) were detected only in the micromere lineages (Figs. 3 and 4, Paper II), comparably with the ectodermal fate of the micromeres, observed in the fate maps of *N. fusca* and *I. pulchra* [(Henry et al., 2000) and Paper I in this thesis]. In addition, the temporal expression of the examined ectodermal markers at different developmental stages indicates the possible specification time of the micromeres. In particular, the expression of the three ectodermal genes (*dlx, emx, gata1/2/3*) at the early cleavage stages (4-, 6-, 8-, 10- and 12-cell stages) in the micromere duets suggests the possible specification of these micromeres already during early developmental stages, while the expression of the ectodermal and neuronal markers *nk2.1,* and *six3/6* at late stages possibly indicates a late deployment of these genes for the specification of neuronal fates. Similarly, I found comparable endomesodermal fates in the vegetal macromeres of *C. macropyga* embryos. The fate maps of *I. pulchra* and *N. fusca* revealed that endomesodermal fates arise from the second and third macromere duet (2A, 2B) (Figs. 7 and 10, Paper I) (Henry et al., 2000) . Comparably, the expression of endodermal (*foxA, gata4/5/6a*) and mesodermal (*foxF*) markers in the macromeres of *C. macropyga* (Fig. 5, Paper II), is consistent with endomesodermal fates of the macromeres and also indicates the temporal specification of the macromeres. Interestingly, while the endodermal marker *foxA* and the mesodermal markers *foxC* are expressed after gastrulation, the endomesodermal marker *gata4/5/6a* is expressed earlier, during the cleavage stages in the vegetal macromeres. These expression patterns suggest that the vegetal macromeres are specified early to became endomesoderm, as seen from the expression of *gata4/5/6a,* but endoderm and mesoderm are probably differentiated later, only after the ingression of the macromeres into the embryo.

Such general conservation of cells fates in species belonging to the same taxon is not surprising since numerous bilaterian taxa display a comparable conservation of cell fate of early blastomeres, as seen in Acoels. This is particularly true in the large group of Spiralia, which includes numerous taxa with very different adult body forms such as annelids, molluscs, bryozoans, brachiopods, phoronids, platyhelminths and

nemerteans, and shows comparable cell fates of the early blastomeres. In these animals, virtually all animal micromeres give rise to ectoderm while the vegetal macromeres give rise to endoderm and mesoderm, besides specific exceptions (Gline et al., 2011; Hejnol, 2010; Hejnol et al., 2007; Henry et al., 2008; Lyons et al., 2015; Meyer and Seaver, 2010; Nielsen, 2004, 2005; Özpolat et al., 2017). This general conservation of cell fates is observed even in species that have lost the spiral cleavage pattern, such as bryozoans (Vellutini et al., 2017). A peculiar case of cell fate conservation is found in nematodes. This group includes numerous species that display very different cleavage pattern, from the well-known invariant, stereotypic and determinate cleavage pattern of *Caenorhabditis elegans,* to the variable and indeterminate cleavage pattern of *Enoplus brevis* and *Pontonema vulgare.* Still, it is possible to identify conserved cell fates among early blastomeres Even in the most variable and indeterminate cleavage pattern of the Enoplida group, for instance, there is always one cell, produced at the third division round, that forms the gut (Goldstein, 2001). Comparable blastomere fates are also found among deuterostomes groups, such as echinoderms and hemichordates (Ambulacraria). In both these taxa, the ectodermal and endomesodermal fates are distributed in the cells along the animal-vegetal embryonic axis (Colwin and Colwin, 1951; Darras et al., 2011; Davidson, 1989). Thus, the conservation of blastomere fates, followed by the distribution of ectoderm and endomesoderm along the animal-vegetal axis, seems to be an evolutionary conserved feature across bilaterian embryos, and acoels are no different. The comparison with cnidarians, sister group to Bilateria, shows that there is a change in the axial distribution of the fates, i.e. ectodermal fates are found at the vegetal pole while endodermal fates are at the animal pole, which seems to be associated with the change of the site of gastrulation (Martindale, 2005; Martindale and Hejnol, 2009). However, a comparison of cell fates within the cnidarians seems to be more difficult, due to their indeterminate and variable development (Fritzenwanker et al., 2007).

Fate map analyses on more acoels species, including early branching species, such as *Hofstenia miamia* or *Paratomella rubra*, are needed to expand our knowledge of acoel

embryogenesis and to support such conservation of cellular fates within the acoel lineage.

4.2 Variability and plasticity of the blastomeres

The fate map analysis of *I. pulchra* revealed that, although all the micromeres possess ectodermal fate, a certain degree of variability is found in their descendants. This is seen in the epidermal areas, that show a variable spatial localisation among juveniles (probably due to the different number of epidermal cells derived from one micromere) and in the variable ectodermal derivatives of the third micromere duet (Paper I). On the other hand, I did not observe variability in the distribution of endomesodermal derivatives of the two injected macromeres, suggesting that only the micromere descendants possess such variability. Also, ectodermal fates are observed only in micromeres, while endomesodermal fates are observed only in macromeres. This indicates that the fates are invariably and stereotypically assigned to the early blastomeres. A slight variation in the micromere progeny was also observed in the fate map of *N. fusca*, and it was proposed that it is due to the highly regulative potential of these embryos (Henry et al., 2000). Ablation experiments in *N. fusca* showed that the juveniles produced after the ablation of both macromeres, still displayed a central cavity, although they were missing the statocyst and the muscles (macromeres derivatives). This and the variability in the micromeres descendants led Henry and colleagues to hypothesise that the micromeres possess a high degree of plasticity, being able to produce some endodermal fates, even after the ablation of both endomesodermal precursors (Boyer, 1971; Henry et al., 2000). In *I. pulchra*, I did not find any endodermal derivative in juveniles produced after ablating both endomesodermal precursors at the 12-cell stage, but the ablation of the micromeres at the 4-cell stage showed that the resulting juveniles developed all the ectodermal structures (Paper I). This formation of a normal juvenile after the ablation of a micromere indicates that the other blastomeres retain a certain plasticity within themselves, so that they are able to adapt to this perturbation and replace the missing structures. This possibly indicate that, because the essence of the regulative potential

are the cellular interactions that specify cell fates, micromeres are specified by the neighbour cells to become ectoderm. In this context, the variability of the micromere progeny possibly indicates that, despite the micromeres are specified for ectodermal fate, they still retain a certain degree of flexibility and can give rise to different ectodermal structures.

The gene expression analyses of *C. macropyga* embryos seems to contrast with the results on *I. pulchra*, as they revealed that all the early micromeres express ectodermal markers, while the early macromeres expressed endomesodermal marker (Paper II). The expression of the germ layer markers in such early micromeres and macromeres not only characterise the fates of the early blastomeres but also indicate a possible early specification of these. Nevertheless, an early specification does not necessarily mean an early commitment of the cell. Assuming that the regulative potential can be conserved also in the species *C. macropyga*, then the cell fate specification is governed by cell-cell interactions. In this context, the expression of germ layer markers could indicate that, despite an early specification of the ectodermal and endodermal fates, the early blastomeres are still not committed towards their final derivatives, possibly retaining some plasticity.

4.3 Regulative development of embryos with stereotypic and invariant cleavage pattern

How is the regulative development connected to the stereotypic cleavage? Studies on the embryonic development of several animals and the comparison of their cell lineages showed that there is no clear-cut dichotomy between mosaic versus regulative development (Lawrence and Levine, 2006). This is demonstrated by several examples of animals that possess an invariant stereotypic cleavage pattern with cell fates determined early during development, and still, they possess some cells that need interactions to be determined, implying that cell-cell interactions are important for the determination of cell fates, as seen in *C. elegans* (Sommer, 1997), *P. hawaiensis*, (Extavour, 2005; Price et al., 2010) *C. teleta* (Yamaguchi et al., 2016) and *H. roretzi*

(Nishida and Satoh, 1989). If embryologists want to hold on to this historical and yet dated terminology, then the terms mosaic or regulative should be referred to specific cell lineages and not to the embryo as a *whole*, as Davidson suggested (Davidson, 1990). Although *I. pulchra* embryos adapt to the deletion of micromeres or macromeres, it is unclear which blastomere is able to replace the deleted one. For instance, in the case of the deletion of a micromere and the consequent replacement by another micromere (intra-germ layer compensation), then one could say that the micromere lineage possesses a regulative ability. On the other hand, the replacement of the deleted micromere by a macromere (inter-germ layer compensation), would indicate that the macromere lineage possesses the regulative ability. Therefore, with these unclear results it is not possible to assign the regulative ability to a specific lineage in *I. pulchra* embryos, although they definitely show a regulative potential.

The early studies on cell lineages and perturbation experiments led many embryologists to the generalisation that embryos with invariant and *determinate* cleavage pattern, possess a *mosaic* development, as seen in ascidians (Conklin, 1905a), while *regulative* development is often found in embryos with a variable and *indeterminate* cleavage pattern, as seen mainly in vertebrates embryos (Conklin, 1905a). Nevertheless, already in those early times of the developmental biology, embryologists understood the close relationship of the cleavage pattern with the cell fates specification (and consequently with the regulative or mosaic ability) giving to the cleavage pattern a causative role for the specification of cell fates (Stent, 1985). Nowadays, this relationship is still accepted, but now the question is whether the *determinate* cleavage pattern is also *determinative,* i.e. if the precise and repetitive position of the cells causes their specification and differentiation (Scholtz, 1997). In this regard, acoels represent an interesting case of study as they display an invariant stereotypic cleavage pattern, a determinate cell lineage but they also display the regulative ability to compensate for deletion of specific blastomeres. *I. pulchra* fate map and ablation analyses revealed how the cell fates are distributed across the early blastomeres and how the blastomeres contribute to the juvenile tissues, while the gene expression in *C. macropyga* embryos revealed an early specification of cell fates. From

these results it seems clear that the stereotypic and invariable cleavage pattern is definitely connected with the specification of cell by giving specific position to the cells. Nevertheless, because the regulative ability implies that cellular interactions are needed for the cell fate specification (*conditional* specification), it seems that the *determinate* cleavage pattern does not have a *determinative* role in the specification of cell fates itself. This means that the determinate and invariable cleavage pattern gives the positional information to the cells but the factor that mostly influences the cell fate specification are the genes and signalling molecules, which govern the cellular interactions. Therefore, *I. pulchra* and *C. macropyga* embryos, with their stereotypic cleavage pattern but conditional specification, represent an example in which the stereotyped cell lineage seems to not have a determinative role in the specification of cell fates.

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