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# The Annelid *Platynereis dumerilii* as an Experimental Model for Evo-Devo and Regeneration Studies

Quentin Schenkelaars, Eve Gazave

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# **Annelids, *Platynereis dumerilii***

Quentin Schenkelaars, Eve Gazave

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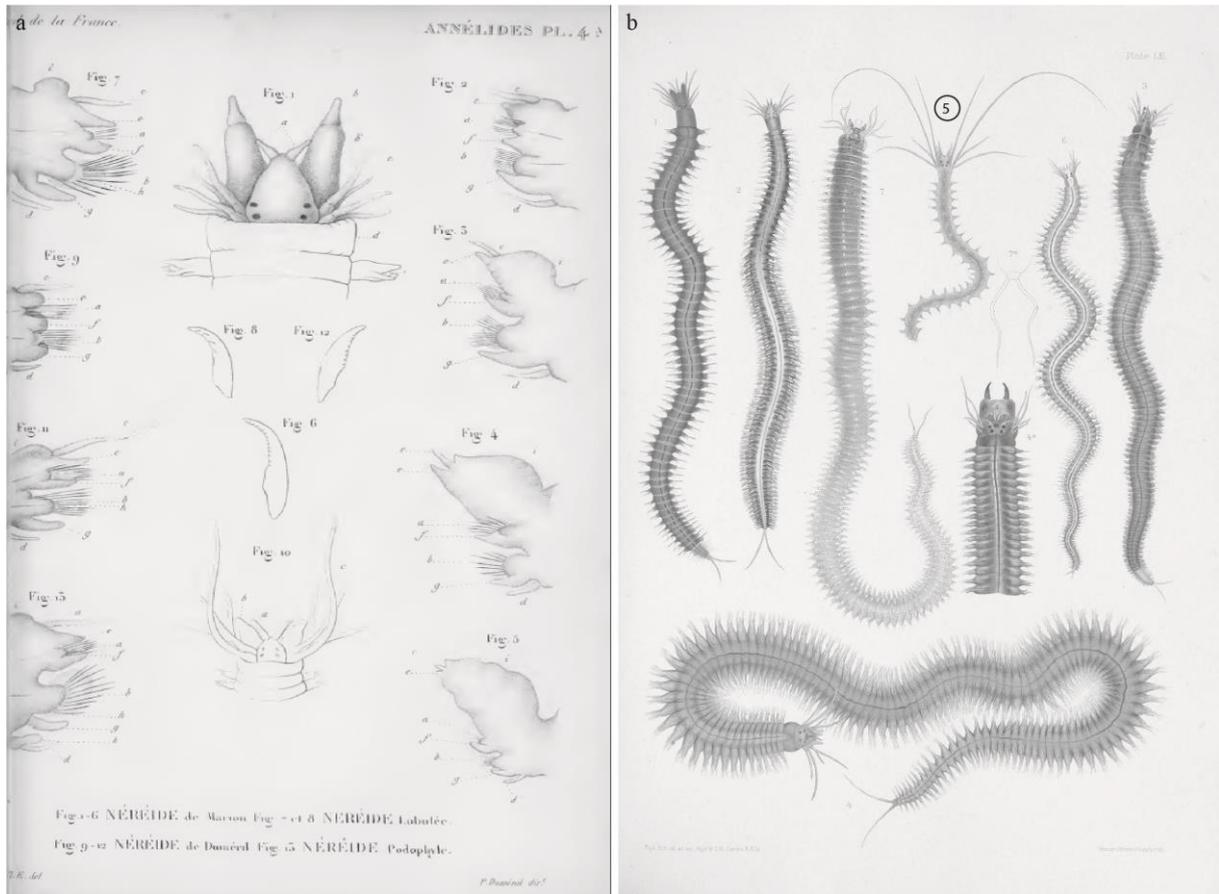
## **Bibliography**

### 13.1 History of the model

Annelids, also known as segmented worms, are a major group of non-vertebrate bilaterian animals. Annelid name comes from the Latin *annellus* meaning “little ring” and refers to their segmented or metamerized body plan. Annelids represent a large number of species and an ecologically diversified animal taxon with over 18,950 described species living in various ecosystems from deep sea to rainforest canopy (Brusca and Brusca 2003). They are especially abundant in sea water but also occupy humid terrestrial and freshwater habitats. Some species are parasitic, mutualist, or commensal (Rouse and Pleijel 2001; Piper 2015). Annelid species present a huge diversity of body forms coexisting with various life history strategies, being either scavengers, bioturbators, predators, or filter feeders. They also harbor a multitude of (sometimes), extravagant forms of sexual and asexual reproduction (Caspers 1984; Fischer 1999; Schroeder et al. 2017). The annelid phylum, like Mollusca, Platyhelminthes, Bryozoa and more, is part of the Lophotrochozoa clade (Laumer et al. 2019), which together with Ecdyzoa form the large group of Protostomia within Bilateria. Annelid phylogenetic relationships were, for a long time, mostly based on morphological characteristics and thus were difficult to ascertain (Weigert and Bleidorn 2016). The first classification of annelids separated them in three main groups, Polychaeta, Oligochaeta and Hirudinae (Lamarck 1818; Weigert and Bleidorn 2016). Briefly, Polychaeta, or bristle worms, referred to a large and diverse group of worms presenting numerous bristles, or chaetae, hence the name “polychaeta”. In contrast, worms having very few or reduced chaetae were grouped together into Oligochaeta, while Hirudinae referred to worms with no chaetae and presenting a sucker. In addition, a multitude of other groups of “invertebrates” such as Sipuncula and Echiura were, at that time, considered to be closely related to annelids. During the 20<sup>th</sup> century, new morphology-based classifications proposed the separation of annelids into two main groups,

Polychaeta and Clitellata, the latter containing the Hirudinae (Weigert and Bleidorn 2016). Polychaeta were themselves divided into two groups, the Errantia and the Sedentaria, based on worm lifestyles. Free moving and predatory worms were encompassed in Errantia, while sessile and tube-dwelling worms formed the Sedentaria group (de Quatrefarges 1865; Fauvel 1923; 1927). This Errantia/Sedentaria separation was dismissed with the advent of morphological cladistic analysis (Rouse and Fauchald 1997). Indeed, in 1997, Rouse and Fauchald proposed to separate Polychaeta into the clades Palpata and Scolecida based on the presence or absence of palps, respectively. Over the last 20 years, with the rise of molecular biology, the phylogenetic relationships of annelids were regularly reassessed. A recent seminal phylogenomic study highlighted the division of annelids into two main subgroups, reviving the ancient Errantia and Sedentaria nomenclature, in addition to a couple of early branching lineages such as Sipuncula (Struck et al. 2011). Errantia and Sedentaria together form the Pleistoannelida (Struck 2011). Internal relationships among those two groups are quite well-defined (Struck 2011; Weigert et al. 2014; Weigert and Bleidorn 2016). Notably, the Sedentaria now also includes the Clitellata and the Echiura. The Polychaeta term is consequently no longer valid, as “polychaete worms” are present in both the Errantia and Sedentaria groups. In contrast, the phylogenetic affiliations of early branching annelid lineages (notably Sipuncula) are not yet stable (Struck et al. 2011; Weigert et al. 2014; Andrade et al. 2015; Weigert and Bleidorn 2016). Recent discovery of new annelid fossils and reassessment of their discrete morphological characters allowed for the reconciliation of annelid fossil records and new molecular phylogenetic relationships (Parry et al. 2016; Chen et al. 2020). Thanks to their huge diversity and rich phylogenetic and evolutionary histories, annelids represent an key source of potential model species to investigate a variety of biological questions, notably the evolution of developmental mechanisms (Ferrier 2012).

Among Errantia, the nereididae *Platynereis dumerilii* (Audouin and Milne Edwards 1833), is an important annelid model species developed by the scientific community to address key biological questions. *Platynereis dumerilii* also named “*Néréide de Dumeril*” was discovered thanks to an oceanographic campaign around the French North coast of the English Channel (Granville, Chausey Island and Saint Malo) that occurred from 1826 to 1829. Jean Victor Audouin and Henri Milne Edwards subsequently described the type species (deposited in the La Rochelle museum) and named it as *Nereis dumerilii* in their “*Classification des Annélides et description de celles qui habitent les côtes de la France*” book chapter containing dozens of new annelid species descriptions, especially for the Nereididae family (Audouin and Milne Edwards 1833; 1834) (Figure 13.1A). *Platynereis dumerilii* and Nereididae in general have been the subject of intense studies in the past century, especially regarding embryology, reproduction strategies and regeneration. Their fascinating nuptial dance behavior observed before reproduction (Just 1929; Boilly-Marer 1973; Zeeck et al. 1990), the influence of a brain hormone on their reproduction, regeneration and growth processes (Hauenschild 1956; 1960; Hofmann 1976), and their oogenesis and spiral embryonic development (Fischer 1974; Dorresteijn et al. 1987; Dorresteijn 1990) were the main scientific questions addressed at that time. Those pioneer studies still provide important information for current challenging research questions (see section 13.8 Challenging questions). Carl Haeuenschild established the first *Platynereis* year-round laboratory culture in 1953 in Germany from a Mediterranean population (Caspers 1971). Since then, *Platynereis* culture procedures have been slightly refined, allowing them to be easily bred in a dozens of research laboratories all over the world (Kuehn et al. 2019).



**Fig 13.1: Original and historical drawings of *Platynereis* (initially named *Nereis*) *dumerilii*.**

(a) Drawings of the original description of *Platynereis dumerilii* mentioned as “*Nereide de dumeril*” from (Audouin and Milne Edwards 1833; 1834), (Plate 4A, drawings 9 to 12: 9= parapodia; 10= anterior part and head sensory structures; 11= parapodia; 12= denticulated jaw).

(b) Drawing of annelids (M'Intosh et al. 1910) (5= *Nereis dumerilli*, from a sketch drawn at Lochmaddy).

### 13.2 Geographical location

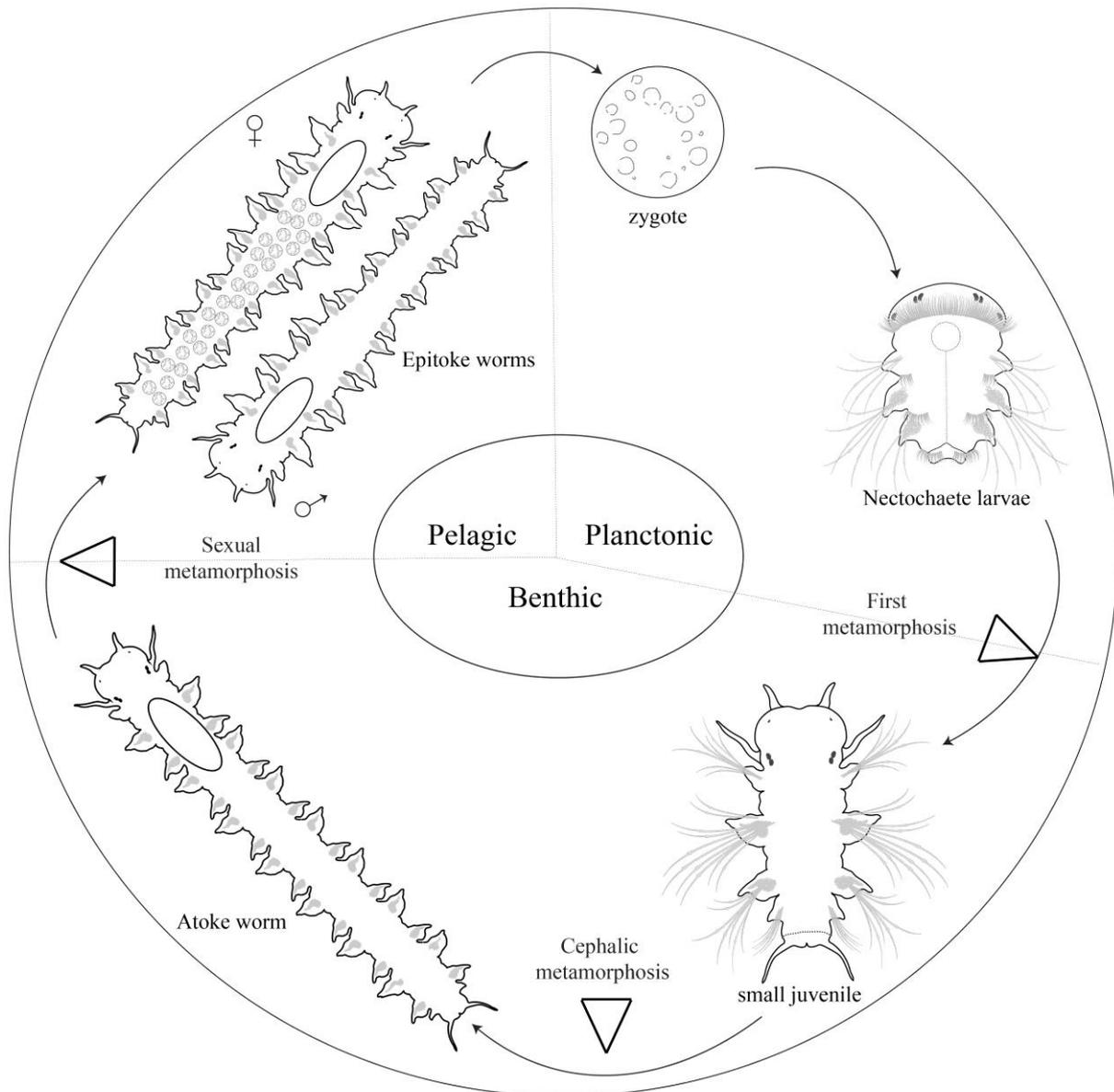
*Platynereis dumerilii* worms live in coastal marine waters, especially inhabiting shallow (usually between 0 to 5 meters' depth), hard-bottom, algae-covered substrates. They can also directly live on seaweeds and marine plant leaves such as *Posidonia oceanica* and *Zostera*

*marina* (Jacobs and Pierson 1979). As mentioned before, *Platynereis dumerilii* was first described from the French north coast of the English Channel. Surprisingly, *Platynereis dumerilii* is also found in many other locations, from temperate to tropical zones: they are often encountered throughout the Mediterranean Sea (Gambi et al. 2000) but also in the North Sea, the English Channel (Figure 13.1b), the Atlantic down to the Cape of Good Hope, the Black Sea, the Red Sea, the Persian Gulf, the Gulf of Mexico, Cuba, the Sea of Japan, the Pacific, the Kerguelen Islands, and the coasts of Mozambique and South Africa (Read and Fauchald 2018; Kara et al. 2020). As a consequence of this very broad geographical distribution, *Platynereis dumerilii* is considered to be a cosmopolitan species (Fischer and Dorresteijn 2004; Read and Fauchald 2018). However, cosmopolitan species rarely exist since they actually often pool together sibling species or a species complex with (nearly) identical morphologies (Knowlton 1993). As shown for many other marine non-vertebrates species, recent population genetic studies from the Mediterranean Sea (Italian coast) and South Africa revealed that *Platynereis dumerilii*, in those localities, is in fact a species complex. In Italy, *P. dumerilii* is frequently mistaken with its sibling species *P. massiliensis* (Moquin-Tandon 1869), (Valvassori et al. 2015; Wäge et al. 2017). In South Africa, *P. dumerilii* lives in sympatry with *P. australis* (Schmarda 1861), another morphologically sibling species (Kara et al. 2020). Population genetic analysis of specimens found in South Africa initially identified as *P. dumerilii* are probably a new species, *P. entshonae*, highlighting the fact that only rigorous and broad-scale population genetic studies worldwide will help to uncover the real geographic distribution of *P. dumerilii* (Kara et al. 2020) and the diversity of the *Platynereis* genus that currently contains 41 valid species (Read and Fauchald 2018).

### **13.3 Life cycle**

*Platynereis*' life cycle exhibits several interesting features and encompasses three phases separated by metamorphosis events (Fischer and Dorresteijn 2004) (Figure 13.2). Like many other marine animals, such as corals, sea urchins and even fishes, *Platynereis* sexual maturation and reproduction are synchronized with the natural moon phases (Bentley et al. 1999). This fascinating biological characteristic of lunar-controlled reproductive periodicity, regulated thanks to an endogenous oscillator, is called a circalunar life cycle (Tessmar-Raible et al. 2011; Raible et al. 2017). Each worm will reproduce only once and the timing of this reproduction is tightly regulated by this clock (Zantke et al. 2013). The number of animals reaching sexual maturity is maximal shortly after the new moon and minimal during the full moon (Hauenschild 1955; Zantke et al. 2013). In nature, this reproductive period occurs between May and September in the Mediterranean Sea (Giangrande et al. 2002). When *Platynereis dumerilii* worms are ready to spawn, usually at night, males and females reach the surface, start an elegant nuptial dance and synchronously release eggs and sperm in a massive spawning event. This external fertilization induces the formation of thousands of small zygotes and ultimately implies the death of the reproducing males and females (Figure 13.2). By the third day, the zygote develops into small, segmented, planktonic larva named nectochaetae (see section 13.5 Embryogenesis and larval development). Nectochaete larvae live on their own nutritive stock and move thanks to marine currents and ciliary belts. After five to seven days of planktonic life, small juvenile worms, while still able to swim, switch to a benthic/errant life mode following metamorphosis (Fischer et al. 2010). This first metamorphosis event corresponds to the disappearance of ciliated belts. Juvenile worms then continue to grow throughout their lives at a rate that is highly dependent on food availability. At some point, a second metamorphosis occurs, inducing profound morphological modifications of the head and first segment. Additionally, worms start to produce silk, important for the building of a tube in which they will live for several months. They continue to grow, to regenerate following injury, and to grow

posteriorly until they initiate their last metamorphosis, corresponding to the appearance of sexual traits (Fischer et al. 2010). This sexual metamorphosis is moon-dependent and implies drastic morphological changes (see section 13.4 Anatomy) to allow production of thousands of gametes. During this very short reproductive period, worms become pelagic.



**Fig 13.2: *Platynereis dumerilii* life cycle.** The zygote gives rise to a small planktonic nectochaete larva within three days. After five to seven days post fertilization (dpf), the small juvenile worm starts to feed and become benthic. Shortly thereafter, the small worm undergoes cephalic metamorphosis. The atoke worm lives inside its tube and grows continuously until

sexual maturation. The sexually mature or epitoke worm then leaves its tube and swims into the water column until they perform mass spawning.

This eventful life cycle can be reproduced in laboratory settings with culture rooms maintained at 18°C and a daily artificial illumination regime (16 hours of light / 8 hours of darkness). To induce sexual maturation, a low-light lamp is used to mimic the lunar stimulus seven days per month. A couple of days after this week of artificial full moon, juvenile (or atoke) worms start sexual maturation for a two-week period, allowing the production of sexually-mature (or epitoke) worms every day (Fischer and Dorresteijn 2004; Kuehn et al. 2019; Vervoort and Gazave in press).

## **13.4 Anatomy**

Like many other annelid species, *Platynereis dumerilii* worms have a complex body plan with various tissues, structures, and organs that are described below for both atoke and epitoke forms.

### 13.4.1 External anatomy of *Platynereis dumerilii* juvenile (atoke) worms

*Platynereis dumerilii* juvenile worm size can be up to 90 mm for around 100 segments (Figure 13.3a). Their color is highly variable, from yellowish and reddish to greenish, and this coloration mostly relies on pigmented cells, or chromatophores (Arboleda et al. 2019), that shine at the surface of the epidermis itself, secreting a cellular cuticle. While their sex is genetically determined, at the juvenile stage, male and female animals are indistinguishable. The morphology of Nereid annelids such as *Platynereis* is often described in zoological textbooks as representative of the typical annelid body plan, composed of three main parts: (i) an anterior region, the head with a substantial cephalization, (ii) the segmented or metamerized

trunk composed of many identical units called segments, with appendages named parapodia, (iii) and a post-segmental terminal part, containing the pygidium, a differentiated structure notably containing the anus (Figure 13.3a) (Fischer and Dorresteijn 2004; Fischer et al. 2010). *Platynereis*' head is composed of different structures, many of them being sensory (Chartier et al. 2018). These structures ensure crucial functions for the worm's life (Purschke 2005) (Figure 13.3b and c). To begin, *Platynereis* possesses two pairs of pigmented cup brown adult eyes, in a trapezoid arrangement, only visible on the dorsal part of the worm (Figure 13.3b). These pairs of adult eyes represent a distinct type of eyes in comparison to larval eyes, as revealed by their specific developmental program (Arendt et al. 2002; Guhmann et al. 2015). They also harbor a very specific cellular structure with rhabdomeric photoreceptor extensions traversing the pigmented cell layer (Arendt et al. 2002). These eyes are localized on a specific structure of the head, named the prostomium (Figure 13.3b). The prostomium also bears a pair of highly chemosensory antennae localized at the front of the head (Chartier et al. 2018) (Figure 13.3b). A pair of sensory palps are present near the antennae; based on their cellular ultrastructure (Dorsett and Hyde 1969) and a physiological experiment (Chartier et al. 2018), they have been proposed to be chemosensory as well (Figure 13.3c). The head is also composed of four pairs of long sensory and photosensitive tentacular cirri (namely anterior/posterior dorsal/ventral tentacle cirrus) (Figure 13.3c). They are involved in the worm's "shadow reflex", a defensive behavior triggered by a decrease in illumination (Ayers et al. 2018). At the posterior dorsal margin of the prostomium are nuchal organs, a pair of ciliated cavities considered to be important chemosensory structures too (Schmidtberg and Dorresteijn 2010; Chartier et al. 2018) (Figure 13.3b). Finally, the aforementioned structures are located on a specific segment with no appendages, named the peristomium (Figure 13.3b). The peristomium also contains a large structure, visible only on the ventral side of the worm, which is the stomium or mouth (Figure 13.3c). Following the mouth, the pharynx contains a pair of chitinous and denticulated

jaws, invaginated in the first segment of the worm, and which are evaginated to catch food (Figure 13.3d). This eversible pharynx corresponds to the anterior part of the digestive tract (Verdonschot 2015).

*Platynereis*' trunk is composed of identical segments (Figure 13.3a). Its segmentation is thus named homonomous (Fischer and Dorresteijn 2004). Each segment is externally composed of an outer annulus and parapodia (Scholtz 2002). Parapodia are paired appendages, found in many annelids, and which have locomotion, respiratory, and sensory functions (Figure 13.3e). They notably allow the worm to crawl and swim (Grimmel et al. 2016). Parapodia are biramous and thus composed of two parts, the notopodium in the dorsal side of the animal and the neuropodium in its ventral side (Figure 13.3e). Each rami is composed of cirri (dorsal and ventral cirrus), a lobe, and a beam of extracellular chitinous structures named chaetae or bristles (Verdonschot 2015) (Figure 13.3f). The latter are surrounded by specific glands that secrete material for tube or cocoon synthesis and are named spinning glands (Fischer et al. 2010). These external chaetae are constantly produced by internal structures named chaetal sacs (Gazave et al. 2017). Chaetae have a terminal articulated portion at their tips (Figure 13.3f). In addition, a robust, skeletal, internal, peculiar chaeta, named the acicula, stabilizes each lobe (Figure 13.3e). Interestingly, in annelid systematics, the shape of the parapodia and the type of chaetae are informative characteristics to determine species (Zakrzewski 2011).

The terminal part of the worm is named the pygidium (Starunov et al. 2015) (Figure 13.3g). The pygidium contains the anus and presents two sensory anal or pygidial cirri in its ventral part (Ayers et al. 2018).

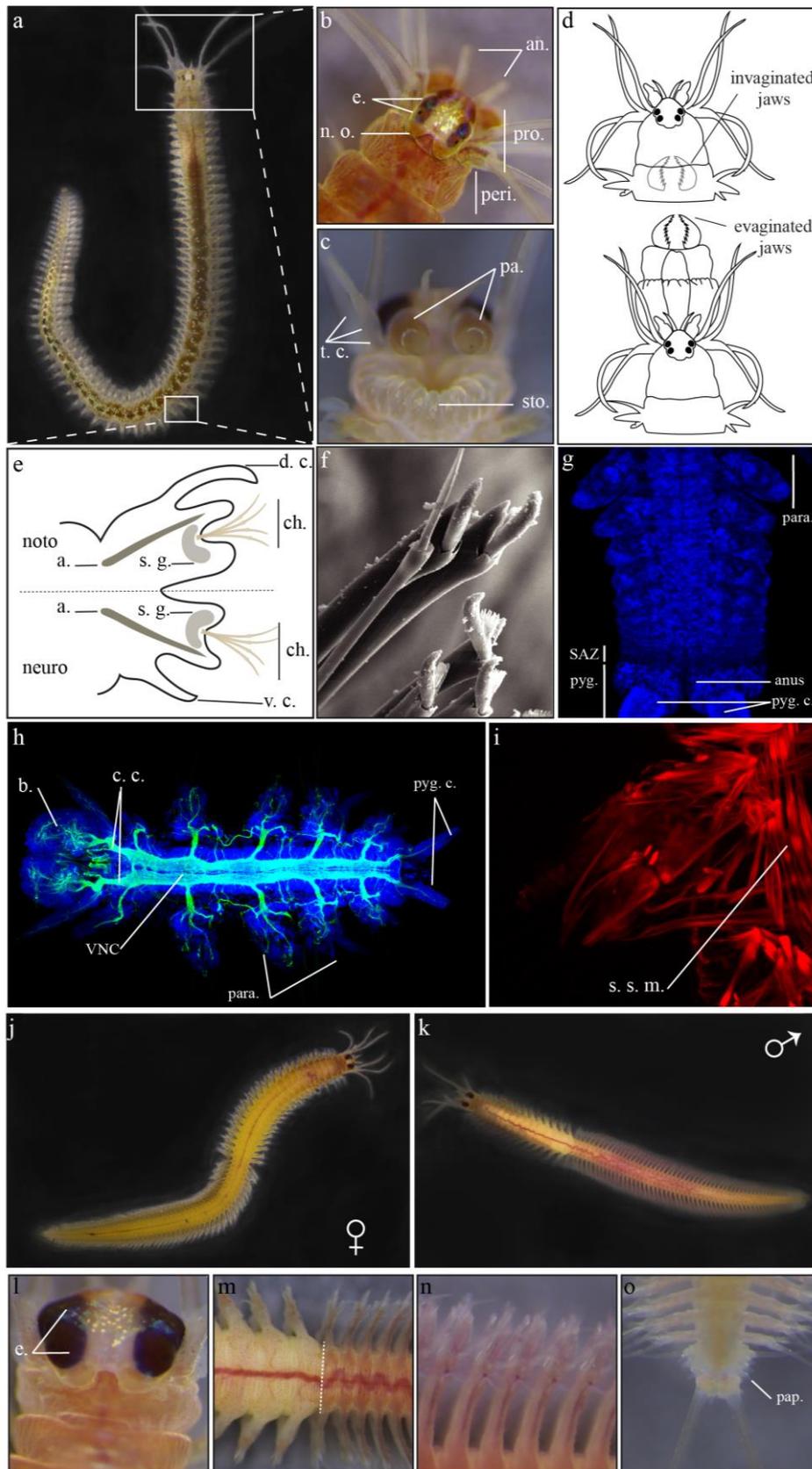


Fig 13.3: Anatomical features of *Platynereis dumerilii* atoke and epitoke worms.

(a) Juvenile of *Platynereis dumerilii*, head (see b to d) and parapodia (see e and f) are framed. (b) Dorsal closed view of the head, bearing sensory structures. (c) Ventral closed view of the head, bearing the mouth or stomodeum. (d) Process of jaw evagination to catch prey. (e) Schematic representation of parapodia, the dorsal part is facing up. (f) Scanning Electron Microscopy (SEM) of chaetae, photo courtesy of N. Dray, PhD. (g) Posterior part of the worm, showing the parapodia, segment addition zone, and pygidium. Hoechst nuclear staining in blue. (h) Nervous system of small juvenile worm. Ventral view, anterior is to the left. Nerves are labelled by acetylated-Tubulin antibody in green and hoechst nuclear staining is in blue. (i) Musculature of small juvenile worm parapodia. Muscles are labelled with Phalloidin. (j) *Platynereis* mature female. (k) *Platynereis* mature male. (l) Enlarged *Platynereis* eyes during sexual maturation. (m) Boundary between anterior (left) and posterior (right) segments of mature worms. (n) Important blood network within the posterior parapodia of mature male. (o) Male pygidium presenting extra papillae. **Abv:** a.= acicula, an.= antennae, b.= brain, c. c.= circumpharyngeal connectives, ch.= chaetae, d. c.= dorsal cirri, e.= eye, neuro= neuropodium; n. o.= nuchal organ, noto.= notopodium, pa.= palps, pap.= papillae, para.= parapodia, peri.= peristomium, pro.= prostomium, pyg.= pygidium, pyg. c.= pygidial cirri, SAZ= segment addition zone, s. g.= spinning gland, s. s. m.= somatic striated muscle, sto.= stomium, t. c.= tentacular cirri, v. c.= ventral cirri, VNC= ventral nerve chord

#### 13.4.2 Internal anatomy of *Platynereis dumerilii* juvenile (atoke) worms

As for its external anatomy, *Platynereis*' internal body plan is segmented and presents a repetition of internal structures within each segment. Indeed, each body unit contains a body cavity or coelom (separated from the next one by an incomplete inter-segmental piece of tissue called septa), each containing a part of the (i) nervous system, (ii) circulatory system, (iii) musculature and (iv) excretory system (Verdonschot 2015). The non-metamerized digestive tract (v) runs along the antero-posterior axis of the worm.

#### 13.4.2.1 Nervous system:

*Platynereis* worms possess a central nervous system (CNS) and a peripheral nervous system (PNS). One main elements of the CNS is the highly developed brain that resides, in a dorsal position, within the head (Starunov et al. 2017) (Figure 13.3h). Another important element of the CNS is a paired serial chain of spherical ganglia, in a ventral position (named the ventral nerve chord or VNC) that runs all along the length of the worm's body, making a ladder-shaped structure (Figure 13.3h). The brain is connected to the VNC by circumpharyngeal connectives, which surround the pharynx (Verdonschot 2015) (Figure 13.3h). *Platynereis*' brain contains prominent dorsal neuropile arrangements named mushroom bodies (Tomer et al. 2010), a structure shared with arthropods (Heuer et al. 2010). Interestingly, the mushroom body anlagen in *Platynereis* larvae expresses a similar molecular signature that developing mushroom bodies in *Drosophila melanogaster*, thus providing strong evidence in favor of an evolutionary relatedness of insect and annelid mushroom bodies (Tomer et al. 2010). *Platynereis*' brain is also an important neurosecretory center. Its developing forebrain expresses the neuropeptides FMRFa and vasotocin (Tessmar-Raible et al. 2007), plus a diversity of other neuropeptides recently identified, such as somatostatin, galanin etc... (Williams et al. 2017). Interestingly, this annelid brain region shares a common molecular signature with the vertebrate hypothalamus, furthering the hypothesis of an evolutionary relationship between those two structures (Williams and Nagy 2017). In addition, *Platynereis*' brain produces a brain hormone responsible for the switch from a growing juvenile to a sexually mature worm (Hauenschild 1956). This hormone, whose activity suppresses reproduction, was recently identified as Methylfarnesoate (Schenk et al. 2016). In addition to the brain, the VNC is also a complex structure that has been shown to harbor around 200 distinct types of neurons, expressing specific combinations of transcription factors in a small juvenile (Vergara et al. 2017).

*Platynereis*' PNS is prominent in the head, being associated with the many sensory structures it contains. The PNS also contains the parapodial and pygidial nerve extensions. Indeed, the terminal part of the worm is highly innervated with nerve projections into the pygidium and the anal cirri (Starunov et al. 2015) (Figure 13.3h).

#### 13.4.2.2 Circulatory system

*Platynereis* has a closed circulatory system mainly composed of two vessels and capillary networks. The dorsal and ventral vessels are connected together by a capillary network forming a ring around the intestine. The dorsal pulsatile vessel is the main pump of the circulatory system, pumping the blood anteriorly (from the tail to the head) while the ventral vessel pumps the blood posteriorly. Segmental lateral vessels irrigate the parapodia in each segment in order to ensure their respiratory function (Saudemont et al. 2008; Verdonschot 2015). A circular blood sinus is present in the pygidium (Starunov et al. 2015).

#### 13.4.2.3 Musculature

*Platynereis* has two main types of muscles, smooth and striated, which together ensure precise movements of the worm's body structures (Brunet et al. 2016) (Figure 13.3i). Some somatic striated muscles run longitudinally from the head to the tail of the animal. Additional somatic striated muscles control the movements of parapodia thanks to ventral oblique and parapodial fibers (Figure 13.3i). In contrast, visceral muscles are mainly smooth muscle (with the noticeable exception of the anterior part of the gut that contains striated visceral muscles). They formed a specific muscular structure, the orthogon, which is composed of both circular and longitudinal fibers (Brunet et al. 2016). Smooth muscles are also associated with the pulsatile dorsal vessel (Brunet et al. 2016). A peculiar somatic striated and longitudinal muscle, the axochord, is found between the VNC and the dorsal vessel and is proposed to be at the origin

of the chordate notochord (Lauri et al. 2014; Brunet et al. 2015). The pygidium musculature is also highly complex, mainly composed of a strong array of circular muscles that plays the role of the anal sphincter (Starunov et al. 2015).

#### 13.4.2.4 Excretory system

*Platynereis* atoke worms possess in each segment, except the pygidium, a pair of metanephridia that connects the coelomic compartment to the exterior to ensure the excretion of waste products (Hasse et al. 2010; Verdonschot 2015).

#### 13.4.2.5 Digestive system

The digestive system of *Platynereis* is mainly composed of three successive elements called the foregut, the midgut, and the hindgut (Fischer et al. 2010; Zidek et al. 2018). The foregut is comprised of the mouth, the eversible pharynx and the jaws, in charge of collecting and grinding the food. Digestive enzymes are secreted and active in the midgut, where food absorption occurs. The last section of the digestive system is a hindgut connecting the midgut to the anus and producing digestive enzymes too (Verdonschot 2015; Williams et al. 2015).

#### 13.4.3 External and internal anatomy of *Platynereis dumerilii* adult (epitoke) worms

As mentioned before, one of the main event in the *Platynereis* life cycle is sexual maturation (epitoky) since it induces not only drastic morphological modifications but also changes in behavior (Fischer and Dorresteijn 2004). A striking difference between mature and juvenile worms is the difference in body color (Figure 13.3j and k): while juveniles mainly show a sex-independent brownish color, sexual dimorphism appears during epitoky as females become bright yellow (Figure 13.3j) and males display white anterior and red posterior body regions (Figure 13.3k). During sexual maturation, worms stop food intake, and their gut thus regresses

and becomes non-functional. The trunk of the animal is progressively modified to become a “bag” full of gametes, visible through the body wall which loses its pigmentation (Fischer and Dorresteijn 2004; Fischer et al. 2010). The yellow oocytes and white spermatozooids both contribute to the main color of the female and male anterior parts, respectively. Among other morphological changes, the eyes enlarge dramatically (Figure 13.31) and the homonomous segmentation present in juveniles is lost. Indeed, while anterior segments are not modified, posterior segments are substantially reshaped and a clear boundary between these two parts of the trunk becomes visible (between the 15<sup>th</sup> and 20<sup>th</sup> segments, depending on the sex, Figure 13.3m) (Schulz et al. 1989; Fischer 1999). In modified posterior segments, parapodia flatten and develop paddle-shaped chaetae in both sexes. In males, posterior parapodia show a significant increase in vascularization, conferring its red color to the posterior part (Figure 13.3n). Muscles present in juvenile worms degenerate and are replaced by new muscle fibers which are specific to sexually-mature animals. This dramatic reorganization of the body enables the formerly benthic juvenile worms an ability to swim quickly to ensure the nuptial dance required for sexual reproduction (Fischer and Dorresteijn 2004; Fischer et al. 2010). Finally, while the terminal part of the female is not modified, the male pygidium presents extra papillae, allowing the sperm to be released in many directions (Figure 13.3o) (Starunov et al. 2015). In *Platynereis*, the switch from a growing worm to its reproductive life stage is controlled by brain hormone activity. Interestingly, worm decapitation (*i.e.* artificial reduction of brain hormone) induces worms’ sexual maturation similarly to natural conditions (Schenk et al. 2016).

### **13.5 Embryogenesis and larval development**

More than a century ago, Edmund B. Wilson retraced an incredibly relevant and reliable cell-lineage of embryo blastomeres in order to depict the origin of the germ-layers in annelids

(Wilson 1892). To do so, he took advantage of the transparency of *Nereis limbata* (now *Alitta succinea*) and *Nectonereis megalops* (now *Platynereis megalops*) embryos and of their stereotypic development. Indeed, as all embryos develop in exactly the same way, they provide an ideal framework to link cell division to blastomere formation and cell fate. Interestingly, since his publication, his work has been reasserted by the description of *Platynereis dumerilli* embryogenesis in the early nineties (Dorresteijn 1990), and his assumptions regarding blastomere cleavage and fate remain a reference in the field of annelid development. Micro-injection of individual blastomeres at different embryonic stages with fluorescent dyes has more recently confirmed previous observations (Fischer and Dorresteijn 2004; Ackermann et al. 2005). Hereafter, we have mainly compiled the above-mentioned publications to depict the main events of embryogenesis and larval development (Fischer et al. 2010).

### 13.5.1 Embryo development

#### 13.5.1.1 Unfertilized eggs

Unfertilized eggs are packed within the coelomic cavity of the mature female, causing their polymorphous shapes. Upon laying, the pressure is released and the eggs rapidly undergo a massive shape change to become ellipsoid (the short axis of the unfertilized egg corresponds to the future animal-vegetal axis of the zygote). At that stage, their cytoplasm is organized, in a concentric fashion, around the central nucleus which is wrapped in yolk-free cytoplasm. The latter is surrounded by a shell of yolk containing large lipid droplets (in particular in the equatorial plane where they are bigger) and a thick outer layer of cortical granules (sensory organelles found within oocytes). Finally, the egg is itself protected within a vitelline envelope. Interestingly, in *Platynereis*, eggs are in fact oocytes blocked in metaphase and as such, the release of polar bodies occurs after fertilization.

### 13.5.1.2 Fertilization

Upon fertilization, the fertilizing spermatozoid sticks to the cell surface until the emission of the first polar body (a small haploid cell). As soon as this contact is established, substantial changes in the cytoplasmic organization of the oocyte occur. The cortical granules are released to form an external jelly layer (0-23 minutes post fertilization, mnpf). As a consequence, the yolk granules are less packed within the spherical egg and more broadly distributed while the lipid droplet pattern remains as a readout of the equatorial plan. When the vitelline envelope breaks down (18 mnpf), a small area is progressively cleared from yolk at one pole of the egg compared to the equatorial location of lipid droplets. This area marks the future animal pole where the first polar body is formed (60 mnpf, Figure 13.4a). The sperm pronucleus finally enters into the ooplasm and a second polar body is formed (80 mnpf). Yolk granules migrate towards the vegetal pole allowing the rapid expansion of the clear cytoplasm, the female pronucleus forms, and karyogamy (fusion of the two nuclei) occurs (90-100 mnpf). Subsequently, the animal pole is completely cleared from yolk granules and the first cleavage is initiated.

### 13.5.1.3 First cleavages (120-420 mnpf)

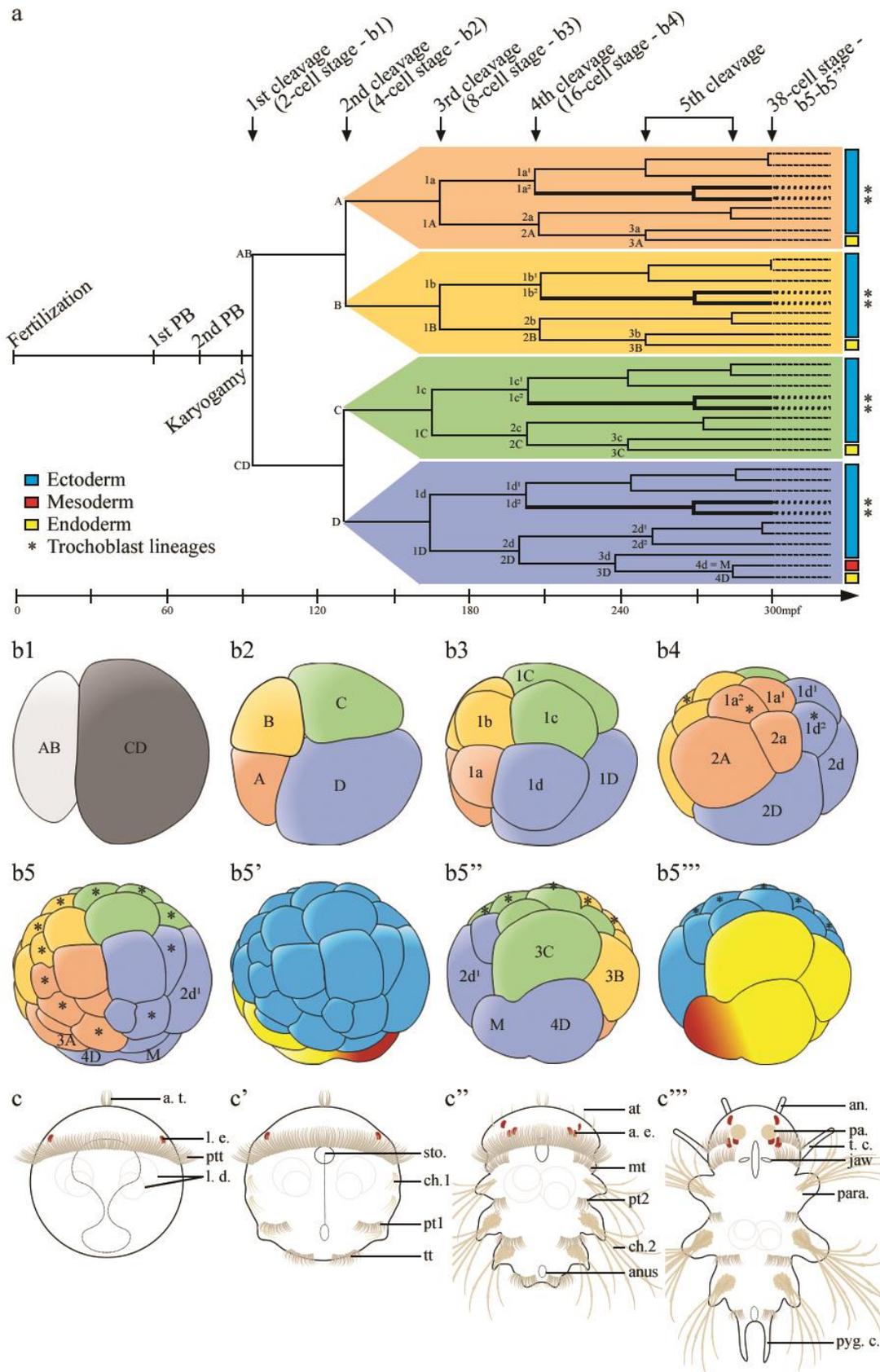
The first cleavage is unequal, giving rise to a small AB blastomere and a large CD blastomere (73% of the volume, 100 mnpf, Figure 13.4b1). This unequal cleavage induces a new axis, perpendicular to the vegetal/animal axis, which nearly corresponds to the dorsoventral axis. The second cleavage is slightly asynchronous (Figure 13.4a), unequal in the CD blastomere (D blasomere inheriting 50% of the total egg volume) and equal in the small AB blastomere (135 mnpf, Figure 13.4a, 4b2). Each of these four macromeres is the founder of a distinct quadrant (*e.g.* A-quadrant corresponds to the offspring of A macromere). The third cleavage is slightly

asynchronous as well (Figure 13.4a) and corresponds to the first “spiral” cleavage (clockwise), producing two batches of two nearly identical micromeres (1a and 1b versus 1c and 1d) (170 mmpf, Figure 13.4b3). Before the fourth asynchronous cleavage (Figure 13.4a), yolk granules are segregated at the vegetal pole of each blastomere. As a result, after completion of the fourth cleavage (*i.e.* 16-cell stage), blastomeres 1a<sup>1</sup>-1d<sup>1</sup> contain less yolk than blastomeres 1a<sup>2</sup>-1d<sup>2</sup>. The latter are called the primary trochoblasts and give rise to the equatorial ciliated belt, or prototroch, of the trochophore larva (Figure 13.4b4). The 2d blastomere is by far the largest micromere since its size even exceeds that of the macromeres 2A-2C. After the fourth cleavage, cleavages become highly asynchronous (except in the trochoblasts) and the cleavage strategy of the D quadrant strongly differs from the others with the short cell cycle of 2d<sup>1</sup> and 4d cell lines (Figure 13.4a). At the 38-cell stage, the fate of the three germ-layers is established. The four macromeres (3A-3C and 4D) give rise to the endoderm, the mesoderm mainly arises from 4d micromere (also called mesoblast or “M”), as well as the germ line, while all other micromeres form the ectoderm (Figure 13.4a, 4b5 to 4b5’’’’).

13.5.1.4 Stereoblastula / stereogastrula / Protrochophore larva (7-24 hours post fertilization of hpf)

After the 38-cell stage, micromeres no longer undergo spiral cleavage but rather progressively follow a bilateral symmetry. They rapidly divide and initiate their epibolic movement toward the vegetal pole, thus covering macromeres. This movement of micromeres results in the final equatorial position of trochoblasts, thus forming the prototroch. At the vegetal pole, cells arising from the cleavage of 4d micromere submerge beneath the large cells produced by the 2d micromere and start to form the mesodermal bands (4d<sup>122</sup> and 4d<sup>222</sup> lines the dorsal rim of the blastopore), gastrulation is thus initiated. In *Platynereis*, this process shows the amphistome mode, meaning that the blastopore gives rise to both the mouth and the anus (Figure 13.4c)

(Steinmetz et al. 2007). During this massive rearrangement of embryonic cells, the D-quadrant plays a key role, especially in the formation of trunk tissues. Indeed, the 4D blastomere participates in midgut anlage, the 2d offspring forms the somatic plate and the entire trunk ectoderm (*i.e.* epidermis and nervous system) while the 4d lineage provides the full trunk mesoderm, including the four quiescent putative primordial germ cells (*i.e.* 1ml, 1mr, 2ml and 2mr resulting from two asymmetric divisions of M-daughter cells) but also the cells composing the growth zone where new segments are added after the larval stage (Fischer and Arendt 2013; Ozpolat et al. 2017). During gastrulation and later on, the presence of four lipid droplets appears as a good readout of the proper development of the embryo. After the gastrulation stage, the embryo, often called the protochophore larva (13-24hpf) despite the persisting jelly, is slowly rotating within the jelly thanks to the prototroch (Figure 13.4c). It develops an apical tuft (apical ciliated organ) and the stomodeal field (*i.e.* the mouth anlage) starts to develop. At around 17 hpf, the first serotonergic neuron differentiates at the posterior extremity of the protochophore larvae (Starunov et al. 2017).



**Fig 13.4: Embryogenesis and larval development in *Platynereis dumerilii*.**

(a) Dendrogram summarizing the stereotypic steps following fertilization, including the emission of the two polar bodies (PB), karyogamy and the first cleavages (see b1 to b5''') that give rise to a 38-cell embryo in which the fate of the three germ layers is established (see legend on the panel) as well as trochoblast lineages (1a<sup>2</sup>-1d<sup>2</sup>). colored backgrounds represent each quadrant (*i.e.* A, B, C and D). Blastomere names are provided above and below nodes (in capital letters for macromeres), including the highly proliferative 2d<sup>1</sup> and M micromeres. Time frame is provided below the dendrogram. Based on (Dorresteijn 1990). (b1-b5''') Schematic representation of embryo following the five first cleavages. Color codes are similar than in panel a. (b1-b3, b5, b5') Animal views. (b4) Animal pole at the top. (b5'', b5''') Vegetal views. Based on (Dorresteijn 1990). (c-c''') Schematic representation of larval development. Ventral view of (c) 24-hour post fertilization (hpf) larva, (c') 48-hpf larva, (c'') 72-hpf larva and (c''') 6-day post fertilization larva. **Abv:** a. e.= adult eye; an.= antenna, a. t.= apical tuft, at= akrotrich, ch.1/2= chaeta within/outside the body wall, l. e.= larval eye, l. d.= lipid droplets, mnpf= minutes post fertilization, mt= metatroch, para= functional parapodia, pa.= palpa, pt1/2= paratroch 1 et 2, ptt= prototroch, pyg.c.= pygidial cirrus, sto.= stomodeum, t.c.= tentacular cirrus, tt= telotroch.

### 13.5.2 Larvae development

#### 13.5.2.1 Trochophore larva (24-48hpf)

The trochophore is a phototactic swimming larva possessing two pigmented eyes that become more and more prominent (Figure 13.4c). With age, the spherical larva elongates and three segments start to appear. Consistent with this first sign of segmentation, three pairs of ectodermal bulges develop laterally from 2d descendants to form the ventral chaetal sac pairs (Figure 13.3c'). An additional band of ciliated cells, called the telotroch, is formed at the posterior end, marking the edge between the pygidium and the rest of the trunk (26hpf).

Regarding the establishment of the digestive tract, the number of stomodeal cells slightly increases and they start to form a ring (*i.e.* the stomodeal rosette). The stomodeal field progressively moves toward the anterior pole and the rosette opens just below the prototroch to form the mouth (40hpf). Meanwhile, the overall nervous system rapidly develops (also from 2d micromeres) in part along with the increase in ciliated structures. From 24 hpf, various nervous connections are also implemented. Indeed, the apical ganglion at the posterior pole, containing the pioneer neuron of the VNC, is linked to the prototroch nerve ring by two ventral connectives. These connectives of the ventral nerve cord represent the two first axons of the brain. Immediately thereafter, the dorsal root of the circumesophageal connectives develop as well, followed by the ventral root (26hpf) to connect the VNC to the brain. At the same time, the single asymmetric unpaired dorsal axon and the first cerebral commissures appear. Later on, three additional serotonergic cells arise at the apical part (30-34hpf) as well as one pair at the first ventral commissure (40hpf). Finally, the second ventral commissure appears (44hpf) (Starunov et al. 2017). Similarly, muscles appear and develop during the trochophore stage. The dorsal longitudinal muscles develop first (28hpf), followed by ventral longitudinal muscles (32hpf) while the oblique and parapodial muscles start to be visible at the late trochophore stage (46-48hpf). The excretory system appears also at the trochophore stage, with the emergence of small, lateral, non-ciliated tubules (Hasse et al. 2010).

#### 13.5.2.2 Metatrochophore larva (48-66hpf)

The metatrochophore larval stage is marked by the appearance of the two adult eye pairs (Figure 13.4c''). In addition, the three first segments appear more defined due to the formation of non-functional parapodia and the significant growth of the chaetae outside the body wall (these segments are so called the chaetigerous segments). In addition, the first paratroch appears between the second and the third chaetigerous segments (48hpf) and a second one is visible

later on between the first and the second chaetigerous segments (56hpf), thus participating in segment delimitation. Then, above the prototroch, an additional ciliated structure progressively develops - the akrotroch - close to the apical tuft (60hpf). The stomodeal rosette size increases with an additional ring of cells (52hpf). The stomodeum invaginates, resulting in the larval foregut that elongates towards the posterior part. The nervous system also rapidly develops. A third commissure appears (48hpf) and all commissures thicken (54hpf). Axon projections from the VNC are observed laterally and redirected ventrally toward the surface. The circumesophageal connectives get closer to each other and the prototroch nerve ring moves toward the brain. All these phenomena participate in the formation and growth of the brain (52hpf). Additionally, the number of serotonergic cells along the ventral nerve cord increases with the occurrence of three additional pairs. Finally, the ventral medial longitudinal muscle appears (56hpf) and elongates up to the posterior border of the third segment. Similarly, oblique and parapodial muscles also elongate (Figure 13.4c''). Excretory system development continues as non-ciliated tubules elongate laterally toward the developing stomodeum (Hasse et al. 2010).

#### 13.5.2.3 Nectochaete larva (66hpf-5dpf)

The nectochaete larva corresponds to a major lifestyle transition. Indeed, the pelago-benthic larva starts crawling on the substrate thanks to functional parapodia, and to eat. The sensory organs, including antenna, palps, tentacular antero-dorsal cirri and anal cirri, appear and develop (75hpf) (Figure 13.4c'''). The trunk continues to elongate, providing a worm-like shape to the larva and a constriction distinguishes the trunk from the head. The two adult eyes found on both sides of the head increase in size and become extremely close. Lipid droplets progressively move toward the posterior part. Ciliogenesis progresses with the establishment of the metatroch, an additional line of ciliated cells that develops below the prototroch and fuses

with this latter on the lateral sides. The midgut forms as well as the proctodeum (anal region), and the stomodeum/foregut continues to elongate toward the posterior part, resulting in a fully functional digestive tract (75hpf-4dpf). Furthermore, the jaws develop within the foregut (4dpf) and a pair of primary teeth appears (5dpf). Meanwhile, the brain continuously grows, the convergence of the circumesophageal connective roots progresses, axon numbers increase in connectives and commissures, and additional serotonergic cells arise in the ventral nerve cord (66-72hpf) and in the brain (4-5dpf). The overall musculature develops as well, especially around the stomodeum to form the pharynx. Additionally, muscles and nerves associated with the development of antennae and cirri increase. 72hpf larvae possess a pair of antero-lateral non-ciliated tubules named “head kidneys” located close to the episphere. These larval structures are transitory since they disappear before 96hpf. In parallel, larval nephridia or protonephridia, formed from ciliated tubules and localized between segments, start to appear (Hasse et al. 2010).

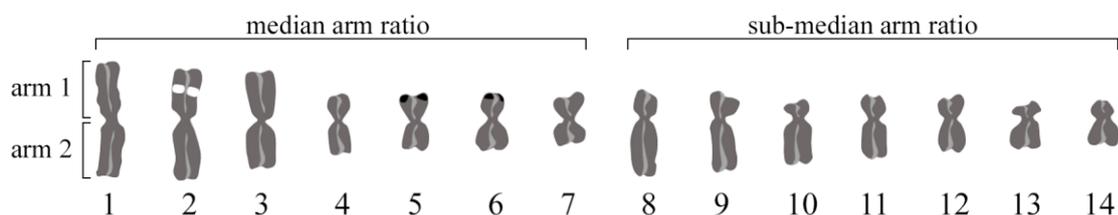
#### 13.5.2.4 Young errant juvenile

At this stage, the development of animals is no longer synchronous. Very young worms start to sequentially produce additional segments through posterior elongation, a process relying on a thin row of cells (presumably stem cells) that forms the segment addition zone (SAZ) in front of the pygidium (Gazave et al. 2013). Worms also lose several larva-specific features such as the prototroch, apical tuft, larval eyes, and lipid droplets. The excretory system is composed of segmented protonephridia until the worms reach the size of 20 segments, at which stage metanephridia appear (Hasse et al. 2010). In addition, the first chaetigerous segment fuses with the head. This important morphological transition, called cephalization, consists in the transformation of the first pair of parapodia into tentacular posterior-dorsal cirri and the

progressive loss of chaetae. Finally, spinning glands develop and produce mucus, allowing worms to build their first cocoon network.

### 13.6 Genomic data

As in many animals, counting chromosomes during metaphase revealed that *Platynereis dumerilii* is diploid ( $2n=28$ ) (Jha et al. 1995). More precisely, the *Platynereis* karyotype encompasses seven chromosome pairs showing a median arm ratio while the seven other pairs show a sub-median ratio (Figure 13.5). Different regular staining techniques were used to further characterize chromosome pairs. For instance, *Chromosome 2* shows a clear C-band-positive region, revealing a constitutive heterochromatin region not localized at the centromeric region while nuclear organizer regions (NORs) are found at the terminal positions of *Chromosomes 5* and *6*, thus revealing the localization of genes coding for ribosomal RNA.



**Fig 13.5: Schematic representation of *Platynereis* chromosomes.** *Platynereis* possesses  $2n=28$  chromosomes, five pairs showing a median position of centromeres (*Chromosome 1* to *7*). Black and white areas represent heterochromatin (C-band-positive band) and ribosomal RNA genes (NOR staining), respectively.

The precise genetic content of these chromosomes is in the course of being uncovered. Indeed, the *Platynereis dumerilii* genome has been recently sequenced by the D. Arendt laboratory (EMBL, Germany) from sperm. Although this genome is currently being refined with the aim

of obtaining a chromosome-level assembly, a high-quality draft version is already available, upon request, for the whole community working on *Platynereis*. Preliminary data revealed that *Platynereis* genome appears less compact than in other annelids (~1 to 2 Gpb) (Zantke et al. 2014), and a previous analysis comparing bacterial artificial chromosome sequencing (BAC) and expressed sequence tags (EST) on a subset of 30 randomly-detected genes suggested that *Platynereis* genes are intron-rich, surprisingly with two-thirds of introns shared between *Platynereis* and human orthologs (Raible et al. 2005). Various additional transcriptomic databases have been acquired during the past years (Table 13.1), including bulk RNA-seq data for all key stages of embryonic and larval development, juveniles of different ages, and adults (Conzelmann et al. 2013; Chou et al. 2016). These data have been grouped altogether and are now publicly available on a website *Pdumbase* platform (Chou et al. 2018) ([pdumbase.gdcb.iastate.edu](http://pdumbase.gdcb.iastate.edu)). *Platynereis* is still being actively studied by an active scientific community in the field of evolution and developmental biology, and as such, additional transcriptomic databases are constantly produced. For instance, Achim and collaborators shed light on the transcriptomic landscape of cell diversity in 48hpf-larvae using a single-cell RNA-seq (scRNA-seq) approach (Achim et al. 2015; Williams et al. 2017; Achim et al. 2018). In addition, bulk RNA-seq were acquired to unravel the dynamic of gene expression during circalunar-dependent sexual maturation (Schenk et al. 2019) and posterior regeneration (Vervoort's Lab, unpublished data).

Table 13.1 *Platynereis* genomic (BAC) and transcriptomic (EST and RNA-seq) databases

	Stage	Sequencing information	Repository	References	
BAC	Sperm of mature Male	Sanger (shotgun)	15 contigs	Genbank: CT030666 - CT030681	Raible et al., 2005
EST	Larvae (48hpf)	Sanger (3730xl)	1,484 expressed sequence tags	Genbank: CT032248 - CT033731	Raible et al., 2005
	Larvae and juvenile stages	Sanger + 454 Roche	77,419 expressed sequence tags	Genbank: JZ391525 - JZ468943	Conzelmann et al., 2013
Bulk RNA-seq	Fertilized eggs, larvae (24, 36, 48, 72hpf and 4dpf), juveniles (10, 15dpf, 1, 3mpf) and adults (males and females)	Illumina (HiSeq 2000)	351,625 reads, 87,686 contigs (>500bp), 28,067 (>1000bp), 51,767 ORFs (>120aa)	Supp. Data	Conzelmann et al., 2013
	Embryonic development (2, 4, 6, 8, 10, 12hpf) and larvae (14hpf)	Illumina (HiSeq)	273,087 contigs, 51,260 ORFs (>100aa)	<a href="https://github.com/hsienchao/pdu_sqs/fin/master">https://github.com/hsienchao/pdu_sqs/fin/master</a>	Chou et al., 2016
	Head samples under various circalunar conditions and maturation stages	Illumina (HiSeq 2000)	52,059 contigs (>500bp)	ENA repository: PRJEB27496	Schenk et al., 2019
scRNA-seq	47hpf-larva epispheres	Fluidigm C1 Single-Cell Auto Prep System / Illumina (HiSeq 2000)		ArrayExpress: E-MTAB-2865	Achim et al., 2015
	48hpf-larvae	Fluidigm C1 Single-Cell Auto Prep System / Illumina (HiSeq 2000)		ArrayExpress: E-MTAB-2865 and E-MTAB-5953	Achim et al., 2018

BAC= bacterial artificial chromosome sequencing; EST= expressed sequences tags

Finally, in addition to the significant *Platynereis* resources acquired during the past decade, the availability of genome sequences of the Sedentaria *Capitella teleta*, *Helobdella robusta* (Simakov et al. 2013), *Spirobranchus lamarcki* (Kenny et al. 2015), *Lamellibrachia luymesii* (Li et al. 2019), *Eisenia Andrei* (Shao et al. 2020), *Eisenia fetida* (Bhambri et al. 2018) as well as the Dinophiliformia (sister group to Sedentaria + Errantia) *Dimorphilus gyrociliatus* (Martin-Duran et al. 2020) allow for comparative analyses within annelids.

## 13.7 Functional approaches: tools for molecular and cellular analyses

In addition to its scientific relevance and its easy maintenance in laboratory, the success of *Platynereis* as a new model system also strongly relies on the efforts that have been done to develop a large panel of molecular and cellular tools to successfully tackle interesting biological questions in evolutionary and developmental biology (Backfisch et al. 2014; Williams and Jekely 2016).

### 13.7.1 Descriptive approaches

#### 13.7.1.1 Detection of mRNA: whole mount *in situ* hybridization (WMISH)

As mentioned in the genomic data section, several high-quality bulk RNA-seq and scRNA-seq were recently used to investigate modulations in gene expression during various processes in *Platynereis*. Nevertheless, bulk RNA-seq average information from various cell populations and scRNA-seq remains expensive and their interpretation relies on a comprehensive description of cell populations *in vivo*. Accordingly, despite important breakthroughs in sequencing technologies, WMISH remains an indispensable molecular approach to localize gene expression. WMISH has been established in *Platynereis* to investigate gene expression during early embryonic / larval stages (Arendt et al. 2001), posterior elongation (Prud'homme et al. 2003), regeneration (Planques et al. 2019), and the adult stage (Backfisch et al. 2013) using the regular NBT/BCIP colorimetric staining (Figure 13.6a and a'). Similarly, fluorescent *in situ* hybridization (FISH, Figure 13.6b and b') has been established (Tessmar-Raible et al. 2005) while current efforts are now also dedicated to implement hybridization chain reactions (HCR) (Choi et al. 2018), thus allowing multiple transcript detection to be required for co-expression analysis. Finally, the stereotypic development of embryo and larva coupled with *in situ* hybridizations allows for image registration (Figure 13.6c) which consists of a virtual atlas

of expression patterns for their systematic comparison (Tomer et al. 2010; Asadulina et al. 2012).

#### 13.7.1.2 Detection of proteins: immunohistochemistry (IHC) and western blot (WB)

The *in vivo* detection of proteins has been developed as well. The first detection of proteins in *Platynereis* date back to the early 90s with the visualization of the nervous system, ciliated cells, and the entire epidermis during early development using various antibodies (Abs) raised against *Nereis diversicolor* (Annelida) proteins, *Drosophila* Engrailed and Antennapedia, respectively (Dorresteyn et al. 1993). Since, antibodies such as those against acetylated-Tubulin Abs are now routinely used to depict the *Platynereis* nervous system (Figure 13.6d). In contrast to WMISH that can be performed on virtually all genes, IHC suffers from the lack of appropriate Abs developed against *Platynereis* proteins (or proteins from closely related species). Accordingly, WMISH remains the preferred approach used as a proxy of protein location while IHC are often restricted to highly conserved proteins (*e.g.* proteins from the cytoskeleton and Histones). Nevertheless, IHC against other proteins such as MIP peptides,  $\beta$ -catenin or neuropeptides have also proven to be successful (Schneider and Bowerman 2007; Conzelmann et al. 2011; Williams et al. 2015; Gazave et al. 2017). WBs have been also developed from whole cell extract (Schneider and Bowerman 2007) and nuclear extracts (Figure 13.6e, unpublished data Vervoort's Lab), thus allowing for the quantification of specific proteins in different tissues or upon various conditions.

#### 13.7.2.3 Tracking cell, cell components, and monitoring key cellular processes

Staining approaches: Various staining using commercially-available dyes are pertinent to study for instance muscles (phalloidin), chaetae (wheat germ agglutinin), or to stain cell membranes

(mCLING–ATTO 647N, FM-464) either on fixed or live animals, depending on the dye used (Lauri et al. 2014; Williams et al. 2015; Gazave et al. 2017; Chartier et al. 2018). Staining to monitor key cellular processes has also been developed in *Platynereis*. For instance, EdU (5-ethynyl-2'-deoxyuridine, Figure 13.6f and f') and BrdU (Bromo-desoxyuridine) incorporations followed by chasing are used to highlight proliferative cells and their progenies, a key approach to characterize putative stem cells and their lineage during early development (Rebscher et al. 2012; Demilly et al. 2013), posterior elongation (Gazave et al. 2013), and regeneration (Planques et al. 2019). Cell death can be assessed as well, using real-time apoptosis detection (TUNEL) (Demilly et al. 2013; Lauri et al. 2014; Zidek et al. 2018).

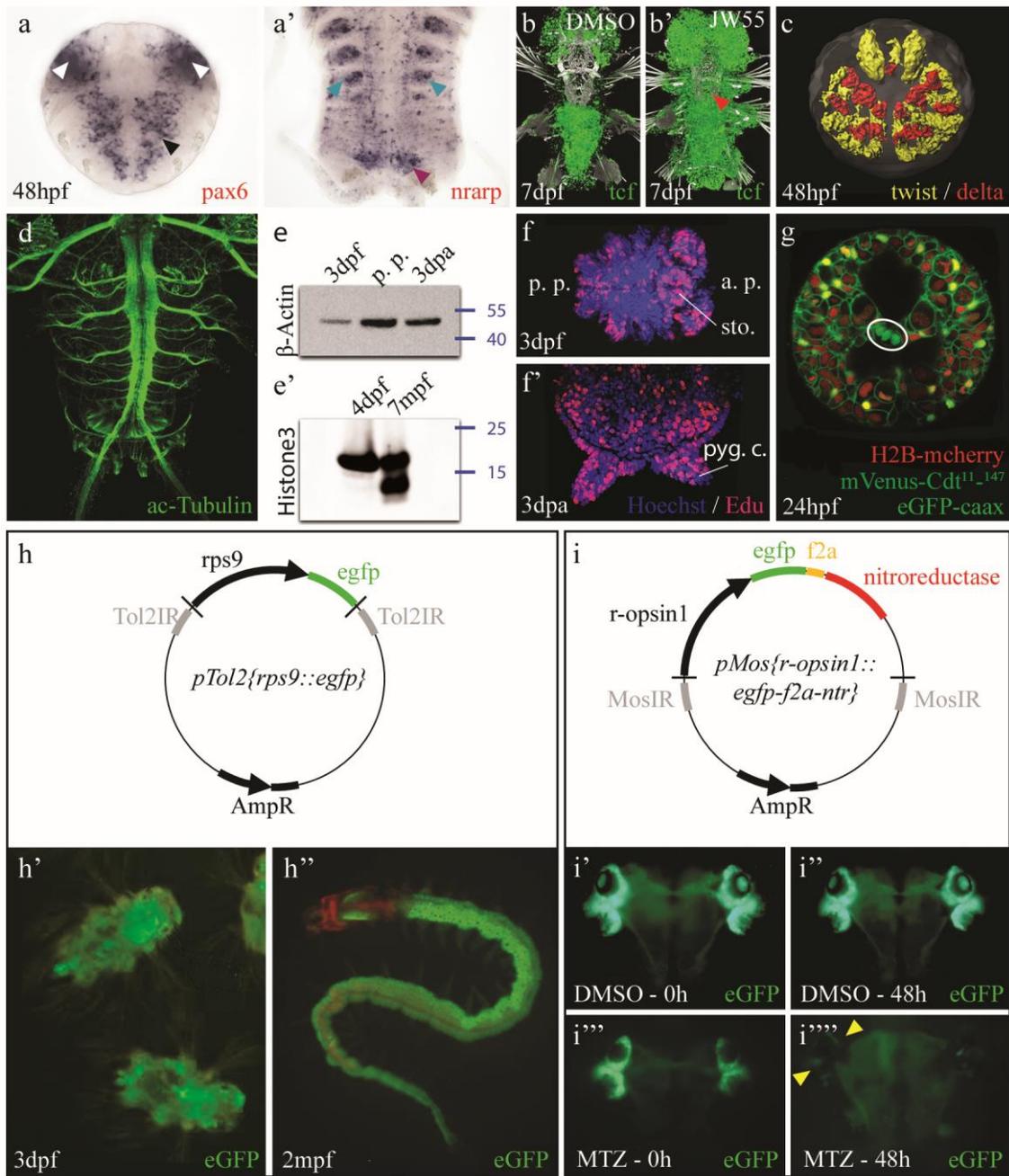
Microinjection of dyes: As reported in the *Embryogenesis and larval development* section, Ackermann and colleagues injected *Platynereis* embryos at the 2-, 4- and 8-cell stages with fluorescent dyes (*e.g.* FITC-dextrane) to trace blastomere lineages and their respective contribution to tissue in young worms (Fischer and Dorresteijn 2004; Ackermann et al. 2005).

Microinjection of mRNA: mRNA were also successfully injected into fertilized embryos to induce the expression of different fluorescent proteins such as the photoactivable mCherry (PAmCherry1) and the photoconvertible Kikume Green-Red (KIKGR) protein for cell tracking (Lauri et al. 2014; Veraszto et al. 2017), biomarkers to mark nucleus (*H2A-mCherry* ; *H2B-eGFP*) and cell membranes (*egfp-caax* ; *mYfp*) (Lauri et al. 2014; Ozpolat et al. 2017; Kuehn et al. 2019) or biosensors to monitor cell cycle progression (mVenus-cdt1<sup>aal-147</sup>) (Ozpolat et al. 2017) (Figure 13.6g) and neuronal activity (GCaMP6, calcium imaging) (Veraszto et al. 2017; Chartier et al. 2018) in live animals.

Transposon-based transgenesis of reporter cassettes: While mRNA represent an incredible useful technique for biomarker and biosensor expression during early development, transgenic animals allow for a tight control of gene expression. In *Platynereis*, two transposon-mediated systems (*i.e.* Tol2 and Mos-based constructs) were efficiently developed for both transient and

stable transgenesis (Backfisch et al. 2014). To implement this approach, the promoter of the ribosomal protein Rps9 has been used to drive ubiquitous expression of the *enhanced green fluorescent protein* (*egfp*) (Figure 13.6h to h’). Interestingly, comparison of Tol2- and Mos-based systems using similar constructs (*i.e.* *pTol2(rps9::egfp)* and *pMos(rps9::egfp)*) revealed that whereas embryos injected with Tol2-based plasmids tend to show a higher frequency of genome integration than with Mos-based plasmids, transgenes are heritable to progeny only through Mos-mediated transgenesis (Backfisch et al. 2014). Additional promoters to *rps9* have been developed to target specific cell populations such as the *r-opsin1* promoter for adult eye cells and their neuronal projections (Backfisch et al. 2013; Veedin-Rajan et al. 2013) (Figure 13.6i to i’), a specific *alpha-tubulin* promoter (*tuba*) for cells with motile cilia in larvae, a *maf* promoter for a subtype of nerve cells in the larval brain (Backfisch et al. 2014) and a *guanylyl cyclase-β* promoter for the cholinergic motoneurons (Veraszto et al. 2017).

Serial section transmission electron microscopy (ssTEM): By imaging and assembling numerous serial sections (around 1,700 sections for a head and the first chaetigerous segment or 5,000 sections for a full individual) and manually tracing all neurons, researchers were able to reconstruct a comprehensive 3-dimension cell atlas of the visual neuronal circuit in 72hpf larvae, including 106 neurons (*i.e.* photoreceptor cells, interneurons and motoneurons) and their synaptic connectivity (Randel et al. 2014; Randel et al. 2015). This sophisticated approach has been more recently extended to other circuits such as the neurosecretory connectome (Williams et al. 2017).



**Fig 13.6: Molecular and cellular tools for functional approaches in *Platynereis*.**

(a-a') Whole mount *in situ* hybridizations using NBT/BCIP colorimetric staining showing (a) the expression pattern of *pax6* in brain hemispheres (white arrowheads) and ventral neurectoderm (black arrowhead) and (a') *nrarp* expression in chaetal sacs (blue arrowheads) and cells of growth zone (purple arrowhead) during posterior elongation. (b-b') Fluorescent *in situ* hybridization showing the effect of Wnt/ $\beta$ -catenin pathway inhibition on *tcf* expression. Upon JW55 treatment (Axin2 stabilization), *tcf* expression is extended to other tissues (red

arrowhead) in addition to its regular expression in brain ganglia and midgut (Zidek et al. 2018). (c) Image registration showing *Platynereis twist* and *delta* expression in mesoderm and chaetal sacs, respectively. Ventral view. (d) Acetylated-Tubulin immunohistochemistry revealing the ventral nervous system in posterior part. (e-e') Western blot of *Platynereis* using (e) whole cell extracts ( $\beta$ -Actin) and (e') nuclear extracts (Histone 3). (f-f') EdU staining to investigate proliferative cells (f) in larva and (f') posterior part regeneration. (g) Co-injection of *H2B-mcherry* (nuclear marker), *mvenus-cdt<sup>11-147</sup>* (cell cycle biosensor) and *egfp-caax* (membrane marker) mRNAs in fertilized embryo used to follow cell cycle progression during embryo development. The green staining of putative primordial germ cells (white circle) suggests that they no longer divide (Ozpolat et al. 2017). (h-h'') Tol2 transposase system for transient transgenesis using the promoter of the ribosomal protein Rps9 (*rps9*) to ubiquitously express *enhanced green fluorescent protein (egfp)* (h') in larvae and (h'') young worms (Backfisch et al. 2014). (i-i''') Mos transposase system for heritable transgenesis using the promoter of *r-opsin1* to co-express *egfp* and bacterial *nitroreductase* in (i') adult eyes. (i'-i''') Upon metronidazole 48h-treatment (MTZ), Nitroreductase convert MTZ into a toxic compound leading to the death of positive cells (yellow arrowheads) (Backfisch et al. 2014). **Abv:** a. p.= anterior part, dpa= days post amputation, hpf/dpf/mpf= hours/days/month post fertilization, p. p.= posterior part, pyg. c.= pygidial cirrus, sto= stomodeum.

### 13.7.3 Functional approaches

#### 13.7.2.1 Gene knock-down: translation-blocking morpholinos (MOs)

Although they are used unfrequently, MOs represent an interesting knock-down approach to assess gene functions during early development. Indeed, in a study aiming to show the implication of myoinhibitory (MIP) peptides on larval settlement, two MIP-receptor MOs were

successfully used. Indeed, in embryos injected with MOs, MIP treatment-induced settlement was no longer observed (Conzelmann et al. 2013).

#### 13.7.2.4 Protein inhibition / activation: pharmacological and peptide treatments

Although concerns regarding putative off-target effects have been raised with the pharmacological approaches – often addressed by the use of different molecules in parallel – the treatment using inhibitors is an easy approach to assess the function of specific proteins in live animals, especially in water-dwelling animals such as *Platynereis*. In addition, this approach allows researchers to interfere with proteins at specific timepoints and during processes that cannot be reached using MOs (*e.g.* post-larval and regeneration processes). Accordingly, a broad range of studies has developed this approach for instance to investigate the function of key signaling pathways such as Wnt/ $\beta$ -catenin (Schneider and Bowerman 2007; Steinmetz et al. 2007; Demilly et al. 2013; Marlow et al. 2014; Zidek et al. 2018) (Figure 13.6b and b'), Planar cell polarity (Steinmetz et al. 2007), Notch (Gazave et al. 2017), Hedgehog (Dray et al. 2010), or to assess the role of key cellular processes such as cell proliferation (Planques et al. 2019). Similarly, successful results were obtained by incubating *Platynereis* larvae with zebrafish BMP4 peptides (Denes et al. 2007), *Platynereis* synthetic neuropeptide (Conzelmann et al. 2011), or *Platynereis* synthetic MIB peptides (Conzelmann et al. 2013; Williams et al. 2015).

#### 13.7.2.5 Genome editing

Transgenesis: Transgenesis in *Platynereis* has been so far mainly used to monitor gene expression and to study specific cell populations (see above). However, this technique now opens a broad range of subsequent functional approaches, including conditional knock-down and ectopic expression. In *Platynereis*, transgenesis has been used for effective targeted cell

ablation. Indeed, the use of *r-opsin1* promoter allowed the expression of the bacterial nitroreductase enzyme (Ntr) in *Platynereis* adult eyes (Veedin-Rajan et al. 2013). This enzyme converts metronidazole (MTZ) into a toxic product that induces the death of the corresponding cells (Figure 13.6i to i'''''). Thus, transgenic animals expressing nitroreductase represent a great alternative to laser ablation to specifically remove a subset of cells.

Transcriptional activator-like nuclease (TALEN): In *Platynereis*, TALEN has been established as an efficient tool to induce heritable mutagenesis (Bannister et al. 2014) and this approach has been recently used to highlight the involvement of Gonadotropin Releasing Hormone (GnRH, known to integrate environmental stimuli for vertebrate sexual maturation and breeding) in the regulation of growth and sexual maturation by lunar phases. Indeed, maturation, growth and regeneration were reduced in animals where mutations leading to *corazonin1/gnrhl1* knock-outs were performed (Andreatta et al. 2020).

CRISPR/Cas9: CRISPR/Cas9 also has been recently used in *Platynereis*. In planktonic larvae, the startle response is mediated by collar receptor neurons expressing *polycystin* genes (*PKD1-1* and *PKD2-1*). Interestingly, this freezing response is abolished in both *PKD1-1* and *PKD2-1* mutants (Bezares-Calderon et al. 2018).

## 13.8 Challenging questions

*Platynereis* has been successfully developed as a powerful marine model thanks to the development of many tools (see section 13.7 Functional approaches: tools for molecular and cellular analyses), allowing researchers to address a variety of biological questions, mostly related to evolutionary developmental biology (Ferrier 2012). Several of these questions have been already raised earlier in this chapter and have been the subject of detailed recent reviews, notably (i) biological rhythms and clocks (Tessmar-Raible et al. 2011; Raible and Falciatore

2014; Raible et al. 2017; Andreatta and Tessmar-Raible 2020), (ii) neuronal connectomics and plankton behavior (Jekely et al. 2018; Williams and Jekely 2019; Bezares-Calderon et al. 2020; Marinkovic et al. 2020), and (iii) cell types evolution (Brunet et al. 2015; Arendt, Musser, et al. 2016; Arendt, Tosches, et al. 2016; Arendt 2018; Nielsen et al. 2018; Arendt et al. 2019). Here, we chose to introduce two additional lines of research that are currently (re)emerging: the regeneration processes and the epigenetic modifications during embryonic and post-embryonic development.

### 13.8.1 Regeneration

Animal regeneration is defined as the ability to restore a lost or damaged body part (Poss 2010). This fascinating process has intrigued scientists for centuries and we recently observed a strong re-emergence of the regeneration field thanks to the availability of new tools for less conventional models (Gazave and Rottinger 2019). Injury-induced regeneration is a widespread phenomenon harbored by species of all the major lineages of Metazoa. In addition, the extent of what can be regenerated after an injury greatly varies among animals (Grillo et al. 2016). The origin and evolution of animal regeneration is a long-standing debate and the questions of why and how regeneration abilities evolved are still poorly understood (Bely 2010). Annelids show amazing regenerative capabilities, as most species are able to regenerate the posterior part of their body and their parapodia following an amputation, as well as, for some species, their anterior part (including the head) (Ozpolat and Bely 2016). Experimental and descriptive morphological studies of annelid regeneration have provided important knowledge (Boilly 1969a; Boilly 1969b) (for recent reviews see (Kostyuchenko and Kozin 2020; Nikanorova et al. 2020)). Nowadays, some cellular and molecular aspects of these processes have been addressed in a limited number of models (Myohara 2012; Sugio et al. 2012; de Jong and Seaver 2018; Ribeiro et al. 2019) notably *Platynereis* (Planques et al. 2019).

*Platynereis* is able to regenerate its posterior part as well as various body outgrowths, such as tentacles and parapodia, but not its head. Its posterior regeneration was recently carefully described at the morphological, cellular, and molecular levels (Planques et al. 2019). After amputation of the posterior part of their body (segments, growth zone and pygidium), *Platynereis* worms rapidly regenerate both the posterior-most part of the body, the pygidium, and the stem cell-rich growth zone, the latter then producing new segments through posterior elongation (Gazave et al. 2013). Interestingly, both complex differentiated structures and stem cell populations are regenerated during this event (Gazave et al. 2013). In precise conditions of worm age/size and a specific amputation procedure, *Platynereis* posterior regeneration follows five well-defined stages, which correspond to particular timepoints after amputation. Briefly, (i) wound healing is achieved one day post-amputation (1 dpa), (ii) a proliferating blastema appears around 2 dpa, (iii) at 3 dpa, this blastema shows a conspicuous antero-posterior and dorso-ventral organization, (iv) a well-differentiated pygidium is formed at 4 dpa and (v) from 5 dpa new morphologically visible segments are produced by the growth zone (Planques et al. 2019). While several parameters such as the size of the worms, the position of amputation, and the realization of serial amputations affect the timing of the process, posterior regeneration is always successful (except when the amputation is performed close to the pharynx and in sexually mature animals). Further characterization of posterior regeneration using various labellings and *in situ* hybridizations for tissue patterning genes indicates that regeneration is a rapid process: important cell and tissue differentiation starts at 3 dpa and at this stage the growth zone is already re-established and starts to produce segments. Thanks to EdU incorporations, cell cycle marker labellings, and the use of an inhibitor of cell divisions, it has been also shown that cell proliferation is strictly required for regeneration (Planques et al. 2019). These findings pave the way for a better understanding of *Platynereis* posterior regeneration while many pressing questions remain unanswered.

An important question in the regeneration field concerns the initiation and control of regeneration (Ricci and Srivastava 2018). Recent studies have suggested that cell death could be a crucial event by triggering cell proliferation (Perez-Garijo and Steller 2015). Cell death seems to be itself stimulated by the production of reactive oxygen species (ROS), essential for regeneration in several models (*Hydra*, *Drosophila* ...) through the activation of various signaling pathways (Vriz et al. 2014). Whether the cascade ROS → apoptosis → proliferation may represent a general principle of regeneration is however not known. In annelids, this question has not been addressed yet but preliminary data for *Platynereis* strongly suggest the occurrence of cell death at 1 and 2 dpa, concomitantly with a peak of cell proliferation (Vullien, Renaud, Vervoort and Gazave, unpublished data).

Thanks to recently developed tools for molecular and cellular analyses in *Platynereis*, it is now possible to characterize the *in vivo* distribution of apoptotic cells and to detect the ROS production cells using fluorescent dyes or genetically encoded biosensors. This in-depth description of the processes at play, combined with functional tools and transcriptomic analysis, will certainly in near future uncover the initiation and control mechanisms of *Platynereis* posterior regeneration.

Another key question is to determine the origin and fate of blastema cells, which gives rise to the regenerated structures (Morgan 1901; Tanaka 2016). They can derive from pre-existing stem cells present in the body before the amputation and/or being produced by dedifferentiation of cells at the amputation site (Tanaka and Reddien 2011). These cells could be pluripotent stem cells and/or more tissue-restricted progenitor cells with limited potency. In annelids, the source of cells involved in posterior and anterior regeneration has been partially addressed in a couple of species such as *Nereis diversicolor* (Boilly 1969c), *Enchytraeus japonensis* (Myohara 2012; Sugio et al. 2012) or *Capitella teleta* (de Jong and Seaver 2018). During *Platynereis* posterior regeneration, EdU pulse and chase experiments strongly support the idea that blastema cells

mostly derive from dedifferentiation of cells coming from the segment abutting the amputation plane, with the notable exception of the gut which probably regenerates from pre-existing gut stem cells (Planques et al. 2019). In addition, blastema cells, from very early stages express a collection of genes belonging to the GMP signature (Juliano et al. 2010) whose orthologs in other species are expressed in pluripotent/multipotent somatic stem cells and primordial germ cells. This suggests that blastema may contain multi or pluripotent progenitors/stem cells, even if this need to be confirmed. To better assess the origin and fate of blastema cells, it would be highly valuable to perform blastema cell lineage tracing experiments. This would allow us to clearly define the respective contribution of resident stem cells and local dedifferentiation events to blastema formation in *Platynereis* as well as the fate of blastema cells.

#### 13.8.2 Epigenetic modifications during embryonic / larval development and regeneration

Development and regeneration are highly dynamic processes both requiring important changes in gene expression to handle the establishment of various cell populations (Gerber et al. 2018; Cao et al. 2019; Pijuan-Sala et al. 2019; Shao et al. 2020). These cell fate trajectory, allowing cells to progressively acquire their molecular and functional identities, imply dynamic modulations of epigenetic marks. Nowadays, in developmental biology and cell biology, epigenetics includes any alteration of gene expression that is not associated with changes in the DNA sequence but is due to other molecular mechanisms such as changes in the chromatin structure, histone post-translational modifications, and non-coding RNAs (Nicoglou and Merlin 2017). By revealing how each locus is activated or down-regulated, epigenetics represents a tremendous step forward by allowing comprehensive overviews of biological processes. Among epigenetic marks, DNA methylation (5-methyl-cytosine, 5mC) appears to be the most extensively studied one (Greenberg and Bourc'his 2019). Basically, two different DNA methylation patterns exist, both occurring at CpG sites (CG motif in the DNA sequence)

(Zemach et al. 2010). On one hand, high levels of methylation at CpG islands (DNA regions where CpG sites are abundant) of promoter regions tends to be associated with low gene expression while low methylation corresponds to active genes. Although this regulatory-promoter methylation is well-identified in vertebrates, only few cases have been reported in non-vertebrates so far (de Mendoza et al. 2019). On the other hand, gene body methylation (GBM, *i.e.* methylation on coding regions, exons and introns) is found in vertebrates, non-vertebrate animals, and other multicellular organisms (Suzuki and Bird 2008; Zemach et al. 2010). However, the function of this type of methylation remains largely unknown. Beyond DNA methylation, epigenetics also strongly relies on Histone mark modifications (*e.g.* acetylation, methylation, phosphorylation, ubiquitination). For instance, the study of Histone methylation and acetylation in vertebrates allowed researchers to describe 37 specific marks of active and inactive genes (Karlic et al. 2010; Dai and Wang 2014). Among them, Histone 3 (H3) tri-methylation (me3) at lysine 4 (H3K4me3), H3K36me, and H3 acetylation at K27 (H3K27ac) coincide with gene activation during embryonic development in sponges, cnidarians, planarians and vertebrates, while H3K9me3 and H3K27me3 represent repressive marks (Karlic et al. 2010; Schwaiger et al. 2014; Cunliffe 2016; Gaiti et al. 2017; Dattani et al. 2018). Accordingly, epigenetics represents one of the most active domains in biology, especially in the context of biological phenomena such as cell differentiation and development. However, epigenetics is often restricted to vertebrates and a few non-vertebrate organisms (*e.g.* cnidarians and poriferans) while no data has been acquired for other lineages such as annelids, thus calling for comparative studies. In *Platynereis*, gene coding for orthologous proteins of all main actors of 5mC DNA methylation / demethylation machinery were found (Planques et al. 2020). In addition, computational analyses (CpG observed/expected) and assays with methylation-sensitive restriction enzymes revealed a high level of DNA methylation during embryonic and larval development. Interestingly, treatment with a hypomethylating agent

(Decitabine / 5-aza-2'deoxycytidine) during larval development impairs parapodia, chaetae, and pygidium formation, and eventually leads to the death of juvenile worms, suggesting a fundamental role of DNA methylation during larval development. Similarly, Decitabine greatly delays worm regeneration and sometimes leads to abnormal posterior elongation (*i.e.* no or reduced number of new segments, abnormal parapodia and cirri) after drug removal. This suggests that the regenerated growth zone is affected by Decitabine-mediated hypomethylation, leading to persistent defects of its function thereafter. Now, additional data are required to assess the precise methylation patterns in *Platynereis* (*e.g.* genome-wide bisulfite sequencing) and the link between modulations in methylation patterns and changes in gene expression. Furthermore, extending research to other epigenetic mechanisms such as the role of post-translational Histone marks and non-coding RNA would bring additional clues to questions on the tight mechanisms controlling cell fate trajectories during dynamic processes, especially in non-vertebrate animals and during regeneration for which studies remains highly scarce.

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