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Abstract

The urochordate *Oikopleura dioica* is emerging as a nonclassical animal model in the field of evolutionary developmental biology (a.k.a. evo-devo) especially attractive for investigating the impact of gene loss on the evolution of mechanisms of development. This is because this organism fulfills the requirements of an animal model (i.e., has a simple and accessible morphology, a short generation time and life span, and affordable culture in the laboratory and amenable experimental manipulation), but also because *O. dioica* occupies a key phylogenetic position to understand the diversification and origin of our own phylum, the chordates. During its evolution, *O. dioica* genome has suffered a drastic process of compaction, becoming the smallest known chordate genome, a process that has been accompanied by exacerbating amount of gene losses. Interestingly, however, despite the extensive gene losses, including entire regulatory pathways essential for the embryonic development of other chordates, *O. dioica* retains the typical chordate body plan. This unexpected situation led to the formulation of the so-called inverse paradox of evo-devo, that is, when a genetic diversity is able to maintain a phenotypic unity. This chapter reviews the biological features of *O. dioica* as a model animal, along with the current data on the evolution of its genes and genome. We pay special attention to the numerous examples of gene losses that have taken place during the evolution of this unique animal model, which is helping us to understand to which the limits of evo-devo can be pushed off.

Chapter 4
Oikopleura dioica: An Emergent Chordate Model to Study the Impact of Gene Loss on the Evolution of the Mechanisms of Development

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Abstract The urochordate *Oikopleura dioica* is emerging as a nonclassical animal model in the field of evolutionary developmental biology (a.k.a. evo-devo) especially attractive for investigating the impact of gene loss on the evolution of mechanisms of development. This is because this organism fulfills the requirements of an animal model (i.e., has a simple and accessible morphology, a short generation time and life span, and affordable culture in the laboratory and amenable experimental manipulation), but also because *O. dioica* occupies a key phylogenetic position to understand the diversification and origin of our own phylum, the chordates. During its evolution, *O. dioica* genome has suffered a drastic process of compaction, becoming the smallest known chordate genome, a process that has been accompanied by exacerbating amount of gene losses. Interestingly, however, despite the extensive gene losses, including entire regulatory pathways essential for the embryonic development of other chordates, *O. dioica* retains the typical chordate body plan. This unexpected situation led to the formulation of the so-called inverse paradox of evo-devo, that is, when a genetic diversity is able to maintain a phenotypic unity. This chapter reviews the biological features of *O. dioica* as a model animal, along with the current data on the evolution of its genes and genome. We pay special attention to the numerous examples of gene losses that have taken place during the evolution of this unique animal model, which is helping us to understand to which the limits of evo-devo can be pushed off.

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4.1 Introduction

In 1872, the Swiss marine biologist Hermann Fol during his stay in Messina (Sicilia, Italy) described a new planktonic species of Urochordates (a.k.a. Tunicates), the appendicularian (a.k.a. Larvacean) *Oikopleura dioica* (Fol 1872). From the beginning, *O. dioica* exhibited interesting biological features because, using Fol's own words, "Je n'ai jamais trouvé sur un meme individu les organes male et femelle. Notre espece est réellement un tunicier a sexes distincts" (I have never found on the same individual the male and female organs. Our species is really a tunicate with distinct sexes) (Fol 1872). This was a remarkable finding because until then, it was assumed that Urochordates were hermaphrodites. At the beginning of the twentieth century, the Russian embryologist Vladimir Vladimirovich Salensky (commonly known as W. Salensky) described the anatomy of different Appendicularia species (Salensky 1903, 1904, 1905). Although in 1903 Richard Benedict Goldschmidt provided a first brief description of the larva of *O. dioica* (Goldschmidt 1903), the Dutch biologist Hendricus Christoffel Delsman can be considered the father of *O. dioica* embryogenesis. He, analyzing embryos directly collected from the field, was able to laboriously reconstruct its cleavage pattern with amazing exactitude (Delsman 1910, 1912), which a century later has been confirmed by confocal microscopy and live imaging (Fujii et al. 2008; Stach et al. 2008).

During the 1960s and 1970s, the *O. dioica* anatomy was described in deep detail, paying special attention to some conspicuous structures such as the notochord or the endostyle (Olsson 1963, 1965a, b; Welsch and Storch 1969) and to the organs required for the construction and use of the "house," a filtering complex structure that traps the food (Galt 1972). Almost at the same time, the first *O. dioica* cultures in the laboratory were successfully maintained through numerous generations (Paffenhöfer 1973), and over the next 30 years, numerous ecological and biogeographical studies were reported (e.g., Galt 1978; King et al. 1980; Gorsky et al. 1984; Bedo et al. 1993; Acuña et al. 1995; Hopcroft and Roff 1995; Uye and Ichino 1995; Nakamura et al. 1997; Hopcroft et al. 1998) along with multiple morphological, anatomical, and physiological descriptions (e.g., Last 1972; Bone and Mackie 1975; Olsson 1975; Fenaux 1976, 1986; Flood and Afzelius 1978; Fredriksson and Olsson 1981, 1991; Holmberg 1982, 1984; Fredriksson et al. 1985; Bollner et al. 1986; Georges et al. 1988; Holland et al. 1988; Olsson et al. 1990; Nishino and Morisawa 1998; Lopez-Urrutia and Acuña 1999; Acuña and Kiefer 2000).

All this accumulated knowledge led *O. dioica* to the twenty-first century, when shotgun sequencing techniques (Seo et al. 2001; Denoeud et al. 2010) together with the establishment of protocols for gene expression analysis (Bassham and Postlethwait 2000; Nishino et al. 2000) paved the groundwork for *O. dioica* as a promising nonclassical model species for different biological disciplines, including comparative genomics, molecular genetics, or evolutionary developmental biology (evo-devo). Here, we will review the main biological features of *O. dioica* as chordate model at the morphological, physiological, embryonic, ecological, methodological, evolutionary, genetic, and genomic levels, paying special attention to the

fact that *O. dioica* has shrunk and compacted its genome retaining the archetypical chordate body plan. We will finish by analyzing the impact of gene loss, especially the loss of genes related to embryonic development, on the evolution of *O. dioica*.

4.2 *Oikopleura dioica* as a Nonclassical Model System for Evo-Devo

A model organism is a simplified and accessible system that represents a more complex entity, based on the notion summarized by Jacques Monod: "what is true for *E. coli* is true for the elephant." This notion reflects the evolutionary principle that all organisms share some degree of functional and genetic similarity due to common ancestry. There are several classical model species in cell and developmental biology, from non-vertebrates such as the roundworm *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster* to vertebrates such as zebrafish and mouse. The choice of a particular model organism depends, essentially, on the specific scientific question that needs to be investigated, and new scientific questions usually demand new model systems. Thus, when we became interested to understand the impact of the gene loss on the evo-devo of chordates, we had to look for a new "nonclassical" model system suitable for our studies. *O. dioica* was our first choice because it has many technical and biological advantages that made it attractive for our objectives: (1) *O. dioica* has a simple and accessible morphology, maintaining the typical chordate body plan throughout its life; (2) its generation time and life span is short with a fast, invariant, and determinative embryonic development; (3) *O. dioica* is easily accessible and affordably cultured; (4) *O. dioica* can be experimentally manipulated; (5) *O. dioica* occupies a privileged phylogenetic position within the chordates to better understand the evolution of our own phylum; and (6) its genome is small, compacted, and prone to lose genes. In the next sections, we will summarize these technical and biological advantages.

4.2.1 O. dioica *Is a Morphologically Simple Animal with Typical Chordate Body Plan*

O. dioica has a simple but typical chordate body plan with organs and structures that are unequivocally homologous to those in vertebrates and other chordates, including a notochord anchoring muscle cells along a postanal tail, a hollow neural tube that becomes a central nervous system (CNS) organized in two big ganglia and a nerve cord, as well as a pair of gill slits and an endostyle in the pharyngeal region, which connects with the digestive tract (Fig. 4.1). The body of *O. dioica* is divided in two parts, the trunk (0.12–0.85 mm), which contains most of the organs, and the tail (1.2–3.2 mm), which contains the notochord, the tail nerve cord, and two rows of

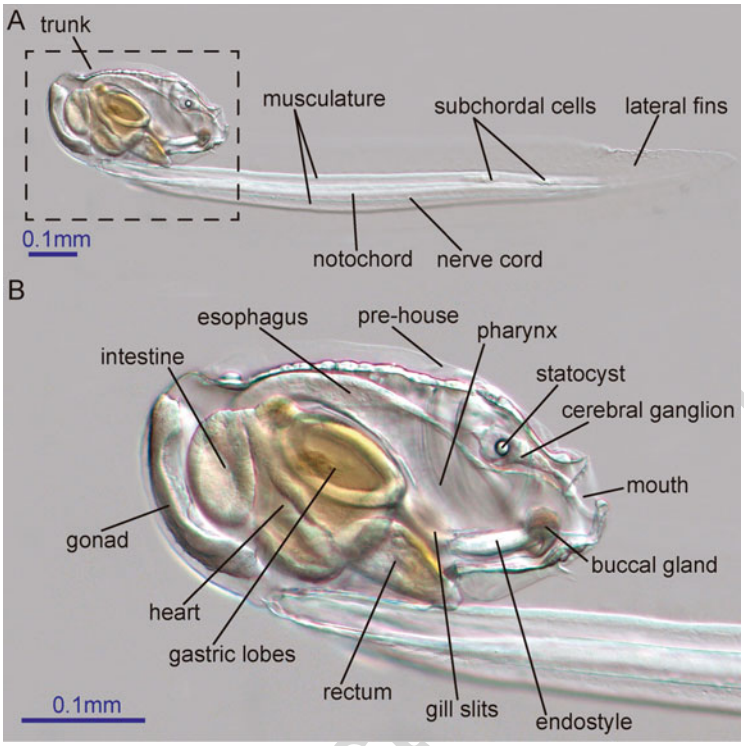

Fig. 4.1 Immature adult specimen of *O. dioica*. (**a**) Differential interference contrast (DIC) micrograph in right lateral view with anterior to the left and ventral down. (**b**) Magnification of the trunk showing the main organs and structures. Bar: 0.1 mm

paired muscle cells. The whole body is transparent so the inner structures can be visualized by simply changing the focal plane of the microscope.

4.2.1.1 Nervous System

The CNS of *O. dioica* is made by only approximately 100 cells. Despite this extremely simple structure, *O. dioica* CNS shows homology with the vertebrate forebrain, hindbrain, and spinal cord of vertebrates (but not the midbrain) (Cañestro et al. 2005). The CNS of *O. dioica* comprises a nerve cord running along the tail and two big ganglia (i.e., the cerebral ganglion in the most anterior part of the trunk and the caudal ganglion in the most proximal region of the tail) (Holmberg 1984). Both ganglia secrete gamma-aminobutyric acid (GABA) (Soviknes et al. 2005). The cerebral ganglion, proposed to be homologous to the vertebrate forebrain (Cañestro et al. 2005), is made of approximately 70 neurons and includes a statocyst, a balance sensory receptor in which a mineralized mass called statolith pushes a group of ciliary cells in response to gravity, providing therefore feedback to the animal on

change in orientation (Holmberg 1984) (Fig. 4.1). The cerebral ganglion connects with sensory cells in the mouth, the pharynx, and the ventral organ (a simple structure of about 30 cells homologous to the olfactory placode and the pituitary of vertebrates) (Bollner et al. 1986; Cañestro et al. 2005) and integrates the information received from the sensory cells. The cerebral ganglion also connects to the caudal ganglion through the trunk nerve cord, which turns to the right before entering the tail. Caudal ganglion and trunk nerve cord have been proposed to be homologous to at least part of vertebrate hindbrain (Cañestro et al. 2005). The caudal ganglion and the nerve cord that runs along the tail consist of approximately 30 neurons. Along the nerve cord, neuronal nuclei are collected in groups of two to four forming small ganglia. Small fibers arising from these ganglia innervate the symmetric musculature of the tail with cholinergic motoneurons and coordinate its movements (Galt 1972; Soviknes and Glover 2007).

4.2.1.2 Epidermis and Secreted "House"

The epidermis is a monolayered epithelium without any underlying mesodermal tissue that covers the whole body of *O. dioica* (Nishida 2008). The epidermis of the trunk, called oikoblast or oikoplastic epithelium, contains a fixed number of cells (approximately 2000) organized in different domains defined by the shape of the cells and the morphology of their nuclei and showing a complex bilateral symmetric pattern (Thompson et al. 2001; Kishi et al. 2017; Mikhaleva et al. 2018) (Fig. 4.2a). The oikoplastic epithelium secretes the so-called house, a filter-feeding device made of glycopolysaccharides, mucopolysaccharides, and cellulose (Fig. 4.2b, c) (Kimura et al. 2001; Spada et al. 2001; Thompson et al. 2001). The distribution of the epidermal cells of the trunk has a direct correlation with the different architectonic structures of the house (Fig. 4.2a) (Spada et al. 2001; Thompson et al. 2001; Kishi et al. 2017). The adult specimens of *O. dioica* live inside this mucous house (Fig. 4.2b, c) that works as a food-trapping device by filtering picoplankton particles of different sizes (from 0.1 to 50 μm) from the water stream propelled by stylish and grooving tail movements (Fenaux 1986; Thompson et al. 2001). The house is abandoned when the filters are obstructed, and a new one is immediately inflated, which happens between four and ten times a day increasing with higher water temperatures (Flood and Deibel 1998; Sato et al. 2003). This energetically exhausting strategy has been proposed as an evolutionary adaptation to poor food environments (Acuña et al. 2002).

4.2.1.3 Pharyngeal Region and Digestive Tract

The food particles caught by the house enter through the mouth situated at the anterior region of the trunk to the pharynx, thanks to the water flux generated by the spiracles, a pair of gill slits with beating cilia that generate a water stream into the body to draw the food (Fenaux 1998a) (Fig. 4.3). Within the pharynx, food particles

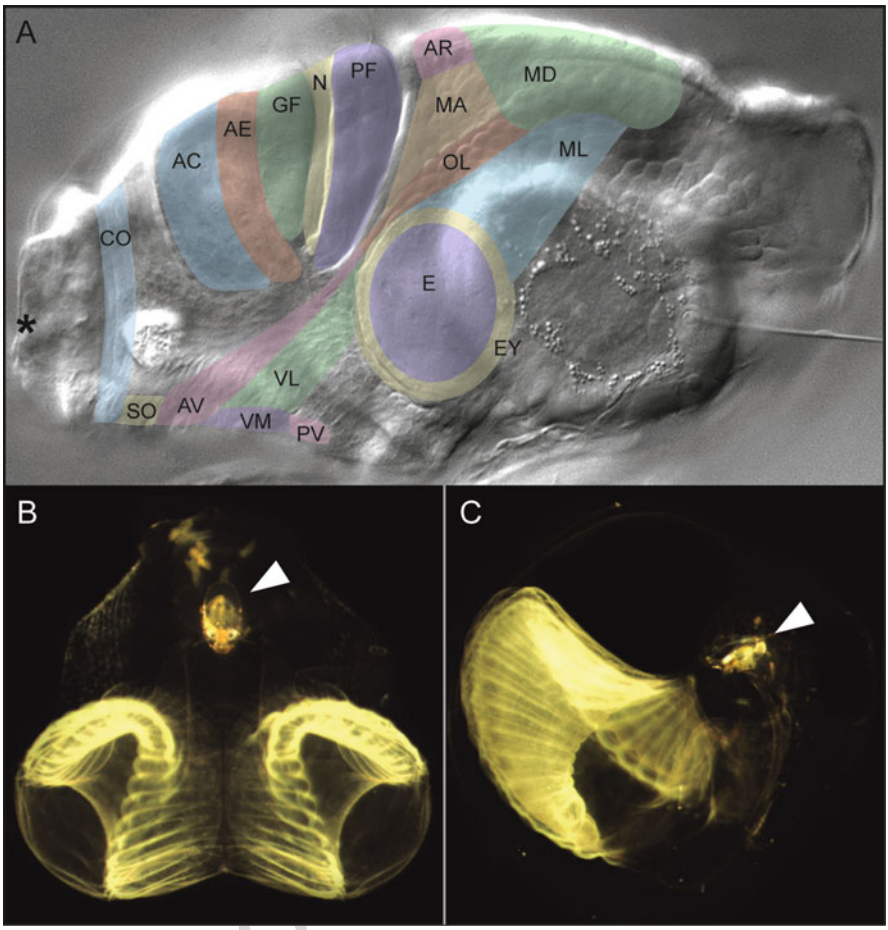

Fig. 4.2 The *O. dioica* epidermis and the secreted house. Lateral view of an *O. dioica* trunk, in which the main domains of the oikoplastic epithelium that correlate with the different architectonic structures of the house are depicted (**a**): *AC* anterior crescent, *AE* anterior elongated region, *AR* anterior rosette, *AV* anterior ventral domain, *CO* circumoral domain, *E* Eisen giant cells, *EY* Eisen's yard, *GF* giant Fol, *MA* martini, *ML* mid-lateral domain, *MD* middorsal domain, *N* nasse, *OL* oblique line, *PF* posterior fol, *PV* posterior ventral domain, *SO* sensory organ, *VL* ventrolateral domain, *VM* ventromedial domain. AC, AE, GF, N, and PF form the fol domain (Kishi et al. 2017). Anterior is to the left and ventral down. The asterisk indicates the mouth. Frontal (**b**) and lateral (**c**) views of the *O. dioica* house, which is visible thanks to the algae trapped in the structure. The trunk of the animal inside the house is also visible (arrowhead)

are trapped by the mucus that covers its walls. The mucus is secreted by the endostyle, an organ homologous to the thyroid of vertebrates, and localized on the ventral side of the pharynx (Olsson 1963; Cañestro et al. 2008). This mucus is conducted to the digestive tract by two symmetric rows of cilia at the lateral walls of the pharynx that finally meet at the superior region (Fenaux 1998a). The digestive

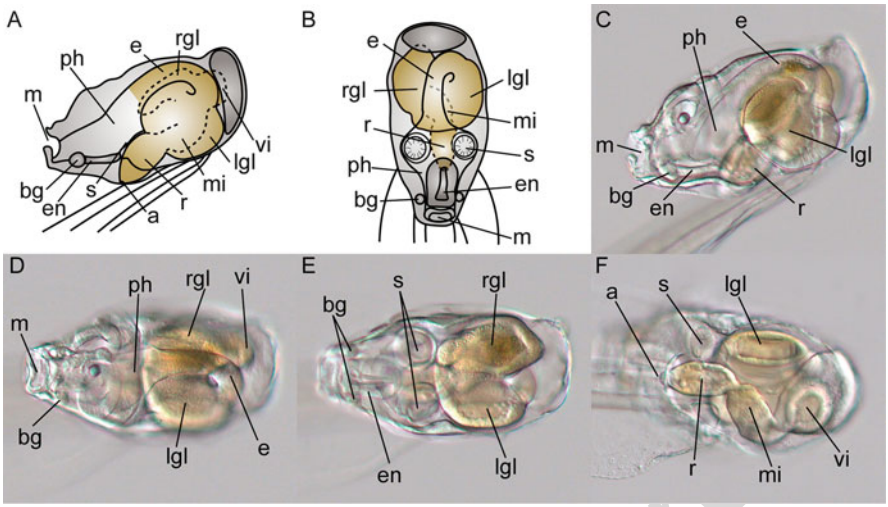

Fig. 4.3 Pharyngeal region and digestive system in the *O. dioica* trunk. Diagrams of the trunk and anatomy of the pharyngeal region and digestive tract of *O. dioica* from the left (**a**) and dorsal (**b**) views. DIC micrographs of the trunk of an *O. dioica* adult specimen in left lateral view (**c**), dorsal views at two different focal planes (**d**, **e**), and ventral view (**f**). Anterior is to the left. Anus (a), buccal gland (bg), endostyle (en), esophagus (e), left gastric lobe (lgl), mid-intestine (mi), mouth (m), pharynx (ph), rectum (r), right gastric lobe (rgl), spiracles (s), and vertical intestine (vi)

tract is composed of an esophagus, two gastric lobes (right and left stomachs), and a bent intestine divided in vertical, mid, and distal intestine (or rectum) (Burighel and Brena 2001; Cima et al. 2002) (Fig. 4.3). Although the absorption of liquids, ions, and small molecules takes place all over the digestive system (Burighel and Brena 2001), different compartments have different functions. Digestive enzymatic activity, for instance, is led mainly by the left gastric lobule where different enzymes such as α-amylase, acid phosphatase, nonspecific esterase, 5′-nucleodidase, and aminopeptidase M are released (Cima et al. 2002). In contrast, accumulation of lipid droplets in ciliated cells of the right gastric lobule and the vertical and mid-intestines indicates a storage role for these compartments (Cima et al. 2002). Finally, fecal pellets are formed in the vertical intestine and transit along the intestine allowing protein absorption by the rectal granular cells (Burighel and Brena 2001; Cima et al. 2002), until reaching the anus where they are released. Functional compartmentalization is further supported by spatiotemporal differences in the onset of gene expression during the development of the digestive system (Martí-Solans et al. 2016), likely reflecting processes of functional differentiation and specialization of digestive cells.

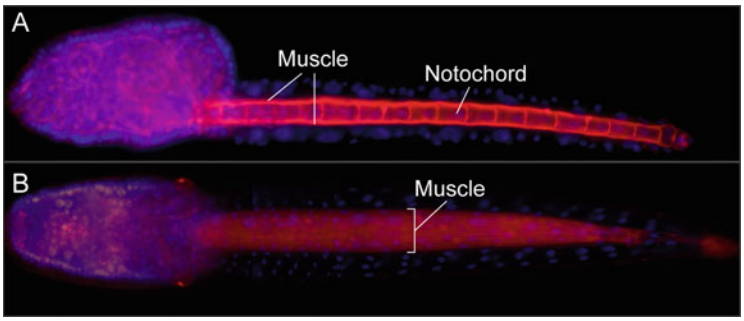

Fig. 4.4 Notochord and tail musculature in an *O. dioica* larva. These two tissues stand out over the general phalloidin staining of the actin filaments (in red), and they spread from the base of the trunk to the posterior tip. In lateral view, notochord is in the center of the tail flanked by two strips of striated muscle cells located on dorsal and ventral sides (**a**). In ventral view, sarcomeres are distinguished as repeated structures all along the strip of muscle cells (**b**). Nuclei are stained with Hoechst (in blue). Anterior is to the left

4.2.1.4 Notochord and Subchordal Cells

The notochord is a synapomorphic structure of the chordate phylum (Satoh et al. 2012) that has a dual function: providing structural support to the developing embryo and secreting patterning signals required for tissue specification and organogenesis (Cleaver and Krieg 2001). In *O. dioica*, the notochord is a row of cells or "stack of coins" that runs along the tail (Figs. 4.1 and 4.4). During larval development, notochord cells form vacuoles that eventually coalesce to form a hollow lumen (Soviknes and Glover 2008). In the adult, the notochord is a tube of cells with thin walls, whose cells secrete proteins rich in sulfur to the extracellular media to keep the stiffness of the notochord, but also flexible enough to facilitate swimming and other movements (Olsson 1965a). The genetic toolkit to build the notochord in *O. dioica* has been investigated, revealing not only similarities but also significant differences in the complement of genes employed by different chordates (Ferrier 2011; Kugler et al. 2011). The evolutionary origin of the notochord is a hot topic of discussion, and the analysis of the *O. dioica* notochord has contributed to corroborate that the urochordate noncontractile actin-expressing notochord may represent the ancestral condition in stem chordates (Almazán et al. 2018; Inoue and Satoh 2018).

At the right side of the notochord, there is a pair of conspicuous cells, the so-called subchordal cells (Fol 1872; Delsman 1910; Lohmann 1933) (Fig. 4.1). These cells migrate during *O. dioica* development from the trunk to the tip of the tail, at the same time that the endodermal strand cells enter the trunk following the same path but in opposite directions (Kishi et al. 2014). Subchordal cells together with endodermal strand cells and oral gland cells are three cell populations exhibiting long-distance migration during *O. dioica* development (Kishi et al. 2014), two of them—the subchordal and the endodermal strand cells—likely sharing the same origin (Fredriksson and Olsson 1991). The function of the subchordal cells remains elusive, although it has been proposed that they may be involved in the exchange of

materials with the body fluid, from which they pick and transform substances in a hepatic-like process (Fredriksson and Olsson 1991). These substances can either be changed into low molecular weight products merely for detoxification of harmful substances or be metabolized into new, useful molecules that are released back to the hemolymph (Fredriksson and Olsson 1991; Fenaux 1998a).

4.2.1.5 Heart and Tail Musculature

In *O. dioica*, muscle cells are only found in the heart and in the tail. They are in both cases striated muscle cells showing the characteristic repeating functional units called sarcomeres (Onuma et al. 2017; Almazán et al. 2018) (Fig. 4.4b), while the existence of smooth muscle cells in *O. dioica* has not been established so far. *O. dioica* heart is the simplest chordate heart, and assisted by the tail movement, it contributes to the circulation of the hemolymph between the ectoderm and the internal organs in an open circulatory system (Fenaux 1998a). The heart consists of a posterior ventral bag wedged between the left stomach wall and the intestine (Fig. 4.1). The internal lumen of this bag is the only coelomic space in *O. dioica*, and it is completely lined by two types of flat mesodermal tissues, the myocardium composed by muscle fibers and the nonmuscular pericardium (Stach 2009; Onuma et al. 2017). Myocardial cells are connected by cell junctions, and their cytoplasm is full of actin filaments (Stach 2009; Onuma et al. 2017; Almazán et al. 2018). This muscle-structured tissue produces peristaltic contractions that periodically reverse, causing the hemolymph to course between the myocardium and the stomach wall reaching the rest of the organs in the trunk and circulating through the tail (Fenaux 1998a).

The muscular tissue of the tail spreads posteriorly from the base of the trunk in two strips of striated cells distributed in the dorsal and ventral sides of the notochord (Nishino et al. 2000; Nishino and Satoh 2001; Almazán et al. 2018) (Fig. 4.4). This paraxial musculature, combined with the notochord, provides the ability to drive movement to the tail, which is fundamental not only for locomotion but also for *O. dioica* feeding. The tail musculature of *O. dioica* consists of only ten multinucleated striated cells on the dorsal and ventral sides of the notochord (Nishino et al. 2000; Nishino and Satoh 2001; Soviknes et al. 2007; Almazán et al. 2018). In just hatched larvae, eight mononucleated muscular cells are easily recognizable along the anterior–posterior axes of the tail. Along the larval development, two additional small muscle cells appear at the tip of the tail although their origin remains unknown (Nishino and Satoh 2001).

Structurally, the actomyosin contractile complexes of the muscle cells in *O. dioica* have not been fully characterized, but actin and myosin genes can be identified in the *O. dioica* database (Almazán et al. 2018). *O. dioica* has four muscular-actin genes that appear to be expressed only in the muscle cell lineage (Almazán et al. 2018). The four muscular actins show differences in their expression domains during embryonic development, which suggests differences in their spatio-temporal regulation (Almazán et al. 2018). Muscular actin expression can be

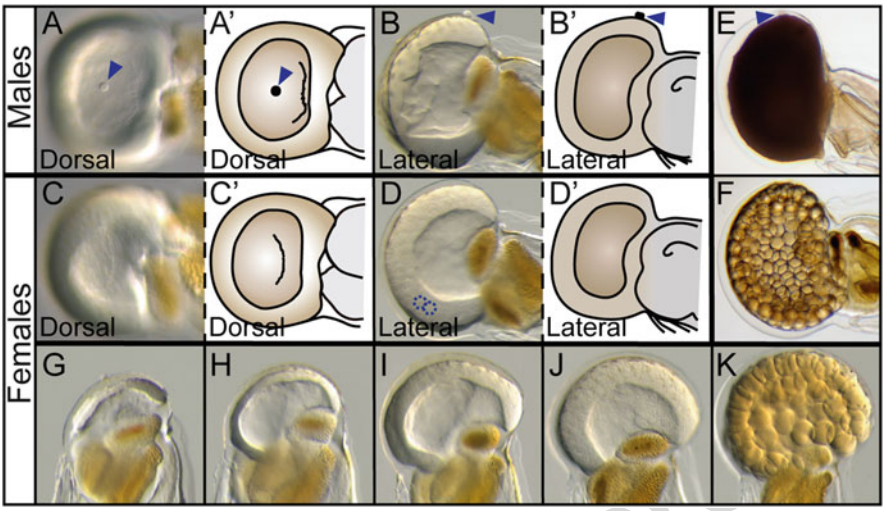

Fig. 4.5 Maturation of male and female gonads. DIC micrographs and schematic representations of dorsal (**a–a'**) and lateral (**b–b'**) views of a premature male gonad. The sperm duct appears as a small protuberance in the most dorsal part of the male gonad (arrowhead). DIC micrographs and schematic representations of dorsal (**c–c'**) and lateral (**d–d'**) views of a premature female ovary. Pro-oocytes can be distinguished inside the gonad (dashed circles). DIC micrographs of mature male (**e**) and female (**f**) gonads. Mature male gonads become brown and opaque as spermatogenesis progresses, while female gonads become yellowish and refringent until the round eggs are clearly visible (**g–k**)

detected as early as at 64-cell stage, although muscle fibers are formed later in the larvae. They are restricted to the inner part of the cell, while the epidermal side is full of mitochondria generating the energy for the tail movement (Nishino et al. 2000; Almazán et al. 2018). Comparative analyses among different chordate species, including *O. dioica*, have suggested that muscular actins originated from a cytoplasmic actin at the base of chordates, which was followed by the recruitment of the myosin motor-machinery that conferred contractile capability to muscle cells (Kusakabe et al. 1997; Almazán et al. 2018; Inoue and Satoh 2018).

4.2.1.6 Reproductive System

O. dioica is the sole dioecious species so far reported in the urochordate subphylum (Fol 1872; Nishida 2008). The gonad is localized in the posterior region of the trunk and is the only apparent character that allows us to differentiate males from females: male gonad is full of sperm and homogenously brown and dark, whereas the female one is yellowish and refringent as it is full of eggs (Fig. 4.5). The growth of the gonad is parallel to the growth of the animal, acquiring their final size and maturity in only some hours, when gametogenesis occurs (Nishino and Morisawa 1998; Ganot et al. 2007). Spermatogenesis takes place in the testicle, a syncytium with a

substantial number of identical nuclei that become individual spermatozoa (Martinucci et al. 2005; Onuma et al. 2017). The ovary has also an initial phase of syncytial nuclear proliferation. Then, it becomes a coenocyst where half of the nuclei enter meiosis, whereas the other half became highly polyploid nurse nuclei (Ganot et al. 2007). Inside the coenocyst, a ramified structure composed of filamentous actin surrounds each of the pro-oocytes that are connected to the common cytoplasm via intercellular bridges termed also ring canals (Ganot et al. 2007). During oogenesis a subset of pro-oocytes grows by transferring the cytoplasm through the ring canals while the others degenerate (Ganot et al. 2007). Typically, a mature female spawns between 100 and 400 eggs, although as a clutch manipulator, *O. dioica* females might increase or decrease the oocyte number depending on favorable or unfavorable environmental conditions, respectively (Troedsson et al. 2002). This feature can be useful to manipulate the clutch size in laboratory conditions.

4.2.2 O. dioica *Has a Short Generation Time and a Determinative Embryonic Development*

A convenient characteristic shared by many model organisms is that their generation time (i.e., the time between two consecutive generations) is short, facilitating genetic analyses through generations. The generation times of *D. melanogaster* and *C. elegans*, for instance, are of only 9 and 3 days, respectively. The generation time of *O. dioica* is also short, from 5 days at 19 °C to 10 days at 13 °C (Nishida 2008; Bouquet et al. 2009; Martí-Solans et al. 2015). After the external fertilization, the zygote starts embryonic development, which lasts 3.5–6.0 h at 19 °C and 13 °C, respectively, and terminates when a larva hatches breaking the chorion (Fig. 4.6). Next, larval development starts; it takes 6–13 h (at 19 °C–13 °C, respectively) and terminates with a 120° twist of the tail at the tailshift stage. Ten minutes later, the juvenile makes its first house and starts feeding. During the next few days, animals filtrate water and feed arriving to the 1.5–4.0 mm of body size for an adult animal. At that point, the gonads complete their maturation, and males and females spawn the gametes and, as semelparous animals, die afterwards (Fig. 4.6).

In vitro fertilization protocols (Fenaux 1976; Holland et al. 1988; Nishino and Morisawa 1998) have enabled the understanding of processes that lead the egg to become a larva. Cleavage patterns, cell lineages, and morphogenetic movements, which are especially important for the understanding of the embryology of organisms that follow an invariant and determinative embryonic development such as those of *O. dioica*, have been extensively studied (reviewed in Nishida and Stach 2014). Thus, fate maps from one cell to the tail bud stage have been established (Fig. 4.7), similarly to what has been made in other organisms as the nematode *C. elegans* or the ascidian *Ciona robusta* (formerly *C. intestinalis*) (Sulston et al. 1983; Nishida 1987; Fujii et al. 2008; Stach et al. 2008; Nishida and Stach 2014; Stach and Anselmi 2015). After fertilization, the surface of the egg becomes rough,

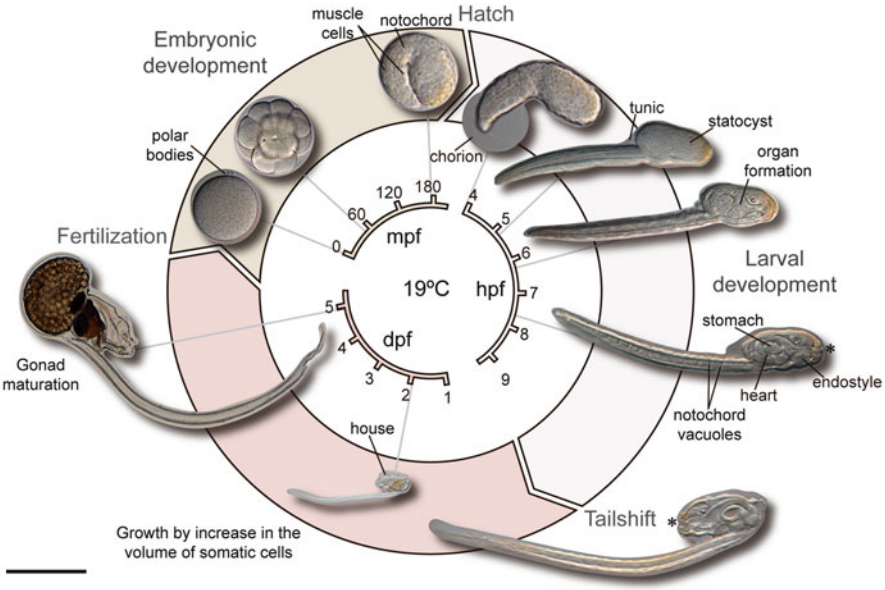

Fig. 4.6 Schematic representation of *O. dioica* life cycle. Embryonic development starts with the fertilization of the oocyte and ends when a larva hatches breaking the chorion (≈3.6 hpf at 19 °C). Larval development lasts for ≈6 h (at 19 °C) and ends when the tail of the larva changes 120° its orientation in a process called tailshift. Notice the change of the position of the mouth (asterisk) relative to the tail in juvenile animals. During the next ≈4.5 days (at 19 °C), juvenile animals feed, grow, and become mature males or females, which spawn the gametes closing the cycle. Scale bar represents 100 μm for all stages except for day 2 and mature adults (1 mm)

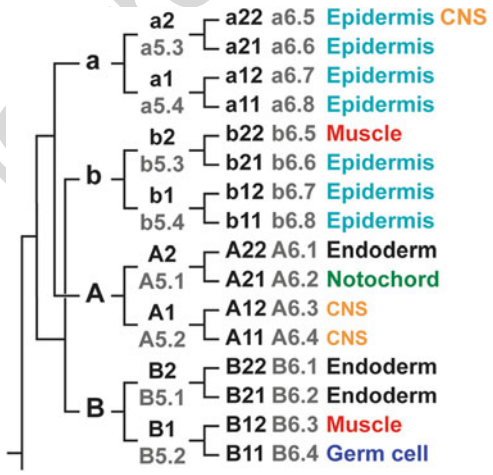

Fig. 4.7 Cell-lineage tree of *O. dioica* embryos. The tree is bilaterally symmetric, and therefore, only one-half is shown. The nomenclature agrees with that of Delsman (1910) and reviewed in Nishida (2008). The ascidian nomenclature system is shown in gray according to Stach et al. (2008) and Nishida and Stach (2014). Developmental fates of cells at 32-cell stage are indicated. CNS, central nervous system

and 9 min later, two polar bodies appear as an obvious signal of successful fertilization (Fig. 4.6) (Fenaux 1998a; Fujii et al. 2008). At 19 °C, the first division takes place after 23 min postfertilization (mpf), generating two morphologically equal cells that correspond to the right and left sides of the animal. At 32 mpf, another symmetric division takes place. This division is meridional and perpendicular to the previous one giving place to the anterior and posterior hemispheres. The third division (at 40 mpf) is asymmetric and leads to the formation of 8 cells, 4 big cells in the animal pole and 4 small cells in the vegetal pole. The morula stage appears 52 mpf, after the fourth division generates an embryo composed of 16 cells. Gastrulation starts 67 mpf, at 32-cell stage, and it consists in the ingression of the vegetal blastomeres that remain covered by the animal blastomeres (Nishida 2008). Neurulation starts at the 64-cell stage 80 mpf, when 8 cells from the anterior region are aligned in two rows of 4 cells that form a matrix that is internalized. Tail bud stage starts 135 mpf, when tail and trunk differentiate. During this stage, the tail elongates, and the embryo bents ventrally. Notochord cells align in a single row and become evident at 180 mpf. At 3.6 h postfertilization (hpf), a larva hatches, and embryogenesis terminates (Fig. 4.6) (Delsman 1910; Cañestro et al. 2005; Fujii et al. 2008; Nishida 2008; Stach et al. 2008).

Larval development is subdivided into six stages (I–VI) (Nishida 2008) that last 6 h at 19 °C, from the hatchling until the juvenile stage (Fig. 4.6). At stage I, the larva elongates and occasionally moves by tail beats. At stage II, the boundaries of the organs begin to appear, and at stage III, the organs are perceptible. At stage IV, organs are clearly recognizable, mouth opens, and the cilia of the digestive system and the ciliary rings of the gill slits start moving. Buccal glands locate at each side of the endostyle, and the heart starts beating. Notochord vacuoles fuse, and the tail flattens to form the lateral fins. At stage V, water current starts inside the larva, the lumen of all organs is continuous, and the trunk epidermis (oikoplastic epithelium) secretes house materials (pre-house). Swimming movements are vigorous. At stage VI, after a few seconds of intense movement, the tail changes its orientation 120° during a process called tailshift (Delsman 1910; Galt and Fenaux 1990; Fenaux 1998b; Nishida 2008). The tailshift event occurs when the larva reaches the juvenile stage, and it is thought comparable to metamorphosis in other urochordates (Fig. 4.6), although in *O. dioica* the definitive organs develop earlier than in ascidian species (Galt and Fenaux 1990).

After the tailshift, juvenile animals inflate the first house and start to feed. During the next days, animals filtrate water and feeds at the time that they grow and their gonads mature. From day 1 to day 3, the trunk grows from 0.17 to 0.35 mm (Bouquet et al. 2009), and the gonad expands in the posterior and ventral part of the trunk to fill the whole posterior region (Fig. 4.5). On day 4, the gonad increases its size becoming wider than the trunk and becomes visible to the unaided eye. At day 5, the trunk of *O. dioica* measures almost 1 mm and the whole animal between 3 and 4 mm (Bouquet et al. 2009). At that time, the gonad is fully developed, and males and females are easily distinguishable as the gonad is full of sperm or eggs, respectively (Figs. 4.5 and 4.6).

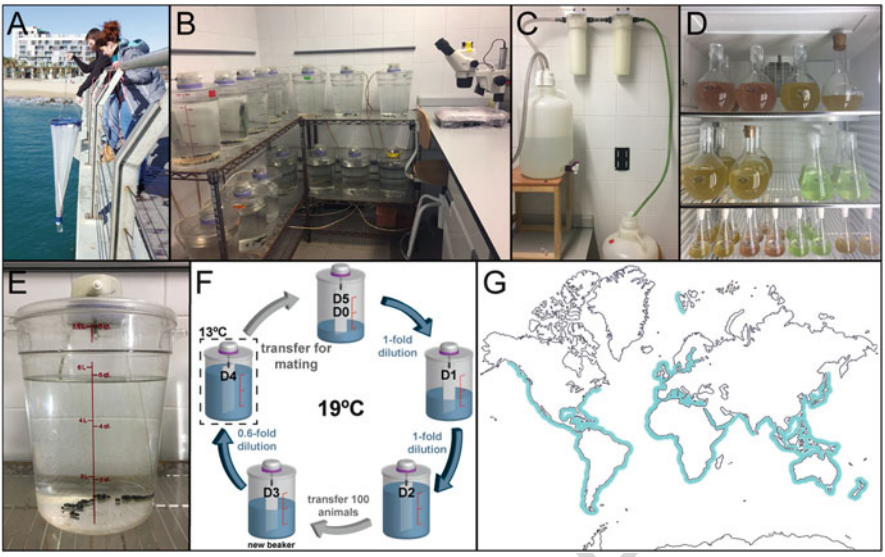

Fig. 4.8 *O. dioica* culture and geographic distribution. Animals might be collected in the coast using a plankton net or directly with a bucket (**a**) and maintained in the laboratory at 19 °C using a standard air conditioning device (**b**). Seawater is filtered through 10–0.5 μm polypropylene filters to remove major particles and organisms and sterilized using 0.2 μm filters (**c**). Three algal species and a cyanobacteria are individually cultured in a 13 °C incubator with a 12 h light/dark photoperiod and used to feed the animals (**d**). *O. dioica* animals are cultured at 19 °C in polycarbonate backers and maintained in suspension by the rotation (5–15 rpm) of a paddle driven by an electric motor mounted on the lid of the backer (**e**). A new animal culture starts by mating 20 females and 10 males close to spawn (D0) in a baker with 1.5 L of sterilized seawater (**f**, top). Next 2 days (D1 and D2), the culture is just diluted adding 1.5 and 3 L of sterilized seawater, respectively (**f**, right). At day 3 (D3), ≈100 animals are transferred to a new backer with 4 L of fresh sterilized seawater (**f**, left-down), and at day 4 (D4), cultures are diluted adding 2 L of sterilized seawater and moved to a 13 °C incubator (**f**, left-top). In the morning of day 5 (D5), most of the animals are mature and ready to set a new mating for new generation (**f**, top). For additional details, see Martí-Solans et al. (2015). Geographic distribution of the *O. dioica* species in the coast of most of the oceans (blue line) (reviewed in Fenaux et al. 1998, and supplemented with Bary 1960; Fenaux 1972; Costello and Stancyk 1983; Larson 1987; Gaughan and Potter 1994; Hwan Lee et al. 2001; Aravena and Palma 2002; Walkusz et al. 2003; Menéndez et al. 2011) (**g**) AU4

4.2.3 O. dioica *Is Easily Accessible, with a Wide Geographic Distribution and Suitable for Lab Culturing*

4.2.3.1 Geographic Distribution and Abundance

O. dioica is a semi-cosmopolite free-swimming species present in the Atlantic, Pacific, and Indian Oceans as well as in the Mediterranean and Red Seas, but not in extreme latitude oceans as Arctic or Antarctic (Fenaux et al. 1998) (Fig. 4.8). Although *O. dioica* is a widespread species, its populations may vary in a seasonal way (Essenberg 1922; Raduan et al. 1985; Uye and Ichino 1995; Fenaux et al. 1998;

Tomita et al. 2003). In the west Mediterranean Sea, for instance, its maximum abundance is found during spring and autumn seasons (Raduan et al. 1985). *O. dioica* along with other appendicularian species are so abundant that they occupy an important trophic position in food webs. Appendicularians are the second most abundant species, after copepods, in marine mesozooplankton (Gorsky and Fenaux 1998; Captanio et al. 2008), and they graze about 10% of the ocean's primary production (Acuña et al. 2002). Acting as an important short-circuit that allows a rapid energy transfer from colloidal carbon and phytoplankton primary producers to zooplanktivorous predators such as fish larvae (Flood and Deibel 1998; Fernández et al. 2004), they contribute to at least 8% of the vertical carbon transport to the deep ocean (Davoll and Youngbluth 1990; Robison et al. 2005; Troedsson et al. 2013). Appendicularians are, indeed, a major contributor of "marine snow" (i.e., biological debris that originates from the top layers of the ocean and drifts to the seafloor) in euphotic and mesopelagic zones through the production of discarded houses and fecal pellets (Robison et al. 2005). Those particles, full of nutrients, contribute to 28–39% of total particulate organic carbon export to the deep oceans (Alldredge 2005). The ecological relevance of appendicularians is so high that human activities affecting their populations [e.g., global warming or an increase of toxins in oceans (Bouquet et al. 2018; Torres-Aguila et al. 2018)] might impact on marine food webs and in vertical carbon flux at a world scale. Therefore, the *O. dioica* populations might be valuable sentinels for monitoring marine ecosystems. In summary, *O. dioica* is an ecological relevant organism, abundant in the neritic zone in almost all marine costs, and easily accessible using ordinary plankton nets (Fig. 4.8a, g).

4.2.3.2 *O. dioica* Lab Culturing

In addition to its accessibility directly from nature, *O. dioica* cultures can be maintained in the laboratory (Fig. 4.8b, f). *O. dioica* was first cultured by Paffenhöfer in 1973, but the bases for a long-term maintenance system were developed by Fenaux and Gorsky (1985). Nowadays, to our knowledge, the procedures to culture *O. dioica* in the laboratory all year around have been published from three facilities in the world located in Norway, Japan, and Spain (our facility) (Nishida 2008; Bouquet et al. 2009; Martí-Solans et al. 2015). We culture the animals at 19 °C in 8 L polycarbonate backers containing 6 L of seawater and 10 grams of activated charcoal (see legend of Fig. 4.8 for details). Animals are maintained in suspension by the rotation of a polyvinyl carbonate paddle connected to an electric motor (5–15 rpm). We add 2 or 3 pearls of 1-hexadecanol to reduce surface tension and therefore avoiding that animals get trapped by the surface of the water. For feeding, a cocktail of three different species of algae (*Chaetoceros calcitrans*, *Isochrysis* sp., *Rhinomonas reticulata*) and a cyanobacterium (*Synechococcus* sp.) is added to the animal cultures every day. Because growing *O. dioica* specimens can trap only foods of a proper size, the amount of each alga and of the cyanobacterium in the cocktail is adjusted according to their cell sizes along the *O. dioica* life cycle. Animal cultures are a source of mature male and female

specimens from which developmentally synchronic embryos can be obtained using several in vitro fertilization protocols (Clarke et al. 2007; Martí-Solans et al. 2015; Mikhaleva et al. 2015). Embryos and larvae at different developmental stages can be thereby easily collected, fixed, and stored for experiments on demand.

4.2.4 O. dioica *Can Be Experimentally Manipulated*

4.2.4.1 Pharmacological Treatments

Pharmacological treatments are a powerful tool to perform functional studies due to its experimental simplicity, and embryos are just transferred to a solution with the drug. The fact that we can easily obtain hundreds of synchronously developing *O. dioica* embryos by in vitro fertilization allows us to perform numerous treatments at different drug concentrations in a fast, simple, and reproducible way. The small size of the *O. dioica* embryos facilitates the diffusion of the drugs to the inner tissues, and their transparency allows the observation of the phenotypic effects of the treatments by DIC microscopy in live developing specimens without the need to perform histological sections. In addition, gene expression responses might be investigated by whole-mount in situ hybridization techniques or by high-density tiling arrays. Pharmacological treatments have been performed, for instance, with developmental morphogens such as all-trans-retinoic acid (RA), which yielded a range of morphological abnormalities, from mild to severe, depending on developmental stage, duration, and concentration of RA exposure (Cañestro and Postlethwait 2007). Treatments with xenobiotic compounds such as the carcinogenic polycyclic aromatic hydrocarbon BaP or with the lipid-lowering agent clofibrate have been also assayed to investigate the transcriptional regulation of the xenobiotic defense mechanisms and to identify the *O. dioica* chemical defensome (Yadetie et al. 2012). Finally, treatments with biotoxins such as *trans*,*trans*-2,4-decadienal, a model for polyunsaturated aldehydes produced during diatom blooms, have been used to analyze the impact on marine food webs of possible future intensification of algal blooms associated with climate change (Torres-Aguila et al. 2018).

4.2.4.2 Techniques for Altering Gene Function by Morpholino, dsRNA, or dsDNA Injections

Different knockdown approaches for altering gene function have been developed in *O. dioica* by injecting morpholinos (Sagane et al. 2010), double-stranded RNA (dsRNA; RNA interference-RNAi) (Omotezako et al. 2013; Mikhaleva et al. 2015), or double-stranded DNA (dsDNA; DNA interference-DNAi) (Omotezako et al. 2015, 2017), and new techniques for genome editing based on CRISPR-Cas9 are currently being developed (Deng et al. 2018). Most techniques rely on the injection of a given molecule into the gonad of premature females, when it is still

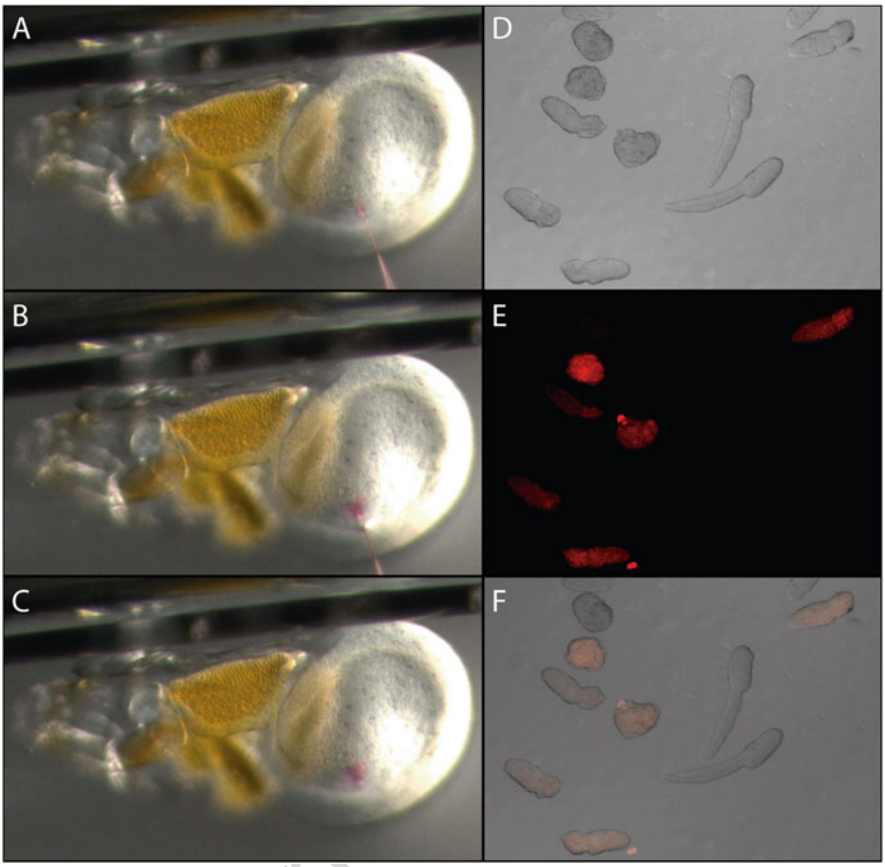

Fig. 4.9 Knocking down by dsDNA injection into the gonad of premature females. A dsDNA against the *brachyury* gene is injected into a premature female gonad (syncytium) (**a–c**), along with phenol red and *pSD64Flife-act-mCherry-mRNA*. Phenol red allows us to visualize the injected solution (red dot in panels **b** and **c**), while mCherry fluorescence highlights the embryos containing mRNA and, therefore, most likely also dsDNA after the cellularization process. In this regard, notice that malformed embryos showing the *brachyury*-dsDNA phenotype are fluorescent (compare panels **d–f)**

a syncytium of meiotic nuclei surrounded by common cytoplasm (Ganot et al. 2007). Then, the injected molecule might spread along the forming oocytes, generating tens of knockdown embryos with a single injection (Fig. 4.9). Alternatively, injections in spawned eggs are also possible (Mikhaleva et al. 2015, 2018; Deng et al. 2018), though they are more difficult and less productive.

Injection of morpholinos, synthetic molecules of approximately 25 nucleotides in length that bind to complementary sequences of RNA blocking translation or altering splicing, was the first knockdown approach in *O. dioica* (Sagane et al. 2010). Morpholinos were used to knock down a cellulose synthase gene necessary to build the house, obtaining different phenotypes related to the production of

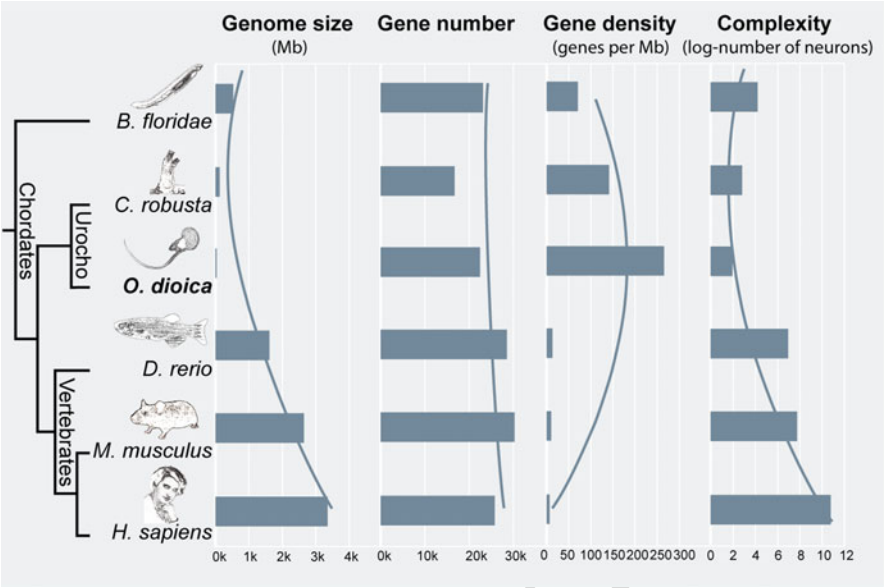

Fig. 4.10 Comparison of genome size, gene number, gene density, and biological complexity among selected chordate species arranged according to their phylogenetic relationships. Bar plots illustrate that *O. dioica* has the smallest chordate genome but has a number of genes rather similar to other chordate species, which implies that *O. dioica* gene density is the highest one. As a rough estimate of biological complexity, we plotted the number of neurons of each organism in logarithmic scale. Lines represent the overall tendency within each plot

cellulose fibrils (Sagane et al. 2010). In 2013, RNA interference by dsRNA injection was used to investigate the development of the tail and the function of the CNS in tail movement (Omotezako et al. 2013; Mikhaleva et al. 2015). Finally, in 2015, DNA interference by dsDNA injection, a gene silencing technique reported in plants, ciliates, and archaea, was successfully assayed in *O. dioica*, being the first case reported in any metazoan organism (Omotezako et al. 2015). Although the molecular bases for DNAi are unknown, *O. dioica* experiments suggest that dsDNA can induce sequence-specific transcription blocking and mRNA degradation.

4.2.5 O. dioica *Occupies a Privileged Phylogenetic Position Within the Chordate Phylum*

The urochordate (or tunicates), group to which the appendicularian *O. dioica* belongs, is the sister subphylum of the vertebrates, which together with basally branching cephalochordates form the chordate phylum (but see Satoh et al. (2014) for the chordate superphylum hypothesis) (Fig. 4.10). Urochordates are an extremely diverse taxonomic group that appears to have undergone a rapid evolution and

speciation, spreading over many marine habitats, from shallow waters to deep sea (Holland 2016). Urochordates are classically divided into three classes, Appendicularia (larvaceans, ≈70 species including *O. dioica*), Ascidiacea (sea squirts, ≈3000 species), and Thaliacea (salps, doliolids, and pyrosomes, ≈100 species), although it has been recently proposed that Thaliacea species are nested within the Ascidiacea class (Delsuc et al. 2018; Kocot et al. 2018). In any case, Appendicularians—also known as Larvaceans because they maintain the larval morphology, including the chordate synapomorphies, throughout the entirely life cycle—appear to be the sister clade of all other urochordates, representing a branch of the subphylum that split from all other urochordates 450 million years ago (Delsuc et al. 2018). Appendicularians are divided into three families. The Fritillariidae family that includes 3 genera and 30 species, the Kowalevskiidae family, the smallest one with just 1 genus and 2 species, and the Oikopleuridae family, the most diverse group with 11 genera and 37 species (Bone 1998).

O. dioica occupies a privileged phylogenetic position within urochordates for evo-devo studies because it can be morphologically, functionally, genetically, and genomically compared with many other urochordate species (more than 10 ascidians have been totally or partially sequenced; http://www.aniseed.cnrs.fr/; http://octopus.obs-vlfr.fr/public/botryllus/blastbotryllus.php), including well-studied ascidian species of the *Ciona* or *Halocynthia* genera (Corbo et al. 2001; Dehal et al. 2002; Nishida 2002; Cañestro et al. 2003; Satoh 2003; Satoh et al. 2003; Brozovic et al. 2016, 2017). In addition, from a more general perspective, comparisons of *O. dioica* and ascidians with diverse vertebrate species (there are more than 100 vertebrate genome projects) are relevant for detecting subphylum-specific traits, which might be classified as evolutionary innovations or losses depending on their presence in the external cephalochordate subphylum (there are three *Branchiostoma* genome projects and one *Asymmetron* RNA-Seq project).

4.2.6 O. dioica *Has Small and Compacted Genome*

With only 70 Mb, *O. dioica* genome is the smallest chordate genome and one of the smallest genomes in metazoans (Seo et al. 2001; Denoeud et al. 2010; Danks et al. 2013), only surpassed by the small genomes of some parasite animals (Burke et al. 2015; Chang et al. 2015; Mikhailov et al. 2016) (Table 4.1) (Fig. 4.10). The small size of the *O. dioica* genome is the result of a process of compaction mostly due to three factors: (1) a reduction of the length of intergenic regions, partly because of numerous operons; (2) a reduction of the abundance of transposable elements; and (3) the reduction of the size of introns (Denoeud et al. 2010; Berna and Alvarez-Valin 2014; reviewed in Chavali et al. 2011). As a result of the genome compaction, the gene density in *O. dioica* genome (1 gene per 3.9 kb) has become very high, 3 times higher than in ascidian *C. robusta*, 6 times higher than in cephalochordate amphioxus, and 35 times higher than in human genome (Table 4.1) (Fig. 4.10).

t1.1 **Table 4.1** Main features of *O. dioica* genes and genome (Volff et al. 2004; Denoeud et al. 2010; Chalopin et al. 2015) AU5

t1.2	*Genome*	
t1.3	Size	70 Mb
t1.4	Size of intergenic regions	53% of intergenic distances <1 kb
t1.5	Population mutation rate	$\theta = 4N_e\mu = 0.0220$
t1.6	Conserved synteny	Negligible
t1.7	Diversity of transposable elements	4 class I retroelements + 3 class II DNA transposons
t1.8	Abundance of transposable elements	10–20% genome (at most few hundreds of each TE)
t1.9	*Genes*	
t1.10	Number	18,020 predicted genes
t1.11	Density	1 gene per 3.9 kb (257 genes per Mb)
t1.12	Genes in operons	27% (1800 operons)
t1.13	Number of introns per Gene	4.1
t1.14	Intron size	<50 nt in 62% of the genes; >1 kb in 2.4% of the genes
t1.15	Intron position	76% intron positions specie-specific
t1.16	Noncanonical splicing sites	In 12% introns (9% GA-AG, 2.7% GC-AG, 0.7% GG-AG)

509 4.2.6.1 Intergenic Regions and Operons

510 *O. dioica* genes are densely packed, with 53% of their intergenic sequences smaller
511 than 1 kb (Table 4.1). Contributing to the intragenic reduction, many *O. dioica* genes
512 are organized in polycistronic transcription units (operons). The majority of operons
513 are bicistronic (60%), but a substantial proportion contains three or more genes, and
514 some have up to 11 genes. Operon organization reduces the intergenic space because
515 it reduces the DNA segments containing transcription initiation and regulation
516 signals. The fact that 27% of *O. dioica* genes are predicted to be organized in around
517 1800 operons (Denoeud et al. 2010), together with the observation that most genes
518 have relatively small intergenic spaces, may favor the genome compaction of this
519 species (Table 4.1).

520 4.2.6.2 Transposable Elements

521 Transposable elements (TEs or transposons) are main components of eukaryote
522 genomes that can replicate and change to new locations. *O. dioica* genome is
523 relatively poor in TEs, in terms of quantity and diversity, and most of the so-called
524 pan-animal transposon families, many of them even present in *C. robusta* (Cañestro
525 and Albalat 2012), are absent (Denoeud et al. 2010). *O. dioica* genome contains TEs
526 of only six superfamilies—*Ty3/Gypsy*, *DIRS1*, and *Penelope*-like retrotransposons
527 (Volff et al. 2004) and *Tc-Mariner*, *PyggyBac*, and *Maverik* DNA transposons
528 (Chalopin et al. 2015)—plus a new family of non-LTR retrotransposon named
529 *Odin* (Volff et al. 2004) (Table 4.1). Although the copy number of each TE is low

(at most few hundreds), LTR retrotransposons appear to be the most abundant (>60%), while non-LTR retrotransposons and DNA transposons represent less than 20% each. The extent of TEs on the *O. dioica* genome size has been estimated about 10–20% (Chalopin et al. 2015). In summary, a massive purge of pan-animal TEs has occurred in *O. dioica* genome probably associated with an intense process of genome compaction. Some TEs show a low level of sequence corruption, suggesting a rather recent activity, but their low copy number and uneven genome distribution indicate that TE expansion in *O. dioica* is under tight genetic control (Denoeud et al. 2010).

4.2.6.3 Introns

Another factor contributing to *O. dioica* genome compaction is the small size of the introns of the majority of the genes (Table 4.1). Despite *O. dioica* having a typical number of introns per gene (4.1) as in other vertebrates, it has reduced the size of the introns to less than 50 nucleotides in 62% of the genes, and only 2.4% of the introns being larger than 1 kb (Seo et al. 2001; Denoeud et al. 2010). *O. dioica* shows a high intron turnover: 76% of the introns are in positions unique to *O. dioica* (newly acquired introns) and only 17% are in ancestral positions (7% remain unclassified) (Denoeud et al. 2010) (Table 4.1). Several mechanisms have been proposed for the gain and loss of introns in *O. dioica* (Denoeud et al. 2010), but since newly acquired introns tend to be shorter than old introns, intron turnover might have favored genome compaction. Surprisingly, canonical splicing signal (GT-AG intron boundaries) is not observed in about 12% of introns (1% in other species), which mostly (9%) have noncanonical GA-AG (GC-AG, 2.7%, or GG-AG, 0.7%) sequences (Edvardsen et al. 2004; Denoeud et al. 2010). Because *O. dioica* lacks the minor spliceosome and only has one type of each spliceosomal component (Denoeud et al. 2010), a single major spliceosome, permissive in terms of splicing signals, would take care of all splicing processes. It has been proposed that such permissive splicing could favor the intron turnover (Denoeud et al. 2010) and, hence, the compaction of the *O. dioica* genome.

4.3 Evolution of *O. dioica* Genes

The *O. dioica* genome project did not only reveal that this species has an extremely compacted genome with small intergenic regions, tiny introns, and few TEs, but that its genes have suffered many evolutionary changes affecting the conservation of the sequence, organization, and number of genes.

4.3.1 Gene Sequence Evolution: High Evolutionary Rates

The sequencing of the genome confirmed that *O. dioica* is a very fast evolver and its genes always show surprisingly long branches in phylogenetic tree reconstructions (Edvardsen et al. 2005; Denoeud et al. 2010). This high rate of sequence evolution appears to be the result of two independent evolutionary forces acting at different levels and with different strength.

First, affecting at a global genome scale with a moderate strength, we found that most genes show a high evolutionary rate (Table 4.1) as a result of a combination of a high population mutation rate and a reduced negative (purifying) selection. An estimate of *O. dioica* population mutation rate ($\theta = 4N_e$, $\mu = 0.0220$) shows it is high, which is consistent with a large effective population size (N_e) and/or a high mutation rate per generation (μ) (Denoeud et al. 2010). In addition, the short generation time of this species implies that the effective mutation rate per year is substantially increased. A reduced negative selection for *O. dioica* has been proposed based on the homogeneity of its amino acid rates that is a symptom of relaxation of selective—structural and/or functional—constraints (Berna et al. 2012; Berna and Alvarez-Valin 2014). Relaxation of selective constraints associated with rapid rates of sequence evolution has been actually proposed for other fast-evolving animal species such as *Ciona robusta* and *Caenorhabditis elegans* (Holland and Gibson-Brown 2003).

Second, some particular genes have even a higher evolutionary rate due to positive selection that contributes to the adaptation to new environments or to new challenges (Berna and Alvarez-Valin 2014). Many of these genes with this higher evolutionary rate have been described to be involved in regulatory and developmental functions (Berna et al. 2012; Berna and Alvarez-Valin 2014).

4.3.2 Evolution of Gene and Genome Organization

4.3.2.1 Negligible Synteny Conservation

Conserved synteny describes the colocalization of homologous genes on homologous chromosomic regions of different species. *O. dioica* genome appears to have suffered numerous chromosomal rearrangements during evolution, and thereby, it lacks any chromosomal synteny conservation with other animal genomes (Denoeud et al. 2010). Conservation of local synteny is also almost negligible since local gene order is indistinguishable from random for distances smaller than 30 genes, and a low level of conserved synteny is only detectable at a wider distance span (Denoeud et al. 2010; Irimia et al. 2012). These observations suggest that constraints that maintain gene order in metazoans may actually be relaxed in *O. dioica.*

4.3.2.2 Disintegration of *Hox* Cluster

Another significant feature of *O. dioica* genome is the disintegration of the cluster of *Hox* genes, which is generally well conserved in all bilaterians. *Hox* genes are a subset of homeobox genes involved in establishing morphological identities along the anterior–posterior axis, and although the biological significance of *Hox* clustering remains unclear, functional and structural explanations leading to a spatiotemporal coordinated transcriptional regulation of *Hox* genes have been proposed (Kmita and Duboule 2003). In many species, the position of the *Hox* genes in the cluster correlates with their temporal and spatial sequential expression along the anterior–posterior axis (reviewed in Duboule 2007). *O. dioica* does not have a *Hox* cluster since its complement of nine *Hox* genes—three anterior *Hox* genes (*Hox1*, *Hox2*, and *Hox4*) and six posterior genes (*Hox9A*, *Hox9B*, *Hox10*, *Hox11*, *Hox12*, and *Hox13*)—is totally dispersed in its genome (Seo et al. 2004). The disorganization of the *Hox* cluster in *O. dioica* has been related to the lineage-driven mode of development of *O. dioica*, in which *Hox* genes would contribute to tissue specification with separated domains of *Hox* expressions, rather than to axial patterning with overlapping *Hox* expressions (Seo et al. 2004).

4.3.2.3 Evolution of Operon Organization

A third important genomic feature in the organization of *O. dioica* genes is their grouping in operons (Table 4.1). The operons do not only contribute to the compaction of the genome (see above) but might also serve to group genes that have to be either efficiently coregulated—repressing or activating them as a group—or ubiquitously expressed with a low degree of transcriptional regulation (Blumenthal 2004). *O. dioica* has around 1800 operons transcribed as polycistronic pre-mRNAs, most of them processed to mature monocistronic mRNAs via spliced-leader RNA (SL RNA) *trans* splicing (Ganot et al. 2004; Denoeud et al. 2010). Functional annotation of the gene set in operons shows that they are significantly enriched for genes involved in housekeeping functions or general metabolic processes such as RNA, protein, DNA, lipid, and carbohydrate processing and transport. Genes involved in developmental processes such as morphogenesis and organogenesis are, in contrast, significantly underrepresented in the operon gene set (Denoeud et al. 2010).

4.3.3 Gene Number Evolution

Despite its reduced size, *O. dioica* genome contains 18,020 predicted genes, a similar number to other urochordates (e.g., *C. robusta* ≈15,300 genes) and only slightly below other chordates such as the cephalochordate *Branchiostoma floridae*

(≈22,000 genes) or the vertebrate *Fugu rubripes* (≈18,300 genes) (Table 4.1) (Fig. 4.10). The current number of genes is the result of the balance between gene gains and losses impacting a certain set of ancestral genes, and because *O. dioica* appears to be prone to lose genes (see next section), it has to be also prone to gain them.

Gene duplications have been proposed to be a major driving force for gene gains (Ohno 1970; Cañestro et al. 2013), and *O. dioica* seems to have retained many lineage-specific duplicates. For instance, among homeobox genes, *O. dioica* shows a high incidence of retention of lineage-specific duplicates of genes such as *Irx*, *Not*, and *Pax3/7* (Edvardsen et al. 2005). Additional examples of such duplicates are found in g-type lysozyme genes (Nilsen et al. 2003), caspase genes (Weill et al. 2005), metallothionein genes (Calatayud et al. 2018), notochord *Noto15* and *Noto9* genes (Kugler et al. 2011), *RdhE2* and *Cco* genes (Martí-Solans et al. 2016), and actin as well as other muscle structural genes (Almazán et al. 2018; Inoue and Satoh 2018), among many others predicted from the genome project analysis (Denoeud et al. 2010). Lineage-specific duplicates might contribute to both general evolutionary adaptations of the organisms and innovations associated with the unique biology of the lineage. For example, homeobox genes expressed—and possibly patterning—in the oikoplastic epithelium, which represents a significant novelty of appendicularians for secreting the mucous house, mostly belong to the duplicated groups (Denoeud et al. 2010; Mikhaleva et al. 2018), while gene families with cell adhesion roles are overrepresented in *O. dioica*, most likely because of the extensive and assorted interactions required for building the house (Chavali et al. 2011).

4.4 Evolution by Gene Loss: *O. dioica* as a Model System for Evo-Devo Studies

Urochordate genomes (e.g., *C. robusta*) appear to have a "liberal" evolutionary pattern of gene loss (Dehal et al. 2002; Holland and Gibson-Brown 2003; Hughes and Friedman 2005; Somorjai et al. 2018), which contrasts with the "conservative" pattern of cephalochordates and vertebrates (Somorjai et al. 2018). *O. dioica* appears to have pushed this urochordate trend to its limits (Ferrier 2011) by having lost many genes or entire genetic pathways (Table 4.2). In this section, we review these losses grouped into three categories: Sect. 4.4.1, losses of genes involved in general characteristics of gene/genomic structure and expression, Sect. 4.4.2, losses of genes related to particular cellular and physiological functions, and Sect. 4.4.3, losses of genes essential for embryonic development in chordates.

Table 4.2 Absent genes[a] in *O. dioica* genome

Category	Genes	References
cNHEJ repair system	*Xrcc5* (*Ku80*), *Xrcc6* (*Ku70*), *Xrcc4*, *Lig4*, *NHEJ1* (*Xlf*), *DNA-PKc*, *Dclre1c*	Denoeud et al. (2010)
Epigenetic machinery	*Dnmt1*, *Dnmt3*, *Mbd4/MeCP2*, *Gcn5/PcaF*, *Hat1*, *Mll1*, *Ash1*, *Rnf1*, *Suz12*, *Pcgf*, *Scmh1*, *kdm2b*	Albalat et al. (2012), Navratilova et al. (2017)
Spliceosome machinery	snRNA U11, snRNA U12, snRNA U4atac, snRNA U6atac	Denoeud et al. (2010)
Caspase family	*Csp1/4/5*, *Csp6*, *Csp2/9*, *Csp8/10*	Weill et al. (2005)
Immune system	*NLRs*, *RLHs*, *MyD88-like*, *Sarm1-like*, *Tirap-like*, *Ticamp2-like*	Denoeud et al. (2010)
Defensome system	*AhR*, *AhRR*, *Nr1C* (*Ppar*), CYP1 genes	Yadetie et al. (2012)
Peroxins	*Pex1*, *Pex2*, *Pex3*, *Pex5*, *Pex6*, *Pex7*, *Pex10*, *Pex11*, *Pex12*, *Pex13*, *Pex14*, *Pex16*, *Pex19*, *Pex26*	Zarsky and Tachezy (2015)
Retinoic acid signaling	*Rdh10*, *Rdh16*, *Bco1*, *Aldh1a*, *Cyp26*, *RAR*, *PPAR*	Cañestro et al. (2006), Cañestro and Postlethwait (2007), Martí-Solans et al. (2016)
Homeobox genes	*Hox3*, *Hox5*, *Hox6*, *Hox7*, *Hox8*, *Gbx*, *Nk3*, *Nk6*, *TGIF*, *POU VI*, *Lhx6/7*, *Vax*, *Cux*, *SATB*, *ZFH1*, *Sax*, *Xlox*, *Mox*, *Hlx*, *Bsh*, *Chox10*, *Otp*, *Prx*, *goosecoid*, *Prox*, *Tlx*	Seo et al. (2004), Edvardsen et al. (2005)
Sox genes	*SoxC*, *SoxE*, *SoxF*, *SoxH*	Heenan et al. (2016)
miRNA	miR-9, miR-29, miR-33, miR-34, miR-96, miR-126, miR-133, miR-135, miR-153, miR-182, miR-183, miR-184, miR-196, miR-200, miR-216, miR-217, miR-218, miR-367	Wang et al. (2017)
Notochord genes	*Entactin*, *fibrinogen-like*, *multidom*, *myomegalin*, *Noto1*, Noto2, *Noto3*, *Noto4*, *Noto5*, *Noto6*, *Noto7*, *Noto8*, *Noto11*, *Noto12*, *Noto13*, *Noto14*, *Noto16*, *perlecan*, *Ptp*, *Slc*, *Swipi*, *tropomyosin-like*, *tune*, *Ube2*	Kugler et al. (2011)
General repair system	*Polb*, *Apex2*, *Lig3*, *Msh3*, *Atm*, *Chek2*, *Aptx*, *Nbn*, *Rad52*	Denoeud et al. (2010)
Apoptotic genes	*Bax*, *Bak*, *BCL-X*$_L$	Robinson et al. (2012)
Cyclins and CDK	*Cyclin J*, *cyclin G*, *cyclin F*, *Cdk14/15*	Campsteijn et al. (2012)
Rab GTPases	*Rab4*, *Rab7L1*, *Rab9*, *Rab15*, *Rab19/43*, *Rab20*, *Rab21*, *Rab22*, *Rab24*, *Rab26/37*, *Rab28*, *Rab30*, *Rab32LO*, *Rab40*, *Ift27*, *Rasef*, *EFcab44/Rab44*, *RabX1*, *RabX4*, *RabX6*	Coppola et al. (2019)

[a]Genes are not found in *O. dioica*, and, thereby, are likely lost during the evolution of the *O. dioica* lineage

4.4.1 *Loss of Genes Involved in Gene/Genomic Structure and Expression*

4.4.1.1 Loss of Non-homologous End Joining Repair Genes

All organisms have the ability to repair the double-strand breaks (DSBs) that normally happen in DNA due to numerous external and internal factors. One fundamental mechanism present in all eukaryotes to repair these breaks is the canonical non-homologous end joining (cNHEJ) repair system. Unexpectedly, *O. dioica* (Denoeud et al. 2010) and other six species of appendicularians (Deng et al. 2018; Ferrier and Sogabe 2018) do not have the cNHEJ machinery (Table 4.2), meaning this repair system became dispensable during the evolution of this chordate lineage. In fact, *O. dioica* seems to use the alternative NHEJ (aNHEJ) system (a.k.a. alternative end joining, aEJ, Pannunzio et al. 2018) as a compensatory system that overcomes DSBs, although the existence of another so far undescribed pathway cannot be excluded (Deng et al. 2018). Because the aNHEJ system seems to be less faithful than cNHEJ (it often involves deletion of some intervening nucleotides and commonly leads to chromosome rearrangements, for example, translocations, (Deriano and Roth 2013)), the loss of the cNHEJ system might have had important consequences in the genome architecture. It may have contributed, for instance, to the compaction of the *O. dioica* genome by either favoring the deletions caused by the aNHEJ repair system or by restricting replication of autonomous retrotransposons that uses the cNHEJ system for their propagation (Deng et al. 2018). In addition, this loss may have also contributed to the reorganization of the genome through accumulated rearrangements, leading to negligible synteny conservation (Deng et al. 2018) that may be associated with modifications of the mechanisms of gene regulation, changing from long-range mechanisms acting on topologically associated genomic blocks that include several genes to short-range and gene-specific systems for gene regulation (Cañestro et al. 2007; Ferrier and Sogabe 2018).

AU8

4.4.1.2 Loss of Genes of the Epigenetic Machinery

Histone modifications and DNA methylation are epigenetic marks mainly associated with regulation of gene expression, replication, DNA repair, recombination, chromosome segregation, and other meiotic and mitotic processes. Histone proteins, responsible of packing DNA into nucleosomes, provide multiple sites for posttranslational modifications by evolutionarily conserved histone modifiers that establish the so-called histone code. Among the proteins that modify histones, *O. dioica* has lost several genes for histone acetyltransferases such as *Gcn5/Pcaf* and *Hat1* (Navratilova et al. 2017) (Table 4.2). In addition, homologs of core components of the canonical Polycomb complexes (*Rnf1*, *Suz12*, *Pcgf*, and *Scmh1*) and genes for several proteins of the trithorax group (*Mll1*, *Ash1*, and *Kdm2b*) have neither been

found in *O. dioica*'s genome (Navratilova et al. 2017). Regarding DNA methylation, *O. dioica* has lost the two main DNA methyltransferases *Dnmt1* and *Dnmt3*, and one of the two *Mbd* genes, *Mbd4/MeCP2* (Cañestro et al. 2007; Albalat et al. 2012) (Table 4.2). Overall, it is through that the loss of components of the epigenetic machinery might has favored changes in the genome architecture and modifications of the mechanisms of gene regulation, leading to compacted regulatory spaces, reduced chromatin state domain widths, evolution of operons, and loss of synteny and dispersion of the Hox cluster (Cañestro et al. 2007; Navratilova et al. 2017). AU9

4.4.1.3 Loss of Minor Spliceosome Genes

While the major spliceosome containing the snRNAs U1, U2, U4, U5, and U6 removes introns with canonical GT-AG boundaries, the minor spliceosome, which contains the snRNAs U11, U12, U4atac, U5, and U6atac, mainly acts on noncanonical AT-AC boundaries (Patel and Steitz 2003; Sheth et al. 2006). Both major and minor spliceosomes are present across most eukaryotic lineages tracing them back to the origin of eukaryote evolution. Although *O. dioica* has many noncanonical introns (see above), no AT-AC introns have been detected in cDNA resources (Denoeud et al. 2010), which may explain that none of the minor splicesomal snRNAs are found in *O. dioica* (Table 4.2). It seems therefore that in the absence of AT-AC introns, the components of the minor spliceosome became dispensable and lost during *O. dioica* evolution and at the same time that the major spliceosome became permissive and able to remove all—canonical and noncanonical—*O. dioica* introns, although with different efficiency (Denoeud et al. 2010). Whether these differences in splicing efficiency could reflect specific usage of noncanonical introns for gene expression regulation and what evolutionary impact these changes had needs further investigation.

4.4.2 Loss of Genes for Cellular and Physiological Functions

4.4.2.1 Loss of Genes of the Caspase Family

Caspases are a family of protease enzymes that play an important role not only in apoptosis but also in maturation of different immunity system proteins and in the control of the proliferation and differentiation of specific cell types (Weill et al. 2005). Caspases are present in all kingdoms (Uren et al. 2000), but their number has a taxon-dependent diversity ranging from 3 members in *C. elegans* to 10–13 in vertebrates and 17 in *C. robusta* (Weill et al. 2005). *O. dioica* has only three caspase genes deriving from a single founder distantly related to *Caspase 3/7* (Weill et al. 2005) (Table 4.2). *O. dioica* seems, therefore, to have lost all of the components of the caspase family except one. The reduced complexity of the caspase family might be associated with a low cell number at the adult stage, the absence of a major

metamorphosis event, and a minimized immune system in this species (Weill et al. 2005).

4.4.2.2 Loss of Genes of the Immune System

As a marine organism, *O. dioica* should have an innate immune system prepared to fight against viruses and bacteria present in high amounts in seawater, but its short life span along with the absence of hemolymph cells—hemocytes or macrophages—points to a rapid immune system based on transcriptional upregulation response or on constitutively expressed effectors, rather than to a more slow system reliant on cell proliferation triggered by immune receptors (Denoeud et al. 2010). In agreement, *O. dioica* has lost almost all genes with domains corresponding to typical immune receptors or immune effectors and lacks homologous genes to interleukins or cytokines involved in the immunity response of more complex chordates (Denoeud et al. 2010) (Table 4.2). Interestingly, it has been proposed that a simplified immune system may be compensated by the antibacterial function of some oikosins with phospholipase A2 domains, which hydrolyze glycerophospholipids present in bacterial cell walls (Hosp et al. 2012). In summary, *O. dioica* seems to have a highly derived and simplified strategy of defense that together with its high fertility, short life cycle, and high polymorphism contributes to the survival of the *O. dioica* populations (Denoeud et al. 2010).

4.4.2.3 Loss of *Cyp* Genes for Xenobiotic Defensome Systems

The first step for elimination or inactivation of xenobiotic compounds often involves the oxidative modification of the toxic chemicals, mostly performed by enzymes of the cytochrome P450 (CYP) family, some of which are also required for embryonic development (Goldstone et al. 2006). Due to its variable functions, Cyp enzymes are quite abundant in animal genomes—up to 120 *Cyp* genes in sea urchin (Goldstone et al. 2006), 94 in zebrafish (Goldstone et al. 2010), or 236 in amphioxus (Nelson et al. 2013). *O. dioica*, however, has lost many *Cyp* genes, and with only 23 *Cyp* genes, this species has the smallest *Cyp* repertoire among sequenced metazoan genomes (Yadetie et al. 2012). *O. dioica* lacks, for instance, the CYP1 family genes, which play a central role in the metabolism of environmental toxicants (Table 4.2). *O. dioica* also lacks the aryl hydrocarbon receptor (*AhR*) gene (and its repressor *AhRR*) (Yadetie et al. 2012), which is the transcriptional regulator of CYP1 family genes and a major xenobiotic sensing receptor activated by pollutants. Overall, these data suggest the absence of an AhR-mediated xenobiotic biotransformation signaling pathway in *O. dioica* and suggest the evolution of alternative mechanisms of response to environmental xenobiotic compounds (Yadetie et al. 2012).

4.4.2.4 Loss of Peroxin Genes and Absence of Peroxisomes

Peroxisomes are single membrane-bound organelles with important functions in detoxification of reactive oxygen species, long-chain fatty acid beta-oxidation, plasmalogen synthesis, amino acid degradation, and purine metabolism. Peroxisome biosynthesis and protein import is mediated by a group of 13 highly conserved eukaryotic proteins called peroxins (Gabaldon et al. 2006; Schluter et al. 2006; Zarsky and Tachezy 2015). Peroxisomes are ubiquitous in eukaryotes, and only some groups of anaerobic protist and parasitic helminths lack peroxisomes (Schluter et al. 2006; Gabaldon and Capella-Gutierrez 2010). It is therefore extraordinary that *O. dioica* has lost all peroxin genes (Table 4.2), becoming the only known aerobic non-parasitic organism that does not have peroxisomes (Zarsky and Tachezy 2015). The evolutionary and physiological conditions in which *O. dioica* was able to lose the peroxisomes are still a matter of debate as this organism has high oxygen consumption and oxidizes fatty acids for ATP synthesis, unlike the anaerobic parasitic lineages missing these organelles (Zarsky and Tachezy 2015). It has been proposed that the loss of peroxisomes might be evolutionary adaptive, since it has been associated with reduced genomes and traits of r-reproductive strategies such as high fecundity, early maturation, and simplified ontogenesis, which *O. dioica* shares with parasite organisms as well as with some selective advantages by rendering organisms resistant to xenobiotics that become activated in the peroxisomal lumen by redox reactions (Zarsky and Tachezy 2015).

4.4.3 Loss of Genes Essential for Embryonic Development in Chordates

4.4.3.1 Loss of the RA Genetic Machinery

All-*trans*-RA is a vitamin A-derived compound that acts as a crucial signaling system involved in the differentiation and outgrowth of neurons in many metazoans (reviewed in Albalat 2009), which was adopted for Hox-controlled anterior–posterior patterning in the chordate phylum (Handberg-Thorsager et al. 2018). Despite the crucial role of RA in chordate axial patterning, it has been shown that *O. dioica* has lost most of the genes for RA production, degradation, and signaling (i.e., the RA genetic machinery, including *Rdh10*, *Aldh1a*, *Cyp26*, and *RAR* genes) (Cañestro et al. 2006, 2007; Cañestro and Postlethwait 2007; Martí-Solans et al. 2016) (Table 4.2) (Fig. 4.11). In the absence of mutational robustness (i.e., alternative pathways) capable of compensating them, these gene losses were accompanied by the loss of the RA signaling in this species (Martí-Solans et al. 2016).

The loss of the RA genetic machinery in *O. dioica* probably took place in the context of regressive evolution associated with the disintegration of the Hox cluster that, as mentioned in Sect. 4.3.2, has been related with the shift to a determinative mode of development. This shift would have released the restrictions to maintain the

Fig. 4.11 The loss of the retinoic acid genetic machinery in *O. dioica* exemplifies how functionally linked genes are co-eliminated during evolution. In the absence of mutational robustness capable of compensating the loss, the co-elimination was accompanied by the loss of the RA signaling. *O. dioica* has lost genes for the Rdh10, Rdh16, Bco1, Aldh1a, and Cyp26 enzymes, as well as genes for the RAR and PPAR nuclear receptors (strikethrough in lighter boxes). The surviving genes (i.e., *Adh1/4*, *RdhE2*, *Aldh8a1*, and *Cco* in dark boxes) (Cañestro et al. 2010; Martí-Solans et al. 2016) do not constitute an alternative pathway for RA synthesis because neither RA nor RA precursors have been detected at concentrations that are likely to play any role in developmental or physiological processes of this species (strikethrough)

integrity of the Hox cluster (Seo et al. 2004) and led extracellular signals such as RA that establish embryonic coordinates and regulate the expression of *Hox* genes by gradually increasing the portion of the cluster exposed to transcription machinery over time to become dispensable and eventually lost (Cañestro and Postlethwait 2007; Cañestro et al. 2007).

4.4.3.2 Loss of Homeobox Gene Families

Homeobox genes encode transcription factors involved in many developmental processes in eukaryotes. Homeobox genes are classified into 11 classes and over 100 gene families/groups (Holland et al. 2007), some of them vertebrate innovations. *O. dioica* has lost 36 homeobox groups, 12 of which probably lost during the early urochordate evolution and 14 specifically lost in the *O. dioica* lineage (Edvardsen et al. 2005) (Table 4.2). Noteworthy, among the homeobox families, *O. dioica* has lost the anterior *Hox3* and all the central *Hox* genes of the cluster (Seo et al. 2004). In parallel with the losses, homeobox genes have also duplicated during the evolution of *O. dioica* lineage (Edvardsen et al. 2005). Interpreting these gains and losses in terms of the evolution of developmental mechanisms remains difficult until the function of *O. dioica* homeobox genes is revealed. It has been proposed that family losses may be the result of a relaxed selective pressure to conserve the full set of homeobox families in the *O. dioica* (or in the urochordate) lineage due to a simplification of its body plan in comparison with that of the chordate ancestor (Edvardsen et al. 2005). On the other hand, the preservation of duplicates in some

families might be the result of a re-diversification of homeobox genes after major group losses (Edvardsen et al. 2005).

4.4.3.3 Loss of *Sox* Genes

Sox (Sry-type HMG box) genes are a family of transcription factors defined by a conserved sequence called the high-mobility group (HMG) box. *Sox* genes are involved in a number of essential functions during embryonic development, including sex determination and neuronal development (Wegner 2010). Ten different Sox groups have been described (A to J), some of them (B, C, D, E, F) conserved in almost all metazoans, and some groups being specific of certain lineages (A, G, and I of vertebrates, H of chordates, and J of nematodes) (Bowles et al. 2000; Heenan et al. 2016). *O. dioica*, however, has only four *Sox* genes, two of them belonging to the *SoxD* group and the other two to the *SoxB* group (Heenan et al. 2016; Torres-Aguila et al. 2018). These results imply that *O. dioica* has lost three ancient metazoan Sox groups (C, E, F) and the chordate Sox group (H) (Heenan et al. 2016) (Table 4.2). As in other examples reviewed above, the evolutionary impact of the gains and losses in the Sox family on the developmental mechanisms remains difficult to interpret since the expression of only two SoxB genes has been described (Torres-Aguila et al. 2018).

4.4.3.4 Loss of miRNAs

MicroRNAs (miRNAs) are noncoding RNAs of about 22 nucleotides involved in the regulation of different biological processes, including embryo development, cell differentiation, and growth. miRNA innovation has been correlated with increased developmental complexity during animal evolution (Hertel et al. 2006). Analysis of the miRNA repertoire of *O. dioica* has shown that this species has lost (or derived to the point they cannot be recognized anymore) at least eighteen highly conserved bilaterian miRNA families (Fu et al. 2008; Wang et al. 2017) (Table 4.2). On the other hand, at least 29 new miRNA families would have appeared in appendicularians (Fu et al. 2008), suggesting a profound reorganization of the miRNA repertoire due to recurrent events of gene losses and gains during *O. dioica* evolution. This scenario is consistent with the general notion that animal miRNAs are poorly conserved between distant taxa (Wang et al. 2017) and, therefore, that changes in miRNA repertoires have been important in shaping animal evolution (Fu et al. 2008). It is thought that the reorganization of *O. dioica* miRNA repertoire might have impacted on the temporal robustness of the rapid developmental program of this urochordate lineage (Fu et al. 2008), which would be in agreement with the idea that modifications in the miRNA repertoires have been important in adapting radically different life-history strategies from a common larval body plan (Fu et al. 2008).

4.4.3.5 Loss of Notochord Genes

The notochord, one of defining features of the chordate phylum, is a stiff rod of tissue located ventral to the neural tube that provides a rigid, but still bendy, structure for muscle attachment, as well as an important source of embryonic developmental signals (Stemple 2005). Actually, developing chordate embryos require a notochord as an organizer for secreting signals that pattern several organs such as the somites, heart, or pancreas. Comparison of a set of notochord genes known to be targets of the notochord-specific Brachyury transcription factor revealed that from 50 notochord genes of *C. robusta* used as a reference, 24 are absent in *O. dioica* (likely lost, e.g., *Noto2*), and 15 are not expressed (8 genes, e.g., *Asak*) or expressed at low levels (7 genes, e.g., *Noto10*) in the notochord (Kugler et al. 2011) (Table 4.2). Taken together, these results suggest a considerable divergence in the genetic toolkit for notochord development in *O. dioica*, which it would have been impacted by events of gene loss or by the loss of the notochord function of some genes. Considering the morphological similarities between urochordate notochords, the divergence in the genetic toolkits used to develop them is surprising.

4.4.4 *O. dioica, Gene Loss, and the Inverse Paradox*

The original paradox in the field of evo-devo arose by the discovery that similar genetic toolkits were able to build a wide variety of morphologies in disparate animals, implying that the same genes (genetic unity) were used to build such different forms (phenotypic diversity) (Jacob 1977). The discovery of the pervasiveness of gene loss along evolution (Albalat and Cañestro 2016) affecting relevant developmental genes led to the formulation of the so-called inverse paradox of evo-devo. This hypothesis proposes that organisms might develop fundamentally similar morphologies (phenotypic unity) despite important differences in their genetic toolkits (genetic diversity) (Cañestro et al. 2007) (Fig. 4.12). Because differences between the genetic toolkits are often a consequence of the loss of some of their crucial genes, the study of *O. dioica* has become fundamental to build the new conceptual framework as this species has been able to maintain a phylotypic chordate-body plan after having lost many genes thought to be crucial for the archetypal chordate development (see Sect. 4.4.3). In this regard, the characterization of the *O. dioica* "lossosome" (the complete catalogue of gene losses in a phylogenetic context; Cañestro and Roncalli 2018) and the analysis of its functional consequences should provide the framework to investigate how gene loss might have been an important evolutionary force for generating differences in the developmental genetic toolkits of chordates and, thereby, to understand the evolution of the mechanisms of development of our own phylum.

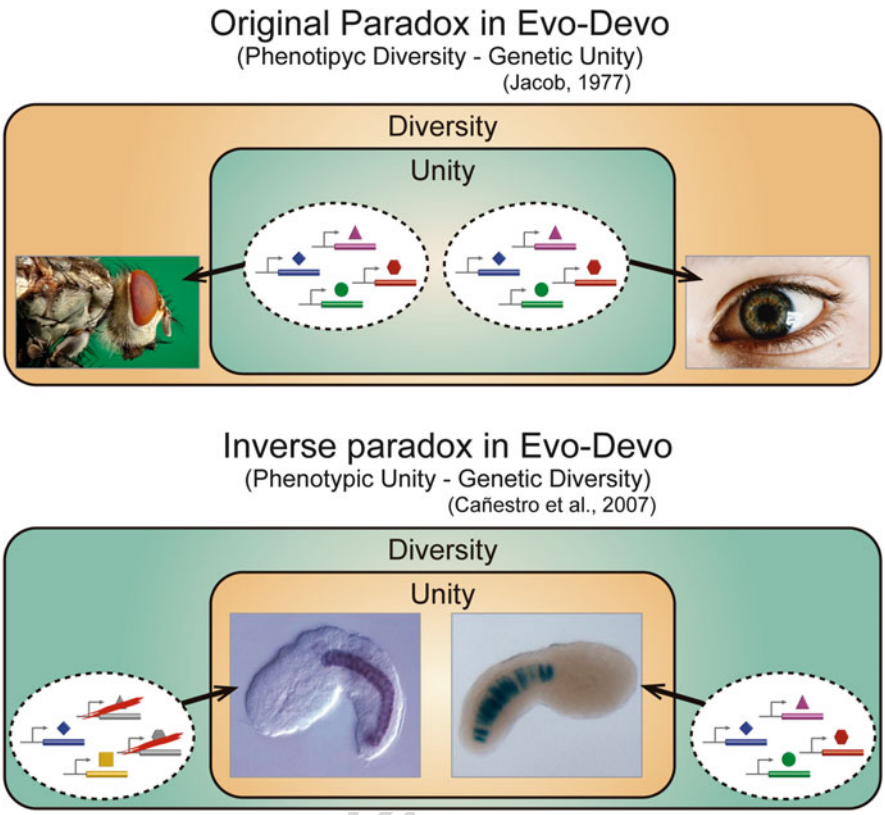

Fig. 4.12 In contrast to the original paradox, the inverse paradox in evo-devo proposes that organisms might develop fundamentally similar morphologies (phenotypic unity) despite important differences in their genetic toolkits (genetic diversity). Because differences in the genetic toolkits often are the consequence of the gene losses, the study of *O. dioica* has been fundamental to build this new conceptual framework

4.5 Future Directions

The tiny planktonic chordate *Oikopleura dioica* appears as an emerging nonclassical animal model, not only attractive to better understand the radiation of our own phylum in the field of evo-devo but also attractive in many other biological fields, such as ecology, environmental toxicology, biomedicine, or basic research. In ecology and toxicology, for instance, the relevance of *O. dioica* and other larvacean species in the marine food webs makes the study of its defense and detoxification mechanisms significant for understanding the impact of industrial pollutants or the effect of the climate change on marine environments, trophic webs, and ocean production. Monitoring *O. dioica* populations has been proposed to be used as valuable sentinels for following the health of marine ecosystems and the expression of its defensome genes as molecular biosensors that marine biologists could use to

monitor stress situations of natural populations. For biomedical applications, the chordate condition of *O. dioica* makes it closer to humans than classical model animals such as fruit flies or worms, but genetically and functionally more tractable than classical vertebrate models as mouse or zebrafish. The homology of organs, tissues, and structures between *O. dioica* and vertebrates (i.e., human) is unquestionable and therefore a good proxy to understand the genetic bases of many human disorders. In addition, the genetic, functional, and structural similarities of *O. dioica* and vertebrates make this species a promising model system for pharmacological screenings and other functional tests by amenable knockdown systems such as RNAi or DNAi approaches. Moreover, the relative small and compact genome of *O. dioica* makes it a good system for basic research in genetics. In the field of gene regulation analysis, for example, its small intergenic regions should facilitate the identification of *cis*-regulatory elements that control gene transcription. And in the field of evolutionary biology, the high mutation rate of *O. dioica*, its propensity to lose genes, and its miniature genome that can be affordably sequenced in many individuals should facilitate studies of population genomics for better understanding evolutionary forces in natural populations, searching for adaptive interpopulation differences, or investigating microevolution processes. Thus, *O. dioica* research has a brilliant present, but an even more promising future.

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References

Acuña JL, Kiefer M (2000) Functional response of the appendicularian *Oikopleura dioica*. Limnol Oceanogr 45:608–618

Acuña JL, Bedo AW, Harris RP, Anadón R (1995) The seasonal succession of appendicularians (Tunicata: Appendicularia) off Plymouth. J Mar Biol Assoc UK 75:755–758

Acuña JL, Deibel D, Saunders P, Booth B, Hatfield E, Klein B, Mei ZP, Rivkin R (2002) Phytoplankton ingestion by appendicularians in the North Water. Deep Sea Res Part II Top Stud Oceanogr 49:5101–5115

Albalat R (2009) The retinoic acid machinery in invertebrates: ancestral elements and vertebrate innovations. Mol Cell Endocrinol 313:23–35

Albalat R, Cañestro C (2016) Evolution by gene loss. Nat Rev Genet 17:379–391

Albalat R, Martí-Solans J, Cañestro C (2012) DNA methylation in amphioxus: from ancestral functions to new roles in vertebrates. Brief Funct Genomics 11:142–155

Alldredge AL (2005) The contribution of discarded appendicularian houses to the flux of particulate organic carbon from oceanic surface waters. In: Gorsky G, Youngbluth MJ, Deibel D (eds) Response of marine ecosystems to global change: ecological impact of appendicularians. Éditions Scientifiques, Paris

Almazán A, Ferrández-Roldán A, Albalat R, Cañestro C (2018) Developmental atlas of appendicularian *Oikopleura dioica* actins provides new insights into the evolution of the notochord and the cardio-paraxial muscle in chordates. Dev Biol. https://doi.org/10.1016/j.ydbio.2018.09.003

Aravena GP, Palma S (2002) Taxonomic identification of appendicularians collected in the epipelagic waters off northern Chile (Tunicata, Appendicularia). Rev Chilena Hist Nat 75:307–325

Bary BM (1960) Notes on ecology, distribution, and systematics of Pelagic Tunicata from New Zealand. Pac Sci 14(2):101–121

Bassham S, Postlethwait J (2000) Brachyury (T) expression in embryos of a larvacean urochordate, *Oikopleura dioica*, and the ancestral role of T. Dev Biol 220:322–332

Bedo AW, Acuña JL, Robins D, Harris RP (1993) Grazing in the micron and the sub-micron particle size range: the case of *Oikopleura dioica* (Appendicularia). Bull Mar Sci 52:2–14

Berna L, Alvarez-Valin F (2014) Evolutionary genomics of fast evolving tunicates. Genome Biol Evol 6:1724–1738

Berna L, D'onofrio G, Alvarez-Valin F (2012) Peculiar patterns of amino acid substitution and conservation in the fast evolving tunicate *Oikopleura dioica*. Mol Phylogenet Evol 62:708–717

Blumenthal T (2004) Operons in eukaryotes. Brief Funct Genomic Proteomic 3:199–211

Bollner T, Holmberg K, Olsson R (1986) A rostral sensory mechanism in *Oikopleura dioica* (Appendicularia). Acta Zool (Stockholm) 67:235–241

Bone Q (1998) The biology of pelagic tunicates. Oxford University Press, New York

Bone Q, Mackie GO (1975) Skin impulses and locomotion in *Oikopleura* (tunicata: larvacea). Biol Bull 149:267–286

Bouquet JM, Spriet E, Troedsson C, Ottera H, Chourrout D, Thompson EM (2009) Culture optimization for the emergent zooplanktonic model organism *Oikopleura dioica*. J Plankton Res 31:359–370

Bouquet JM, Troedsson C, Novac A, Reeve M, Lechtenborger AK, Massart W, Skaar KS, Aasjord A, Dupont S, Thompson EM (2018) Increased fitness of a key appendicularian zooplankton species under warmer, acidified seawater conditions. PLoS One 13:e0190625

Bowles J, Schepers G, Koopman P (2000) Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev Biol 227:239–255

Brozovic M, Martin C, Dantec C, Dauga D, Mendez M, Simion P, Percher M, Laporte B, Scornavacca C, Di Gregorio A, Fujiwara S, Gineste M, Lowe EK, Piette J, Racioppi C, Ristoratore F, Sasakura Y, Takatori N, Brown TC, Delsuc F, Douzery E, Gissi C, Mcdougall A, Nishida H, Sawada H, Swalla BJ, Yasuo H, Lemaire P (2016) ANISEED 2015: a digital framework for the comparative developmental biology of ascidians. Nucleic Acids Res 44:D808–D818

Brozovic M, Dantec C, Dardaillon J, Dauga D, Faure E, Gineste M, Louis A, Naville M, Nitta KR, Piette J, Reeves W, Scornavacca C, Simion P, Vincentelli R, Bellec M, Aicha SB, Fagotto M, Gueroult-Bellone M, Haeussler M, Jacox E, Lowe EK, Mendez M, Roberge A, Stolfi A, Yokomori R, Brown CT, Cambillau C, Christiaen L, Delsuc F, Douzery E, Dumollard R, Kusakabe T, Nakai K, Nishida H, Satou Y, Swalla B, Veeman M, Volff JN, Lemaire P (2017) ANISEED 2017: extending the integrated ascidian database to the exploration and evolutionary comparison of genome-scale datasets. Nucleic Acids Res 46:D718–D725

Burighel P, Brena C (2001) Gut ultrastructure of the appendicularian *Oikopleura dioica* (Tunicata). Invertebr Biol 120:278–293

Burke M, Scholl EH, Bird DM, Schaff JE, Colman SD, Crowell R, Diener S, Gordon O, Graham S, Wang X, Windham E, Wright GM, Opperman CH (2015) The plant parasite *Pratylenchus coffeae* carries a minimal nematode genome. Nematology 17:621

Calatayud S, Garcia-Risco M, Rojas NS, Espinosa-Sanchez L, Artime S, Palacios O, Cañestro C, Albalat R (2018) Metallothioneins of the urochordate *Oikopleura dioica* have Cys-rich tandem repeats, large size and cadmium-binding preference. Metallomics 10:1585–1594

Campsteijn C, Ovrebo JI, Karlsen BO, Thompson EM (2012) Expansion of cyclin D and CDK1 paralogs in *Oikopleura dioica*, a chordate employing diverse cell cycle variants. Mol Biol Evol 29:487–502

Cañestro C, Albalat R (2012) Transposon diversity is higher in amphioxus than in vertebrates: functional and evolutionary inferences. Brief Funct Genomics 11:131–141

Cañestro C, Postlethwait JH (2007) Development of a chordate anterior-posterior axis without classical retinoic acid signaling. Dev Biol 305:522–538

Cañestro C, Roncalli V (2018) Gene losses did not stop the evolution of big brains. elife 7:e41912

Cañestro C, Bassham S, Postlethwait JH (2003) Seeing chordate evolution through the *Ciona* genome sequence. Genome Biol 4:208–211

Cañestro C, Bassham S, Postlethwait JH (2005) Development of the central nervous system in the larvacean *Oikopleura dioica* and the evolution of the chordate brain. Dev Biol 285:298–315

Cañestro C, Postlethwait JH, Gonzalez-Duarte R, Albalat R (2006) Is retinoic acid genetic machinery a chordate innovation? Evol Dev 8:394–406

Cañestro C, Yokoi H, Postlethwait JH (2007) Evolutionary developmental biology and genomics. Nat Rev Genet 8:932–942

Cañestro C, Bassham S, Postlethwait JH (2008) Evolution of the thyroid: Anterior-posterior regionalization of the *Oikopleura* endostyle revealed by Otx, Pax 2/5/8, and Hox1 expression. Dev Dyn 237:1490–1499

Cañestro C, Albalat R, Postlethwait JH (2010) *Oikopleura dioica* alcohol dehydrogenase class 3 provides new insights into the evolution of retinoic acid synthesis in chordates. Zool Sci 27:128–133

Cañestro C, Albalat R, Irimia M, Garcia-Fernandez J (2013) Impact of gene gains, losses and duplication modes on the origin and diversification of vertebrates. Semin Cell Dev Biol 24:83–94

Captanio FL, Curelovich J, Tresguerres M, Negri RM, Viñas MD, Esnal GB (2008) Seasonal cycle of appendicularians at a coastal station (38°28′S, 57°41′W) of the SW Atlantic Ocean. Bull Mar Sci 82:171–184

Chalopin D, Naville M, Plard F, Galiana D, Volff JN (2015) Comparative analysis of transposable elements highlights mobilome diversity and evolution in vertebrates. Genome Biol Evol 7:567–580

Chang ES, Neuhof M, Rubinstein ND, Diamant A, Philippe H, Huchon D, Cartwright P (2015) Genomic insights into the evolutionary origin of Myxozoa within Cnidaria. Proc Natl Acad Sci USA 112:14912–14917

Chavali S, Morais DA, Gough J, Babu MM (2011) Evolution of eukaryotic genome architecture: insights from the study of a rapidly evolving metazoan, *Oikopleura dioica*: non-adaptive forces such as elevated mutation rates may influence the evolution of genome architecture. BioEssays 33:592–601

Cima F, Brena C, Burighel P (2002) Multifarious activities of gut epithelium in an appendicularian (*Oikopleura dioica*: Tunicata). Mar Biol 141:479–490

Clarke T, Bouquet JM, Fu X, Kallesoe T, Schmid M, Thompson EM (2007) Rapidly evolving lamins in a chordate, *Oikopleura dioica*, with unusual nuclear architecture. Gene 396:159–169

Cleaver O, Krieg PA (2001) Notochord patterning of the endoderm. Dev Biol 234:1–12

Coppola U, Ristoratore F, Albalat R, D'aniello S (2019) The evolutionary landscape of the Rab family in chordates. Cell Mol Life Sci (In press) AU10

Corbo JC, Di Gregorio A, Levine M (2001) The ascidian as a model organism in developmental and evolutionary biology. Cell 106:535–538

Costello J, Stancyk SE (1983) Tidal influence upon appendicularian abundance in North Inlet estuary, South Carolina. J Plankton Res 5:263–277

Danks G, Campsteijn C, Parida M, Butcher S, Doddapaneni H, Fu B, Petrin R, Metpally R, Lenhard B, Wincker P, Chourrout D, Thompson EM, Manak JR (2013) OikoBase: a genomics and developmental transcriptomics resource for the urochordate *Oikopleura dioica*. Nucleic Acids Res 41:D845–D853

Davoll P, Youngbluth M (1990) Heterotrophic activity on appendicularian (Tunicata: Appendicularia) houses in mesopelagic regions and their potential contribution to particle flux. Deep-Sea Res 37:285–294

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomoso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein DM, Harafuji N, Hastings KEM, Ho I, Hotta K, Huang W, Kawashima T, Lemaire P, Martinez D, Meinertzhagen IA, Necula S, Nonaka M, Putnam N, Rash S, Saiga H, Satake M, Terry A, Yamada L, Wang H-G, Awazu S, Azumi K, Boore J, Branno M, Chin-Bow S, Desantis R, Doyle S, Francino P, Keys DN, Haga S, Hayashi H, Hino K, Imai KS, Inaba K, Kano S, Kobayashi K, Kobayashi M, Lee B-I, Makabe KW, Manohar C, Matassi G, Medina M, Mochizuki Y, Mount S, Morishita T, Miura S, Nakayama A, Nishizaka S, Nomoto H, Ohta F, Oishi K, Rigoutsos I, Sano M, Sasaki A, Sasakura Y, Shoguchi E, Shin-I T, Spagnuolo A, Stainier D, Suzuki MM, Tassy O, Takatori N, Tokuoka M, Yagi K, Yoshizaki F, Wada S, Zhang C, Hyatt PD, Larimer F, Detter C, Doggett N, Glavina T, Hawkins T, Richardson P, Lucas S, Kohara Y, Levine M, Satoh N, Rokhsar DS (2002) The draft genome of *Ciona intestinalis:* insights into chordate and vertebrate origins. Science 298:2157–2167

Delsman HC (1910) Beiträge zur Entwicklungsgeschichte von *Oikopleura dioica*. Verh Rijksinst Onderz Zee 3:1–24

Delsman HC (1912) Weitere beobachtungen über die entwicklung von *Oikopleura dioica*. Tijdschr ned dierk Ver 12:199–206

Delsuc F, Philippe H, Tsagkogeorga G, Simion P, Tilak MK, Turon X, Lopez-Legentil S, Piette J, Lemaire P, Douzery EJP (2018) A phylogenomic framework and timescale for comparative studies of tunicates. BMC Biol 16:39

Deng W, Henriet S, Chourrout D (2018) Prevalence of mutation-prone microhomology-mediated end joining in a chordate lacking the c-NHEJ DNA repair pathway. Curr Biol 28(3337–3341): e3334

Denoeud F, Henriet S, Mungpakdee S, Aury JM, Da Silva C, Brinkmann H, Mikhaleva J, Olsen LC, Jubin C, Cañestro C, Bouquet JM, Danks G, Poulain J, Campsteijn C, Adamski M, Cross I, Yadetie F, Muffato M, Louis A, Butcher S, Tsagkogeorga G, Konrad A, Singh S, Jensen MF, Cong EH, Eikeseth-Otteraa H, Noel B, Anthouard V, Porcel BM, Kachouri-Lafond R, Nishino A, Ugolini M, Chourrout P, Nishida H, Aasland R, Huzurbazar S, Westhof E, Delsuc F, Lehrach H, Reinhardt R, Weissenbach J, Roy SW, Artiguenave F, Postlethwait JH, Manak JR, Thompson EM, Jaillon O, Du Pasquier L, Boudinot P, Liberles DA, Volff JN, Philippe H, Lenhard B, Roest Crollius H, Wincker P, Chourrout D (2010) Plasticity of animal genome architecture unmasked by rapid evolution of a pelagic tunicate. Science 330:1381–1385

Deriano L, Roth DB (2013) Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. Annu Rev Genet 47:433–455

Duboule D (2007) The rise and fall of Hox gene clusters. Development 134:2549–2560

Edvardsen RB, Lerat E, Maeland AD, Flat M, Tewari R, Jensen MF, Lehrach H, Reinhardt R, Seo HC, Chourrout D (2004) Hypervariable and highly divergent intron-exon organizations in the chordate *Oikopleura dioica*. J Mol Evol 59:448–457

Edvardsen RB, Seo HC, Jensen MF, Mialon A, Mikhaleva J, Bjordal M, Cartry J, Reinhardt R, Weissenbach J, Wincker P, Chourrout D (2005) Remodelling of the homeobox gene complement in the tunicate *Oikopleura dioica*. Curr Biol 15:R12–R13

Essenberg CE (1922) The seasonal distribution of the Appendicularia in the region of San Diego, California. Ecology 3:55–64

Fenaux R (1972) A historical survey of the appendicularians from the area covered by the IIOE. Mar Biol 16:230–235

Fenaux R (1976) Cycle vital d'un appendiculaire: *Oikopleura dioica* Fol, 1872. Ann Inst Océanogr Paris 52:89–101

Fenaux R (1986) The house of *Oikopleura dioica* (Tunicata, Appendicularia): structure and functions. Zoomorphology 106:224–231

Fenaux R (1998a) Anatomy and functional morphology of the Appendicularia. In: Bone Q (ed) The biology of pelagic tunicates. Oxford University Press, New York
Fenaux R (1998b) Life history of the Appendicularia. In: Bone Q (ed) The biology of pelagic tunicates. Oxford University Press, Oxford
Fenaux R, Gorsky G (1985) Nouvelle technique d'élevage des appendiculaires. Rapp Comm Int Mer Médit 29:291–292
Fenaux R, Bone Q, Deibel D (1998) Appendicularian distribution and zoogeography. In: Bone Q (ed) The biology of pelagic tunicates. Oxford University Press, Oxford
Fernández D, López-Urrutia A, Fernández A, Acuña JL, Harris R (2004) Retention efficiency of 0.2 to 6 µm particles by the appendicularians *Oikopleura dioica* and *Fritillaria borealis*. Mar Ecol Prog Ser 266:89–101
Ferrier DE (2011) Tunicates push the limits of animal evo-devo. BMC Biol 9:3
Ferrier DEK, Sogabe S (2018) Genome biology: unconventional DNA repair in an extreme genome. Curr Biol 28:R1208–R1210
Flood PR, Afzelius BA (1978) The spermatozoon of *Oikopleura dioica* Fol (Larvacea, Tunicata). Cell Tissue Res 191:27–37
Flood PR, Deibel D (1998) The appendicularian house. In: Bone Q (ed) The biology of pelagic tunicates. Oxford University Press, Oxford
Fol H (1872) Etudes sur les Appendiculaires du Détroit de Messine. Mem Soc Physique Hist Nat Geneve 21:445
Fredriksson G, Olsson R (1981) The oral gland cells of *Oikopleura dioica* (Tunicata Appendicularia). Acta Zool (Stockholm) 62:195–200
Fredriksson G, Olsson R (1991) The subchordal cells of *Oikopleura dioica* and *Oikopleura albicans* (Appendicularia, Chrodata). Acta Zool (Stockholm) 72:251–256
Fredriksson G, Ofverholm T, Ericson LE (1985) Ultrastructural demonstration of iodine binding and peroxidase activity in the endostyle of *Oikopleura dioica* (Appendicularia). Gen Comp Endocrinol 58:319–327
Fu X, Adamski M, Thompson EM (2008) Altered miRNA repertoire in the simplified chordate, *Oikopleura dioica*. Mol Biol Evol 25:1067–1080
Fujii S, Nishio T, Nishida H (2008) Cleavage pattern, gastrulation, and neurulation in the appendicularian, *Oikopleura dioica*. Dev Genes Evol 218:69–79
Gabaldon T, Capella-Gutierrez S (2010) Lack of phylogenetic support for a supposed actinobacterial origin of peroxisomes. Gene 465:61–65
Gabaldon T, Snel B, Van Zimmeren F, Hemrika W, Tabak H, Huynen MA (2006) Origin and evolution of the peroxisomal proteome. Biol Direct 1:8
Galt CP (1972) Development of *Oikopleura dioica* (Urochordata: Larvacea): ontogeny of behavior and of organ systems related to construction and use of the house. PhD, University of Washington, Seattle
Galt C (1978) Bioluminescence: dual mechanism in a planktonic tunicate produces brilliant surface display. Science 200:70–72
Galt CP, Fenaux R (1990) Urochordata larvacea. In: Adiyodi KG, Adiyodi RG (eds) Reproductive biology of invertebrates. Oxford and IBH, New Delhi
Ganot P, Kallesoe T, Reinhardt R, Chourrout D, Thompson EM (2004) Spliced-leader RNA trans splicing in a chordate, *Oikopleura dioica*, with a compact genome. Mol Cell Biol 24:7795–7805
Ganot P, Bouquet JM, Kallesoe T, Thompson EM (2007) The Oikopleura coenocyst, a unique chordate germ cell permitting rapid, extensive modulation of oocyte production. Dev Biol 302:591–600
Gaughan D, Potter IC (1994) Relative abundance and seasonal changes in the macrozooplankton of the lower estuary in South-Western Australia. Rec West Aust Mus 16:461–474
Georges D, Holmberg K, Olsson R (1988) The ventral midbrain cells in *Oikopleura dioica* (Appendicularia). Acta Embryol Morphol Exp 9:39–47
Goldschmidt R (1903) Notiz über die Entwickekung der Appendicularien-Biologisches Centralblatt, Band

Goldstone JV, Hamdoun A, Cole BJ, Howard-Ashby M, Nebert DW, Scally M, Dean M, Epel D, Hahn ME, Stegeman JJ (2006) The chemical defensome: environmental sensing and response genes in the *Strongylocentrotus purpuratus* genome. Dev Biol 300:366–384

Goldstone JV, Mcarthur AG, Kubota A, Zanette J, Parente T, Jonsson ME, Nelson DR, Stegeman JJ (2010) Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics 11:643

Gorsky G, Fenaux R (1998) The role of Appendicularia in marine food webs. In: Bone Q (ed) The biology of pelagic tunicates. Oxford University Press, Oxford

Gorsky G, Fisher N, Fowler S (1984) Biogenic debris from the pelagic tunicate *Oikopleura dioica* and its role in the vertical transport of a transuranium element. Est Coast Shelf Sci 18:13–23

Handberg-Thorsager M, Gutierrez-Mazariegos J, Arold ST, Kumar Nadendla E, Bertucci PY, Germain P, Tomancak P, Pierzchalski K, Jones JW, Albalat R, Kane MA, Bourguet W, Laudet V, Arendt D, Schubert M (2018) The ancestral retinoic acid receptor was a low-affinity sensor triggering neuronal differentiation. Sci Adv 4:eaao1261

Heenan P, Zondag L, Wilson MJ (2016) Evolution of the Sox gene family within the chordate phylum. Gene 575:385–392

Hertel J, Lindemeyer M, Missal K, Fried C, Tanzer A, Flamm C, Hofacker IL, Stadler PF, Students of Bioinformatics Computer Labs A (2006) The expansion of the metazoan microRNA repertoire. BMC Genomics 7:25

Holland LZ (2016) Tunicates. Curr Biol 26:R146–R152

Holland LZ, Gibson-Brown J (2003) The *Ciona intestinalis* genome: when the constraints are off. BioEssays 25:529–532

Holland L, Gorsky G, Fenaux R (1988) Fertilization in *Oikopleura dioica* (Tunicata, Appendicularia): acrosome reaction, cortical reaction and sperm-egg fusion. Zoomorphology 108:229–243

Holland PW, Booth HA, Bruford EA (2007) Classification and nomenclature of all human homeobox genes. BMC Biol 5:47

Holmberg K (1982) The ciliated brain duct of *Oikopleura dioica* (Tunicata, Appendicularia). Acta Zool 63:101–109

Holmberg K (1984) A transmission electron microscopic investigation of the sensory vesicle in the brain of *Oikopleura dioica* (Appendicularia). Zoomorphology 104:298–303

Hopcroft RR, Roff JC (1995) Zooplankton growth rates: extraordinary production by the larvacean *Oikopleura dioica* in tropical waters. J Plankton Res 17:205–220

Hopcroft RR, Roff JC, Bouman HA (1998) Zooplankton growth rates: the larvaceans *Appendicularia*, *Fritillaria* and *Oikopleura* in tropical waters. J Plankton Res 20:539–555

Hosp J, Sagane Y, Danks G, Thompson EM (2012) The evolving proteome of a complex extracellular matrix, the *Oikopleura* house. PLoS One 7:e40172

Hughes AL, Friedman R (2005) Loss of ancestral genes in the genomic evolution of *Ciona intestinalis*. Evol Dev 7:196–200

Hwan Lee J, Chae J, Kim W-R, Won Jung S, Man Kim J (2001) Seasonal variation of phytoplankton and zooplankton communities in the Coastal Waters off Tongyeong in Korea. Ocean Polar Res 23:245–253

Inoue J, Satoh N (2018) Deuterostome genomics: lineage-specific protein expansions that enabled chordate muscle evolution. Mol Biol Evol 35:914–924

Irimia M, Tena JJ, Alexis MS, Fernandez-Minan A, Maeso I, Bogdanovic O, De La Calle-Mustienes E, Roy SW, Gomez-Skarmeta JL, Fraser HB (2012) Extensive conservation of ancient microsynteny across metazoans due to cis-regulatory constraints. Genome Res 22:2356–2367

Jacob F (1977) Evolution and tinkering. Science 196:1161–1166

Kimura S, Ohshima C, Hirose E, Nishikawa J, Itoh T (2001) Cellulose in the house of the appendicularian *Oikopleura rufescens*. Protoplasma 216:71–74

King KR, Hollibaugh JT, Azam F (1980) Predator-prey interactions between the larvacean *Oikopleura dioica* and bacterioplankton in enclosed water columns. Mar Biol 56:49–57

Kishi K, Onuma TA, Nishida H (2014) Long-distance cell migration during larval development in the appendicularian, *Oikopleura dioica*. Dev Biol 395:299–306
Kishi K, Hayashi M, Onuma TA, Nishida H (2017) Patterning and morphogenesis of the intricate but stereotyped oikoplastic epidermis of the appendicularian, *Oikopleura dioica*. Dev Biol 428:245–257
Kmita M, Duboule D (2003) Organizing axes in time and space; 25 years of colinear tinkering. Science 301:331–333
Kocot KM, Tassia MG, Halanych KM, Swalla BJ (2018) Phylogenomics offers resolution of major tunicate relationships. Mol Phylogenet Evol 121:166–173
Kugler JE, Kerner P, Bouquet JM, Jiang D, Di Gregorio A (2011) Evolutionary changes in the notochord genetic toolkit: a comparative analysis of notochord genes in the ascidian *Ciona* and the larvacean *Oikopleura*. BMC Evol Biol 11:21
Kusakabe T, Araki I, Satoh N, Jeffery WR (1997) Evolution of chordate actin genes: evidence from genomic organization and amino acid sequences. J Mol Evol 44:289–298
Larson RJ (1987) Daily ration and predation by medusae and ctenophores in Saanich Inlet, B.C., Canada. Neth J Sea Res 21:35–44
Last JM (1972) Egg development, fecundity and growth of *Oikopleura Dioica* Fol in the North Sea. ICES J Mar Sci 34:232–237
Lohmann H (1933) Erste Klasse der Tunicaten: Appendiculariae. In: Kükenthal W, Krumbach T (eds) Handbuch der Zoologie. Walter De Gruyter, Berlin
Lopez-Urrutia A, Acuña JL (1999) Gut throughput dynamics in the appendicularian *Oikopleura dioica*. Mar Ecol Prog Ser 191:195–205
Martinucci G, Brena C, Cima F, Burighel P (2005) Synchronous spermatogenesis in appendicularians. In: Gorsky G, Youngbluth M, Deibel D (eds) Response of marine ecosystems to global changes: ecological impact of appendicularians. Éditions des Archives Contempoaines, Paris
Martí-Solans J, Ferrández-Roldán A, Godoy-Marín H, Badia-Ramentol J, Torres-Aguila NP, Rodríguez-Marí A, Bouquet JM, Chourrout D, Thompson EM, Albalat R, Cañestro C (2015) *Oikopleura dioica* culturing made easy: a low-cost facility for an emerging animal model in EvoDevo. Genesis 53:183–193
Martí-Solans J, Belyaeva OV, Torres-Aguila NP, Kedishvili NY, Albalat R, Cañestro C (2016) Coelimination and survival in gene network evolution: dismantling the RA-signaling in a chordate. Mol Biol Evol 33:2401–2416
Menéndez M, Biancalana F, Berasategui A, Fernández Severini MD, Hoffmeyer MS, Esteves JL (2011) Mesozooplankton composition and spatial distribution, Nuevo Gulf, Patagonia, Argentina. Check List 7:101–107
Mikhailov KV, Slyusarev GS, Nikitin MA, Logacheva MD, Penin AA, Aleoshin VV, Panchin YV (2016) The genome of *Intoshia linei* affirms orthonectids as highly simplified spiralians. Curr Biol 26:1768–1774
Mikhaleva Y, Kreneisz O, Olsen LC, Glover JC, Chourrout D (2015) Modification of the larval swimming behavior in *Oikopleura dioica*, a chordate with a miniaturized central nervous system by dsRNA injection into fertilized eggs. J Exp Zool B Mol Dev Evol
Mikhaleva Y, Skinnes R, Sumic S, Thompson EM, Chourrout D (2018) Development of the house secreting epithelium, a major innovation of tunicate larvaceans, involves multiple homeodomain transcription factors. Dev Biol 443:117–126
Nakamura Y, Suzuki K, Suzuki S, Hiromi J (1997) Production of *Oikopleura dioica* (Appendicularia) following a picoplankton 'bloom' in a eutrophic coastal area. J Plankton Res 19:113–124
Navratilova P, Danks GB, Long A, Butcher S, Manak JR, Thompson EM (2017) Sex-specific chromatin landscapes in an ultra-compact chordate genome. Epigenetics Chromatin 10:3
Nelson DR, Goldstone JV, Stegeman JJ (2013) The cytochrome P450 genesis locus: the origin and evolution of animal cytochrome P450s. Philos Trans R Soc Lond Ser B Biol Sci 368:20120474

Nilsen IW, Myrnes B, Edvardsen RB, Chourrout D (2003) Urochordates carry multiple genes for goose-type lysozyme and no genes for chicken- or invertebrate-type lysozymes. Cell Mol Life Sci 60:2210–2218

Nishida H (1987) Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III Up to the tissue restricted stage. Dev Biol 121:526–541

Nishida H (2002) Patterning the marginal zone of early ascidian embryos: localized maternal mRNA and inductive interactions. BioEssays 24:613–624

Nishida H (2008) Development of the appendicularian *Oikopleura dioica*: culture, genome, and cell lineages. Develop Growth Differ 50(Suppl 1):S239–S256

Nishida H, Stach T (2014) Cell lineages and fate maps in tunicates: conservation and modification. Zool Sci 31:645–652

Nishino A, Morisawa M (1998) Rapid oocyte growth and artificial fertilization of the Larvaceans *Oikopleura dioica* and *Oikopleura longicauda*. Zool Sci 15:723–727

Nishino A, Satoh N (2001) The simple tail of chordates: phylogenetic significance of appendicularians. Genesis 29:36–45

Nishino A, Satou Y, Morisawa M, Satoh N (2000) Muscle actin genes and muscle cells in the appendicularian, *Oikopleura longicauda*: phylogenetic relationships among muscle tissues in the urochordates. J Exp Zool 288:135–150

Ohno S (1970) Evolution by gene duplication. Springer, New York

Olsson R (1963) Endostyles and endostylar secretions: a comparative histochemical study. Acta Zool (Stockholm) 44:299–329

Olsson R (1965a) Comparative morphology and physiology of the *Oikopleura* notochord. Isr J Zool 14:213–220

Olsson R (1965b) The cytology of the endostyle of *Oikopleura dioica*. Ann NY Acad Sci 118:1038–1051

Olsson R (1975) Primitive coronet cells in the brain of *Oikopleura* (Appendicularia, Tunicata). Acta Zool (Stockholm) 56:155–161

Olsson R, Holmberg K, Lilliemark Y (1990) Fine structure of the brain and brain nerves of *Oikopleura dioica* (Urochordata, Appendicularia). Zool Morphol 110:1–7

Omotezako T, Nishino A, Onuma TA, Nishida H (2013) RNA interference in the appendicularian *Oikopleura dioica* reveals the function of the Brachyury gene. Dev Genes Evol 223:261–267

Omotezako T, Onuma TA, Nishida H (2015) DNA interference: DNA-induced gene silencing in the appendicularian *Oikopleura dioica*. Proc Biol Sci 282:20150435

Omotezako T, Matsuo M, Onuma TA, Nishida H (2017) DNA interference-mediated screening of maternal factors in the chordate *Oikopleura dioica*. Sci Rep 7:44226

Onuma TA, Isobe M, Nishida H (2017) Internal and external morphology of adults of the appendicularian, *Oikopleura dioica*: an SEM study. Cell Tissue Res 367:213–227

Paffenhöfer G-A (1973) The cultivation of an appendicularian through numerous generations. Mar Biol 22:183–185

Pannunzio NR, Watanabe G, Lieber MR (2018) Nonhomologous DNA end-joining for repair of DNA double-strand breaks. J Biol Chem 293:10512–10523

Patel AA, Steitz JA (2003) Splicing double: insights from the second spliceosome. Nat Rev Mol Cell Biol 4:960–970

Raduan A, Blanco C, Soler E, Del Rio JG, Raga JA (1985) The seasonal distribution of the Appendicularia in the Bay of Cullera, Spain. Rapp Comm Int Mer Médit 29:293–294

Robinson AJ, Kunji ER, Gross A (2012) Mitochondrial carrier homolog 2 (MTCH2): the recruitment and evolution of a mitochondrial carrier protein to a critical player in apoptosis. Exp Cell Res 318:1316–1323

Robison BH, Reisenbichler KR, Sherlock RE (2005) Giant larvacean houses: rapid carbon transport to the deep sea floor. Science 308:1609–1611

Sagane Y, Zech K, Bouquet JM, Schmid M, Bal U, Thompson EM (2010) Functional specialization of cellulose synthase genes of prokaryotic origin in chordate larvaceans. Development 137:1483–1492

Salensky W (1903) Etudes anatomiques sur les Appendiculaires. I *Oikopleura vanhoeffeni* Lohmann. Mem Acad Sci St Petesbourg Ser 13:1–44
Salensky W (1904) Etudes anatomiques sur les Appendiculaires. II *Oikopleura refescens*. Fol Mem Acad Sci St Petesbourg Ser 15:1–54
Salensky W (1905) Zur Morphologie der Cardialorgane der Appendicularien. Sixième Congrès International de Zoologie, Berne. Kandiget fils. Genève, 381–383
Sato R, Tanaka Y, Ishimaru T (2003) Species-specific house productivity of appendicularians. Mar Ecol Prog Ser 259:163–172
Satoh N (2003) The ascidian tadpole larva: comparative molecular development and genomics. Nat Rev Genet 4:285–295
Satoh N, Satou Y, Davidson B, Levine M (2003) *Ciona intestinalis*: an emerging model for whole-genome analyses. Trends Genet 19:376–381
Satoh N, Tagawa K, Takahashi H (2012) How was the notochord born? Evol Dev 14:56–75
Satoh N, Rokhsar D, Nishikawa T (2014) Chordate evolution and the three-phylum system. Proc Biol Sci 281:20141729
Schluter A, Fourcade S, Ripp R, Mandel JL, Poch O, Pujol A (2006) The evolutionary origin of peroxisomes: an ER-peroxisome connection. Mol Biol Evol 23:838–845
Seo H-C, Kube M, Edvardsen RB, Jensen MF, Beck A, Spriet E, Gorsky G, Thompson EM, Lehrach H, Reinhardt R, Chourrout D (2001) Miniature genome in the marine chordate *Oikopleura dioica*. Science 294:2506
Seo HC, Edvardsen RB, Maeland AD, Bjordal M, Jensen MF, Hansen A, Flaat M, Weissenbach J, Lehrach H, Wincker P, Reinhardt R, Chourrout D (2004) Hox cluster disintegration with persistent anteroposterior order of expression in *Oikopleura dioica*. Nature 431:67–71
Sheth N, Roca X, Hastings ML, Roeder T, Krainer AR, Sachidanandam R (2006) Comprehensive splice-site analysis using comparative genomics. Nucleic Acids Res 34:3955–3967
Somorjai IML, Martí-Solans J, Diaz-Gracia M, Nishida H, Imai KS, Escriva H, Cañestro C, Albalat R (2018) Wnt evolution and function shuffling in liberal and conservative chordate genomes. Genome Biol 19:98
Soviknes AM, Glover JC (2007) Spatiotemporal patterns of neurogenesis in the appendicularian *Oikopleura dioica*. Dev Biol 311:264–275
Soviknes AM, Glover JC (2008) Continued growth and cell proliferation into adulthood in the notochord of the appendicularian *Oikopleura dioica*. Biol Bull 214:17–28
Soviknes AM, Chourrout D, Glover JC (2005) Development of putative GABAergic neurons in the appendicularian urochordate *Oikopleura dioica*. J Comp Neurol 490:12–28
Soviknes AM, Chourrout D, Glover JC (2007) Development of the caudal nerve cord, motoneurons, and muscle innervation in the appendicularian urochordate *Oikopleura dioica*. J Comp Neurol 503:224–243
Spada F, Steen H, Troedsson C, Kallesoe T, Spriet E, Mann M, Thompson EM (2001) Molecular patterning of the oikoplastic epithelium of the larvacean tunicate *Oikopleura dioica*. J Biol Chem 276:20624–20632
Stach TG (2009) Anatomy of the trunk mesoderm in tunicates: homology considerations and phylogenetic interpretation. Zoomorphology 128:97–109
Stach T, Anselmi C (2015) High-precision morphology: bifocal 4D-microscopy enables the comparison of detailed cell lineages of two chordate species separated for more than 525 million years. BMC Biol 13:113
Stach T, Winter J, Bouquet JM, Chourrout D, Schnabel R (2008) Embryology of a planktonic tunicate reveals traces of sessility. Proc Natl Acad Sci USA 105:7229–7234
Stemple DL (2005) Structure and function of the notochord: an essential organ for chordate development. Development 132:2503–2512
Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Dev Biol 100:64–119

Thompson EM, Kallesøe T, Spada F (2001) Diverse genes expressed in distinct regions of the trunk epithelium define a monolayer cellular template for construction of the oikopleurid house. Dev Biol 238:260–273

Tomita M, Shiga N, Ikeda T (2003) Seasonal occurrence and vertical distribution of appendicularians in Toyama Bay, southern Japan Sea. J Plankton Res 25:579–589

Torres-Aguila NP, Martí-Solans J, Ferrandez-Roldan A, Almazan A, Roncalli V, D'aniello S, Romano G, Palumbo A, Albalat R, Cañestro C (2018) Diatom bloom-derived biotoxins cause aberrant development and gene expression in the appendicularian chordate *Oikopleura dioica*. Commun Biol 1:121

Troedsson C, Bouquet J-M, Aksnes DL, Thompson EM (2002) Resource allocation between somatic growth and reproductive output in the pelagic chordate *Oikopleura dioica* allows opportunistic response to nutritional variation. Mar Ecol Prog Ser 243:83–91

Troedsson C, Bouquet J-M, Lobon C, Novac A, Nejstgaard J, Dupont S, Bosak S, Jakobsen H, Romanova N, Pankoke L, Isla A, Dutz JR, Sazhin A, Thompson E (2013) Effects of ocean acidification, temperature and nutrient regimes on the appendicularian *Oikopleura dioica*: a mesocosm study. Mar Biol 160:2175–2187

Uren AG, O'rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell 6:961–967

Uye S-I, Ichino S (1995) Seasonal variations in abundance, size composition, biomass and production rate of *Oikopleura dioica* (Fol) (Tunicata: Appendicularia) in a temperate eutrophic inlet. J Exp Mar Biol Ecol 189:1–11

Volff JN, Lehrach H, Reinhardt R, Chourrout D (2004) Retroelement dynamics and a novel type of chordate retrovirus-like elements in the miniature genome of the tunicate *Oikopleura dioica*. Mol Biol Evol 21:2022–2033

Walkusz W, Storemark K, Skau T, Gannefors C, Lundberg M (2003) Zooplankton community structure; a comparison of fjords, open water and ice stations in the Svalbard area. Pol Polar Res 24:149–165

Wang K, Dantec C, Lemaire P, Onuma TA, Nishida H (2017) Genome-wide survey of miRNAs and their evolutionary history in the ascidian, *Halocynthia roretzi*. BMC Genomics 18:314

Wegner M (2010) All purpose Sox: the many roles of Sox proteins in gene expression. Int J Biochem Cell Biol 42:381–390

Weill M, Philips A, Chourrout D, Fort P (2005) The caspase family in urochordates: distinct evolutionary fates in ascidians and larvaceans. Biol Cell 97:857–866

Welsch U, Storch V (1969) On the fine structure of the chorda dorsalis in lower chordata. (*Dendrodoa grossularia* (v. Beneden) and *Oikopleura dioica* Fol). Z Zellforsch Mikrosk Anat 93:547–549

Yadetie F, Butcher S, Forde HE, Campsteijn C, Bouquet JM, Karlsen OA, Denoeud F, Metpally R, Thompson EM, Manak JR, Goksoyr A, Chourrout D (2012) Conservation and divergence of chemical defense system in the tunicate *Oikopleura dioica* revealed by genome wide response to two xenobiotics. BMC Genomics 13:55

Zarsky V, Tachezy J (2015) Evolutionary loss of peroxisomes-not limited to parasites. Biol Direct 10:74

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