

## Cell types, morphology and evolution of animal excretory organs

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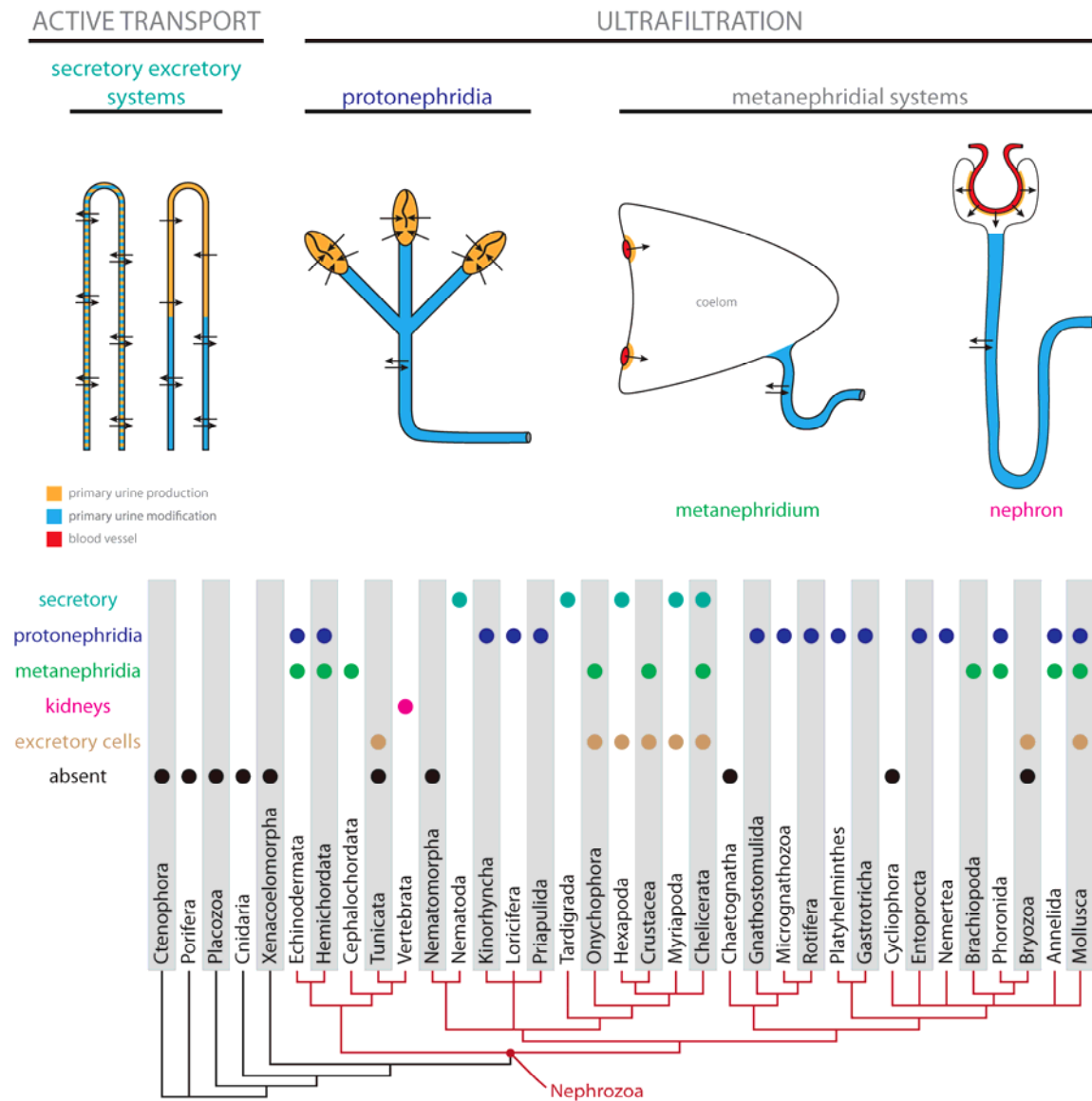
### **Abstract**

*Excretion and osmoregulation are fundamental processes of the organism, as they prevent the accumulation of toxic waste products in the body and control the osmotic differences between the cells and the environment. In most of the animals these phenomena are taking place through specialized organs, namely excretory organs, composed of diverse cell types that are performing tasks such as secretion and ultrafiltration. Although the morphology and embryology of excretory organs can differ dramatically, the common spatial arrangement of structural proteins and transporters as well as the similar transcriptional developmental programs underlying their formation suggests the homology of their cell types. In this chapter we discuss the current understanding of the evolution of excretory organs from a comparative morphological, developmental and functional perspective, flanked by an additional, cell-type perspective. We argue that a putative homologization of certain excretory cell types does not necessarily reflect the homology of the resulting organs, and that integrating all different levels of comparison is crucial for addressing evolutionary questions.*

### **1. Introduction**

All animals (Metazoa) need to excrete metabolic waste products from their body (Larsen et al., 2014; Schmidt-Nielsen, 1997; Schmidt-Rhaesa, 2007). On a cellular level, this process is taking place via transmembrane proteins that are specialized for transporting these products in the context of ion gradients (Ichimura and Sakai, 2017; Larsen et al., 2014; O'Donnell, 2010; Schmidt-Nielsen, 1997; Weihrauch and O'Donnell, 2017). It is widely believed that non-bilaterian animals excrete via passive diffusion through their integument, although this hypothesis has been challenged by a recent work on cnidarian and xenacoelomorph species (Andrikou et al., 2019). However, most bilaterians possess specialized excretory organs that remove metabolites more efficiently (Fig. 1). These organs are diverse, and their evolutionary relationship has puzzled many zoologists since their discovery (Bartolomaeus and Ax, 1992; Bartolomaeus and Quast, 2005; Goodrich, 1945; Ichimura and Sakai, 2017; Koch et al., 2014; Ruppert, 1994; Ruppert and Smith, 1988; Schmidt-Rhaesa, 2007). Nephridia, kidneys,

Malpighian tubules etc. are composed out of different cell types that can be discriminated by their morphology and function (e.g. (Goodrich, 1945; Ruppert and Smith, 1988; Schmidt-Rhaesa, 2007)). The embryology of these organs is also intriguing, because it varies between species and also involves, in some cases (e.g. metanephridia), an interaction between cells of different germ layers, such as mesoderm and ectoderm (Bartolomaeus, 1989; Goodrich, 1945; Lüter, 1995; Ruppert, 1994; Schmidt-Rhaesa, 2007). When and how many times these specialized organs evolved remains unclear. In the context of the placement of the Xenacoelomorpha as sister group to all remaining Bilateria, a new taxon name for Protostomia + Deuterostomia has been introduced, namely Nephrozoa (Jondelius et al., 2002). This refers to the presence of excretory organs (i.e. nephridia) in the last common ancestor of this clade that would also be an apomorphy of Nephrozoa (Fig. 1). Here we aim to describe the astonishing variability seen in excretory organs from a cell-type perspective, for which the diversity in morphology, development and functional composition can be particularly challenging. We interpret this diversity from an evolutionary perspective and discuss problems in homologization on different levels.



**Figure 1. Excretory systems distribution across the animal tree.** Schematic drawings and typology of basic types of excretory organs with color coded sites of primary urine production and modification (see text for details). Distribution of different types of excretory organs in nephrozoan clades.

## 2. Diversity of cell types in excretory organs: a morphological perspective

Excretion in most of the Nephrozoa is a two-stage process (Ichimura and Sakai, 2017; Ruppert, 1994; Ruppert and Smith, 1988; Schmidt-Nielsen, 1997; Schmidt-Rhaesa, 2007). Initially the body fluid (e.g. blood, haemolymph or interstitial fluid) is roughly filtered from large proteins and cells to produce the so-called primary urine (Ichimura and Sakai, 2017; Schmidt-Rhaesa, 2007). This initial product becomes later secondarily modified (e.g. the ion concentration or water volume can be specifically changed), resulting in the finite secondary urine, which is eventually expelled from the body (Schmidt-Nielsen, 1997; Schmidt-Rhaesa, 2007). As there are many ways in which those two processes can be

performed, the excretory organs vary greatly regarding their morphology and physiology and, as a consequence, in the diversity and spatial arrangement of the particular cell types, which built them.

Traditionally, zoologists group excretory organs based on their physiological and structural properties into categories, which reflect rather functional than evolutionary similarities (Fig. 1; (Bartolomaeus and Ax, 1992; Ichimura and Sakai, 2017; Ruppert and Smith, 1988; Schmidt-Rhaesa, 2007)). The most basic division relates to the mechanism of primary urine production – in the secretory excretory organs the active intracellular transport is used for that purpose, whereas in the ultrafiltration-based systems the primary urine is filtered through semipermeable extracellular membrane – a filter composed of either extracellular matrix (ECM) or slit diaphragm or both (Fig. 1; (Ichimura and Sakai, 2017; Schmidt-Nielsen, 1997; Schmidt-Rhaesa, 2007)). The latter category includes protonephridia, where ultrafiltration is driven by the ciliary action, and the metanephridial system in which blood pressure is used to produce primary urine (Bartolomaeus and Ax, 1992; Ichimura and Sakai, 2017; Ruppert, 1994; Ruppert and Smith, 1988; Schmidt-Rhaesa, 2007). Among metanephridial systems it is possible to distinguish between vertebrate kidneys, in which ultrafiltration and secondary urine modification occurs in the single structural unit (i.e. nephron with glomerulus) and invertebrate metanephridial systems in which ultrafiltration and secondary urine modification are spatially separated, the former happening in the coelom lining and the latter in the metanephridium itself (Ichimura and Sakai, 2017; Schmidt-Nielsen, 1997; Schmidt-Rhaesa, 2007).

#### *Secretory excretory organs (Malpighian tubules and others)*

Among the best-known examples of the secretory excretory organs are the Malpighian tubules, the prevalent excretory organs in many Panarthropoda taxa. Although Malpighian tubules are found in representatives of Eutardigrada, Chelicerata, Myriapoda and Hexapoda (Fig. 1), it remains unknown if they are all homologous to each other or if they rather evolved independently in particular lineages (Bitsch and Bitsch, 2004; Greven, 1982; Paulus, 2000). Those organs are tubular invaginations of the gut (originating close to the midgut-hindgut transition), which penetrate the haemocoel; distally they are blindly ended and proximally they open to the gut lumen (Berridge and Oschman, 1969; Eichelberg and Wessing, 1975; Nocelli et al., 2016; Schmidt-Nielsen, 1997; Schmidt-Rhaesa, 2007). Malpighian tubules are surrounded by a basal lamina, which serves as a barrier for cells and large proteins. Both their external and internal surface is greatly increased by respectively basal infoldings and microvillar structures, which enhance cellular uptake and secretion (Berridge and Oschman, 1969; Maddrell, 1980). In insects the organs are additionally

equipped with contractile muscle fibers and tracheae (e.g. (Bradley, 1983; Garayoa et al., 1992; Li et al., 2015; Taylor, 1971b; Wall et al., 1975).

Often there are two basic cell types in Malpighian tubules – e.g. many insects possess 1) primary cells (known as well as type I cells), which produce primary urine and are characterized from numerous mitochondria-rich, external, basal infoldings and internal microvilli and 2) less abundant secondary or stellate cells (known as well as type II cells) with less developed microvilli, which are likely involved in the secondary urine modification (e.g. (Berridge and Oschman, 1969; Dow and Davies, 2001; Kapoor, 1994; Li et al., 2015; Nocelli et al., 2016; Pal and Kumar, 2012; Taylor, 1971a, b; Wall et al., 1975). In some animals Malpighian tubules seem to include additional cell types – e.g. in tardigrades "supportive" cells are present next to primary and secondary cells (Møbjerg and Dahl, 1996; Węglarska, 1980).

It is evident that not only cell type diversity, but also their spatial distribution along the organ is crucial for the evolution of Malpighian tubules. The two basic cell types – the primary urine producing and the secondarily modifying ones – can be a) uniformly distributed along the tubule as it is case in many insects (Berridge and Oschman, 1969; Dow and Davies, 2001; Kapoor, 1994; Nocelli et al., 2016; Pal and Kumar, 2012; Taylor, 1971a, b; Wall et al., 1975), or b) restricted to the respectively proximal and distal portion of the tubule, resulting in a clear division of the organ into distinct parts responsible for urine production and secondary modification, as in e.g. tardigrades (Møbjerg and Dahl, 1996; Schill, 2019; Węglarska, 1980), millipedes (Johnson and Riegel, 1977) or some insects (Arab and Caetano, 2002; Bradley, 1983; Green, 1979, 1980; Li et al., 2015; Nicholls, 1983; Nocelli et al., 2016). The subdivision of Malpighian tubules into distinct regions with different cell types has been also shown in spiders, however the function of the cells in each section is not clear (Hazelton et al., 2002; Seitz, 1987).

Secretory excretory organs are also found among nematodes in which the two different basic types can be recognized (reviewed in (Chitwood and Chitwood, 1950)). Some of the free-living, mostly marine forms (once united into the group Aphasmodia=Adenophorea) have a single large glandular cell used for excretion, the so-called ventral gland, which opens to the exterior by the single pore. Members of the clade Phasmodia (=Secernentea), which include most of the parasitic forms, as well as a model species *Caenorhabditis elegans*, possess a specialized excretory organ, which consists of several cells, each with different ultrastructure and function (e.g. (McLaren, 1974; Nelson et al., 1983; Waddell, 1968). The exact number and types of cells might vary but generally the organ always represents some modification of the so-called H-system (Chitwood and Chitwood, 1950). There is an unpaired sinus cell which opens to the exterior by a ventromedian terminal duct lined with cuticle. From the sinus two pairs of longitudinal, lateral

canals extend in the lateral hypodermal cords to the anterior and posterior portion of the animal. Each canal is built by a single cell with intracellular lumen (sometimes canals are projections of the sinus cell, e.g. (Nelson et al., 1983)), which can show spatial differentiation into smooth and microvillar portions (McLaren, 1974; Waddell, 1968). Additional glandular, sphincter or lip cells can be associated with the terminal duct (Chitwood and Chitwood, 1950; McLaren, 1974; Nelson et al., 1983; Waddell, 1968). The molecular phylogeny of nematodes shows that species with H-systems (or its modification) represents a monophyletic group whereas species with single cell excretory gland are forming a basal grade (Holterman et al., 2006; Smythe et al., 2019), hence the latter should be considered as plesiomorphic nematode arrangement, whereas the former represent synapomorphy of Secernentea. Both systems are nematode specific and represent derived excretory organs not easily comparable with any structures found in remaining Nephrozoa.

Interestingly, the putative unicellular secretory excretory organs were also described in the acoel *Paratomella rubra* (Ehlers, 1992), representing likely an evolutionary gain of excretory organs independent from nephrozoan lineage (Andrikou et al., 2019). The so-called dermonephridia are specialized epidermal cells, randomly distributed in the epidermis, which lack cilia but possess modified long microvilli on their apical surface and intracellular lacunar system of tubules and vacuoles, which communicate with the exterior. Taking into account that dermonephridia are known only from a single acoel species they probably represent a recent evolutionary novelty.

### *Protonephridia*

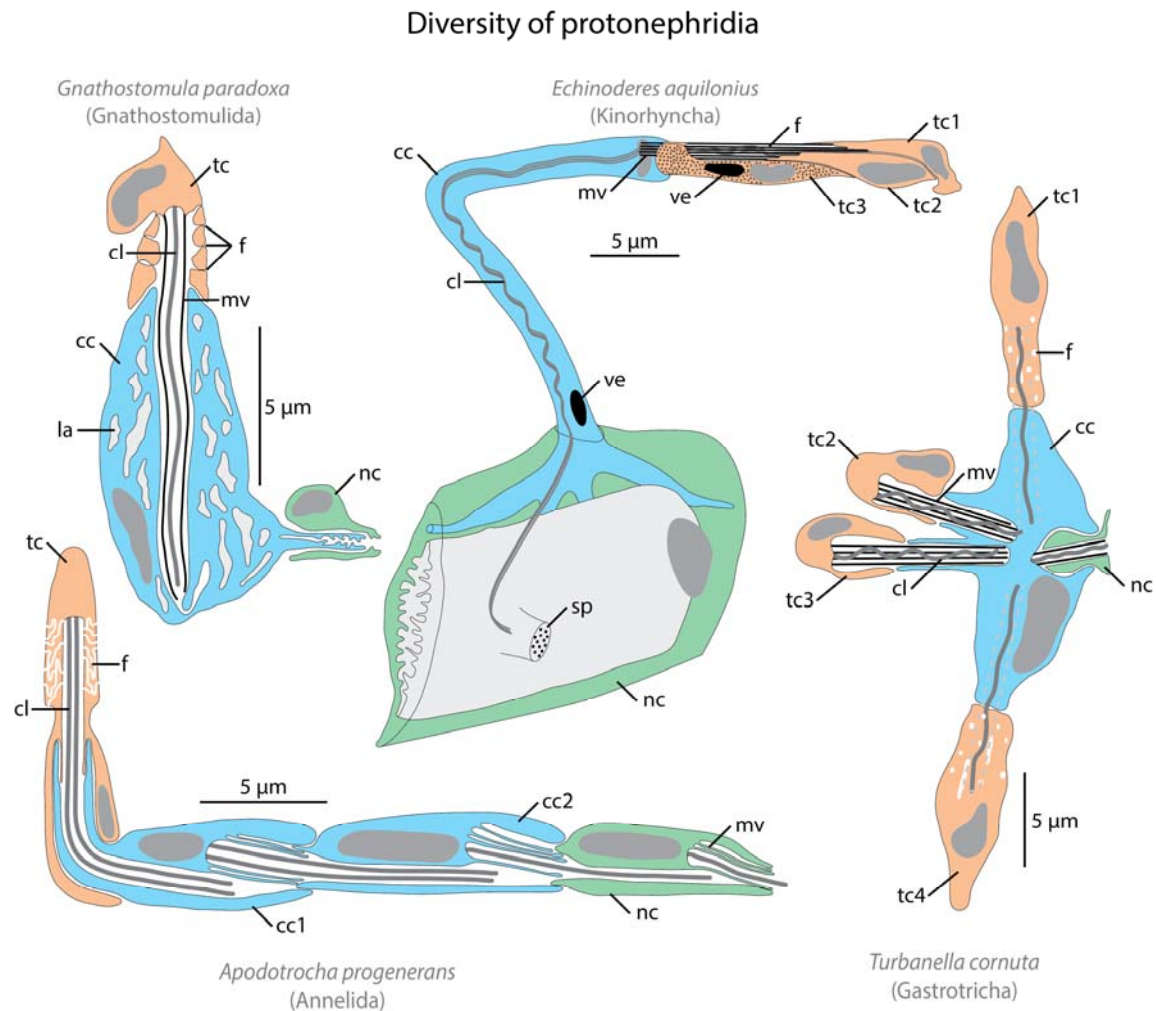
Protonephridia use ultrafiltration through extracellular filters driven by ciliary action (Bartolomaeus and Ax, 1992; Ichimura and Sakai, 2017; Ruppert, 1994; Ruppert and Smith, 1988; Schmidt-Nielsen, 1997; Schmidt-Rhaesa, 2007) (Figs. 1). Some of the simplest protonephridia among entire animal kingdom are found in Gnathostomulida (Fig. 2; (Lammert, 1985)), a group of microscopic marine worms closely related with rotifers. Each of the gnathostomulid protonephridium consists of only three cells and can be used as an example of the minimal and most basic cellular architecture required for this type of organs (Fig. 2; (Lammert, 1985)). The most proximal (or terminal) cell has single cilium surrounded by the cytoplasmic portion (so-called filtering area) traversed by irregular clefts closed with a filtering membrane (i.e. slit diaphragm). The tubular filtering area tightly adjoins to the second cell of the system – the canal cell – that harbors the intracellular canal into which the cilium of the terminal cell protrudes. The canal cell is non-ciliated and has a complicated lacunar system and microvilli facing the canal lumen, which both greatly increase the cellular surface. Laterally, the canal cell adjoins the nephroporus cell, which is a modified epidermal cell,

through which a canal of the protonephridium communicates with the exterior. The terminal cell provides the filter as well as the negative pressure necessary for the ultrafiltration and is therefore the site of primary urine production. The canal cell, on the other hand, is responsible for the secondary modification of the urine. Importantly, both components of the organ show ultrastructural characteristics of epidermal cells, indicating that the entire organ has an ectodermal origin (Lammert, 1985).

There are two ways in which this extremely simple system can be complicated in other protonephridia-bearing animals. First of all, particular cells can be multiplied resulting in e.g. many terminal cells opening to the single canal cell (e.g. in some gastrotrichs, Fig. 2, (Kieneke et al., 2008; Kieneke and Hochberg, 2012; Teuchert, 1973) or some kinorhynchs, Fig. 2, (Kristensen and Hay-Schmidt, 1989)), one terminal cell can open to the canal built of many similar canal cells (e.g. in annelid *Apodotrocha*, Fig. 2; (Westheide, 1985)) or both elements can be multiplied (e.g. in some Kinorhyncha and Loricifera; (Neuhaus, 1988; Neuhaus and Kristensen, 2007) or in Nemertea (Bartolomaeus, 1985)). When terminal cells are multiplied, each of them might form a separate filtering unit (Bartolomaeus, 1985; Teuchert, 1973) or they can be merged together, with the filtering area formed by two or more adjacent cells (Kieneke et al., 2008; Kieneke and Hochberg, 2012; Kristensen and Hay-Schmidt, 1989; Neuhaus, 1988; Neuhaus and Kristensen, 2007).

Apart from cell multiplication, additional cell types might be present in the protonephridial organs of some animals, increasing spatial diversification and functional specification of the nephridium. For instance, in some platyhelminths the multicellular canal is divided into the proximal ciliated zone to which the terminal cells open and a distal zone, built exclusively by cells with extensive microvilli on the luminal surface (Rohde and Watson, 1993; Scimone et al., 2011; Vu et al., 2015). Additionally, it has been demonstrated, that in planarians those two parts express different sets of solute carrier transporters and apparently have different roles in the secondary urine modification (Vu et al., 2015). In microscopic rotifers the canal is built of two or three distinct cell types (Ahlrichs, 1993a; Ahlrichs, 1993b; Warner, 1969) and additionally in monogononts it opens to the muscular bladder, which collects urine from paired protonephridial systems (Ahlrichs, 1993b; Warner, 1969). The cellular complexity of the rotifer excretory system is further increased due to the fact that some of its parts are of syncytial nature.





**Figure 2. Morphological diversity of protonephridia.** Schematic reconstruction of various protonephridial systems as inferred from TEM studies, based on (Kristensen and Hay-Schmidt, 1989; Lammert, 1985; Teuchert, 1973; Westheide, 1985). In all drawings primary urine filtering cells (terminal cells) are in orange, primary urine modifying cells (canal cells) in blue, nephroporus cells in green, cell nuclei in dark grey and intracellular cavities in light gray. Abbreviations: **cc** canal cell, **cl** cilium, **f** protonephridial filter, **la** lacunar system, **mv** microvilli, **nc** nephroporus cell, **sp** sieve plate (cuticular opening for protonephridium), **tc** terminal cell, **ve** vesicle. Number after abbreviation indicate multiplication of particular cell type.

Protonephridia are present in many invertebrate taxa (sometimes only in larvae e.g. in phoronids, some molluscs and annelids; see (Baeumler et al., 2011; Bartolomaeus, 1989; Bartolomaeus and Quast, 2005; Goodrich, 1945; Hay-Schmidt, 1987; Koch et al., 2014; Ruthensteiner et al., 2001; Temereva and Malakhov, 2006; Todt and Wanninger, 2010) and even though they are often considered plesiomorphic (e.g. (Bartolomaeus and Ax, 1992) they show remarkable diversity and evolutionary variation related with the fact that their particular elements can be easily organized in various ways (Ichimura and Sakai, 2017). For example, the filter might be built by a single cell with irregular openings, a single cell with slits, two adjacent terminal cells with interdigitating processes or a terminal cell and a canal cell forming a common weir (Kieneke et al., 2008). Additionally, it might stand with or without



diaphragm, with or without supporting microvilli, microvilli might be differentiated or uniform etc.. The terminal cell might be monociliated (also called solenocyte), multiciliated with independent cilia (then known as cyrtocyte) or multiciliated with all the cilia forming a single structure, the so-called flame as in e.g. rotifers or some platyhelminthes (Riemann and Ahlrichs, 2010; Rohde, 1991). The canal might be intracellular or multicellular, with or without cilia, spatially diversified or uniform etc. However, despite all their diversity, protonephridia are always straightforwardly comparable to each other (Bartolomaeus and Ax, 1992) and the primary homology statements regarding their particular portions can be readily made. The distribution of the protonephridia-bearing animals on the phylogenetic tree (Fig. 1), suggests that those organs evolved either twice, once in Scaldiphora and once in Spiralia, or are homologous between those two groups. This makes protonephridia an excellent model for studying the evolution of complexity and functionality of the excretory organs over long evolutionary time, combining morphological, cellular and molecular levels.

### *Metanephridial systems*

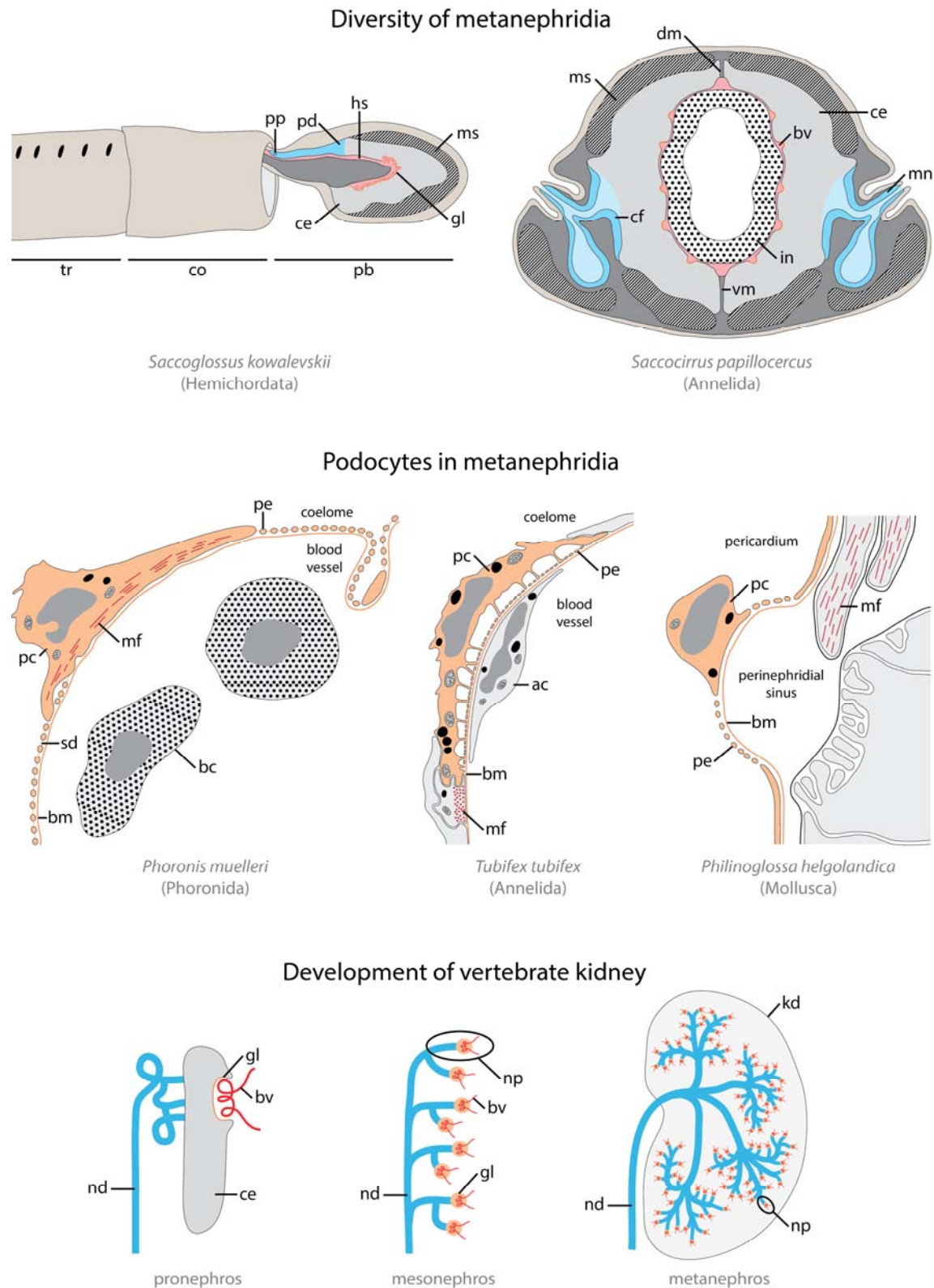
The typical metanephridia (Figs. 1, 3) are present in some annelids (e.g. (Bartolomaeus and Quast, 2005; Goodrich, 1945), in brachiopods (Kuzmina and Malakhov, 2015; Lüter, 1995), adult phoronids (Bartolomaeus, 1989; Storch and Herrmann, 1978; Temereva and Malakhov, 2006), cephalochordates (Ichimura and Sakai, 2017; Moller and Ellis, 1974; Ruppert, 1994) and adult hemichordates (Balsler and Ruppert, 1990; Dilly et al., 1986; Mayer and Bartolomaeus, 2003). Furthermore, the onychophoran nephridia (Mayer, 2006), coxal glands of Chelicerata (Briggs and Moss, 1997; Koch et al., 2014), antennal gland of Crustacea (Bartolomaeus et al., 2009; Khodabandeh et al., 2005), molluscan heart-kidneys (Baeumler et al., 2011; Bartolomaeus, 1997) and the axial organ of echinoderms (Balsler and Ruppert, 1993; Ezhova et al., 2013, 2014; Welsch and Rehkemper, 1987; Ziegler et al., 2009) can be also considered as metanephridial excretory systems, at least from the functional point of view. The ultrafiltration occurs in invertebrate metanephridial systems (Fig. 3) between blood vessels (or their functional equivalents, e.g. haemocoelic sinus) and the coelomic lining where specialized cells – podocytes (Fig. 3) – are present (Bartolomaeus and Ax, 1992; Ichimura and Sakai, 2017; Ruppert, 1994; Ruppert and Smith, 1988; Schmidt-Rhaesa, 2007). The primary urine is therefore synonymous with the coelomic fluid in those animals (Bartolomaeus and Ax, 1992; Ruppert, 1994; Schmidt-Rhaesa, 2007). The fluid leaves the coelom through the spatially independent structure, a metanephridium (Fig. 3), which often consists of the proximal dilated portion (i.e. ciliated funnel) and the distal region (i.e. nephroduct), which might be further subdivided into regions with differing functions and cell type composition (Goodrich, 1945; Schmidt-Rhaesa, 2007). Therefore,

compared to protonephridia, many different cell types build metanephridial systems and their exact number and qualitative composition varies from taxon to taxon, showing diverse ultrastructural, developmental and functional characteristics. In some animals – e.g. phoronids or some annelids - the metanephridia are ontogenetically predated by protonephridia (Bartolomaeus, 1989; Bartolomaeus and Quast, 2005; Goodrich, 1945; Koch et al., 2014; Temereva and Malakhov, 2006). In such instances the terminal cells of the protonephridium degenerate during metamorphosis, and the adult metanephridium develops from the larval protonephridial canal, whereas the podocytes develop *de novo* from the myoepitheliocytes (Bartolomaeus, 1989; Bartolomaeus and Quast, 2005; Bartolomaeus et al., 2009; Storch and Herrmann, 1978; Temereva and Malakhov, 2006). The term nephromixium is sometimes used to refer to such definite organ of dual origin (Goodrich, 1945; Temereva and Malakhov, 2006). On the other hand in Panarthropoda taxa, the podocyte-bearing cavity, called sacculus, does not seem to be formed by the coelom but rather by dilatation of the blind end of the developing nephroduct (summarized and discussed in (Koch et al., 2014)). Nephridia in Arthropoda are further distinguishable from those found in other animals (including onychophorans) by lacking any ciliation (Mayer, 2006).

In the vertebrate kidney, the excretion is carried out in the structural unit called nephron (Fig. 1; e.g. (Gérard, 1936; Ichimura and Sakai, 2017; Ruppert, 1994; Vize et al., 1997)), which due to its importance in human physiology is very well studied on morphological, physiological, developmental and molecular levels (e.g. (Bates, 2016; Desgrange and Cereghini, 2015; Dressler, 2006; Lindström et al., 2018; Little et al., 2010; McMahon, 2016; Quaggin and Kreidberg, 2008)). There are three types of nephrons found among vertebrates: the closed glomerular (present in e.g. mammalian kidney), opened glomerular (e.g. in salamanders) and aglomerular ones (found exclusively in some teleosts) (Gérard, 1936; Schmidt-Nielsen, 1997). The first two are relatively similar: both have a glomerular portion (also known as Bowman capsule), where filtering cells – podocytes – tightly surround capillaries lined with the extremely thin-walled fenestrated endotheliocytes (Bates, 2016; Ichimura and Sakai, 2017; Koriyama et al., 1992; Schmidt-Nielsen, 1997; Wolff and Merker, 1966). The primary ultrafiltrate passes through the endothelium-ECM-podocytes barrier and is subsequently accumulated inside the capsule, from where it is drained by the proximal nephron tubule. Aside from the podocytes and endotheliocytes two additional cell types of predominantly supportive function are found in the glomerulus – mesangial cells, which support capillaries (but also contribute to filtration of some molecules directly from the bloodstream) and parietal cells, which exclusively build the external wall of Bowman capsule (Dressler, 2006; Ichimura and Sakai, 2017; Quaggin and Kreidberg, 2008; Vize et al., 1997). The nephron tubule leads from the glomerulus to the collective duct, which eventually opens

to the urinary bladder. The tubule is differentiated into proximal, intermediate and distal regions, which differs in function and consequently in cell types of which they are composed (Bates, 2016; Desgrange and Cereghini, 2015; Lindström et al., 2018; Little et al., 2010; Schmidt-Nielsen, 1997). The main difference between closed and opened glomerular nephrons is that in the latter the additional ciliated canal (composed of yet another cell type) connects its proximal tubule with the peritoneal cavity (Gérard, 1936). The aglomerular nephron, on the other hand, is found only in some, mostly marine, teleosts (Gérard, 1936; Ichimura and Sakai, 2017; Schmidt-Nielsen, 1997). It lacks glomerular capsule and in fact utilize only active transport through the cells for primary urine production (Bulger, 1965; Dobbs and Devries, 1975; Schmidt-Nielsen, 1997), which makes it actually an example of a secretory excretory organ. It is, however, homologous to the glomerular nephron and, in some species, it ontogenetically develops from the glomerular condition (Gérard, 1936).

Depending on the arrangement of nephrons in the organ it is possible to distinguish between a mesonephros and a metanephros (Fig. 3; (Bates, 2016; Ichimura and Sakai, 2017; Vize et al., 1997)). In amniotes, they form an ontogenetic series with the metanephros being a definite excretory organ and the mesonephros is found only in the embryonic or larval stages (Bates, 2016; Vize et al., 1997). The earliest developmental stage of the vertebrate kidney – pronephros – does not have separate nephrons: the glomerulus and nephric duct are spatially separated, whereas the primary urine is accumulated inside coelom (Fig. 3; e.g. (Møbjerg et al., 2000; Vize et al., 1997)); from the functional perspective it can be therefore categorized as the proper metanephridial system (Ichimura and Sakai, 2017).



**Figure 3. Morphological diversity of metanephridial systems.** Schematic drawings of metanephridial systems in hemichordate (longitudinal section) and annelid (cross section), based on (Balsler and Ruppert, 1990) and (Goodrich, 1945). Schematic drawings of podocytes present in various invertebrates reconstructed from TEM sectioning, based on (Bartolomaeus, 1997; Peters, 1977; Storch and Herrmann, 1978). Schematic organization of three developmental stages of vertebrate excretory organs adapted from (Vize et al., 1997). In all drawings blood vessels are in red, sites of primary urine filtration in orange and

sites of primary urine modification in blue. Abbreviations: **ac** ameocyte, **bc** blood cell, **bm** basal membrane, **bv** blood vessel, **ce** coelom, **cf** ciliated funnel, **co** collar region, **dm** dorsal mesentery, **gl** glomerulus, **hs** heart sinus, **in** intestine, **kd** kidney, **mf** myofibrils, **mn** metanephridium, **ms** musculature, **nd** nephric duct, **np** nephron, **pb** proboscis, **pc** podocyte, **pd** protocoel duct, **pe** pedicle, **pp** proboscis pore, **sd** slit diaphragm, **tr** trunk region, **vm** ventral mesentery.

The most distinctive cell type that seems to be shared by all metanephridial systems (including kidneys) is a podocyte (Ichimura and Sakai, 2017; Ruppert, 1994). Cells of this type are divided into the cell body and pedicles – long projections, which interdigitate with each other (Fig. 3). On the junctions of the pedicles the proteins anchored in the podocyte cell membrane might form the filtering membrane – a slit diaphragm (Fig. 3; e.g. (Gerke et al., 2003; Ichimura and Sakai, 2017; Quaggin and Kreidberg, 2008; Storch and Herrmann, 1978; Tryggvason and Wartiovaara, 2001). Interestingly, a filtering region of terminal protonephridial cells of planarians shares some ultrastructural and molecular similarities with the filtering portion of the podocyte (Ichimura and Sakai, 2017; Vu et al., 2015). Taking into account that metanephridia probably evolved independently at least few times in the animal kingdom (Fig. 1; (Bartolomaeus, 1997; Bartolomaeus and Ax, 1992; Koch et al., 2014)), the podocytes of various animal groups represent analogous cell types (Bartolomaeus and Ax, 1992), which likely evolved by independent modification of the same ancestral filtering mechanism (e.g. from terminal cells of protonephridia) (Ruppert, 1994).

### *Excretory cells*

In addition to multicellular excretory organs (and sometimes instead of them) some animals possess specialized cells, which serve for excretory purposes (Haszprunar, 1996; Ruppert, 1994). Instead of expelling toxic waste products from the animal body, these cells accumulate the excreted substances inside their cytoplasm. Such accumulative excretory cells have been reported in a wide range of animals (Fig. 1) and although they are generally referred as nephrocytes, their ultrastructure and function differ from taxon to taxon.

The most studied excretory cells among nephrocytes are cells present in arthropods and onychophorans (Coons et al., 1990; Crossley, 1972; El-Shoura, 1989; Hessler and Elofsson, 1995; Seifert and Rosenberg, 1977; Shatrov, 1998; Vandenbulcke et al., 1998; Weavers et al., 2009). These cells, located inside haemolymph-filled cavities, are surrounded by ECM and resemble isolated podocytes with diaphragm-like structure. The waste products and other toxic compounds are filtered through the ECM and diaphragm and accumulate inside the nephrocytes (Vandenbulcke et al., 1998; Weavers et al., 2009). Insects have two sets of nephrocytes with similar ultrastructural properties – garland cells around esophagus and pericardial cells located on the heart walls (Crossley, 1972; Weavers et al., 2009). Cells with similar ultrastructure and function have been also reported in mollusks, where they are

called rhogocytes (Haszprunar, 1996; Rivest, 1992; Ruthensteiner et al., 2001). It has been proposed, based on molecular similarities (homology of the proteins forming diaphragm) and intermediate morphological forms, that both arthropod nephrocytes and molluscan rhogocytes are actually homologues of the filtering nephridial cells, which became spatially separated from an ancestral excretory organ (Haszprunar, 1996; Hessler and Elofsson, 1995; Rivest, 1992; Ruppert, 1994; Weavers et al., 2009).

A very different cell type is the nephrocyte in tunicates. Tunicate nephrocytes represent a fraction of blood cells, which accumulate nitrogenous waste products inside voluminous vacuoles (Ballarin and Cima, 2005; Cima et al., 2014; George, 1939). A similar type of excretory cells is also present in the coelomic fluid of Bryozoa (Schwaha et al., 2020). Contrary to the excretory cells of insects and mollusks, nephrocytes in tunicates and bryozoans lack both ECM and a slit diaphragm and they represent specialized haemo- and coelomocytes, therefore they are evolutionarily unrelated to other excretory organs.

#### *Excretory organs and adaptations to new environments*

Excretory organs are not only responsible for the expulsion of metabolic waste products but they are also the primary organs for osmoregulation and ion balance (Larsen et al., 2014; Schmidt-Nielsen, 1997; Schmidt-Rhaesa, 2007). The maintenance of water balance is especially challenging in environments such as freshwater and terrestrial habitats, where water is excessive or sparse, respectively (Schmidt-Nielsen, 1997). Another problem related to osmoregulation is faced by the organisms inhabiting the brackish and intertidal realms, where salinity can rapidly and dramatically change in a short time span, which requires ability to accommodate to different salinity regimes (Schmidt-Nielsen, 1997). Moreover, differences in the environmental salinity and water availability result in adaptations regarding the mechanisms of ammonia (one of the most toxic metabolites) excretion, such as transforming it into less harmful nitrogenous end products (e.g. urea or uric acid) (Larsen et al., 2014; Needham, 1935; Schmidt-Nielsen, 1997; Weihrauch and O'Donnell, 2017). Therefore, modifications of excretory organs are believed to be crucial for colonizing new environments (Schmidt-Rhaesa, 2007). In fact, studies on various nephrozoans have shown that species inhabiting terrestrial, freshwater, brackish and intertidal environments exhibit species-specific adaptations in the morphology (e.g. (Krishnamoorthi, 1963; Randsø et al., 2019; Smith, 1984; von Nordheim and Schrader, 1994) and physiology (e.g. (Generlich and Giere, 1996; Needham, 1935; Schmidt-Nielsen, 1997; Smith, 1970; Werntz, 1963) of their excretory organs and these adaptations are not necessarily reflecting their evolutionary relationship.



### 3. Molecular identity of excretory organs: from development to function

In contrast to the large number of detailed morphological descriptions of excretory organs in a variety of animals, molecular data are scarce. Most of the gene expression studies have been conducted in vertebrates (mainly mammals, fish and frogs) (summarized in (Desgrange and Cereghini, 2015) and a handful of invertebrates (mainly planarians and flies) (Scimone et al., 2011; Vu et al., 2015; Weavers et al., 2009) and have revealed not only common transcriptional programs governing the development of excretory organs, but also analogous sub-localization of solute transporters and structural proteins within the differentiated excretory compartments (Figure 4). Moreover, physiological studies in a large array of animals have shown that a number of ammonium transporters and proton pumps have conserved roles in excretory processes (Weihrauch and O'Donnell, 2017).

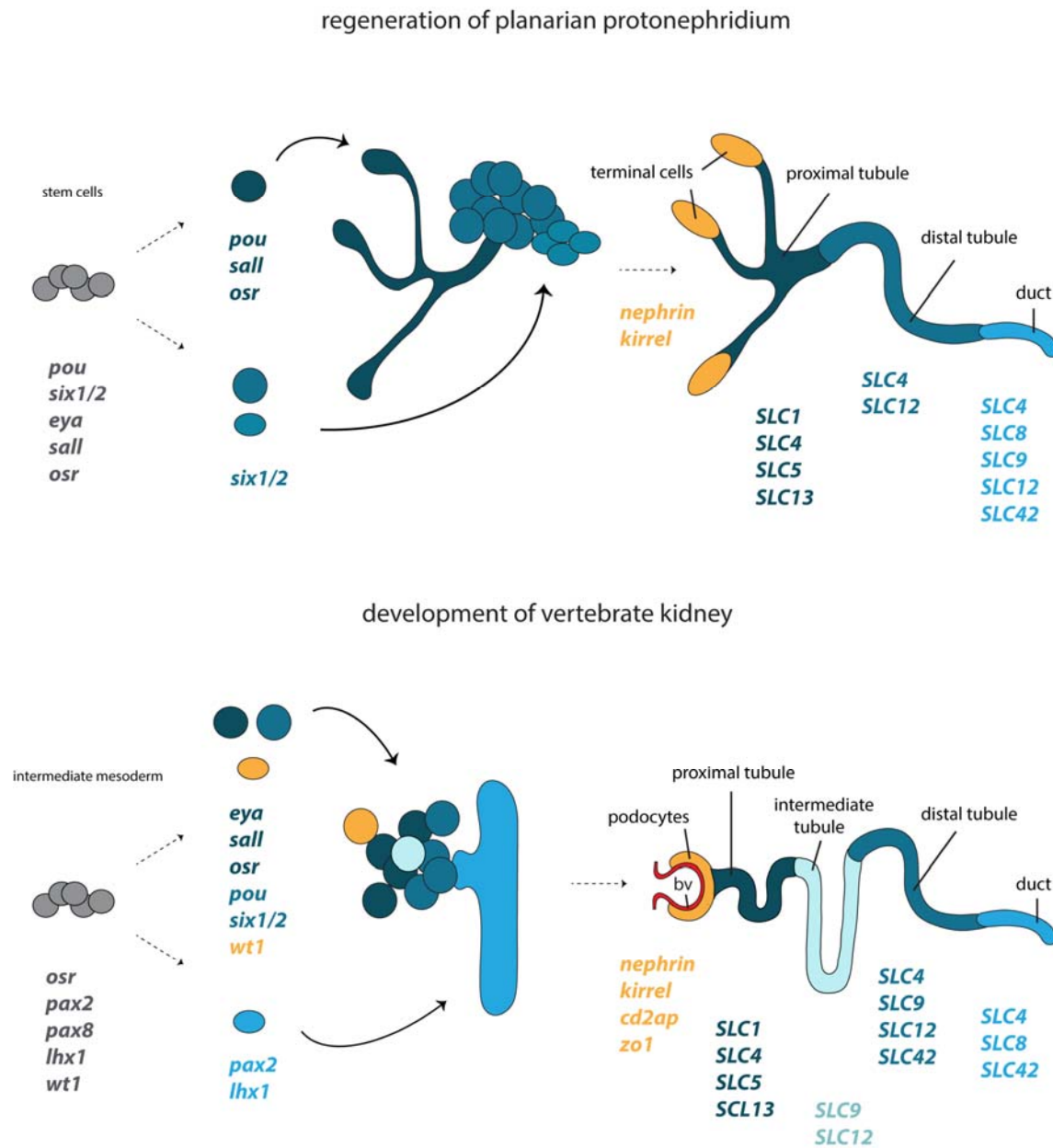
#### *Nephron development*

Kidneys, the excretory organs of vertebrates, emerge from the intermediate mesoderm and develop through a sequential formation of up to three organs: pronephros, mesonephros and metanephros (Figure 3) (Saxén and Saxén, 1987). Pronephros and mesonephros are only transient structures in amniotes and function as the fetal excretory organs, whilst metanephros is the definitive adult kidney. In fish and frogs, the adult kidney is the mesonephros that replaces the embryonic pronephros. Both mesonephros and metanephros are composed from a basic structural and functional unit, the nephron, which shows a comparable regional organization (Wingert and Davidson, 2008). The formation of kidneys is dictated by similar genetic interactions and morphogenetic events (Desgrange and Cereghini, 2015) (Figure 4).

Nephron development in mammals starts with a mesenchyme-to-epithelial transition (MET) of the anterior intermediate mesoderm (IM) and the specification of renal progenitor cells. This process is induced by the interplay of an ectodermal BMP4 signaling (James and Schultheiss, 2005) and the expression of *odd skipped related gene (osr1)* (James et al., 2006) and *lhx1* transcription factors (Tsang et al., 2000). *Pax2* and *pax8* genes are activated shortly after and act redundantly in the nephric lineage specification (Bouchard et al., 2002). The renal progenitor cells will form an epithelial tubule, the future nephric duct. As the tubule extends posteriorly, a number of transcription factors, such as *hox11*, *six1/2/4*, *eya*, *sall*, *pax2*, *foxc* and *wt1* (Brophy et al., 2001; Kobayashi et al., 2008; Kreidberg et al., 1993; Kume et al., 2000; Nishinakamura et al., 2001; Sajithlal et al., 2005; Wellik et al., 2002; Xu et al., 2003) are expressed along its anterior-posterior axis and induce the expression of *gdnf* (Gong et al., 2007; Moore et al., 1996). Ret/Gdnf signaling is crucial for the outgrowth and



branching of the ureteric bud (UB) at the posterior end of the nephric duct, which grows into medially positioned metanephric mesenchyme, which in turn gives rise to a renal vesicle (Costantini and Shakya, 2006). Other signaling pathways involved in UB formation and branching include Wnt (Bridgewater et al., 2008; Carroll et al., 2005; Kispert et al., 1998), sonic hedgehog (Shh) (Cain and Rosenblum, 2011), bone morphogenic protein (Bmp) (Nishinakamura and Sakaguchi, 2014) and fibroblast growth factor (Fgf) (Bates, 2011). Eventually, each renal vesicle forms a nephron through a series of morphogenetic movements and patterning events. *Mafb*, *wt1* and *lhx1b* drive podocyte specification (Miner et al., 2002; Moriguchi et al., 2006) and Notch signaling together with *pou3f3*, *hnf1b*, *irx1* and *irx2* are responsible for the proximal tubule fates (Cheng et al., 2007; Heliot et al., 2013; Nakai et al., 2003). The specification of distal tubule is controlled from the molecular interplay of several transcription factors, including *lef1*, *sox9* and *lhx1* (Mugford et al., 2009).



**Figure 4. Development and structural correspondences of protonephridia and kidneys.** Cartoon depiction of the molecular programs governing the regeneration of planarian protonephridia and the development of vertebrate kidneys, based on Scimone et al 2011 and Vu et al 2015. The corresponding structural components of protonephridia (terminal cell, tubule, duct) and kidneys (podocyte, tubule, duct) and the expression domains of orthologous genes in relation to their components, are color coded. Abbreviations: **bv** blood vessel.

In the zebrafish *Danio rerio*, the renal progenitor field forms from the lateral-most IM that express the transcription factors *pax2a* and *pax8* (Pfeffer et al., 1998). *Osr1* has also a conserved expression since its endodermal expression during gastrulation promotes renal lineages at the expense of blood/vascular ones (Mudumana et al., 2008). The renal progenitors adopt an epithelial state through MET and form an epithelial tubule. Cells located

in the anterior-most domain express *wt1a*, *wt1b*, *osr1*, *foxc1a* and *lhx1a* (O'Brien et al., 2011; Perner et al., 2007; Tomar et al., 2014) and will form the podocytes, whilst the remaining cells will give rise to the proximal and distal tubule and express *jagged*, *irx3b*, *evil* and *pou3f3a/pou3f3b* (Li et al., 2014; Ma and Jiang, 2007; Wingert et al., 2007). The transcriptional interplay responsible for the formation of this boundary consists from *pax2a*, which forms a negative feedback loop with *wt1a* (Majumdar et al., 2000) and *hnf1b*, a suppressor of *pax2a* (Naylor et al., 2013). Tubule regionalization is also regulated by Retinoic Acid (Ra) signaling pathway (Wingert et al., 2007).

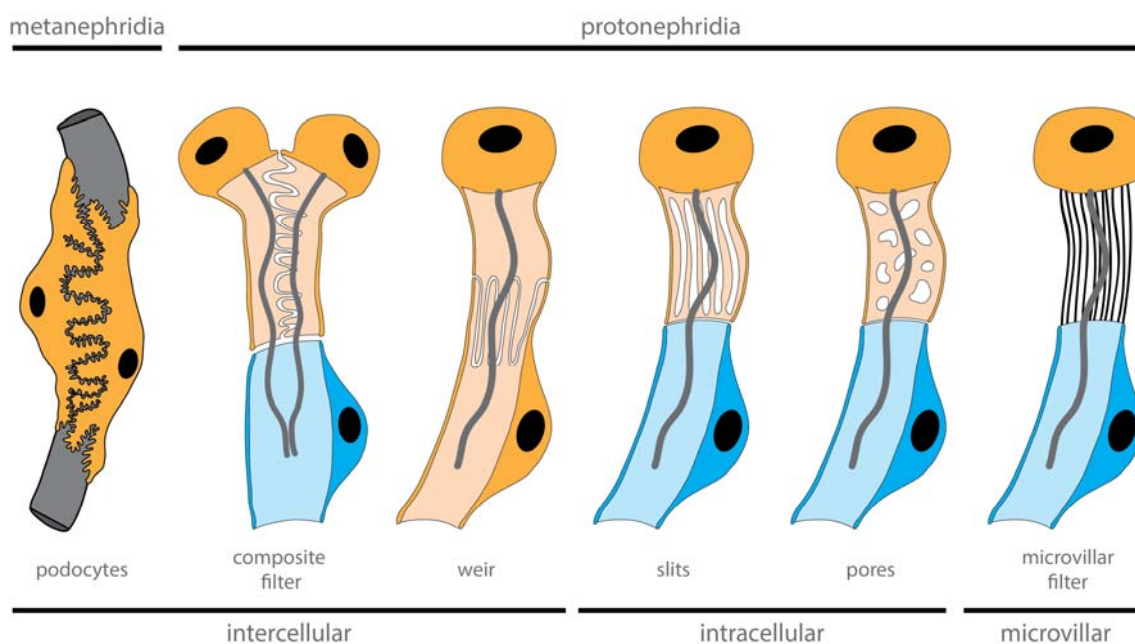
In the frog *Xenopus*, the renal progenitors emerge after a MET of the caudo-lateral IM resulting in the formation of a tubule. Once again, *osr1* and *osr2* are upstream of the specification of the renal progenitor field (Tena et al., 2007). The first renal molecular markers are *lhx1* and *pax8*, followed by the expression of *pax2*, *wt1* and *hnf1 $\beta$*  (Buisson et al., 2015; Carroll and Vize, 1996, 1999; Carroll et al., 1999; Wild et al., 2000). *Wt1* specifies the future glomerulus whilst *pax2* is restricted to the future tubular region. The subdivision of the nephron into segments is governed by *evi1* expression in the distal tubule and pronephric duct (Van Campenhout et al., 2006) and *irx1*, *irx2* and *irx3* expression in the proximal and intermediate tubule (Alarcon et al., 2008). The developing podocytes are specified from a cross talk between *wt1*, *foxc2*, *lhx1b* and *mafB* genes (Haldin et al., 2008; White et al., 2010). Signaling pathways with crucial roles in *Xenopus* nephrogenesis involve Wnt (Lavery et al., 2008; Saulnier et al., 2002; Tetelin and Jones, 2010), Fgf (Urban et al., 2006), Bmp (Bracken et al., 2008), Notch (McLaughlin et al., 2000; White et al., 2010) and Ra (Cartry et al., 2006).

### *Protonephridial development*

Although there are several morphological descriptions on protonephridial development (Baeumler et al., 2011; Bartolomaeus, 1985; Hasse et al., 2010; Rohde et al., 1988; Temereva and Malakhov, 2006; Wenning et al., 1993), molecular studies are extremely limited. The most detailed work has been performed on the planarian *Schmidtea mediterranea* (Scimone et al., 2011) that has shown a remarkable conservation of the molecular programs between the regeneration of planarian protonephridia and the development of vertebrate kidneys (Figure 4). Planarian protonephridia consists of four cells types, the flame (terminal) cell, the ciliated tubule cell type (proximal tubule), the tubule-associated cell type (distal tubule) and the duct. Regeneration and RNAi experiments on amputated animals showed a conserved function of *eya*, *six1/2*, *pou3*, *hunchback*, *sall* and *osr* genes in the regeneration and maintenance of protonephridia. *Eya*, *six1/2*, *pou3*, *sall* and *osr* are expressed in the specified progenitor cells, whose fate segregates and results in the

formation of the ciliated tubule cell type and the tubule-associated cell type. The ciliated tubule cell type continues to express *pou3*, *sall* and *osr*, whilst the tubule-associated cells express *six1/2* (Scimone et al., 2011).

The conserved role of *pou3* is also reported in the nematode *C. elegans*, where an orthologous gene, *ceh-6* is required for the formation and function of its excretory cell (Burglin and Ruvkun, 2001). Other conserved nephrogenesis-related transcription factors has been shown to be *pax3* in the developing nephridia of the leech *Helobdella robusta* (Woodruff et al., 2007), *pax2/5/8* in the nephridium of the cephalochordate *Branchiostoma floridae* (Kozmik et al., 1999) and *sall* in the protonephridium precursors of the polychaete *Hydroides elegans* (Arenas-Mena, 2013).



**Figure 5. Diversity of filter-forming cells within nephridia.** The filter apparatus can be formed by different cell features. Intercalated cells form the filtration apparatus between cells, filtration can be performed by cellular pores and slits or microvilli can build up the filtration apparatus. Cells forming extracellular filter are depicted in orange, while nephroduct cells in blue.

#### *Development of insect Malpighian tubules*

The Malpighian tubules of insects consist of two cell types; the primary cell (PC) and the secondary (stellate) cell (SC). In *Drosophila*, these two cell types have a different developmental origin; while the PC derive from an ectodermal primordium at the hindgut/midgut junction and form the Malpighian tubule epithelium, the SC originate from the posterior mesoderm and invades the tubule epithelium through MET (Denholm et al., 2003). The molecular patterning of Malpighian tubule development appears quite different compared to the protonephridia and kidneys. *Krüppel* and *cut* expression in a cluster of cells

of the hindgut marks the onset of PC development due to a Wnt signaling. Once specified, these cluster of cells start to form bud-like branches under the control of Decapentaplegic (BMP) signaling and Brinker (Hatton-Ellis et al., 2007). Later on, a regulatory interplay between *cut*, *barr*, *three rows*, *pebble*, *pimples*, *trachealess*, *ribbon*, *raw*, *crooked neck*, *faint sausage*, *pant* and *schnurri*, under a DPP/BMP signal, will result in the formation and morphogenesis of tubules (Hatton-Ellis et al., 2007; Jack and Myette, 1999; Shim et al., 2001). As the tubules elongate, a caudal mesodermal population that will become SC, express the transcription factors *tiptop* and *teashirt*, undergo MET and intercalate in the tubule (Denholm et al., 2003). The developmental process of Malpighian tubule development is overall conserved, since studies in *Tribolium* have shown that the onset, morphogenesis and molecular profile are similar. An important difference is the fact that in *Drosophila* only differentiated PC are expressing *cut*, whilst in *Tribolium* *cut* is expressed in both PC and SC (King and Denholm, 2014).

#### *Molecular basis of functional compartmentalization*

The terminal differentiated excretory organs are highly specialized and compartmentalized into discrete segments, composed of distinct epithelial cell types that carry out different functions. Each region is characterized by the expression of a set of structural proteins and transporters, involved in excrete ultrafiltration and modification, such as glucose and solute transport, and homeostasis. The spatial distribution of these proteins on the different segments of excretory organs is, once more, remarkably conserved (Figure 4) (Kozmik et al., 1999; Vu et al., 2015; Weavers et al., 2009).

In vertebrates, the nephron is divided in five segments: the glomerulus, the proximal, intermediate and distal tubule, and the collecting duct. Glomerulus has a central role in ultrafiltration and is expressing a set of membrane-associated proteins, such as Nephtrin (NPHS1), KIRREL1 (NEPH1), CD2AP, ZO1, Nck and Stomatin/Podocin (summarised in (Patari-Sampo et al., 2006)). The tubular segments are further subdivided in smaller regions; each of them specialized in different aspects or excrete modification. The segmental organization is highly conserved, with the solute carrier (SLC) protein family to be sequentially expressed along the tubular segments of different types of nephrons, defining their boundaries (Desgrange and Cereghini, 2015). SLCs are membrane transporters, composed from 52 families, which can transport a number of different substrates (Hediger et al., 2013). In both mammalian metanephros and *Xenopus* pronephros, subsets of cells of the proximal tubule are specialized in reabsorbing: a) salts, expressing members from the bicarbonate transporter SLC4, sodium- and chloride-dependent neurotransmitter transporter SLC6, sodium/proton exchanger SLC9, sodium-sulfate/carboxylate cotransporter SLC13 and

organic cation/anion/zwitterion transporter SLC22 families; b) amino acids, expressing members from the glutamate and neutral amino acid transporter SLC1, heavy subunits of the heteromeric amino acid transporter SLC3 and cationic/glycoprotein-associated amino acid transporter SLC7 families; and c) glucose, expressing members from the sodium-glucose cotransporter SLC5 family (Landowski et al., 2008; Raciti et al., 2008). SLC9a3 expression is also marking the proximal tubule of the pronephros of *D. rerio*, suggesting a similar function of this segment in reabsorbing sodium (Wingert et al., 2007). The intermediate tubule of nephron (Henle's loop) is facilitating the concentration of excreted and does not share an extensive molecular conservation among the different nephron types (Desgrange and Cereghini, 2015). Cells of the distal tubule are specialized in the reabsorption and secretion of ions, ammonium and water through the SLC9 and the electroneutral cation-chloride cotransporter SLC12. Common transporters characterizing the distal tubule of mammalian metanephros and *Xenopus* pronephros are members of SLC12, monocarboxylate transporter SLC16, type III sodium-phosphate cotransporter SLC20 and ammonium transporter SLC42/Rhesus families (Landowski et al., 2008; Raciti et al., 2008). Cells comprising the distal tubule of *Xenopus* are additionally expressing SLC4a4 (Raciti et al., 2008), whilst in *D. rerio* only members of the SLC12 family are seen (Wingert et al., 2007). Finally, the collecting duct consists from highly specialized, electrically tight cell types with dedicated roles in reabsorption and secretion of salts and water, and the concentration and preparation of the urine. In mammals, the collecting duct expresses members of the sodium/potassium exchanger SLC8, SLC16, SLC20, SLC22 and SLC42/Rhesus families (Landowski et al., 2008), while in *Xenopus* the analogous segment, the collecting tubule, expresses additionally SLC12a3 and doesn't express SLC42/Rhesus (Raciti et al., 2008). Even bigger differences we encounter in *D. rerio*, where the pronephric duct is not expressing any of these transporters (Wingert et al., 2007). The fact that the distal tubule of *D. rerio* and *Xenopus* pronephros expresses SLC12a3 and *Xenopus* do not express SLC42/Rhesus in their collecting tubule but only in their distal tubule, suggests that the function of distal tubule of *D. rerio* and *Xenopus* might be analogous to a mammalian tubule/duct hybrid (Desgrange and Cereghini, 2015). Another family of transporters that exhibit a nephron segment-specific expression is the one of aquaporins, which transports mainly water, urea and glycerol (Gomes et al., 2009). In mammals, the proximal tubule is expressing the water/nitrate/chloride transporter AQ1 (Nielsen et al., 1993), the aquaglyceroporin AQ7 (Nejsum et al., 2000), the water/ammonia transporter AQ8 (Elkjaer et al., 2001) and the super-aquaporin AQ11 (Morishita et al., 2005), while the collecting duct expresses the water transporters AQ2 (Fushimi et al., 1993) and AQ4 (Terris et al., 1995), the aquaglyceroporin AQ3 (Ecelbarger et al., 1995), the water/nitrate/chloride transporter AQ6 (Yasui et al., 1999) and AQ8. In *Xenopus*, a similar distribution of the AQ2 and AQ3



mammalian orthologs has been shown in the collecting duct. However, AQ1 expression was restricted in glomerulus, in contrast to what is observed in mammals (Pandey et al., 2010). The situation looks even more different in teleosts where the proximal tubules express one AQ8, one AQ10-like and one AQ3 paralog (Engelund and Madsen, 2015; Santos et al., 2004). Moreover, two copies of AQ1 have a renal expression but have acquired different functions; AQ1a is expressed in the proximal tubule and AQ1b in the distal tubule (Engelund and Madsen, 2015; Madsen et al., 2011). Finally, one AQ11 and one AQ12 ortholog are also expressed in the teleost nephron but the exact expression domain has not yet been revealed (Kim et al., 2014; Madsen et al., 2014).

In the planarian *S. mediterranea* protonephridia are divided in four major compartments: the flame (terminal) cells, the proximal tubule, the distal tubule and the duct. The flame cells are performing ultrafiltration and express Nephtrin/Kirrel. In a similar fashion with the metanephridial systems, the tubular compartments are further subdivided in smaller specialized domains with different cell types being defined by the expression of a suite of SLC transporters and having diverse roles in excrete modification (Vu et al., 2015). For instance, the proximal tubule, primarily responsible for the recovery of filtered substances and reabsorption of salts, is expressing members from the SLC1, SLC5, SLC4, SLC6, SLC13 and SLC22 families. The distal tubule has a central role in homeostasis and expresses members from the SLC4 and SLC12 families, which are also marking the duct. The duct is additionally expressing SLC42/Rhesus, as seen in mammals but not in *D. rerio* and *Xenopus*, and a number of other SLCs, which mark the proximal tubule of vertebrates, such as SLC6, SLC7, SLC9 indicating a role of the planarian duct not only in the urine concentration but also in reabsorption of salts and amino acids. Interestingly, aquaporins are not expressed in any of the protonephridial compartments, suggesting divergent functions of these transporters in planarians (Vu et al., 2015).

Malpighian tubules of some insects (e.g. the fly *Drosophila melanogaster* and the mosquito *Aedes aegypti*) are also divided in distinct compartments with different physiological functions: the initial, transitional, main and lower segments, and the ureter (Beyenbach et al., 1993; Sozen et al., 1997). Each segment is populated by a different numeral and positional combination of PC and SC cells, depending on the investigated species. Overall, the initial and transitional segments are not participating in secretion but rather act as storage segments and transport (Dow et al., 1994; Dube et al., 2000). The main segment has both secretory and absorptive roles of salts and water and generates the primary urine. The PC cells of the main segment express a basolateral sodium/potassium transporter (Torrie et al., 2004), an inward-rectifier potassium transporter (Kir) (Evans et al., 2005), sodium/proton exchangers of the NHA and NHE (SLC9) families (Pullikuth et al., 2006; Rheault et al., 2007) and an aquaporin (Kaufmann et al., 2005). In contrast, the SC cells, which exhibit mostly an



absorptive role, express mainly a chloride transporter and an aquaporin (Kaufmann et al., 2005; Kolosov and O'Donnell, 2020; O'Connor and Beyenbach, 2001; O'Donnell et al., 1998). The expression of SLC4 exchanger has also been reported in the SC cells of some species (Linser et al., 2012; Piermarini et al., 2010). Finally, the lower segment and rectum are mostly dedicated in the reabsorption of salts and water and the concentration of urine (O'Donnell and Maddrell, 1995).

Molecular similarities found at the building blocks of the ultrafiltration sites of proto- and metanephridial systems are also seen in individual ultrafiltration cells. The membrane-associated protein complex composed of Nephrin, Kirrel, Cd2ap, Zo1 and Stomatin/Podocin is also forming the slit diaphragm of nephrocytes of the fly *Drosophila melanogaster* (Weavers et al., 2009; Zhuang et al., 2009) that performs filtration of the haemolymph (Weavers et al., 2009; Wigglesworth, 1943; Zhang et al., 2013). Nephrocytes are not only involved in ultrafiltration but also in protein reabsorption, via receptors such as Cubilin and Amnionless (AMN), similarly to mechanisms encountered in the renal proximal tubule cells (Zhang et al., 2013). Moreover, Nephrin/Kirrel expression has been described in the ultrafiltration apparatus of rhogocytes of the snail *Biomphalaria glabrata* (Kokkinopoulou et al., 2014), through which proteins and ions are filtered (Kokkinopoulou et al., 2015).

### *Nitrogenous waste excretion*

One of the main and most toxic products of excretion in animals is nitrogenous waste. Nitrogenous waste products are the result of the amino acid catabolism (Campbell, 1991) and exist in three forms: ammonia, urea, or uric acid. Most aquatic species excrete ammonia, due to its high solubility in water, whilst semi-aquatic and terrestrial species usually convert ammonia to less hazardous and less soluble forms, such as urea and uric acid. Ammonia can occur either in its gaseous ( $\text{NH}_3$ ) or in its ionic form ( $\text{NH}_4^+$ ); however, due to the high pK of  $\text{NH}_3$ , in physiological solutions the vast amount of ammonia exists as  $\text{NH}_4^+$ . The excretory process of ammonia has been investigated in a large array of animals and shown extensive conservation of the repertoire of the ammonia transporters and proton pumps, independently of the presence of specialized excretory organs (Weihrauch and O'Donnell, 2017).

In kidneys, the epithelial cells of the proximal tubule secrete ammonia apically into the luminal fluid. A significant fraction of this ammonia secretion occurs via  $\text{Na}^+/\text{NH}_4^+$  exchange by the sodium/proton exchangers SLC9 (NHE) and some also takes place via diffusion (Bourgeois et al., 2010; Knepper et al., 1989; Preisig and Alpern, 1990). Almost all secreted ammonia is reabsorbed by cells of the thick ascending limb of Henle's loop into the interstitium, both by  $\text{K}^+/\text{NH}_4^+$  exchange of the apical cation-chloride cotransporter SLC12

(NKCC) (Good, 1994) and Na<sup>+</sup>/NH<sub>4</sub><sup>+</sup> exchange of the basolateral SLC9 exchangers (Blanchard et al., 1998). Another cotransporter that plays an important role in NH<sub>4</sub><sup>+</sup> reabsorption is the basolateral bicarbonate transporter SLC4, which drives NH<sub>3</sub> diffusion across the basolateral membrane due to the bicarbonate transport into the cell and the subsequent rise of the intracellular pH (Good et al., 1984; Lee et al., 2010). The accumulated ammonia in the interstitium is forming a gradient that drives diffusion across the epithelium of the cells of the collecting duct and secretion of the concentrated ammonia to the lumen. This process is also supported by an proton gradient formed by the apical vacuolar H<sup>+</sup>-ATPase, which creates an acidic environment facilitating NH<sub>4</sub><sup>+</sup> entrapment (Flessner et al., 1991; Star et al., 1987) and a basolateral Na<sup>+</sup>/K<sup>+</sup> ATPase (NKA) that actively transfers ammonia by Na<sup>+</sup>/NH<sub>4</sub><sup>+</sup> exchange (Wall, 1996). Finally, the Rhesus ammonia transporters (RhBG and RhCG), spatially restricted to the collecting duct, also participate to the secretion to the lumen and preparation of the urine (Mak et al., 2006).

Species-specific excretory organs/sites have also recruited these transporters for excreting ammonia. In Malpighian systems, ammonia is firstly secreted into the Malpighian tubules and then actively absorbed by the hindgut and midgut, which express the ammonia transporter Rhesus, sodium/proton exchangers (NHE) and a vacuolar H<sup>+</sup>-ATPase (Blaesse et al., 2010; Weihrauch, 2006). Similarly, in the posterior rectum (anal papillae) of the aquatic mosquito larvae *Aedes aegypti*, the ammonia transporters Rhesus and the Rhesus-related AMTs, as well as a basal NKA and an apical vacuolar H<sup>+</sup>-ATPase are involved in ammonia excretion (Chasiotis et al., 2016; Durant and Donini, 2018). In the excretory H-system of the nematode *C. elegans*, ammonia enters the excretory cells via the basolateral NKA and potassium transporters and then diffuses across the apical membrane through an acid-trapping mechanism (Adlimoghaddam et al., 2015). Rhesus and the vacuolar H<sup>+</sup>-ATPase are likely also involved in ammonia excretion in the plicate organ of mussels of the *Mytilus* family (Thomsen et al., 2016). Other animal-specific ammonia excretory sites include the gills of several crustacean species and the branchial appendages of the marine annelid *Eurythoe complanata*, which all seem to express NKA, the vacuolar H<sup>+</sup>-ATPase, Rhesus, as well as AMTs (in the case of the *E. complanata*) (Si et al., 2018; Thiel et al., 2017; Weihrauch et al., 2017). Interestingly, NKA, NHE, Rhesus and the vacuolar H<sup>+</sup>-ATPase are also reported to be involved in ammonia excretion even when this process is occurring through the integument, as shown in the leech *Nepheleopsis obscura* (Quijada-Rodriguez et al., 2015), the planarian *S. mediterranea* (Weihrauch et al., 2012) and the nematode *C. elegans* (Adlimoghaddam et al., 2016), or through digestive-associated tissues, as suggested in members of the Xenacoelomorpha and Cnidaria (Andrikou et al., 2019).

The astonishing reported molecular similarities, not only at the developmental level but also at the structural and functional level of excretory systems, has led some authors to

propose their common evolutionary origin (Haszprunar, 1996; Ruppert, 1994; Scimone et al., 2011; Vu et al., 2015; Weavers et al., 2009). However, given the fact that correspondences in molecular patterning cannot be the only criterion for supporting homology, especially in the level of a complex organ system, such as a nephridium, these interpretations need to be handled with caution.

#### 4. The problem of homology

Although excretory organs are present across the Nephrozoa, their emergence and evolutionary relationship remain unsolved. When mapping nephridia on the recent animal phylogeny it is likely that the protonephridia are the ancestral form, from which at least the metanephridia evolved (Bartolomaeus and Ax, 1992; Ruppert, 1994). The evolution of metanephridia has likely happened multiple times independently, given that coeloms emerged convergently at different animal lineages (Koch et al., 2014). The fact that protonephridia can directly develop into metanephridia in some lineages indicates a close evolutionary relationship between these two types of excretory organs (Bartolomaeus and Ax, 1992; Ruppert, 1994). The homology of protonephridia and metanephridia is partly supported by similar transcription factors that seem to be involved in patterning both structures (Scimone et al., 2011). However, the taxon sampling is, so far, very narrow and therefore needs to be extended to solidify this interpretation.

So far, the cell type perspective does not contribute much to the question on the homology of excretory organs, mainly because it is dealing with a different level of homology (Abouheif, 1997). Firstly, excretory organs are composed of different cells that perform diverse functions. At what time in evolutionary history these cells originated and - if homologous - have been assembled to a functional organ, remains unclear. Secondly, the different cells that build a nephridium can perform similar functions with very different structures, which could speak for their convergence. When comparing for example the terminal ultrafiltration cell with other cell types that possess a “collar” or slit-like openings, it is evident that these collar-like structures within nephridia are highly diverse and therefore difficult to compare between species (Figure 5). Sometimes the filters are formed between the cells, sometimes the openings are within the cells, and in other cases these slits are formed by microvilli. All variations perform a similar task, namely the filtering of the primary urine. Do these differences speak for a convergence of the cells or even of the whole organ? On which level can these cellular structures be homologous? Can one use the presence of microvilli to state cell type homology or can we only homologize the microvilli themselves? It is clear that a cell is composed out of many different substructures that might have to be treated separately when investigating their origin (Carvalho-Santos et al., 2011; Sebe-Pedros

et al., 2013). This raises the question of how many substructures are necessary to characterize a “cell type” and whether these are sufficient to homologize these cell types between species?

Additional problems are introduced when transcriptomic similarities are used for characterizing a cell type. The transcriptomic differences between cell-types and cell-states is a continuum, meaning that clear boundaries are established by the observer, and are therefore artificial (Trapnell, 2015). It is furthermore in the nature of a cell that transcriptomic noise (and in some cases technical noise), which can be to some extent stochastic, obscures the potential signal that could be used to characterize the cell type/state (Ballouz et al., 2019). But what kind of signal are we looking for? As mentioned already, subcellular structures that are plesiomorphic for a clade (e.g. cilia, microvilli for Metazoa) may provide a signal, but cannot be used to homologize cell-types. Some authors propose the use of a combination of transcription factors and effector genes to detect cell types within transcriptomes (Arendt et al., 2016). Considering what we know about gene regulatory networks and their flexibility and evolutionary exchangeability of key-regulators within these networks, using “this gene combination” as definitions of cell type can lead to wrong conclusions, especially when taking into account false positives and false negatives. Moreover, the concentration of transcription factors on the protein level may impact the output of a gene regulatory network and cannot yet be detected with the current single-cell methods (Marx, 2019). Finally, given the fact that the consideration of sets of co-expression of regulators and effectors without functional testing is very arbitrary, how do we then discriminate between homoplasy and homology? (Shafer, 2019; Tschopp and Tabin, 2017). In principle, we face similar problems in the homologization of cell types that we face with the homologization of other biological levels. Noise, drift of underlying structures and methodological problems may obscure the conclusions. The coming years of data harvesting, comparative analyses and developments in these technologies will guide the way for cell and organ comparisons between animals.

In summary, excretory systems with their structural and functional variation, diverse cellular composition and variable embryology are an ideal showcase to test different approaches currently used for unravelling the origin of organ systems.

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